Thesis for the Degree of Master of Science

Uncultured bacterial diversity and genomic analysis of two strains from a seawater recirculating aquaculture system

by

Da Eun Lee

Department of Microbiology

The Graduate School

Pukyong National University

August 2016

Uncultured bacterial diversity and genomic analysis of two strains from a seawater recirculating aquaculture system
(해수 순환여과양식시스템의 미생물 다양성 및 분리된 두 균주의 유전체 분석)

Advisor: Prof. Kyoung-Ho Kim

by

Da Eun Lee

A thesis submitted in partial fulfillment of the requirements

for the degree of

Master of Science

in Department of Microbiology, The Graduate School,

Pukyong National University

August 2016

Uncultured bacterial diversity and genomic analysis of two strains from a seawater recirculating aquaculture system

A dissertation

by

Da Eun Lee

Approved by:

(Chairman) Young-Jae Jeon

(Member) Tae-Jin Choi

(member) Kyoung-Ho Kim

August 2016

Contents

ABSTRACT

The recirculating aquaculture system (RAS), one of the land-based facilities, uses a method of purifying and recycling water through the biological filtration tanks. The bacterial communities present in the rearing water and sludge in the systems involved in the purification process. In this study, we analyzed the bacterial diversity present in a seawater RAS and analyzed the genomes of two strains isolated from an experimental seawater RAS. The seawater RAS was composed of six tanks and operated at nine conditions (combinations of three temperature and three salinity) during the experimental period. The bacterial diversity was analyzed using 16S rRNA amplicon sequencing. Most common bacterial groups were *Proteobacteria* and *Bacteroidetes* (total 67.2 to 99.4%). Among these six RAS tanks, the bacterial community present in three tanks including packed bed biofilters, mesh biofilters and maturation biofilters showed higher bacterial diversity than those from other RAS tanks such as aerated biofilers, floating bed biofilters and fish tanks with 97% operational taxonomic units (OUT) level. Two major taxonomic groups, family *Rhodobacteraceae* and *Flavobacteriaceae*, were widely distributed in the samples. Nitrifying bacteria were also detected frequently and abundantly in the three biofilters. Among them, denitrifying bacteria that contribute to removal of ammonia in the rearing water are especially worth to be investigated for increasing efficiency of purification process in RAS. The genome sequence analysis and its annotation were performed for the two denitrifying strains, RR3-28 and RR3-57, which were isolated from mesh biofilter samples. The strain RR3-57 showed 16S rRNA sequence similarity of 96.22% compared with the *Halioglobus pacificus* S1-72(T). The whole genome was consisted 5,003,575 base pair length, average G+C content 57.5 mol%. It contains 4,627 coding genes and 49 RNAs. The other strain RR3-28 showed 95.45% 16S rRNA sequence similarity compared

with the *Nitratireductor basaltis* J3(T). The 3,513,368 base pair genome of strain RR3-28 contains 3,410 coding genes and 43 RNAs with an average G+C content of 58.3 mol%. The isolates, RR3-28 and RR3-57 harbored 18 and 21 predicted genes associated with denitrification, respectively. Comparative genomic analysis of strain RR3-28 was performed with five previously reported *Nitratireductor* genome sequences (NCBI genome ID: 13983, 15054, 15055, 15056, 15083), but RR3-58 was not performed that because there was no previously reported genome sequence of the genus *Halioglobus*. The present study summarize the bacterial diversity in a seawater RAS for understanding the roles of bacterial communities in the system. Furthermore, genomic sequence analysis of two denitrifying strains isolated from the system would contribute to the further understanding with of the potential denitrifying strategies on genome level.

INTRODUCTION

A recirculating aquaculture system (RAS) is to re-use the rearing water by circulation, filtration, and purification through filtration tanks in the aquaculture facility without waste of rearing water. It can resolve coastal pollution, the disadvantage of other land-based aquaculture, caused by outflow of waste rearing water. In land-based aquaculture, fishes are reared under the artificially controlled condition including temperature, salinity, etc., so it is possible to enhance the economic efficiency and productivity (Zohar et al. 2005). Recently, due to the unpredictable changes of environment in the coastal area with the increasing occurrence of disasters like red tides, the advantage of land-based aquaculture system was unveiled and many have much interest in RAS accordingly.

The biofilters used for purification of rearing water in RAS are closely related to microorganisms and bacterial communities mainly affects purification ability of the biofilters (Auffret et al. 2013). Of these, heterotrophic bacteria remove organic carbons from unused fish feed and fish feces. Nitrifying and denitrifying bacteria remove inorganic nitrogen after organic carbon degradation (Brown et al. 2013). Nitrifying bacteria (i.e. ammonium and nitrite oxidizing bacteria) were known to exist on aerobic condition and denitrifying bacteria on anaerobic condition while forming a biofilm in the biological filtration tank of the RAS (Gao et al. 2012). Therefore, the effects of increasing efficiency of marine fish production mediated by aerobic and anaerobic microorganisms in the filtration process of biological filtration tank of the RAS can be expected. Bacteria communities formed in biological filtration tanks of RAS affect their community structure and diversity by various factors of the system itself (Im gi et al. 1997), and water quality and microbial community of the RAS also affects the quality of farmed fish (Interdonato 2012). So, in-depth understanding of the relationship

between the RAS biofilter systems and the microbial communities is essential in order to improve the efficiency, stability and economic of the RAS process. For this purpose, many researches were proceeded to investigate identification for the dominant heterotrophic bacteria in the RAS (Leonard et al. 2000; Michaud et al. 2009), microbial community diversity and physiology of the RAS biofilter (Schreier et al. 2010; Gao et al. 2012), and a variety of nitrifying bacteria involved in the nitrification of ammonia removal (Tal et al. 2003; van Kessel et al. 2010; van Kessel et al. 2011), but there is still lack of studies sufficient enough to understand the microbial diversity in the RAS. For RAS microbial community diversity studies, 16S rRNA gene clone library methods (Sugita et al. 2005; Itoi et al. 2006; Gao et al. 2012; Zhu et al. 2012), DGGE (Matos et al. 2011; Martins et al. 2013; Wold et al. 2014), and pyrosequencing (Martins et al. 2013; Ruan et al. 2015) are mainly applied.

Sequencing technology has developed rapidly recently and new platforms had been announced (Quail et al. 2012). Whole genome sequencing is a method for analyzing the information of bacteria genome efficiently (Gupta 2008). For more facile assembly of the long reads, an automated approach, the Pacific Biosciences RS (PacBio) platform was used (English et al. 2012).

In this study, to analyze the diversity of bacterial communities and genome sequencing data of some nitrifying bacteria exists in RAS filtration tank, we used molecular biological methods mentioned above, and the results of this study will be provided as basic sources for RAS microbial research in future.

REFERENCES

Zohar, Y., Y. Tal, et al. 2005. "10 Commercially Feasible Urban Recirculating Aquaculture: Addressing the Marine Sector." *Urban Aquaculture. CAB International*: 159-171.

Auffret, M., É. Yergeau, et al. 2013. "Impact of water quality on the bacterial populations and off-flavours in recirculating aquaculture systems." *FEMS microbiology ecology* **84**(2): 235-247.

Brown, M. N., A. Briones, et al. 2013. "Ammonia-oxidizing archaea and nitrite-oxidizing nitrospiras in the biofilter of a shrimp recirculating aquaculture system." *FEMS microbiology ecology* **83**(1): 17-25.

Gao, X.-Y., Y. Xu, et al. 2012. "Bacterial diversity, community structure and function associated with biofilm development in a biological aerated filter in a recirculating marine aquaculture system." *Marine Biodiversity* **42**(1): 1-11.

Im Gi, J., S. Maeng Hyun, et al. 1997. "해수 순환 여과 양식 시설의 여과 재료별 암모니아 제거 능력." JOURNAL OF AQUACULTURE **10**(3): 261-271.

Interdonato, F. 2012. Recirculating aquaculture system (RAS) biofilters: focusing on bacterial communities complexity and activity, Università degli studi di Messina.

Itoi, S., A. Niki, et al. 2006. "Changes in microbial communities associated with the conditioning of filter material in recirculating aquaculture systems of the pufferfish Takifugu rubripes." Aquaculture **256**(1): 287-295.

Keuter, S. 2012. Characterization of nitrifying bacteria in marine recirculation aquaculture systems with regard to process optimization. Hamburg, Staats- und Universitätsbibliothek Hamburg.

Leonard, N., J. P. Blancheton, et al. (2000). "Populations of heterotrophic bacteria in an experimental recirculating aquaculture system." Aquacultural Engineering **22**(1-2): 109-120.

Martins, P., D. F. Cleary, et al. (2013). "Molecular analysis of bacterial communities and detection of potential pathogens in a recirculating aquaculture system for Scophthalmus maximus and Solea senegalensis."

Matos, A., M. T. Borges, et al. (2011). "A molecular and multivariate approach to the microbial community of a commercial shallow raceway marine recirculation system operating with a moving bed biofilter." Aquaculture Research **42**(9): 1308-1322.

Michaud, L., A. Lo Giudice, et al. (2009). "Phylogenetic characterization of the heterotrophic bacterial communities inhabiting a marine recirculating aquaculture system." Journal of applied microbiology **107**(6): 1935-1946.

Ruan, Y.-J., X.-S. Guo, et al. (2015). "Bacterial Community Analysis of Different Sections of a Biofilter in a Full-Scale Marine Recirculating

Aquaculture System." North American Journal of Aquaculture 77(3): 318-326.

Schreier, H. J., N. Mirzoyan, et al. (2010). "Microbial diversity of biological filters in recirculating aquaculture systems." Curr Opin Biotechnol 21(3): 318-325.

Sugita, H., H. Nakamura, et al. (2005). "Microbial communities associated with filter materials in recirculating aquaculture systems of freshwater fish." Aquaculture 243(1): 403-409.

Tal, Y., H. J. Schreier, et al. (2009). "Environmentally sustainable land-based marine aquaculture." Aquaculture 286(1-2): 28-35.

Tal, Y., J. E. Watts, et al. (2006). "Anaerobic ammonium-oxidizing (anammox) bacteria and associated activity in fixed-film biofilters of a marine recirculating aquaculture system." Applied and environmental microbiology 72(4): 2896-2904.

Tal, Y., J. E. M. Watts, et al. (2003). "Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system." Aquaculture 215(1-4): 187-202.

van Kessel, M. A., H. R. Harhangi, et al. (2011). "Anammox bacteria in different compartments of recirculating aquaculture systems." Biochemical Society Transactions 39(6): 1817.

van Kessel, M. A. H. J., H. R. Harhangi, et al. (2010). "Biodiversity of N-cycle bacteria in nitrogen removing moving bed biofilters for freshwater recirculating aquaculture systems." Aquaculture 306(1-4): 177-184.

Wold, P.-A., A. B. Holan, et al. (2014). "Effects of membrane filtration on bacterial number and microbial diversity in marine recirculating aquaculture system (RAS) for Atlantic cod (Gadus morhua L.) production." Aquaculture 422-423: 69-77.

Zhu, P., Y. Ye, et al. (2012). "Characterizing the structural diversity of a bacterial community associated with filter materials in recirculating aquaculture systems of Scortum barcoo." Canadian journal of microbiology 58(3): 303-310.

Quail, M. A., M. Smith, et al. (2012). "A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers." BMC Genomics 13(1): 1.

Gupta, P. K. (2008). "Single-molecule DNA sequencing technologies for future genomics research." Trends in biotechnology 26(11): 602-611.

English, A. C., S. Richards, et al. (2012). "Mind the gap: upgrading genomes with Pacific Biosciences RS long-read sequencing technology." PLoS One 7(11): e47768.

CHAPTER 1. Uncultured bacterial diversity in a seawater recirculating aquaculture system revealed by 16S rRNA gene amplicon sequencing

[1]

ABSTRACT

Bacterial diversity in a seawater recirculating aquaculture system (RAS) was investigated using 16S rRNA amplicon sequencing to understand the roles of bacterial communities in the system. The RAS was operated at nine different combinations of temperature (15° C, 20° C, and 25° C) and salinity (20‰, 25‰, and 32.5‰). Samples were collected from five or six RAS tanks (biofilters) for each condition. Fifty samples were analyzed. *Proteobacteria* and *Bacteroidetes* were most common (sum of both phyla: 67.2% to 99.4%) and were inversely proportional to each other. Bacteria that were present at an average of ≥ 1% included Actinobacteria (2.9%) Planctomycetes (2.0%), Nitrospirae (1.5%), and *Acidobacteria* (1.0%); they were preferentially present in packed bed biofilters, mesh biofilters, and maturation biofilters. The three biofilters showed higher diversity than other RAS tanks (aerated biofilters, floating bed biofilters, and fish tanks) from phylum to operational taxonomic unit (OTU) level. Samples were clustered into several groups based on the bacterial communities. Major taxonomic groups related to family *Rhodobacteraceae* and *Flavobacteriaceae* were distributed widely in the samples. Several taxonomic groups like [*Saprospiraceae*], *Cytophagaceae*, *Octadecabacter*, and *Marivita* showed a cluster-oriented distribution. *Phaeobacter* and *Sediminicola*-related reads were detected frequently and abundantly at low temperature. Nitrifying bacteria were detected frequently and abundantly in the three biofilters.

This paper was published in Journal of Microbiology (2016) Vol. 54, No. 4, pp. 296-304.

Phylogenetic analysis of the nitrifying bacteria showed several similar OTUs were observed widely through the biofilters. The diverse bacterial communities and the minor taxonomic groups, except for *Proteobacteria* and *Bacteroidetes*, seemed to play important roles and seemed necessary for nitrifying activity in the RAS, especially in packed bed biofilters, mesh biofilters, and maturation biofilters.

Keywords: bacterial diversity, recirculating aquaculture system, biofilter, amplicon sequencing, 16S rRNA gene

INTRODUCTION

Recirculating aquaculture systems (RASs) have been recognized for their potential in aquaculture as a way of overcoming limited water supplies, increasing production capacity, reducing heat loss, and minimizing waste effluent (Losordo and Hobbs, 2000). Carbon and nitrogen compounds from fish manure and undigested feed are metabolized in a RAS by diverse microbial communities. Carbon compounds are degraded by heterotrophic bacteria and nitrogen-containing organic compounds are degraded with nitrogen released as ammonia. Even low levels of ammonia are a detriment in aquaculture; lethal concentrations, expressed as median 96 h LD50, of 1.7 mg/L for seabass and 2.5-2.6 mg/L for seabream and turbot (Ruyet et al., 1995). Ammonia is converted to nitrite, which is converted to nitrate, by ammonia- and nitrite-oxidizing bacteria, respectively. Most studies have focused on the role of biofilters in mediating nitrogen cycles (Schreier et al., 2010) with the aim of reducing ammonia concentration in the fish tank. Little is known of the overall bacterial diversity in RAS. A recent study that used bar code pyrosequencing to investigate the overall diversity of RAS bacterial communities used to rear turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis*) demonstrated differences in the bacterial communities in the two systems (Martins et al., 2013). Using a combination of bar code primers and high throughput sequencing, it is possible to obtain thousands of 16S rRNA gene sequences per sample from dozens of samples, which allows the determination of uncultured bacterial diversity in given environments (Binladen et al., 2007). We applied 16S rRNA gene amplicon sequencing to investigate the bacterial diversity in an experimental RAS system rearing convict groupers (*Epinephelus septemfasciatus*). Knowledge of bacterial diversity depending on operating conditions is important to enhance the design and operational efficiency of biofilters, but the data are limited. We

investigated the bacterial diversity in different biofilter types during different operating conditions of salinity and water temperature in an experimental RAS using 16S rRNA gene amplicon sequencing.

MATERIALS AND METHODS

RAS operational conditions

The RAS used in this study is depicted schematically in Fig. 1. In the packed bed biofilter (A; 1.5 m length, 0.8 m width, and 0.6 m height), solid materials in the effluent from a fish tank (F; 1.5 m diameter, 1 m height, and 0.65 m water depth) were precipitated using low pressure hydro-cyclone process and water was treated while passing through columns containing plastic media that served as the colonization surface. In the aerated biofilter (B; 1.2 m diameter and 2.4 m height), an air blower supplied air bubbles (1 m3/min) to remove carbon dioxide (CO2) and dispersed solids into fine particles. In the fluidized bed biofilter (C; 1.2 m diameter, 2 m height), the plastic medium was fluidized by aeration at 0.8 m3/min. At the sixth sampling, the aerated biofilter was removed from the system. The mesh biofilter (D; 2 m length, 1 m width, 1.6 m height) was composed of 50 pieces of mesh media (1 m × 1 m) to which microbes attached. The maturation biofilter (E; 1.5 m diameter, 1 m height, and 0.65 m water depth) had the same dimensions as the fish tank, which aided in the acclimation of microbes prior to entering the fish tank used to rear up to 50–70 kg of *E. septemfasciatus*. During the experimental periods, concentration of ammonia, nitrite, nitrate, and CO2 in the experimental sea water RAS was maintained at less than 0.5 mg/L, 0.08 mg/L, 30 mg/L, and 6 mg/L, respectively, within the pH range of 6.5–7.9. The 60%–85% nitrification rates determined here were similar and comparable to the rates in RAS systems reported from previous studies (Rusten et al., 2006; Pfeiffer and Wills, 2011; Terjesen et al., 2013).

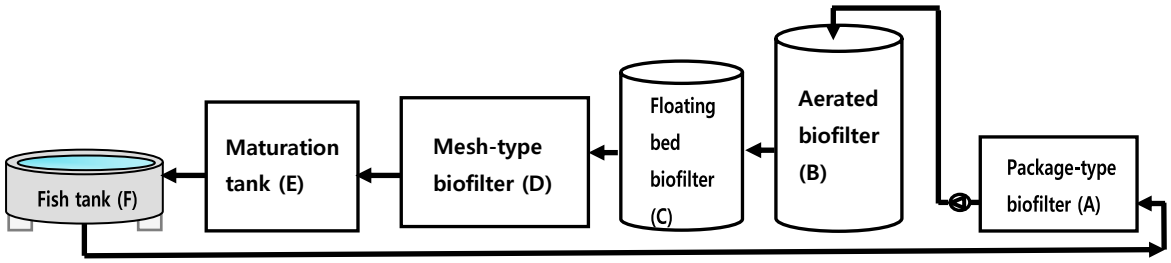


Fig. 1. Schematic representation of the experimental recirculating aquaculture system. The letter in parentheses indicates each biofilter.

Sample collection and DNA extraction

The experimental RAS was developed and operated in the National Institute of Fisheries Science to rear convict groupers (*E. septemfasciatus*). Samples were collected during the different operating conditions involving nine combinations of temperature (15° C, 20° C, and 25° C) and salinity (20‰, 25‰, and 32.5‰) (Table 1). Water or slurry samples were collected from the packed bed biofilter (A), aerated biofilter (B), floating bed biofilter (C), mesh biofilter (D), maturation tank (E), and fish tank biofilter (F) in the experimental recirculating aquaculture system using sterilized bottles. Samples were transferred on ice to the laboratory and processed immediately. After the fifth sampling, the aerated biofilter was removed so that no sample was available. In total, 50 samples were collected. Bacteria in the water samples were collected with a 0.2 μm membrane filter (Sartorius). Bacteria in slurry samples were collected by centrifugation at 4,500 rpm for 10 min. A soil DNA extraction kit (Intron) was used for DNA extraction.

Chemical analysis of water quality

Ammonia, nitrite, nitrate, and CO2 concentrations were measured colorimetrically with HachTM reagents by the salycilate method (Hach #8155), diazotization method (Hach #8507), cadmium reduction method (Hach #8039), and digital titration using sodium hydroxide (Hach #8205), respectively, which are specific for seawater samples (Verbeeten et al., 1999). Dissolved oxygen and pH levels were determined using YSI 85-10FT and a model HQ 30d pH meter (Hach), respectively. The respective levels were maintained at 5.8- 7.2 mg/L and 7.1-8.1.

Bar code PCR and amplicon sequencing

The V1 to V2 regions of the 16S rRNA genes was amplified using primer sets containing oligonucleotide tags (27F: 5-X- TC-AGAGTTTGATCCTGGCTCAG-3Gand 338R: 5-X-CA-TGCTGCCTCCCGTAGGAGT-3' ; × indicates tag sequences composed of various combinations of eight nucleotides). Each primer (10 pmol) was added to PCR premix (Accupower PCR premix, Bioneer). After an initial denaturation at 94° C for 7 min, 32 cycles of PCR (denaturation at 94° C for 30 sec, annealing at 60° C for 30 sec, and extension at 72° C for 45 sec) and final extension at 72° C for 10 min were performed. PCR products were pooled and sequenced with the Ion Torrent platform (Life Technologies).

Bioinformatic analysis of reads

After high throughput sequencing, sequences from 300 bp to 400 bp with average quality score more than 25 were screened with the Mothur program (Schloss et al., 2009). Reads were sorted based on tags and the primer and tag sequences were excised using the QIIME program. (Kuczynski et al., 2012). Reverse complement reads were changed to the same orientation. The chimer.uchime (Edgar et al., 2011) of the Mothur program (Schloss et al., 2009) was used to check chimera sequences. QIIME v1.8.0 was used for analysis of reads (Kuczynski et al., 2012). The uclust approach and an open-reference operational taxonomic unit (OTU) picking strategy of the QIIME package was used for OTU clustering and representative sequence determination based on 97% sequence similarity. Taxonomic assignment was carried out by comparing with the 97_otus and the 97_otu_taxonomy files of the gg_13_08 version of the Greengenes database (McDonald et al., 2012). Sequences assigned to chloroplasts and mitochondria were discarded. Values

of the Chao1, Shannon, and Simpson diversity index were determined with subsamples adjusted to contain the same number of sequences.

Phylogenetic analysis of representative sequences

Some representative sequences were compared against the database of the Ribosomal Database Project (Cole et al., 2014) and the EzTaxon server (http://www.ezbiocloud.net/) (Chun et al., 2007), and reference sequences were collected for the construction of phylogenetic trees. A neighbor joining tree was constructed with the MEGA6 program (Tamura et al., 2013); the Kimura 2 parameter method (Kimura, 1980) was used to calculate a distance matrix.

Table 1. Pyrosequencing results and diversity indexes

Sample ID	Sampling time	Type of tank	Temperature (°C)	Salinity (‰)	Total reads	No. reads subsampled	OTUs	Chao1	Shannon	Simpson
A1	1st	packed bed biofilter	15	32.5	7099	1200	541	1,272	8.36	0.99
B1	1st	aerated biofilter	15	32.5	6541	1200	134	339	3.32	0.66
C1	1st	floating bed biofilter	15	32.5	3206	1200	378	1,055	5.72	0.84
D1	1st	mesh biofilter	15	32.5	5249	1200	365	980	5.67	0.85
E1	1st	maturation tank	15	32.5	3879	1200	224	720	3.83	0.65
F1	1st	fish tank	15	32.5	2924	1200	107	344	2.12	0.41
A2	2nd	packed bed biofilter	20	32.5	4307	1200	367	1,000	6.98	0.98
B2	2nd	aerated biofilter	20	32.5	2021	1200	349	817	6.99	0.98
C2	2nd	floating bed biofilter	20	32.5	7490	1200	346	963	6.75	0.97
D2	2nd	mesh biofilter	20	32.5	12786	1200	545	1,660	8.20	0.99
E2	2nd	maturation tank	20	32.5	11898	1200	380	1,017	6.86	0.97
F2	2nd	fish tank	20	32.5	3550	1200	162	424	4.31	0.83
A3	3rd	packed bed biofilter	20	25	2784	1200	470	1,195	7.92	0.99
B3	3rd	aerated biofilter	20	25	5068	1200	167	543	3.95	0.77
C3	3rd	floating bed biofilter	20	25	3233	1200	412	1,120	7.29	0.98
D3	3rd	mesh biofilter	20	25	15923	1200	451	1,284	7.24	0.97
E3	3rd	maturation tank	20	25	12604	1200	150	573	4.05	0.85
F3	3rd	fish tank	20	25	5118	1200	140	388	4.04	0.87
A4	4th	packed bed biofilter	20	20	38674	1200	440	1,245	7.72	0.99
B4	4th	aerated biofilter	20	20	42370	1200	205	712	4.91	0.90
C4	4th	floating bed biofilter	20	20	41870	1200	164	597	4.64	0.89
D4	4th	mesh biofilter	20	20	28614	1200	470	1,124	7.94	0.99
E4	4th	maturation tank	20	20	13150	1200	429	990	7.79	0.99
F4	4th	fish tank	20	20	9731	1200	141	487	4.26	0.87
A5	5th	packed bed biofilter	25	20	8969	1200	406	1,000	7.51	0.99
B5	5th	aerated biofilter	25	20	15900	1200	225	647	4.72	0.84
C5	5th	floating bed biofilter	25	20	7378	1200	142	448	3.38	0.73
D5	5th	mesh biofilter	25	20	40262	1200	337	913	6.77	0.98
E5	5th	maturation tank	25	20	84399	1200	287	853	5.80	0.92
F5	5th	fish tank	25	20	167676	1200	141	492	3.34	0.71
A6	6th	packed bed biofilter	25	25	3991	1200	359	842	7.15	0.98
C6	6th	floating bed biofilter	25	25	9266	1200	186	472	4.49	0.83
D6	6th	mesh biofilter	25	25	12202	1200	302	753	6.64	0.97
E6	6th	maturation tank	25	25	5959	1200	303	702	6.84	0.98
F6	6th	fish tank	25	25	9125	1200	107	255	3.38	0.74
A7	7th	packed bed biofilter	25	32.5	1695	1200	410	872	7.36	0.98
C7	7th	floating bed biofilter	25	32.5	3006	1200	311	653	6.50	0.97
D7	7th	mesh biofilter	25	32.5	2598	1200	328	802	6.81	0.98
E7	7th	maturation tank	25	32.5	1207	1200	389	1,024	7.19	0.98
F7	7th	fish tank	25	32.5	33132	1200	147	408	4.78	0.93
A8	8th	packed bed biofilter	15	25	15322	1200	256	686	5.84	0.95
C8	8th	floating bed biofilter	15	25	37744	1200	182	430	5.01	0.93
D8	8th	mesh biofilter	15	25	6250	1200	440	984	7.80	0.99
E8	8th	maturation tank	15	25	3946	1200	494	1,203	8.04	0.99
F8	8th	fish tank	15	25	7468	1200	114	295	4.08	0.89
A9	9th	packed bed biofilter	15	20	4664	1200	194	511	4.67	0.90
C9	9th	floating bed biofilter	15	20	9620	1200	195	538	5.19	0.93
D9	9th	mesh biofilter	15	20	30113	1200	529	1,209	8.34	0.99
E9	9th	maturation tank	15	20	29088	1200	152	427	4.47	0.90
F9	9th	fish tank	15	20	10651	1200	162	418	5.00	0.93

RESULTS AND DISCUSSION

Rarefaction curves and diversity indexes

Fifty samples obtained from the combinations involving three different temperatures and three different salinities were analyzed. Rarefaction curves revealed different bacterial diversity profiles. All samples of F, most of B, and half of C showed saturated rarefaction curves, but many of D, A, and E were less saturated (Fig. 2A). This biofilter-dependent pattern was also observed from diversity indexes (Fig. 2B). Diversity indexes including observed OTUs and Shannon indexes showed the bacterial diversity of fish tanks (F) was the lowest, followed by aeration biofilters (B). Bacterial diversity was greatest in the packed bed (A) and mesh biofilters (D) followed by the maturation tank biofilter (E). The influences of temperature and salinity on bacterial diversity were not clear, but the average values were somewhat lower than other conditions concerning the lowest temperature and salinity (15° C and 20‰). Low diversity in ammonia oxidizing archaea in low temperature was reported in the previous study (Urakawa et al., 2008).

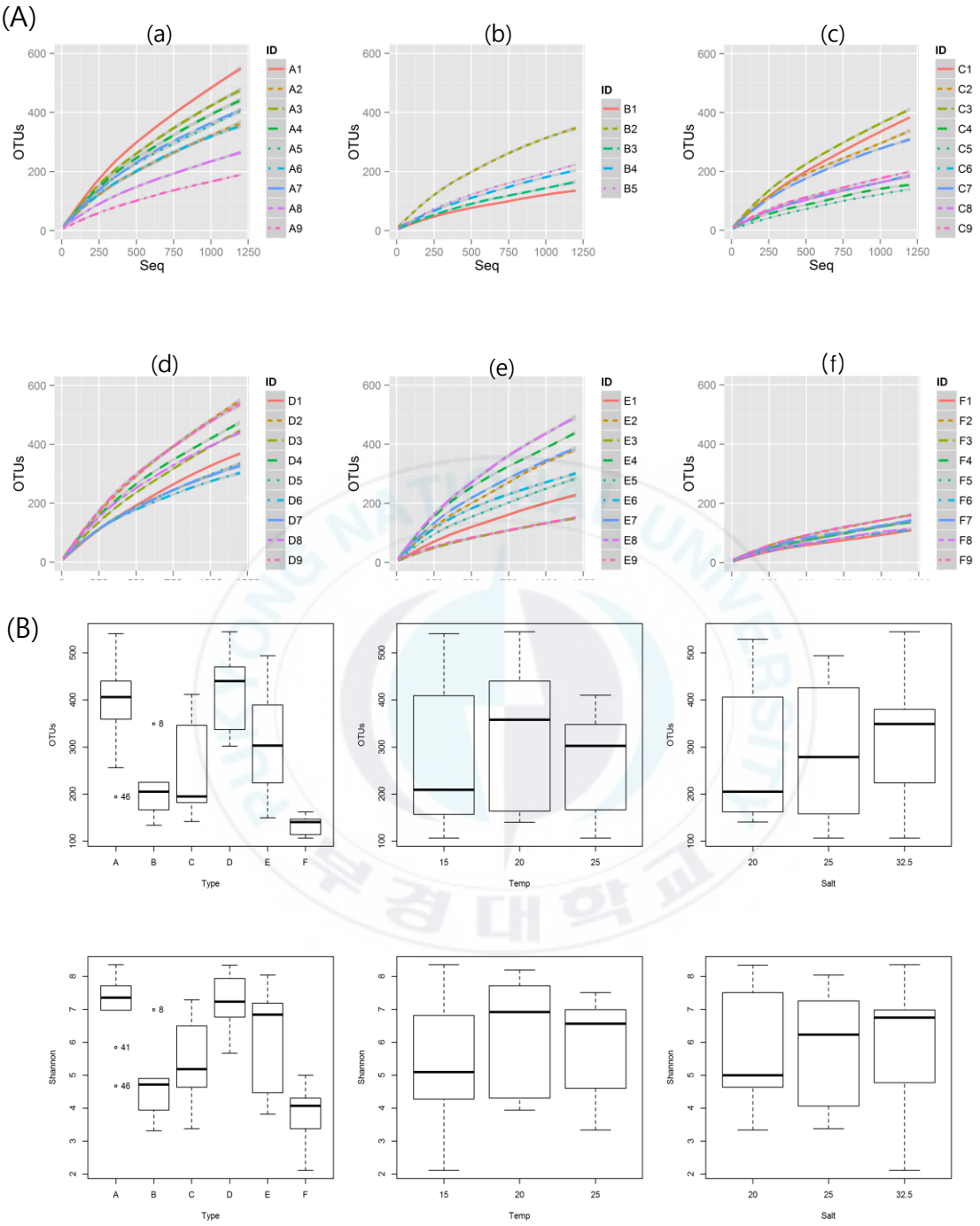
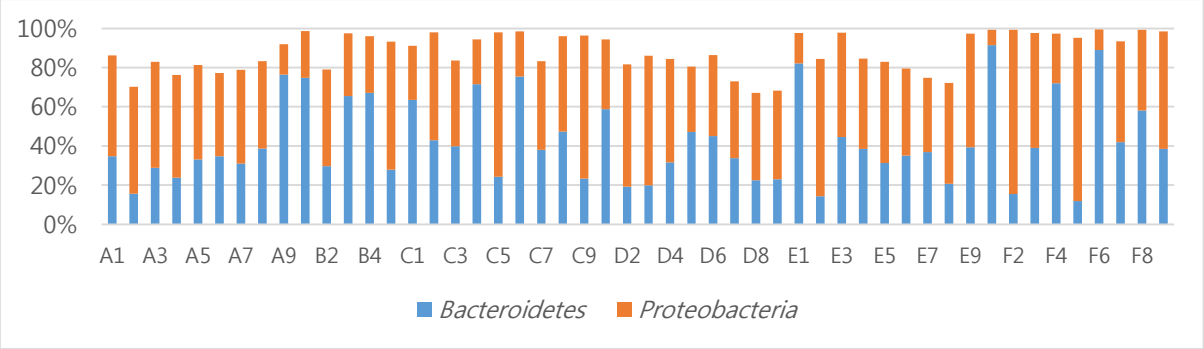


Fig .2. Observed OTUs and Shannon index values. (A) Rarefaction curves for observed OTUs. Curves were presented according to biofilter types. (B) Box plots based on diversity indexes on different tank types, temperature, and salinity. Observed OTUs (upper) and Shannon index (lower) were shown.

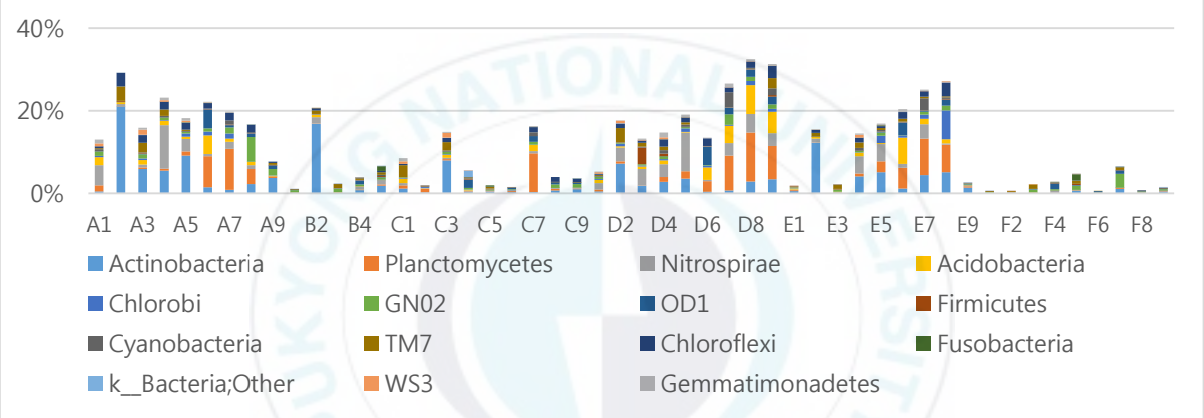
Overall diversity at phylum level

Bacteroidetes and *Proteobacteria* were the most abundant phyla. The composition of each phylum ranged from 11.9% to 91.4% (average 42.2%) for *Bacteroidetes*, and 7.9% to 84.0% (45.9%) for *Proteobacteria*, but the sums of the two phyla were constant in the samples (67.2%–99.4%). *Bacteroidetes* and *Proteobacteria* levels were inversely proportional to each other with significantly high Spearman's rank correlation coefficient of −0.80. The high absolute value of the coefficient with inverse correlation between two phyla might imply a relationship between the roles of these phyla in this RAS. In a previous study (Martins et al., 2013), *Proteobacteria* was the most abundant phylum (70%–90%) and *Bacteroidetes* second (7%–26%). Dissimilar to the present study, the proportion of *Proteobacteria* was much higher than that of *Bacteroidetes*. Minor groups with a prevalence of ≥ 1% included *Actinobacteria* (2.9%), *Planctomycetes* (2.0%), *Nitrospirae* (1.5%), and *Acidobacteria* (1.0%). The compositions of these minor groups varied markedly between samples, but the total amount of the other groups differed according to the type of biofilter (Fig. 3B). Higher proportions of minor groups were observed in packed bed biofilters (A; 18.4% on average), mesh biofilters (D; 19.3%), and maturation biofilters (E. 14.0%), but were very low in fish tank biofilters (F; 2.2%). Average abundance of the minor groups was lowest in the lowest temperature and salt condition, but was statistically not significant.

(A)



(B)



(C)

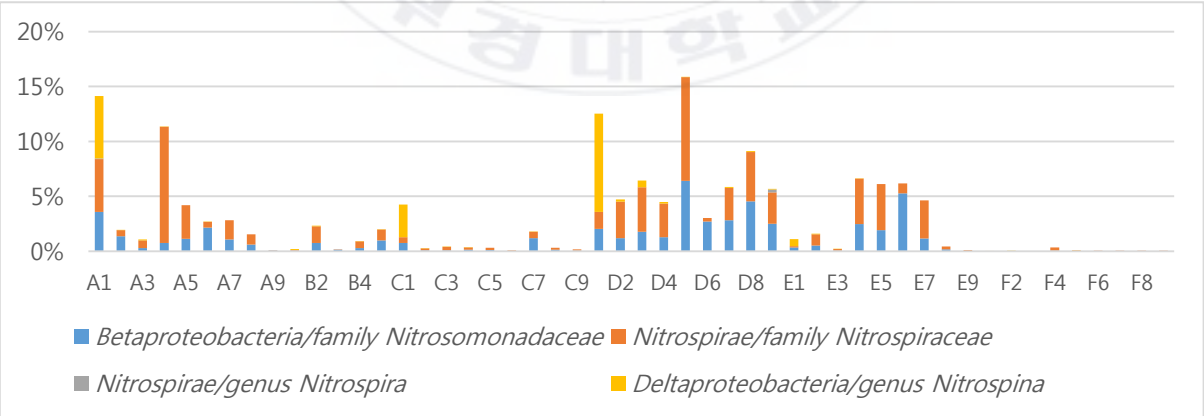


Fig. 3. Taxonomic assignment results at the phylum level and for nitrifying bacteria. (A) Two major phyla, Bacteroidetes and Proteobacteria. (B) The other phyla. (C) Proportions of nitrifying bacteria. The taxa known to contain ammonia− and nitrite−oxidizing bacteria are shown.

Overall diversity in lower levels

Alphaproteobacteria (average 33.4%) and *Flavobacteriia* (37.3%) were the major classes in most of the biofilters. A heat map showing bacterial diversity at genus or higher level is presented in Fig. 4. A few taxonomic groups related to family *Rhodobacteraceae* in *Alphaproteobacteria*, and family *Flavobacteriaceae* in *Flavobacteriia* were very abundant in most of the biofilters. Samples were clustered into several groups based on the patterns of taxonomic groups. The small boxes in Fig. 4 show examples in which similar bacterial communities were observed in the same operation period. Large boxes in the figure depict major clusters that contain specific taxonomic groups. Group A, B, and C contained the genus *Marivita*, genus *Octadecabacter*, and unknown *Alphaproteobacteria* and family *Saprospiraceae*, respectively. The C group contained more diverse taxonomic groups than other samples, with bacteria obtained mainly from the A, D, and E biofilters. Genus *Phaeobacter* and *Sediminicola* were detected in high proportions in several samples and correlated with each other. Many of these samples were from the low temperature conditions. *Sediminicola* grow at low temperature; the two species of the genus, S. arcticus and *S. luteus*, have an optimum growth temperature of 15° C and 20° C, respectively (Khan et al., 2006; Hwang et al., 2015). *Phaeobacter* also contain psychotrophic members, such as P. arcticus and *P. leonis*, of which optimum growth temperature was 20oC and 17– 22° C, respectively (Zhang et al., 2008; Gaboyer et al., 2013).

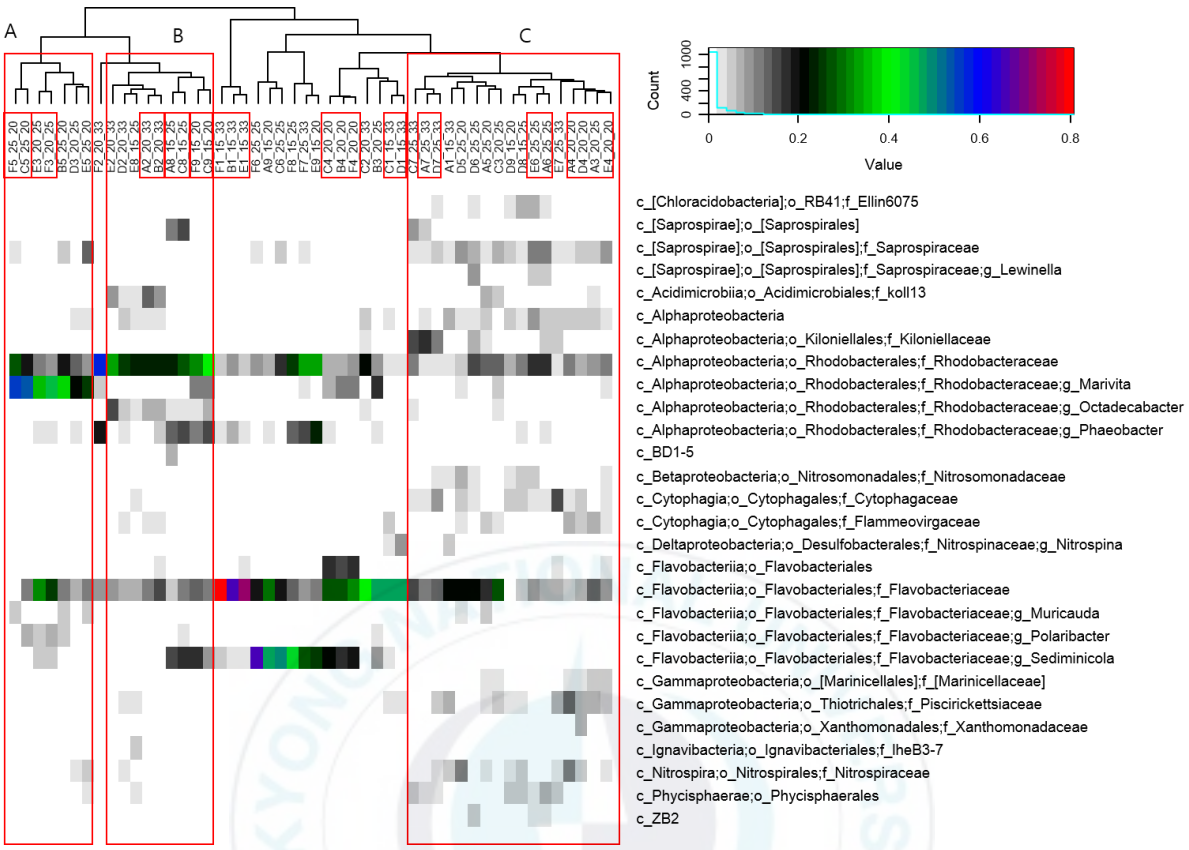


Fig. 4. Heat map showing bacterial diversity at genus or higher level. Samples were clustered based on the distributions of taxonomic groups and the dendrogram was shown at top of the heat map. Samples names were expressed as (ID)_(temperature, ° C)_(salinity, ‰) and 33 in salinity means 32.5‰. In the taxonomic groups, c_, o_, f_, and g_ mean that class, order, family and genus. Red boxes indicate noticeable groups.

Diversity and phylogenetic analysis of nitrifying bacteria

Proportions of ammonia-oxidizing and nitrite-oxidizing bacteria also showed biofilter type-dependent patterns (Fig. 3C). Packed bed biofilter (A) and mesh biofilter (D) harbored appreciable amounts of nitrifying bacteria. Phylotypes related to Nitrosomonadaceae which contains most of the ammonia oxidizing bacteria occupied 0.06%-3.56% (average 1.22%) in A biofilters and 1.19%-6.41% (average 2.80%) in D biofilters. Nitrospirae group was also detected from 0.02%-10.56% (average 2.55%) and from 0.32%-9.45% (3.57%) in A and D biofilters, respectively. Nitrospina, nitrite oxidizing group belonging to Deltaproteobacteria, was detected up to 9.0% in several samples mainly collected at the temperature and salinity condition of 15° C and 32.5‰, respectively. Nitrifying bacteria represented a significant portion of bacterial communities (up to 16%). Packed bed and mesh biofilters were influential in removing ammonia in RAS. All OTUs related to nitrifying bacteria were associated with Nitrosomonas, Nitrospira, and Nitrospina. Major OTUs related to nitrifying bacteria (summed amount in A, D, and E biofilters > 1%) were used to reconstruct a phylogenetic tree (Fig. 5). The major OTUs occupied 82% on average (50%- 100%) among the total nitrifying bacteria-related OTUs, which implies that several OTUs are dominant in in A, D, and E biofilters. In the case of Nitrosomonas-related group, few OTUs were detected. New.ReferenceOTU207 was detected most abundantly and frequently, most commonly at 25° C showing a temperature-dependent occurrence. The OTU had 96.7% similarity with Nitrosomonas marina strain Nm22 (NR_104815) isolated from the South Pacific Sea water (Koops et al., 1991), and might play an important role in overall conditions in RAS. Nitrospina-related OTUs were detected only in the temperature-salinity condition of 15° C and 32.5‰. In the Nitrospirae-related group, New.ReferenceOTU157, OTU199127, New.ReferenceOTU135, and OTU2958053 were frequently detected in

significant amounts. Many OTUs were distantly related with cultivated strains. Nitrospina-related OTUs showed a 91%-92% similarity to *Nitrospina gracilis* Nb-211 (NR_104821) isolated from the Atlantic Ocean (Teske et al., 1994). In OTUs belonging to order Nitrospirales, only one OTU (OTU1991270) showed high similarity (99.4%) to *Nitrospira marina* Nb-295 (X82559) isolated from a corroded iron pipe (Ehrich et al., 1995) and the others displaying very low similarity (87.9%-93.7%). In the Nitrosomonas-related group, OTU112204 showed a close relationship (99.3%) with *Nitrosomonas aestuarii* Nm36 (NR_ 104818) isolated from seawater of the North Sea (Koops et al., 1991). Other OTUs showed distant relationships (95.4%- 96.7%) with cultivated strains *Nitrosomonas marina* Nm22 (NR_104815), *Nitrosomonas cryotolerans* Nm55 (AF272423) isolated from the Kasitsna Bay seawater (Koops et al., 1991), and *Nitrosospira lacus* APG3T (CAUA01000011) isolated from sandy lake sediment (Urakawa et al., 2015).

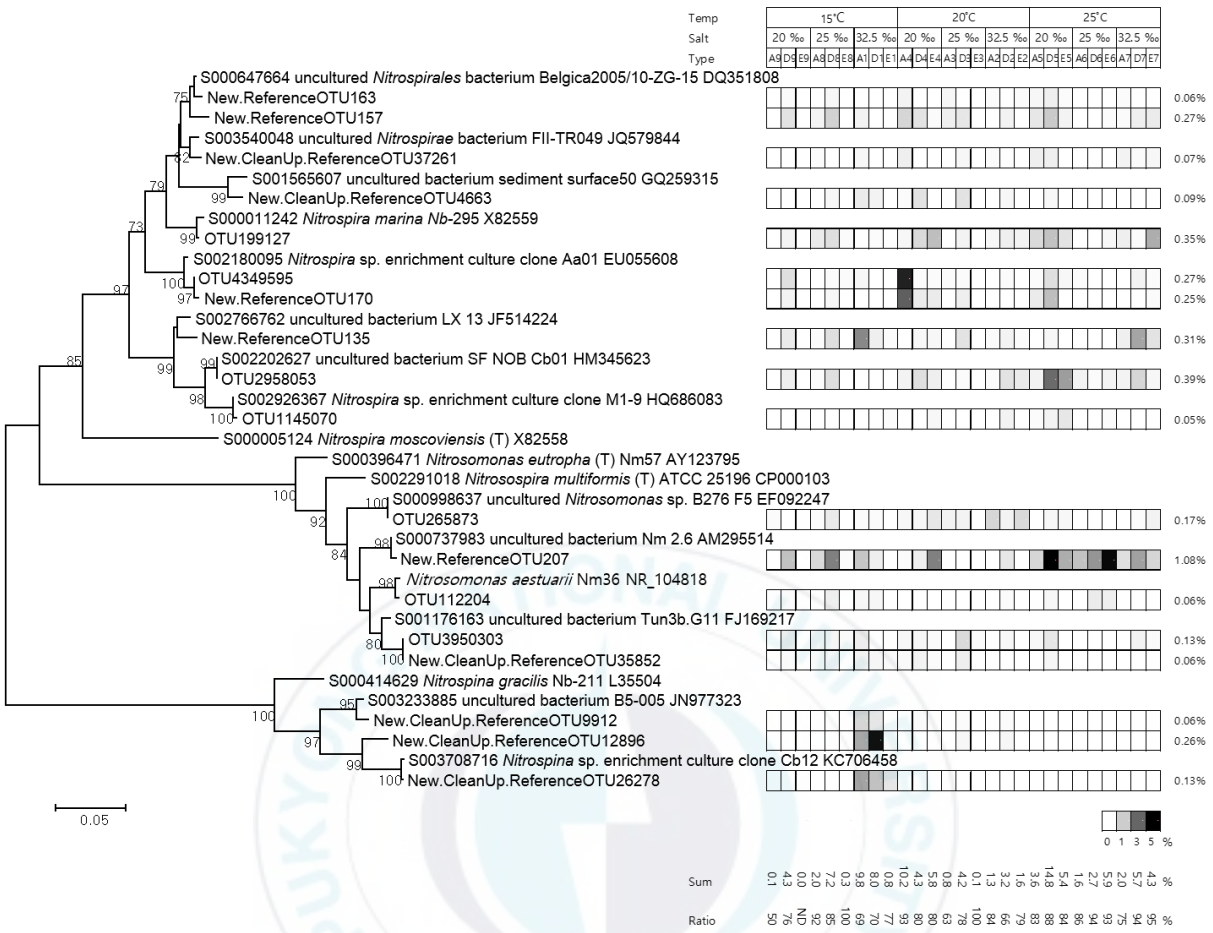


Fig. 5. Neighbor joining tree and heat map of major OTUs related to nitrifying bacteria. Bootstrap values more than 70% are shown in the tree. Temperature, salinity, and type of samples are indicated at the top of heat map. The % value on the right side is an average value of each OTU per sample. The % value in upper line at the bottom (Sum) is a sum of the ratios of major nitrifying bacteria-related OTUs among total OTUs. The % value in lower line at the bottom (Ratio) is a ratio of the major OTUs to the total OTUs in the nitrifying bacteria-related OTUs

CONCLUSION

16S rRNA amplicon sequencing revealed the bacterial diversity of a seawater RAS in detail. Packed bed biofilters, mesh biofilters, and maturation biofilters showed high diversities from the phylum to OTU level, and were enriched for nitrifying bacteria through different operation conditions, implying their significant roles in the RAS. Proteobacteria and Bacteroidetes were the major taxonomic groups in RAS, but as shown in the three biofilters, diverse bacterial communities and other minor taxonomic groups seemed to play important roles and were necessary for nitrifying activity in RAS.

ACKNOWLEDGEMENTS

This research was supported from the research project (R2016021) in National Institute of Fisheries Science.

REFERENCES

Binladen, J., Gilbert, M.T., Bollback, J.P., Panitz, F., Bendixen, C., Nielsen, R., and Willerslev, E. 2007. The use of coded PCR primers enables high-throughput sequencing of multiple homolog amplification products by 454 parallel sequencing. *PLoS ONE* **2**, e197.

Chun, J., Lee, J.H., Jung, Y., Kim, M., Kim, S., Kim, B.K., and Lim, Y.W. 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int. J. Syst. Evol. Microbiol.* **57**, 2259-2261.

Cole, J.R., Wang, Q., Fish, J.A., Chai, B., McGarrell, D.M., Sun, Y., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., and Tiedje, J.M. 2014. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* **42**, D633-642.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194-2200.

Gaboyer, F., Tindall, B.J., Ciobanu, M.C., Duthoit, F., Le Romancer, M., and Alain, K. 2013. *Phaeobacter leonis* sp. nov., an alphaproteobacterium from Mediterranean Sea sediments. *Int. J. Syst. Evol. Microbiol.* **63**, 3301-3306.

Hwang, C.Y., Lee, I., Cho, Y., Lee, Y.M., Jung, Y.J., Baek, K., Nam, S.I., and Lee, H.K. 2015. *Sediminicola arcticus* sp. nov., a psychrophilic bacterium isolated from deep-sea sediment, and emended description of the genus *Sediminicola*. *Int. J. Syst. Evol. Microbiol.* **65**, 1567-1571.

Khan, S.T., Nakagawa, Y., and Harayama, S. 2006. *Sediminicola luteus* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. *Int. J. Syst. Evol. Microbiol.* **56**, 841-845.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **16**, 111-120.

Kuczynski, J., Stombaugh, J., Walters, W.A., Gonzalez, A., Caporaso, J.G., and Knight, R. 2012. Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Curr. Protoc. Microbiol.* **Chapter 1**, Unit 1E 5.

Losordo, T.M., and Hobbs, A.O. 2000. Using computer spreadsheets for water flow and biofilter sizing in recirculating aquaculture production systems. *Aquacult. Eng.* **23**, 95-102.

Martins, P., Cleary, D.F., Pires, A.C., Rodrigues, A.M., Quintino, V., Calado, R., and Gomes, N.C. 2013. Molecular analysis of bacterial communities and detection of potential pathogens in a recirculating aquaculture system for Scophthalmus maximus and Solea senegalensis. *PLoS One* **8**, e80847.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., and Hugenholtz, P. 2012. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* **6**, 610-618.

Pfeiffer, T.J., and Wills, P.S. 2011. Evaluation of three types of structured floating plastic media in moving bed biofilters for total ammonia nitrogen removal in a low salinity hatchery recirculating aquaculture system. *Aquacult. Eng.* **45**, 51-59.

Rusten, B., Eikebrokk, B., Ulgenes, Y., and Lygren, E. 2006. Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacult. Eng.* **34**, 322-331.

Ruyet, J.P.-L., Chartois, H., and Quemener, L. 1995. Comparative acute ammonia toxicity in marine fish and plasma ammonia response. *Aquaculture* **136**, 181-194.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., *et al.* 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **75**, 7537-7541.

Schreier, H.J., Mirzoyan, N., and Saito, K. 2010. Microbial diversity of biological filters in recirculating aquaculture systems. *Curr. Opin. Biotechnol.* **21**, 318-325.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol. Biol. Evol.* **30**, 2725-2729.

Terjesen, B.F., Summerfelt, S.T., Nerland, S., Ulgenes, Y., Fjæra, S.O., Megård Reiten, B.K., Selset, R., Kolarevic, J., Brunsvik, P., Bæverfjord, G., *et al.* 2013. Design, dimensioning, and performance of a research facility for studies on the requirements of fish in RAS environments. *Aquacult. Eng.* **54**, 49-63.

Verbeeten, B.E., Carter, C.G., and Purser, G.J. 1999. The combined effect of feeding time and ration on growth performance and nitrogen metabolism of greenback flounder. *J.Fish Biol.* **55**, 1328-1343.

Zhang, D.C., Li, H.R., Xin, Y.H., Liu, H.C., Chi, Z.M., Zhou, P.J., and Yu, Y. 2008. *Phaeobacter arcticus* sp. nov., a psychrophilic bacterium isolated from the Arctic. *Int. J. Syst. Evol. Microbiol.* **58**, 1384-1387.

CHAPTER 2. Genomic analysis of the denitrifying strain RR3-57 isolated from a seawater recirculating aquaculture system.

ABSTRACT

The strain RR3-57 was isolated from a seawater recirculating aquaculture system and showed a 16S rRNA sequence similarity of 96.22% compared to the *Halioglobus pacificus* type strain S1-72(T), a member of the class *Gammaproteobacteria*, which was identified as a denitrifying bacterium. Here, we describe information of the draft genome sequence of RR3-57. The RR3-57 strain has a genome of total 5,003,575 base pair (sum of contig 1: 4,847,776 bp and contig 2: 155,799 bp). For gene prediction analyses and functional annotation, we used a Rapid Annotation using the Subsystem Technology (RAST) pipeline. The genome contains 4,627 coding sequences, 43 tRNAs and 6 rRNAs with an average G+C content of 57.5 mol%. The strain carried 18 predicted genes associated with denitrification. As there was no previously reported genome sequence of the genus *Halioglobus*, the genomes will show novel information in this group.

INTRODUCTION

The genus *Halioglobus* in the class *Gammaproteobacteria* was first proposed by (Park et al. 2012), and the genus comprises only two species, *Halioglobus japonicus* and *Halioglobus pacificus*. Strain RR3-57 was isolated from a biofilter of a seawater recirculating aquaculture system (RAS) in Busan, Korea. The RASs are closed-loop facilities that treat the water within the system, and nitrogenous wastes in these systems such as fish manure, undigested feed can be eliminated through nitrifying and denitrifying bacteria (Leonard et al. 2000). In nitrification process, ammonia (NH_3) is first oxidized to nitrite (NO_2), and nitrite is oxidized to nitrate (NO_3) sequentially. These nitrite and nitrate are reduced to nitrogen (N_2) or nitrogen dioxide (N_2O), this process is called denitrification. Two bacteria, *H. japonicus* and *H. pacificus* were known to reduce nitrate to nitrogen (Park et al. 2012). The strain RR3-57 showed 96.22% sequence similarity with *H. pacificus*, and also it was confirmed that the NO_X removal capability. The purpose of this study was to analyze the genome sequence of the new strain of the genus *Halioglobus*, strain RR3-57.

MATERIALS AND METHODS

Genome sequencing and assembly

The strain RR3-57 was isolated from a sludge sample collected from a seawater recirculating aquaculture system in Busan, Korea. It was grown in modified marine agar with 2.5% (w/v) NaCl at 25℃ for two weeks. Grown colonies were gathered in a 1.5 ml tube. For washing, we add PBS buffer 1 ml and vortex 30 seconds and spin-down, discard the buffer, leaving only pellets. The washed cells was used by a NGS service company (Macrogen, Korea) for sequencing and sequence assembly. PacBio system was used for sequencing (Au et al. 2012), and HGAP3 software was used for assembly (Chin et al. 2013).

Gene prediction analyses and functional annotation

Rapid Annotation using the Subsystem Technology (RAST) pipeline (Aziz et al. 2008) was used for gene prediction and annotation. To identify prophages, we used a web server PHAge Search Tool (PHAST) (Zhou et al. 2011).

Phylogenetic analysis based on 16S rRNA gene sequences

For confirming taxonomic position of strain RR3-57, its 16S rRNA gene sequence was based on. The 16S rRNA gene sequence of strain RR3-57 was compared with some sequences of the closest strains in the EzTaxon-e database (Chun et al. 2007). To determine the phylogenetic relationships between strain RR3-57 and other strains, a phylogenetic tree was constructed with sequences from NCBI databases (Pruitt et al. 2007). Their 16S rRNA gene sequences were aligned using CLUSTAL W program (Thompson et al. 1994), and the phylogenetic tree was constructed using the neighbor-joining (Saitou and Nei 1987) method with 1000 randomly selected bootstrap replicates using MEGA 6 program (Tamura et al. 2013).

RESULTS AND DISCUSSION

The 16S rRNA sequences of strain RR3-57 and other closest strains were used to generate a phylogenetic tree (figure 1). The phylogenetic tree indicated that the strain RR3-57 belongs to the genus *Halioglobus*. It showed sequence similarity less than 97% (96.22%) with *Halioglobus pacificus* (table 1), the strain RR3-57 should be classified as new species in the genus *Halioglobus*.

Table 1. 16S rRNA sequence similarity of strain RR3-57 and other strains.

Rank	Name	Strain	Pairwise similarity (%)
1	*Halioglobus pacificus*	S1-72(T)	96.22
2	*Haliea mediterranea*	7SM29(T)	95.77
3	*Haliea salexigens*	DSM 19537(T)	95.71
4	*Halioglobus japonicus*	S1-36(T)	95.46
5	*Chromatocurvus halotolerans*	EG19(T)	94.75
6	*Congregibacter litoralis*	KT71(T)	94.30
7	*Luminiphilus syltensis*	NOR5-1B(T)	94.03
8	*Pseudohaliea rubra*	DSM 19751(T)	94.03
9	*Oceanicoccus sagamiensis*	PZ-5(T)	92.34

Showed nine closest strains on the ez-biocloud server.

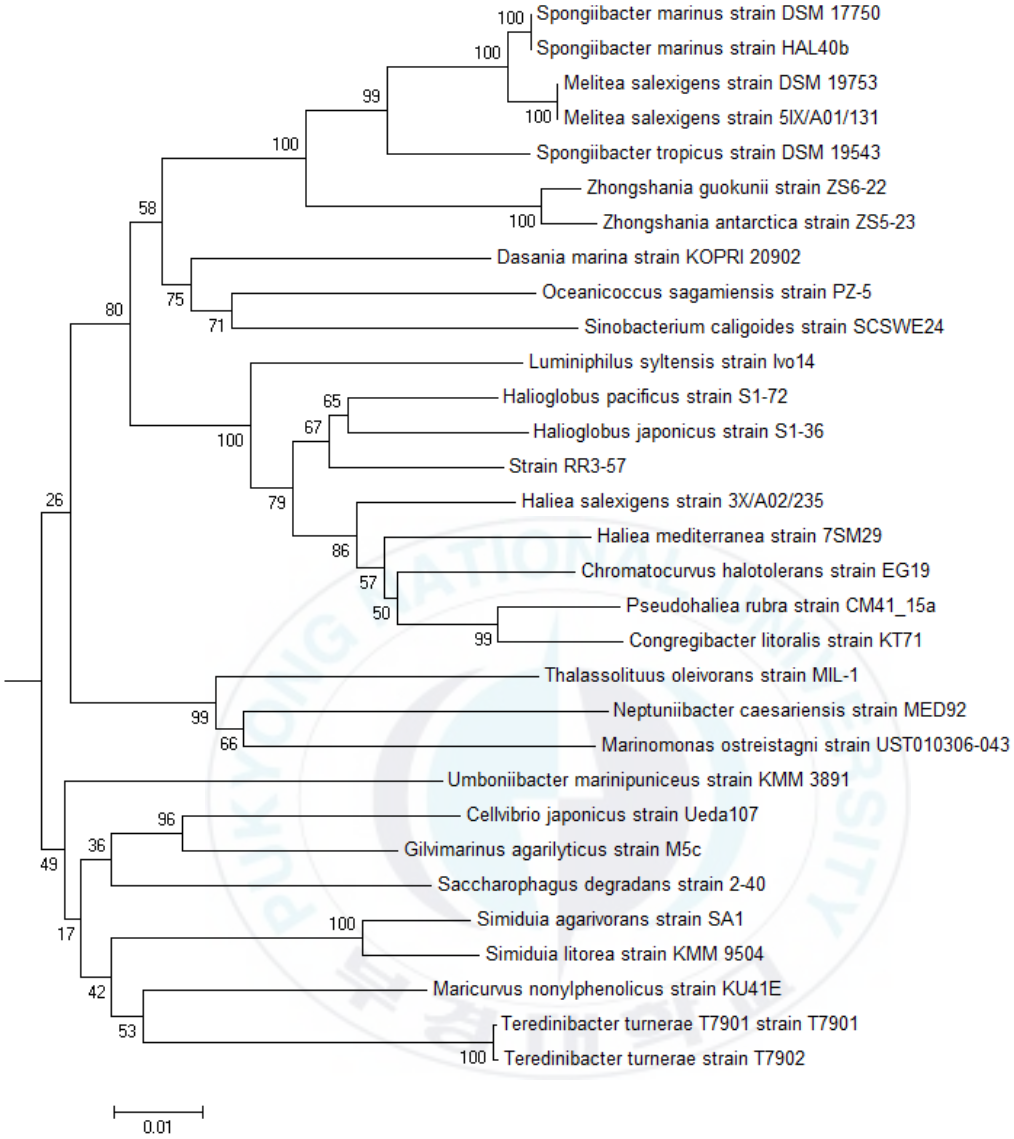


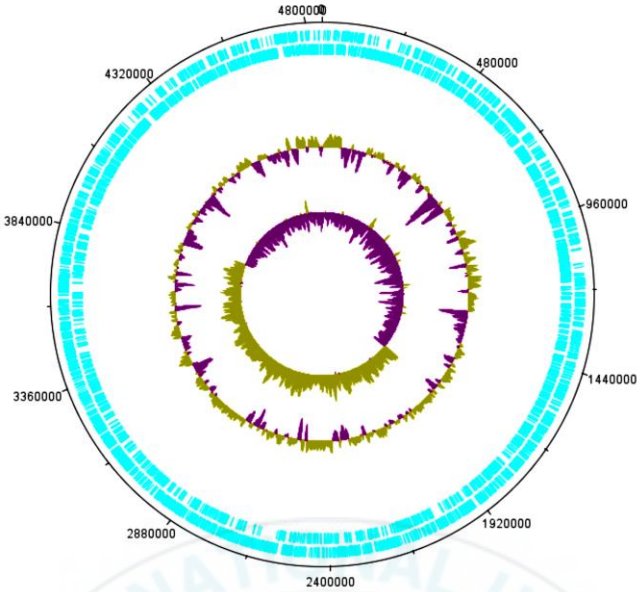
Figure 1. Phylogenetic tree of strain RR3-57 and nearest strains in NCBI databases. It conducted based on the aligned sequences using the neighbor-joining methods.

Two circular contigs was formed by assembling the whole genome sequence (figure 2). The length of each contig was 4,847,776 (contig 1) and 155,799 (contig 2) base pairs, respectively (figure 3, 4). The smaller of the two is expected that a plasmid, including genome of cobalt-zinc-cadmium resistance, programmed cell death and toxin-antitoxin systems, stress response, etc. The whole genome has total 4,572 coding sequences. Of these, 2,015 were in subsystems (44%) and 2,557 were not in subsystems (56%) of the RAST system (Aziz, Bartels et al. 2008). The predicted genes were classified under 26 subsystem categories. The number of RNA genes were 49 including 43 tRNA genes and 6 rRNA genes.

The genome of strain RR3-57 encodes 18 predicted genes associated with denitrification, such as nitric oxide reductase activation protein and respiratory nitrate reductase (table 2). 61 genes associated with flagellar motility were predicted, such as flagellar basal-body rod proteins, flagellar biosynthesis proteins, flagellar motor switch proteins, flagellin proteins, etc. Strain RR3-57 encodes 90 genes associated with stress response, such as osmotic stress (6), cold shock (3), heat shock (16), detoxification (4), periplasmic stress (4), oxidative stress (36), and the others (21). Also the strain RR3-57 encodes one gene of sporulation-associated proteins with broader functions. To identify prophages, we used PHAST. One questionable prophage has detected in a 13.2 kb (with 47.79 % GC percentage) region containing 13 CDS.

Strain RR3-57 contains 76 predicted genes associated with membrane transporter systems, such as ABC transporters, cation transporters, protein and nucleoprotein secretion systems, protein translocation across cytoplasmic membrane, and TRAP transporters. These genes, including osmoregulation genes would have the roles required to the strain survive in seawater.

(A)



(B)

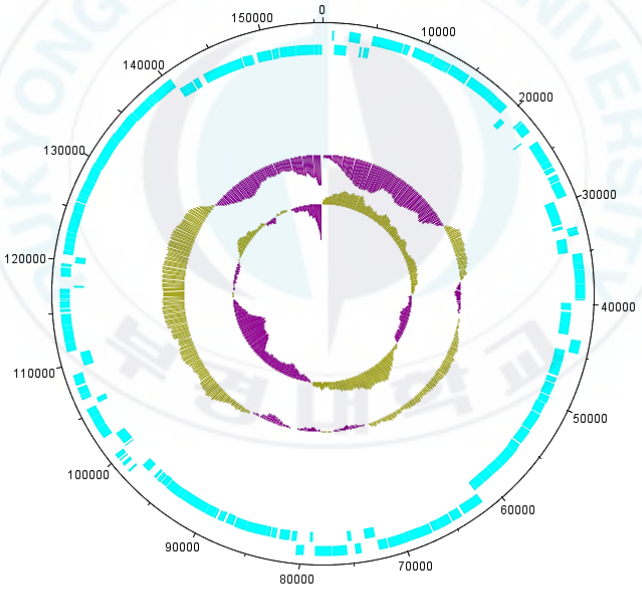


Figure 2. Circular map of (A) contig 1, (B) contig 2. Marked characteristics are shown from outside to the center: CDS on forward strand, CDS on reverse strand, GC content, and GC skew.

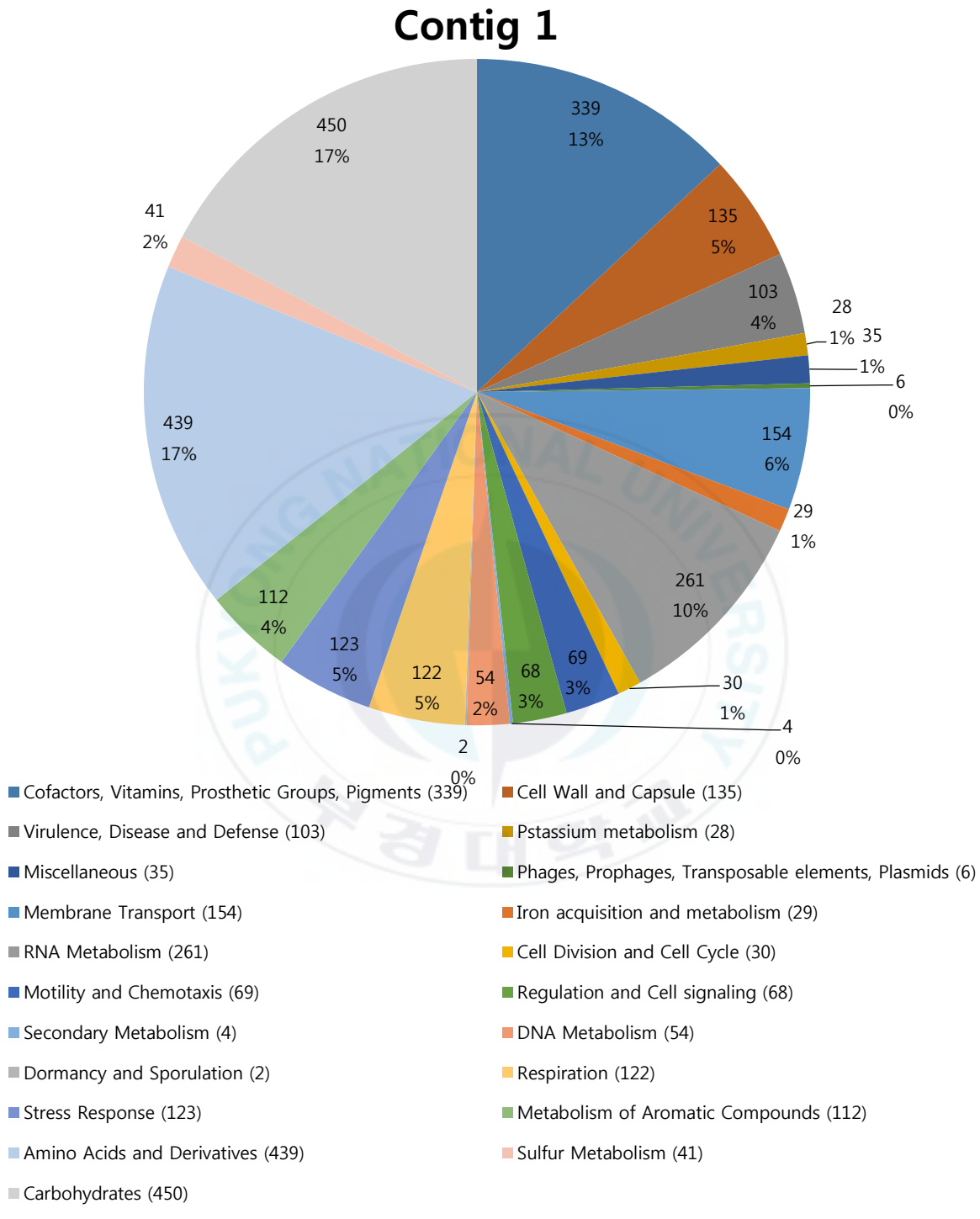


Figure 3. Subsystems statistics of contig 1

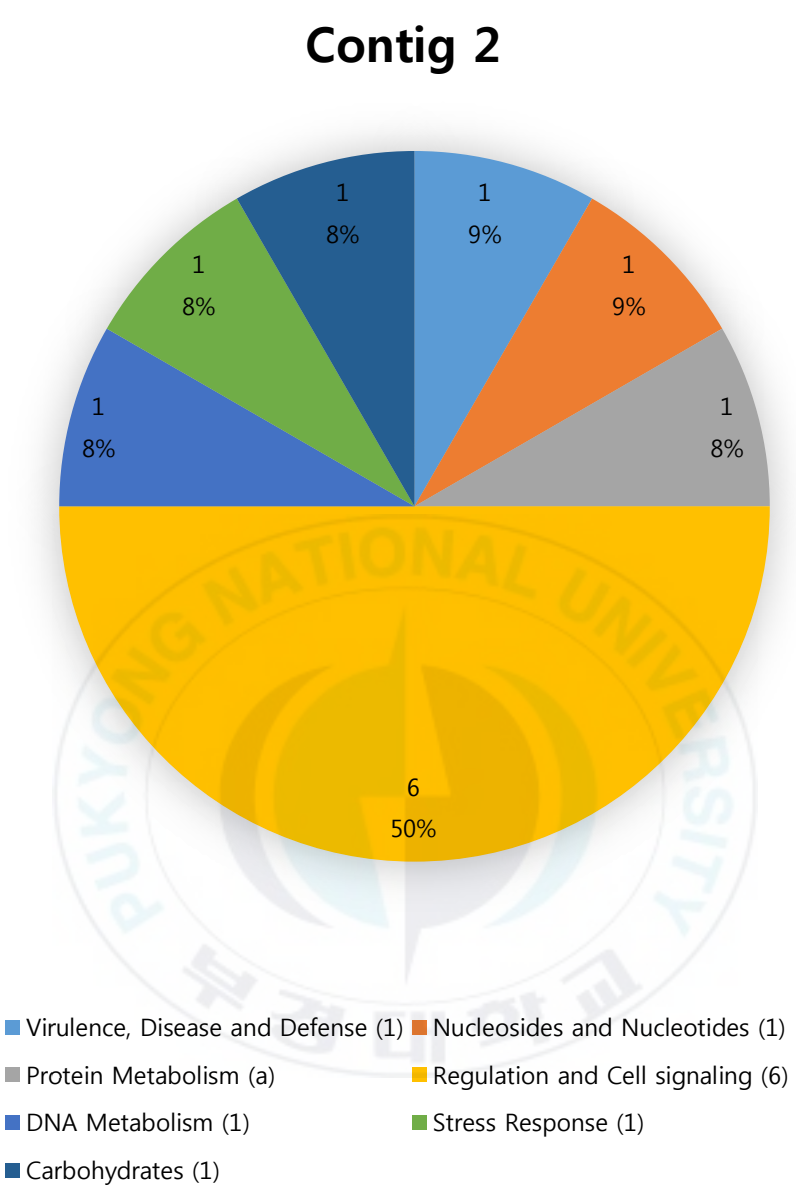


Figure 4. Subsystems statistics of contig 2

Table 2. Genes associated with denitrification within the genome of RR3-57

Category	Subsystem	Role
Denitrification	Denitrifying reductase gene clusters	Nitric oxide reductase activation protein NorE
		Respiratory nitrate reductase alpha chain (EC 1.7.99.4)
		Nitric-oxide reductase subunit C (EC 1.7.99.7)
		Nitric oxide reductase activation protein NorQ
		Nitric oxide reductase activation protein NorD
		Respiratory nitrate reductase gamma chain (EC 1.7.99.4)
		Respiratory nitrate reductase beta chain (EC 1.7.99.4)
		Respiratory nitrate reductase delta chain (EC 1.7.99.4)
		Nitrous oxide reductase maturation protein NosR
		Nitric-oxide reductase subunit B (EC 1.7.99.7)
	Denitrification	Nitric oxide reductase activation protein NorE
		Nitric oxide reductase activation protein NorD
		NnrS protein involved in response to NO
		Nitric-oxide reductase subunit C (EC 1.7.99.7)
		Cytochrome cd1 nitrite reductase (EC:1.7.2.1)
		Nitrous oxide reductase maturation protein NosR
		Nitric oxide reductase activation protein NorQ
		Nitric-oxide reductase subunit B (EC 1.7.99.7)

CONCLUSION

The results of 16S rRNA gene sequence-based phylogenetic analysis, it was confirmed that the strain RR3-57 can be a new (third) species in genus *Halioglobus*. Additional tests will be needed to identify this strain. Also the genome of the strain RR3-57 was first reported genome sequence of the genus *Halioglobus*. These results will provide more information about genus *Halioglobus*.

REFERENCES

Au, K. F., J. G. Underwood, et al. (2012). "Improving PacBio long read accuracy by short read alignment." PLoS One 7(10): e46679.

Chin, C.-S., D. H. Alexander, et al. (2013). "Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data." Nature methods 10(6): 563-569.

Chun, J., J.-H. Lee, et al. (2007). "EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences." Int. J. Syst. Evol. Microbiol. 57(10): 2259-2261.

Pruitt, K. D., T. Tatusova, et al. (2007). "NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins." Nucleic acids research 35(suppl 1): D61-D65.

Park, S., S. Yoshizawa, et al. (2012). "Halioglobus japonicus gen. nov., sp. nov. and Halioglobus pacificus sp. nov., members of the class Gammaproteobacteria isolated from seawater." Int J Syst Evol Microbiol 62(Pt 8): 1784-1789.

Leonard, N., J. P. Blancheton, et al. (2000). "Populations of heterotrophic bacteria in an experimental recirculating aquaculture system." Aquacultural engineering 22(1-2): 109-120.

Zhou, Y., Y. Liang, et al. (2011). "PHAST: a fast phage search tool." Nucleic acids research: gkr485.

Thompson, J. D., D. G. Higgins, et al. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." Nucleic acids research 22(22): 4673-4680.

Tamura, K., G. Stecher, et al. (2013). "MEGA6: molecular evolutionary genetics analysis version 6.0." Mol. Biol. Evol.: mst197.

Saitou, N. and M. Nei (1987). "The neighbor-joining method: a new method for reconstructing phylogenetic trees." Mol. Biol. Evol. 4(4): 406-425.

Aziz, R. K., D. Bartels, et al. (2008). "The RAST Server: rapid annotations using subsystems technology." BMC Genomics 9(1): 75.

CHAPTER 3

Genomic analysis of the denitrifying strain RR3-28 isolated from a seawater recirculating aquaculture system.

ABSTRACT

Strain RR3-28 was isolated from a biofilter of seawater recirculating aquaculture system. The strain showed 95.45% 16S ribosomal RNA sequence similarity compared to the *Nitratireductor basaltis* type strain J3(T). The genus *Nitratireductor* belongs to the class *Alphaproteobacteria*. *N. basaltis* strain J3 was known as denitrifying bacteria, and also the strain RR3-28 was able to reduce nitrate to nitrite, and nitrite to nitrogen. In this study, the genome sequence of the strain RR3-28 was analyzed and compared with genomes of other five strains which are phylogenetically closed and have sequenced genomes. In NCBI server, there were five previously reported genome sequences of the genus *Nitratireductor* including *Nitratireductor aquibiodomus*, *Nitratireductor indicus*, *Nitratireductor pacificus*, *Nitratireductor basaltis* and unnamed *Nitratireductor* sp. ZZ-1. For gene prediction analyses and functional annotation, Rapid Annotation using the Subsystem Technology (RAST) pipeline was used. A contig was formed (3,357,577 bp). The genome contains total 3,410 coding sequences and 443 RNAs. G+C content was 58.6 mol%. The strain RR3-28 carried 21 predicted genes associated with denitrification. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain RR3-28 was a member of the genus *Nitratireductor*, but shows low sequence similarities to *Nitratireductor basaltis* J3(T) (95.45%), *Nitratireductor indicus* C115(T) (95.22%) and *Nitratireductor pacificus* pht-3B(T) (95.15%).

INTRODUCTION

The genus *Nitratireductor* is one kind of denitrifying bacteria, which has nitrate removal capacity from nitrate-contaminated water (Nguyen et al. 2015). In this study, strain RR3-28, a *Nitratireductor*-like strain isolated from a biofilter of a seawater recirculating aquaculture system (RAS) in Busan, Korea, was analyzed through genetic analysis. The RAS was composed of six tanks and biofilters including fish tank, maturation tank, mesh biofilter, floating bed biofilter, aerated biofilter, packed bed biofilter, and these facilities should allow the reuse of the contaminated water through biological treatment (Van Rijn 1996). We summarized the genetic features and differences between this strain RR3-28 and the other strains in genus *Nitratireductor*.

MATERIALS AND METHODS

Genome sequencing and assembly

The strain RR3-57 was isolated from a sludge sample collected from a seawater recirculating aquaculture system in Busan, Korea. It was grown in modified marine agar with 2.5% (w/v) NaCl at 25℃ for two weeks. Grown colonies were gathered in a 1.5 ml tube. For washing, we add PBS buffer 1 ml and vortex 30 seconds and spin-down, discard the buffer, leaving only pellets. The washed cells was used by a NGS service company (Macrogen, Korea) for sequencing and sequence assembly. PacBio system was used for sequencing (Au et al. 2012), and HGAP3 software was used for assembly (Chin et al. 2013).

Gene prediction analyses and functional annotation

Rapid Annotation using the Subsystem Technology (RAST) pipeline (Aziz et al. 2008) was used for gene prediction and annotation. To identify prophages, we used a web server PHAge Search Tool (PHAST) (Zhou et al. 2011).

Phylogenetic analysis based on 16S rRNA gene sequences

For confirming taxonomic position of strain RR3-57, its 16S rRNA gene sequence was based on. The 16S rRNA gene sequence of strain RR3-57 was compared with some sequences of the closest strains in the EzTaxon-e database (Chu et al. 2007). To determine the phylogenetic relationships between strain RR3-57 and other strains, a phylogenetic tree was constructed with sequences from NCBI databases (Pruitt et al. 2007). Their 16S rRNA gene sequences were aligned using CLUSTAL W program (Thompson et al. 1994), and the phylogenetic tree was constructed using the neighbor-joining (Saitou and Nei 1987) method with 1000 randomly selected bootstrap replicates using MEGA 6 program (Tamura et al. 2013).

RESULTS AND DISCUSSION

By 16S rRNA sequence analysis, strain RR3-28 showed 95.45% similarity with *N. basaltis* (table 1). But the phylogenetic tree indicated that strain RR3-28 does not belong in the genus *Nitratireductor* and closest related to *Pseudahrensia aquimaris* (figure 1). The *P. aquimaris* HDW-32(T) exhibited 95.1% 16S rRNA gene sequence similarity with *N. basaltis* J3(T), and the strain HDW-32 was distinguished from representatives of the genera *Nitratireductor* by differences in fatty acids and polar lipids (Jung, Park et al. 2012). Therefore, the strain RR3-28 will be able to determine the exact taxonomic position after a series of phylogenetic, chemotaxonomic and phenotypic analytical procedures.

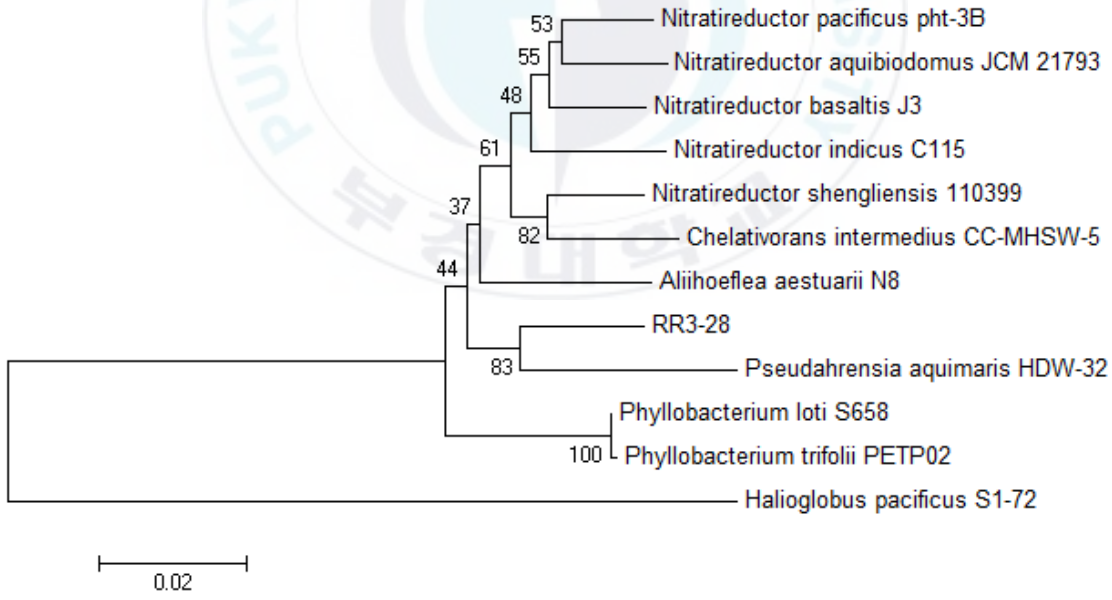


Figure 1. Phylogenetic tree of strain RR3-28 and closest strains. It was constructed based on the aligned sequences using the neighbor-joining methods. *Halioglobus pacificus* S1-72 is an outgroup.

Table 1. 16S rRNA sequence similarity of strain RR3-28 and other strains. Nine closest strains on the ez-biocloud server were shown

Rank	Name	Strain	Pairwise similarity (%)
1	*Nitratireductor basaltis*	J3(T)	95.45
2	*Nitratireductor indicus*	C115(T)	95.22
3	*Phyllobacterium loti*	S658(T)	95.22
4	*Nitratireductor pacificus*	pht-3B(T)	95.15
5	*Pseudahrensia aquimaris*	HDW-32(T)	95.15
6	*Nitratireductor shengliensis*	110399(T)	95.08
7	*Nitratireductor aquibiodomus*	JCM 21793(T)	95.01
8	*Aliihoeflea aestuarii*	N8(T)	94.94
9	*Phyllobacterium trifolii*	PETP02(T)	94.87

One circular contig was obtained by assembling the whole genome sequence (figure 2). The length of the contig was 3,357,577 bp. The G+C contents were 58.6 and 53.2 mol%, respectively.

The genomes have the capacity to code for a total of 3,410 predicted genes (figure 3). In annotation results of contig 1, the genome contains 3,410 coding sequences and 43 RNAs. A total of 2,331 genes were assigned to putative functions, and the remaining 1,079 genes were annotated as hypothetical proteins. The predicted genes were classified under 26 categories.

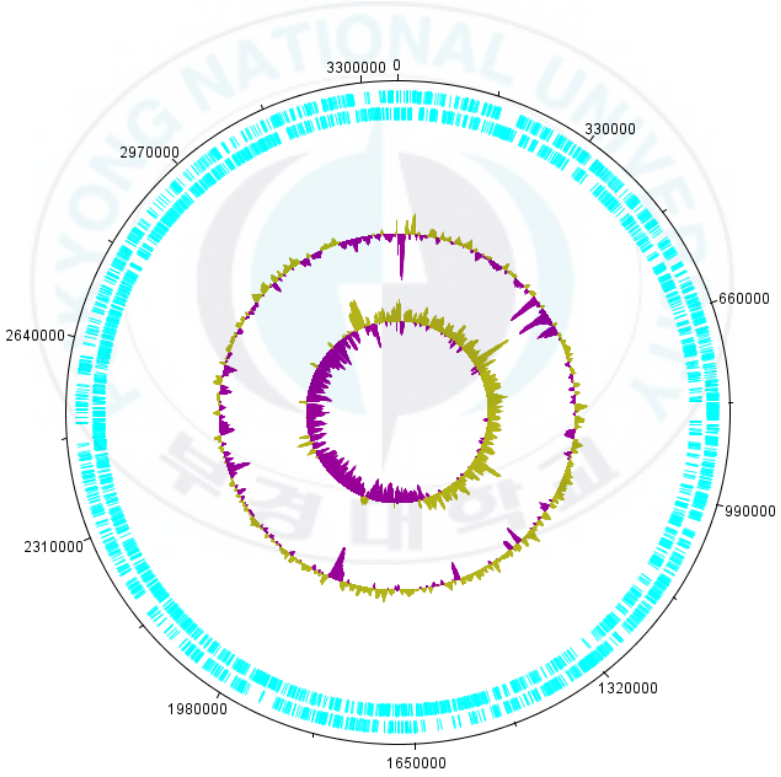


Figure 2. Circular map of contig 1: Marked characteristics are shown from outside to the center: CDS on forward strand, CDS on reverse strand, GC content and GC skew.

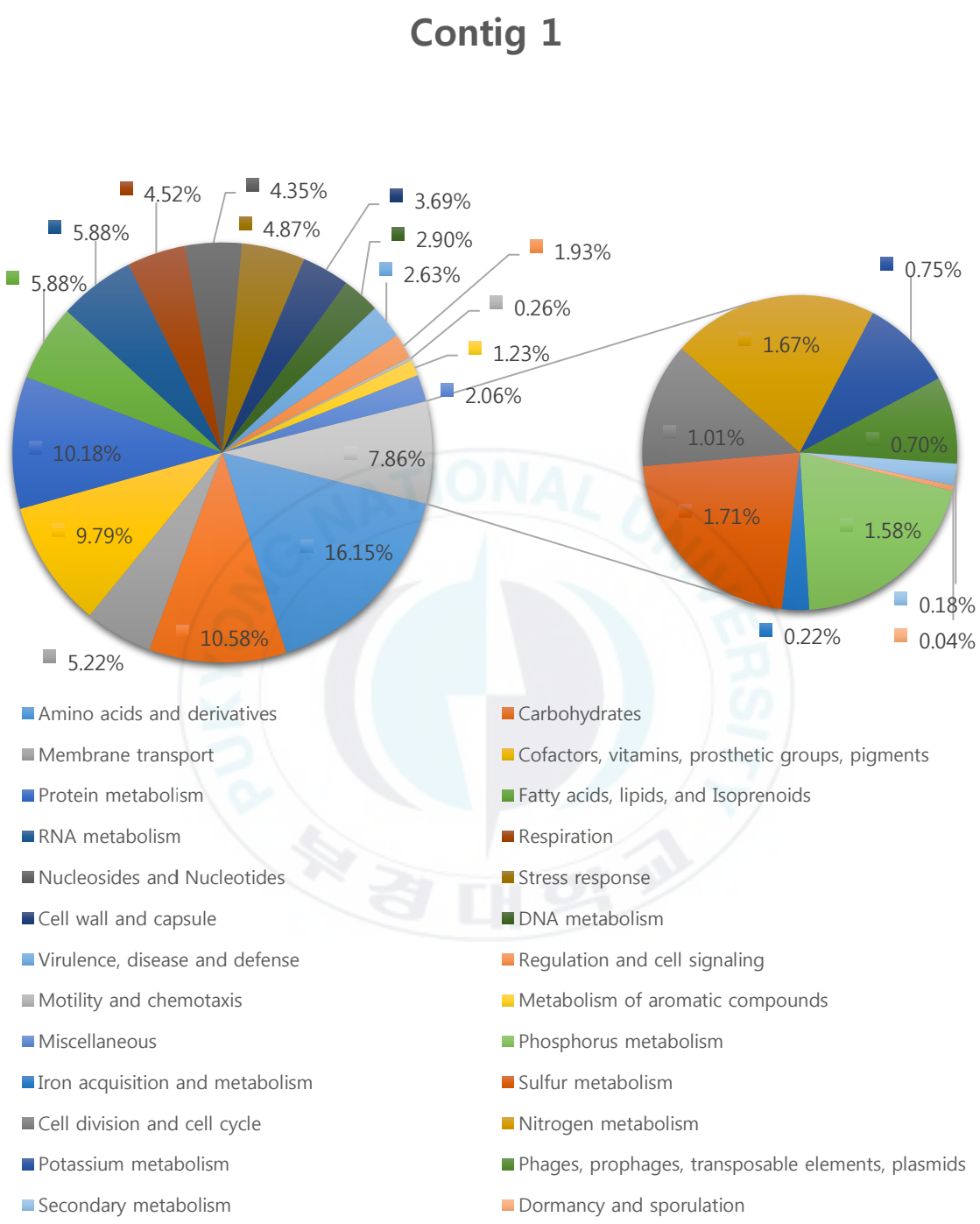


Figure 3. Subsystems statistics of contig 1

The genome of strain RR3-28 contains 21 predicted genes associated with denitrification, such as nitrous oxide reductase-related proteins (table 2).

Table 2. Genes associated with denitrification within the genome of RR3-28

Category	Sybsystem	Role
Denitrification	Denitrifying reductase gene clusters	Respiratory nitrate reductase alpha chain (EC 1.7.99.4) Nitrous oxide reductase maturation periplasmic protein NosX Nitrous oxide reductase maturation protein, outer-membrane lipoprotein NosL Nitrous oxide reductase maturation protein NosD Nitrous oxide reductase maturation protein NosF (ATPase) Respiratory nitrate reductase gamma chain (EC 1.7.99.4) Respiratory nitrate reductase delta chain (EC 1.7.99.4) Respiratory nitrate reductase beta chain (EC 1.7.99.4) Nitrous oxide reductase maturation protein NosR Nitrous oxide reductase maturation transmembrane protein NosY Nitrous-oxide reductase (EC 1.7.99.6)
	Denitrification	NnrS protein involved in response to NO Nitrous oxide reductase maturation periplasmic protein NosX Nitrous oxide reductase maturation protein, outer-membrane lipoprotein NosL Nitrous oxide reductase maturation protein NosD Nitrous oxide reductase maturation protein NosF (ATPase) NnrU family protein, required for expression of nitric oxide and nitrite reductases (Nir and Nor) Nitric oxide -responding transcriptional regulator NnrR (Crp/Fnr family) Nitrous oxide reductase maturation protein NosR Nitrous oxide reductase maturation transmembrane protein NosY Nitrous-oxide reductase (EC 1.7.99.6)

Strain RR3-28 encodes five predicted genes associated with motility and 15 genes for Gene Transfer Agent (GTA). Twenty-one genes associated with resistance to antibiotics and toxic compounds were predicted, such as beta-lactamase, mercuric reductase, cobalt-zinc-cadmium resistance protein, multicopper oxidase, etc. Strain RR3-28 encodes 86 predicted genes associated with stress response, including osmotic stress, detoxification, cold shock, heat shock, oxidative stress, periplasmic stress, etc. Strain RR3-28 encodes 79 predicted genes associated with membrane transport system such as ABC transporters, cation transporters, TRAP transporters, protein secretion system Type II, protein and nucleoprotein secretion system Type IV, protein translocaion across cytoplasmic membrane. Due to these genes, this strain would have been able to survive in frequently condition-changed seawater RAS facility. To identify prophages, PHAST was used. One intact prophage was identified in a 15.8 Kb (with G+C content 61.2 mol%) region containing 22 CDS (table 3).

Region	1
Region length	15.8 Kb
Completeness(score)	intact(120)
Specific keyword	terminase, portal, head, capsid, tail
Region position	824712-840539
Total protein number	22
Phage hit protein number	14
Hypothetical protein percentage	8
Phage+hypo protein percentage	100%
Bacterial protein number	0
Phage species number	9
Most common phage name (hit genes count)	PHAGE_Paraco_vB_PmaS_IMEP1_NC_026608(4) PHAGE_Rhodob_RcRhea_NC_028954(4) PHAGE_Roseob_1_NC_015466(4) PHAGE_Entero_phiP27_NC_003356(3) PHAGE_Geobac_GBK2_NC_023612(3) PHAGE_Paenib_Fern_NC_028851(2) PHAGE_Shigel_SfIV_NC_022749(2) PHAGE_Rhodob_RcapNL_NC_020489(2) PHAGE_Pseudo_Pq0_NC_029100(2) PHAGE_Entero_Sfl_NC_027339(2) PHAGE_Tetras_SI1_NC_020861(2) PHAGE_Paenib_Xenia_NC_028837(2) PHAGE_Bacter_Rani_NC_029084(2) PHAGE_Idioma_1N2_2_NC_025439(1) PHAGE_Nitrin_1M3_16_NC_024217(1) PHAGE_Salmon_ST64B_NC_004313(1) PHAGE_Vibrio_vB_VpaS_MAR10_NC_019713(1) PHAGE_Strept_mu1_6_NC_007967(1) PHAGE_Azospi_Cd_NC_010355(1) PHAGE_Xantho_Xp10_NC_004902(1) PHAGE_Sodali_SO_1_NC_013600(1) PHAGE_Siphov_YD_2008.s_NC_027383(1) PHAGE_Colwel_9A_NC_018088(1) PHAGE_Feldma_species_virus_NC_011183(1) PHAGE_phiJL001_NC_006938(1) PHAGE_Bacter_Sitara_NC_028854(1) PHAGE_Burkho_KS9_NC_013055(1) PHAGE_Pseudo_YMC11/02/R656_NC_028657(1) PHAGE_Bacter_Diva_NC_028788(1) PHAGE_Caulob_CcrRogue_NC_019408(1) PHAGE_Xantho_OP1_NC_007709(1) PHAGE_Vibrio_pYD38_B_NC_021561(1) PHAGE_Rhizob_16_3_NC_011103(1) PHAGE_Paenib_HB10c2_NC_028758(1) PHAGE_Entero_mEp460_NC_019716(1) PHAGE_Shigel_SfII_NC_021857(1) PHAGE_Tetras_S20_NC_020840(1) PHAGE_Burkho_phi1026b_NC_005284(1) PHAGE_Gordon_GTE6_NC_028665(1)
First most common phage number	4
First most common phage percentage	18.18%
CG percentage	61.20%

Table 3. Detail information of a prophage detected in the genome of RR3-28

Heretofore, five *Nitratireductor* strains, *N. basaltis*, *N. indicus*, *N. pacificus*, *N. aquibiodomus* and *Nitratireductor* sp. ZZ-1, have been sequenced and validated (Lai et al. 2012; Lai et al. 2012; Singh et al. 2012; Danish-Daniel et al. 2014; Chen et al. 2015). Compared with other strains, strain RR3-28 showed lowest values in all categories such as genome size, G+C content, number of CDS and subsystems (table 4).

Table 4. Assembly and annotation results of strain RR3-28 and other strains in genus *Nitratireductor*.

Strain	Genome size	G+C content	CDS	Subsystems	RNAs
RR3-28	3,513,368	58.3	3,410	416	43
N. aquibiodomus RA22	4592790	61.3	4,495	448	53
N. pacificus pht-3B	4466205	65.5	4,292	450	50
N. basaltis UMTGB225	3551806	59.8	3,482	433	50
N. indicus C115	4992479	60.9	4,948	458	48
Nitratireductor sp. ZZ-1	5125657	64.1	5,004	456	48

The functional profile of the six *Nitratireductor* species annotated with the SEED databases is presented in figure 5. These strains in genus *Nitratoreductor* showed a similar trend in the overall. Strain RR3-28 indicated low values for most categories, but it is most similar to the *N. basaltis* than the other strains. In addition, RR3-28 and *N. basaltis* had similar genome size which was shorter than that of the other strains. Also on the ezbiocloud database (www.ezbiocloud.net/), 16S rRNA sequence of strain RR3-28 showed the closest homology with *N. basaltis*. There results indicated that the strain RR3-28 shares more genomic features with *N. basaltis* than with other strains in genus *Nitratireductor*.

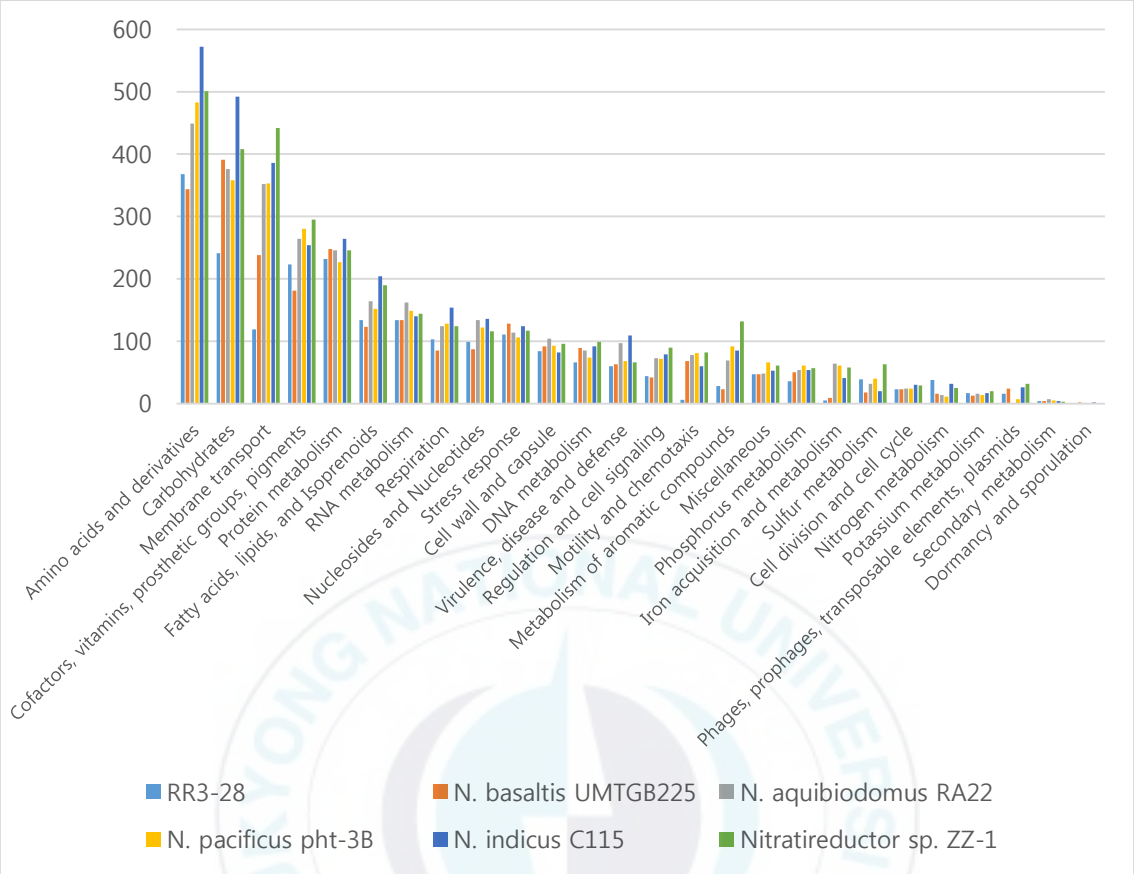


Figure 4. SEED-based functional profiles of strain RR3-28, *N. aquibiodomus* RA22, *N. pacificus* pht-3B, *N. basaltis* UMTGB225, *N. indicus* C115, *Nitratireductor* sp.ZZ-1. The graph indicates the abundance (%) of each category in total annotated CDS.

CONCLUSION

Strain RR3-28 is a denitrifying bacterium derived from seawater recirculating aquaculture system. The genome sequence analysis of strain RR3-28 will provide more knowledge about differences between this strain and the other strains in genus *Nitratireductor*. To be a novel species belonging to a genus *Nitratireductor* or *Pseudahrensia* or novel genus, further studies identifying this bacterium are required.

REFERENCES

Au, K. F., J. G. Underwood, et al. (2012). "Improving PacBio long read accuracy by short read alignment." PLoS One 7(10): e46679.

Chin, C.-S., D. H. Alexander, et al. (2013). "Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data." Nature methods 10(6): 563-569.

Chun, J., J.-H. Lee, et al. (2007). "EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences." Int. J. Syst. Evol. Microbiol. 57(10): 2259-2261.

Pruitt, K. D., T. Tatusova, et al. (2007). "NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins." Nucleic acids research 35(suppl 1): D61-D65.

Au, K. F., J. G. Underwood, et al. (2012). "Improving PacBio long read accuracy by short read alignment." PLoS One 7(10): e46679.

Aziz, R. K., D. Bartels, et al. (2008). "The RAST Server: rapid annotations using subsystems technology." BMC Genomics 9(1): 75.

Chen, Q., H.-Y. Ni, et al. (2015). "Nitratireductor soli sp. nov., isolated from phenol-contaminated soil." Antonie Van Leeuwenhoek 108(5): 1139-1146.

Chin, C.-S., D. H. Alexander, et al. (2013). "Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data." Nature methods 10(6): 563-569.

Chun, J., J.-H. Lee, et al. (2007). "EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences." Int. J. Syst. Evol. Microbiol. 57(10): 2259-2261.

Danish-Daniel, M., H. Y. Gan, et al. (2014). "Genome sequence of Nitratireductor basaltis strain UMTGB225, a marine bacterium isolated from a green barrel tunicate in Bidong Island, Malaysia." Genome announcements 2(5): e01015-01014.

Jeanmougin, F., J. D. Thompson, et al. (1998). "Multiple sequence alignment with Clustal X." Trends Biochem Sci 23(10): 403-405.

Jia, W. Z., Z. Li, et al. (2008). "[Genetic variation and clustal analysis of Trichomonas vaginalis cysteine proteases]." Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi 26(3): 191-196, 202.

Jung, Y.-T., S. Park, et al. (2012). "Pseudahrensia aquimaris gen. nov., sp. nov., isolated from seawater." Int. J. Syst. Evol. Microbiol. 62(9): 2056-2061.

Lai, Q., G. Li, et al. (2012). "Genome sequence of Nitratireductor pacificus type strain pht-3B." J. Bacteriol. 194(24): 6958-6958.

Lai, Q., G. Li, et al. (2012). "Genome sequence of Nitratireductor indicus type strain C115." J. Bacteriol. 194(24): 6990-6990.

Lehtonen, S. (2008). "Phylogeny estimation and alignment via POY versus Clustal + PAUP*: a response to Ogden and Rosenberg (2007)." Syst Biol 57(4): 653-657.

Leonard, N., J. P. Blancheton, et al. (2000). "Populations of heterotrophic bacteria in an experimental recirculating aquaculture system." Aquacultural engineering 22(1-2): 109-120.

Mansur, M. A., G. P. Cardozo, et al. (2008). "SNUFER: A software for localization and presentation of single nucleotide polymorphisms using a Clustal multiple sequence alignment output file." Bioinformation 3(2): 63-64.

Nguyen, V. K., S. Hong, et al. (2015). "Autotrophic denitrification performance and bacterial community at biocathodes of bioelectrochemical systems with either abiotic or biotic anodes." Journal of bioscience and bioengineering 119(2): 180-187.

Park, S., S. Yoshizawa, et al. (2012). "Halioglobus japonicus gen. nov., sp. nov. and Halioglobus pacificus sp. nov., members of the class Gammaproteobacteria isolated from seawater." Int J Syst Evol Microbiol 62(Pt 8): 1784-1789.

Pruitt, K. D., T. Tatusova, et al. (2007). "NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins." Nucleic acids research 35(suppl 1): D61-D65.

Saitou, N. and M. Nei (1987). "The neighbor-joining method: a new method for reconstructing phylogenetic trees." Mol. Biol. Evol. 4(4): 406-425.

Sievers, F., A. Wilm, et al. (2011). "Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega." Mol Syst Biol 7: 539.

Singh, A., P. K. Jangir, et al. (2012). "Genome sequence of Nitratireductor aquibiodomus strain RA22." J. Bacteriol. 194(22): 6307-6307.

Tamura, K., G. Stecher, et al. (2013). "MEGA6: molecular evolutionary genetics analysis version 6.0." Mol. Biol. Evol.: mst197.

Thompson, J. D., T. J. Gibson, et al. (1997). "The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools." Nucleic Acids Res 25(24): 4876-4882.

Thompson, J. D., D. G. Higgins, et al. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." Nucleic acids research 22(22): 4673-4680.

Van Rijn, J. (1996). "The potential for integrated biological treatment systems in recirculating fish culture—a review." Aquaculture 139(3): 181-201.

Zhou, Y., Y. Liang, et al. (2011). "PHAST: a fast phage search tool." Nucleic acids research: gkr485.

국문 초록

육상 수조 양식법 중 하나인 순환 여과양식시스템(recirculating aquaculture system, RAS)에서는 양식수를 생물여과조를 통해 정화시켜 재사용하는 방식을 이용하며, 이 때 시스템 내에 존재하는 세균이 양식수의 정화 과정에 관여한다. 본 연구에서는 부산 기장 수산과학연구원의 해수 RAS 내에 존재하는 세균의 다양성을 확인하고, 여기에서 분리된 두 탈질 균주의 유전체 분석도 수행하였다. 실험에 이용된 해수 RAS 는 여섯 개의 탱크로 구성되었으며 실험기간 동안 염도와 온도를 다르게 조합한 아홉 가지 조건 하에 가동되었다. 세균 다양성은 16S rRNA amplicon sequencing 을 이용하여 분석하였다. *Proteobacteria* 와 *Bacteroidetes* 문의 세균 그룹이 67.2-99.4%로 가장 많은 비율을 차지하였다. 여섯 개의 탱크 중에 팩키지여과조, 메쉬여과조 그리고 수조숙성조에서 97% OTU 수준에서 다른 여과조들보다 높은 세균 다양성을 확인하였다. Nitrifying bacteria 도 이 세 여과조에서 많이 확인되었고, *Rhodobacteraceae* 와 *Flavobacteriaceae* 과에 속하는 세균 그룹은 전 시료에 골고루 분포한 것을 확인하였다. 이 중에서도, denitrifying bacteria 는 양식수 내의 암모니아 제거에 기여함으로써 RAS 의 정화 프로세스의 효율성 증진을 위하여 특히 연구 가치가 있다고 보여진다. 메쉬여과조에서 분리된 두 탈질 균주, RR3-28 과 RR3-57 에 대하여 genome sequencing 과 annotation 을 수행하였다. RR3-57 은 *Halioglobus pacificus* S1-72(T)와 96.22%의 16S rRNA sequence similarity 를 보였고, whole genome 은 총 5,003,575 bp 길이, GC 함량 57.5 mol%이며 4,627 개의 CDS 와 49 개의 RNA 를 포함한 것으로 확인되었다. RR3-28 은 *Nitratireductor basaltis* J3(T)과 95.45%의 16S rRNA sequence similarity 를 보였고, whole genome 은 총 3,513,368 bp 길이, GC 함량 58.3 mol%이며 3,410 개의 CDS 와 43 개의 RNA 를 포함한 것으로 확인되었다. RR3-28 과 RR3-57 은 각각 18 개와 21 개의 탈질 관련 유전자를 가지는 것으로 확인되었다. RR3-28 은 기존에 유전체 분석이 완료된 *Nitratireductor* 속의 균주들과 유전체 비교를 했지만, RR3-57 은 *Halioglobus* 속의 균주 중 유전체 분석이 진행된 균주가 없는 관계로 유전체

비교는 하지 않았다. 본 연구에서는 해수 RAS 내에서 세균 군집이 가지는 역할을 이해하기 위하여 세균 다양성을 연구하였다. 또한, 이 시스템에서 분리된 두 탈질 균주의 유전체 서열 분석은 시스템 내에서 잠재적으로 일어나는 탈질 기작을 유전자 수준에서 이해하는 데에 기여할 것이다.

Supplementary: the published paper containing the content of Chapter 1.

Journal of Microbiology (2016) Vol. 54, No. 4, pp. 296–304
DOI 10.1007/s12275-016-5571-4

eISSN 1976-3794
pISSN 1225-8873

Uncultured bacterial diversity in a seawater recirculating aquaculture system revealed by 16S rRNA gene amplicon sequencing

Da-Eun Lee[1†], Jinhwan Lee[2†], Young-Mog Kim[3], Jeong-In Myeong[2], and Kyoung-Ho Kim[1*]

[1]*Department of Microbiology, Pukyong National University, Busan 48513, Republic of Korea*
[2]*Aquaculture Research Division, National Institute of Fisheries Science, Busan 46083, Republic of Korea*
[3]*Department of Food Science and Technology, Pukyong National University, Busan 48513, Republic of Korea*

(Received Nov 17, 2015 / Revised Mar 7, 2016 / Accepted Mar 14, 2016)

Bacterial diversity in a seawater recirculating aquaculture system (RAS) was investigated using 16S rRNA amplicon sequencing to understand the roles of bacterial communities in the system. The RAS was operated at nine different combinations of temperature (15°C, 20°C, and 25°C) and salinity (20‰, 25‰, and 32.5‰). Samples were collected from five or six RAS tanks (biofilters) for each condition. Fifty samples were analyzed. *Proteobacteria* and *Bacteroidetes* were most common (sum of both phyla: 67.2% to 99.4%) and were inversely proportional to each other. Bacteria that were present at an average of ≥ 1% included Actinobacteria (2.9%) Planctomycetes (2.0%), Nitrospirae (1.5%), and Acidobacteria (1.0%); they were preferentially present in packed bed biofilters, mesh biofilters, and maturation biofilters. The three biofilters showed higher diversity than other RAS tanks (aerated biofilters, floating bed biofilters, and fish tanks) from phylum to operational taxonomic unit (OTU) level. Samples were clustered into several groups based on the bacterial communities. Major taxonomic groups related to family *Rhodobacteraceae* and *Flavobacteriaceae* were distributed widely in the samples. Several taxonomic groups like [*Saprospiraceae*], *Cytophagaceae*, *Octadecabacter*, and *Marivita* showed a cluster-oriented distribution. *Phaeobacter* and *Sediminicola*-related reads were detected frequently and abundantly at low temperature. Nitrifying bacteria were detected frequently and abundantly in the three biofilters. Phylogenetic analysis of the nitrifying bacteria showed several similar OTUs were observed widely through the biofilters. The diverse bacterial communities and the minor taxonomic groups, except for *Proteobacteria* and *Bacteroidetes*, seemed to play important roles and seemed necessary for nitrifying activity in the RAS, especially in packed bed biofilters, mesh biofilters, and maturation biofilters.

Keywords: bacterial diversity, recirculating aquaculture system, biofilter, amplicon sequencing, 16S rRNA gene

† These authors contributed equally to this work.
*For correspondence. E-mail: kimkh@pknu.ac.kr; Tel.: +82-52-629-5611; Fax: +82-52-629-5618

Introduction

Recirculating aquaculture systems (RASs) have been recognized for their potential in aquaculture as a way of overcoming limited water supplies, increasing production capacity, reducing heat loss, and minimizing waste effluent (Losordo and Hobbs, 2000).

Carbon and nitrogen compounds from fish manure and undigested feed are metabolized in a RAS by diverse microbial communities. Carbon compounds are degraded by heterotrophic bacteria and nitrogen-containing organic compounds are degraded with nitrogen released as ammonia. Even low levels of ammonia are a detriment in aquaculture; lethal concentrations, expressed as median 96 h LC50, of 1.7 mg/L for seabass and 2.5–2.6 mg/L for seabream and turbot (Ruyet *et al.*, 1995). Ammonia is converted to nitrite, which is converted to nitrate, by ammonia- and nitrite-oxidizing bacteria, respectively. Most studies have focused on the role of biofilters in mediating nitrogen cycles (Schreier *et al.*, 2010) with the aim of reducing ammonia concentration in the fish tank. Little is known of the overall bacterial diversity in RAS. A recent study that used bar code pyrosequencing to investigate the overall diversity of RAS bacterial communities used to rear turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis*) demonstrated differences in the bacterial communities in the two systems (Martins *et al.*, 2013).

Using a combination of bar code primers and high throughput sequencing, it is possible to obtain thousands of 16S rRNA gene sequences per sample from dozens of samples, which allows the determination of uncultured bacterial diversity in given environments (Binladen *et al.*, 2007). We applied 16S rRNA gene amplicon sequencing to investigate the bacterial diversity in an experimental RAS system rearing convict groupers (*Epinephelus septemfasciatus*). Knowledge of bacterial diversity depending on operating conditions is important to enhance the design and operational efficiency of biofilters, but the data are limited. We investigated the bacterial diversity in different biofilter types during different operating conditions of salinity and water temperature in an experimental RAS using 16S rRNA gene amplicon sequencing.

Materials and Methods

RAS operational conditions

The RAS used in this study is depicted schematically in Fig. 1. In the packed bed biofilter (A; 1.5 m length, 0.8 m width, and