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[3] Diseases Caused By Bacterial Pathogens In Inland Water

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Glossary

LPS: Lipopolysaccharide
ECPs: Extracellular products
CBB: sCoomassie brilliant blue

3. BACTERIAL GILL DISEASE

Hisatsugu Wakabayashi

3.1. Synopsis

Bacterial gill disease (BGD) caused by *Flavobacterium branchiophilum* has been reported from various cultured freshwater fish species, in particular salmonids, worldwide. The bacterium is Gram-negative slender rods measuring 0.5 × 5-8 µm that are non-motile and showed neither gliding movement nor swarming growth on cytohaga agar (CA). When an outbreak of BGD occurs, *F. branchiophilum* first appears abundantly on the surfaces of the gills. A proliferative hyperplasia develops in the epithelium and progresses to clubbing and fusion of gill lamellae. Several chemical disinfectants including sodium chloride have been used to treat BGD. When the bacteria on the gills are removed by treatment at the early stage of infection, fish recover rapidly.

3.2. Introduction

Bacterial gill disease (BGD) is characterized by the presence of numerous filamentous bacteria on the surface of the gill epithelium. Davis (1926, 1927) first observed it in fry and fingerling brook trout (*Salvelinus fontinalis)* and rainbow trout (*Oncorhyncus mykiss*) in hatcheries in Vermont, USA. He called the condition bacterial gill disease, but did not attempt to isolate or identify the bacteria. Rucker et al. (1952) and Bullock (1972) isolated several strains of yellow-pigmented bacteria, referred to myxobacteria from infected gill tissue but none could be shown to be the causative agent. Kimura et al. (1978) and Wakabayashi et al. (1980) isolated a different yellow-pigmented bacterium from gill lesions of several species of salmonids in Japan and USA, and experimentally produced BGD with this organism. The bacterium was named *Flavobacterium branchiophila* by Wakabayashi et al. (1989). Later, this name was corrected to *F. branchiophilum* by von Graevenitz, (1990). BGD with *F. branchiophilum* has been reported from various cultured freshwater fish species, especially salmonids, in Japan, USA, Hungary (Farkas 1985), Canada (Ferguson et al. 1991, and Korea (Ko and Heo 1997). Probably the cause of most BGD outbreaks in salmonids is *F. branchiophilum* (Bullock 1990, Turnbull 1993). However, similar pathology could result from the presence of a low grade opportunist pathogen in extreme environmental conditions, or the presence of a highly pathogenic bacterium in marginal environmental conditions (Turnbull 1993).

3.3. Disease Agent

3.3.1. Flabobacterium Branchiophilum

Cells of *F. branchiophilum* from cytophaga broth (CB) (Anacker and Ordal 1959) are Gram-negative slender rods measuring 0.5 × 5-8 µm and usually occurred in chains of

two or three cells (Figure 3.1). They are non-motile and showed neither gliding movement nor swarming growth on cytohaga agar (CA). Growth on CA is slow and colonies are rarely visible in less than 2 day incubation. They are light yellow, round, transparent, smooth and 0.5 – 1 nm in diameter after incubation for 5 days at 18°C. The bacteria grow well at 10 to 25°C and in the presence of 0.25% NaCl or in the absence of NaCl. Some strains are also able to grow at temperatures as low as 5°C and as high as 30°C and in the presence of higher concentrations of NaCl (0.05 to 0.1%).

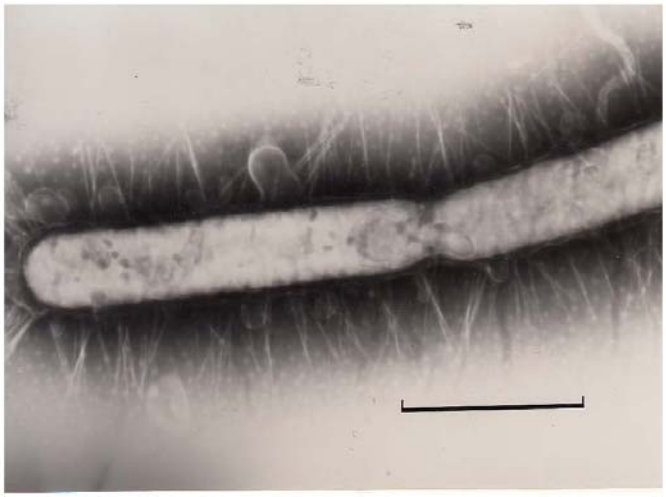

Figure 3.1. Electron micrograph of negative stained cells of Flavobacterium branchiophilum ATCC35035^{T}. Bar – 1 µm.

Wakabayashi et al (1989) described the biochemical characteristics of 16 strains from Japan, USA and Hungary as follows. All strains produced catalase and cyctochrome oxidase, and hydrolyzed gelatin, casein, and starch. They were negative for production of hydrogen sulfide and indole, reduction of nitrate, and degradation of chitin. Some of the strains tested were unable to grow in test medium for nitrate reduction. Also, there was no growth on DNase medium. In the cellulose digestion test, the bacteria grew on the surface of the filter paper, but disintegration of the strip did not occur. Acid but no gas was produced from plucose, fructose, sucrose, maltose, trehalose, cellobiose, melibiose, raffinose, and inulin, but not from galactose, lactose, arabinose, xylose, rhamnose, adonitol, mannitol, dulcitol, sorbitol, inositol, or salicin. The DNA G+C contents of three of the strain ranged from 29 to 31 mol% (mean, 30%). These characteristics accord with those of *F. branchiophilum* strains from Canadian salmonids with BGD (Ostland et al. 1994). However, Bernardet et al (1996) reported a G+C content range of 33-34 mol%.
The strains isolated in Japan, USA, Canada, and Hungary possessed common antigens detectable by agglutination and precipitation tests (Wakabayashi et al. 1980, Huh and Wakabayashi 1989, Ostland et al. 1994). However, the Japanese strains were distinguished from USA and Hungarian strains by agglutinin-adsorption tests, and the precipitation tests revealed one antigen specific for Japanese strains and two antigens specific for both USA and Hungarian strains (Huh and Wakabayashi 1989).

Negative-stained preparation of *F. branchiophilum* cells demonstrated fimbriae-like appendages extending from their surface (Wakabayashi et al. 1989, Ostland et al. 1994).

Ototake and Wakabayashi (1985) found that the infectivity of the bacteria was significantly reduced by a mechanical agitation of the bacterial suspension with a blender. The adherence of the bacteria to the gill tissue surface was thought to be mediated by fimbriae (pili), but the hemagglutins contained in the supernatant of the agitated bacterial suspension were non-fimbrial agglutinin (Ototake and Wakabayashi 1985). Heo et al. (1990) tried to purify and characterize the fimbriae of *F. branciophilum* ATCC35035^T. The fimbriae mechanically detached from the bacterial cells were purified by using ion-exchange chromatography on DEAE-cellulose. A main purified fimbrial subunit had a molecular weight of 23,000 dalton and a maximum absorption at 276 nm in ultraviolet absorption spectrum. In serological analysis, four strains of *F. branchiophilum* produced a single common precipitin line against rabbit anti-fimbriae serum. Although a significant reduction in the attachment of *F. branchiophilum* strains was not achieved after immersion of fish in the crude fimbrial extracts (CFEs), it was suggested that inhibition by CFE was dose dependent (Ostland et al. 1997).

In virulence studies with eight selected strains of *F. branchiophilum* strains, Ostland et al. (1995) found that all of the strains were fimbriated and adhered to the gills of rainbow trout, but that only five strains succeeded in proliferating and further colonizing the gills to cause mortality. They suggested that an additional virulence factor(s) might facilitate bacterial proliferation resulting in further branchial colonization, to induce mortality. Kudo and Kimura (1983c), and Ototake and Wakabayashi (1985) reported that *F. branchiophilum* produced an extracellular hyperplasia inducing factor which could reproduce lesions histopathologically similar to those in BGD.

3.4. Other Bacteria Associated with BGD

Thunbull (1993) summarized phenotypic characteristics of bacteria recovered from BGD. In addition, Ostland et al. (1999) reported a new form of BGD affecting intensively reared salmonids in Ontario Canada. The outbreaks occurred at water temperature less than 10 ℃. Shorten and somewhat stubby lamellae covered with swollen epithelial cells occurred in the sequel to infection, while overt epithelial hyperplasia, lamellar fusion and filamental clubbing were not common. The predominant bacterium recovered from affected gills was a short, Gram-negative rods which shared phenotypic characteristics with *Pseudomonas fluorescens*. An attempt to reproduce using the isolates was unsuccessful.

3.5. Diagnostic Methods

Diseased fish become suddenly lethargic and anorexic. They remain near the water surface or gather at the inflow site. Opercula of these fish are bilaterally flared with an irregularly eroded margin. Respiratory rates are markedly increased, and some fish exhibit labored and accentuated use of the buccal and opercular pumps (Spear and Ferguson 1989). A proliferative hyperplasia develops in the epithelium of the gill lamellae. As the disease progresses, the epithelium proliferates, causing clubbing and fusing of gill lamellae (Bullock 1990). The hyperplasia is usually seen first at the distal tips of the lamellae. Bacterial colonization begins at these lamellar tips before spreading proximally (Wood and Yasutake 1957, Kudo and Kimura 1983a, Spears et al. 1991a). The relationship between the severity of lamellar lesions and the abundance of bacteria is not

always clear since, in some cases, the bacteria are more numerous in less severely affected areas of the gills (Daoust and Ferguson 1983). However, Ostland et al. (1990) reported that statistical analysis on the severity of lesions and bacterial recovery indicated a strong association between the severity of lesions and the presence of filamentous bacteria.

Wakabayashi et al. (1980) made a scanning electron microscopic observation of the gill filaments following bath challenge of juvenile rainbow trout with pure cultures of *F. branchiophilum*. All of the cultures were able to establish abundant growth on the surface of the tissues within 18 to 24 hours (Figure3.2 and 3.3). The gill epithelium assumed hyperplastic appearance 4 days after exposure, though the bacterial cells decrease in number on the surface of the gill filaments. The general pathology of BGD is most likely caused by restrictions on the respiratory and excretory functions of the gills (Snieszko 1981). The oxygen response thresholds for fish experimentally infected with *F. branchiophilum* were 1.5 – 2.5 times higher than those of uninfected fish, and their respiratory functions seemed to be impaired 2 days after infection and became worse after 5 days (Wakabayashi and Iwado 1985a). Changes in glycogen, pyruvate and lactate concentrations in the muscle tissue of juvenile rainbow trout with BGD suggested that a breakdown in gas exchange at the gills caused the failure in circulation to provide oxygen enough to remove excess lactate from the muscle, even though the level of muscle lactate was not so high as that of healthy fish (Wakabayashi and Iwado 1985b).

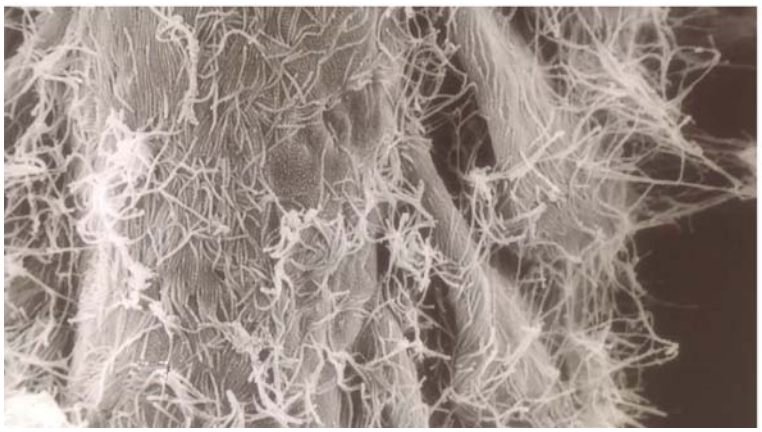

Figure 3.2. Scanning electron micrograph of *Flavobacterium branciophilum* and lamellae 18 hours after exposure of a rainbow trout fingerling to the bacterial suspension in an aquarium.

Because outbreaks of BGD occur suddenly, the disease usually cannot be diagnosed until mortalities begin. Diagnosis is based on the clinical signs along with the examination of wet mounts of the gill tissue for hyperplasia and the presence of filamentous bacteria. Phase contrast microscopy is recommended for enhanced observation of wet mount gill filaments. The causative agent is identified as *F. branchiophilum* by isolation and application of definitive biochemical tests. But *F. branchiophilum* is usually not isolated from internal organs.

A fluorescent antibody test (FAT) was used to detect from infected fish and their environment in a trout hatchery (Huh and Wakabayashi 1987, Heo et al. 1990)). An

enzyme-linked immunosorbent assay (ELISA) was developed to estimate the quantity of *F. branchiophilum* in crude gill extracts from rainbow trout following bath exposure to the bacterium (MacPhee et al. 1995a, Ostland et al. 1995). Toyama et al. (1996) reported that the PCR with a pair of a specific primer BRA1 and a universal primer 1500R succeeded in specifically amplifying 16S rDNA from *F. branchiophilum*.

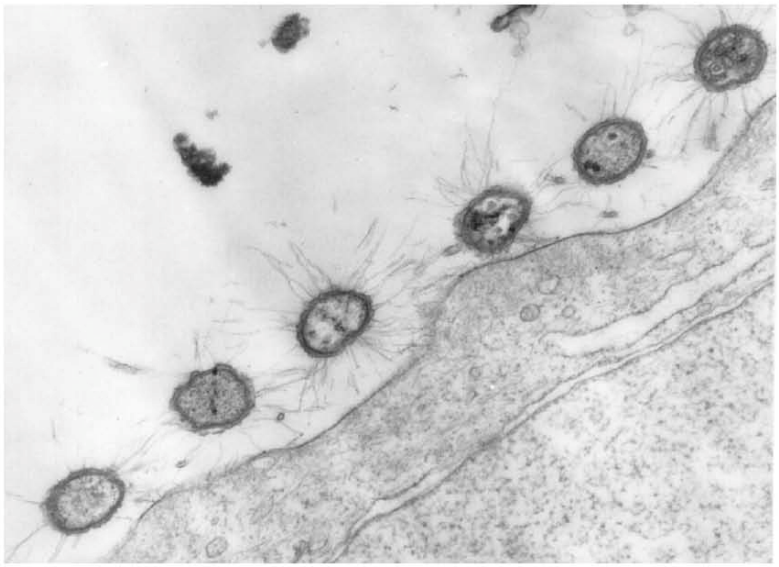

Figure 3.3. Transmitting electron micrograph of *Flavobacterium branchiophilum* cells on the gill epithelium 18 hours after exposure of a rainbow trout fingerling to the bacterial suspension in an aquarium.

3.6. Control

According to Bullock (1990), several chemical disinfectants have been used to treat BGD in salmonid hatcheries in the USA. The most widely used are quarterly ammonium compounds, such as benzalkonium chlorides. Another chemical is the herbicide Diquat. However, none of these chemicals are approved by the U. S. Food and Drug Administration for disease control in food fishes. Efforts are under way to have chloramine-T registered as a treatment for BGD (Bullock et al. 1991, Bowker and Garty 2008, 2011). Hydrogen peroxide (H_2O_2) represents a more environmentally friendly alternative because of its low regulatory priority (Lusmsden et al. 1998, Derksen et al. 1999). Rach et al. (2000) reported that two static bath treatment regimens were effective in the control of BGD: H_2O_2 administered at concentrations of 56 – 110 mg/L as a 60 min exposure or H_2O_2 administered at a concentration of 56 – 230 mg/L as a 30 min exposure. Sodium chloride (NaCl) is widely employed to treat BGD in hatchery salmonids in Japan: NaCl is used at a concentration of 1 – 5 % as a 1 – 2 min bath. Kudo and Kimura (1983a, b) demonstrated that this treatment was very effective for the removal of bacterial cells and subsequent recovery.

3.7. Recent Topics

Touchon et al. (2011) reported the complete genome sequence of *F. branciophilum* strain

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,