[3] Diseases Caused By Bacterial Pathogens In Inland Water

FL-15 isolated from a diseased sheatfish (*Silurus glanis*) in Hungary. The FL-15 genome encodes 20 predicted adhesion precursors that could be implicated in cell-cell and cell-surface interactions. However, no genes encoding known pilus or fimbrial proteins were identified in the FL-15 genome. The authors mentioned that, if present, the corresponding genes may be hidden within those encoding hypothetical proteins; it is also possible that production of pilus-like structures is a strain-dependent feature absent from strain FL-15. It is also interesting that the FL-15 genome contains two distinct groups of genes, *gld* and *spr*, which are involved in gliding motility. This suggests that *F. branchiophilum* may actually be motile, but experimental conditions used so far failed to mimic natural conditions where gliding motility is expressed.

4. COLUMNARIS DISEASE

Hisatsugu Wakabayashi

4.1. Synopsis

Columnaris disease, caused by *Flavobacterium columnare,* affects cultured, ornamental and wild-fish populations in freshwater worldwide. The disease is characterized by external infections in the fish body surface, gills or fins. The bacterial cells are long, flexible, Gram-negative rods that are motile by gliding. They grow well on low nutrient media producing pale yellow rizoid colonies. Traditional culture techniques require several days for a definitive diagnosis. Therefore, molecular techniques such as the polymerase chain reaction (PCR) have been used. Because *F. columnare* primarily attacks gills, skin and fins of fish, most treatments proposed for columnaris disease are surface-acting disinfectants used as baths.

4.2. Introduction

Columnaris disease was first described by Davis (1922), who observed it in warm-water fish from the Missisippi River, USA. Although Davis did not succeed in isolating the pathogen, he named it *Bacillus columnaris* because, when pieces of infected tissues from diseased fish were examined microscopically in wet mount preparation, column-like masses of the bacteria were observed along the periphery of the tissues (Figure 4.1).

The etiological agent was first isolated by Ordal and Rucker (1944) from hatchery reared sockeye salmon (*Oncorhynchus nerka*) in the summer of 1943 and renamed *Chondrococcus columnaris.* At the same time Garnjobst (1945) isolated the organism from some warm water fishes and assigned it to the genus *Cytophaga*. The precise taxonomy of the agent has been the subject of continuing discussion (Leadbetter 1974, Reichenbach 1989). This taxonomical confusion was resolved when Bernardet *et al.* (1996) transferred the bacterium to the genus *Flavobacterium* (Bernardet and Bowman 2011).

Flavobactrium columnare, the causative agent of columnaris disease, is ubiquitous in freshwater environments and an opportunistic pathogen that causes skin and gill infections in freshwater fishes worldwide (Nigrelli and Hutner 1945, Wakabayashi and Egusa 1966, Anderson and Conroy 1969, Bowser 1973, Wobeser and Atton 1973, Bootsma and Clerx 1976, Fergson 1977, Morrison et al. 1981, Chun et al. 1985,

Bernardet 1989, Berno 1989, Koski et al. 1993, Figueiredo et al. 2005, Welker et al. 2005, Tien et al. 2012).

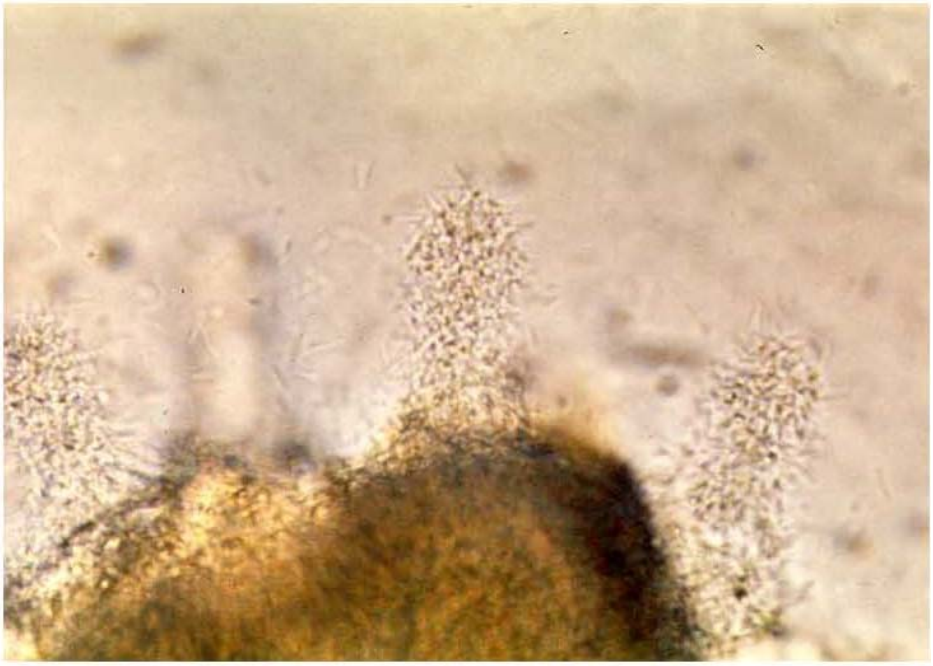

Figure 4.1. Columnar formations of *F. columnare* along the margin of a piece of the tissue.

4.3. Disease Agent

Cytophaga agar (CA) (Anacker and Ordal 1959a) is the most frequently used medium for cultivation of *F. columnare*. It contains trypton 0.05%, yeast extract 0.05%, sodium acetate 0.02%, beef extract 0.02% and agar 0.9% and is adjusted to pH7.2-7.4. The colonies are of a spreading nature with irregular margins and they adhere to the agar. Under the microscope (×40), the edge of the colonies appeared rhizoid (Figure 4.2). *F. columnare* contains flexirubin type pigments, so that the colonies change their color from yellow to brown when flooded with 20% KOH solution. In static culture in cytophaga broth (CB) the bacteria form clusters or a pellicle of cells on the surface of the broth. When gently agitated, however, they usually grow homogeneously.

The cells are Gram-negative, slender and rather long bacilli, 0.3 - 0.5 μm wide and 3 - 8μm. The filamentous cells display an active flexing movement in wet mount preparation. They have no flagella but exhibit gliding motility on a wet surface. Bernaldet and Grimont (1989) described the physiological characteristics of eight strains of *F. columnare* isolated Europe, USA and Japan as follows. Growth occurs in CB supplemented with 0.1% or 0.5% NaCl and at 10-33 C. Catalase and cytochrome oxidase are produced; nitrate is reduced to nitrite; hydrogen sulfide is produced. Cellulose, carboxymethyl cellulose, chitin, starch, aesculin and agar are not hydrolysed. No acid is produced from carbohydrates in ammonium salt-sugar medium. Gelatin, casein (skim milk agar), and tyrosine are hydrolysed. Lysine, arginine and ornithine are not decarboxylated. Tributyrin, lecithin (egg yolk), Tween 20, Tween 80 and DNA are

hydrolysed. These characteristics accord with those reported by other workers such as Garnjobst (1945), Wakabayashi et al (1970) and Bootsma and Clerx (1976).

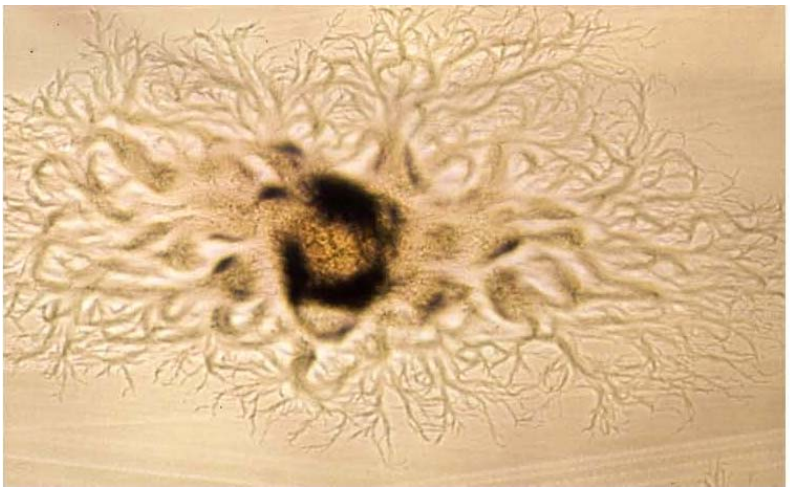

Figure 4.2. A colony of *F. columnare* growing on cytophaga agar, showing the rhizoid edges to the colony.

The DNA base composition has been variously defined as between 29.8 and 35.9 mol % G+C (Mitchell), or 32.6 and 42.9 mol % G+C (Bootsma and Clerx 1976). According to Bernardet and Grimont (1989) the base composition of their strains were 32.0 and 33.2 mol % G+C and the relatedness in DNA-DNA hybridization was more than 76%. Song et al. (1988) compared the isolates from Western North America and other areas of the Pacific Rim. They reported that the mol % G+C of the DNA was 29.6 - 32.5 and that 19 of the 22 strains were 81-98% homologous with their type strain isolated from fish in Oregon, USA. Triyanto and Wakabayashi (1999a,b) described three genomovars among the species based on the analysis of the 16S rDNA gene restriction fragment length polymorphisms (16S rDNA-RFLP). The 16S rDNA sequence provided enough variability to defined three genomovars within the species. Genomovar I is defined by the type strain IAM14301^{T} (=ATCC23463^{T}) isolated from Chinook salmon in USA and including 10 Japanese and 8 French strains from various fishes, a Chinese strain from grass carp (*Ctenopharyngodon idella*), and an Indonesian strain from common carp (*Cyprinus carpio*). Genomovar II is defined by EK28 (=IAM14820) isolated from eel (Anguilla japonica) in Japan and including a Japanese strain from loach (*Misgurnus anguillicaudatus*). Genomovar III is defined by a single strain PH97028 (=IAM14821) isolated from ayu (*Plecoglossus altivelis*) in Japan. Michel et al (2002) found that two neon tetra (*Paracheirodon innesi*) isolates and two blackmolly (*Poecilia sphenops*) isolates belonged to genomovar II, while all North American and French fresh water fish isolates belonged to genomovar I. They thought that genomovar II or Asian type strains might have been brought to Europe through ornamental fish imports. Two isolates from warm water fishes, i.e. catfish (*Ictalurus* sp.) and baitfish (*Notropis* sp.) in USA were added to the original Genomovar III isolate PH97028 (Scheck and Caslake 2006). Amplified fragment length polymorphism (AFLP) fingerprinting further subdivided the species without losing genetic hierarchy of genomovar division (Arias et al. 2004). Olivares-Fuster et al. (2007) reported that both the 16S - single strand conformation polymorphism (16S-SSCP) and the intergenic spacer region - single strand conformation

polymorphism (ISR-SSCP) improved resolution when compared with standard RFLP. The SSCP analysis of rRNA genes proved to be a simple, rapid, and most effective method for routine fingerprinting of *F. columnare* (Olivares-Fuster et al. 2007).

Anacker and Ordal (1959b) reported that the strains isolated from fish in the USA possessed a common species specific antigen and several other antigens. On the basis of their antigenic composition, strains were separated into four serotypes and one miscellaneous group. However, no correlation between serotype, geographical origin, or species of host-fish and virulence was found.

The difference in virulence among *F. columnre* strains was reported on the basis of experimental infection (Pacha and Ordal 1963). Some authors have suggested that one of the virulence factors of *F. columnre* is the extracellular proteases produced by the bacterium (Griffin 1991, Bertolini and Rohovec 1992, Teska 1993, Newton et al. 1997). Chondroitin lyase activity was found to be significantly related to the virulence of eight *F. columnare* strains (Suomalainen et al. 2006). Zhang et al. (2006) compared lipopolysaccharide (LPS) and total protein profiles from four *F. columnare* isolates and reported that it was possible to discriminate the attenuated mutant FC-RR strain from other virulent strains. Some researchers demonstrated the relationship between adhesion of *F. columnare* and virulence (Decostere et al. 1999ab, Olivares-Fuster et al. 2011), while others found no such association (Suemalainen et al. 2006, Kunttu et al. 2009).

4.4. Diagnostic Method

The first indication of the infection is generally the appearance of a white spot on some part of the head, gills, fins or body. This is usually surrounded by a zone with a distinct reddish tinge, leading to under-running of adjacent skin. Lesions on the gills or fins extend principally from the distal end towards the base, and the tissues are eroded and destroyed (Figure 4.3). Lesions are covered with a yellowish white mucoid exudate consisting largely of swarms of *F. columnare*. The bacteria are not usually found systemically until a relatively large amount of external skin or gill damage has taken place; thus it would appear that the bacteria enter the blood stream through the external lesions and are probably not directly involved in causing death (Wood 1979).

High water temperature enhances the outbreaks of columnaris disease (Fish and Rucker 1943, Ordal and Rucker 1944, Holt et al. 1975, Decostere et al. 1999, Suomalainen et al. 2005a). Wakabayashi and Egusa (1972) studied the effect of water temperature on columnaris disease in loach. Fish were challenged by immersion in water containing *F. columnare* at about 10^6 cfu/ml, then held at temperatures ranging from 5 to 35 ^oC in 5 ^oC intervals. No mortalities occurred in fish held 5 or 10 ^oC, twenty five percent of those held at 15 C died and all of the exposed fish held at 20 - 35 ^oC died. The mean times to death were 7.0, 3.0, 1.8, 1.0 and 1.0 days at 15, 20, 25, 30 and 35 ^oC, respectively. During the period 1955 – 9, the incidence of columnaris disease in salmon, especially sockeye salmon, in the Columbia River Basin, increased with increasing water temperature (Pacha and Ordal 1970). Field surveys of Fraser River spawning areas in 1963, 1964, and 1965 revealed that pre-spawning losses of sockeye salmon by columnaris disease in 1964 were comparatively very small (less than 5%) owing to the lower temperature in that year (Colgrove and Wood 1966).

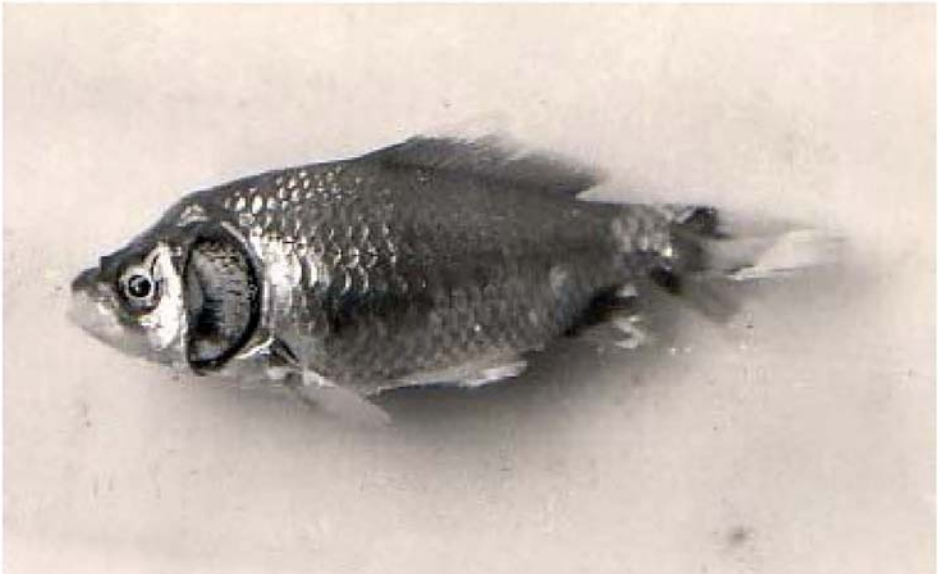

Figure 4.3. Goldfish infected with *F. columnare*. Lesion on the periphery of gills and fins gradually extending towards the body surface.

The influence of water quality on F. columnare infection has been studied by many authors. Fijan (1968) indicated that *F. columnare* could persist for long periods in water of high hardness and organic matter content. Survival and growth of *F. columnare* are affected by the ionic composition of water (Wakabayashi and Egusa 1972, Chowdhury and Wakabayashi 1988a,b). Columnaris disease did not occur in 3.0 or 9.0‰ salinity, and 31–39% fewer channel catfish (*Ictalurus punctatus*), goldfish (*Carassius auratus*) and striped bass (*Morone saxatilis*) died in 1.0‰salinity than in freshwater (Altinok and Grizzle 2001). Hanson and Grizzle (1985) indicated that nitrite at a concentration of 5 ppm enhanced *F. columnare* infection. Sugimoto et al. (1981) found that *F. columnare* grew very well on particles of fresh meal derived from the break-up of pelleted diets in water. In an experiment, transmission of columnaris disease to healthy fish was enhanced by adding a small quantity of feed pellets to an aquarium containing both diseased and healthy fish, but no transmission occurred without addition of the particulate feed matter (Sugimoto et al. 1981).

Bacterial culture and biochemical characterization has been employed for diagnosis of columnaris disease. However, this traditional culture technique requires several days to complete. Immunological techniques such as agglutination test, enzyme-linked immunoassay (ELISA) (Shoemaker et al. 2003) and indirect fluorescent antibody test (Panangala et al. 2006) have been used for detection of *F. columnare* infection. Several researchers have developed identification methods for *F. columnare* using PCR targeted 16S ribosomal DNA (Toyama et al. 1996, Bader et al. 2003). A PCR detection method based on the 16S-23S rDNA intergenic spacer region (IRS) of the ribosomal RNA operon was used for detection of *F. columnare* in channel catfish tissues and in tank water (Welker et al. 2005). Panangala et al. (2007) developed a multiplex PCR (m-PCR) for simultaneous detection of three bacterial fish pathogens, *F. columnare, Edwardsiella tarda,* and *Aeromonas hydrohila* in warm water aquaculture. In their study, they found that *A. hydrophila* outcompetes not only *F. columnare* but also *E. ictaluri.*

4.5. Control

Environmental control along with good rearing practice might provide a means of controlling columnaris disease. Auxiliary cold water, if available, is extremely beneficial even if it cools the water by only a few degrees (Wood 1979). Both salt (4 and 2%) and acidic baths failed to prevent fish mortality, but the mortality rate was lower in rainbow trout treated with 4% salt bath compared to a control group (Suomalainen et al. 2005b). The incidence of *F. columnare* in fish might even be reduced by adding significant numbers of competitive bacteria susceptible fish ponds before *F. columnare* became established on the fish body (Chowdhury and Wakabayashi 1989, Sumalainen et al. 2005c). Suomalainen et al. (2005a) showed that high rearing density, together with high temperature, were the main factors influencing mortality during a *F. columnare* outbreak. Shoemaker et al. (2003) demonstrated that in the absence of natural food juvenile channel catfish should be fed at least once every other day to apparent satiation to maintain normal physiological function and improve resistance to *F. columnaris*.

An excellent review on chemotherapeutics and compounds which had been commonly used for treating columnaris disease was provided by Amend (1970). Heavy metals such as copper sulfate ($CuSO_4$), potassium permanganate ($KMnO_4$), PMA (pyridylmercuricacetate) were used for many years, but their use as therapeutants is now restricted in most countries including UK, Japan and USA because they accumulate in the tissues of treated fish. An immersion flush exposure of NH_4Cl at 46.3mg/L (normally yielding 15mg/L total ammonia nitrogen) served to lower the channel catfish mortality caused by *F. columnare* (Farmer et al. 2011). Chloramine-T (*n*-chloro-*para*-toluene sulfonamide sodium salt) or Diqat (6,'-dihydrodipyrido 1,2-a: 2', 1'-c pyrazidinium dibromide) have been used extensively for treating columnaris disease in the USA (Altinok 2004, Thomas-Jinu and Goodwin 2004, Darwish and Mitchell 2009). For systemic infections, sulfonamide or antibiotics are added to the food. Administration of florfenicol at a dosage of 10 or 15mg/kg body weight for 10 days was efficacious for the control of mortality from *F. columnare* infection in channel catfish (Gaunt and Gao 2010, Darwish et al. 2012).

Studies on oral, parenteral and immersion vaccination of channel catfish against *F. columnare* were carried out but results were inconclusive (Schachte and Mora 1973). Moor et al. (1990) demonstrated the feasibility of immunizing channel catfish against columnaris disease by immersion vaccination with formalin-inactivated bacterins. A commercial vaccine against columnaris disease is available under the registered name Aquavac-Col (Intervet / Schering-Plough Animal Health) and the main active ingredient for the vaccine is a rifampin-resistant mutant of *F. columnare* (Olivares-Fuster and Arias 2011).

4.6. Recent Topics

Shoemaker et al. (2008) carried out immersion challenge experiments to ascribe virulence of genomovar I and II isolates to channel catfish. Their results demonstrated that genomovar II ($n = 4$) isolates were significantly more virulent to channel catfish fry (92-100% mortality) than genomovar I ($n = 3$) isolates. Klesius et al. (2008) reported that genomovar II isolates were more strongly chemotactic to channel catfish mucus than

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