Thesis for the Degree of Master of Science

Isolation and taxonomic study of novel bacteria from a recirculating aquaculture system

By

Yeon Hee Jang

Department of Microbiology

The Graduate School

Pukyong National University

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(순환여과양식 시스템에서 신균의 분리 및 분류학적 연구)

Advisor: Prof. Kyoung-Ho Kim

By

Yeon Hee Jang

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Yeon-Hee Jang

Approved by:

(Chairman) Gun-Do Kim

(Member) Young-Jae Jeon

(Member) Kyoung-Ho Kim

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CONTENTS

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ABSTRACT

A recirculating aquaculture system (RAS) is a means which can make it possible to maintain water quality of an inland fish farm in suitable condition. A RAS has physical, chemical and biological treatment processes to remove fodder debris, fish excreta and toxic nitrogen compounds such as ammonia, nitrate and nitrite. In the biological process, bacteria take an important role. Bacteria have nitrification and denitrification metabolism system that can convert harmful nitrogen compounds to harmless. In this study, denitrifying bacteria were screened and some of them were candidates for novel genera and species. All strains have low sequence similarity with their closely related species as follows; RR4-38, *Ulvibacter antarcticus* IMCC3103(T) (95.05%); RR4-40, *Ulvibacter marinus*

IMCC1208(T) (94.17%); RR4-41, *Kangiella geojedonensis* YCS-5(T) (98.08%); RR4-56, *Halovulum dunhuangense* YYQ-30(T) (92.84%); RR4-68, *Formosa spongicola* A2(T) (97.35%) respectively. Phylogenetic trees for each strain showed their phylogenetic positions in each taxonomic level. All strains have rod-shaped cell body with motility and showed optimal growth at 30℃, pH 7.0-7.5 and 2.5-3.0% of NaCl concentration. Oxidase, catalase and biochemical tests were conducted for each strain. Physiological tests such as carbon assimilation and enzyme production were also conducted. On the basis of physiological and phylogenetic analyses, three strains are candidates for new genus and two strains for novel species. The type strains are RR4-40, RR4-56, RR4-38, RR4-41 and RR4-68 respectively. Thus several novel strains could be obtained from the RAS. The results showed that a RAS is an unexplored environment and good resources for finding new taxon. This study will contribute to understand RASs and to improve their efficiency.

INTRODUCTION

The recirculating aquaculture system (RAS) is a method building the aquafarm on the ground which supply the breeding water and filtrate the breeding water. The used breeding water is filtered gradually and supplied to the aquafarm again by pump (Nazar, *et al.*, 2013).

The most important thing in the system of farming fish is water quality management. Preventing the penetration of pathogens and getting rid of them by filtering the waste caused by fish in a farm and solid matter, which is not taken but left to sink to the bottom, are the basis of water quality management in the fish farm. (Au, *et al.*, 2012) RAS is one of fish-farming methods that process wastes coming from raising fish through recirculating rearing water. RAS aims to maintain water quality, keep fish from catching a disease, and improve fish quality. (Chin, *et al.*, 2013)

Organic carbon and organic nitrogen are generated from the scraps of fodder and filth in fish tanks. Since ammonia nitrogen and nitrate nitrogen from organic nitrogen severely affects organism, controlling remaining ammonia nitrogen and nitrate nitrogen in circulated water is most important for stable running and management. (Chin, *et al.*, 2013) Stable nitrogen cleaning process is essential in RASs. For RAS, cleaning process of organic nitrogen is done by microorganism in biofilter. (Harwanto and Jo, 2010) Especially nitrifying microorganisms (nitrifiers) convert ammonia to nitrate nitrogen via

nitrous acid and denitrifying microorganisms (denitrifiers) convert nitrate nitrogen to nitrogen gas. (Schreier, *et al.*, 2010) Therefore, biological processing of RAS is so critical in terms of economic feasibility and efficiency (Interdonato, 2012).

The RAS model used in this study was operated in the National Institute of Fisheries Science designed to rear convict groupers (*Epinephelus septemfasciatus*) using seawater. The system was composed of fish-rearing tank, packed bed biofilter, fluidized bed biofilter, mesh biofilter, and maturation biofilter, in which the tanks were connected sequentially and water was circulated by pump. The water from maturation biofilter was recirculated to the fish-rearing tank. In the previous study, the uncultured microbial diversity of the system was investigated using the 16S rRNA amplicon sequencing method (Lee*, et al.*, 2016).

Nitrifying bacteria or ammonia oxidizing bacteria are important members in RAS being in charge of nitrification, the bottleneck process in nitrogen removal. They are autotrophic microorganisms and generally regarded to be difficult to cultivate (Urakawa, *et al.*, 2008). Several kinds of novel nitrifiers existing in the RAS system used in this system thorough the culture-independent study (Lee, *et al.*, 2016).

Denitrifying bacteria are also important members in RAS. They degrade organic carbons through nitrate respiration and discard nitrate from the system by transforming into nitrogen gas (Schreier

et al., 2010). Various kinds of novel heterotrophic bacteria were identified in the system and some of them might be denitrifiers (Lee, *et al.*, 2016).

In this study, cultivation of heterotrophic bacteria were conducted to isolate heterotrophic and/or denitrifying bacteria, Oligotrophic cultivation was used for growing denitrification bacteria to increase cultivability. Oligotrophic culture was adopted for culturing the strains that adapted to slow growth rate and low concentration of organic matter.

The isolates were identified using partial 16S rRNA genes sequencing. Several novel strains were selected and their 16S rRNA gene full sequences were used for phylogenetic analysis. Five candidates were selected for novel species or genera and their taxonomic analyses have been done. Thus several novel strains could be obtained from the RAS. The results showed that a RAS is an unexplored environment and good resources for finding new taxon. This study will contribute to understand RASs and to improve their efficiency.

MATERIALS AND METHODS

Sample preparation

The RAS was operated at 25‰ of salt concentration and 25℃ of temperature. The RAS was designed to rear convict groupers (*E. septemfasciatus*), marine fish. Samples for isolating bacteria were collected from two biofilters, packed bed biofilter and mesh biofilter because they were known to have important roles in the RAS system (Lee, *et al.*, 2016). Detailed scales of the five biofilters were described in the previous study (Lee, *et al.*, 2016). Sludge from packed bed biofilter and cells collected from mesh were used as inoculum.

Isolation of strains

The sludge from two biofilters was serially diluted and inoculated on BTB agar (Wu, *et al.*, 2013) and diluted marine agar. To create an oligotrophic condition, marine broth was diluted 5 times and salt concentration was adjusted to 25‰. The salt concentration was also adjusted to 25‰ for BTB agar. Each 100ul of the 10, 100, 1,000, and 10,000-times diluted samples in PBS were spread on the agar plate and cultured at the temperature of 25℃ for 7 days. The generation of colony was checked daily and a new colony was moved to a new

medium for pure culture. Single colony was transferred to fresh agar 3-4 times to verify pure culture.

Colony PCR of 16S rRNA gene

One colony was taken and added to PCR tube containing PCR premix. Each 1 μl of primer 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-CGG TTA CCT TGT TAC GAC TT-3') were added to PCR premix (HiPi PCR premix, Elpis, Korea) and adjusted up to a total of 20 μl PCR reaction solution using distilled water. PCR process was performed as follows: The templates were pre-denaturated at the temperature of 95℃ for 10 minutes. They were denaturated at 95℃ for 30 seconds, annealed at 60℃ for 30 seconds, and extended at 72℃ for one and a half minutes. This process was repeated 32 times. Final extension step was performed at 72℃ for 10 minutes.

PCR product was checked on electrophoresis in 1% agarose gel. The amplified PCR product went through DNA sequencing using the primer 8F. The sequences with high quality was selected to yield about 400~600 bp of nucleotides and compared with the database at EzTaxon server (http://www.ezbiocloud.net/) to find out the most similar bacterial strains verified taxonomically.

Five strains distantly related to known bacteria were selected to further phylogenetic analysis. The full sequencing of the selected strain was made by using additional primers such as 1492R (5'-CGG

TTA CCT TGT TAC GAC TT-3'), 518R (5'-GTA TTA CCG CGG CTG CTG G-3'), and 1100F (5'-YAA CGA GCG CAA CCC-3'). The merged sequence was analyzed again at EzTaxon server and used to phylogenetic analysis.

Phylogenetic analysis of novel strains

16S rRNA genes of close strains were collected from ExTaxon server to be used for phylogenetic analysis. The Clustal W program was used to align sequences (Chun, *et al.*, 2007). The aligned sequences were used to construct phylogenetic tree with MEGA 6 (Tamura *et al.* 2013). The Kimura 2 parameter method was used to calculate a distance matrix (Kimura, 1980) and neighbor joining tree was constructed with 1,000 bootstrap resampling. Maximum likelihood tree was also constructed to confirm the phylogenetic position with 100 bootstrap resampling.

Growth condition tests

Growth conditions of five novel strains were conducted. Growth on different media such as mueller hinton broth (MHB; Difco), luria-bertani broth (LB; Difco), tryptic soy broth and nutrient broth (KFTA) was checked. All media were adjusted up to 25‰ NaCl concentration. 100 µl culture medium were inoculated to 5 ml of media in 15 ml conical tubes and incubated at 25℃ for 7 days. Culture solution was transferred to 96 well plates and absorbance was measured using

amicroplate reader (VERSAmax, Molecular Devices). Growth on different pH (from 3 to 11) was tested in marine broth (MB; Difco) adjusted to each pH. Different range of temperature (4, 25, 30, 35, and 40 ℃) and NaCl concentrations (0, 0.5, 1, 2, 2.5, and 3%) were tested in marine broth at 25℃ for 7 days.

Motility test

Motility was examined by stabbing each colony into the center of a semi-solid agar medium (0.3% agar) and observing the diffusing colonies for 4 days. (Luna, *et al.*, 2005)

Physiological tests

Physiological tests were performed using API 20NE (bioMérieux) and API 50CH (bioMérieux) kits and enzyme activities were tested using an API ZYM kit (bioMérieux) according to manufacturer's instructions.

Whole cell fatty acid analysis

Total cellular fatty acids were analyzed using the Microbial Identification (MIDI; Newar, Delaware) system by the standardized procedure described by Miller and Berger (1985). Approximately 40 to 50 mg of concentrated whole cell was incubated for 30 min at 100℃ after addition of 1ml of 15% (wt/vol) NaOH in 50% aqueous methanol.

The samples were then acidified to pH 2 by adding 6N HCl in CH_3OH, and the methylated fatty acids were further extracted with 1.25 ml of 1:1 (vol/vol) solution of methyl-*tert*-buthyl ether-hexane. The organic extract was washed with 3 ml of 1.2% (wt/vol) NaOH and transfered to new sample bottle.

Fatty acid methyl esters (FAMEs) were analyzed by gas-liquid chromatography on an HP 6890A gas chromatograph (Hewlett-Packard Co. USA) equipped with a flame ionization detector. A fused-silica capillary column (0.2mm×25m; cross-linked 5% methyl phenyl silicone [HewlettPackard Co. USA] with ultrahigh-purity hydrogen as the carrier gas was used. The details of the gas-liquid chromatography conditions are as follows: injector temperature, 250℃; detector temperature, 300℃; initial column temperature, 170℃, increasing by 5℃/min to 270℃ in 20 min; carrier gas flow rate, 50℃/min; sample volume, 1ul. FAMEs were calibrated against a standard mixture of known fatty acids provide by MIDI. The peak retention time and peak area values were recorded, analyzed and named with a Sherlock Computer System. The result was expressed as percentages relative to the total peak area.

Table1. List of isolates and their closest match at the EzTaxon server

No	Strain	Closest match	Similarity (%)	Class	Family
1	RR4-1	*Roseovarius sediminilitoris M-M10(T)*	99.11	*Alphaproteobacteria*	*Rhodobacteraceae*
2	RR4-2	*Mycobacterium conceptionense CIP 108544(T)*	99.66	*Actinobacteria*	*Mycobacteriaceae*
3	RR4-3	*Mycobacterium conceptionense CIP 108544(T)*	99.62	*Actinobacteria*	*Mycobacteriaceae*
4	RR4-6	*Algibacter aquimarinus KYW589(T)*	97.21	*Flavobacteriia*	*Flavobacteriaceae*
5	RR4-7	*Maribacter forsetii KT02ds18-6(T)*	94.71	*Flavobacteriia*	*Flavobacteriaceae*
6	RR4-8	*Formosa spongicola A2(T)*	96.51	*Flavobacteriia*	*Flavobacteriaceae*
7	RR4-10	*Pelagicola litorisediminis D1-W8(T)*	97.5	*Alphaproteobacteria*	*Rhodobacteraceae*
8	RR4-11	*Gilvibacter sediminis Mok-1-36(T)*	94.71	*Flavobacteriia*	*Flavobacteriaceae*
9	RR4-12	*Sphingopyxis italica SC13E-S71(T)*	97.05	*Alphaproteobacteria*	*Sphingomonadaceae*
10	RR4-13	*Ruegeria conchae TW15(T)*	100	*Alphaproteobacteria*	*Rhodobacteraceae*
11	RR4-14	*Oceanospirillum linum ATCC 11336(T)*	92.96	*Gammaproteobacteria*	*Oceanospirillaceae*
12	RR4-15	*Ruegeria atlantica IAM 14463(T)*	99.79	*Alphaproteobacteria*	*Rhodobacteraceae*
13	RR4-16	*Algibacter mikhailovii LMG 23988(T)*	96.04	*Flavobacteriia*	*Flavobacteriaceae*
14	RR4-17	*Gilvibacter sediminis Mok-1-36(T)*	93.89	*Flavobacteriia*	*Flavobacteriaceae*
15	RR4-18	*Thalassotalea agariperforans M-M1(T)*	100	*Gammaproteobacteria*	*Colwelliaceae*
16	RR4-19	*Sphingorhabdus marina FR1087(T)*	98.93	*Alphaproteobacteria*	*Sphingomonadaceae*
17	RR4-20	*Microbacterium awajiense YM13-414(T)*	99.05	*Actinobacteria*	*Microbacteriaceae*
18	RR4-21	*Altererythrobacter aestuarii*	97.84	*Alphaproteob*	*Erythrobacte*

		KYW147(T)		*acteria*	*raceae*
19	RR4-23	*Marinicella litoralis KMM 3900(T)*	98.35	*Gammaproteobacteria*	*Marinicella*
20	RR4-24	*Formosa spongicola A2(T)*	96.55	*Flavobacteriia*	*Flavobacteriaceae*
21	RR4-25	*Sphingorhabdus marina FR1087(T)*	98.93	*Alphaproteobacteria*	*Sphingomonadaceae*
22	RR4-27	*Maritalea porphyrae LCM-3(T)*	97.21	*Alphaproteobacteria*	*Hyphomicrobi aceae*
23	RR4-28	*Microbacterium awajiense YM13-414(T)*	99.13	*Actinobacteria*	*Microbacteriaceae*
24	RR4-29	*Psychroserpens mesophilus KOPRI 13649(T)*	98.01	*Flavobacteriia*	*Flavobacteriaceae*
25	RR4-30	*Erythrobacter odishensis JA747(T)*	99.2	*Alphaproteobacteria*	*Erythrobacteraceae*
26	RR4-31	*Sabulilitoribacter multivorans M-M16(T)*	96.51	*Flavobacteriia*	*Flavobacteriaceae*
27	RR4-32	*Sneathiella chungangensis CAU 1294(T)*	97.2	*Alphaproteobacteria*	*Sneathiellaceae*
28	RR4-33	*Formosa spongicola A2(T)*	96.63	*Flavobacteriia*	*Flavobacteriaceae*
29	RR4-35	*Sabulilitoribacter multivorans M-M16(T)*	96.46	*Flavobacteriia*	*Flavobacteriaceae*
30	RR4-38	*Ulvibacter antarcticus IMCC3101(T)*	94.34	*Flavobacteriia*	*Flavobacteriaceae*
31	RR4-40	*Ulvibacter marinus IMCC 12008(T)*	94.39	*Flavobacteriia*	*Flavobacteriaceae*
32	RR4-41	*Kangiella geojedonensis YCS-5(T)*	96.85	*Gammaproteobacteria*	*Alcanivoracaceae*
33	RR4-42	*Pseudoalteromonas agarivorans KMM 255(T)*	100	*Gammaproteobacteria*	*Pseudoalteromonadaceae*
34	RR4-43	*Muricauda ruestringensis DSM 13258(T)*	95.54	*Flavobacteriia*	*Flavobacteriaceae*
35	RR4-44	*Formosa spongicola A2(T)*	99.6	*Flavobacteriia*	*Flavobacteriaceae*
36	RR4-45	*Winogradskyella damuponesis F081-2(T)*	99.85	*Flavobacteriia*	*Flavobacteriaceae*
37	RR4-46	*Altererythrobacter luteolus SW-*	97.12	*Alphaproteob*	*Erythrobacte*

		109(T)		*acteria*	*raceae*
38	RR4-49	*Defluviimonas aestuarii BS14(T)*	98.41	*Alphaproteob acteria*	*Rhodobactera ceae*
39	RR4-51	*Altuibacter lentus JL2010(T)*	97.51	*Flavobacterii a*	*Flavobacteria ceae*
40	RR4-52	*Defluviimonas aestuarii BS14(T)*	98.18	*Alphaproteob acteria*	*Rhodobactera ceae*
41	RR4-53	*Hwangdonia seohaensis HD-3(T)*	95.36	*Flavobacterii a*	*Flavobacteria ceae*
42	RR4-55	*Fretibacter rubidus JC2236(T)*	96.87	*Alphaproteob acteria*	*Hyphomonad aceae*
43	RR4-56	*Rhodobacter azotoformans KA25(T)*	91.95	*Alphaproteob acteria*	*Rhodobactera ceae*
44	RR4-57	*Psychroserpens mesophilus KOPRI 13649(T)*	98.28	*Flavobacterii a*	*Flavobacteria ceae*
45	RR4-58	*Roseovarius halocynthiae MA1-10(T)*	97.17	*Alphaproteob acteria*	*Rhodobactera ceae*
46	RR4-60	*Pelagicola litorisediminis D1-W8(T)*	98.04	*Alphaproteob acteria*	*Rhodobactera ceae*
47	RR4-61	*Psychroserpens mesophilus KOPRI 13649(T)*	98.19	*Flavobacterii a*	*Flavobacteria ceae*
48	RR4-62	*Ruegeria atlantica IAM 14463(T)*	99.84	*Alphaproteob acteria*	*Rhodobactera ceae*
49	RR4-63	*Arenitalea lutea P7-3-5(T)*	99.88	*Flavobacterii a*	*Flavobacteria ceae*
50	RR4-64	*Fretibacter rubidus JC2236(T)*	96.11	*Alphaproteob acteria*	*Hyphomonad aceae*
51	RR4-65	*Ruegeria atlantica IAM 14463(T)*	99.74	*Alphaproteob acteria*	*Rhodobactera ceae*
52	RR4-66	*Defluviimonas aestuarii BS14(T)*	98.05	*Alphaproteob acteria*	*Rhodobactera ceae*
53	RR4-67	*Maribacter forsetii KT02ds18-6(T)*	94.14	*Flavobacterii a*	*Flavobacteria ceae*
54	RR4-68	*Formosa spongicola A2(T)*	95.77	*Flavobacterii a*	*Flavobacteria ceae*
55	RR4-71	*Pseudoalteromonas arctica A 37-1-2(T)*	100	*Gammaprote obacteria*	*Pseudoaltero monadaceae*
56	RR4-74	*Pseudoalteromonas agarivorans*	100	*Gammaprote*	*Pseudoaltero*

		KMM 255(T)		*obacteria*	*monadaceae*
57	RR4-75	*Pseudoalteromonas agarivorans KMM 255(T)*	100	*Gammaproteobacteria*	*Pseudoalteromonadaceae*
58	RR4-76	*Pseudoalteromonas agarivorans KMM 255(T)*	100	*Gammaproteobacteria*	*Pseudoalteromonadaceae*
59	RR4-77	*Pseudoalteromonas agarivorans KMM 255(T)*	100	*Gammaproteobacteria*	*Pseudoalteromonadaceae*
60	RR4-78	*Pseudoalteromonas agarivorans KMM 255(T)*	100	*Gammaproteobacteria*	*Pseudoalteromonadaceae*
61	RR4-79	*Psychrobacter nivimaris 88/2-7(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
62	RR4-80	*Psychrobacter piscatorii T-3-2(T)*	99.85	*Gammaproteobacteria*	*Moraxellaceae*
63	RR4-81	*Psychrobacter nivimaris 88/2-7(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
64	RR4-82	*Psychrobacter nivimaris 88/2-7(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
65	RR4-83	*Psychrobacter piscatorii T-3-2(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
66	RR4-84	*Psychrobacter nivimaris 88/2-7(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
67	RR4-85	*Psychrobacter nivimaris 88/2-7(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
68	RR4-86	*Psychrobacter nivimaris 88/2-7(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
69	RR4-89	*Psychrobacter piscatorii T-3-2(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
60	RR4-91	*Pseudoalteromonas agarivorans KMM 255(T)*	100	*Gammaproteobacteria*	*Pseudoalteromonadaceae*
61	RR4-93	*Psychrobacter piscatorii T-3-2(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
62	RR4-95	*Psychrobacter piscatorii T-3-2(T)*	100	*Gammaproteobacteria*	*Moraxellaceae*
63	RR4-100	*Williamsia deligens IMMIB RIV-956(T)*	99.15	*Actinobacteria*	*Nocardiaceae*
64	RR4-101	*Nitratireductor pacificus pht-3B(T)*	97.73	*Alphaproteobacteria*	*Phyllobacteriaceae*
65	RR4-	*Halomonas alkaliphila 18bAG(T)*	99.69	*Gammaprote*	*Halomonadac*

	102			*obacteria*	*eae*
66	RR4-103	*Halomonas alkaliphila 18bAG(T)*	99.71	*Gammaproteobacteria*	*Halomonadaceae*
67	RR4-105	*Halomonas alkaliphila 18bAG(T)*	99.7	*Gammaproteobacteria*	*Halomonadaceae*
68	RR4-106	*Marinomonas foliarum IVIA-Po-155(T)*	98.68	*Gammaproteobacteria*	*Oceanospirillaceae*
69	RR4-107	*Halomonas alkaliphila 18bAG(T)*	99.73	*Gammaproteobacteria*	*Halomonadaceae*
70	RR4-110	*Pseudoalteromonas agarivorans KMM 255(T)*	99.67	*Gammaproteobacteria*	*Pseudoalteromonadaceae*

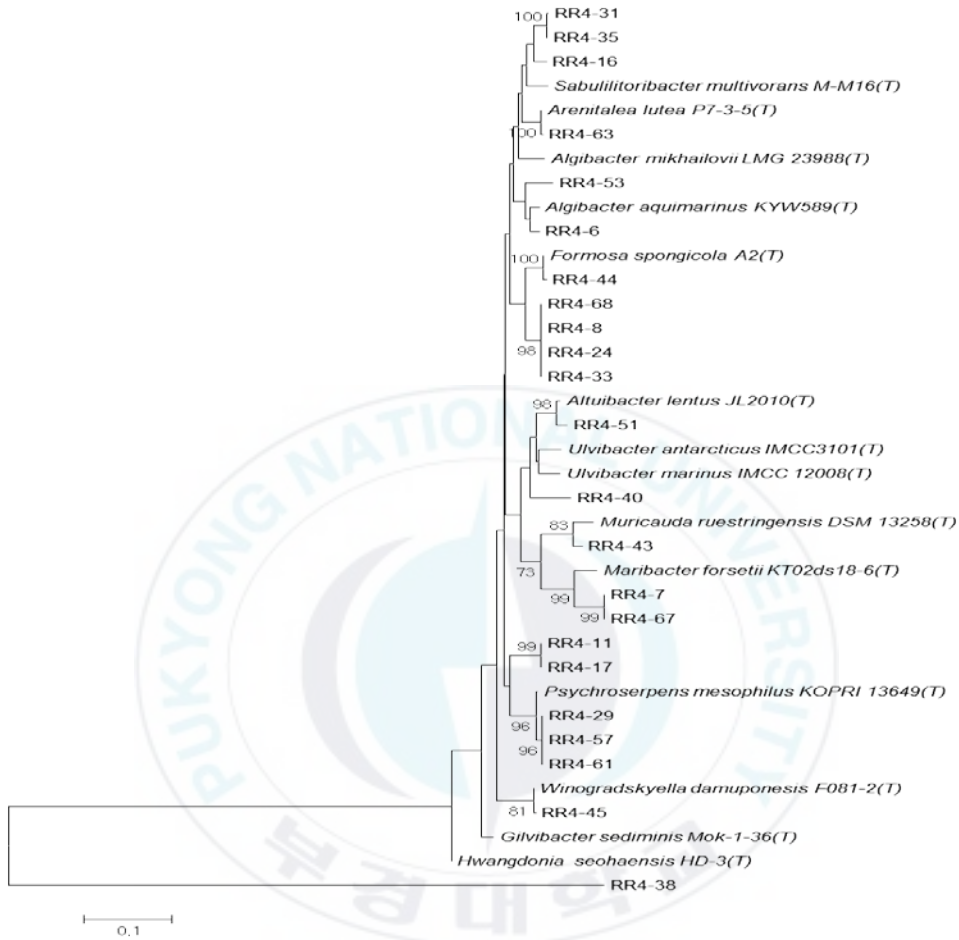

Figure 1. Phylogenetic tree of isolated strains belonging to the phylum *Bacteroidetes*

Tree was constructed based on the aligned 16S rRNA gene sequences using the neighbor-joining methods. The strain RR4-38, 40, 68 were selected for further analysis because they were candidates for novel taxa. All of them belong to family *Flavobacteriaceae* in class *Flavobacteriia*.

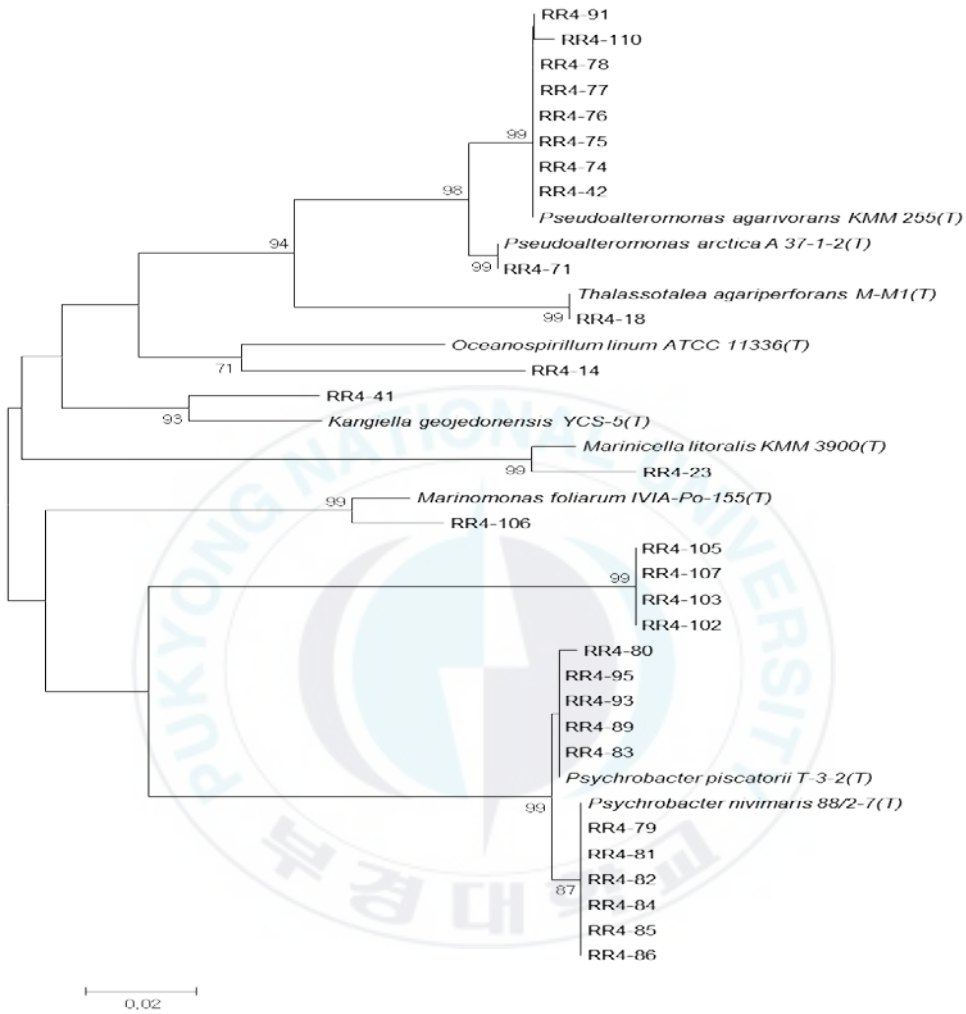

Figure 2. Phylogenetic tree of isolated strains belonging to the class *Gammaproteobacteria*

Tree was constructed based on the aligned 16S rRNA gene sequences using the neighbor-joining methods. The strain RR4-41 was selected for further analysis because it was a candidate for novel taxon.

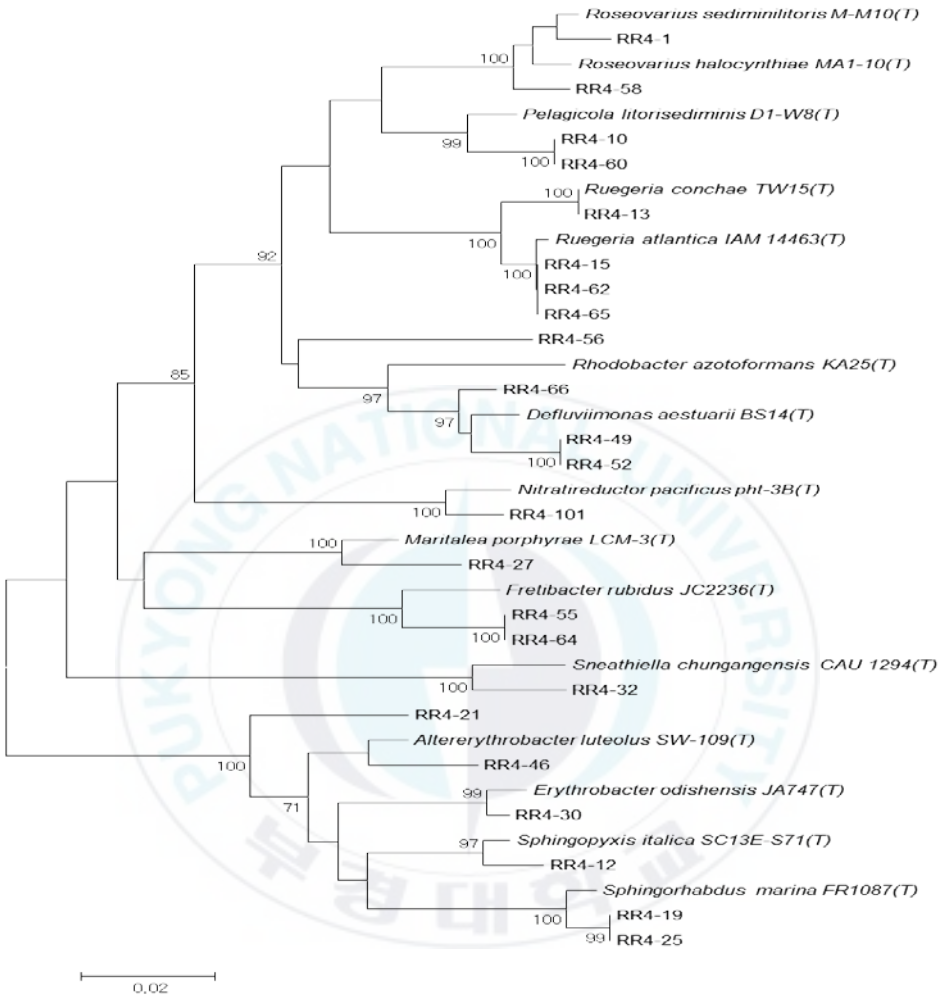

Figure 3. Phylogenetic tree of isolated strains belonging to the class *Alphaproteobacteria*

Tree was constructed based on the aligned 16S rRNA gene sequences using the neighbor-joining methods. The strain RR4-56 was selected for further analysis because it was a candidate for novel taxon.

CHAPTER 1. Taxonomic study of the strain RR4-38

INTRODUCTION

The genus Ulvibacter, first proposed by Nedashkovskaya et al. (2004), currently was affiliated to the branch of the family Flavobacteriaceae. The genus was mostly isolated from different marine environments and had menaquinone-6 (MK-6) as a major respiratory quinone (Nedashkovskaya et al., 2004). The genus Ulvibacter recently got new species, Ulvibacter antarcticus, isolated from Antarctic costal seawater. The family Flavobacteriaceae is included in Cytophaga-Flavobacterium-Bacteroides (Nedashkovskaya et al., 2004). The phylum Cytophaga-Flavobacterium-Bacteroides is one of the dominant group in the marine environment (Suzuki, et al., 2001) and has ability of degradation macromolecule (Makoto et al., 2001). The family Flavobacteriaceae have underwent expansion as new genera have been discovered (Bowman et al., 2006).

Strain RR4-38 was isolated from a biofilter from a seawater recirculating aquaculture system. On the basis of 16S rRNA analysis, strain RR4-38 was mostly related to Ulvibacter antarcticus (95.05%). In this study, strain RR4-38 was proposed as a novel genus belonging to the Phylum Bacteroidetes based on phenotypic and biochemical difference, and phylogenetic feature.

RESULTS AND DISCUSSION

Cells are Gram-negative, non-spore forming, motile, facultative aerobic, rod-shaped. Cells are 1㎛ in length. Colonies are circular, entire edge, glistening and yellow grown on MA for 7 days. Optimal growth occurs at 30℃ (growth range, 4-30℃). Optimal pH for growth occurs at pH 7.0-7.5 (growth range, pH 4-8). Optimal growth occurs in the presence 2.5-3.0% NaCl (w/v). Catalase and oxidase are negative. In API 20NE strips (Table 1-4), esculin and gelatin hydrolysis and reduction of nitrate is positive. Glucose assimilation and fermentation are negative. In the API ZYM system (Table 1-2), Leucine arylamidase and naphtol-AS-BI-phosphohydrolase activities are positive, but alkaline phosphatase, esterase (C4), lipase(C14), α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosidase, α-mannosidase and α-fucosidase activities are negative.

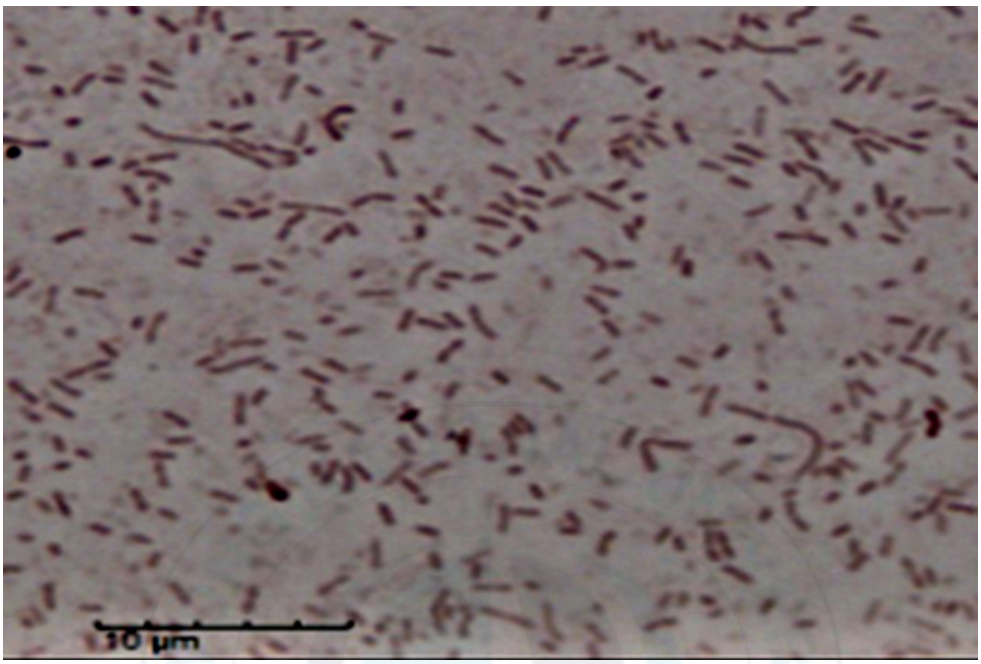

Figure 1-1. Microscopy of strain RR4-38 after Gram staining

The 16S rRNA sequences of strain RR4-38 and other closest strains were used to generate a phylogenetic tree (Figure 1-2, 1-3). Identification of sequences was done at the EzTaxon server (http://www.ezbiocloud.net/).

Table 1-1. 16S rRNA sequence similarity of strain RR4-38 and other strains.

Rank	Name	Strain	Pairwise similarity (%)
1	*Ulvibacter antarcticus*	IMCC3101(T)	95.05
2	*Altuibacter lentus*	JLT2010(T)	94.91
3	*Aureitalea marina*	S1-66(T)	94.76
4	*Ulvibacter litoralis*	KMM 3912(T)	93.94
5	*Gilvibacter sediminis*	Mok-1-36(T)	93.41
6	*Aureisphaera galaxeae*	04OKA003-7(T)	93.27
7	*Ulvibacter marinus*	IMCC12008(T)	93.12
8	*Winogradskyella multivorans*	T-Y1(T)	93.01
9	*Jejudonia soesokkakensis*	SSK1-1(T)	93.01
10	*Winogradskyella crassostreae*	TYO-19(T)	92.89

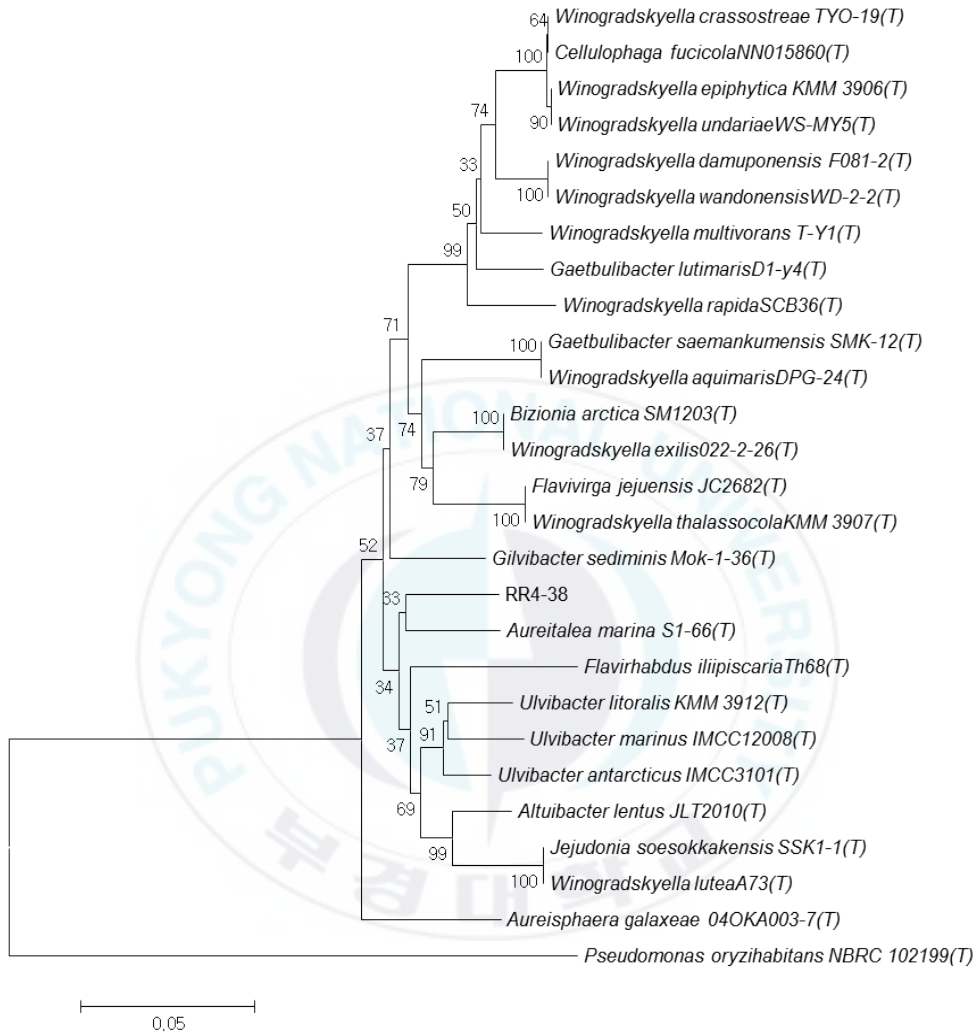

Figure 1-2. Neighbor-joining phylogenetic tree of strain RR4-38 and reference strains

It was conducted based on the aligned sequences using the neighbor-joining methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

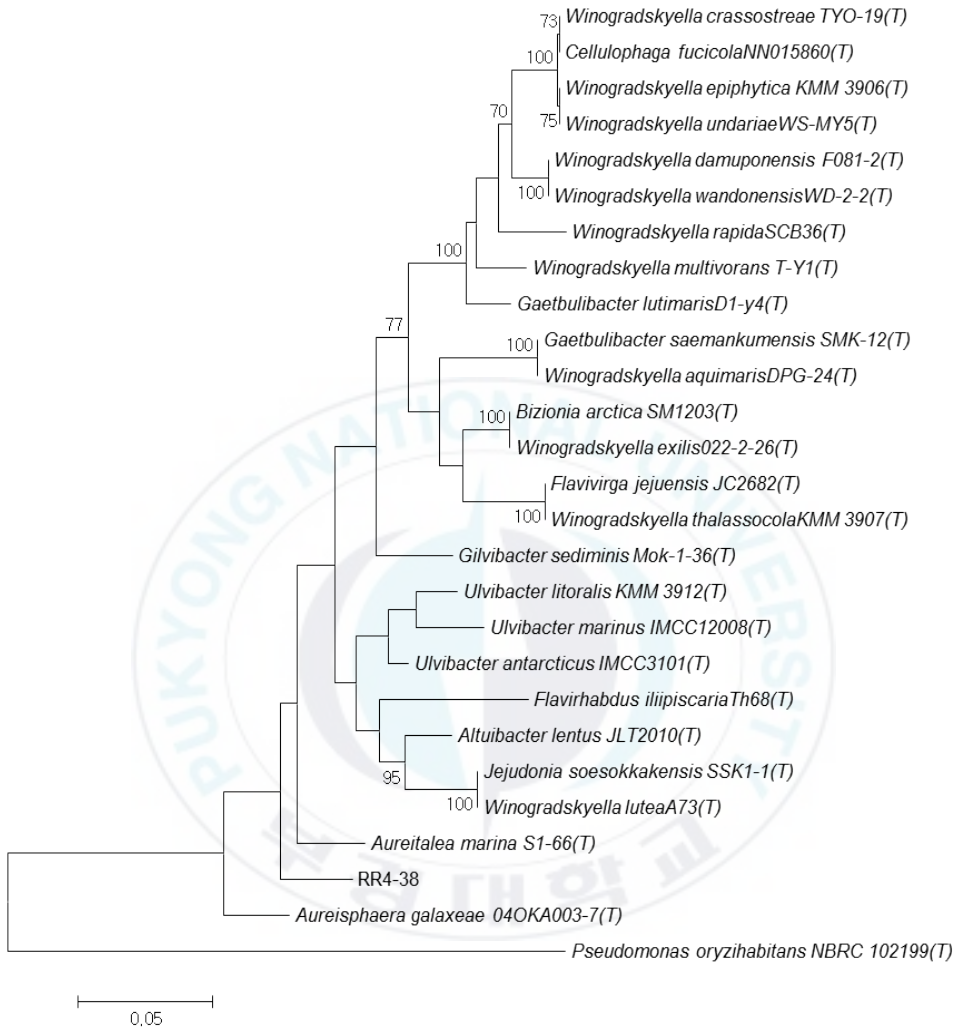

Figure 1-3. Maximum-likelihood phylogenetic tree of strain RR4-38 and reference strains

It was conducted based on the aligned sequences using the maximum-likelihood methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

Table 1-2. API ZYM results of RR4-38.

No.	Enzyme Assayed For	Substrate	pH	Result		RR4-38
				positive	negative	
1	Control			Colorless or color of the sample if it has an intense coloration		-
2	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	Colorless or very pale Yellow	-
3	Esterase (C4)	2-naphthyl butyrate	6.5	Violet		-
4	Esterase Lipase (C8)	2-naphthyl caprylate	7.5	Violet		-
5	Lipase (C14)	2-naphthyl myristate	7.5	Violet		-
6	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	Orange		+
7	Valine arylamidase	L-Valyl-2-naphthylamide	7.5	Orange		-
8	Crystine arylamidase	L-cystyl-2-naphthylamide	7.5	Orange		-
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange		-
10	α-chymotrypsin	N-glutaryl-phenylanine-2-naphthylamide	7.5	Orange		-
11	Acid phospatase	2-naphtyl phosphate	5.4	Violet		-
12	Naphtol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5.4	Blue		+
13	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	5.4	Violet		-
14	β-galactosidase	2-naphthyl-βD-galactopyranoside	5.4	Violet		-
15	β-glucuronidase	Naphthol-AS-BI-βD-glucuronide	5.4	Blue		-
16	α-glucosidase	2-naphthyl-αD-glucopyranoside	5.4	Violet		-
17	β-glucosidase	6-Br-20naphthyl-βD-glucopyranoside	5.4	Violet		-
18	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	5.4	Brown		-
19	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4	Violet		-
20	α-fucosidase	2-naphthyl-α-fucopyranoside	5.4	Violet		-

Table 1-3. API 50CH results of RR4-38

50CH Tube	Test	Active ingredients	RR4-38	50CH Tube	Test	Active ingredients	RR4-38
0		Control	-	25	ESC	Esculin ferric citrate	+
1	GLY	Glycerol	-	26	SAL	Salicin	-
2	ERY	Erythritol	-	27	CEL	D-celiobiose	-
3	DARA	D-arabinose	-	28	MAL	D-maltose	-
4	LARA	L-arabinose	-	29	LAC	D-lactose(Bovine origin)	-
5	RIB	D-rinose	-	30	MEL	D-MELibiose	-
6	DXYL	D-xylose	-	31	SAC	D-SACcharose (sucrose)	-
7	LXYL	L-xylose	-	32	TRE	D-TREhalose	-
8	ADO	D-adonitol	-	33	INU	INUlin	-
9	MDX	Methyl-βD-xylopyranoside	-	34	MLZ	D-MeLeZitose	-
10	GAL	D-galactose	-	35	RAF	D-RAFfinose	+
11	GLU	D-glucose	-	36	AMD	AmiDon (starch)	-
12	FRU	D-fructose	-	37	GLYG	GLYcoGen	-
13	MNE	D-mannose	-	38	XLT	XyLiTol	-
14	SBE	L-sorbose	-	39	GEN	GENtiobiose	-
15	RHA	L-rhamnose	-	40	TUR	D-TURanose	-
16	DUL	Dulcitol	-	41	LYX	D-LYXose	-
17	INO	Inositol	-	42	TAG	D-TAGatose	-
18	MAN	D-manitol	-	43	DFUC	D-FUCose	-
19	SOR	D-sorbitol	-	44	LFUC	L-FUCose	-
20	MDN	Methyl-αD-Mannopyranoside	-	45	DARL	D-ARabitoL	-
21	MDG	Methyl-αD-Glucopyranoside	-	46	LARL	L-ARabitoL	-
22	NAG	N-Acetylglucosamine	-	47	GNT	potassium GlucoNaTe	-
23	AMY	Amygdalin	-	48	2KG	potassium 2-KetoGluconate	-
24	ARB	Arbutin	+	49	5KG	potassium 5-KetoGluconate	+

Table 1-4. API 20NE (48h) results of RR4-38.

Active ingredients	Reactions / Enzymes	NO.	Tests	판독 기준 및 결과		
				Negative	Positive	RR4-38
potassium nitrate	reduction of nitrate to nitrites	1	NO_3	colorless	pink-red	
	reduction of nitrate to nitrogen			pink	colorless	+
L-tryptophane	indole production(TRyptoPhane)	2	TRP	colorless	pink	-
				pale green/yellow		
D-glucose	fermentation(GLUcose)	3	GLU	blue to green	yellow	-
L-arginine	Arginine DiHydrolase	4	ADH	yellow	orange/pink/red	-
urea	UREase	5	URE	yellow	orange/pink/red	-
essulin	hydrolysis(β-glucosidase) (ESCulin)	6	ESC	yellow	grey/brown/black	+
ferric citrate						
gelatine	hydrolysis(protease) (GELatin)	7	GEL	no pigment	diffusion of	+
(bovine origin)				diffusion	black pigment	
4-nitrophenyl-βD-galactopyranoside	β-galactosidase(Para-NitroPhenyl-βD-Galactopyranosidase)	8	PNPG	colorless	yellow	-
D-glucose	assimilation (glucose)	9	GLU	transparent	opaque	-
L-arabinose	assimilation (arabinose)	10	ARA	transparent	opaque	-
D-mannose	assimilation (mannose)	11	MNE	transparent	opaque	-
D-mannitol	assimilation (mannitol)	12	MAN	transparent	opaque	-
N-acetyl-glucosamine	assimilation (N-acetyl-glucosamine)	13	NAG	transparent	opaque	-
D-maltose	assimilation (maltose)	14	MAL	transparent	opaque	-
potassium gluconate	assimilation (potassium gluconate)	15	GNT	transparent	opaque	-
capric acid	assimilation (capric acid)	16	CAP	transparent	opaque	-
adipic acid	assimilation (adipic acid)	17	ADI	transparent	opaque	-
malic acid	assimilation (malate)	18	MLT	transparent	opaque	-
trisodium citrate	assimilation (trisodium citrate)	19	CIT	transparent	opaque	-
phenylacetic acid	assimilation (phenylacetic acid)	20	PAC	transparent	opaque	-
		Oxidase		-		

CHAPTER 2. Taxonomic study of the strain RR4-40

INTRODUCTION

The genus *Ulvibacter* is required Na^+ ions, non-motile. Yellow-orange pigments are produced and flexirubin is produced (Nedashkovskaya, 2004). The genus *Ulvibacter* has contained two species, *Ulvibacter litoralis*, isolated from the green alga *Ulva fenestrata*, and *Ulvibacter antarcticus*, isolated from Antarctic coastal seawater. Recently, new species *Ulvibabacter marinus* isolated from coastal seawater has been proposed to the genus *Ulvibacter* (Baek *et al.*, 2014). This species is chemoheterotrophic, strictly aerobic and Gram negative, but nitrate reduction is positive. Flexirubin-type pigments, first isolated from a strain *Flexibacter elegans*, are produced (FAUTZ *et al.*, 1980). This pigment was extracted from many species. One was *Flexibacter elegans* and another was *Cytophaga johnsonae*. *Flexibacter* pigments can resist photooxidation. An important regulatory factor is pH as specific pigment was made under acidic condition than neutral conditions. Carotenoid is not produced (Reichenbach, 1974). Whereas, *C. jonhsonae* produces carotenoid 20 μg (Achenbach *et al.*, 1978). In the study, the strain RR4-40 is proposed to novel genus belonging to the Phylum *Bacteroidetes* based on biochemical, physiological and phylogenetic differences

RESULTS AND DISCUSSION

Cells are Gram-negative, motile, facultative aerobic, rod-shaped. Cells are observed from 1㎛ in length. Colonies are circular, entire edge, raised margin, glistening and yellow grown on MA for 7days. Optimal growth occurs at 30℃ (growth range 20-30℃). Optimal growth occurs at pH 7.0-7.5 (growth range, pH 6-8). Optimal growth occurs in the presence 2.5-3.0% NaCl (w/v). Catalase and oxidase are negative. In the API 20NE strips (Table 2-4), nitrate and gelatin hydrolysis is positive, but reduction of indole production, glucose fermentation, D-glucose, L-arabinose, D-mannose, D-mannitol, L-acetyl-glucosamine and D-maltose assimilation is negative. In API ZYM system (Table 2-2), esterase (C4), leucine arylamidase, valine arylamidase, crystine arylamidase and acid phosphatase acitivities are positive, but alkaline phosphatase, esterase lipase (C8), lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosidase, α-mannosidase and α-fucosidase activities are negative.

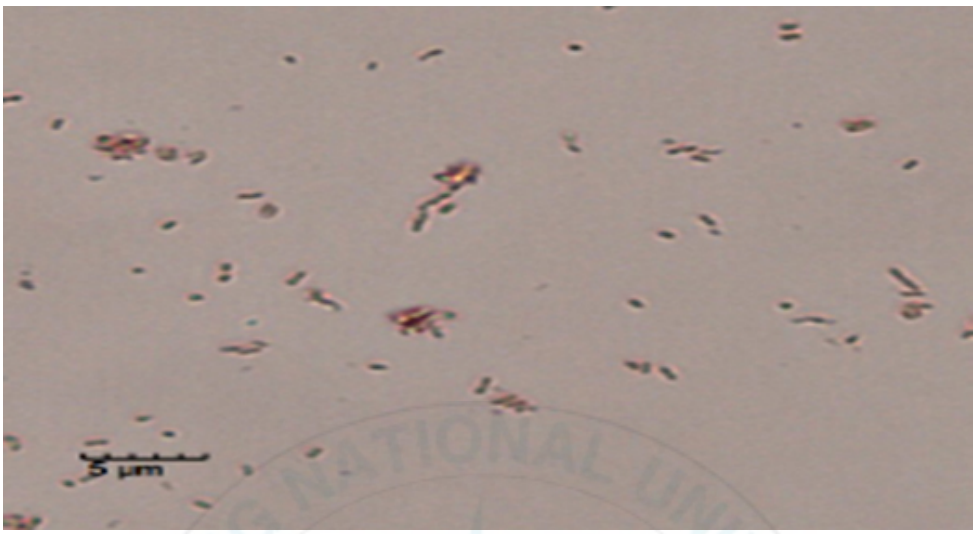

Figure 2-1. Microscopy of strain RR4-40 after Gram staining

The full 16S rRNA sequences of strain RR4-40 and other closest strains were used to generate a phylogenetic tree (Figure 2-2, 2-3). Identification of sequences was done at the EzTaxon server (http://www.ezbiocloud.net/) .

Table 2-1. 16S rRNA sequence similarity of strain RR4-40 and other strains.

Rank	Name	Strain	Pairwise similarity (%)
1	*Ulvibacter marinus*	IMCC12008(T)	94.17
2	*Altuibacter lentus*	JLT2010(T)	94.10
3	*Ulvibacter antarcticus*	IMCC3101(T)	93.94
4	*Jejudonia soesokkakensis*	SSK1-1(T)	93.83
5	*Aureitalea marina*	S1-66(T)	93.74
6	*Ulvibacter litoralis*	KMM_3912(T)	93.65
7	*Gilvibacter sediminis*	Mok-1-36(T)	93.25
8	*Aureisphaera galaxeae*	04OKA003-7(T)	93.00
9	*Marixanthomonas ophiurae*	KMM_3046(T)	92.44
10	*Bizionia hallyeonensis*	T-y7(T)	92.30

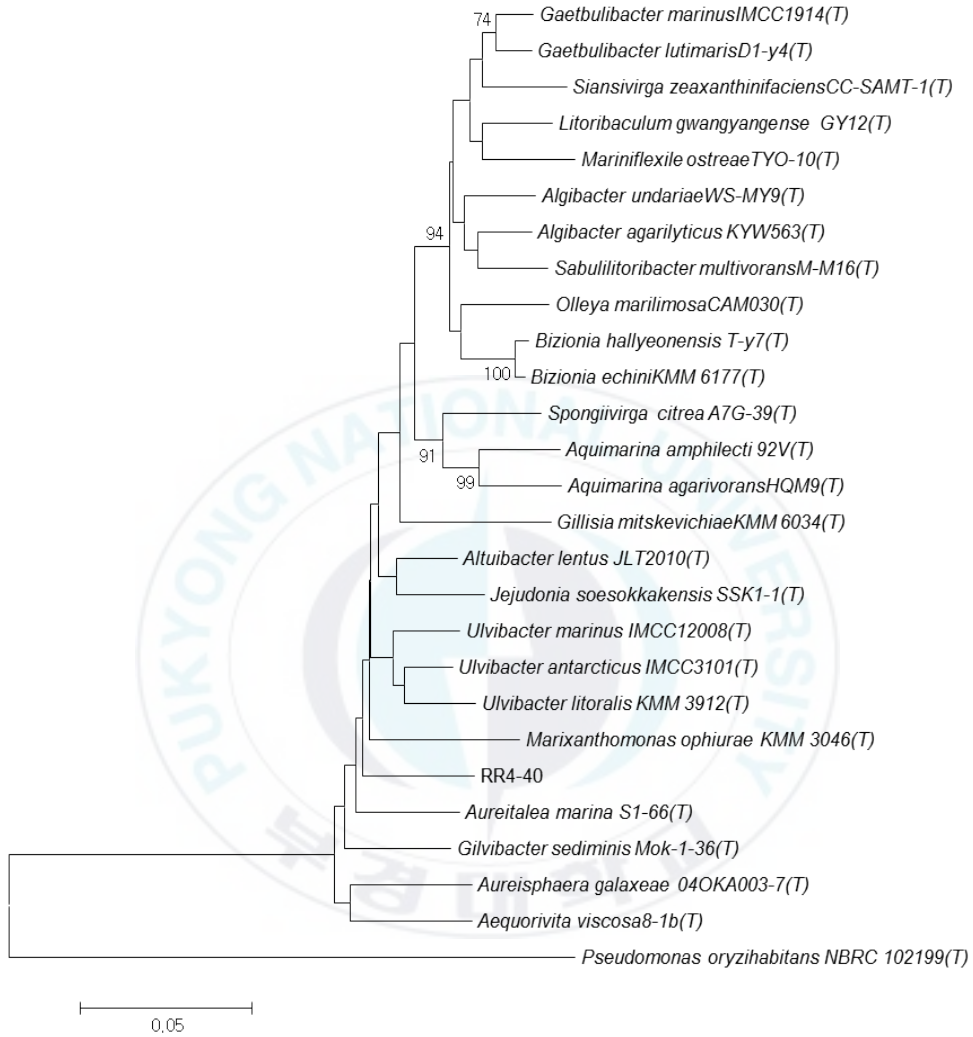

Figure 2-2. Neighbor-joining phylogenetic tree of strain RR4-40 and reference strains

It was conducted based on the aligned sequences using the neighbor-joining methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

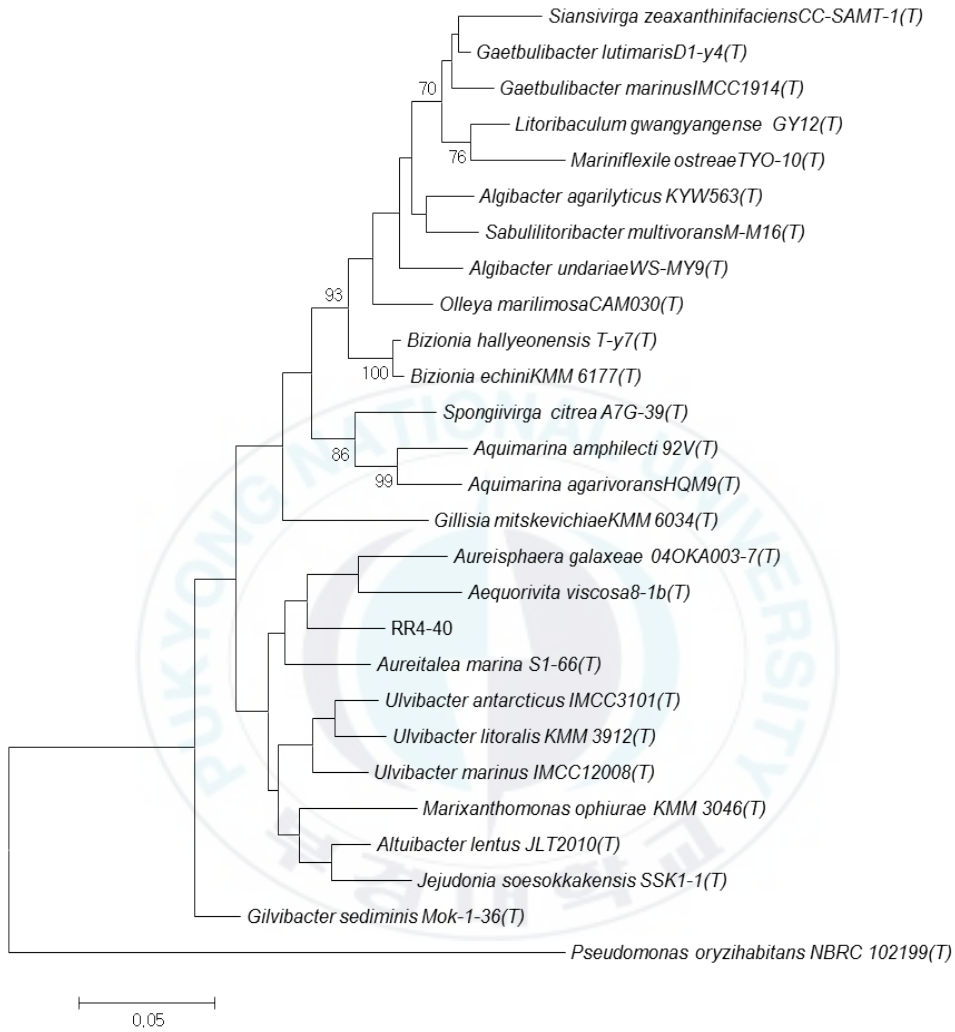

Figure 2-3. Maximum-likelihood phylogenetic tree of strain RR4-40 and reference strains

It was conducted based on the aligned sequences using the maximum-likelihood methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

Table 2-2. API ZYM results of RR4-40.

No.	Enzyme Assayed For	Substrate	pH	Result		RR4-40
				positive	negative	
1	Control			Colorless or color of the sample if it has an intense coloration		-
2	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	Colorless or very pale Yellow	-
3	Esterase (C4)	2-naphthyl butyrate	6.5	Violet		+
4	Esterase Lipase (C8)	2-naphthyl caprylate	7.5	Violet		-
5	Lipase (C14)	2-naphthyl myristate	7.5	Violet		-
6	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	Orange		+
7	Valine arylamidase	L-Valyl-2-naphthylamide	7.5	Orange		+
8	Crystine arylamidase	L-cystyl-2-naphthylamide	7.5	Orange		+
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange		-
10	α-chymotrypsin	N-glutaryl-phenylanine-2-naphthylamide	7.5	Orange		-
11	Acid phospatase	2-naphtyl phosphate	5.4	Violet		+
12	Naphtol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5.4	Blue		-
13	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	5.4	Violet		-
14	β-galactosidase	2-naphthyl-βD-galactopyranoside	5.4	Violet		-
15	β-glucuronidase	Naphthol-AS-BI-βD-glucuronide	5.4	Blue		-
16	α-glucosidase	2-naphthyl-αD-glucopyranoside	5.4	Violet		-
17	β-glucosidase	6-Br-20naphthyl-βD-glucopyranoside	5.4	Violet		-
18	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	5.4	Brown		-
19	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4	Violet		-
20	α-fucosidase	2-naphthyl-α-fucopyranoside	5.4	Violet		-

Table 2-3. API 50CH results of RR4-40.

50CH Tube	Test	Active ingredients	RR4-40	50CH Tube	Test	Active ingredients	RR4-40
0		Control	-	25	ESC	Esculin ferric citrate	+
1	GLY	Glycerol	-	26	SAL	Salicin	-
2	ERY	Erythritol	-	27	CEL	D-celiobiose	-
3	DARA	D-arabinose	-	28	MAL	D-maltose	-
4	LARA	L-arabinose	-	29	LAC	D-lactose(Bovine origin)	-
5	RIB	D-rinose	-	30	MEL	D-MELibiose	-
6	DXYL	D-xylose	-	31	SAC	D-SACcharose (sucrose)	-
7	LXYL	L-xylose	-	32	TRE	D-TREhalose	-
8	ADO	D-adonitol	-	33	INU	INUlin	-
9	MDX	Methyl-βD-xylopyranoside	-	34	MLZ	D-MeLeZitose	-
10	GAL	D-galactose	-	35	RAF	D-RAFfinose	-
11	GLU	D-glucose	-	36	AMD	AmiDon (starch)	-
12	FRU	D-fructose	-	37	GLYG	GLYcoGen	-
13	MNE	D-mannose	-	38	XLT	XyLiTol	-
14	SBE	L-sorbose	-	39	GEN	GENtiobiose	-
15	RHA	L-rhamnose	-	40	TUR	D-TURanose	-
16	DUL	Dulcitol	-	41	LYX	D-LYXose	-
17	INO	Inositol	-	42	TAG	D-TAGatose	-
18	MAN	D-manitol	-	43	DFUC	D-FUCose	-
19	SOR	D-sorbitol	-	44	LFUC	L-FUCose	-
20	MDN	Methyl-αD-Mannopyranoside	-	45	DARL	D-ARabitoL	-
21	MDG	Methyl-αD-Glucopyranoside	-	46	LARL	L-ARabitoL	-
22	NAG	N-Acetylglucosamine	-	47	GNT	potassium GlucoNaTe	-
23	AMY	Amygdalin	-	48	2KG	potassium 2-KetoGluconate	-
24	ARB	Arbutin	-	49	5KG	potassium 5-KetoGluconate	+

Table 2-4. API 20NE (48h) results of RR4-40.

Active ingredients	Reactions / Enzymes	NO.	Tests	판독 기준 및 결과		
				Negative	Positive	RR4-40
potassium nitrate	reduction of nitrate to nitrites	1	NO_3	colorless	pink-red	
	reduction of nitrate to nitrogen			pink	colorless	+
L-tryptophane	indole production(TRyptoPhane)	2	TRP	colorless	pink	-
				pale green/yellow		
D-glucose	fermentation(GLUcose)	3	GLU	blue to green	yellow	-
L-arginine	Arginine DiHydrolase	4	ADH	yellow	orange/pink/red	-
urea	UREase	5	URE	yellow	orange/pink/red	-
essulin	hydrolysis(β-glucosidase) (ESCulin)	6	ESC	yellow	grey/brown/black	-
ferric citrate						
gelatine	hydrolysis(protease) (GELatin)	7	GEL	no pigment	diffusion of	+
(bovine origin)				diffusion	black pigment	
4-nitrophenyl-βD-galactopyranoside	β-galactosidase(Para-NitroPhenyl-βD-Galactopyranosidase)	8	PNPG	colorless	yellow	-
D-glucose	assimilation (glucose)	9	GLU	transparent	opaque	-
L-arabinose	assimilation (arabinose)	10	ARA	transparent	opaque	-
D-mannose	assimilation (mannose)	11	MNE	transparent	opaque	-
D-mannitol	assimilation (mannitol)	12	MAN	transparent	opaque	-
N-acetyl-glucosamine	assimilation (N-acetyl-glucosamine)	13	NAG	transparent	opaque	-
D-maltose	assimilation (maltose)	14	MAL	transparent	opaque	-
potassium gluconate	assimilation (potassium gluconate)	15	GNT	transparent	opaque	-
capric acid	assimilation (capric acid)	16	CAP	transparent	opaque	-
adipic acid	assimilation (adipic acid)	17	ADI	transparent	opaque	-
malic acid	assimilation (malate)	18	MLT	transparent	opaque	-
trisodium citrate	assimilation (trisodium citrate)	19	CIT	transparent	opaque	-
phenylacetic acid	assimilation (phenylacetic acid)	20	PAC	transparent	opaque	-
		Oxidase		-		

CHAPTER 3. Taxonomic study of the strain RR4-41

INTRODUCTION

The genus *Kangiella* was first described by Yoon *et al* (2004). The genus *Kangiella* belongs to the class *Gammaproteobacteria*. The genus has ability of nitrate reduction under anaerobic conditions (Yoon *et al*., 2004). The class *Gammaproteobacteria* has mostly been retrieved from marine environment and has five clades. The clades contains OM60, BD1-7, KI89A, OM182 and SAR92 clade (Cho *et al*, 2004), Two species proposed first, *Kangiella koreensis* and *Kangiella aquimarina* were isolated from a tidal flat of the Yellow sea in Korea (Yoon *et al*., 2004). Other species, *Kangiella japanica* (Romanenko *et al*., 2010) and *Kangiella spongicola* (Anh *et al*., 2011), were isolated from sediment and the sponge *Chondrilla nucula*, respectively. A novel species, *Kangiella geojedonensis*, is proposed by Yoon *et al*. (2012). This novel species has ability of nitrate reduction. In this study, strain RR4-41 was described by novel species belonging to *Kangiella* based on biochemical, physiological and phylogenetic differences.

RESULTS AND DISCUSSION

Cells are Gram-negative, motile, obligate aerobic and rod-shaped. Cells are 0.3㎛ in length. Colonies are slightly irregular form, entire edge, raised, glistening and white grown on MA for 7days. Optimal growth occurs at 30℃ (growth range, 20-30℃). Optimal growth occurs at pH 7.0-7.5. Optimal growth occurs in the presence 2.5-3.0% NaCl (w/v). Positive for catalase but negative for oxidase. In the API 20NE systems (Table 3-4), gelatin hydrolysis is positive but reduction of nitrate and glucose fermentation are negative. Also assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine is negative. In API ZYM system (Table 3-2), alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, α-chymotrysin and naphtol-AS-BI-phosphohydrolase activities are positive, but lipase (C14), trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase and β-glucosidase activities are negative.

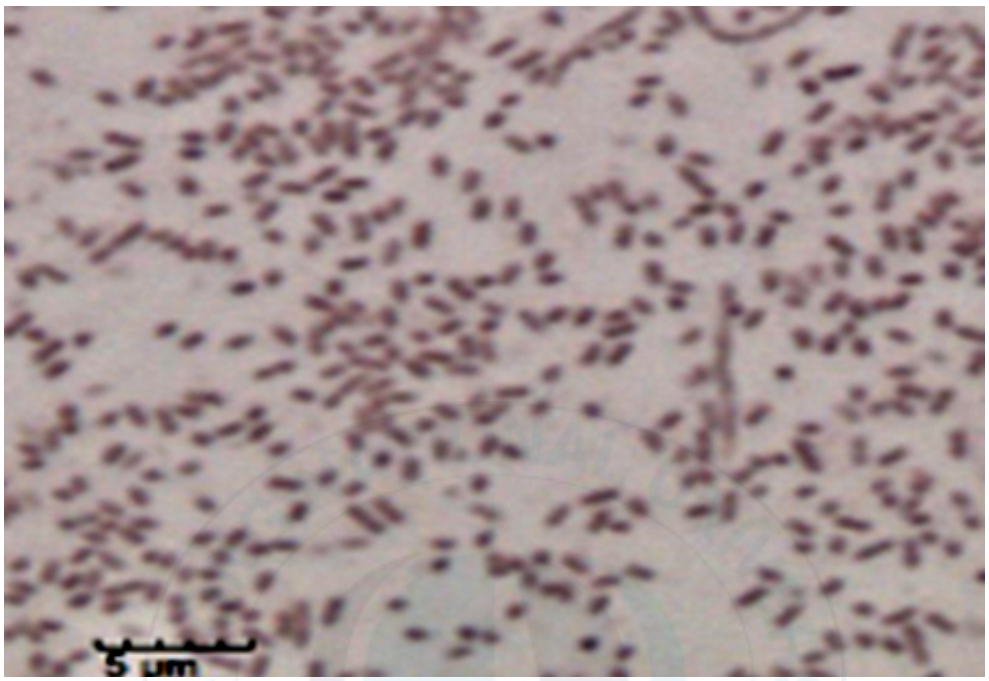

Figure 3-1. Microscopy of strain RR4-41 after Gram staining

The 16S rRNA sequences of strain RR4-41 and other closest strains were used to generate a phylogenetic tree (Figure 3-2, 3-3). The phylogenetic tree indicated that the strain RR4-41 belongs to the genus *Kangiella*. It showed sequence similarity 98.08% with *Kangiella geojedonensis* (Table 3-1), the strain RR4-68 is classified as new species in the genus *Kangiella*.

Table 3-1. 16S rRNA sequence similarity of strain RR4-41 and other strains.

Rank	Name	Strain	Pairwise similarity (%)
1	*Kangiella geojedonensis*	YCS-5(T)	98.08
2	*Kangiella spongicola*	A79(T)	97.19
3	*Kangiella japonica*	KMM_3899(T)	97.03
4	*Kangiella koreensis*	DSM_16069(T)	96.95
5	*Kangiella marina*	KM1(T)	96.97
6	*Kangiella taiwanensis*	KT1(T)	96.85
7	*Kangiella sediminilitoris_BB*	Mw22(T)	96.88
8	*Kangiella profundi*	FT102(T)	96.83
9	*Kangiella aquimarina_DSM*	16071(T)	96.22
10	*Kangiella chungangensis*	CAU_1040(T)	95.62

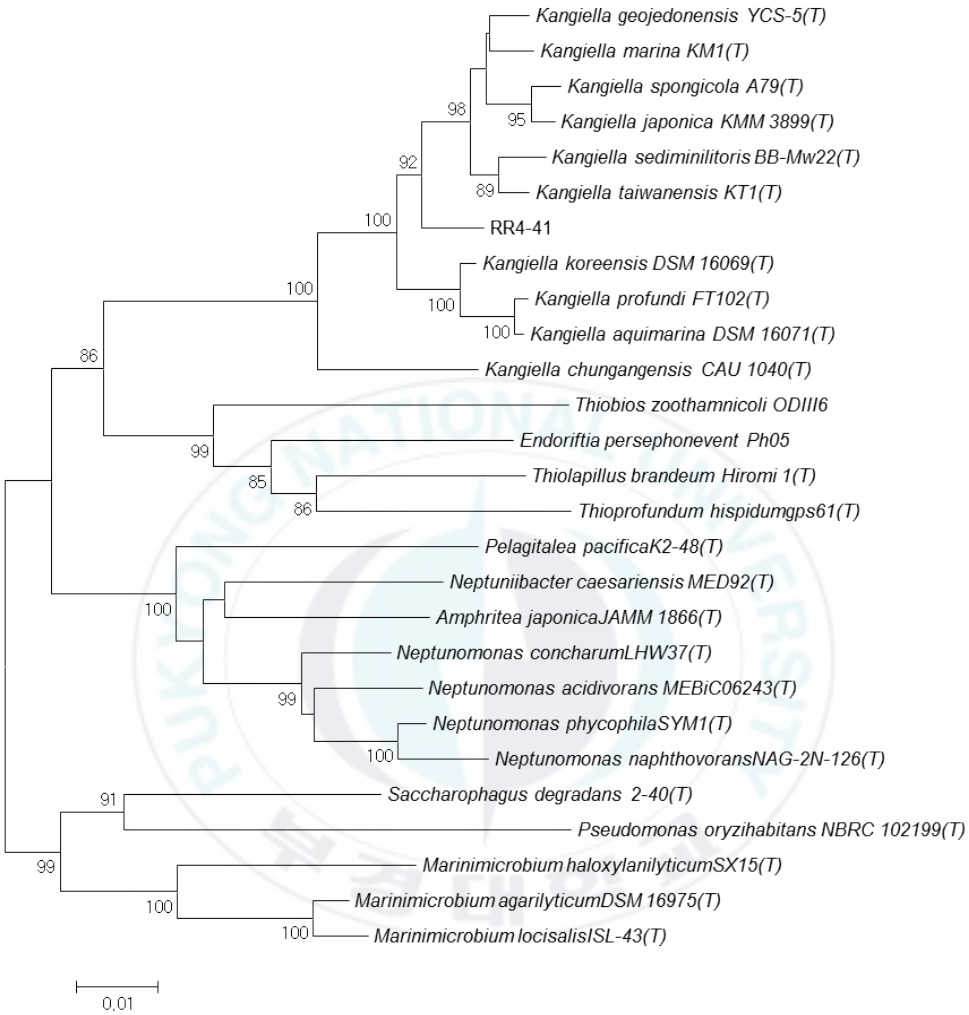

Figure 3-2. Neighbor-joining phylogenetic tree of strain RR4-41 and reference strains

It was conducted based on the aligned sequences using the neighbor-joining methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

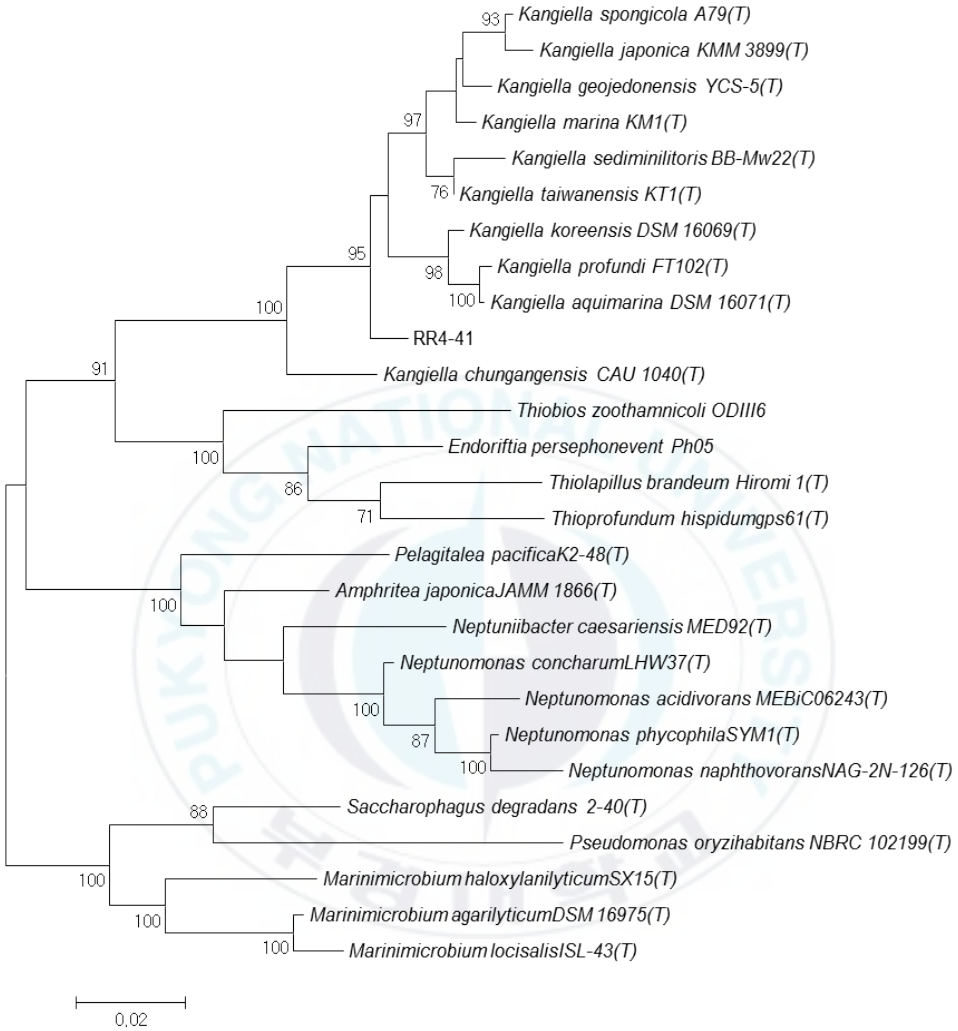

Figure 3-3 Maximum-likelihood phylogenetic tree of strain RR4-41 and reference strains

It was conducted based on the aligned sequences using the maximum-likelihood methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

Table 3-2. API ZYM results of RR4-41.

No.	Enzyme Assayed For	Substrate	pH	Result		RR4-41
				positive	negative	
1	Control			Colorless or color of the sample if it has an intense coloration		-
2	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	Colorless or very pale Yellow	+
3	Esterase (C4)	2-naphthyl butyrate	6.5	Violet		+
4	Esterase Lipase (C8)	2-naphthyl caprylate	7.5	Violet		+
5	Lipase (C14)	2-naphthyl myristate	7.5	Violet		-
6	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	Orange		+
7	Valine arylamidase	L-Valyl-2-naphthylamide	7.5	Orange		-
8	Crystine arylamidase	L-cystyl-2-naphthylamide	7.5	Orange		-
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange		-
10	α-chymotrypsin	N-glutaryl-phenylanine-2-naphthylamide	7.5	Orange		+
11	Acid phospatase	2-naphtyl phosphate	5.4	Violet		-
12	Naphtol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5.4	Blue		+
13	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	5.4	Violet		-
14	β-galactosidase	2-naphthyl-βD-galactopyranoside	5.4	Violet		-
15	β-glucuronidase	Naphthol-AS-BI-βD-glucuronide	5.4	Blue		-
16	α-glucosidase	2-naphthyl-αD-glucopyranoside	5.4	Violet		-
17	β-glucosidase	6-Br-20naphthyl-βD-glucopyranoside	5.4	Violet		-
18	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	5.4	Brown		-
19	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4	Violet		-
20	α-fucosidase	2-naphthyl-α-fucopyranoside	5.4	Violet		-

Table 3-3. API 50CH results of RR4-41

50CH Tube	Test	Active ingredients	RR4-41	50CH Tube	Test	Active ingredients	RR4-41
0		Control	-	25	ESC	Esculin ferric citrate	+
1	GLY	Glycerol	-	26	SAL	Salicin	+
2	ERY	Erythritol	-	27	CEL	D-celiobiose	-
3	DARA	D-arabinose	-	28	MAL	D-maltose	-
4	LARA	L-arabinose	-	29	LAC	D-lactose(Bovine origin)	-
5	RIB	D-rinose	-	30	MEL	D-MELibiose	-
6	DXYL	D-xylose	-	31	SAC	D-SACcharose (sucrose)	-
7	LXYL	L-xylose	-	32	TRE	D-TREhalose	-
8	ADO	D-adonitol	-	33	INU	INUlin	-
9	MDX	Methyl-βD-xylopyranoside	-	34	MLZ	D-MeLeZitose	-
10	GAL	D-galactose	-	35	RAF	D-RAFfinose	-
11	GLU	D-glucose	-	36	AMD	AmiDon (starch)	-
12	FRU	D-fructose	-	37	GLYG	GLYcoGen	+
13	MNE	D-mannose	-	38	XLT	XyLiTol	-
14	SBE	L-sorbose	-	39	GEN	GENtiobiose	-
15	RHA	L-rhamnose	-	40	TUR	D-TURanose	-
16	DUL	Dulcitol	-	41	LYX	D-LYXose	-
17	INO	Inositol	-	42	TAG	D-TAGatose	-
18	MAN	D-manitol	-	43	DFUC	D-FUCose	-
19	SOR	D-sorbitol	-	44	LFUC	L-FUCose	-
20	MDN	Methyl-αD-Mannopyranoside	-	45	DARL	D-ARabitoL	-
21	MDG	Methyl-αD-Glucopyranoside	-	46	LARL	L-ARabitoL	-
22	NAG	N-Acetylglucosamine	-	47	GNT	potassium GlucoNaTe	-
23	AMY	Amygdalin	-	48	2KG	potassium 2-KetoGluconate	-
24	ARB	Arbutin	-	49	5KG	potassium 5-KetoGluconate	+

Table 3-4. API 20NE (48h) results of RR4-41.

Active ingredients	Reactions / Enzymes	NO.	Tests	판독 기준 및 결과		
				Negative	Positive	RR4-41
potassium nitrate	reduction of nitrate to nitrites	1	NO_3	colorless	pink-red	
	reduction of nitrate to nitrogen			pink	colorless	+
L-tryptophane	indole production(TRyptoPhane)	2	TRP	colorless	pink	-
				pale green/yellow		
D-glucose	fermentation(GLUcose)	3	GLU	blue to green	yellow	-
L-arginine	Arginine DiHydrolase	4	ADH	yellow	orange/pink/red	-
urea	UREase	5	URE	yellow	orange/pink/red	-
essulin	hydrolysis(β-glucosidase) (ESCulin)	6	ESC	yellow	grey/brown/black	-
ferric citrate						
gelatine	hydrolysis(protease) (GELatin)	7	GEL	no pigment	diffusion of	+
(bovine origin)				diffusion	black pigment	
4-nitrophenyl-βD-galactopyranoside	β-galactosidase(Para-NitroPhenyl-βD-Galactopyranosidase)	8	PNPG	colorless	yellow	-
D-glucose	assimilation (glucose)	9	GLU	transparent	opaque	-
L-arabinose	assimilation (arabinose)	10	ARA	transparent	opaque	-
D-mannose	assimilation (mannose)	11	MNE	transparent	opaque	-
D-mannitol	assimilation (mannitol)	12	MAN	transparent	opaque	-
N-acetyl-glucosamine	assimilation (N-acetyl-glucosamine)	13	NAG	transparent	opaque	-
D-maltose	assimilation (maltose)	14	MAL	transparent	opaque	-
potassium gluconate	assimilation (potassium gluconate)	15	GNT	transparent	opaque	-
capric acid	assimilation (capric acid)	16	CAP	transparent	opaque	-
adipic acid	assimilation (adipic acid)	17	ADI	transparent	opaque	-
malic acid	assimilation (malate)	18	MLT	transparent	opaque	-
trisodium citrate	assimilation (trisodium citrate)	19	CIT	transparent	opaque	-
phenylacetic acid	assimilation (phenylacetic acid)	20	PAC	transparent	opaque	-
		Oxidase		-		

CHAPTER 4. Taxonomic study of the strain RR4-56

INTRODUCTION

Strain RR4-56 was isolated from a biofilter from seawater recirculating aquaculture system. The 16S rRNA sequences of strain RR4-56 and other closest strains were used to generate a phylogenetic tree (Figure 4-2, 4-3). The strain RR4-56 is relative to two genera, *Halovulum* and *Rhodobacter* (respectively, similarity 92.84% and 91.60%). Two genera were rod-, oval-shaped, facultative anaerobic and photoheterotrophic. *Rhodobacter* strains can grow by anaerobic-dark respiration with nitrate, nitrous oxide, di-methylsulfoxide, or trimethylamine N-oxide as a terminal electron acceptor (Hiraishi and Veda, 1994). Two genera, *Rhodobacter* and *Halovulum*, which are complete denitrifiers have been separated from the biological wastewater processing system. *Rhodobacter* can play a significant role in the environmental nitrogen cycle. Also, all marine creatures except bacteriochlorophyll, spheroidene series, and carotenoids have recently been in the genus for freshwater and marine life (Hiraishi and Veda, 1994). On the basis of 16S rRNA sequence, the strain was most closely related to the genus *Rhodobacter*, but was affiliated to the genus *Halovulum* based on phylogenetic tree. After comparing taxonomic characteristics with

other various bacterial strains and analyzing gene sequence of 16S rRNA, strain RR4-56 seems to form novel genus different from *Halovulum (Sun, et al., 2015)* belonging to the *Rhodobacteraceae* family.

RESULTS AND DISCUSSION

Cells are Gram-negative, motile, rod-shaped, facultative aerobic. Cells are 0.3㎛ in length. Colonies are small, circular form, entire edge, flat margin and white grown on MA for 7days. Optimal growth occurs at 30℃ (growth range, 20-30℃). Optimal pH for growth occurs at pH 7.0-7.5 (growth range, pH 5-8; but weak growth at pH 7), Optimal growth occurs in the presence 2.5-3.0% NaCl (w/v). Catalase and oxidase are negative. In the API 20NE strips (Table 4-4), reduction of nitrite is positive, but reduction of nitrate, glucose fermentation, gelatin hydrolysis, assimilations of D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose, N-acetyl-glucosamine, D-glucosamine and malate are negative. In the API ZYM system (Table 4-2), esterase (C4), esterase lipase (C8), leucine arylamidase, crystine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase activities are positive, but alkaline phosphatase, lipase (C14), valine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase and α-mannosidase activities are negative.

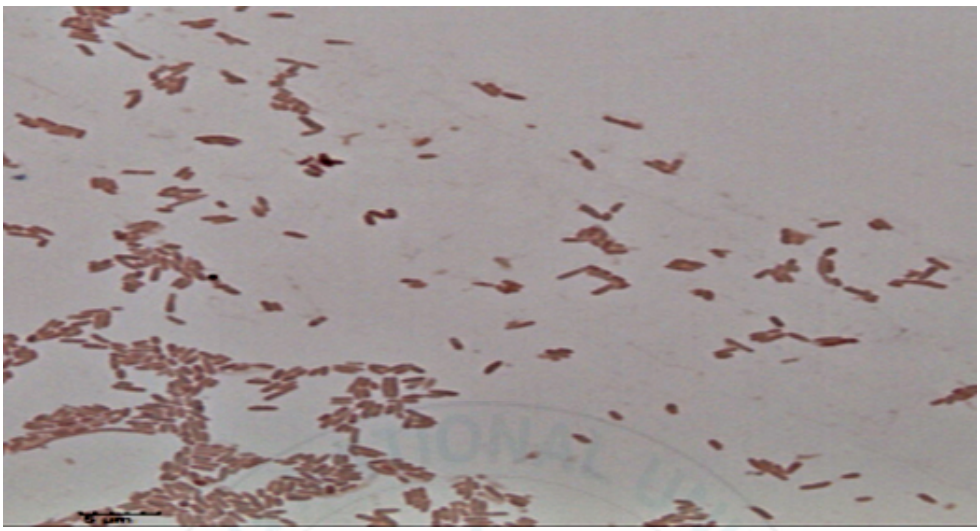

Figure 4-1. Microscopy of strain RR4-56 after Gram staining

The full 16S rRNA sequences of strain RR4-56 and other closest strains were used to generate a phylogenetic tree (Figure 4-2, 4-3). It showed sequence similarity 92.84% with *Halovulum dunhuangense* (Table 4-1). The phylogenetic tree indicated that the strain RR4-56 was classified as new genus.

Table 4-1. 16S rRNA sequence similarity of strain RR4-56 and other strains.

Rank	Name	Strain	Pairwise similarity (%)
1	*Halovulum dunhuangense*	*YYQ-30T*	92.84
2	*Rhodobacter azotoformans*	*KA25T*	91.60
3	*Defluviimonas aquaemixtae*	*CDM-7T*	91.46
4	*Pararhodobacter aggregans*	*D1-19T*	91.32
5	*Jannaschia aquimarina*	*GSW-M26T*	91.25
6	*Albidovulum inexpectatum*	*FRR-10T*	91.23
7	*Defluviimonas indica*	*20V17T*	91.20
8	*Albidovulum xiamenense*	*YBY-7T*	91.20
9	*Pontivivens insulae*	*GYSW-23T*	91.18
10	*Roseivivax isoporae*	*LMG_25204T*	91.17

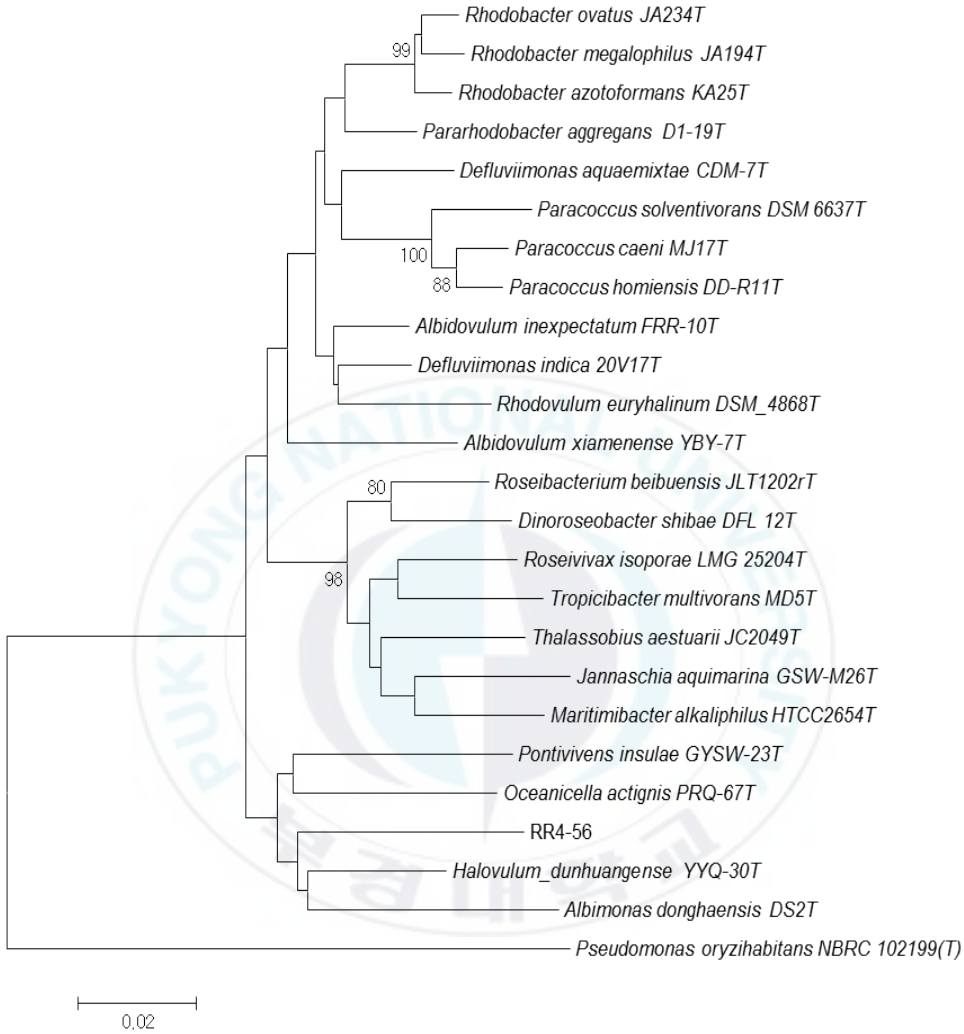

Figure 4-2. Neighbor-joining phylogenetic tree of strain RR4-56 and reference strains

It was conducted based on the aligned sequences using the neighbor-joining methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

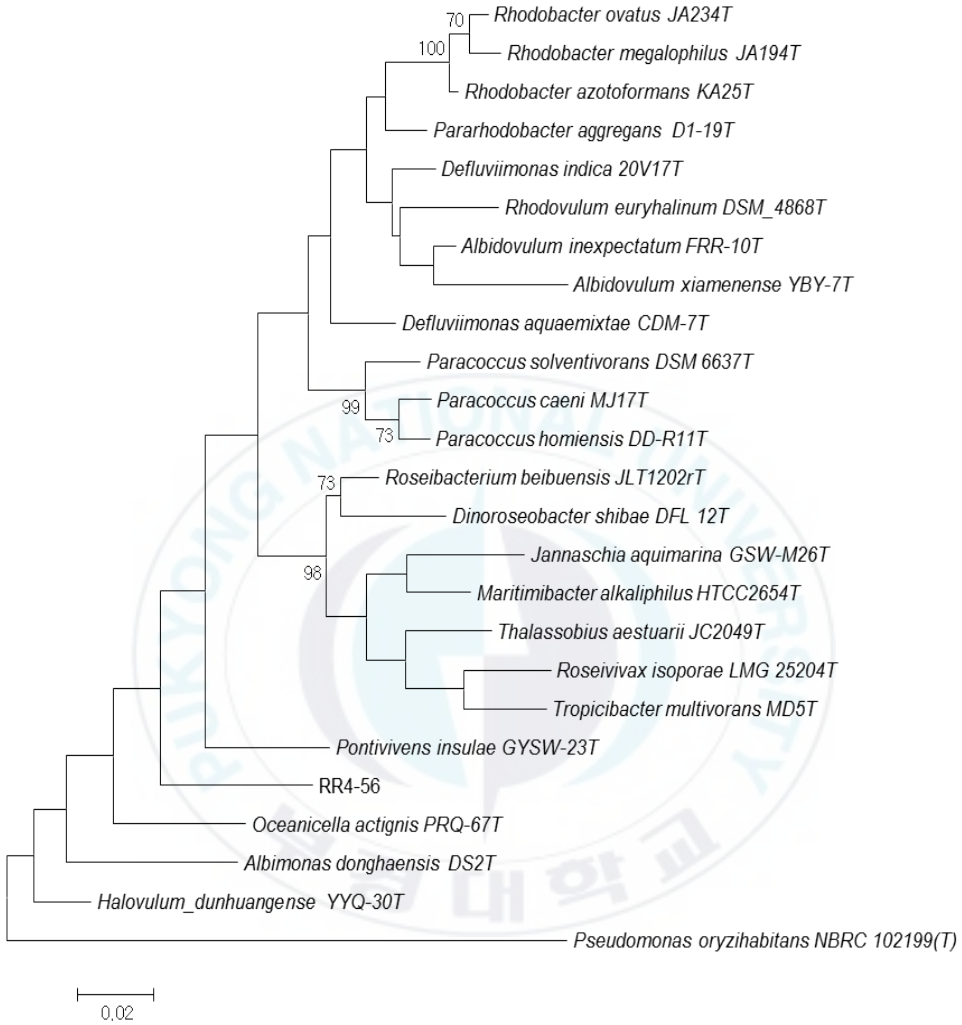

Figure 4-3. Maximum-likelihood phylogenetic tree of strain RR4-56 and reference strains

It was conducted based on the aligned sequences using the maximum-likelihood methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

Table 4-2. API ZYM results of RR4-56.

No.	Enzyme Assayed For	Substrate	pH	Result		RR4-56
				positive	negative	
1	Control			Colorless or color of the sample if it has an intense coloration		-
2	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	Colorless or very pale Yellow	-
3	Esterase (C4)	2-naphthyl butyrate	6.5	Violet		+
4	Esterase Lipase (C8)	2-naphthyl caprylate	7.5	Violet		+
5	Lipase (C14)	2-naphthyl myristate	7.5	Violet		-
6	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	Orange		+
7	Valine arylamidase	L-Valyl-2-naphthylamide	7.5	Orange		-
8	Crystine arylamidase	L-cystyl-2-naphthylamide	7.5	Orange		+
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange		-
10	α-chymotrypsin	N-glutaryl-phenylanine-2-naphthylamide	7.5	Orange		-
11	Acid phospatase	2-naphtyl phosphate	5.4	Violet		+
12	Naphtol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5.4	Blue		+
13	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	5.4	Violet		-
14	β-galactosidase	2-naphthyl-βD-galactopyranoside	5.4	Violet		-
15	β-glucuronidase	Naphthol-AS-BI-βD-glucuronide	5.4	Blue		-
16	α-glucosidase	2-naphthyl-αD-glucopyranoside	5.4	Violet		-
17	β-glucosidase	6-Br-20naphthyl-βD-glucopyranoside	5.4	Violet		-
18	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	5.4	Brown		-
19	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4	Violet		-
20	α-fucosidase	2-naphthyl-α-fucopyranoside	5.4	Violet		-

Table 4-3. API 50CH results of RR4-56

50CH Tube	Test	Active ingredients	RR4-56	50CH Tube	Test	Active ingredients	RR4-56
0		Control	-	25	ESC	Esculin ferric citrate	+
1	GLY	Glycerol	-	26	SAL	Salicin	-
2	ERY	Erythritol	-	27	CEL	D-celiobiose	-
3	DARA	D-arabinose	-	28	MAL	D-maltose	-
4	LARA	L-arabinose	-	29	LAC	D-lactose(Bovine origin)	-
5	RIB	D-rinose	-	30	MEL	D-MELibiose	-
6	DXYL	D-xylose	-	31	SAC	D-SACcharose (sucrose)	-
7	LXYL	L-xylose	-	32	TRE	D-TREhalose	-
8	ADO	D-adonitol	-	33	INU	INUlin	-
9	MDX	Methyl-βD-xylopyranoside	-	34	MLZ	D-MeLeZitose	-
10	GAL	D-galactose	-	35	RAF	D-RAFfinose	-
11	GLU	D-glucose	-	36	AMD	AmiDon (starch)	-
12	FRU	D-fructose	-	37	GLYG	GLYcoGen	-
13	MNE	D-mannose	-	38	XLT	XyLiTol	-
14	SBE	L-sorbose	-	39	GEN	GENtiobiose	-
15	RHA	L-rhamnose	-	40	TUR	D-TURanose	-
16	DUL	Dulcitol	-	41	LYX	D-LYXose	-
17	INO	Inositol	-	42	TAG	D-TAGatose	-
18	MAN	D-manitol	-	43	DFUC	D-FUCose	-
19	SOR	D-sorbitol	-	44	LFUC	L-FUCose	-
20	MDN	Methyl-αD-Mannopyranoside	-	45	DARL	D-ARabitoL	-
21	MDG	Methyl-αD-Glucopyranoside	-	46	LARL	L-ARabitoL	-
22	NAG	N-Acetylglucosamine	-	47	GNT	potassium GlucoNaTe	-
23	AMY	Amygdalin	-	48	2KG	potassium 2-KetoGluconate	-
24	ARB	Arbutin	-	49	5KG	potassium 5-KetoGluconate	+

Table 4-4. API 20NE (48h) results of RR4-56.

Active ingredients	Reactions / Enzymes	NO.	Tests	판독 기준 및 결과		
				Negative	Positive	RR4-56
potassium nitrate	reduction of nitrate to nitrites	1	NO_3	colorless	pink-red	+
	reduction of nitrate to nitrogen			pink	colorless	
L-tryptophane	indole production(TRyptoPhane)	2	TRP	colorless	pink	-
				pale green/yellow		
D-glucose	fermentation(GLUcose)	3	GLU	blue to green	yellow	-
L-arginine	Arginine DiHydrolase	4	ADH	yellow	orange/pink/red	-
urea	UREase	5	URE	yellow	orange/pink/red	-
essulin	hydrolysis(β-glucosidase) (ESCulin)	6	ESC	yellow	grey/brown/black	-
ferric citrate						
gelatine	hydrolysis(protease) (GELatin)	7	GEL	no pigment	diffusion of	-
(bovine origin)				diffusion	black pigment	
4-nitrophenyl-βD-galactopyranoside	β-galactosidase(Para-NitroPhenyl-βD-Galactopyranosidase)	8	PNPG	colorless	yellow	-
D-glucose	assimilation (glucose)	9	GLU	transparent	opaque	-
L-arabinose	assimilation (arabinose)	10	ARA	transparent	opaque	-
D-mannose	assimilation (mannose)	11	MNE	transparent	opaque	-
D-mannitol	assimilation (mannitol)	12	MAN	transparent	opaque	-
N-acetyl-glucosamine	assimilation (N-acetyl-glucosamine)	13	NAG	transparent	opaque	-
D-maltose	assimilation (maltose)	14	MAL	transparent	opaque	-
potassium gluconate	assimilation (potassium gluconate)	15	GNT	transparent	opaque	-
capric acid	assimilation (capric acid)	16	CAP	transparent	opaque	-
adipic acid	assimilation (adipic acid)	17	ADI	transparent	opaque	-
malic acid	assimilation (malate)	18	MLT	transparent	opaque	-
trisodium citrate	assimilation (trisodium citrate)	19	CIT	transparent	opaque	-
phenylacetic acid	assimilation (phenylacetic acid)	20	PAC	transparent	opaque	-
		Oxidase		-		

CHAPTER 5. Taxonomic study of the strain RR4-68

INTRODUCTION

The genus *Formosa* was first described by Ivanova *et al*. (2004) with the novel species *Formosa* algae. This genus has ability of anaerobic growth by D-glucose fermentation and belongs to the family *Flavobacteriaceae*. Currently, the genus contains one species, *Formosa spongicola* by Yoon *et al* (2011). The novel species produces acid from glucose and reduces nitrate to gas nitrogen. (Yoon *et al*., 2011) Strain RR4-68 is isolated from a biofilter of a seawater recirculating aquaculture system and related to the species, *Formosa spongicola*. (97.35% similarity) based on the analysis of 16S rRNA sequence. In this study, the strain RR4-68 is proposed as the novel species based on biochemical, physiological and phylogenetic differences.

RESULTS AND DISCUSSION

Cells are Gram-positive, motile, facultative aerobic, rod-shaped. Cells are 1㎛ in length. Colonies grown on MA for 7days are circular form, convex margin, entire edge and yellow. Optimal growth occurs at 30℃ (growth range, 20-30℃). Optimal growth occurs in the presence 2.5-3.0% NaCl (w/v). oxidase- and catalase-positive. In the API 20NE strips (Table 5-4), gelatin hydrolysis, nitrate, assimilations of glucose and maltose are positive, but reduction of glucose fermentation, assimilations of L-arabinose, D-mannose, D-mannitol, D-maltose, malate are negative. In the API ZYM system (Table 5-2), alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, crystine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-glucosidase activities are positive, but lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase activities are negative.

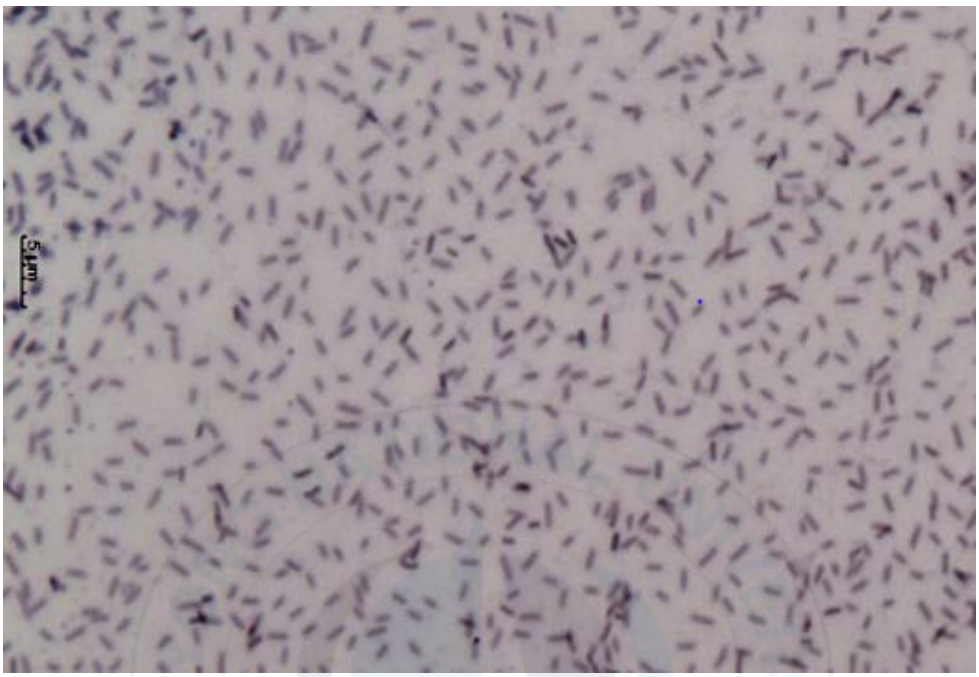

Figure 5-1. Microscopy of strain RR4-68 after Gram staining

The 16S rRNA sequences of strain RR4-68 and other closest strains were used to generate a phylogenetic tree (Figure 5-2, 5-3). The phylogenetic tree indicated that the strain RR4-68 belongs to the genus *Formosa*. It showed sequence similarity 97.35% with *Formosa spongicola* (Table 5-1), the strain RR4-68 is classified as new species in the genus *Formosa*.

Table 5-1. 16S rRNA sequence similarity of strain RR4-68 and other strains.

Rank	Name	Strain	Pairwise similarity (%)
1	*Formosa spongicola*	A2(T)	97.35
2	*Ichthyenterobacterium magnum*	Th6(T)	95.88
3	*Bizionia echini*	KMM_6177(T)	95.86
4	*Bizionia psychrotolerans*	PB-M7(T)	95.84
5	*Bizionia argentinensis*	JUB59(T)	95.80
6	*Formosa agariphila*	KMM_3901(T)	95.80
7	*Bizionia paragorgiae*	KMM_6029(T)	95.78
8	*Bizionia hallyeonensis*	T-y7(T)	95.77
9	*Olleya namhaensis*	WT-MY15(T)	95.77
10	*Algibacter aquimarinus*	KYW589(T)	95.71

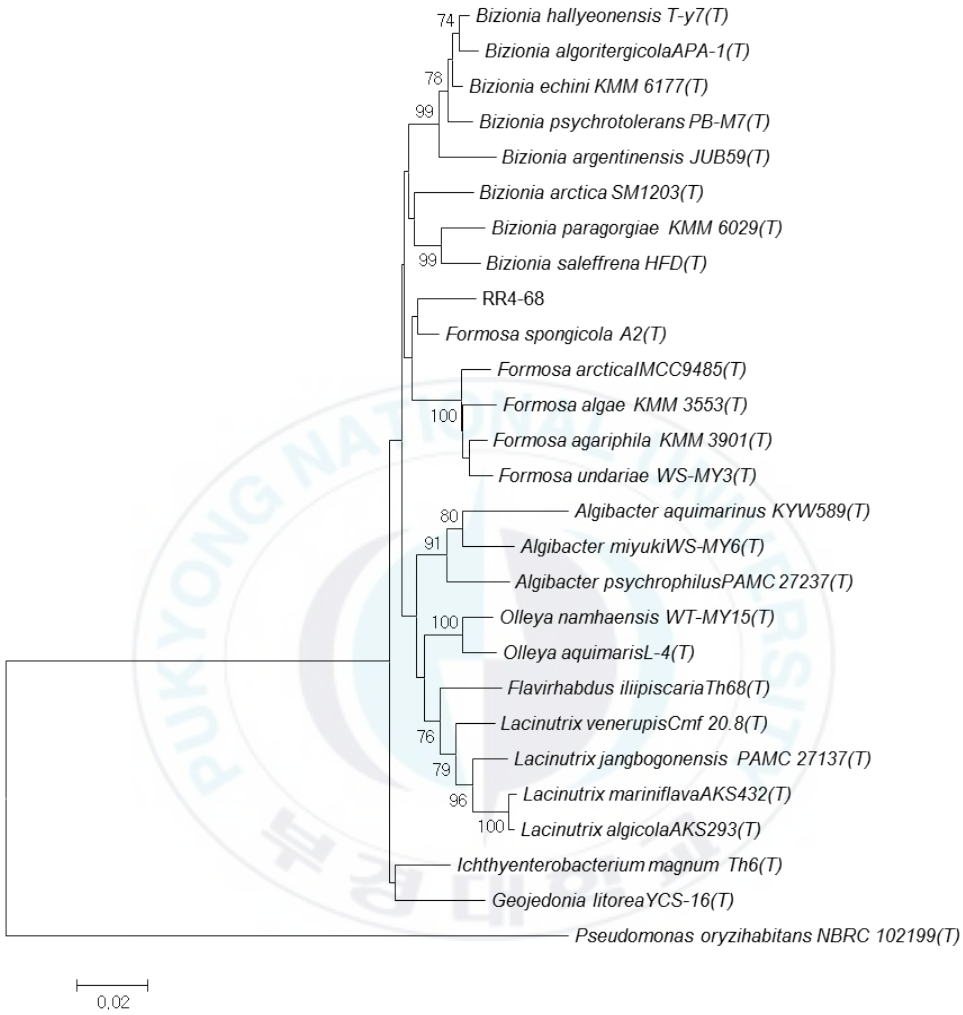

Figure 5-2. Neighbor-joining phylogenetic tree of strain RR4-68 and reference strains

It was conducted based on the aligned sequences using the neighbor-joining methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

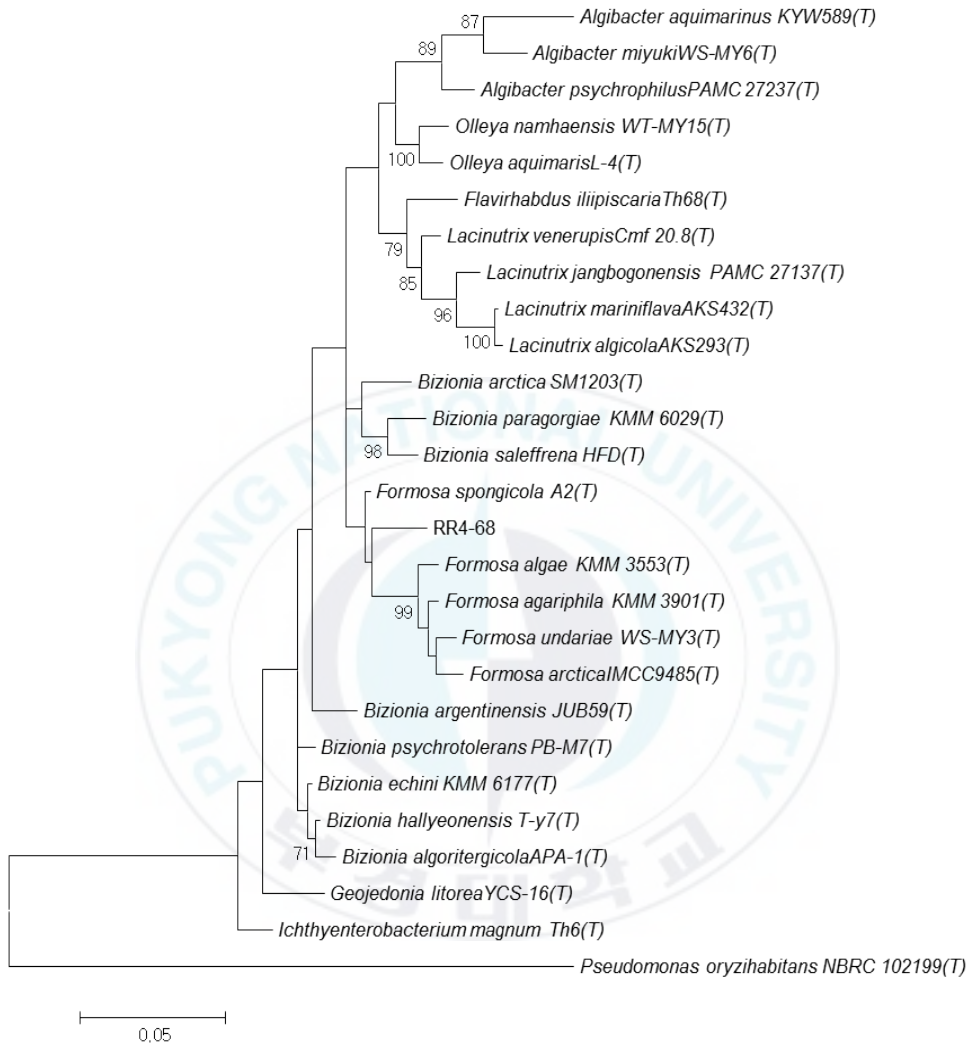

Figure 5-3. Maximum-likelihood phylogenetic tree of strain RR4-68 and reference strains

It was conducted based on the aligned sequences using the maximum-likelihood methods. *Pseudomonas oryzihabitans* NBRC 102199(T) is an outgroup.

Table 5-2. API ZYM results of RR4-68.

No.	Enzyme Assayed For	Substrate	pH	Result		RR4-68
				positive	negative	
1	Control			Colorless or color of the sample if it has an intense coloration		-
2	Alkaline phosphatase	2-naphthyl phosphate	8.5	Violet	Colorless or very pale Yellow	+
3	Esterase (C4)	2-naphthyl butyrate	6.5	Violet		+
4	Esterase Lipase (C8)	2-naphthyl caprylate	7.5	Violet		+
5	Lipase (C14)	2-naphthyl myristate	7.5	Violet		-
6	Leucine arylamidase	L-leucyl-2-naphthylamide	7.5	Orange		+
7	Valine arylamidase	L-Valyl-2-naphthylamide	7.5	Orange		+
8	Crystine arylamidase	L-cystyl-2-naphthylamide	7.5	Orange		+
9	Trypsin	N-benzoyl-DL-arginine-2-naphthylamide	8.5	Orange		-
10	α-chymotrypsin	N-glutaryl-phenylanine-2-naphthylamide	7.5	Orange		-
11	Acid phospatase	2-naphtyl phosphate	5.4	Violet		+
12	Naphtol-AS-BI-phosphohydrolase	Naphthol-AS-BI-phosphate	5.4	Blue		+
13	α-galactosidase	6-Br-2-naphthyl-αD-galactopyranoside	5.4	Violet		-
14	β-galactosidase	2-naphthyl-βD-galactopyranoside	5.4	Violet		-
15	β-glucuronidase	Naphthol-AS-BI-βD-glucuronide	5.4	Blue		-
16	α-glucosidase	2-naphthyl-αD-glucopyranoside	5.4	Violet		+
17	β-glucosidase	6-Br-20naphthyl-βD-glucopyranoside	5.4	Violet		-
18	N-acetyl-β-glucosaminidase	1-naphthyl-N-acetyl-βD-glucosaminide	5.4	Brown		-
19	α-mannosidase	6-Br-2-naphthyl-αD-mannopyranoside	5.4	Violet		-
20	α-fucosidase	2-naphthyl-α-fucopyranoside	5.4	Violet		-

Table 5-3. API 50CH results of RR4-68.

50CH Tube	Test	Active ingredients	RR4-68	50CH Tube	Test	Active ingredients	RR4-68
0		Control	-	25	ESC	Esculin ferric citrate	+
1	GLY	Glycerol	-	26	SAL	Salicin	-
2	ERY	Erythritol	-	27	CEL	D-celiobiose	-
3	DARA	D-arabinose	-	28	MAL	D-maltose	-
4	LARA	L-arabinose	-	29	LAC	D-lactose(Bovine origin)	-
5	RIB	D-rinose	-	30	MEL	D-MELibiose	-
6	DXYL	D-xylose	-	31	SAC	D-SACcharose (sucrose)	-
7	LXYL	L-xylose	-	32	TRE	D-TREhalose	-
8	ADO	D-adonitol	-	33	INU	INUlin	-
9	MDX	Methyl-βD-xylopyranoside	-	34	MLZ	D-MeLeZitose	-
10	GAL	D-galactose	-	35	RAF	D-RAFfinose	-
11	GLU	D-glucose	-	36	AMD	AmiDon (starch)	-
12	FRU	D-fructose	-	37	GLYG	GLYcoGen	-
13	MNE	D-mannose	-	38	XLT	XyLiTol	-
14	SBE	L-sorbose	-	39	GEN	GENtiobiose	-
15	RHA	L-rhamnose	-	40	TUR	D-TURanose	-
16	DUL	Dulcitol	-	41	LYX	D-LYXose	-
17	INO	Inositol	-	42	TAG	D-TAGatose	-
18	MAN	D-manitol	-	43	DFUC	D-FUCose	-
19	SOR	D-sorbitol	-	44	LFUC	L-FUCose	-
20	MDN	Methyl-αD-Mannopyranoside	-	45	DARL	D-ARabitoL	-
21	MDG	Methyl-αD-Glucopyranoside	-	46	LARL	L-ARabitoL	-
22	NAG	N-Acetylglucosamine	-	47	GNT	potassium GlucoNaTe	-
23	AMY	Amygdalin	-	48	2KG	potassium 2-KetoGluconate	-
24	ARB	Arbutin	-	49	5KG	potassium 5-KetoGluconate	+

Table 5-4. API 20NE (48h) results of RR4-68.

Active ingredients	Reactions / Enzymes	NO.	Tests	판독 기준 및 결과		
				Negative	Positive	RR4-68
potassium nitrate	reduction of nitrate to nitrites	1	NO_3	colorless	pink-red	
	reduction of nitrate to nitrogen			pink	colorless	+
L-tryptophane	indole production(TRyptoPhane)	2	TRP	colorless	pink	-
				pale green/yellow		
D-glucose	fermentation(GLUcose)	3	GLU	blue to green	yellow	-
L-arginine	Arginine DiHydrolase	4	ADH	yellow	orange/pink/red	-
urea	UREase	5	URE	yellow	orange/pink/red	-
essulin	hydrolysis(β-glucosidase) (ESCulin)	6	ESC	yellow	grey/brown/black	-
ferric citrate						
gelatine	hydrolysis(protease) (GELatin)	7	GEL	no pigment	diffusion of	+
(bovine origin)				diffusion	black pigment	
4-nitrophenyl-βD-galactopyranoside	β-galactosidase(Para-NitroPhenyl-βD-Galactopyranosidase)	8	PNPG	colorless	yellow	-
D-glucose	assimilation (glucose)	9	GLU	transparent	opaque	+
L-arabinose	assimilation (arabinose)	10	ARA	transparent	opaque	-
D-mannose	assimilation (mannose)	11	MNE	transparent	opaque	-
D-mannitol	assimilation (mannitol)	12	MAN	transparent	opaque	-
N-acetyl-glucosamine	assimilation (N-acetyl-glucosamine)	13	NAG	transparent	opaque	-
D-maltose	assimilation (maltose)	14	MAL	transparent	opaque	+
potassium gluconate	assimilation (potassium gluconate)	15	GNT	transparent	opaque	-
capric acid	assimilation (capric acid)	16	CAP	transparent	opaque	-
adipic acid	assimilation (adipic acid)	17	ADI	transparent	opaque	-
malic acid	assimilation (malate)	18	MLT	transparent	opaque	-
trisodium citrate	assimilation (trisodium citrate)	19	CIT	transparent	opaque	-
phenylacetic acid	assimilation (phenylacetic acid)	20	PAC	transparent	opaque	-
		Oxidase		-		

국문초록

순환 여과 양식 시스템(RAS)은 적절한 상태인 내륙 어류 양식장의 수질을 유지할 수 있도록 하는 수단이다. RAS는 사료의 잔해, 어류 배설물, 그리고 암모니아, 질산염, 아질산염과 같은 독성이 있는 질소 화합물을 제거하기 위한 물리적, 화학적, 생물학적 처리 과정을 거친다. 생물학적 처리 중, 박테리아는 중요한 역할을 한다. 박테리아는 질화 및 탈 질소 대사 작용 시스템을 지니고 있어서, 유해한 질소 화합물을 무해한 것으로 전환할 수 있다. 본 연구에서, 질소를 제거하는 박테리아를 선별했고 그 중 일부는 새로운 속(genera)들과 종들의 후보군이었다. 모든 균주는 긴밀히 연관된 종들 즉, RR4-38, *Ulvibacter antarcticus* IMCC3103(T) (95.05%); RR4-40, *Ulvibacter marinus* IMCC1208(T) (94.17%); RR4-41, *Kangiella geojedonensis* YCS-5(T) (98.08%); RR4-56, *Halovulum dunhuangense* YYQ-30(T) (92.84%); RR4-68, *Formosa spongicola* A2(T) (97.35%)와 서열의 유사성이 낮다. 각각의 균주에 대한 계통수는 분류학적 차원에서 균주들의 계통발생학적 위치를 나타낸다. 모든 균주들은 운동성을 지닌 막대 모양의 세포체를 지니며, 30℃, pH 7.0-7.5, NaCl 농도 2.5-3.0%에서 최적의 성장을 나타냈다. 각 균주에 대해 Oxidase, catalase 및 생화학적 검사와 탄소 동화 및 효소 생산과 같은 생리 학적 검사도 수행되었다.

생리학적, 계통 발생적 분석을 토대로, 세 가지 균주는 새로운 속에 대한 후보군이며, 두 가지 균주는 새로운 종에 대한 후보군인 것으로 나타났다. 그러한 유형의 균주는 각각 RR4-40, RR4-56, RR4-38, RR4-41 and RR4-68이다. 요컨대 몇몇 새로운 균주들을 RAS로부터 얻을 수 있었다. 그 결과들은 RAS가 탐구한 적이 없는 환경이며, 두 가지 분류군을 발견하기 위한 좋은 자원이란 점을 보여주었다. 본 연구는 RAS를 이해하고 그것들의 효율성을 개선하는 데 기여할 것이다.

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