Original Article

Diversity of Non-*Flavobacterium columnare* Bacteria Associated with Columnaris-like Diseased Fish

Ha Thanh Dong[1] Vuong Viet Nguyen[1] Wanniwat Mata[1] Pattanapon Kayansamruaj[1]

Saengchan Senapin[2,3] Dachrit Nilubol[1] Channarong Rodkhum[1*]

Abstract

Numerous yellow and pink pigmented bacterial isolates had been recovered from diseased fish exhibiting columnaris-like disease in tilapia (*Oreochromis* spp.) farms in Thailand. The predominant species had been identified as *Flavobacterium columnare* while taxonomic classification of the remaining isolates and their pathogenic potential remain undetermined. An additional yellow bacterial strain had also been obtained from a koi carp sample showing columnaris-like symptoms. To continue our previous work, this study described the identification of ten representatives of unknown culturable non-*Flavobacterium columnare* bacteria based on a combination of phenotypic characteristics and nucleotides homology of 16S rRNA gene and subsequently investigated their pathogenicity in Nile tilapia (*Oreochromis niloticus*) fingerlings. The majority of the yellow pigmented bacteria were identified as *Chryseobacterium* spp. while the remainders were identified as [*Flexibacter*] *aurantiacus* subsp. *excathedrus* and *Flavobacterium indicum*. The pink pigmented bacteria were identified as *Flectobacillus roseus*. Five representative species of the identified bacteria isolated from diseased tilapia were individually subjected to a pathogenicity test in healthy Nile tilapia fingerlings. The experimental challenge results within 14 days revealed that the tested bacteria exhibited low or no virulence to the fish (0-20% cumulative mortality). This suggests that the identified bacteria merely served as opportunistic pathogens that may require stressors for disease manifestation.

Keywords: 16S rRNA, non-*Flavobacterium columnare* bacteria, columnaris-like

[1]*Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, THAILAND*

[2]*National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani 12120, THAILAND*

[3]*Center of Excellence for Shrimp Molecular Biology and Biotechnology, Mahidol University, 272 Rama VI Road, Bangkok 10400, THAILAND*

***Correspondence**: channarong_r@yahoo.com, Channarong.R@chula.ac.th*

Introduction

Columnaris disease caused by *Flavobacterium columnare* has been reported in over 37 fresh water fish species worldwide (Anderson and Conroy, 1969; Noga, 2010). Diseased fish usually exhibit typical external lesions such as eroded fins and epidermis ulcers or necrotic gills, which results in mass mortality and severe financial losses for aquaculture producers (Davis, 1922; Figueiredo et al., 2005; Dong et al., 2015a). With respect to disease diagnosis in most fish disease laboratories, suspected cases of columnaris disease are generally subjected for *F. columnare* isolation using recommended selective media such as Anacker and Ordal's agar (Anacker and Ordal, 1955) or modified Shield agar (Decostere et al., 1997). Our previous study (Dong et al., 2015a) was performed in the same manner and found that numerous yellow and pink pigmented bacterial isolates were concurrently recovered from diseased fish in Thai tilapia farms exhibiting columnaris-like symptoms. The predominant species was identified as *F. columnare* and exhibited high virulence through experimental challenge using the fry red tilapia model (Dong et al., 2015a, d). Taxonomic classification of the remaining isolates and their ability to cause disease in fish remains undetermined. The reality in intensively cultured fish farms is that the manifestation of disease involves multiple potential pathogens (Dong et al., 2015c, b). Therefore, beside the well-described pathogens, the presence of other pathogens and their potential contribution to disease manifestation should be uncovered. The objectives of this study, therefore, were to identify the remaining unknown culturable non-*Flavobacterium columnare* bacteria, which are involved in the columnaris-like disease in fish, and investigate their pathogenicity in Nile tilapia fingerlings.

Materials and Methods

Bacterial isolates and growth conditions: Eight yellow and two pink pigmented bacterial isolates previously recovered from necrotic gills, skin ulcers, or kidney of red tilapia (*Oreochromis* sp.), Nile tilapia (*Oreochromis niloticus*), and koi carp (*Cyprinus carpio*) exhibiting columnaris-like disease (Dong et al., 2015d) were subjected for species identification in this study. All isolates used in this study were designated with the organism code, CUVET as summarized in Table 1. Isolation of the bacteria was described in an earlier work (Dong et al., 2015a). Briefly, a sterile loop that was inserted into the diseased fish tissues was primarily streaked on an Anacker and Ordal's agar (AOA) (Anacker and Ordal, 1955) supplemented with Neomycin (Sigma) 0.5 mg mL^{-1} and Polymycin B (Sigma-Aldrich) 200 IU mL^{-1} (Anacker and Ordal, 1955) or modified Shield agar added with Tobramycin (Sigma) 1 µg mL^{-1} (Decostere et al., 1997). Suspected colonies were then sub-cultured for further isolation on AOA without antibiotic. Bacterial preservation was performed using AO broth containing 20% glycerol and preserved in -80°C until needed. Prior to phenotypic and experimental challenge assays, fresh isolates were recovered by spreading 30 µL of bacterial stock on AOA supplemented with 0.5 g $MgSO_4.7H_2O$ and 0.5 g $CaCl_2.2H_2O$ L^{-1}, incubated at 28°C for 24-36 h.

Phenotypic tests: Conventional biochemical characteristics of the ten bacterial isolates were performed as described by Bernardet (1989) and Dong et al. (2015a). The isolates were examined for colony morphology, bacterial cell shape, Gram stain, ability to grow on MacConkey and tryptic soy agar (TSA) media, catalase, oxidase, and the presence of flexirubin pigment. Carbohydrate metabolism was determined using modified Anacker and Ordal's medium (AO) (Anacker and Ordal, 1955) that contained 0.5% tryptone, 0.5% yeast extract, 0.2% phenol red as indicator, and 1% carbohydrate (arabinose, glucose, sucrose, maltose, mannitol, lactose and trehalose). AO agar supplemented with gelatin (1%), skim milk (5%) or starch (5%) was used to test for degradation of gelatin, casein, and starch, respectively. AO agar supplemented with 0.1% esculin and 10% bile salts was prepared for esculine hydrolysis test. Sodium chloride tolerance was performed by growing the isolates in AO broth containing 0, 1, 1.5, and 2% NaCl for 5 days at 28°C (Table 2).

Table 1 Description of yellow and pink pigmented bacterial isolates obtained from fish exhibiting columnaris-like disease

Isolates/Color	Host	Organ	16S rRNA GenBank accession number	Most closely related species	Identity (%)
1.**CUVET1205**Yellow	NT	Skin	KJ190166	*Candidatus Chryseobacterium massiliae* (AF531766)	99.2
2.**CUVET1206***Pale yellow	NT	Gill	KJ190167	[*Flexibacter*] *aurantiacus* subsp. *excathedrus* (AB078045)	99.9
3.CUVET1211*Pale yellow	NT	Gill	KJ190172	[*Flexibacter*] *aurantiacus* subsp. *excathedrus* (AB078045)	99.9
4.**CUVET1217**Yellow	RT	Skin	KJ190174	*Chryseobacterium taichungense* (JX042458)	98.1
5.CUVET1218Yellow	RT	Kidney	KJ190175	*Chryseobacterium taichungense* (JX042458)	98.1
6.**CUVET1219**Yellow	RT	Kidney	KJ190176	*Chryseobacterium indologenes* (EU221399)	99.1
7.CUVET1220Yellow	RT	Kidney	KJ190177	*Candidatus Chryseobacterium massiliae* (AF531766)	99.3
8.CUVET1225Yellow	KC	Skin ulcer	KJ190180	*Flavobacterium indicum* (NR074422)	99.7
9.**CUVET1207**Pink	NT	Skin ulcer	KJ190168	*Flectobacillus roseus* GFA-11 (EU420062)	99.8
10.CUVET1227Pink	NT	Gill	KJ190182	*Flectobacillus roseus* GFA-11 (EU420062)	99.8

NT, Nile tilapia; RT, red tilapia; KC, koi carp
Grey shade marks fish that was also infected with *F. columnare* (Dong et al., 2015a).
Underlined bold-faced codes represent isolates subjected for experimental challenge.
* indicates isolates subjected for ISR sequence analysis

Table 2 Biochemical characteristics of yellow and pink pigmented bacterial isolates in this study

Phenotypic profile	I	II	III	IV	V	VI
Isolate	*Flavobacterium indicum* (CUVET1225)	[*Flexibacter*] *aurantiacus* subsp. *excathedrus* (**CUVET1206** & CUVET1211)	*Chryseobacterium indologenes* (**CUVET1219**)	*Candidatus Chryseobacterium massiliae* (**CUVET1205** & CUVET1220)	*Chryseobacterium* sp. (**CUVET1217** & CUVET1218)	*Flectobacillus roseus* (**CUVET1207** & CUVET1227)
Colony	Yellow	Yellow	Yellow	Yellow	Yellow	Pink
Gram	Negative	Negative	Negative	Negative	Negative	Negative
Oxidase	-	+	+	+	+w	+
Catalase	-	-	+	+	+	+
Flexirubin pigment	+	-	+	+	+	ND
TSA	-	-	+	+	+	-
MacConkey	-	-	-	-	-	-
Glucose	+	-	+	+	+	+
Sucrose	-	-	-	v	-	+
Maltose	+	ND	+	+	+	ND
Mannitol	+	ND	-	-	+	ND
Lactose	-	ND	-	-	+	-
Trehalose	-	ND	+	-	+	ND
Arabinose	-	ND	-	-	+	ND
Degradation of						
Gelatin	ND	+	-	v	+	+
Casein	+	-	+	+	+	-
Starch	-	-	+	v	+w	-
Esculin	-	ND	+	+	+	ND
Sodium chloride tolerance						
NaCl 0%	+	+	+	+	+	+
NaCl 1%	-	-	+	+	+	-
NaCl 1.5%	-	-	+	+	+	-
NaCl 2%	-	-	+	-	+	-

ND, not determined; v, variable; w, weak
Underlined bold-faced codes represent isolates subjected for experimental challenge.

Table 3 Cumulative mortality in Nile tilapia fingerlings upon 14 day-experimental challenge with different bacterial isolates

Biochemical characteristics group	Bacteria administrated	Number of fish	Infection route	Challenge dose (CFUs fish^{-1})	Cumulative percentage mortality (%)
II	[*Flexibacter*] *aurantiacus* subsp. *excathedrus* CUVET1206	10	i.m.	2.7×10^7	10
III	*Chryseobacterium indologenes* CUVET1219	10	i.m.	8.2×10^8	20
IV	*Candidatus Chryseobacterium massiliae* CUVET1205	10	i.m.	9.3×10^8	10
V	*Chryseobacterium* sp. CUVET1217	10	i.m.	4.1×10^8	0
VI	*Flectobacillus roseus* CUVET1207	10	i.m.	0.7×10^8	10
Control	0.85% NaCl	10	i.m.	0.1 mL	0

CFUs, colony forming units; i.m., intramuscular

16S rRNA amplification, DNA sequencing, and phylogenetic analysis: Genomic DNA from each isolate was extracted as described by Arias et al. (2004). Briefly, a single colony of each pure isolate was suspended in 100 µL nuclease-free water, boiled for 10 min, cooled rapidly on ice, and then briefly centrifuged. Supernatant containing genomic DNA was used as template for PCR reactions. A fragment of the 16S rRNA gene from the bacterial isolates was amplified using universal primers UN20 (5'-AGA GTT TGA TCM TGG CTC AG-3') and R1438 (5'-GCC CTA GTT ACC AGT TTT AC-3') according to Darwish and Ismaiel (2005). PCR reaction mixtures were prepared in 50 µL volume containing 25 µL Master Mix (GoTaq®Green, Promega, USA), 0.2 µM of each primer, 5 µL genomic DNA templates and 18 µL nuclease-free water. PCR amplification was performed in a thermocycler (TC-96/G/H(b), Bioer, China) as follows: denaturation at 94°C for 5 min, followed by 30 cycles of amplification at 94°C for 30 sec, 45°C for 30 sec, 72°C for 2 min and final extension at 72°C for 4 min (Darwish and Ismaiel, 2005). Amplified products were purified using NucleoSpin® Extract II Kit (Macherey-Nagel, Germany) and subjected for sequencing (1st

BASE Pte Ltd) using UN20 and R1438 primers as mentioned above. Assembly of forward and reverse sequences of each isolate was conducted using ContigExpress software (Invitrogen Corporation, 2006). Nucleotide sequences were deposited in the GenBank database (Table 1). Similarity of the 16S rRNA gene sequences was compared with published sequences available in the GenBank database using Nucleotide BLAST program from National Center for Biotechnology Information (NCBI). Isolates were identified at species level based on at least 99% identity of the 16S rRNA gene with sequences of type strains or published studies, whereas isolates that exhibited lower than 99% identity were only identified at genus level. A phylogenetic tree was constructed based on 1302 nucleotides of 16S rRNA gene sequences (position 87-1388 *E. coli* numbering) of the 10 isolates in this study and closely related taxa retrieved from GenBank. The 16S rRNA gene sequence of *Aeromonas hydrophila* ATCC7966 (NR074841) was used as outgroup. The phylogenetic tree was generated by the neighbor-joining method using p-distance model of MEGA 5.2 package (Tamura et al., 2011) after discarding gaps and unidentified bases (complete deletion option). The tree topology was evaluated by bootstrap analysis of 1000 replicates.

16S-23S rRNA intergenic spacer region (ISR) amplification and sequencing: Two isolates, designated as CUVET1206 and CUVET1211, were suspected for a novel potential pathogen first discovered in the present study. To provide more genetic characteristics and evidence for bacterial identification, ISR of these isolates was further investigated. The universal primer 16S14F (5'-CTT GTA CAC ACC GCC CGT C-3') and 23S1R (5'-GGG TTT CCC CAT TCG GAA ATC-3') targeted to 16S rRNA and 23S rRNA genes, respectively, were used to amplify full length of ISR (Zavaleta et al., 1996). Preparation of PCR reaction mixtures was performed in the same manner mentioned above. Thermocycler conditions were as follows: denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min and a final extension at 72°C for 3 min (Zavaleta et al., 1996). Purification of PCR products and DNA sequence analysis were performed in the same manner described above.

Experimental challenge: The potential of the yellow and pink pigmented bacteria to cause disease in tilapia was investigated by experimental challenge using an intramuscular injection method. Five representative bacterial isolates were cultured on modified AO agar (AOA supplemented with 0.5 g L^{-1} $MgSO_4.7H_2O$ and 0.5 g L^{-1} $CaCl_2.2H_2O$) at 28°C for 36 h. Colonies of each bacterial isolate were harvested and suspended in sterile normal saline (0.85% NaCl) to reach approximately 10^9 CFUs mL^{-1}. Bacterial density was then verified by the plate count method and the exact injection doses of each bacterium are summarized in Table 3. Sixty apparently healthy Nile tilapia fingerlings (mean weight, 14.5 ± 1.5 g) were divided into 6 groups. Each group of 10 fish was intramuscularly injected with 0.1 mL volume containing either *Candidatus Chryseobacterium massiliae* CUVET1205, [*Flexibacter*] *aurantiacus* subsp. *excathedrus* CUVET1206, *Chryseobacterium* sp. CUVET1217, *Chryseobacterium indologenes* CUVET1219 or *Flectobacillus roseus* CUVET1207 (Table 3). The control group was injected with 0.1 mL normal saline water. Fish were fed daily on tilapia feed (CP, Thailand) and observed for 14 days. Water temperature during the experiment was 26.6 ± 1.4°C.

Results

Bacterial identification: In the present study, 1, 4, and 5 bacterial isolates obtained from the koi carp (*Cyprinus carpio*), red tilapia (*Oreochromis* sp.), and Nile tilapia (*Oreochromis niloticus*) exhibiting columnaris-like disease (Table 1) were selected for further investigation. The ten bacterial isolates were Gram negative, formed yellow, pale yellow or pink colonies on AO agar and were unable to grow on MacConkey agar (Table 2). Most of the isolates were positive for oxidase, catalase, flexirubin pigment, and some were able to grow on TSA agar. Detailed biochemical results are presented in Table 2. Based on the phenotypic tests, ten bacterial isolates were classified into six different phenotypic profiles. Colony morphologies of the six representative isolates from each of the biochemical phenotypes on modified AO agar are shown in Fig. 1.

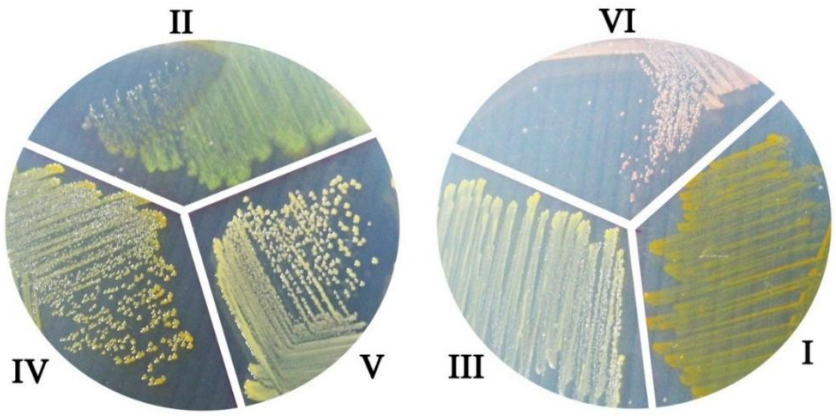

Figure 1 Colony morphology of bacterial species on modified AO agar after 36 h incubation at 28°C. The roman numerals represent six individual biochemical characteristics. See Table 2 for a key to isolates classified into biochemical characteristics I to VI.

Further characterization was performed by sequencing approximately 1300 bp of the 16S rRNA gene of all ten bacterial isolates. Consistent with the phenotypic tests, homology search by BLAST analysis indicated that the ten bacterial isolates belonged to six different species including *Candidatus Chryseobacterium massiliae, Chryseobacterium* sp., *C. indologenes*, *Flectobacillus roseus*, [*Flexibacter*] *aurantiacus* subsp *excathedrus*, and *Flavobacterium indicum* (Tables 1-2). Specifically, the isolates CUVET1205 and CUVET1220 were putatively identified as *Candidatus Chryseobacterium massiliae* based on > 99% nucleotide identity to *C. massiliae* (AF531766). The isolates CUVET1219 and CUVET1225 were putatively identified as *Chryseobacterium indologenes* and *Flavobacterium indicum* based on 99.1% and 99.7% nucleotide identity to *C. indologenes* H2S10 (EU221399) and *F. indicum* **GPTSA100-9** (NR074422), respectively. The two pale yellow pigmented isolates, designated as CUVET1206 and CUVET1211, were putatively identified as [*Flexibacter*] *aurantiacus* subsp. *excathedrus* IFO 16024 (AB078045) based on 99.9% identity. The isolates CUVET1217 and CUVET1218 exhibited the highest 16S rRNA sequence identity to *Chryseobacterium* sp. and were most closely related to *C. taichungense* (JX042458) with 98.1% nucleotide identity. The isolates CUVET1207 and CUVET1227 were identified as *Flectobacillus roseus* based on 99.8% identity to *F. roseus* GFA-11 (EU420062). All ten 16S rRNA sequences of the bacterial isolates were deposited in the GenBank database and their accession numbers are presented in Table 1. Additionally, the 16S-23S rRNA intergenic spacer region sequences of the two [*Flexibacter*] *aurantiacus* subsp. *excathedrus* isolates (CUVET1206 and CUVET1211) were first sequenced in the present study and deposited in GenBank under accession numbers KM977904 and KM977905, respectively. These sequences exhibited the highest similarity to ISR sequences of *Flavobacterium columnare* isolates (85-88%) in the GenBank database.

Note that the isolates identified as *Chryseobacterium* sp., *C. indologenes*, *C. massiliae*, *F. indicum* and *F. roseus* (CUVET1217 - CUVET1220, CUVET1225, CUVET1227) in the present study were recovered from diseased fish that exhibited columnaris-like disease in which *F. columnare* was also isolated (Dong et al., 2015a) (Table 1). The isolates identified as [*Flexibacter*] *aurantiacus* subsp. *excathedrus* (CUVET1206), *C. massiliae* (CUVET1205) and *F. roseus* (CUVET1207) were concurrently recovered from Nile tilapia that exhibited columnaris-like disease, but *F. columnare* was not isolated from these fish (Table 1).

16S rRNA phylogenetic analysis: Together with the phenotypic and molecular analysis described above, eight yellow pigmented bacteria in this study were composed of 2 *Candidatus Chryseobacterium massiliae,* 2 *Chryseobacterium* sp., 1 *C. indologenes*, 1 *Flavobacterium indicum*, and 2 [*Flexibacter*] *aurantiacus* subsp *excathedrus* while 2 isolates of the pink bacteria belonged to *Flectobacillus roseus* (Tables 1-2). The phylogenetic analysis based on the 16S rRNA gene sequences of the ten bacterial isolates and closely related species retrieved from GenBank clearly separated into two distinct phylogenetic groups each with members of previously reported and newly identified yellow and pink bacteria (Fig. 2). Within the yellow bacterial group, there were two clusters consisting of bacteria in the genera a) *Candidatus Chryseobacterium* and *Chryseobacterium* and b) *Flavobacterium* and [*Flexibacter*] (Fig. 2).

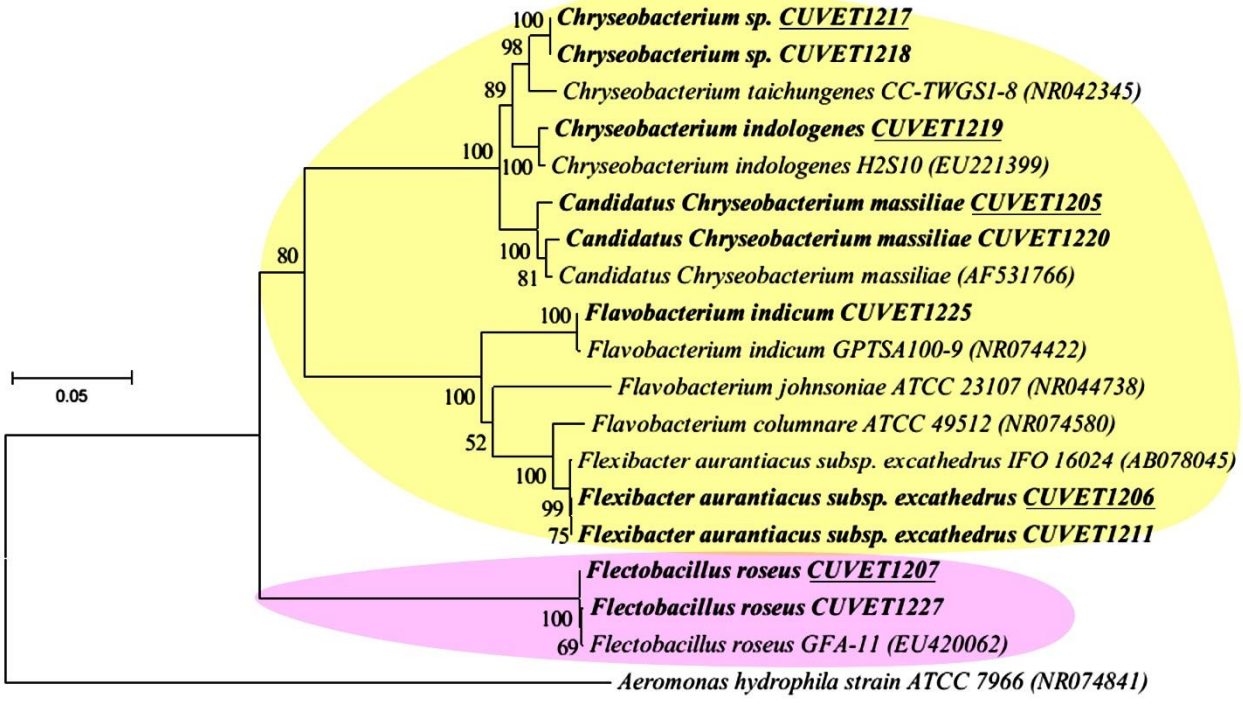

Figure 2 Phylogenetic tree was constructed based on 16S rRNA gene sequences (position 87-1388 *E. coli* numbering) of eight yellow and two pink pigmented bacteria in this study and closely related taxa by the neighbor-joining method using p-distance model. The tree topology was evaluated by bootstrap analysis of 1000 replicates. *Aeromonas hydrophila* ATCC 7966 was used as outgroup. The underlined bold-faced codes represent isolates subjected for experimental challenge.

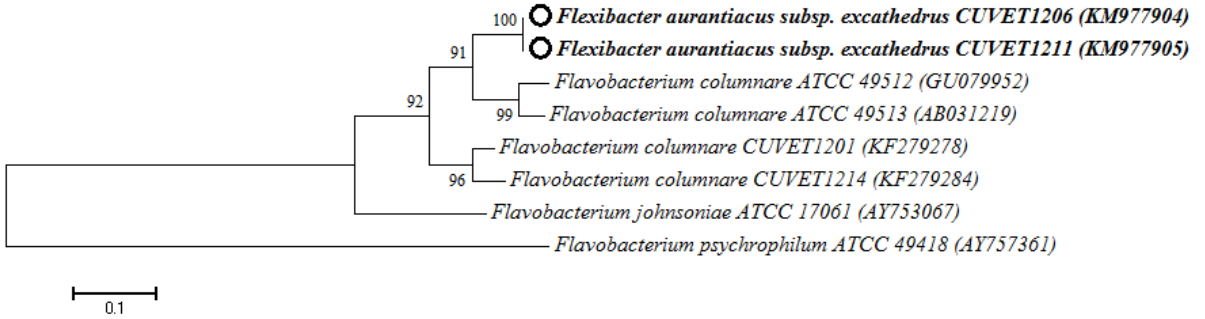

Figure 3 Neighbor-joining tree was constructed from complete 16S-23S rRNA intergenic spacer region sequences of two [*Flexibacter*] *aurantiacus* subsp. *excathedrus* isolates (CUVET1206 and CUVET1211) and closely related taxa. The tree topology was evaluated by bootstrap analysis of 1000 replicates.

Figure 4 Experimental fish exposed to *Chryseobacterium indologenes* CUVET1219 (upper) and *Candidatus Chryseobacterium massiliae* CUVET1205 (lower) exhibited discoloration areas on the body surface resembling columnaris disease.

Evaluation of pathogenic potential of yellow and pink pigmented bacteria: In the present study, bacterial isolates from phenotypic profiles II to VI were chosen for evaluation of the pathogenic potential in Nile tilapia fingerlings. This study mainly focused on bacteria recovered from diseased tilapia, the koi carp isolate (phenotypic profile I), therefore, was not selected for the challenged test. After 14 days post challenge by injection method, the highest cumulative percentage mortality of 20% was observed in the group administered with the bacterial isolate *Chryseobacterium indologenes* CUVET1219 (Table 3). Three groups which received either *Candidatus Chryseobacterium massiliae* CUVET1205, [*Flexibacter*] *aurantiacus* subsp. *excathedrus* CUVET1206 or *Flectobacillus roseus* CUVET1207 exhibited only 10% cumulative percentage mortality while no mortality was observed in the group injected with *Chryseobacterium* sp. CUVET1217 and saline buffer (Table 3). All dead fish were observed within the first 7 days of the experimental period. The moribund fish which received *C. indologenes* CUVET1219 or *Candidatus Chryseobacterium massiliae* CUVET1205 exhibited discoloration areas on the body surface resembling columnaris disease (Fig. 4) and yellow pigmented bacteria were re-isolated from these fish. Observation of clinical signs and bacterial isolation were not performed with the dead fish from two groups (injected with CUVET1206 and CUVET1207) because only one fish died (10% mortality) and this happened during the night.

Discussion

Since *Flavobacterium columnare* (previously known as *Bacillus columnaris*, *Chondrococcus columnaris*, *Cytophaga columnaris* or *Flexibacter columnaris*) was first discovered as the aetiological agent of columnaris diseases (Davis, 1922), most later disease laboratory studies have typically aimed at different aspects of single *F. columnare* infection and other bacteria involved are comparatively ignored in literature. Our recent studies first revealed concurrent infections of multiple pathogens in natural diseased Nile tilapia (*O. niloticus*) and striped catfish (*Pangasianodon hypophthalmus*) which exhibited clinical signs resembling columnaris disease (Dong et al., 2015c, b), and have proposed the concept that "The reality of natural disease outbreaks in fish farms caused by multiple pathogen infections probably outweighs single infection". The present study, therefore, aimed

at further investigation into other bacterial infections, with an emphasis on unknown culturable yellow and pink pigmented bacteria associated with columnaris diseased fish samples. The ten representative yellow and pink pigmented bacteria isolated from tilapia and koi carp exhibiting columnaris-like disease belonged to six different species including *Candidatus Chryseobacterium massiliae, Chryseobacterium* sp., *C. indologenes*, *Flavobacterium indicum*, [*Flexibacter*] *aurantiacus* subsp *excathedrus*, and *Flectobacillus roseus*. To the best of our knowledge, all bacteria identified in our study have never been reported in tilapia and koi carp.

Regarding bacterial taxonomy, [*Flexibacter*] *aurantiacus* Lewin 1969 contains two subspecies, [*Flexibacter*] *aurantiacus* subsp. *excathedrus* and [*Flexibacter*] *aurantiacus* subsp. *copepodarum* (Lewin, 1969). Based on fatty acid profiles and DNA-DNA hybridization assay, the reference strains of [*Flexibacter*] *aurantiacus* have been transferred to *Flavobacterium johnsoniae* (Bernardet et al., 1996). However, phylogenetic analysis based on 16S rRNA (Fig. 2) and 16S-23S rRNA intergenic spacer region (Fig. 3) of the isolates (CUVET1206 and CUVET1211) in this study and closely related taxa clearly indicated that the [*Flexibacter*] *aurantiacus* subsp. *excathedrus* isolates were the closest relatives of *Flavobacterium columnare* ATCC 49512. This suggests that the taxonomy of [*Flexibacter*] *aurantiacus* subsp. *excathedrus* or [*Flavobacterium*] *johnsoniae* subsp. *excathedrus* should be reconsidered as [*Flavobacterium columnare*] subsp. *excathedrus*.

The majority of the collected unknown yellow pigmented bacteria were identified as *Chryseobacterium* spp. Over the last decade, a large number of new *Chryseobacterium* species have been described from diseased fish (Bernardet et al., 2005; de Beer et al., 2006; Ilardi et al., 2009; Kämpfer et al., 2011; Zamora et al., 2012a, b, c, d; Pridgeon et al., 2013; Loch and Faisal, 2014). For example, *C. indologenes* was reported as the pathogenic bacteria causing disease in American yellow perch, *Perca flavescens* (Mitchill) (Pridgeon et al., 2013). The pink pigmented bacterium, *F. roseus*, was originally isolated from a freshwater environment (Sheu et al., 2009) and was recently reported as the causative agent of a new disease (Flectobacillosis) in roho labeo (*Labeo rohita*) fingerlings (Adikesavalu et al., 2015).

In contrast to published reports (Pridgeon et al., 2013; Adikesavalu et al., 2015), *C. indologenes* and *F. roseus* identified in the present study exhibited low virulence to the healthy Nile tilapia fingerlings even when the high dose of different bacterial isolates was used. These contradictory findings might be implicated in different host organisms and their susceptibility to the pathogens. With respect to bacterial pathogenicity, the challenged experiment revealed that single bacterial infection failed to establish their virulence to result in high mortality in the challenged fish. However, the collaboration of multiple bacteria in disease manifestation according to our concurrent infection concept has not yet been evaluated. In addition, it might be possible that the previously identified *F. columnare* isolates could play a role as primary instigator of the disease outbreaks followed by secondary infections of the yellow and pink pigmented bacteria. If this proves to be the case, the newly identified bacteria might serve as opportunistic pathogens that need stressors (e.g. primary pathogens and environmental factors) for disease manifestation in tilapia farms.

It is interesting to note that concurrent infections of three bacterial species were first addressed in this study. [*Flexibacter*] *aurantiacus* subsp. *excathedrus* (CUVET1206), *C. massiliae* (CUVET1205), and *F. roseus* (CUVET1207) were recovered from the same fish specimen of Nile tilapia showing clinical signs resembling columnaris disease in which *F. columnare* seemed to be absent. The causative agent(s) of this case was not revealed through single infections within our current experimental challenges. However, it might be worthwhile to establish an experiment for multiple concurrent infections in fish model and to survey these bacteria as potential pathogens in natural outbreaks in order to gain better understanding of the reality of natural disease manifestation in fish farms.

Herein, this study first reported various non-*Flavobacterium columnare* bacteria involved in columnaris-like diseased fish and primarily investigated their pathogenic potential to tilapia fingerlings without stressors. Future works will investigate virulence of the bacteria through different routes of infections under environmental stress conditions or multiple infections.

Acknowledgements

This study was financially supported by the Chulalongkorn University (CU) – Veterinary Science Research Fund (RG8/2555) and Ratchadapiseksomphot Endowment Fund. H.T. Dong has been supported by the 100th year anniversary of CU fund for doctoral scholarship and the 90th anniversary of CU fund. The authors would like to thank Dr. Benjamin LaFrentz of USDA-ARS (Auburn, AL, USA) for critical comments of the manuscript and Mr. Daniel Montefusco for providing proofreading.

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บทคัดย่อ

ความหลากหลายของเชื้อแบคทีเรียชนิดอื่นนอกจากเชื้อฟลาโวแบคทีเรียม คอลัมนาแนร์ ซึ่งแยกได้จากปลาที่ป่วยซึ่งแสดงอาการของโรคคอลัมนาริส

ฮา ทานห์ ดง1 เวื่อง เวียด เหวียน1 วันนิวัฒน์ มะทะ1 พัฒนพล ขยันสำรวจ1
แสงจันทร์ เสนาปิน2,3 เดชฤทธิ์ นิลอุบล1 ชาญณรงค์ รอดคำ1*

เชื้อฟลาโวแบคทีเรียม คอลัมแนร์ เป็นเชื้อแบคทีเรียหลักซึ่งแยกได้จากปลานิลในฟาร์มแห่งหนึ่งในประเทศไทยและปลาคาร์ฟป่วยจำนวน 1 ตัวซึ่งแสดงอาการของโรคคอลัมนาริส นอกจากนี้ ยังมีเชื้อแบคทีเรียโคโลนีสีเหลืองและโคโลนีสีชมพูซึ่งแยกได้จากปลาป่วยดังกล่าวด้วย แต่เนื่องจากแบคทีเรียทั้งสองชนิดที่แยกได้นั้นยังไม่มีข้อมูลอนุกรมวิธาน การศึกษานี้จึงเป็นการศึกษาต่อยอดเพื่อระบุชนิดของเชื้อแบคทีเรียดังกล่าวด้วยการใช้วิธีเปรียบเทียบสัณฐานวิทยา การทดสอบชีวเคมีและการเปรียบเทียบลำดับนิวคลีโอไทด์ของยีน 16S rRNA รวมถึงเพื่อศึกษาความรุนแรงของการก่อโรคในลูกปลานิลทดลอง (โอรีโอโครมิส นิโลทิคัส) การศึกษาพบว่าเชื้อที่มีโคโลนีสีเหลืองประกอบด้วยเชื้อแบคทีเรียคริซิโอแบคทีเรียม เชื้อเฟล็กซิแบคเตอร์ ออแรนเทียคัสซับสปีชีส์เอ็กซ์คาทีดัส และเชื้อฟลาโวแบคทีเรียม อินดิคัม ส่วนเชื้อที่มีโคโลนีสีชมพู คือเชื้อเฟลคโตบาซิลลัส โรเซียส จากการทดสอบความรุนแรงในการก่อโรคในลูกปลานิลเป็นเวลา 14 วัน พบว่าเชื้อแบคทีเรียเหล่านี้มีความรุนแรงในการก่อโรคต่ำจนถึงไม่สามารถก่อโรคได้ (อัตราการตายสะสมร้อยละ 0 ถึง 20) ซึ่งแสดงให้เห็นว่าเชื้อดังกล่าวอาจมีบทบาทเป็นเชื้อฉวยโอกาสซึ่งอาศัยปัจจัยเรื่องความเครียดร่วมด้วยจึงทำให้เกิดโรคขึ้น

คำสำคัญ: 16S rRNA เชื้อแบคทีเรียนอกเหนือจากฟลาโวแบคทีเรียม คอลัมแนร์ โรคคอลัมนาริส

[1]ภาควิชาจุลชีววิทยา คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ปทุมวัน กรุงเทพมหานคร 10330 ประเทศไทย
[2]ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ (ศช.) สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ อุทยานวิทยาศาสตร์ประเทศไทย คลองหลวง ปทุมธานี 12120 ประเทศไทย
[3]หน่วยวิจัยเพื่อความเป็นเลิศเทคโนโลยีชีวภาพกุ้ง มหาวิทยาลัยมหิดล 272 ถนนพระราม 6 กรุงเทพฯ 10400 ประเทศไทย
***ผู้รับผิดชอบบทความ** E-mail: channarong_r@yahoo.com, Channarong.R@chula.ac.th*