

Original Research Article

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Flavobacteriosis in Cultured Freshwater Ornamental Goldfish *Carassius auratus*

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ABSTRACT

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Isolation of *Flavobacterium* sp. from the gill of telescopic eye goldfish *Carassius auratus* with white patches on gill along with its phenotypic, molecular and phylogenetic characteristics are described in this report. The diseased goldfish from a cemented tank (6.75 m³) had gill rot, white patches on gill with excess mucus secretion. On selective cytophaga agar, inocula from the gills yielded yellow pigmented colonies. A bacterial strain (WPGT1) isolated from the gill of goldfish formed a monophyletic group with other strains of *Flavobacterium* sp. based on phylogenetic analyses. The strain *Flavobacterium* sp. WPGT1 (NCBI accession number KP997191) was Gram negative long rod and weakly pathogenic to *C. auratus*. It caused only 14.29% mortality at a level of 3.50x10⁶ cells/ml through immersion challenge after skin wounding; while no mortality was recorded in intraperitoneally injected goldfish even at 3.50x10⁸ cfu/fish. Understanding the pathology and pathogenesis of this emerging pathogen in cultured goldfish would help manage flavobacteriosis.

Introduction

Aquarium keeping is amongst the most popular hobbies with millions of enthusiast's worldwide (Livengood and Chapman, 2011). Among ornamental fish, goldfish *C. auratus* is the most common and of international significance. A variety of diseases including bacterial diseases have been reported and characterized in gold fish (Citarasu *et al.*, 2011). Among the bacterial diseases, flavobacteriosis is regarded as a predominant disease of ornamental fish (Moyer and Hunnicutt, 2007). Flavobacteriosis debilitates a wide ecological and phylogenetic spectrum of

temperate and tropic freshwater fish (Loch and Faisal, 2015). Various commercially important fish species such as salmonids, eels, carps, goldfish, tilapia and channel catfish are also susceptible to this disease (Suomalainen *et al.*, 2009). Flavobacteriosis in fish are caused by multiple bacterial species within the family Flavobacteriaceae and are responsible for devastating losses in wild and farmed fish stocks around the world (Loch and Faisal, 2015). Members of the genus *Flavobacterium* are Gram negative rods that range from 0.3 to 0.5 µm in diameter and from 1.0 to 40.0 µm in

length. These organisms are known for its opportunistic pathogenic role in fish (Bernardet and Bowman, 2011). Ornamental fish are cultured on a large scale in various localities of West Bengal in earthen and cemented tanks. Aquarists are facing varied types of infectious and non-infectious diseases. The present study reports the phenotypic and molecular characterization of *Flavobacterium* sp. isolated from diseased *C. auratus* with gill rot and its pathogenicity on goldfish *C. auratus*.

Materials and Methods

Isolation and Phenotypic Characterization of Bacteria

During the routine fish disease surveillance in December 2015, the telescopic eye variety of goldfish *C. auratus* (≈ 50 g; 14-15 cm) with gill rot, white patches on gill and excessive mucus secretion from a ornamental fish farm located in Jafarpur (Lat. 22°19'38.9"N; Long. 88°14'48.4"E), South 24 Parganas district, West Bengal, India were examined. At site, the behavioral abnormalities, gross and clinical signs of diseased *C. auratus* were recorded. Diseased fish (n=5) as well as apparently healthy fish (n=5) from the affected cemented tank (6.75 m³) were collected and brought to the laboratory within 2 h of collection in oxygen filled polythene bags. Prior to sample collection for bacteriology, the fish were rinsed in sterile saline and wiped with sterile paper towel. Inocula from the gills of infected and apparently healthy goldfish were streaked on to selective cytophaga agar supplemented with neomycin 5 µg/ml and polymyxin B 200 IU/ml (SCA; Hawke and Thune, 1992) and incubated at 30°C for 48 h. The SCA plates of infected fish predominantly yielded yellow pigmented colonies of 1–2 mm size. The SCA plates of healthy goldfish had no yellow pigmented

colonies. Representative colonies from infected fish were randomly picked and Gram stained for preliminary observations on lengthy rods. For further studies, a yellow pigmented, round, convex colony on SCA showing lengthy rods was picked aseptically, purified by subculturing on cytophaga agar without antibiotics (CA) and maintained on CA slants at 30°C. Phenotypic characterization was done by VITEK 2 compact system (BioMerieux, France).

Molecular Characterization of Yellow Pigmented Strain WPGT1 Isolated from the Gill

The genomic DNA of the yellow pigmented colony was isolated using Genomic DNA isolation kit (Macherey-Nagel, Germany) as per manufacturer's protocol. The 16S small subunit ribosomal RNA (16S rRNA) was amplified by Eppendorf Master Cycler Pro S using a set of universal prokaryotic primers 8F, 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R, 5'-GGTTACCTTGTTA CGACTT-3' (Eden *et al.*, 1991). The PCR master-mix contained 50 ng of genomic DNA, 10 µM of each primer and 2× PCR TaqMixture (HiMedia, India). Amplification was done by initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing of primers at 44°C for 30 sec and extension at 72°C for 60 sec. The final extension was at 72°C for 5 min. The PCR product was analysed on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide in 1× Tris-acetate-EDTA (TAE) buffer.

Sequencing and Phylogenetic Analyses

The PCR amplified product was sequenced at the Genomics Division, Xcelris Labs Ltd, Ahmedabad, India. The edited sequence was compared against the GenBank database of

the National Institute of Biotechnology Information (NCBI) by using the BLAST (Basic Local Alignment Search Tool) program (<http://blast.ncbi.nlm.nih.gov>). Twenty two more gene sequences comprising 10 *Flavobacterium* spp. and 12 other strains of Gram negative long rods, viz., *Flexibacter aurantiacus*, *Flectobacillus roseus*, *Chryseobacterium indologenes*, *Flavobacterium sasangense*, *Flavobacterium cucumis*, *Tenacibaculum maritimum* and *Sphingobacterium thalpophilum* were selected from the NCBI GenBank database. Data analysis and multiple alignments were performed by using ClustalW 1.6 (MEGA6). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2011). The nucleotide sequence of *Flavobacterium* sp. WPGT1 has been deposited in NCBI GenBank under the accession number KP997191.

Pathogenicity of *Flavobacterium* sp. WPGT1 on *C. auratus* Juveniles by Intraperitoneal Injection and Skin Wounding

Farm grown goldfish *C. auratus* (weight: 3.85±0.66 g; length: 7.99±1.12 cm) were brought from Piyarapur (Lat. 22° 47'49"N;

Long 88° 18'18"E), Hooghly district, India to the laboratory in oxygen filled polythene bags. First the fish were disinfected by placing in 5 ppm KMnO₄ solution for 15 min and maintained in FRP tanks of 500L capacity @ 75 numbers/tank. The weak fish were removed immediately. All fish were maintained in FRP tanks for 20 days and fed daily with balanced basal dry pellet feed (CP9931, CP Pvt. Ltd., Andhra Pradesh, India) twice daily @ 2% of the body weight. Accumulated wastes and faeces were removed once in three days and 50% water exchanged. Fourteen glass aquaria (60 x 30 x 30 cm) were used for injected, skin wounded and control groups. All glass aquaria, after through washing and drying, were filled with clean bore-well water to a volume of 30L each and conditioned for three days. Seven each of the healthy fish were released into the experimental glass aquaria and acclimatized for three days with continuous aeration. All fish were fed with pellet feed and maintained under optimal condition.

Flavobacterium sp. WPGT1 maintained as glycerol stock at -20°C was revived on CA at 30±2°C for 24 h to get young culture. One colony was aseptically picked, transferred to 10 ml of Cytophaga broth (CB; Song *et al.*, 1988) separately and incubated at 30±2°C for 24 h. Mass culture was done in 500 ml of CB at 30±2°C for 24 h and centrifuged at 7500 rpm at 20°C for 10 min. The pellet thus obtained was washed thrice with sterile physiological saline (0.85% w/v sodium chloride) and suspended in 10ml of saline. Pathogenicity of *Flavobacterium* sp. WPGT1 was tested by two methods, viz., intraperitoneal injection and immersion of skin wounded *C. auratus* in *Flavobacterium* sp. WPGT1 cell suspension at predetermined doses in duplicate (Adikesavalu *et al.*, 2015; Abraham *et al.*, 2016). Aliquots (0.1 ml each) of

Flavobacterium sp. WPGT1 cell suspensions from 10^0 to 10^{-3} dilutions were intraperitoneally (i/p) injected in such a way so as to get 10^8 - 10^5 cells/fish, respectively. The control fish (i/p) received 0.1 ml each of sterile saline. In case of skin wounded fish, scales of all the fish from each aquaria were scrapped off gently with a scalpel from caudal peduncle to the pectoral fin, i.e., in the opposite direction (skin wounded). The skin wounded fish from each aquarium were immersed in a bacterial cell suspension (1000 ml) containing *Flavobacterium* sp. WPGT1 at a level of 3.50×10^6 cells/ml for 30 min. The fish along with the suspending medium were then transferred to the respective aquaria and observed for 28 days. The control group was neither skin-wounded nor dipped in *Flavobacterium* sp. WPGT1 suspension. The challenged and control groups were maintained in the respective aquaria for 28 days. The behavioral abnormalities, external signs of infection and mortality, if any were recorded daily.

Results and Discussion

The isolation of *Flavobacterium* sp. in diseased *C. auratus* from a ornamental fish farm with gill rot as well as white patches

and excessive mucus secretion on gill (Fig. 1A) during the winter season indicated that it can be an opportunistic fish pathogen capable causing mortalities in immunosuppressed fish at low water temperature. Due to its colonial morphology and phenotypic reaction (Fig. 1B), it was considered to be one of the species of the genus *Flavobacterium*. *Flavobacterium* spp. have been reported to be pathogenic to fish causing gill disease with similar symptoms (Loch and Faisal, 2015). Bowman and Nowak (2004) also detected a *Flavobacterium* sp. from the gills of net-penned Atlantic salmon that concurrently suffered from amoebic gill disease. The phenotypic characteristics of the bacterium as assessed by VITEK 2 system are presented in Table 1. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the novel sequence belonged to the family Flavobacteriaceae, phylum Bacteroidetes, and fell within the evolutionary radiation of the genus *Flavobacterium*. The 16S rRNA gene sequence of this bacterium was closely related to *Flavobacterium* sp. KJ461684 with cent percent node value and 98% DNA homology (Fig. 2).

Fig.1 [A] White patches on the gill of goldfish *Carassius auratus*; [B] Gram reaction and morphology of the bacterium *Flavobacterium* sp. WPGT1 isolated from the diseased *Carassius auratus* gill

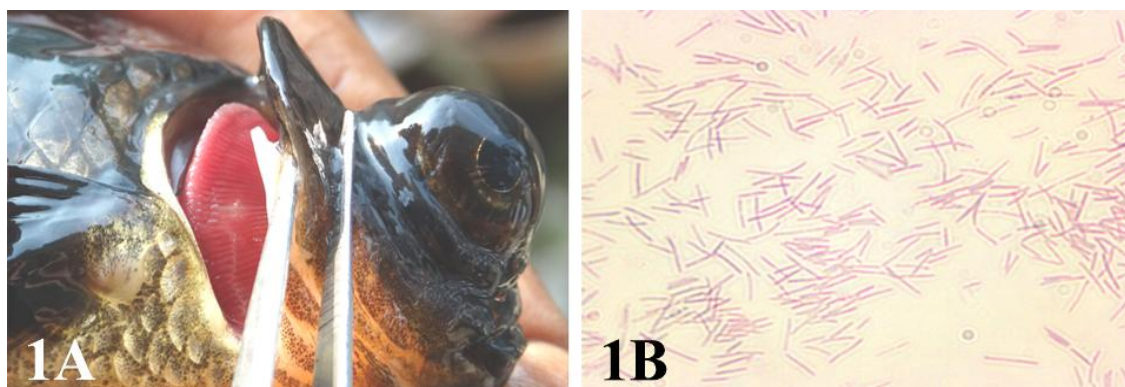
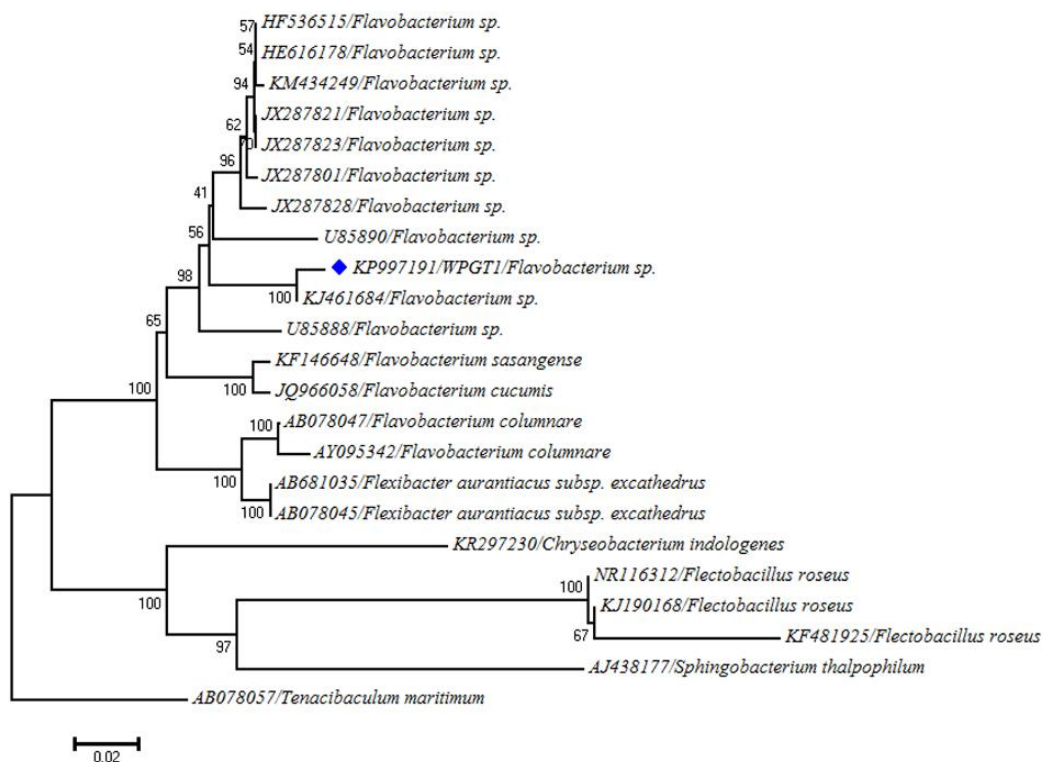


Table.1 Biochemical Characteristics of *Flavobacterium* sp. WPGT1 Strain as Assessed by Conventional Biochemical Tests and VITEK 2 Compact (Biomerieux, France)

Biochemical characteristics	Reaction	Biochemical characteristics	Reaction
Conventional biochemical tests		Vitek 2-Compact system	
Colony colour	Yellow	D-Cellobiose (dCEL)	-
Colony morphology	R, C, I	D-Glucose (dGLU)	-
Gram reaction	-	Glu-Gly-Arg-arylamidase (GGAA)	-
Morphology	LR	Glutamyl arylamidase pNA (AGLTp)	-
Oxidase	(+)	Glycine arylamidase (GlyA)	-
Oxidative/Fermentative reaction	-/-	H ₂ S production (H ₂ S)	-
Casein hydrolysis	+	L Pyrrolydonyl-arylamidase (PyrA)	-
Chondroitin sulphate degradation	-	L-Arabitol (IARL)	-
Congo red reaction	-	L-Histidine assimilation (IHISa)	-
Fibrinogen hydrolysis	-	Lipase (LIP)	-
Flexirubin pigment presence	-	L-Lactate alkalisation (ILATk)	-
Gelatin hydrolysis	-	L-Lactate assimilation (ILATa)	-
Growth in selective cytophaga agar#	+	L-Malate assimilation (IMLTa)	-
Vitek 2-Compact system		L-Proline arylamidase (ProA)	-
5-Keto D-gluconate (5KG)	-	Lysine decarboxylase (LDC)	-
Adonitol (ADO)	-	Malonate (MNT)	-
Ala-Phe-Pro-arylamidase (APPA)	+	O/129 Resistance (O129R)	-
Alpha-galactosidase (AGAL)	-	Ornithine decarboxylase (ODC)	-
Alpha-glucosidase (AGLU)	+	Palatinose (PLE)	-
Beta-alanine arylamidase pNA (BALap)	-	Phosphatase (PHOS)	-
Beta-galactosidase (BGAL)	-	Saccharose/Sucrose (SAC)	-
Beta-glucuronidase (BGUR)	-	Succinate alkalisation (SUCT)	-
Beta-glucosidase (BGLU)	+	Tyrosine arylamidase (TyrA)	+
Beta-xylosidase (BXYL)	-	Urease (URE)	-
Citrate (sodium) (CIT)	-	β -N-acetyl-galactosaminidase (NAGA)	-
Coumarate (CMT)	-	β -N-Acetyl-glucosaminidase (BNAG)	-

#: Growth in the presence of neomycin sulphate and polymyxin B; LR: Long rod; R: Round; C: Convex; I: Irregular edge

Fig.2 Molecular phylogenetic tree produced by Neighbor-Joining method. Numbers at nodes indicate bootstrap confidence values (1000 replicates). The GenBank accession number is provided for each species. Units of evolutionary distance are in the number of base substitutions per site



Although the gross and clinical signs observed on the diseased fish were similar to columnaris, the molecular characterization of the test strain revealed that the isolated bacterium belonged to *Flavobacterium* sp. This observation confirmed that the disease was related to a condition called flavobacteriosis.

Challenged fish had white patches on gill, tail rot, body discoloration, scale loss and skin peeling at caudal peduncle site as reported in earlier studies on flavobacteriosis (Jansson *et al.*, 2012; Loch and Faisal, 2015). No mortality was observed in fish i/p challenged with *Flavobacterium* sp. WPGT1 at a level of 10^8 cfu/fish. The LD₅₀ value of *Flavobacterium* sp. WPGT1 was determined to be $>3.50 \times 10^8$ cfu/fish. The pathogenicity of *Flavobacterium* sp. WPGT1 on *C. auratus* as assessed by skin wounding-bath

experiment resulted 14.29% mortality in 6 days of challenge. The strain was found to be weakly pathogenic to *C. auratus*. This result suggested that *Flavobacterium* sp. can cause mortalities in fish that carry physical or mechanical injuries and confirms the earlier observations (Moyer and Hunnicutt, 2007; Loch and Faisal, 2015). Our results suggested that *Flavobacterium* sp. may be involved in the pathogenesis of fish as with other potential pathogens in conjunction with adverse environmental or stressful conditions in the culture systems or during the periods of immuno-suppression.

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