Brazilian Journal of Microbiology 46, 4, 969-976 (2015)
ISSN 1678-4405
DOI: http://dx.doi.org/10.1590/S1517-838246420131081

Research Paper

New host record of five *Flavobacterium* species associated with tropical fresh water farmed fishes from North India

Dev Kumar Verma[1], Gaurav Rathore[2]

[1]National Bureau of Fish Genetic Resource, Lucknow, India.
[2]Central Institute of Fisheries Education, Mumbai, India.

Submitted: September 11, 2013; Approved: August 4, 2014.

Abstract

Yellow pigmented, filamentous, Gram-negative bacteria belonging to genus *Flavobacterium* are commonly associated with infections in stressed fish. In this study, inter-species diversity of *Flavobacterium* was studied in apparently healthy freshwater farmed fishes. For this, ninety one yellow pigmented bacteria were isolated from skin and gill samples (n = 38) of three farmed fish species *i.e. Labeo rohita*, *Catla catla* and *Cyprinus carpio*. Among them, only twelve bacterial isolates (13.18%) were identified as *Flavobacterium* spp. on the basis of morphological, biochemical tests, partial 16S rDNA gene sequencing and phylogenetic analysis. On the basis of 16S rDNA gene sequencing, all the 12 isolates were 97.6-100% similar to six different formally described species of genus *Flavobacterium*. The 16S rDNA based phylogenetic analysis grouped these strains into six different clades. Of the 12 isolates, six strains (Fl9S1-6) grouped with *F. suncheonense*, two strains (Fl6I2, Fl6I3) with *F. indicum* and the rest four strains (Fl1A1, Fl2G1, Fl3H1 and Fl10T1) clustered with *F. aquaticum*, *F. granuli*, *F. hercynium* and *F. terrae*, respectively. None of these species except, *F. hercynium* were previously reported from fish. All the isolated *Flavobacterium* species possessed the ability of adhesion and biofilm formation to colonize the external surface of healthy fish. The present study is the first record of tropical freshwater farmed fishes as hosts to five environmentally associated species of the *Flavobacterium*.

Key words: *Flavobacterium*, fish, species diversity, 16S rDNA, phylogenetic analysis.

Introduction

Heterotrophic bacteria including Bacteroidetes are major contributors to biogeochemical cycles and influence water quality. This phylum is also known as CFB (*Cytophaga-Flavobacteria-Bacteroides*) and thrives in a variety of aquatic environments, sediments, hydrothermal vents and polar regions (Kirchman, 2002). These bacteria have been found both free living and attached to organic aggregates, and can be associated with phytoplanktons (Alonso *et al.*, 2007). *Flavobacterium* is recognized as an important genus in the phylum Bacteriodetes and are considered as major mineralizers of organic matter. Data compilation shows that till date, genus *Flavobacterium* comprises of 106 species isolated from diverse ecological niches, including diseased animals (Bernardet *et al.*, 1996), sediments (Fu *et al.*, 2011), soil (Weon *et al.*, 2007), water (Cousin *et al.*, 2007), Antarctic regions (Yi *et al.*, 2005) and glacier samples (Zhu *et al.*, 2013). Of these, seven novel species of *Flavobacterium* have been reported from India till now. Among them, three species were isolated from soil (Madhaiyan *et al.*, 2010; Jit *et al.*, 2008; Lata *et al.*, 2012), two from fresh water (Saha and Chakrabarti, 2006; Subhash *et al.*, 2013), and two from marine environment (Kaur *et al.*, 2012; Nupur *et al.*, 2013).

The major concerns regarding some members of this genus are their ability to cause disease in a variety of aquaculture settings. Fish infections caused by pathogenic *Flavobacterium* spp. are a major problem in the aquaculture industry worldwide, often leading to large economic losses. A number of *Flavobacterium* spp. are pathogenic or regarded as opportunistic pathogens and

Send correspondence to G. Rathore. AEHM Division, Central Institute of Fisheries Education, Off Yari Road, Versova, Andheri, 400061 Mumbai, India. E-mail: rathore69@rediffmail.com.

cause disease in a wide variety of organisms. This genus accounts for 13% of total bacterial fish pathogens (Zhang, 2007). *F. columnare*, *F. psychrophilum*, *F. branchiophilum*, *F. aquatile* and *F. johnsoniae* have been associated with fish disease and have also been detected in surrounding water in the presence of disease outbreaks (Bernardet *et al.*, 2005). *F. aquatile*, *F. hydatis* and *F. succinicans* have been also occasionally isolated from diseased fish (Bernardet *et al.*, 1996). Many new species of *Flavobacterium* spp. were isolated from diseased fish in Europe and South America, including *F. oncorhynchi* (Zamora *et al.*, 2012, 2013), *F. chilense* and *F. araucananum* (Kampfer *et al.*, 2012). Most recently, 21 *Flavobacterium* species were reported from diseased as well as apparently healthy wild, feral and farmed fish of Michigan, North America (Loch *et al.*, 2013). However, no information is available on the diversity of fish associated *Flavobacterium* species from India. So the present study was an attempt to describe the phenotypic, genotypic and phylogenetic diversity of *Flavobacterium* species associated with tropical fresh water farmed fish species samples collected from India.

Materials and Methods

Bacterial isolation

Live freshwater farmed fishes (~500-600 g) *i.e.* twelve number (n = 12) common carp (*Cyprinus carpio*), (n = 10) catla (*Catla catla*) and (n = 16) rohu (*Labeo rohita*) were collected from different four fish farms of north India. Samples of gills and muscles were collected aseptically and homogenized in normal saline (0.85% NaCl). Processed samples were serially diluted in sterile saline and an aliquot of 100 µL from the 10^{-4}, 10^{-5}, and 10^{-6} dilutions in duplicate were plated onto Anacker and Ordal (AO) agar. Plates were incubated at 28 °C for 24-48 h and bacterial growth was recorded. Yellow pigmented flat or very thin colonies, spreading, with uneven, rhizoid, or filamentous margins were selected and subcultured for phenotypic and molecular analysis. For cryopreservation, purified colonies were grown in AO broth and stored at -80 °C after supplementation with 10% (v/v) glycerol.

Biochemical identification

Gram-negative yellow pigmented rod shaped isolates were differentiated from other Flavobacteria using the following tests: presence of non-diffusible carotenoid or flexirubin pigments; production of oxidase, catalase and indole; and decomposition of gelatin and casein (Bernardet *et al.*, 1996). Proteolytic activity was assayed by using skim milk-enriched AO agar for casein proteolysis and gelatin AO agar deeps for gelatin hydrolysis. Characterized isolates were biochemically identified as *Flavobacterium* spp. These isolates were additionally characterized for more phenotypic characteristics according to Bernardet *et al.* (2002).

Molecular identification of *Flavobacterium* species

Genomic DNA extraction

For molecular identification of *Flavobacterium* species, total genomic DNA was isolated from freshly grown broth culture of biochemically identified *Flavobacterium* spp. according to the protocol of Marmur (1961), with minor modifications. In brief, the cells were pelleted and resuspended in an equal volume of TES buffer (50 mM Tris buffer, 1 mM EDTA, 8.56% wt/vol sucrose) pH 8.0 and sodium dodecyl sulphate was added to the mixture. The solution was treated once with chloroform-isoamyl alcohol (24/1; v/v) and once with a mixture of phenol, chloroform and isoamyl alcohol (25/24/1; v/v/v). The DNA was precipitated by an equal volume of isopropanol and dissolved in 1x Tris-EDTA buffer and stored at -20 °C for further use.

PCR amplification of bacterial 16S rDNA gene and sequencing

Amplification of 16S rDNA of biochemically confirmed *Flavobacterium* strains were performed by using universal primers 20F and 1492R (Weisburg *et al.*, 1991). PCR amplification was carried in a 50 µL reaction mixture containing: 100 ng of purified DNA as template, 1x*Taq* DNA polymerase buffer, 10 mM dNTPs, 1.5 mM $MgCl_2$, and 0.4 µL of *Taq* DNA polymerase (MBI Fermentas) in gradient mastercycler (Eppendorf, Germany). The PCR reaction was incubated for 2 min denaturation at 95 °C, followed by 30 cycles at 95 °C for 30 s, annealing at 50 °C for 60 s, and extension at 72 °C for 60 s, with a final extension step of 10 min at 72 °C. PCR products were analysed by electrophoresis in 1% (w/v) agarose gel in 1x Tris Acetate-EDTA buffer. PCR products were analyzed at constant voltage of 7V cm^{-1} on 1% agarose gel containing (0.5 µg mL^{-1}) ethidium bromide and DNA marker (Lambda DNA *Eco*RI/*Hind*III marker, Genei Pvt. Ltd, Bangalore, India). PCR products were gel purified by using the QIAquick Purification Kit (Qiagen, Limburg, Netherlands) according to the manufacturer's protocol. Purified amplicons were sequenced bidirectionally with the 27F and 1492R primers.

Phylogenetic affiliation of sequences

All obtained sequences were checked for chimeric artifacts by the Check-Chimera program (Maidak *et al.*, 2001). Bacterial identity was deduced by BLAST search to ascertain its closest related sequences. Percent identity of the isolates with other *Flavobacterium* spp. was calculated with Ez-Taxon server. Phylogenetic tree was constructed by neighbour-joining method, and topology was evaluated by bootstrap analysis of 1000 dataset using MEGA version 5.2 software (Tamura *et al.*, 2011). The 16S rDNA se-

quences from *Chryseobacterium piscium* (AM040439), *Chryseobacterium soldanellicola* (AY883415), *Cytophaga hutchinsonii* (NR102866) were taken as the minor outgroup, while *E. coli* (EU014689) was used as major outgroup for rooting of the tree.

Nucleotide sequence accession number

The partial 16S rDNA sequence (~1400 bp) data of *Flavobacterium* species determined in this study were deposited in the GenBank database and appear in the DDBJ, EMBL, and NCBI nucleotide sequence databases under accession numbers shown in Table 1. The accession numbers of reference organisms used in phylogenetic analysis are shown in Figure 1.

Micro plate adherence assay

Micro plate adherence assay was done to assess the biofilm forming capacity of *Flavobacterium* species as per method described by Stepanovic *et al.* (2000), with minor modifications. All *Flavobacterium* isolates were cultured for 4 days in AO broth and centrifuged for 5 min at 12,000 *g*. Cell pellets were washed and resuspended in phosphate-buffered saline to a turbidity equivalent to 0.5 McFarland standards. For measuring bacterial microtitre plate adherence, wells of sterile 96-well plates were each filled with 90 µL AO broth and inoculated with 10 µL of *Flavobacterium* cell suspensions in triplicate. Negative control wells containing only broth were included in each assay, while *F. columnare* isolate RDC-1 was used as a positive control. Plates were incubated aerobically at room temperature 28 °C for 4 days for bacterial growth. Contents of each well were aspirated, washed three times with 250 µL sterile PBS, and adherent cells were fixed with 200 µL of methanol for 15 min. After air-drying, wells were stained with 150 µL of 2% crystal violet for 15 min. Dye bound to adherent cells was resolubilized with 200 µL of 96% (v /v) ethanol acid, and biofilm formation was quantified after 10 minu by measuring the optical density (OD) of each well at 595 nm. Based on the optical densities of bacterial films, all *Flavobacterium* species were classified according to Abdallah *et al.* (2009), into the following categories: no biofilm producers or non-adherent (< 0.10), weak (0.20-0.40), moderate (0.40-0.80), or strong biofilm producers (> 0.80).

Statistical analysis

Data on biofilm formation on micro plates were analyzed by one-way analysis of variance (ANOVA) using SPSS 16.0 software. Significant difference was set at $p < 0.05$ by using tukey's test. Composite dataset including the OD_{595} nm values from all isolated *Flavobacterium* species and *F. columnare* strain RDC-1 (Verma and Rathore, 2013) served as input for pair wise comparisons.

Results

Bacterial isolation and biochemical characterization

A total of 38 fish samples were processed during 2009-2011 for isolation and assessing the diversity of *Flavobacterium* species. Ninety one yellow pigmented colonies were selected and purified for biochemical characterization. Non-pigmented colonies were excluded from the study. Of the 91 yellow pigmented isolates analyzed, only twelve isolates (13.18%) were identified as *Flavobacterium* spp. based on the following characteristics; Colonies were circular, yellow-pigmented, smooth and entire on AO agar after 24-48 h of incubation at 28 °C incubation. Cells were Gram-negative rods and non-endospore forming. The strains grew well in aerobic condition at temperature 18-30 °C with optimal growth at approximately 28 °C, while no growth was observed at 37-40 °C.

Table 1 - Similarity values among the isolated strains and reference strains on the basis of 16S rDNA

S. No	Strain	Source tissue	Host species	GenBank accession number	Closest related species in the GenBank database	% similarity with EZ-taxon
1	Fl10T1	Gills	*Cyprinus carpio*	KJ635879	*Flavobacterium terrae*	97.62
2	Fl9S1	Skin	*Labeo rohita*	KJ635878	*Flavobacterium suncheonense*	97.91
3	Fl6I3	Gills	*Cyprinus carpio*	KJ635877	*Flavobacterium indicum*	98.60
4	Fl1A1	Gills	*Catla catla*	KJ635870	*Flavobacterium aquaticum*	100.0
5	Fl9S2	Skin	*Cyprinus carpio*	KJ635876	*Flavobacterium suncheonense*	99.09
6	Fl3H1	Skin	*Labeo rohita*	JQ966057	*Flavobacterium hercynium*	98.72
7	Fl2G1	Gills	*Catla catla*	JQ994263	*Flavobacterium granuli*	98.55
8	Fl9S3	Gills	*Catla catla*	KJ635875	*Flavobacterium suncheonense*	99.11
9	Fl9S4	Skin	*Labeo rohita*	KJ635874	*Flavobacterium suncheonense*	99.08
10	Fl9S5	Skin	*Labeo rohita*	KJ635873	*Flavobacterium suncheonense*	98.28
11	Fl9S6	Gills	*Cyprinus carpio*	KJ635872	*Flavobacterium suncheonense*	99.16
12	Fl6I2	skin	*Labeo rohita*	KJ635871	*Flavobacterium indicum*	98.82

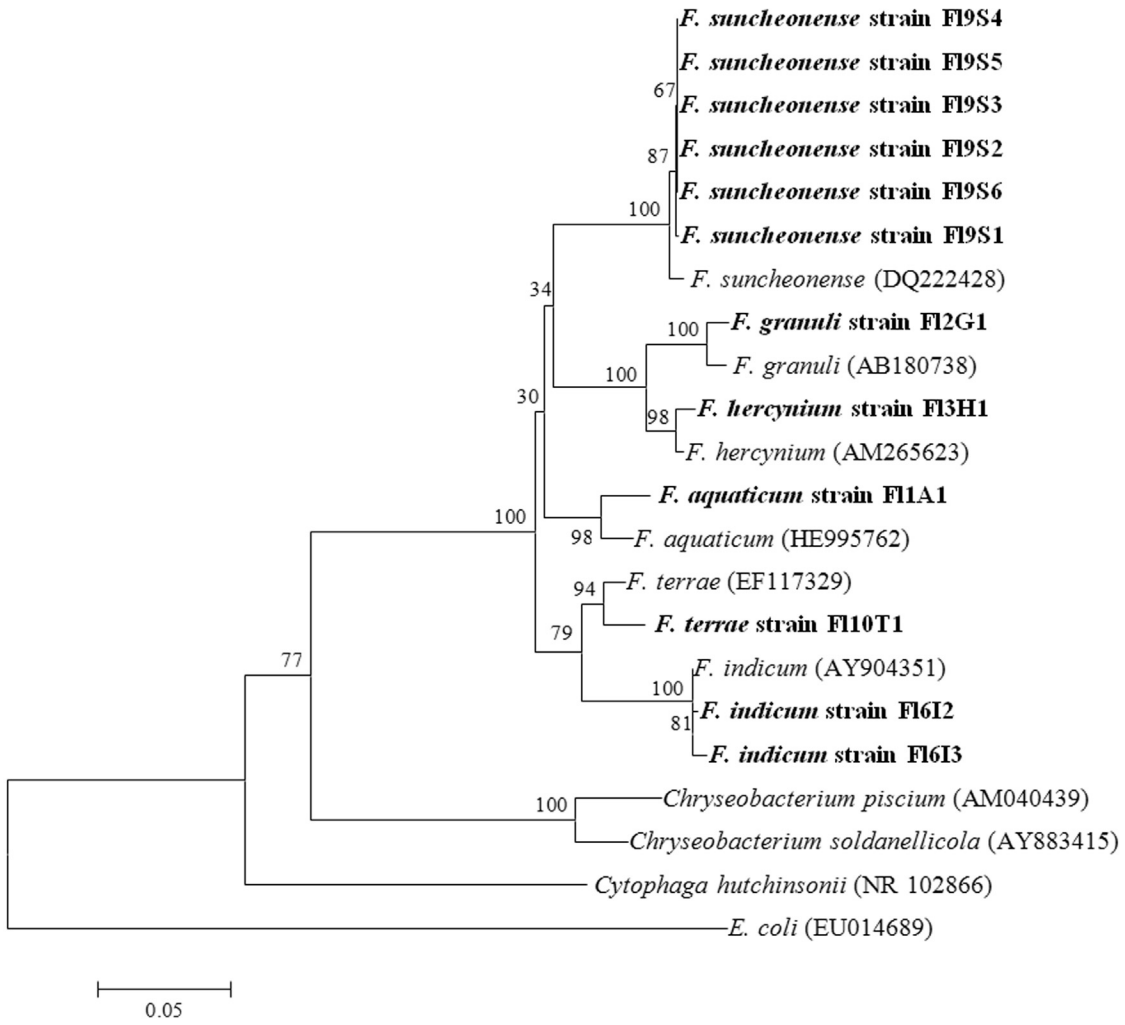

Figure 1 - Neighbour-joining phylogenetic tree based on 16S rDNA gene sequences for isolated *Flavobacterium* species and other closely related *Flavobacterium* species. Numbers at branch nodes are bootstrap percentages based on 1000 re-samplings. *E. coli* was used as major out-group for the rooting of the tree. Bar represents 0.05 changes per sequence position. The sequences obtained in this study are shown in bold.

Identified isolates produced catalase, oxidase, flexirubin or carotenoid pigment. Gelatin and casein was hydrolyzed while indole was not produced by any isolate. The majority of biochemical reactions of the *Flavobacterium* isolates showed similarity with their reference strains (Bernardet *et al.*, 2002). Biochemical characteristics of these isolates are recorded in Table 2.

Molecular identification of *Flavobacterium* spp. by 16S rDNA sequencing

The 16S rDNA sequences of all twelve isolates were analysed individually by BLAST search to obtain its closest reference sequence available in GenBank. All sequences of *Flavobacterium* were aligned with the existing reference sequences in the NCBI database. Results revealed that all the strains shared closed homology with the different species of the genus *Flavobacterium* (Table 1). Percent sequence identity of the isolated strain of *Flavobacterium* was calculated with respect to its reference strain by Ez-Taxon server. All the twelve strains were 97.6-100% similar to six formally described species of *Flavobacterium*. The constructed phylogenetic tree confirmed the phylogenetic positions of these strains in the genus *Flavobacterium* (Fig.1). The 16S rDNA based phylogenetic analysis grouped these strains into six different clades. Six strains (Fl9S1-6) grouped with *F. suncheonense*, two strains (Fl6I2, Fl6I3) with *F. indicum* and the rest four strains (Fl1A1, Fl2G1, Fl3H1, and Fl10T1) clustered with *F. aquaticum*, *F. granuli*, *F. hercynium* and *F. terrae*, respectively (Fig. 1). Partial sequences of the amplified 16S rDNA gene of the isolates were submitted to NCBI under the accession numbers KJ635870-79, JQ966057 and JQ994263, respectively.

Table 2 - Differential characteristics between Indian strains of *Flavobacterium* and the reference strains of closely related species.

Biochemical tests	*F. terre*		*F. granuli*		*F. hercynium*		*F. suncheonense*		*F. aquaticum*		*F. indicum*	
	Weon *et al.*, 2007	Strain Fl10T1	Aslam *et al.*, 2005	Strain Fl2G1	Cousin *et al.*, 2007	Strain Fl3H16	Kim *et al.*, 2006	Strain Fl9S1-6	Subhash *et al.*, 2013	Strain Fl1A1	Saha and Chakrabarti 2006	Strain Fl6I2, 13
Motility	-	-	-	-	-	-	-	-	-	-	-	-
Congo red absorption	-	-	-	-	-	-	-	-	-	-	-	-
Flexirubin type pigment	+	+	-	-	+	+	-	-	-	-	-	-
Growth on Nutrient agar	+	+	+	+	+	+	+	+	+	+	(+)	+
Trypticase soya agar	(+)	+	+	+	+	+	(+)	+	(+)	+	-	-
Glucose utilization	+	+	+	+	+	+	-	-	-	-	-	-
Acid produced from Fructose	-	+	+	+	-	+	-	+	+	+	+	+
Degradation of Gelatin	+	+	-	+	+	+	+	+	+	+	+	+
Degradation of Casein	+	+	-	+	+	+	+	+	+	+	+	+
Degradation of Starch	+	+	-	-	-	-	-	-	+	+	+	+
Production of cytochrome oxidase	+	+	+	+	-	+	-	+	+	+	+	+
Hydrogen sulphide	-	-	-	-	-	-	-	-	-	-	-	-
Catalase production	-	+	+	+	+	+	+	+	+	+	+	+
Indole production	-	-	-	-	-	-	-	-	-	-	-	-

+ = positive; (+) = weak positive; - = negative.

Comparison of biofilm formation among strains

All the *Flavobacterium* species were able to adhere to polystyrene plates. Isolated stains showed moderate biofilm formation ability as compared to *F. columnare*, which showed the strongest adhesion pattern. Significant difference was observed between the different isolates (Figure 2).

Discussion

Aquaculture as a means of farmed production is a rapidly growing industry in India. The bacterial species isolated in present study ascribed to the genus *Flavobacterium*. These bacteria are usually associated with aquatic environment, which represents their principal reservoir. Flavobacteria are also known to belong to the microbiota of fish and fish eggs (Bernardet and Bowman, 2006). The main purpose of this study was to investigate the extent of species diversity of fish associated *Flavobacterium* in tropical freshwater farmed fishes from India and examines their phylogenetic relationships to previously characterized strains from other part of the world.

In this study, Gram-negative, yellow pigmented bacterial isolates were obtained on AO agar from three important farmed fish species of India, *i.e.* common carp (n = 12), catla (n = 10) and rohu (n = 16) collected from different fish farms. These isolates were screened for diversity of *Flavobacterium* species by microbiological and molecular techniques. For this, pure cultures were tested using a battery of biochemical tests. Of the ninety one Gram-negative, yellow-pigmented *Flavobacterium* isolates analyzed in this study, only twelve were identified as *Flavobacterium* spp. Phenotypic heterogeneity was detected among these *Flavobacterium* isolates as six different phylotypes were obtained.

During course of study, no known species of *Flavobacterium* pathogenic to fish *i.e. F. columnare*, *F. psychrophilum* and *F. branchiophilum* were recovered from the test farmed fishes. *F. columnare* is known to cause columnaris disease in warm water fishes and was successfully isolated from diseased catla fish in our previous study from India (Verma and Rathore, 2013). Similarly, *F. psychrophilum* another important fish pathogen of cold water fish and causative agent of cold water disease was also not recovered from the common carp collected from cold water environment. Additionally, it is also noteworthy that *F. branchiophilum*, the etiological agent of bacterial gill disease was also found to be absent in the screened fish in accordance with the (Loch *et al.*, 2013). Absence of pathogenic *Flavobacterium* species in the tested fish indicates that the above mentioned species of *Flavobacterium* are opportunistic pathogens and colonize only when the fish is under stress (Kumar *et al.*, 1986).

Molecular methods are now routinely being used to detect and confirm the identity of prokaryotic organisms.

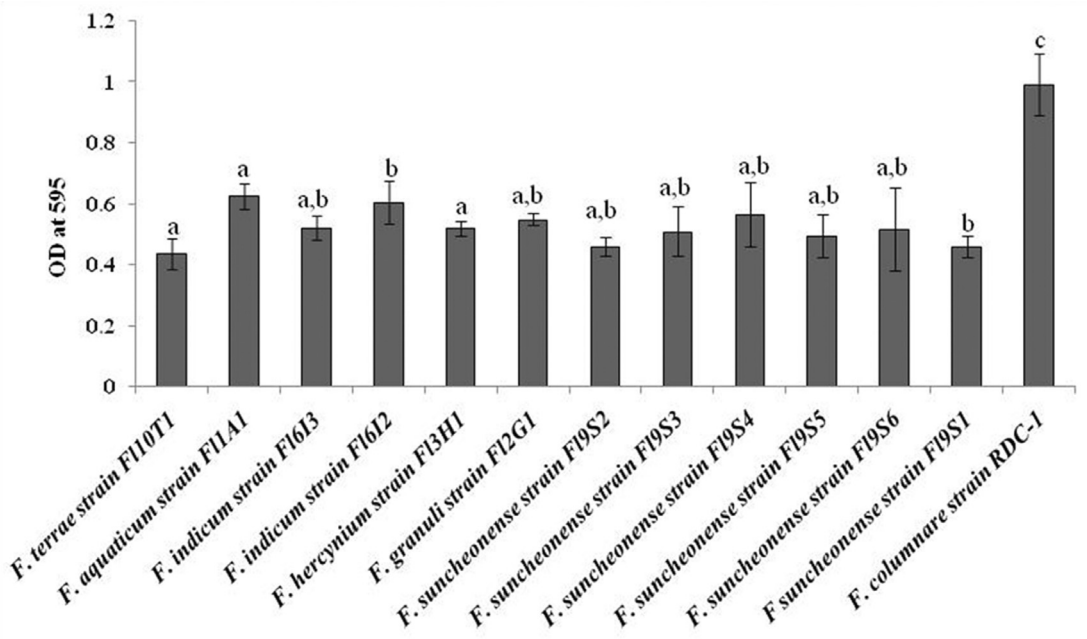

Figure 2 - Adhesion (mean absorbance ± SE) of different *Flavobacterium* species to microplate. Different letters indicate significantly difference ($p < 0.05$) in cell attachment.

For this, housekeeping genes *viz.* ribosomal RNA are preferably used as the target DNA for identification of bacteria because they contain highly conserved regions and variable species-specific regions (Weisburg *et al.*, 1991). Likewise, we have also used 16S rDNA sequencing for the molecular identification of all the bacterial isolates belonging to *Flavobacterium*. The reported species/strains were identified up to 100% sequence similarity with the reference *Flavobacterium* species on the basis of partial 16S rDNA. Phylogenetic analysis along with cultural, morphological and biochemical characteristics provide evidence that most prevalent *Flavobacterium* species was *F. suncheonense* in healthy farmed fishes. This species has a wide host range as it was isolated from all three fish species. All the six isolates (strain Fl9S1-6) belonged to one phylotype and exhibited substantial sequence resemblance (97.9-99.2%) to F. suncheonense reference strain isolated from soil in Korea (Kim *et al.*, 2006). Previously, there are no reports on isolation of *F. suncheonense* from fish worldwide. Next most frequently isolated species was *F. indicum* as this species was recovered from rohu and common carp. This bacterium was previously isolated from warm spring water in Assam, India (Saha and Chakrabarti, 2006), but not from any fish host. The other *Flavobacterium* species recovered in this study was *F. hercynium*, *F. aquaticum*, *F. granuli* and *F. terrae.* Of these *F. hercynium* (33 nos.) was the most prevalent species of *Flavobacterium* recently reported from diseased fishes of Michigan, North America (Loch *et al.*, 2013). Reference strain of *F. hercynium* was previously recovered from freshwater of the hard-water creek, Germany (Cousin *et al.*, 2007). This shows that this species has wide spread distribution ranging from diseased fish to freshwater aquatic environment of several geographical locations. Remaining three species of *Flavobacterium* are not known to occur in fish as *F. granuli* was isolated from granules used in the wastewater treatment plant of a beer-brewing factory in Republic of Korea (Aslam *et al.*, 2005); and *F. terrae* were isolated from greenhouse soils in Korea (Weon *et al.*, 2007); while *F. aquaticum* was recently isolated from water samples of same geographic region (Subhash *et al.*, 2013). The present study is the first record of tropical freshwater farmed fishes as hosts to five environmentally associated species of the *Flavobacterium i.e. F. suncheonense*, *F. indicum F. aquaticum*, *F. granuli* and *F. terrae*.

In the present study, gill and muscle tissues were used for isolation of the adhered *Flavobacterium* species. Bacterial adhesion to the external surfaces and subsequent colonization would lead to formation of biofilms which can confer resistance to mucosal immune defenses and antibiotic resistance. It is also possible that the protective function of biofilm could enhance potential for survival of these bacteria in aquatic environment (Cai *et al.*, 2013). Therefore, testing of a bacterial isolate for biofilm formation is known to be a useful marker for assessing the bacterial pathogenicity and also their potential to colonize biotic and abiotic surfaces (Stepanovic*et al*., 2000; Jacobs and Chenia, 2009). In the present study, microplate adherence assay was performed to test the ability of adhesion of *Flavobacterium* species. The results showed that *Flavobacterium* species possessed the ability of adhesion and therefore they would be able to form biofilm and colonize the external surfaces of healthy fish. Previously, only two

members of the genus *Flavobacterium i.e. F. johnsoniae* and *F. columnare* were known to possess strong tendency to colonize surfaces (Rickard *et al.*, 2003; Cai *et al.*, 2013). More work would be needed to study the full range of symbiotic interactions of these *Flavobacterium* species with their fish hosts.

Another aspect of this study is that there are no standard molecular approaches that can be used easily and accurately to study the diversity of *Flavobacterium* species in fishes. As a result the study on distribution of this complex group of organisms in aquatic environment is limited. Development of genus specific primers/probe may help in rapid identification of the members of this genus.

Acknowledgments

Authors are thankful to NBAIM, Mau for funding and U. P. Technical University, Lucknow for supporting this work. We are also thankful to Dr. J. K. Jena, Director, NBFGR, Lucknow for providing necessary facilities to carry out this work.

References

Abdallah FB, Chaieb K, Zmantar T *et al.* (2009) Adherence assays and slime production of *vibrio alginolyticus*and *Vibrio parahaemolyticus*. Braz J Microbiol 40:394-398.

Alonso C, Warnecke F, Amann R *et al.* (2007) High local and global diversity of Flavobacteria in marine plankton. Environ Microbiol 9:1253-66.

Aslam Z, Im WT, Kim MK et al. (2005) *Flavobacterium granuli* sp. nov., isolated from granules used in a wastewater treatment plant. Int J Syst Evol Microbiol 55:747-751.

Bernardet JF, Bowman JP (2006) The genus *Flavobacterium*. In The Prokaryotes: a Handbook on the Biology of Bacteria, 3rd Edn. Vol 7, New York, pp 481-531.

Bernardet JF, Nakagawa Y, Holmes B (2002) Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. Int J Syst Evol Microbiol 52:1049-1070.

Bernardet JF, Vancanneyt M, Matte-Tailliez O *et al.* (2005) Polyphasic study of *Chryseobacterium* strains isolated from diseased aquatic animals. Syst Appl Microbiol 28:640-660.

Bernardet JF, Seger P, Vancanneyt M *et al.* (1996) Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (Basonym, *Cytophaga aquatilis* Strohl and Tait 1778). Int J Syst Bacteriol 46:128-148.

Cai W, Fuente LDL, Arias CR (2013) Biofilm formation by the fish pathogen *Flavobacterium columnare*: development and parameters affecting surface attachment. Appl Environ Microbiol 73:5633-5642.

Cousin S, Pauker O, Stackebrandt E (2007) *Flavobacterium aquidurense* sp. nov. and *Flavobacterium hercynium* sp. nov. from a hard-water creek. Int J Syst Evol Microbiol 57:243-249.

Fu Y, Tang X, Lai Q *et al.* (2011) *Flavobacterium beibuense* sp. nov., isolated from marine sediment. Int J Syst Evol Microbiol 61:205-209.

Jacobs A, Chenia HY (2009) Biofilm-forming capacity, surface hydrophobicity and aggregation characteristics of *Myroides odoratus* isolated from South African *Oreochromis mossambicus* fish. J Appl Microbiol 107:1957-1966.

Jit S, Dadhwal M, Prakash O *et al.* (2008) *Flavobacterium lindanitolerans* sp. nov., isolated from hexachloro cyclohexane-contaminated soil. Int J Syst Evol Microbiol 58:1665-1669.

Kampfer P, Lodders N, Martin K *et al.* (2012) *Flavobacterium chilense* sp. nov. and *Flavobacterium araucananum* sp. nov. isolated from food salmonid fish. Int J Syst Evol Microbiol 62:1402-1408.

Kaur I, Kaur C, Khan F *et al.* (2012) *Flavobacterium rakeshii* sp. nov., isolated from marine sediment, and emended description of *Flavobacterium beibuense* Fu *et al.*, 2011. Int J Syst Evol Microbiol 62:2897-2902.

Kim BY, Weon HY, Cousin S *et al.* (2006) *Flavobacterium daejeonense* sp. nov and *Flavobacterium suncheonense* sp. nov., isolated from greenhouse soils in Korea. Int J Syst Evol Microbiol 56:1645-1649.

Kirchman DL (2002) The ecology of *Cytophaga-Flavobacteria* in aquatic environments. FEMS Microbio Ecol 39:91-100.

Kumar D, Suresh K, Dey RK *et al.* (1986) Stress mediated columnaris disease in rohu, *Labeo rohita* (Hamilton). J Fish Dis 9:87-89.

Lata P, Lal D, Lal R (2012) *Flavobacterium ummariense* sp. nov., isolated from hexachlorocyclohexane-contaminated soil, and emended description of *Flavobacterium ceti* Vela *et al.*, 2007. Int J Syst Evol Microbiol 62:2674-2679.

Loch TP, Fujimoto M, Woodiga SA *et al.* (2013) Diversity of Fish-Associated Flavobacteria of Michigan. J Aquatic Anim Health 25:149-164.

Madhaiyan M, Poonguzhali S, Lee JS *et al.* (2010) *Flavobacterium glycines* sp. nov., a facultative methylotroph isolated from the rhizosphere of soybean. Int J Syst Evol Microbiol 60:2187-2192.

Maidak BL, Cole JR, Lilburn TG *et al.* (2001) The RDP-II (Ribosomal Database Project). Nucl Acid Res 29:173-174.

Marmur JA (1961) Procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:208-218.

Nupur BV, Srinivas TNR, Kumar PA (2013) *Flavobacterium nitratireducens* sp. nov., an amylolytic bacterium of the family *Flavobacteriaceae* isolated from coastal surface sea water. Int J Syst Evol Microbiol 63:2490-2496.

Rickard AH, Gilbert P, High NJ *et al.* (2003) Bacterial coaggregation: an integral process in the development of multi-species biofilms. Trends Microbiol 11:94-100.

Saha P, Chakrabarti T (2006) *Flavobacterium indicum* sp. nov., isolated from warm spring water in Assam, India. Int J Syst Evol Microbiol 56:2617-2621.

Stepanovic S, Ranin L, Irkovic CI *et al.* (2000) Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol 38:428-432.

Subhash Y, Sasikala C, Ramana CV (2013) *Flavobacterium aquaticum* sp. nov., isolated from a water sample of a rice field. Int J Syst Evol Microbiol 63:3463-3469 .

Tamura K, Peterson D, Peterson N *et al.* (2011) MEGA5 Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Bio Evol 28:2731-2739.

Verma DK, Rathore G (2013) Molecular characterization of *Flavobacterium columnare* isolated from natural outbreak of columnaris disease in food fish, *Catla catla* from India. J Gen Appl Microbiol 59: 417-424.

Weisburg WG, Barns SM, Pelletier DA *et al.* (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173:697-703.

Weon HY, Song MH, Jung A *et al.* (2007) *Flavobacterium terrae* sp. nov. and *Flavobacterium cucumis* sp. nov., isolated from green house soil. Int J Syst Evol Microbiol 57:1594-1598.

Yi H, Oh HM, Lee JH *et al.* (2005) *Flavobacterium antarcticum* sp. nov., a novel psychrotolerant bacterium isolated from the Antarctic. Int J Syst Evol Microbiol 55:637-641.

Zamora L, Fernández-Garayzábal JF, Sánchez-Porro C *et al.* (2013) *Flavobacterium plurextorum* sp. nov. isolated from food rainbow trout (*Oncorhynchus mykiss*) PLoSONE 8:e67741.

Zamora L, Fernandez-Garayzabal JF, Svensson-Stadler LA *et al.* (2012) *Flavobacterium oncorhynchi* sp. nov. a new species isolated from rainbow trout (*Oncorhynchus mykiss*). Syst Appl Microbiol 35:86-91.

Zhang Y (2007) Molecular characterization of *Edwardsiella* spp. and *Flavobacterium columnare*. Ph.D. thesis, Auburn University, Alabama.

Zhu L, Liu Q, Liu H *et al.* (2013) *Flavobacterium noncentrifugens* sp. nov., a psychrotolerant bacterium isolated from Hailuogou Glacier, South west China. Int J Syst Evol Microbiol 63:2032-2037.

Associate Editor: Cynthia Canêdo da Silva