Phylogenetic studies on marine bacteria within the phylum *Proteobacteria* and *Bacteroidetes*

（*Proteobacteria* 門および *Bacteroidetes* 門に属する海洋細菌の系統分類に関する研究）

Sanghwa Park
朴　相和

Phylogenetic studies on marine bacteria within the phylum *Proteobacteria* and *Bacteroidetes*

（*Proteobacteria* 門および *Bacteroidetes* 門に属する海洋細菌の系統分類に関する研究）

東京大学農学生命科学研究科

応用生命工学　専攻

平成２０年度博士課程入学

氏　　名　　朴　相和

指導教員名　　横田　明

Phylogenetic studies on marine bacteria within the phylum *Proteobacteria* and *Bacteroidetes*

by

Sanghwa Park

A dissertation submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Department of Biotechnology
Graduate School of Agricultural and Life Sciences
The University of Tokyo
January 2011

CONTENTS

CHAPTER I.

GENERAL INTRODUCTION

CHAPTER I. GENERAL INTRODUCTION

1. Polyphasic taxonomy and phylogenetics of bacteria

The term bacterial taxonomy could be defined as classification, nomenclature, and identification of bacteria. Along with the rapid progress of molecular techniques, bacterial taxonomy has entered in a phase of rapid and radical changes. Throughout the history of bacterial taxonomy, it has gradually developed from traditional morphology, physiological, biochemical and chemical studies to the period molecular studies. The term "polyphasic taxonomy" was introduced 30 years ago by Colwell (1970) to refer to a taxonomy that assembles and assimilates many levels of information, from molecular to ecological, and incorporates several distinct portions of information extractable from a nonhomogeneous system to yield a multidimensional taxonomy. Nowadays, polyphasic taxonomy refers to a consensus type of taxonomy and aims to utilize all the available data in delineating consensus groups, decisive for the final conclusions. Early bacterial classifications were based mainly on the morphological characteristics. In contrast to eukaryotes, prokaryotes lack the morphological, developmental and fossil evidences for tracing their phylogenetic relationships. To overcome this intrinsic weakness, physiological and biochemical characteristics (e.g., composition of the polar lipids, cellular fatty acid and isoprenoid quinone) were used extensively for classification and identification of bacterial strains, and this approach produced more phenotypic information helpful in bacterial taxonomy. The development of molecular biological techniques such as DNA sequencing, determination of DNA G+C content and DNA-DNA hybridization and the improvement of tools of chemical analysis have dramatically changed bacterial determinative taxonomy toward bacterial phylogenetics. The small subunit ribosomal RNA (16S rRNA) gene is highly conserved

molecule and universally exists for every bacterial species. For this reason, recently, the comparative analysis of 16S rRNA gene sequence has been recognized as the most powerful method in molecular phylogenetics of bacteria (Woese, 1987, 1992). However, 16S rRNA gene is often inconvenient for resolving bacterial strains at the species level because of its slow evolution (Fox *et al*., 1992; Clayton *et al*., 1995). For the purpose of phylogenetic analysis, gene used as molecular taxonomic markers should be universal, not be transferred horizontally, and not show any mutation to specific conditions (Yamamoto & Harayama, 1995). Also development of whole-genome sequencing project still going. Except for the well-known 16S rRNA genes and other housekeeping genes, there're still other newly genome-base parameters, such as the average amino acid identity (AAI) or average nucleotide identity (ANI) (Konstantinidis *et al*., 2005) have introduced. The whole genomic world is met great excitement and changing. Certainly, with the rapid growth in genome information, emerging evolutionary and ecological insight, the application using in bacterial taxonomy will be more complete in the future.

2. Marine bacteria

The oceans occupy 7l% of earth's surface, with a volume of 1.46 x 10^9 km^3, an average depth of 4,000 m, and a maximal depth of approximately 11,000 m (Rumney, 1968). The huge water masses of the oceans have an important moderating effect on the climate of earth, being the ultimate reservoir and receptacle of the global water cycle. The main problem in performing autecological studies on marine bacteria lies in the methods used to obtain viable cultures. Whether the isolates obtained by current culture methods represent the major indigenous population is unknown. Determining the representative physiological studies that can be performed on the vast numbers of marine bacteria that have yet to be cultured is still a matter of guesswork (Schute *et al*., 1993). Historically based on the cultivation dependent methods only a few phylogenetic groups such as the members of the phylum *Proteobacteria* and the phylum *Bacteroidetes* are isolated and incorporated within the marine bacterial group. However, recently, the small subunit ribosomal RNA (16S rRNA) genes have become universal phylogenetic markers and are the main criteria by which microbial plankton groups are identified and named (Giovannoni *et al*., 2000). Molecular approaches for characterizing microbial species and assemblage have significantly influenced understanding of microbial diversity and ecology (Delong, 2005). Most of the marine microbial groups were first identified by sequencing rRNA genes cloned from seawater (Britschgi & Giovannoni, 1991; Fuhrman *et al*., 1992) and remain uncultured today, indicating that a large amount of potentially interesting bacterial diversity has yet to be cultured (O'Sullivan *et al*., 2004). Although most of the major microbial plankton clades have cosmopolitan distributions, new marine microbial plankton clades continue to emerge from studies that focus on unique hydrographic features.

3. Marine sponge bacteria

Sponges (phylum *Porifera*) are among the oldest metazoan animals, with a fossil record dating back more than 580 million years to the Precambrian. Sponges contribute significantly to, and sometimes even dominate, the tropical reef fauna in terms of biomass, but are also found in polar and deep oceans and in freshwater lakes and streams. Eighty-five percent of the 6000 formally described living species belong to the class *Demospongiae* (demosponges), with the other species being represented by the classes *Hexactinellida* (glass sponges) and *Calcarea* (calcareous sponges) (Hooper & van Soest, 2002). The growth habits of sponges encompass various shapes (e.g. encrusting, rope, ball, tube, barrel, vase), colours (e.g. white, yellow, green, blue, purple, brown, black) and sizes (a few millimetres to nearly two metres), and can be quite variable in response to environmental conditions (Brusca & Brusca, 1990).

Despite the fact that sponges feed on microorganisms, large numbers of extracellular bacteria populate many demosponges. These types of sponge have been termed 'bacteriosponges' or 'high-microbial-abundance sponges' (Vacelet & Donadey, 1977; Hentschel *et al*., 2003). However, the mesohyl of other sponges that coexist in the same habitat are essentially devoid of microorganisms ('low-microbial-abundance sponges'). In the bacteriosponges, bacterial population densities may reach 10^8–10^{10} bacteria per gram of sponge wet weight, exceeding seawater concentrations by 2–4 orders of magnitude, whereas in the low-microbial-abundance sponges, they are within the range of natural seawater (10^5–10^6 bacteria per gram of sponge wet weight). Until several years ago, the microbial communities of sponges were only identified by electron microscopy (Vacelet & Donadey, 1977; Wilkinson, 1978; Rűtzler, 1990). While early works indicated high microbial diversity and the presence of unusual microorganisms, it was not until the application of molecular tools, specifically 16S rRNA

gene library construction, FISH, and denaturing gradient gel electrophoresis (DGGE), that more precise insights into the microbial community composition could be gained. Nearly a dozen studies have now been undertaken, with sometimes strikingly congruent results (Hentschel *et al.*, 2003; Hill, 2004). 16S rRNA gene library construction using universal bacterial PCR primers revealed a common microbial signature in many sponges that is phylogenetically complex yet highly sponge-specific and distinctly different from that of marine plankton (Fig. 1). 16S rRNA gene phylotypes affiliated with the phyla *Acidobacteria, Chloroflexi, Actinobacteria, Proteobacteria* (*Alphaproteobacteria, Gammaproteobacteria, Deltaproteobacteria*), *Nitrospira, Cyanobacteria, Bacteriodetes,* and *Gemmatimonadetes* were recovered.

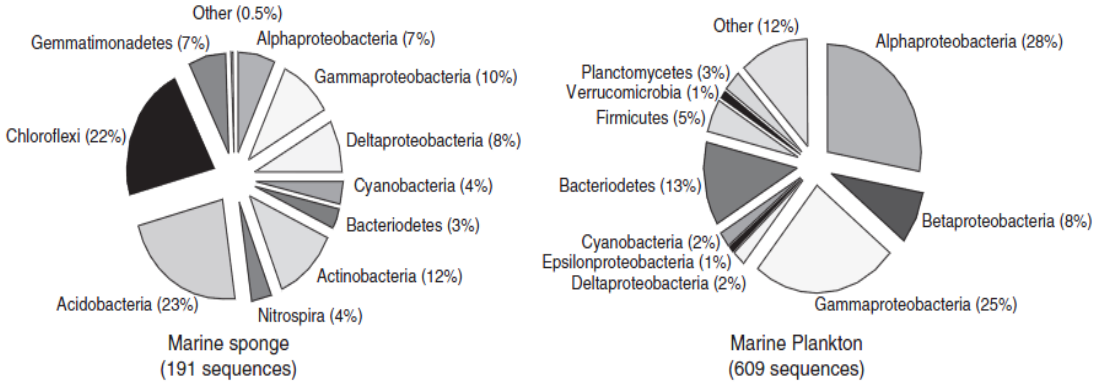


Figure 1. 16S rRNA gene sequences derived from marine sponges compared with those from marine bacterioplankton based on PCR amplification with universal bacterial primers

4. Aims of this study

In this study, an attempt has been made to isolate novel bacterial strains from marine sponges collected obtained from Off Jeju Island, Korea by using culture dependent methods (medium P and Medium SN). Also bacterial strains isolated from seawater, Japan were charcterized to obtain a better understanding about the taxonomic position of the bacterial strains within the phylum *Proteobacteria* and *Bacteroidetes* using a polyphasic approach including 16S rRNA gene sequence, molecular, physiological, biochemical and chemotaxonomic analyses.

CHAPTER II.

PHYLOGENETIC STUDIES ON THE NOVEL MARINE SPONGE BACTERIA IN THE CLASS *ALPHAPROBACTERIA*

CHAPTER II. PHYLOGENETIC STUDIES ON THE NOVEL MARINE SPONGE BACTERIA IN THE CLASS *ALPHAPROBACTERIA*

1. Introduction

Alphaproteobacteria exhibit an enormous diversity in their morphological and metabolic characteristics and the class is presently recognized solely as a clade in the 16S rRNA gene-based phylogeny (Stackebrandt *et al*., 1988). Based on 16S rRNA gene trees, the *Alphaproteobacteria* has been divided into seven orders: *Caulobacterales, Rhizobiales, Rhodobacterales, Rhodospirillales, Rickettsiales, Sphingomonadales* and *Parvularculales* (Kersters *et al*., 2006). The *Alphaproteobacteria* includes important bacteria that are widely studied, including for example, the most important genera of soil bacteria able to live in symbiosis with leguminous plants (order *Rhizobiales*). The genus *Sphingomonas* was first proposed by Yabuuchi *et al* (1990) in the class *Sphingomonadales*. At present the genus *Sphingomonas* comprises approximately 45 species with validly published names, including the recently described species *sphingomonas chagbensis* (Zhang *et al*., 2010) and *Sphingomonas histidinilytica* (Nigam *et al.*, 2010). Members of the genus *Sphingomonas* sensu strict have been isolated from a variety of natural sources, rhizospheres, soil, aquatic habitats and clinical material. But little is known about *Sphingomonas* strains dwelling in marine environments, particularly those associated with animals.

In Chapter II, I tried to isolate novel bacterial strain from 20 species of marine sponge using culture dependent methods and the pylogenetic studies were performed using a polyphasic taxonomic approach on novel marine sponge bacteria strain MS-31^{T}.

2 .Materials and methods

2.1. Marine sponges investigated in this study

20 species of marine sponge were collected Jeju Island Korea

Marine sponge	Sampling place	Date
Raspailia hirrsuta	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 20 m).	Oct, 2008
Spirastrella panis	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2008
Spirastrella panis	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2008
Halichondria panicea	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2008
Halichondria sinapium	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2008
Petrosia corticata	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2008
Cliona lobata	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 20 m).	Aug, 2008
Disccodermia sp	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 20 m).	Oct, 2008
Erylus nobilis	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 20 m).	Oct, 2008
Petrosia corticata	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 20 m).	Oct, 2008
Tedania ignis	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2009
Lissodendorys isodictyalis	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2009
Halichondria okadai	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2009
Halichondria permollis	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2009
Cliona celata	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2009
Hymeniacidon flavia	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 20 m).	Aug, 2009
Homaxinella sp	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2009
Scrcotragus sp	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2009
Apongosorites sp	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2009
Asteropus simplex	Off Jeju Island, Korea (33°13' N, 126°33' E; depth, 0 m).	Aug, 2009

Investigated marine sponges were provided by professor Jin-Suk Park, Hannam University, Korea

2.2. Compositions of media used in this study

2.2.1. Medium ‘P’

Composition

Metal mix X (for composition see below)	250 ml
Distilled Water	650 ml
Agar	20 g
‘P’ mix (for composition see below)	100 ml
Cycloheximide	50 mg
Griseofulvin	25 mg

Composition of metal mix X (pH 7.6)

NaCl	500 g
$MgSO_4 \cdot 7H_2O$	180 g
$CaCl_2 \cdot 2H_2O$	2.8 g
KCl	14 g
$Na_2HPO_4 \cdot 12H_2O$	5 g
$FeSO_4 \cdot 7H_2O$	200 mg
PII metals (for composition see below)	600 ml
S2 metals (for composition see below)	100 ml
Distilled Water	4300 ml

Composition of ‘P’ mix (pH 7.6)

NZ Amine (Sigma)	0.1 g
Bactopeptone (Difco)	0.1 g
Tryptone peptone (Difco)	0.1 g
Yeast extract (Difco)	0.2 g
Malt Extract (Difco)	0.1 g
Bacto Soytone (Difco)	0.1 g
Potato dextrose broth (Difco)	0.1 g
Extract Ehlrich (Wako)	0.2 g

Heart infusion broth (Difco)	0.1 g
Gelatin (Difco)	0.1 g
Cellulose	0.1 g
Skimmed milk	0.1 g
Pharmamedia (Traders Protein)	0.1 g
Glycerol	0.1 g
C solution (for composition see below)	0.5 ml
Distilled Water	100 ml

Composition of PII metals (pH 7.5)

Na_2 EDTA	1 g
H_3BO_3	1.13 g
Fe solution	1 ml of $FeCl_3 \cdot 6H_2O$ (2.42 g 50 ml^{-1})
Mn solution	1 ml of $MnCl_2 \cdot 4H_2O$ (7.2 g 50 ml^{-1})
Zn solution	1 ml of $ZnCl_2$ [0.52 g 50 ml^{-1} (+HCl)]
Co solution	1 ml of $CoCl_2 \cdot 6H_2O$ (0.2 g 50 ml^{-1})
Distilled Water	996 ml

Composition of S2 metals (pH 7.5)

NaBr	1.28 g
Mo solution	10 ml of $Na_2MoO_4 \cdot 2H_2O$ (0.63 g 50 ml^{-1})
Sr solution	10 ml of $SrCl_2 \cdot 6H_2O$ (3.04 g 50 ml^{-1})
Rb solution	10 ml of RbCl (141.5 mg 50 ml^{-1})
Li solution	10 ml of LiCl (0.61 g 50 ml^{-1})
I solution	10 ml of KI (6.55 mg 50 ml^{-1})
V solution	10 ml of V_2O_5 (1.785 mg 50 ml^{-1} (+NaOH))
Distilled Water	940 ml

Composition of C solution (pH 7.6, filter sterilized)

Na-pyruvate	25 g
Mannitol	50 g
Glucose	50 g
Distilled Water	500 ml

2.2.2. Artificial seawater (Lyman & Fleming, 1940)

Composition

NaCl	23.5 g
$MgCl_2$	4.9 g
Na_2SO_4	3.9 g
$CaCl_2 \cdot 2H_2O$	1.1 g
KCl	0.66 g
$NaHCO_3$	0.19 g
KBr	0.096 g
H_3BO_3	0.026 g
$SrCl_2$	0.024 g
NaF	0.003 g
Distilled water	1000 ml

2.2.3. Medium SN

Composition

$NaNO_3$	750 mg
K_2HPO_4	15.9 mg
$Na_2EDTA\ 2H_2O$	5.6 mg
Na_2CO_3	10.4 mg
Vitamin B_{12}	1.0 mg
Cyano trace metal solution	1.0 mg
Artificial seawater	750ml
Distilled water	250ml

Composition of Cyano trace metal solution

Citric acid H_2O	6.25 g
Ferric ammonium citrate	1.4 g
$MnCl_2 \cdot 4H_2O$	6.0 g
$Na_2MoO_4 \cdot 2H_2O$	0.39 g
$Co(NO3)_2 \cdot 6H_2O$	0.025 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
Distilled water	1L

2.3. Morphology

Cell morphology was observed by using light microscopy (BX60; Olympus) and transmission electron microscopy (TEM). For TEM observation, cells were mounted on Formvar-coated copper grids and negatively stained with 2% (w/v) aqueous uranyl acetate. Grids were observed in an H-7000 transmission electron microscope (Hitachi) operated at 75 kV.

2.4. Genotypic characterizations

2.4.1. PCR amplification and sequencing of 16S rRNA gene

An approximately 1500 bp fragment of the 16S rRNA gene was amplified from extracted DNA by using bacterial universal primers specific to the 16S rRNA gene: 27F and 1492R (*Escherichia coli* numbering system; Weisburg *et al.*, 1991). Crude DNA was prepared from single colony by using InstaGene Matrix (BioRad). The 16S rRNA gene fragment covering positions 27–1492 in the *E. coli* 16S rRNA gene was amplified using the Bacteria-universal primers Bac27F (5'- AGA GTT TGA TCC TGG CTC AG -3') and Bac1492R (5'- GGC TAC CTT GTT ACG ACT T -3'). PCR reaction was performed by using TaKaRa LA Taq polymerase (Takara, Japan) by the following conditions; one cycle of 95°C for 5 min, followed by 35 cycles of 95 °C for 30 s and 58 °C for 1 min, followed by 72°C for 1 min 30s, followed by a final 5-min incubation at 72°C. PCR products were purified by MontagePCR96 (Millipore). Sequencing was performed using primers; Bac27F, Bac341F (5'-CTC CTA CGG GAG GCA GCA G -3'), Bac534R (5'- ATT ACC GCG GCT GCT GG -3'), Bac906R (5'- CCG TCA ATT CCT TTG AGT TT -3'), Bac926F (5'- AAA CTC AAA GGA ATT GAC GG -3') and Bac1492R, by BigDye Terminator v. 3.1 cycle sequencing kit (ABI). Sequencing products purified by MontageSEQ96 (Millipore) were analyzed by 3730 DNA analyzer (ABI). Sequence data was edited and assembled by using ChromasPro v. 1.34 (Technelysium).

2.4.2. Phylogenetic analysis

To ascertain the phylogenetic position of the novel isolates, the 16S rRNA gene sequences were compared with the sequences obtained from GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). Multiple alignments of the sequences were performed using CLUSTAL_X (version 1.83) (Thompson *et al*., 1997). Alignment gaps and ambiguous bases were not taken into consideration when the bases of 16S rRNA gene nucleotides were compared. Aligned sequences were analysed using MEGA4 software (Kumar *et al*., 2004). The evolutionary distances (distance options according to the Kimura two-parameter model; Kimura, 1983) and clustering with the neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-parsimony (MP) (Fitch, 1971) methods were determined by using a bootstrap analysis based on 1000 replications (Felsenstein 1985). Phylogenetic relationships by using maximum-likelihood (ML) method (Felsenstein, 1981) were also determined using the Ratchet model (Sikes & Lewis, 2001) of evolution in PAUP* 4.0b10 (Swofford, 2002). A bootstrap analysis was performed by using 1000 trial replications to provide confidence estimates for tree topologies. The similarity values were calculated using MEGA4.

2.4.3. Determination of G+C content

DNA extraction for G+C content DNA used for the determination of G+C content was isolated according to the procedure described my Marmur (1960) with some modifications. Bacterial cells were collected in 2 ml tube and stored in 20°C overnight. 500 ul lytic solution (Lysozyme 0 .75 in 1ml of 10 mM Tris-HCl buffer, pH 8.0) was added to the thawed phase

cells and mixed well. The mixture was incubated at 37°C at least 3 hr. 200 ul TES buffer (pH 8.0) and pre-warmed (60°C) 600 ul Tris-SDS solution (1M Tris-HCl+10% SDS) are added and mixed gently by using sterile toothpick. The mixture was incubated at 55°C degree overnight after adding 10 µl proteinase K (10 mg/ml) . It was then cooled and 250 µl of cold phenol and chloroform were added and mixed well at 15 rpm using a mixer (Iwaki Glass Co., Japan) for 30 min. The mixture was then centrifuged at 15,000 rpm, 4°C for 15 min. The supernatant was transferred to a new tube and the extraction process with phenol and chloroform was repeated. The supernatant was then transferred to a new tube and extracted with 500 µl chloroform and mixed for 30 min. After centrifuged at 14,000 rpm for 10 min, the supernatant was collected in 50 ml sterile entrifuge tube and 30 ml ice cooled 99.5% ethanol was added. The precipitated DNA was collected using glass bar and washed again with the cooled 99.5% ethanol. The DNA adhered glass ban was transferred in a 15 ml centrifuge tube containing 4.5 ml 0.1X SSC with the tube sealing by Parafilm and stored at 10°C overnight after the DNA that adhered on the glass bar was dried at room temperature. The 0.1XSSC solution was adjusted to 1XSSC after the DNA has dissolved. A 20 µl RNase A (10 mg/ml) and 100 µl RNAse T1 (400 U/ml , pH 7.5) was added and incubate at 37 °C for 1 hour. A 20 µl proteinase K (10 mg /ml), was added and further incubated for 90 min at 37°C. After the enzyme treatment, the mixture was separated to 2 ml tubes and extracted with phenol and chloroform treatment as described before, Supernatant were collected and added with 99.5% ethanol then washed with 70% and 99.5% ethanol respectively. The extracted DNA was dissolved in 1ml TE buffer (pH 8,0) and stored at 4°C after the precipitated DNA was vacuum dried.

2.4.4. DNA-DNA hybridization

DNA-DNA hybridization was performed with photobiotin-labelled probes in microplate wells as previously described (Ezaki *et al*. 1989) to identify differences in the species level.

Immobilization of genomic DNAs in microplate

Denatured DNA (1 µg/well) was added into microplate (100 µl/well). After the microplate was maintained at 28°C for 3 h, the DNA solution was discarded and the microplate was dried at 45 ˚C for overtime.

Preparation of probe DNA

DNA solution 10 µl (1 µg/µl) was mixed with 10 µl of photobiotin solution (Vector Lab. Inc.). The mixture was irradiated in ice bath by a sunlump (300 W) above 10 cm distance for 20 min. 180 µl of 0.1M Tris-HCl (pH 9.0) was added and mixed. 100 µl of 1-butanol was added. After centrifugation (15000 rpm) for 5 seconds, the supernatant was obtained as probe DNA.

Hybridization

Pre-hybridization solution[*1] was added (200 µl/well) and maintained at 37˚C for 30 min. The excess solution was discarded and hybridization solution[*2] (100 µl/well) with probe DNA (0.15 µg/well) were added and maintained at 55°C for 3 h. Hybridization temperature is calculated as 69.3+[G+C (mol%)]×0.41-45.

Binding of enzyme

The excess hybridization solution was discarded and wells were washed with 1×SSC for three times. 100 µl of β-D-galactosidase-streptoavidin solution[*3] (1 unit/ml 0.5 % bovin serum albumin PBS solution) was added and the microplate was maintained at 37°C for 10min. The excess solution was discarded and wells were washed for three times with 1×SSC.

Measurement

4-Methylumbelliferyl-β-D-galactopyranoside solution[*4] (100 µl, 0.1 mg/ml PBS) was added

and the microplate was measured at 15 min interval by fluorescence reader, model series 4000 (PE Biosystems).

Calculation of hybridization values

Hybridization value = 100×(X-N) / (Max-N)

X: detected value of target DNA

N: detected value of negative control DNA

Max: detected maximum value

Chemical composition used in DNA-DNA hybridization

Pre-hybridization solution[*1]

20×SSC	3ml
50×Denhardt	3ml
Denatured salmon DNA (10mg/ml)	0.3ml (boiled for 5min and cooled on ices)
Formamide	15ml
DDW	8.7ml

Hybridization solution[*2]

Pre-hybridization solution	10ml
Dextran sulfate	0.25g

□ βD-galactosidase-streptoavidin solution[*3]

Streptoavidin -β-galactosidase conjugate (1 unit/μl)	10μl
Bovine serum albumin	0.05g
1×PBS	10ml

4-Methylumbelliferyl- β-D-galactopyranoside solution[*4]

4-Methylumbelliferyl-β-D-galactopyranoside (10mg/ml dimethyl formamide)	100μl
0.2M $MgCl_2$	50μl
1×PBS	10ml

2.5. Physiological characterizations

The temperature range and pH range for growth were determined by incubating the isolates on its respective growth medium. The NaCl concentration for growth was determined using a salt-tolerance test medium: containing 1% tryptone, 0.3% yeast extract, 0.9% $MgCl_2 \cdot 6H_2O$, 0.9% $MgSO_4 \cdot 7H_2O$, 0.2% $CaCl_2 \cdot 2H_2O$, 0.06% KCl and 1.5% agar, with 0-10% (w/v) NaCl. Gram-staining was performed as described by Murray *et al*. (1994). Growth under anaerobic conditions was determined after 2 weeks of incubation in an AnaeroPack (Mitsubishi Gas Chemical Co., Inc.) on its appropriate growth medium. Catalase activity was determined by the observation of bubble formation in a 3% H_2O_2 solution. Oxidase activity was determined by cytochrome oxidase paper (Nissui Pharmaceutical Co., Ltd). API 20E, API 20NE, API 50CH and API ZYM strips (bioMérieux) were used to determine physiological and biochemical characteristics. All suspension media for API test strips were supplemented with 2% (w/v) NaCl solution (final concentration). The API 20E, API 20NE and API 50CH tests were read after 2- 5 days and the API ZYM tests were scored after 1- 2 days incubation at individual strain optimum temperature

2.6. Chemotaxonomic analyses

2.6.1. Analysis of isoprenoid quinones

Isoprenoid quinones were analyzed with modifications as described by Collins & Jones (1981). The strains were heavily inoculated in TSA plates at 15-25 °C for around 1-3 weeks (depend on the growth rate) and the bacterial cells were vacuum freeze dried in freeze dryer after collected and transferred in 50 ml centrifuge tube. About 200 mg lyophilized cells were then dissolved in 20 ml chloroform-methanol (2:1) solvent and stirred for more than 2 hrs at room temperature, The mixture was filtered with filter paper and the extraction process was repeated. The collected filtrates were then evaporated in vacuum at 30°C. Crude quinines were dissolved with small amount of acetone and evaporated again.

A small amount of ethanol was added, and the quinones fractions were separated with thin-layer chromatography (TLC, Silica gel 60 F250, Merck) using a chromatographic chamber containing 100 ml toluene as a solvent. Quinones spots were detected under UV light, then scrapped and collected the pieces. After extracted with 150 ul acetone twice, the quinones samples were analyzed by HPLC (Shimadzu), which was performed under the following conditions, column: Cosmil 5C18R (Nacalai, Japan), 40°C as oven temperature, methanol-2-propanol (3:1) as solvent, pumping rate at 1.0 ml/min and read at 270 nm.

2.6.2. Analysis of whole cellular fatty acids

Fatty methyl esters were extracted and analyzed according to the Sherlock Microbial Identification Systems (MIDI). The strains were cultured at individual strain optimum temperature in around two week (depend on the growth rate) on TSA (Difco). About 10 ul bacterial cells growing from the third quadrant were coated on the bottom part of the tube. The tube was boiled for 5 min after 1 ml Sherlock Reagent 1 was added and vortex for 5 seconds. The tube was then re-vortex for another 5 seconds and boiled further 25 min. The cooled mixture was methylated with 2 ml Sherlock Reagent 2. And then rotated and heated at 80 °C for 10 min then cooled rapidly. A 1.25 ml of Sherlock Reagent 3 was added and the tube was mix lightly for 10 min. After the lower phase was discarderd, the remaining upper phase was washed with Sherlock Reagent 4, the tube was mix gently for 10 min again. Then the upper layer (around 300 ul) was transferred to a GC vial and the extracted fatty acid methyl esters were analyzed by a gas chromatograph (Hewlett Packard 5890 series II) equipped with an Ultra2 capillary column.

2.6.3. Analysis of polar lipid composition

Polar lipids were extracted and purified from 100 mg of dried cells. The dried cells were put into a glass tube with a cap and added 4 ml of aqueous methanol (10 ml of 0.3 % aqueous NaCl added to 100 ml of methanol) and 4 ml of hexane and shaked for 15 min. And the suspended was centrifuged at 3000 rpm for 10 min and the upper layer was removed. 2 ml of ether was added and shaked for 15 min. After centrifugation at 3000 rpm for 10 min, the upper layer was removed. The under layer was hermetically boiled for 5 min and cooled at 37 ˚C for 5 min in a water bath. 4.6 ml of chloroform: methanol: water (90: 100: 300) was added and shaked for 1 h. After centrifugation at 3000 rpm for 10 min, the upper layer was transferred to another clean tube. In the biomass and lower layer, 1.5 ml of chloroform: methanol: water (90: 100: 300) was added and shaked for 30 min. After centrifugation at 3000 rpm for 10 min, the upper layer was transferred to another clean tube which previous upper layer was in. The procedure which was extraction with 1.5 ml of chloroform: methanol: water (90: 100: 300) was repeated. 2.6 ml of respective chloroform and water was added to the upper layer which was collected. It was shaked, centrifuged and the upper layer was removed. The lower layer was dried with a flow of N_2 gas and solved with 120 µl of chloroform: methanol (2:1). The solution was analyzed by two-dimentional TLC, first developing with chloroform-methanohl-water (65:25:4, by vol.) and second developing with chloroform-acetic acid-methanol-water (80:18:12:5, by vol.). The solution was spotted to the bottom of thin-layer plate coated with silica gel (silica gel 60, Merck, Darmstadt, Germany) which required four plates for one sample. After development, the four plates were dried. The plates were put into iodine to detect whole lipids and these spots were marked. Each plate was used to identify some kinds of lipids as follow.

First plate: It was sprayed with ninhydrin (Tokyo Kasei Co., Ltd.) and heated at 120 ˚C for 2 min to identify lipid spots containing free amino groups as purple spots. After marking, it was sprayed with Dittmer-Lester regent*[1] to identify all lipids and marked.

Second plate: It was sprayed with Dragendorff regent*[2] to identify phosphatidylcholine and marked. And then it was sprayed with Dittmer-Lester regent*[1] to identify all lipids and marked.

Third plate: It was sprayed with Anisaldehyde regent*[3] and heated at 110˚C for 15 min to identify sugar lipids.

Fourth plate: the plate was sprayed with a solution of sodium metaperiodate (1 %, w/v) until saturated and leave at room temperature for 10 min to oxidize, and washed with water. And the plate was decolonized with sulphur dioxide gas to remove excess periodate. It was sprayed with Schiff's regent*[4]

*[1]**Dittmer-Lester regent:** Molybdenum trioxide (4.01 g) was added into 100 ml of 25N H_2SO_4 and boiled until all residue dissolved (solution A). Powered molybdenum (1.5 g) was added into 50 ml of solution A, and boiled the mixture gently for 15 min and leave to cool. (solution B). Equal volumes of solution A and B were mixed and diluted with two-fold volume of distilled water.

*[2]**Dragendorff regent:** $Bi(OH)_2NO_3$ (0.5 g) was dissolved into 200 ml of 20% acetic acid (solution A). KI (10 g) was dissolved into 25 ml of distilled water (solution B). 20 ml of solution A and 5 ml of solution B were mixed and diluted with 70 ml of distilled water.

*[3]**Anisaldehyde regent:** Ethanol (90 ml), H_2SO_4 (5 ml), *p*-Anisaldehyde (5 ml) and acetic acid (1 ml) were mixed.

*[4]**Schiff's regent:** 1% Pararosaniline base solution was decolonized through sulphur dioxide gas.

3. Results and discussion

3.1. A new species of a genus *Sphingomonas*

3.1.1 Results and discussion

To isolate novel marine sponge bacteria, twenty species of marine sponges were obtained from off Jeju Island in Republic of Korea. Individual marine sponges (0.5–1 cm^3) were homogenized with glass rod 5 ml sterile seawater. 50 µl homogenate were spread onto medium P (Yoon et al., 2007) and medium SN (Kurahashi et al., 2009). After cultivation at 15˚C for 4 weeks, colonies were picked and then re-isolated on individual mediums. Approximately 300 colonies of partial 16S rRNA gene sequence (1100nt) were amplified by PCR and sequenced. Almost isolated bacterial strains showed above 97% partial 16S rRNA gene sequence similarity. On the medium P screen results (107 colonies), 27 genera (*Aeromicrobium, Agrococcus, Arthrobacter, Brachybacterium, Bacillus, Brevibacterium, Collimonas, Dermacoccus, Dietzia, Halomonas, Kineococcus, Kocuria, Kytococcus, Marihabitans, Microbulbifer, Microbacterium, Paenibacillus, Paracoccus, Planococcus, Pelagiobacter, Pseudovibrio, Pseudomonas, Psychrobacter, Rhodococcus, Serinicoccus, Sphingomonas* and *Staphylococcus*) in the 5 classes were isolated from marine sponges (Fig. II.1). The other hands on the medium SN screen results (132 colonies), 29 genera (*Agrococcus, Alicyclobacillus, Arthrobacter, Bacillus, Cellulosimicrobium, Curtobacterium, Demequina, Dietzia, Enterococcus, Exiguobacterium, Halobacillus, Janibacter, Kocuria, Microbacterium, Microbulbifer Netunomonas, Nocardia, Paenibacillus, Paraconglomeratum, Pelagiobacter, Pseudovibrio Psychrobacter, Rhodococcus, Ruegeria, Serinicoccus Sphingomonas, Sphingopyxis, Staphylococcus* and *Tetrasphaera*) in the 4 classes were obtained (Fig. II.2).

Interestingly i couldn’t find colony within the phylum *Bacteroidetes* and other class that

privieusly reported using culture independent methods and large number of gram positive bacterial strains were obtained. I thought that medium P and SN containing artificial seawater are not proper to isolation of novel sponge gram negative bacteria.

Isolated bacterial strains showed below 96% partial 16S rRNA gene sequence similarity that were re-sequenced of full 16S rRNA gene sequences and compared to database (Genbank). Respectively phylogenentic tree of five candidates were constructed. (Fig. II.3, 4, 5, 6, 7, 8). Finally, *Bacillus*-like strain and *Sphingomonas*-like strain, two colonies were obtained that showed 95% similarity of 16S rRNA gene sequence. I focused on *Sphingomonas*-like strain MS-31^T in the class *Alphaproteobacteria* on medium P and it was analyzed polyphasic taxonomic studies. The strain MS-31^T was maintained and experimented on Trypticase soy agar (TSA: Difco) at 25°C. Strain MS-31 ^{T} was closely related to *S. koreensis* JSS26^T (95.6%), *S. dokdonensis* DS-4^T (95.5%), *S. changbaiensis* V2M44^T (95.4%), *S. asaccharolytica* NBRC 15499^T (95.2%), *S. pituitosa* EDIV^T (95.0%) and *S. molluscorum* KMM 3882^T (95.0). The phylogenetic tree based on NJ generated comparisons of 16S rRNA gene sequences revealed that the strain MS-31^T formed a cluster with *S. changbaiensis* V2M44^T but with low bootstrap confidence values (<50) in the NJ method (Fig. II.8). Cells of strain MS-31^T grown on TSA at 25°C for a week were rod shaped, approximately 0.5- 0.7 μm wide and 1.5- 6 μm long. Cells are non-motile. Spores were not formed. Cultural, physiological and biochemical characteristics of strain MS-31^T were compared to related species within the genus *Sphingomonas* (Table II.1). Strain MS-31^T showed negative activity of oxidase and positive activity for a indole production and nitrate reduction. These results could be distinguished from strain MS-31^T and related species. No growth was observed under anaerobic conditions in TSA. Growth occurred only under aerobic conditions between 15- 37 °C. The optimal temperature and pH for the growth of the strain MS-31^T were 20- 25 °C and pH 6- 8,

respectively. The DNA G+C content of the strain MS-31^T was 69.4% which is within the range of values (60.7–69.9 %) previously reported for the genus *Sphingomonas* (Zhang et al., 2010). The major respiratory quinone was ubiquinone-10. As shown in Table II. 2, the major fatty acids of strain MS-31^T were summed feature 7 (comprising $C_{18:1}$ ω7c, $C_{18:1}$ ω9t and/or $C_{18:1}$ ω12t, 39.7 %), $C_{16:0}$ (16.3 %) and summed feature 3 (comprising $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH 11.7%). The hydroxy fatty acid was $C_{14:0}$ 2OH (15.9 %). No 3-hydroxy fatty acids were detected in this strain. Strain MS-31^T contained 11 methyl-$C_{18:1}$ ω7c (5.6%) while *S. koreensis* JSS26^T (Lee *et al*., 2001), *S. dokdonensis* DS-4^T (Yoon *et al*., 2006), *S. asaccharolytica* NBRC 15499^T (Takeuchi *et al*., 1995) and *S. molluscorum* KMM 3882^T (Romanenko *et al*., 2007) did not. The polar lipids profile comprised sphingoglycolipid, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol and an unidentified glycolipid (Fig. II.9). Phosphatidylmonomethylethanolamine and phosphatidylcholine were not detected in the strain MS-31^T which distinguishes strain MS-31^T from *S.changbaiensis* (Zhang *et al*., 2010), *S. pituitosa* (Denner *et al*., 2001), *S. dokdonensis* (Yoon *et al*., 2006) and *S. molluscorurn* (Romansenko *et al*., 2007). Based on the results of the phylogenetic analysis and biochemical and physiological properties, the strain MS-31^T isolated from the marine sponge *Hymenicacidon flavia* represents a novel species of the genus *Sphingomonas*, for which the name *Sphingomonas jejuensis* sp. nov. is proposed.

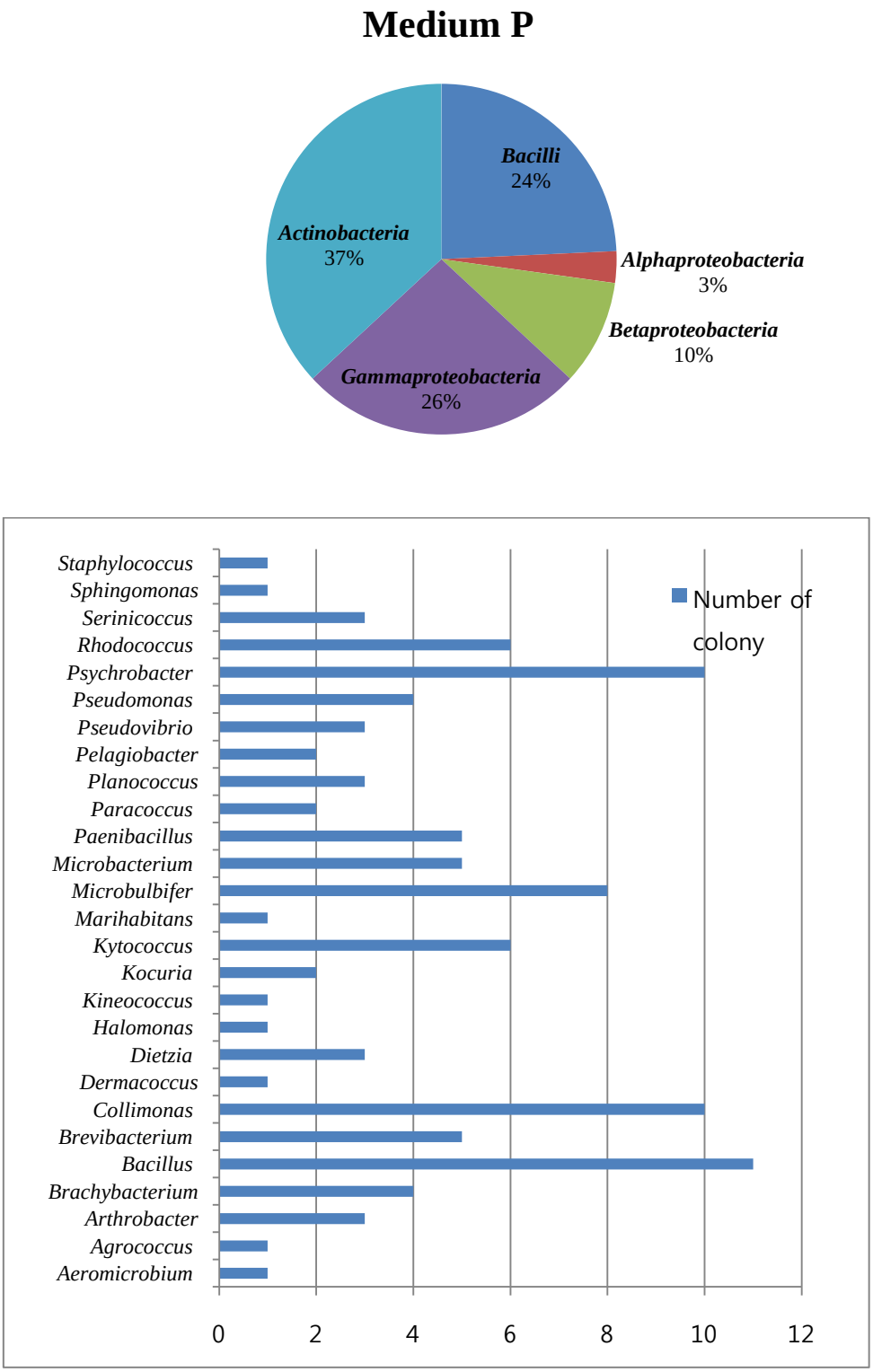


Figure II.1. Diversity of marine sponge bacteria used medium P based on partial 16S rRNA gene sequence (1100 nt).

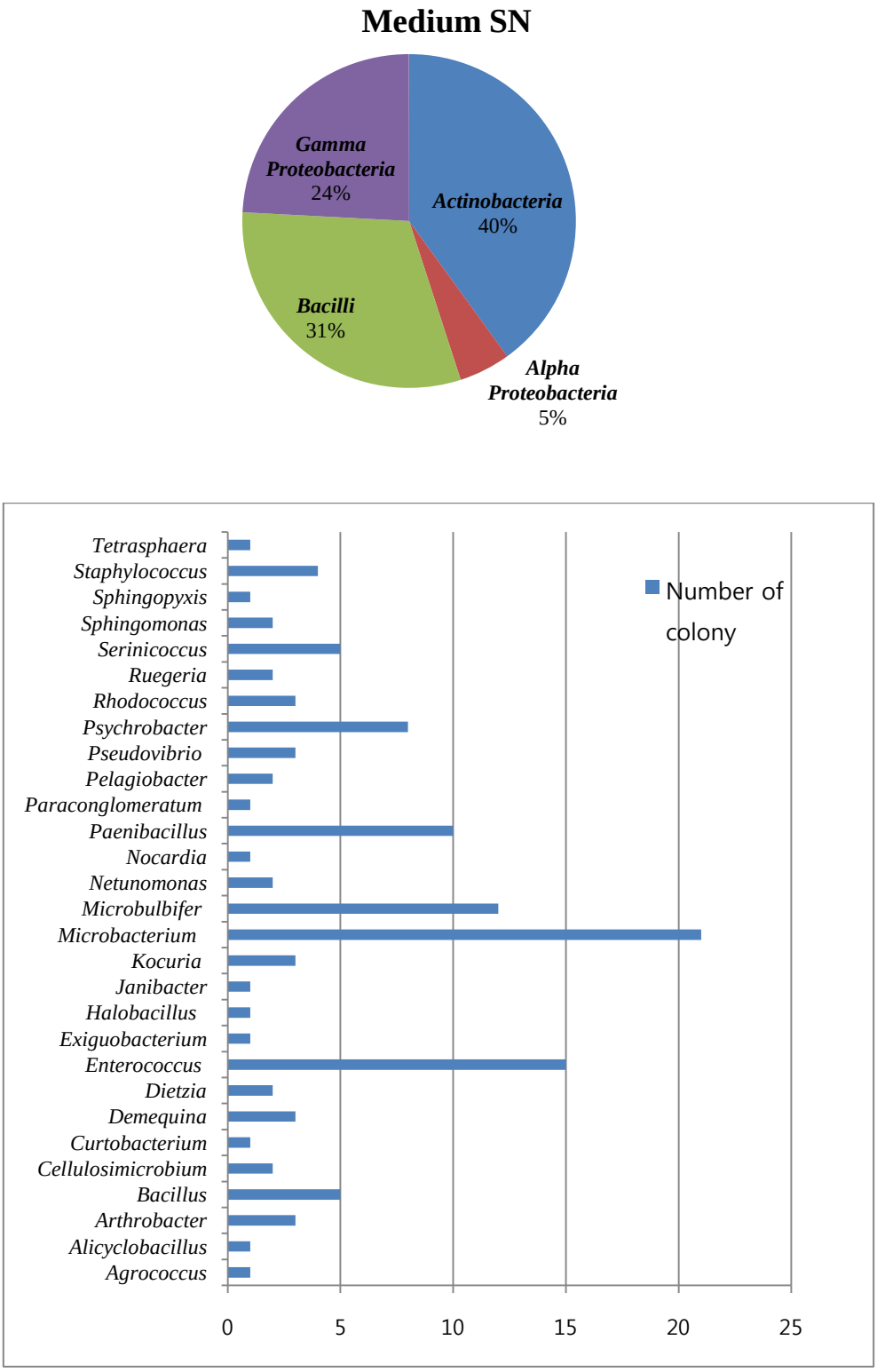


Figure II.2. Diversity of marine sponge bacteria used medium SN based on partial 16S rRNA gene sequence (1100 nt).

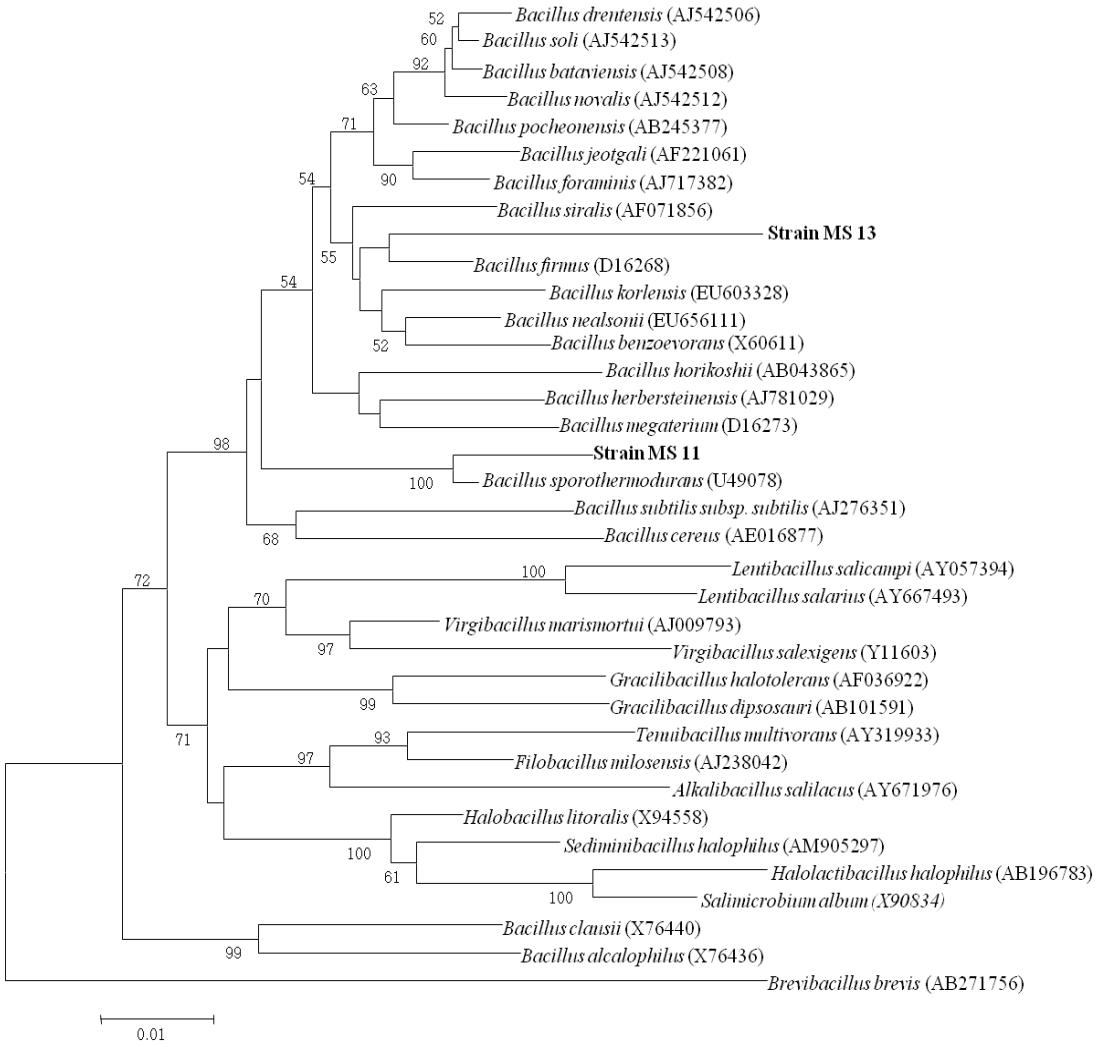


Figure II.3. Neighbour-joining phylogenetic tree of the genus *Bacillus* like novel candidate based on 16S rRNA gene sequence.

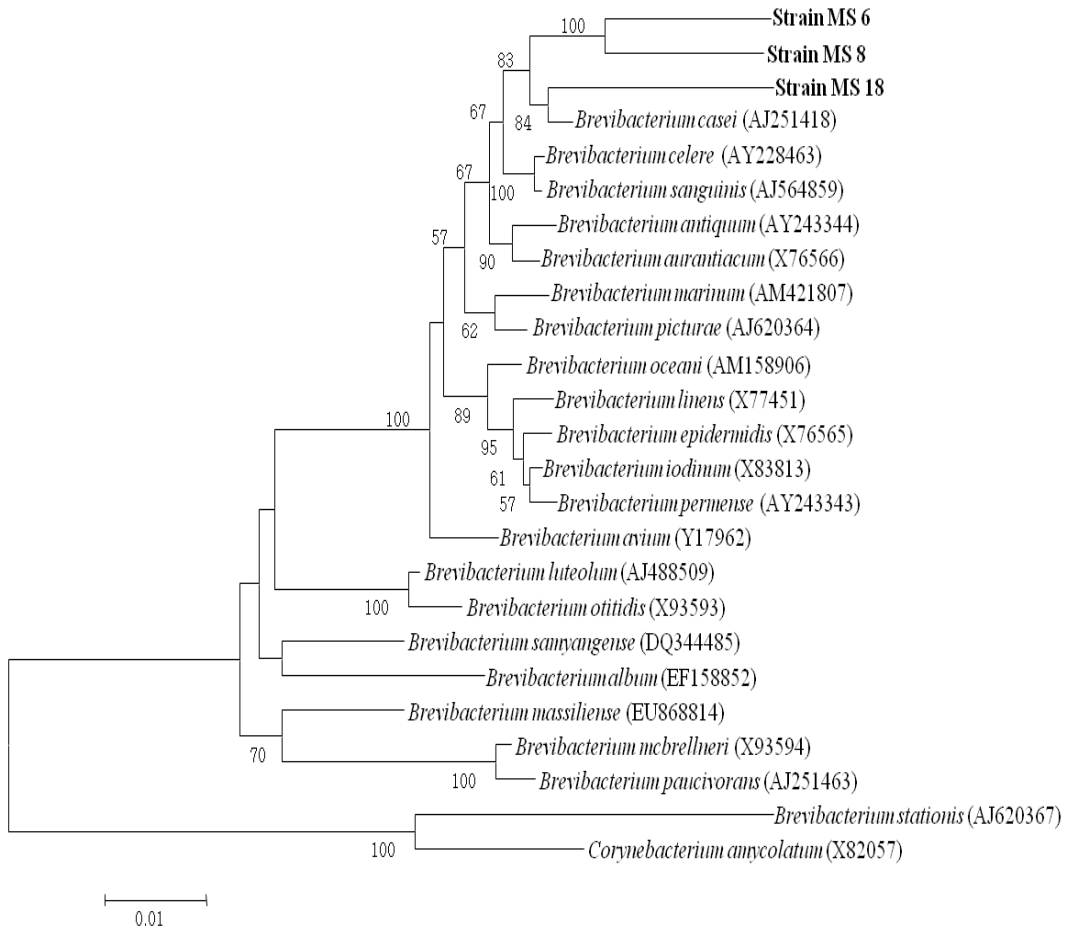


Figure II.4. Neighbour-joining phylogenetic tree of the genus *Brevibacterium* like novel candidate based on 16S rRNA gene sequence.

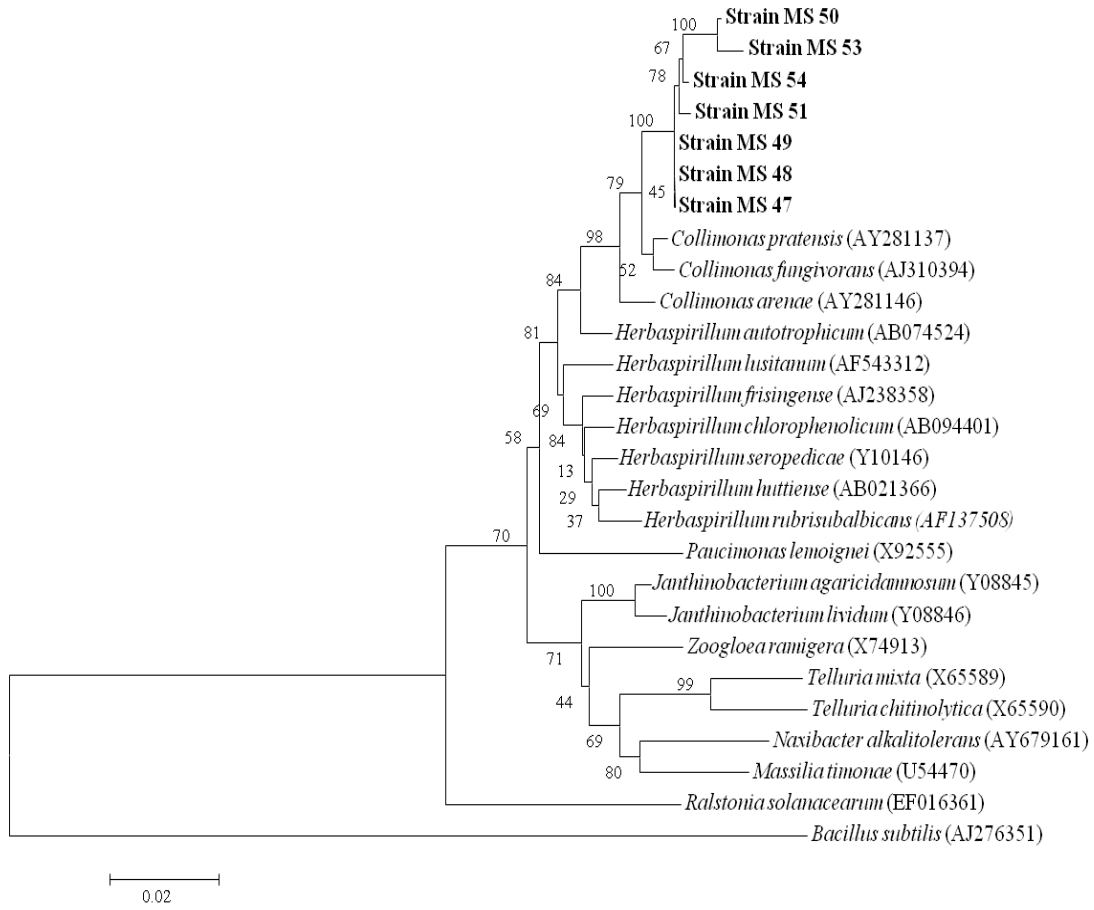


Figure II.5. Neighbour-joining phylogenetic tree of the genus *Collimonas* like novel candidate based on 16S rRNA gene sequence.

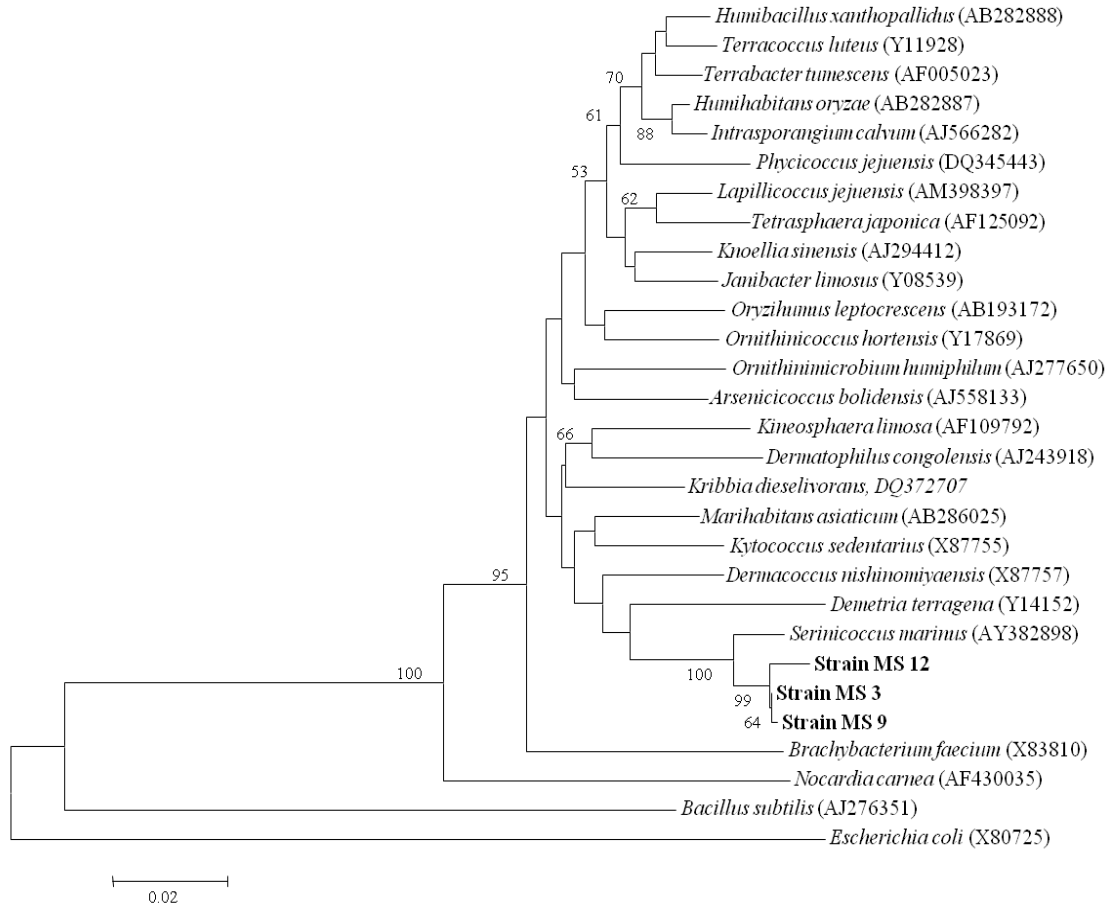


Figure II.6. Neighbour-joining phylogenetic tree of the genus *Serinicoccus* like novel candidate based on 16S rRNA gene sequence.

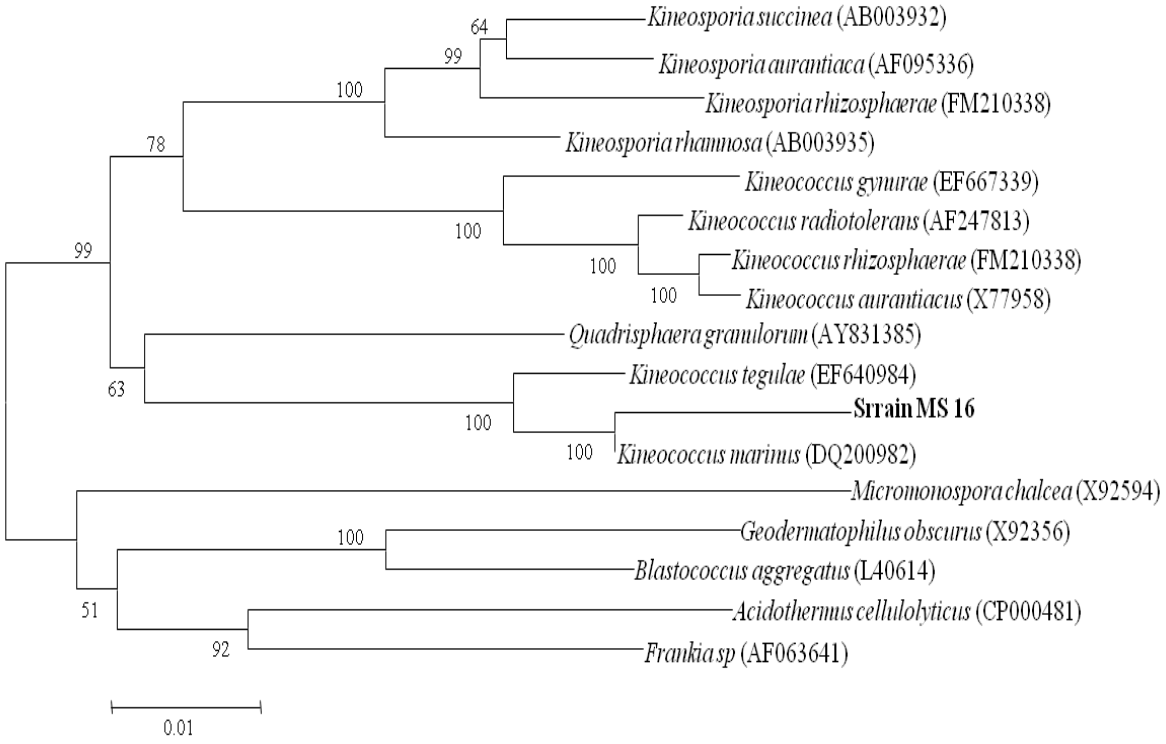


Figure II.7. Neighbour-joining phylogenetic tree of the genus *Kineococcus* like novel candidate based on 16S rRNA gene sequence.

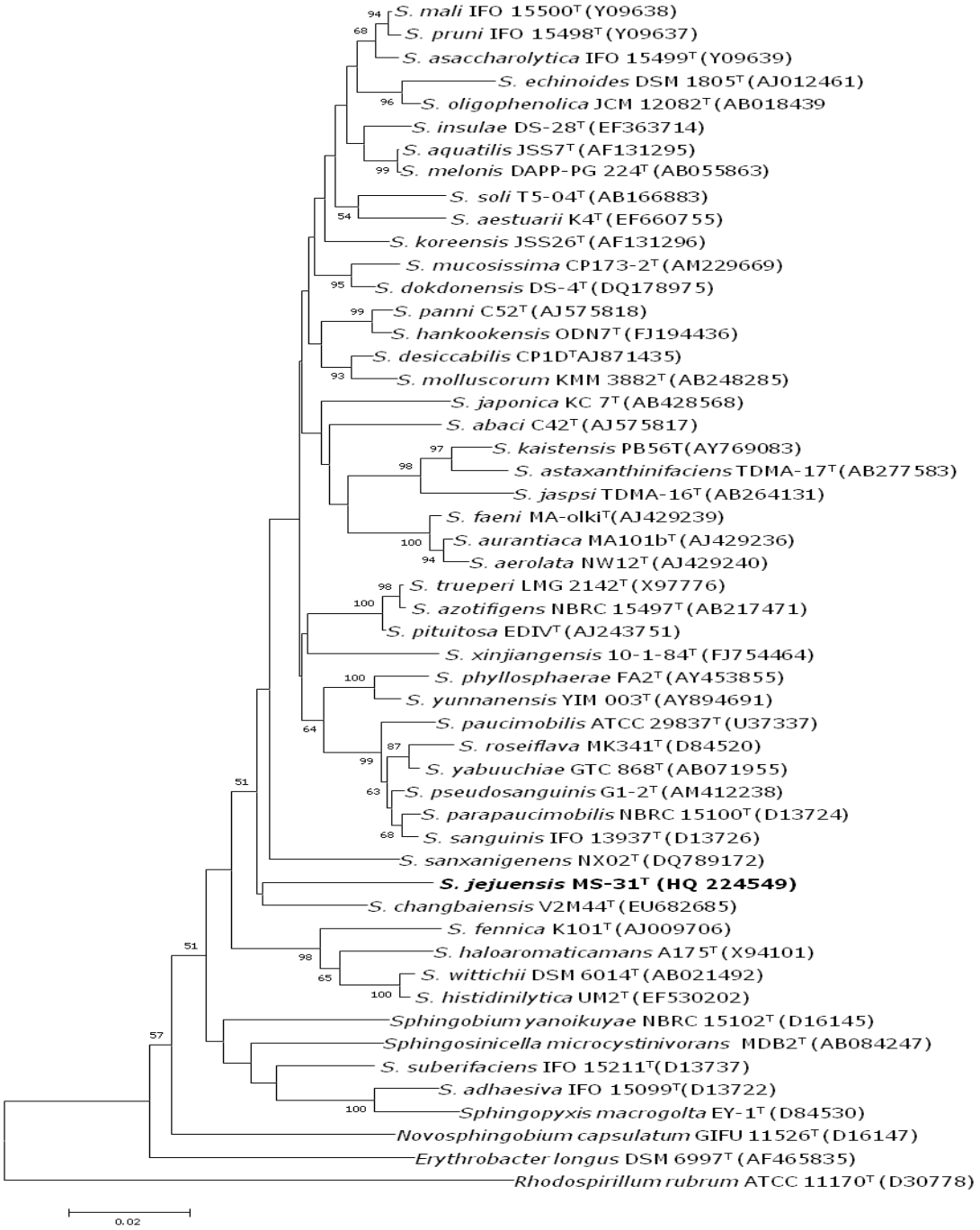


Figure II.8. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence showing the position of MS-31^T among the currently known and related species of the genus *Sphingomonas.* Numbers at nodes are bootstrap percentages derived from 1000 replications. The sequence of *Rhodospirillum rubrum* ATCC 11170^T (D30778) was used as an outgroup. Scale bar, 0.02 substitutions per nucleotide position.

Table II.1. Differential characteristics of strain MS-31^T and related species.

Strains: 1, *S. jejuensis* MS-31^T; 2, *S. koreensis* JSS26^T (Lee *et al*., 2001); 3, *S. dokdonensis* DS-4^T (Yoon *et al.*, 2006); 4, *S. changbaiensis* V2M44^T (Zhang *et al* 2010); 5, *S. asaccharolytica* NBRC 15499^T (Takeuchi *et al*., 1995); 6, *S. pituitosa* EDIV^T (Denner *et al*., 2001); 7, *S. molluscorum* KMM 3882^T (Romanenko *et al*., 2007). +, Positive; –, negative; (+), weakly positive; ND, not determined or no data; Y, yellow; LY, light yellow; DY, dark yellow; OR, orange.

Characteristic	1	2	3	4	5	6	7
Colony color	OR	Y	Y	Y	LY	DY	Y
Motility	-	+	+	+	+	+	-
Oxidase activity	-	+	+	+	ND	-	+
Nitrate reduction	+	-	-	-	-	-	+
Indole production	+	-	-	-	-	-	-
Gelatin hydrolysis	+	-	+	+	-	-	-
Aesculin hydrolysis	+	-	+	+	+	+	+
β-Galactosidase	+	+	-	-	+	+	+
Assimilation of:							
N-Acetyl-D-glucosamine	-	+	-	-	+	+	+
Adipate	-	-	+	+	-	-	-
L-Arabinose	+	-	-	-	(+)	+	+
Citrate	-	-	+	-	-	-	-
Gluconate	-	-	-	+	-	-	+
D-Glucose	+	+	+	(+)	(+)	+	+
Malate	-	+	-	(+)	-	+	-
Maltose	-	+	-	-	+	+	+
D-Mannose	-	-	+	-	(+)	+	+
DNA G+C content (mol%)	69.4	66	66.9	65.8	64.8	64.5	68.3

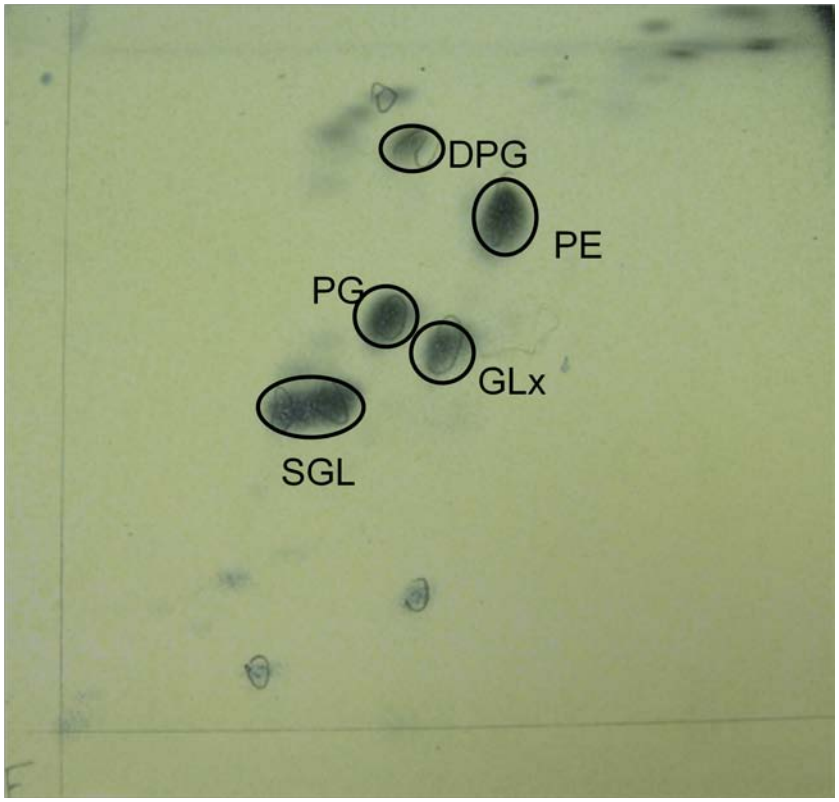
Table II.2. Cellular fatty acid content (%) of the MS-31^T and related species.

Strains: 1, *S. jejuensis* MS-31^T; 2, *S. koreensis* JSS26^T (Lee *et al*., 2001); 3, *S. dokdonensis* DS-4^T (Yoon *et al*., 2006); 4, *S. changbaiensis* V2M44^T (Zhang *et al*., 2010); 5, *S. asaccharolytica* NBRC 15499^T (Takeuchi *et al*., 1995); 6, *S. pituitosa* EDIV^T (Denner *et al*., 2001); 7, *S. molluscorum* KMM 3882^T (Romanenko *et al*., 2007).

Fatty acid	1	2	3	4	5	6	7
Saturated							
$C_{14:0}$	1.1	1.9	3.0	-	1.6–1.9	-	tr
$C_{15:0}$	-	tr		2.3	-	-	tr
$C_{16:0}$	16.3	13.0	10.0	5.3	12.0–15.8	19.0	13.4
$C_{17:0}$	-	-		3.8	-	-	tr
$C_{18:0}$	-	tr		tr	-	-	6.1
$C_{19:0}$ cyclo ω8*c*	-	-		-	-	-	1.0
Hydroxy fatty acids							
$C_{14:0}$ 2OH	15.9	9.4	19.0	1.3	8.7–14.1	12.1	4.5
$C_{15:0}$ 2OH	-	tr	-	4.4	-	-	-
$C_{16:0}$ 2OH	-	tr	-	1.8	-	-	-
Unsaturated							
$C_{16:1}$	-	-	6.0	-	-	-	-
$C_{16:1}$ ω5*c*	2.4	1.3		1.4	1.2–2.0	1.3	-
$C_{17:1}$	-	-	1.0	-	-	-	-
$C_{17:1}$ ω6*c*	3.0	1.5	-	38.9	-	-	6.8
$C_{17:1}$ ω8*c*	-	tr	-	5.2	-	-	-
$C_{18:1}$ ω5*c*	-	1.8	-	1.2	-	-	3.7
11 methyl-$C_{18:1}$ ω7*c*	5.6	-	-	2.0	-	6.3	-
Summed features							
3*	11.7	2.1	-	1.0	24.6–26.8	14.8	18.1
7*	39.7	67.0	56.0	31.5	38.5–46.0	45.7	37.8

*Summed feature 3 comprises $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH.
*Summed feature 7 comprises $C_{18:1}$ ω7c, $C_{18:1}$ ω9t and/or $C_{18:1}$ ω12t.

Figure II.9. Polar lipids of strain MS-31^{T} after separation by two-dimensional TLC. SGL, sphingoglycolipid: PG, phosphatidylglycerol: PE, phosphatidylethanolamine: DPG, diphosphatidylglycerol: GLx, unidentified glycolipid.



3.1.2 Description

Description of *Sphingomonas jejuensis* sp. nov.

Sphingomonas jejuensis (je.ju.en'sis. N.L. fem. adj. *jejuensis* pertaining to off Jeju Island in the Republic of Korea, from where the type strain was isolated).

Cells are orange-pigmented, rod shape, Gram-negative and obligately aerobic. Cells are non-motile and non-spore-forming. Cells are approximately 0.5 -0.7 μm wide and 1.5 -2.0 μm long. The temperature range for growth is 15 –37°C, with the optimal temperature growth being 20 -25°C. No growth occurs above 37°C. The pH range for growth is 6.0– 9.0 and the optimum pH range is 6-8. NaCl is not required for growth and can be tolerated up to 2 % (w/v). Cells are oxidase-negative but catalase -positive. Nitrate reduction to nitrite is positive. Alkaline phosphatase, esterase (C4), esterase lipase(C8), leucine arylamidase, trypsin, *α*-chymotrpsin, *α*-glucosidase and *β*-glucosidase are positive, valine arylamidase, cystine arylamidase are weekly positive but lipase (C14), acid phosphatase, naphthol-AS-BI-phosphohydrolase, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *N*-acetyl-*β*-glucosaminidase, *α*-mannosidase and *α*-fucosidase are negative. Agar is not hydrolyzed. Indole production is positive. Esculin is hydrolyzed. In the API 50 CH test system, acids are produced from D-fructose, D-arabinose, aesculin and D-turanose but negative are 2-keto-gluconate, glycerol, ribose, glucose, sucrose, mannitol, galactose, , arbutin, melezitose, glycogen, glucose, *N*-acetyl-glucosamine, ferric citrate, melibiose, mannose, D-tagatose, D-lyxose, 5-keto-gluconate, maltose, D-xylose, L-arabinose, L-xylose, methyl-β-D-xylopyranoside, rhamnose, methyl-α-D-mannnopyranoside, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose, inulin, raffinose, trehalose, gentiobiose, D-fucose, L-fucose, erythritol, adonitol, sorbose, dulcitol, inositol, sorbitol, starch, xylitol, D-

arabitol, L-arabitol, gluconate and 2-keto-gluconate. Major cellular fatty acids are summed feature 7 (comprising $C_{18:1}$ ω7c, $C_{18:1}$ ω9t and/or $C_{18:1}$ ω12t, 39.7 %), $C_{16:0}$ (16.3 %), $C_{14:0}$ 2-OH (15.9 %), summed feature 3 (comprising $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH 11.7%) and 11 methyl-$C_{18:1}$ω7c (5.6 %) The major respiratory quinone is ubiquinone 10. The DNA G+C content of the genomic DNA is 69.4 mol%. The polar lipids profile comprised sphingoglycolipid, phosphatidylglycerol, phosphatidylethanolamine, diphosphatidylglycerol and four of unidentified glycolipids The type strain, MS-31^T (=KCTC 23321^T=NBRC 107775^T), was isolated from marine sponge *Hymenicacidon flavia.*

CHAPTER III.

PHYLOGENETIC STUDIES ON THE NOVEL MARINE BACTERIA IN THE CLASS *GAMMAPROTEOBACTERIA*

CHAPTER III. PHYLOGENETIC STUDIES ON THE NOVEL MARINE BACTERIA IN THE CLASS *GAMMAPROTEOBACTERIA*

1. Introduction.

The class *Gammaproteobacteria* constitutes a very large and diverse group of bacteria that exhibits enormous variety in terms of their phenotype and metabolic capabilities (Woese *et al*., 1985; Stackebrandt *et al*., 1988; Brenner *et al*., 2005; Kersters *et al*., 2006). In the current taxonomic scheme based on 16S rRNA gene sequences, the *Gammaproteobacteria* are recognized as a class within the phylum *Proteobacteria* (Stackebrandt *et al*., 1988; De Ley, 1992; Brenner *et al*., 2005; Kersters *et al*., 2006). In phylogenetic trees, the class *Gammaproteobacteria* shows a close relationship to the class *Betaproteobacteria* and the other three classes of *proteobacteria* (*Alphaproteobacteria, Deltaproteobacteria* and *Epsilonproteobacteria*) are more distantly related (Gupta, 2000; Ludwig & Klenk, 2005; Kersters *et al*., 2006; Gupta & Sneath, 2007). Based on their branching in the 16S rRNA gene trees, the class *Gammaproteobacteria* has been divided into 14 main orders or subgroups: the *Enterobacteriales, Pseudomonadales, Alteromonadales, Vibrionales, Pasteurellales, Chromatiales, Xanthomonadales, Thiotrichales, Legionellales, Methylococcales, Oceanospirillales, Acidithiobacillales, Cardiobacteriales* and *Aeromonadales* (Garrity *et al*., 2005; Brenner *et al*., 2005; Kersters *et al*., 2006). Although gammaproteobacteria are among the most extensively studied bacterial groups, they are presently defined solely on the basis of their clustering and branching pattern in phylogenetic trees (Woese *et al*., 1985). No unique morphological, molecular or biochemical characteristic has been identified that can distinguish members of the class *Gammaproteobacteria* or its main orders from other bacteria. The family *Pseudoalteromonadaceae* currently comprised only two genera, *Pseudoalteromonas* and *Algicola* (Ivanova *et al*., 2004) in the class *Alteromonadaceae*. The

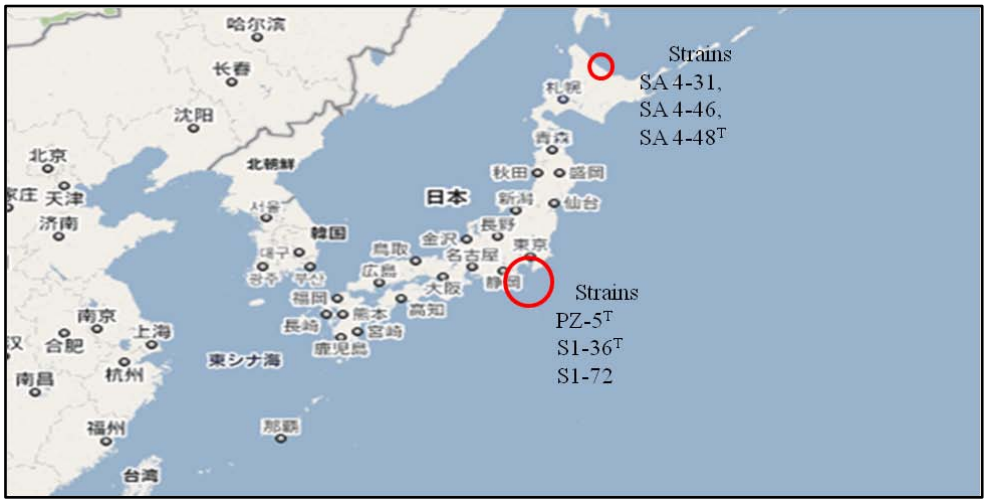
genus *Pseudoalteromonas* (type genus of the family *Pseudoalteromonadaceae*) (Gauthier *et al*., 1995; Ivanova *et al*., 2004) currently comprises 34 recognized species, with *Pseudoalteromonas haloplanktis* as the type species. These bacteria play an important role in marine environments owing to their abundance and high metabolic activities. *Pseudoalteromonas* are highly capable of surviving in nutrient-poor marine environment by adjustment of their biochemical pathways and production of a wide variety of metabolites, including biologically active compounds and enzymes (Ivanova *et al*., 2003).On the other hand, the genus *Algicola* has been proposed to resolve the phylogenetic relationships among the marine *Alteromonas*-like proteobacteria (Ivanova *et al*., 2004) and currently comprised only two species, *Algicola bacteriolytica* and *Algicola sagamiensis.*Type order of the class *Gammaproteobacteria*, the order *Pseudomonadales*, was proposed by Orla-Jensen (1921) and includes two families, *Pseudomonadaceae* and *Moraxellaceae*, and the genus *Dasania* (Garrity *et al.*, 2005: Lee *et al.*, 2007). The family *Pseudomanadaceae* contains the genera *Pseudomonas, Azomonas, Azotobacter, Cellvibrio, Mesophilobacter, Rhizobacter, Rugamonas*, and *Serpens*. The family *Moraxellaceae* includes *Moraxella*, *Acinetobacter, Alkanindiges, Enhydrobacter*, and *Psychrobacter* (Bogan *et al.*, 2003; Garrity *et al.*, 2005). The genus *Dasania* was proposed as a member of the order *Pseudomonadales* (Lee *et al.*, 2007) but affiliation at the family level has not yet been determined.

In the chapter III, 6 novel marine bacterial strains were investigated using polyphasic taxonomic approch in the *Gammaproteobacteria.*

2 Material and methods

2.1. Strains investigated in this study

6 bacterial strains were isolated from Lake Saroma and Western North Pacific Ocean.



Strains	Sources	Sampling place	Date
PZ-5^{T}	Seawater	Sagami Bay at station P (35°00'N, 139°20'E; depth 100m)	July, 2009
SA 4-31	Brackish water	Lake Saroma (44°07’ N, 143°58’E; depth below sea ice)	Feb, 2009
SA 4-46	Brackish water	Lake Saroma (44°07’ N, 143°58’E; depth below sea ice)	Feb, 2009
SA 4-48^{T}	Brackish water	Lake Saroma (44°07’ N, 143°58’E; depth below sea ice)	Feb, 2009
S1-36^{T}	Seawater	Western North Pacific Ocean (30°11' N, 145°05' E; depth, 100m)	Feb, 2010
S1-72 ^{T}	Seawater	Western North Pacific Ocean (30°11' N, 145°05' E; depth, 100m)	Feb, 2010

Investigated strains were provided by Lab of Marine Microbiology, Atmosphere and Ocean Research Institute, The University of Tokyo.

2.2.Composition of media used in this study

2.2.1. Marine agar 2216 medium

Composition

Agar	15 g
$MgCl_2$	8.8 g
Peptone	5 g
Na_2SO_3	3.24 g
$CaCl_2$	1.8 g
Yeast extract	1 g
KCl	0.54 g
$NaHCO_3$	1.6 g
Ferric citrate	1 g
KBr	0.08 g
$SrCl_2$	0.03 g
H_3BO_3	0.02 g
Na_2HPO_4	8 mg
Na_2SiO_3	4 mg
NaF	2.4 mg
NH_4NO_3	1.6 mg
Distilled water	1000 ml

2.2.2. Zobell agar medium

Composition

Agar	15 g
Peptone	5 g
Yeast extract	1 g
Natural seawater	800 ml
Distilled water	200 ml

2.3. Morphology

(Same materials and methods as Chap II.2.3)

2.4. Genotypic characterizations

(Same materials and methods as Chap II.2.4)

2.5. Physiological characterizations

(Same materials and methods as Chap II.2.5)

2.6. Chemotaxonomic analyses

(Same materials and methods as Chap II.2.6)

3 .Results and discussion

3.1. A news species of a novel genus *Oceanicoccus*

3.1.1. Results and discussion

An aerobic bacterium was isolated from seawater samples obtained from Sagami Bay in Japan (35°00' N, 139°20' E; depth, 100 m) during KT-09-11 (2- 6 July 2009) of research ship 'Tansei Maru' (Ocean Research Institute, The University of Tokyo and Japan Agency for Marine-Earth Science and Technology [JAMSTEC]). The seawater samples (200 µl) were applied to 1/10- strength ZoBell agar (agar 15 g, polypeptone 0.5 g, yeast extract 0.1g/L) containing 80% natural seawater and the agar plates were incubated at 15°C for 4 weeks. The bacterial strain PZ-5^T was isolated from these plates and maintained on 1/2- strength marine agar 2216 (MA: Difco) containing 2% NaCl at 15°C. An almost-complete 16S rRNA gene sequence for strain PZ-5^T was determined and a FASTA search in GenBank showed that the strain belongs to the class *Gammaproteobacteria.* Strain PZ-5^T showed 96.3% and 96.2% 16S rRNA gene sequence similarity to marine gammaproteobacterium clone HTCC2143 (GenBank accession no. AY386333) and uncultured gammaproteobacterium clone 20m-45 (GU061297). However, all species with validly published names exhibited <94% sequence similarity to the determined sequence. Strain PZ-5^T displayed the highest 16S rRNA gene sequences similarities to *Dasania marina* KOPRI 20902^T (GenBank accession no. AY771747, 93.9%), *Spongiibacter marinus* HAL40b^T (AM117932, 92.9%), *Melitea salexigens* 5IX/A01/131^T (AY576729, 92.7%), *Haliea salexigens* 3X/A02/235^T (AY576769, 91.8%) and *Congregibacter litoralis* KT71^T (AAOA01000004. 89.4%). It also displayed 87.5% sequence similarity to *Pseudomonas aeruginosa* DSM 50071^T(X06684) (type genus of family *Pseudomonadaceae*). The NJ- and ML-trees based on the 16S rRNA gene sequences revealed that the strain formed a monophyletic clade with *D. marina* KOPRI 20902^T and two

unidentified strains within the class *Gammaproteobacteria* with bootstrap confidence values of 96% in the NJ method and 74% in the ML method (Fig. III.1). Cells of strain PZ-5^T grown on 1/2 MA agar containing 2% NaCl at 15°C for 4 weeks were coccoid and amorphous shaped, approximately 0.5-0.7 μm wide and 0.6-0.8 μm long. Colonies after 4 weeks of incubation on 1/2 marine agar were 1-2 mm in diameter. Cells were motile by means of a flagellum (Fig. III.2). Spores were not formed. Cultural, physiological and biochemical characteristics of PZ-5^T were compared to related genera within the class *Gammaproteobacteria* (Table III.1). No growth was observed under anaerobic conditions in 1/2 MA with added 1% NaCl. Growth occurred only under aerobic conditions between 10-30°C. Optimal temperature and pH for the growth of PZ-5 ^{T} was 15-20°C and pH 6-8, respectively. DNA G+C content of the strain PZ-5^T was 49.8% and the major respiratory quinone was ubiquinone-8.The strain required NaCl for growth and grew in 1-5% NaCl. As shown in Table III. 2, the predominant cellular fatty acids of strain PZ-5^T were $C_{15:0}$ ISO 2OH (19%), $C_{16:1}$ ω7c (17.4%), $C_{17:1}$ ω8c (16.2%), $C_{11:0}$ 3OH (7.5%) and $C_{15:1}$ ω8c (6.5%). The presence of unsaturated fatty acids $C_{15:1}$ ω8c (6.5%), $C_{15:1}$ ω6c (3.8%) and 3-hydroxy fatty acid $C_{11:1}$ 3OH (7.5%), distinguishes strain PZ-5^T from type species of related genera. Strain PZ-5^T showed features clearly different from related genera. Strain PZ-5^T has a coccoid and amorphous shape, while related genera are rod shaped. DNA G+C content of strain PZ-5^T was 49.8 mol% which is much different from the genera *Dasania* (37 mol%), *Melitea* (57 mol%), *Spongiibacter* (57.7- 69.1 mol%), *Haliea* (61.4- 64.8 mol%) and *Congregibacter* (57.8 mol %).In the fatty acids analysis, PZ-5^T showed a different fatty acid profile from related genera. Strain PZ-5^T contains a substantial amount of unsaturated fatty acid $C_{15:1}$ ω8c (6.5%) while related genera only contain a small amount (≤0.7%) or none. Also PZ-5^T showed a different fatty acid profile from related genera. Based on the results of the

phylogenetic analysis and biochemical and physiological properties, the strain PZ-5^T isolated from Sagami Bay in Japan represents a new genus and a novel species of the class *Gammaproteobacteria*, for which the name *Oceanicoccus sagamiensis* gen. nov., sp. nov., is proposed. Although the affiliation of the genus *Oceanicoccus* at the family level remains uncertain, the genus together with genera *Dasania*, *Melitea*, *Spongiibacter, Haliea* and *Congregibacter* may form a family, separate from *Pseudomonadaceae* and *Alteromonadaceae*.

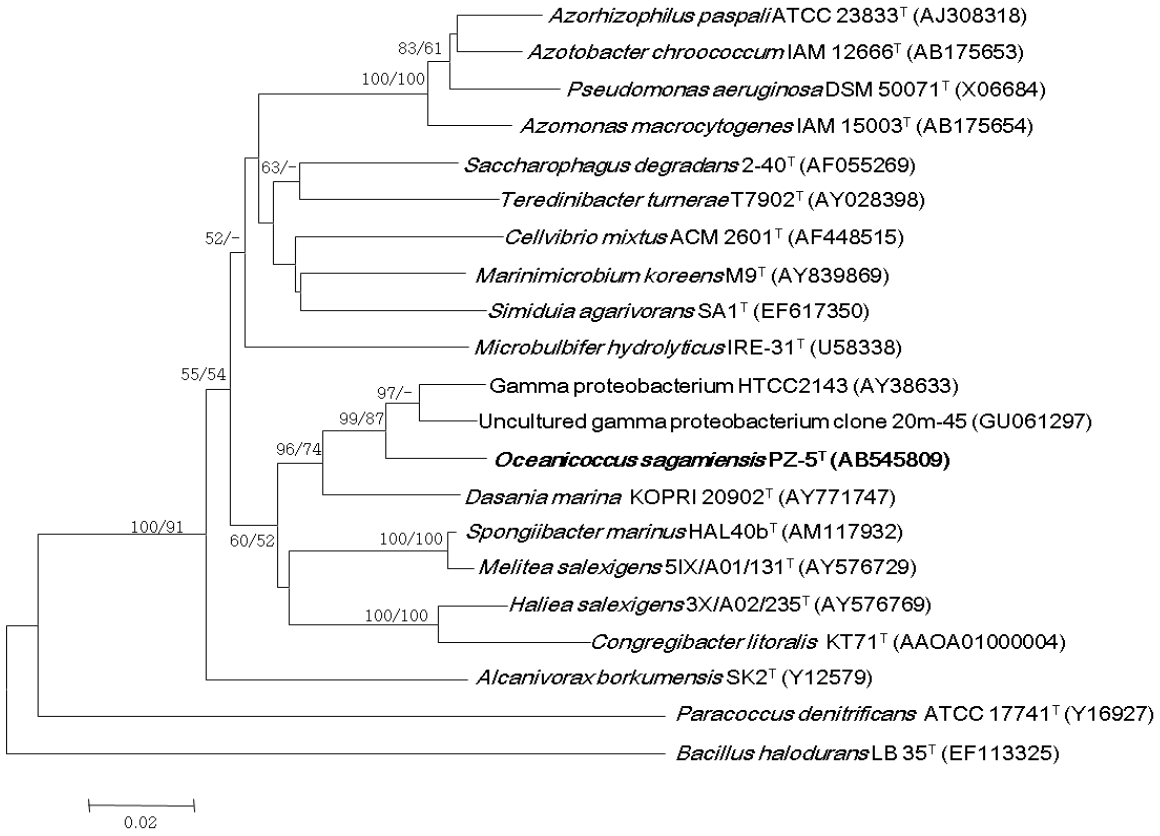


Figure III.1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequence showing the position of PZ-5^T among the currently known and related species of the class *Gammaproteobacteria.* Numbers at nodes are bootstrap percentages derived from 1000 replications (NJ/ML). The sequence of *Bacillus halodurans* LB 35^T (EF113325) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position. "-" indicates recovered nodes with <50% bootstrap values in the ML tree.

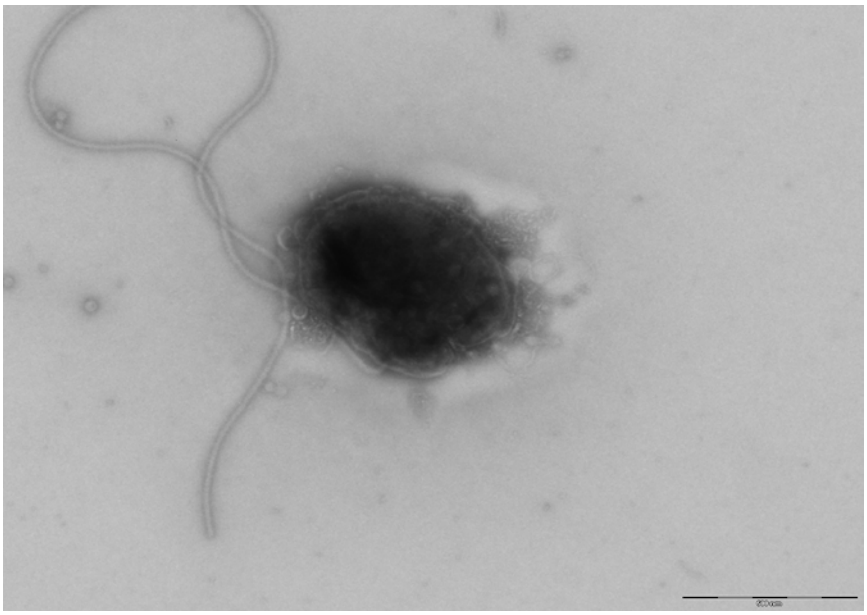


Figure III.2. Transmission electron micrograph of strain PZ-5^{T}, showing coccoid and amorphous -shaped cells with a flagellum. Bar, 500 nm.

Table III.1. Differential characteristics of strain PZ-5^T and related genera

Strain: 1. Strain PZ-5^T; 2. *Dasania* (data from Lee *et al.*, 2007); 3. *Melitea* (Urios *et al.*, 2008); 4. *Spongiibacter* (Graeber *et al.*, 2008: Hwang *et al.*, 2009); 5. *Haliea* (Urios *et al.*, 2008b: Urios *et al.*, 2009: Lucena *et al.*, 2010); 6. *Congregibacter* (Spring *et al.*, 2009). ND, No data available, +/- means variable.

	1	2	3	4	5	6
Shape	Coccus/ Amorphous	Irregular rod	Rod	Rod	Rod	Pleomorphic
Temperature for growth (°C)	10-30	4-30	15-37	10-40	10-44	9-33
Opt temperature (°C)	15-20	17-22	30	20-35	25-30	28
NaCl (%)	1-5	1-9	0.7-7	1-9	0.4-15	1-7
Catalase	-	+	+	+	+	+
Esterase	+	-	-	+	-	ND
N-Acetyl-β-glucosaminidase	-	-	-	+/-	+/-	ND
Utilization of ;						
Arabinose	+	-	-	+/-	-	ND
Glucose	+	-	+	ND	+/-	-
DNA G+C content (mol%)	49.8	37	57	57.7-69.1	61.4-64.8	57.8

Table III.2. Cellular fatty acid content (%) of the PZ-5^T and related genus type species.

Strain: 1. Strain PZ-5^T; 2, *Dasania marina* KOPRI 20902^T (Data from Lee *et al*., 2007); 3. *Melitea salexigens* 51X/A01/131^T (Urios *et al.,* 2008); 4. *Spongiibacter marinus* HAL40b^T (Graeber *et al.,* 2008); 5. *Haliea salexigens* 3X/A02/235^T (Urios *et al.,* 2008b); 6. *Congregibacter litoralis* KT71^T (Spring *et al.,* 2009). "–"= Not detected

	1	2	3	4	5	6
Saturated						
$C_{10:0}$	2.1	1.4	-	-	-	0.5
$C_{14:0}$	0.8	5.9	0.8	0.3	1.3	2.0
$C_{16:0}$	1.9	18.4	3.9	2.4	2.0	5.4
$C_{17:0}$	-	-	13.0	9.6	9.3	3.1
$C_{18:0}$	0.7	1.8.	0.5	0.4	-	0.6
Unsaturated						
$C_{15:1}$ ω6c	3.8	-	-	0.4	5.8	2.0
$C_{15:1}$ ω8c	6.5	-	-	0.7	-	0.3
$C_{16:1}$ ω7c	17.4	-	-	-	21.2	23.1
$C_{17:1}$ ω8c	16.2	-	34.1	51.7	23.9	8.1
$C_{18:1}$ ω7c	1.4	4.1	11.4	7.8	17.5	29.4
Hydroxy						
$C_{10:0}$ 3-OH	4.0	10.4	1.7	1.3	1.8	2.1
$C_{11:0}$ 3-OH	7.5	-	4.6	4.1	3.3	0.5
$C_{15:0}$ ISO 2OH	19.0	-	-	-	-	-
Summed feature 3	-	45.3	9.0	5.9	-	-

*Summed feature 3 comprises $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH.

3.1.2. Description

Description of *Oceanicoccus* gen. nov.

Oceanicoccus (O.ce.a.ni.coc'cus. L. masc. n. *oceanus* the great sea, outer sea, ocean; N.L. masc. n. *coccus* from Gr. n. *kokkos* a berry; unit; N.L. masc. n**.** *Oceanicoccus,* coccus from sea).

Cells are non-pigmented, coccoid and ellipsoid shape, Gram-negative and obligately aerobic. Cells are motile with a single flagellum and are non-spore-forming. The major respiratory quinone is ubiquinone 8. The DNA G+C content of the genomic DNA is 49.8 mol%. Predominant cellular fatty acids are $C_{15:0}$ ISO 2OH (19%), $C_{16:1}$ ω7c (17.4%), $C_{17:1}$ ω8c (16.2%) and $C_{11:0}$ 3OH (7.5%). The type species is *Oceanicoccus sagamiensis.*

Description of *Oceanicoccus sagamiensis* sp. nov.

Oceanicoccus sagamiensis (sa.ga.mi.en'sis. N.L. adj. *sagamiensis* referring to Sagami Bay, the site of isolation).

It exhibits the following properties in addition to those given in the genus description. Cells are approximately 0.5-0.7 μm wide and 0.6-0.8 μm long. Temperature range for growth is 10–30°C, with optimal temperature growth of 15-20°C. No growth occurs above 30°C. The pH range for growth is 5.0 –9.0 and with the optimum being 6-8. NaCl is required for growth and can be tolerated up to 5 % (w/v). Cells are catalase-negative but oxidase-positive. Nitrate reduction to nitrite is positive. Alkaline phosphatase, esterase (C4), esterase lipase(C8), acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive, leucine arylamidase and trypsin are weakly positive but lipase (C14), valine arylamidase, *N*-acetyl-*β*-

glucosaminidase, α-galactosidase, β-galactosidase, α-glucosidase, cystine arylamidase, chymotrypsin, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase are negative. Agar is not hydrolyzed. Acid is produced from glycerol, ribose, glucose, sucrose, mannitol, esculin and melezitose but not from galactose, fructose, arbutin, glycogen, *N*-acetyl-glucosamine, ferric citrate, melibiose, mannose, D-turanose, 5-keto-gluconate, D-arabinose, maltose, D-tagatose, D-xylose, L-arabinose, L-xylose, methyl-β-D-xylopyranoside, rhamnose, methyl-α-D-mannnopyranoside, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose, inulin, raffinose, trehalose, gentiobiose, D-fucose, L-fucose, erythritol, adonitol, sorbose, dulcitol, inositol, sorbitol, starch, xylitol, D-arabitol, L-arabitol, gluconate and 2-keto-gluconate. Indole production is positive. Esculin is hydrolyzed. Major cellular fatty acids are $C_{15:0}$ ISO 2OH, $C_{16:1}$ ω7c, $C_{17:1}$ ω8c, $C_{11:0}$ 3OH, $C_{17:1}$ ω8c, $C_{11:1}$ 3OH and $C_{15:1}$ ω8c. The DNA G+C content of the type strain is 49.8 mol%. The type strain, PZ-5^T (=NBRC 107125^T =KCTC 23278^T), was isolated from Sagami Bay in Japan (35°00' N, 139°20' E; depth 100 m).

3.2. A news species of a novel genus *Psychrosphaera*

3.2.1 Results and discussion

Three novel aerobic bacteria were isolated from brackish water samples just below ice. The samples were collected from Lake Saroma in Hokkaido, Japan on 22 February 2009 (44°07' N, 143°58' E; temperature, -1.5 °C; salinity, 34.0 PSU; surface brackish water below sea ice). Lake Saroma is a brackish lake and surface layer is iced over in winter season. The seawater sample (100 µl) was inoculated on 1/20 strength ZoBell agar containing 80% seawater and incubated at 4°C for 30 days. Isolated strains were maintained on 1/2 Marine agar 2216 (MA: Difco) containing 2% NaCl under 15°C. The phylogenetic tree based on neighbour-joining-(NJ) and maximum-parsimony-(MP) generated comparisons of the 16S rRNA gene sequences revealed that the strains studied formed a monophyletic clade within the family *Pseudoalteromonadaceae* with bootstrap confidence values of 99% in the NJ method (Fig. III.3) and 96% in the MP method (data not shown). Strains SA4-30, SA4-46 and SA4-48^T formed a single cluster with sequence similarities ranging from 99.7% to 100 % among the strains (Fig. III.3) and showed 89.5-93.2% similarities with strains in the family *Pseudoalteromonadaceae* with validly published names. They showed 92.9%, 92.9% and 92.7% similarity with *Pseudoalteromonas haloplanktis* (type species of the genus) and 89.5%, 89.5% and 89.8% similarity with *Algicola bacteriolytica*, respectively. Most closely related species in the family *Pseudoalteromonadaceae*, were *Pseudoalteromonas atlantica* (93.2%, 93.2% and 93.1% respectively) and *Pseudoalteromonas espejiana* (93.2%, 93.2% and 93.1%). The DNA hybridization values among strains SA4-31, SA4-46 and SA4-48 ^{T} showed higher than 80% each others (data not shown). This result strongly suggests that strains SA4-31, SA4-46 and SA4-48^T should be classified as representing one species (Wayne *et al*., 1987) The strain SA4-48^T grown on 1/2 MA agar containing 2% NaCl at 15°C for 14 days were

coccoid- or ellipsoidal-shaped, approximately 0.5- 0.7 μm wide and 0.75- 1 μm long. Cells were motile by means of a polar flagellum. Spores were not observed under the microscope (Fig. III.4). Cultural, physiological and biochemical characteristics of SA4-31, SA4-46 and SA4-48^T were compared to related genera in the family *Pseudoalteromonadaceae* (Table III.3). No growth was observed under anaerobic conditions in 1/2 MA containing 2% NaCl. Growth occurred only under aerobic conditions at between 4-30 °C. Optimal temperature and pH for strain SA4-31, SA4-46 and SA4-48^T growth were 15-20°C and pH 6-9, respectively. The strains required NaCl for growth and grew in 1- 5%. As shown in Table III.4, the predominant cellular fatty acids of strains SA4-31, SA4-46 and SA4-48^T were $C_{16:0}$ (19.4%, 19.8% and 21% respectively), $C_{18:1}$ ω7c (25.3%, 20.9% and 20.4%) and $C_{16:1}$ ω7c (16.76%, 16.4% and 18.8%). They contained $C_{12:1}$ 3OH (4.52%, 5.71% and 6.2%) as 3-hydroxy fatty acid which distinguishes them from cultivated members of the family *Pseudoalteromonadaceae*. Also, based on the detailed profiles of fatty acids, these strains could be separated into an independent group. Based on the results of the phylogenetic analysis and their biochemical and physiological properties, these novel three strains, SA4-31, SA4-46 and SA4-48^T, isolated from Lake Saroma in Hokkaido, Japan, represent a novel genus and a novel species within the family *Pseudoalteromonadaceae*, for which the name *Psychrosphaera saromensis* gen. nov., sp. nov., is proposed.

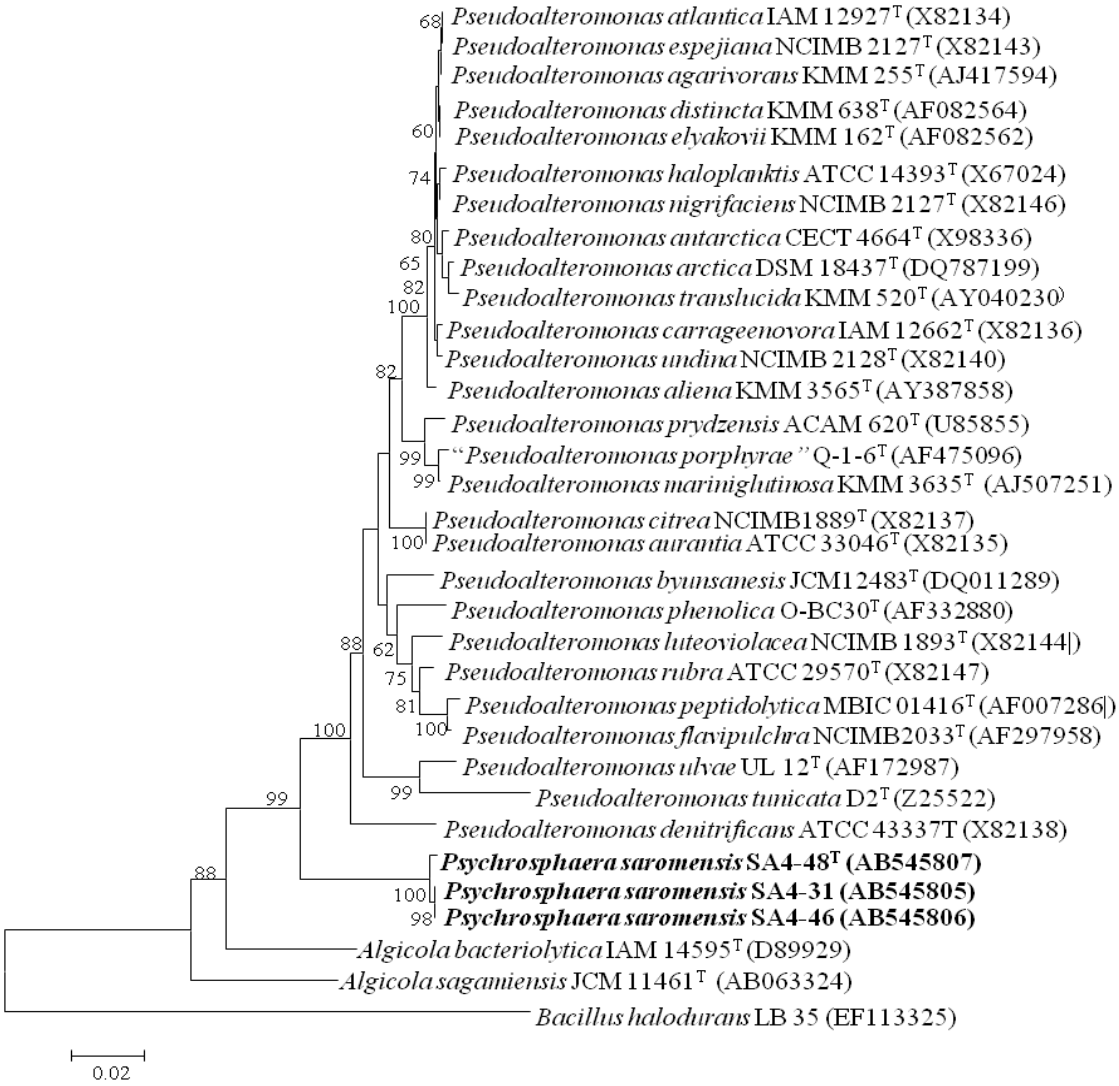


Figure III.3. Neighbour-joining(NJ) phylogenetic tree based on 16S rRNA gene sequence showing the positions of strains SA4-31, SA4-46 and SA4-48^T among the currently known and cultivated species of the family *Pseudoalteromonadaceae*. Numbers at nodes are bootstrap percentages derived from 1000 replications (frequencies less than 50% are not shown). The sequence of *Bacillus halodurans* LB 35 (EF113325) was used as an outgroup. Bar, 2% sequence divergence.

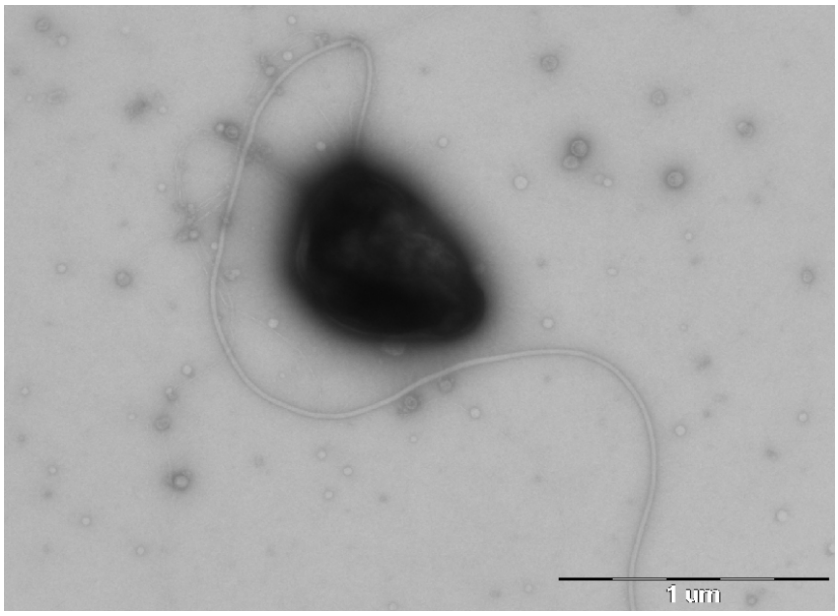


Figure III.4. Transmission electron micrograph of negatively stained strain SA4-48^{T}, showing ellipsoidal shape with a polar flagellum.

Table III.3. Comparative characteristics of strains SA 4-31, SA 4-46, SA 4-48^T and related members of the family *Pseudoalteromonadaceae*

+, Positive; -, negative; +/-, variable reaction; V, reaction varies between strains.

*, data from Ivanova, *et al.* (2004)

Characteristic	**SA -31**	**SA-46**	**SA-48^T**	***Pseudoalteromonas****	***Algicola****
Morphology	Coccoid/ ellipsoid	Coccoid/ ellipsoid	Coccoid/ ellipsoid	rod shape	rod shape
Pigmentation	–	–	–	+/-	+
Flagellation:					
Single polar	+	+	+	+	+
Bipolar	–	–	–	+/-	–
Halotolerance (% NaCl)	5	5	5	15	6
Growth at:					
4°C	+	+	+	+	–
37°C	–	–	–	+	–
42°C	–	–	–	–	–
Temperature range for growth	4-30	4-30	4-30	4-37	15-30
Hydrolysis of:					
chitin	–	–	–	V	–
agar	–	–	–	V	–
gelatin	–	–	+	+	+
Utilization of:					
D-glucose	–	+	+	+	+
D-fructose	–	–	–	V	V
D-mannose	–	–	–	V	V
L-rhamnose	–	–	–	V	ND
cellobiose	+	+	+	V	–
lactose	–	–	–	V	V
glycerol	–	–	+	V	–
DNA G+C content (mol%)	38.9	39.6	38.7	37-50	44-46
Quinone	Q-8	Q-8	Q-8	Q-8	Q-8

Table III.4. Comparative fatty acids content (%) of the strains SA 4-31, SA 4-46 an SA 4-48^T and related strains belonging to the family *Pseudoalteromonadaceae*

Strains: 1, SA 4-31; 2, SA 4-46; 3, SA 4-48^T; 4, *A. bacteriolytica* IAM14595^T ; 5, *A. sagamiensis* B-10-31^T; 6, *P. haloplanktis* CECT 4188^T (Khudary *et al*., 2008); 7 *P. arctica* A 37-1-2^T (Khudary *et al*., 2008); 8, *P. tetraodonis* IAM 14160^T (Ivanova *et al*., 2001); 9, *P. atlantica* IAM 14164 (Bozal *et al*., 1997): 10, *P.lipolytica* LMEB 39^T (Xue-Wei *et al*., 2009); 11, *P. byunsanensis* JCM 12483^T (Xue-Wei *et al*., 2009); 12, *P. undina* DSM 6065^T (Xue-Wei *et al*., 2009) -, Not detected.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
straight-chain												
$C_{12:0}$	0.4	0.4	0.3	2.3	2.5	2	2.4	1.5	2.5	2.5	0.9	3.4
$C_{14:0}$	0.8	0.9	0.9	2.2	1.2	2	1.1	2	0.7	0.8	0.6	2
$C_{15:0}$	3.0	4.0	4.9	0.1	0.6	3.3	2.2	8	2.7	2.4	1.2	1.7
$C_{16:0}$	19.4	19.8	21.0	12	8.9	30.1	12.7	18.2	19.4	15.3	23.9	14.5
$C_{17:0}$	4.2	5.2	5.2	-	0.4	3.9	1.5	5.5	9.6	5	3.1	1.5
unsaturated												
$C_{15:1}$ ω8c	4.5	5.7	6.2	-	0.3	2.3	1.8	6.3	-	0.8	0.2	1.5
$C_{16:1}$ ω7c	16.8	16.4	18.8	-	-	40.5	40.2	35	22	-	-	-
$C_{17:1}$ ω8c	2.9	3.1	3.1	0.1	1	6	5.5	10.7	8.2	9.3	3.4	4
$C_{18:1}$ ω7c	25.3	20.9	20.4	4.2	11.5	2.4	7.8	2.5	-	12.1	16.4	5.6
hydroxy												
$C_{12:0}$ 3OH	-	-	-	21	4.8	1.9	6.4	0.9	3.8	9.8	5	8
$C_{12:0}$ ISO 3OH	-	-	-	-	0.5	-	-	-	-	2.1	0.2	2
$C_{12:1}$ 3OH	5.8	5.5	6.4	0.3	0.2	-	-	-	-	-	0.3	0.2
Summed feature 3	-	-	-	32.5	15.46	-	-	-	-	23.9	27.5	42.3

Summed feature 3 comprises $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH.

3.2.2. Description

Description of *Psychrosphaera* gen. nov.

Psychrosphaera (Psy.chro.sphae'ra. Gr. adj. *psychros* cold; M.L. fem. n. *sphaera* sphere; N.L. fem. n. *Psychrosphaera* a cold sphere).

Cells are coccoid or ellipsoid shape, Gram-negative and obligately aerobic. Cells are motile. Non-spore-forming. The major respiratory quinone is isoprenoid quinone 8. The G+C content of the genomic DNA is 38.7-39.6 mol%. Predominant cellular fatty acids are $C_{16:0}$, $C_{16:1}$ ω7c**,** $C_{18:1}$ ω7c and $C_{12:1}$ 3OH. The type species is *Psychrosphaera saromensis.*

Description of *Psychrosphaera saromensis* sp. nov.

Psychrosphaera saromensis (sa.ro.men'sis N. L. fem. adj. *saro-mensis*, pertaining to Lake Saroma, where organisms were collected).

Exhibits the following properties in addition to those given in the genus description. Cells are not pigmented and approximately 0.5-0.7 μm wide and 0.75-1 μm long. Cells were motile by means of a polar flagellum and are non-spore-forming. Temperature range for growth is 4–30°C. Optimal temperature for growth is 15-20°C. No growth occurs at above 37°C. pH range for growth is 6.0–9.0. NaCl is required for growth and can be tolerated up to 5% (w/v). Catalase- negative but oxidase- positive. Alkaline phosphatase, esterase (C4), esterase lipase(C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive, but *N*-acetyl-*β*-glucosaminidase, *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, cystine arylamidase, chymotrypsin, *β*-glucuronidase, *β*-glucosidase, *α*-mannosidase and *α*-fucosidase are negative. Gelatin is hydrolyzed. Acid is produced from glycerol, L-arabinose, β-methyl-α-D-mannnopyranoside, D-xylose, galactose,

glucose, fructose, mannose, amygdalin, esculin, salicin, cellobiose, maltose and gentiobiose but not from ribose, glucose, *N*-acetyl-glucosamine, ferric citrate, melibiose, sucrose, trehalose, D-tagatose , D-turanose, D-lyxose, and 5-keto-gluconate, D-arabinose, D-tagatose, starch, xylitol, L-xylose, methyl-β-D-xylopyranoside, rhamnose, methyl-α-D-glucopyranoside, arbutin, lactose, melezitose, inulin, raffinose, D-fucose, L-fucose, erythritol, adonitol, sorbose, glycogen, dulcitol, inositol, mannitol, sorbitol, starch, xylitol, D-arabitol, L-arabitol, gluconate and 2-keto-gluconate. Major fatty acid components (>5.0 %) include $C_{16:0}$, $C_{16:1}$ ω7c, $C_{18:1}$ ω7c, $C_{12:1}$ 3OH and $C_{15:1}$ ω8c. The DNA G+C content of the type strain is 38.7 mol%. The type strain, SA4-48^{T} (=NBRC 107123^{T}= KCTC 23240^{T}), was isolated from Lake Saroma in Hokkaido, Japan.

3.3. Two new species of a novel genus *Halicoccus* in the family *Dasaniaceae* fam. nov.

3.3.1. Results and discussion

These aerobic bacteria were isolated from the seawater samples collected from the western North Pacific Ocean (30°11' N, 145°05' E; depth, 100m) during the R/V Mirai on Feb. 9, 2010 (MR10-01 cruise). The seawater (200 μl) sample was inoculated on medium 1/10 strength Zobell agar (0.5 g peptone, 0.1 g yeast extract, 15 g agar in 1 L of 80% aged natural seawater) and incubated at 20°C for 30 days. Cells of strains S1-36^T and S1-72^T grown on MA at 25°C for 3 weeks were coccoid- shaped, approximately 0.4-0.6 μm diameter. Flagellum and spores were not observed microscopically (Fig. III.5). No growth was observed under anaerobic conditions in MA. Growth occurred only under aerobic conditions between 15-30°C. Optimal temperature and pH range were 20- 25°C and 6-9, respectively. The strains required NaCl for growth and grew in 1- 4% NaCl (Table III.5). The major cellular fatty acids of strain S1-36^T and S1-72^T were summed feature 3 (comprising $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH; 24.6%, 25.4% respectively), $C_{18:1}$ ω7c (18.9%, 16.6%) and $C_{17:1}$ ω8c (9%, 11.1%). Present of two saturated fatty acids, $C_{10:0}$ and $C_{11:0}$ approximately 12% of the total fatty acids which distinguishes S1-36^T and S1-72^T from related genera of the class *Gammaproteobacteria* (Table III.6). Cultural, physiological and biochemical characteristics of strain S1-36^T and S1-72^T were compared with related genera *Spongiibacter* (Graeber *et al.*, 2008), *Melitea* (Urios *et al.*, 2008), *Dasania* (Lee *et al*., 2007), "*Oceanicoccus*" (Park *et al*., 2011), *Haliea* (Urios *et al.*, 2008), and *Congregibacter* (Spring *et al.*, 2009) in the class *Gammaproteobacteria* (Table III.5). An almost complete 16S rRNA gene sequence for strain S1-36^T and S1-72^T were determined and a FASTA search in the GenBank showed that the strain belongs to the Class *gammaproteobacteria.* The strain S1-36^T showed 96.1% sequence similarity with strain S1-72^T and displayed sequence similarity to uncultured bacterium clone

S1-29 (GenBank accession no. FJ545459, 95.8%), uncultured bacterium clone SHFG (FJ203223, 95.4%), uncultured bacterium clone JSSS04 (EU707305, 95.4%), uncultured gammaproteobacterium clone (DQ351790, 95.4%), uncultured gammaproteobacterium clone (GU230345, 95.4%), uncultured gammaproteobacterium clone b29 (GQ452899, 95.3%). However, all species with validly published names exhibited <93.4% sequence similarity to the determined sequence. Strain S1-36^T and S1-72^T showed 16S rRNA gene sequences similarities to *Haliea salexigens* 3X/A02/235^T (GenBank accession no. AY576769, 93.3%, 94.7% similarity), *Congregibacter litoralis* KT71^T (AAOA01000004, 92.3%, 93.8), *Dasania marina* KOPRI 20902^T (AY771747, 91.7%, 91.6%), *"Oceanicoccus sagamiensis"* PZ-5^T (AB545809, 91.4%, 92.5%), *S. marinus* HAL40b^T (AM117932, 91.4%, 91.5%) and *Melitea salexigens* 51XA01/131^T (AY576729, 90.7%, 91.4%). Phylogenetic trees obtained by the neighbour-joining (NJ) method (Fig. III.6) and maximum-likelihood (ML) methods revealed clear affiliations of the novel isolates S1-36^T and S1-72^T to the cluster of *H. salexigens* 3X/A02/235^T, *H. mediterranea* 7SM29^T. *H. rubra* CM41_15a^T *C. litoralis* KT71^T and six uncultured clones. Also, it formed another cluster with *D. marina* KOPRI 20902^T, *"O. sagamiensis"* PZ-5^T, *S. marinus* HAL40b^T and *M. salexigens* 51XA01/131^T 85% (NJ) and 97% (ML) bootstrap confidence values. To confirm in the family level, representatives in several family or closed relative genera were chosen in the Gammaproteobacteria and phylogenetic tree was reconstructed by NJ and ML methods (Fig III.7). Strain S1-36^T and S1-72^T formed a cluster with *H. salexigens* 3X/A02/235^T, *C. litoralis* KT71^T, *D. marina* KOPRI 20902^T, *"O. sagamiensis* PZ-5^T*" S. marinus* HAL40b^T and *M. salexigens* 51XA01/131^T 84% (NJ) and 95% (ML) bootstrap confidence values. Although the affiliation of this cluster at order level is unclear but showed 100% bootstrap confidence of NJ and ML methods in class *Gammaproteobacteria* (Fig III.7).

Physiologic and chemotaxonomic differences among strain S1- 36^T, S1-72^T and related genera are shown in Table III.5 and 6. The data presented showed that strain S1- 36^T, S1-72^T are sufficiently different from related genera and represents a novel genus as *Halicoccus marinus* gen. nov., sp. nov. and *Halicoccus pacifica* sp. nov., in the family *Dasaniaceae* fam .nov. belong to the class *Gammaproteobacteria*. In the family *Dasaniaceae*, genera *Spongiibacter*, *Haliea*, *Melitea*, *Dasania*, *Congregibacter*, *Halicoccus* and "*Oceanicoccus*" are included.

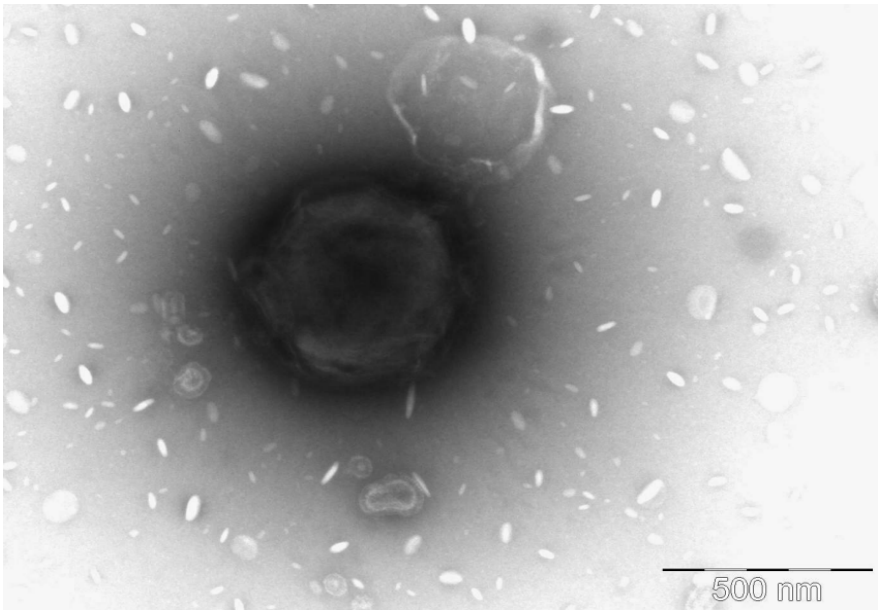


Figure III.5. Transmission electron micrograph of strain S1-36^{T}, showing coccoid cells.

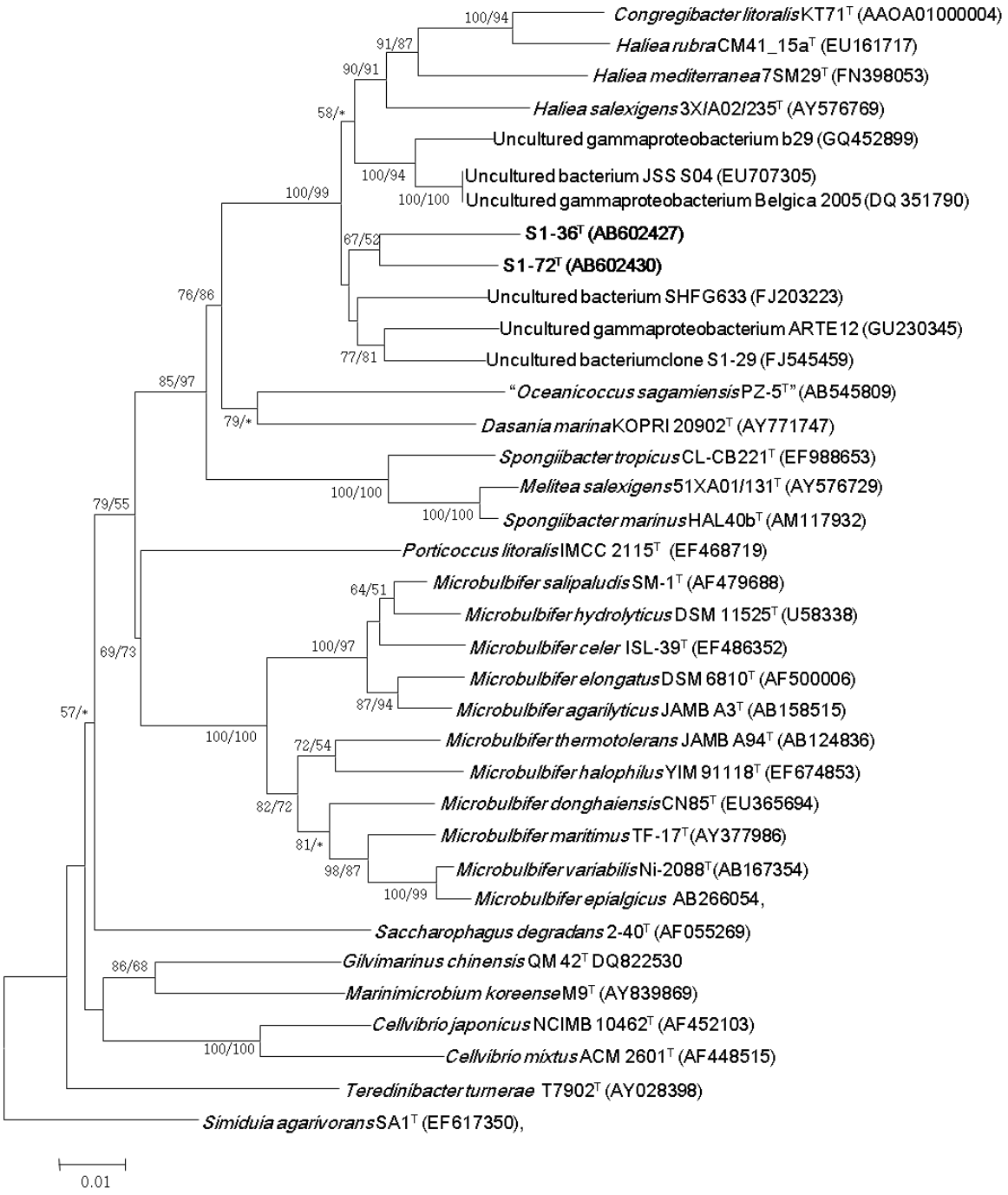


Figure III.6. Neighbour-joining (NJ) phylogenetic tree based on the 16S rRNA gene sequence showing the position of strain S1-36^T among the currently known and related species of the class *Gammaproteobacteria*. Numbers at nodes are bootstrap percentages derived from 1000 replications (NJ/ML). Sequences of *Simiduia agarivorans* SA1^T (EF617350) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position. " * " indicates recovered nodes with <50% bootstrap values in the Maximum-likelihood (ML) tree.

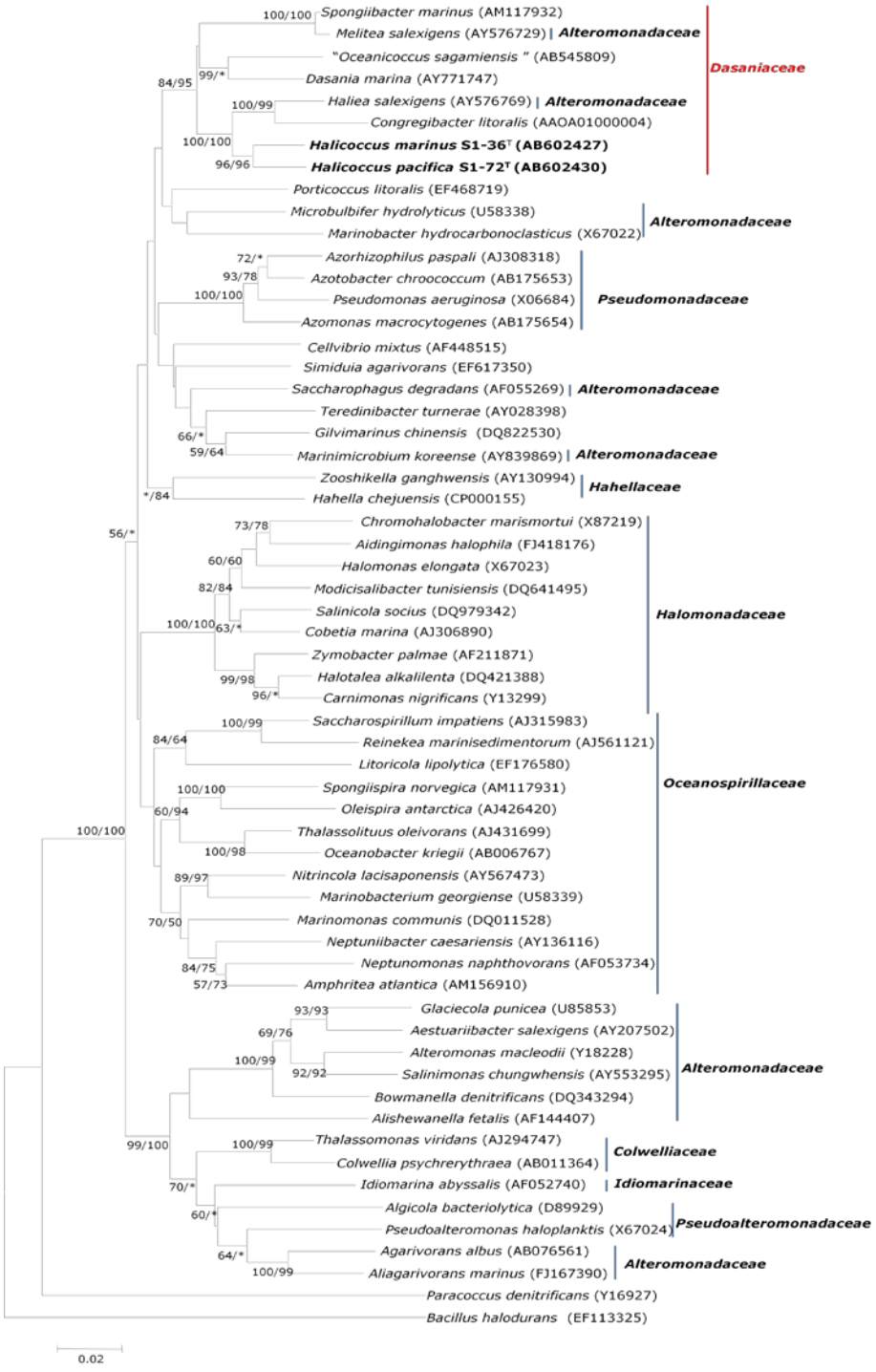


Figure III.7. Neighbour-joining (NJ) phylogenetic tree based on the 16S rRNA gene sequence showing the position of strain S1-36^T among the currently known and related species of the class *Gammaproteobacteria*. Numbers at nodes are bootstrap percentages derived from 1000 replications (NJ/ML). Sequences of *Bacillus halodurans* (EF113325) was used as an outgroup. Bar, 0.02 substitutions per nucleotide position. " * " indicates recovered nodes with <50% bootstrap values in the Maximum-likelihood (ML) tree.

Table III.6. Differential characteristics of strain S1-36^T, S1-72^T and related genera in the class *Gammaproteobacteria.*

1. Stain S1-36^T; 2. Strain S1-72^T; 3. *Haliea* (Data from Urios *et al.*, 2008; Urios *et al.*, 2009: Lucena *et al.*, 2010); 4. *Congregibacter litoralis* KT71^T (Spring *et al.*, 2009); 5. *Dasania marina* KOPRI 20902^T (Lee *et al*., 2007); 6. "*Oceanicoccus sagamiensis*" PZ-5^T (Park *et al*., 2011); 7. *Spongiibacter* (Graeber *et al.*, 2008: Hwang *et al.*, 2009); 8. *Melitea salexigens* 51X/A01/131^T (Urios *et al.*, 2008). "–"= negative or none. "ND"= No data available.

	1	2	3	4	5	6	7	8
Shape	Coccus	Coccus	Rod	pleomorphic	Irregular rod	Coccus/ amorphous	Rod	Rod
Flagellation	-	-	+	+	+	+	+	+
Temperature for growth (°C)	10-30	10-30	10-44	9-33	4-30	10-30	10-40	15-37
Opt temperature (°C)	20-25	20-25	25-30	28	17-22	15-20	20-35	30
NaCl (%)	1-4	1-5	0.4-15	1-7	1-9	1-5	1-9	0.7-7
Catalase	-	-	+	+	+	-	+	+
Esterase	+	+	-	ND	-	+	+	-
N-Acetyl-β-glucosaminidase	+	-	+/-	ND	-	-	+/-	-
Utilization of								
Arabinose	-	-	-	ND	-	+	+/-	-
Glucose	+	+	+/-	-	-	+	ND	+
DNA G+C content (mol%)	59.6	59.5	61.4-64.8	57.8	37	49.8	57.7-69.1	57

Table III.7. Major cellular fatty acid composition of strain S1-36^T, S1-72^T and related genera in the Class *Gammaproteobacteria*

1. Stain S1-36^T; 2. Strain S1-72^T; 3. *Haliea salexigens* 3X/A02/235^T (Data from Urios *et al.*, 2008); 4. *Congregibacter litoralis* KT71^T (Spring *et al.*, 2009); 5. *Dasania marina* KOPRI 20902^T (Lee *et al*., 2007); 6. *"Oceanicoccus sagamiensis"* PZ-5^T (Park *et al*., 2011); 7. *Spongiibacter marinus* HAL40b^T (Graeber *et al.*, 2008); 8. *Melitea salexigens* 51X/A01/131^T (Urios *et al.*, 2008). "–"= negative or none. "ND"= No data available.

	1	2	3	4	5	6	7	8
Saturated								
$C_{10:0}$	5.4	8.3	-	0.5	1.4	2.1	-	-
$C_{11:0}$	6.1	4.3	1.0	1.0	ND	1.7	0.2	0.7
$C_{12:0}$	1.2	-	1.6	2.2	-	0.5	1.4	0.3
$C_{14:0}$	2.1	3.4	1.3	2.0	5.9	0.8	0.3	0.8
$C_{15:0}$	5	4.6	4.5	4.9	-	1.9	4.1	5.3
$C_{16:0}$	4.5	2.2	2.0	5.4	18.4	1.9	2.4	3.9
$C_{17:0}$	5.4	1.8	9.3	3.1	-	-	9.6	13.0
$C_{18:0}$	0.4	0.2	-	0.6	1.8.	0.7	0.4	0.5
Unsaturated								
$C_{15:1}$ ω6c	2.0	0.9	5.8	2.0	-	3.8	0.4	-
$C_{15:1}$ ω8c	1.5	2.2	-	0.3	-	6.5	0.7	-
$C_{16:1}$ ω7c	-	-	21.2	23.1	-	17.4	-	-
$C_{17:1}$ ω8c	9	11.1	23.9	8.1	-	16.2	51.7	34.1
$C_{18:1}$ ω7c	18.9	16.6	17.5	29.4	4.1	1.4	7.8	11.4
Hydroxy								
$C_{10:0}$ 3OH	3.3	2.9	1.8	2.1	10.4	4.0	1.3	1.7
$C_{11:0}$ 3OH	2.2	2.9	3.3	0.5	-	7.5	4.1	4.6
$C_{15:0}$ ISO 2OH	-	--	-	-	-	19	-	-
Summed feature 3	24.6	25.4	-	-	45.3	-	5.9	9.0

Summed feature 3 comprises $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH.

3.3.2 Description

Description of *Dasaniaceae* fam. nov

Dasaniaceae (Da.sa'ni.a'ce.ae. N.L. fem. n. *Dasania* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Dasaniaceae* the Dasania family)

Cells are gram-negative, non-spore-forming. Growth not observed in the absence of sodium chloride. Segregation of these organisms into a new family is justified by their distinct phyletic lineage based on the 16S rRNA gene. The family contains the genera *Spongiibacter*, *Melitea*, *Dasania*, "*Oceanicoccus*", *Haliea*, *Congregibacter* and *Halicoccus*.
type genus is *Dasania.*

Description of *Halicoccus marinus* gen. nov.

Halicoccus (ha.li. coc'cus. Gr. adj, *halios* belonging to the sea or marine; N.L. n. coccus, from Gr. masc. n. kokkos a berry; unit; N.L. masc. n. *Halicoccus*, coccus from marine).

Cells are coccoid shaped, Gram- negative and obligately aerobic. The major respiratory quinone is Q-8. Predominant cellular fatty acids are summed feature 3 (comprising $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH), $C_{18:1}$ ω7c and $C_{17:1}$ ω8c. The type species is *Halicoccus marinus.*

Description of *Halicoccus marinus* sp. nov.

Halicoccus marinus (ma.ri'nus. L. masc. adj. *marinus* of the sea, marine).

Halicoccus marinus exhibits the following properties in addition to those given in the genus description. Cells are 0.3-0.5μm diameter. Temperature range for growth is 15–30 °C.

Optimal temperature for growth is 20-25 °C. No growth occurs above 37 °C. pH range for growth is 6.0–9.0. NaCl is required for growth and can be tolerated at up to 4 % (w/v). Catalase- negative but oxidase- positive. Production of acetoin and reduction of nitrate to N_2 are observed. Gelatin and aesculin are hydrolyzed but agar and starch are not hydrolyzed. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-*β*-glucosaminidase are positive but *β*-glucosidase, *α*-galactosidase, *β*-galactosidase, leucine arylamidase, *α*-glucosidase, *β*-glucuronidase, *α*-mannosidase and *α*-fucosidase are negative. Acid is produced from ribose, glucose, fructose, esculin, maltose, sucrose, trehalose and glycogen but not from glycerol, galactose, *N*-acetyl-glucosamine, ferric citrate, melibiose, mannose, D-tagatose, D-turanose, 5-keto-gluconate, D-arabinose, L-arabinose, starch, xylitol, D-xylose, L-xylose, methyl-β-D-xylopyranoside, rhamnose, methyl-α-D-mannnopyranoside, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose, melezitose, inulin, raffinose, gentiobiose, D-fucose, L-fucose, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, D-arabitol, L-arabitol, gluconate and 2-keto-gluconate. Major cellular fatty acids are were summed feature 3 (comprising $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH; 24.6%), $C_{18:1}$ ω7c (18.9%), $C_{17:1}$ ω8c (9%), $C_{11:0}$ (6.1%), $C_{10:0}$ (5.4%) and $C_{17:0}$ (5.4%). The DNA G+C content of the type strain is 59.6 mol%. The type strain, S1-36^T (=NBRC XXXX^T =KCTC 23429^T), was isolated from western North Pacific Ocean near Japan.

Description of *Halicoccus pacifica* sp. nov.

Halicoccus pacifica (pa.ci’fi.ca. L. fem. adj. *pacificus* peaceful, pertaining to the Pacific Ocean).

Halicoccus pacifica exhibits the following properties in addition to those given in the genus description. Cells are 0.3-0.5 μm diameter. Temperature range for growth is 15–30 °C. Optimal temperature for growth is 20-25 °C. No growth occurs above 37 °C. pH range for growth is 6.0–10.0. NaCl is required for growth and can be tolerated at up to 4 % (w/v). Catalase- negative but oxidase- positive. . Production of acetoin and reduction of nitrate to N_2 are observed. Gelatin is hydrolyzed but agar, starch, aesculin are not hydrolyzed. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, and acid phosphatase are positive but leucine arylamidase, and *β*-glucosidase *α*-galactosidase, *β*-galactosidase, leucine arylamidase, *α*-glucosidase, *β*-glucuronidase, *α*-mannosidase, naphthol-AS-BI-phosphohydrolase, *N*-acetyl-*β*-glucosaminidase and *α*-fucosidase are negative. Acid is produced from glycerol, ribose, glucose, fructose, sucrose, arbutin and glycogen but not from galactose, *N*-acetyl-glucosamine, ferric citrate, melibiose, mannose, D-tagatose, D-turanose, 5-keto-gluconate, D-arabinose, maltose, D-xylose, L-arabinose, L-xylose, methyl-β-D-xylopyranoside, rhamnose, methyl-α-D-mannnopyranoside, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose, melezitose, inulin, raffinose, trehalose, gentiobiose, D-fucose, L-fucose, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, starch, esculin, xylitol, D-arabitol, L-arabitol, gluconate and 2-keto-gluconate. Major cellular fatty acids are were summed feature 3 (comprising $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH; 25.4%), $C_{18:1}$ ω7c (16.6%), $C_{17:1}$ ω8c (11.1%) and $C_{11:0}$ (8.3%). The DNA G+C content of the type strain is 59.5 mol%. The type strain, S1-72^T (=NBRC XXXX^T =KCTC 23430^T), was isolated from western North Pacific Ocean near Japan.

CHAPTER IV.

PHYLOGENETIC STUDIES ON THE NOVEL MARINE BACTERIA IN THE PHYLUM *BACTEROIDETES*

CHAPTER IV. PHYLOGENETIC STUDIES ON THE NOVEL MARINE BACTERIA IN THE PHYLUM *BACTEROIDETES*

1. Introduction

Members of the phylum '*Bacteroidetes*' (previously known as *Cytophaga–Flavobacterium–Bacteroides)* are seemingly omnipresent in aquatic environments (Reichenbach, 1992). Members have been identified in a diverse range of freshwater and marine habitats, including temperate rivers (Bőckelmann *et al*., 2000), freshwater lakes (McCammon *et al*., 1998), marine sediments (Humphry *et al*., 2001), sea ice (Brown & Bowman, 2001), hypersaline lakes (Dobson *et al*., 1993) and saline hot springs (Tenreiro *et al*., 1997). Fluorescent in situ hybridization studies indicate that members of the *'Bacteroidetes'* are second only to the Proteobacteria in terms of aquatic abundance (Glockner *et al*., 1999; Simon *et al*., 1999; Cottrell & Kirchman, 2000). Bacteroidetes have been implicated as major utilizers of high-molecular-mass dissolved organic matter in marine ecosystems (Cottrell & Kirchman, 2000) and are often abundant in nutrient-rich waters where biomacromolecules accumulate (Reichenbach, 1989). Despite their abundance and apparent ecological significance, bacteroidetes are underrepresented in culture collections when compared with other abundant phyla such as the Proteobacteria. The family *Flavobacteriaceae*, belonging to the phylum *Bacteroidetes* and subsequently described and emended by Bernardet *et al.* (1996, 2002). Currently the family *Flavobacteriaceae* comprises more than 91 genera (http://www.bacterio.cict.fr). Most members of the family have been isolated from diverse marine environments including the deep sea (Romanenko *et al.*, 2007), sediment (Khan *et al*., 2008), Antarctic water (Macián *et al*., 2002) and marine plants or animals (Nedashkovskaya *et al*., 2005; Bae *et al*., 2007). The family "*Rhodothermaceae"* belong to the class "*Cytophagia*" in phylum *Bacteroidetes* (Ludwig *et al.*, 2008) includes three genera,

Rhodothermus (Alfredsson *et al.,* 1988), *Salinibacter* (Anton *et al.,* 2002) and the recently proposed genus *Salisaeta* (Vaisman *et al.,* 2009). The strains among these genera, have been isolated from extreme environments: *Rhodothermus marinus* was isolated from submarine hot springs in Iceland, *Salinibacter ruber* was isolated from hypersaline crystallizer ponds in Spain and *Salisaeta longa* was isotated from the Dead Sea (Israel/Jordan). They therefore exhibit extreme thermophilic or halophilic characteristics. The family "*Rhodothermaceae"* is not validated in the phylum *Bacteroidetes* at present.

In CHAPTER IV the pylogenetic studies were performed using a polyphasic taxonomic approach on two marine bacterial strains which isolated from western North Pacific within the phylum *Bacteroidetes*.

2 .Materials and methods

2.1.Strains investigated in this study. . . . . ..

2 bacterial strains were isolated from Western North Pacific Ocean.

Strains	Sources	Sampling place	Date
SG-29^T	Seawater	Western North Pacific Station S (30°40'N, 138°00'E; depth, 50m)	July, 2009
S1-66^T	Seawater	Western North Pacific Ocean (30°11' N, 145°05' E; depth, 100m)	Feb, 2010

Investigated strains were provided by Lab of Marine microbiology, Atmosphere and Ocean Research Institute, The University of Tokyo.

2.2. **Composition of media used in this study**

(Same materials and methods as Chap II.2.2)

2.3. Morphology

(Same materials and methods as Chap II.2.3)

2.4. Genotypic characterizations

(Same materials and methods as Chap II.2.4)

2.5. Physiological characterizations

(Same materials and methods as Chap II.2.5)

2.6.Chemotaxonomainc analysis

(Same materials and methods as Chap II.2.6)

3 .Results and discussion

3.1. A news species of a novel genus *Rubricoccus*

3.1.1. Results and discussion

This aerobic bacterium was isolated from the seawater samples collected from western the North Pacific Ocean (30°40' N, 138°00' E; depth, 50m) during KT-09-11 (2-6 July 2009) of RV 'Tansei Maru' (Ocean Research Institute, The University of Tokyo, and Japan Agency for Marine-Earth Science and Technology [JAMSTEC]). The seawater (200 µl) sample was inoculated on medium G (0.5 g peptone, 0.1 g yeast extract, 5 g Gelrite in 1 L of 80% aged seawater) and incubated at 15°C for 30 days. The isolated strain was maintained on 1/2-strength marine agar 2216 (MA: Difco) containing 2 % NaCl at 20°C. Cells of strain SG-29^T grown on 1/ 2 MA containing 2 % NaCl at 20 °C for 3 weeks were coccoid- and amorphous-shaped, reddish pigmented, approximately 0.3-0.5 µm wide and 0.3-0.5 µm long. Spores were not observed microscopically (Fig. IV.1). A reddish pigment accumulated in the cells was extracted by acetone after the freeze-drying treatment of the cells and analyzed using Beckman Du 530 UV-VIS Spectrophotometer. This pigment showed UV-visible spectra with absorption maxima at 474 nm and 446 nm. No growth was observed under anaerobic conditions in 1/2 MA containing 2% NaCl. Growth occurred only under aerobic conditions between 5-37°C. Optimal temperature and pH range were 20- 30°C and 5-9, respectively. The strain required sodium-ions for growth and grew in 1- 5% NaCl (Table IV.1). The major cellular fatty acids of strain SG-29^T were $C_{17:1}$ ISO ω9c (21.9%), $C_{17:1}$ ω8c (16.9%), $C_{17:0}$ ISO (14.5%), $C_{16:0}$ (7.6%) and $C_{17:0}$ (6.9%) which distinguishes SG-29^T from three related genera of the phylum *Bacteroidetes* (Table IV.2).

Cultural, physiological and biochemical characteristics of SG-29 ^{T} were compared with related species *Salisaeta longa* S 4-4^T, *Salinibacter ruber* M8^T and *R. marinus* DSM 4252^T in

the phylum *Bacteroidetes* (Table IV.1). An almost complete 16S rRNA gene sequence for strain SG-29^T was determined and a FASTA search in the GenBank showed that the strain belongs to the phylum *Bacteroidetes.* All species with validly published names exhibited <87% sequence similarity to the determined sequence. Strain SG-29^T displayed 16S rRNA gene sequences similarities to *Rhodothermus obamensis* OKD7^T (GenBank accession no. AF217493, 86.1% similarity), *R. marinus* DSM 4252^T (AF217494, 85.9%) (type species of genus *Rhodothermus*), *Salisaeta longa* S4- 4^T (EU426570, 85.4%), *Rhodothermus profundi* PRI 2902^T (FJ624399, 85.3%) and *Salinibacter ruber* M8 ^{T} (EF323500, 84.8%). Phylogenetic trees obtained by the neighbour-joining (NJ) method (Fig. IV.2) and maximum-likelihood (ML) (data not shown) methods revealed clear affiliations of the novel isolate SG-29^T to the cluster of *Salisaeta longa* S 4-4^T, *Salinibacter ruber* ^{T} M8 and *R. marinus* DSM 4252^T, and the cluster including SG-29^T was placed on a separate branch within the phylum *Bacteroidetes.*

Phenotypic differences among strain SG-29^T and related genera are shown in Table IV.1 and IV.2. The data presented showed that strain SG-29^T is sufficiently different from related genera and represents a novel genus as *Rubricoccus marinus* gen. nov., sp. nov. in the family "*Rhodothermaceae"*.

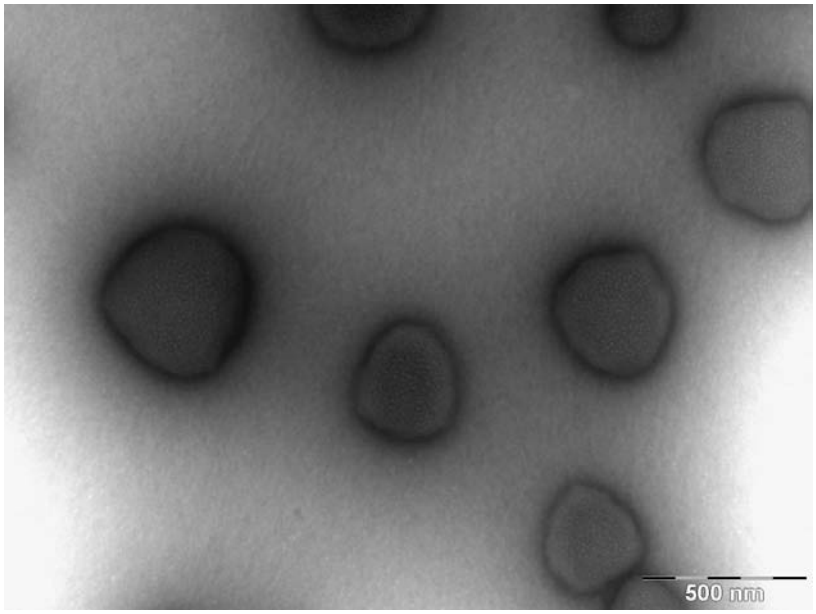


Figure IV.1. Transmission electron micrograph of strain SG-29^{T}, showing coccoid and amorphous cells.

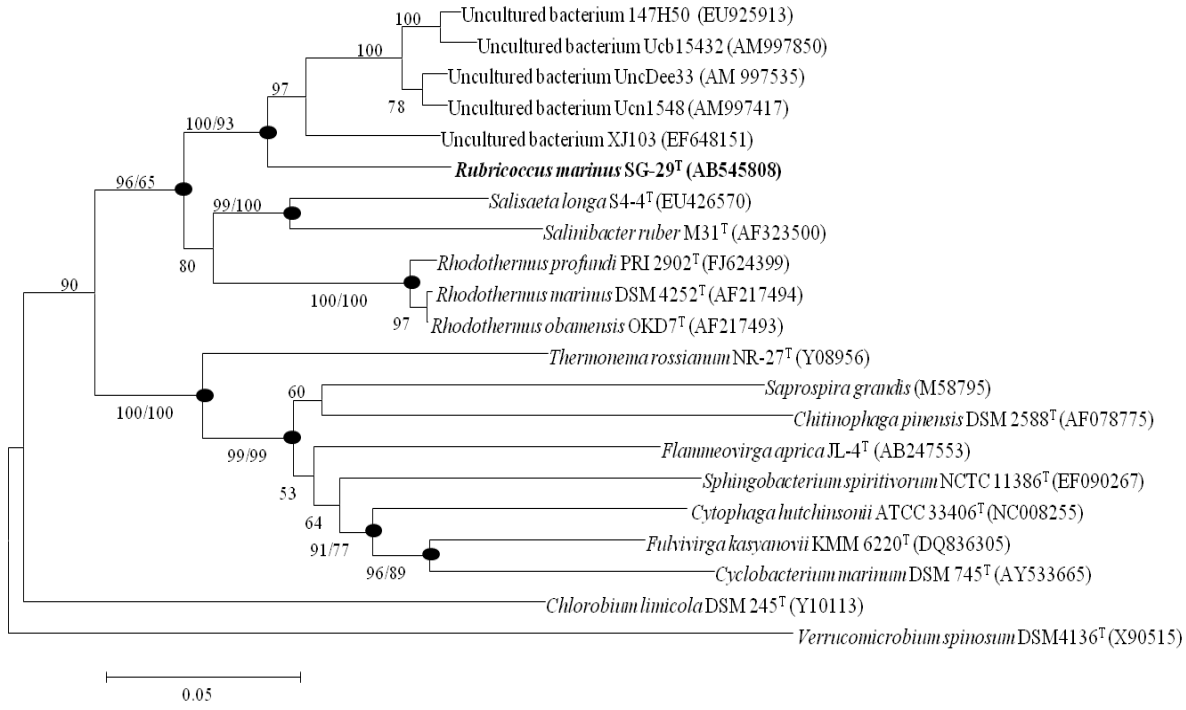


Figure IV. 2. Neighbour-joining (NJ) phylogenetic tree based on the 16S rRNA gene sequence showing the position of strain SG-29^T among the currently known and related species of the phylum *Bacteroidetes*. Numbers at nodes are bootstrap percentages derived from 1000 replications (NJ/ML). Sequences of *Chlorobium limicola* DSM 245^T (Y10113) and *Verrucomicrobium spinosum* DSM 4136^T (X90515) were used as an outgroup. Bar, 0.05 substitutions per nucleotide position. Dots indicate recovered nodes with >50% bootstrap values in the Maximum-likelihood(ML) tree.

Table IV.1. Differential characteristics of strain SG-29^T and related genera in the phylum *Bacteriodetes.*

Data were taken from *Rhodothermus marinus* DSM 4252^T (Sako *et al.*,1996), *Salinibacter ruber* M31^T (Antón *et al.*, 2002), *Salisaeta longa* S4-4^T (Vaisman *et al.*, 2009) and this study.

Characteristic	SG-29^T	*Salisaeta longa* S4-4^T	*Salinibacter ruber* M3^T	*Rodothermus marinus* DSM 4252^T
Pigment	reddish	red	red	reddish
Cell length (μm)	0.3-0.5	15–30	0.4–2.6	2-2.5
Temperature (°C)	5-37	37-46	32–47	54-77
Opt Temp (°C)	20-30	37-46	37–47	65
NaCl (%,w/v)	1-5	5–20	15–30	2-6
DNA G+C content (mol%)	68.85	62.9	66.5*	64.4
Catalase	+	+	+	+
Oxidase	-	+	+	+

Table IV.2. Major cellular fatty acid composition of strain SG-29^T and related genera in the phylum *Bacteriodetes*

Data were taken from *Rhodothermus marinus* DSM 4252^T (Marteinsson *et al*. 2010), *Salinibacter ruber* M31^T (Bardavid *et al*., 2007), *Salisaeta longa* S4-4^T (Vaisman *et al.*, 2009) and this study.

	SG-29^T	*Salisaeta longa* S4-4^T	*Salinibacter ruber* M3^T	*Rodothermus marinus* DSM 4252^T
$C_{15:0}$ ISO	3.9	8.5	25	6
$C_{15:0}$ ANTEISO	0.2	3.8	3.9	12.3
$C_{16:0}$	7.6	ND	9.8	4.1
$C_{16:0}$ ISO	2.2	26-30	-	27.8
$C_{16:1}$ ω9c	0.5	35-38	29	ND
$C_{17:0}$ ISO	14.5	ND	0.9	20.7
$C_{17:0}$ ANTEISO	0.4	ND	1.1	13.9
$C_{17:1}$ ω8c	16.9	ND	ND	ND
$C_{17:1}$ ISO ω9c	21.9	ND	ND	ND
$C_{18:1}$ ω11c	-	ND	24.4	ND

ND: no data,**-:** not detected

3.1.2 Description

Description of *Rubricoccus* gen. nov.

Rubricoccus (Ru.bri.coc'cus. L. adj, *ruber* reddish; N.L. n. coccus, from Gr. masc. n. kokkos a berry; unit; N.L. masc. n. *Rubricoccus,* reddish coloured coccus).

Cells are reddish pigmented, coccoid- and amorphous- shaped, non-gliding, Gram- negative and obligately aerobic. The major respiratory quinone is MK-7. The G+C content of the genomic DNA is 68.9 mol%. Predominant cellular fatty acids are C17:1 ISO ω9c, C17:1 ω8c and C17:0 ISO. The type species is *Rubricoccus marinus.*

Description of *Rubricoccus marinus* sp. nov.

Rubricoccus marinus (ma.ri'nus. L. masc. adj. *marinus* of the sea, marine).

Rubricoccus marinus exhibits the following properties in addition to those given in the genus description. Cells are 0.3-0.5 μm wide and 0.3-0.5 μm long. Temperature range for growth is 5–37 °C. Optimal temperature for growth is 20-30 °C. No growth occurs above 45 °C. pH range for growth is 6.0–9.0. NaCl is required for growth and can be tolerated at up to 5 % (w/v). Catalase- positive but oxidase- negative. Esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are positive, but alkaline phosphatase, *N*-acetyl-*β*-glucosaminidase, *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, *β*-glucuronidase, *β*-glucosidase, *α*-mannosidase and *α*-fucosidase are negative. Production of acetoin and reduction of nitrate to N_2 are observed. Gelatin is weekly hydrolyzed. Acid is produced from esculin, 2-keto-gluconate, D-turanose

and D-arabinose but not from glycerol, ribose, glucose, sucrose, mannitol, galactose, fructose, arbutin, melezitose, glycogen, glucose, *N*-acetyl-glucosamine, ferric citrate, melibiose, mannose, D-tagatose, D-lyxose, 5-keto-gluconate, maltose, D-xylose, L-arabinose, L-xylose, methyl-β-D-xylopyranoside, rhamnose, methyl-α-D-mannnopyranoside, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose, inulin, raffinose, trehalose, gentiobiose, D-fucose, L-fucose, erythritol, adonitol, sorbose, dulcitol, inositol, sorbitol, starch, xylitol, D-arabitol, L-arabitol, gluconate and 2-keto-gluconate. Major cellular fatty acids are $C_{17:1}$ ISO ω9c (21.9%), $C_{17:1}$ ω8c (16.9%), $C_{17:0}$ ISO (14.5%), $C_{16:0}$ (7.6%) and $C_{17:0}$ (6.9%). The DNA G+C content of the type strain is 68.9 mol%. The type strain, SG-29^T (=NBRC 107124^T =KCTC 23197^T), was isolated from western North Pacific Ocean near Japan.

3.2. A news species of a novel genus *Aureimonas*

3.2.1. Result and discussion

This aerobic bacterium was isolated from the seawater samples collected from the western North Pacific Ocean (30°11' N, 145°05' E; depth, 100m) during the R/V Mirai on Feb. 9, 2010 (MR10-01 cruise). The seawater (200 μl) sample was inoculated on medium 1/10 strength Zobell agar (0.5 g peptone, 0.1 g yeast extract, 15 g agar in 1 L of 80% aged natural seawater) and incubated at 20°C for 30 days. The isolated strain was maintained on 1/2-strength marine agar 2216 (MA: Difco) containing 2 % NaCl at 20°C. An almost complete 16S rRNA gene sequence for strain S1-66^T was determined and a FASTA search in the GenBank showed that the strain belongs to the family *Flavobacteriaceae*. All species with validly published names exhibited <95% sequence similarity to the determined sequence. Strain S1-66^T displayed 16S rRNA gene sequences similarities to *Ulvibacter antarcticus* IMCC3101^T (GenBank accession no. EF554364, 94.2% similarity), *Ulvibacter litoralis* KMM 3912^T (AY243096, 93.9 %), *Marixanthomonas ophiurae* KMM 3046^T (AB261012, 92.6 %), *Vitellibacter vladivostokensis* KMM 3516^T (AB071382, 90.1 %), *Aequorivita antarctica* SW49^T (AY027802, 89.7 %) and *Croceibacter atlanticus* HTCC 2559^T (AY163576, 87.8%). Phylogenetic trees obtained by the neighbour-joining (NJ) method and maximum-likelihood (Fig. IV.3) methods revealed clear affiliations of the novel isolate S1-66^T to the cluster of *U. antarcticus* IMCC3101^T, *U. litoralis* KMM 3912^T, *M. ophiurae* KMM 3046^T, *V. vladivostokensis* KMM 3516^T, *A. antarctica* SW49^T and *C. atlanticus* HTCC 2559^T. Strain S1-66^T formed a cluster with *U. antarcticus* IMCC3101^T and *U. litoralis* KMM 3912^T with 87% (NJ) and 76% (ML) bootstrap confidence values.

Cultural, physiological and biochemical characteristics of S1-66 ^{T} were compared with related genera *Ulvibacter*, *Marixanthomonas*, *Vitellibacter*, *Aequorivita* and *Croceibacter*.

Cells of strain S1-66^T grown on 1/ 2 MA containing 2% NaCl at 20°C for 3 weeks were irregular rod shaped, yellow pigmented, approximately 0.5-1 μm wide and 0.5-6 μm long. Spores were not observed microscopically. A yellow pigment accumulated in the cells was extracted by acetone after the freeze-drying treatment of the cells and analyzed using Beckman Du 530 UV-VIS Spectrophotometer. This pigment showed UV-visible spectra with absorption maxima at 343 nm, 452 nm and 487 nm. Flexirubin-type pigments was not produced that was investigated by using the bathochromatic shift test with a 20% (w/v) KOH solution (Bernardet *et al*., 2002). No growth was observed under anaerobic conditions in 1/2 MA containing 2% NaCl. Growth occurred only under aerobic conditions between 15- 30°C. Optimal temperature and pH range were 20- 25°C and 7- 8, respectively. Oxidase is positive and catalase in negative. DNA G+C content of the strain S1-66^T was 48.1 % which distinguishes strain S1-66^T from related genera *Ulvibacter*, *Marixanthomonas*, *Aequorivita* and *Croceibacter* .The strain S1-66^T required NaCl for growth and grew in 1- 4% NaCl (Table IV.3). The major cellular fatty acids of strain S1-66^T were $C_{15:0}$ ISO (26.4 %), $C_{15:1}$ ISO (20.3 %), $C_{17:0}$ ISO 3OH (14.2%). summed feature 3 (comprising $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH; 5.9 %) and $C_{16:0}$ ISO (5.2%). Only two $C_{15:0}$ ISO (26.4 %) and $C_{15:1}$ ISO (20.3 %) branched fatty acids represented 46.3% of the total fatty acids and it could distinguishes S1-66^T from related genera of the family *Flavobacteriaceae.* (Table IV.4)

Phenotypic differences among strain S1- 66^T and related species are shown in Table IV.3 and IV.4. The data presented showed that strain S1- 66^T is sufficiently different from related genera and represents a novel genus and species as *Aureimonas marinus* gen. nov., sp. nov. in the family *Flavobacteriaceae*

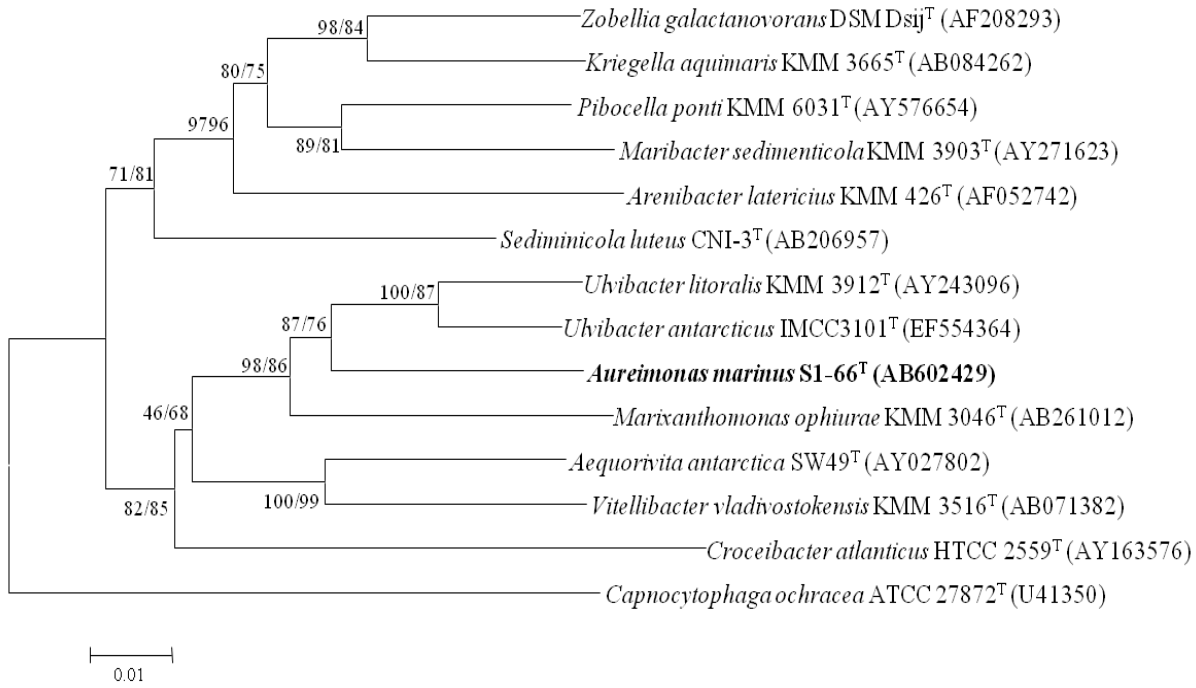


Figure IV.3. Neighbour-joining (NJ) phylogenetic tree based on the 16S rRNA gene sequence showing the position of strain S1-66^T among the currently known and related species of the family *Flavobacteriaceae*. Numbers at nodes are bootstrap percentages derived from 1000 replications (NJ/ML). Sequences of *Capnocytophaga ochracea* ATCC 27872^T (U41350) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table IV.3. Differential characteristics of strain S1-66^T and related genera in the family *Flavobacteriaceae.*

genera: 1, Strain S1-66^T: 2, *Ulvibacter* (Nedashkovskaya *et al.*, 2004: Choi *et al*., 2007): 3, *Marixanthomonas* (Romanenko *et al*., 2007): 4, *Vitellibacter* (Nedashkovskaya *et al*., 2003: Kim *et al*., 2010): 5, *Aequorivita* (Bowman *et al*., 2002: Park *et al*., 2009): 6, *Croceibacter* (Cho *et al*., 2003) Abbreviations: -, negative; +, positive; V,variable; ND, not determined.

Characteristic Strain	1	2	3	4	5	6
Flexirubin pigment	-	+	-	+	-	-
Cell length (μm)	0.5-6	0.5-7.3	2.2-2.8	3-10	0.5-20	1.2-3.1
Temperature (°C)	15-30	3-36	5-32	4-43	-2-40	10-28
Opt Temp (°C)	20-25	21-25	25-28	28-35	20-25	0.5-15
NaCl (%,w/v)	1-4	1-6	1-12	1-6	1-8	0.5-15
Hydrolysis of:						
starch	+	V	-	-	V	+
Catalase	-	+	+	V	+	+
Oxidase	+	+	+	+	-	-
DNA G+C content (mol%)	48.1.	36.7-38.0	37.3	41.3-48.7	33.5-39.1	34.8

Table IV. 4. Cellular fatty acid composition of strain S1-66^T and related species in the family *Flavobacteriaceae.*

Species: 1, Strain S1-66^T: 2, *Ulvibacter antarcticus* IMCC 3101^T (Choi *et al*., 2007): 3, *Ulvibacter litoralis* KMM 3912^T (Nedashkovskaya *et al.*, 2004): 4, *Marixanthomonas ophiurae* KMM 3046^T (Romanenko *et al*., 2007): 5, *Vitellibacter vladivostokensis* KMM 3516^T (Kim *et al*., 2010): 6, *Aequorivita antarctica* SW49^T (Bowman *et al*., 2002: Park *et al*., 2009): 7, *Croceibacter antarcticus* HTCC 2559^T (Cho *et al*., 2003)

	1	*2*	*3*	4	5	*6*	7
$C_{15:0}$	4.6	1.2	3.9	2.4	4.3	-	3.9
$C_{16:0}$	0.9	-		6.9	1.7	1.9	0.6
$C_{18:0}$	-	-		5.2	-	-	0.2
$C_{15:0}$ ISO	26.4	15.3	21.3	2.9	32.4	7.6	13.3
$C_{15:0}$ ANTEISO	3.8	1.3	2.3	-	3.8	15.7	1.6
$C_{15:1}$ ISO	20.3	3.9	12.5	-	2.4	-	9.2
$C_{15:1}$ ISO ω10c	-	-	-	-	-	9.5	-
$C_{15:1}$ ANTEISO ω10C	-	-	-	-	-	16	-
$C_{16:0}$ ISO	5.3	11.0	2.8	1.8	2.7	1.4	1.5
$C_{16:1}$ ISO	2.6	5.4	4.2	-	-	-	1.72
$C_{15:0}$ ISO 3OH	3.3	4.1	3.3	-	3.5	5.4	4.3
$C_{16:0}$ ISO 3OH	2.9	9.2	8.7	41.2	1.4	9.2	4.4
$C_{17:0}$ ISO 3OH	14.2	19.4	17.2	16.4	23.2	2.0	28.0
$C_{17:0}$ ANTEISO 3OH	-	-	-	5.1	-	2.9	-
$C_{17:1}$ ISO ω9c	-	2.2	3.7	-	9	-	9.36
summed feature 3	7.8	5.9	5.7	-	6.9	-	5.1

Summed feature 3 (comprising $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH).

ND: no data,**-:** not detected

3.2.2 Description

Description of *Aureimonas* gen. nov.

Aureimonas (Au.re.i. mo'nas. L. adj, *aureus,* golden; N.L. masc. n. *monas,* a unit in bacteriology, a monad; N.L. masc. n. *Aureimonas,* goleden color monad).

Cells are yellow pigmented, flexiruburin pigment are not produced. Irregular rod shaped, Do not form endospores, Gramstain- negative and obligately aerobic. The major respiratory quinone is MK-6. Predominant cellular fatty acids are were $C_{15:0}$ ISO, $C_{15:1}$ ISO, $C_{17:0}$ ISO 3OH. The type species is *Aureimonas marinus.*

Description of *Aureimonas marinus* sp. nov.

Aureimonas marinus (ma.ri'nus. L. masc. adj. *marinus* of the sea, marine).

Aureimonas marinus exhibits the following properties in addition to those given in the genus description. Cells are 0.4 -0.6 μm wide and 0.5-6 μm long. Non gliding motility. Temperature range for growth is 15–30 °C. Optimal temperature for growth is 20-25 °C. No growth occurs above 30 °C. pH range for growth is 6.0–9.0. NaCl is required for growth and can be tolerated at up to 4 % (w/v). Catalase-negative but oxidase-positive. Production of acetoin and reduction of nitrate to N_2 are observed. Gelatin and starch are hydrolyzed but agar is in not hydrolyzed. Alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *β*-glucosidase are positive, but esterase (C4), esterase lipase (C8), lipase (C14), *N*-acetyl-*β*-glucosaminidase, *α*-galactosidase, *β*-galactosidase, *α*-glucosidase, *β*-glucuronidase, *α*-mannosidase and *α*-fucosidase are negative. Acid is produced from glycerol, ribose, glucose, fructose, *N*-acetyl-glucosamine, esculin, maltose, D-tagatose, starch, xylitol, sucrose,

trehalose but not from galactose, ferric citrate, melibiose, mannose, D-turanose, and 5-keto-gluconate, D-arabinose, L-arabinose, D-xylose, L-xylose, methyl-β-D-xylopyranoside, rhamnose, methyl-α-D-mannnopyranoside, methyl-α-D-glucopyranoside, amygdalin, arbutin, salicin, cellobiose, lactose, melezitose, inulin, raffinose, gentiobiose, D-fucose, L-fucose, erythritol, adonitol, sorbose, dulcitol, inositol, mannitol, sorbitol, glycogen, xylitol, D-arabitol, L-arabitol, gluconate and 2-keto-gluconate. Major cellular fatty acids are $C_{15:0}$ ISO (26.4 %), $C_{15:1}$ ISO (20.3 %), $C_{17:0}$ ISO 3OH (14.2 %), summed feature 3 (comprising $C_{16:1}$ ω7c and/or $C_{15:0}$ ISO 2OH; 5.9 %), $C_{16:0}$ ISO (5.2 %). The DNA G+C content of the type strain is 48.1 mol%. The type strain, S1-66^T (=NBRC XXXX^T =KCTC 23434^T), was isolated from we stern North Pacific Ocean near Japan.

CHAPTER V.

GENERAL CONCLUSION

CHAPTER V. GENERAL CONCLUSION

Total 9 novel bacterial strains were isolated from marine water and marine sponge and investigated using polyphasic taxonomy. Based on the results I proposed 7 novel species of 5 new genera within the phylum *Proteobacteria* and *Bacteroidetes.* Individually, it was concluded that SA4-31, SA4-46, SA4-48^{T} should be classified as representing a new genus and species of the family *Pseudoalteromonadaceae,* for which the name *Psychrosphaera saromensis* gen. nov., sp. nov. is proposed. Strain PZ-5^{T} represents a novel genus and species, for which the name *Oceanicoccus sagamiensis* gen. nov., sp. nov., is proposed. Two strains S1-36 ^{T} and S1-72^{T} present a novel genus and two species as *Halicoccus marinus* and *H. pacifica* were proposed. Interestingly strain PZ-5^{T}, S1-36 ^{T} and S1- 72^{T} were found to belong to the novel family *Dasaniaceae* with in the class *Gammaproteobacteria*. Strain SG-29^{T} is classified as a novel genus and species, for which the name *Rubricoccus marinus* gen. nov., sp. nov., within family '*Rhodothermaceae*', is proposed. Strain S1-66^{T} represent a novel genus and species as *Aureimonas marinu* was proposed. 20 species of marine sponge were investigated to find novel bacterial strains using medium P and medium SN. Approximately 300 colonies were obtained and sequenced partial 16S rRNA gene sequences (1100nt). According to the 16S rRNA gene sequence searching results, about 30 genera in the class *Bacilli, Actinobacteria, Alphaproteobacteria, Betaproteobacteria* and *Gammaproteobacteria* were obtained, but we couldn't find any bacterial strain which the genus level unknown and sponge specific. It means that media used screening (medium P and SN contained artificial seawater) are not good methods. But in species level, strain MS-31^{T} was isolated from marine sponge and classified as *Sphingomonas Jejuensis* sp. nov., within the genus *Sphingomonas* and it is first report about isolation of *Sphingomonas* from marine sponge.

ACKNOWLEDGEMENTS

Firstalbly, I would like to express my deep gratitude and appreciation to my academic supervisor, Professor Dr. Akira Yokota. Institute of Molecular and Cellular Biosciences, The University of Tokyo, all for his guidance and constant encouragement has contributed to my study. I am thankful to Professor Kazuhiro Kogure, Dr Koji Hamasaki, Dr Susumu Yoshizawa and Kentaro Inomata engaged Lab of marine microbiology, Atmosphere and Ocean Research Institute, The University of Tokyo for providing valuable strains investigated in this study. Also I appreciate to Professor Jin-Suk Park Hannam University, Korea for providing marine sponges. I would to extend my great appreciation to Mr Oohasi, Mrs Akimoto and the members of the Laboratory of Bioresources, Department of Biotechnology, The University of Tokyo, for their friendship and assistance. Finally I wish to express my sincere gratitude to my parents and my brother who gave me endless encouragement.

But he knows the way that I take, when he has tested me, I will come forth as gold.

[job 23 :10]

REFERENCE

Alfredsson, G. A., Kristjansson, J. K., Hjorleifsdottir, S., & Stetter, K. O. (1988). *Rhodothermus marinus* gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine hot springs in Iceland. *J Gen Microbiol* **134**, 299–306.

Antón, J., Oren, A., Benlloch, S., Rodrı´guez-Valera, F., Amann, R., & Rosselló Mora, R. (2002). *Salinibacter ruber* gen. nov., sp. nov., a novel, extremely halophilic member of the Bacteria from saltern crystallizer ponds. *Int J Syst Evol Microbiol* **52,** 485–491.

Bae, S. S., Kwon, K. K., Yang, S. H., Lee, H. S., Kim, S. J., & Lee, J. H. (2007). *Flagellimonas eckloniae* gen. nov., sp. nov., a mesophilic marine bacterium of the family *Flavobacteriaceae*, isolated from the rhizosphere of Ecklonia kurome. *Int J Syst Evol Microbiol* **57,** 1050–1054.

Bernardet, J.-F., Nakagawa, Y., & Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* 52, 1049–1070.

Bernardet, J.-F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K., & Vandamme, P. (1996). Cutting a Gordian knot: emended classification and description of the genus *Flavobacterium,* emended description of the family *Flavobacteriaceae,* and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl & Tait 1978). *Int J Syst Bacteriol* **46,** 128–148.

Bőckelmann, U., Manz, W., Neu, T. R., & Szewzyk, U. (2000). Characterization of the microbial community of lotic organic aggregates (‘river snow’) in the Elbe River of Germany by cultivation and molecular methods. *FEMS Microbiol Ecol* **33,** 157–170.

Bogan, B. W., Sullivan, W. R., Kayser, K. J., Derr, K. D., Aldrich, H. C., & Paterek, J. R. (2003). *Alkanindiges illinoisensis* gen. nov., sp.nov., an obligately hydrocarbonoclastic, aerobic squalane-degrading bacterium isolated from oilfield soils. *Int J Syst Evol Microbiol* **53,** 1389-1395.

Bowman, J. P. & Nichols, D. S. (2002). *Aequorivita* gen. nov., a member of the family *Flavobacteriaceae* isolated from terrestrial and marine Antarctic habitats. *Int J Syst Evol Microbiol* **52,** 1533–1541.

Bozal, N., Tudela, E., Rosselló-Mora, R., Lalucat, J., & Guinea, J. (1997) *Pseudoalteromonas antarctica* sp. nov., **isolated** from an Antarctic coastal environment. *Int J Syst Bacteriol* **47,** 345–351

Brenner, D. J., Krieg, N. R., Staley, J. T., & Garrity, G. M. (2005). Bergey’s Manual of Systematic Bacteriology. New York: Springer.

Britschgi, T. B. & Giovannoni, S. J. (1991). Phylogenetic analysis of a natural marine bacterioplankton population by rRNA gene coding and sequencing. *Appl Environ Microbiol* **57,** 1313-1318.

Brown, M. V. & Bowman, J. P. (2001). A molecular phylogenetic survey of sea-ice microbial communities (SIMCO). *FEMS Microbiol Ecol* **35,** 267–275.

Brusca, R. C. & Brusca, G. J (1990). Phylum *Porifera*: the sponges. Invertebrates (Sinauer AD, ed), pp. 181–210. Sinauer Press,Cambridge, MA.

Cho, J. C. & Giovannoni, S. J. (2003). *Croceibacter atlanticus* gen. nov., sp. nov., a novel marine bacterium in the family *Flavobacteriaceae*. *Syst Appl Microbiol* **26,** 76–83.

Choi, T-H., Lee, H. G., Lee, K., & Cho, J-C. (2007). *Ulvibacter antarcticus* sp. nov., isolated from Antarctic coastal seawater. *Int J Syst Evol Microbiol* **57,** 2922 - 2925.

Clayton, R. A., Sutton, G., Hinkle, P. S. Jr., Bult, C., & Fields, C. (1995). Intraspecific variation in smalls ubunitr RNA sequences in Genbank: Why single sequences may not adequately represent prokaryotic taxa. *Int J Sys Btacteriol* **45,** 595-599

Colwell , R. R. (1970). Polyphasic taxonomy of the genus *Vibrio*: numerical taxonomy of *Vibrio cholerae*,*Vibrio parahaemolyticus*,and related *Vibrio* species. *J Bacteriol* **104,** 470-433

Cottrell, M. T. & Kirchman, D. L. (2000a). Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. *Appl Environ Microbiol* **66,** 1692–1697.

Cottrell, M. T. & Kirchman, D. L. (2000b). Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. *Appl Environ Microbiol* **66,** 5116–5122.

De Ley, J. (1992). The Proteobacteria: ribosomal RNA cistron similarities and bacterial taxonomy. In The Prokaryotes, pp. 2111–2140. Edited by A. Balows, H. G. Tru¨per, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer-Verlag.

Delong, E. F. (2005). Microbial community genomics in the ocean. *Nature Rev Microbiol* **3,** 459-469.

Denner, E. B., Paukner, S., Kampfer, P., Moore, E. R., Abraham, W. R., Busse, H. J., Wanner, G., & Lubitz, W. (2001). *Sphingomonas pituitosa* sp. nov., an exopolysaccharide-producing bacterium that secretes an unusual type of sphingan. *Int J Syst Evol Microbiol* **51,** 827–884.

Dittmer, J. C. & Lester, L. A. (1964). A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J Lipid Res* **15,** 126–127.

Dobson, S. J., Colwell, R. R., McMeekin, T. A., & Franzmann, P. D. (1993). Direct sequencing of the polymerase chain reaction-amplified 16S rRNA gene *of Flavobacterium gondwanense* sp. nov. and *Flavobacterium salegens* sp. nov., two new species from a hypersaline Antarctic lake. Int *J Syst Bacteriol* **43**, 77–83.

Elevi Bardavid, R., Ionescu, D., Oren, A., Rainey, F. A., Hollen, B. J., Bagaley, D. R., Small, A. M., & McKay, C. (2007). Selective enrichment, isolation and molecular detection of *Salinibacter* and related extremely halophilic Bacteria from hypersaline environments. *Hydrobiologia* **576,** 3–13.

Ezaki, T., Hashimoto, Y., & Yabuuchi, E. (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39,** 224–229.

Fautz, E. & Reichenbach, H. (1980). A simple test for flexirubin-type pigments. FEMS *Microbiol Lett* **8,** 87–91.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17,** 368–376.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39,** 783–791.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20,** 406–416.

Fox, G, E., Wisotzkey, J. D., & Jurtshuk, P. J. Jr. (1992). How close is close: 16S rRNA sequence identity may not be sufficient to guarantees species identity. *Int J Syst Bacteriol* **42**,

166-170.

Garrity, G.M., Bell, J. A., & Lilburn, T (2005). Order IX. *Pseudomonadales* Orla-Jensen 1921, 270AL. Brenner, p. 323. In *Bergey's Manual of Systematic Bacteriology,* 2nd ed, Vol. 2,D.J. Brenner, N.R. Krieg, &J.T. Staley (eds.), Springer, New York.

Gauthier, G., Gauthier, M., & Christen, R. (1995). Phylogenetic analysis of the genera *Alteromonas, Shewanella,* and *Moritella* using genes coding for small-subunit rRNA sequences and division of the genus *Alteromonas* into two genera, *Alteromonas* (Emended) and *Pseudoalteromonas* gen. nov., and proposal of twelve new species combinations. *Int J Syst Bacteriol* **45,** 755 - 761.

Giovannoni, S. J. & Rappe, M. S. (2000). Microbial Ecology of the Oceans (ed. Kirchman, D. L.),47-85.(Wiley-Liss,N ew York, 2000).

Glőckner, F. O., Fuchs, B. M., & Amann, R. (1999). Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Appl Environ Microbiol* **65,** 3721–3726.

Graeber, I., Kaesler, I., Borchert, M. S., Dieckmann, R., Pape, R., Lurz, R., Nielsen, P., Döhren, H. V., Michaelis, W., & Szewzyk, U. (2008) *Spongiibacter marinus* gen. nov., sp. nov., a halophilic marine bacterium isolated from the boreal sponge *Haliclona* sp. *Int J Syst Evol Microbiol* **58,** 585 - 590.

Guindon, S. & Gascuel, O. (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52,** 696–704.

Gupta, R. S. (2000). The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiol Rev* **24**, 367– 402.

Gupta, R. S. & Lorenzini, E. (2007). Phylogeny and molecular signatures (conserved proteins and indels) that are specific for the bacteroidetes and chlorobi species. *BMC Evol Biol* **7,** 71.

Hagstrom, A., Pommier, T., Rohwer, F., Simu, K., Stolte, W., Svensson, D., & Zweifel, U. L. (2002). Use of 16S ribosomal DNA for delineation of marine bacterioplankton species. *Appl Environ Microbiol* **68**, 3628–3633

Hentschel, U., Hopke, J., Horn, M., Friedrich, AB.,Wagner, M., Hacker, J., & Moore, BS. (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microbiol* **68,** 4431–4440.

Hentschel, U., Fieseler, L.,Wehrl, M., Gernert, C., Steinert, M., Hacker, J., & Horn, M. (2003). Microbial diversity of marine sponges. In *Molecular Marine Biology of Sponges,* Muller WEG(ed), pp. 60–88. Springer Verlag, Heidelberg.

Hill, R. T. (2004). Microbes from marine sponges: a treasure trove of biodiversity for natural products discovery. In *Microbial Diversity and Bioprospecting* (Bull AT, ed), pp. 177–190.

ASM Press,Washington, DC.

Hooper, N. J. A. & van Soest, R. W. M. (2002). Systema Porifera: A Guide to the Classification of Sponges. Kluwer Academic/Plenum Publishers, New York.

Humphry, D. R., George, A., Black, G. W., & Cummings, S. P. (2001). *Flavobacterium frigidarium* sp. nov., an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. *Int J Syst Evol Microbiol* **51**, 1235–1243.

Hwang, C. Y. & Cho, B. C. (2009).*Spongiibacter tropicus* sp. nov., isolated from a *Synechococcus* culture. *Int J Syst Evol Microbiol* **58,** 2176 - 2179.

Ivanova, E. P., Bakunina, I. Y., Nedashkovskaya, O. I., Gorshkova, N. M., Alexeeva, Y. V., Zelepuga, E. A., Zvaygintseva, T. N., Nicolau, D. V., & Mikhailov, V. V. (2003). Ecophysiological variabilities in ectohydrolytic enzyme activities of some *Pseudoalteromonas* species, *P. citrea, P. issachenkonii,* and *P. nigrifaciens*. *Curr Microbiol* **46,** 6–10.

Ivanova, E. P., Flavier, S., & Christen, R. (2004). Phylogenetic relationships among marine Alteromonas-like proteobacteria: emended description of the family *Alteromonadaceae* and proposal of *Pseudoalteromonadaceae* fam. nov., *Colwelliaceae* fam. nov., *Shewanellaceae* fam. nov., *Moritellaceae* fam. nov., *Ferrimonadaceae* fam. nov., *Idiomarinaceae* fam. nov. and *Psychromonadaceae* fam. nov. *Int J Syst Evol Microbiol* **54,** 1773–1788.

Ivanova, E. P., Romanenko, L. A., Matte, M. H., Matte, G. R., Lysenko, A. M., Simidu, U., Kita-Tsukamoto, K., Sawabe, T., Vysotskii, M. V., Frolova, G. M., Mikhailov, V., Christen, R., & Colwell, R. R. (2001). Retrieval of the species *Alteromonas tetraodonis* Simidu et al. 1990 as *Pseudoalteromonas tetraodonis* comb. nov. and emendation of description *Int J Syst Evol Microbiol* **51,** 1071 - 1078.

Kersters, K., De Vos, P., Gillis, M., Swings, J., Vandamme, P., & Stackebrandt, E. (2006). Introduction to the Proteobacteria. In *The Prokaryotes: a Handbook on the Biology of Bacteria*, 3rd edn, vol. 5, pp. 3–37. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer and E. Stackebrandt. New York: Springer.

Khan, S. T., Nakagawa, Y., & Harayama, S. (2008). *Fulvibacter tottoriensis* gen. nov., sp. nov., a member of the family *Flavobacteriaceae* isolated from marine sediment. *Int J Syst Evol Microbiol* **58,** 1670–1674.

Khudary, R. A., Stößer, N. I., Qoura, F., & Antranikian, G.(2008). *Pseudoalteromonas arctica* sp. nov., an aerobic, psychrotolerant, marine bacterium isolated from Spitzbergen. *Int J Syst Evol Microbiol* **58,** 2018 - 2024.

Kim, B-S., Kim, O-S., Moon, E. Y., & Chun. J. (2010). *Vitellibacter aestuarii* sp. nov., isolated from tidal-flat sediment, and an emended description of the genus *Vitellibacter Int J Syst Evol Microbiol* **60,** 1989 - 1992.

Kimura, M. (1983). The Neutral Theory of Molecular Evolution. Cambridge: Cambridge

University Press.

Konstantinidis, K. T. & Tiedje, J. M (2005). Genomic insights that advance the species definition for prokaryotes. *PNAS* **102.** 7.2567-2572.

Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19,** 161–207.

Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics,* pp. 115-147. Edited by E. Stackebrandt & M.Goodfellow: John Wiley and Sons, New York.

Lee, J. S., Shin, Y. K., Yoon, J. H., Takeuchi, M., Pyun, Y. R., & Park, Y. H. (2001). *Sphingomonas aquatilis* sp. nov., *Sphingomonas koreensis* sp. nov., and *Sphingomonas taejonensis* sp. nov., yellow-pigmented bacteria isolated from natural mineral water. *Int J Syst Evol Microbiol* **51,** 1491–1498.

Lee, Y. K., Hong, S. G., Cho, H. H., Cho, K. H., & Lee. H. K. (2007). *Dasania marina* gen. nov., sp. nov., of the order *Pseudomonadales*, isolated from Arctic marine sediment. *J Microbiol*. **45,** 505-509.

Lucena, T., Pascual, J., Garay, E., Arahal, D. R., Macián, M. C., & Pujalte, M. J.(2010). *Haliea mediterranea* sp. nov., a marine gammaproteobacterium *Int. J Syst Evol Microbiol* **60,**1844-1848.

Ludwig, W. & Klenk, H.-P. (2005). Overview: a phylogenetic backbone and taxonomic framework for prokaryotic systematics. In *Bergey's Manual of Systematic Bacteriology*, pp. 49–65. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. Berlin: Springer-Verlag.

Ludwig, W., Euzéby, J., & Whitman, W. B. (2008). Draft taxonomic outline of the *Bacteroidetes, Plantomycetes, Chlamydiae, Spirochaetes, Fibrobacteres, Fusobacteria, Acidobacteria, Verrucomicrobia, Dictyoglomi,* and *Gemmatimonadadetes.* http://www.bergeys.org/outlines/Bergeys_Vol_4_Outline.pdf

McCammon, S. A., Innes, B. H., Bowmann, J. P., Franzmann, P. D., Dobson, S. J., Holloway, P. E., Skerratt, J. H., Nichols, P. D., & Rankin, L. M. (1998). *Flavobacterium hibernum* sp. nov., a lactoseutilizing bacterium from a freshwater Antarctic lake. *Int J Syst Bacteriol* **48,** 1405–1412.

Macián, M. C., Pujalte, M. J., Márquez, M. C., Ludwig, W., Ventosa, A., Garay, E., & Schleifer, K. H. (2002). *Gelidibacter mesophilus* sp. nov., a novel marine bacterium in the family *Flavobacteriaceae*. *Int J Syst Evol Microbiol* **52,** 1325–1329.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3,** 208–218.

Marteinsson, V. T., Bjornsdottir, S. H., Bienvenu, N., Jakob, K., Kristjansson, J. K., &

Birrien, J. L. (2010). *Rhodothermus profundi* sp. nov., a new thermophilic bacterium isolated from a deep sea hydrothermal vent in the Pacific Ocean. *Int J Syst Evol Microbiol* doi;10.1099/ijs.0.012724-0

Mesbah, M., Premachandran, U., & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39,** 159–167.

Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A., & Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2,** 233–241.

Murray, R. G. E., Doetsch, R. N., & Robinow, F. (1994). Determinative and cytological light microscopy. In *Methods for General and Molecular Bacteriology*, pp. 21–41. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Nedashkovskaya, O. I., Kim, S. B., Han, S. K., Rhee, M. S., Lysenko, A. M., Falsen, E., Frolova, G. M., Mikhailov, V. V., & Bae, K. S. (2004). *Ulvibacter litoralis* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae* isolated from the green alga *Ulva fenestrate*. *Int J Syst Evol Microbiol* **54,** 119 - 123.

Nedashkovskaya, O. I., Kim, S. B., Lysenko, A. M., Frolova, G. M., Mikhailov, V. V., & Bae, K. S. (2005). *Bizionia paragorgiae* gen. nov., sp. nov., a novel member of the family

Flavobacteriaceae isolated from the soft coral *Paragorgia arborea*. *Int J Syst Evol Microbiol* **55,** 375–378.

Nedashkovskaya, O. I., Suzuki, M., Vysotskii, M. V., & Mikhailov, V. V. (2003). *Vitellibacter vladivostokensis* gen. nov., sp. nov., a new member of the phylum *Cytophaga–Flavobacterium –Bacteroides*. *Int J Syst Evol Microbiol* **53,** 1281–1286.

Nigam, A., Jit, S., & Lal, R. (2010). *Sphingomonas histidinilytica* sp. nov., isolated from a hexachlorocyclohexane dump site. *Int J Syst Evol Microbiol* **60,** 1038 - 1043.

Orla-Jensen, S. (1921). The main lines of the natural bacterial system.*J. Bacteriol*. **6**, 263 - 273.

O'Sullivan, L. A., Fuller, K. E., Thomas, E. M., Tirrley, C. M., Fry, J. C., & Weightman, A. J.(2004). Distribution and culturability of the uncultivated 'AGG58 cluster' of the *Bacteroidetes* phylum in aquatic environments *FEMS Microbiol EcoI* **47**,359-370.

Park, S. C., Baik, K. S., Kim, M. S., Kim, S. S., Kim, S. R., Oh, M-J., Kim, D., Bang, B-H., & Seong, C. M. (2009). *Aequorivita capsosiphonis* sp. nov., isolated from the green alga *Capsosiphon fulvescens*, and emended description of the genus *Aequorivita*. *Int J Syst Evol Microbiol* **59,** 724 - 728.

Park, S., Yoshizawa, S., Kogure, K & Yokota, A. (2011). *Oceanicoccus sagamiensis* gen. nov., sp. nov., a gammaproteobacterium isolated from sea water of Sagami Bay in Japan. *J.*

Microbiol TJOM-D-10-00368R2, In Press.

Reichenbach, H. (1989). Nonphotosynthetic, nonfruiting gliding bacteria. Genus 1. Cytophaga Winogradsky 1929, 577AL, emend. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 3, pp. 2015–2050. Edited by J. Staley, M. P. Bryant, N. Pfennig & J.G. Holt. Baltimore: Williams & Wilkins.

Reichenbach, H. (1992). The order *Cytophagales*. In *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn, pp. 3631–3675. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. New York: Springer.

Romanenko, L. A., Uchino, M., Frolova, G. M., & Mikhailov, V. V. (2007). *Marixanthomonas ophiurae* gen. nov., sp. nov., a marine bacterium of the family *Flavobacteriaceae* isolated from a deep-sea brittle star. *Int J Syst Evol Microbiol* **57,** 457 - 462.

Romanenko, L. A., Uchino, M., Frolova, G. M., Tanaka, N., Kalinovskaya, N. I., Latyshev, N., & Mikhailov, V. V. (2007). *Sphingomonas molluscorum* sp. nov., a novel marine isolate with antimicrobial activity. *Int J Syst Evol Microbiol* **57,** 358–363.

Rumney, G. R. (1968). Climatology and theWorld's Climate. Macmillan, New York.

Romanenko, L. A., Zhukova, N. V., Rohde, M., Lysenko, A. M., Mikhailov, V. V., & Stackebrandt, E. (2003). *Pseudoalteromonas agarivorans* sp. nov., a novel marine

agarolytic bacterium. *Int J Syst Evol Microbiol* **53,** 125–131.

Rűtzler, K. (1990) Associations between Caribbean sponges and photosynthetic organisms. In *New Perspectives in Sponge Biology* (Rűtzler K, ed), pp. 455–466. Smithsonian Institute Press, Washinton, DC.

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

Sako, Y., Takai, K., Ishida, Y., Uchida, A., & Katayama, Y. (1996). *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. *Int J Syst Bacteriol* **46,** 1099 - 1104.

Sasser, M. (1990). Identification of bacteria by gas chromatography of cellular fatty acids, MIDI Technical Note 101. MIDI Inc. Newark, DE, USA.

Schut, F., de Vreis, E . J., Gottschal J. C, Robertson. B. R., Harder, H., Prins, R., & Button, D. K. (1993). Isolation of typical marine bacteriab y dilution culture: Growth, maintenance, and characteristic of isolates under laboratory conditions. *Appl Environ Microbiol* **59**,2150-2160

Simon, M., Glockner, F. O., & Amann, R. (1999). Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. *Aquat Microb Ecol* **18,** 275–284.

Spring, S., Lünsdorf, H., Fuchs, B. H., & Tindall B. J. (2009). The photosynthetic apparatus and its regulation in the aerobic gammaproteobacterium *Congregibacter litoralis* gen. nov., sp. nov. *PLoS One* **4**, e 4866.

Stackebrandt, E., Murray, R. G. E. & Trűper, H. G. (1988). *Proteobacteria* classis nov., a name for the phylogenetic taxon thatincludes the ‘‘purple bacteria and their relatives’’. *Int J Syst Bacteriol* **38,** 321–325.

Swofford, D.L. (2002). PAUP* Phylogenetic analysis using parsimony (* and other methods). Sunderland, MA: Sinauer Associates.

Takeuchi, M., Sakane, T., Yanagi, M., Yamasato, K., Hamana, K., & Yokota, A. (1995). Taxonomic study of bacteria isolated from plants:proposal of *Sphingomonas rosa* sp. nov., *Sphingomonas pruni* sp. nov., *Sphingomonas asaccharolytica* sp. nov., and *Sphingomonas mali* sp. nov. *Int J Syst Bacteriol* **45,** 334–341.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24,** 1596-1599.

Tenreiro, S., Nobre, M. F., Rainey, F. A., Miguel, C., & da Costa, M. S. (1997). *Thermonema rossianum* sp. nov., a new thermophilic and slightly halophilic species from saline hot springs in Naples, Italy. *Int J Syst Bacteriol* **47**, 122–126.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25,** 4876–4882.

Urios, L., Agogué, H., Intertaglia, L., Lesongeur, F., & Lebaron, P. (2008). *Melitea salexigens* gen. nov., sp. nov., a gammaproteobacterium from the Mediterranean Sea. *Int J Syst. Evol Microbiol* **58,** 2479 - 2483.

Urios, L., Intertaglia, L., Lesongeur, F., & Lebaron, P. (2008). *Haliea salexigens* gen. nov., sp. nov., a member of the *Gammaproteobacteria* from the Mediterranean Sea. *Int J Syst Evol Microbiol* **58,** 1233–1237.

Urios, L., Intertaglia, L., Lesongeur, F., & Lebaron, P. (2009). *Haliea rubra* sp. nov., a member of the *Gammaproteobacteria* from the Mediterranean Sea. *Int J Syst Evol Microbiol* **59,** 1188 - 1192.

Vacelet, J. & Donadey, C. (1977). Electron microscope study of the association between some sponges and bacteria. *J Exp Mar Ecol* **30**, 301–314.

Vaisman, N. & Oren, A. (2009). *Salisaeta longa* gen. nov., sp. nov., a red, halophilic member of the Bacteroidetes. *Int J Syst Evol Microbiol* **59,** 2571 – 2574.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E., Stackebrandt, E., Starr, M. P.,

& Truper, H. G. (1987). Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Int J Syst Bacteriol* **37,** 463 - 464.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173,** 697–703.

Wilkinson, C. R. (1978). Microbial associations in sponges. III. Ultrastructure of the in situ associations in coral reef sponges. *Mar Biol* **49**, 177–185.

Worliczek, H. L., Kämpfer, P., Rosengarten, R., Tindall, B. J., & Busse, H. J. (2007). Polar lipid and fatty acid profiles-re-vitalizing old approaches as a modern tool for the classification of mycoplasmas? *Syst Appl Microbiol* **30,** 355–370

Woese, C. R., Weisburg, W. G., Hahn, C. M., Paster, B. J., Zablen, L. B., Lewis, B. J., Macke, T. J., Ludwig, W., & Stackebrandt, E. (1985). The phylogeny of purple bacteria: the gamma subdivision. *Syst Appl Microbiol* **6,** 25–33.

Woese, C. R. (1987). Bacteriael volution. *Microbiol Rev* **51**,221-277.

Woese, C. R. (1987). Prokaryotic systematic: The evolution of a science. In *the Prokarytotes* 2 nd. edn (Ed. by Balow, A., Triiper, H. G., Dworkin, M., Harder, W. & SchleiferK, H.). Springer-Verlag, 3-18

Xie, C. & Yokota, A. (2003). Phylogenetic analysis of *Lampropedia hyalina* based on the

16S rRNA gene sequence. *J Gen Appl Microbiol* **49,** 345–349.

Xu, X-W., Wu, Y-H., Wang C-H., Gao, X-H., Wang, X-G., &Wu, M. (2010). *Pseudoalteromonas lipolytica* sp. nov., isolated from the Yangtze River estuary. *Int J Syst Evol Microbiol,* **60,** 2176 - 2181.

Yabuuchi, E., Yano, I., Oyaizu, H., Hashimoto, Y., Ezaki, T., & Yamamoto, H. (1990). Proposals of *Sphingomonas paucimobilis* gen. nov. and comb. nov., *Sphingomonas parapaucimobilis* sp. nov., *Sphingomonas yanoikuyae* sp. nov., *Sphingomonas adhaesiva* sp. nov., *Sphingomonas capsulata* comb. nov., and two genospecies of the genus *Sphingomonas*. *Microbiol Immunol* **34,** 99–119.

Yamamoto, S. & Harayama, S. (1995). PCR amplification and direct sequencing of gyrB genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl EnvironM icrobio1*. **61,** 1104 -1109.

Yan, S., Fuchs, B. M., Lenk, S., Harder, J., Wulf, J., Jiao, N.-Z., & Amann, R. (2009). Biogeography and phylogeny of the NOR5/OM60 clade of *Gammaproteobacteria*. *Syst Appl Microbiol* **32,** 124–139.

Yoon, J. H., Lee, M. H., Kang, S. J., Lee, S. Y., & Oh, T. K. (2006). *Sphingomonas dokdonensis* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **56,** 2165–2169.

Yoon, J., Yasumoto-Hirose, M., Matsuo, Y., Nozawa, M., Matsuda, S., Kasai, H., &

Yokota, A. (2007). *Pelagicoccus mobilis* gen. nov., sp. nov., *Pelagicoccus albus* sp. nov. and *Pelagicoccus litoralis* sp. nov., three novel members of subdivision 4 within the phylum *'Verrucomicrobia'*, isolated from seawater by in situ cultivation. *Int J Syst Evol Microbiol* **57,** 1377–1385.

Zhang, J., Liu, X., & Liu, S. (2010). *Sphingomonas changbaiensis* sp. nov., isolated from forest soil. *Int J Syst Evol Microbiol* **60,** 790 - 795.

PUBLICATIONS

Sanghwa Park, Susumu Yoshizawa, Kazuhiro Kogure and Akira Yokota (2011). *Oceanicoccus sagamiensis* gen. nov., sp. nov., a gammaproteobacterium isolated from sea water of Sagami Bay in Japan. *J Microbiol.* doi 10.1007/S12275-011-0368-y, In Press.

Sanghwa Park, Susumu Yoshizawa, Kazuhiro Kogure and Akira Yokota (2011). *Rubricoccus marinus* gen. nov., sp. nov., isolated from seawater in the family "*Rhodothermaceae" Int J Syst Evol Microbiol.* doi:10.1099/ijs.0.026294-0 In Press.

Sanghwa Park, Susumu Yoshizawa, Koji Hamasaki, Kazuhiro Kogure and Akira Yokota (2011). *Psychrosphaera saromensis* gen. nov., sp. nov., within the family *Pseudoalteromonadaceae,* isolated from Lake Saroma, Japan. *J Gen Appl Microbiol.* Vol.56, No.6. pp.475-480.

Jaewoo Yoon, Kyoko Adachi, **Sanghwa Park**, Hiroaki Kasai and Akira Yokota (2011) *Aureibacter tunicatorum* gen. nov., sp. nov., a novel marine bacterium isolated from a coral reef sea spuirt, and description of *Flammeovirgaceae* fam. nov. *Int J Syst Evol Microbiol.* doi:10.1099/ijs.0.027573-0. In Press.

Sanghwa Park, Akira Yokota, Takashi Itoh, Choon-soo Im and Jin-Sook Park (2011). *Sphingomonas jejuensis* sp. nov**.,** isolated from marine sponge *Halichondra flava. J. Microbiol.* Submitted.

Sanghwa Park, Susumu Yoshizawa, Kentaro Inomata, Kazuhiro Kogure and Akira Yokota (2011) *Aureimonas marinus* gen. nov., sp. nov., isolated from seawater within the family *Flavobacteriaceae. Int J Syst Evol Microbiol.* Submitted.

Sanghwa Park, Susumu Yoshizawa, Kentaro Inomata, Kazuhiro Kogure and Akira Yokota (2011). *Halicoccus marinus* gen. nov., sp. nov., and *Halicoccus pacifica* sp. nov., isolated from seawater in the family *Dasaniaceae* fam. nov., within the class *Gammaproteobacteria Int J Syst Evol Microbiol.* Submitted.

論 文 の 内 容 の 要 旨

応用生命工学　専攻
平成２０年度博士課程　入学
氏　　名　　朴　相和
指導教員名　横田　　明

論文題目

Phylogenetic studies on marine bacteria within the phylum *Proteobacteria* and *Bacteroidetes*

（*Proteobacteria* 門及び *Bacteroidetes* 門に属する海洋細菌の系統分類に関する研究）

The oceans occupy 71% of earth's surface, with a volume of 1.46×10^9 Km^3 and average depth of 4,000 m, and maximal depth of approximately 11,000 m. The main problem in studying species distribution of on marine bacteria lies in the methods used to obtain viable cultures. Whether the isolates obtained by current culture methods represent indigenous population is unknown. Determining the representative physiological studies that can be performed on the vast numbers of marine bacteria that have yet to be cultured is still a matter of guesswork (Schute et al.,1993). Marine sponges are the most ancient multicellular animals, dating back to the Precambrian period, more than 635 million years ago. Their natural product diversity is among the highest found in nature. There is growing evidence that bacterial symbionts play a crucial role as producers of sponge-derived metabolites. In many sponge species, termed "high microbial abundance" (HMA) sponges, up to half the biomass can consist of microbial symbionts, whereas "low microbial abundance" (LMA) species,

which live in the same habitats, contain much fewer symbionts. Often, distantly related HMA sponges from different oceans share remarkably similar microbial communities.

In this study I attempted to elucidate the taxonomic position of nine novel marine bacterial strains that were isolated from marine seawater and marine sponges within the phylum *Proteobacteria* and *Bacteroidetes* by using polyphasic taxonomic approach.

The phylogenetic trees based on the neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) generated comparisons of the 16S rRNA gene sequences revealed individual taxonomic positions. Also molecular (DNA-DNA hybridization test and determination of the DNA G+C content), physiological (API 20E, API 20NE, API 50CH and API ZYM tests) and biochemical and chemotaxonomic analyses (determination of respiratory quinone system and cellular fatty acid content) were investigated on the novel isolates.

As results, six of them were determined to be four new species of three novel genera (*Oceanicoccus, Psychrosphaera, Halicoccus*) in the *Gammaproteobacteria*. Individualy, it was concluded that strains SA4-31, SA4-46 and SA4-48^T should be classified as representing a new genus and species of the family *Pseudoalteromonadaceae,* for which the name *Psychrosphaera saromensis* gen. nov., sp. nov. is proposed. Strain PZ-5^T represents a novel genus and species, for which the name *Oceanicoccus sagamiensis* gen. nov., sp. nov., is proposed. Two strains S1-36 ^{T} and S1- 72^T present a novel genus and two species as *Halicoccus marinus* and *H. pacifica* were proposed. The genus *Oceanicoccus* and the genus *Halicoccus* formed a cluster with genera *Spongiibacter, Melitea, Dasania, Haliea* and *Congregibacter* at a family level, for which the name *Dasaniaceae* the Class *Gammaproteobacteria,* is proposed (Fig. 1). Two strains SG-29^T and S1-66^T were determined to be two species of two novel genera (*Rubricoccus, Aureimonas*) belonging to the phylum *Bacteroidetes.* Also phylogenetic position based on 16S rRNA gene sequence, molecular, physiological and biochemical and chemotaxonomic analyses and cellular fatty acid content) were investigated. Strain SG-29^T was classified as a novel genus and species, for which the name *Rubricoccus marinus* gen. nov., sp. nov., within family '*Rhodothermaceae*', is proposed. Strain S1-66^T represents a species of novel genus as *Aureimonas marina* was proposed.

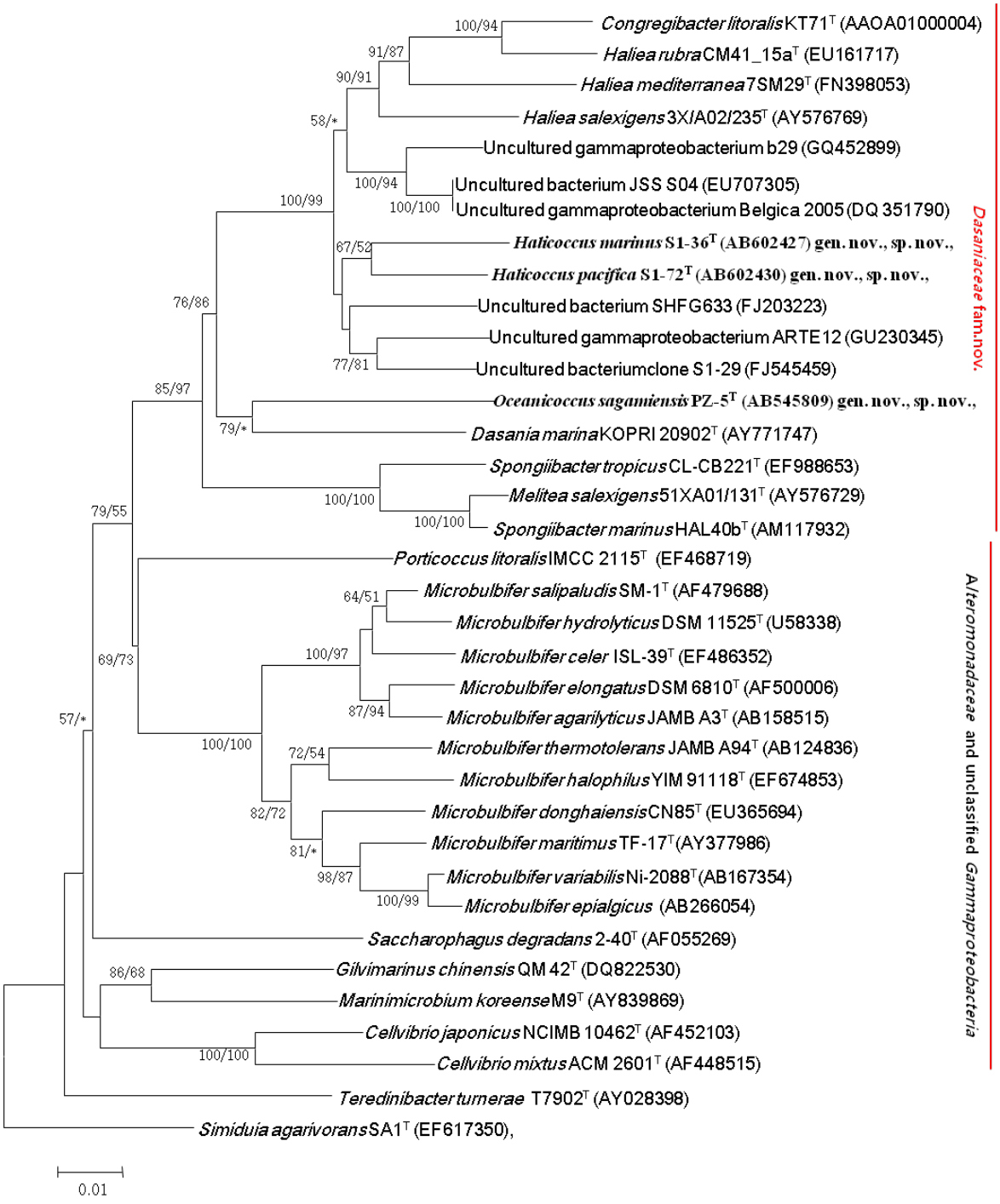


Figure 1. Neighbour-joining(NJ) phylogenetic tree based on the 16S rRNA gene sequence showing the position of strain S1-36^T among the currently known and related species of the class *Gammaproteobacteria*. Numbers at nodes are bootstrap percentages derived from 1000 replications (NJ/ML). Sequences of *Simiduia agarivorans* SA1^T (EF617350) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position. " * " indicates recovered nodes with <50% bootstrap values in the Maximum-likelihood(ML) tree.

The other hands, about 300 colonies were isolated from about 20 species of marine sponge were investigated to find novel bacterial strains using medium P and SN. On the medium P screen results (107 colonies), 27 genera in the 5 classes were isolated from marine sponge. On the medium SN screen results (132 colonies), 29 genera in the 4 classes were obtained. Interestingly I didn't find any strain belong to the phylum *Bacteroidetes* and the other class that previously reported using culture independent methods. But I obtained some 6 candidates showed less than 96% partial 16S rRNA gene sequence similarity were re-sequenced of full 16S rRNA gene sequences and compared to data base information (Genbank) also respectively phylogenentic tree of six candidates were constructed. Finally strain MS-31^{T} isolated from marine sponge *Hymenicacidon flavia* was classified as *Sphingomonas jejuensis* sp. nov., in the *Alphaproteobacteria*.

I attempted to elucidate the phylogenetic position of nine novel isolates using a polyphasic taxonomic approaches 16S rRNA gene sequence, physiological, biochemical and chemotaxonomic analyses to characterize the novel strains. Based on the data it is present that these isolates represent 7 species of the 5 genera in the phylum *Proteobacteria* and *Bacteroidetes*