Characteristics of *Flavobacterium branchiophilum* Isolated from Rainbow Trout in Korea

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Outbreaks of bacterial gill disease (BGD) occurred among cultured rainbow trout (*Oncorhynchus mykiss*) in Chungbuk province of Korea. Gram-negative, filamentous and yellow-pigmented bacteria were isolated from the diseased fish. The morphological, physiological, biochemical, and antigenic properties of the Korean isolates were consistent with those of *Flavobacterium branchiophilum* (ATCC 35035^T and ATCC 35036) isolated from salmonid fish in Japan and Oregon, USA. It was concluded that the Korean isolates were identified as *F. branchiophilum*.

Key words: Korea, BGD, *Flavobacterium branchiophilum*, rainbow trout

Bacterial gill disease (BGD) is an economically significant gill disease of cultured teleost in many countries (Daoust and Ferguson, 1983; Farkas, 1985; Wakabayashi *et al.*, 1980, 1989; Bullock, 1990). The disease caused physical irritation to the fish and impaired the respiratory function of the gills by large numbers of filamentous, Gram-negative bacteria associated with the surface of the gill epithelium.

Traditionally most yellow-pigmented bacteria (YPB) recovered from fish with BGD have been classified as "myxobacteria" and usually identified as members of the genus *Flexibacter* or *Cytophaga*. One specific genus however, did not appear to be common among all BGD outbreaks, and axenic cultures were seldom recovered from natural outbreaks (Rucker *et al.*, 1952; Anderson and Conroy, 1969; Ostland *et al.*, 1990). Filamentous YPB from some salmonid fish with BGD in Japan, the northwestern USA (Oregon), and Hungary were classified as *Flavobacterium branchiophilum* (Wakabayashi *et al.*, 1980, 1989; Farkas, 1985; von Graevenitz, 1990).

A filamentous, gram-negative YPB resembling *F. branchiophilum* was recently isolated from rainbow trout (*Oncorhynchus mykiss*) affected with BGD in Korea. The bacteria appeared on the gills of young rainbow trout within 18-24h after fish were exposed to bacterial suspensions in aquaria. Based on the physiological, biochemical, and antigenic characteristics, three isolates were identified as *F. branchiophilum*.

Materials and Methods

Bacterial strains

During outbreaks of BGD, three strains of filamentous, gram-negative YPB were isolated from commercially reared rainbow trout in Chungbuk province of Korea (Table 1). These YPB were isolated by swabbing several gill arches and streaking the sample directly onto cytophage medium containing 1.5% agar (1.5% CA; Anacker and Ordal, 1959). The isolates were maintained and subcultured on 1.5% CA at 18°C or stored in cytophage broth (CB; pH7.0) containing 15% glycerol at -70°C until required.

Two reference strains (Table 1) of *F. branchiophilum* were used for comparative purposes. These were purchased from the American Type Culture Collection (ATCC, Rockville, Maryland).

Morphology and growth studies

The following criteria of Holmes *et al.* (1984) were used for identification of the YPB from affected gills. Additional criteria and methods of Wakabayashi *et al.* (1980, 1989) were used to compare the biochemical characteristics of the Korean isolates with *F. branchiophilum* isolated in Japan and Oregon. Cells used for the study were grown in CB for 48h at 18°C on a rotary shaker. Unless otherwise indicated, incubations were standardized to 7 days at 18°C. Standard microbiological methods were used to interpret tests

Table 1. Strains of *Flavobacterium branchiophilum* used in this study

Isolate	Year of isolation	Host species
Korean isolates		
CB1	1996	Rainbow trout (<i>Oncorhynchus mykiss</i>)
CB2	1996	Rainbow trout
CB3	1996	Rainbow trout
Reference strains		
ATCC 35035 ^T	1977	Cherry salmon (<i>O. masou</i>) (Japan)
ATCC 35036	1978	Chinook salmon (<i>O. tshawytscha</i>) (Oregon)

(MacFaddin, 1980).

Cell morphology was determined by light microscopy of gram-stained smears, or electron microscopy. Colonial spreading was determined by point inoculation on a freshly poured cytophaga agar plate containing 0.9% agar (0.9% CA) and incubation in a humid chamber.

Motility was determined by phase-contrast microscopy of a hanging drop prepared from a CA culture. The absence of flagella was confirmed by incubating one drop of a 48h CB culture on copper grid coated with collodion and carbon, staining it with 2% phosphotungstic acid, and examining the grid on a transmission electron microscope (TEM). The method of Perry was also used to test for gliding movement (Perry, 1973).

Growth temperature was determined by streaking bacterial samples onto 1.5% CA and incubating the cultures aerobically at 5, 10, 18, 25, 30, and 37°C for 1.4 weeks, depending on the incubation temperature. Anaerobic growth was tested by steel wool method in anaerobic jar (Mitsuoka *et al.*, 1969). The tolerance to NaCl was determined by inoculating CB containing 0, 0.025, 0.05, 0.075, 0.1, or 0.2% NaCl and recording the amount of growth for more than 2 weeks. Growth on tryptic soy agar (TSA), MacConkey's agar (MCA), and nutrient agar (NA) was checked by streaking samples from 1.5% CA cultures.

Biochemical characterization

Standard methods (MacFaddin, 1980) were employed by using CA or CB cultures for biochemical characterization of the strains unless otherwise stated.

To examine aerobic carbohydrate utilization, the low peptone medium described by Wakabayashi *et al.* (1980) was used. The inoculated media were incubated on a rotary shaker (200rpm) and examined for acidification daily for 7 days. The following carbohydrate were tested: dextrose, fructose, lactose, sucrose, maltose, trehalose, cellobiose, arabinose, xylose, rhamnose,

raffinose, mannose, salicin, and galactose.

Penicillin G sensitivity was determined by placing an single antibiotic disk (10 units, BBL SensiDisk susceptibility test disks; Becton Dickinson) on a 1.5% CA plate seeded with 100µl of CB culture. The organism was considered penicillin sensitive if the diameter of inhibition zone was larger than 20mm after 7 days of incubation.

The presence of cell-wall-associated flexirubin-like pigments was determined by flooding 1.5% CA colonies with 20% KOH (w/v).

Hydrolysis of gelatin was detected by mercuric chloride procedure of Frazier (MacFaddin, 1980). Cellulose degradation was determined by adding a sterile strip of Whatman No.1 filter paper to a freshly seeded CB culture and allowing static incubation for 30 days.

DNA base composition

DNA base composition was determined by reversed-phase high-performance liquid chromatography (HPLC). Genomic DNA was resuspended in distilled water after isolated from a 5µl culture. The isolated DNA was hydrolysed into nucleosides with nuclease P1 (Sigma) and bacterial alkaline phosphatase (Sigma). The molar percentage of guanine plus cytosine in the DNA (mol% G+C) was calculated using reversed-phase HPLC (Jin and Komagata, 1984). ATCC 35035^T and ATCC 35036 strains served as controls.

Antigenic characterization

The antigenic relationships among the Korean isolates and the BGD reference strains were examined by microtiter agglutination and double immunodiffusion.

Bacterial antigen: Whole-cell bacterial antigens for immunization were prepared as follows. Several colonies from a 1.5% CA culture of each strain were inoculated into 1l of CB in a 2l flask and grown for 48h (approximated 10⁷ cfu/ml). Cells were killed by adding

100ml of 0.5% (v/v) formalinized PBS (f-PBS) and incubating overnight at 10°C. The following day cells were harvested by centrifugation, resuspended in 25ml of f-PBS and stored at 4°C until required.

Heat-stable antigens were prepared from whole-cell antigens of each strain using methods similar to Wakabayashi *et al.* (1989). A 1l CB culture was steamed for 30min, cooled for 15min at -70°C, and centrifuged at 10,000×g for 20min at 5°C. The pellet was resuspended in 25ml of f-PBS and stored at 4°C until used. For agglutination tests, both whole cell and heat-stable antigens were washed twice in PBS and adjusted to an optical density of 1.0 (550nm) with PBS prior to use.

Preparation of antiserum: One milliliter of formalin-killed bacterial cells in Freund's complete adjuvant was injected subcutaneously into the backs of white rabbits weighing approximately 2.5kg. After 2 weeks, a booster injection with Freund's incomplete adjuvant was given subcutaneously. The blood was collected 2 weeks after the second injection, inactivated at 56°C for 30 min, and frozen at -20°C.

Agglutination test: The microtiter technique for agglutination test was used to examine the serological relationship among the strains.

Immunodiffusion test: For immunodiffusion tests, soluble antigens were prepared by sonication (three times at 200W for 5min) or heat-treatment (121°C, 30min) of f-PBS-killed bacteria.

The double immunodiffusion test was performed with the use of 1% agarose gel. Soluble antigens were added to the duplicate outer peripheral wells and the undiluted antiserum was added to the central well (approximate volume per well, 25µl). Gels were incubated for 2 days at 20°C before examination.

Results

Morphologic, phenotypic, and genomic characteristics

The cells of all the present isolates as well as reference strains were gram-negative slender rods measuring 0.5 by 8 to 15µm, and they usually occurred in chains of two or three cells in CB (Fig. 1). They were nonmotile and showed neither gliding movement nor swarming growth on agar media.

Growth on CA was usually slow, with colonies appearing only after 2 to 5 days of incubation at 18°C. The colonies were light yellow, round transparent, smooth, and about 0.5 to 1mm in diameter after incubation for 5 days. All strains grew well at 10

Fig. 1. Electron micrograph of Korean isolate CB1 demonstrating thin, fimbria-like appendages extending from bacterial surface. Bar=1µm.

through 30°C, and at 0 through 0.1% NaCl. None of the strains grew anaerobically, at 37°C, nor at 0.2% NaCl.

All strains used were sensitive to penicillin G, and zones of inhibition ranged from 55 to 64mm (Table 2). Cytochrome-c oxidase and catalase were positive in all strains. None of the strains displayed typical flagellar or gliding motility, but TEM studies demonstrated fimbria-like structures originating from their cell surface (Fig. 1). The agar colony pigments were not of the flexirubin type. All strains failed to produce indole and H₂S, reduce nitrate or hydrolyze esculin.

No strains gave any reaction anaerobically in the OF medium, but produced acid aerobically and therefore considered to be an oxidative type. All of the strains tested hydrolyzed gelatin, starch, and casein, but not cellulose.

The values for G+C content of all Korean isolates were similar to those of *F. branchiophilum* reference strains (range 30–31mol%) (Table 3).

The carbohydrate utilization profiles were variable. All strains tested produced a strong acid reaction from dextrose and sucrose after 3 days of incubation (Table 4).

Antigenic characteristics

Microtiter agglutination test: Agglutinin titers of antisera for homologous formalin-killed cells ranged from 1024 to 4096 and those for heat-killed cells ranged from 64 to 256 (Table 5). The agglutinin titers of antisera for heterologous formalin-killed cells ranged from 128 to 1024 and those for heat-killed cells from 32 to 128. The Korean isolates shared varying degrees of

Table 2. Morphological and physiological characteristics of Korean isolates and *F. branchiophilum* reference strains

Characteristic	CB1	CB2	CB3	ATCC35035 ^T	ATCC35036
Gram reaction	–	–	–	–	–
Motility	–	–	–	–	–
Gliding movement	–	–	–	–	–
Swarming	–	–	–	–	–
Anaerobic growth	–	–	–	–	–
Growth at 30°C	+	+	+	+	+
Growth in 0.1% NaCl	+	+	+	+	+
Growth on					
TSA	–	–	–	–	–
MCA	–	–	–	–	–
NA	–	–	–	–	–
Penicillin G sensitivity (Zone diameter, mm)	58	61	64	60	55

+: positive or present, (+): weakly positive or poor, –: negative or absent

Table 3. Biochemical and genomic characteristics of Korean isolates and *F. branchiophilum* reference strains

Characteristic	CB1	CB2	CB3	ATCC35035 ^T	ATCC35036
Production of					
Cytochrome-c oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Hydrogen sulfide	–	–	–	–	–
Indole	–	–	–	–	–
Flexirubinn	–	–	–	–	–
Degradation of					
Gelatin	+	+	+	+	+
Starch	+	+	+	+	+
Casein	+	+	+	+	+
Esculin	–	–	–	–	–
Cellulose degradation	–	–	–	–	–
Fermentation in	–	–	–	–	–
O-F glucose medium					
Nitrate reduction	–	–	–	–	–
Mol% G+C	30	30	30	31	30

+: positive or present, (+): weakly positive or poor, –: negative or absent

antigenic similarity with ATCC 35035^T and ATCC 35036.

Immunodiffusion test: The Korean BGD isolates and the BGD reference strains (ATCC 35035^T and ATCC 35036) shared many lines of precipitation in double immunodiffusion gels in both tests using formalin-killed antigens and heat-killed antigens.

Discussion

The results obtained indicate that the yellow-pigmented, filamentous, gram-negative bacteria isolated from BGD outbreaks of rainbow trout in Korea are strains of *Flavobacterium branchiophilum*. Korean isolates share morphological, physiological, biochemical, antigenic, and genomic similarities with the reference

Table 4. Carbohydrate reactions of Korean isolates and *F. branchiophilum* reference strains

Carbohydrate	CB1	CB2	CB3	ATCC35035 ^T	ATCC35036
Dextrose	++	++	++	++	++
Fructose	++	++	++	++	++
Lactose	(+)	—	—	—	—
Sucrose	++	++	++	++	++
Maltose	++	++	+	++	+
Trehalose	+	+	++	+	+
Cellubiose	+	—	(+)	+	(+)
Arabinose	(+)	—	—	(+)	(+)
Xylose	—	—	—	—	—
Rhamnose	—	—	—	—	—
Raffinose	—	(+)	—	—	—
Mannose	—	—	—	—	—
Salicin	—	—	+	—	+
Galactose	(+)	—	—	—	—

Acid production from carbohydrate; ++: strong, +: moderate, (+): weak, —: none

Table 5. Agglutinin titers of five rabbit anti-*F. branchiophilum* sera against homologous and heterologous antigens

Agglutininogen	Agglutinin titers of antisera to				
	CB1	CB2	CB3	ATCC35035 ^T	ATCC35036
Formalin-killed cells					
CB1	1024	512	512	512	1024
CB2	512	1024	512	512	256
CB3	512	512	1024	256	512
ATCC35035	1024	512	1024	4096	128
ATCC35036	256	256	512	256	1024
Heat-killed cells (121°C for 30 min)					
CB1	64	32	64	32	32
CB2	32	128	32	64	32
CB3	64	32	64	32	32
ATCC35035	64	32	32	128	64
ATCC35036	64	64	128	64	256

strains of *F. branchiophilum* isolated from salmonid fish in Japan (ATCC 35035^T) and Oregon (ATCC 35036). Further studies of DNA homology between the Korean isolates and reference strains would help establish their genetic relationship.

Electron microscopic studies of Korean isolates of *F. branchiophilum* demonstrated large numbers of thin, fimbria-like structures present on the bacterial surface (Fig. 1). Similar structures were seen on both ATCC 35035^T and ATCC 35036; these structures have been

purified and characterized from ATCC 35035^T and appear to be antigenically related among strains of *F. branchiophilum* (Heo *et al.*, 1990).

The antigenic characteristics of Korean BGD isolates were examined. The BGD isolates shared many lines of precipitation in double immunodiffusion gels and high agglutinin titer with the two reference strains. It was reported that Japanese strains and Oregon strains were serologically distinct although they possessed common antigens based on a cross absorption test (Wakabayashi

et al., 1980; Huh and Wakabayashi, 1989). It can not be concluded by the present results to which type, Japanese or Oregon, the Korean isolates are serologically related.

BGD has been known to occur in fry of rainbow trout and has caused a great economic loss in Korea but etiological agent was not isolated and identified before. This is the first case that the Korean isolates from BGD outbreaks were confirmed to be *F. branchiophilum*.

The disease occurs from February to July in case of ground water and from May to June in case of natural water when water temperatures are about 18°C in Korea. Our current recovery rate of *F. branchiophilum* from Korean BGD of rainbow trout still remains poor. The reason for this poor recovery is unknown, but it may be that this bacterium has particular growth requirements that are not met with cytophaga agar. The development of a selective or enrichment medium for isolation of *F. branchiophilum* would improve our understanding of the ecology of this mucosal pathogen.

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