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Microbial diversity of intestinal contents and mucus in yellow catfish (*Pelteobagrus fulvidraco*)

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ABSTRACT

In this study, traditional culture-based techniques and the 16S rDNA sequencing method were used to investigate the microbial community of the intestinal contents and mucosal layer in the intestine of yellow catfish (*Pelteobagrus fulvidraco*). Eleven phylotypes were detected from culturable microbiota, and their closest relatives were *Plesiomonas*, *Yersinia*, *Enterobacter*, *Shewanella*, *Aeromonas*, *Vibrio*, and *Myroides*. Forty-four phylotypes were retrieved from 100 positive clones from intestinal contents (library C), and 21 phylotypes were detected in the 57 positive clones from intestinal mucus (library M), most of which were affiliated with Proteobacteria (>50% of the total). However, the bacterial groups OP10 and Actinobacteria detected in library C were not found in library M, suggesting that the abundance and diversity of bacterial populations in mucus might be different from the microbiota in gut contents, and that some microbial species poorly colonized the gut mucosal layer.

1. Introduction

In recent years, intestinal microbiota investigations of fish have been motivated by the possible use of the biological significant bacteria as so-called probiotica (Gatesoupe, 1999). Studies on the intestinal microflora of fishes have revealed that the bacterial population structure of the intestine influences the establishment of pathogenic microorganisms in the intestinal tract (Van der Waaij, 1989; Singer and Nash, 2000; Ringø et al., 2003). The stability of the intestinal flora is an extremely important factor in the natural resistance of fish to infections produced by bacterial pathogens in the digestive tract (Ringø et al., 2003). Knowledge about the fish microbiota would help to understand the disturbances, if there are any, brought about during disease outbreaks, and it has received increasing attention.

The bacterial flora of fish, particularly the microflora of the digestive tract, has been surveyed by many researchers. In early studies, conventional culture-dependent techniques have been used. Works using these conventional methods demonstrated that fish possesses specific intestinal microbiota consisting of aerobic, facultative anaerobic, and obligate anaerobic bacteria, but the bacterial composition may vary with age, individuals, nutritional status, environmental conditions, and the complexity of the fish digestive system (Cahill, 1990; Ringø et al., 1995, 2003). Conventional culture-dependent techniques, have however limited the understanding of the complexity of the fish gut ecosystem, resulting in biased results. In natural environments, most microorganisms cannot be easily isolated and cultivated on traditional agar substrates (Van Elsas and Van Overbeek, 1993), and only an estimated 0.1–10% of bacteria can be cultivated (Amann et al., 1995). Recently, molecular methods, including the 16S rDNA clone library technique, have been successfully used to analyze the complex microbial community of fish intestine. Studies using molecular methods have retrieved many novel sequences, which have not been identified as part of the intestinal flora of fish. These results suggest that fish gut harbors a larger bacterial diversity than previously recognized and that previous understandings on microbiota should be revised (Holben et al., 2002; Martin et al., 2006; Hovda et al., 2007; Kim et al., 2007).

Yellow catfish, *Pelteobagrus fulvidraco*, is a teleost fish belonging to the order Siluriformes. It is restricted to freshwater habitats, mostly in Asia. The fish has become one of the most important freshwater aquaculture species in South China and has a high market value because of its excellent meat quality (Lee and Lee, 2005). In 2006, the total output of yellow catfish was nearly 100,000 MT (http://www.fao.org/fishery/statistics). While under intensive cultural circumstance, bacterial infectious diseases often occur, causing serious losses (Ye et al., 2009). Despite its importance in aquaculture, so far, there is no information on the microbiota of this fish.

The present study is therefore designed to analyze the 16S rDNA sequences from DNA isolated from both cultivated and noncultivated

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microorganisms from the intestinal contents and mucus of yellow catfish.

2. Materials and methods

2.1. Sample collection and isolation of culturable intestinal microbiota

In October 2008, yellow catfish was obtained from the Niushan Lake in Hubei Province, Central China. Fifty fish, with an average weight of approximately 80 g, were killed using high doses of anaesthesia (Tricaine methanesulfonate, Finquel MS 222, Sigma Inc.).

Culturable microbiota was isolated from intestinal contents and mucus of the fish. The intestine was aseptically removed from the fish abdominal cavity, and the contents were squeezed out and mixed. One gram (wet weight) of the gut contents was homogenized for 5 min using tissue grinders and vortexed severely in 9 ml of sterile phosphate buffered saline (PBS; 0.1 mol l^{-1}, pH 7.2). Then, 0.1 ml of the dilutions 10^{-5}, 10^{-6}, and 10^{-7} were spread over triplicate plates of tryptone soya agar (TSA; Becton, Dickinson and Company, USA). The plates were incubated at 20 °C for 2 days in order to determine the aerobic plate count of the cultivable bacteria present. Forty-seven colonies, as determined by colony morphology, Gram-staining, and catalase-reactions, were selected, and these isolates were purified by streaking and re-streaking on fresh media. Pure cultures were stored at −70 °C in tryptone soya broth (TSB) supplemented with 15% (v/v) glycerol as cryopreservant.

Separately, intestinal mucus was collected from the same 50 fish. After removing the gut content for cultivation, the digestive tracts were rinsed five times in sterile PBS. The mucus was then scraped off with a sterilized scalpel and collected together into a sterile microcentrifuge tube. Similarly, the mucus was homogenized and serially diluted to 10^{-4}, 10^{-5}, and 10^{-6} in PBS. Finally, the homogenous intestinal mucus was plated for triplicate plates on TSA and incubated at 20 °C for 2 days. Overall, twenty-seven isolates in the case of colony morphology and Gram-staining reaction were purified and examined for 16S rDNA sequencing.

2.2. DNA extraction, PCR amplification and DNA sequencing for cultured bacteria from intestine samples

For cultured microbiota, 74 colonies (47 from intestinal contents and 27 from mucus) were selected for the following analyses. The colonies were cultivated in TSB at 37 °C for 24 h and harvested by centrifugation at 4000 *g* for 20 min. DNA was extracted by using a bacterial genomic DNA extraction kit (BioTeke Corporation, China) following the manufacturer's protocols. To ensure extraction of DNA from Gram-positive bacteria, a lysozyme treatment was performed in advance. The extracted DNA was eluted into 25 μl TE buffer (pH 8.0) and stored at −20 °C.

The two universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1491R (5′-GTTTACCTTGTTACGACTT-3′), corresponding to the nucleotides 27–1491 on the 16S rDNA of *Escherichia coli*, were used to amplify the 16S rDNA from the isolates (Hovda et al., 2007). To each PCR tube, final concentrations of the following reagents were included; 20 ng bacterial genomic DNA, 0.4 μM of the two primers, 0.2 mM of each dNTP solution, 1 × PCR reaction buffer, 2.4 U of TaKara Ex Taq DNA polymerase (TaKaRa Corporation Ltd., Dalian, China) and double-distilled water to a final volume of 100 μl. The PCR reactions were performed on a PTC-100TM Programmable Thermal Controller (MJ Research, Inc., Watertown, USA) with the following cycling conditions: initial denaturation at 94 °C for 4 min, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 53 °C, and 90 s elongation at 72 °C, and a final 10 min extension at 72 °C. PCR amplification products were visualized on ethidium bromide-stained 1.0% agarose-TAE gels under ultraviolet (UV) light and purified with spin columns (Wizard PCR Prep, Promega, USA). The purified products were cloned into pMD18-T vector (TaKaRa Corporation Ltd., Dalian, China) following the manufacturer's protocol. Finally, clones were sequenced on an automatic DNA sequencer (model 3730, ABI Applied Biosystems, Foster City, California, USA) by using plasmid flanking sequence primers M13(+)/M13(−).

2.3. Direct DNA extraction from intestine samples and construction of 16S rDNA clone libraries

The mixed samples from intestinal contents (library C) and mucus (library M) were also used to construct two different 16S rDNA clone libraries. DNA was extracted using a bacterial genomic DNA extraction kit (BioTeke Corporation, China) following the manufacturer's protocols. The genomic DNA of the intestinal content and of the mucus were each extracted twice, and the same DNA sample was pooled together to minimize bias in sampling. For the same reason, the following PCR reaction was also performed twice, and the same PCR products were pooled together as one sample. The PCRs were performed as described using primers 27F and 1491R. The purified PCR products were cloned into the pGEM-T vector system (Promega, Southampton, U.K.) according to the manufacturer's instructions, and they were transformed to *E. coli* Top10 strain. The presence of inserts was distinguished using blue white screening. One hundred and fifty-seven white colonies (ampicillin-resistant transformants) were retrieved from the samples of gut contents and mucus, respectively. These colonies were cultured in LB broth overnight at 37 °C, and 2 mL LB broth containing bacteria was used to amplify the 16S rDNA fragment using the primers 27F and 1491R. Samples containing insert DNA were then fully bidirectionally sequenced on an ABI 3730 DNA automatic sequencer (Applied Biosystems, Inc., USA).

2.4. Sequence analysis

The 16S rDNA sequences were checked for chimeric constructs by using the CHECK_CHIMERA program of the ribosomal database project (RDP) web site (http://rdp.cme.msu.edu/) (Cole et al., 2005). Sequence similarity analysis was performed on MatGAT 2.0 (Campanella et al., 2003), with 99% minimum similarity as the threshold for any pair of sequences in a phylotype. The phylogenetic relationships of representative microbes (different phylotypes) covered in the present study were determined by comparing individual rDNA sequences with those published in the GenBank database through the BLAST program (Altschul et al., 1990).

3. Results

A total of 231 nucleotide sequences were collected from the bacterial flora of yellow catfish intestine. The total contained 74 sequences retrieved from the cultured microflora isolated from intestinal contents (47 sequences) and mucus (27 sequences), and 157 sequences were from library C (100 sequences) and library M (57 sequences). Of these sequences, 76 phylotypes were recognized. These phylotypes were compared using the BLAST program in the GenBank website and deposited in the GenBank database under accession numbers GQ359955–GQ360030. In the present study, no sequence was found to be chimeric.

3.1. Enumeration of the intestinal microflora

A number of average aerobic and facultative anaerobic bacteria were 3.4×10^8 colony-forming units (cfu)/g in the intestinal content and 2.1×10^7 cfu/g in the intestinal mucus, respectively.

3.2. Characterization of pure cultures

The 74 colonies cultured on the TSA were sequenced by determining the 16S rDNA sequence. These sequences were analyzed using 99% minimum similarity as the threshold for any pair of sequences in a phylotype. Eleven phylotypes were found in total, and they were compared by the BLAST program. The results are presented in Tables 1 and 2. According to the results, the closest relatives were from Enterobacteriaceae, Aeromonadaceae, and Shewanellaceae for the cultures from the intestinal contents, and from Enterobacteriaceae, Shewanellaceae, Aeromonadaceae, Flavobacteriaceae, and Vibrionaceae for the isolates from the gut mucus. Concretely, the bacteria isolated from intestinal contents comprised of the genera *Plesiomonas*, *Yersinia*, *Enterobacter*, *Shewanella*, and *Aeromonas*, while the bacteria isolated from the gut mucus comprised of the genera *Plesiomonas*, *Vibrio*, *Myroides*, *Aeromonas*, and *Shewanella*.

3.3. Characterization of 16S rDNA libraries

Forty-four phylotypes were observed in the 100 positive clones from library C. Within these bacterial clones, 62 were represented by 6 phylotypes including at least 2 clones, while the remaining 38 clones were represented by sequence types that included a single clone. The nearest relatives to the phylotypes were obtained using the BLAST program, and are presented in Table 3. As some of the phylotypes could not be identified to the genus level by the program, they were analyzed by using the Seqmatch program in the RDP website. Our results showed that the microbiota of library C contained a diverse range of bacteria, including *Cetobacterium*, *Agrobacterium*, *Sphingomonas*, *Ochrobactrum*, *Ralstonia*, *Herbaspirillum*, *Aeromonas*, *Plesiomonas*, *Escherichia*, *Edwardsiella,* *Acinetobacter*, *Enterococcus*, *Clostridium*, *Anoxybacillus*, *Lactococcus*, "*Peptostreptococcaceae Incertae Sedis*", *Microbacterium*, *Corynebacterium*, and *Chryseobacterium*. However, two clones, IC29 and IC76, could only be identified at the family level (Lachnospiraceae); one phylotype IC45 could only be identified at the order level (Bacteroidales); and three cultures IC20, IC61, and IC95 could only be ascribed to bacteria.

The sequences related to *C. somerae* (26/100) and *P. shigelloides* (23/100) were most frequent in the library. Isolate IC1, the most abundant phylotype in the library, included 25 clones that were nearly related (99.8% similarities) to *C. somerae*. The second most abundant phylotype IC9 contained 17 clones that were related (99.5% similarities) to *P. shigelloides*. The third most abundant phylotype IC2, representing 11 clones, was related (99.9% similarities) to *Agrobacterium tumefaciens*.

Twenty-one phylotypes were detected from the 57 positive clones of intestinal mucus (library M). Within these clones, 44 were represented by 8 phylotypes including at least 2 clones, while the remaining 13 clones were represented by a phylotype with a single clone. The closest relatives to the phylotypes were obtained through the GenBank database and Seqmatch program, and they are shown in Table 4. Unlike library C, these phylotypes were grouped into five genera, and their closest relatives were *Edwardsiella*, *Plesiomonas*, *Aeromonas*, *Cetobacterium*, and *Clostridium*. In addition, phylotype IM3 could only be identified at the family (Porphyromonadaceae) level, and clones IM18 and IM29 could only be identified at the order level (Bacteroidales).

The sequences related to *C. somerae* (18/57) and *P. shigelloides* (17/57) were most common in the library. Clone IM4, the most abundant phylotype in the library, comprised 16 clones closely related (99.6% similarities) to *P. shigelloides*. The phylotype IM2 included 13 bacterial clones, displaying 99.5% similarity to *C. somerae*, and represented the second most abundant phylotype. The four cultures represented by IM11, the third most abundant phylotype, were most closely related (99.9% similarities) to *A. veronii*.

Both libraries were compared by using the classifier tool in RDP, as shown in Table 5. These results show that the dominant group was Proteobacteria (>50%) in both libraries, whereas Actinobact (2.0%) and OP10 (1.0%) and an unclassified_bacteria (3.0%) were only found in library C.

4. Discussion

Previous studies have demonstrated that bacterial counts varied between individual fish (Trust and Sparrow, 1974; Spanggaard et al., 2000). Thus, this study takes samples from 50 fish to minimize the influence of these variations. We found that the total viable counts (TVCs) were 10^7–10^8 cfu/g in the intestine. Generally, this result is consistent with those in the previous studies (Trust and Sparrow 1974; Ringø et al., 2006; Kim et al., 2007; Hovda et al., 2007). However, these values are significantly lower compared with those reported for humans and terrestrial animals (approximately 10^{11} cfu/g) (Moore and Holdeman, 1974; Mead, 1997). As the present study can only enumerate the number of aerobic and facultative anaerobic bacteria, the discrepancy may probably reflect the much higher number of anaerobes (10^{10}–10^{11} cfu/g) (Suau et al., 1999; Marteau et al., 2001) in the intestine of endotherms when compared with that of fish.

The present study showed that the TVCs were 3.4×10^8 cfu/g in the intestinal content and 2.1×10^7 cfu/g in the intestinal mucus. This agrees with Spanggaard et al. (2000) and Austin and Al-Zahrani

Table 1
Representative of cultured bacteria isolated from the intestinal contents of yellow catfish.

Phylogenetic group	Isolate code	Closest relative (obtained from BLAST search)	Identity	Length	No. of clones	Accession no.
Shewanellaceae	PIC1	*Shewanella putrefaciens* CN-32, complete genome (CP000681)	99.4	1504	1	GQ359955
Aeromonadaceae	PIC2	16S rRNA *Aeromonas allosaccharophila*, strain CECT 4199, rRNA, 1502 nt (S39232)	99.6	1506	32	GQ359956
Enterobacteriaceae	PIC3	*Plesiomonas shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	99.7	1502	11	GQ359957
	PIC8	*Yersinia ruckeri* 16S ribosomal RNA gene, partial sequence (EU401667)	99.5	1504	1	GQ359958
	PIC15	*Enterobacter* sp. 638, complete genome (CP000653)	99.1	1501	2	GQ359959

Table 2
Representative sequences of cultured microbiota isolated from the intestinal mucus of yellow catfish.

Phylogenetic group	Isolate code	Closest relative (obtained from BLAST search)	Identity	Length	No. of clones	Accession no.
Shewanellaceae	PIM1	*Shewanella* sp.S4 16S ribosomal RNA gene, partial sequence (FJ589031)	99.5	1504	6	GQ359960
Aeromonadaceae	PIM2	*Aeromonas veronii* strain WE08 16S ribosomal RNA gene, partial sequence (EU770306)	99.7	1507	6	GQ359961
Enterobacteriaceae	PIM3	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	99.1	1504	11	GQ359962
	PIM23	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	99	1493	1	GQ359965
Vibrionaceae	PIM9	*Vibrio cholerae* O395 chromosome 2, complete genome (CP000627)	96.1	1504	1	GQ359963
Flavobacteriaceae	PIM10	*Myroides odoratimimus* strain GJ1-8 16S ribosomal RNA gene, partial sequence (EU331413)	98.8	1479	2	GQ359964

Table 3
Representative sequences of clone library C isolated from the intestinal contents of yellow catfish.

Phylogenetic group	Isolate code	Closest relative (obtained from BLAST search)	Identity	Length	No. of clones	Accession no.
Rhizobiaceae	IC2	*Agrobacterium tumefaciens* 16S ribosomal RNA gene, partial sequence (DQ468100)	99.9	1446	11	GQ359967
	IC66	*A. tumefaciens* 16S ribosomal RNA gene, partial sequence (DQ468100)	98.3	1449	1	GQ359996
	IC78	*A. tumefaciens* 16S ribosomal RNA gene, partial sequence (DQ468100)	97.8	1446	1	GQ359999
	IC91	*A. tumefaciens* 16S ribosomal RNA gene, partial sequence (DQ468100)	97	1437	1	GQ360006
Sphingomonadaceae	IC19	*Sphingomonas* sp. MN57.2a partial 16S rRNA gene, isolate MN57.2a (AM159534)	96.2	1481	1	GQ359975
Brucellaceae	IC79	*Ochrobactrum* sp. 1605 16S ribosomal RNA gene, partial sequence (DQ989292)	99.2	1444	1	GQ360000
Burkholderiaceae	IC4	*Ralstonia pickettii* 12 J chromosome 2, complete sequence (CP001069)	99.9	1499	7	GQ359968
	IC54	*R. pickettii* 12 J chromosome 2, complete sequence (CP001069)	99.7	1500	2	GQ359991
Oxalobacteraceae	IC30	*Herbaspirillum* sp. R740 16S ribosomal RNA gene, partial sequence (EU090893)	99.8	1494	1	GQ359980
Aeromonadaceae	IC8	*Aeromonas punctata* strain 219c 16S ribosomal RNA gene, partial sequence (FJ168776)	99.7	1506	1	GQ359971
	IC52	*A. veronii* strain LMG13695 16S ribosomal RNA gene, partial sequence (EU488698)	97	1504	1	GQ359990
	IC88	*A. veronii* strain WE11 16S ribosomal RNA gene, partial Sequence (EU770307)	99.3	1508	1	GQ360004
	IC40	Uncultured bacterium clone aaa96f04 16S ribosomal RNA gene, partial sequence (DQ817154)	95.7	1508	1	GQ359986
Enterobacteriaceae	IC9	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	99.5	1503	17	GQ359972
	IC27	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	98.5	1471	1	GQ359978
	IC31	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	99	1469	1	GQ359981
	IC36	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	98.5	1505	1	GQ359983
	IC71	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	99.5	1492	1	GQ359997
	IC84	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	98.3	1504	1	GQ360002
	IC100	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	96.5	1506	1	GQ360009
	IC56	Uncultured bacterium clone KD3-89 16S ribosomal RNA gene, partial sequence (AY218620)	86.7	1492	1	GQ359993
	IC35	*Escherichia coli* BW2952, complete genome (CP001396)	99.9	1496	1	GQ359982
	IC39	*Edwardsiella ictaluri* glutamate racemase (murI) gene, partial cds; rrnH operon and tRNA-Asp genes, complete sequence; and putative LysR family transcriptional regulatory protein gene, partial cds (EU285525)	97	1505	1	GQ359985
Moraxellaceae	IC13	*Moraxella* sp. D30C2A 16S ribosomal RNA gene, partial sequence (AY162144)	99.3	1493	1	GQ359974
	IC86	*Acinetobacter* sp. EH 28 16S ribosomal RNA gene, partial sequence (EU703817)	99.7	1447	1	GQ360003
Fusobacteriaceae	IC1	*Cetobacterium somerae* 16S rRNA gene, strain WAL 14325 (AJ438155)	99.8	1474	25	GQ359966
	IC22	*C. somerae* gene for 16S rRNA, partial sequence, strain: AG39 (AB353123)	99.9	1254	1	GQ359977
	IC12	*Cetobacterium ceti* 16S rRNA gene, strain M-3333; NCFB 3026 (X78419)	98.2	1474	1	GQ359973
Enterococcaceae	IC6	*Enterococcus saccharominimus* 16S rRNA gene, strain LMG 22197 (AJ626904)	95.4	1500	1	GQ359969
Clostridiaceae	IC7	*Clostridium* sp. PN6-15 16S ribosomal RNA gene, partial sequence (FJ357796)	98	1475	1	GQ359970
	IC93	*Clostridium* sp. zx5 16S ribosomal RNA gene, partial sequence (EF052864)	99.5	1452	1	GQ360007
Bacillaceae	IC48	*Anoxybacillus flavithermus* clone LK4 16S ribosomal RNA gene, partial sequence (EU816689)	99.9	1517	1	GQ359988
Streptococcaceae	IC55	*Lactococcus garvieae* isolate M79 16S ribosomal RNA gene, partial sequence (AY699289)	99.8	1510	1	GQ359992
Lachnospiraceae	IC29	Uncultured bacterium clone HH_aai35f10 16S ribosomal RNA gene, partial sequence (EU459290)	96	1484	1	GQ359979
	IC76	Uncultured bacterium clone p-5278-2Wa3 16S ribosomal RNA gene, partial sequence (AF371938)	96.3	1487	1	GQ359998
Peptostreptococcaceae	IC37	Uncultured bacterium partial 16S rRNA gene, clone SMG72 (AM930298)	97.1	1464	1	GQ359984
	IC82	Uncultured bacterium clone JSC8-B12 16S ribosomal RNA gene, partial sequence (DQ532227)	98	1466	1	GQ360001
Microbacteriaceae	IC50	*Microbacterium lacticum* strain 3075 16S ribosomal RNA gene, partial sequence (EU714351)	99.7	1364	1	GQ359989
Corynebacteriaceae	IC89	*Corynebacterium* sp. CIP107067 partial 16S rRNA gene (AJ438051)	99.9	1479	1	GQ360005
Flavobacteriaceae	IC58	*Chryseobacterium haifense* strain H38 16S ribosomal RNA gene, partial sequence (EF204450)	98.2	1478	1	GQ359994
Bacteroidales	IC45	Uncultured bacterium clone AA3 16S ribosomal RNA gene, partial sequence (EU884933)	90.8	1485	2	GQ359987
Bacteria; environmental samples	IC20	Uncultured bacterium clone D5 16S ribosomal RNA gene, partial sequence (AY375144)	99.4	1453	1	GQ359976
	IC61	Uncultured bacterium clone HB101 16S ribosomal RNA gene, partial sequence (EF648093)	90.5	1471	1	GQ359995
	IC95	Uncultured bacterium clone MFBC1A04 16S ribosomal RNA gene, partial sequence (EU592819)	95.6	1475	1	GQ360008

(1988), who demonstrated that the number of average aerobic and facultative anaerobic bacteria was higher in intestinal content than in intestinal mucus. However, these results are inconsistent with those reported by Kim et al. (2007), who found that the number of bacteria from the intestinal content and the mucus was almost equal ($4.8 \pm 6.4 \times 10^6$ and $3.0 \pm 4.6 \times 10^6$).

It is difficult to identify the representatives from the intestinal microbiota by using traditional morphological and biochemical criteria (Kim et al., 2007). The present study used 16S rDNA sequencing to differentiate between the isolates. A total of 74 clones isolated from TSA were sequenced. The results showed that the contents and mucus shared the microflora of the genera *Shewanella*, *Aeromonas*, and *Plesiomonas*, while members of the genera *Yersinia* and *Enterobacter* were associated with the content, and members of the genera *Vibrio* and *Myroides* were isolated from the mucus. Species of these genera have been isolated and cultured from various fishes (Pond et al., 2006; Hovda et al., 2007; Kim et al., 2007). Of the species/groups identified in this study, aeromonads are the most common organisms in the guts of freshwater fish and are commonly isolated from normal healthy fish. Some certain strains of these bacteria possess the virulence factors necessary to induce disease (Cahill, 1990). *Vibrio* species have previously been detected in gut of a wide variety of fishes, such as halibut larvae (Verner-Jeffreys et al., 2003; Jensen et al., 2004), hybrid tilapia (*Oreochromis niloticus*) (Al-Harbi and Uddin, 2004), Atlantic cod (*Gadus morhua*) (Korsnes et al., 2006; Ringø et al., 2006), Atlantic salmon (*Salmo salar*) (Hovda et al., 2007) and so on, and the importance of the genera *Vibrio* in aquaculture have been discussed (Thompson et al., 2004; Austin, 2006). *Shewanella putrefaciens* is a potential pathogen of fish, and has been isolated from the intestine of rainbow trout (*Oncorhynchus mykiss*) (Huber et al., 2004; Koziñska and Pêkala, 2004; Pond et al., 2006). *Plesiomonas shigelloides* has been isolated from aquacultured striped bass (*Morone saxatilis*) (Nedoluha and Westhoff, 1997), wild pike (*Esox lucius*) (González et al., 1999), tilapia (Sugita et al., 1987, 1988), and rainbow trout (Pond et al., 2006). Sugita et al. (1988) suggested that *P. shigelloides* is a transient bacterium that appears in intestinal samples without being established. However, our results indicate that *P. shigelloides* occurs in the mucus and may be regarded as an indigenous species. The genus *Myroides* contains two species, namely *M. odoratus* (type species) and *M. odoratimimus* (Vancanneyt et al., 1996). *Myroides* has only been detected in the case of marine fish (Engelbrecht, 1992), and the present study represents the first report of the bacteria from

Table 4
Representative sequences of clone library M isolated from the intestinal mucus of yellow catfish.

Phylogenetic group	Isolate code	Closest relative (obtained from BLAST search)	Identity	Length	No. of clones	Accession no.
Enterobacteriaceae	IM1	*E. ictaluri* glutamate racemase (murI) gene, partial cds; rrnH operon and tRNA-Asp genes, complete sequence; and putative LysR family transcriptional regulatory protein gene, partial cds. (EU285525)	99.1	1505	1	GQ360010
	IM4	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 X60418)	99.6	1505	16	GQ360013
	IM14	*P. shigelloides* 16S rRNA gene, strain NCIMB9242 (X60418)	97.3	1504	1	GQ360019
Aeromonadaceae	IM9	*A. veronii* strain LMG13695 16S ribosomal RNA gene, partial sequence (EU488698)	95.8	1504	2	GQ360016
	IM12	*A. veronii* strain WE11 16S ribosomal RNA gene, partial sequence (EU770307)	97.8	1506	1	GQ360018
	IM19	*A. veronii* bv. veronii 16S rRNA gene, strain ATCC 35624 (X60414)	97.1	1504	1	GQ360022
	IM26	*A. veronii* strain IQ105 16S ribosomal RNA gene, partial sequence (EU770287)	95.8	1506	1	GQ360024
	IM11	*A. veronii* strain WE08 16S ribosomal RNA gene, partial sequence (EU770306)	99.9	1507	4	GQ360017
	IM32	*A. veronii* strain WE08 16S ribosomal RNA gene, partial sequence (EU770306)	97.2	1506	1	GQ360026
	IM42	*A. veronii* strain WE08 16S ribosomal RNA gene, partial sequence (EU770306)	96	1505	1	GQ360028
	IM53	*A. veronii* strain IH118 16S ribosomal RNA gene, partial sequence (EU770282)	97.2	1497	1	GQ360030
Fusobacteriaceae	IM2	*C. somerae* 16S rRNA gene, strain WAL 14325 (AJ438155)	99.5	1474	13	GQ360011
	IM5	*C. somerae* 16S rRNA gene, strain WAL 14325 (AJ438155)	97.8	1474	1	GQ360014
	IM16	*C. somerae* 16S rRNA gene, strain WAL 14325 (AJ438155)	92.5	1472	2	GQ360020
	IM52	*C. somerae* 16S rRNA gene, strain WAL 14325 (AJ438155)	92.1	1471	1	GQ360029
	IM7	*C. somerae* gene for 16S rRNA, partial sequence, strain: AG39 (AB353123)	99.8	1482	1	GQ360015
Clostridiaceae, Clostridium	IM21	Uncultured bacterium clone aaa74e11 16S ribosomal RNA gene, partial sequence (DQ817375)	97.2	1478	1	GQ360023
	IM34	Uncultured bacterium clone PBF_b50 16S ribosomal RNA gene, partial sequence (FJ375819)	97.8	1476	2	GQ360027
Porphyromonadaceae	IM3	Uncultured bacterium clone WF16S_154 16S ribosomal RNA gene, partial sequence (EU939416)	91.2	1488	3	GQ360012
Bacteroidales	IM18	Uncultured bacterium clone AA3 16S ribosomal RNA gene, partial sequence (EU884933)	91	1486	1	GQ360021
	IM29	Uncultured bacterium clone AA3 16S ribosomal RNA gene, partial sequence (EU884933)	91.1	1486	2	GQ360025

freshwater fish. *Yersinia ruckeri* is the causative agent of enteric redmouth disease (ERM) or yersiniosis, a serious infectious disease of fish that has caused high economic losses in the rainbow trout farming industry in many countries (Furones et al., 1993). It has been recovered from rainbow trout (Austin et al., 2003), Silver carp (*Hypophthalmichthys molitrix*), and bighead carp (*Aristichthys nobilis*) (Xu et al, 1991). Members of the genus *Enterobacter* are common in the digestive tract of freshwater and marine fishes. For example, they have been found in the intestine of rainbow trout (Pond et al., 2006), hybrid tilapia (He et al., 2009), and yellow grouper (*Epinephelus awoara*) (Zhou et al., 2009).

In the present study, 65 phylotypes were obtained from library C and library M. Of these phylotypes, some were unique and had not previously been detected in fish. Phylotypes are as follows: *Cetobacterium ceti*, *A. tumefaciens*, *Herbaspirillum* sp., *Anoxybacillus flavithermus*, *Microbacterium lacticum*, and *Corynebacterium* sp. In addition to these unique phylotypes, the following unknown bacteria with isolate codes: IC40, IC56, IC29, IC76, IC45, IC20, IC61, IC95, IM3, IM18, and IM29 were recovered. These findings are inconsistent with Ringø et al. (1995) and Ringø and Birkbeck (1999), who suggested that the intestinal tract microbiota of fish are simpler than those of endothermic animals. However, our results are in accordance with Hovda et al. (2007) and Ringø et al. (2006), who demonstrated that the alimentary microflora of fish was more complex than earlier recognized.

Some phylotypes that are closely related to *Sphingomonas* sp. (Isolate IC19, similarity = 96.2%), *Aeromonas* (IC40, similarity = 95.7%), *P. shigelloides* (IC100, similarity = 96.5%), *Plesiomonas* (IC56, similarity = 86.7%), *E. saccharominimus* (IC6, similarity = 95.4%), *A. veronii* (IM9, similarity = 95.8%; IM26, similarity = 95.8%; IM42, similarity = 96%), and *C. somerae* (IM16, similarity = 92.5%; IM52, similarity = 92.1%) might be assigned to new groups. Stackebrandt and Goebel (1994) suggested that the prokaryote in which its 16S rDNA sequence differed by more than 3% and 5–7% from that of all other organisms of one group might be regarded as a new species and genus, respectively.

Of the phylotypes of library C and library M, *Edwardsiella ictaluri* and *Lactococcus garvieae* are important in aquiculture. *E. ictaluri* is a Gram-negative, rod-shaped bacterium belonging to the family Enterobacteriaceae. The bacterium may cause enteric septicemia of channel catfish (Hawke, 1979; Hawke et al., 1981; Waltman et al., 1986), the most important disease in commercially raised channel catfish (Morrison and Plumb, 1994). Outbreaks of edwardsiellosis caused by *E. ictaluri* have occurred in a variety of fish species including channel catfish (*Ictalurus punctatus*), rosy barb (*Puntius conchonius*), white catfish (*I. catus*), brown bullhead (*I. bebulosus*), green knife fish (*Eigemannia virens*), danio (*Danio devario*), walking catfish (*Clarias batrachus*), freshwater catfish (*Pangasius hypophthalmus*), tadpole madtom (*Noturus gyrinus*), rainbow trout, and yellow catfish (Ye et al., 2009). *Lactococcus garvieae* is a Gram-positive, coccal bacterium

Table 5
Comparison between library C and library M analysed by the classifier program in ribosomal database project.

Library C (n = 100)	%	Phylum	%	Library M (n = 57)
	1.0	OP10	0	
	2.0	Actinobacteria	0	
	3.0	Bacteroidetes	10.5	
	9.0	Firmicutes	5.3	
	55.0	Proteobacteria	52.6	
	27.0	Fusobacteria	31.6	
	3.0	Unclassified_Bacteria	0	

belonging to the family Streptococcaceae. The bacterium is the etiological agent of "lactococcosis," which has become a major economic problem in the fish farming industry, particularly during the summer, given its association with high water temperature. This disease affects cultured freshwater and marine fish with special incidence in rainbow trout (Eldar et al., 1999), yellowtail (*Seriola quinqueradiata*) (Kusuda and Kawai, 1998), and flounder (*Platichthys flesus*) (Fang et al., 2006).

In the present study, the microfloras of intestinal contents and mucus were investigated. Our results suggested that the dominant bacterial species from both library C and library M belonged to Proteobacteria. This is consistent with Kim et al. (2007) and Zhou et al. (2009), who demonstrated that the most common bacteria from fish intestine were affiliated with Proteobacteria. However, compared with library M, library C showed much higher microflora diversities as the bacterial groups OP10 and Actinobacteria occurred. The representative sequences most closely related to *C. ceti*, *A. tumefaciens*, *Ochrobactrum* sp., *Ralstonia pickettii*, *Herbaspirillum* sp., *Aeromonas punctata*, *E. coli*, *Moraxella* sp., *Acinetobacter* sp., *Clostridium* sp., *A. flavithermus*, *L. garvieae*, *M. lacticum*, *Corynebacterium* sp., and *Chryseobacterium haifense* detected in library C were not obtained from intestinal mucus. These findings are in line with Kim et al. (2007), who suggested that the abundance and diversity of bacterial populations in mucus might be different from the microbiota in gut contents, and that some microbial species would poorly colonize the gut mucosal layer.

Of particular interest is that most of the bacteria, such as *S. putrefaciens*, *E. ictaluri* and *L. garvieae*, found in the intestinal content and mucus are opportunistic pathogens. This is in accordance with Pond et al. (2006), who proposed that fish digestive tract is a reservoir for many pathogens. The overgrowth of pathogens and the transmission of disease may then break out following a breach of intestinal microflora due to a stressor such as intensive farming cultivation (Ringø and Birkbeck, 1999).

Overall, the present study shows that the molecular approach using 16S rDNA clone library is a very useful tool to determine the microbial diversity of yellow catfish intestine. Our results indicate that the alimentary microflora of fish is more complex than earlier recognized and that most of the bacteria found in intestinal content and mucus are opportunistic pathogens.

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