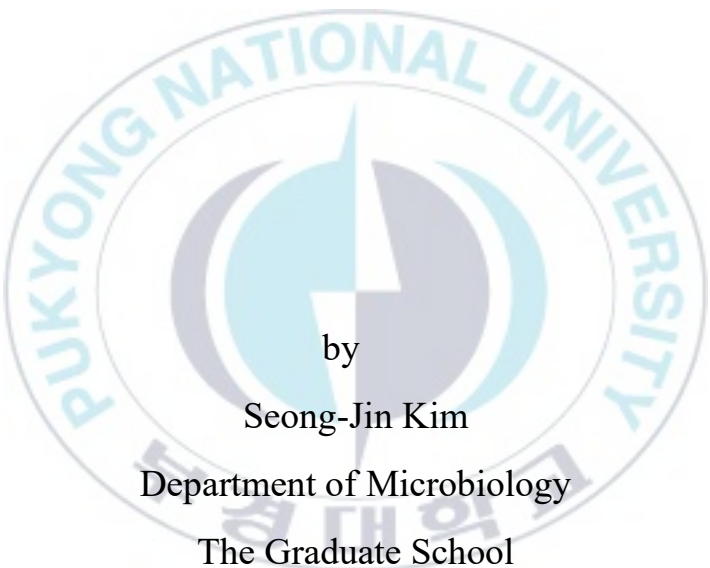
Thesis for the Degree of Master of Microbiology

Microbiome of ascidian '*Halocynthia roretzi*' and taxonomic study of strain RR4-40



by

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Department of Microbiology

The Graduate School

Pukyong National University

August 2020

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멍게 (*Halocynthia roretzi*)의 마이크로바이옴과 균주 RR4-40의 분류학적 연구

Advisor: Prof. Kyoung-Ho Kim

by

Seong-Jin Kim

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A dissertation

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Abstract

A Korean edible ascidian, '*Halocynthia roretzi'*, is one of most important economic invertebrates which has been mostly consumed in South Korea and Japan. Studies of *H. roretzi* are, however, focused mainly on farming systems of the ascidian and tunic-softness syndrome. In this thesis, microbial communities of three digestive parts, gill-slit, digestive gland, and intestine, from a Korean ascidian, *H. roretzi* were studied through high throughput parallel sequencing with tagged PCR. A total of 2,215 OTUs were grouped and taxonomically assigned to 23 phyla, 46 classes, 79 orders, 139 families and 203 genera. Comparison analysis showed that three digestive organs shared core-microbiome such as *Parcubacteria* as a predominant phylum. Minor core-microbiome were genus of *Gammaproteobacteria* and *Entomoplasmatales* in three digestive organs. Different bacterial taxa were identified in different organ as predominant groups as follows: an unidentified genus of *Gammaproteobacteria* (17.85%) in the gill-slit, *Hepatoplasma* (14.73%) and *Entomoplasmatales* (26.15%) in the digestive gland, and *Bacillus* (15.29%) in the intestine. In beta diversity,

intestine generally showed higher values of species richness and Shannon index than the other organs. Gill-slit, on the other hand, comprised the simplest microbial composition. *H. roretzi* absorbs planktonic organisms as food. We predicted limited foods of *H. roretzi* lead to simple microbial diversity. In contrast, intestine supports to remove residue from host through atrial siphon and is adjacent organs to atrial siphon. Therefore, functions and positions of three digestive organs affect to microbial composition. Analysis of microbial diversity will improve to comprehend the nutrition and physiology of *H. roretzi*.

Chapter 1. Microbiome of ascidian '*Halocynthia roretzi*'

1.1 Introduction

Marine animals are classified as marine vertebrates and invertebrates depending on presence of backbone. Marine invertebrates harbor a variety of microorganisms with symbiotic relationship from mutualism to commensalism. Microbial community including bacteria, archaea and eukaryotic microbes provides beneficial functions to host such as metabolic cycle including carbon and sulfate, aiding to digest feeding and host's defense by bioactive metabolites (Kelecom 2002, Ugarelli, Chakrabarti et al. 2017). Prokaryotic symbionts in digestive tract associated with invertebrates which live in marine habitat supply energy to their host and can hydrolyze organic materials (Meziti, Kormas et al. 2007, Hirose 2015). Studies of microbial diversity in marine invertebrates have been progressed mainly on that of coral and marine sponges. Molecular methods have been used for investigating the marine invertebrates-microbe assemblages of corals and marine sponges (Dunphy, Gouhier et al. 2019, Turon, Caliz et al. 2019).

Microbial communities are totally spread in the diverse environment and they have evolved with their hosts. Assessments of microorganisms belonging to the habitat were investigated by traditional culturing technique, however, more than 99% of microbial symbionts are not observed on agar plate or in broth medium (Pei, Mi et al.

2016). Culture-independent methods made it possible to reveal the microbiome of a specific environment thoroughly. Microbiome is a term of 'total microbial community associated with host or environment' and 'their genomes' (Paul A. O Brien 2019). Environmental DNA is extracted from a specific sample and is analyzed by culture independent tools such cloning, community fingerprinting and high throughput sequencing. Polymerase Chain Reaction (PCR)-based community fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP) and terminal restriction fragment length polymorphism (T-RFLP) have been applied to assess microbial community (Muyzer and Smalla 1998, Bardakci 2001). DNA fragments were subjected to Sanger sequencing but this traditional platform has limitation in terms of sequencing throughput and cost. High-throughput sequencing (HTS) platforms, on the other hand, are capable to overcome this limitation by reading millions of sequences at the same time and economical cost (Kircher and Kelso 2010). Sequencing data from molecular technologies were processed by programs of microbial analysis such as QIIME (Quantitative Insight Into Microbial Ecology) and Mothur which have been used to identify the sequences by comparing to database and estimate the relationships among samples associated with various hosts. Operational Taxonomic Units (OTUs) are defined by grouping highly similar sequences in one sample and represent phylogenetic species to be used to estimate microbial diversity in sequencing-based

tools (Nguyen, Warnow et al. 2016). These technologies lead to comprehend symbiotic relationships between microbiome and host with high resolution.

Ascidians (commonly called as sea squirts or tunicates) belong to the class Ascidiacea, the phylum Chordata. Ascidians have features between vertebrates in larval stage and invertebrate in adults (Nho, Jang et al. 2014). They comprise over 2,000 species and are classified to two types, colonial and solitary ascidians, based on life history (Kott 1989). There is a distinct characteristic between colonial and solitary ascidians. Colonial ascidians form a group and solitary ones, on the other hand, live as a separate individual. They are enveloped by tunic which protects ascidians from predators and contains polysaccharide including various celluloses (Goodbody 1975). They are marine-sessile and filter-feeding animals like marine sponges. For filter-feeding seawater enters to oral siphon where larger particles are excluded using tentacles from seawater (Hecht 1915). Particle is sieved from gill-slit (or called as pharynx and branchial basket) which captures particles with mucus net secreted from endostyle (Petersen 2007). The food captured is transferred by cilia in gill-slit (Bone, Carré et al. 2003) and then is digested in stomach by various enzymes. Digestive gland is connected to stomach and releases various hydrolytic enzymes including amylase, lipase and weak protease in ascidians (Koch and Marsh 1972, Goodbody 1975). Digestion is performed in stomach, intestine and anus. Then residue of food particle is eliminated through atrial siphon (Nishida 2000).

Sea squirt '*H. roretzi*' which is known as 'meongge' (sea pineapple) is an edible ascidian mostly consumed in Korea and northern region in Japan. In South Korea, 'meongge' has been cultured mainly in the South Sea since the aquafarming method was established in 1970s (Jang 1979). Aquaculture is conducted by attaching embryos to artificial structure and each embryo is cultured as surrounded by seawater. (Oh, Kim et al. 1997). Mature bodies are used for edible seafood. Consumers eat raw internal organs containing muscle, fascia in sea squirt body and the taste is related to glycogen and nitrogen compounds (Park, Takashi et al. 1990, Oh, Kim et al. 1997). Also, they contain saturated and unsaturated alcoholic materials such as cynthiol and trans-2, cis-7-decadien-1-ol. These alcoholic materials are responsible for unique scent and flavor which are attractive to consumers (Shin, Jun et al. 2011). A study reported that the chemical materials related to the scent and flavor were metabolites of alkylsulfate degraded by alkylsulfohydrolase in intestine (Oh, Kim et al. 1997). *H. roretzi* has digestive gland that is connected to outer wall of intestine, not stomach. This organ is involved in controlling osmosis, digesting and storage of glycogen. Moreover, digestive gland can perform lipid metabolism and remove toxicity in individual that has a similar role to liver in vertebrate and hepatopancreas in mollusk and crustacean (Shin, Jung et al. 2014).

Traditional microscopic and culture-base methods were frequently used for studies of microbial symbionts associated with ascidians (Chen, Fu et al. 2016). Observation was usually applied to tunic-associated microorganisms (Hirose, Aoki et al. 1996).

Culture-dependent method was used to confirm bacterial strain in ascidian host and used for obtaining bioactive bacteria. Advance of culture-independent methods provided new perspective to our understanding in microbial diversity of ascidians (Chen, Fu et al. 2016). Although various molecular technologies have been progressed currently, many studies on microbiome of ascidians have been focused on microbial diversity associated with specific organ, tunic associated with solitary and colonial sea squirts (Groepler and Schuett 2003, Schuett, Doepke et al. 2005, Erwin, Pineda et al. 2014). Core microbiome has been analyzed by comparing various species of ascidians and geographically distributed samples in a species (Erwin, Carmen Pineda et al. 2012, Dror, Novak et al. 2019). Among them, there is no study to analyze bacterial community of *H. roretzi* as we know. Most of studies on *H. roretzi* have been published based on development of aquaculture-method, food science and tunic-softness syndrome (Oh, Kim et al. 1997, Lee, Kim et al. 2013).

In this study, we investigated microbial diversity of organs involved in feeding, gill-slit, digestive gland, and intestine, associated with Korean edible sea squirt, *H. roretzi*. This study showed that each feeding organ of edible sea squirt harbored microorganisms.

1.2 Materials and Methods

1.2.1 Sample collection and dissection

Mature ascidian which were cultured under 10-20 m benthic area were collected from Tongyoung, South Korea (34.82° N, 128.36° E) in April, 2018 (Fig. 1 (A) and (B)). Collected ascidian was transferred to laboratory and stored at -80 °C. Before dissection of ascidians samples, weight and length were estimated. 30-72 g samples were used to analyze microbial communities in three digestive organs per each sample. Dissecting scissors and tweezers were sterilized with 95% ethyl alcohol and tough tunic and latticed tissue were removed by cleaving. Three regions were used for analyses of microbial communities of three digestive organs, gill-slit, digestive gland, and intestine. Total 45 samples were obtained from 15 ascidian specimens.

1.2.2 Total DNA extraction

Dissected 45 samples were extracted using E.Z.N.A soil extraction kit (Omega Bio-tek). The genomic DNA was extracted from the content of organ obtained by squeezing to reduce the contamination of host DNA. 0.1–0.25g contents of three major organs which were involved in degradation of food particles were used for total DNA extraction. Contents from organs were mixed to lysis buffer and bead and homogenized by using Bead 3.0 for breaking cell walls at 4,000 rpm for 3 minutes.

Protein precipitation process was conducted by using solution and cooling in ice and centrifuged at 15,000 rpm for 1 minute. Supernatant from centrifuged content and XP1 buffer (Omega Bio-tek) were mixed in equal amount in new tube and transferred to HiBind® DNA Mini Column (Omega Bio-tek). Mixed aliquot was centrifuged at 13,000 rpm for 1 minute and this process was repeated by transferring to the column samples completely. Total DNA binding to column with elution buffer was stored at room temperature for 5 minutes to improve efficiency and DNA samples were eluted by using centrifuging at 13,000 rpm for 1 minute.

1.2.3 Tagged PCR and high-throughput sequencing using Illumina Miseq technology

To recognize DNA samples from individuals, extracted 45 DNA samples were amplified by tagged PCR technology. Tagged PCR was conducted with primers 1047F (5'- GGWGBTGCATGGYYG-3') and U1492R (5'-AAGTCGTAACAAGGTW-3') which amplify bacterial, archaeal and micro eukaryotic DNA simultaneously with barcoded sequences attached to each sample under conditions as follows; initial pre-denaturation at 95 °C for 5 min, followed by 40 cycles (denaturation at 95 °C for 10 sec, annealing at 58 °C for 10 sec, extension 72 °C for 10 sec), one final extension step at 72 °C for 5 min. Tagged PCR products were purified using FavorPrep GEL/ PCR Purification Kit (Favorgen). PCR products were measured concentration with Qubit 3.0 (Invitrogen), 3 times and average of concentration of each sample was

calculated. Total amount sample with the lowest concentration was transferred to new tube and other purified samples with different concentration for making equal concentration. DNA library was constructed by using TruSeq Nano DNA Sample Prep Kit (Illumina) and sequenced by Illumina Miseq technique.

1.2.4 Data processing using QIIME 2 program

QIIME 2 (Quantitative Insights Into Microbial Ecology 2 (ver. 2020.2)) program was used to analyze microbial compositions of samples. Single-end raw sequences were used and processed. and barcoded sequences were removed. Two samples (PSS3 and DgSS10) with small number of reads were discarded from further analyses. Each sequence was separated from pooled amplicons and barcode sequences attaching to sequencing data were detected to identify individual samples and removed. Singletons and chimera sequences of single-end sequences were excluded by using DADA 2.

Representative sequences were clustered using operational taxonomic units (OTUs). Eukaryotic sequences were recognized by the silva database and removed from further analyses. Taxonomic assignment was done by classifier of the greengenes database and mitochondrial and chloroplast sequences were discarded. Evenness, abundance and richness of microbial communities were estimated by three alpha diversity indices including Shannon, Simpson and Chao 1. Principal coordinate analyses (PcoA) were calculated using Bray-Curtis dissimilarity.

1.3 Result

1.3.1 Microbiome associated with 15 *H. roretzi* individuals

Microbial communities were sequenced by using Miseq platform (Illumina) and analyzed with QIIME 2 program. Raw sequences were 3,503,302 reads of microbial communities associated with three organs (gill-slit, digestive gland and intestine). DADA 2 were used to remove chimera and singleton sequences.

Figure 1-3 describe the bacterial diversity of 15 *H. roretzi* individuals. Total 2,215 OTUs contained 23 phyla, 139 families and 203 genera. Overall, the core microbial community comprised 9 phyla, 12 classes, 14 orders, 12 families and 12 genera in 15 individuals. Samples were observed abundant core bacteria in phylum, class and genus levels. OD1 (37.41%) is the highest abundant taxon. *Firmicutes*, *Tenericutes* and *Proteobacteria* were shared phyla among 15 individuals (Figure 5). Less abundant microorganisms were genus of *Entomoplasmatales* (11.15%), *Bacillus* (7.31%), genus of *gammaproteubacteria* (6%) candidatus *Hepatoplasma* (4.45%) and genus of *Rhodobacteraceae* (1.3%). We identified that 15 individuals contatined genus of *Bacteroidales* (0.7%), *Synechococcus* (0.25%), genus of *Pirellulaceae* (0.25%), and genus of *Sphingomonadaceae* (0.23%) as bacteria of less than 1%.

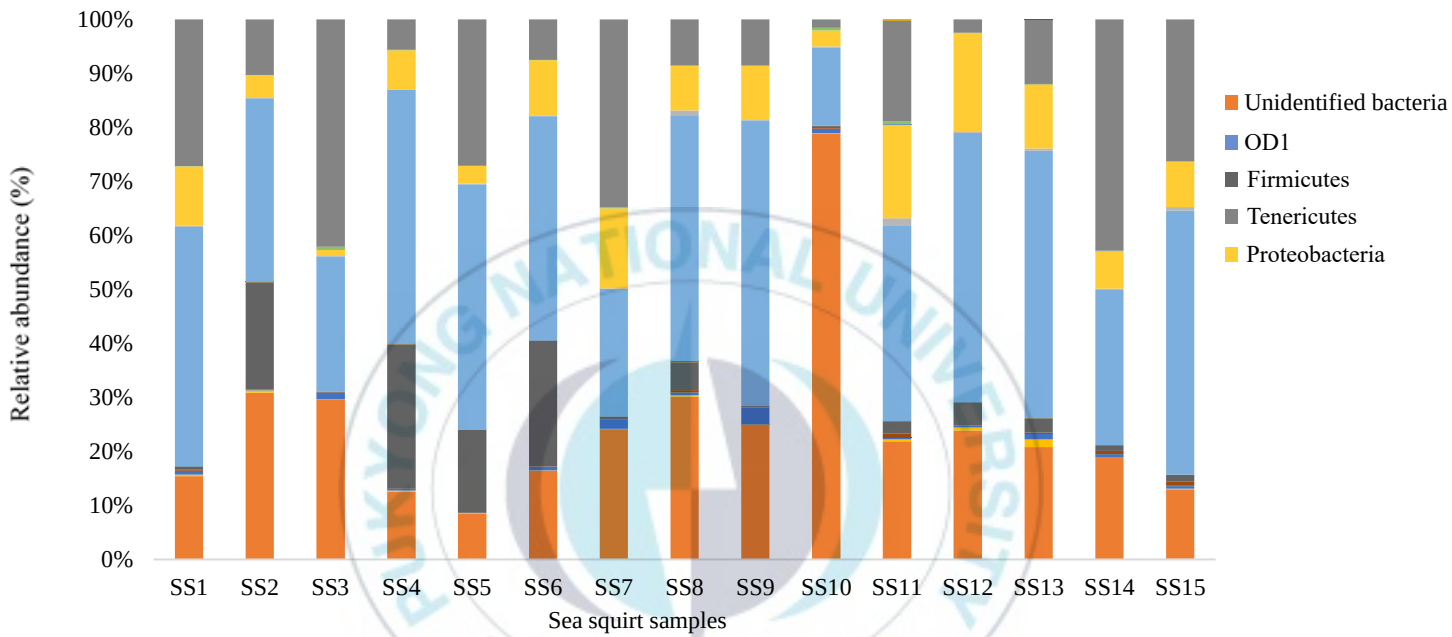

Figure 1-1. Microbial communities associated with 15 ascidian individuals in phylum level. 15 ascidian individuals contained unidentified class of OD1, *Firmicutes*, *Tenericutes* and *Proteobacteria* in phylum level.

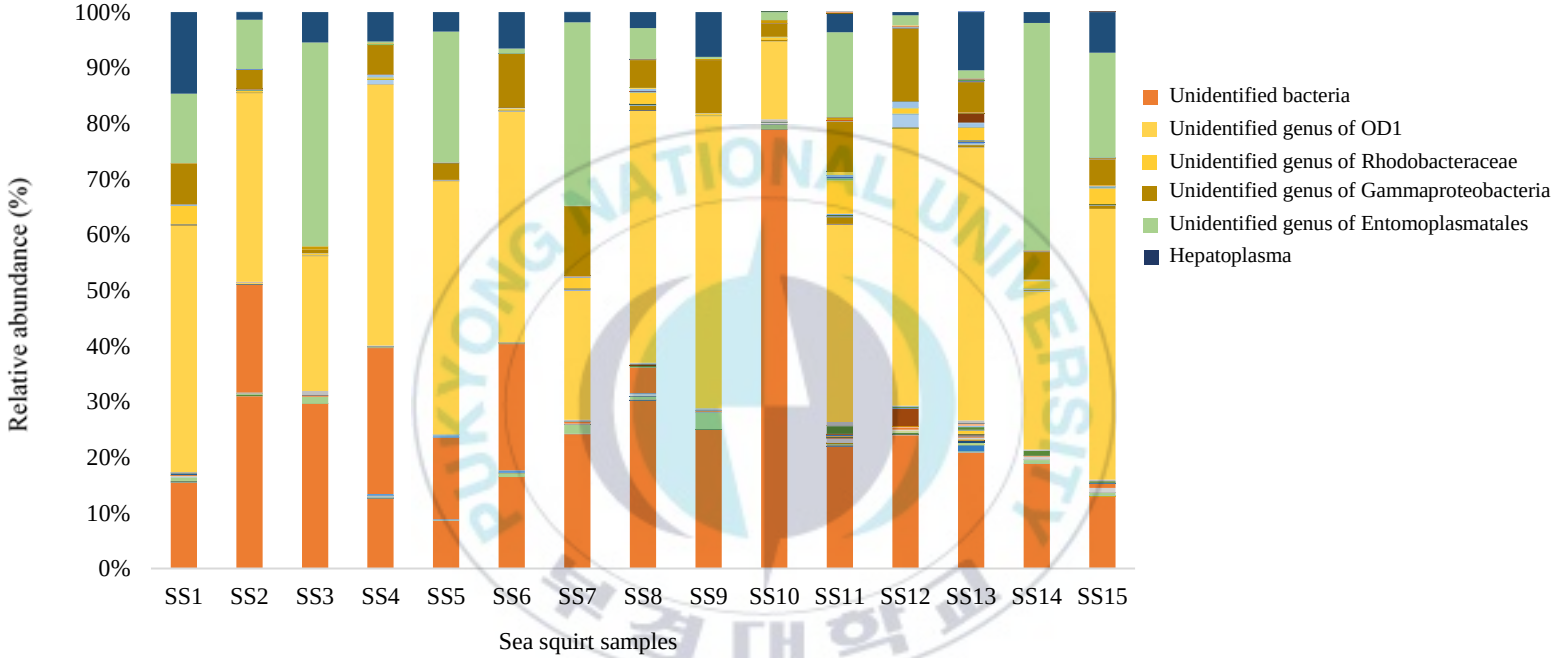

Figure 1-2. Microbial communities associated with 15 ascidian individuals in genus level. 15 ascidian individuals contained OD1, *Synechococcus, Bacillus,* the genus of *Pirellulaceae,* the genus of *Rhodobacteraceae,* the genus of *Sphingomonadaceae*, the genus of *Entomoplasmatales* in genus level in class level.

1.3.2 Core bacteria of gill-slit, digestive gland and intestine.

43 samples contained *Parcubacteria* (candidatus phylum OD1) at the highest proportion (Figure 1-3 (A)). Three digestive organs contained *Tenericutes*, *Proteobacteria* and *Firmicutes* at different proportion in phylum level (Figure 1-3 (B)).

Proteobacteria was identified in gill-slit and digestive gland contained *Tenericutes* as higher proportions (18.66% and 40.91%, respectively). Intestine contained *Firmicutes* in phylum level (16.27%; figure 1-4).

Three organs of 15 individuals contained the core communities and three organs had *Parcubacteria* and lower abundant groups were *Gammaproteobacteria* within the *Proteobacteria* and *Entomoplasmatales* within the phylum *Tenericutes*. *Parcubacteria* occupied in three organs as similar percentages. Microbial symbionts were observed in thee organs as different proportions. Three feeding organs contained *Gammaproteobacteria*, *Entomoplasmatale* and *Bacillus*. There were no significant differences in phylum level, while differences of relative abundance presented in the genus level (Figure 1-5).

1.3.3 Specific microbial symbionts in three organs

Relative abundance showed that microbial symbionts were identified for each sea squirt's organ as higher proportion than other two organs. Gill-slit contained *Gammaproteobacteria* (17.85%). *Entomoplasmatales* and *Hepatoplasma* were identified in digestive gland (26.15% and 14.73%, respectively) and intestine contained *Bacillus* (15.29%). These bacteria distinguished in each digestive organ (Figure 1-6 and Table 1-1).

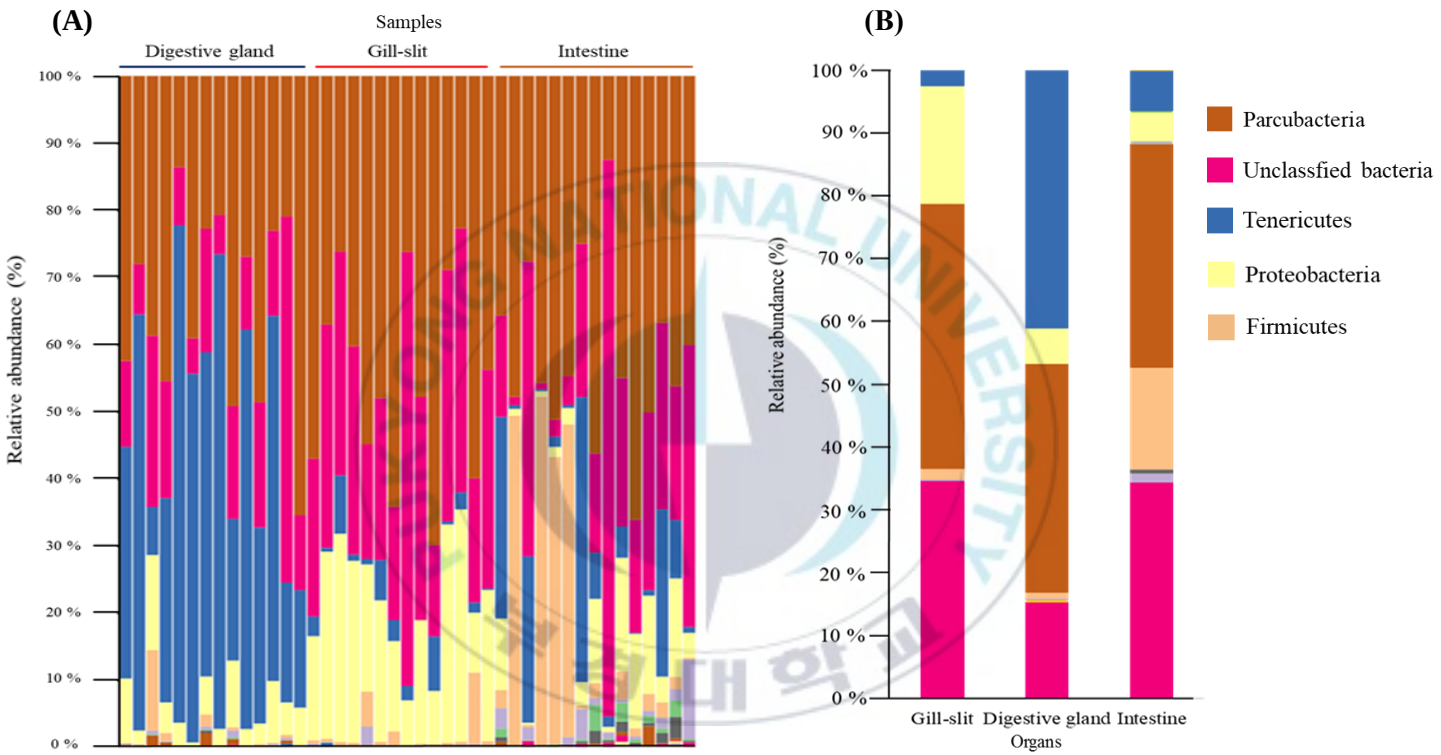

Figure 1-3. (A), Microbiome of 43 samples associated with *H. roretzi* ; (B), Microbiome of three organs.

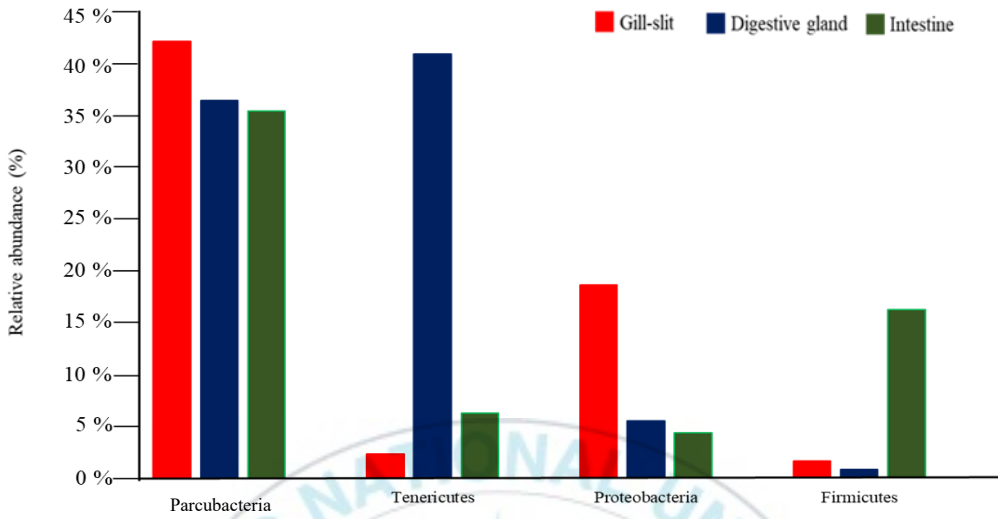

Figure 1-4. Distinction of major microbial groups in three organs. Different colors encompass three organs, gill-slit (red), digestive gland (blue) and intestine (green).

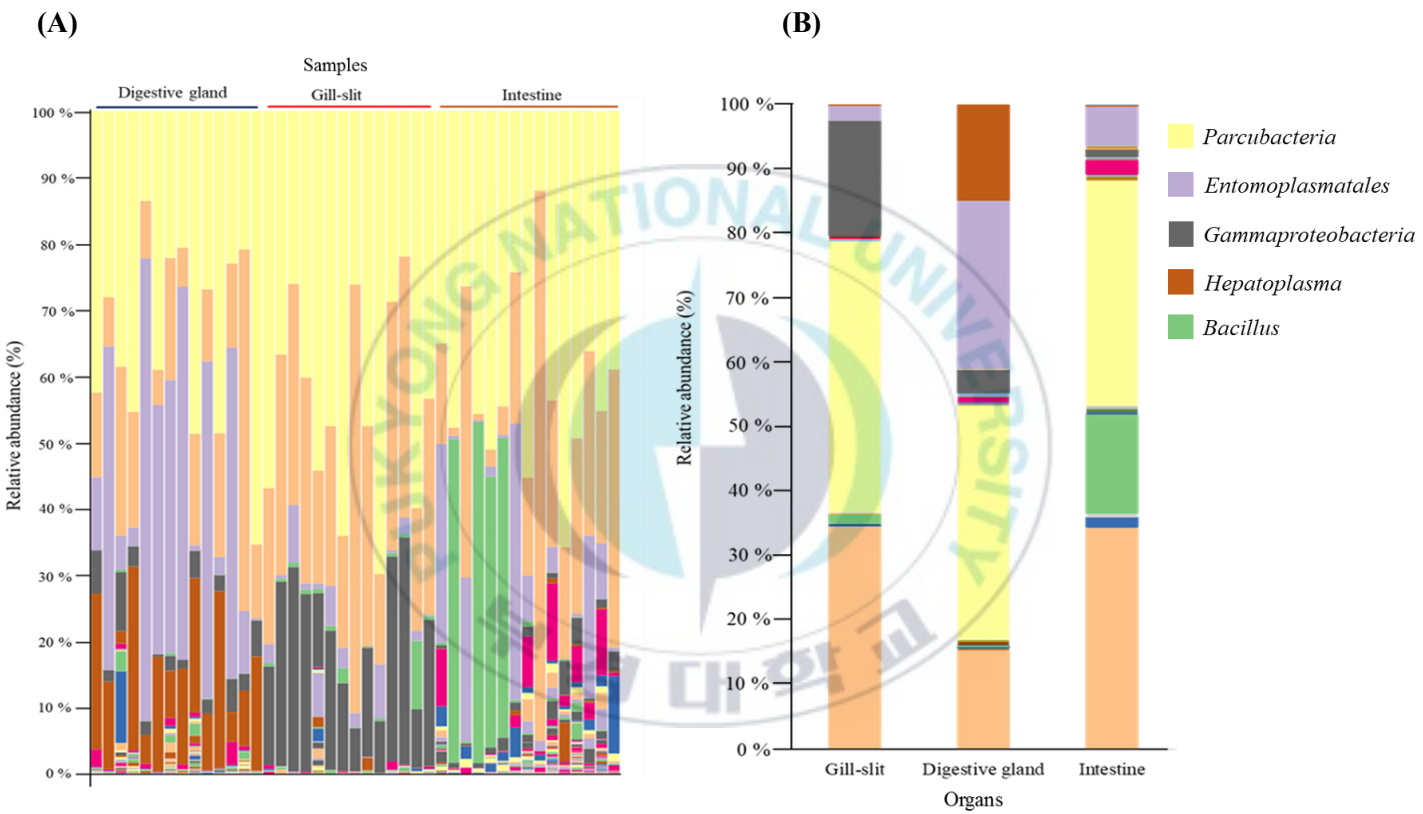

Figure 1-5. (A), Microbiome of 43 samples in genus level, (B), Microbiome of three organs in genus level.

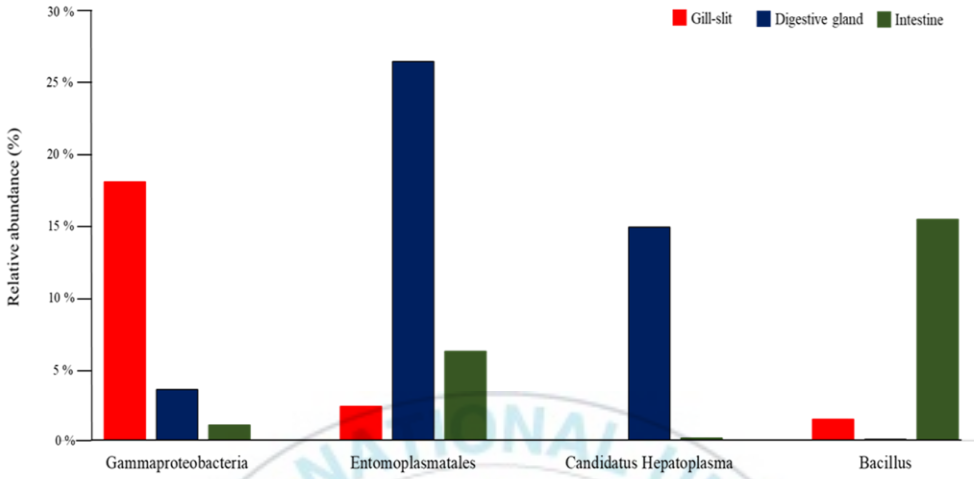

Figure 1-6. Distinct bacterial group of three digestive organs. Different colors indicate each organ, gill-slit (red), digestive gland (blue), and intestine (green).

Table 1-1. Major specific symbionts in relative abundance of three organs in *H. roretzi*.

Phylum	Lowest taxonomy	Relative abundance (%)		
		Gill-slit	Digestive gland	Intestine
OD1	-	42.06	36.43	35.42
Proteobacteria	Gammaproteobacteria	17.85	3.53	1.06
Tenericutes	Entomoplasmatales	2.38	26.15	6.14
Tenericutes	Hepatoplasma	0.01	14.73	0.16
Firmicutes	Bacillus	1.47	0.08	15.29

1.3.4 Observed OTU in three digestive organs of *H. roretzi*

Operational taxonomic units (OTUs) group sequences in same taxa. Total OTUs are 2,215 OTUs in 15 ascidian samples. Alpha rarefaction curve based on observed OTUs presents in Figure 1-5. When sequencing depth was 10,000, three organs (gill-slit, digestive gland and intestine) gill-slit contained 51 OTUs, 82 OTUs were identified in digestive gland and intestine contained 155 OTUs. The highest value of observed OTUs showed organs, gill-slit, digestive gland and intestine, harbored diverse microorganisms. The most abundant microorganisms associated with three organs are intestine and it has high diversity among three organs.

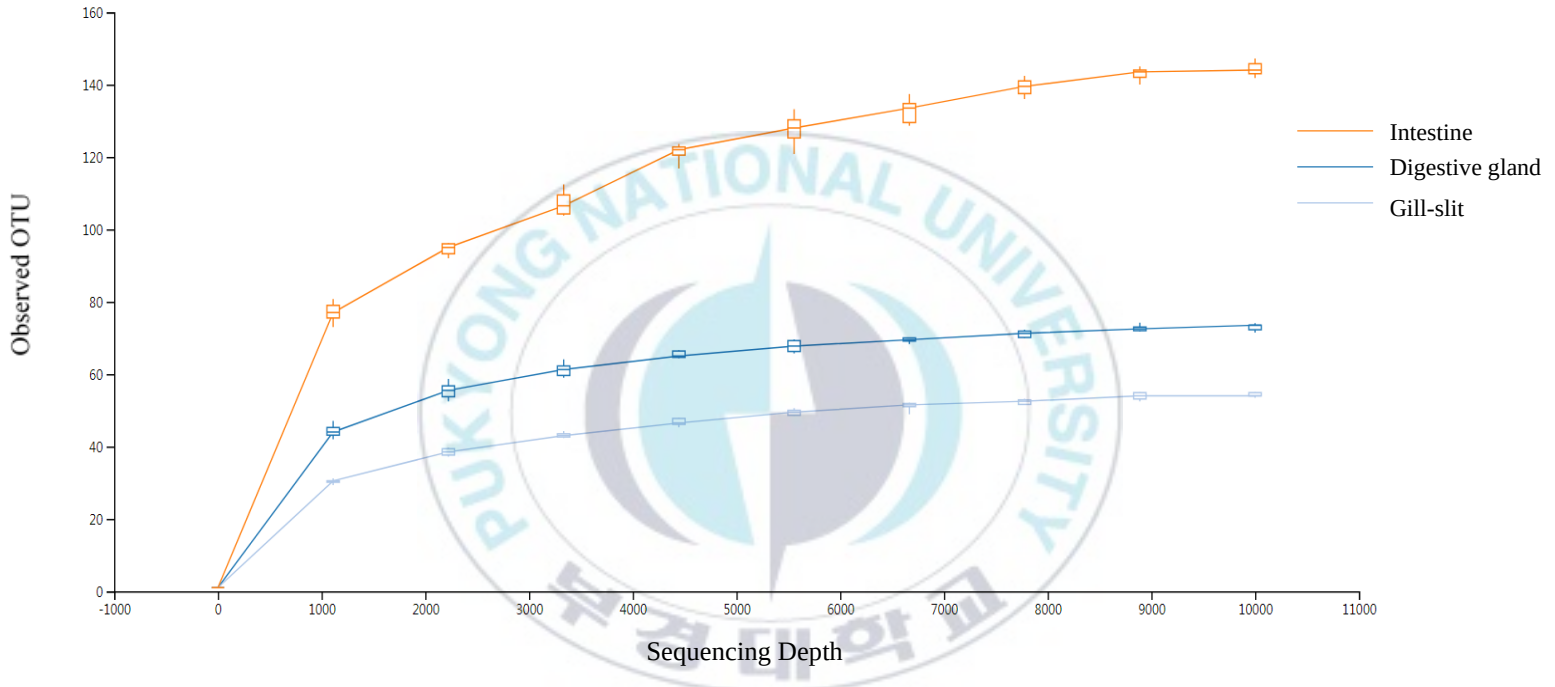

Figure 1-5. Alpha rarefaction curve of three digestive organs. Alpha rarefaction curve indicated that relationship between observed OTUs and sequencing depth in intestine, digestive gland and gill-slit. Different colors encompass each organ; Intestine (orange), digestive gland (blue) and gill-slit (azure).

1.3.5 Alpha diversity of three organs in *H. roretzi*

Three indices including Chao1, Shannon and Simpson showed a significant difference of microbial alpha diversity in three organs. Chao1 estimates richness of rare OTUs in microbial community. When value of Chao1 was higher, microbial symbiont was more diverse among hosts. Value of Chao1 of intestine was significantly high compared to other two organs (161.87) and indicated that microbial symbionts of intestine contained higher number of rare OTUs. In contrast, gill-slit was significantly lower value of Chao1 (52.94). The result showed that microbial community of intestine was higher diversity compared to other two organs gill-slit, on the other hand, comprised simplest microbial composition among three digestive organs (Figure 1-6 and Table 1-2).

Evenness and abundance of microbial communities in three organs associated with *H. roretzi* were assessed by both two indices (Shannon and Simpson, Figure 1-7). The average Shannon index of intestine was 4.52 and value of gill-slit was 2.49. Diversity was assessed by Faith's phylogenetic diversity (PD; figure 1-6 (B)). When organ estimated higher score of Faith's PD, microbial symbionts contained higher diversity. Value of Faith's PD in intestine was higher than other two organs and this implied microbial community of intestine was phylogenetic diversity. Gill-slit, on the other hand was lower value. This result showed that bacterial diversity of gill-slit was

simpler than other two microbial compositions. In contrast, microbial composition of intestine contained higher abundance.

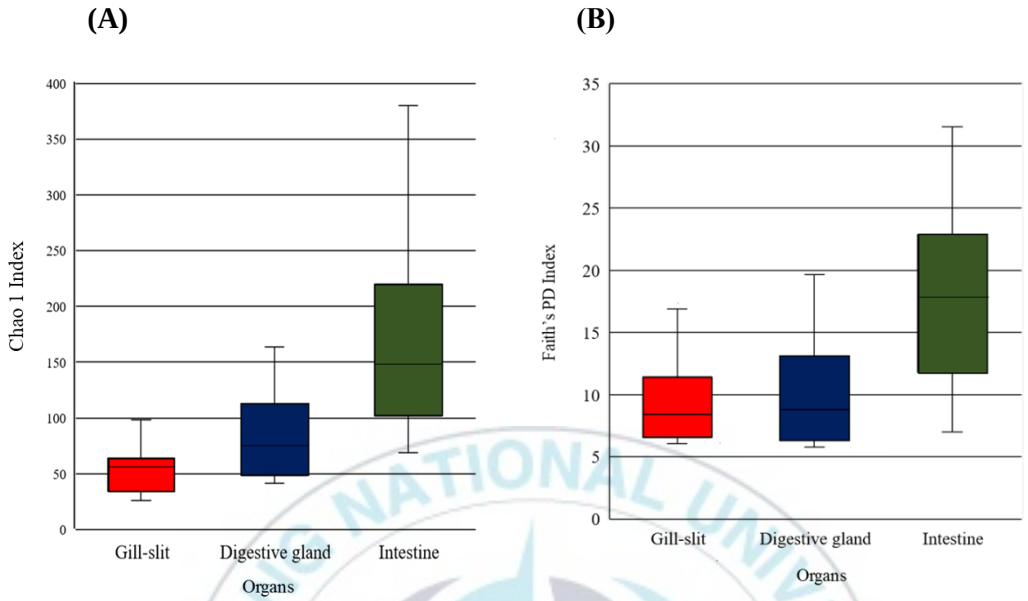

Figure 1-6. Richness and diversity of microbial symbiont estimate ((A): Chao1 and (B): Faith's PD) in three organs (p-value < 0.001).

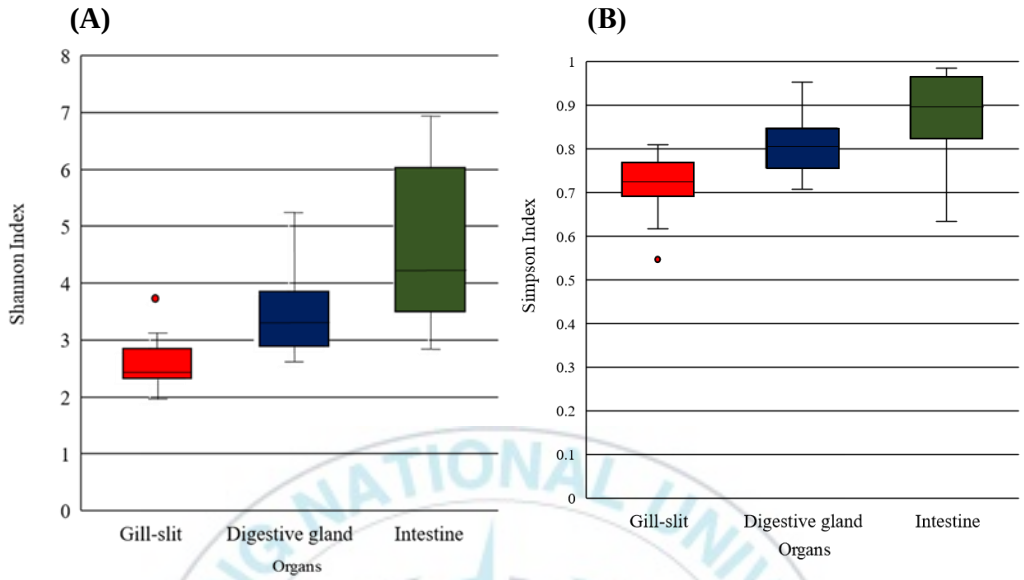

Figure 1-7. Boxplots illustrating of Shannon index (A) and Simpson index (B) in three organs (p-value < 0.001).

Table 1-2. Indices of alpha diversity in three organs

Source	OTUs	Chao 1	Shannon	Simpson	Faith's PD
Gill-slit	51.71	52.94	2.49	0.72	9.21
Digestive gland	82.43	85.39	3.37	0.81	10.12
Intestine	155.20	161.87	4.52	0.87	18.17

1.3.6. 2D PcoA plot result in three organs

Principal coordinate analysis (PcoA) was measured by Bray-Curtis distance of microbial communities in samples of three organs (Figure 1-8). Microbial symbionts from same organs were clustering. Microbial compositions of samples in gill-slit (blue stars) were similar microbial species. In contrast, microbial compositions of intestinal samples had higher diversity (brown diamonds).

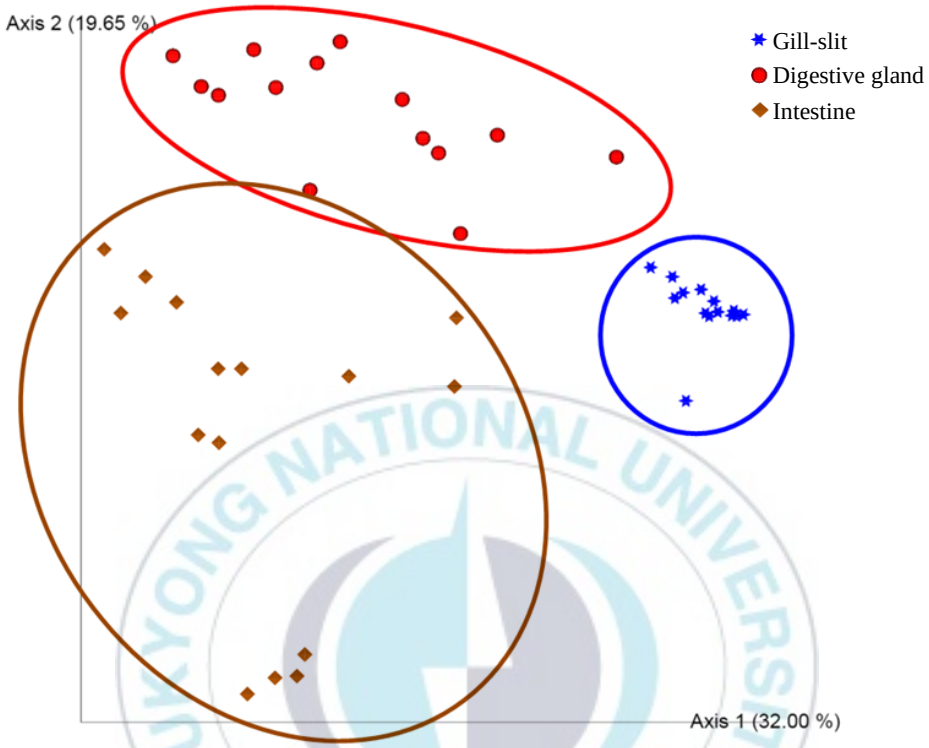

Figure 1-8. PcoA plot measured by Bray-Curtis distance of microbial communities associated with gill-slit, digestive gland, and intestine. Gill-slit (blue stars), digestive gland (red circles) and intestine (brown diamonds). Circles indicate same samples of individuals within each organ.

1.4 Discussion

We conducted analyses of microbial communities associated with digestive tract in Korean edible ascidians, *Halocynthia roretzi* by using high-throughput technology. Gill-slit is crucial organ to digestion of ascidians because they are filter-feeding organisms (Petersen 2007) and intestine is organ which finally digest and performs removal of residue from food particle through exhalent siphon. Digestive gland assists to digest and conduct metabolite of glycogen. Digestive gland is similar to hepatopancreas in marine invertebrates and liver in vertebrates (Shin, Jung *et al*. 2014). These results showed that three digestive regions have organ-core microbial symbiont. (Petersen 2007)(Shin, Jung et al. 2014)These three organs shared dominant bacterial species, OD1 and unassigned bacteria.

OD1 group is candidate phylum and live in diverse habitats as symbiotic bacteria (Harris, Kelley *et al*. 2004). OD1 group are characterized as anoxic bacteria that they were microbial symbionts which lack abilities to biosynthesize amino acid, nucleotides, vitamins and polar lipids and assist fermentative metabolite to host in anaerobic environment (Nelson and Stegen 2015).

Results showed that bacteria were identified in each organ as higher proportions. *Gammaproteobacteria* was identified in gill-slit. *Entomoplasmatales* and *Hepatoplasma* were identified in digestive gland and *Bacillus* in intestine.

The genus affiliated with *Gammaproteobacteria* was specific group in gill-slit. This group has been found in several organs containing tunic, zooid (in colonial ascidians) and gill-slit of ascidians (Martinez-Garcia, Diaz-Valdes *et al*. 2007, Schreiber, Kjeldsen *et al*. 2016) . In published study, *Gammaproteobacteria* group was detected in pharyngeal samples of ascidian. *Endozoicomonas* within *Gammaproteobacteria* colonized in all of ascidian samples. Ascidian-symbiotic *Endozoicomonas* was reported that the bacterium produces bioactive materials (Schreiber, Kjeldsen *et al.* 2016).

Entomoplasmatales and *Hepatoplasma* were identified in digestive gland for higher percentages (26.15% and 14.73%, respectively) compared to two other organs. Both two bacterial groups belong to the class *Mollicutes* and lack cell walls. *Entomoplasmatales* bacteria sometimes have been found in guts of deep-sea shrimp and mud shrimp (Zbinden and Cambon-Bonavita 2003, Demiri, Meziti *et al*. 2009). They are parasitic and heterotrophic bacteria. Although clear roles of them were not speculated, they were found in healthy shrimp and non-toxic for shrimp (Zbinden and Cambon-Bonavita 2003).

Hepatoplasma (candidatus genus) was identified in digestive gland mainly. Candidatus *Hepatoplasma* genus belonging to the class *Mollicutes* is symbiont of invertebrates and has been found in hepatopanceas of isopods (Leclercq, Dittmer *et al*. 2014). Candidatus genus almost infects hepatopancreas and is transmitted from one individual to others, horizontally (Wang, Brune *et al*. 2007). In previous study, researchers reported that effect of this symbiont was related to survival of starving host because they detected no effect to host in enough nutrition (Fraune and Zimmer 2008).

Bacillus genus was specific group in intestine. Ascidian-specific *Bacillus* group have biological and chemical diversity. Chemical compounds such as polyketides, terpenoids and peptides were produced by *Bacillus* group isolated from several ascidians. Previous study showed that these materials performed roles of cytotoxic antimicrobial and antioxidant activities (Chen, Hu *et al*. 2018).

Microbial diversity of Tongyeong bay was studied by using pyrosequencing in March and May. *Proteobacteria* and *Bacteroidetes* were identified as dominant phyla in both months. *Verricumicrobia* and *Bacillariophyta* were identified as minor symbionts (Suh, Park et al. 2014). Ascidian, however, contained *Parcubacteria* as dominant phylum and *Tenericutes*, *Proteobacteria* and

Firmicutes phyla were identified in ascidian samples. Result showed that ascidian, *H. roretzi*, contained endemic bacteria.

Gill-slit is organ which captures specific food particles using mucus net. Digestive gland is connected to outer wall of intestine and releases digestive enzymes assisting to degrade food particles. (Shin, Jung *et al*. 2014). Then digestion is performed in intestine in final stage. Also, intestine supports to remove residue from host through exhalent siphon (Nishida 2000) and is adjacent organs to atrial siphon. Therefore, surrounding seawater can enter to intestine through atrial siphon. Microbial composition of intestine was higher diversity. Intestinal bacteria were reported that they removed waste from host. These bacteria don't need to compete with host (Pagan-Jimenez, Ruiz-Calderon *et al*. 2019). We predict that understanding of relationship microbial diversity and functions of three digestive organs will assist to study of effects from microbial symbionts to *H. roretzi*

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1.6 국문요약

한국의 식용으로 사용되는 '*Halocynthia roretzi*'는 한국과 일본에서 주로 소비되어 온 경제적인 무척추 동물 자원 중 하나이다. 하지만 *H. roretzi* 에 대한 연구는 멍게 물렁증과 멍게의 양식 시스템에 집중되어 있다. 본 논문에서는 한국 멍게류인 *H. roretzi* 의 세 소화기관, 아가미, 소화샘, 장의 미생물 군집을 바코드 PCR 을 이용하여 차세대 염기서열 분석을 통하여 연구하였다. 전체 2215 OTU 를 얻었으며, 분류학적으로 23 개의 문, 46 개의 강, 79 개의 목, 139 개의 과와 203 개의 속을 포함한다. 세 기관이 공통의 세균을 가지고 있으며, 각 기관별로 다른 세균분포를 보였다. 공통의 세균은 OD1 이 우세한 세균 그룹이며, 소수의 공통 세균은 *Gammaproteobacteria* 와 *Entomoplasmatale* 이다. 세균은 기관별로 확인되며 다음과 같다. 아가미에서는 Gammaproteobacteria (17.85%), 소화샘에서는 *Hepatoplasma* (14.73%)와 *Entomoplasmatales* (26.15%) 그리고 장에서는 *Bacillus* 속이 (15.29%) 다른 두 기관과 비교하였을 때 높은 비율을 차지하였다. 장은 다른 기관에 비하여 보통 Shannon 지수와 종의 풍부성 지수가 다른 두 기관보다 높게 나타났다. 반면, 아가미는 가장 단순한 미생물 구성을 가지고 있다. 우리는 *H. roretzi* 가 한정된 먹이를 섭취하는 것이 아가미의 단순한 미생물 구성과 관련이 있으며, 반면 장은 먹이 찌꺼기를 멍게로부터 출수공으로 제거하는 일을 하는데 도움을 주며, 출수공과 가장 가까운 위치에 존재한다. 따라서 이러한 기능과 위치가 미생물의 구성에 영향을 줄 것이라 예상하였다. 이러한 결과는 공생 미생물들이 기관들의 위치와 기능들에 따라 다르다는 것을 보여준다. 이 세 기관의 미생물 다양성 분석은 *H. roretzi* 의 영양과 생리를 이해를 향상시킬 것이다.

Chapter 2: Taxonomic study of strain RR4-40

Abstract

A novel strain RR4-40^T isolated from a recirculating aquaculture system was classified to member belonging to the *Flavobacteriaceae* family by using a polyphasic approach. Phylogenetic, genomic and biochemical characteristics of strain RR4-40^T were analyzed and compared with six reference strains. Cells of RR4-40^T were aerobic, gram-negative, non-motile and irregular short-rod. Strain RR4-40^T was catalase and oxidase negative and growth occurred at 15–35°C (optimum: 25–30 °C), pH 5–9.5 (optimum: pH 8.5) and 0.5–6% NaCl (optimum: 3% NaCl; w/v). Phylogenetic features based on analysis of 16S rRNA gene sequences indicated that RR4-40^T was most closely to *Marinirhabdus gelatinilytica* NH83^T (94.16%). Whole genome sequences showed that novel bacterium, designated RR4-40^T, have phylogenetic distance from its reference strains and we suggest that strain RR4-40^T is novel genus within the *Flavobacteriaceae* through these results.

2.1 Introduction

Spatial and polluted water which were used in farming industry constraints assist to develop innovative aquaculture technology based on reusing seawater (Gao, Xu et al. 2011). Recirculating aquaculture systems (RAS) are interior or exterior technology and effective treatments of waste factors assure water quality in this technology. Reusing water is determined by depending on capture of solid materials through physical filtering, lower concentration of carbon dioxide, dissolved oxygen concentration and toxic ammonia and nitrite (Michaud, Lo Giudice et al. 2009). Biofilter is essential implement to eliminate pollutant and microflora surface on biofilter provide to this system with removal of toxic ammonia through nitrogenous metabolisms such as nitrification, denitrification and anaerobic ammonia oxidation (anammox) (Schreier, Mirzoyan et al. 2010, van Kessel, Harhangi et al. 2011). Previous studies of microbial communities in this technology focused on harmful pathogens and antibiotic activities. Recent microbial studies on biofilter in RAS are performed to focus on activities involved in purification of waste materials and culture-dependent and culture-independent approaches have been used by investigating microbial communities in RAS. These analyses supply to many characteristics and provide detailed species-specific information of microflora (Michaud, Lo Giudice et al. 2009).

Bacteria belonging to the *Flavobacteriaceae* family have been found in diverse environments such as soil, freshwater, seawater and symbionts in various host including animals and plants (Choi, Lee et al. 2007, Joung, Jang et al. 2019). The family comprises currently over 90 genera. Although members within the family live in various habitats, they share several characteristics. They form yellow colonies, common respiratory quinone, menaquinone-6 (MK-6) and are non-spore forming (McBride 2014). The study of effects of microbial communities in RAS which treated farming water of white leg shrimp (*Litopenaeus vannamei*) showed that bacterial genus within the *Flavobacteriaceae* family surface on bioreactor which was used in RAS was investigated by using metagenomic analysis. Role of bacteria the family found on bioreactor, however, has not been revealed in RAS (Chen, Chang et al. 2019).

In this study, we aimed to investigate novel bacterium, designated RR4-40^T, through polyphasic analysis and suggest that strain RR4-40^T is new genus within the *Flavobacteriaceae* family by comparing genomic, phenotypic and biochemical characteristics with six reference strains belonging to the family.

2.2 Materials and Methods

2.2.1 Sample collection and isolation

Novel bacterium, strain RR4-40^T, was isolated from biofilter in recirculating aquaculture system, Busan in South Korea. Sample was diluted by general diluted method with phosphate buffered saline (PBS) and spread on marine agar (MA, Difco). Inoculated samples were incubated at 28 ℃ for 5 days and yellow-pigmented colony was isolated on MA by streaking method. To amplify 16S rDNA, colony PCR was conduct with a pair of universal bacterial primer, 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGYTACCTTGTTACGACTT-3') and PCR product was purified. Sequences were read by using traditional Sanger sequencing and compared with sequences of other taxa in Ezbiocloud and strain RR4-40^T was identified to novel genus within the *Flavobacteriaceae* family.

2.2.2 Genomic and phylogenetic analyses

To obtain nearly complete 16S rRNA gene sequence, 16S rRNA gene was amplified with universal primers, 8F and 1492R and PCR product was inserted into TA-cloning vector (pLUG-Prime® TA-cloning Vector Kit II, iNtRON). Nearly complete 16S rDNA of 1443bp was obtained and compared with data

from Ezbiocloud (https://www.ezbiocloud.net/). Phylogenetic trees were constructed by using neighbor-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) methods with Kimura 2-parameter. Bootstrap analysis was used to assess topology with 1000 replications in NJ and 100 replications in ML method. To analyze complete genome of RR4-40^T, genomic DNA of RR4-40^T was extracted with HiGene™ Genomic DNA Prep Kit (BIOFACT) according to the manufacturer's instruction and purified. Genomic DNA was subjected to the library construction and sequenced by using single-molecule real-time sequencing (SMRT; MACROGEN KOREA) applied to PacBio RS II platform. Sequence was obtained by de novo assembly using Hierarchical Genome Assembly Process (HGAP, Version 3.0) and annotated by NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Subsystems were analyzed using Rapid Annotation using Subsystems Technology (RAST; https://rast.nmpdr.org/rast.cgi). Genomic distances among RR4-40^T and related taxa were calculated by Average Nucleotide Identity (ANI; https://www.ezbiocloud.net/tools) and digital DNA-DNA Hybridization (dDDH; http://ggdc.dsmz.de/ggdc.php#) calculators and distances of amino acids sequences were calculated using Average Amino acid Identity calculator (AAI; http://enve-omics.ce.gatech.edu/aai/index).

2.2.3 Phenotypic and biochemical investigation

For microscopic analysis, motility was investigated using wet-mount method and hanging drop method and observed under optical microscope. Strain RR4-40^T was grown on MA at 28 °C for 3 days and colony morphology was observed. Gram-stain was conducted by Ramel gram-stain kit (Thermofisher) and cell morphology was observed under microscope. To determine growth condition, strain RR4-40^T was inoculated on MA and incubated at 5–40 °C (intervals of 5 °C) for 3 days and the growth of NaCl-tolerance was tested by using NaCl-free marine broth (MB; Difco) with various NaCl concentration, 0, 0.5 and 1–15% NaCl (w/v; intervals of 1%). The growth condition of pH range was confirmed by inoculating the cells in MB of pH 4–12 (intervals of 0.5). The API 20NE, API 20E, API ZYM and API 50CH strips (bioMérieux) were used to analyze biochemical features such as assimilation, acid production and enzyme activities according to manufacturer's instruction. The oxidase test was performed by using 1% (w/v) tetramethyl *p*-phenylenediamine (bioMérieux) and catalase activity was investigated by observing bubble using 3% (v/v) hydrogen peroxide.

2.2.4 Analysis of cell component

To identify fatty acids and polar lipids of the cells, strain RR4-40^T was inoculated on MA and incubated at 28 °C, 4 days for expotential growth

according to the Microbial Identification System (MIDI). The fatty acids of novel bacterium were investigated by using gas chromatography (GC; Hewlett Packard 6890) and analyzed with Sherlock software (version 6.3). The bacterial cells were dissolved in chloroform-methanol solvent (2:1, v/v) and spotted on thin layer chromatography plates (TLC Silica gel 60 F_{254}; Millipore). Inoculated TLC plates were separated in chloroform-methanol-water solvent (65:24:4, v/v/v) and second separations of TLC plates were performed in chloroform-acetic acid-methanol-water solvent (80:15:14:4, v/v/v/v). Individual polar lipids were stained with reagents and total polar lipids were observed by spraying 10% (w/v) ethanolic molybdophosphoric acid. Amino lipids were identified by using ninhydrin reagent and molybdenum blue reagent was used to detect phospholipids. Glycolipids and carbohydrate were observed by α-naphthol and schiff's reagent, respectively. Dragendorff reagent stained choline group that was spotted on TLC plate. Respiratory quinone was detected by high performance liquid chromatography (HPLC; Waters e2695). Major quinone of the cell was extracted according to the MIDI protocol and detected by using methanol-heptane solvent (90:10, v/v). Quinone of RR4-40^T was compared with standard quinones, MK-6, MK-7 and MK-8.

2.3 Result

2.3.1 Phylogenetic and genomic characteristics

Nearly complete 16S rDNA of strain RR4-40^T was 1443bp and novel bacterium was most closely relative to *Marinirhabdus gelatinilytica* NH83^T (94.73%), followed by *M. citrea* MEBiC09412^T (94.53%) and *Ulvibacter marinus* IMCC12008^T (94.17%). Phylogenetic tree using RR4-40^T and type species of relative taxa showed that strain RR4-40^T which belongs to the *Flavobacteriaceae* family formed lineage with *M. gelatinilytica* (Figure 7). The length of complete genome was 3,388,965 bp with G+C content of 37.4% and detailed map of genome is presented in figure 8. The whole genome of RR4-40^T contains total 3018 genes, 2973 coding sequences (CDSs), total 45 RNAs including 6 rRNAs, 35 tRNAs, 4 ncRNAs and 14 pseudogenes in NCBI PGAP (Table 3).Values of Average Nucleotide Identity (ANI), digital DNA-DNA Hybridization (dDDH) and Average Amino acid Identity (AAI) of complete sequences indicate that RR4-40^T shares low sequences with members within the *Flavobacteriaceae* (Table 2-2)

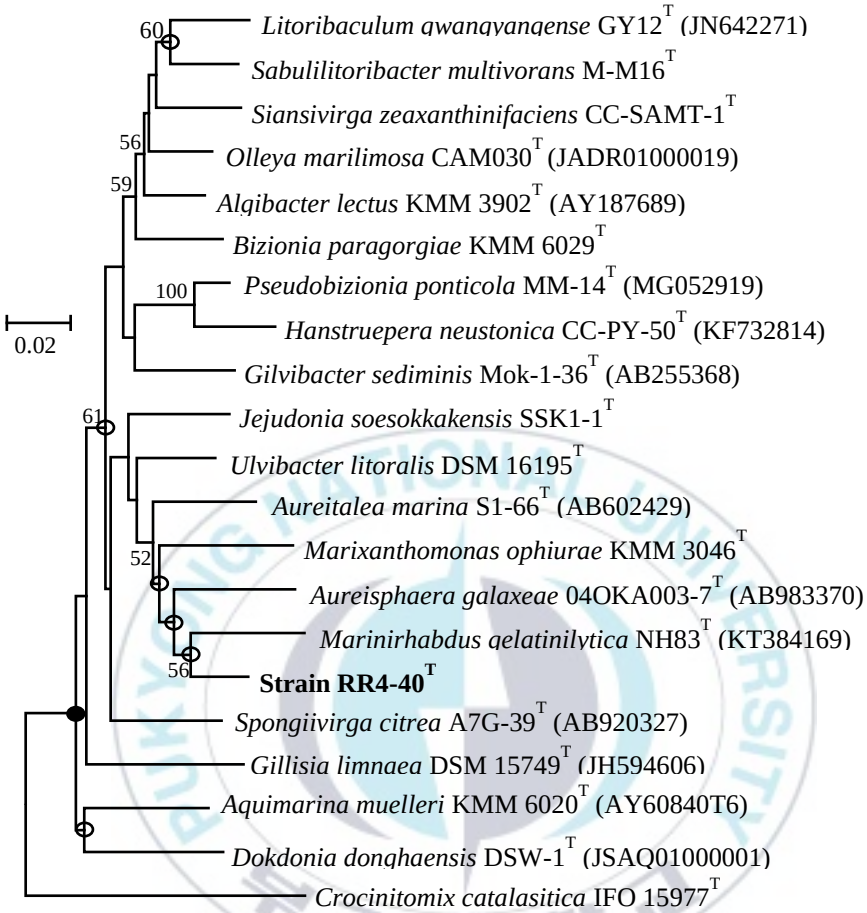

Figure 2-1. Phylogenetic tree of Strain RR4-40^T and related taxa within the family *Flavobacteriaceae* using Neighbor-Joining Method (NJ). NJ tree indicates that strain RR4-40^T formed lineage with Marinirhabdus gelatinilytica NH 83^T. Filled circle is used for illustrating same topology in phylogenetic tree using NJ, maximum-likelihood (ML) and maximum-parsimony (MP) methods and unfilled circles show that same positions in NJ and ML methods. Numbers indicate bootstrap value over 50%. Crocinitomix catalasitica IFO 15977^T was used for outgroup and bar, 0.02 substitutions per nucleotide site.

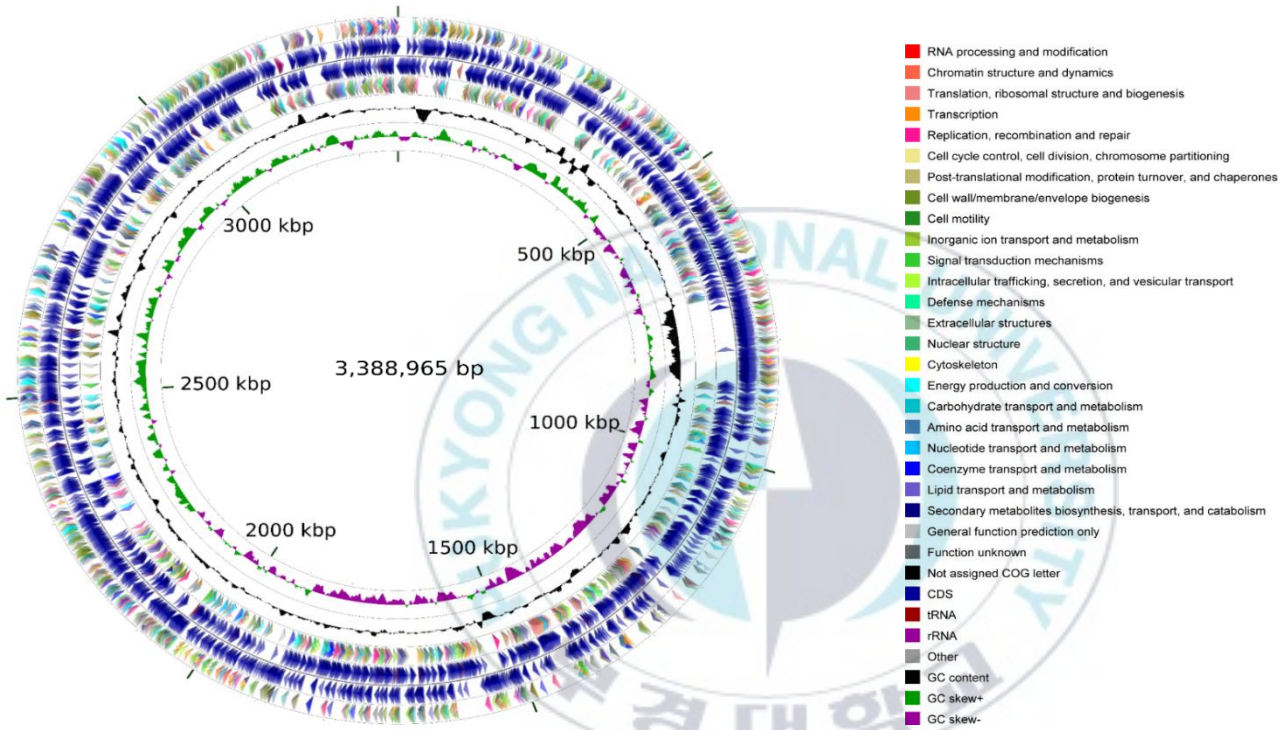

Figure 2-2. The circular genome map of RR4-40^T Total length of strain RR4-40^T is 3,388,965 bp and GC skew (green and purple), G+C content (black).

Table 2-1. Genomic features of strain RR4-40^{T}.

Feature	Value
Genome size (bp)	3,388,965
G+C content (%)	37.4
Total gene	3,018
Total CDS	2,973
Genes assigned to COGs	1,902
rRNA (5S, 16S, 23S)	2, 2, 2
tRNA	35
ncRNA	4
Pseudogene	14

Table 2-2. Values of average nucleotide identity (ANI), digital DDH and average amino acid identity (AAI) among RR4-40^{T} and related taxa. All values of related taxa were compared with RR4-40^{T}.

Strains	Total length (bp)	G+C content (mol%)	ANI Value (%)	DDH (%)	AAI (%)
RR4-40^{T}	3,388,440	37.4	100	100	100
Marinirhabdus gelatinilytica	3,280,320	41	72.91	18.2	76.84
Marinirhabdus ophiurae	3,299,700	41	71.3	18.1	68.82
Ulvibacter litoralis	3,791,340	36.7–38.0	71.3	18.7	67.82
Aureitalea marina	3,072,240	48.1	67.56	20.5	63.44

2.3.2 Phenotypic and chemotaxonomic characteristics

Shape of RR4-40^T is irregular short-rod and cell size is 0.6–0.7X0.8–2 μm. Novel bacterium is gram-negative, aerobic, non-spore forming and motility was not observed through hanging drop, wet-mount and flagella staining methods. Oxidase and catalase tests of cells are negative. Colonies of RR4-40^T on MA form yellow-pigmented, circular, glister, slightly convex shape and 1–2 mm in diameter. Cells grow at 15–35 °C, pH 5.0–9 and 0.5–6 % (w/v). Optimal growth occurs at 25–30 °C, pH 8.5 and 3 % (w/v). In chemical features, RR4-40^T has ability to hydrolyze gelatin however, urea and esculin are not degraded. Novel taxon is negative for nitrate reduction, indole production, arginine dihydrolase, β-galactosidase, lysine decarboxylase, H_2S production, tryptophan deaminase and acetoin production in API 20NE and 20E kits. Enzymes released from RR4-40^T are alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, and naphtol-AS-BI-phosphohydrolase, trypsin and α-chymotrypsin are weakly on the other hand, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannose and α-fucosidase are absent in RR4-40^T. Acid is produced from potassium 5-ketogluconate as sole carbon source and distinct phenotypic and chemical characteristics of RR4-40^T and relative taxa are explained (Table 5). The major fatty acids of RR4-40^T (≥10%) are iso-$C_{15:0}$ (31.34%), $C_{17:0}$ 3-OH (13.65%), iso-$C_{17:0}$ 3-OH (10.61%) and iso-$C_{15:1}$ G (10.38%) and minorities are

summed feature 3 ($C_{16:1}$ ω7c and/or $C_{16:1}$ ω6c; 8.08%) and iso-$C_{16:0}$ (6.11%). Percentages of fatty acids from RR4-40^T and six reference strains are presented in table 2-4. Quinone of RR4-40^T is MK-6 and polar lipids comprise diphosphoglycerol, phosphoglycerol, phosphatidylethanolamine, two unidentified glycolipids, unidentified phospholipid, two unidentified aminolipids and one unidentified lipid using 10% (w/v) ethanolic molybdophosphoric acid. Detailed features were presented in table 2-3.

Table 2-3. Phenotypic and biochemical features of strain RR4-40^T and reference strains. Table shows different characteristics of six taxa and strain RR4-40^T. All data are from previous studies. Strain: 1, **RR4-40^T**; 2, *Marinirhabdus gelatinilytica* NH83^T (Wu, Xamxidin et al. 2016); 3, *Aureisphaera galaxeae* 04OKA003-7^T (Yoon, Yasumoto-Hirose et al. 2015); 4, *Marixanthomonas ophiurae* KMM 3046^T (Romanenko, Uchino et al. 2007); 5, *Ulvibacter litoralis* KMM 3912^T (Nedashkovskaya, Kim et al. 2004); 6, *Aureitalea marina* S1-66^T (Park, Yoshizawa et al. 2012); 7, *Jejudonia soesokkakensis* SSK1-1^T (Park, Lee et al. 2013). +, positive; –, negative; w, weak positive; ND, No available data. *PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GL, glycolipid; APL, aminophospholipid; UL, unidentified lipid; LPE, lysophosphatidylethanolamine; SL, sphingolipid.

Biochemical features	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Cell shape	Irregular short rod	Rod	Coccus	Rod	Rod	Irregular rod	Rod
Length X width (μm)	0.8–2 X 0.6–0.7	2.5–4.5 X 0.5–0.8	0.4–0.5	2.2–2.8 X 0.4–0.5	2.5–7.3 X 0.4–0.5	0.5–6 X 0.4–0.6	0.7 – (>10.0) X 0.2–0.5
Growth conditions							
NaCl (%, w/v; optimum)	0.5–6 (3)	0.5–7.5 (2.0–5.0)	2.5–5.5 (ND)	1.0–12 (3–5)	1–6 (1.5–2)	1–4 (ND)	1–5 (2.0)
pH (optimum)	pH 5.0-9.5 (pH 8.5)	pH 6.5-7.5 (pH 7.0)	pH 7–9 (pH 7.0)	pH 6–10 (pH 6.5–8.5)	pH 5.5–10.0 (pH 7.1–8.3)	pH 6.0–9.0 (ND)	pH 7.0–7.5 (ND)
Temperature (°C, optimum)	15–30 (25–28)	4–37 (30)	20–30 (28)	5–32 (25–28)	4–36 (21–23)	15–30 (20–25)	10–30 (30)
Chemical properties							
Oxidase	–	–	–	+	+	+	+
Catalase	–	+	–	+	+	–	+
Urease	–	–	+	–	–	–	–
Gelatinase	+	+	+	+	+	+	–

Nitrate reduction	–	–	–	–	+	+	–
Enzyme activity							
Esterase (C4)	+	+	–	+	–	–	–
Esterase lipase (C8)	+	+	–	+	+	–	+
Trypsin	w	+	–	–	–	+	–
Cystine arylamidase	+	+	–	+	–	+	–
α-chymotrypsin	w	–	–	+	+	+	–
Acid production							
D-fructose	–	+	–	–	–	+	–
Polar lipids	PE, DPG, PG, GL, UAL, UL, PL	PE, AL, APL, UL	PE, AL, UL	PE, LPE, APL, SL, AL, UL	PE, AL, GL, UL	PE, AL, GL, UL	PE, UL
G+C content (mol%)	40T.38	41.0	41.0	37.3	36.7–38.0	48.1	39.9

Table 2-4. Percentage of fatty acid of strain RR4-40^T and relative six strains.

Strain: 1, **RR4-40^T**; 2, *Marinirhabdus gelatinilytica* NH83^T (Wu, Xamxidin et al. 2016); 3, *Aureisphaera galaxeae* 04OKA003-7^T (Yoon, Yasumoto-Hirose et al. 2015); 4, *Marixanthomonas ophiurae* KMM 3046^T (Romanenko, Uchino et al. 2007); 5, *Ulvibacter litoralis* KMM 3912^T (Nedashkovskaya, Kim et al. 2004); 6, *Aureitalea marina* S1-66^T (Park, Yoshizawa et al. 2012); 7, *Jejudonia soesokkakensis* SSK1-1^T (Park, Lee et al. 2013). –, Not detected; ND, No available data. *Summed feature 3 comprises $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$ that cannot be separated by MIDI system.

Fatty acid	1	2	3	4	5	6	7
Saturated							
$C_{15:0}$	–	4.8	2.4	–	3.2	4.6	ND
$C_{16:0}$	0.62	1.1	6.9	1.9	3	0.9	4
Branched							
Iso-$C_{15:0}$	31.34	39	2.9	29.9	26.2	26.4	18.1
Anteiso-$C_{15:0}$	3.62	3.1	2.8	1.4	3.4	3.8	11.7
Iso-$C_{16:0}$	6.11	7.1	1.8	1.6	2.9	5.3	11.1
Unsaturated							
Iso-$C_{15:1}$ G	10.38	6.4	7.2	7.7	16.4	17.8	4.1
Iso-$C_{16:1}$ G	3.04	0.8	–	tr	1.9	2.9	1.3
$C_{17:1}\omega6c$	1.45	0.6	0.6	ND	2.9	tr	1.1
Hydroxy							
$C_{15:0}$ 2-OH	0.92	0.5	3.3	tr	1	0.8	1.1
Iso-$C_{15:0}$ 3-OH	3.9	3.6	3.1	7	2.5	3.3	1.1
Iso-$C_{16:0}$ 3-OH	10.61	4.6	41.2	4.2	2	2.9	9.3
Iso-$C_{17:0}$ 3-OH	13.65	11	16.4	31.1	30.2	14.2	6.7
$C_{17:0}$ 2-OH	1.58	0.5	1.1	1.1	2.5	2.0	9.4
Summed feature*							
3	8.08	6	6.9	5.9	3.8	7.8	4.7

2.4 Conclusion

Phenotypic, phylogenetic and biochemical characteristics such as fatty acid composition and polar lipids and genomic distance between novel bacterium and reference strains indicates that strain RR4-40^{T} is novel bacterium distinguished from other taxa belonging to the *Flavobacteriaceae*. Values of ANI, dDDH and AAI calculated distances of complete genome between RR4-40^{T} and four related strains have lower percentage (Table 2-2).

2.5 References

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2.6 국문요약

해수 순환 여과 시스템에서 분리된 균주 RR4-40 는 다상적 방법을 통하여 *Flavobacteriaceae* 과의 멤버로 분류되었다. RR4-40 의 계통학적, 유전적, 생화학적 특성을 6 개의 참조균주와 비교하여 분석하였다. 균주 RR4-40 은 호기성의, 그람음성, 운동성이 없는 불규칙적인 단간균이다. RR4-40 은 카탈라아제와 옥시데이즈 검사에서 모두 음성이 나왔으며, 생장은 15–35 °C (최적온도: 25–30 °C), pH 는 5-9.5 (최적 pH: pH 8.5), 0.5–6 %의 NaCl (최적 NaCl 농도: 3%; w/v)에서 일어난다. 16S rRNA 유전자를 기반으로 한 계통학적인 특성은 RR4-40 이 *Marinirhabdus gelatinilytica* NH83^{T} (94.16%)와 가장 유사하다는 사실을 보여주었다. 유전체 염기서열은 RR4-40 이 참조균주와의 계통학적 거리를 보여준다. 우리는 이러한 결과를 통해 균주 RR4-40 이 *Flavobacteriaceae* 과에 속하는 새로운 속이라는 것을 제안한다.

감사의 글

이 논문을 쓸 수 있게 2 년간 저를 학문적으로 이끌어주신 김경호 교수님, 대학원 수업에서 부족했던 저를 성장하게 해주시고 부족한 석사 논문을 검토해 주시며 아낌없이 조언 해주신 이명숙 교수님, 김영태 교수님, 김군도 교수님, 최태진 교수님, 전용재 교수님께 감사드립니다.

또한 실험실 생활을 함께 해주시며 저에게 선배로써 조언을 아끼지 않으셨던 영삼 선배한테도 감사드립니다. 실험실 생활을 즐겁게 보낼 수 있게 해준 상언 선배, 현경이, 지은이, 민주한테도 고맙습니다.

다른 연구실임에도 불구하고 동료로서 때로는 학교 선배로서, 후배로서 고민상담과 격려를 아끼지 않았던 유승현, 아름이 언니, 중인이 오빠, 준호 오빠, 예린이, 미선이 한테도 고맙습니다.

제가 힘든 일이 있을 때마다 위로와 격려를 아끼지 않았던 저의 친구들과 언니들 감사합니다. 학교 일에 모르는게 있을 때마다 짜증 한 번 안 내고 도와줬던 종욱이 오빠, 혜숙이 언니한테도 감사합니다.

마지막으로 끝이 보이지 않는 공부를 마칠 수 있게 변함없는 지지와 자랑스럽다 해 주셨던 저희 부모님과 언니한테도 감사드립니다.