Isolation and Characterization of *Flavobacterium johnsoniae* from Farmed Rainbow Trout *Oncorhynchus mykiss*

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Abstract

Flavobacterium johnsoniae was isolated from farmed rainbow trout *Oncorhynchus mykiss* in Korea, and its biochemical and molecular characterization was determined. Yellow-pigmented bacterial colonies were isolated from 18 of 64 fish samples (28.1%) on trypticase soy agar plates, and their biochemical profiles were characterized by API 20E and API 20NE test kits. *F. johnsoniae* was identified by biochemical phenotyping of factors including rapid gliding motility, Gram-negative condition, oxidase- and catalase-positive status, Congo red absorption, nitrate reduction, β -galactosidase production, acid production from glucose, and gelatin and casein hydrolysis. PCR and subsequent sequencing of 16S rRNA confirmed that the yellow-pigmented colonies were most similar to *F. johnsoniae*. The alignment analysis of 16S rRNA sequences also showed that all 18 rainbow trout isolates had highly similar homologies (97-99% identity). One isolate was selected and named FjRt09. This isolate showed 98% homology with previously reported *F. johnsoniae* isolates, and in phylogenetic analysis was more closely grouped with *F. johnsoniae* than with *F. psychrophilum*, *F. columnare*, or *F. branchiophilum*. This is the first report on the occurrence and biochemical characterization of *F. johnsoniae* isolated from rainbow trout in Korea.

Key words: *Flavobacterium johnsoniae*, *Oncorhynchus mykiss*, Rainbow trout, PCR

Introduction

Flavobacterium johnsoniae (formerly *Cytophaga johnsonae*) belongs to a large, diverse group of aerobic Gram-negative bacteria known as the *Cytophaga-Flavobacterium-Bacteroides* group (Bernardet et al., 1996). *F. johnsoniae* commonly exists in soil and is also frequently isolated from the external lesions of several fish species (Flemming et al., 2007). Generally, *F. johnsoniae* is not considered a serious fish pathogen, but the species has been associated with several disease outbreaks. For example, *F. johnsoniae* infection in farmed barramundi *Lates carcalifer* with 2-5% mortality was reported in Australia (Carson et al., 1993). Thereafter, these bacteria have been found in Atlantic salmon *Salmo salar*, sea trout *S. trutta* m. *trutta* and brown trout *S. trutta* m. *lucustris* with more than 5% mortality during outbreaks in Finland

(Rintamäki-Kinnunen et al., 1997). Recently, *F. johnsoniae* was also isolated from the external lesions of fish from aquaculture systems in South Africa (Flemming et al., 2007).

The rainbow trout *Oncorhynchus mykiss* is the most important freshwater fish species in aquaculture in Korea, with annual production increasing annually (Yoon, 2008). The species is susceptible to the fish-pathogenic bacteria of the genus *Flavobacterium*, i. e., *F. columnare* (Suomalainen et al., 2005), *F. psychrophilum* (Misaka and Suzuki, 2007), and *F. branchiophilum* (Ko and Heo, 1997), but *F. johnsoniae* has not been reported in rainbow trout to date.

The purpose of this study is to report the isolation and characterization of *F. johnsoniae* from farmed rainbow trout in Korea.

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Materials and Methods

Fish samples

Sixty-four juvenile rainbow trout (0.4-0.7 kg body weight) were collected from four hatcheries in Gangwon Province, South Korea, in 2010 for routine disease monitoring. Fish were packed individually in plastic bags, transported to the laboratory, and processed immediately. Gill and kidney tissues were aseptically removed from individual fish and placed in 1.5-mL microcentrifuge tubes.

Isolation of bacteria and biochemical characterization

Gill and kidney tissue samples (100 mg each) were homogenized with 0.9% (w/v) NaCl and then spread on trypticase soy agar (TSA) plates at 15°C for 2-7 days. Among the colonies that appeared, yellow-pigmented colonies, characteristic of the genus *Flavobacterium*, were selected. These colonies were then subcultured on TSA plates at 15°C for an additional 2-7 days and examined for biochemical characterization. A presumptive identification was carried out by Gram staining and oxidase and catalase tests, as described by Austin and Austin (2007). Colonies were tested for the presence of flexirubin pigments by being exposed to 10 N KOH, followed by neutralization with 12 N HCl. The biochemical characteristics of the bacteria were then determined using API 20E and 20NE kits (BioMerieux, Marcy l'Etoile, France) with incubation at 15°C for 48-72 h. The chitinolytic activity (Richter and Pate, 1988), Congo red absorption (Crump et al., 2001), and gelatin and casein hydrolysis (Cepeda et al., 2004) of the colonies were also tested. The published biochemical characteristics of *F. columnare*, *F. psychrophilum*, *F. johnsoniae*, and *F. branchiophilum* were used as references (Ko and Heo, 1997; Tamaki et al., 2003; Austin and Austin, 2007; Kubilay et al., 2008; Karatas et al., 2010).

Extraction of DNA and amplification of 16S ribosomal RNA (rRNA) genes

Genomic DNA was extracted from 5 mL of cultured bacterial cells by the AccuPrep Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea). Extracted nucleic acids were concentrated to a final concentration of 100 ng/μL. PCR targeting the 16S rRNA was conducted using the universal bacterium-specific primer set, 20F (5'-AGA GTT TGA TCA TGG CTC AG-3') and 1500R (5'-GGT TAC CTT GTT ACG ACT T-3') (Weisburg et al., 1991). PCR was performed using the lyophilized AccuPower PCR PreMix tube (Bioneer) containing 20 μL of reaction mixture, which comprised 25 μM of each primer, 5 μL of extracted DNA and DEPC-treated water, with a DNA Engine Peltier Thermal Cycler (BioRad, Hercules, CA, USA). Amplification was carried out with an initial cycle at

95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 51°C for 1 min, and 72°C for 2 min, with a final extension step at 72°C for 5 min.

Sequencing and phylogenetic analysis of the 16S rRNA genes

PCR products were purified by an AccuPrep Gel Purification Kit (Bioneer). Then, 10 ng/μL of purified PCR products were directly sequenced (ABI 3730xl; PE Applied Biosystems, Foster City, CA, USA; available at the National Instrumentation Center for Environmental Management, Seoul National University, Korea). The nucleotide sequences of the 16S rRNA genes were aligned with those available in the GenBank database (NCBI) according to Clustal W by MEGA version 4 (Tamura et al., 2007). This alignment was used to infer phylogenetic relationships among the sequences by the neighbor-joining algorithm with bootstrap values determined by 1,000 replicates. The 16S rRNA gene sequences of several *F. psychrophilum*, *F. columnare*, and *F. branchiophilum* strains were included as references, because these species are genetically very close to *F. johnsoniae*. The nucleotide sequence was deposited in GenBank under accession no. GU461280.

Results and Discussion

From 64 fish samples, a total of 231 colonies were observed on TSA plates after 72 h at 15°C, and 18 fish samples (28.1%) from two hatcheries had yellow-pigmented colonies, which were selected for further identification (Table 1). All 18 isolates showed the same biochemical types with characteristics including rapid gliding motility, Gram-negative rods, oxidase-positive, catalase-positive, flexirubin pigment, nitrate reduction, β-galactosidase-positive, acid production from glucose, Congo red absorption, and hydrolysis of gelatin and casein. Growth occurred with 1% NaCl in tryptic soy broth (TSB) but not with 1.5% NaCl in TSB and no growth occurred at 4°C. The biochemical characteristics of the bacteria are summarized in Table 2.

Table 1. Prevalence of *Flavobacterium johnsoniae* in rainbow trout collected from four hatcheries in Gangwon Province, Korea

Hatchery sites	No. of samples	Prevalence	
		Gill	Kidney
A	10	6 (60.0)	6 (60.0)
B	10	0 (0)	0 (0)
C	15	0 (0)	0 (0)
D	29	12 (41.4)	12 (41.4)
Grand total	64	18 (28.1)	18 (28.1)

Values are presented as number (%).

The amplification of 16S rRNA from all 18 yellow-pigmented colonies showed a band of approximately 1,300 bp (data not shown). Highly conserved sequences (97-99%) were found among the 18 isolates (Fig. 1), and one isolate was selected and named FjRt09 (GenBank accession no. GU461280). Phylogenetic tree analysis showed that FjRt09 had 98% homology and grouped more closely with *F. johnsoniae* than with *F. columnare*, *F. psychrophilum*, and *F. branchiophilum* (Fig. 2).

Gram-negative yellow-pigmented bacteria belonging to the genus *Flavobacterium* have been reported from external

lesions on fish, e.g., gill erosion, fin or tail rot, and saddle-like lesions (Rintämäki-Kinnunen et al., 1997). However, the symptoms are usually not specific and sometimes individuals present with no external symptoms. Thus, it is difficult to identify the bacteria, and they are frequently misidentified (Austin and Austin, 2007).

Flavobacterium johnsoniae is known as an opportunistic skin pathogen in certain fish species (Carson et al., 1993). Rintämäki-Kinnunen et al. (1997) showed that brown trout with lesions caused by this bacteria have a high risk of mortality, which can be accelerated by high water temperature

Table 2. Phenotypic characteristics of *Flavobacterium johnsoniae* isolate from rainbow trout in Korea

Characteristics	FjRt09	<i>F. columnare</i>	<i>F. psychrophilum</i>	<i>F. branchiophilum</i>	<i>F. johnsoniae</i>
Colony pigment	Y	GY	Y	LY	Y
Gram reaction	-	-	-	-	-
Morphology	Rods	Rods	Rods	Rods	Rods
Gliding motility	+	+	(+)	-	+
Production of:					
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Flexirubin-type pigment	+	+	+	-	+
Congo red absorption	+	+	-	NA	V
Nitrate reduction	+	+	-	-	+
β -Galactosidase	+	-	V	-	+
Arginine dihydrolase	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-
Urease production	-	-	-	-	-
Tryptophane deaminase	-	-	-	-	-
Indole	-	-	-	-	-
Voges Proskauer reaction	-	-	-	-	+
H ₂ S production	+	+	-	-	+
Degradation of:					
Casein hydrolysis	+	+	+	+	+
Gelatin degradation	+	+	+	+	+
Utilization of:					
Citrate utilization	-	-	-	-	+
Acid production from:					
Glucose	+	-	-	+	V
Mannitol	-	-	-	-	-
Inositol	-	-	-	-	-
Sorbitol	-	-	-	-	-
Rhamnose	-	-	-	-	-
Sucrose	-	-	-	+	-
Melibiose	-	-	-	+	-
Amygdalin	-	-	-	-	-
Arabinose	-	-	-	(+)	-

F. columnare data from Tamaki et al. (2003); Kubilay et al. (2008), *F. psychrophilum* data from Tamaki et al. (2003); Austin and Austin (2007), *F. branchiophilum* data from Austin and Austin (2007); Ko and Heo (1997), *F. johnsoniae* data from Tamaki et al. (2003); Karatas et al. (2010).

-, negative response; +, positive response; (+), weak or delayed positive response; Y, yellow; GY, greenish yellow; LY, light yellow; V, variable among strains; NA, not available data.

Fig. 1. Continued.

Fig. 2. The phylogenetic tree showing the genetic relationships of *Flavobacterium columnare*, *F. psychrophilum*, *F. branchiophilum*, *F. johnsoniae* and FjRt09 (accession no. GU461280) isolates based on the 16S rRNA gene sequences. The numbers on the branches are bootstrap values with 1,000 replicates.

and rearing density. *F. johnsoniae* has been detected in barramundi, Atlantic salmon, sea trout, brown trout, brook trout, and even in the freshwater environment (Carson et al., 1993; Rintämäki-Kinnunen et al., 1997; Hahn et al., 2004; Clark et al., 2009; Seo et al., 2009). However, there has been no report of the isolation of *F. johnsoniae* from rainbow trout to date. Hence, this is the first report documenting *F. johnsoniae* in rainbow trout. Of the four hatcheries tested, two were found to harbor *F. johnsoniae*, based on rainbow trout gill and kidney samples. It is still unclear if these bacteria strains are pathogenic to rainbow trout in those hatcheries, because the susceptibility of rainbow trout to infection by these bacterial isolates was not tested in this study. *F. johnsoniae* can be opportunistic under certain conditions, such as a sudden change in water temperature, stress caused by handling, or negative environmental factors (Carson et al., 1993; Karatas et al., 2010).

In this study, 18 out of 64 rainbow trout sample plates developed yellow-pigmented colonies. The affected samples originated from two hatcheries, although the fish showed no external clinical signs. Biochemical characterization was conducted on the 18 yellow-pigmented colonies, and the biochemical characteristics of the bacterial isolates corresponded to those of *F. johnsoniae* (Carson et al., 1993; Karatas et al., 2010). PCR and subsequent sequencing of 16S rRNA genes also identified all 18 yellow-pigmented colonies as *F. johnsoniae* with similarities of 97-99%; thus, one strain was selected and named FjRt09. A phylogenetic tree based on 16S rRNA gene sequences was constructed for FjRt09 and other *F. johnsoniae*, *F. columnare*, *F. psychrophilum*, and *F. branchiophilum*. The phylogenetic tree demonstrated that the FjRt09 strain clustered in the *F. johnsoniae* group and shared a branch with *F. johnsoniae* P550 isolated from brook trout in the United

States (Clark et al., 2009). It remains unclear how FjRt09 shares a close relationship with P550, and it would be interesting to investigate the relationship between these isolates from geographically distant areas, because there have been no further reports of *F. johnsoniae* in the Asia-Pacific region to date. Moreover, the pathogenic potential of the *F. johnsoniae* isolate in Korea also needs to be studied.

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