การคัดแยกเชื้อ *Flavobacterium columnare* และเชื้อ *Bacillus* sp. จากปลานิลและปลาดุก

Isolation of *Flavobacterium columnare* and *Bacillus* sp. from Nile tilapia and Catfish

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บทคัดย่อ

การวิจัยนี้มีวัตถุประสงค์ในการคัดแยกเชื้อ *Flavobacterium columnare* และเชื้อ *Bacillus* sp. จากปลานิล (*Oreochromis niloticus*) และปลาดุก (*Clarias* spp.) เพื่อคัดเลือกเชื้อ *Bacillus* sp. ที่มีกิจกรรมการเจริญต่อต้านเชื้อ *F. columnare* สูงที่สุด เก็บตัวอย่างปลานิลจำนวน 140 ตัวและตัวอย่างปลาดุกจำนวน 70 ตัวจากฟาร์มปลาในพื้นที่จังหวัดอุบลราชธานี ระหว่างเดือนมีนาคมถึงเดือนพฤศจิกายน 2556 เพื่อคัดแยกเชื้อ *F. columnare* และเชื้อ *Bacillus* spp. พบเชื้อที่โคโลนีมีสีเหลือง เกาะติดแน่นกับผิวหน้าอาหาร ขอบโคโลนีมีลักษณะเป็นแบบ rhizoid รูปร่างเป็นท่อนผอมยาวซึ่งเป็นลักษณะของเชื้อ *Flavobacterium* spp. จำนวน 36 ไอโซเลต นำเชื้อกลุ่มนี้ 1 ไอโซเลตที่มีลักษณะเหมือนกันและมีการเจริญอย่างหนาแน่นไปคัดแยกให้บริสุทธิ์ 7 ครั้ง จากนั้นจัดจำแนกเชื้อโดยใช้ลักษณะทางฟีโนไทป์และทางชีวโมเลกุล พบว่าเชื้อมีลักษณะเป็น *F. columnare* KJ720207 ผลการคัดแยกเชื้อ *Bacillus* spp. และทดสอบสมบัติการยับยั้งการเจริญต่อเชื้อ *F. columnare* พบว่าคัดแยกได้เชื้อ *Bacillus* spp. จำนวน 24 ไอโซเลต เมื่อจัดจำแนกไอโซเลตที่แสดงสมบัติยับยั้งการเจริญของเชื้อ *F. columnare* ได้สูงสุดด้วยลักษณะทางฟีโนไทป์และทางชีวโมเลกุล พบว่าเชื้อมีลักษณะเป็น *B. amyloliquefaciens* KJ720206.

คำสำคัญ: การคัดแยกเชื้อ, ปลานิล, ปลาดุก, *Flavobacterium columnare, Bacillus*

Abstract

The aims of this research were to isolate *Flavobacterium columnare* and *Bacillus* sp. from Nile tilapia (*Oreochromis niloticus*) and Catfish (*Clarias* spp.) and to select the isolated *Bacillus* sp. showing the highest antagonistic activity against *F. columnare*. The 140 Nile tilapia and 70 Catfish samples were collected from fish farms in Ubon Ratchathani province during March-November, 2013 and were screened for *F. columnare* and *Bacillus* spp.. The 36 isolates showing yellow-orange pigmented colonies, adhering tightly to the agar medium and rhizoid edges, slender and long bacilli were classified as *Flavobacterium* spp., then one of them which showed purity and highest growth density in 7 times subculturing was selected and identified by phenotypic and

molecular characterization as *F. columnare* KJ720207. The isolation of *Bacillus* spp. were performed and tested for the inhibitory effect against *F. columnare*. Of the 24 isolated *Bacillus* spp., the one which exhibited the highest inhibitory activity was identified by phenotypic and molecular characterization as *B. amyloliquefaciens* KJ720206.

Key words: Isolation, Nile tilapia, Catfish, *Flavobacterium columnare, Bacillus*

Introduction

F. columnare is the causative agent of freshwater fish disease known as columnaris disease, saddleback disease, cotton-wool disease and fin rot (Mohamed & Refat, 2011). This disease has been recognized as one of the most infected bacterial worldwide problem in both natural and cultured freshwater fish in aquaculture industry (Prasad *et al*., 2011; Decercq *et al.,* 2013). It can be a cause of large economic losses resulting from massive mortality of the infected fish (Elkamel & Mohamed, 2012). Generally, this organism is distributed in freshwater environments and also found in mucus and skin of many fish species as normal-flora. Outbreaks of the disease are spontaneous, but are influenced under negative factors of culturing, such as sudden water temperature changes or on a daily fluctuations, high organic and inorganic matter in the water, stress environmental conditions due to high stocking density, grading selection and transportation (Kubilay *et al*., 2008; Pilarski *et al*., 2008). *F. columnare* is contagious disease that can infect any fish at any age during any season. Symptoms of the infected fish were dependent on the virulence of the pathogenic strains. The strains with low virulence cause massive tissue damage before death occurred while the strains with high virulence kill fish fry within 12-14 hours without clinical signs at the time of death (Decercq *et al*., 2013). The infection appears as gray-white discoloration on some part of head, mouth and body, grayish dots or yellow areas of erosion and ulceration that might be surrounded by hyperemic reddish zone on the head, body surface, fins and gills (Sebastião *et al*., 2010) and finally resulting in tissue destruction.

In Ubon Ratchatani province, there are more than 7,000 cultured fish farms which mostly are Nile tilapia (*Oreochromis niloticus*) and Catfish (*Clarias batrachus*). For treating bacterial disease outbreaks, antibiotics are popular used as disease controlling in fish farms. However, the use of antibiotics can contribute to the emergence of antibiotic-resistant bacteria population (Naviner *et al*., 2006). Moreover, several antibiotics were banned in the European Union (Deeseenthum *et al.,* 2007). In recent years, alternative methods, such as probiotics based therapeutic and prophylactic methods have been used as means of disease control and immune

booster. Probiotics are viable and edible microorganism which can show health beneficial effects to consumers. Among these the genus *Bacillus* represents about half of the commercially available biocontrol probiotics because they are spore-forming bacteria that high tolerance toward unsuitable culture conditions. Chantharasophon *et al.* (2011) isolated the safe *Bacillus* probiotic for Nile tilapia cultivation, they reported that *B. brevis* was possibly the first isolate from aquaculture isolation which showed bioactive compound production against *Aeromonas hydrophila* and no hemolytic activity on blood agar medium. However, the use of probiotics to control the disease caused by *F. columnare* is not popular due to the lack of potential probiotic strains. Therefore, this research aimed to isolate *F. columnare* and probiotic bacillus from Nile tilapia and Catfish samples in order to find out the best probiotic bacillus for columnaris disease control.

Materials and Methods

Sampling

The 140 Nile tilapia and 70 Catfish samples were randomly collected from fish farms and Moon River in Ubon Ratchathani province, Thailand, during March-November 2013. All samples were transferred to the laboratory within 1 hour.

Primary screening for *F. columnare*

For *Flavobacterium* spp. screening, fish samples displaying skin ulcers or not were swabbed and streaked on cytophaga agar supplemented with Neomycin (5 µg/ml) and polymycin B (10 U/ml). All plates were incubated at 25°C for 72 h then morphological characteristics, colony appearance on cytophaga agar, cell morphology and Gram stains were determined as described by Kubilay *et al.* (2008) and Sebastião *et al*. (2010). The primary screened colonies were checked on yellow-orange pigmented colonies, adherence to the agar and rhizoid edges, Gram negative slender and long bacilli were chosen in primary screening. Then the selected colonies were purified by cross streak technique for 7-10 times, and observation of cell morphology under microscope that was filamentous and thin long rod bacilli was checked further characterization.

Biochemical and physiological characterization of *F. columnare*

Biochemical and physiological properties of the isolated bacterial strain were carried out as described by Kubilay *et al*. (2008) and Sebastião *et al*. (2010). The bacterial identification was determined according to the following characteristics including cytochrome oxidase activity, catalase production, production of indole, production of flexirubin type of pigments, nitrates reduction, acetoin and H_2S, oxidation gas from glucose, hydrolysis of gelatin, casein and starch,

utilization of glucose, sucrose and citrate, growth on cytophaga agar supplemented with polymysin and neomycin, growth on 0.5% and 1% NaCl, growth on tryptic soy agar and antibiotic sensitivity tests to Penicillin (10 µg), Kanamycin (30 µg) and Streptomycin (25 µg) for bacterial preliminary diagnosis (Kubilay *et al*., 2008; Pilarski *et al*., 2008; and Sebastião *et al.,* 2010). The isolated bacterial strain was also identified by the 16S rDNA sequence analysis conducted at Mahidol University-Osaka University Collaborative Research Center for Bioscience and Biotechnology (MU-OU : CRC), Faculty of Science, Mahidol University, Thailand.

Isolation, selection and identification of probiotic *Bacillus* sp.

For probiotic *Bacillus* isolation, the same fish samples from *Flavobacterium* screening were dissected for their gastrointestinal tracts. After grinding and boiling at 100 °C for 3 min, all samples were screened and purified 7 times for spore forming bacteria by cross streak technique in tryptic soy agar. The isolate which was showed the highest growth inhibition against *F. columnare* was selected and characterized by biochemical properties which were Gram reaction, motility, production of catalase and acetoin, nitrates reduction, hydrolysis of starch, citrate utilization, growth on 6.5% NaCl, and antibiotic sensitivity tests to Penicillin, Kanamycin and Streptomycin (Chantharasophon *et al*., 2011) and by 16S rDNA sequence analysis conducted at MU-OU : CRC, Faculty of Science, Mahidol University, Thailand.

Results

Primary screening of *F. columnare*

The 140 Nile tilapia and 70 Catfish samples were collected from earthen ponds fish farms and cage culture farm in Moon River in Ubon Ratchathani province during June-November, 2013 and bacterial strains were screened from the skin lesions or yellow areas of Nile tilapia and Catfish samples on cytophaga agar supplemented with polymycin B and neomycin. Among 36 bacterial isolates, 9 (from Nile tilapia) and 27 (from Catfish) isolates showed yellow-orange pigmented colonies, adhering tightly to the agar medium and rhizoid edges, slender and long bacilli. By gram staining, they were Gram negative and filamentous bacteria. Of the 36 isolates, only one showed purity and highest growth density in 7 times subculturing, therefore, it was selected and identified by phenotypic characterization. The selected strain exhibited no growth at 4°C little growth at 20°C, optimum growth at 25°C and growth at 30°C, colonies appear to be flat, dry, spreading and strongly adhere to agar medium, yellowish orange color (flexirubin-type), the strain in broth

exhibited a pellicle as a ring at the medium glass interface and most of its filamentous tufts were floated on the top of the tube. This isolate was chosen for further characterization.

Phenotypic characteristics identification and antibiotics sensitivity test of *Flavobacterium* sp.

For identification, biochemical and physiological characteristics of the selected *Flavobacterium* sp. are given in Table 1. This strain produced oxidase, catalase, indole, flexirubin-type pigment, nitrates reduction, but not produced acetoin, gas from glucose, and H_2S. It hydrolyzed gelatin and casein but not starch, and utilized glucose and sucrose but not citrate. In addition, it grew on cytophaga agar supplemented with polymycin and neomycin, 0.5% NaCl but not on tryptic soy agar and 1% NaCl. Antibiotic sensitivity tests showed that the selected strain was sensitive to Penicillin (10 µg), Kanamycin (30 µg) and Streptomycin (25 µg) (Table 1). The characteristics of this isolated strain is in agreement with that of *F. columnare*.

Table 1 Phenotypic characteristics and antibiotics sensitivity test of the selected *Flavobacterium* sp.

Characteristics	Response	Characteristics	Response
Production of:		Utilization of:	
-Oxidase	+	-Glucose	+
-Catalase	+	-Sucrose	+
-Indole	+	-Citrate	-
-Flexirubin-type pigment	+	Growth on:	
-Nitrates reduction	+	-cytophaga agar	+
-Acetoin	-	-0.5% NaCl	+
-H_2S	-	-1% NaCl	-
-Gas from glucose	-	-Tryptic soy agar	-
Hydrolysis of:		Sensitive to:	
-Gelatin	+	-Penicillin (10 µg),	+
-Casein	+	-Kanamycin (30 µg)	+
-Starch	-	-Streptomycin (25 µg)	+

(+) positive response (-) negative response

Molecular identification

The isolated bacterial strain was characterized by molecular biology assays at the species level by Nucleotide sequencing, 16S rDNA (Partial sequence; 1,500 bp). DNA sequencing of the isolated strain was showed in Figure 4 which was closely to *F. columnare* IAM 14821 (99%

similarity). Therefore, it was identified as *F. columnare* and the 16S rDNA sequence of this isolate was deposited in the GenBank database with accession number *F. columnare* KJ720207 (Figure 1).

Figure 1 DNA sequence of *F. columnare* KJ720207 by 16S rDNA sequence (Partial sequence; 1,500 base pairs)

Isolation and selection of probiotic *Bacillus* sp.

All fish samples were dissected for their gastrointestinal tracts which were used to screen for spore forming bacteria in tryptic soy agar. Among 24 bacteria isolates, 21 of them were derived from Nile tilapia samples and the rest were from Catfish samples. The 24 isolated bacteria were tested for the inhibitory effect against *F. columnare* KJ720207 and the widest of inhibition diameter was 19.5 mm. As a result, the isolate of *Bacillus* sp. which was showed 19.5 mm of inhibition diameter against *F. columnare* KJ720207 was selected for further use.

Identification of the selected *Bacillus* sp.

Biochemical and physiological properties and antibiotic sensitivity tests of the selected strain *Bacillus* sp. are given in Table 2. This strain was Gram positive, motile, showed positive results on the production of catalase and acetoin, nitrates reduction, hydrolysis of starch, utilization of citrate, growth on 6.5% NaCl. It was sensitive to Penicillin (10 µg), Kanamycin (30 µg), and Streptomycin (25 µg).

Table 2 Phenotypic characteristics and antibiotics sensitivity test of the selected *Bacillus* sp.

Characteristics	Response	Characteristics	Response
Gram	+	Utilization of citrate	+
Motility	+	Growth on 6.5% NaCl	+
Production of:		Sensitive to:	
-Catalase	+	-Penicillin (10 µg),	+
-Acetoin	+	-Kanamycin (30 µg)	+
Nitrates reduction	+	-Streptomycin (25 µg)	+
Hydrolysis of starch	+		

(+) positive response (-) negative response

The selected *Bacillus* sp. was characterized by molecular biology assays at the species level by Nucleotide sequencing, 16S rDNA (Partial sequence; 1,500 bp). DNA sequencing of the isolated strain was showed in Figure 4 which was similar to *B. amyloliquefaciens* YH-22 (100% similarity). Therefore, it was identified as *B. amyloliquefaciens* and the 16S rDNA sequence of this isolate was deposited in the GenBank database with scientific name and accession number *B. amyloliquefaciens* KJ720206 (Figure 2)

Figure 2 DNA sequence of *B. amyloliquefaciens* KJ720206 by 16S rDNA sequence (Partial sequence; 1,500 base pairs)

Discussion

F. columnare is a causative agent of bacterial disease which is an important infectious disease in farmed fish (Prasad *et al*., 2011). In Ubon Ratchathani province, Thailand, columnaris disease is a cause of significant loss to aquaculture industry. Even though this outbreaks disease was found in short winter season and under temporary stress conditions of culturing or transportation, but it always causes in term of mass mortality of cultured fish especially in fingerlings. Antibiotics are widely used as disease control but not succeed for columnaris disease cause of rapid outbreak incidences. The use of probiotics as disease control in aquaculture is now acceptable and more interesting, moreover, probiotics can use as digestion aids and immune booster, so the prevention of sudden outbreak disease as columnare by probiotics using may be suitable for Nile tilapia cultivation. Probiotic *Bacillus* strains were regarded as no toxicity when they were used as feed supplemented (Coa *et al*., 2011; Chantharasophon *et al*., 2011). For that reason, the selected isolate of probiotic bacillus must be tested for its inhibitory effect to the pathogen *F. columnare*.

In this research, phenotypic characteristics which were studied by conventional microbiological methods and DNA sequencing of the selected columnaris disease bacterial isolate were expressed as *F. columnare* KJ720207. These results are consistent with Kubilay *et al*. (2008) and Sebastião *et al*. (2010) whose were reported that conventional microbiological methods can be used to classify *F. columnare* as well as the use of API 20 E test system and molecular technique (PCR-RFLP technique). The accession number KJ720207 was showed only 99% homology with *F. columnare* IAM 14821 and the other strains of *F. columnare* from the GenBank database, it may be that KJ720207 strain was normal flora strain of Ubon Ratchathani province, Thailand, therefore 1% DNA sequencing was not homology to GenBank database strains.

For probiotic bacillus screening, after inhibitory effect of fish gastrointestinal tracts bacilli against *F. columnare* KJ720207 were performed in order to select the best strain of probiotic bacillus for the identification. Phenotypic characteristics and 16S rDNA sequencing method were used for this probiotic bacillus characterization at the species level and the selected strain was found to be *B. amyloliquefaciens* KJ720206 which was showed 100% homology with the strain YH-22 from the GenBank database. Normally, bacilli are transient bacteria, the strain KJ720206 from fish gut might be come from surrounding water or soil. This study was in agreement with that of Coa *et al*. (2011) who reported that *B. amyloliquefaciens* isolate G1 from the brackish water sediment samples displayed a significant *in vitro* inhibitory effect against the eel-pathogenic

A. hydrophila and other *A. hydrophila* strains which were opportunistic pathogens in aquaculture. Many researchers reported the potential of *B. amyloliquefaciens*. Arguelles-Arias *et al*. (2009) found that it could synthesize biocontrol agents, particularly, the dipeptide antibiotic bacilysin which could display biocontrol of plant pathogens. It showed clear zone against fire blight, a serious disease of orchard trees caused by *Erwinia amylovora* (Chen *et al*., 2013); tomato bacterial wilt, *Ralstonia solanacearum* (Tan et al., 2013); fugal wilt, *Fusarium solani* (Ajilogba *et al*., 2013); phytopathogenic fungi (Ji *et al*., 2013); root and rots disease (Xue *et al*., 2014); fish pathogens, *Edwardsiella tarda, A. hydrophila, Vibrio parahaemolyticus* and *V. harveyi* (Das *et al*., 2013). Furthermore, Xie et al. (2013) isolated and used *B. amyloliquefaciens* HN for remediation of aquaculture water and reported that it expressed high tolerance towards 80 mg/L of nitrile-N and ammonia-N and removed 20 mg/L of nitrile-N.

Singh *et al*. (2013) and Liu *et al*. (2012) reported that the use of phenotypic characteristics and 16S rDNA sequencing which are widely practiced methods could accurately classify *B. amyloliquefaciens*. In this study, phenotypic characteristics were used as bacterial preliminary diagnosis then the 16S rDNA sequencing technique was used to identify at the species level.

Conclusions

F. columnare and probiotic bacillus were isolated from Nile tilapia and Catfish samples and identified by phenotypic characteristics and 16S rDNA sequencing method. The selected strains were classified as *F. columnare* KJ720207 and *B. amyloliquefaciens* KJ720206. Moreover, the selected probiotic bacillus, strain KJ720206 could show antagonistic effect against *F. columnare* KJ720207. These results suggested that *B. amyloliquefaciens* KJ720206 may be used as a potential probiotic for columnaris disease controlling in farmed fish.

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