ชุมชนแบคทีเรียในลำไส้ของกุ้งกุลาดำ *Penaeus monodon*

นายพยุงศักดิ์ มงคล

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BACTERIAL COMMUNITY IN INTESTINE OF BACK TIGER SHRIMP *Penaeus monodon*

Mr. Phayungsak Mongkol

A Thesis Submitted in Partial Fulfillment of the Requirements
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พยุงศักดิ์ มงคล : ชุมชนแบคทีเรียในลำไส้ของกุ้งกุลาดำ *Penaeus monodon*. (BACTERIAL COMMUNITY IN INTESTINE OF BLACK TIGER SHRIMP *Penaeus monodon*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.ดร. เปี่ยมศักดิ์ เมนะเศวต , อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. เสจ ไชยเพ็ชร, ดร. ศิราวุธ กลิ่นบุหงา, 116 หน้า.

กุ้งกุลาดำ (*Penaeus monodon*) เป็นสัตว์เศรษฐกิจที่สำคัญชนิดหนึ่งของประเทศไทย ในปัจจุบันผลผลิตของกุ้งกุลาดำมีแนวโน้มลดลงเนื่องจากอุตสาหกรรมการเพาะเลี้ยงกุ้งกุลาดำประสบกับปัญหาต่างๆ เช่น ปัญหา การขาดแคลนพ่อแม่พันธุ์ ปัญหากุ้งโตช้าและแคระแกรนรวมถึงผลกระทบจากสิ่งแวดล้อมและการจัดการด้วยโรค จุลินทรีย์ ที่อาศัยอยู่ในระบบทางเดินอาหารของสัตว์น้ำมีส่วนร่วมในการเพิ่มกิจกรรมของเอนไซม์ในการย่อยอาหาร และอาจช่วยต่อต้านโรคต่างๆ และส่งผลให้กุ้งมีสุขภาพดี เจริญเติบโตได้อย่างรวดเร็วทนต่อโรคต่างๆ ได้มากขึ้น ความรู้ความเข้าใจเกี่ยวกับโครงสร้างและองค์ประกอบของกลุ่มแบคทีเรียที่อาศัยอยู่ในลำไส้ของกุ้งยังมีจำนวนจำกัด การศึกษาชุมชนแบคทีเรียในลำไส้กุ้งจึงมีความสำคัญต่อการเพิ่มประสิทธิภาพการเลี้ยงกุ้งให้สูงยิ่งขึ้น

วัตถุประ สงค์ของวิทยานิพนธ์ เพื่อศึกษาชุมชนแบคทีเรียในลำไส้ ของกุ้งกุลาดำ โดยประกอบด้วย (1) ศึกษาความแตกต่างของชุมชนแบคทีเรียในลำไส้ของกุ้งกุลาดำในระยะวัยรุ่นอายุเท่ากันที่มีขนาดต่างๆ กัน (2) ศึกษาองค์ประกอบและ ความหลากหลายของชุมชนแบคทีเรียในระบบ ทางเดินอาหารส่วน ต่างๆ ของกุ้งพ่อแม่พันธุ์ (3) ศึกษาการเปลี่ยนแปลง ของชุมชนแบคทีเรียในลำไส้กุ้งกุลาดำหลังถูกกระตุ้นด้วยเชื้อ *V. harveyi* ที่เวลาต่างๆ และทำการคัดกรองแบคทีเรีย ในลำไส้ของกุ้งกุลาดำที่มีคุณสมบัติในการยับยั้งการเจริญเติบโตของ *V. harveyi* และ *V. parahaemolyticus* เชื้อ 2 สายพันธุ์ที่ก่อโรคในกุ้ง

ชุมชนแบคทีเรียในลำไส้ของกุ้งกุลาดำในระยะวัยรุ่นอายุเท่ากันที่มีขนาดต่างๆ กัน จำนวน 60 ตัว และลำไส้ส่วนต่างๆ จำนวน 5 ส่วน (กระเพาะอาหาร ลำไส้ส่วนกลางตอนต้น ลำไส้ส่วนกลาง ลำไส้ส่วนกลางตอนท้าย ลำไส้ส่วนท้าย และ มูลกุ้ง) ของกุ้งพ่อแม่พันธุ์จำนวน 8 ตัว ถูกวิเคราะห์โดยวิธีพีซีอาร์ - ดีจีจีอี (PCR –DGGE) และจำแนกสายพันธุ์ของแบคทีเรียที่ได้จากวิธีดีจีจีอีด้วยยีน 16S rDNA โดยทำการโคลนและหาลำดับนิวคลีโอไทด์เพื่อเปรียบเทียบกับฐานข้อมูล พบความหลากหลายของชุมชนแบคทีเรีย 3 ถึง 4 ไฟลัมในลำไส้ของกุ้งกุลาดำได้แก่ ไฟลัม Proteobacteria (β-, δ-, γ- และα-) Fusobacteria Firmicutes และ Bacteroidetes พบแบคทีเรียในกลุ่ม γ - Proteobacteria มากสุดและพบในทุกตัวอย่างโดยไม่ขึ้นกับแหล่งที่มาของกุ้ง ในขณะที่กลุ่มอื่นๆ พบน้อยและมีการจำกัดในการกระจายตัว พบแบคที เรียพวก *Vibrio* spp. และ *Photobacterium* sp. เป็นจำนวนมากและพบในกุ้งระยะวัยรุ่นและกุ้งพ่อแม่พันธุ์ ไม่พบความแตกต่างระหว่างชุมชนแบคทีเรียในกุ้งระยะวัยรุ่นที่มีอายุเท่ากันแต่ขนาดต่างกัน (เล็ก กลาง และ ใหญ่) แต่พบความแตกต่างระหว่าง 2 เดือนและ 5 เดือน พบองค์ประกอบและความหลากหลายของชุมชนแบคทีเรียในส่วนต่างๆ ของระบบทางเดินอาหารในกุ้งพ่อแม่พันธุ์ การเปลี่ยนแปลงของชุมชนแบคทีเรียในลำไส้เมื่อถูกกระตุ้นด้วยเชื้อ *V. harveyi* สามารถตรวจพบได้หลังจากการกระตุ้นเป็นระยะเวลา 48 ชั่วโมง เชื้อบริสุทธิ์ทั้งหมดที่คัดแยกได้ พบ 17 เชื้อ สามารถยับยั้งเชื้อก่อโรคในกุ้งทั้ง 2 ชนิดได้ (*V. harveyi* และ *V. parahaemolyticus*) โดยวิธี co-culture ทำการคัดเลือก 12 เชื้อจากทั้งหมด 17 เชื้อที่สามารถยับยั้งเชื้อก่อโรคได้สูงมาศึกษาคุณลักษณะ ของเชื้อเพิ่มเติม พบว่า 8 เชื้อเป็นสายพันธุ์ *Vibrio* spp. 2 เชื้อเป็นสายพันธุ์ *Shewanella* spp. และอย่างละ 1 เชื้อเป็นสายพันธุ์ของ *Pseudomonas* sp. และ *Pseudoalteromonas* sp. ตามลำดับ

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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PHAYUNGSAK MONGKOL : BACTERIAL COMMUNITY IN INTESTINE OF BLACK TIGER SHRIMP *Penaeus monodon*.
ADVISOR : PROF. PIAMSAK MENASVETA, Ph.D., CO-ADVISOR : SAGE CHAIYAPECHARA, Ph.D., SIRAWUT KLINBUNGA, Ph.D., 116 pp.

The black tiger shrimp *Penaeus monodon* is an important aquaculture species for Thailand. In recent years, the industry faced with several problems such as a lack of suitable broodstocks, environmental impact, and diseases. Intestinal bacteria of aquatic animals play a crucial role in the host-animals well-being such as improving digestive system and disease resistance, but the understanding of shrimp-bacteria interaction is limited.

The objective of this thesis was to examine several aspects of the intestinal bacterial community associated with black tiger shrimp (*Penaeus monodon*) including (1) the differences in the intestinal bacterial community of farmed juvenile shrimp of different sizes in the same cohort, (2) the diversity of the intestinal bacteria community in different segments of the gastrointestinal tract, and (3) the variation in the intestinal bacterial community upon a challenge with shrimp pathogen *Vibrio harveyi*. In addition, pure culture isolates collected from the intestines of *P. monodon* were also screened for inhibitory effects against *V. harveyi* and *V. parahaemolyticus*.

Bacterial communities from the intestinal tracts of 60 farmed-raised juveniles and 5 different segments of the GI tracts of 8 wild-caught adult shrimps were analyzed using PCR-DGGE, cluster analysis, and 16S rDNA sequencing. The intestinal bacterial communities of *P. monodon* are a diverse group of bacteria that generally included 3 to 4 main phyla including Proteobacteria (β-, δ-, γ- and α-), Fusobacteria, Firmicutes and Bacteroidetes. Regardless of the source or life-history of the shrimp, γ- Proteobacteria is the most abundant in the intestinal community and can be found in all segments. Other phyla were less abundant and limited to certain segments in their distribution. *Vibrio* spp. or *Photobacterium* sp. are dominant genera that can be found in farm-raised juvenile and wild-caught adults. Bacterial communities from farm-raised juvenile shrimp of different sizes showed no distinct clustering pattern by size at either 2 or 5 month. However, the differences in bacterial communities between 2 and 5 month old juveniles were pronounced. Bacterial communities in different segments of the GI tract of wild-caught adults were different in both the diversity and composition. The change in the intestinal bacterial community upon challenge with *V. harveyi* can usually be observed after 48 hrs. Seventeen pure culture isolates were shown to inhibit the growth of both *V. harveyi* and *V. parahaemolyticus* using co-culture methods. Twelve isolates that were selected for further characterization were closely related to *Vibrio* spp. (8 isolates), *Shewanella* spp. (2 isolate), *Pseudomonas* sp. (1 isolate), and *Pseudoalteromonas* sp. (1 isolate).

Field of Study : Biotechnology Student's Signature .....
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LIST OF ABBREVIATIONS

α	Alpha
BLAST	Basic Local Aligment Search Tool
bp	Base pair
β	Beta
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CFU	Colony Forming Unit
cm	Centimeter
CTAB	Cetyl trimethylammonium bromide
°C	Degree celcius
δ	Delta
DGGE	Denatured gradient gel electrophoresis
DNA	Deoxyribonucleic acid
$dNTP_s$	Deoxynucleotide triphosphates
ε	Epsilon
e.g.	*Exempli gratia*, Latin: "for example"
et, al	*Et. alli* (Latin), and others
EDTA	Ethylene diamine tetra-acetic acid

Fig	Figure
γ	Gamma
g	Gram
HCl	Hydrochloric acid
hr	Hour (s)
IPTG	Isopropyl-β-D-thio-galactopyranoside
Kb	Kilobase (1,000 bp)
lbs	Pound
M	Molar
MEGA	Molecular Evolution Genetic Analysis
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
µg	Microgram
µl	Microliter
µM	Micromolar
NaCl	Sodium chloride

ng	Nanogram
nM	Nanomolar
OD	Optical density
OTU	Operational Taxonomic Unit
PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction denatured gradient gel electrophoresis
pH	Potential of hydrogen ion
ppm	Parts per million
ppt	Parts per thousand
%	Percent
RDP	Ribosomal Database Project
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
sec	Second (s)
sp	Species
spp	Species (plural)
$\times g$	Times gravity
T	Type strain
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA

Tris	Tris (hydroxyl methyl) aminomethane
U/ml	Unit per milliliter
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultraviolet
V	Volt
v/v	Volume by volume
w/v	Weight by volume
X-gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

CHAPTER I

INTRODUCTION

1.1 Background information

The black tiger shrimp *Penaeus monodon* is a major agricultural product of high economic values for Thailand in the past decade (Department of Fisheries, 2010). However, production of *P. monodon* in Thailand has decreased dramatically since 2004-2008 because there are many challenges associated with *P. monodon* aquaculture such as a lack of suitable captive broodstock, management of disease and the increasing popularity of *Litopenaeus vannamei* (Tanticharoen *et al*., 2008). At present, *L. vannamei* is dominant aquaculture species and the production in 2008 is approaching 500,000 metric ton. In contrast, production of *P. monodon* is approximately 8,000 metric ton (Fig. 1.1).

Shrimp aquaculture production in much of the world is affected by diseases particularly those caused by luminous *Vibrio harveyi* and viruses. *V. harveyi*-the cause of luminescent bacterial disease, white sport syndrome virus and yellow virus (YHV) are the three pathogens that account for the majority of losses in Thai shrimp aquaculture by causing sudden and massive shrimp mortality (Flegel *et al*., 1992; Spaargaren, 1996; Lightner and Redman, 1998). The use of antibiotics can lead to the emergence of more virulent pathogens, which may transfer of antibiotic resistance to human pathogens. Furthermore, the excessive use of excessive antibiotic can also lead to antibiotic residue in shrimp product, which can affect market acceptance and leaving negative impact on the environment (Holmström *et al*., 2003). Alternative methods to control diseases such as the use of beneficial bacteria to displace pathogens by competitive processes or to inhibit their proliferation should be encouraged and applied whenever applicable (Verschuere *et al*., 2000; Rengpipat *et al*., 2003).

There are many studies on the intestinal bacterial community in commercially important aquaculture species such as rainbow trout *Oncoryhnchus mykiss Walbaum* (Merifield *et al*., 2009), common carp *Cyprinus carpio* (Al-Harbi and Uddin, 2012),

European sea bass *Dicentrarchus labrax* (Schryver *et al*., 2011) and channel catfish *Ictalurus punctatus* (Burr *et al*., 2012).

The interaction between bacteria and their host animal have been recognized as an important element in the host animal well-being. Intestinal bacteria play a major role in many aquaculture species. Their roles include helping with digestion by contributing exogenous enzymes, enhancing shrimp growth and survival by improving its intestinal microbial balance and water quality, and improving disease resistance by suppressing the pathogens (Li *et al*., 2008; Rawls *et al*., 2004; Ringø and Birkbeck 1999; Wang, 2007; Zhou *et al*., 2009). The knowledge in the literature about this intestinal bacterial community and their roles in Penaeid shrimp are limited. There might be many benefits in learning about the interaction of bacteria and shrimp in aquaculture.

1.2 Objective of this thesis

The objective of this study was to examine several aspects of the intestinal bacterial community associated with the black tiger shrimp (*Penaeus monodon*). Using traditional plate counting, 16S PCR-DGGE and 16S rDNA clone library methods, experiments were conducted to determine (1) the differences in the intestinal bacterial community of farmed- raised juvenile shrimps of different sizes in the same cohort, (2) the diversity of the intestinal bacteria communities in different segments of the gastrointestinal tract of wild-caught adult shrimps, and (3) the variation in the intestinal bacterial community upon a challenge with shrimp pathogen *Vibrio harveyi*. In addition, pure culture isolates collected from the intestines of *P. monodon* were also screened for inhibitory effects against two shrimp pathogens (*V. harveyi* and *V. parahaemolyticus*), and selected isolates were further characterized using phenotypic tests.

1.3 General introduction

Asians have been farming fish and crustaceans in coastal areas using traditional technique for at least 3000 years (Stickney, 1979). Thailand started farming shrimp in the 1970s, using locally available *P. monodon* broodstock captured from the sea to produce post-larvae (PL) in land-based hatcheries for pond stocking. Farming activity of *P. monodon* in Thailand has rapidly increased reflected by a large annual production. Aquaculture of *P. monodon* increases national revenue, therefore *P. monodon* is an economically important species in Thailand. During 1991s-1994s, Thailand emerged as the world's leader in farmed shrimp producer and exporter based on *P. monodon* production (Rosenberry, 1995). Exports increased from 153,000 tons in 1991 to 250,000 tons in 1994, representing a value of nearly 2,000 billion U.S. dollars.

Thailand has been regarded as the leading shrimp producer of farmed shrimp for over a decade. The major global producers of *P. monodon* are Thailand, China, Vietnam, Indonesia, India, Malaysia and Philippines (Table 1.1).

Table 1.1 Total shrimp production (metric tons) from the aquaculture during 2004 - 2010 by country.

Country	**2004**	**2005**	**2006**	**2007**	**2008**	**2009**	**2010**
Thailand	360,000	380,000	500,000	530,000	495,000	563,000	640,000
China	352,000	380,000	400,000	480,000	523,000	560,000	600,000
Vietnam	106,000	115,000	150,000	170,000	200,000	200,000	224,000
Indonesia	205,000	230,000	260,000	210,000	230,000	180,000	140,000
India	100,000	100,000	103,000	110,000	870,000	100,000	120,000
Malaysia	28,000	32,000	42,000	62,000	68,000	92,000	105,000
Philippines	35,000	35,000	36,000	38,000	29,000	35,000	410,000
United state	275,000	304,000	395,000	495,000	397,000	412,000	387,000
Other	125,000	125,000	55,000	55,000	55,000	50,000	65,000
Total	**1,586,000**	**1,701,000**	**1,941,000**	**2,150,000**	**2,867,000**	**2,192,000**	**2,691,000**

(Source: http://www.thaiahpa.com/Feed5.pdf)

The important markets for shrimp are the United States, Japan, Canada, South Korea, and Australia (Table 1.2). Currently, nearly 70% of shrimp produced in Thailand comes from aquaculture. In 2000, Thailand produced 240,000 tons of the World's 680,000 tons output. The export from Thailand grew to 380,000 tons black tiger prawns (Source: http://www.thaiahpa.com/Feed5.pdf).

In recent years the industry faced with several major challenges such as a lack of suitable captive broodstocks, long-term sustainability of feed ingredients, environmental impact, and management of diseases. Diseases in shrimp, mostly due to bacteria (especially the luminous *V. harveyi*) and viruses, led to slow growth, high mortality rate, and can have a large negative economic impact on shrimp farmers (Alam *et al*., 2007; Valderrama and Engle 2004). These problems has eventually lead the farming to decline and a replacement of the giant tiger shrimp with Pacific white shrimp *Litopenaeus vannamei*. At present, *L. vannamei* is the dominant aquacultured species and production in 2008 is approaching 500,000 metric ton. In contrast, production of *P. monodon* is 8,000 metric ton in 2008 (Fig. 1.1).

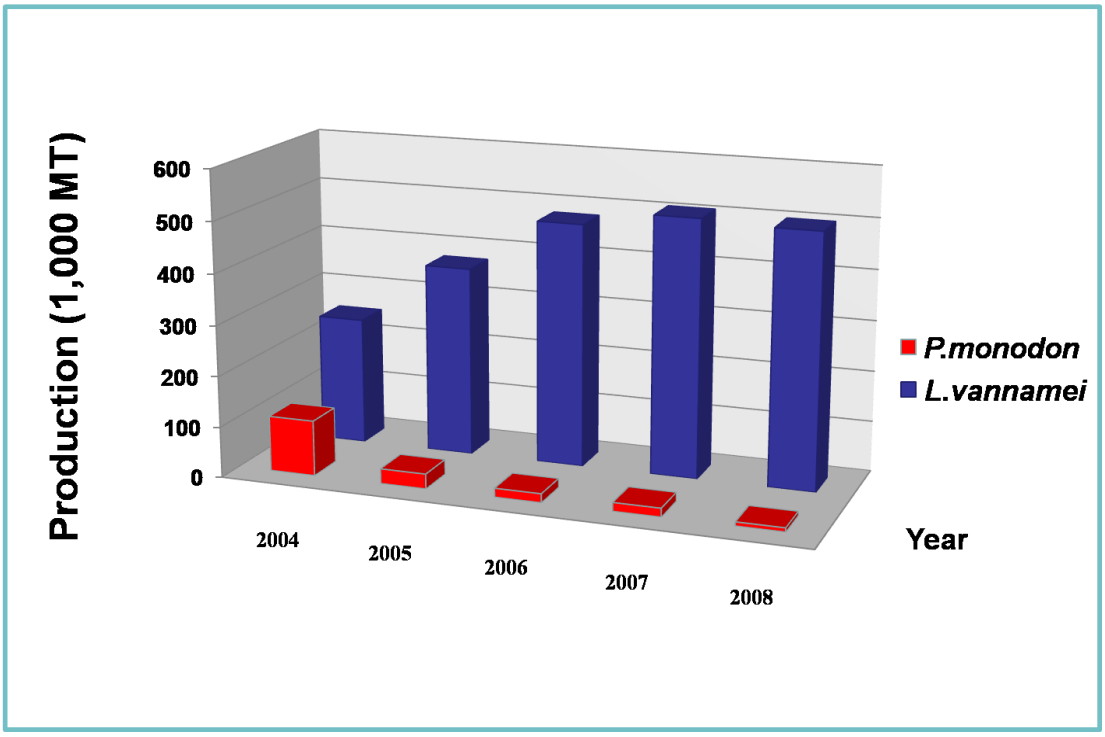

Figure 1.1 Annual production of *P. monodon* and *L. vannamei* in Thailand during the 2004-2008 period. (Source: Department of Fisheries, 2010)

Table 1.2 Black Tiger Shrimp Export from Thailand during 2006 - 2011.

Country	2006		2007		2008		2009		2010		2011	
	Quantity	**Value**	**Quantity**	**Value**	**Quantity**	**Value**	**Quantity**	**Value**	**Quantity**	**Value**	**Quantity**	**Value**
USA	157,648	39,222	196,827	50,416	180,974	41,737	177,847	42,496	183,455	44,750	192,513	47,208
Japan	49,358	15,590	53,867	15,413	58,982	14,458	63,154	16,374	70,585	19,133	76,954	20,373
EU27	11,669	2,798	20,574	5,342	30,989	7,722	39,667	9,699	52,165	12,357	66,150	14,924
Canada	14,640	3,706	16,661	4,252	25,206	5,525	20,447	4,837	21,428	5,039	22,437	5,447
Australia	10,348	2,387	9,334	2,287	8,876	2,031	7,251	1,740	9,579	2,231	10,290	2,473
Korea, republic of	13,219	2,853	15,472	3,347	14,952	2,968	12,728	2,321	10,638	1,943	9,423	1,825
China	3,312	595	2,889	585	3,924	728	5,105	681	4,615	793	11,665	1,599
ASEAN10	5,584	1,127	5,879	1,039	7,217	1,193	7,994	1,113	10,439	1,485	9,452	1,527
Taiwan	3,929	818	2,809	600	3,166	639	5,192	936	5,665	994	6,281	1,070
Hong kong	3,977	988	4,219	1,250	4,504	1,143	3,764	906	3,185	875	3,204	835
OTHER	5,650	1,270	8,280	1,743	11,299	2,189	11,157	2,182	11,288	2,309	11,780	2,327
Total	**279,334**	**71,354**	**336,810**	**86,275**	**350,089**	**80,332**	**354,305**	**83,285**	**383,042**	**91,909**	**420,149**	**99,609**

Source: Office of Agricultural Economics, Ministry of Agriculture and Cooperatives, 2006

1.4 Penaeid shrimp biology

1.4.1 Taxonomy of *P. monodon*

Penaeid shrimp belong to the largest phylum in the animal kingdom, the Arthopoda. This group of animals is characterized by the presence of paired appendages and a protective cuticle or exoskeleton that covers the whole animal. The subphylum Crustacea is made up of 42,000, predominantly aquatic, species that belong to 10 different classes. Within the class Malacostraca, shrimp, together with crayfish, lobsters and crabs, belong to the order Decapoda (Fig. 1.2). Taxonomical recognition of *P. monodon* is illustrated below (Bailey-Brock and Moss, 1992).

Kingdom Animalia

Phylum Arthropoda

Subphylum Crustacea

Order Decapoda

Family Penaeidae (Rafinesque, 1985)

Genus *Penaeus* (Fabricius, 1798)

Subgenus *Penaeus*

Species *monodon*

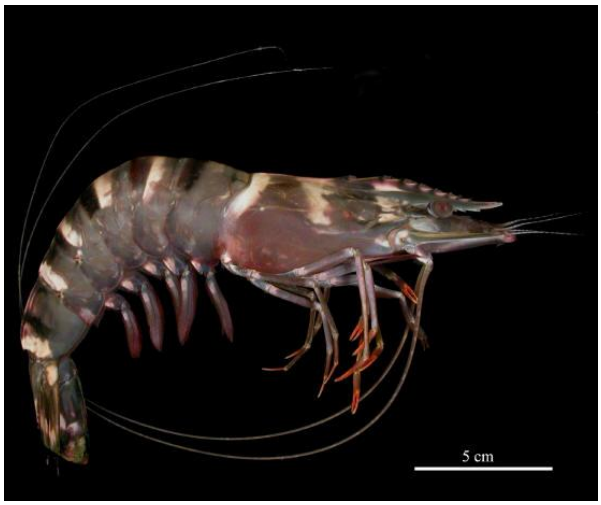

Figure 1.2 Taxonomy of the black tiger shrimp, *P. monodon* (Fabricius, 1798) (Brusca and Brusca, 1990)**.**

1.4.2 Morphology

The external morphology of penaied shrimp is separated into 2 parts; cephalothorax (in which head and thorax are fused and covered with carapace) and abdomen (Fig. 1.3). Most organs such as gills, digestive system and heart are located in the cephalothorax such as gills, digestive system and heart. In the abdomen has the muscles while, five pairs of p1eopods (swimming legs) are found on the abdomen (Baily-Brock and Moss, 1992). The most distinct features for identification of species are hepatic carina horizontally straight, fifth pereiopods without exopod and gastro-orbital carina occupying the posterior half of the distance between hepatic spine and postorbital margin of carapace (Holthuis, 1980).

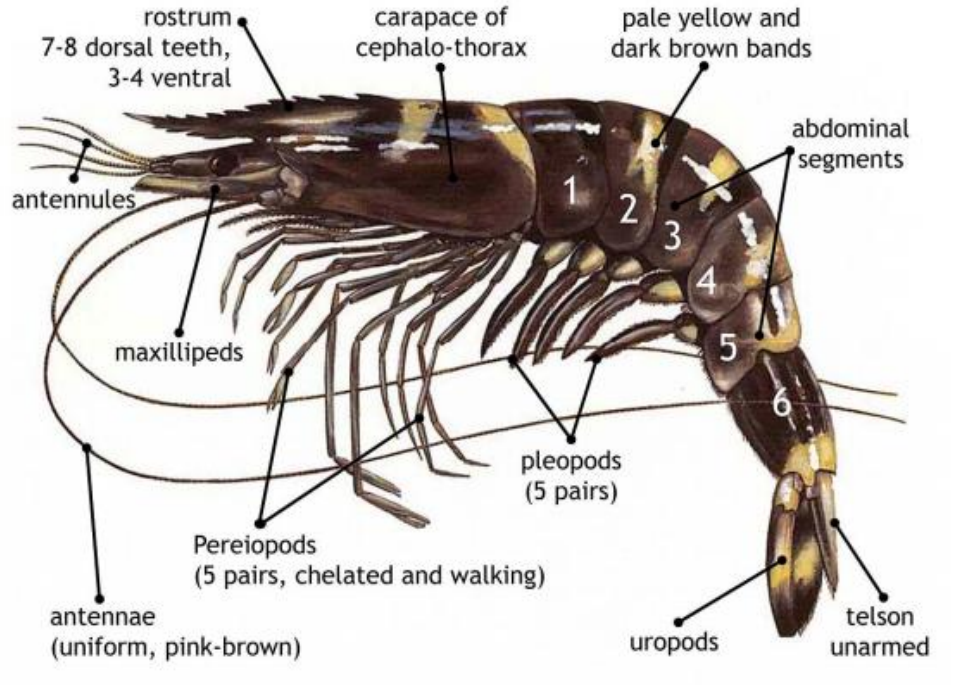

Figure 1.3 Lateral view of the external morphology of *P. monodon*.

1.4.3 Digestive system

The internal morphology of penaeid shrimp is showed in Figure 1.4. Penaeid shrimp have a morphologically typical decapod digestive tract (Dall *et al*., 1990). Detection of feed begins with sight and touch, but shrimp also have numerous chemoreceptors on their appendages (e.g. the mandible, maxillule, maxilla, lateral antennular flagellum, dactyls of maxilliped 3 and periopods, merus of periopods, maxillipeds and the branchial chamber) (Lee and Meyers, 1997). The digestive tract of shrimp is divided into three main parts, the foregut, midgut and hindgut (Ceccaldi, 1997). The foregut (proventriculus, stomach) comprises the oesophagus and the part of the stomach where mastication occurs. The midgut gland or hepatopancreas secretes digestive enzymes, absorbs digested products and maintains mineral reserves. It also functions in lipid and carbohydrate metabolism, the distribution of stored reserves and the catabolism of some organic compounds (Ceccaldi, 1997). The hindgut is a chitin-lined straight tube running from the cephalothorax dorsally through the abdomen to the rectum (Dall *et al*., 1990; Ceccaldi, 1997).

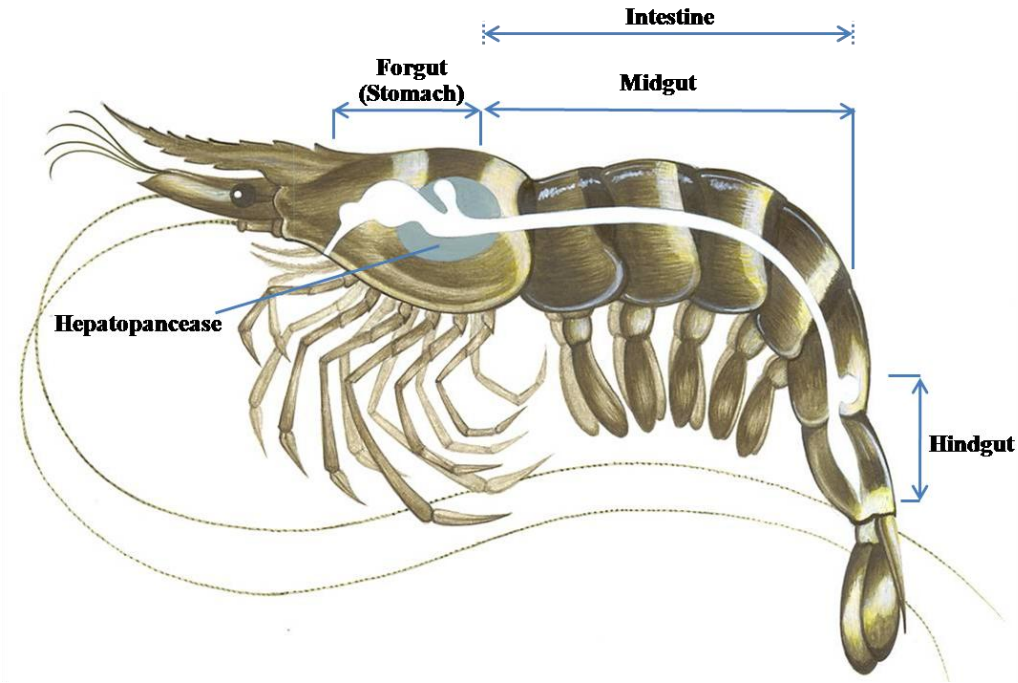

Figure 1.4 Digestive system of *P. monodon*.

1.5 Shrimp pathogens and antibiotics

Shrimp aquaculture production in much of the world is affected by diseases, particularly those caused by luminous *Vibrio* and viruses (Flegel *et al.*, 1992). Member of the genus *Vibrio* are autochthonous bacterial flora in the aquatic ecosystem, and quite a few of them are associated with infections in human and aquatic animals. *Vibrio* are the normal bacteria flora of shrimp and the aquaculture environment (Jiravanichpaisal *et al*., 1994; Otta *et al*., 1999), but they often act as secondary or opportunistic pathogens that cause mortality (ranging from few to 100 % mortality) in populations under stress (Lightner, 1998). Vibriosis has been implicated as the cause of major mortality in juvenile penaeid shrimp (Lightner and Redman, 1994) and has been connected to a decrease in the shrimp production and intensive rearing system (Karunasagar *et al*., 1994). *V. harveyi* and *V. parahaemolyticus* are two major bacterial pathogens that can cause high mortality among economically important species of farmed marine fish and shrimp in Thailand (Nash *et al*., 1992). Recently, the production of *P. monodon* from shrimp farming has decreased dramatically due to massive mortality of farmed shrimp caused by *Vibrio* bacteria (Chythanya *et al*., 2002).

In the past, shrimp farmers widely used antibiotics to promote shrimp growth and to treat shrimp diseases (Holmström *et al*., 2003). Flaherty *et al*. (2000) reported that most commercial shrimp feeds are enriched with antibiotic. The most commonly used antibiotics were norfoxacine, oxytetracycline, enrofloxacin and different sulphonamides (Holmström *et al*., 2003). The use of antibiotics during shrimp cultivation has led to problem, including antibiotic resistant pathogens and that these microbes can infect both human and domesticated animals (Gräslund *et al*., 2002; Khachatourians, 1998; Wegener *et al*., 1999; Willis, 2000) and evidence of transfer of resistance encoding plasmids between aquaculture environmental and human (Rhodes *et al*., 2000). In 2002 the EU community rejected exported Thai shrimp that was contaminated with chloramphenicol and nitrofuran. Control and management of these diseases without resorting to excessive use of antibiotics should be encouraged.

1.6 Interactions between bacteria with aquatic animals in aquaculture

Aquatic organisms constantly come into contact with bacteria whose concentration can be as high as 10^6 to 10^7 mL^{-1} in their environments including in shrimp aquaculture ponds (Abraham *et al*., 2004; Burford *et al*., 2003; Maeda, 2002; Sakami *et al*., 2008). The microbes in the gastrointestinal tract (GI) tract are of high importance for the health of the host. They are believed to play a unique part to in host animal gastrointestinal tract development, nutrition, immune responses, and disease resistance (Li *et al*., 2008; Rawls *et al*., 2004; Ringø and Birkbeck, 1999; Wang, 2007; Zhou *et al*., 2009). In addition, the microorganisms in the GI tract can be involved in the protection against pathogens by the production of inhibitory compounds and competition for nutrients and space. For example, probiotics are micro-organisms, often *Bacillus* spp., intentionally added to the ponds, e.g. with the purpose to outcompete the pathogenic bacteria and thereby decrease the risk for disease outbreaks (Moriarty, 1998). Queiroz and Boyd (1998) and Moriarty (1998) found that *Bacillus* improved the survival of larvae, increased food absorption by enhancing protease levels and gave better growth in catfish and shrimp pound. The probiotic decreased the number of suspected pathogenic bacteria in the gut. Also, the *enterococci* influenced the microflora of the intestine, reducing the incidence of *Escherichia coli*, *Staphylococcus aureus* and *Clostridium* spp. Irianto and Austin (2002) reported that cultures of *Aeromonas* and *Vibrio* were effective at controlling infections by *A. salmonicida* in rainbow trout. In addition, the dominant antagonist was *Pseudomonas*, which improved the survival of rainbow trout against vibriosis following the addition of cultures to water.

The knowledge about this intestinal bacterial community in Penaeid shrimp is limited and researches in this area are still at an early stage. Data from other aquatic species and existing results suggested that this bacterial community could have some beneficial roles in shrimp similar to other animals.

1.7 Beneficial effects of probiotics and its application in aquaculture

Application of probiotics in shrimp aquaculture along with other aquatic animals has been gaining attention in the past decade (Balcázar *et al*., 2006; Wang *et al*., 2008). The source of many probiotics comes from microorganisms associated with the host animal either from the animal itself or the living environment (Verschuere *et al*., 2000; Vine *et al*., 2006). Successful competition and adequate ability to persist in the intestinal tract of the host animal is one of the keys to success as probiotic whose main function is to prevent infection via the intestinal route (Verschuere *et al*., 2000; Vine *et al*., 2006). Others key functions of probiotics includes production of inhibiting compounds, competition for resources and nutrients, competition for adhesion sites, and improving the host animal immune response (Verschuere *et al*., 2000). A selection scheme for probiotic has been suggested by several researchers (Verschuere *et al*., 2000; Vine *et al*., 2006). The first recommended step is to obtain bacterial strains for screening. Often bacteria associated with healthy animals and the rearing habitat can provide a significant source of probiotic bacteria that can survive best in the environment of the target animal (Vine *et al*., 2006).

Most research in probiotic uses in shrimp aquaculture focused on commercial shrimp species such as *L. vannamei* and *P. monodon*. Probiotic bacteria were shown to improve growth and survival, increase activities of digestive enzymes and immune responses, and reduced mortality after challenges with pathogens. Many of these probiotic species are gram-positive such as *Bacillus* spp. (Balcázar and Rojas-Luna, 2007; Decamp *et al*., 2008; Le *et al*., 2005; Rengpipat *et al*., 1998; Rengpipat *et al*., 2003; Tseng *et al*., 2009; Vaseeharan and Ramasamy, 2003; Wang, 2007; Ziaei-Nejad *et al*., 2006). *Paenibacillus* sp. (Ravi *et al*., 2007), *Lactobacillus* (Chiu *et al*., 2007), and *Arthrobacter* (Li *et al*., 2008; Li *et al*., 2006). Gram-negative bacteria such as *V. alginolyticus* (Balcázar and Rojas-Luna, 2007; Rodríguez *et al*., 2007), *Rhodobacter* (Wang, 2007), *Roseobacter* (Balcázar and Rojas-Luna, 2007), *Pseudomonas* (Balcázar and Rojas-Luna, 2007), and *Halomonas* (Zhang *et al*., 2009) have also been reported to have probiotic activity in shrimp. Common probiotic application methods included mixing with the feed or applied to rearing water, depending on the size and life-stage of the shrimp and the intended purpose of the probiotic. The interaction of

these probiotic bacteria to the existing bacterial community in the intestinal tract was rarely investigated due to both the lack of understanding and the lack of baseline for comparison. The characterization of bacteria in the intestinal tract of Penaeid shrimp is therefore needed.

1.8 Molecular strategies for monitoring bacterial communities

Traditionally, the occurrence of microorganisms in a given environment has been examined by a culture–based approach (culturing technique or plate count). However, several studies performed on many environments estimated that more than 99% of organisms observed microscopically cannot be cultivated on traditional culture media. Therefore, researchers have come to the conclusions that microbial diversity is much greater than that previously anticipated, and that culture techniques are insufficient for exploring the enormous "reservoir" of hidden diversity in natural habitats (Amann, 1995; Muyzer, 1999).

In the last two decades, the increased development and routine application of molecular-based techniques has made possible a more accurate evaluation of the biodiversity of microbial communities. Since its introduction in the mid-1980s, PCR has become a fundamental aspect of molecular ecology, and several PCR-based techniques have been developed to study microbial communities.

These methods involve an initial PCR amplification step achieved by means of primers that are specific to the organisms of interest. The second step involves the detection of sequence variations in the PCR fragments either by a cloning or sequencing analysis, which provides a complete characterization of the fragments, or by an electrophoretic analysis, which provide a visual separation of the mixture of fragments according to sequence polymorphism (denaturing or temperature gradient gel electrophoresis, single strand conformation polymorphism) or length polymorphism (terminal-restriction fragment length polymorphism, automated ribosomal intergenic spacer analysis) (Fig. 1.5).

The PCR-based techniques most widely used for assessing the genetic diversity of microbial communities will be discussed in this section to illustrate the principles, advantages and shortcomings of PCR-DGGE.

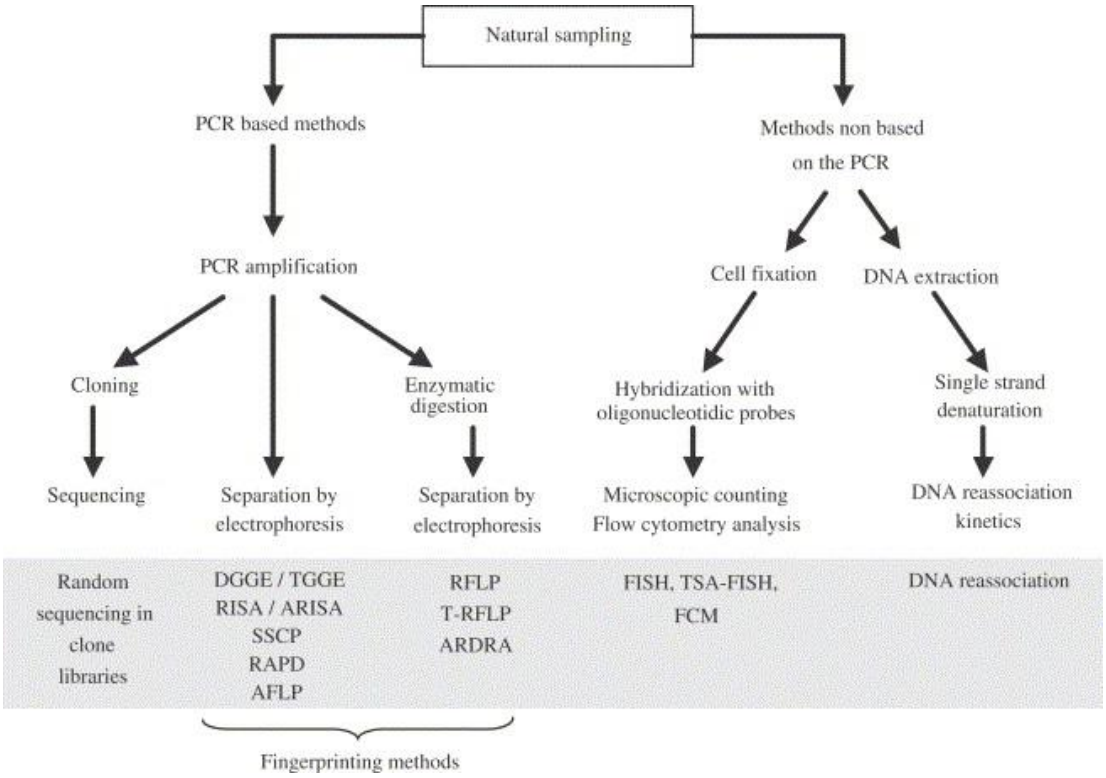

Figure 1.5 Diagram of different molecular approaches for assessing the genetic diversity of natural microbial communities (Dorigo *et al*., 2005).

Denatured Gradient Gel Electrophoresis (DGGE) is a preferred method for many studies of complex bacterial community due to its versatility and large existing database for 16S rDNA. DGGE profiles complex microbial community based on the differences in the denaturing property of 16S rDNA sequences associated with each bacteria (Muyzer *et al*., 1993). The increasing concentration of denaturant along the length of the acrylamide gel (horizontal gradient) used during electrophoresis separates 16S rDNA segments with different nucleotide sequences along the gradient. The resulting DNA bands can be excised and sequenced to determine their identities. Comparing to other profiling methods, DGGE has both advantages and disadvantages. DGGE profile reflects the predominant community and is not as sensitive as 16S rDNA clone library to detect rare species. Liu *et al*. (2009) reported that species comprised of less than 3% of total community cannot be detected by DGGE. DGGE

has an advantage over automated ribosomal intergenic spacer analysis (ARISA) with a larger database of 16S rDNA compared to intergenic spacer database. Cherif *et al*. (2008) suggested that ARISA is more appropriate for rapid assessment of bacterial community when large numbers of sample need to be compared, and the exact identity of bacteria was not crucial to the analysis. One main disadvantage associated with DGGE as with other gel based analysis is the variation between each gel, run conditions, and results interpretation. The use of internal standards, fluorescent-labeled primers, and standardized run condition can be helpful in enhancing the normalization of DGGE (Bruggemann *et al*., 2000; Neufeld and Mohn, 2005; Powell *et al*., 2005; Sigler *et al*., 2004). The incorporation of a nested PCR step to focus the analysis on a specific bacterial community can further enhance the discriminating power of DGGE (Boon *et al*., 2002; Dar *et al*., 2005).

1.9 Intestinal bacteria of other shrimps

There were a few reports in the literature on the intestinal bacterial community of *P. monodon,* but there reports rely on culture-based method to analyze the community. The intestinal bacterial community of related shrimp species such as white leg shrimp *L. vannamei,* Chinese shrimp *Fenneropenaeus chinensis*, banana shrimp *Fenneropenaeus indicus*, and pink shrimp *Penaeus duorarum* have also been reported in the literature.

The characterization of intestinal bacteria of *P. monodon* using cultivation method has been done. Shakibazadeh *et al*. (2009) examined the intestinal bacterial community along with several body parts of hatchery reared *P. monodon* using 4 types of agar media including tryptone soy agar (TSA), MacCongey agar (*Enterobactericeae* specific), Thiosulphate Citrate Bile Salt (TCBS) agar (*Vibrio* and *Aeromonas* specific) and *Pseudomonas*-isolating agar. They reported that the bacterial concentration in the digestive tract of *P. monodon* is 1.1 x 10^6 CFU/ g (on TSA medium), and the intestinal bacterial community of juvenile *P. monodon* consisted of *Vibrios, Shewanella, Burkholderia Clavibacter, Staphylococcus, Corynebacterium* and *Brevibacterium* (Shakibazadeh *et al*., 2009).

The only report examining the intestinal bacterial community using molecular methods in shrimp is that of white leg shrimp *L. vannamei.* Johnson *et al*. (2008) studied the intestinal bacteria of *L. vannamei* reared in recirculating water system

using DGGE. They reported that the foregut of *L. vannamei* was populated by *Mycobacterium* spp., *Propionibacterium* spp. and *Desulfocapsa* spp. and the hindgut was dominated by *Vibrio* spp. (Johnson *et al*., 2008). In addition, while the biomass of bacteria was lower in the rearing water than in the intestinal tract of shrimp, bacterial diversity was higher in the rearing water (Johnson *et al*., 2008).

Liu *et al*. (2011) conducted a study to identify intestinal microbial diversity of Chinese shrimp (*F. chinensis*) using PCR–DGGE and clone library analyses methods, and compare the results obtained by two methods. They found that the gut microbiota of was composed of Firmicutes, Proteobacteria and Bacteroidetes. The predominant bacterial population in the intestine of Chinese shrimp was Proteobacteria, and *Vibrio* sp. was the most abundant bacteria. In addition, they concluded that both PCR-DGGE and clone library gave similar results.

Oxley *et al*. (2002) found that both wild and cultured Banana prawns *F. indicus* supported remarkably similar bacterial floral compositions, which included members of *Aeromonas, Plesiomonas, Photobacterium, Pseudoalteromonas, Pseudomonas* and *Vibrio,* with members of the genus *Vibrio* being quantitatively dominant.

Esiobu *et al*. (2003) also reported that all healthy and live pink shrimp *Penaeus duorarum* guts were heavily colonized by *Vibrio* species, especially *V. harveyi* and *V. logei*.

Other studies of intestinal bacterial community in shrimp focused on specific groups of bacteria that are relevant to the objectives of the studies or the effects of dietary manipulation, and most of these studies used only culture-based techniques. The bacterial community in the rearing environment of shrimp including both earthen ponds and recirculation tank were reported (Burford *et al*., 2003; Johnson *et al*., 2008).

CHAPTER II

MATERIALS AND METHODS

2.1 Juvenile *P. monodon* (small, medium and large)

2.1.1 Sample collection

Samples of healthy (showing no sign of disease) juvenile shrimp from a concurrent genetic family in a rearing pond were collected from the Marine Technology Center, Burapha University (Chantaburi, Thailand). At two and five month after initial stocking, approximately 250 shrimp each were size-graded by weight. The highest and lowest 10% of the shrimp were labeled “large” and “small”, while the rest of the shrimp was considered medium. For each sampling month, ten shrimp of each size class (large, medium and small) was randomly selected, and placed on ice for 5-10 minute before further processing. The body surface was washed three times with 30 ml sterile saline solution and disinfected using 70% ethanol for 30 second. Each whole intestine was aseptically dissected and transferred into 2 ml cryogenic vials. All samples were homogenized with 1.0 ml of 2% sterile saline solution using sterile glass rod and vortex mixer before being analyzed. A 0.5 ml aliquot of the intestinal homogenate was mixed with 0.25 ml of 50% glycerol for bacterial enumeration and the rest of homogenate was stored at -80 °C for DNA analysis. The vials were frozen in nitrogen before being transported to the laboratory.

2.1.2 Bacterial enumeration

Bacterial enumeration was performed from the homogenate of a whole intestinal tract of each shrimp using drop plate technique (Herigstad *et al*., 2001). One milliliter of each collected sample was added to 9 ml of 2% (w/v) sterile saline solution. A series of 10 fold dilutions of each samples were prepared and 10 µl of the 10^{-4}, 10^{-5}, and 10^{-6} dilution were dropped on Marine 2216 agar plates (MA2216; Appendix A, Table 1). The plates were incubated at 28 $^{\circ}$C for 1 day. After incubation, 20-30 colonies were selected from MA2216 plate and streaked onto a fresh MA2216 plates until pure culture isolates were obtained. The isolates were maintained at 4 $^{\circ}$C

on MA2216 agar slant for further testing. For long term preservation, pure culture isolates were kept in 15% glycerol at -80 °C for until use.

2.1.3 DNA extraction

Total bacterial genomic DNA from each intestinal homogenate was extracted by using CTAB and chloroform-isoamylalcohol extraction method according to Zhou *et al*. (1996) with some modifications. Briefly, 0.5 ml homogenate of each sample was thawed at room temperature for 15 min and centrifuged at 11,000×*g* for 10 min. The supernatant was discarded, and then 600 µl extraction buffer (final concentration 100 mM Tris-HCl pH 8.0, 100 mM sodium EDTA pH 8.0, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB) and 10 µl proteinase K (10 mg mL^{-1}) were added to the ground tissue in the tube. After mixing, the mixture was incubated at 65 °C for 2 hrs under continuous agitation at 250 rpm. After 2 hrs, 75 µl 20% sodium dodecyl sulfate (20% SDS) was added to the mixture and incubated for 1 hr at 65 °C. During the incubation, the tubes were mixed gently by inversion every 15 min. The supernatant was collected in a new collection tube after centrifugation for 10 min at 11,000×*g* and the pellet was re-extracted with extraction buffer and SDS for 15 min using the same procedure. The supernatant was mixed with equal volume of chloroform-isoamyl alcohol (24:1 ratio) and centrifuged at 6,000×*g* for 1 min at temperature to separate the phase. The aqueous phase was transferred to a new collection tube, and the chloroform-isoamyl alcohol step was repeated. After two round of chloroform-isoamyl alcohol extraction, 0.6x volume of isopropanol was added to the supernatant and stored for 1 h at room temperature. The DNA pellet was obtained by centrifugation for 20 min at 16,000×*g* after washing with 1 ml of 70% cold ethanol. The DNA pellet was air dried at room temperature and re-suspended in 50 µl TE buffer pH 8.0. All DNA was stored at -20 °C until analysis. DNA quality and concentration were measured by gel electrophoresis and spectrophotometer.

2.1.4 PCR amplification

A nested PCR approach was used to amplify eubacterial 16S rDNA gene fragments from total DNA samples. The first PCR amplification of near-complete 16S rDNA gene was performed using the forward primer 8fm and the reverse primer 1492r (Table 2.1) as described by Lane (1991). The first PCR (25 µl) consisted of 1x

Mg-free PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM of each primer, 2 U/mL Taq DNA polymerase and approximately 20-100 ng template. The following conditions were used: 4 min initial denaturation at 95 °C, 30 cycles of 1 min at 95 °C, 30 sec at 55 °C, 2 min at 72 °C, and 7 min final extension at 72 °C. The presence of a 1.5 Kbp fragment was confirmed on a 1.0 % agarose gel electrophoresis. A 1.0 µl 1:10 dilution of product from the first PCR were then used as template for nested PCR using the forward primer 338f-GC and the reverse primer 517r (Table 2.1) to produce 200 bp fragments suitable for DGGE analysis (Muyzer *et al*., 1993) (Table 2.1).

Table 2.1 Primers used in this study.

Primer[a]	Sequence (5' to 3')	Target	Fragment size (bp)	Reference
8fm	AGAGTTTGAT(AC)MTGGCTCAG	universal	1500	Lane, 1991
1492r	G(CT)TACCTTGTTACGACTT	universal		Lane, 1991
338f-GC[b]	(GC)-ACTCCTACGGGAGGCA	universal	200	Muyzer et al., 1993
517r	ATTACCGCGGCTGCTGG	universal		Muyzer *et al*., 1993
567f-GC[c]	(GC)-GGCGTAAAGCGCATGCAGGT	*Vibrio* specific	120	Thompson *et al*., 2004
680r	GAATTCTACCCCCCTCTACAG	*Vibrio* specific		Thompson *et al*., 2004

[a]Target gene of all primers used in this study is 16S rDNA

[b]This primer has the following GC clamp at its 5'end:
5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG- 3' (Muyzer *et al*., 1993)

[c]This primer has the following GC clamp at its 5'end:
5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG3' (Thompson *et al*., 2004)

The PCR reaction mixtures for the nested PCR contained 1x Mg-free PCR buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTPs, 200 nM of each primer, 2 U/mL Taq DNA polymerase and approximately 20-100 ng template, in a total volume of 50 µl. The PCR condition was followed: 4 min initial denaturation at 95 °C, 25 cycles of 30 sec at 95 °C, 30 sec at 60 °C, 30 sec at 72 °C, and 3 min final extension at 72 °C. A 200 bp fragment was confirmed on a 1.5% agarose gel electrophoresis.

2.1.5 DGGE analysis

The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) by using DCode™ universal mutation detection system (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturer's instruction and the procedure described by Muyzer *et al*. (1993). A 8% (w/v) polyacrylamide gel (acrylamide: N, N-methylenebisacrylamide, 37.5:1) with denaturing gradient ranging from 25% to 50% was used. A 100% denaturing solution contained 8 M urea and 40% (v/v) deionized formamide. PCR samples (10 µl) were loaded in each well of 8% polyacrylamide gel that contained a 25% to 50% linear gradient of a denaturing solution. The gels were run for 5 hrs at 200 V in 1X TAE buffer at 60°C. After electrophoresis, the gels were stained for 15 min in the staining solution containing 1:10000 dilution of SYBR® Gold stain (Invitrogen, UK), destained in water for 15 min and then visualized using Pharos FX™ Molecular Imager (Bio-Rad Laboratories Inc., Hercules, CA).

2.1.6 Cluster analysis

Analysis of DGGE profile, the DGGE band profile of a gel was analyzed using the InfoQuest™ software (Bio-Rad Laboratories Inc., Hercules, CA). Each DGGE profiles were subjected to normalization among different gels using custom ladders and reference sample. Cluster analysis was performed with the unweighted pair-group method using arithmetic averages (UPGMA) to contract a dendrogram. Relative signal intensities of detected bands, in each individual DGGE profile, were calculated from the peak area of the densitometric curves determined using the InfoQuest™ software (Bio-Rad Laboratories Inc., Hercules, CA). The number of DGGE bands present in each sample was exported for analysis. The DGGE bands of interest was extracted, cloned and sequenced to identify the bacteria.

2.1.7 Construction of 16S rDNA clone libraries

Based on the results of cluster analysis, 16S rDNA clone libraries from selected individual intestinal sample of each cluster were created as below.

2.1.7.1 PCR amplification

Amplification was carried out by using DNA Thermal Cycler (PTC-200, Bio-Rad) in a 25 µl of total volume. PCR reaction and PCR condition were prepared according to the procedure described in section 2.1.4. To increase yield in PCR amplification, BSA (10 mg/mL) was added into the reaction. After amplification, aliquots (2 µl) of PCR product (1500 bp) was confirmed on a 1.0% (w/v) agarose gel

2.1.7.2 Purification of PCR products

PCR product was purified using illustra^{TM} GFX^{TM} Microspin^{TM} columns (GE Healthcare, UK) according to manufacturer's recommendation. After elution (20 µl), aliquots (2 µl) of purified PCR product (1500 bp) was confirmed on a 1.0% (w/v) agarose gel.

2.1.7.3 Ligation of PCR products to the pGEM – T Easy vector

Purified products from each sample were ligated to the pGEM – T Easy vector (Promega, USA). The ligation mixture (10 µl) contained 5 µl 2x Rapid Ligation Buffer, 1.0 µl T4 DNA ligase (3 U/mL), 0.5 µl pGEM^{R} – T easy vector (50 ng/mL) and 3.5 µl DNA insert. The reaction mixture was incubated overnight at 4 °C.

2.1.7.4 Preparation of competent *E. coli* cell

E. coli JM109 was streaked on LB plate (Appendix A, Table 2) and incubated overnight at 37 °C. A single colony of *E. coli* JM109 was inoculated into 10 ml of LB broth (Appendix A, Table 3) and incubated overnight at 37 °C under continuous agitation of 250 rpm. The next day, 1 ml of culture was inoculated into 50 ml of LB broth and the incubation continued at 37 °C with vigorous shaking until the culture reached an optical density (OD_{600}) of 0.5 to 0.8. The culture was chilled on ice for 10 min, and the cells were recovered by centrifugation at 2700×*g* for 10 min at 4°C. The cell pellets were then resuspended in 30 ml of ice-cold $MgCl_2$/$CaCl_2$ solution (80 mM $MgCl_2$ and 20 mM $CaCl_2$) and centrifuged again as above. The last resuspension was performed in 2 ml of ice-cold 0.1 M $CaCl_2$, and the concentrated cell suspension was aliquoted into 100 µl portion in an ice-cooled 1.5 ml microcentrifuge tubes to prevent

the cells from warming. These competent cells was either used immediately or stored at -80 °C for subsequent use.

2.1.7.5 Transformation of ligation products to *E. coli* host cells

The competent cells were thawed in ice for 5 min. Four microliters of the ligation mixture were added and gently mixed by pipetting and incubated in ice for 30 min. After incubation, the mixture was heat-shocked at 42°C for exactly 45 sec and immediately placed in ice for 2-3 min. Then the mixture was transferred into 1 ml of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) and incubated at 37 °C for 90 min continuous. The mixture were centrifuged for 20 sec at room temperature, and gently resuspended in 100 µl of SOC medium and spreaded onto a selective LB plates containing 50 µg / ml of ampicillin, 25 µg / ml of IPTG and 20 µg/ml of X-gal and further incubated at 37°C overnight (Sambrook and Russell, 2001). White colonies were picked and replicated on LB plate containing 50 µg / ml of ampicillin for confirmation of positive transformant clones by colony PCR.

2.1.7.6 Colony PCR of the recombinant clones

Recombinant clones were selected by a lacZ′ system following standard protocols (Sambrook and Russel, 2001). Colony PCR amplification was performed to identify the expect sizes of positive clones. Colony PCR was carried out in a 25 µl reaction mixture, containing 1µl PCR product, 1 µl 10x Mg-free PCR buffer, 2.5 µl $MgCl_2$ (25 mM), 2.5 dNTPs (1 mM), 2 µM of each primer including 1.25 µl pUC1 (5′-TTC GGC TCG TAT GTT GTG TGG A-3′) and 1.25 µl pUC2 (5′-GTG GTG CAA GGC GAT TAA GTT GG-3′), 19.4 µl sterile deionized water and 0.1µl Taq DNA Polymerase (5U/mL). A colony was picked by a sterile toothpick and served as the template in the reaction, PCR was carried out in a thermocycler consisting of denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 1 min and extension at 72 °C for 2 min. The final extension was carried out at the same temperature for 7 min. The colony PCR products were checked in 1.0% agarose gel and visualized after ethidium bromide staining for 15 min. The presence of insert of the expected size was collected.

2.1.7.7 Isolation of positive transformant clones by DGGE

The colony PCR product of positive clones was collected to be used as the template (1:10 dilution) for nested PCR. The PCR reaction was prepared according to section 2.1.4. The PCR condition was followed: 4 min initial denaturation at 95 °C, 25 cycles of 30 sec at 95 °C, 30 sec at 60 °C, 30 sec at 72 °C, and 3 min final extension at 72 °C. After amplification, aliquots (2 µl) of PCR product (200 bp) was confirmed on a 1.5% (w/v) agarose gel in comparison with 100 bp ladder at 100 V for 25 min, stained with ethidium bromide for 15 min. The rest of PCR product was screened using DGGE following section (2.1.5). After DGGE electrophoresis, the pattern of each band from each clone was selected for plasmid extraction.

2.1.7.8 Extraction of recombinant plasmid DNA

Plasmid DNA was isolated using illustra^{TM} plasmidPrep Mini spin kit (GE Healthcare, UK). The single colony from transformed bacteria was inoculated into 3 ml of LB broth supplemented with 50 µg/ml of ampicillin and incubated with vigorous shaking (250 rpm) at 37 °C overnight. After incubation, the culture was collected by centrifugation (14,000 rpm) at room temperature for 1 min. The cell pellet was resuspended in 200 ml of ice-cold lysis buffer type 7, mixed by pipetting and incubated at room temperature for 5 min. The 200 ml of lysis buffer type 8 was added and mixed by gently inverting for 4-5 time. After that, 400 ml of lysis buffer type 9 was added and mixed as above. The sample was centrifuged (14,000 rpm) for 15 min at room temperature. The supernatant was transferred to the mini column and centrifuged at the same speed for 60 sec. The flow-through was discarded. The mini column was placed back in the collection tube. The column was washed by adding 400 µl of lysis buffer type 1 and centrifuged at 16,000×*g* for 1 min. After discarding the flow-through, 400 µl of lysis buffer type 1 was added and centrifuged as above. The flow-through was discarded. The spin tube was centrifuge for 2 min at full speed (14,000 rpm) to remove the residual lysis buffer type 1. The dried mini column was placed in a new 1.5 ml microcentrifuge tube and 20 µl of the Elution buffer type 4 was added at the center of the column to elute the extracted plasmid DNA. The column was incubated at room temperature for 2 min, centrifuged at 14,000 rpm for 2

min and stored at -20 °C until use. The concentration of extracted plasmid DNA was spectrophotometrically measured.

2.1.8 DNA sequencing and phylogenetic analysis

The recombinant clones were unidirectional sequenced using the 27F forward primer on an automatic sequencer at Macrogen Inc. (Seoul, Korea). Each sequence was uploaded to the Ribosomal Database Project (RDP) for further analysis. Sequence similarity searches were performed using the BLAST network service of the NCBI database and Seqmatch tool of the RDP. For the phylogenetic analysis, the obtained 16S rDNA gene sequences were aligned using the ClustalX program (Thompson *et al*., 1994). The phylogenetic trees were constructed using the neighbor-joining method by MEGA5 (Tamura *et al*., 2007) and the bootstrap analysis of 500 replicates also was performed using the same software.

2.2 Wild *P. monodon*

2.2.1 Sample collection

Wild-caught broodstock size (N = 8) *P. monodon* were caught from the Gulf of Thailand, and they were transported on ice to the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. After body surface disinfection with 70% ethanol for 30 sec, the stomach, intestine, and feces (squeezed out of intestine) of the shrimp were aseptically dissected and homogenized with 1.0 ml of 2% sterile saline solution. The homogenate was stored at -80 °C for DNA analysis.

2.2.2 DNA extraction

DNA extraction was performed as described in section 2.1.3.

2.2.3 PCR amplication and DGGE analysis

PCR amplification of the 16S rDNA gene and nested PCR and DGGE procedures were performed as described in sections 2.1.4 and 2.1.5.

2.2.4 Construction of 16S rDNA clone libraries

A clone library of 16S rDNA PCR product from each segment of the GI tract (pooled samples; N = 8) was created as described in section 2.1.7.

2.2.5 DNA sequencing and phylogenetic analysis.

Phylogenetic analysis was performed using the RDP Classification Algorithm and constructed by the neighbor-joining method (MEGA software), were performed as described in section 2.1.8.

2.3 *Vibrio* challenge test

2.3.1 Shrimp

Juvenile *P. monodon* shrimp (average length and weight: 17±2 cm and 13±2 g, respectively) were purchased from local farms in Patumtanee province, Thailand, on 17 May 2011. They were transported live to the Center of Excellence for Marine and Biotechnology (CEMB), Chulalongkorn University. The shrimp were kept for 1 day in continuously aerated water at 28±2 °C with salinity of 20 parts per thousand (20 ppt). Only healthy shrimps were used for the experiment.

2.3.2 Pathogenic bacteria and culture condition

The pathogenic bacterium strain used for this experiment was *Vibrio harveyi*. It was maintained at 28 °C on tryptic soy agar containing additional 2% (w/v) sodium chloride (TSA+2% NaCl; Appendix A, Table 4) and cultured in tryptic soy broth containing additional 2% (w/v) sodium chloride (TSB+2% NaCl; Appendix A, Table 5). For long term preservation, the culture was kept in 15% glycerol at -80 °C for subculture analysis.

2.3.3 Preparation of *Vibrio harveyi*

V. harveyi was cultured at 28 °C on TSA+2% NaCl for 1 day. The single colony on plate was selected and pre-cultured in 25 ml of TSB+2% NaCl under continuous agitation of 250 rpm at 28 °C for 16 hrs. Then 1 ml of pre-culture was inoculated into 25 ml of TSB+2% NaCl under the same condition for 16 hrs and centrifuged (3600×*g*) at 4 °C for 10 min. The supernatant was discarded and the bacterial pellet was washed twice with 2% (w/v) sterile saline solution. Bacterial concentration was adjusted to the optical density at OD_{600} = 1 (approximately 10^8 CFU/mL). The bacterial concentration was adjusted and verified by viable plate count according to standard methods as described in Collins and Lyne (1976), The *V.*

harveyi inoculum concentration was adjusted to 10^7 CFU/mL with 2.0% (w/v) sterile saline solution.

2.3.4 Preparation of *Artemia*

Adult *artemia* were purchased from Jatujak Weekend Market and transported live to the National Center for Genetic Engineering and Biotechnology (BIOTEC). Adult *artemia* were treated with formalin solution (100 ppm) for 2 hrs, and followed by a mixture of antibiotics (Norfoxacin 200 ppm, Erytothromycin 25 ppm and Oxytetracyclin 25 ppm) for overnight. Adult *artemia* were then washed tree times with 2% (w/v) sterile saline solution to remove trace antibiotics. The remaining adult *artemia* were then divided into 2 groups. Adult *artemia* in group I were exposed to *V. harveyi* at the final concentration approximately 1.0 x 10^7 CFU/ml for 2 hrs before they were offered to the shrimp. Adult *artemia* in group II was used as control and were not exposed to additional bacteria. Adult *artemia* sample from each group was analyzed for bacterial concentration before and after the antibiotic treatment using drop plate method. Samples (N =10) were collected and homogenized in sterile screw-capped tube containing 2.0% (w/v) sterile saline solution and serial diluted 10-fold were performed. Fifty µl of each dilution was dropped over duplicate plates on MA2216, and the plates were incubated at 28 °C and colony forming units (CFUs) enumerated after 16-18 hrs.

2.3.5 *Vibrio* challenge test

The experimental design for this study consisted of 2 treatments, namely control treatment (fed with adult *artemia* without *V. harveyi*) and challenge treatment (fed with adult *artemia* disposed with *V. harveyi*). Twenty shrimp were distributed into 20 rearing plastic tanks (43.5×27.5×25.5 cm), that contained 1.5 liters of seawater (30 ppt). Ten tanks were assigned to each treatment. The shrimp was individually placed into each tank. The shrimp were allowed to acclimate to the tank and starved for 24 hrs before the experiment begin. The shrimps were fed twice with adult *artemia* (N = 20), each time approximately 1 hr apart (N = 10). Two replicates were carried out. Hepatopancreas, intestine and hindgut from shrimp in 2 tanks for each treatment were aseptically collected at 0, 3, 12, 24 and 48 hrs. Each were weighted and homogenized with 1.0 ml of 2% (w/v) sterile saline solution using sterile glass

rod and vortex mixer before analysis. Each sample was serially diluted and 0.1 ml aliquots of homogenate was used for bacterial enumeration, and the rest of homogenate was stored at -80 °C for DNA analysis.

2.3.5.1 Bacterial enumeration

Bacterial enumeration was performed for samples in each treatment at pre-trial level and at 0, 12 and 24 hrs using drop plate technique. Each sample was serially diluted 10-fold with sterilized 2% (w/v) saline solution and 50 µl of each dilution was dropped on duplicate plates on thiosulfate citrate bile sucrose agar (TCBS, Appendix A, Table 6) for enumeration of *V. harveyi* and MA2216 agar for enumeration other bacteria, respectively. Bacterial colonies were counted after incubation at room temperature for 1 day.

2.3.5.2 DNA extraction

DNA extraction was performed as described in section 2.1.3.

2.3.5.3 PCR amplication

For *Vibrio*-specific PCR-DGGE, a 1.0 µl 1:10 dilution of the PCR products from the first 16S rDNA amplification (1500 bp) was used as a template for the second PCR with 200 nM of each primer (567f-GC – 680r pair; Table 2.1) in a total volume of 50 µl under the same PCR condition (described in section 2.1.4).

The positive control for first PCR and nested PCR was prepared using 1 µl of *V. harveyi* DNA under the reaction condition described above. The negative control of each PCR reaction had no template. The PCR products (120 bp) were confirmed on a 1.5% agarose gel electrophoresis.

2.3.5.4 DGGE analysis and cluster analysis

DGGE analysis and cluster analysis were performed as described in sections 2.1.5 and 2.1.6.

2.4 Screening for inhibitory effects against two shrimp pathogens (*V. harveyi* and *V. parahaemolyticus*), and selected phenotypic tests

2.4.1. Shrimp pathogens

Vibrio harveyi (Karunasager *et al*., 1994; Saeed, 1995; Liu *et al*., 1996; Ruangsri *et al*., 2004) and *V. parahaemolyticus*(Brock and Lightner, 1990; Ishimaru *et al*., 1995), two previously known *P. monodon* pathogens, were kindly provided by Dr. Pikul Jiravanichpaisal. These strains were maintained at 4 °C in tryptic soy agar slant containing with additional 2% (w/v) sodium chloride and were sub-cultured in fresh media once a month. Stock cultures were kept in 15% glycerol at -80 °C.

2.4.2 Isolation of potential antagonistic bacteria from intestinal tract of shrimp and rearing water.

Bacteria used for the screening of antagonistic activity against shrimp pathogens were isolated from the gastrointestinal tract of shrimp and rearing water from shrimp ponds. These isolates were collected as part of this master's research project and another experiment to study the intestinal bacterial community of *P. monodon* (Chaiyapechara *et al*., 2012).

Shrimp samples included juvenile *P. monodon* and wild *P. monodon* from three different locations in Thailand (Chachoengsao province, Chanthaburi province and Suratthani province) as shown in Table 3.7. The individual gastrointestinal tract of each samples were homogenized with 1.0 ml of 2% (w/v) sterile saline solution. A 0.5 ml portion of the homogenate was stored on ice for serial dilutions and used in this study. One and a half liters of water samples were collected from the edge pond at Chachoengsao province, Thailand, and were used for bacterial isolation directly.

A series of 10 fold dilutions of each samples were prepared using 2% (w/v) sterile saline solution, and 100 microliter of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions were dropped on Marine 2216 agar plates. The plates were incubated at 28 °C for 1 day. After incubation, 20-30 colonies were randomly selected from MA2216 plate and streaked onto MA2216 plates. Bacterial isolates were subcultured until pure cultures were obtained. These pure cultures were maintained at 4 °C on MA2216 agar slant for

further testing. For long term preservation, pure culture isolates were kept in 15% glycerol at -80 °C for until use.

2.4.3 Screening inhibitory effect of potential antagonistic bacteria against shrimp pathogens by co-culture method.

2.4.3.1 Preparation of the tested organisms for inhibitory testing

Two pathogenic bacteria were grown overnight at 28 °C in TSA+2% NaCl. After that, 2-3 well isolated colonies with the same appearance were inoculated into tubes containing 5 ml TSB+2% NaCl. The tubes were incubated at 28 °C with shaking (250 rpm) for 16 hrs. After incubation, the inoculated was swabbed on TSA+2% NaCl agar surface for co-culture analysis.

2.4.3.2 Co-culture method

Modified co-culture method based on that described by Euanorasetr *et al*. (2010) was used to screening inhibitory effect of potential antagonistic bacteria against pathogenic bacteria. Pure culture test isolates were grown in MA2216 media at 28 °C for 7 days. Then, a patch of colonies from each agar plates were cut into an agar plug by using the blunt end of the 1ml pipet tips (7 mm in diameter), and the plug were placed right side up on the surface of the TSA+2% NaCl agar plate previously swabbed with pathogenic bacteria. After incubation at 28 °C overnight, inhibitory results were observed by measuring the diameters of the zones of inhibition (including diameter of agar plug and record inhibition zone (in mm).

2.4.3.3 Plate screening of phytase activity

The method described by Yanke *et al*. (1998) was modified to apply for indicating clear zone on modified phytase screening medium plate (MPSM; Appendix A, Table 7) and the phytase activity of the colony was visually indicated by clear zone of the agar made by extracellular phytase production. Briefly, the isolated were incubated at 28 °C on MPSM plates for 7 days. And then, screening of phytase plate was carried out by washing the colonies from the agar surface and flooded the plate with 2% (w/v) cobalt chloride solution. After incubation at room temperature for

30 min, the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of 6.25% (w/v) ammonium molybdate solution and 0.42% (w/v) ammonium vanadate solution. Following 5 min incubation the solution was removed. Finally, the plates were examined for zones of clearing indicative of phytase activity. The microorganisms were classified by the size of clear zone in cm.

2.4.4 API 20NE test

2.4.4.1 Bacterial strains

Twelve isolates with strong antagonistic activity against shrimp pathogen identified using co-culture method (from section 2.4.3.2) were subjected to further phenotypic characterization using API 20NE strip (Biomerieux, Marcy L'Etoile, France). The isolates were grown on MA2216 agar plates at 28 ºC overnight for the purpose of preparation of inocula.

2.4.4.2 Biochemical phenotypic tests using API 20NE

Phenotypic tests were performed using the API 20NE test kit (Biomerieux, Marcy L'Etoile, France) according to the manufacturer's instructions. The inoculum of each isolated strains were inoculated into a strip panel of API test. The API test panels were incubated at 28°C. Examination of the strips in each panel was read as follows: the nitrate reduction (NO_3), indole (TRP), and glucose fermentation reaction were read after 24 hrs incubation, and read all the tests again for a further 48 hrs, except the first 3 (NO_3 , TRP and GLU) which should only be read once at 24 hrs. The development of reaction was recorded using reading table (Appendix B). Results for each test were recorded as positive (+), negative (-) or weak (+/-) as shown in Fig. 2.1. Based on the results sheet (Fig. 2.2), the tests were separated into groups of 3 and a number 1, 2, or 4 was indicated for each. By adding the numbers corresponding to positive reaction within each group, a seven digit number was obtained with constitutes the numeric profile (see Fig. 2.2 and Table 2.2).

The identification of strains was carried out according to the API 20NE identification manual by comparing the numerical profile to those of species listed in a profile index. For these purposes, a weak reaction was recorded as positive.

Fig. 2.1 shows all positive results (A) and all negative results (B) for utilization of carbon source in API 20 NE tests. (http://www.tgw1916.net/Tests/api.html; March 27, 2012)

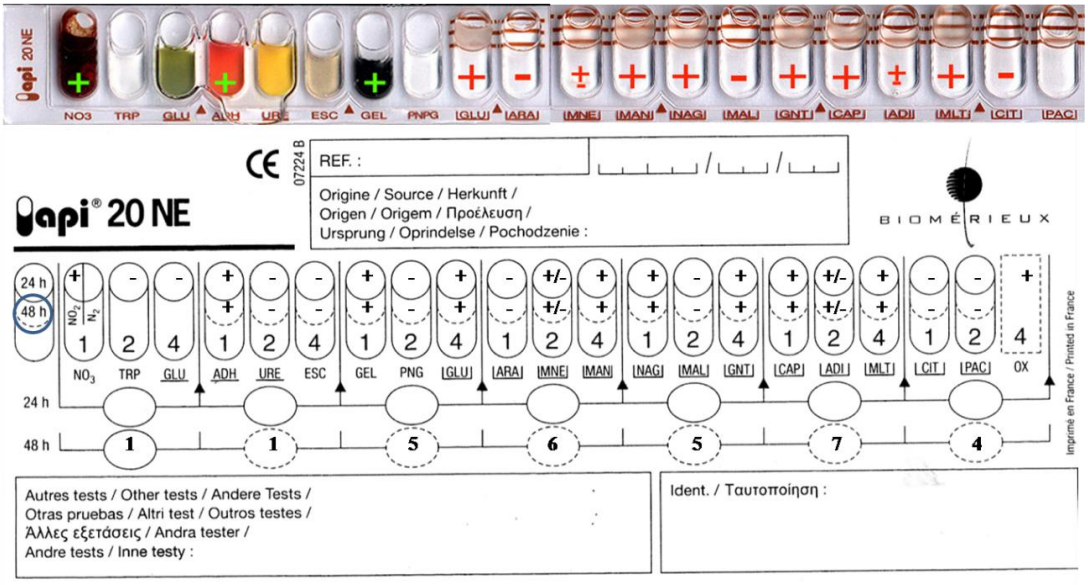

Fig. 2.2 shows the evaluation sheet of API 20 NE test strip.

(http://everest.bic.nus.edu.sg/lsm2104/stu/a-sci10637/start.htm; March 27, 2012)

Table 2.2 Example of numerical profile and identification on API 20 NE test

I			**II**			**III**			**IV**			**V**			**VI**			**VII**		
No_3	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARE	MNE	MA	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX
+	-	-	+	-	-	+	-	+	-	+	+	+	-	+	+	+	+	-	-	+
1	2	4	**1**	2	4	**1**	2	**4**	1	**2**	**4**	**1**	2	**4**	**1**	**2**	**4**	1	2	**4**
1			**1**			**5**			**6**			**5**			**7**			**4**		
Identification code: 1156574 (*Burkholderia pseudomallei*)																				

For example (Table 2.2), according to real figure 2.2

CHAPTER III

RESULTS

3.1 Juvenile *P. monodon* (small, medium and large)

3.1.1 Bacteria enumeration

Average weight for small, medium and large shrimp at Month 2 were 3.3, 6.0 and 12.7 g/shrimp, and average weight for small, medium and large shrimp at Month 5 were 6.3, 11.9 and 20.5 g/shrimp, respectively (Table 3.1). Table 3.1 shows bacterial concentration in the intestinal tract of 5 month-old shrimp. Average bacterial concentrations in the intestinal tract of small, medium and large shrimp at Month 5 were 1.12 x 10^6, 1.93 x 10^6 and 1.53 x 10^6 CFU/g intestine, respectively (Table 3.1). Bacterial concentration in the intestinal tract of 2 month-old shrimp was not determined.

Table 3.1 Average weight, average bacterial concentration, and average number of operational taxonomic units (OTUs) based on DGGE profiles of bacteria from the intestinal tract of shrimp.

List	Month 2			Month 5		
	Small (*N*=10)	Medium (*N*=10)	Large (*N*=10)	Small (*N*=10)	Medium (*N*=10)	Large (*N*=10)
Average weight (g/shrimp)	3.3±0.23	6.0±0.26	12.7±1.5	6.3±0.35	11.9±0.18	20.5±0.91
Average bacterial concentration (CFU/g intestine)[a]	ND[c]	ND[c]	ND[c]	$1.12x10^6$	$1.93x10^6$	$1.53x10^6$
Average number of OTUs (OTUs/shrimp)[b]	4.6±0.54	5.2±0.70	7.6±0.67	7.7±0.90	6.6±0.75	5.8±0.61

[a] CFU; colony-forming unit,

[b] OTU; operational taxonomic units (= number of detectable DGGE bands)

[c] ND = not determined

3.1.2 16S rDNA PCR- DGGE analysis and cluster analysis

The shrimp intestinal tract samples (*N* = 60) were collected according to size (small, medium and large) and age (2 and 5 month-old shrimp). The total bacterial genomic DNA was successfully extracted with the modified protocol as visualized by electrophoresis gel. The size of bacterial genomic DNA was approximately 23 kb (Fig 3.1). The DNA (approximately 20-100 ng) samples were successfully amplified with PCR. The size of the PCR product after the first amplification was about 1500 bp (Fig. 3.2A), and the size of the PCR product after nested PCR was about 200 bp (Fig. 3.2B). The PCR products of the nested PCR were successfully fingerprinted by DGGE.

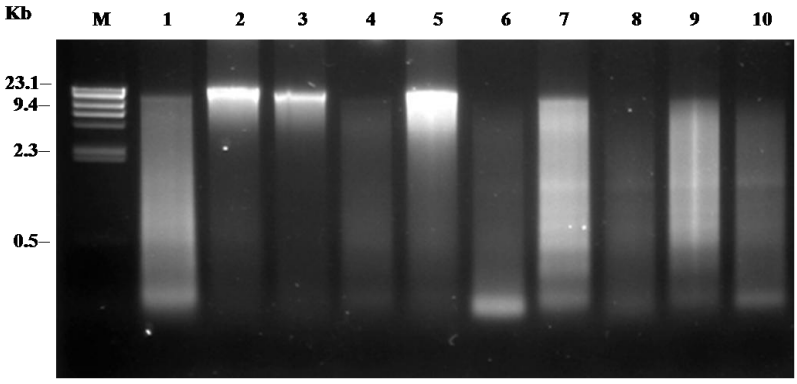

Figure 3.1 Agarose gel electrophoresis of bacterial genomic DNA extracted from the intestinal tract of (small, *N*=10) shrimp at Month 2. Total DNA extracted from the intestinal tract of shrimp at Month 2 was analyzed by electrophoresis through a 1.0 % agarose gel. Electrophoresis was for 30 min at 100 V. DNA was visualized under UV after staining with ethidium bromide. Lanes: M, λ *Hin*dIII; 1–10, DNA extracted of small shrimp at Month 2 (no.1-10), respectively. Numbers on the left side of the gel refer to the size of DNA markers in Kb.

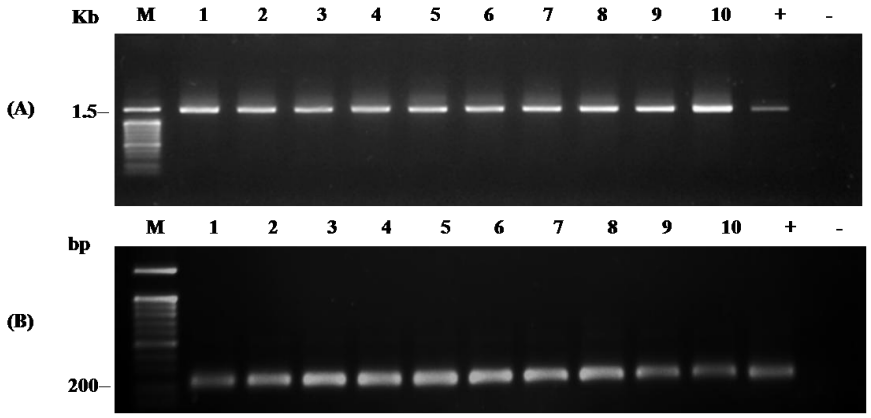

Figure 3.2 16S rDNA PCR product on 1.0 % agarose gel (A) and nested DGGE PCR product on 1.5% agarose gel (B). Lanes: M, 100 bp marker; 1–10, DNA extracted from small shrimp at Month 2 (no.1-10), respectively. Numbers on the left side of the gel refer to the size of DNA markers in Kb and bp.

After DGGE analysis, cluster analyses of the profiles of intestinal bacterial from individual shrimp were performed for each month of sampling separately for Month 2 (Fig. 3.3) and Month 5 (Fig. 3.4). Profiles of the small, medium and large shrimp are represented in green, blue and red squares, respectively. Average number of OTUs in the intestinal tract of small, medium and large shrimp at Month 2 were 4.6, 5.2 and 7.6 OTUs/shrimp, and average number of OTUs in the intestinal tract of small, medium and large shrimp at Month 5 were 7.7, 6.6 and 5.8 OTUs/shrimp, respectively (Table 3.1).

Based on Month 2 shrimp alone, the results of cluster analysis showed 4 major clusters: cluster I, II, III, and IV. Bacterial profiles of shrimp in three different sizes do not form a district cluster based on size at Month 2.

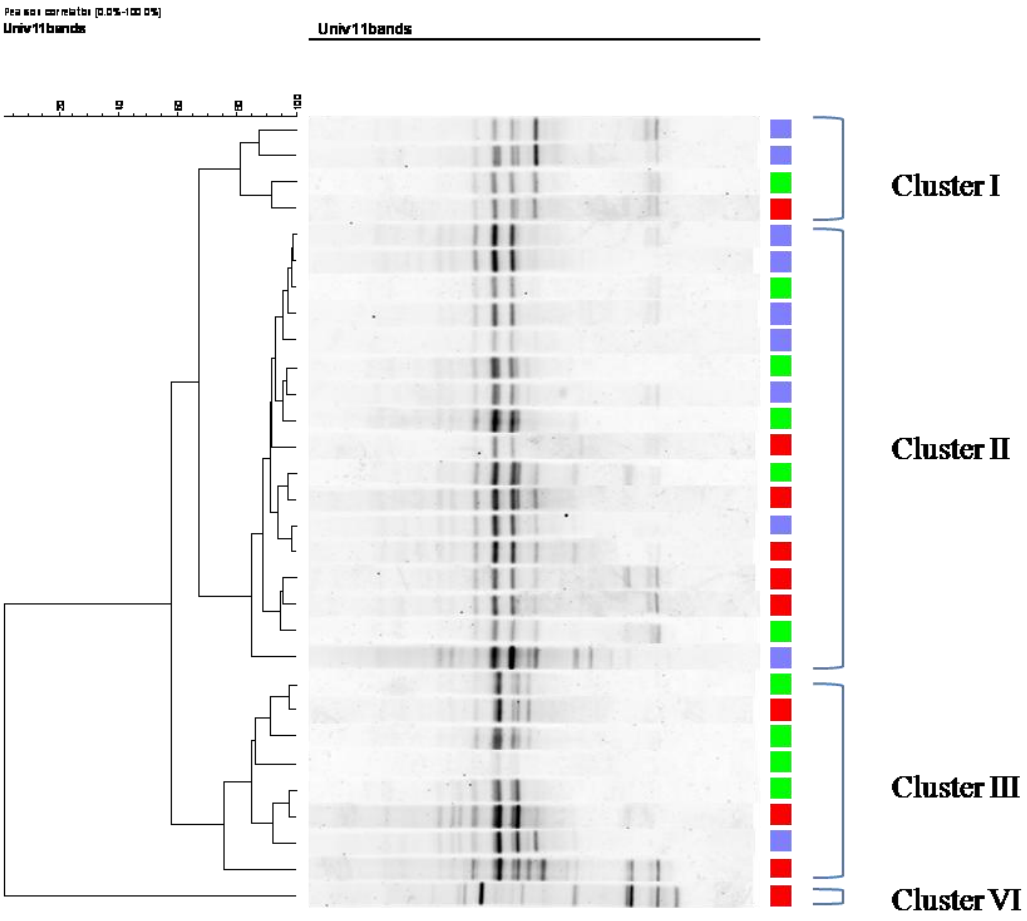

Figure 3.3 Cluster analysis results of DGGE profile of bacterial community of 2 month-old shrimps : (■) small, (■) medium and (■) large with *N*=10 each. Cluster analysis was performed using UPGMA method based on Pearson correlation of densitometric curves.

Based on Month 5 shrimp alone, the results of cluster analysis showed 3 major clusters. While bacterial profiles of shrimp in three sizes do not form a district cluster based on size at Month 5, but there was no large shrimp were represented in cluster I.

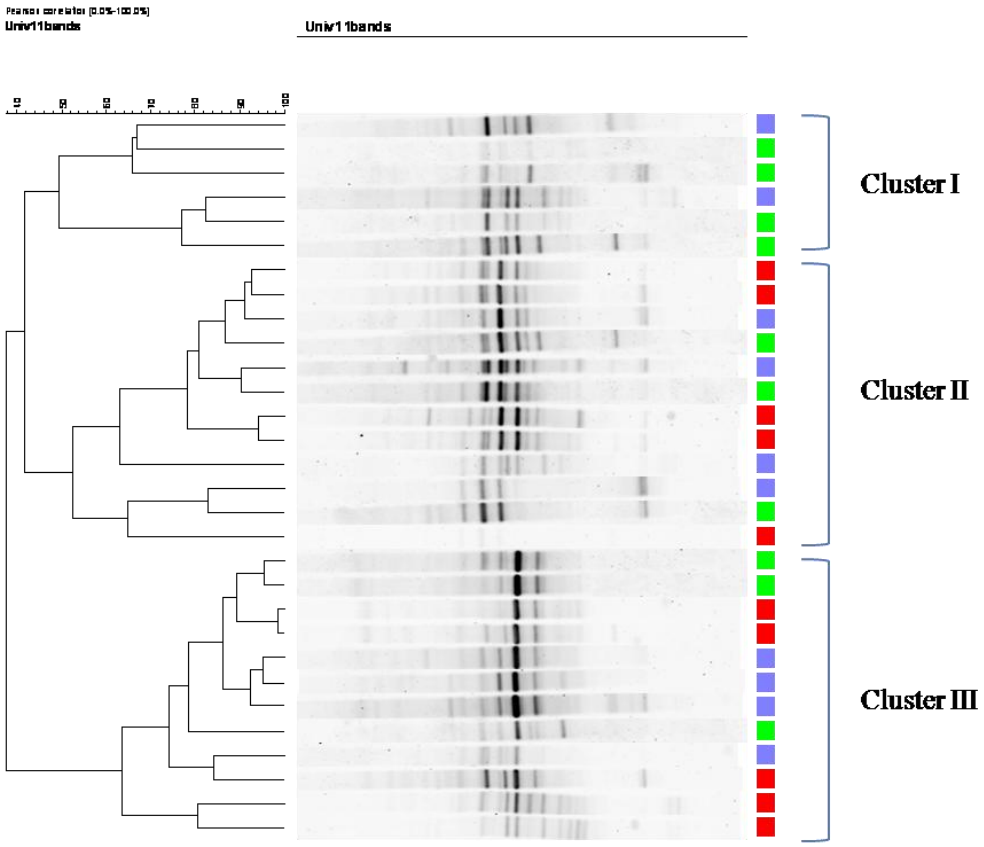

Figure 3.4 Cluster analysis results of DGGE profile of bacterial community of 5 month-old shrimps: ( ) small, ( ) medium and ( ) large with *N*=10 each. Cluster analysis was performed using UPGMA method based on Pearson correlation of densitometric curves.

When analyzing the bacterial community profiles from 2 and 5 month shrimp together, DGGE profiles of intestinal bacteria at 2 months old shrimp (green square) and 5 months old shrimp (red square) clustered in to 5 major clusters (Fig. 3.5). Cluster I and cluster III contained bacterial profiles of shrimp from both Month 2 and Month 5. Cluster II and cluster IV only had bacterial profiles from Month 5 shrimp only, and cluster V contained bacterial profile from Month 2 shrimp only.

DGGE profiles of 7 shrimp with the most number of bands in each cluster were selected for clone libraries constructions. They were designated as Shrimp M2 from Cluster I (one individual from Month 2, medium size); Shrimp M5 from Cluster II (one individual from Month 5, medium size); Shrimp L2-1 and Shrimp S2 from Cluster III (two individuals from Month 2, large and small size); Shrimp L5 and Shrimp S5 from Cluster IV (two individuals from Month 5, large and small size) and Shrimp L2-2 from Cluster V (one individual from Month 2, large size), respectively (Fig. 3.5).

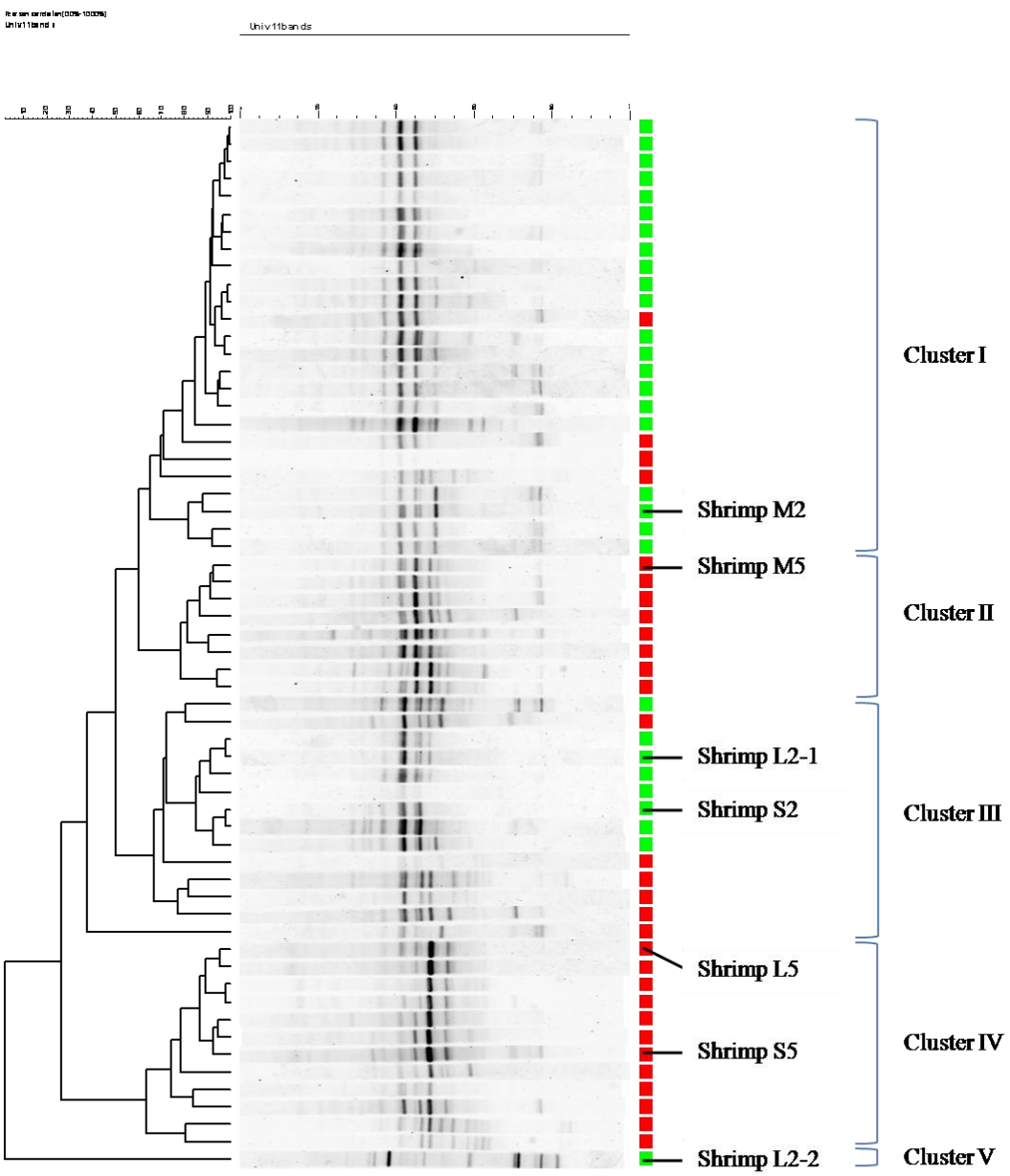

Figure 3.5 Cluster analysis results of individual DGGE profiles of 2 (■) and 5 month-old shrimp (■). Cluster analysis was performed using UPGMA method base on Pearson correlation representative. Profiles of individual shrimp from each cluster that were selected for clone libraries constructions were designated (Shrimp M2, M5, L2-1, S2, L5, S5 and L2-2).

3.1.3 16S rDNA clone libraries, sequence analysis and phylogenetic analyisis.

One hundred and thirteen out of 840 clones of seven shrimp were sequenced. Among these, 96 out of 113 sequences yielded acceptable results. Phylogenetic tree was constructed, and 10 clustered were formed (Fig. 3.6, 3.6A and 3.6B). Results were summarized in Table 3.2. The results of phylogenetic analysis from 16S rDNA clones libraries were contained sequences closely relate to the (β-, δ-, ε-, γ- and α-) Proteobacteria (78 out of 96 clones), Fusobacteria (1 out of 96 clones), Cyanobacteria (1 out of 96 clones), Firmicutes (5 out of 96 clones), Bacteroidetes (7 out of 96 clones) and Chlorobi (4 out of 96 clones) (Fig. 3.6). Proteobacteria were the most abundant, and they were represented in all the 7 libraries. *Vibrio* spp. was the most common bacteria represented in the total number of clones (37 out of 96 clones) from the intestinal tract of juvenile giant tiger shrimp. The total number of phyla each clone libraries ranged from 1 to 5 and the total number of species ranged from 3 to 10 (Table 3.2). Shrimp L2-1 had the most diverse intestinal bacterial composition (9 species in 5 phyla).

The detailed results of all sequences from 16S rDNA clones libraries of 7 selected shrimps are summarized in Table 3.3. Sequences consisted of bacteria from 23 genera including *Achromobacter, Bacillus, Brevundimonas, Crinalium, Cytophaga, Desulfovibrio, Ferrimona, Fusibacter, Granulosicoccus, Helicobacter, Iganavibacterium, Ilyobacter, Jeogalicoccus, Legionella, Oceanobacillus, Photobacterium, Sphingomonas, Shewanella, Thiobaca, Thiohalospira, Thioreductor, Turicibacter* and *Vibrio.*

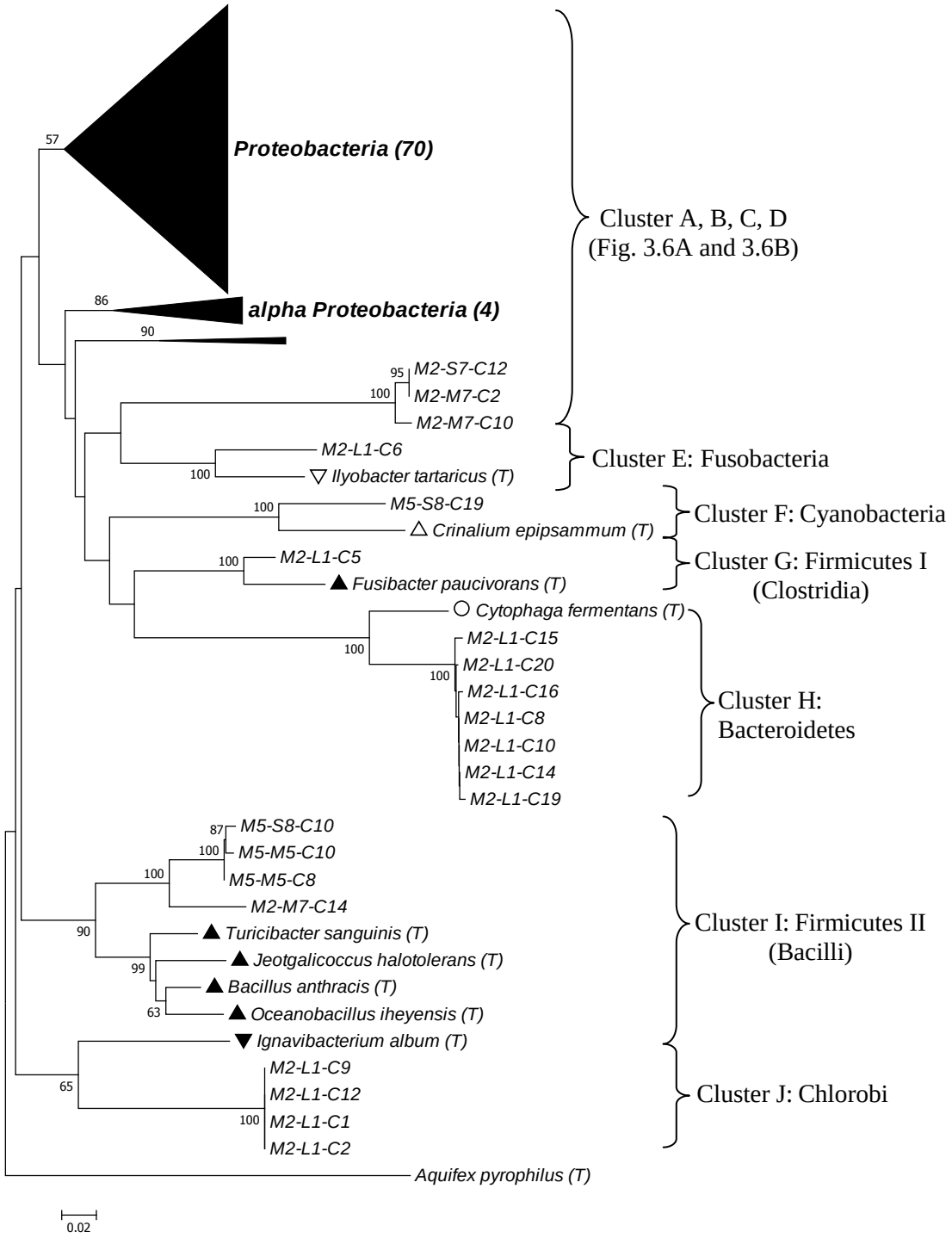

Figure 3.6 Phylogenetic tree of 16S rDNA sequences of bacteria obtained from the intestinal tracts of seven selected shrimps of different sizes at Month 2 and Month 5. Phylogenetic analyses were conducted in MEGA5 (Tamura *et al*., 2007) using the Neighbor-joining method (Jukes-Cantor model) and 500 replicates bootstrap test (value under 50 omitted). *Aquifex pyrophilus* was used as the outgroup. The Proteobacteria branches (total number of sequences in parentheses) were collapsed (Fig. 3.6A).

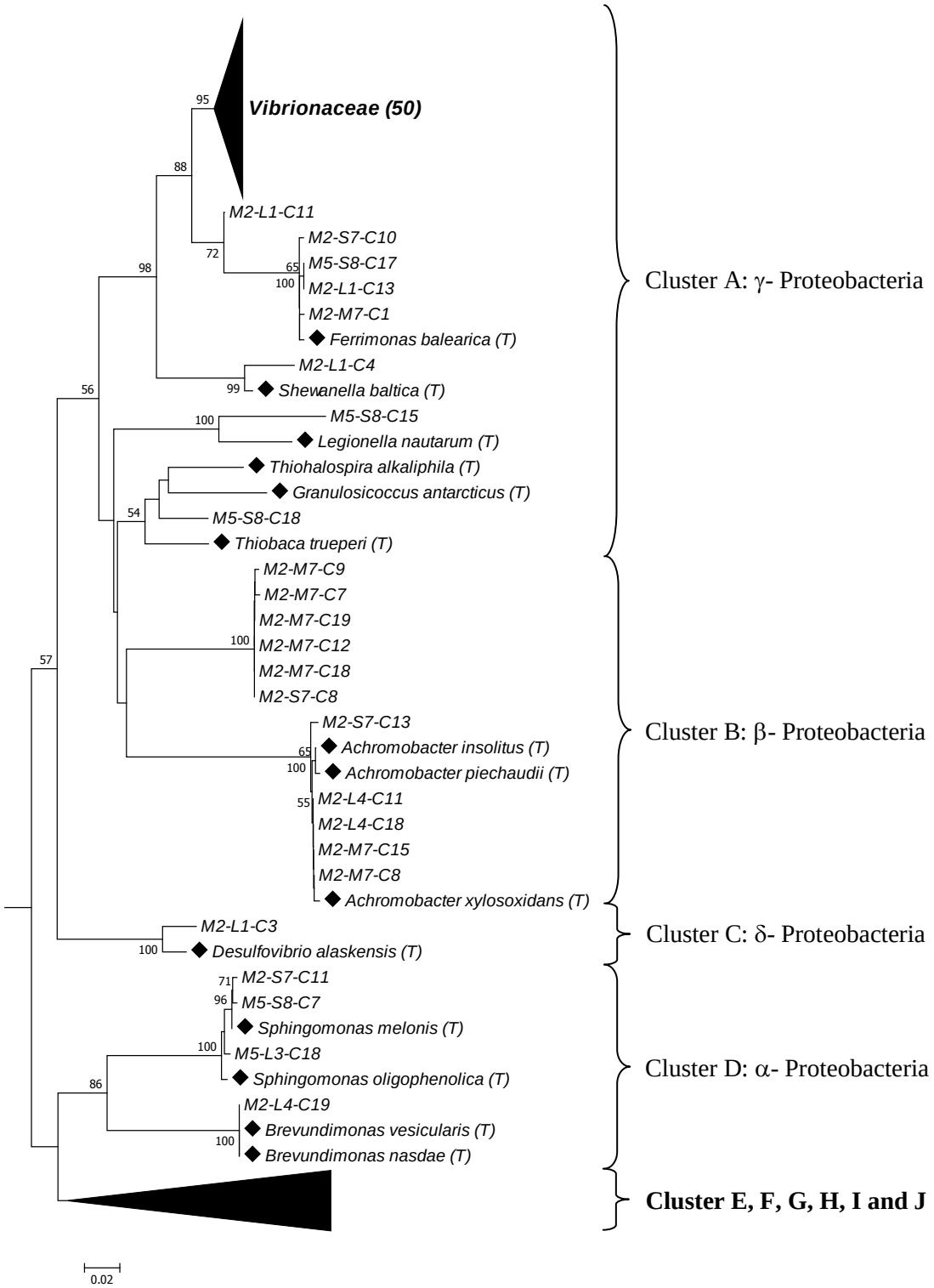

Figure 3.6A Phylogenetic tree of 16S rDNA sequences of Proteobacteria obtained from the intestinal tracts of seven selected shrimps of different sizes at Month 2 and Month 5. Phylogenetic analyses were conducted in MEGA5 (Tamura *et al*., 2007) using the Neighbor-joining method (Jukes-Cantor model) and 500 replicates bootstrap test (value under 50 omitted). The Vibrionaceae family branches (total number of sequences in parentheses) were collapsed (Fig. 3.6B).

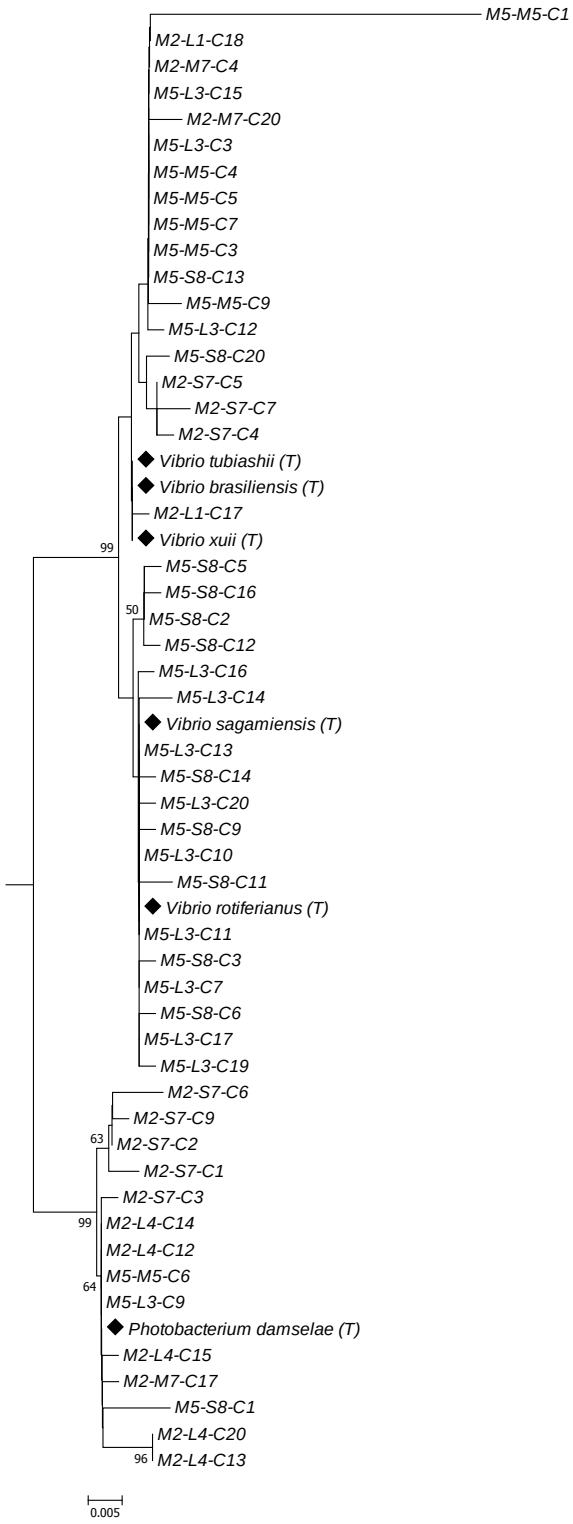

Figure 3.6B Phylogenetic tree of 16S rDNA sequences of the Vibrionaceae family obtained from the intestinal tracts of seven selected shrimps of different sizes at Month 2 and Month 5. Phylogenetic analyses were conducted in MEGA5 (Tamura *et al*., 2007) using the Neighbor-joining method (Jukes-Cantor model) and 500 replicates bootstrap test (value under 50 omitted).

Table 3.2 Distribution of bacterial species based on 16S rDNA sequences of the intestinal bacteria obtained from seven selected individual (M2, M5, L2-1, S2, L5, S5, and L2-2) shrimp of different sizes (small "S", medium "M" and large "L") at Month 2 and Month 5 into DGGE profile cluster (I, II, III, IV and V ; Fig. 3.5) and phylogenetic cluster (A, B, C, D, E, F, G, H, I, and J; Fig. 3.6, 3.6A, and 3.6B). Numbers of DGGE profiles represented in each DGGE cluster are in parentheses.

Presumptive Identification (no. of clones)	**DGGE Profile Cluster**							**Phylogenetic Cluster**									
	I (*N* = 25)	**II** (*N* = 8)	**III** (*N* = 14)		**IV** (*N* = 12)		**V** (*N* = 1)										
	M2	**M5**	**L2-1**	**S2**	**L5**	**S5**	**L2-2**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Proteobacteria																	
γ -Proteobacteria																	
Vibrio brasiliensis (14)	3	6	1		3	1		×									
Vibrio sagamiensis (10)						10		×									
Vibrio rotiferianus (9)					9			×									
Vibrio tubiashii (3)				3				×									
Vibrio xuii (1)			1					×									
Photobacterium damselae (14)	1	1		5	1	1	5	×									
Granulosicoccus antarcticus (2)	2							×									
Ferrimonas balearica (5)	1		2	1		1		×									
Legionella nautarum (1)						1		×									
Thiobaca trueperi (1)						1		×									
Thiohalospira alkaliphila (4)	3			1				×									
Shewanella baltica (1)			1					×									
β -Proteobacteria																	
Achromobacter xylosoxidans (1)	1								×								
Achromobacter insolitus (2)							2		×								

Table 3.2 continued

Presumptive Identification (no. of clones)	DGGE Profile Cluster							Phylogenetic Cluster									
	I (*N* = 25)	II (*N* = 8)	III (*N* = 14)		IV (*N* = 12)		V (*N* = 1)										
	M2	M5	L2-1	S2	L5	S5	L2-2	A	B	C	D	E	F	G	H	I	J
Achromobacter piechaudii (1)				1					×								
δ -Proteobacteria																	
Desulfovibrio alaskensis (1)			1							×							
α-Proteobacteria																	
Brevundimonas nasdae (1)	1										×						
Brevundimonas vesicularis (1)							1				×						
Sphingomonas oligophenolica (1)					1						×						
Sphingomonas melonis (2)				1		1					×						
ε-Proteobacteria																	
Helicobactor anseris (1)	1											×					
Thioreductor micatisoli (2)	1			1								×					
Bacteroidetes																	
Cytophaga fermentans (7)			7												×		
Cyanobacteria																	
Crinalium epipsammum (1)						1							×				
Erysipelotrichai																	
Turicibacter sanguinis (1)	1															×	
Fusobacteria																	
Ilyobacter tartaricus (1)			1									×					
Firmicutes																	
Bacillus anthracis (1)						1										×	

Table 3.2 continued

Presumptive Identification (no. of clones)	DGGE Profile Cluster							Phylogenetic Cluster									
	I (*N* = 25)	II (*N* = 8)	III (*N* = 14)		IV (*N* = 12)		V (*N* = 1)										
	M2	M5	L2-1	S2	L5	S5	L2-2	A	B	C	D	E	F	G	H	I	J
Fusibacter paucivorans (1)			1											×			
Jeogalicoccus halotolerans (1)		1														×	
Oceanobacillus iheyensis (1)		1														×	
Ignavibacteria																	
Iganavibacterium album (4)			4														×

Table 3.3 Nearest type strain matches based on bacterial 16S rDNA sequences, similarity scores, S_ab score, length, GenBank accession number, and phylum affiliation from the intestinal tracts of seven selected individual shrimps of different sizes at Month 2 and Month 5.

Sample	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank Accession Number	Taxonomic Affiliation
Month 2						
Small - Shrimp S2						
M2-S7-C1	*Photobacterium damselae* (T)	0.96	0.79	1323	X78105	γ-Proteobacteria
M2-S7-C2	*Photobacterium damselae* (T)	0.97	0.82	1323	X78105	γ-Proteobacteria
M2-S7-C3	*Photobacterium damselae* (T)	0.96	0.81	1323	X78105	γ-Proteobacteria
M2-S7-C4	*Vibrio tubiashii* (T)	0.95	0.78	1303	X74725	γ-Proteobacteria
M2-S7-C5	*Vibrio tubiashii* (T)	0.94	0.76	1303	X74725	γ-Proteobacteria
M2-S7-C6	*Photobacterium damselae* (T)	0.96	0.80	1323	X78105	γ-Proteobacteria
M2-S7-C7	*Vibrio tubiashii* (T)	0.94	0.74	1303	X74725	γ-Proteobacteria
M2-S7-C8	*Thiohalospira alkaliphila* (T)	0.89	0.59	1241	EU169227	γ-Proteobacteria
M2-S7-C9	*Photobacterium damselae* (T)	0.96	0.82	1323	X78105	γ-Proteobacteria
M2-S7-C10	*Ferrimonas balearica* (T)	0.91	0.67	1441	X93021	γ-Proteobacteria
M2-S7-C11	*Sphingomonas melonis* (T)	0.97	0.80	1350	AB055863	α-Proteobacteria
M2-S7-C12	*Thioreductor micantisoli* (T)	0.77	0.30	1357	AB175498	ε-Proteobacteria
M2-S7-C13	*Achromobacter piechaudii* (T)	0.97	0.82	1218	AB010841	β-Proteobacteria
Medium- Shrimp M2						
M2-M7-C1	*Ferrimonas balearica* (T)	1.00	0.94	1441	X93021	γ-Proteobacteria
M2-M7-C2	*Thioreductor micantisoli* (T)	0.79	0.33	1357	AB175498	ε-Proteobacteria
M2-M7-C4	*Vibrio brasiliensis* (T)	0.99	0.93	1380	AJ316172	γ-Proteobacteria
M2-M7-C6	*Brevundimonas nasdae* (T)	0.94	0.22	1353	AB071954	α-Proteobacteria
M2-M7-C7	*Granulosicoccus antarcticus* (T)	0.91	0.58	1399	EF495228	γ-Proteobacteria
M2-M7-C9	*Thiohalospira alkaliphila* (T)	0.91	0.62	1241	EU169227	γ-Proteobacteria

Table 3.3 continued

Sample	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank Accession Number	Taxonomic Affiliation
M2-M7-C10	*Helicobacter anseris* (T)	0.77	0.31	1407	DQ415545	ε-Proteobacteria
M2-M7-C11	*Vibrio brasiliensis* (T)	0.98	0.54	1380	AJ316172	γ-Proteobacteria
M2-M7-C12	*Thiohalospira alkaliphila* (T)	0.91	0.62	1241	EU169227	γ-Proteobacteria
M2-M7-C14	*Turicibacter sanguinis* (T)	0.85	0.45	1410	AF349724	Erysipelotrichai
M2-M7-C15	*Achromobacter xylosoxidans* (T)	1.00	0.94	1396	Y14908	β-Proteobacteria
M2-M7-C17	*Photobacterium damselae* (T)	1.00	0.98	1437	AB032015	γ-Proteobacteria
M2-M7-C18	*Granulosicoccus antarcticus* (T)	0.91	0.61	1399	EF495228	γ-Proteobacteria
M2-M7-C19	*Thiohalospira alkaliphila* (T)	0.91	0.61	1241	EU169227	γ-Proteobacteria
M2-M7-C20	*Vibrio brasiliensis* (T)	0.98	0.90	1380	AJ316172	γ-Proteobacteria
Large - Shrimp L2-1						
M2-L1-C1	*Ignavibacterium album* (T)	0.81	0.45	1391	AB478415	Ignavibacteria
M2-L1-C2	*Ignavibacterium album* (T)	0.82	0.43	1391	AB478415	Ignavibacteria
M2-L1-C3	*Desulfovibrio alaskensis* (T)	0.97	0.82	1430	Y11984	δ-Proteobacteria
M2-L1-C4	*Shewanella baltica* (T)	0.88	0.54	1432	AJ000214	γ-Proteobacteria
M2-L1-C5	*Fusibacter paucivorans* (T)	0.93	0.65	1437	AF050099	Clostridia
M2-L1-C6	*Ilyobacter tartaricus* (T)	0.91	0.63	1430	AJ307982	Fusobacteria
M2-L1-C8	*Cytophaga fermentans* (T)	0.91	0.60	1324	M58766	Sphingobacteria
M2-L1-C9	*Ignavibacterium album* (T)	0.81	0.42	1391	AB478415	Ignavibacteria
M2-L1-C10	*Cytophaga fermentans* (T)	0.92	0.58	1324	M58766	Sphingobacteria
M2-L1-C11	*Ferrimonas balearica* (T)	0.96	0.78	1441	X93021	γ-Proteobacteria
M2-L1-C12	*Ignavibacterium album* (T)	0.81	0.44	1391	AB478415	Ignavibacteria
M2-L1-C13	*Ferrimonas balearica* (T)	0.96	0.87	1441	X93021	γ-Proteobacteria

Table 3.3 continued

Sample	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank Accession Number	Taxonomic Affiliation
M2-L1-C14	*Cytophaga fermentans* (T)	0.90	0.57	1324	M58766	Sphingobacteria
M2-L1-C15	*Cytophaga fermentans* (T)	0.91	0.57	1324	M58766	Sphingobacteria
M2-L1-C16	*Cytophaga fermentans* (T)	0.92	0.60	1324	M58766	Sphingobacteria
M2-L1-C17	*Vibrio xuii* (T)	1.00	0.95	1348	AJ316181	γ-Proteobacteria
M2-L1-C18	*Vibrio brasiliensis* (T)	0.98	0.87	1380	AJ316172	γ-Proteobacteria
M2-L1-C19	*Cytophaga fermentans* (T)	0.92	0.57	1324	M58766	Sphingobacteria
M2-L1-C20	*Cytophaga fermentans* (T)	0.92	0.57	1324	M58766	Sphingobacteria
Large- Shrimp L2-2						
M2-L4-C11	*Achromobacter insolitus* (T)	1.00	0.95	1364	AY170847	β-Proteobacteria
M2-L4-C12	*Photobacterium damselae* (T)	1.00	0.99	1437	AB032015	γ-Proteobacteria
M2-L4-C13	*Photobacterium damselae* (T)	0.99	0.97	1437	AB032015	γ-Proteobacteria
M2-L4-C14	*Photobacterium damselae* (T)	1.00	0.99	1437	AB032015	γ-Proteobacteria
M2-L4-C15	*Photobacterium damselae* (T)	1.00	0.97	1437	AB032015	γ-Proteobacteria
M2-L4-C18	*Achromobacter insolitus* (T)	1.00	0.97	1364	AY170847	β-Proteobacteria
M2-L4-C19	*Brevundimonas vesicularis* (T)	0.99	0.97	1344	AJ227780	α-Proteobacteria
M2-L4-C20	*Photobacterium damselae* (T)	0.99	0.96	1437	AB032015	γ-Proteobacteria
Month 5						
Small- Shrimp S5						
M5-S8-C1	*Photobacterium damselae* (T)	0.96	0.81	1323	X78105	γ-Proteobacteria
M5-S8-C2	*Vibrio sagamiensis* (T)	0.98	0.85	1224	AB428909	γ-Proteobacteria
M5-S8-C3	*Vibrio sagamiensis* (T)	0.97	0.85	1224	AB428909	γ-Proteobacteria
M5-S8-C5	*Vibrio sagamiensis* (T)	0.97	0.84	1224	AB428909	γ-Proteobacteria

Table 3.3 continued

Sample	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank Accession Number	Taxonomic Affiliation
M5-S8-C6	*Vibrio sagamiensis* (T)	0.98	0.89	1224	AB428909	γ-Proteobacteria
M5-S8-C7	*Sphingomonas melonis* (T)	0.97	0.80	1350	AB055863	α-Proteobacteria
M5-S8-C9	*Vibrio sagamiensis* (T)	0.98	0.86	1224	AB428909	γ-Proteobacteria
M5-S8-C10	*Bacillus anthracis* (T)	0.82	0.43	1236	AB190217	Bacilli
M5-S8-C11	*Vibrio sagamiensis* (T)	0.98	0.84	1224	AB428909	γ-Proteobacteria
M5-S8-C12	*Vibrio sagamiensis* (T)	0.97	0.82	1224	AB428909	γ-Proteobacteria
M5-S8-C13	*Vibrio brasiliensis* (T)	0.97	0.87	1380	AJ316172	γ-Proteobacteria
M5-S8-C14	*Vibrio sagamiensis* (T)	0.97	0.87	1224	AB428909	γ-Proteobacteria
M5-S8-C15	*Legionella nautarum* (T)	0.90	0.58	1309	Z49728	γ-Proteobacteria
M5-S8-C16	*Vibrio sagamiensis* (T)	0.96	0.83	1224	AB428909	γ-Proteobacteria
M5-S8-C17	*Ferrimonas balearica* (T)	0.94	0.73	1441	X93021	α-Proteobacteria
M5-S8-C18	*Thiobaca trueperi* (T)	0.92	0.63	1302	AJ404006	γ-Proteobacteria
M5-S8-C19	*Crinalium epipsammum* (T)	0.87	0.49	1367	AB115964	Cyanobacteria
M5-S8-C20	*Vibrio sagamiensis* (T)	0.95	0.78	1224	AB428909	γ-Proteobacteria
Medium- Shrimp M5						
M5-M5-C1	*Vibrio brasiliensis* (T)	0.96	0.79	1380	AJ316172	γ-Proteobacteria
M5-M5-C3	*Vibrio brasiliensis* (T)	1.00	0.96	1380	AJ316172	γ-Proteobacteria
M5-M5-C4	*Vibrio brasiliensis* (T)	0.99	0.94	1380	AJ316172	γ-Proteobacteria
M5-M5-C5	*Vibrio brasiliensis* (T)	0.99	0.95	1380	AJ316172	γ-Proteobacteria
M5-M5-C6	*Photobacterium damselae* (T)	1.00	0.99	1437	AB032015	γ-Proteobacteria
M5-M5-C7	*Vibrio brasiliensis* (T)	1.00	0.96	1380	AJ316172	γ-Proteobacteria
M5-M5-C8	*Oceanobacillus iheyensis* (T)	0.87	0.49	1441	AB010863	Bacilli

Table 3.3 continued

Sample	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank Accession Number	Taxonomic Affiliation
M5-M5-C9	*Vibrio brasiliensis* (T)	0.99	0.95	1380	AJ316172	γ-Proteobacteria
M5-M5-C10	*Jeotgalicoccus halotolerans* (T)	0.86	0.48	1434	AY028925	Bacilli
Large- Shrimp L5						
M5-L3-C3	*Vibrio brasiliensis* (T)	1.00	0.96	1380	AJ316172	γ-Proteobacteria
M5-L3-C7	*Vibrio rotiferianus* (T)	0.99	0.93	1380	AJ316187	γ-Proteobacteria
M5-L3-C9	*Photobacterium damselae* (T)	0.99	0.97	1437	AB032015	γ-Proteobacteria
M5-L3-C10	*Vibrio rotiferianus* (T)	1.00	0.97	1380	AJ316187	γ-Proteobacteria
M5-L3-C11	*Vibrio rotiferianus* (T)	1.00	0.96	1380	AJ316187	γ-Proteobacteria
M5-L3-C12	*Vibrio brasiliensis* (T)	0.99	0.92	1380	AJ316172	γ-Proteobacteria
M5-L3-C13	*Vibrio rotiferianus* (T)	1.00	0.95	1380	AJ316187	γ-Proteobacteria
M5-L3-C14	*Vibrio rotiferianus* (T)	0.99	0.97	1380	AJ316187	γ-Proteobacteria
M5-L3-C15	*Vibrio brasiliensis* (T)	1.00	0.96	1380	AJ316172	γ-Proteobacteria
M5-L3-C16	*Vibrio rotiferianus* (T)	0.99	0.95	1380	AJ316187	γ-Proteobacteria
M5-L3-C17	*Vibrio rotiferianus* (T)	0.99	0.94	1380	AJ316187	γ-Proteobacteria
M5-L3-C18	*Sphingomonas oligophenolica* (T)	0.99	0.93	1224	AB018439	α-Proteobacteria
M5-L3-C19	*Vibrio rotiferianus* (T)	1.00	0.97	1380	AJ316187	γ-Proteobacteria
M5-L3-C20	*Vibrio rotiferianus* (T)	1.00	0.98	1380	AJ316187	γ-Proteobacteria

[a]- Similarity score = Percent sequence identity

[b]- S_ab score = seqmatch score = number of (unique) 7-base oligomers shared between a sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences (Cole *et al*., 2005)

(T) = Type strain

3. 2 Wild *P. monodon*

The size of genomic DNA extracted from eight wild *P. monodon* tissue samples (stomach, anterior midgut, midgut, posterior midgut, hindgut and feces) was approximately 23.1 kb (Fig. 3.7). The size of the product from the first PCR (near complete 16S rDNA) was 1500 bp, and the size of the product from the nested PCR was 200 bp, as shown in Fig. 3.8A and 3.8B.

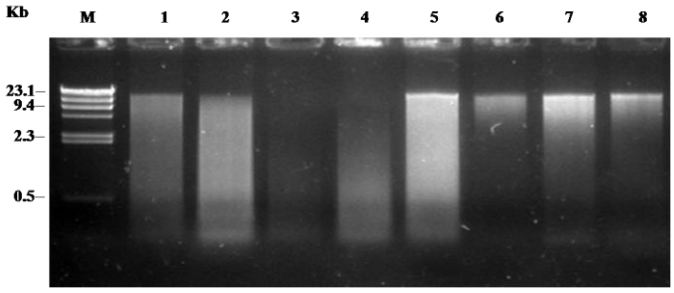

Figure 3.7 Genomic DNA extracted from the hindgut of 8 wild *P. monodon* samples on a 1.0 % agarose gel. Electrophoresis was performed for 30 min at 100 V. DNA was visualized under UV after staining with ethidium bromide. Lanes: M, λ *Hin*dIII; 1–8, DNA extracted from hindgut of shrimp no.1-8, respectively. Numbers on the left side of the gel refer to the size of DNA markers in Kb.

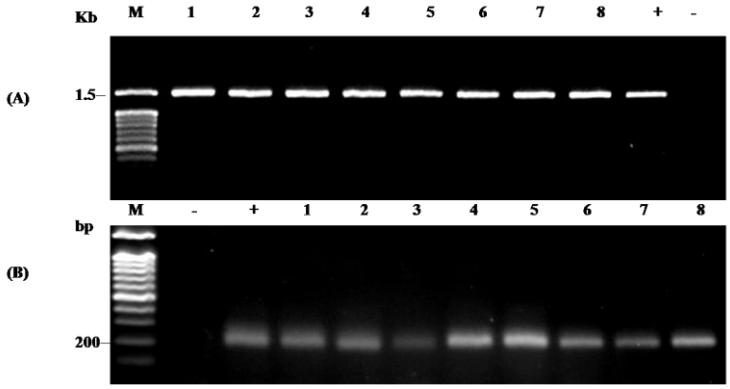

Figure 3.8 16S rDNA PCR product on 1.0 % agarose gel (A) and nested DGGE PCR product on 1.5% agarose gel (B). Lanes: M, 100 bp marker; 1–8, DNA extracted from hindgut of shrimp no.1-8, respectively. Numbers on the left side of the gel refer to the size of DNA markers in Kb and bp.

PCR-DGGE analysis was used to determine the bacterial community composition and diversity between different segments of the gastrointestinal tract (GI). The PCR-DGGE profiles of bacteria from all segments of the GI tract revealed the presence of 2-12 OTUs (bands) (figure not show). Some OTUs are common to all segments of the GI tract. The total numbers of OTUs from feces, stomach and hindgut showed higher diversity (more bands) than that of midgut (all three segments).

Five hundred and twenty six out of 720 clones were screened using DGGE analysis. Among them, 494 were produced positive results (200 bp). One hundred out of 494 clones were selected for 16S rDNA sequencing (section 2.1.7.7).

The nearest type-strain matches of these sequences based on the RDP-II database, accession numbers and the distribution of bacteria are summarized in Table 3.5. Most acceptable sequences ranged between 1224 to 1459 bp in length, and had similarity scores between 0.79 to 1.00 to sequences in the RDP-II database. The S_ab score, a more stringent measure of similarity, ranged from 0.33 to 1.00. Most sequences (58 sequences) had similarity scores greater than 0.98 when compared to type strains of recognized species in the RDP-II database (Table 3.5). Fourteen sequences had low similarity score of less than 0.93 with type strain. There sequences were WstC4 (0.88), WAC1 (0.85), WAC7 (0.85), WAC12 (0.92), WAC13 (0.90), WAC15 (0.86), WAC21 (0.92), WAC24 (0.89), WAC25 (0.92), WHC11 (0.85), WHC33 (0.79), WFC6 (0.92), WFC7 (0.92) and WFC13 (0.90). The sequences with less than 93% similarity were identified to the phylum level. Most sequences belonged to the phylum Proteobacteria. *Photobacterium damselae* and *Vibrio* spp. were the most common bacteria represented in the total number of clones from all parts in the gastrointestinal tract (GI) of wild *P. monodon*.

Phylogenetic tree and the distribution of bacterial species from each sample are shown in Fig. 3.9 and Table 3.4, respectively. There were 34 bacterial species in 3 phyla represented in the sequences (Table 3.5). These bacteria were clustered into 4 major groups including γ-Proteobacteria (cluster A; 81 out of 100 sequences), α-Proteobacteria (cluster B; 6 out of 100), ε-Proteobacteria (cluster C; 1 out of 100), Firmicutes (cluster D and F; 6 out of 100), and Bacteroidetes (cluster E; 6 out of 100).

Cluster A consisted of 81 sequences in the γ- Proteobacteria class, that was obtained from the stomach, anterior midgut, hindgut, and feces of the shrimp. These sequences were identified as members of the genera *Acinetobacter*, *Aeromonas*, *Endozoicomonas*, *Escherichia/Shigella*, *Ferrimonas*, *Marinobacter*, *Marinomonas*, *Methylophaga*, *Neptunomona*, *Photobacterium*, *Pseudoalteromonas* and *Vibrio*. Twenty of them were identified as *Vibrio* sp. and consisted of 10 species including *V. brasiliensis, V. communis, V. hepatarius, V. mytili, V. neptunius, V. nigripulchritudo, V. rotiferianus, V. sagamiensis, V. sinaloensis and V. tubiashii.* Thirty - three out of the total number of sequence clones belonged to *Photobacterium damselae.*

Cluster B consisted of 6 sequences in the α-Proteobacteria class, that was obtained from the stomach, anterior midgut, hindgut, and feces of the shrimp. There were 4 different species in cluding *Afipia massiliensis, Shimia isoporae, Shimia marina* and *Silicibacter lacuscaerulensis.*

Cluster C consisted of 1 sequence in the phylum ε-Proteobacteria. The sequence was closely related to *Thioreductor micantisoli*, and was found only in the hindgut.

Cluster D consisted of 3 sequences in the phylum Firmicutes I (Clostridia). The 3 sequences were closely related to *Fusibacter paucivorans*, and were found only in the hindgut and feces.

Cluster E consisted of 6 sequences in the phylum Bacteroidetes. These 6 sequences which were found in the anterior midgut, hindgut and feces, belonged to the following into 4 species: *Actibacter sediminis, Gaetbulibacter marinus, Leptobacterium flavescens* and *Mesoflavibacter zeaxanthinifaciens,* respectively.

Cluster F consisted of 3 sequences in the phylum Firmicutes II (Bacilli). The 3 sequences were closely related to *Jeotgalicoccus halotolerans, Paenibacillus wynnii,* and were found only in the anterior midgut.

The distribution of bacterial species in the GI tract of shrimp was not uniform. While bacteria in the α-Proteobacteria and γ-Proteobacteria were distributed throughout all segments of the GI tract, those in the phylum Bacteroidetes were not represented in the stomach (anterior midgut, hindgut and feces only). Bacteria in the phylum Firmicutes were found in the anterior midgut, hindgut and feces only. The hindgut, stomach and feces samples demonstrated greater bacterial diversity more than the anterior midgut as measured by the number of species. The hindgut and stomach were dominated by sequence belonging to *Vibrio* sp., while the feces and anterior midgut were dominated by *Photobacterium damselae*. *Vibrio* sp. was not found in the anterior midgut sample.

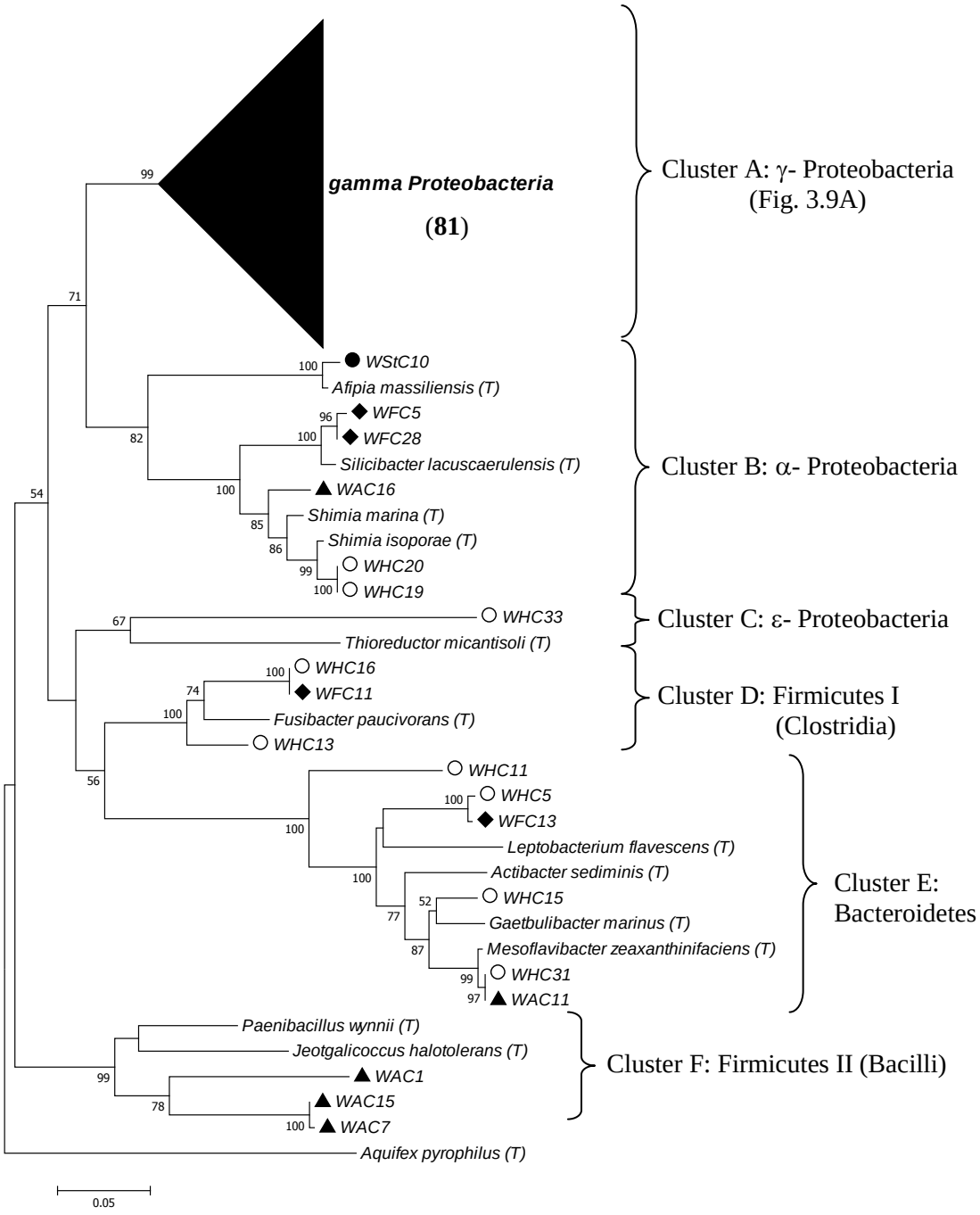

Figure 3.9 Phylogenetic tree of 16S rDNA sequences of bacteria obtained from the stomach (●), midgut-anterior (▲), hindgut (○), and feces (♦) from the intestinal tracts of wild broodstock shrimps. Phylogenetic analyses were conducted in MEGA5 (Tamura *et al*., 2007) using the Neighbor-joining method (Jukes-Cantor model) and 500 replicates bootstrap test (value under 50 omitted). *Aquifex pyrophilus* was used as the outgroup. The γ-Proteobacteria branch (total number of sequences in parentheses) was collapsed (Fig. 3.9A).

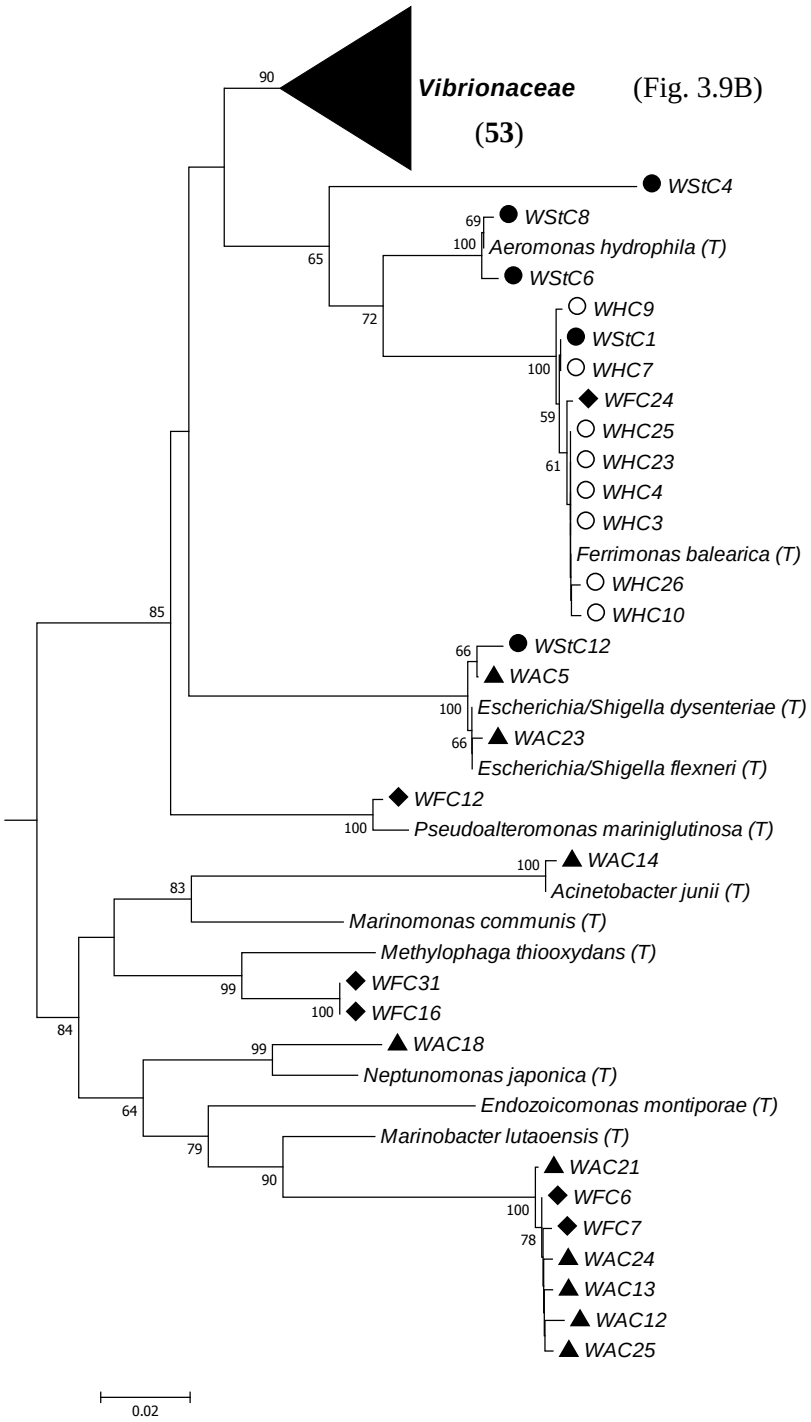

Figure 3.9A Phylogenetic tree of 16S rDNA sequences of γ-Proteobacteria obtained from the stomach (●), midgut-anterior (▲), hindgut (○), and feces (♦) from the intestinal tracts of wild broodstock shrimps. Phylogenetic analyses were conducted in MEGA5 (Tamura *et al.*, 2007) using the Neighbor-joining method (Jukes-Cantor model) and 500 replicates bootstrap test (value under 50 omitted) The Vibrionaceae family branch (total number of sequences in parentheses) were collapsed (Fig. 3.9B).

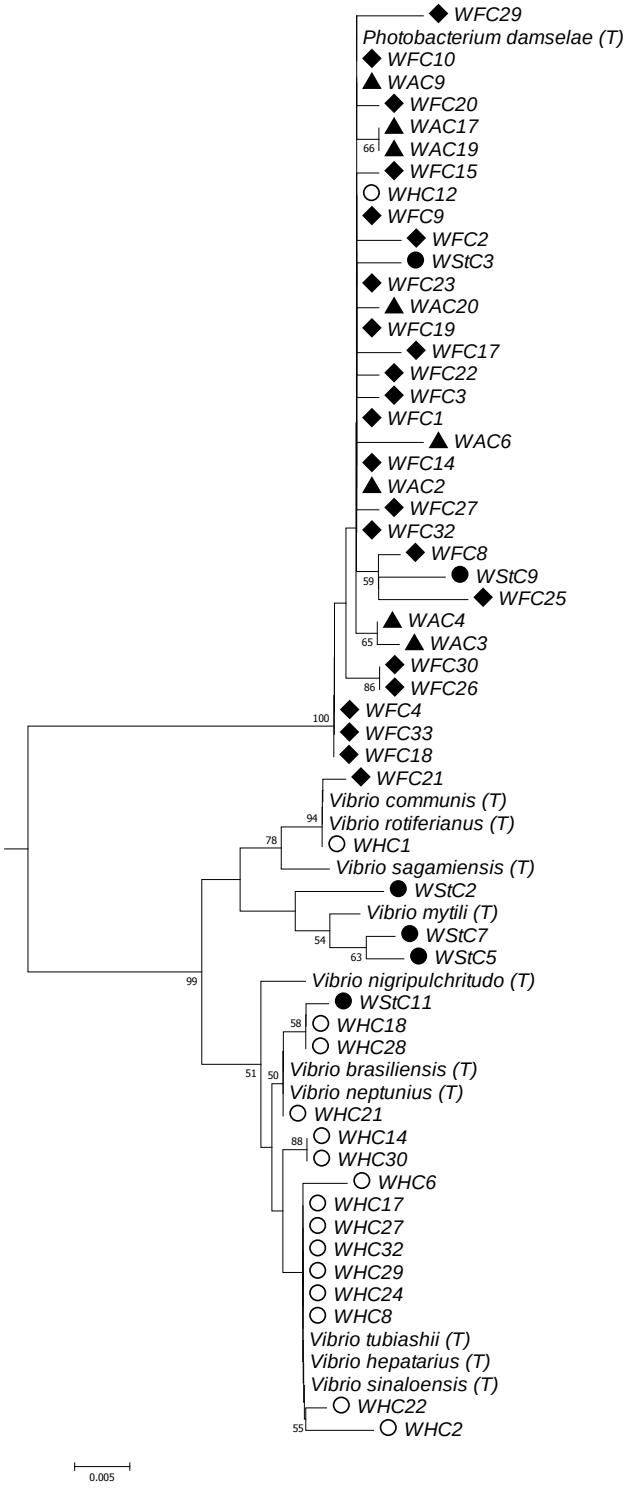

Figure 3.9B Phylogenetic tree of 16S rDNA sequences of the Vibrionaceae family obtained from the stomach (●), midgut-anterior (▲), hindgut (○), and feces (♦) from the intestinal tracts of wild broodstock shrimps. Phylogenetic analyses were conducted in MEGA5 (Tamura *et al*., 2007) using the Neighbor-joining method (Jukes-Cantor model) and 500 replicates bootstrap test (value under 50 omitted).

Table 3.4 Distribution of bacterial species based on 16S rDNA sequences of the stomach, midgut-anteroir, hindgut, and feces from the tract of wild broodstock shrimps and their respective phylogenetic cluster (A, B, C, D, E and F; Fig. 3.9, 3.9A, and 3.9B). Numbers of sequences observed in each GI tract segments were in parentheses.

Presumptive Identification	Number of Sequences				Cluster					
	Stomach	Anterior midgut	Hindgut	Feces	A	B	C	D	E	F
Proteobacteria										
α-Proteobacteria										
Afipia massiliensis	1					×				
Shimia isoporae			2			×				
Shimia marina		1				×				
Silicibacter lacuscaerulensis				2		×				
γ-Proteobacteria										
Acinetobacter junii		1			×					
Aeromonas hydrophila	3				×					
Endozoicomonas montiporae		1			×					
Escherichia/Shigella dysenteriae		1			×					
Escherichia/Shigella flexneri	1	1			×					
Ferrimonas balearica	1		8	1	×					
Marinobacter lutaoensis		3		2	×					
Marinomonas communis		1			×					
Methylophaga thiooxydans				2	×					
Neptunomonas japonica		1			×					
Photobacterium damselae	2	8	1	22	×					
Pseudoalteromonas mariniglutinosa				1	×					

Table 3.4 continuted

Presumptive Identification	Number of Sequences				Cluster					
	Stomach	Anterior midgut	Hindgut	Feces	A	B	C	D	E	F
Vibrio brasiliensis			5		×					
Vibrio communis			1		×					
Vibrio hepatarius			7		×					
Vibrio mytili	1				×					
Vibrio neptunius			1		×					
Vibrio nigripulchritudo	1				×					
Vibrio rotiferianus				1	×					
Vibrio sagamiensis	1				×					
Vibrio sinaloensis			1		×					
Vibrio tubiashii	1				×					
ε-Proteobacteria										
Thioreductor micantisoli			1				×			
Bacteroidetes										
Actibacter sediminis			1						×	
Gaetbulibacter marinus			2						×	
Leptobacterium flavescens				1					×	
Mesoflavibacter zeaxanthinifaciens		1	1						×	
Firmicutes										
Fusibacter paucivorans			2	1				×		
Jeotgalicoccus halotolerans		1								×
Paenibacillus wynnii		2								×
Total (100)	**(12)**	**(22)**	**(33)**	**(33)**						

Table 3.5 Nearest type strain matches based on bacterial 16S rDNA sequences, similarity scores, S_ab score, length, GenBank accession number, and phylum affiliation from the stomach, midgut-anterior, hindgut, and feces from the intestinal tract of wild broodstock shrimps.

Designation	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank accession number	Taxonomic affiliation
Stomach						
WStC1	*Ferrimonas balearica* (T)	0.90	0.64	1441	X93021	γ-Proteobacteria
WStC2	*Vibrio nigripulchritudo* (T)	0.95	0.75	1320	X74717	γ-Proteobacteria
WStC3	*Photobacterium damselae* (T)	0.97	0.87	1437	AB032015	γ-Proteobacteria
WStC4	*Aeromonas hydrophila* (T)	0.88	0.64	1459	DQ207728	γ-Proteobacteria
WStC5	*Vibrio mytili* (T)	0.96	0.85	1354	X99761	γ-Proteobacteria
WStC6	*Aeromonas hydrophila* (T)	0.95	0.79	1459	DQ207728	γ-Proteobacteria
WStC7	*Vibrio sagamiensis* (T)	0.94	0.76	1224	AB428909	γ-Proteobacteria
WStC8	*Aeromonas hydrophila* (T)	0.96	0.71	1459	DQ207728	γ-Proteobacteria
WStC9	*Photobacterium damselae* (T)	0.96	0.85	1437	AB032015	γ-Proteobacteria
WStC10	*Afipia massiliensis* (T)	0.97	0.82	1295	AY029562	α-proteobacteria
WStC11	*Vibrio tubiashii* (T)	0.94	0.77	1303	X74725	γ-Proteobacteria
WStC12	*Escherichia/Shigella flexneri* (T)	0.97	0.80	1405	X96963	γ-Proteobacteria
Midgut (anterior)						
WAC1	*Jeotgalicoccus halotolerans* (T)	0.85	0.47	1434	AY028925	Bacilli
WAC2	*Photobacterium damselae* (T)	1.00	0.98	1437	AB032015	γ-Proteobacteria
WAC3	*Photobacterium damselae* (T)	0.97	0.89	1437	AB032015	γ-Proteobacteria
WAC4	*Photobacterium damselae* (T)	0.99	0.97	1437	AB032015	γ-Proteobacteria
WAC5	*Escherichia/Shigella flexneri* (T)	0.99	0.96	1405	X96963	γ-Proteobacteria
WAC6	*Photobacterium damselae* (T)	1.00	0.97	1437	AB032015	γ-Proteobacteria
WAC7	*Paenibacillus wynnii* (T)	0.85	0.42	1421	AJ633647	Bacilli

Table 3.5 continued

Designation	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank accession number	Taxonomic affiliation
WAC9	*Photobacterium damselae* (T)	0.99	0.98	1437	AB032015	γ-Proteobacteria
WAC11	*Mesoflavibacter zeaxanthinifaciens* (T)	0.98	0.94	1361	AB265181	Flavobacteria
WAC12	*Marinobacter lutaoensis* (T)	0.92	0.57	1432	AF288157	γ-Proteobacteria
WAC13	*Endozoicomonas montiporae* (T)	0.90	0.56	1394	FJ347758	γ-Proteobacteria
WAC14	*Acinetobacter junii* (T)	1.00	0.98	1371	X81664	γ-Proteobacteria
WAC15	*Paenibacillus wynnii* (T)	0.86	0.44	1421	AJ633647	Bacilli
WAC16	*Shimia marina* (T)	0.96	0.85	1310	AY962292	α-proteobacteria
WAC17	*Photobacterium damselae* (T)	0.99	0.97	1437	AB032015	γ-Proteobacteria
WAC18	*Neptunomonas japonica* (T)	0.97	0.80	1403	AB288092	γ-Proteobacteria
WAC19	*Photobacterium damselae* (T)	1.00	0.96	1437	AB032015	γ-Proteobacteria
WAC20	*Photobacterium damselae* (T)	0.99	0.97	1437	AB032015	γ-Proteobacteria
WAC21	*Marinobacter lutaoensis (T)*	0.92	0.58	1432	AF288157	γ-Proteobacteria
WAC23	*Escherichia/Shigella dysenteriae* (T)	0.99	0.94	1397	X96966	γ-Proteobacteria
WAC24	*Marinomonas communis* (T)	0.89	0.58	1371	DQ011528	γ-Proteobacteria
WAC25	*Marinobacter lutaoensis* (T)	0.92	0.57	1432	AF288157	γ-Proteobacteria
Hindgut						
WHC1	*Vibrio communis* (T)	1.00	0.96	1384	GU078672	γ-Proteobacteria
WHC2	*Vibrio hepatarius* (T)	1.00	0.93	1371	AJ345063	γ-Proteobacteria
WHC3	*Ferrimonas balearica* (T)	1.00	0.97	1441	X93021	γ-Proteobacteria
WHC4	*Ferrimonas balearica* (T)	1.00	0.98	1441	X93021	γ-Proteobacteria
WHC5	*Gaetbulibacter marinus* (T)	0.93	0.66	1398	EF108219	Flavobacteria
WHC6	*Vibrio sinaloensis* (T)	0.99	0.93	1374	DQ451211	γ-Proteobacteria
WHC7	*Ferrimonas balearica* (T)	1.00	0.95	1441	X93021	γ-Proteobacteria

Table 3.5 continued

Designation	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank accession number	Taxonomic affiliation
WHC8	*Vibrio hepatarius* (T)	1.00	0.95	1371	AJ345063	γ-Proteobacteria
WHC9	*Ferrimonas balearica* (T)	0.99	0.94	1441	X93021	γ-Proteobacteria
WHC10	*Ferrimonas balearica* (T)	1.00	0.96	1441	X93021	γ-Proteobacteria
WHC11	*Actibacter sediminis* (T)	0.85	0.51	1316	EF670651	Flavobacteria
WHC12	*Photobacterium damselae* (T)	1.00	0.98	1437	AB032015	γ-Proteobacteria
WHC13	*Fusibacter paucivorans* (T)	0.93	0.66	1437	AF050099	Clostridia
WHC14	*Vibrio hepatarius* (T)	1.00	0.94	1371	AJ345063	γ-Proteobacteria
WHC15	*Gaetbulibacter marinus* (T)	0.97	0.83	1398	EF108219	Flavobacteria
WHC16	*Fusibacter paucivorans* (T)	0.93	0.70	1437	AF050099	Clostridia
WHC17	*Vibrio hepatarius* (T)	1.00	0.95	1371	AJ345063	γ-Proteobacteria
WHC18	*Vibrio brasiliensis* (T)	0.99	0.92	1380	AJ316172	γ-Proteobacteria
WHC19	*Shimia isoporae* (T)	0.98	0.90	1320	FJ976449	α-proteobacteria
WHC20	*Shimia isoporae* (T)	0.98	0.90	1320	FJ976449	α-proteobacteria
WHC21	*Vibrio brasiliensis* (T)	1.00	0.95	1380	AJ316172	γ-Proteobacteria
WHC22	*Vibrio brasiliensis* (T)	1.00	0.96	1380	AJ316172	γ-Proteobacteria
WHC23	*Ferrimonas balearica* (T)	1.00	0.97	1441	X93021	γ-Proteobacteria
WHC24	*Vibrio neptunius* (T)	1.00	0.93	1364	AJ316171	γ-Proteobacteria
WHC25	*Ferrimonas balearica* (T)	1.00	0.98	1441	X93021	γ-Proteobacteria
WHC26	*Ferrimonas balearica* (T)	1.00	0.98	1441	X93021	γ-Proteobacteria
WHC27	*Vibrio hepatarius* (T)	1.00	0.94	1371	AJ345063	γ-Proteobacteria
WHC28	*Vibrio brasiliensis* (T)	0.98	0.93	1380	AJ316172	γ-Proteobacteria
WHC29	*Vibrio hepatarius* (T)	1.00	0.95	1371	AJ345063	γ-Proteobacteria
WHC30	*Vibrio brasiliensis* (T)	0.99	0.94	1380	AJ316172	γ-Proteobacteria
WHC31	*Mesoflavibacter zeaxanthinifaciens* (T)	0.99	0.97	1361	AB265181	Flavobacteria

Table 3.5 continued

Designation	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank accession number	Taxonomic affiliation
WHC32	*Vibrio hepatarius* (T)	1.00	0.94	1371	AJ345063	γ-Proteobacteria
WHC33	*Thioreductor micantisoli* (T)	0.79	0.33	1357	AB175498	ε-Proteobacteria
Feces						
WFC1	*Photobacterium damselae* (T)	1.00	0.98	1323	X78105	γ-Proteobacteria
WFC2	*Photobacterium damselae* (T)	1.00	0.98	1437	AB032015	γ-Proteobacteria
WFC3	*Photobacterium damselae* (T)	1.00	0.99	1437	AB032015	γ-Proteobacteria
WFC4	*Photobacterium damselae* (T)	1.00	0.98	1437	AB032015	γ-Proteobacteria
WFC5	*Silicibacter lacuscaerulensis* (T)	0.98	0.90	1268	U77644	α-proteobacteria
WFC6	*Marinobacter lutaoensis* (T)	0.92	0.59	1432	AF288157	γ-Proteobacteria
WFC7	*Marinobacter lutaoensis* (T)	0.92	0.58	1432	AF288157	γ-Proteobacteria
WFC8	*Photobacterium damselae* (T)	0.99	0.98	1437	AB032015	γ-Proteobacteria
WFC9	*Photobacterium damselae* (T)	1.00	1.00	1437	AB032015	γ-Proteobacteria
WFC10	*Photobacterium damselae* (T)	1.00	1.00	1437	AB032015	γ-Proteobacteria
WFC11	*Fusibacter paucivorans* (T)	0.93	0.66	1437	AF050099	Clostridia
WFC12	*Pseudoalteromonas mariniglutinosa* (T)	0.99	0.90	1400	AJ507251	γ-Proteobacteria
WFC13	*Leptobacterium flavescens* (T)	0.90	0.61	1368	AB362212	Flavobacteria
WFC14	*Photobacterium damselae* (T)	1.00	1.00	1437	AB032015	γ-Proteobacteria
WFC15	*Photobacterium damselae* (T)	1.00	0.99	1437	AB032015	γ-Proteobacteria
WFC16	*Methylophaga thiooxydans* (T)	0.96	0.74	1368	DQ660915	γ-Proteobacteria
WFC17	*Photobacterium damselae* (T)	1.00	0.98	1437	AB032015	γ-Proteobacteria
WFC18	*Photobacterium damselae* (T)	1.00	0.98	1437	AB032015	γ-Proteobacteria
WFC19	*Photobacterium damselae* (T)	1.00	0.99	1437	AB032015	γ-Proteobacteria
WFC20	*Photobacterium damselae* (T)	1.00	0.99	1437	AB032015	γ-Proteobacteria

Table 3.5 continued

Designation	Presumptive Identification	Similarity score[a]	S_ab score[b]	Length (bp)	GenBank accession number	Taxonomic affiliation
WFC21	*Vibrio rotiferianus* (T)	1.00	0.98	1380	AJ316187	γ-Proteobacteria
WFC22	*Photobacterium damselae* (T)	1.00	0.97	1323	X78105	γ-Proteobacteria
WFC23	*Photobacterium damselae* (T)	1.00	0.99	1323	X78105	γ-Proteobacteria
WFC24	*Ferrimonas balearica* (T)	1.00	0.96	1441	X93021	γ-Proteobacteria
WFC25	*Photobacterium damselae* (T)	0.99	0.96	1437	AB032015	γ-Proteobacteria
WFC26	*Photobacterium damselae* (T)	1.00	0.98	1437	AB032015	γ-Proteobacteria
WFC27	*Photobacterium damselae* (T)	1.00	0.99	1437	AB032015	γ-Proteobacteria
WFC28	*Silicibacter lacuscaerulensis* (T)	0.98	0.91	1268	U77644	α-proteobacteria
WFC29	*Photobacterium damselae* (T)	0.99	0.96	1323	X78105	γ-Proteobacteria
WFC30	*Photobacterium damselae* (T)	1.00	0.98	1437	AB032015	γ-Proteobacteria
WFC31	*Methylophaga thiooxydans* (T)	0.96	0.74	1368	DQ660915	γ-Proteobacteria
WFC32	*Photobacterium damselae* (T)	1.00	0.99	1437	AB032015	γ-Proteobacteria
WFC33	*Photobacterium damselae* (T)	1.00	0.97	1437	AB032015	γ-Proteobacteria

a- Similarity score = Percent sequence identity

b- S_ab score = seqmatch score = number of (unique) 7-base oligomers shared between a sequence and a given RDP sequence divided by the lowest number of unique oligos in either of the two sequences (Cole *et al*., 2005)

(T) = Type strain

3.3 *Vibrio* challenge test

3.3.1 Bacterial enumeration

Average total bacterial and total *Vibrio* concentrations from the digestive tract of control (unchallenged) and *V. harvei*-challenged shrimps were shown in Table 3.6. Average total bacterial concentrations (based on the enumeration on Marine 2216 agar) in the hepatopancreas and intestines increased slightly from the pre-trial level for both the controlled and infected group upon feeding of *Artemia* as soon as the 0 hour post- challenged, while the increase was not observed in hindgut. In contrast, average total *Vibrio* concentrations (based on the enumeration on TCBS agar) in the control group at 0 hr were slightly lower than that at the pre-trial level (Table 3.6), while total *Vibrio* concentration of the hepatopancreas and intestine of the challenged group was in the same order of magnitude as that of the pre-trial shrimp. The total *Vibrio* concentration in the hindgut dropped slightly for both control and challenged group at 0 hr from the pre-trial level.

There was no significant difference in average total bacteria or *Vibrio* concentrations in the digestive tract of the control and the challenge group. Average total bacterial concentration in the hepatopancreas of shrimp fed *Artemia* with *V. harveyi* at 0 and 24 hr post-challenged (5.7×10^5 and 5.4×10^3 CFU/ hepatopancreas, respectively) were slightly higher than that of the shrimp fed *Artemia* without *V. harveyi* (8.5×10^4 and 3.0×10^2 CFU/ hepatopancreas at 0 and 24 post-challenge, respectively) (Table 3.6). The difference was not observed at 12 hr post-challenge. The elevated total bacterial concentration in the challenged group was not observed in the hindgut at 0 hr post-challenge, and only a slight increase in total bacterial concentration (in the same order of magnitude) was observed at 12 and 24 hr post-challenge (Table 3.6). Total *Vibrio* concentrations in the hepatopancreas and intestines of challenged shrimps at 0 hr were slightly higher than that of the control shrimp. However, similar trend did not continue at 12 and 24 hr post-challenge, and the total *Vibrio* concentrations were inconsistent with treatments. In addition, total *Vibrio* concentration in the hindgut was consistently high (greater than 10^3 CFU/ tissue) throughout the entire experiment, while the concentration in the hepatopancreas and the intestine decreased.

Table 3.6 Average total bacterial and total *Vibrio* concentrations (CFU/ tissue on Marine 2216 Agar and TCBS agar, respectively; $N = 2$ shrimp) of three tissues (hepatopancreas, intestine, and hindgut) from the gastrointestinal tract of *P. monodon* at pre-trial level and at 0 h, 12 hrs and 24 hrs after being fed with *Artemia* containing *V. harveyi*.

	Total Bacterial Concentration (CFU/ tissue; Marine 2216 agar)						Total *Vibrio* Concentration (CFU/ tissue; TCBS agar)					
Time	Hepatopancreas		Intestine		Hindgut		Hepatopancreas		Intestine		Hindgut	
(h)	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected	Control	Infected
Pre-trial	2.9×10^4 (1.4 ± 10^4)	N/A[a]	7.4×10^5 (7.2 ± 10^4)	N/A[a]	9.6×10^5 (9.1 ± 10^5)	N/A[a]	1.0×10^5 N/A[a]	N/A[a]	1.1×10^6 (1.1×10^6)	N/A[a]	1.5×10^7 (1.5×10^7)	N/A[a]
0	8.5×10^4 (3.5 ± 10^4)	5.7×10^5 (5.4×10^5)	4.3×10^6 (4.2×10^6)	2.0×10^7 (4.2×10^6)	8.2×10^5 (3.0×10^5)	2.6×10^5 (2.4×10^5)	$< 10^3$ est[b]	1.5×10^5 N/A[a]	2.6×10^5 N/A[a]	5.3×10^6 (5.2×10^6)	6.7×10^6 N/A[a]	5.2×10^5 (4.8×10^5)
12	2.0×10^4 N/A[a]	$< 10^3$ est[b]	2.0×10^4 N/A[a]	2.0×10^4 N/A[a]	2.0×10^4 N/A[a]	3.3×10^4 N/A[a]	$< 10^3$ est[b]	$< 10^3$ est[b]	2.0×10^4 N/A[a]	$< 10^3$ est[b]	2.7×10^4 (6.7×10^3)	5.0×10^4 (1.7×10^4)
24	3.0×10^2 (1.0×10^2)	5.4×10^3 (3.1×10^3)	4.7×10^2 (2.7×10^2)	1.7×10^4 (1.6×10^4)	7.9×10^3 (3.8×10^3)	9.9×10^3 (6.4×10^3)	< 10 est[b]	4.5×10^3 N/A[a]	< 10 est[b]	< 10 est[b]	2.0×10^4 (2.0×10^4)	3.6×10^3 (9.0×10^2)

All values is mean and standard errors in parentheses below

[a] N/A = Not applicable

[b] est = estimated value. Less than 10 CFU/ drop was detected

3.3.2 16S rDNA PCR- DGGE analysis and cluster analysis

In duplicate, the shrimp intestinal tract (hepatopancreas, intestine and hindgut) samples from *P. monodon* for each treatment were collected and pooled at pre-trial, 0, 3, 12, 24 and 48 hrs (in duplicate). Bacterial communities were determined using Denatured Gradient Gel Electrophoresis (DGGE) targeting the 16S- rDNA V3 region using 338f-GC - 517r primer pair for total bacteria concentration and using 567f-GC - 680r for total *Vibrio* concentration (120 bp). After DGGE analysis, cluster analysis of profiles of total bacterial community of three tissues (hepatopancreas, intestine and hindgut) from the gastrointestinal tract of *P. monodon* at pre-trial, 0, 3, 12, 24 and 48 hrs was performed. Results from cluster analysis for hepatopancreas, intestine, and hindgut are shown in Fig. 3.10, Fig. 3.11 and Fig. 3.12, respectively.

Based on the results of cluster analysis of total bacteria, the DGGE profiles of bacteria in all 3 tissues from the challenged shrimp at 48 hrs post-challenge consistently formed a separate cluster from the majority of the profiles. The DGGE band representing *V. harveyi* (as referred from a reference band from *V. harveyi* isolate, not shown) was present in hepatopancreas (1 replicate), intestine (1 replicate), and hindgut (both replicates) at 48 hrs post-challenge. Variations in the DGGE profiles of bacterial community were quite high, and most of the DGGE profiles of bacterial community from control and challenged shrimp mixed together in one large cluster.

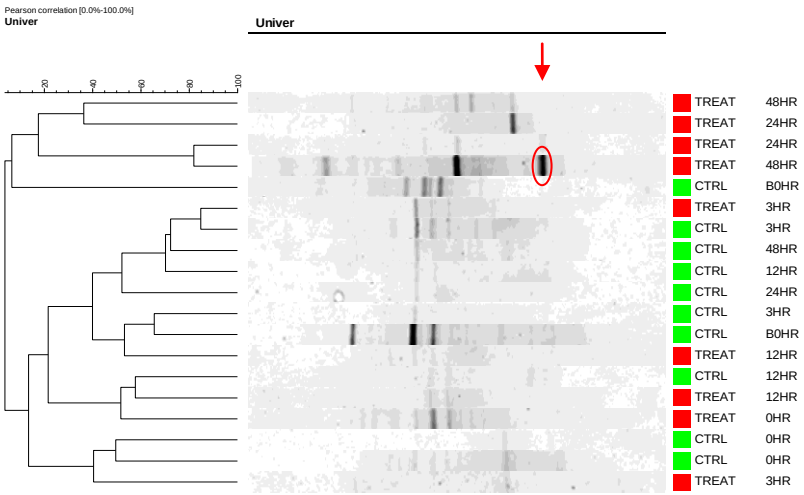

Figure 3.10 Cluster analysis results of DGGE profile of bacterial community from the hepatopancreas of control ( ) and *V. harveyi* challenged shrimp ( ) at pre-trial, 0, 3, 12, 24 and 48 hrs. Both duplicate are included. Cluster analysis was performed using UPGMA method based on Pearson correlation of densitometric curves. Red circle and an arrow indicated the DGGE band representing *V. harveyi*.

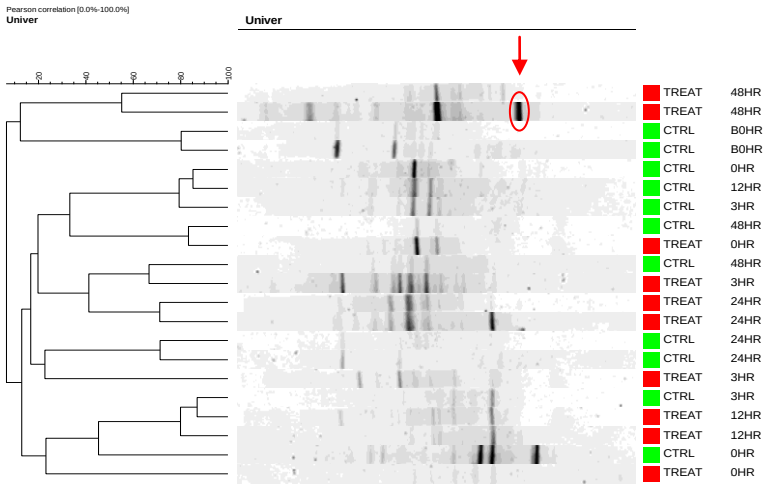

Figure 3.11 Cluster analysis results of DGGE profile of bacterial community from the intestines of control ( ) and *V. harveyi* challenged shrimp ( ) at pre-trial, 0, 3, 12, 24 and 48 hrs. Both duplicate are included. Cluster analysis was performed using UPGMA method based on Pearson correlation of densitometric curves. Red circle and an arrow indicated the DGGE band representing *V. harveyi*.

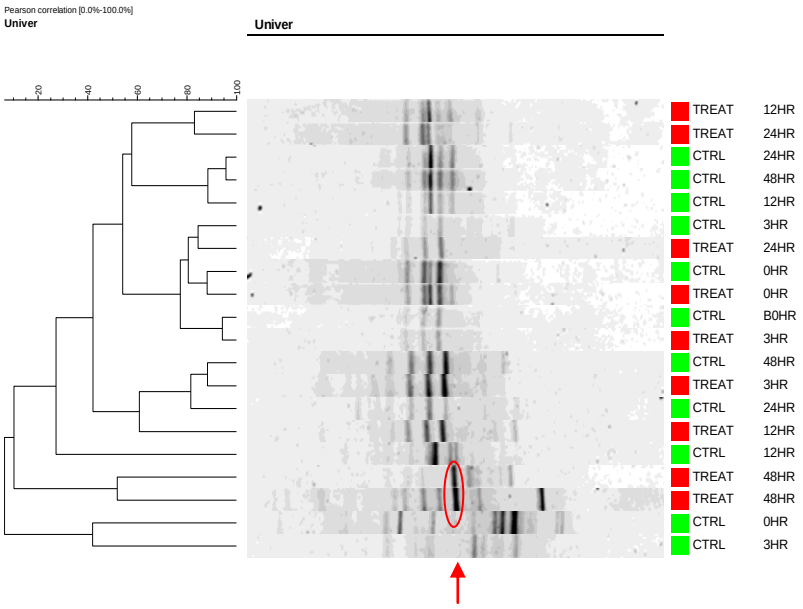

Figure 3.12 Cluster analysis results of DGGE profile of bacterial community from the hindgut of control ( ) and *V. harveyi* challenged shrimp ( ) at pre-trial, 0, 3, 12, 24 and 48 hrs. Both duplicate are included. Cluster analysis was performed using UPGMA method based on Pearson correlation of densitometric curves. Red circle and an arrow indicated the DGGE band representing *V. harveyi*.

Cluster analysis of profiles of *Vibrio* only community of three tissues (hepatopancreas, intestine and hindgut) from the gastrointestinal tract of *P. monodon* at pre-trial, 0, 3, 12, 24 and 48 hrs was performed, and the results are shown in Fig. 3.13, Fig. 3.14 and Fig. 3.15, respectively. The clustering pattern was inconsistent between control and *V. harveyi*-challenged group with no clear pattern emerging. However, the presence of *V. harveyi* in the hepatopancreas and intestine could be detected in both the control and challenged shrimp at various sampling times (Fig. 3.13 and 3.14). *Vibrio* community profile of the hindgut for both control and challenged shrimp were similar at most sampling times, except for the challenged shrimp at 48 hrs post-challenge due to the presence of *V. harveyi* in the hindgut.

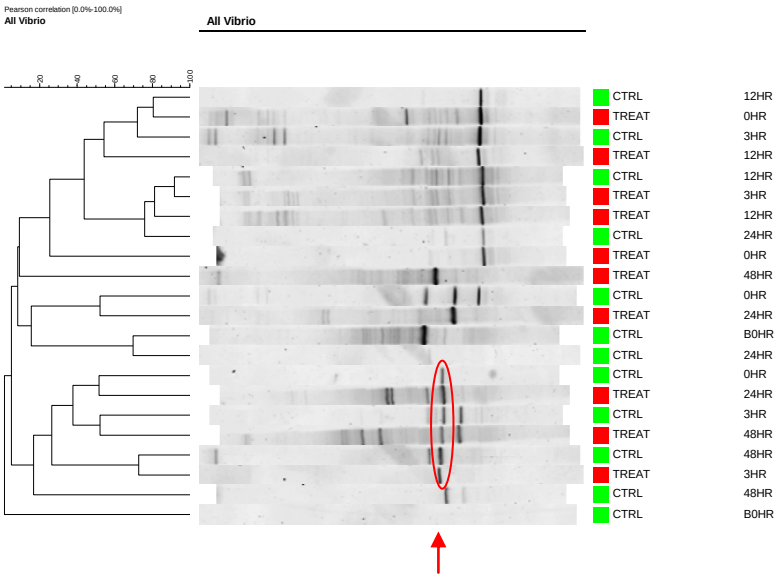

Figure 3.13 Cluster analysis results of DGGE profile of *Vibrio* community from the hepatopancreas of control ( ) and *V. harveyi* challenged shrimp ( ) at pre-trial, 0, 3, 12, 24 and 48 hrs. Both duplicate are included. Cluster analysis was performed using UPGMA method based on Pearson correlation of densitometric curves. Red circle and an arrow indicated the DGGE band representing *V. harveyi*.

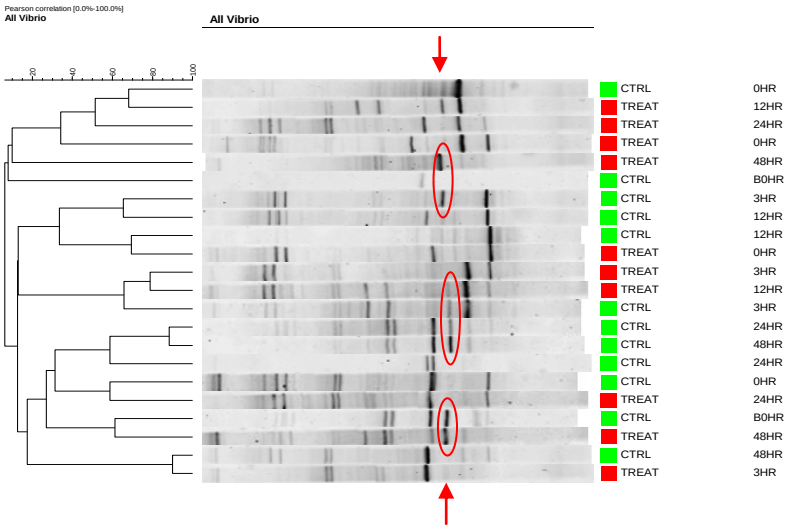

Figure 3.14 Cluster analysis results of DGGE profile of *Vibrio* community from the intestine of control ( ) and *V. harveyi* challenged shrimp ( ) at pre-trial, 0, 3, 12, 24 and 48 hrs. Both duplicate are included. Cluster analysis was performed using UPGMA method based on Pearson correlation of densitometric curves. Red circle and an arrow indicated the DGGE band representing *V. harveyi*.

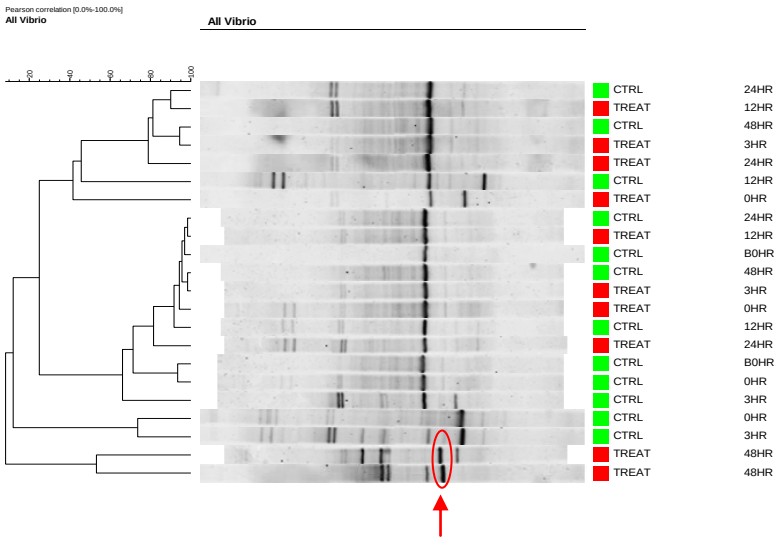

Figure 3.15 Cluster analysis results of DGGE profile of *Vibrio* community from the hindgut of control ( ) and *V. harveyi* challenged shrimp ( ) at pre-trial, 0, 3, 12, 24 and 48 hrs. Both duplicate are included. Cluster analysis was performed using UPGMA method based on Pearson correlation of densitometric curves. Red circle and an arrow indicated the DGGE band representing *V. harveyi*.

3.4 Screening for inhibitory effects against two shrimp pathogens (*V. harveyi* and *V. parahaemolyticus*), and selected phenotypic tests.

3.4.1 Isolation of strains from intestinal tract and rearing pond.

Sixty eight isolates were isolated from gastrointestinal tract of wild *P. monodon* samples collected from the Gulf of Thailand (section 2.2). Other isolates were collected from previous experiments (Chaiyapechara *et al.*, 2011). Forty-nine isolates were obtained from water samples collected from commercial farms (four farms; farm A, B_1, B_2, D and E) in Chachoengsao province, Thailand. A total of 148 isolates were obtained from gastrointestinal tract of juvenile *P. monodon* samples collected from commercial farms (eight farms; farm A_1, B, D, E, F, G, H and I) in Chachoengsao province and Chanthaburi province (one farm; farm A_2), Thailand. The list and details of isolates was summarized in Table 3.7.

3.4.2 Antagonistic bacteria isolated from intestinal tract and rearing pond by co-culture method.

Bacterial strains from all sources including rearing water, juvenile *P. monodon* and wild *P. monodon* isolated were tested for their inhibitory effect against antagonistic bacteria against shrimp pathogen including *V. harveyi* and *V. parahaemolyticus* by co-culture method. Among 265 isolated strains, 17 (6.42%) and 78 (29.44%) showed inhibitory effect *Vibrio harveyi* and *Vibrio parahaemolyticus*, respectively whereas 17 (6.42%) could inhibit both (Table 3.9).

Among the isolates from water, 4 and 29 (out of 49 isolates) could inhibit *V. harveyi* and *V. parahaemolyticus*, respectively. Four isolates from water could inhibit both pathogens. The isolate WB45.1 showed highest activity against all tested bacteria from water (Table 3.8 and see Fig. 3.16).

Four and 39 isolates from juvenile *P. monodon* could inhibit *V. harveyi* and *V. parahaemolyticus*, respectively. Four isolates from juvenile shrimp could inhibit both pathogens. Isolate InE86.2 showed highest activity against all tested bacteria from (Table 3.8).

In wild *P. monodon* isolates, of 68 bacteria isolated, 9 and 10 could inhibit *V. harveyi* and *V. parahaemolyticus*, respectively. Nine isolates could inhibit both pathogens. The wild *P. monodon* isolated In-WB_4-17 showed highest activity against all tested bacteria (Table 3.8)

Table 3.7 Total isolates of bacteria obtained from water shrimp pond, gastrointestinal tract of juvenile *P. monodon and* wild *P. monodon* in different locations in Thailand.

No.	Location	No. of isolates	Designation	Screening
1	Water			
	Chachoengsao province			
	Farm A	24	WA_2M_4 2-4 – WA_2M_4 2-30	Antagonism
	Farm B_1	3	WB_1.F3, WB_4.F1 and WB_4.F2	against
	Farm B_2	6	WB_2.H2, WB45.1 – WB45.5	shrimp
	Farm D	8	WD50.1 – WD50.8	pathogen
	Farm E	8	WE86.1 - WE86.8	
2	Intestinal tract (juvenile)			
	Chachoengsao province			
	Farm A_1	30	A_2M_4-1 – A_2M_4-30	
	Farm B	7	InB45.1 – InB45.4, InB45.9.1 – InB45.9.3	
	Farm D	2	InD50.1 and InD50.3	
	Farm E	5	InE86.1 – InE86.5	
	Farm F	25	InF1 – InF25	
	Farm G	25	InG1 – InG25	
	Farm H	25	InH1 – InH25	
	Farm I	25	InI1 – InI25	
	Chanthaburi province			
	Farm A_2	4	InA45.1 – InA45.4	
3	Intestinal tract (wild)			
	Gulf of Thailand			
	In-WB_4	32	In-WB_4-1 - In-WB_4-32	
	In-WB_4-MPSM	8	In-WB_4-MPSM.1 - In-WB_4-MPSM.8	Phytase
	In-WB_5-MPSM	28	In-WB_5-MPSM.1 - In-WB_5-MPSM.28	Phytase
	Total	**265**		

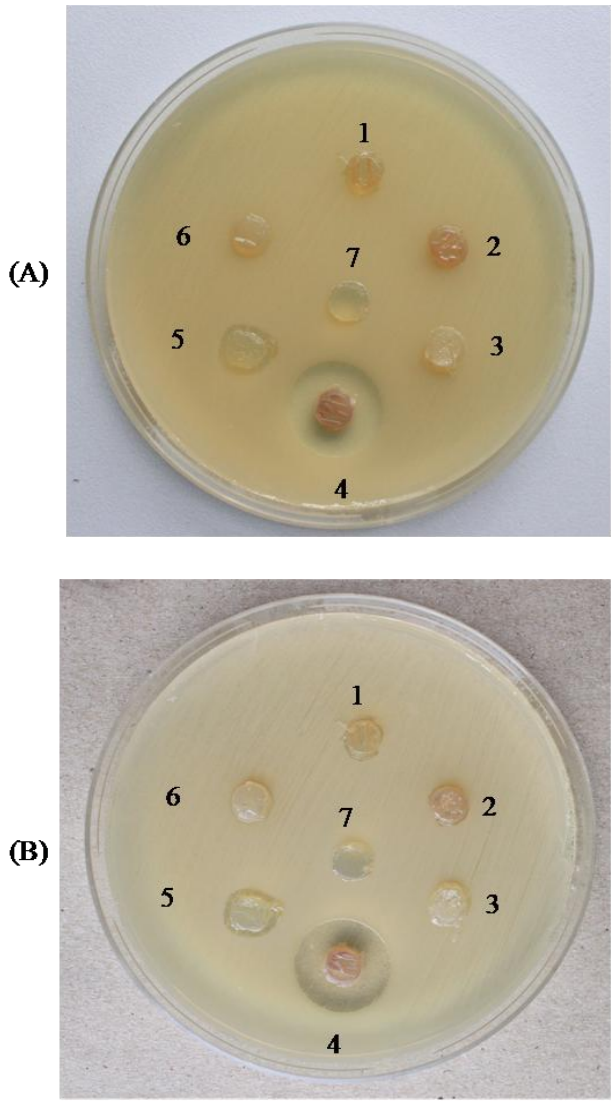

Fig. 3.16 The example of antagonistic against *V. harveyi* (A) and *V. parahaemolyticus* (B) of isolated from water by co-culture. No. 1-7 represented culture of $WA_2M_4$2-4, WB_4 F1, WB_4 F2, WB45.1, WB_2H2, WD50.1 and control, respectively.

Table 3.8 Screening of potential antagonistic bacteria against shrimp pathogens by co-culture method.

No.	Isolate designation	Diameter of inhibition zone (in mm)	
		Vibrio harveyi	*Vibrio parahaemolyticus*
1	**Water isolates**		
	Farm A		
	WA_2M_4 2-4	-	16.0
	WA_2M_4 2-5	-	-
	WA_2M_4 2-6	-	14.0
	WA_2M_4 2-7	-	15.0
	WA_2M_4 2-8	-	16.0
	WA_2M_4 2-10	-	15.0
	WA_2M_4 2-11	-	17.0
	WA_2M_4 2-12	-	15.0
	WA_2M_4 2-13	-	-
	WA_2M_4 2-14	-	15.0
	WA_2M_4 2-15	-	-
	WA_2M_4 2-16	-	-
	WA_2M_4 2-17	-	15.0
	WA_2M_4 2-18	-	-
	WA_2M_4 2-20	-	14.0
	WA_2M_4 2-21	-	-
	WA_2M_4 2-22	-	13.0
	WA_2M_4 2-23	-	-
	WA_2M_4 2-24	-	-
	WA_2M_4 2-25	-	14.0
	WA_2M_4 2-26	-	14.0
	WA_2M_4 2-27	-	-
	WA_2M_4 2-29	-	15.0
	WA_2M_4 2-30	-	15.0
	Farm B_1		
	WB_1.F3	8.0 uc	8.0
	WB_4.F1	-	-
	WB_4.F2	-	-
	Farm B_2		
	WB_2.H2	9.0	8.0
	WB45.1	20.0	16.0
	WB45.2	-	-
	WB45.3	-	-
	WB45.4	-	-
	WB45.5	-	8.0 uc
	Farm D		
	WD50.1	-	-
	WD50.2	-	-

Table 3.8 (continued)

No.	Isolate designation	Diameter of inhibition zone (in mm)	
		Vibrio harveyi	*Vibrio parahaemolyticus*
	WD50.3	-	8.0 uc
	WD50.4	-	8.0 uc
	WD50.5	-	8.0 uc
	WD50.6	14.0	11.0 uc
	WD50.7	-	-
	WD50.8	-	7.0 uc
	Farm E		
	WE86.1	-	-
	WE86.2	-	8.0 uc
	WE86.3	-	-
	WE86.4	-	8.0
	WE86.5	-	-
	WE86.6	-	8.0 uc
	WE86.7	-	8.0 uc
	WE86.8	-	8.0 uc
2	**Intestinal (juvenile)**		
	Farm A_1		
	A_2M_4-1	-	8.0
	A_2M_4-2	-	-
	A_2M_4-3	-	8.0
	A_2M_4-4	-	10.0
	A_2M_4-5	-	8.0
	A_2M_4-6	-	8.0
	A_2M_4-7	-	8.0
	A_2M_4-8	-	8.0
	A_2M_4-9	-	8.0
	A_2M_4-10	-	8.0
	A_2M_4-11	-	8.0
	A_2M_4-12	-	8.0
	A_2M_4-13	-	8.0
	A_2M_4-14	-	8.0
	A_2M_4-15	-	8.0
	A_2M_4-16	-	10.0
	A_2M_4-17	-	8.0
	A_2M_4-18	-	8.0
	A_2M_4-19	-	8.0
	A_2M_4-20	-	7.0
	A_2M_4-21	-	8.0
	A_2M_4-22	-	7.0
	A_2M_4-23	-	7.0
	A_2M_4-24	-	8.0
	A_2M_4-25	-	8.0

Table 3.8 (continued)

No.	Isolate designation	Diameter of inhibition zone (in mm)	
		Vibrio harveyi	*Vibrio parahaemolyticus*
	A_2M_4-26	-	10.0
	A_2M_4-27	-	10.0
	A_2M_4-28	-	8.0
	A_2M_4-29	-	8.0
	A_2M_4-30	-	8.0
	Farm B		
	InB45.1	-	8.0 uc
	InB45.2	9.0	8.0 uc
	InB45.3	-	8.0 uc
	InB45.4	9.0	9.0 uc
	InB45.9.1	-	-
	InB45.9.2	-	-
	InB45.9.3	-	-
	Farm D		
	InD50.1	-	-
	InD50.3	-	-
	Farm E		
	InE86.1	-	-
	InE86.2	10.0 uc	8.0
	InE86.3	8.0 uc	8.0
	InE86.4	-	8.0 uc
	InE86.5	-	-
	Farm F		
	InF1	-	-
	InF2	-	-
	InF3	-	-
	InF4	-	-
	InF5	-	-
	InF6	-	-
	InF7	-	-
	InF8	-	-
	InF9	-	-
	InF10	-	-
	InF11	-	-
	InF12	-	-
	InF13	-	-
	InF14	-	-
	InF15	-	-
	InF16	-	-
	InF17	-	-
	InF18	-	-
	InF19	-	-
	InF20	-	-

Table 3.8 (continued)

No.	Isolate designation	Diameter of inhibition zone (in mm)	
		Vibrio harveyi	*Vibrio parahaemolyticus*
	InF21	-	-
	InF22	-	-
	InF23	-	-
	InF24	-	-
	InF25	-	-
	Farm G		
	InG1	-	-
	InG2	-	-
	InG3	-	-
	InG4	-	-
	InG5	-	-
	InG6	-	-
	InG7	-	-
	InG8	-	-
	InG9	-	-
	InG10	-	-
	InG11	-	-
	InG12	-	-
	InG13	-	-
	InG14	-	-
	InG15	-	-
	InG16	-	-
	InG17	-	-
	InG18	-	-
	InG19	-	-
	InG20	-	-
	InG21	-	-
	InG22	-	-
	InG23	-	-
	InG24	-	-
	InG25	-	-
	Farm H		
	InH1	-	-
	InH2	-	-
	InH3	-	-
	InH4	-	-
	InH5	-	-
	InH6	-	-
	InH7	-	-
	InH8	-	-
	InH9	-	-
	InH10	-	-
	InH11	-	-
	InH12	-	-

Table 3.8 (continued)

No.	Isolate designation	Diameter of inhibition zone (in mm)	
		Vibrio harveyi	*Vibrio parahaemolyticus*
	InH13	-	-
	InH14	-	-
	InH15	-	-
	InH16	-	-
	InH17	-	-
	InH18	-	-
	InH19	-	-
	InH20	-	-
	InH21	-	-
	InH22	-	-
	InH23	-	-
	InH24	-	-
	InH25	-	-
Farm I			
	InI1	-	-
	InI2	-	-
	InI3	-	-
	InI5	-	-
	InI6	-	-
	InI7	-	-
	InI8	-	-
	InI9	-	-
	InI10	-	-
	InI11	-	-
	InI12	-	-
	InI13	-	-
	InI14	-	-
	InI15	-	-
	InI16	-	-
	InI17	-	-
	InI18	-	-
	InI19	-	-
	InI20	-	-
	InI21	-	-
	InI22	-	-
	InI23	-	-
	InI24	-	-
	InI25	-	-
Farm A2			
	InA45.1	-	8.0 uc
	InA45.2	-	8.0 uc
	InA45.3	-	8.0 uc
	InA45.4	-	-

Table 3.8 (continued)

No.	Isolate designation	Diameter of inhibition zone (in mm)	
		Vibrio harveyi	*Vibrio parahaemolyticus*
3	**Intestine (wild)**		
	In-WB_4-1	-	-
	In-WB_4-2	9.0	8.0
	In-WB_4-3	-	-
	In-WB_4-4	-	-
	In-WB_4-5	-	-
	In-WB_4-6	9.0	8.0
	In-WB_4-7	9.0	8.0
	In-WB_4-8	-	-
	In-WB_4-9	-	-
	In-WB_4-10	-	-
	In-WB_4-11	-	-
	In-WB_4-12	-	-
	In-WB_4-13	-	-
	In-WB_4-14	11.0	8.0
	In-WB_4-15	-	8.0
	In-WB_4-16	-	-
	In-WB_4-17	9.0	10.0
	In-WB_4-18	-	-
	In-WB_4-19	7.0	10.0
	In-WB_4-20	-	-
	In-WB_4-21	-	-
	In-WB_4-22	-	-
	In-WB_4-23	-	-
	In-WB_4-24	7.0	9.0
	In-WB_4-25	-	-
	In-WB_4-26	-	-
	In-WB_4-27	9.0	8.0
	In-WB_4-28	-	-
	In-WB_4-29	-	-
	In-WB_4-30	-	-
	In-WB_4-31	7.0	9.0
	In-WB_4-32	-	-

uc : unclear inhibition zone, - : negative, Diameter including the diameter of produce strains agar block (7.0 mm)

Table 3.9 Summary of potential antagonistic bacteria from intestinal tract of shrimp and rearing water by co-culture method.

Sample	No. of isolates	No. of isolates with potential antagonistic bacteria		
		V. harveyi	*V. parahaemolyticus*	Inhibit Both
Water	49	4 (1.51%)	29 (10.94%)	4 (1.51%)
Intestinal tract (juvenile)	148	4 (1.51%)	39 (14.72%)	4 (1.51%)
Intestinal tract (wild)	68	9 (3.40%)	10 (3.77%)	9 (3.40%)
Total	265	17 (6.42%)	78 (29.43%)	17 (6.42%)

3.4.3 Plate screening for phytase-producing strains

Thirty six from wild *P. monodon* were collected from MSPM plates initial screening for phytase-producing strains. All isolates were incubated on MPSM plates, and clear zone by phytase activity of the colony was visually indicated by the modified method of Yanke *et al*. (1998). The results of plate screening for phytase-producing strains were summarized in Table 3.10. Eleven out of thirty six strains showed the ability to produces extracellular phytase production with ranged 0.5-1.5 cm and showed the size of clear zone was as follow: 1 isolate was for +++, 6 for +++, 4 for + and 25 isolates did not make clear zone. In-WB_5-MPSM.27 showed the strong activity (1.5 cm) of phytase production as shown in Fig.3.17.

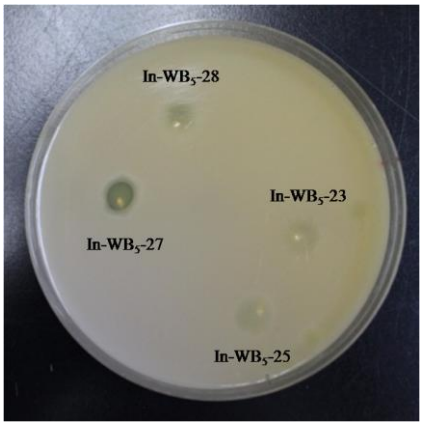

Figure 3.17 The example of clear zone by phytase-producing bacteria on MPSM plate. Left is a strong clear zone by phytase-producing bacteria In-WB_5-MPSM.27.

Table 3.10 Results of plate screening for phytase-producing strains from the intestinal tract of wild *P. monodon*

Designation	Phytase production (cm)
In-WB_4-MPSM.1	-
In-WB_4-MPSM.2	-
In-WB_4-MPSM.3	-
In-WB_4-MPSM.4	-
In-WB_4-MPSM.5	+
In-WB_4-MPSM.6	-
In-WB_4-MPSM.7	++
In-WB_4-MPSM.8	-
In-WB_5-MPSM.1	-
In-WB_5-MPSM.2	-
In-WB_5-MPSM.3	-
In-WB_5-MPSM.4	-
In-WB_5-MPSM.5	-
In-WB_5-MPSM.6	-
In-WB_5-MPSM.7	-
In-WB_5-MPSM.8	-
In-WB_5-MPSM.9	-
In-WB_5-MPSM.10	-
In-WB_5-MPSM.11	++
In-WB_5-MPSM.12	-
In-WB_5-MPSM.13	-
In-WB_5-MPSM.14	+
In-WB_5-MPSM.15	+
In-WB_5-MPSM.16	-
In-WB_5-MPSM.17	-
In-WB_5-MPSM.18	-
In-WB_5-MPSM.19	-
In-WB_5-MPSM.20	-
In-WB_5-MPSM.21	++
In-WB_5-MPSM.22	++
In-WB_5-MPSM.23	++
In-WB_5-MPSM.24	-
In-WB_5-MPSM.25	++
In-WB_5-MPSM.26	-
In-WB_5-MPSM.27	+++
In-WB_5-MPSM.28	+

The isolates were classified by the size of clear zone; above 1.5 cm of diameter as +++, above 1.0 cm as ++, above 0.5 cm as + and -, no production of phytase - producing bacteria

3.4.4 API 20 NE test

The API 20 NE strip tests consisted of 20 tubes which contained dehydrated media including substrates for eight conventional and twelve assimilation tests. This strip was designed to identify non-enteric bacteria. Table 3.11, 3.12 and Figure 3.18 show the API 20 NE test results for the twelve isolates. All isolates were positive for aesculin hydrolysis and cytochrome oxidase. Also, all isolates were negative for ureas hydrolysis and assimilation of phenyl acetate. Only one isolate (WB_1.F3) was negative for gelatinase, while it is the only isolate that tested positive for arginine dihydrolase. Seven out of twelve isolates (A_2M_4-4, A_2M_4-16, InB45.2, InE86.2, In-WB_4-24, In-WB_4-27 and $WA_2M_4$2-12) were positive for β−galactosidase. Eight out of twelve isolates (A_2M_4-4, A_2M_4-16, InE86.2, In-WB_4-24, In-WB_4-27, $WA_2M_4$2-7, $WA_2M_4$2-12, and WB_2.H2) were positive for glucose fermentation. Nitrate reduction and indole production were observed for A_2M_4-4, A_2M_4-16, InE86.2, In-WB_4-24, In-WB_4-27 and WB_2.H2 isolates. Five isolates (In-WB_4-27, $WA_2M_4$2-7, $WA_2M_4$2-12, WD50.6 and WB_1.F3) were negative for twelve assimilations tests, while isolate of $WA_2M_4$2-7 and $WA_2M_4$2-12 were positive for L-malate. The remaining 7 tests showed variable results for the various isolates.

Table 3.11 Source and presumptive identification of twelve isolates in phenotypic characteristics using API 20 NE

Designation	Source	Presumptive Identification (partial 16S rDNA sequence)	S_ab score	GenBank accession number	*In vitro* Antagonistm[a]
A_2M_4-4	Intestine (farmed juvenile)	*Vibrio rotiferanus* (T)	0.965	AJ316187	Vp
A_2M_4-16	Intestine (farmed juvenile)	*Vibio sagamiensis* (T)	0.855	AB428909	Vp
InB45.2	Intestine (farmed juvenile)	*Shewanella amazonensis* (T)	0.823	AF005248	Vh, Vp
InE86.2	Intestine (farmed juvenile)	*Vibrio tubiashii* (T)	0.878	X74725	Vh, Vp
In-WB_4-24	Intestine (wild adult)	*Vibrio brasiliensis* (T)	0.923	AJ316172	Vh, Vp
In-WB_4-27	Intestine (wild adult)	*Vibio sagamiensis* (T)	0.836	AB428909	Vh, Vp
$WA_2M_4$2-7	Water (farm)	*Vibrio hepatarius* (T)	0.934	AJ345063	Vp
$WA_2M_4$2-12	Water (farm)	*Vibrio furnissii* (T)	0.936	X76336	Vp
WB45.1	Water (farm)	*Pseudomonas otitidis* (T)	0.739	AY953147	Vh, Vp
WD50.6	Water (farm)	*Pseudoalteromonas piscicida* (T)	0.985	AB090232	Vh, Vp
WB_1.F3	Water (farm)	*Shewanella haliotis* (T)	0.917	EF178282	Vh, Vp
WB_2.H2	Water (farm)	*Vibrio neptunius* (T)	0.933	AJ316171	Vh, Vp

[a] *In vitro* antagonism against shrimp pathogens as screened by co-culture method; Vp = *Vibrio parahaemolyticus*, Vh = *Vibrio harveryi*

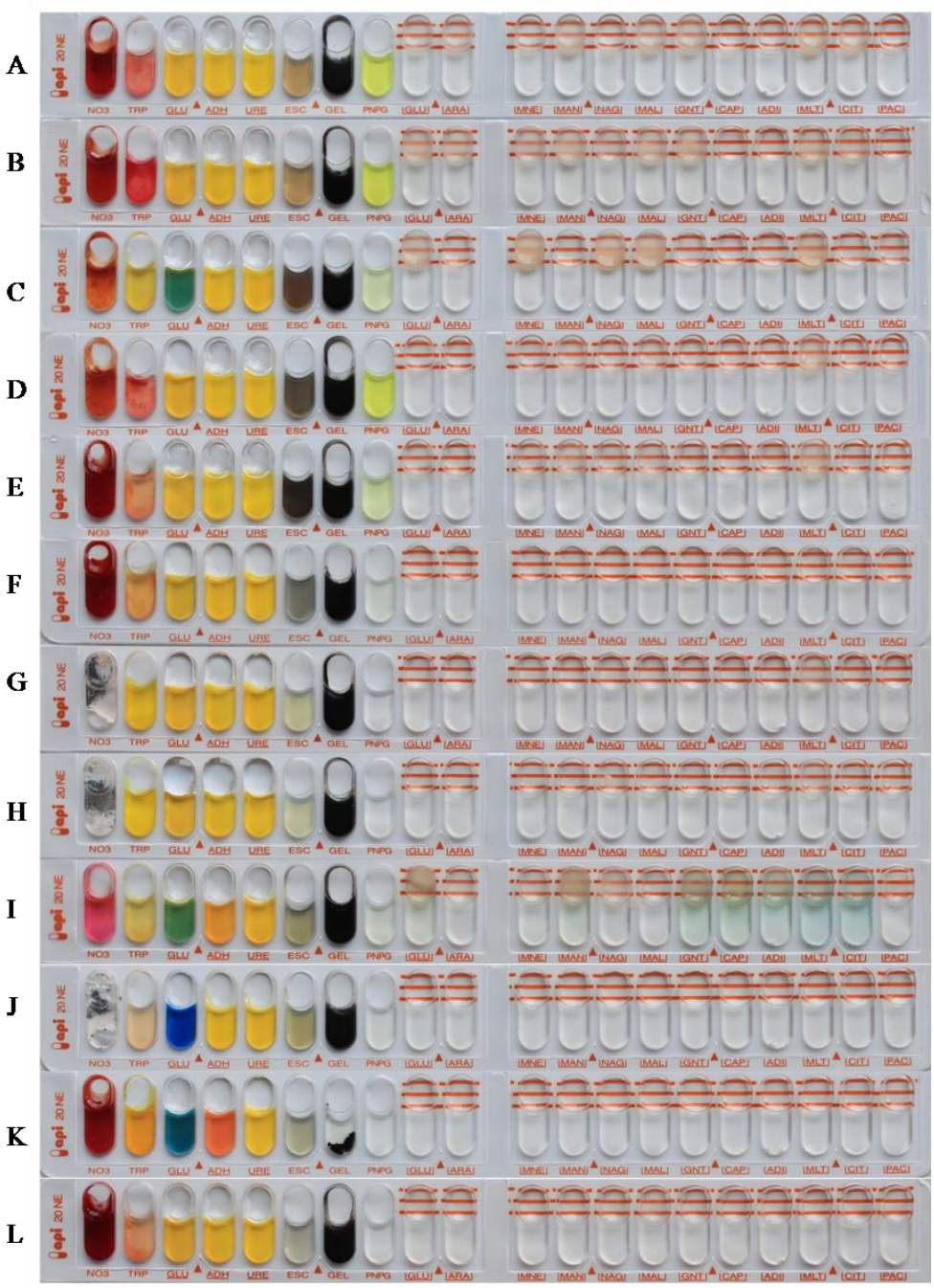

Fig. 3.18 shows results of characteristic for 12 isolates on API 20 NE tests; A = A_2M_4-4 isolate; B = A_2M_4-16 isolate; C = InB45.2 isolate; D = InE86.2 isolate; E = In-WB_4-24 isolate; F = In-WB_4-27 isolate; G = $WA_2M_4$2-7 isolate; H = $WA_2M_4$2-12; I = WB45.1 isolate; J = WD50.6 isolate; K = WB_1.F3 isolate and L = WB_2.H2 isolate.

Table 3.12 Characteristic of 12 isolates on API 20 NE test for 24 hrs incubation[a]

Test	A A_2M_4-4	B A_2M_4-16	C InB45.2	D InE86.2	E In-WB_4-24	F In-WB_4-27	G $WA_2M_4$2-7	H $WA_2M_4$2-12	I WB45.1	J WD50.6	K WB_1.F3	L WB_2.H2
Nitrate reduction	+	+	+	+	+	+	-	-	-	-	+	+
Indole production	+	+	-	+	+/-	+	-	-	-	-	-	+/-
Glucose fermentation	+	+	-	+	+	+	+	+	-	-	-	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	+	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-
Aesculin hydrolysis	+	+/- (+)	+	+	+	+/- (+)	+/-	+/-	+	+/- (+)	+/- (+)	+
Gelatinase	+	+	+	+	+	+	+/- (+)	- (+)	+	+	-	+
β–galactosidase	+	+	+/-	+	+/-	- (+/-)	-	-	- (+/-)	-	-	-
Assimilation of:												
D-Glucose	+	+	+/- (+)	-	+	-	-	-	+	-	-	- (+/-)
L-Arabinose	+/- (+)	+/- (+)	-	-	-	-	-	-	- (+/-)	-	-	-
D-Mannose	+	+	+	-	+/-	-	-	-	- (+/-)	-	-	-
D-Mannitol	+	+	-	- (+)	- (+)	-	-	-	+	-	-	-
N-Acetyl-D-glucosamine	+/- (+)	+/- (+)	+	-	+/-	-	-	-	+/-	-	-	- (+/-)
Maltose	+	+	+	- (+)	- (+)	-	-	-	+	-	-	- (+/-)
Gluconate	+	+	-	-	-	-	-	-	+	-	-	- (+/-)
Caprate	-	-	-	-	-	-	-	-	+	-	-	-
Adipate	-	+/- (+)	-	-	-	-	-	-	+/- (+)	-	-	-
L-Malate	+	+	+	+/- (+)	- (+)	-	+/-	- (+)	+	-	-	-
Citrate	+/- (+)	+/-	-	-	-	-	-	-	+	-	-	-
Phenyl-acetate	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+

[a] In brackets are shown 48 hrs designation if different from 24 hrs results, positive (+), negative (-) or weak positive (+/-)

CHAPTER IV

DISCUSSION

Bacterial communities in the intestines of juvenile farm-raised black tiger shrimp (*P. monodon*) were examined using two culture-independent methods, PCR-DGGE and 16S rDNA clone libraries. The results showed that bacteria belonged to β-, δ-, ε-, γ- and α-Proteobacteria, Bacteroidetes, Fusobacteria, Cyanobacteria, Firmicutes and Chlorobi were found in the intestinal tract of juvenile shrimp. *Vibrio* spp. were the most dominant group and were found in most shrimps.

Bacterial communities in shrimps of different sizes (from the same pond) were not significantly different at either 2 or 5 month-old, but the bacterial communities varied significantly between different sampling months. The difference in size among different groups at each sampling time might not be large enough to affect the bacterial communities. At Month 2, shrimp sizes ranged from 3.3 g to 12.7 g for small and large shrimp, respectively (Table. 3.1), and at Month 5 shrimp size ranged from 6.3 g to 20.5 g for small and large shrimp, respectively (Table. 3.1). Other factors such as rearing environment, shrimp health, diet and disease status could override the effect that came from sizes. Chaiyapechara *et al*. (2011) reported that the γ-Proteobacteria were the commonly bacterial group found in the intestine tracts of shrimp from all farms and that members of the genus in the intestine population of each shrimp varied among different farms. Similar to the results observed in Chaiyapechara *et al*. (2011), *Vibrio* and *Photobacterium* were found in the present study. However, other genera that were found in Chaiyapechara *et al*. (2011) such as *Aeromonas, Propionigenium, Actinomyces, Anaerobaculum, Haospirulina, Pseudomonas, Mycoplasma and Shewanella* were not observed in the present study. The effect of rearing environment was shown in a study by Moss *et al*. (2000). They reported that the guts from well shrimp (*L. vannamei*) were dominated by *Vibrio* and *Aeromonas*, while the guts from pond shrimp exposed a greater bacterial diversity and were dominated by *Vibrio, Aeromonas and Pseudomonas*. Esiobu *et al*. (2003) also reported that all healthy and live *Penaeus duorarum* shrimp guts were heavily colonized by *Vibrio* species, especially *V. harveyi* and *V. logei*. The relationship

between the intestinal microbiota and nutrient concentration and the effect of diets and role of microorganism within the shrimp pound community could be clarified.

In the present study, the bacterial community composition and diversity between different segments of the gastrointestinal tract (GI) from eight wild *P. monodon* shrimp were analyzed by PCR-DGGE. The 16S rDNA from selected samples were also amplified, cloned and sequenced. The dominant group of bacteria in the (GI) tract of wild *P. monodon* was γ–Proteobacteria, and the dominant genera found in (GI) tract are *Photobacterium damselae* and *Vibrio* sp. (Table 3.4). Other genus found in significant proportion in the gastrointestinal tract (GI) included *Ferrimonas* and *Marinobacter* (Table 3.4). It has been reported that the most commonly observed genera of gut microflora in aquatic invertebrates are *Vibrio, Pseudomonas, Flavobacterium, Micrococcus and Aeromonas* (Harris, 1993). Shakibazadeh *et al*. (2009) conducted a study to identify microflora of digestive system, body surface, and muscle of juvenile *P. monodon* together with rearing water and sediment using culture-based method and found similar species such as *Vibrios Shewanella, Burkholderia, Clavibacter, Staphylococcus, Corynebacterium* and *Brevibacterium*. Oxley *et al*. (2002) found that both wild and cultured prawns supported remarkably similar bacterial floral compositions, which included members of *Aeromonas, Plesiomonas, Photobacterium, Pseudoalteromonas, Pseudomonas* and *Vibrio,* with members of the genus *Vibrio* being quantitatively dominant. Some non-dominant phyla of bacteria observed in the present study such as Bacteroidetes and Firmicutes were not previously reported in other studies that used culture-dependent methods to examine shrimp intestinal bacteria. Compared to the results from the intestine of juvenile black tiger shrimp *L. vanamei* performed using PCR-DGGE technique (Johnson *et al*., 2008) where several species of *Vibrio*, *Desulfocapsa* , *Mycobacterium* and *Propionibacterium* were obtained, none of those species except *Vibrio* was observed in this present study. The distribution of bacterial species in the GI tract of shrimp was not uniform throughout the GI tract in this study. For examples, bacteria in the α-Proteobacteria and γ-Proteobacteria were distributed throughout all segments of the GI tract, but bacteria in the phyla ε- Proteobacteria, Bacteroidetes and Firmicutes were not. Bacteria in the phylum Bacteroidetes were not

represented in the stomach but only observed in the anterior midgut, hindgut and feces only. Four Bacteroidetes species found in the anterior midgut, hindgut and feces from the present study were *Mesoflavibacter zeaxanthinifaciens, Actibacter sediminis, Gaetbulibacter marinus* and *Leptobacterium flavescens*. The oxygen tension of the intestinal environment could contribute to the localization of bacteria in the Bacteroidetes group in certain segments of the intestines. Sakata *et al*. (1980) reported that the salmonid gut could be totally anaerobic due to the ingestion of oxygen with food, and that obligate anaerobic (Bacteroides) have been previously isolated in rainbow trout. Bacteria in the phylum Firmicutes were found in the anterior midgut, hindgut and feces only. The hindgut, stomach and feces samples demonstrated greater bacterial diversity more than the anterior midgut as measured by the number of species. The bacterial community in hindgut and stomach were dominated by sequence belonging to *Vibrio* sp., while *Vibrio* sp. was not found in the anterior midgut sample. The hindgut is a chitin-lined straight tube running from the cephalothorax dorsally through the abdomen to the rectum (Dall *et al*., 1990; Ceccaldi, 1997). The hindgut organisms were phylogenetically grouped with *Vibrio* sp., which have the unique ability to survive on chitin in vitro and in seawater, their association with chitin may be a key to their survival in marine and estuarine environments. Huq *et al*. (1986) report that the *V. cholera* was demonstrated to attach exclusively to crab hindguts, which are lined with chitin, as opposed to the crab midguts, which are endodermal and not lined with chitin. On the other hand, the bacterial community in the feces and anterior midgut was dominated by *Photobacterium damselae*. Gomez-Gil *et al*. (1998) reported that the stomach (foregut) of *P. vannamei* was found a similar bacterial density to that of the intestine (midgut and hindgut), while the digestive gland supported bacterial density less than that of the intestine (midgut and hindgut). However, Oxley *et al*. (2002) reported that the posterior regions (midgut and hindgut) supported the highest densities and the anterior regions (foregut and digestive gland) the lowest. Nevertheless, such observation may reflect the influence of gut structure and function on bacterial colonization in the crustacean digestive system. The intestinal bacterial community of fish had been shown to be highly dependent on the bacterial colonization during early development, environmental conditions and dietary changes (Ringø *et al*., 2003;

Ringø and Brikbeck, 1999; Olafsen, 2001). The presence of γ-Proteobacteria are found in a higher percentage of the total bacteria in GI tract than other groups. *Fusibacter* sp. in these phyla can be involved with sulfate-reducing activity in biological filters used in marine aquaculture (Ravot *et al*., 1999). Future studies of the functions of these bacteria in the GI tract of shrimp would be helpful in understanding their roles of these bacteria on nutrition, health and disease resistance in shrimp.

Pure culture isolates collected from the intestines of *P. monodon* and rearing water were also screened for inhibitory effects against two shrimp pathogens (*V. harveyi* and *V. parahaemolyticus*), and selected isolates were further characterized using phenotypic tests. The results of the antagonistic action of the intestines of *P. monodon and* rearing water against the shrimp pathogens are given in the Table 3.8. In total, among 265 isolated strains, 17 (6.42%) and 78 (29.44%) showed inhibitory effect against *V. harveyi* and *V. parahaemolyticus*, respectively. Seventeen isolates (6.42%) could inhibit both (Table 3.9). The presence of antagonistic isolates against specific pathogens had been observed previously. Earlier reports showed that only about 1-10% of intestinal bacteria from both freshwater and marine fish possessed inhibitory action against fish bacterial pathogen (Sugita *et al*., 1998; Onarheim and Raa 1990). Sugita *et al*. (2002) reported that more than 10% of the isolates from intestinal tract of Japanese flounder exhibited antagonistic action. The gut microflola of *P. monodon and* rearing water showed wide antibacterial activity were *Pseudomonas* sp., *Pseudoalteromonas* sp., *Shewanella* sp. and *Vibrio* sp. with a zone of inhibition of 7-20 mm against shrimp pathogen. Smith and Davey (1993) reported that *Pseudomonas fluorescences* reduced disease caused *A. salmonicida*. Similary, Vijayan *et al*. (2006) reported that a *Pseudomonas* spp. isolated from a brackish water lagoon showed significant probiotic activity against a number of shrimp pathogenic vibrios. Some *Shewanella* species such as *S. marisflavi* (Li *et al*., 2010) and *S. alga* (Beleneva and Kukhlevskii, 2009) and freshwater *S. putrefaciens* have been described as pathogens of marine organisms. There is information about the role that some strains of these species play as antimicrobial against certain fish pathogen and improve the tolerance of crowding stress induced by culture at high densities (Varela *et al*., 2010). Sugita *et al*. (1997) reported that a strain of *Vibrio* isolated from fish

intestine exhibited a wide antibacterial spectrum against *V. vulnificus, Pasteurella piscicida, Escherchia coli* and *Edwardsiella seriolicida.* Future studies should also be conducted to understand the mechanisms of inhibition for bacterial isolates with antagonistic activity against shrimp pathogens.

The API test kit consist of enzymatic and carbon compound assimilation tests element, the number of which varies depending on the type of API test kit used. Several studies have been performed for evaluation of the abilities of the API systems to identify the type collection strains or, alternatively, unknown environmental bacterial strains or isolates of medical interest (Inglis *et al*., 1998; Amy *et al*., 1992; Busse *et al*., 1992; Yohalem and Lorbeer, 1994). The results of the API 20 NE identifications are listed in Table 3.11. On API 20NE strip, all strains were positive for the following tests: oxidase and aesculin dihydrolysis, and negative for the following test: urease and phenyl- acetate (Table. 3.12). The six *V. rotiferanus, V. sagamiensis, V. tubiashii, V.brasiliensis, V. sagamiensis* and *V. neptunius* strains were positive for the following test: nitrate reduction, indole production and glucose fermentation. The three *V. sagamiensis, V.hepatarius* and *V. furnissii* strains were negative for D-Glucose, L-Arabinose, D-Mannose, D-Mannitol, N-Acetyl-D-glucosamine, Maltose, Gluconate, Caprate and Adipate. Two *vibrio (V. rotiferanus and V. sagamiensis)* strains were positive for: D-Glucose, L-Arabinose, D-Mannose, D-Mannitol, N-Acetyl-D-glucosamine, Maltose, Gluconate and L-Malate. For exoenzymes production, all *Vibrio* spp. (N = 8) strains, *Shewanella amazonennsis* and *Pseudomonas otitidid* several hydrolytic enzyme including: Gelatinase and β−galactosidase. *Pseudomonas* WB45.1 was the most versatile and can assimilate 11 out of 12 nutrients tested. Furthermore, it was the only isolate capable of assimilate caprate. *Shewanella* WB_1.F3 is the only isolate with arginine dihydrolase.

CHAPTER V

CONCLUSION

In order to better manage the health and disease of the black tiger shrimp *P. monodon* in aquaculture, the description of intestinal bacterial community of the species is needed. This present study examined the intestinal bacterial community of *P. monodon* from two different source and life stages (farmed-raised juvenile and wild adult) as well as the intestinal bacterial community in response to an infection event. Lastly, the ability of selected bacterial isolates from the intestine of shrimp and rearing environment to inhibit the growth of know shrimp pathogen *in vitro* were screened. The following conclusions were the main findings of the study:

1. Bacterial communities from farm-raised juvenile shrimp of different sizes showed no distinct clustering pattern at either 2 or 5 month suggesting that there was no difference in bacterial community among shrimps of different sizes. However, the differences in bacterial communities between 2 and 5 month old juveniles were more pronounced. Twenty-three genera in 6 phyla including Proteobacteria (β-, δ-, ε-, γ- and α-), Fusobacteria, Cyanobacteria, Firmicutes, Bacteroidetes and Chlorobi were represented in the 16S rDNA clones library results from farmed raised juveniles. The most abundant genera represented in farm raised juvenile shrimps are *Vibrio* spp.

2. Bacterial communities in different segments of the GI tract of wild-caught adults were different in both the diversity and composition. The number of bacterial OTUs (as DGGE bands) in the stomach, hindgut, and feces-associated were higher than that of the 3 intestinal segments. Twenty-three genera in 3 phyla Proteobacteria (ε-, γ- and α-), Firmicutes and Bacteroidetes. The distribution of these species was not uniformed. While γ- and α-Proteobacteria can be found all throughout the GI tract, the distribution was more limited for Firmicutes (anterior midgut, hindgut and feces-associated) and Bacteroidetes (all segments except stomach). Two dominant genera in the GI tract were *Photobacterium* sp. and *Vibrio* spp.

3. There was slight difference in average total bacteria or *Vibrio* concentrations in the digestive tract of the control and the challenge group. The change in the intestinal bacterial community upon challenge with *V. harveyi* can usually be observed after 48 hrs.

4. Seventeen pure culture isolates were shown to inhibit the growth of both *V. harveyi* and *V. parahaemolyticus* using co-culture methods. Seventy-eight isolates can inhibit either pathogen. Twelve isolates that were selected for further characterization were closely related to *Vibrio* spp. (8 isolates), *Shewanella* spp. (2 isolate), *Pseudomonas* sp. (1 isolate), and *Pseudoalteromonas* sp. (1 isolate).

5. The intestinal bacterial communities of *P. monodon* are a diverse group of bacteria that generally included 3 to 4 main phyla including Proteobacteria (β-, δ-, γ- and α-), Fusobacteria, Firmicutes and Bacteroidetes. Regardless of the source or life-history of the shrimp, γ- Proteobacteria is generally the most abundant in the community with *Vibrio* spp. or *Photobacterium* sp. as dominant genera. The distribution of the bacteria in the GI tract was not uniform. Several bacteria isolates from the intestinal tract of shrimp and the rearing were capable of *in vitro* inhibiting the growth of shrimp pathogens.

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APPENDICES

APPENDIX A
MEDIA PREPARATION

Table 1. Composition of Marine 2216 agar (MA2216; Himedia, India)

Component	Concentration (g/L)
Pepic digest of animal tissue	5.00
Yeast extract	1.00
Ferric citrate	0.10
Sodium chloride	19.45
Magnesium chloride	8.80
Sodium sulphate	3.24
Calcium chloride	1.80
Potassium chloride	0.55
Sodium bicarbonate	0.16
Potassium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Sodium fluorate	0.0024
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Agar	15.00
	pH 7.6±0.2

Suspend 55.25 grams of the powder in 1000 ml distilled water and mix thoroughly. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Table 2. Composition of Luria agar (LA; OXOID, England)

Component	Concentration (g/L)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0
Agar	15.0
	pH 7.0

Suspend 35.0 grams of the powder in 1000 ml distilled water and mix thoroughly. Warm slightly to completely dissolve the powder. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Table 3. Composition of Luria broth (LB; OXOID, England)

Component	Concentration (g/L)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	5.0
	pH 7.0

Suspend 20.0 grams of the powder in 1000 ml distilled water and mix thoroughly. Warm slightly to completely dissolve the powder. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Table 4. Composition of Tryptic soy agar (TSA+2% NaCl; Becton, France)

Component	Concentration (g/L)
Pancreatic digest of casein	17.0
Papaic digest of soybean	3.0
Dextrose	2.5
Sodium chloride (2%)	25.0
Dipotassium phosphate	2.5
Agar	15.0
	pH 7.3±0.2

Suspend 65.0 grams of the powder in 1000 ml distilled water and mix thoroughly. Warm slightly to completely dissolve the powder. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Table 5. Composition of Tryptic soy broth (TSB+2% NaCl; Becton, France)

Component	Concentration (g/L)
Pancreatic digest of casein	17.0
Papaic digest of soybean	3.0
Dextrose	2.5
Sodium chloride (2%)	25.0
Dipotassium phosphate	2.5
	pH 7.3±0.2

Suspend 50.0 grams of the powder in 1000 ml distilled water and mix thoroughly. Warm slightly to completely dissolve the powder. Sterilize by autoclaving at 15 lbs pressure (121 °C) for 15 min.

Table 6. Composition of Thiosulfate citrate bile sucrose agar (TCBS; Himedia, India)

Component	Concentration (g/L)
Proteose peptone	10.00
Yeast extract	5.00
Sodium thiosulphate	10.00
Sodium citrate	10.00
Oxgall	8.00
Sucrose	20.00
Sodium chloride	10.00
Ferric citrate	1.00
Bromo thymol blue	0.04
Thymol blue	0.04
Agar	15.00
	pH 8.6±0.2

Suspend 89.0 grams of the powder in 1000 ml distilled water and mix thoroughly. Heat to boiling to dissolve the medium completely. DO NOT AUTOCLAVE. Cool to 50°C and pour into sterile petri plates.

Table 7. Composition of Modified phytate screening medium (MPSM)

Component	Concentration (g/L)
Glucose	10
$(NH_4)_2 SO_4$	1
Urea	1
Citric acid	3
Sodium citrate	2
$MgSO_4 \cdot 7H_2O$	1
Na-phytate (filtrated)[a]	3
1M Tris buffer (pH 8.0)	100 ml
$FeSO_4 \cdot 7H_2O$	0.1
Biotin	50 µg
Thiamine-HCl	20 mg
Agar	20
	pH 7.0

a- For the preparation of MPSM, 0.3 g of sodium phytate was dissolved in 10 ml of deionized H_2O sterilized separately and then combined with 90 ml of sterilized sodium phytate-free MPSM. The pH of phytata-free MPSM was adjusted to 7.0 before sterization at 121 °C for 15 min. (15 pound/inch^2)

APPENDIX B

READING TABLE

Table 1. Reading the strip

Tests	Substrates	Reactions / Enzyme	Results	
			Negative (-)	**Positive (+)**
NO_3	Potassium nitrate	Reduction of nitrates to nitrites	NIT 1 + NIT 2 / 5 min	
			colorless	pink-red
		Reduction of nitrates to nitrogen	Zn / 5 min	
			pink	Colorless
TRP	L-tryptophane	idole production (TRyptoPhane)	JAMES / immediate	
			colorless pale green/yellow	Pink
GLU	D-glucose	Fermentation (GLUcose)	Blue to green	yellow
ADH	L-arginine	Arginine DiHydrolase	yellow	Orange/pink/red
URE	urea	UREase	yellow	Orange/pink/red
ESC	esculin ferric citrate	hydrolysis (β-glucosidase) (ESCulin)	yellow	Grey/brown/black
GEL	gelatin (bovine origin)	hydrolysis (protease) (GELatin)	No pigment diffusion	diffusion of black pigment
PNPG	4-nitrophenyl-βD-galactopyranoside	β-galactosidase (Para-NitroPhenyl-βD-Galactopyranoside)	colorless	yellow
|GLU|	D-glucose	assimilation (GLUcose)	transparent	opaque
|ARE|	L-arabinose	assimilation (AREbinose)	transparent	opaque
|MNE|	D-mannose	assimilation (ManNosE)	transparent	opaque
|MAN|	D-mannitol	assimilation (MANnitol)	transparent	opaque
|NAG|	N-acetyl-glucosamine	assimilation (N-Acetyl-Glucosamine)	transparent	opaque
|MAL|	D-maltose	assimilation (MALtose)	transparent	opaque
|GNT|	potassium gluconate	assimilation (potassium GlucoNate)	transparent	opaque
|CAP|	capric acid	assimilation (CAPric acid)	transparent	opaque
|ADI|	adipic acid	assimilation (ADlpic acid)	transparent	opaque
|MLT|	malic acid	assimilation (MaLaTe)	transparent	opaque
|CIT|	trisodium citrate	assimilation (trisodium CITrate)	transparent	opaque
|PAC|	phenylacetic acid	assimilation (PhenylACetic acid)	transparent	opaque
OX	(see oxidase test package insert)	Cytochrome oxidase	(see oxidase test package insert)	

BIOGRAPHY

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Publications related with this thesis

1. Mongkol, P., Chaiyapechara, S., Jiravanichpaisal, P., Klinbunga, S., and Menasveta, P. (2011). Bacterial community in intestine of the giant tiger shrimp *Penaeus monodon* in rearing pond: Effects of shrimp size. The 23rd Annual Meeting of the Thai Society for Biotechnology *"Systems Biotechnology: Quality & Success"*, February 1-2, Bangkok, THAILAND (Oral presentation).