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

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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 Bacteroidetes 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
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. hyalatis 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, 1xTaq DNA polymerase buffer, 10 mM dNTPs, 1.5 mM MgCl₂ 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-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 EcoRI/HindIII 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 amplions 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 out-group, while *E. coli* (EU014689) was used as major out-group 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 and entire on AO agar after 24-48 h of incubation at 28 °C and entire on AO agar after 24-48 h of incubation at 28 °C. 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. Cells were Gram-negative rods and non-endospore forming. The strains grew well in aerobic condition at temperature 18-30 °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     | F9S1   | Skin          | *Labeo rohita*   | KJ635878                | *Flavobacterium suncheonense*                | 97.91                    |
| 3     | Fl63G3 | Gills         | *Cyprinus carpio* | KJ635877                | *Flavobacterium indicum*                     | 98.60                    |
| 4     | Fl1A1  | Gills         | *Catla catla*    | KJ635870                | *Flavobacterium aquaticum*                   | 100.0                    |
| 5     | F9S2   | 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     | F9S3   | Gills         | *Catla catla*    | KJ635875                | *Flavobacterium suncheonense*                | 99.11                    |
| 9     | F9S4   | Skin          | *Labeo rohita*   | KJ635874                | *Flavobacterium suncheonense*                | 99.08                    |
| 10    | F9S5   | Skin          | *Labeo rohita*   | KJ635873                | *Flavobacterium suncheonense*                | 98.28                    |
| 11    | F9S6   | Gills         | *Cyprinus carpio* | KJ635872                | *Flavobacterium suncheonense*                | 99.16                    |
| 12    | Fl62   | Skin          | *Labeo rohita*   | KJ635871                | *Flavobacterium indicum*                     | 98.82                    |
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 (F9S1-6) grouped with *F. suncheonense*, two strains (F6I2, F6I3) with *F. indicum* and the rest four strains (F1A1, F2G1, F3H1, and F10T1) 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.

![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.](image-url)
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.

| Biochemical test                | F. terre | F. granuli | F. hercynium | F. suncheonense | F. aquaticum | F. indicum |
|--------------------------------|----------|------------|--------------|-----------------|--------------|------------|
| Motility                        | +        | +          | -            | (+)             | -            | +          |
| Congo red absorption            | -        | -          | -            | -               | -            | -          |
| Flexirubin type pigment         | -        | -          | -            | -               | -            | -          |
| Growth on Nutrient agar         | +        | +          | +            | +               | +            | +          |
| Acid produced from Fructose     | -        | -          | -            | -               | -            | -          |
| Degradation of Cellobiose       | -        | -          | -            | -               | -            | -          |
| Degradation of Starch           | -        | -          | -            | -               | -            | -          |
| Degradation of Xylose-oxidise   | -        | -          | -            | -               | -            | -          |
| Hydrogen sulphide               | -        | -          | -            | -               | -            | -          |
| Catalase production             | +        | +          | +            | +               | +            | +          |
| Indole production               | -        | -          | -            | -               | -            | -          |

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

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