博士論文（要約）

論文題目　**Physiological and genetic characteristics of deep-sea bacteria**

（深海細菌の生理的、遺伝的特性に関する研究）

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Abstract

The deep sea is referred to as the ocean below 1,000 m depth and is characterized by high hydrostatic pressure, low temperature, low nutrients and the absence of sunlight. Although it is regarded as one of the extreme environments on the Earth, generally $10^{3\text{-}4}$ cells/mL of prokaryotes are found in entire deep-sea water. It has been expected that those microorganisms might have community structures and characteristics that are unique to the environment and allow them to survive and grow under such conditions. Recent application of molecular techniques to deep-sea microorganisms made it possible to clarify the community structures and genetic characteristics without depending on culture techniques. However, such genetic information does not offer meaningful information unless we have actual cultured strains. Our knowledge on such physiological characteristics, their functions or ecological implications is quite limited, primary due to the paucity of cultures isolated from the deep sea. Therefore, most works have been conducted with very few culturable groups, typically, *Gammaproteobacteria*. It is critical to apply some new technique to isolate more diverse groups of microorganisms and obtain information with those strains. It is then possible to clarify how they respond to the deep-sea environmental conditions and what kind of gene is involved in actual microbial processes for the growth and survival in the extreme environment.

The purpose of this thesis was to clarify physiological and genetic characteristics of deep-sea bacteria in comparison with their surface-sea relatives by using culture-dependent and independent approach, physiological examination and genetic analyses. In order to expand our knowledge, newly isolated strains were used. For this purpose, first, the isolation of deep-sea prokaryotes in diverse phylogenetic groups was tried using

newly designed culture media. Second, culture independent approach was taken to investigate the vertical community structures in the north-western Pacific Ocean. This clarified the phylogenetic and distributional position of my new isolates. Third, the physiological and genetic characteristics of the deep-sea isolates were investigated in comparison with phylogenetically relatives isolated from the surface environments. Whole genome of 7 strains were sequenced and used for the analyses.

The major contents of each chapter are as follows. In chapter 2, total 681 isolates were obtained from the deep-sea water in north-western Pacific Ocean using 1/5 marine agar 2216, 1/10 R2A agar and natural seawater liquid medium. 16S rRNA gene sequences of them revealed their phylogenetic positions. All the deep-sea isolates belonged to the domain *Bacteria* and none for *Archaea*. Among the isolates, strains of phyla *Verrucomicrobia* and *Lentisphaerae* were the first isolates in the phyla from the deep sea. Strains of orders *Arenicellales*, *Thiotrichales*, *Cellvibrionales*, *Kiloniellales* and *Acidimicrobiales* were also the first isolates within the orders. Strains affiliated to 22 genera were considered as novel deep-sea species. Among them, *Rubrivirga marina*, *Rubrivirga profundi*, *Aurantivirga profunda*, and *Lentisphaera profundi* were validated after taxonomical investigations and reported as novel deep-sea species. Approximately 90 % of the identified isolates showed the similarity to the strain isolated from the surface with more than 99 % 16S rRNA sequence similarity, suggesting that the majority of the deep-sea bacterial isolates may have closely related strains in the surface layer.

In chapter 3, the vertical community structures of bacteria in two water columns were investigated using pryoseqeuncing technique for clarifying the presence of depth related groups and also differentiating particle associated (PA) and free living (FL) state. Among the phylotypes affiliated with the deep-sea isolates, *Erythrobacter* phylotypes

were detected in all depths. *Sulfitobacter*, *Paracoccus*, *Sphinogomonas*, *Colwellia*, *Alcanivorax*, *Marinobacter*, *Alteromonas*, *Moritella* and *Rubritalea*-like phylotypes were more retrieved from the deeper layers than the surface layer. Most of the phylotypes affiliated with the deep-sea isolates showed preference toward PA state. PA state suggests the tendency to attach particles and/or to colonize easily. Also, it suggests the possibility to attach sinking particles that are originating in upper water column. In addition, SAR11 and *Sphingomonadales* of *Alphaproteobacteria*, and *Bacteroidetes* were vertically cosmopolitan. *Deltaproteobacteria*, *Deferribactere*, *Planctomycetes*, *Actinobacteria* and *Nitrospirae* were confirmed as specific bacterial lineages in the deep layers. SAR11, *Chromatiales* of *Gammaproteobacteria*, SAR324 of *Deltaproteobacteria*, *Nitrospirae* and *Deferribactere* were found to be more as FL state in the deep sea. *Sphingomonadales* and *Alteromonadales* of *Proteobacteria*, *Planctomycetes*, *Bacteroidetes*, *Lentisphaerae* and *Verrucomicrobia* were more as PA state in the deep sea.

In chapter 4, growth characteristics, cellular membrane composition and hydrolytic enzymes of eight strains within phyla *Proteobacteria*, *Verrucomicrobia* and *Bacteroidetes* were tested in combination with their "surface relatives" to clarify the characteristics of deep-sea bacteria. All the isolates showed decreasing growth at a higher pressure than atmospheric pressure, indicating that they are non-piezophiles. Of the 8 strains, only *Rubritalea* sp. SAORIC-165 of the phylum *Verrucomicrobia* showed optimum growth at 10°C and no growth above 20°C, indicating that the strain is psychrophilic and probably staying in the deep-sea for long time. *Erythrobacter* sp. SAORIC-644 and *Limnobacter* sp. SAORIC-580 showed optimum NaCl concentration at 1 and 0 %, suggesting the origin of low salinity environment. The deep-sea strains commonly contain higher numbers of phospholipids, compared to their surface-relatives. The additional

phospholipids may allow the deep-sea strains to maintain the fluidity of cellular membrane under high pressure.

In chapter 5, whole genome of 7 strains within phyla *Proteobacteria* and *Verrucomicrobia* were sequenced and their genetic features were examined in comparison with those of the surface relatives. Comparisons with metagenome data were also made for genes that appeared unique to the deep-sea. The strains, of which group prefer PA state, contained genes encoding for pili assemble or adherence proteins (FAS1 and von Willebrand A domain), suggesting that the genes are supportive in attachment processes. Some deep-sea strains (more than 3 strains) showed the unique presence or more than 1.5 folds abundance in the numbers of the following genes (51 genes), compared to their surface-sea relatives. These genes were related to respiration, stresses response, cellular structure, metabolism of in- and organic substrates, replication and transcription. Of 51 genes, 39 genes were over-represented in deep-sea metagenomic data, compared to surface-sea metagenomics data. Some of the genes were related with response in high pressure and low temperatures. Although further works are required, genetic (pili, flagella, adhesion proteins and abundant 51 genes) characteristics of the deep-sea isolates appear to support growth and survival in the-deep sea environment.

In conclusion, bacteria from diversified phylogenetic groups were obtained from the deep sea for the first time. Some of them were investigated taxonomically, physiologically and genetically by recent whole-genome sequencing. In addition, their preference to either PA or FL life style was investigated. Their physiological and genetic characteristics allowed to consider their ecology and evolutionary processes as well. In particular, amino acids and lipids metabolism, and osmotic reguration of deep-sea bacteria were newly demonstrated in this study. Further investigation on the isolation and

characterization of more deep-sea bacteria will offer clues to better understand the nature of the deep-sea prokaryotes.

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CHAPTER 1.

General introduction

Feature of the deep-sea

The deep sea is referred as sea below 1000 m depth (Jannasch and Taylor, 1984). It is characterized by high hydrostatic pressure, low temperatures, no sunlight and low nutrients. The ambient hydrostatic pressure increases by 1 atm per 10 m. So, for instance, the ambient hydrostatic pressure at 3000 m is 300 atm. Another unit of pressure, pascal (Pa), defined as one newton per square meter, is widely used for the research in deep-sea environments. One atm corresponds to approximately 0.1 MPa. This unit will be used entirely in this thesis. The average temperature of the deep sea is 2-3℃, except for deep-sea hydrothermal vents. Dissolved organic carbon (DOC) concentration decreases with increasing depth down to approximately 1000 m, below which the concentration is relatively constant. At 1,000 m, DOC is roughly 0.5 mgC/L which is about 50-60 % of those in the surface layer (Ogawa and Tanoue, 2003). In most of ocean, less than 1 % of sunlight penetrates to 100 m depth. Below the euphotic zone which is defined as the zone receiving enough light for primary production, the organic matter available for heterotrophic organisms are supplied from the upper layer. Therefore, downward flux of particulate organic matter from the surface is the major control factor for the biomass and community structures of living organisms in deep-sea (Ducklow *et al*., 2001; Arístegui *et al*., 2002).

Biomass and community structures of prokaryotes in the deep sea

In spite of the extreme conditions, generally $10^{3\text{-}4}$ cells/mL of prokaryotes exist in entire deep-seawater (Morris *et al*., 2002). It is estimated that the number of aquatic prokaryotic cells below 1,000 m depth is approximately 5.1×10^{28} cells and in deep-sea sediments, approximately 2.8×10^{30} cells, comprising one third of the total living biomass on Earth

(Whitman *et al*., 1998). Moreover, they are even found in seawater at 11,000 m or the deepest depths of the entire ocean (Kato *et al*., 1998).

Numerous efforts have been made to elucidate what kind of prokaryotes are present in the deep sea with various approaches. Before molecular techniques were introduced in early 90s, cultivation and identification of isolates were the sole approach. Members belonging to orders of *Alteromonadales*, *Vibrionales* and *Oceanospirillales* in the class *Gammaproteobacteria* have been often recovered (Yayanos *et al*., 1979; Jannasch and Wirsen, 1984; DeLong and Yayanos, 1985; Kato *et al*., 1995; Nogi *et al*., 1998a; Nogi *et al.,* 1998b; Radjasa *et al*., 2001; Cao *et al*., 2014). Members of orders of *Sphingomonadales* and *Rhodobacterales* in the class *Alphaproterobacteria* have been also isolated from deep-sea environment (Gärtner *et al*., 2011). Besides them, members of phyla *Firmicutes* and *Bacteroidetes* were also reported (Gärtner *et al*., 2011; Hwang *et al*., 2015). Because only a portion of deep-sea bacteria are recovered on culture media, it is difficult to assume actual community structures using culture techniques. However, the great advantages are that the isolates can be used for further analyses of their physiology, biochemistry, phylogeny, genetics and so on. Therefore, culture-based approach still has significance for any biological investigations of deep-sea prokaryotes.

The culture-independent approaches based on molecular techniques were first introduced to marine microbiology in early 90s, then the prokaryotic community structures in the deep sea have been intensively investigated. Generally, members of the domain *Archaea* and classes *Alphaproteobacteria* and *Gammaproteobacteria* within the domain *Bacteria* constitute to the majority of deep-sea prokaryotic population (Karner *et al*., 2001; Jing and Ying, 2008). Recent research using the second generation sequencing technology reveled that deep-sea prokaryotic communities are far more diverse than

previously anticipated (Sogin *et al*., 2006; Martín-Cuadrado *et al*., 2007; Eloe *et al*., 2010; Zinger *et al*., 2011; Wang *et al*., 2011).

Factors controlling prokaryotic community structure in the deep sea

In aquatic ecosystems, the community structure is formed as a relative balance between growth and death, and also inflow and outflow of the cells from the system. As for the growth, it is controlled by both physicochemical and biological factors. As is described above, in the deep sea, the cells possessing tolerance to high pressure, low temperature and low nutrient conditions may be able to grow and maintain their population. In addition, quantity, quality and form (dissolved or particulate) of organic compounds are important factors to select specific groups. Virtually, most parts of dissolved organic matter in the deep sea are characterized by low concentration, unknown chemical structure, relatively low molecular weight, and refractory nature. Therefore, only selected groups that are adaptable to these nutritional conditions should grow and survive. As was discussed above, fresh organic matter is usually supplied by the sinking particles from the upper water column (Volk and Hoffert, 1985). As sinking particles are usually colonized by prokaryotes, they are also important as a mechanism to transport prokaryotes from surface to deep layers (Hansel and Ducklow, 2003; Vezzi *et al*., 2005). It is estimated that 10^{10}–10^{12} cells m^{-2} y^{-1} may be transferred to the deep-sea by this way (Turley and Mackie, 1994).

In aquatic environments, predation by flagellates or viruses may be the major death processes of prokaryotes (Danovaro and Serresi, 2000; Anantharaman *et al*., 2014; Morgan-Smith *et al*., 2013). However, very limited investigations have been so far made for predations in the deep sea.

Physiological characteristics of deep-sea prokaryotes

The deep sea is regarded as one of the extreme environments and its features suppress diverse biological processes, causing depression of physiological responses such as metabolic processes or growth rate (Wirsen and Molyneaux, 1999). The pressure generally affects the interactions among high molecular weight subunits and also DNA hydrogen bonds, resulting in difficulty of metabolic processes, replication and transcription (Gross and Jaenicke, 1994; Macgregor, 2002). High pressure leads to the formation of crystalline lipids in cell membrane, which causes lower cell membrane fluidity, making the membrane impermeable to water and other molecules (Braganza and Worcester, 1986; Bartlett, 2002). Therefore, deep-sea prokaryotes tend to have higher proportion of unsaturated fatty acids in their cytoplasmic membranes, allowing the membranes to remain functional at high pressure or at low temperature (Bartlett, 2002; Feller and Gerday, 2003; Siddiqui and Cavicchioli, 2006). Furthermore, high pressure may inhibit motility and cell division of *Escherichia coli* (Bartlett, 2002). Prolonged starvation are reported to cause a reduction of RNA, total lipids and protein content (Mukamolova *et al*., 1995).

Although high pressure constrains normal bacteria's growth, particular groups of deep-sea bacteria show optimum growth at higher pressure than atmospheric pressure. They are called as piezophile (from Greek verb *piezo*, to press). So far, 38 piezophiles have been identified. They belong to genera *Colwellia*, *Moritella*, *Pyschromonas* and *Shewanella* of the order *Alteromonadales* and genus *Photobacterium* of the order *Vibrionales* (Eloe *et al*., 2011b). Some of them do not grow at atmospheric pressure (obligate piezophile), whereas some of them can grow (facultative piezophile or piezotolerant). As for low temperature, most of the piezophilies and particular deep-sea

bacteria exhibited optimum growth below 15℃, and no growth above 20℃. This group is called psychrophile (from Greek verb *psychro*, to cold). Seo *et al*. (2005) and Xiao *et al*. (2007) reported the deep-sea psychrophiles affiliated with genera *Shewanella* and *Photobacterium*. Recently, Hwang *et al*. (2015) first reported a deep-sea psychrophile within the phylum *Bacteroidetes*.

Genetic characteristics of deep-sea prokaryotes

Traditionally, genetic characteristics of deep-sea prokaryotes have been introduced by transcriptional observation. It has been reported that respiratory system (cytochrome C and NADH; Yamada *et al*., 2000; Kato and Qureshi, 1999), outer membrane porin (*ompH*; Bartlett *et al*., 1989), DNA replication and cell division (Campanaro *et al*., 2005) seem to function more under high hydrostatic pressure.

Recent databases of genetic information have facilitated the understanding of the nature of deep-sea prokaryotes. One is a database of metagenomes or information on microorganisms in natural environments, such as Tara Ocean (http://ocean-microbiome.embl.de/companion.html) or iMicrobe (https://www.imicrobe.us/). Another is whole genome of living organisms. For instance, NCBI comprises huge datasets which include genome information of prokaryotes. Currently, nearly 100 thousands sequences are available at NCBI database (https://www.ncbi.nlm.nih.gov/genome/browse/). Although data for deep-sea prokaryotes is rather limited, those information help to understand their unique characters.

The biased presence of particular genes or functions in deep layers compared with surface layers strongly suggests that those genes or functions are important for the microorganisms present in the deep sea. Also abundance of particular genes on genomes

of deep-sea prokaryotes, compared with those of surface dwelling prokaryotes, may suggest the potential importance of those genes among deep-sea prokaryotes. For instance, mobile elements and diverse hydrolytic enzymes were found in deep-sea data of the North Pacific Ocean (Konstantinidis *et al*., 2009). Deep-sea bacterial genomes contained diverse peptidases and amino acid uptake systems for hydrolyzing and metabolizing organic substrates (Hou *et al*., 2004; Wang *et al*., 2008; Qin *et al*., 2010). Furthermore, the deep-sea bacterial genomes showed higher numbers of signal transduction and flagella biosynthesis genes, compared to their surface sea counterparts. It is suggested that the genes facilitate quick response to sporadic influxes of nutrients supplied via sinking particles (Lauro and Bartlett, 2008; Qin *et al*., 2010).

Limitations

Our knowledge on the characteristics, functions or ecological implications of deep-sea prokaryotes is still quite limited. There are several reasons. First, evidently, the sampling itself is not easy. It requires the cruise to the open ocean area and devises to collect seawater samples from deep layers. If one wishes to isolate piezophiles, special sampling and incubation devices to maintain the ambient hydrostatic pressure are required. Obviously, the cost for those instruments is not small. These situations results in the presence of relatively few researcher in this field.

Second, the indigenous microbial population has extremely long generation times (> 600 hr) under *in situ* pressure and nutrient conditions (Wirsen and Molyneaux, 1999). Hence, it takes long time to treat deep-sea isolates to obtain enough cell biomass. If growth characteristics that enable high growth rates are found, we will be able to obtain more information on various deep-sea microorganisms. Therefore, it is necessary to work

with deep-sea strains for clarifying their optimum growth condition, physiological responses to environmental conditions, and specific genes that are involved in various physiological responses.

Finally, although culture independent approaches shows the presence of prokaryotes of various phylogenetic groups, most of actual isolates from deep-sea fall into classes *Alphaproteobacteria* and *Gammaproteobacteria* (Karner *et al*., 2001; Jing and Ying, 2008). This makes it difficult to investigate the representative strains to elucidate their characteristics. For instance, so far only about 50 genomes of prokaryotes have been subjected at NCBI database. Except for the ones from hydrothermal vents, which are not common deep-sea habitat, the prokaryotic genomes derived from deep-seawater or sediment are only 27 genomes. This situation would be ascribed to the few number of isolates from deep-sea and also their biased compositions.

One of the reasons of this biased isolation may be the use of "traditional" media. Typical media such as ZoBell 2216E for marine microorganisms contain nearly 4g/L carbon compounds that far exceed natural concentration (approx. 1mgC/L). Only the microorganisms that can tolerate such drastic change may be able to start growing. It is also doubtful whether the composition is suitable for many microorganisms. Therefore, examination of culture media and condition should be investigated to isolate those that have never been appeared.

The purpose of this doctoral thesis

It is assumed that there are huge number of unknown microorganisms present in the deep sea and they have physiological and genetic characteristics which are different from those in surface layers. Therefore, I had raised the following questions;

1. Which of deep-sea prokaryotes are culturable?
2. What taxonomical position they may possibly have? And are there any new isolates from the deep sea?
3. What about their actural distribution in deep-sea environment?
4. Are they mainly in particles-associated or free living state? This is because particles can be important organic source for deep-sea bacteria.
5. What kind of physiological and genetic characteristics do they have in comparison with those in surface layers?
6. Are there any key functions that make prokaryotes live and survive in the deep sea?

Based on answering these questions, the purpose of this doctoral thesis is to clarify physiological and genetic characteristics of deep-sea bacteria in comparison with their surface-sea relatives by using culture-dependent and independent approaches, physiological examination and genetic analyses. First, I tried to isolate strains from the deep sea by culture dependent approach and checked the taxonomical positions of new isolates (Chapter 2). Subsequently, I described some new species among them (Chapter 2). Second, I investigated the vertical community structures of deep-sea bacteria and their particles-associated or free living state (Chapter 3). Third, some physiological characteristics of the deep-sea isolates were checked by conducting biochemical examinations in comparison with close relatives obtained from surface layers (Chapter 4). Fourth, the whole genome information was obtained for the deep-sea isolates and comparisons with those of close relatives from the surface layers were conducted to clarify the genetic characteristics of the deep-sea isolates (Chapter 5). Sixth, key functions for living and surviving in the deep sea were investigated by reffering to the literatures

and metagenomic data (Chapter5). Finally, general discussion was made for this thesis (Chapter 6).

CHAPTER 2.

Isolation and phylogenetic position of deep-sea bacteria in the north-western Pacific Ocean

Introduction

In order to clarify the distribution, possible ecological contribution and physiological characterizations of microorganisms, there are two major approaches, i.e., culture dependent and independent ones. Because only a small portion of prokaryotes are recovered by any culture methods available at present, culture independent approach is indispensable for investigations of distribution and genetic analyses. One of the most remarkable successes of this approach is the finding of wide distribution of the SAR11 group. This group is present not only in surface layers but also in deep-sea environments (Morris *et al*., 2002; Eloe *et al*., 2010). However, actual behaviour, responses to environmental factors, growth characteristics and physiological features are only available by isolates. Hence, numerous efforts to isolate prokaryotes from the deep-sea have been made. Resultant deep-sea isolates were mainly assigned to orders *Alteromonadales* and *Vibrionales* within the domain *Bacteria* and they have been well characterized (Yayanos *et al*., 1979; Jannasch and Wirsen, 1984; DeLong and Yayanos, 1985; Kato *et al*., 1995; Nogi *et al.,* 1998b). However, the features of orders *Alteromonadales* and *Vibrionales* could not represent the characteristics of deep-sea prokaryotes, since they accounted for less than 1 % of deep-sea bacterial populations (Eloe *et al*., 2010). Except for the members of the orders, most of deep-sea prokaryotes remain uncultured and poorly understood. In particular, the deep-sea member of the SAR11 group and the class *Deltaproteobacteria* have not been cultured yet, members of which making up for a far large proportion of the deep-sea bacterial populations (Martín-Cuadrado *et al*., 2007; Eloe *et al*., 2010),

One of the reasons of lack of isolates is due to the high concentration of organic carbons in culture media. The marine broth 2216 (MA) or 1/2 MA have been widely used

to isolate prokaryotes including for deep-sea ones (Kato *et al*., 1995; Radjasa *et al*., 2001; López-López *et al*., 2005). These media containing high concentration of organic carbons that far exceed the level in marine environments (Button *et al*., 1998; Connon and Giovannoni, 2002). Therefore, application of much lower carbon media would be necessary for the cultivation of prokaryotes in oligotrophic environments including the deep sea. In addition, it is expected that lower carbon media will retard growth of the members of orders *Alteromonadales* and *Vibrionales* which generally grow fast in rather high concentrations of carbon.

Then, what kinds of lower carbon media are available for deep-sea cultures? For cultivating prokaryotes in surface layer, the dilution to extinction culture method based on liquid culture medium with low carbon content (NSLM) has succeeded to culture numerous bacterial groups that had never been cultured before (Rappé & Giovannoni, 2003; Cho *et al.*, 2004a; Cho *et al*., 2004b). Eloe *et al*. (2011b) have applied NSLM with high pressure in the deep-sea microbial cultivation and succeeded in isolating a novel piezophilic *Alphaproteobaterium* phylogenetically close to the SAR11 group. Hence, NSLM would be suitable to culture a deep-sea member of the SAR11 group.

The use of 1/10 R2A media has also yielded many novel isolates in cultivation from marine environments. For instance, *Opitutales* proposed as a novel order within the phylum '*Verrucomicrobia*' (Choo *et al*., 2007), oligotrophic marine *Gammaproteobacteria* group (Cho *et al*., 2004b; Kim *et al*., 2007) and several novel species within *Betaproteobacteria* from a fresh water lake (Song *et al*., 2007). However, there is no report on application to the deep-sea environments. In addition, 1/5 MA was newly designed for this research.

Here, I aimed to obtain diverse prokaryotic isolates from the deep-sea and identify

their phylogenetic position for further studying. I collected 9 deep-seawater samples from various locations in the north-western Pacific Ocean from a range of 1000 to 4000 m depth. For isolating diverse prokaryotes, NSLM, 1/5 marine agar (MA) and 1/10 R2A agar, of which carbon content is lower than MA or 1/2 MA, were applied under atmospheric pressure.

Materials and methods

Samples and isolation

Deep-seawater samples were obtained from the north-western Pacific Ocean during 4 research cruises (KT10-25 and KT12-08 by R/V Tansei Maru, JAMSTEC, and MR11-02 and MR-11-05 by R/V Mirai, JAMSTEC) shown in Table 2-1 and Figure 2-1. Niskin water samplers equipped with a conductivity, temperature, and depth (CTD) device were used to collect seawater samples. Collected seawater samples were immediately stored in darkness at 4℃ until the further inoculation. Total cells of each seawater samples were stained with 4, 6-diamidino-2-phenylindole (DAPI) and counted via epifluorescence microscopy (Nikon 80i, Nikon, Japan).

A portion of 150 μl of each seawater sample was inoculated on 7 plates of 1/5 strength Marine agar 2216E (1/5 MA; BD Difco) (Agar 15 g, Peptone 1 g, Yeast extract 0.2 g, $MgCl_2$ 1.76 g, Na_2SO_3 0.65 g, $CaCl_2$ 0.36g, KCl 0.1 g, $NaHCO_3$ 0.32 g, Ferric citrate 0.2 g, KBr 0.016 g, $SrCl_2$ 6 mg, H_3BO_3 4 mg, Na_2HPO_4 1.6 mg, Na_2SiO_3 0.8 mg, NaF 0.48 mg, NH_4NO_3 0.32 mg in 1 L of 80 % aged seawater) and 1/10 strength marine R2A (1/10 R2A; BD Difco) (Agar 15 g, Peptone 0.05 g, Yeast extract 0.05 g, Casamino Acids 0.05 g, dextrose 0.05 g, Soluble starch 0.05 g, Sodium pyruvate 0.03 g in 1 L of aged seawater). Subsequently, the plates were incubated at 10℃. After incubation for 4

weeks in darkness, all of colonies were transferred on new 1/5 MA or 1/10 R2A and maintained at 10℃. The culture with NSLM was prepared using the protocol by Connon and Giovannoni (2002) after slight modification. NSLM was prepared using deep-sea water filtered by 0.1 μm pore size membranes. The culture medium was supplemented with the following chemicals: 1.0 μM NH_4Cl, 0.1 μM KH_2PO_4, 0.001% (w/v) of D-glucose, D-ribose, glycerol, *N*-acetyl-D-Glugosamine, methylamine, pyruvic acid and ethanol, and a 10^{-4} dilution of a vitamin mixture (Davis & Guillard, 1958). The sampled seawater was diluted to 15-20 cells ml^{-1} with the culture medium and 1ml of that was dispensed into each well of 48-well-polystyrene microtiter plates. After incubation at 10℃ for 8 weeks in darkness, 150 μl of medium in each well was loaded into a custom-made 48-array facilitating filtration with 0.2 μm pore-sized black polycarbonate membranes (48 x 60 mm, Osmonics, USA). After staining with DAPI, the cultured medium was filtered. Cellular growth was checked via epifluorescence microscopy (Nikon 80i, Nikon, Japan). Wells with higher than 2.0 x 10^5 cells ml^{-1} cell densities were considered positive growth, and stored as 10 % (v/v) glycerol suspensions at -80℃ for further analyses. Among the wells, the growth of one more types of organisms in a well is considered a mixed culture.

Phylogenetic position by 16S rRNA gene sequences

DNA was extracted from cells of purified colonies on agar plates or concentrated cells from positive wells in 48-well-polystyrene microtiter plates 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 27F (5'- AGGTTTGA TCCTGGCTCAG -3') and 1492R (5'- GGCTACCTT GTTACGACTT -3') (Lane**,** 1991). PCR reaction was performed by using TaKaRa EX Taq polymerase (Takara, Japan) by

the following condition; one cycle of 95℃ for 5 min, followed by 35 cycles of 95℃ for 30 s and 58℃ for 1 min, followed by 72℃ for 1 min 30s, followed by a final 5-min incubation at 72℃. The PCR products were grouped by restriction fragment length polymorphism (RFLP) analyses employing *HindII* restriction.

Sequencing was performed using primers 27F and 1492R by BigDye Terminator v. 3.1 cycle sequencing kit (ABI). Sequencing products were analyzed by 3730 DNA analyzer (ABI). Sequence data was edited and assembled by using BioEdit software package. To ascertain the phylogenetic position, the resulting 16S rRNA gene sequence of isolates was queried in BLASTn search of GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) and the EzTaxon-e server (Kim *et al.,* 2012). In order to construct phylogenetic trees, multiple sequences alignment was performed using CLUSTAL_X (version 1.83) (Thompson *et al.*, 1997). The aligned sequences without gaps and ambiguous bases were analyzed using MEGA version 7 (Kumar *et al*., 2016) using neighbor-joining (Saitou & Nei, 1987) with Jukes-Cantor correction (Jukes & Cantor, 1969).

Results

Total number of cells in deep-sea water and isolates from the media

Total numbers of cells in each deep-sea water sample are shown in Table 2-1. A total of 681 strains were obtained from 9 deep-sea water samples collected from the north-western Pacific Ocean using 3 kinds of media, 1/5 MA, 1/10 R2A and NSLM. Except for mixed cultures, 617 strains were idendified in this work. Numbers of isolates within classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* of the phylum *Proteobacteria* are shown in Table 2-2, and those within phyla *Bacteroidetes*,

Actinobacteria, *Verrucomicrobia* and *Lentisphaerae* are shown in Table 2-3. Mixed cultures are also shown in Table 2-3 as well.

Deep-sea isolates from KT10-12-S1-3 seawater sample (3000 m)

From KT10-12-S1-3 sample, 69 strains belonging to classes *Alphaproteobacteria* and *Gammaproteobacteria*, and the phylum *Bacteroidetes* were obtained (Table 2-4). Their phylogenetic positions are shown in Figures 2-2 and 2-3. Among the alphaproteobacterial isolates, 14 strains were assigned to the order *Sphingomonadales*. These strains fell into genera *Erythrobacter* (10 strains), *Sphingobium* (3 strains) and *Sphingopyxis* (1 strain). *Erythobacter*-like strains were divided into two subgroups. One subgroup made a monophyletic clade with to the species *E. citreus* RE35F/1^T (>99 %; 16S rRNA gene similarity) isolated from surface seawater in the western Mediterranean Sea (Denner *et al*., 2002). In this study, such phylogenetic position refers to "identical". Another subgroup shows a distinct lineage separated from species *E. citreus* and *E. pelagi* with less than 97 % 16S rRNA gene similarity. *Sphingobium*-like strains were highly close to the species *S. abikonense* NBRC 16140^T (>99 %) isolated from oil-contaminated soil (Kumari *et al*., 2009). *Sphingopyxis*-like strain was close to the species *Sphingopyxis baekryungensis* IAM 12404^T (98.3 %) isolated from surface seawater (Yoon *et al*., 2005). Of 14 strains within the order *Sphingomonadales*, 12 strains were highly close to surface-derived strains with >99 % of 16S rRNA gene similarity. Nine strains assigned to the order *Rhodobacterales* fell into 3 genera, *Oceanicola* (7 strains), *Sagittula* (1 strain) and *Loktanella* (1 strain). *Oceanicola*-like strains were almost identical to *O. nanhaiensis* DSM 18065^T originating from deep-seawater (1100 m) in the Pacific Ocean as well as strains isolated from surface seawater (KP639144). Strains affiliated with *Sagittula* and *Loktanella* were identical to species *S. stellata* E-37 (99.3 %) and *L. aestuariicola* J-TF4^T

(99.1 %), which were isolated from costal environments (Gonzalez *et al*., 1997; Park *et al*., 2014). Other alphaproteobacterial isolates were affiliated with genera *Aurantimonas* of the order *Rhizobiales* (1 strain) and *Phenylobacterium* of the order *Caulobacterales* (1 strain). These isolates also showed higher than 99 % 16S rRNA gene sequences similarity with their relatives originating from the surface sea.

Among the gammaproteobacterial isolates, 25 strains were assigned to the order *Oceanospirillales*. These strains were close to the species *Alcanivorax venustensis* ISO4^{T} (>99 %) isolated at a depth of 200 m in the eastern Mediterranean Sea (Fernández-Martínez *et al*., 2003). Most of *Alcanivorax*-like strains in this sample were retrieved from NSLM and 6 strains were assigned to the order *Alteromonadales*. These strains fell into genera *Alteromonas* (2 strains), *Marinobacter* (2 strains), *Colwellia* (1 strain) and *Pseudoalteromonas* (1 strain). Strains affiliated with genera *Alteromonas* and *Marinobacter* were identical to species *A. macleodii* ATCC 27126^{T} (>99 %) and *M. algicola* DG893^{T} (>99 %) originating from surface-sea environments (Baumann *et al*., 1972; Green *et al*., 2006). Other gammaproteobacterial strains were affiliated with genera *Psychrobacter* (1 strain) and *Acinetobacter* (1 strain) within the order *Pseudomonadales*. These isolates also showed higher than 99 % 16S rRNA gene similarity with strains originating from the surface sea. Strains affiliated with genera *Colwellia*, *Pseudoalteromonas* and *Acinetobacter* showed less than 97 % 16S rRNA gene similarity with known species within the genera.

Among the isolates belonging to the phylum *Bacteroidetes*, isolates within the order *Flavobacteriales* (12 strains) were close to the species *Arenibacter palladensis* KMM 3961^{T} (>99 %) isolated from the green alga (Nedashkovskaya *et al*., 2010). All *Arenibacter*-like strains were retrieved from NSLM. Isolates within the order

Rhodothermales (2 strains) showed less than 97 % 16S rRNA gene sequences similarity with any of the known species within the order.

Deep-sea isolates from MR11-02-K2-2 seawater sample (2000 m)

From MR11-02-K2-2 samples, 12 strains belonging to the class *Gammaproteobacteria*, phyla *Verrucomicrobia* and *Bacteroidetes* were obtained (Table 2-5). Their phylogenetic positions are shown in Figure 2-4. All the strains within the phylum *Verrucomicrobia* were affiliated with the genus *Rubritalea*. These strains were retrieved from 1/10 R2A agar and showed less than 97 % 16S rRNA gene sequences similarity with any of the known species. *Rubritalea*-like strains were highly close to their relative derived from the surface of the Arctic Ocean (GQ452897 and EU919773). Strains belonging to the class *Gammaproteobacteria* were close to either species *Alteromonas macleodii* ATCC 27126^T (99.7 %) or *Pseudoalteromonas shioyasakiensis* SE3^T (100 %), which originated from surface-sea environment (Matsuyama *et al*., 2014). Isolate of the phylum *Bacteroidetes* (1 strain) was affiliated with the genus *Aquimarina*, showing less than 97 % 16S rRNA gene sequences similarity with any of the known species.

Deep-sea isolates from MR11-05-K2-1 seawater sample (1000 m)

From MR11-05-K2-1 sample, 18 strains belonging to classes *Alphaproterobacteria* and *Gammaproteobacteria,* and phyla *Bacteroidetes* were obtained (Table 2-6). Their phylogenetic positions are shown in Figure 2-5. One alphaproterobacterial isolate was identical to a surface-derived bacterium (KJ475182). Its closest species was *Loktanella tamlensis* SSW-35^T (98.2 %). Another alphaproterobacterial isolate was identical to Sulfitobacter sp. H24 (99.2%).

Among the gammaproteobacterial isolates, 9 strains were assigned to the order *Alteromonadales*. These strains fell into genera *Moritella* (7 strains), *Colwellia* (3 strains)

and *Shewanella* (1 strain). *Moritella*-like strains were identical to the species *M. viscosa* NVI 88/478^T (>99 %) isolated form a fish (Lunder *et al*., 2000). *Colwellia*-like strains were divided into two subgroups, which were close to species *C. psychrerythraea* 34H^T (>98 %) and *C. hornerae* ACAM 607^T (>97 %). *Shewanella*-like strain was close to the species *S. Canadensis* (97.8 %). One isolate of the order *Pseudomonadale* was identified and its closest species was *Pseudomonas koreensis* Ps 9-14^T (>99 %) isolated from farm soil (Kwon *et al*., 2003). Other gammaproteobacterial isolates were affiliated with either genera *Arenicella* of the order *Arenicellales* (1 strain) or *Sinobacterium* of the order *Oceanospirillales* (1 strain).

Isolates belonging to the phylum *Bacteroidetes* were assigned to only the order *Flavobacteriales*. One strain affiliated with the genus *Aquimarina* was identical to *A. atlantica* 22II-S11-z7^T (99.6 %) isolated from surface seawater (Li *et al*., 2014b). *Tenacibaculum* and *Dokdonia*-like strains showed less than 97 % 16S rRNA gene sequences similarity with any species within the order.

Deep-sea isolates from MR11-05-S1-1 seawater sample (1000 m)

From MR11-05-S1-1 sample, 96 strains belonging to the class *Alphaproteobacteria* and *Gammaproteobacteria*, and the phylum *Bacteroidetes and Actinobacteria* were obtained (Table 2-7). Their phylogenetic positions are shown in Figures 2-6 and 2-7. Among the alphaproteobacterial isolates, 23 strains were assigned to the order *Rhodobacterales.* These strains were close to either species *Oceanibulbus indolifex* HEL-45^T (>99 %) isolated from surface seawater (Agner-Döbler *et al*., 2004) or *Sulfitobacter pontiacus* ChlG 10 (>99 %) isolated from the Black Sea (Sorokin, 1995). Fourteen strains were assigned to the order *Sphingomonadales* and fell into genera *Erythrobacter* (12 strains) and *Blastomonas* (1 strain). *Erythrobacter*-like strains of this sample were similar with

those of KT10-25-S1-3 sample. *Blastomonas*-like strain showed less than 97 % 16S rRNA gene sequences similarity with known species. As for other alphaproteobacterial isolates, two strains were affiliated with the genus *Thalassospira* of the order *Rhodospirillales* and identical to *Thalassospira*-like strains isolated from soil (AUNC01000051) and 3000 m in the South China Sea (KF906554). One alphaproteobacterial strain was affiliated with the genus *Phenylobacterium* of the order *Caulobacterales* and its phylogenetic position was very close to the species *P. falsum* AC-49^T derived from groundwater (Tiago *et al*., 2005).

Among the gammaproteobacterial isolates, 17 strains were assigned to the order *Oceanospirillales* and affiliated with species *Alcanivorax*. *Alcanivorax*-like strains were divided into two subgroups, which were close to species *A. venustensis* and *A. borkumensis* with higher than 99 % 16S rRNA gene sequences similarity. Fifteen strains were assigned to the order *Alteromonadales*. These strains fell into genera *Marinobacter* (14 strains) and *Pseudomonadales* (1 strain), which were identical to species *M. algicola* CM19^T and *P. shioyasakiensis* SE3^T respectively. Ten strains were assigned to the order *Pseudomonadales* and identical to the species *Pseudomonas aestusnigri* VGXO14^T isolated from crude oil-contaminated intertidal sand (Sánchez *et al*., 2014). Other gammaproteobacterial isolates were 6 strains in the order *Thiotrichales*. Their closest species was the species *Methylophaga nitratireducenticrescens* JAM7^T isolated from biofilm (Villeneuve *et al*., 2013).

Strains within the phylum *Bacteroidetes* were identical to a *Gramella*-like bacterium isolated from soil (EU328069). Strains within the phylum *Actinobacteria* were close to the species *Nocardioides basaltis* J112^T (>99 %) isolated from sand (Kim *et al*., 2009).

Deep-sea isolates from MR11-05-S1-2 seawater sample (2000 m)

From MR11-05-S1-2 sample, 101 strains belonging to two bacterial lineages, classes *Alphaproteobacteria* and *Gammaproteobacteria* were obtained (Table 2-8). Their phylogenetic positions are shown in Figure 2-8. More than 70 % of total isolate in this sample were assigned to the order *Rhodobacterales* of the class *Alphaproteobacteria*. Most of them were affiliated with the genus *Sulfitobacter* (49 strains) and close to the species *Sulfitobacter pontiacus* ChlG 10 (>99 %). Other alphaproteobacterial isolates fell into genera *Hyphomonas* (20 strains), *Oceanibulbus* (4 strains) and *Erythrobacter* (3 strains). *Hyphomonas*-like strains were close to the species *H. atlantica* 22II1-22F38^T (99.0 %) isolated from surface seawater (Li *et al*., 2014a) and mainly retrieved from 1/10R2A and NSLM. *Oceanibulbus* and *Erythrobacter*-like strains were identical to *O. indolifex* HEL-45^T and *E. citreus* RE35F/1^T.

Among the *gammaproteobacterial* isolates, 13 strains were assigned to the order *Alteromonadales* and fell into the genera *Marinobacter* (11 strains) and *Moritella* (2 strains). *Marinobacter*-like strains were divided to two subgroups, which were affiliated with two species, *M. lipolyticus* SM19^T isolated from saline soil (Martín *et al*., 2003) and *M. salaries* R9SW1^T isolated from surface seawater (Ng *et al*., 2014). *Moritella*-like strains were close to the species *M. viscosa* NVI 88/478^T (>99 %) isolated from fish. Isolates affiliated with the species *Alcanivorax atlantica* ISO4^T (9 strains) and *Methylophaga nitratireducenticrescens* JAM7^T (2 strains) within the order *Thiotrichales* were identified.

Deep-sea isolates from MR11-05-S1-3 seawater sample (3000 m)

From MR11-05-S1-3 sample, 20 strains belonging to the classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*, and the phyla *Bacteroidetes* and *Actinobacteria* were obtained (Table 2-9). Their phylogenetic positions are shown in Figure 2-9. Strains belonging to the class *Alphaproteobacteria* fell into genera *Oceanibulbus* (2 strains) and *Erythrobacter* (2 strains). They were similar with those of MR11-05-S1-2 sample.

Among gammaproteobacterial isolates, strains within the order *Pseudomonadales* fell into genera *Alkanindiges* (6 strains) and *Pseudomonas* (1 strain). *Alkanindiges*-like strains were close to *A. illinoisensis* MVAB Hex1^T (97.8 %) and identical to *Alkanindiges* sp. 5-0-9 isolated from soil (LT158291). *Pseudomonas*-like strain were identical to *P. koreensis* Ps 9-14^T (99.7 %). Two strains were assigned to the order *Alteromonadales*. These strains were identical to *Pseudoalteromonas marina* mano4^T isolated from tidal flats (Nam *et al*., 2007). Remarkably, a strain of the class *Betaproterobacteria* was obtained and close to the species *Limnobacter thiooxidans* CS-K2^T (99.9 %) derived from a freshwater lake (Spring *et al*., 2001).

As for the phylum *Bacteroidetes*, two strains of the order *Sphingobacteriales* were close to the species *Pedobacter silvilitoris* W-WS1^T (99.6 %) isolated form a wood fall in coastal area (Park *et al*., 2015). The strain of the order *Rhodothermales* within the phylum showed less than 97 % 16s rRNA gene sequences similarity with any known species. As for the phylum *Actinobacteria*, two strains of the order *Propionibacteriales* were identical to species *Nocardioides furvisabuli* SBS-26^T (99.6 %) and *Nocardioides basaltis* J112^T (99.4 %), respectively. One strain of the order *Micrococcales* was close to the species *Microbacterium lacus* A5E-52^T (98.2 %) and identical to *Microbacterium* sp. SMXB24 isolated from sludge (HF571532). Except for the strains of *Rhodothermales*,

the deep-sea strains shared almost same phylogenetic position with strains originating from surface-sea environments.

Deep-sea isolates from MR11-05-S1-4 seawater sample (4000 m)

From MR11-05-S1-4 sample, 95 strains belonging to classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* of the phylum *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were obtained (Table 2-10). Their phylogenetic positions are shown in Figures 2-10 and 2-11. Among the alphaproteobacterial isolates, 16 strains were assigned to the order *Sphingomonadales*. These strains fell into genera *Erythrobacter* and *Sphingopyxis*. *Erythrobacter*-like strains were close to *E. citreus* RE35F/1^T. *Sphingopyxis*-like strains were identical to *S. chilensis* S37^T (100 %) isolated from the surface sea (Godoy *et al*., 2003). Other alphaproteobacterial isolates fell into genera *Paracoccus* (1 strain) and *Sulfitobacter* (1 strain) of the order *Rhodobacterales*, and *Brevundimonas* (2 strains) of the order *Caulobacterales*. *Paracoccus*-like strain was close to *P. oceanense* JLT1679^T isolated from surface West Pacific (Fu *et al*., 2011) and a deep-sea isolate in the eastern Mediterranean (Gärtner *et al.*, 2011). *Sulfitobacter*-like strain was identical to strains isolated from the deep sea (AB526332) and surface sea (FJ161246). *Brevundimonas*-like strains (2 strains) were identical to the species *B. vesicularis* IHBB 11140^T isolated from lake water (Segers *et al*., 1994). More than 40 % of the isolates (42 strains) in this sample were assigned to the order *Burkholeriales* of the class *Betaproteobacteria*. These strains were close to the species *Limnobacter thioxidans* CS-K2^T (>99 %). Another betaproteobacterial phylotype (1 strain) was close to the species *Achromobacter spanius* LMG 5911^T (100 %) isolated from human clinical sample (Coenye *et al*., 2003).

Among the gammaproteobacterial isolates, 21 strains were assigned to the order *Pseudomonadales.* These strains were affiliated with genus *Pseudomonas* (16 strains), *Alkanindiges* (3 strains) and *Acinetobacter* (2 strains). *Pseudomonas*-like strains were divided into three subgroups, which were close to species *P. stutzeri* ATCC 11607^T, *P. rhodesiae* CIP 104664^T and *P. koreensis* Ps 9-14^T. Seven strains affiliated with *P. stutzeri* were identical to strains isolated from deep-sea sediment in the Pacific Ocean (KR012296, KR012018-KR012025). Two strains were assigned to the order *Oceanospirillales.* One strain was close to the species *Halomonas sulfidaeris* Esulfide1^T (98.5 %) isolated from deep-sea hydrothermal vent environment (Kaye *et al*., 2004) and identical to a strain isolated from deep-sea sediment (AB166966). Another strain was identical to the species *Alcanivorax venustensis* ISO4^T.

Isolates of the phylum *Bacteroidetes* fell into genera *Leeuwenhoekiella* (3 strains), *Flavobacterium* (1 strain) and *Zunongwangia* (1 strain) within the order *Flavobacteriales.* They were affiliated with species *L. aequorea* LMG22550^T, *F. ahnfeltiae* KMM6686^T and *Z. profunda* SMA-87^T. The strains belonging to the phylum *Actinobacteria* were affiliated with genera *Rhodococcus* of the order *Corynebacteriales* and *Microbacterium* and *Brachybacterium* of the order *Micrococcales*.

Deep-sea isolates from KT12-08-OT5 seawater sample (1000 m)

From KT12-08-OT5 sample, 90 strains belonging to the classes *Alphaproteobacteria* and *Gammaproteobacteria*, and the phylum *Bacteroidetes* were obtained (Table 2-11). Their phylogenetic positions are shown in Figures 2-12, 2-13 and 2-14. Among the alphaproteobacterial isolates, 12 strains were assigned to the order *Rhizobiales*. These strains were identical to the species *Aurantimonas coralicida* WP1^T (>99.0 %) isolated from coral and *Aurantimonas* sp. C5-1 isolated form the deep-sea (AB937559). Fourteen

strains assigned to the order *Sphingomonadales* fell into genera *Sphingorhabdus* (5 strains), *Erythrobacter* (3 strains), *Sphingomonas* (3 strains) and *Sphingopyxis* (3 strains). *Sphingorhabdus*-like strains were close to the species *Sphingorhabdus flavimaris* SW-151^T (>99.0 %) isolated from surface seawater (Ogler *et al*., 2013) and identical to a strain isolated from oil amended marine particulate matter (EU239907). *Erythrobacter*-like strains were close to *E. citreus* RE35F/1^T (>99.0 %). *Sphingomonas*-like strains were identical to the species *Sphingomonas paucimobilis* ATCC 51231^T. *Sphingopyxis*-like strains were identical to the species *Sphingopyxis baekryungensis* SW-150^T. Twelve strains assigned to the order *Rhodobacterales* fell into genera *Sulfitobacter* (5 strains), *Oceanicola* (3 strains), *Shimia* (2 strains), *Loktanella* (1 strains), *Octadecabacter* (1 strains) and *Henriciella* (1 strain). *Sulfitobacter* and *Shimia*-like strains showed less than 97 % 16S rRNA gene sequences similarity with validly published species. *Oceanicola*-like strains were close to the species *Oceanicola nanhaiensis* DSM 18065^T (99 %) isolated from sediment at a depth of 1100 m of the South China Sea (Gu *et al*., 2007). *Loktanella* and *Octadecabacter*-like strains was respectively identical to *Loktanella* sp. K4B-4 isolated from Arctic seawater (FJ889559) and uncultured bacterium OA8-30d-034 retrieved during ocean acidification experiment (JN976549). Three strains assigned to the order *Caulobacterales* were affiliated with the genus *Phenylobacterium* and similar with ones of MR11-05-S1-1 seawater sample.

Among gammaproteobacterial strains, deep-sea isolates assigned to the order *Cellvibrionales* fell into genera *Oceanicoccus*, *Dasania* and *Haliea*. All of them showed less than 97 % 16S rRNA gene sequences similarity with validly published species. Twenty strains assigned to the order *Alteromonadales* were affiliated with genera *Colwellia*, *Moritella*, *Psychromonas* and *Shewanella*. *Colwellia*-like strains were

clustered to two groups affiliated with species *C. hornerae* IC035^T (>98.0 %) and *C. aestuarii* SMK-10^T (>98.0 %). *Moritella*-like strains were close to species *Moritella japonica* DSK1^{T}and *Moritella yayanosii* DB21MT-5^T (>97.0 %), which are piezophilic bacteria isolated from Japan Trench sediment (Nogi *et al*., 1998; Nogi and Kato, 1998). Other gammaproteobacterial isolates were genera *Thalassolitus* of the order *Oceanospirillales* (2 strains) and *Photobacterium* of the order *Vibrionales* (2 strains).

All of strains belonging to the phylum *Bacteriotedes* were assigned to the order *Flavobacteriales* including genera *Algibacter*, *Ulvibacter*, *Lutimonas*, *Aquimarina*, *Lewinella* and *Winogradskyella*, Except for 2 strain affiliated with the genus *Lutimonas, Flavobacteriales*-like strains affiliated with showed less than 97 % of 16S rRNA gene sequences similarity with validly published species.

Deep-sea isolates from KT12-08-ON8 seawater sample (1700 m)

From KT12-08-ON8 sample, 115 strains belonging to classes *Alphaproteobacteria* and *Gammaproteobacteria*, and the phylum *Bacteroidetes*, *Actinobacteria, Lentisphaerae* and *Verrucomicrobia* were obtained (Table 2-12). Their phylogenetic positions are shown Figures 2-15, 2-16 and 2-17. Among strains belonging to the class *Alphaproteobacteria,* those belonging to the order *Sphingomonadales* fell into genera *Erythrobacter Sphingomonas*, *Sphingorhabdus* and *Sphingopyxis*. Fourteen strains were close to the species *E. citreus* RE35F/1^T. Fourteen strains were identical to *Sphingomonas pauimobilis* ATCC 51231^T (100 %) and uncultured deep-sea bacterium Bac3B84 (KJ548890). Three *Sphingobium* strains were closely related to clones in Puerto Rico Trench (HM798716). These strains were identical to the species *Sphingobium olei* IMMIB HF-1^T (>98 %). Seven strains were assigned to the order *Rhizobiales*. Of 7 strains, 6 strains were close to *Aurantimonas coralicida* DSM14790 and similar with ones of

KT12-08-OT5 sample. One strain was close to the species *Jiella aquimaris* (>98%) and identical to *Aurantimonas* sp. 5C.5 isolated from Juan de Fuca Ridge basalt (HQ427427). Twelve strains assigned to the order *Rhodobacterales* fell into genera *Paracoccus* (6 strains), *Oceanicola* (3 strains), *Loktanella* (1 strain), *Roseobacter* (2 strains), *Planktotalea* (1 strain), *Sulfitobacter* (1 strain) and *Shimia* (1 strain). Six strains were identical to the species *Paracoccus oceanense* JLT1679^T and an isolate from the Eastern Mediterranean deep-sea (Gärtner *et al*., 2011). All of strains assigned to the order *Caulobacterales* were affiliated with the genus *Phenylobacterium*, which were retrieved from only NSLM. Three *Limnobacter*-like strains were isolated and similar with ones of MR11-05-S1-4 sample.

Among strains belonging to the class *Gammaproteobacteria,* strains assigned to the order *Alteromonadales* fell into genera *Colwellia* (10 strains), *Moritella* (5 strains), *Paraglaciecola* (2 strains), *Psychromonas* (2 strains) and *Shewanella* (1 strain). *Colwellia Moritella* and *Psychromonas*-like strains were similar with ones of other samples. *Paraglaciecola*-like strains were close to *P. psychrophila* 170^T isolated from the Arctic (Zhang *et al*., 2006)

Strains belonging to the phylum *Bacteroidetes* were assigned to orders *Flavobacteriales*. Except for one strain affiliated with the genus *Sabulilitoribacter,* strains affiliated with genera *Winogradsskyella*, *Algibacter*, *Ulvibacter* and *Polaribacter* showed less than 97 % of 16S rRNA gene sequences similarity with validly published species. One strain assigned to the order *Cytophagales* was affiliated with the genus *Reichenbachiella*. Two strains affiliated with the phylum *Lentisphaerae* were close to the species *Lentisphaera marina* IMCC 11369^T (>98 %). One verrucomicrobial strain was

retrieved from 1/10R2A and affiliated with the genus *Coraliomargarita*. The strain showed less than 97 % 16S rRNA gene sequences similarity with known species.

Discussion

In order to clarify the phylogenetic, physiological and genetic characteristics of marine prokaryotes, it is essential to isolate strains from various phylogenetic groups. In this study, a total of 681 isolates were obtained from the 9 deep-seawater samples collected from the north-western Pacific Ocean using 3 kinds of media, NSLM (liquid medium), 1/10 R2A agar and 1/5 MA agar. Based on 16S rRNA gene sequences, first, it was clarified that all the deep-sea isolates belonged to the domain *Bacteria* and none to *Archaea*. Second, over 50 % of the bacterial isolates belonged to the phylum *Proteobacteria* of the domain *Bacteria*. Other isolates were assigned to phyla *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and *Lentisphaerae*. Third, approximately, 90 % of the identified isolates have phylogenetically close relatives from surface layer or other environments. Fourth, among three culture methods applied, there were some differences in phylogenetic groups isolated. For example, strains belonging to the phyla *Verrucomicrobia* and *Lentisphaerae* were isolated by only 1/10 R2A (Figures 2-4 and 2-17). Also *Nocardioides*-like strains were retrieved from 1/10R2A (Figure 2-7). *Hyphomonas*-like strains were mainly isolated from 1/10R2A and NSLM (Table 2-8). NSLM enabled me to isolate *Arenicella*-like strains, which are the first isolates from the deep sea within the orders *Arenicellales* (Figure 2-3). Although no consistent difference was noticed among other phylogenetic groups, combination of different media simultaneously seems to be important to isolate wide groups of prokaryotes. Finally, at the station MR11-05-S1, there were some vertical differences in the phylogenetic groups.

The pattern is, however, not consistent among other stations.

Except for mixed cultures, 617 bacterial strains were identified using 16S rRNA gene sequencing. More than 90 % of them (586 strains) showed > 99 % 16S rRNA gene similiarity with close relatives in surface layers or other environments (Tables 2-4 to 2-12). This suggests that those groups are widely present as "cosmopolitan" groups, probably due to the mechanism of transportations. There may be two major factors, i.e., seawater circulations in the ocean (Kato and Nogi, 2003; Lauro *et al*., 2014) and the vertical flux on sinking particles. Prokaryotic cells inhabiting in surface may adsorb such particles and are transported to deeper layer (Sakiyama and Ohwada, 1998; Vanucci *et al*., 2001). The numbers of cells thus transported may be 10^{10}–10^{12} cells m^{-2} y^{-1} (Turley and Mackie, 1994).

Among the isolates, strains affiliated with genera *Erythrobacter*, *Oceanibulbus* and *Sulfitobacter* within *Alphaproterobacteria*, and *Alcanivorax*, *Colwellia*, *Moritella* within *Gammaproterobacteria* were often isolated from other diverse deep-sea environments (Radjasa *et al*., 2001; Ivars-Martínez *et al*., 2008; Eloe *et al*., 2010; Gärtner *et al*., 2011). On the other hand, some were the first isolates from the deep sea within their phylogenetic groups. Seven strains of the phylum *Verrucomicrobia* and 2 strains of the phylum *Lentisphaerae* were the first ones (Table 2-13) from the deep-sea. Also, strains affiliated with genera *Arenicella*, *Methylophaga*, *Dasania*, *Kinoniella* and *Ilumatobacter* were the first isolates in the orders *Arenicellales*, *Thiotrichales*, "*Cellvibrionales*" and *Kiloniellales* of the class *Gammaproteobacteria*, and *Acidmicrobiales* of the phylum *Actinobacteria*. Furthermore, strains of the genus *Limnobacter* within the family *Burkhoderiaceae* were obtained for the first time. Eloe and colleagues (2010) found the abundance of *Limnobacter*-like sequences from 6000 m in the Puerto Rico Trench. Also

some DNA segments retrieved from 3000 m depth in the Mediterranean matches *Limnobacter* related genomes (Martín-Cuadrado *et al*., 2010), confirming the presence of similar bacterial group. Considering that *Betaproteobacteria* has shown relatively limited abundance in marine environments, the high percentage of betaproteobacterial sequences in the deep sea is an interesting phenomenon.

According to the current taxonomic rule (Stackebrandt and Goebel, 1994), the strains having 16S rRNA gene gene similarity lower than 97 % may be assigned to a novel species. *Rubrivirga marina* SAORIC-28^T (Park *et al*., 2013), *Aurantivirga profunda* SAORIC-234^T (Song *et al*., 2015), *Lentisphaera profundi* SAORIC-696^T (Choi *et al*., 2015) and *Rubrivirga profundi* SAORIC-476^T (Song *et al*., 2016) were validated after taxonomical investigations and I proposed as novel deep-sea species (Figure 2-18).

The success in cultivation of previously unknown strains is ascribed to the use of three newly-designed lower carbon media. Although I did not compare three media with MA and 1/2 MA, the use of the media with lower concentration of carbon contents seems to have some advantages. First, for those living in oligotrophic environments, these media give less stress due to sudden increase of organic concentration. Second, there is less chance that fast growers cover the agar surface and hide colonies of other potentially recoverable strains. The drawback is, however, it may take longer incubation time due to slow growth. For future investigation, the application of agar plate with even lower concentration of organic compounds and/or with different components of nutrients may be effective to isolate more uncultured bacteria. As for NSLM, my first intention was to isolate those in the SAR11 group. Unfortunately, I could not isolate a deep-sea strain within the SAR11 group by NSLM at this time. Some modification will be possible in future. Stingle and colleagues (2007) suggested that the use of Teflon plates cleaned with

metal-free HCl yield new SAR11 isolates. Song and colleagues (2009) suggested that long term incubation at low temperatures improve the culturability of the surface-sea SAR11 strains. In this study, the incubation was performed at 10℃ for 4 weeks in polystyrene microtiter plates. Considering *in situ* conditions, long term incubation at 4℃ may be effective to isolate the SAR11s or other unknown strains from the deep-sea.

In conclusion, deep-sea bacteria from diverse phylogenetic groups were obtained from the north-western Pacific Ocean. 16S rRNA gene sequences of them revealed that most of them are regularly collected from various marine environments. On the other hand, a few of them were the first isolates that have never been cultured from the deep sea before. Among them, 4 strains were described and reported as new species. This result leads to arise some questions. First, what kind of general vertical distribution patterns do prokaryotes, including the groups isolated this time, show in the ocean? Second, what kind of physiological and genetic characteristics do deep-sea microorganisms have? Third, what kind of genetic similarities or dissimilarities do microorganisms have depending on their habitat depth? What kind of improvements of culture method leads to the isolation of more unknown microorganisms? In order to partly answer these questions, the culture independent molecular approach was taken to reveal vertical distribution of prokaryotes in the north-western Pacific Ocean (Chapter 3). Also, physiological and genetic investigations are accomplished by using these isolates in comparison with surface strains (Chapter 4, 5).

Table 2-1. Information of seawater samples and number of isolates.

Sample	Location	Sampling date	Depth (m)	Temperature (℃)	Total number of cells in seawater (cells/ml)	Number of isolates		
						1/5MA	1/10R2A	NSLM
KT10-12-S1-3	32°00' N, 138°13' E	3 July 2010	3000	2.0	1.2×10^5	6	11	61
MR11-02-K2-2	47°00' N, 160°00' E	February 2011	2000	2.0	1.6×10^5	2	10	-
MR11-05-K2-1	47°00' N, 160°00' E	May 2011	1000	2.5	1.8×10^5	8	10	-
MR11-05-S1-1	32°00' N, 145°00' E	May 2011	1000	3.8	1.7×10^5	52	30	17
MR11-05-S1-2	32°00' N, 145°00' E	May 2011	2000	2.0	1.2×10^5	20	44	49
MR11-05-S1-3	32°00' N, 145°00' E	May 2011	3000	1.6	0.6×10^5	10	10	-
MR11-05-S1-4	32°00' N, 145°00' E	May 2011	4000	1.5	0.7×10^5	33	44	25
KT12-08-OT5	39°20' N, 142°20' E	Oct 2012	1000	2.3	1.2×10^5	46	20	32
KT12-08-ON8	38°25' N, 143°00' E	May 2012	1700	2.2	1.8×10^5	39	48	54
Total						216	227	238

1/5MA, 1/5 strength marine agar 2216; 1/10 R2A, 1/10 strength R2A agar; NSLM, natural seawater lipid media. Incubation at 10℃ for 1 month. -, not tested

Table 2-2. Number of deep-sea isolates within classes *Alphaproteobacteria, Betaproteobacteria* and *Gammaproteobacteria* and phylum *Bacteroidetes*.

Sample (depth)	*Alphaproteobacteria*			*Betaproteobacteria*			*Gammaproteobacteria*			*Bacteroidetes*		
	1/5 MA	1/10 R2A	NSLM	1/5 MA	1/10 R2A	NSLM	1/5 MA	1/10 R2A	NSLM	1/5 MA	1/10 R2A	NSLM
KT10-12-S1-3 (3000 m)	4	5	16	0	0	0	0	6	27	2	0	10
MR11-02-K2-2 (2000 m)	0	0	-	0	0	-	1	4	-	1	0	-
MR11-05-K2-1 (1000 m)	2	0	-	0	0	-	3	8	-	3	2	-
MR11-05-S1-1 (1000 m)	23	10	6	0	0	0	27	16	5	2	2	2
MR11-05-S1-2 (2000 m)	12	34	30	0	0	0	8	10	7	0	0	0
MR11-05-S1-3 (3000 m)	2	2	-	0	1	-	6	3	-	2	1	-
MR11-05-S1-4 (4000 m)	8	7	5	13	22	8	10	11	3	1	2	2
KT12-08-OT5 (1000 m)	18	6	21	0	0	0	24	10	3	4	4	0
KT12-08-ON8 (1700 m)	19	25	15	1	1	1	16	11	9	0	7	3
Total	88	89	94	14	24	9	95	79	54	15	18	17

-, not tested.

Table 2-3. Numbers of the deep-sea isolates within phyla *Actinobacteria*, *Verrucomicrobia* and *Lentisphaerae*, and mixed culture showing unclear 16S rRNA gene sequence.

Sample (depth)	*Actinobacteria*			*Verrucomicrobia*			*Lentisphaerae*			Mixed culture		
	1/5 MA	1/10 R2A	NSLM	1/5 MA	1/10 R2A	NSLM	1/5 MA	1/10 R2A	NSLM	1/5 MA	1/10 R2A	NSLM
KT10-12-S1-3 (3000 m)	0	0	0	0	0	0	0	0	0	0	0	8
MR11-02-K2-2 (2000 m)	0	0	-	0	6	-	0	0	-	0	0	-
MR11-05-K2-1 (1000 m)	0	0	-	0	0	-	0	0	-	0	0	-
MR11-05-S1-1 (1000 m)	0	2	0	0	0	0	0	0	0	0	0	3
MR11-05-S1-2 (2000 m)	0	0	0	0	0	0	0	0	0	0	0	12
MR11-05-S1-3 (3000 m)	0	3	-	0	0	-	0	0	-	0	0	-
MR11-05-S1-4 (4000 m)	2	2	0	0	0	0	0	0	0	0	0	6
KT12-08-OT5 (1000 m)	0	0	0	0	0	0	0	0	0	0	0	8
KT12-08-ON8 (1700 m)	3	1	0	0	1	0	0	2	0	0	0	26
Total	5	8	0	0	7	0	0	2	0	0	0	63

-, not tested.

Table 2-4. Bacterial isolates in each phylogenetic group from KT10-12-S1-3 sample (3000 m).

Strains	RFLP	Phylum/class	Closest surface strain	Similarity	Total isolates		
					1/5MA	1/10R2A	NSLM
SAORIC-3000-1-C6, SAORIC-3000-1-D5, SAORIC-3000-1-D8, SAORIC-3000-1-F3, SAORIC-3000-4-F5, SAORIC-3000-7-B4, SAORIC-3000-8-B1	0	*Alphaproteobacteria*	*Erythrobacter citreus* RE35F/1	99	0	0	7
SAORIC-10, SAORIC-32, SAORIC-3000-1-B7	0		*Erythrobacter* sp. H209	99	0	2	1
SAORIC-1, SAORIC-13, SAORIC-16	0		*Sphingobium* sp. 2F5-2	99	1	2	0
SAORIC-3000-1-E4	0		*Sphingpyxis baekryungensis* SW-150	99	0	0	1
SAORIC-3, SAORIC-4, SAORIC-3000-1-C2, SAORIC-3000-2-C4, SAORIC-3000-2-D6, SAORIC-3000-2-E5, SAORIC-3000-7-E2	0		*Ocenicola* sp. LZB062	98	1	1	5
SAORIC-3000-1-C4	0		*Loktanella aestuariicola* J-TF4	99	0	0	1
SAORIC-25	0		*Sagittula stellata* E-37	99	1	0	0
SAORIC-29	0		*Aurantimonas coralicida* DSM14790	99	1	0	0
SAORIC-3000-6-B7	0		Uncultured *Phenylobacterium* EDW07B001	99	0	0	1
SAORIC-17, SAORIC-3000-1-E2, SAORIC-3000-1-F1, SAORIC-3000-2-A7, SAORIC-3000-2-B6, SAORIC-3000-2-C6, SAORIC-3000-2-D2, SAORIC-3000-2-D5, SAORIC-3000-2-E2, SAORIC-3000-2-E4, SAORIC-3000-2-E2, SAORIC-3000-3-C3, SAORIC-3000-4-D2, SAORIC-3000-4-E4, SAORIC-3000-6-C7, SAORIC-3000-6-F8, SAORIC-3000-7-A6, SAORIC-3000-7-C6, SAORIC-3000-7-F2, SAORIC-3000-7-F6, SAORIC-3000-8-A6, SAORIC-3000-8-D3, SAORIC-3000-8-D4, SAORIC-3000-8-E3, SAORIC-3000-8-F2,	0	*Gammaproteobacteria*	*Alcanivorax venustensis* ISO4	99	0	1	24
SAORIC-9, SAORIC-3000-1-E3	0		*Alteromonas macleodii* DSM 6062	99	0	1	1
SAORIC-11, SAORIC-15	0		*Marinobacter algicola* DG893	99	0	2	0
SAORIC-38	0		*Colwellia asteriadis* KMD002	97	0	1	0
SAORIC-3000-1-E1	0		*Pseudoalteromonas phenolica* O-BC30	95	0	0	1
SAORIC-30	0		*Psychrobacter pacificensis* IFO 16279	99	0	1	0

SAORIC-3000-2-C8	0		*Acinetobacter junii* CIP 64.5	97	0	0	1
SAORIC-3000-2-A6, SAORIC-3000-2-F2, SAORIC-3000-7-F1	7	*Bacteroidetes*	*Arenibacter palladensis* VBW129	99	0	0	10
SAORIC-26, SAORIC-28	0		Uncultured *Rubricoccus* EK CK570	96	2	0	0
Total					6	11	52

RFLP, numbers of strains showing same patterns in RFLP analysis

Table 2-5. Bacterial isolates in each phylogenetic group from MR11-02-K2-2 sample (2000 m)

Strains	RFLP	Phylum/class	Closest surface strain	Similarity	Total isolates	
					1/5 MA	1/10 R2A
SAORIC-156, SAORIC-160	0	*Gammaproteobacteria*	*Pseudoalteromonas shioyasakiensis* SE3	99	0	2
SAORIC-154, SAORIC-155, SAORIC-157	0		*Alteromonas macleodii* ATCC 27126	99	1	2
SAORIC-153	0	*Bacteroidetes*	*Aquimarina* sp. RZW4-3-2	95	1	0
SAORIC-159, SAORIC-161, SAORIC-162, SAORIC-163, SAORIC-165, SAORIC-168	0	*Verrucomicrobia*	Uncultured *Rubritalea* strain b36	99	0	6
Total					2	10

Table 2-6. Bacterial isolates in each phylogenetic group from MR11-05-K2-1 sample (1000 m)

Strains	RFLP	Phylum/class	Closest surface strain	Similarity	Total isolates	
					1/5 MA	1/10 R2A
SAORIC-212	0	*Alphaproteobacteria*	*Loktanella* sp. PAMC 27241	99	1	0
SAORIC-220	0		*Sulfitobacter* sp. H24	99	1	0
SAORIC-215, SAORIC-218, SAORIC-236, SAORIC-239	0	*Gammaproteobacteria*	*Moritella viscosa* NVI 88/478	99	2	2
SAORIC-235, SAORIC-237	0		*Colwellia* sp. ANT9381	99	0	2
SAORIC-219	0		*Colwellia psychrerythraea* 34H	99	1	0
SAORIC-238	0		*Shewanella canadensis* HAW-EB2	99	0	1
SAORIC-242	0		*Pseudomonas fluorescens* 9zhy	99	0	1
SAORIC-232	0		Uncultured *Arenicella* strain B78-30	98	0	1
SAORIC-241	0		*Sinobacterium caligoides* SCSWE24	99	0	1
SAORIC-233, SAORIC-234	0	*Bacteroidetes*	Uncultured *Polaribacte*r strain K2S205	99	0	2
SAORIC-211, SAORIC-222	0		Uncultured *Dokdonia* strain OA9-30d-027	99	2	0
SAORIC-214	0		*Aquimarina atlantica* 22II-S11-z7	99	1	0
Total					8	10

Table 2-7. Bacterial isolates in each phylogenetic group from MR11-05-S1-1 sample (1000 m)

Strains	RFLP	Phylum/class	Closest surface strain	Similarity	Total isolates		
					1/5 MA	1/10 R2A	NSLM
SAORIC-246, SAORIC-247, SAORIC-263, SAORIC-278, SAORIC-301, SAORIC-319, SAORIC-332, SAORIC-335, SAORIC-341, SAORIC-348, SAORIC-360, SAORIC-362, SAORIC-1000-20-E1	6	*Alphaproteobacteria*	*Oceanibulbus indolifex* HEL-45	99	11	7	1
SAORIC-245, SAORIC-258, SAORIC-284, SAORIC-290, SAORIC-1000-8-C5	0		*Sulfitobacter pontiacus* ChLG-10	99	4	0	1
SAORIC-248, SAORIC-252, SAORIC-266, SAORIC-285, SAORIC-337, SAORIC-347, SAORIC-359	0		*Erythrobacter citreus* RE35F/1	99	4	3	0
SAORIC-1000-8-D4, SAORIC-1000-10-C1, SAORIC-1000-11-C7, SAORIC-1000-11-C1, SAORIC-1000-11-C3	0		*Erythrobacter pelagi* UST081027-248	99	0	0	5
SAORIC-276	0		*Blastomonas* sp. SSR2A-4-2	98	1	0	0
SAORIC-316	1		*Thalassospira permensis* SMB34	99	2	0	0
SAORIC-257	0		*Phenylobacterium falsum* AC-49	99	1	0	0
SAORIC-250, SAORIC-260, SAORIC-273, SAORIC-283, SAORIC-295, SAORIC-298, SAORIC-313, SAORIC-345, SAORIC-356, SAORIC-1000-7-A8, SAORIC-1000-12-F8	0	*Gammaproteobacteria*	*Alcanivorax borkumensis* SK2	99	7	2	2
SAORIC-251, SAORIC-331, SAORIC-1000-8-D2	3		*Alcanivorax venustensis* ISO4	99	2	3	1
SAORIC-254, SAORIC-255, SAORIC-256, SAORIC-269, SAORIC-304, SAORIC-307, SAORIC-308, SAORIC-333, SAORIC-338, SAORIC-350, SAORIC-352, SAORIC-1000-8-C4	2		*Marinobacter algicola* CM19	99	8	5	1
SAORIC-1000-8-B8	0		*Pseudalteromonas shioyasakiensis* SE3	99	0	0	1
SAORIC-259, SAORIC-277, SAORIC-291, SAORIC-303, SAORIC-317, SAORIC-324, SAORIC-353, SAORIC-361	2		*Pseudomonas aestusnigri* CCUG 64165	99	6	4	0
SAORIC-265, SAORIC-261, SAORIC-270, SAORIC-342, SAORIC-334, SAORIC-344	0		*Methylophaga nitratireducenticrescens* JAM1	99	4	2	0
SAORIC-253, SAORIC-300, SAORIC-346, SAORIC-334, SAORIC-1000-12-C8, SAORIC-1000-12-B3	0	*Bacteroidetes*	Uncultured *Gramella* strain Y114	99	2	2	2
SAORIC-349, SAORIC-354	0	*Actinobacteria*	*Nocardioides basaltis* J112	99	0	2	0

Total				52	30	14

Table 2-8. Bacterial isolates in each phylogenetic group from MR11-05-S1-2 sample (2000 m)

Strains	RFLP	Phylum/class	Closest surface strain	Similarity	Total isolates		
					1/5 MA	1/10 R2A	NSLM
SAORIC-371, SAORIC-386, SAORIC-388, SAORIC-395, SAORIC-402, SAORIC-418, SAORIC-422, SAORIC-428, SAORIC-429, SAORIC-433, SAORIC-435, SAORIC-440, SAORIC-449, SAORIC-2000-19-B2, SAORIC-2000-19-D1, SAORIC-2000-19-E5, SAORIC-2000-19-F1, SAORIC-2000-21-A6, SAORIC-2000-21-B5, SAORIC-2000-21-D1, SAORIC-2000-21-F6, SAORIC-2000-21-F7, SAORIC-2000-22-D8, SAORIC-2000-22-E4, SAORIC-2000-22-F1, SAORIC-2000-23-A7, SAORIC-2000-23-B1, SAORIC-2000-23-B2, SAORIC-2000-23-B3, SAORIC-2000-23-E6,	19	*Alphaproteobacteria*	*Sulfitobacter* sp. MOLA 8	99	10	21	18
SAORIC-398, SAORIC-399, SAORIC-441, SAORIC-2000-20-E1	0		Uncultured *Oceanibulbus* strain SEM1D091	99	0	3	1
SAORIC-374, SAORIC-396, SAORIC-427, SAORIC-430, SAORIC-453, SAORIC-2000-19-F6, SAORIC-2000-20-B5, SAORIC-2000-21-C5, SAORIC-2000-22-A4, SAORIC-2000-23-B8, SAORIC-2000-23-D3, SAORIC-2000-24-C5	8		*Hyphomonas* sp. MCCC 1A05042	99	1	9	10
SAORIC-377, SAORIC-438, SAORIC-2000-23-E5	0		*Erythrobacter citreus* RE35F/1	99	1	1	1
SAORIC-366, SAORIC-368, SAORIC-382, SAORIC-394	4	*Gammaproteobacteria*	*Marinobacter* sp. NBRC 101712	99	5	3	0
SAORIC-375, SAORIC-393, SAORIC-2000-20-C1, SAORIC-2000-23-B7	0		Uncultured *Marinobacter* strain C02-D-2	99	1	1	2
SAORIC-365	0		*Moritella* sp. H130426	99	1	0	0
SAORIC-370	0		*Moritella* sp. J28	99	1	0	0
SAORIC-392, SAORIC-2000-19-B1, SAORIC-2000-20-B3, SAORIC-2000-22-E7, SAORIC-2000-23-F8, SAORIC-2000-24-A8	3		*Alcanivorax venustensis* ISO4	99	0	4	5
SAORIC-412, SAORIC-423	0		*Methylophaga nitratireducenticrescens* JAM1	99	0	2	0

Total					20	44	37

Table 2-9. Bacterial isolates in each phylogenetic group from MR11-05-S1-3 sample (3000 m)

Strains	RFLP	Phylum or class	Closest surface strain	Similarity	Total isolates	
					1/5 MA	1/10 R2A
SAORIC-462	1	*Alphaproteobacteria*	*Oceanibulbus indolifex* HEL-45	99	2	0
SAORIC-483, SAORIC-487	0		*Erythrobacter citreus* RE35F/1	99	0	2
SAORIC-460, SAORIC-465, SAORIC-467, SAORIC-468	2	*Gammaproteobacteria*	*Alkanindiges* sp. 5-0-9	99	4	2
SAORIC-470	0		*Pseudomonas koreensis* Ps 9-14	99	1	0
SAORIC-472, SAORIC-479	0		*Pseudoalteromonas marina* mano4	99	1	1
SAORIC-486	0	*Betaproteobacteria*	*Limnobacter* sp. SSW083	99	0	1
SAORIC-464 SAORIC-466	0	*Bacteoroidetes*	*Pedobacter silvilitoris* W-WS1	99	2	0
SAORIC-476	0		Uncultured *Rubricoccus* EK_CK 570	97	0	1
SAORIC-469	0	*Actinobacteria*	*Nocardioides furvisabuli*	99	0	1
SAORIC-461	0		*Nocardioides* sp. 70071	99	0	1
SAORIC-484	0		*Microbacterium* sp. SMXB24	99	0	1
Total					10	10

Table 2-10. Bacterial isolates in each phylogenetic group from MR11-05-S1-4 sample (4000 m)

Strains	RFLP	Phylum or class	Closest surface strain	Similarity	Total isolates		
					1/5 MA	1/10 R2A	NSLM
SAORIC-498, SAORIC-575, SAORIC-561	0	*Alphaproteobacteria*	*Erythrobacter citreus* RE35F/1	99	1	2	0
SAORIC-519, SAORIC-536	0		*Erythrobacter* sp. CR-36	99	2	0	0
SAORIC-565, SAORIC-4000-46-A3, SAORIC-4000-48-C6, SAORIC-4000-48-F1	1		*Erythrobacter pelagi* UST081027-248	99	0	1	4
SAORIC-510, SAORIC-527, SAORIC-589, SAORIC-590, SAORIC-4000-43-B7, SAORIC-4000-48-A4	1		*Sphingopyxis chilensis* S37	99	2	3	2
SAORIC-582, SAORIC-528	0		*Brevundimonas vesicularis* IHBB 11140	99	1	1	0
SAORIC-496	0		*Paracoccus oceanense* JLT1679	99	1	0	0
SAORIC-531	0		*Sulfitobacter* sp. D4005	99	1	0	0
SAORIC-493, SAORIC-497, SAORIC-509, SAORIC-511, SAORIC-516, SAORIC-518, SAORIC-524, SAORIC-525, SAORIC-526, SAORIC-529, SAORIC-532, SAORIC-539, SAORIC-541, SAORIC-543, SAORIC-544, SAORIC-548, SAORIC-551, SAORIC-553, SAORIC-556, SAORIC-566, SAORIC-579, SAORIC-580, SAORIC-585, SAORIC-596, SAORIC-4000-43-C7, SAORIC-4000-44-A1, SAORIC-4000-44-D6, SAORIC-4000-44-E7, SAORIC-4000-45-B5, SAORIC-4000-45-D2, SAORIC-4000-48-B5, SAORIC-4000-48-C2	9	*Betaproteobacteria*	*Limnobacter* sp. Nb15RA-1	99	12	21	8
SAORIC-557	0		*Achromobacter spanius* LMG 5911	99	0	1	0
SAORIC-506, SAORIC-507, SAORIC-523, SAORIC-547, SAORIC-588, SAORIC-4000-45-D1	6	*Gammaproteobacteria*	*Pseudomonas* sp. SW-76	99	4	7	1
SAORIC-491	0		*Pseudomonas koreensis* Ps 9-14	99	1	0	0
SAORIC-495, SAORIC-530, SAORIC-4000-44-E8	0		*Pseudomonas rhodesiae* CIP 104664	99	2	0	1
SAORIC-490, SAORIC-558, SAORIC-587	0		*Alkanindiges* sp. F22	99	1	2	0
SAORIC-515	0		*Acinetobacter bouvetii* 3-6	99	1	0	0
SAORIC-581	0		*Acinetobacter beijerinckii* CIP 110307	99	0	1	0
SAORIC-540	0		*Halomonas* sp. NT N8	99	0	1	0
SAORIC-4000-48-A6	0		*Alcanivorax venustensis* ISO4	99	0	0	1

SAORIC-514, SAORIC-584, SAORIC-4000-45-A6	0	*Bacteroidetes*	*Leeuwenhoekiella aequorea* LMG 22550	99	1	1	1
SAORIC-4000-45-C3	0		*Zunongwangia* sp. MAR 2010 57	99	0	0	1
SAORIC-560	0		*Flavobacterium ahnfeltiae* KMM 6686	99	0	1	0
SAORIC-583	0	*Actinobacteria*	*Rhodococcus fascians* DSM 20669	99	0	1	0
SAORIC-492, SAORIC-502, SAORIC-537	0		*Microbacterium lacus* A5E-52	99	3	0	0
SAORIC-559	0		*Brachybacterium muris* C3H-21	99	0	1	0
Total					33	44	19

Table 2-11. Bacterial isolates in each phylogenetic group from KT12-08-OT5 sample (1000 m)

Strains	RFLP	Phylum or class	Closest surface strain	Similarity	Total isolates		
					1/5 MA	1/10 R2A	NSLM
SAORIC-753, SAORIC-767, SAORIC-1100-1-B1, SAORIC-1100-1-D7, SAORIC-1100-1-F5, SAORIC-1100-2-A3, SAORIC-1100-2-F4, SAORIC-1100-3-B4, SAORIC-1100-3-C6, SAORIC-1100-3-F4, SAORIC-1100-4-D4, SAORIC-1100-4-F1	0	*Alphaproteobacteria*	*Aurantimonas coralicida* DSM14790	99	2	0	10
SAORIC-770, SAORIC-845	0		*Erythrobacter* sp. CR-36	99	1	1	0
SAORIC-1100-4-1-C4	0		*Erythrobacter citreus* RE35F/1	99	0	0	1
SAORIC-769, SAORIC-786 SAORIC-1100-1-F2	0		*Erythrobacter pelagi* UST081027-248	99	2	0	1
SAORIC-854, SAORIC-855, SAORIC-1100-2-D3	2		*Sphingorhabdus* sp. DG1642	99	0	1	4
SAORIC-762, SAORIC-750, SAORIC-785	0		*Sphingomonas paucimobilis* ATCC 29837	99	3	0	0
SAORIC-760, SAORIC-815	1		*Sphingopyxis baekryungensis* SW-150	99	1	2	0
SAORIC-741, SAORIC-1100-1-A1, SAORIC-1100-4-B1,	0		*Sulfitobacter* sp. BSi20563	99	1	0	2
SAORIC-783, SAORIC-784	0		*Roseobacter* sp. ANT909	99	2	0	0
SAORIC-754, SAORIC-777, SAORIC-794	0		*Oceanicola* sp. LZB062	98	3	0	0
SAORIC-839	0		*Loktanella* sp. K4B-4	99	0	1	0
SAORIC-768, SAORIC-779	0		*Shimia* sp. SK002	99	2	0	0
SAORIC-787	0		Uncultured *Octadecabacter* OA8-30d-034	99	1	0	0
SAORIC-844	0		*Caulobacter* sp. IW1-2CT	99	0	1	0
SAORIC-1100-1-B8, SAORIC-1100-4-E3, SAORIC-1100-4-E8	0		*Phenylobacterium falsum* AC-49	99	0	0	3
SAORIC-732, SAORIC-736, SAORIC-747, SAORIC-752, SAORIC-781, SAORIC-810, SAORIC-817	0	*Gammaproteobacteria*	Uncultured *Dasania strain* OHKB7.46	99	5	2	0
SAORIC-751, SAORIC-782, SAORIC-811, SAORIC-834, SAORIC-852, SAORIC-857, SAORIC-817	0		*Oceanicoccus sp.* HTCC2143	99	2	4	0
SAORIC-819	0		Uncultured *Oceanicoccus* strain EzlYy226	99	1	0	0
SAORIC-788, SAORIC-789	0		Uncultured *Haliea* strain JSS SO4	96	2	0	0
SAORIC-808	0		*Haliea* sp. Woods-Hole a5623	99	1	0	0

SAORIC-826, SAORIC-801	0		*Colwellia* sp. BSi20003	99	0	2	0
SAORIC-764, SAORIC-841	0		Uncultured *Colwellia* strain E11D005E31	99	2	0	0
SAORIC-780	0		Uncultured *Colwellia* strain OS3BD86	99	1	0	0
SAORIC-748, SAORIC-794	0		*Moritella* sp. SC22	99	0	2	0
SAORIC-793, SAORIC-765	0		*Moritella* sp. H130426	99	2	0	0
SAORIC-833, SAORIC-807	0		*Moritella* sp. ODA02	99	2	0	0
SAORIC-1100-2-A4	0		Uncultured *Alkalimarinus* strain S1-53	99	0	0	1
SAORIC-776, SAORIC-791, SAORIC-800	0		Uncultured *Psychromonas strain* SHAN535	99	3	0	0
SAORIC-795	1		*Shewanella sediminis* HAW-EB3	98	2	0	0
SAORIC-1100-1-F8	1		*Thalassolituus oleivorans* MIL-1	99	0	0	2
SAORIC-763	0		*Photobacterium* sp. QY26	98	1	0	0
SAORIC-774, SAORIC-796	0	*Bacteroidetes*	*Algibacter* sp. PAORIC-9	99	2	0	0
SAORIC-834	0		*Lutimonas* sp. PAORIC-13	99	0	2	0
SAORIC-756	0		Uncultured *Ulvibacter* strain s54	99	1	0	0
SAORIC-837	0		*Aquimarina* sp. RZW4-3-2	96	0	1	0
SAORIC-856	0		Uncultured *Lewinella* A6GH	97	0	1	0
SAORIC-773	0		*Winogradskyella* sp. PAMC27136	99	1	0	0
Total					45	21	24

Table 2-12. Bacterial isolates in each phylogenetic group from KT12-08-ON8 sample (1700 m)

Strains	RFLP	Phylum or class	Closest surface strain	Similarity	Total isolates		
					1/5MA	1/10R2A	NSLM
SAORIC-618, SAORIC-619, SAORIC-633, SAORIC-653, SAORIC-645, SAORIC-654, SAORIC-714, SAORIC-644, SAORIC-717, SAORIC-688, SAORIC-694, SAORIC-1700-6-B1, SAORIC-1700-7-C2, SAORIC-1700-5-C5, SAORIC-1700-5-E4, SAORIC-1700-9-D7	0	*Alphaproteobacteria*	*Erythrobacter* sp. sw0106-20	99	7	4	5
SAORIC-600, SAORIC-609, SAORIC-610, SAORIC-613, SAORIC-648, SAORIC-676, SAORIC-704, SAORIC-692, SAORIC-722, SAORIC-672, SAORIC-1700-4-C2, SAORIC-1700-6-A7, SAORIC-1700-9-B6	0		*Sphingomonas paucimobilis* ATCC 29837	99	4	6	3
SAORIC-710, SAORIC-703, SAORIC-693	0		*Sphingobium* sp. CO180	99	0	3	0
SAORIC-657, SAORIC-1700-9-D5	0		*Sphingopyxis baekryungensis*	99	1	0	1
SAORIC-601	0		Uncultured *Sphingorhabdus* strain Woods-Hole a5939	99	1	0	0
SAORIC-662, SAORIC-689, SAORIC-700, SAORIC-713, SAORIC-1700-5-F6, SAORIC-1700-8-B6	0		*Aurantimonas coralicida* DSM14790	99	0	4	2
SAORIC-708	0		*Jiella aquimaris* LZB041	99	0	1	0
SAORIC-682, SAORIC-720, SAORIC-705, SAORIC-679, SAORIC-1700-5-A1,	1		*Paracoccus oceanense* JLT1679	99	0	4	2
SAORIC-652, SAORIC-1700-4-B2	1		*Oceanicola* sp. DongtaiB-3030	96	2	0	1
SAORIC-651, SAORIC-641	0		*Roseobacter* sp. ARK10222	99	2	0	0
SAORIC-695	0		*Planktotalea frisia* SH6-1	99	0	1	0
SAORIC-721, SAORIC-1700-4-E2	0		*Sulfitobacter dubius* KMM3554	99	0	1	1
SAORIC-642	0		*Loktanella* sp. K4B-4	99	1	0	0
SAORIC-659	0		*Shimia* sp. SK002	99	0	1	0
SAORIC-614	0		Uncultured *Kiloniella* strain Woods-Hole a6457	98	1	0	0
SAORIC-643, SAORIC-690, SAORIC-1700-9-C4	0	*Betaproteobacteria*	*Limnobacter thiooxidans* CS-K2	99	1	1	1
SAORIC-602, SAORIC-630, SAORIC-631	0	*Gammaproteobacteria*	Uncultured *Colwellia* strain E11D005E31	99	3	0	0

SAORIC-605, SAORIC-647, SAORIC-725, SAORIC-1700-4-E8	0		*Colwellia* sp. D7	99	2	1	1
SAORIC-629, SAORIC-724	0		*Colwellia* sp. P1 50	97	1	1	0
SAORIC-1700-8-A1	0		Uncultured *Colwellia* strain C146300053	99	0	0	1
SAORIC-640, SAORIC-762, SAORIC-604, SAORIC-719, SAORIC-632	0		*Moritella viscosa* NVI 88/478	99	4	1	0
SAORIC-603, SAORIC-663	0		*Paraglaciecola psychrophilia* 170	99	2	0	0
SAORIC-711	0		*Shewanella hanedai* CIP103207	99	0	1	0
SAORIC-664, SAORIC-655, SAORIC-1700-6-D6	0		Uncultured *Psychromonas* strain SHAN535	99	1	1	1
SAORIC-637, SAORIC-639, SAORIC-660, SAORIC-669, SAORIC-670	0		*Oceanospirillum* sp. HTCC2178	99	1	4	0
SAORIC-1700-4-D6, SAORIC-1700-6-B6,	1		*Dasania* sp. IMCC8910	99	0	0	3
SAORIC-680	0		*Sinobacterium norvegicum* 2CH8	99	0	1	0
SAORIC-671,	0		*Oceanicoccus* sp. HTCC2143	99	0	1	1
SAORIC-1700-3-D5	0		Uncultured *Oceanicoccus* strain EzlYy226	98	1	0	0
SAORIC-638	0		*Halioglobus* sp. FILTER11C211m	95	1	0	0
SAORIC-1700-4-F8	0		Uncultured *Arenicella* sp. LO5SP1r	98	0	0	1
SAORIC-1700-8-A5	0		*Pseudomonas* sp. K65	99	0	0	1
SAORIC-712	0	*Bacteroidetes*	*Winogradsskyella* sp. PAMC 27136	99	0	2	0
SAORIC-665, SAORIC-678	0		*Winogradsskyella* sp. PAMC 27140	99	0	2	0
SAORIC-687, SAORIC-1700-4-F1	0		Uncultured *Algibacter* strain D9	99	0	1	0
SAORIC-1700-6-A1	0		Uncultured *Ulvibacter* strain S1B1S 10-106	96	0	0	1
SAORIC-1700-4-F1	0		Uncultured *Sabulilitoribacter* CB51E04	99	0	0	1
SAORIC-697	0		*Polaribacter* sp. HMF2268	99	0	1	0
SAORIC-683	0		*Psychroserpens* sp. PAMC 27220	99	0	1	0
SAORIC-1700-5-A6	0	*Actinobacteria*	*Reichenbachiella agariperforans* KMM 3525	95	0	0	1
SAORIC-649, SAORIC-650, SAORIC-656	0		*Ilumatobacter fluminis* YM22-133	99	2	1	0
SAORIC-646	0		*Rhodococcus cercidiphylli* 05-Lb0410	99	1	0	0
SAORIC-696, SAORIC-681	0	*Lentisphaera*	*Lentisphaera marina* IMCC11369	98	0	2	0
SAORIC-706	0	*Verrucomicrobia*	Uncultured *Coraliomargarita* strain C114100277	99	0	1	0
Total					39	48	28

Table 2-13. Summary of the deep-sea isolates

Taxonomic level				Sample (depth; m)	Number of isolates	Characteristics		
Phylum or class	Order	Family	Genus			a	b	c
Alphaproterobacteria	*Rhodobacterales*	*Rhodobacteraceae*	*Oceanibulbus*	MR11-05-S1-1 (1000)	20		•	
			Sulfitobacter	MR11-05-S1-2 (2000)	49		•	
	Rhizobiales	*Aurantimonadaceae*	*Aurantimonas*	KT12-08-OT5 (1000)	12		•	
	Sphingomonadales	*Erythrobacteraceae*	*Erythrobacter*	KT12-08-ON8 (1700)	14			
	*Kiloniellales**	*Kiloniellaceae*	*Kiloniella*	KT12-08-ON8 (1700)	1	•		•
Betaproterobacteria	*Burkholderiales*	*Burkholderiaceae**	*Limnobacter*	MR11-05-S1-3 (3000)	1	•		
				MR11-05-S1-4 (4000)	42	•	•	
				KT12-08-ON8 (1700)	3	•		
Gammaproteobacteria	*Alteromonadales*	*Moritellaceae*	*Moritella*	MR11-05-K2-1 (1000)	4		•	
	*Arenicellales**	*Arenicellaceae*	*Arenicella*	KT12-08-ON8 (1700	1	•		
				KT12-08-ON8 (1700)	1	•		
	Oceanospirillales	*Alcanivoracaeae*	*Alcanivorax*	KT10-12-S1-3 (3000)	25		•	
	*Thiotrichales**	*Piscirickettsiaceae*	*Methylophaga*	MR11-05-S1-1 (1000)	6	•		
				MR11-05-S1-2 (2000)	2	•		
	Pseudomonadales	*Moraxellaceae*	*Alkanindiges*	MR11-05-S1-3 (3000)	6		•	
	*Cellvibrionales**	*Spongiibacteraceae*	*Dasania*	KT12-08-OT5 (1000)	7	•		•
				KT12-08-ON8 (1700)	4	•		•
	*Cellvibrionales**	*Spongiibacteraceae*	*Oceanicoccus*	KT12-08-OT5 (1000)	7	•		•
				KT12-08-ON8 (1700)	2	•		•
Actinobacteria	*Acidimicrobiales**	*Acidimicrobiaceae*	*Ilumatobacter*	KT12-08-ON8 (1700)	3	•		
*Lentisphaerae**	*Lentisphaerales*	*Lentisphaeraceae*	*Lentisphaera*	KT12-08-ON8 (1700)	2	•		
*Verrucomicrobia**	*Verrucomicrobiales*	*Rubritaleaceae*	*Rubritalea*	MR11-02-K2-2 (2000)	6	•	•	•
	Puniceicoccales	*Puniceicoccaceae*	*Coraliomargarita*	KT12-08-ON8 (1700)	1	•		•

*, the group containing the first deep-sea isolates; a, first deep-sea isolate in the group; b, predominant group in the sample; c, new species.

Table 2-14. Possible new species.

Taxonomic level			Strains	Sample (depth; m)	Closest strain	Similarity*
Phylum or class	Order	Family				
Alphaproteobacteria	*Sphingomonadales*	*Sphingomonadaceae*	SAORIC-276	MR11-05-K2-1 (1000)	*Novosphingobium hassiacum* W-51^T	95
	Rhodobacterales	*Rhodobacteraceae*	SAORIC-783	KT12-08-OT5 (1000)	*Sulfitobacter brevis* DSM 11443^T	96
	Kiloniellales	*Kiloniellaceae*	SAORIC-614	KT12-08-ON8 (1700)	*Aestuariispira insulae* AH-MY2^T	90
Gammaproteobacteria	*Cellvibrionales*	*Cellvibrionaceae*	SAORIC-637	KT12-08-ON8 (1700)	*Maricurvus nonylphenolicus* KU41E^T	89
			SAORIC-638	KT12-08-ON8 (1700)	*Halioglobus pacificus* S1-72^T	88
			SAORIC-740	MR11-05-S1-3 (3000)	*Oceanicoccus sagamiensis* PZ-5^T	95
			SAORIC-788	KT12-08-OT5 (1000)	*Pseudohaliea rubra* DSM 19751^T	94
			SAORIC-810	KT12-08-OT5 (1000)	*Dasania marina* DSM 21967^T	93
	Altermonadales	*Colwelliaceae*	SAORIC-602	KT12-08-ON8 (1700)	*Colwellia psychrerythraea* ACAM 550^T	95
	Sphingomonadales	*Sphingomonadaceae*	SAORIC-601	KT12-08-ON8 (1700)	*Altererythrobacter confluentis* KEM-4^T	94
	Arenicellales	*Arenicellaceae*	SAORIC-1700-4-F8	KT12-08-ON8 (1700)	*Arenicella xantha* KMM 3895^T	96
Bacteroidetes	*Flavobacteriales*	*Flavobacteriaceae*	SAORIC-153	MR11-02-K2-2 (2000)	*Aquimarina spongiae* A6^T	95
			SAORIC-665	KT12-08-ON8 (1700)	*Winogradsskyella echinorum* KMM 6211^T	95
			SAORIC-211	MR11-05-K2-1 (1000)	*Dokdonia genika* Cos-13^T	95
			SAORIC-234	MR11-05-K2-1 (1000)	*Tenacibaculum adriaticum* B390^T	94
			SAORIC-774	MR11-05-S1-3 (3000)	*Algibacter agarivorans* KYW560^T	96
			SAORIC-837	KT12-08-OT5 (1000)	*Aquimarina spongiae* A6^T	96
	Rhodothermales	*Rubricoccaceae*	SAORIC-28	KT10-12-S1-3 (3000)	*Rubricoccus marinus* SG-29^T	92
			SAORIC-476	MR11-05-S1-3 (3000)	*Rubrivirga marina* SAORIC-28^T	96
	Sphingobacteriales	*Saprospiraceae*	SAORIC-856	KT12-08-OT5 (1000)	*Lewinella persica* DSM 23188^T	95
Verrucomicrobia	*Verrucomicrobiales*	*Rubritaleaceae*	SAORIC-165	MR11-02-K2-2 (2000)	*Rubritalea marina* Pol012^T	95
	Puniceicoccales	*Puniceicoccaceae*	SAORIC-706	KT12-08-ON8 (1700)	*Coraliomargarita akajimensis* DSM 45221^T	93

*, 16S rRNA gene sequence similarit

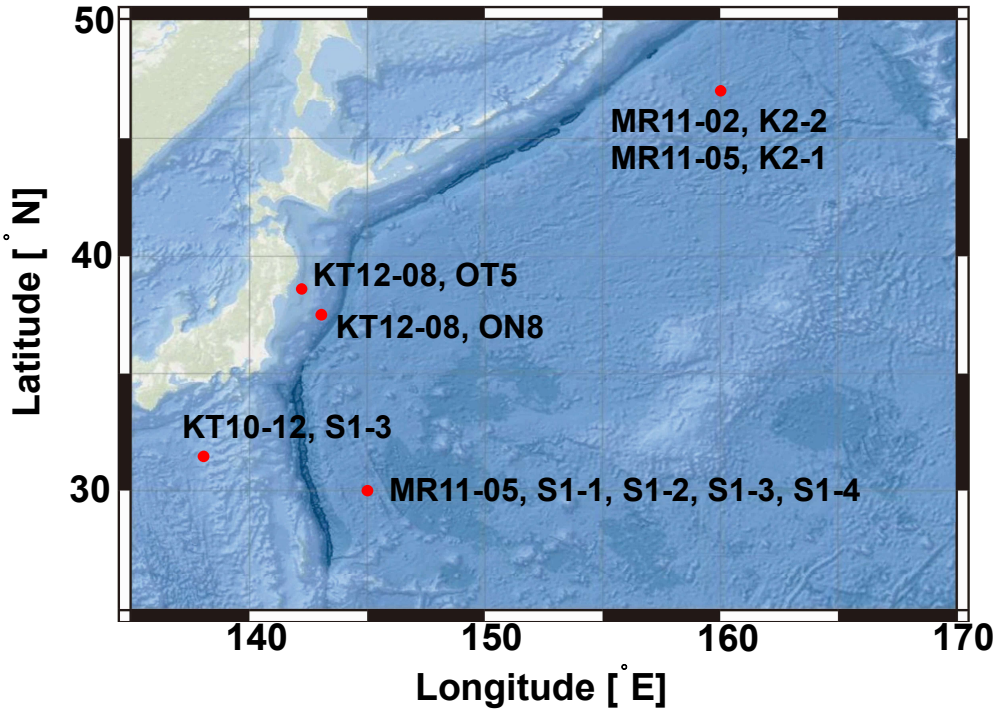

Figure 2-1. Sampling location in the north-western Pacific Ocean.

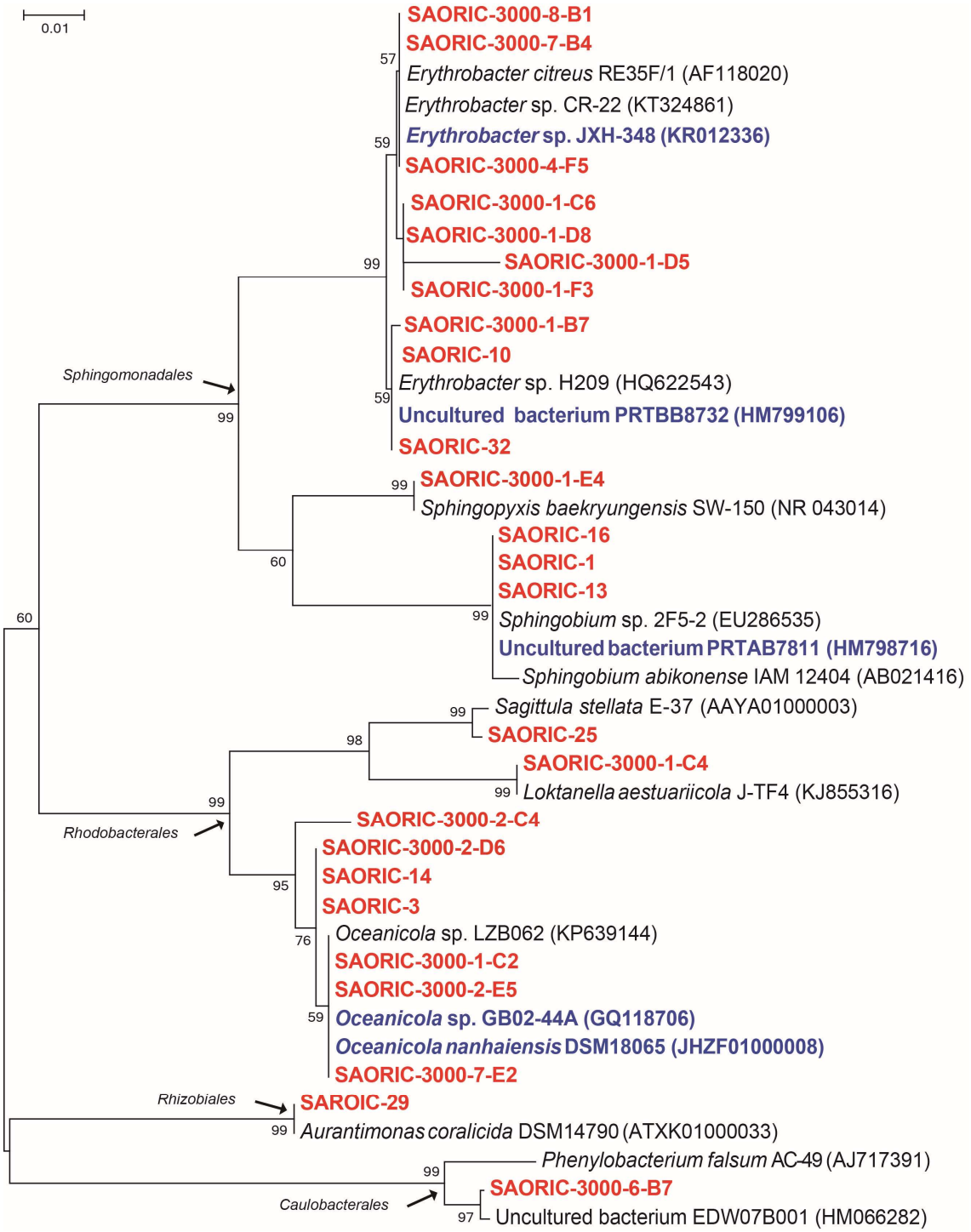

Figure 2-2. Phylogenetic tree of KT10-12-S1-3 isolates in *Alphaproterbacteria*. Blue indicates strains retrieved from the deep sea.

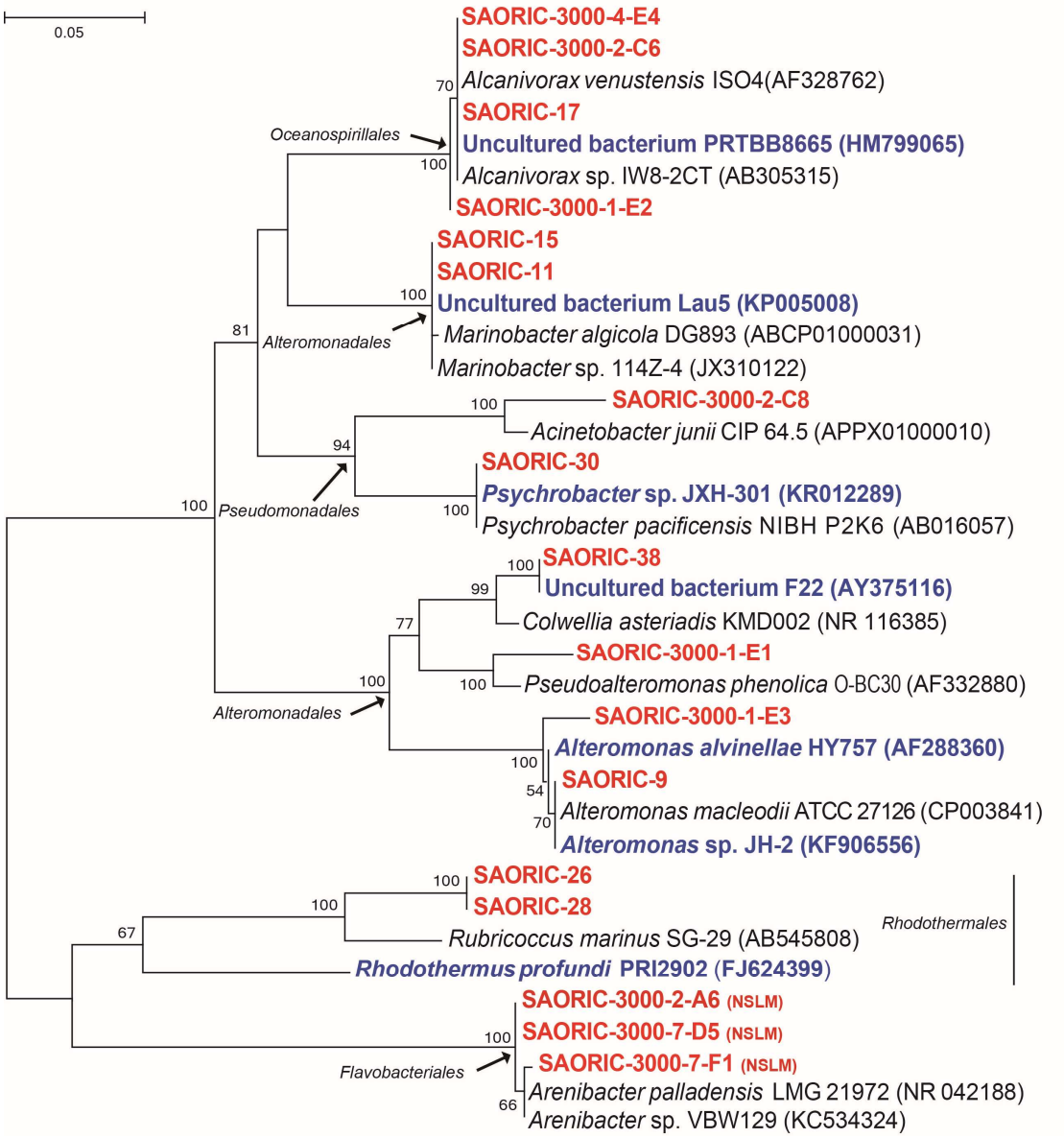

Figure 2-3. Phylogenetic tree of KT10-12-S1-3 isolates in *Gammaproterbacteria* and *Bacteroidetes*.
Blue indicates strains retrieved from the deep sea.
NSLM indicates that strains were isolated from only NSLM

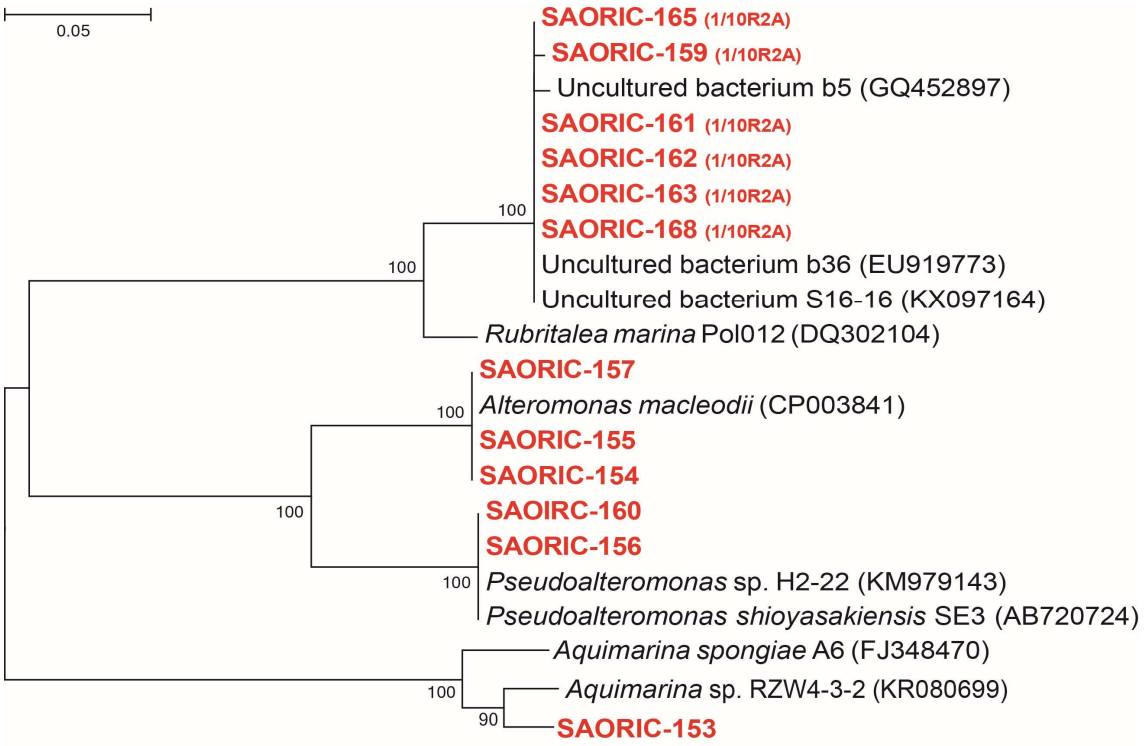

Figure 2-4. Phylogenetic tree of MR11-02-K2-2 isolates

1/10R2A indicates that strains were isolated from only 1/10R2A.

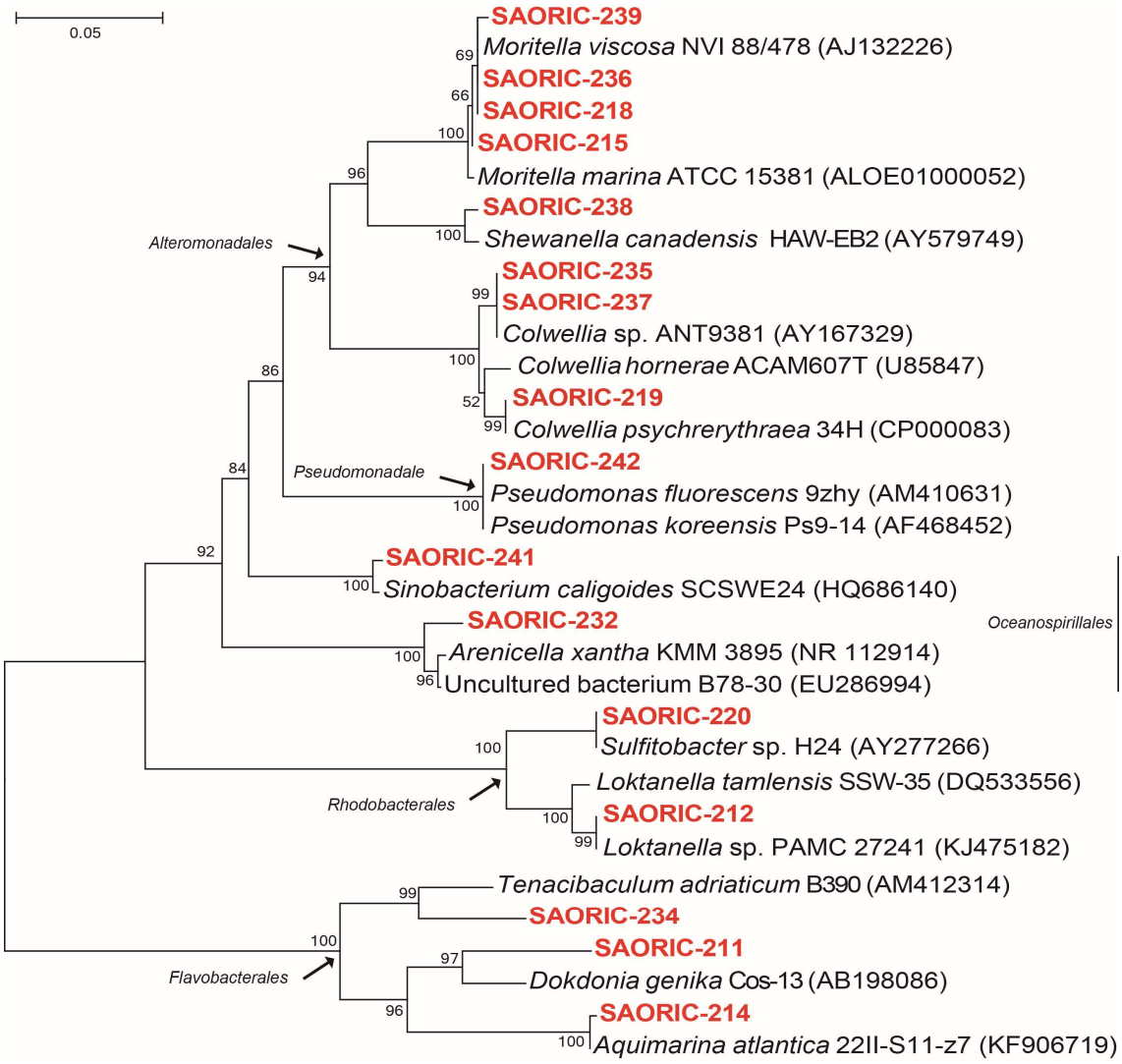

Figure 2-5. Phylogenetic tree of MR11-05-K2-1 isolates.

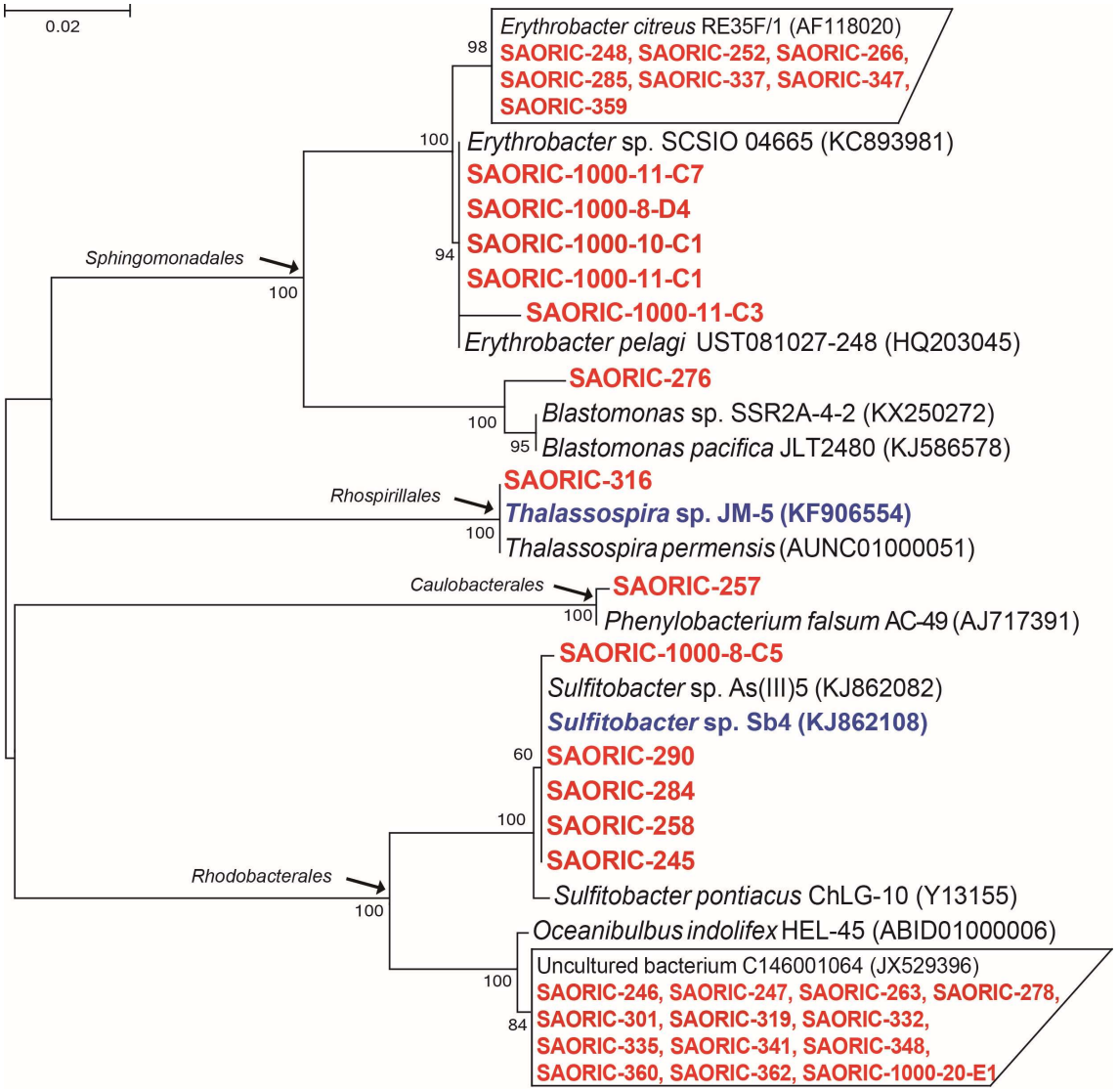

Figure 2-6. Phylogenetic tree of MR11-05-S1-1 isolates in *Alphaproterobacteria*. Blue indicates strains retrieved from the deep sea.

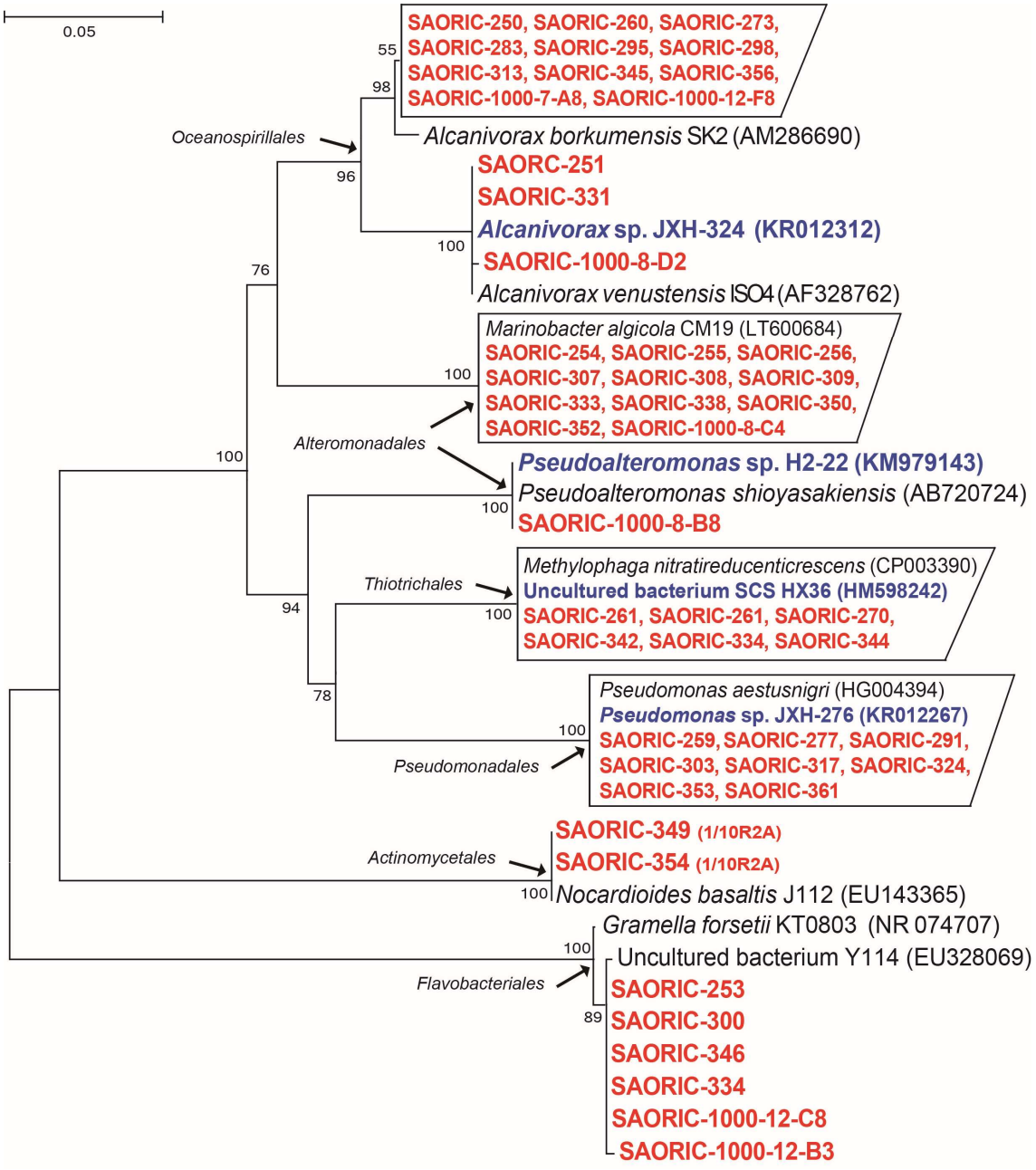

Figure 2-7. Phylogenetic tree of MR11-05-S1-1 isolates in *Gammaproterobacteria*, *Actinobacteria* and *Bacteroidetes*.
Blue indicates strains retrieved from the deep sea.
1/10R2A indicates that strains were isolated from only 1/10R2A.

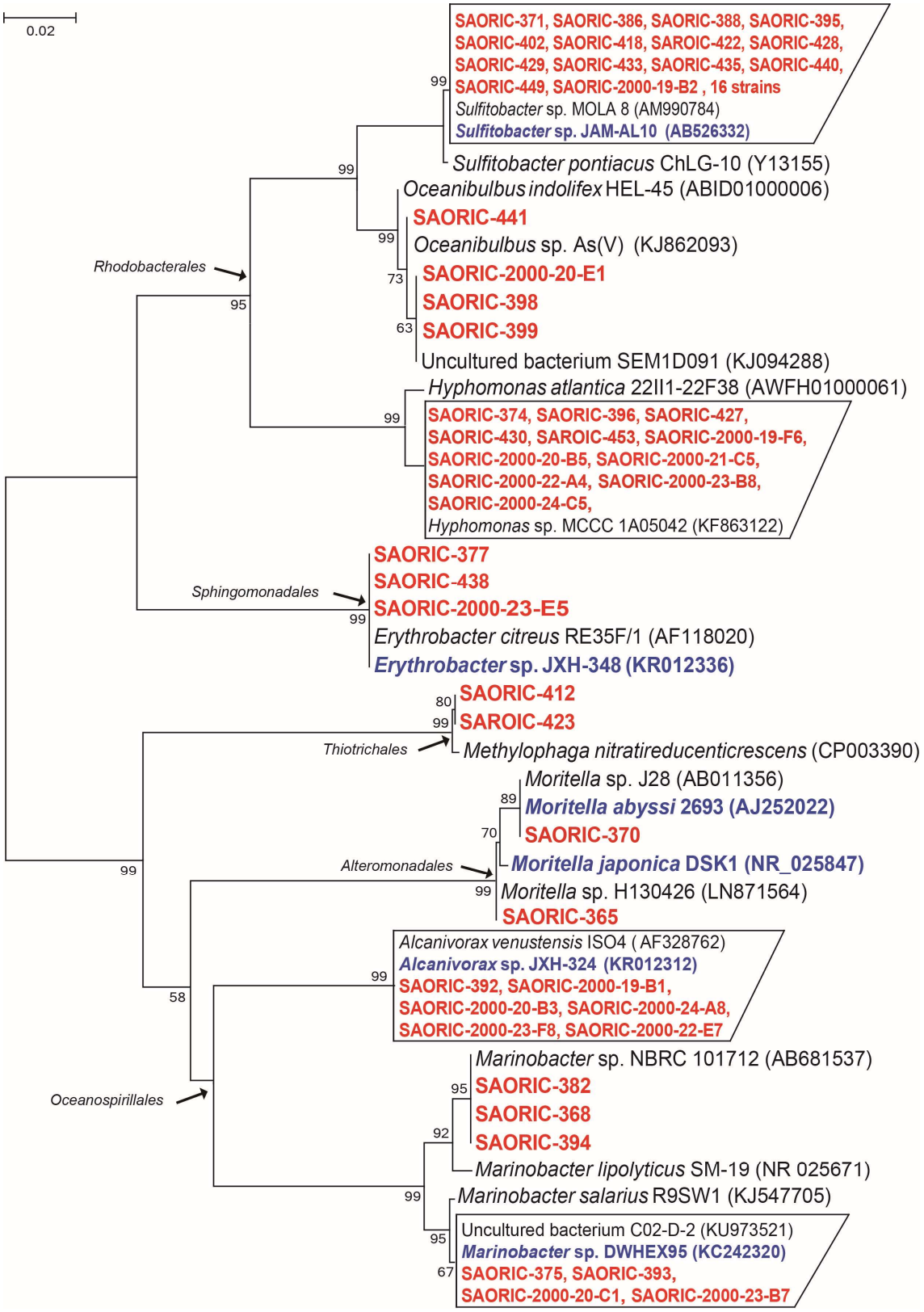

Figure 2-8. Phylogenetic tree of MR11-05-S1-2 isolates
Blue indicates strains retrieved from the deep sea.

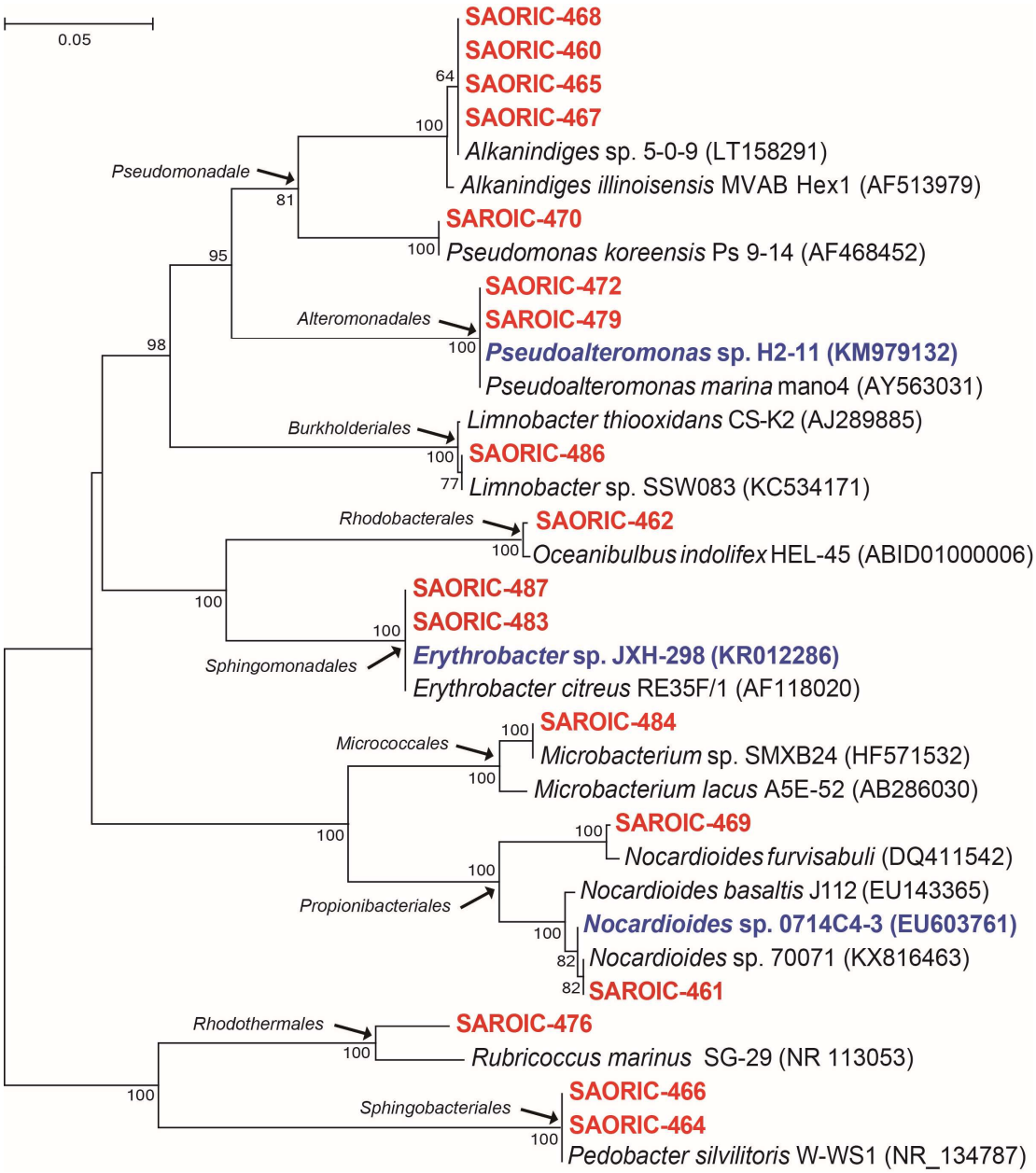

Figure 2-9. Phylogenetic tree of MR11-05-S1-3 isolates
Blue indicates strains retrieved from the deep sea.

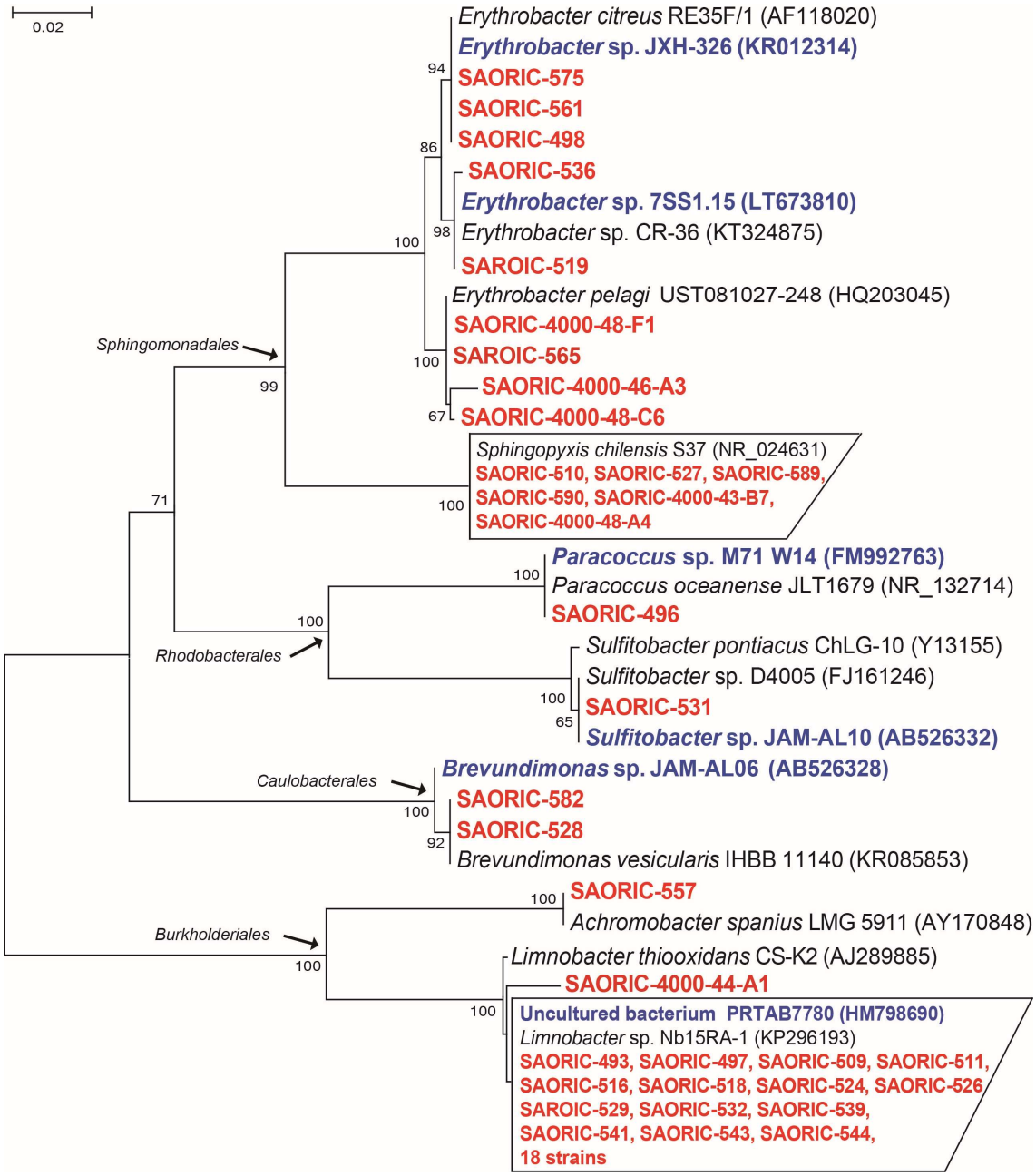

Figure 2-10. Phylogenetic tree of MR11-05-S1-4 isolates in *Alphaproterobacteria* and *Betaproteobacteria*
Blue indicates strains retrieved from the deep sea.

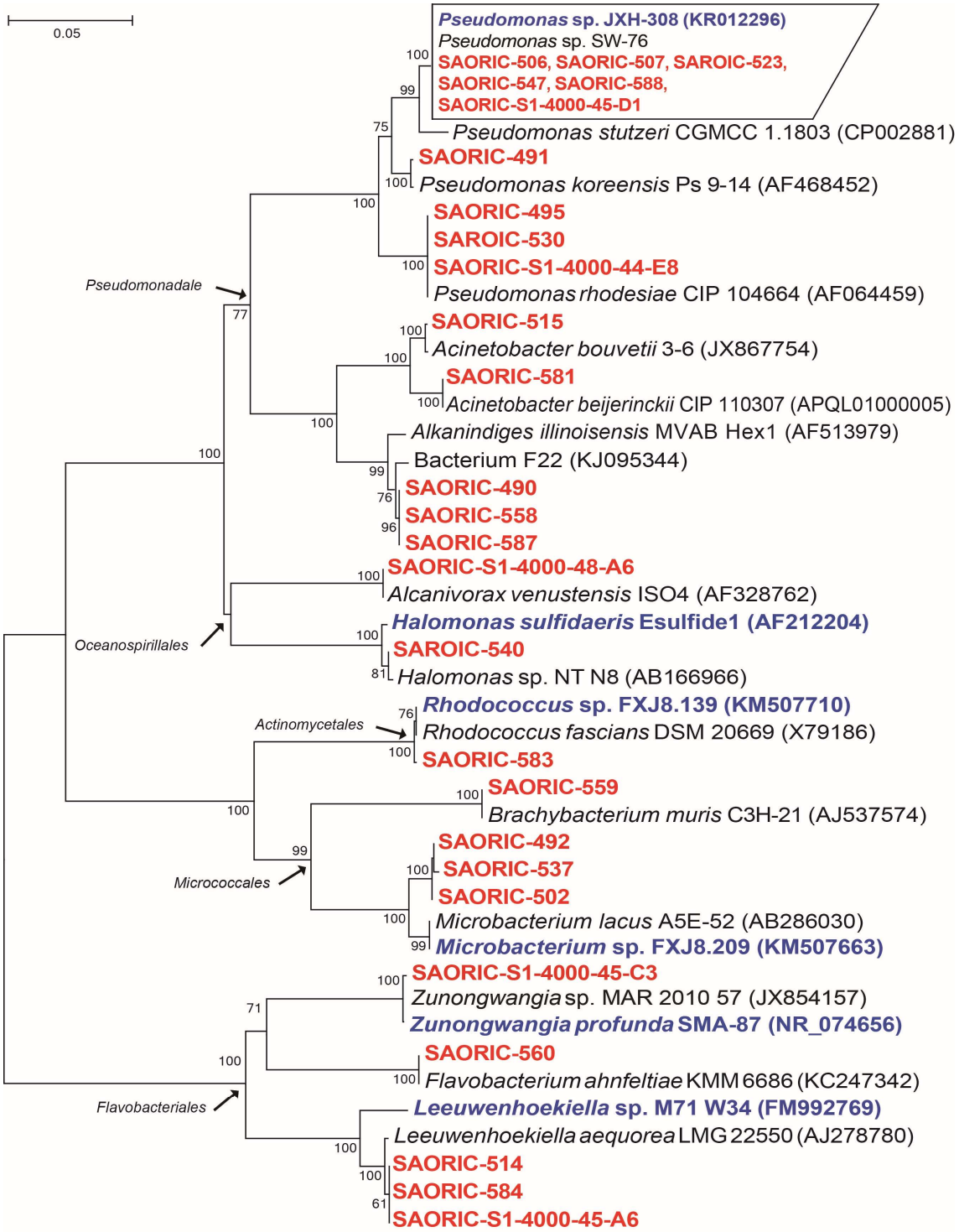

Figure 2-11. Phylogenetic tree of MR11-05-S1-4 isolates in *Gammaproteobacteria*, *Bacteroidetes* and *Actinobacteria*.
Blue indicates strains retrieved from the deep sea.

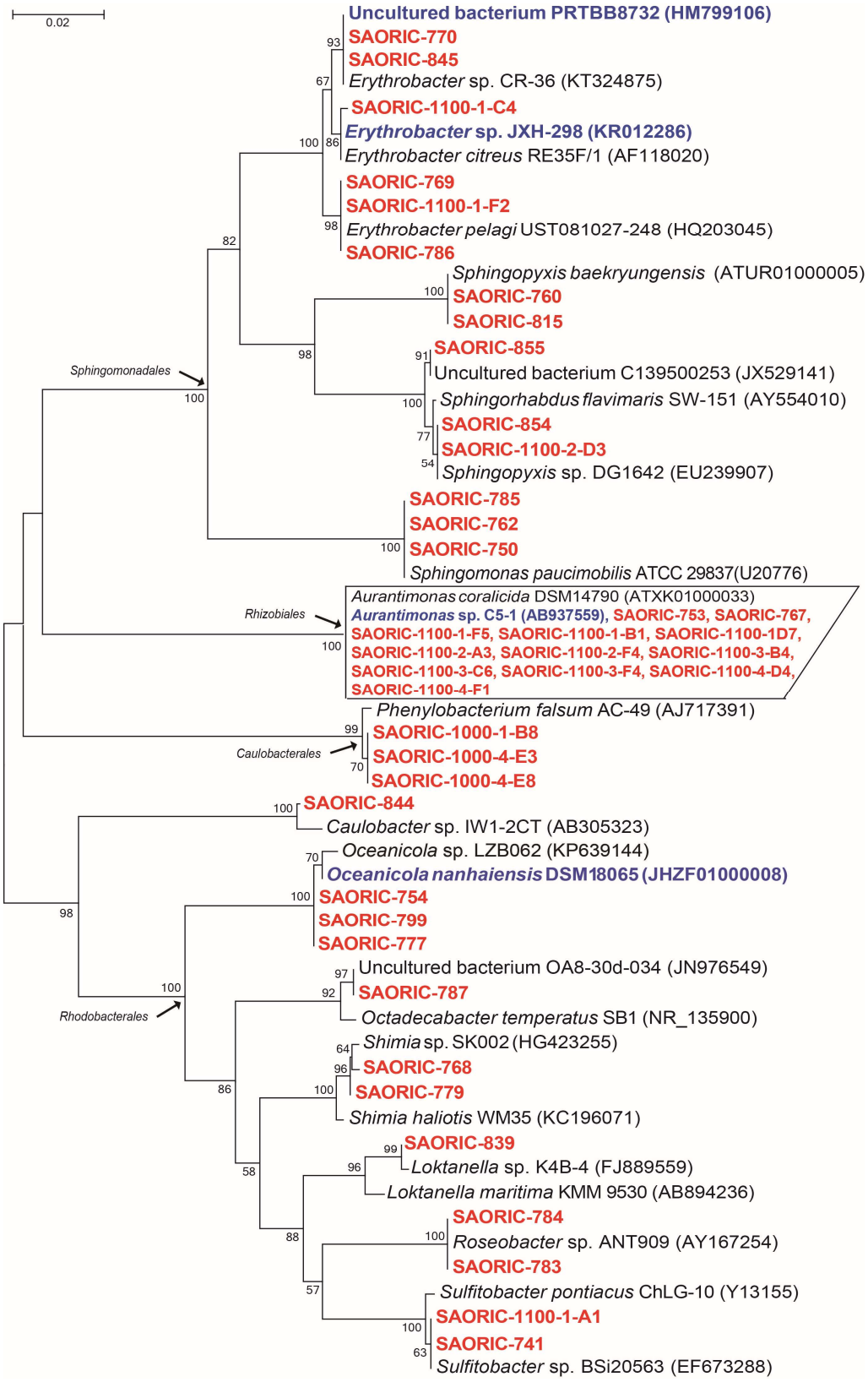

Figure 2-12. Phylogenetic tree of KT12-08-OT5 isolates in *Alphaproterobacteria* Blue indicates strains retrieved from the deep sea.

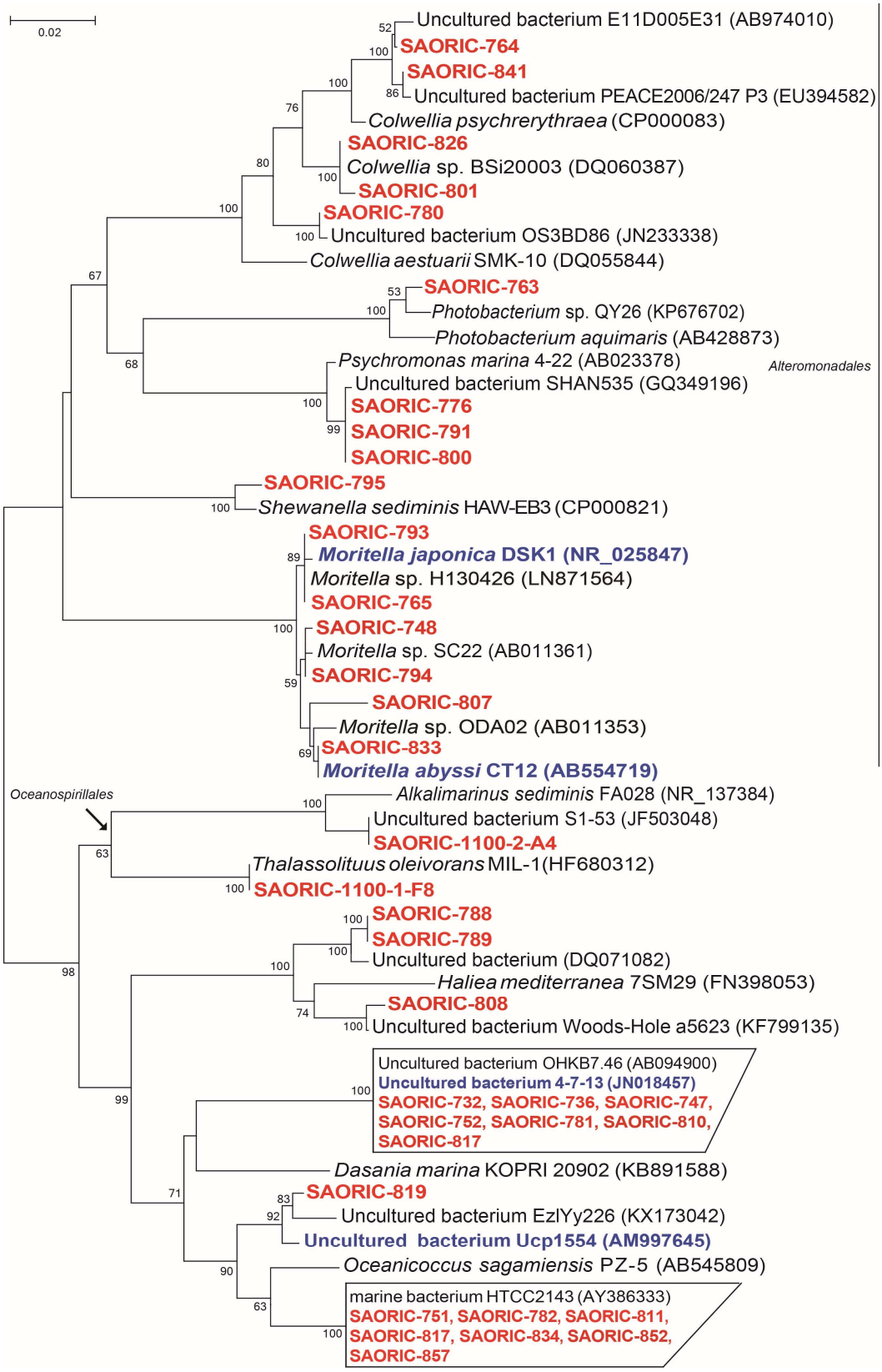

Figure 2-13. Phylogenetic tree of KT12-08-OT5 isolates in *Gammaproterobacteria.* Blue indicates strains retrieved from the deep sea.

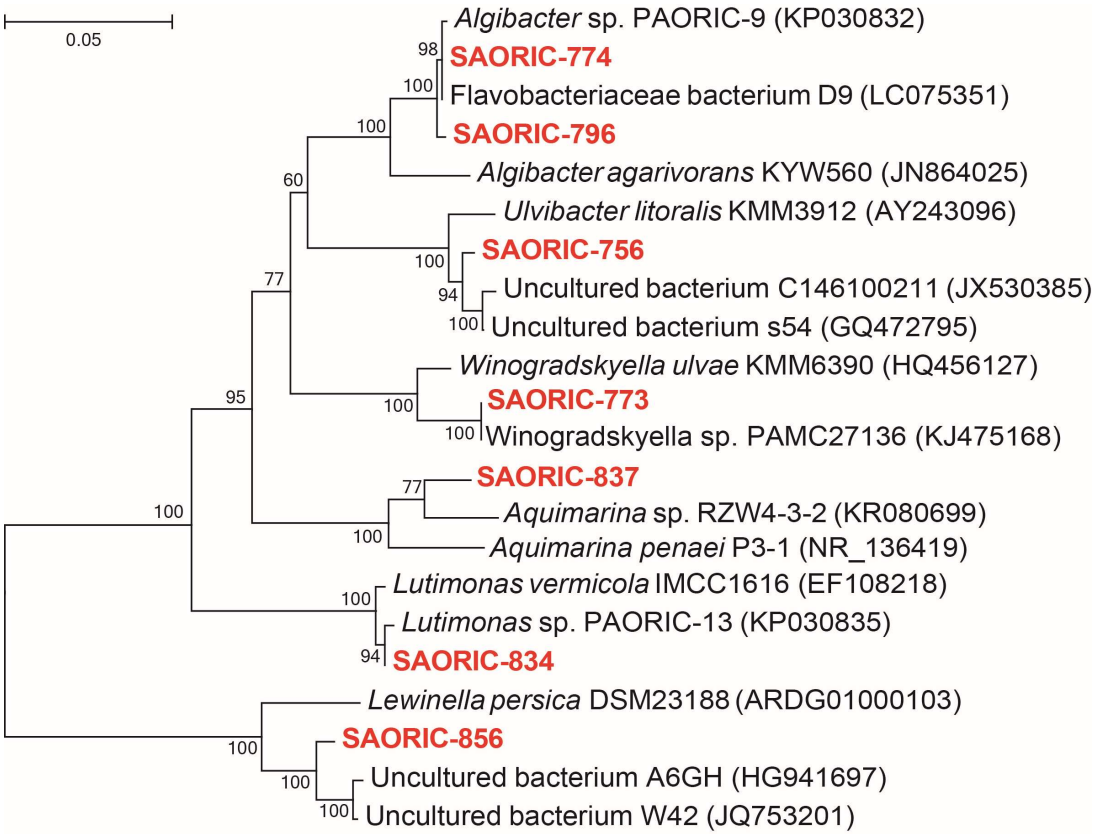

Figure 2-14. Phylogenetic tree of KT12-08-OT5 isolates in *Bacteroidetes*

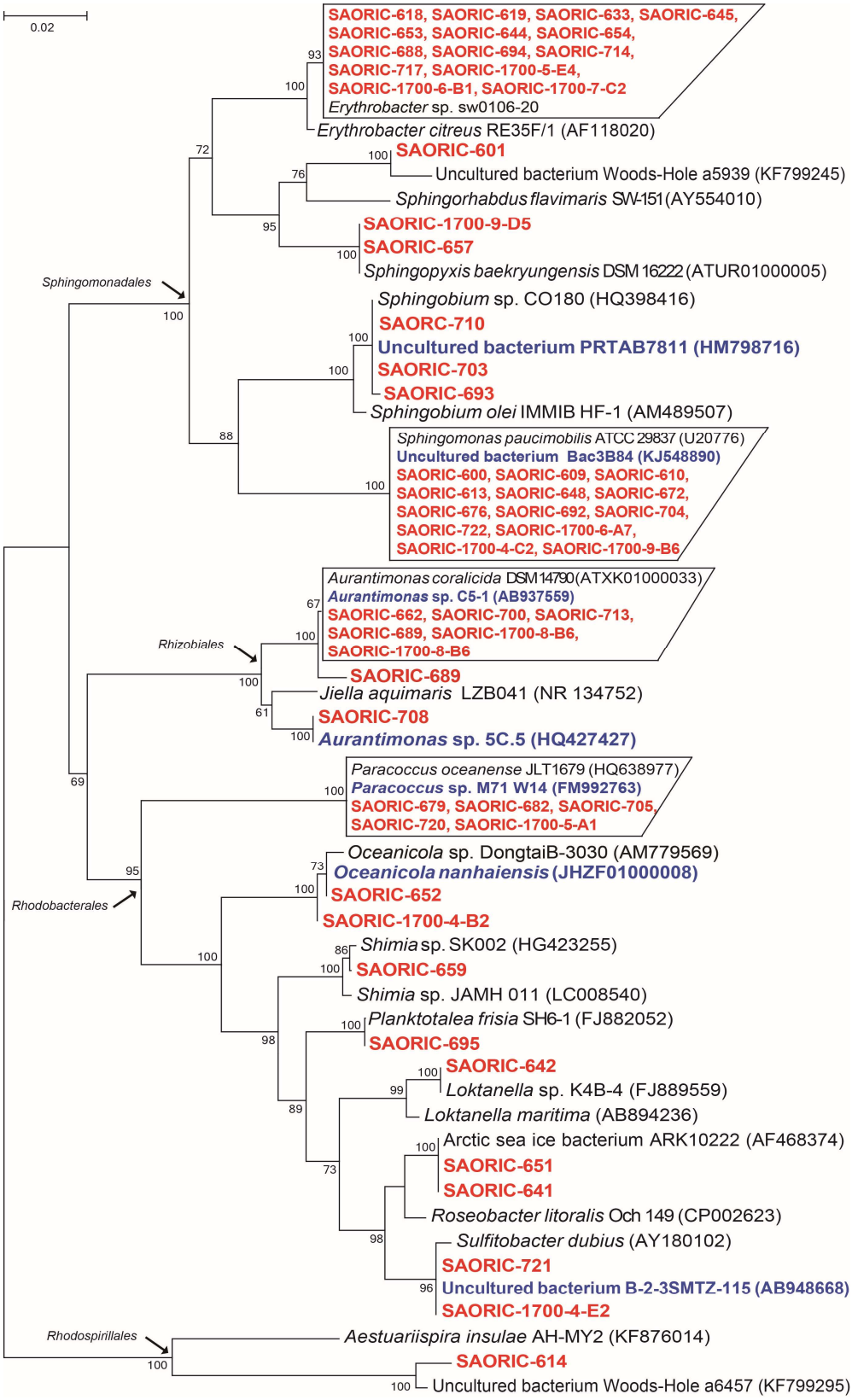

Figure 2-15. Phylogenetic tree of KT12-08-ON8 isolates in *Alphaproterobacteria*. Blue indicates strains retrieved from the deep sea.

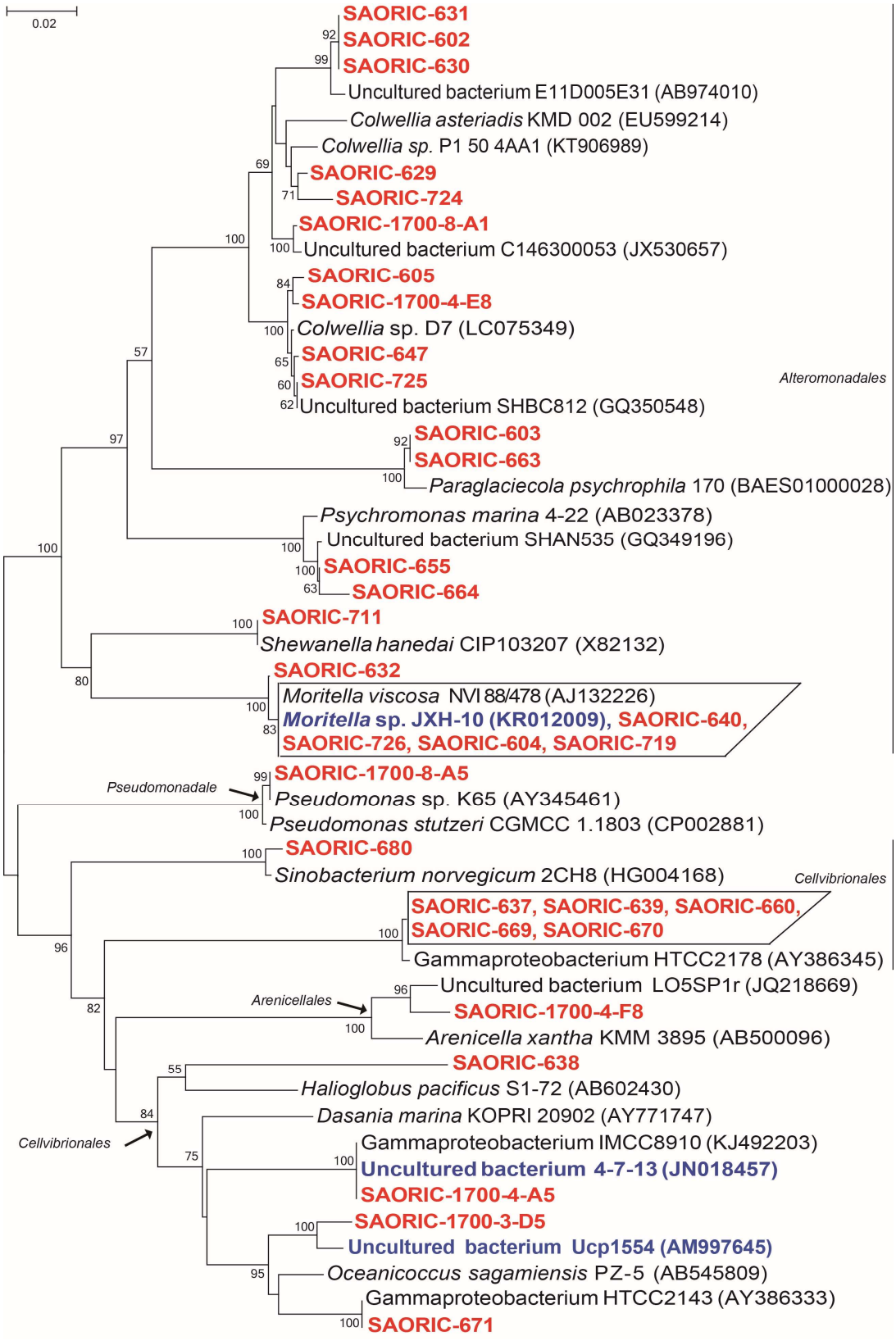

Figure 2-16. Phylogenetic tree of KT12-08-ON8 isolates in *Gammaproterobacteria* Blue indicates strains retrieved from the deep sea.

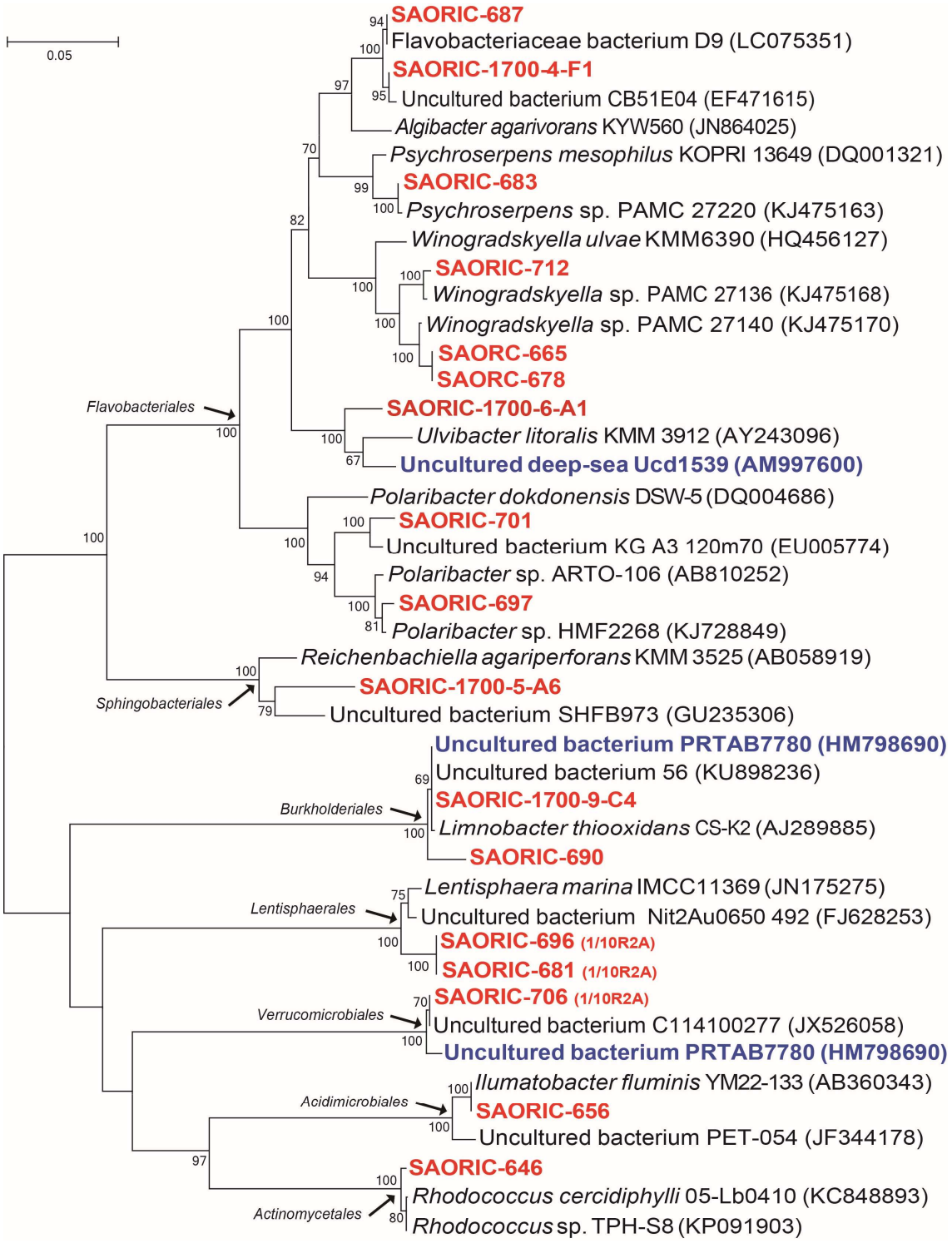

Figure 2-17. Phylogenetic tree of KT12-08-ON8 isolates in *Betaproteobacteria, Bacteroidetes, Verrrucomicrobia, Lentisphaerae* and *Actinobacteria*.

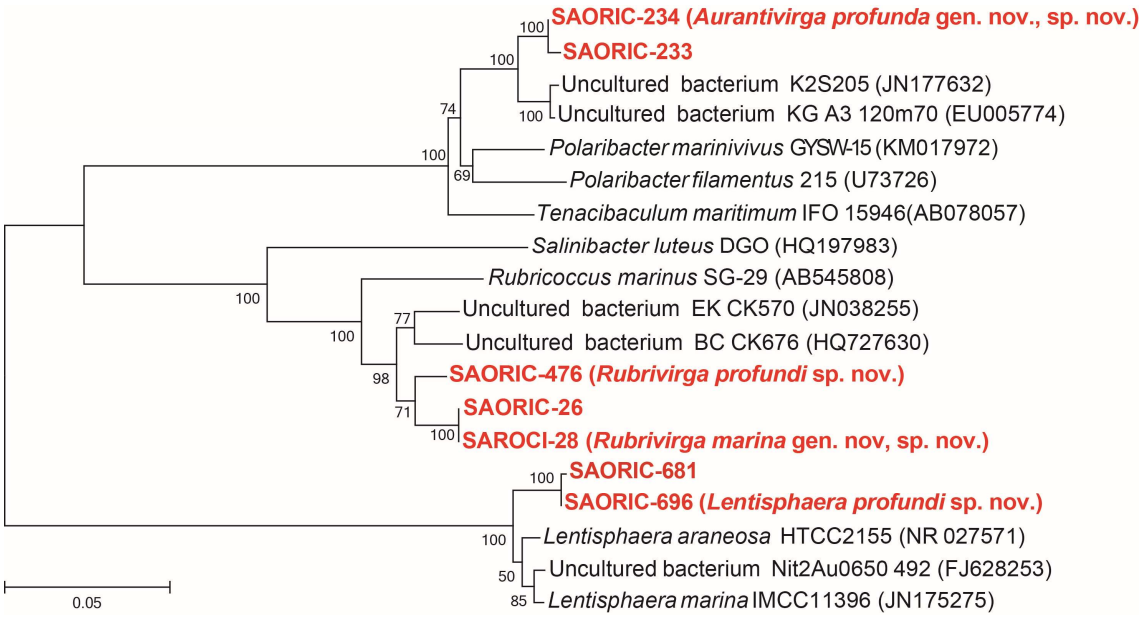

Figure 2-18. Phylogenetic position of validated and reported as novel deep-sea species novel deep-sea species

CHAPTER 3.

Vertical distribution of bacterial community structures in the north-western Pacific Ocean

Introduction

In the previous chapter, bacteria from diverse phylogenetic groups were obtained from 9 deep seawater samples. Approximately 90 % of the total isolates were cosmopolitans, i.e., showing more than 99 % 16S rRNA gene sequence similarities to the strains that had been isolated from the surface. Some strains, however, were novel ones of which 16S rRNA gene sequences had never been reported and/or showed low similarities to any of previous isolates so far. For further examination of my isolates, it is important to know their possible distribution in marine environments, especially vertical distribution in the research area. For this purpose, it is necessary to apply culture independent approach. Recent development of molecular techniques, especially sequencing technologies enabled us to clarify the presence of numerous previously unknown sequences (Sogin *et al*., 2006; Martín-Cuadrado *et al*., 2007; Eloe *et al*., 2010; Zinger *et al*., 2011; Wang *et al*., 2011). Therefore, I tried to apply those sequencing technology to seawater samples in north-western Pacific Ocean.

The findings described in the previous chapter may imply that some prokaryotic groups may be transported vertically due to circulation of seawater in the ocean. Although large scale circulation is known as Broecker's belt conveyor (Broecke, 2010), there should be small scale vertical circulations that bring surface prokaryotic populations to deep layers as well. Another factor for vertical transportation is sinking particles (Sakiyama and Ohwada, 1998; Vanucci *et al*., 2001). Cells on the particles may sink together and distribute in deep environments. However, it is difficult to observe those groups because the collection of sinking particles is not easy. Currently, sediment trap is only the way, but while it is moored in the environment, particles are condensed and eventually degraded by associated prokaryotic cells. Alternatively, separation of particle-

associated (PA) from free-living (FL) populations are often tried by using appropriate size filters (Delong *et al*., 1993; Rösel *et al*., 2012; Suzuki *et al*., 2016). Prokaryotic communities in FL or PA state have repeatedly been proven to differ in diversity and biomass (Kirchman and Mitchell, 1982; Turley and Mackie, 1994; Bidle and Fletcher, 1995; Eloe *et al*., 2010, Suzuki *et al*., 2016). Considering the possible presence of populations on sinking particles, identification of deep-sea prokaryotes in FL or PA state may offer information. Currently, relatively little information is available on the difference between deep-sea prokaryotic communities in PA and FL state (Eloe *et al*., 2010; Salazar *et al*., 2015; Tarn *et al*., 2016).

This chapter aims to clarify the vertical community structures of bacteria in water columns both in PA and FL fractions. Seawater samples were collected at diverse depths (0, 300, 1000, 2000 and 5000 m) at two stations in the north-western Pacific Ocean, and size-fractionated by 3 and 0.22 μm filters. The community structures were profiled using 454-pyroseqeucning. The prokaryotes collected on 3 and 0.22 μm filters refer to particle-associated and free-living ones, respectively. Criterion to determine preference to PA or FL state was based on at least 2 fold differences in relative abundance (Suzuki *et al*., 2016). The results will be discussed with the cultural data in the previous chapter.

Materials and methods

Seawater sampling, genomic DNA extraction, polymerase chain reaction amplification and 454 sequencing were performed by Ryo Kaneko (Okayama University).

Seawater sampling

Seawater samples were obtained from from five depths (0, 300, 1000, 2000 and 5000 m) at 2 stations (K2 and S1) in the north-western Pacific Ocean during MR-11-05 research cruises shown in Table 3-1 and Figure 2-1. Two to 4 L of seawater from each depth were pre-filtered through a 3.0 μm pore size Nuclepore polycarbonate membrane filter (Whatman, Maidstone, UK), and microbial cells were collected onto a 0.22 μm Millipore Sterivex filter unit (EMD Millipore, Darmstadt, Germany). The filters were frozen immediately and stored at -80°C for further analysis in the laboratory.

Genomic DAN extraction

Firstly, the 0.22 μm Sterivex filters were prepared from the cartridge using a sterilized pipe cutter. Genomic DNA was extracted according to the instructions provided with the Charge Switch Forensic DNA purification kit (Invitrogen, Carlsbad, CA, USA) with the following modifications. First, I used a sterile razor blade to cut two types of the filters (3.0 and 0.22 μm pore size) and resultant pieces of the filters were placed into a sterilized 2.0 mL screw cap tube with zirconium beads (ZircoPrep Mini; Nippon Genetics Co. Ltd., Tokyo, Japan) containing Charge Switch Lysis Buffer (L13). Lysis of microbial cells was performed bead beating at 5000 rpm for 30 s using a bead-beater (Micro Smash MS-100R; Tomy Seiko Co., Ltd., Tokyo, Japan) along with proteinase K incubation. After cell wall lysis, 1.0 mL of the supernatant was recovered after spin down at 2000×*g* for 1 min, and the crude DNA in the supernatant was purified according to the manufacturer's instructions. The cell lysis and DNA purification steps were repeated twice to extract

genomic DNA from each sample. The extracted DNA samples were kept at −20℃ until further analysis

Polymerase chain reaction amplification and 454 sequencing

Amplification of the V1–V3 hypervariable regions of bacterial 16S rRNA gene was performed with the bacteria-specific primer 27F (5′-CCATCTCATCCCTGCGTGTCTC CGACTCAGXXXXXXXXXXAGAGTTTGATCMTGGCTCAG-3′; where X's represents the sample-specific multiplex identifier) and the universal primer 519R (5′ GWATTACCGCGGCKGCTG-3′). The forward primer contained the sequence of 454 adapter A (5′-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3′) and the multiplex identifiers (MIDs), and the reverse primer contained the sequence of 454 adapter B (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′). Polymerase chain reaction (PCR) amplifications were performed using 1X Ex TaKaRa Ex Taq® HS Polymerase (Takara Bio., Shiga, Japan) and the following sequence: denaturation at 94℃ for 3 min followed by 27 cycles at 98℃ for 10 s, primer annealing at 57℃ for 30 s and at 72℃ for 50 s, followed by a final extension at 72℃ for 7 min. In order to minimize the potential effect of PCR biases in single reactions (Polz and Cavanaugh 1998), ten independent PCR products were pooled. The pooled PCR product was purified using an Agencourt AMPure XP kit (Beckman Coulter, Brea, CA, USA), according to the manufacturer's instructions. The purity and concentration of PCR products were confrimed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and equal amounts of the PCR products from each samples were mixed. Pyrosequencing was performed using a Roche emPCR Lib-L kit (Roche Diagnostics, Branford, CT, USA) and was carried out using 454 GS-FLX System with Titanium chemistry (Roche Diagnostics).

The resultant high quality sequences were clustered into operational taxonomic

units (OTUs) at a 97 % identity in 16S rRNA gene sequence. Representative 16S rRNA gene sequences from each OTU were assigned to taxonomic categories in Mothur based on the SILVA database (v.119) (Pruesse *et al*. 2007).

Comparison of isolated sequences with 454 pyrosequencing sequences

The V1–V3 hypervariable regions of the 16S rRNA gene seqeucnes obatained from 454 pyrosequencing were compared to the 16S rRNA gene sequences of deep-sea isolates from Chapter 2. The 454 pyrosequencing sequences were used as database. Comparison was performed using BLAST+ software version 2.2.23 (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) on a local compter. Closest related sequences from 454 pyrosequcencing with > 95 % similarity, more than 150 bp, and at least from the same genus based on between the 454-pyrosequencing's SILVA v119.

Results

Table 3-1. Information of sweater samples.

Cruise	Location	Sampling date	Temperature (°C)	Depth (m)
MR11-05-K2	47°00' N, 160°00' E	June, 2011	6.8	0
			3.5	300
			2.5	1000
			1.8	2000
			1.5	5000
MR11-05-S1	32°00' N, 145°00' E	July, 2011	26.5	0
			17.0	300
			3.8	1000
			2.0	2000
			1.5	5000

Table 3-2. Number of sequences collected from station K2.

	Free living					Particle associated				
	0 m	300 m	1000 m	2000 m	5000 m	0 m	300 m	1000 m	2000 m	5000 m

Table 3-3. Number of sequences collected from station S1.

	Free living					Particle associated				
	0 m	300 m	1000 m	2000 m	5000 m	0 m	300 m	1000 m	2000 m	5000 m

Table 3-4. Relative abundance of each group collected from station K2. Significant preference to states was marked in red.

	Relative abundance (Free living) (%)					Relative abundance (Particle associated) (%)				
	0 m	300 m	1000 m	2000 m	5000 m	0 m	300 m	1000 m	2000 m	5000 m

Table 3-5. Relative abundance of each group collected from station S1. Significant preference to state was marked in red.

	Relative abundance (Free living) (%)					Relative abundance (Particle associated) (%)				
	0 m	300 m	1000 m	2000 m	5000 m	0 m	300 m	1000 m	2000 m	5000 m

Table 3-6. Relative abundance of phylotypes including the isolates.

	Free living K2-depths (%)					Particle associated K2-depths (%)					Free living S1-depths (%)					Particle associated S1-depths (%)				
Phylotypes	0	300	1000	2000	5000	0	300	1000	2000	5000	0	300	1000	2000	5000	0	300	1000	2000	5000

Bold numbers indicate >0.5 % relative abundance

Figure 3-1. Bacterial composition at station K2 and S1 at phylum or class level.
Pro-Alpha, *Alphaproteobacteria*; Pro-Gamma*, Gammaproteobacteria*; Pro-Beta, *Betaproteobacteria*; Pro-Delta; *Deltaproteobacteria*; Pro-Epsilon, *Epsilonproteobacteria.*

Figure 3-2. Relative abundance of *Alphaproteobacteria* at station K2 and S1 at order level.

Figure 3-3. Relative abundance of *Gammproteobacteria* at station K2 and S1 at order level.

Figure 3-4. Relative abundance of *Deltaproteobacteria* at station K2 and S1 at order level.

Figure 3-5. Relative abundance of *Flavobacteriales* of *Bacteroidetes*, SAR402 of *Deferribactere* and SAR202 of *Chloroflexi* at station K2 and S1.

Figure 3-6. Relative abundance of phylotypes including the isolates represented at station K2 and S1

CHAPTER 4.

Physiological characteristics of deep-sea bacteria in comparison with surface-sea relatives

Introduction

In the previous chapter, the vertical community structures of bacteria in two water columns were investigated by using culture independent approach. The results indicated the possible distribution patterns of the isolates which were described in Chapter 2. Although *Erythrobacter* phylotypes showed relatively even vertical distribution, other phylotypes of my isolates showed distribution more restricted to deeper layers. Then, are there any characteristics unique to those group thriving in deep-sea? Also, in the previous chapter, particle-associated (PA) or free-living (FL) state of natural populations were examined. It is expected that cells preferring PA state may have a tendency to attach particles, degrade polymeric substances and grow quickly. Again, are these possible characteristics really present and functioning among the isolates? Finally, most of my deep-sea isolates have phylogenetically close strains that had been isolated from surface layers. Does this simply mean, the deep-sea isolates and their surface relatives share most of functional characteristics or are there any genes unique to either deep-sea or surface strains? In order to answer these questions, further study for investigating the characterstics of the deep-sea isolates should be conducted.

Deep-sea bacteria have been well characterised by several physiological characteristics unique to deep-sea conditions. Some of deep-sea bacteria show piezophilic and/or psychrophilic characteristics in response of high hydrostatic pressure and low water temperatures of the deep sea (Lauro and Bartlett, 2008). Therefore, influence of temperature and pressure on the growth is the basic characters to be examined first. Relevantly, the cell membrane composition of the deep-sea bacteria is a point of interest since cell membrane is the primary site affected by high hydrostatic pressure and low temperature (DeLong and Yayanos, 1985; Lauro *et al*. 2007; Grossi *et al*., 2010). It has

been regarded that deep-sea bacteria should maintain their membrane fluidity by tuning the composition, especially, fatty acids. However, so far, most of data on physiological characteristics of deep-sea bacteria are obtained for gammaproteobacterial groups. The information for my deep-sea isolates other than *Gammaproteobacteria* should extend our view on deep-sea bacteria. For instance, *Sulfitobacter* and *Erythrobacter* have been isolated from deep-sea environments but their physiological properties are little known.

In the present chapter, I aimed to identify physiological characteristics of the new deep-sea isolates. The growth patterns of them were examined under a range of pressures, temperatures and NaCl concentration. Subsequently, cell membrane composition (polar lipids and fatty acids) and metabolic (hydrolytic enzyme activity) characteristics of the isolates were identified. Comparisons were made with their surface relatives and the characteristics of deep-sea isolates will be discussed.

In order to identify physiological characteristics, several deep-sea strains from the isolates of Chapter 2 were sellected based on their taxonomical characteristics (Table 2-13). Sellected strains are following; *Rubritalea* sp. SAORIC-165 (the first deep-sea isolate within the phylum *Verrucomicrobia*), *Rubrivirga profunda* SAORIC-234 (novel deep-sea species), *Oceanibulbus* sp. SAORIC-263 (predominant phylotypes in the isolates of MR11-05-S1), *Sulfitobacter* sp. SAORIC-395 (predominant phylotypes in the isolates of MR11-05-S2), *Rubrivirga profundi* SAORIC-476 (novel deep-sea species), *Limnobacter* sp. SAORIC-580 (predominant phylotypes in the isolates of MR11-05-S4 and the first deep-sea isoaltes within the family *Burkholderiaceae*), *Erythrobacter* sp. SAORIC-644 (predominant phylotypes in the isolates of KT12-08-ON8) and *Roseobacter* sp. SAORIC-651 (putative novel deep-sea species).

Materials and methods

Growth profile under high pressure

The growth under different pressure were investigated for the isolates, *Rubritalea* sp. SAORIC-165, *Rubrivirga profunda* SAORIC-234, *Oceanibulbus* sp. SAORIC-263, *Sulfitobacter* sp. SAORIC-395, *Rubrivirga profundi* SAORIC-476, *Limnobacter* sp. SAORIC-580, *Erythrobacter* sp. SAORIC-644 and *Roseobacter* sp., SAORIC-651. In addition, piezophilic bacterial strain, *Photobacterium profundum* DSJ4^T (Nogi *et al*., 1998a) was also investigated for comparision. Cells in mid-log phase on agar plates were suspended into marine broth 2216 (MB; BD Difco, autoclaved and filtered through 0.22 μm membrane filters) or marine R2A broth (BD Difco, autoclaved and filtered through 0.22 μm membrane filters) with 10^4-10^5 cells/ml cell concentration. The broth was divided into 1.5 ml sterilized plastic tubes and sealed with parafilm. The tubes were then incubated under various pressures (0.1, 10, 20, 30, 40 MPa). After incubation for 1 week at 10 or 20℃, total cells of the cultures were counted. Cell counting was performed with an Easy-Cyte flow cytometer (Guava Technologies). A portion of 200 μl of samples was acquired on an Easy-Cyte flow cytometer (Guava Technologies) after 1 h of staining in 1:2,000 diluted SYBR-Green I (Invitrogen).

Growth rate determination

Among the deep-sea isolates, *Rubritalea* sp. SAORIC-165, *Rubrivirga profunda* SAORIC-234, *Oceanibulbus* sp. SAORIC-263, *Sulfitobacter* sp. SAORIC-395, *Limnobacter* sp. SAORIC-580, *Erythrobacter* sp. SAORIC-644 and *Roseobacter* sp., SAORIC-651 were selected. For determination of growth rate, mid-log cultures on agar plates were suspended into marine broth 2216 (MA; BD Difco autoclaved and filtered through 0.22μm membrane filters) or marine R2A broth (MB; BD Difco) with 0.5 x 10^5

cells/ml cell concentration. The broth was incubated at various temperatures (10, 15, 20, 25 and 30). Growth was monitored by measuring turbidity at a wavelength of 660 nm by spectrophotometer every 12 or 24 hours and counting cell numbers by Easy-Cyte flow cytometer (Guava Technologies). Subsequently, their growth rate at each temperature was calculated.

Optimum NaCl concentration

Oceanibulbus sp. SAORIC-263, *Sulfitobacter* sp. SAORIC-395, *Limnobacter* sp. SAORIC-580 and *Erythrobacter* sp. SAORIC-644 were selected. The NaCl concentration for growth was determined on NaCl-free medium based on MA formula. For the test, NaCl-free MB was prepared according to the formula of MB (BD Difco) devoid of NaCl (NaCl-free MB; 5.0g Peptone, 1.0 g Yeast extract, 1.0 g $MgCl_2 \cdot 6H_2O$, 4.0 g Na_2SO_4, 0.7 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl, 0.2 g $NaHCO_3$, 0.1 g KBr, 0.27 g KH_2PO_4, 0.04 g $SrCl_2 \cdot 6H_2O$, 0.025 g H_3BO_3, 0.001 g NaF, 10 ml Tris-Cl (pH 8.0) per 1 L deionized water). NaCl concentration was adjust to 0-5 % (at intervals of 1 %). For inoculation, mid-log cultures on agar plates were suspended into the medium with 10^4-10^5 cells/ml cell concentration. Suspensions was divided into 20 ml sterilized test tubes. After incubation for 1 week at 10 or 20℃, its turbidity was measured at a wavelength of 660 nm by spectrophotometer.

Composition of polar lipids and fatty acids

Rubritalea sp. SAORIC-165, *Rubrivirga profunda* SAORIC-234, *Oceanibulbus* sp. SAORIC-263, *Sulfitobacter* sp. SAORIC-395, *Rubrivirga profundi* SAORIC-476, *Limnobacter* sp. SAORIC-580 and SAORIC-690, *Erythrobacter* sp. SAORIC-644 and *Roseobacter* sp., SAORIC-651 were selected and analyzed along with their surface-sea relatives. Their surface-sea originated relatives were found to *Rubritalea marina* DSM

177716^T (16S rRNA gene similarity; 95.9 % with SAORIC-165), *Polaribacter porphyrae* NBRC 108759^T (94.9% with SAORIC-234), *Sulfitobacter delicates* KCTC12547^T (99.4% with SAORIC-263), *Sulfitobacter pontiacus* JCM 21789^T (99.7% identity with SAORIC-395), *Rubricoccus marinus* SG-29^T (95.9 % with SAORIC-476), *Limnobacter thiooxidans* KCTC 12942^T (99.5% with SAORIC-580), *Erythrobacter citreus* JCM 21816^T (99.7% with SAORIC-644) and *Roseobacter litoralis* Och149^T (99.0 % with SAORIC-651). Deep-sea strains and their surface relatives were maintain at same temperature and under atmospheric pressure.

Polar lipids were extracted according to the procedures described by Minnikin *et al* (1984). Lyophilized cells (50 mg) were put into a glass tube with a Teflon coated cap and 2 ml of aqueous methanol (10 ml of 0.3 % aqueous NaCl added to 100 ml of methanol) and 2 ml of hexane were added and shaken for 15 min. The suspension was centrifuged at 500 rpm for 5 min and the upper layer was removed. Unless otherwise mentioned, centrifugation was performed at 500 rpm for 5 min. The under layer was hermetically boiled for 5 min and cooled at 37°C in a water bath. An aliquot of 4.6 ml of chloroform:methanol:water (90:100:300) was added and shaked for 1 h. After centrifugation, the upper layer was transferred to another clean tube. Susequently, 1.5 ml of chloroform:methanol:water (90:100:300) was added into the clean tube and shaked for 30 min. After centrifugation, the upper layer was transferred to another clean tube. The procedure of the extraction with 1.5 ml of chloroform:methanol:water (90:100:300) was repeated. An aliquot of 2.6 ml of respective chloroform and water was added to the upper layer which was collected. It was 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) for two-dimensional TLC analyzing. The solution was spotted to the bottom of thin-

layer plate coated with silica gel (silica gel 60, Merck, Darmstadt, Germany). First developing with chloroform:methanol:water (65:25:4, by vol.) and second developing with chloroform:acetic acid:methanol:water (80:18:12:5, by vol.) were performed. After development, polar lipids were identified by appropriate detection reagents (Minnikin *et al.*, 1984; Komagata & Suzuki, 1987). Phospholipids were detected with the Zinzadze reagent of Dittmer & Lester (1964). Whole lipid profiles were detected by spraying with molybdatophosphoric acid (5 g molybdatophosphoric acid hydrate in 100 ml ethanol) followed by heating at 150℃ (Worliczek *et al*., 2007).

As for fatty acids, fatty methyl esters were extracted and analyzed according to the Sherlock Microbial Identification Systems (MIDI). Each strains was cultured at optimum temperature in optimum media. The cells growing from the third quadrant streak on the plate were placed in a clean tube. The tube was boiled for 30 min after addition of 1 ml Sherlock Reagent 1 and vigorously vortex for 5-10 seconds. The cooled tubes was methylated with 2 ml Sherlock Reagent 2. The tubes was heated at 80℃ for 10 min then cooled rapidly. After adding 1.25 ml of Sherlock Reagent 3, to the cooled was gently tumblied on a rotator for about 10 min. After the aqueous (lower) phase was discarded, the remaining upper phase was washed with Sherlock Reagent 4. Then about 2/3 of the upper layer (around 300 μl) 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. Fatty acid methyl esters were identified according to the standard protocols provided by the MIDI/Hewlett Packard Microbial Identification system (Sasser, 1990) using TSBA 6.1 database.

Hydrolase enzyme activity

Hydrolytic enzyme activities were tested using API ZYM strips (bioMerieux). All

suspension media for the API test strips were supplemented with artificial seawater (ASW; 25 g NaCl, 1.0 g $MgCl_2 \cdot 6H_2O$, 4.0 g Na_2SO_4, 0.7 g KCl, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl, 0.2 g $NaHCO_3$, 0.1 g KBr, 0.27 g KH_2PO_4, 0.04 g $SrCl_2 \cdot 6H_2O$, 0.025 g H_3BO_3, 0.001 g NaF, 10 ml Tris-Cl (pH 8.0) per 1 L deionized water). Results of API ZYM was recorded by following the manufacturer's instructions after 7 days of incubation.

Results

Table 4-1. Growth rate at different temperature.

	10℃	15℃	20℃	25℃	30℃

Numbers, growth rate (u/h); -, no growth;

Parentheses, predicted growth rate from turbidity at a wavelength of 660 nm

Table 4-2. Cellular fatty acid composition of *Rubritalea* sp. SAORIC-165^T and *Rubritalea marina* DSM 177716^T.

Fatty acid	SAORIC-165	DSM 177716

–, Not detected; tr, trace (less than 1.0 %)

Table 4-3. Cellular fatty acid composition of *Aurantivirga profunda* SAORIC-234^T and *Polaribacter porphyrae* NBRC 108759^T.

Fatty acid	SAORIC-234	NBRC 108759

–, Not detected; tr, trace (less than 1.0 %).

Table 4-4. Cellular fatty acid composition of *Oceanibulbus* sp. SAORIC-263 and *Sulfitobacter delicatus* KCTC12547^T.

Fatty acid	SAORIC-263	KCTC12547

–, Not detected; tr, trace (less than 1.0 %)

Table 4-5. Cellular fatty acid composition of *Sulfitobacter* sp. SAORIC-395 and *Sulfitobacter pontiacus* JCM 21790^{T}.

Fatty acid	SAORIC-395	JCM 21790

–, Not detected; tr, trace (less than 1.0 %).

Table 4-6. Cellular fatty acid composition of *Rubrivirga profunda* SAORIC-476^{T} and *Rubricoccus marinus* SG-29^{T}.

Fatty acid	SAORIC-476	SG-29

–, Not detected; tr, trace (less than 1.0 %).

Table 4-7. Cellular fatty acid composition of strain SAORIC-580 and *Limnobacter thiooxidans* KCTC 12942^{T}.

Fatty acid	SAORIC-580	KCTC 12942

–, Not detected; tr, trace (less than 1.0 %).

Table 4-8. Cellular fatty acid composition of *Erythrobacter* sp. SAORIC-644 and *Erythrobacter citreus* JCM 21816^{T}.

Fatty acids	SAORIC-644	JCM 21816

–, Not detected; tr, trace (less than 1.0 %).

Table 4-9. Cellular fatty acid composition of *Roseobacter* sp. SAORIC-651 and *Roseobacter litoralis* Och 149^{T}.

Fatty acids	SAORIC-651	Och 149

–, Not detected; tr, trace (less than 1.0 %).

Table 4-10. Hydrolytic enzyme activities.

Enzymes	*Rubritalea*		*Aurantivigra*		*Oceanibulbus*		*Sulfitobacter*		*Rubrivirga*		*Limonobacter*		*Erythrobacter*		*Roseobacter*	
	SAORIC-165	DSM 177716*	SAORIC-234	NBRC 108759*	SAORIC-263	KCTC 38213*	SAORIC-395	JCM 21789*	SAORIC-476	SG-29*	SAORIC-580	KACC 13837*	SAORIC-644	JCM 21816*	SAORIC-651	Och149*

*, relatives derived from surface-sea or other environments; +, positive; −, negative.

Table 4-11. Summary of the physiological characteristics of the deep-sea strains and their relatives.

	Rubritalea		*Aurantivigra*		*Oceanibulbus*		*Sulfitobacter*		*Rubrivirga*		*Limonobacter*		*Erythrobacter*		*Roseobacter*	
	SAORIC-165	DSM 177716*	SAORIC-234	NBRC 108759*	SAORIC-263	KCTC 38213*	SAORIC-395	JCM 21789*	SAORIC-476	SG-29*	SAORIC-580	KACC 13837*	SAORIC-644	JCM 21816*	SAORIC-651	Och149*

*, relatives derived from surface-sea or other environments; ND, no data; +, positive; −, negative.

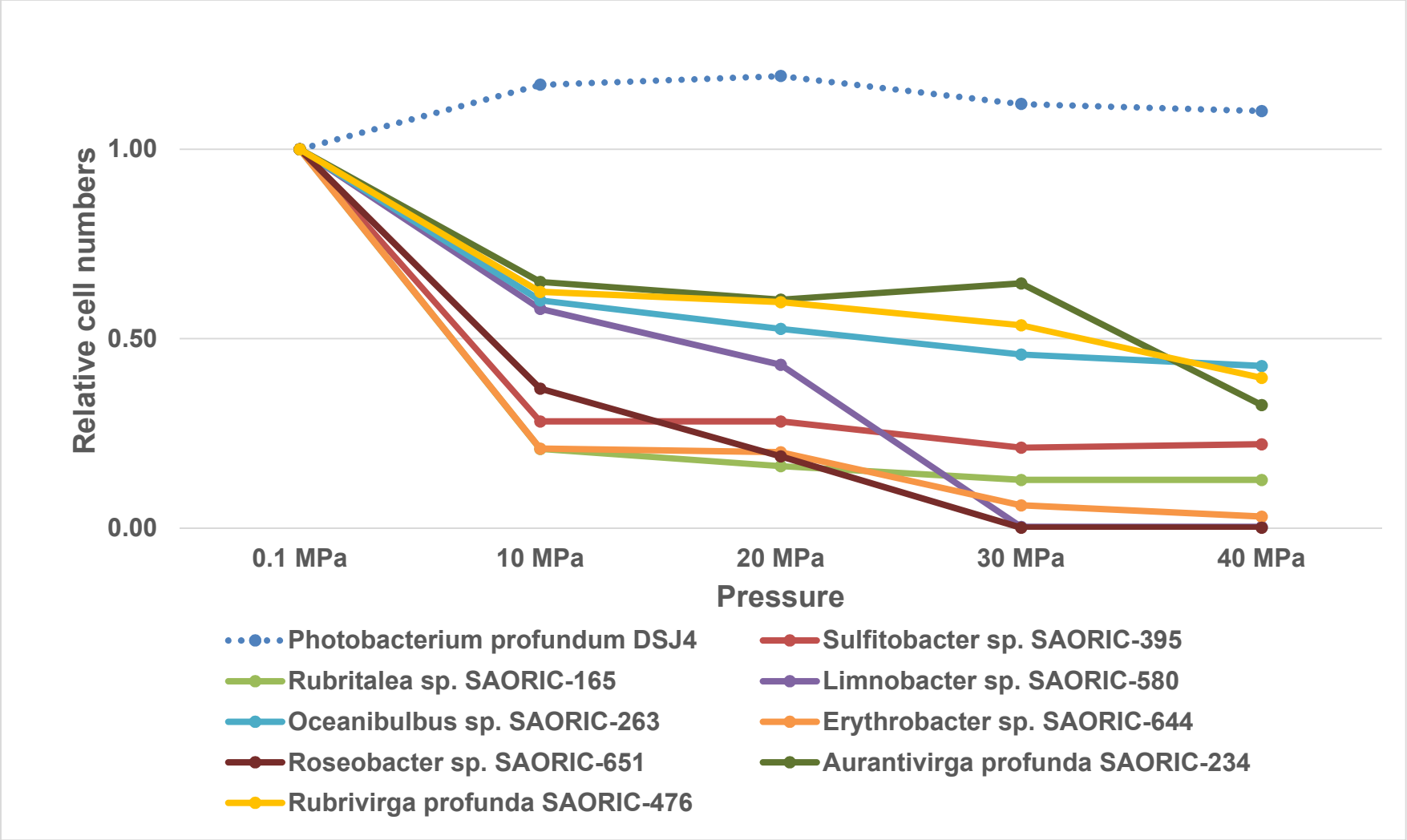

Figure 4-1. Growth profile under high pressure

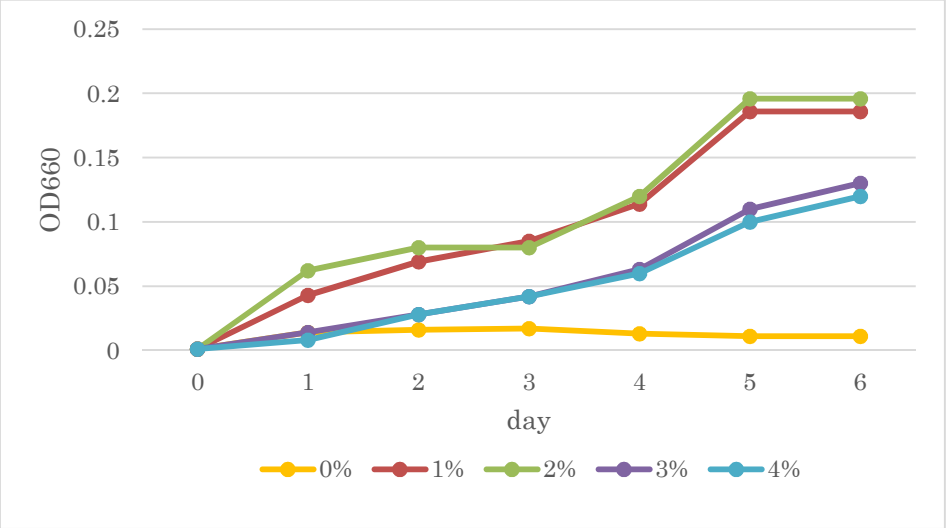

Figure 4-2. Growth profile of *Oceanibulbus* sp. SAORIC-263 on 0, 1, 2, 3, 4 % NaCl concentration

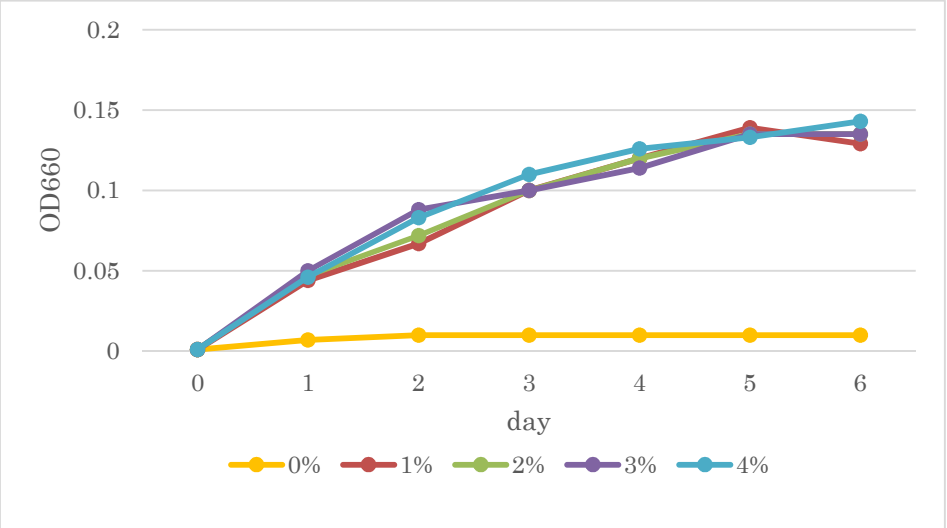

Figure 4-3. Growth profile of *Sulfitobacter* sp. SAORIC-395 on 0, 1, 2, 3, 4 % NaCl concentration

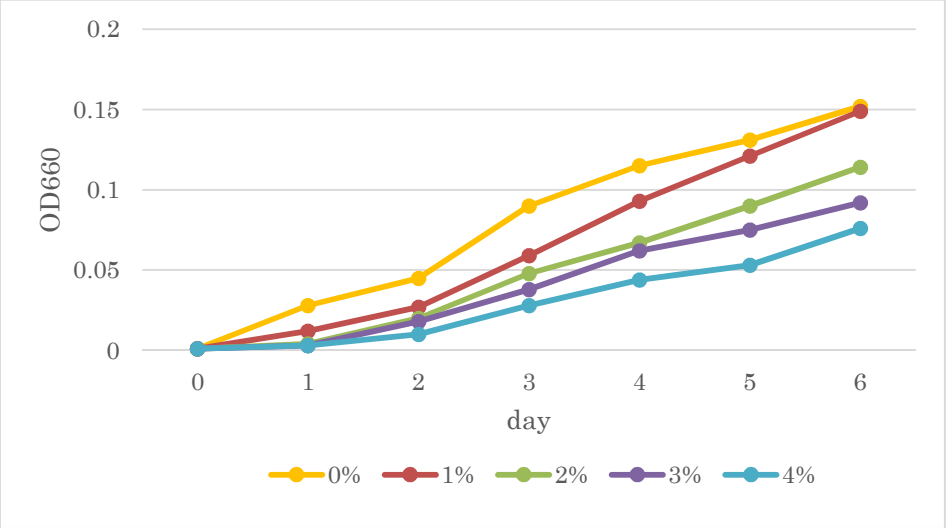

Figure 4-4. Growth profile of SAORIC-580 on 0, 1, 2, 3, 4 % NaCl concentration

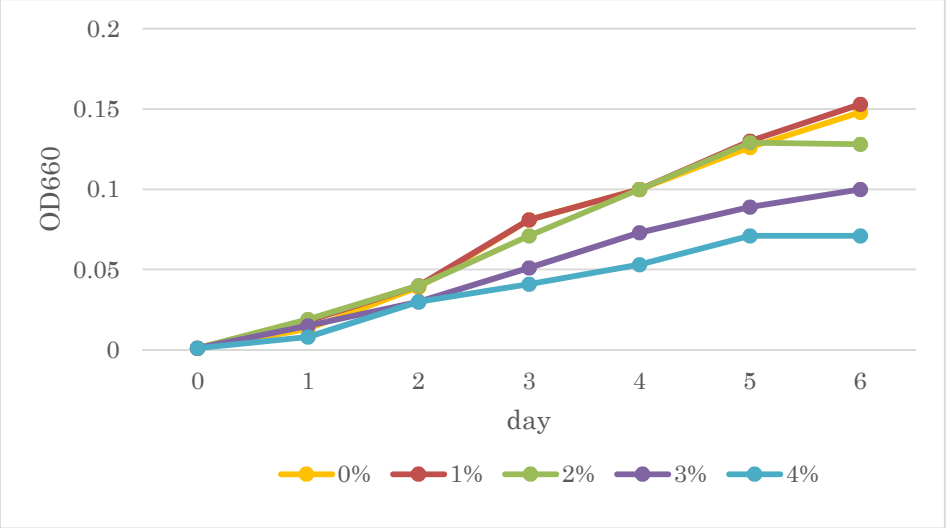

Figure 4-5. Growth profile of SAORIC-644 on 0, 1, 2, 3, 4 % NaCl concentration

Figure 4-6. Two-dimensional thin-layer chromatograph of total polar lipids of *Rubritalea* sp. SAORIC-165 and *Rubritalea marina* DSM 177716^T. PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PL1-4, unknown phospholipids; AL, unknown aminolipids; L1-2, unknown lipids.

Figure 4-7. Two-dimensional thin-layer chromatograph of total polar lipids of strain *Aurantivirga profunda* SAORIC-234^T and *Polaribacter porphyrae* NBRC 108759^T. PE, phosphatidylethanolamine; AL1-2, unknown aminolipids; L1–L3, unknown lipids (Song *et al*., 2015)

Figure 4-8. Two-dimensional thin-layer chromatograph of total polar lipids of *Ocenibulbus* sp. SAORIC-263 and *Sulfitobacter delicatus* KCTC 38213^{T}. PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; AL, unknown amino lipid; PL1-PL3, unknown phospholipids; L1–L3, unknown lipids

Figure 4-9. Two-dimensional thin-layer chromatograph of total polar lipids of strain *Sulfitobacter* sp. SAORIC-395^{T} and *Sulfitobacter pontiacus* JCM 21789^{T}. PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PL1-2, unknown phospholipids; L1–L4, unknown lipids.

Figure 4-10. Two-dimensional thin-layer chromatograph of total polar lipids of *Rubrivirga profundi* SAORIC-476 and *Rubricoccus marinus* SG-29^{T}. PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PL1-3, unknown phospholipids; L1–L3, unknown lipids (Song *et al*., 2016)

Figure 4-11. Two-dimensional thin-layer chromatograph of total polar lipids of *Limnobacter* sp. SAORIC-580 and *Limnobacter thiooxidans* KCTC 12942^{T}. PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PL, unknown phospholipids; L1–L4, unknown lipids.

Figure 4-12. Two-dimensional thin-layer chromatograph of total polar lipids of *Erythrobacter* sp. SAORIC-644 and *Erythrobacter citreus* JCM 21816^{T}. PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PL, unknown phospholipids; L1–L4, unknown lipid

Figure 4-13. Two-dimensional thin-layer chromatograph of total polar lipids of strain SAORIC-651 and *Roseobacter litoralis* Och149^{T}. PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PL1-3, unknown phospholipids; L1–L4, unknown lipid

CHAPTER 5.

Genetic characteristics of deep-sea bacteria in comparison with surface-sea relatives

Introduction

In the previous chapter, some physiological characteristics were examined for several deep-sea isolates that were isolated for this research (Chapter 2). Such information is quite helpful to understand the characteristics of deep-sea bacteria, especially in comparison with those isolated from surface layers. For example, it was clarified that one particular strain, *Rubritalea* sp. SAORIC-165 is a psychrophilic strain. Because various cellular metabolic pathways and cell components are involved in exerting such character, elaborate growth observations under various conditions are required for this kind of findings. On the other hand, such a physiological approach may miss certain characteristics because it is practically impossible to cover all the metabolic processes or all cellular components.

Recent development of molecular techniques have made it possible to obtain the whole genome information within short time. The data are accumulated into database and are available for researchers (e.g., NCBI). Those data give us information on diverse microbial processes which have been overlooked in the "traditional" physiological investigation (Simonate *et al*., 2006; Koeppel *et al*., 2007; Quaiser *et al*., 2011; López-Pérez *et al*., 2013). The comparative genomics or comparisons of multiple genomes allowed us to elucidate presence or absence of particular genes, sequence variations and orders of genes. Such information help us to infer functional characteristics, evolutionary processes and possibly adaptive mechanisms to various environments (Math *et al*., 2012; López-Pérez *et al*., 2013).

Genomic analysis of deep-sea prokaryotes clarified that deep-sea bacteria contain diverse peptidases, amino acid uptake systems for hydrolyzing and metabolizing organic substrates (Hou *et al*., 2004). For instance, deep-sea *Idiomarina loihiensis* contains

metallopeptidases which may be linked to the high concentations of heavy metals in organic substracts. *Shewanella piezotolerans* WP3 has a great number of genes involved in urea and glycan metabolic pathways, compared to its relative, strain MR-1 (Wang *et al*., 2008). It was also revealed that transposable elements, flagella synthesis, heavy metal resistance and signal transduction genes were abundantly present on the deep-sea bacterial genomes or populations compared with those of the surface-sea relativess (Simonato *et al*., 2006; Lauro *et al*., 2008; Ivars-Martinez *et al*., 2008; Qin *et al*., 2010; López-Pérez *et al*., 2013).

In spite of the versatility of the genomic analysis, however, only about 50 genomes of deep-sea prokaryotes have been subjected at the time of writing. If I exclude those from hydrothermal vents, which are not common deep-sea habitat, those from deep-sea water or sediment are only 27 genomes. Furthermore, only several genomic comparisons of deep-sea and surface-sea relatives were carried out. These genomes were mainly assigned to the class *Gammaproteobacteria*. Therefore, more genomes from diverse phylogenetic groups should be sequenced in order to better understand deep-sea prokaryotes.

In the present chapter, I aimed to analyze whole genome of deep-sea bacteria for clarifying characteristics of those strains. I also tried to clarify particular genes related to particle-association, and those possibly involved in the life style in the deep sea. Among the deep-sea isolate, 7 deep-sea strains, of which relatives have genomic data, were selected and belong to the class *Alphaproteobacteria* and *Betaproteobacteria*, and the phylum *Verrucomicrobia*. To my knowledge, this is the first case of whole genome analysis of the deep-sea isolates in genera *Rubritalea*, *Oceanibulbus*, *Limnobacter* and

Roseobacter. Subsequently, comparative genomics was performed using several bioinformatics tools.

Materials and methods

Bacterial strains

Considering dominance and novelty of the deep-sea isoaltes, the following deep-sea strains were analyzed. *Rubritalea* sp. SAORIC-165, *Oceanibulbus* sp. SAORIC-263, *Sulfitobacter* sp. SAORIC-395, *Limnobacter* sp. SAORIC-580, SAORIC-690, *Erythrobacter* sp. SAORIC-644 and *Roseobacter* sp. SAORIC-651.

DNA extraction

Bacterial cells in MA or R2A agar culture medium in exponential phase were collected in 2 ml tubes. A portion of 500 μl lytic solution (Lysozyme 0 .75 in 1ml of 10 mM Tris-HCl buffer, pH 8.0) was added to collected cells and mixed well. The mixture was incubated at 37℃ for least 3 hr. Two hundreds μl TES buffer (pH 8.0) and pre-warmed (60℃) 600 μl Tris-SDS solution (1M Tris-HCl+10% SDS) were added and gently mixed. After adding 10 μl proteinase K (10 mg/ml), the mixture was incubated at 55℃ overnight. An aliquot of 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 centrifuged at 15,000 rpm, 4℃ for 15 min. The supernatant was transferred into a new tube and this extraction process was repeated. The supernatant was then transferred into a new tube and extracted with 500 μl chloroform and mixed for 30 min. After centrifugation at 14,000 rpm for 10 min, the supernatant was collected in 50 ml sterile centrifuge tube and 30 ml ice cooled 99.5 % ethanol was added. The precipitated DNA was collected using a glass rod and washed again with the cooled 99.5 % ethanol. The DNA piled on the glass rod was

transferred into a 15 ml centrifuge tube containing 4.5 ml 0.1X SSC with the tube sealing by Parafilm and stored at 10℃ overnight after the obtained DNA was dried at room temperature. The 0.1XSSC solution was adjusted to 1XSSC after the DNA has dissolved. An aliquot of 20 µl RNase A (10 mg/ml) and 100 µl RNAse T1 (400 U/ml, pH 7.5) was added and incubate at 37℃ for 1 hour. A 20 µl proteinase K (10 mg /ml), was added and further incubated for 90 min at 37℃. After the enzyme treatment, the mixture was separated into 2 ml tubes and extracted with phenol and chloroform treatment as described before, supernatant were collected and mixed 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℃ after the precipitated DNA was vacuum dried

Whole genome sequencing

Genomic sequences were determined using the Illumina MiSeq sequencer in Macrogen (Macrogen, Inc., Korea). The assembly was achieved using the GS De Novo Assembler (v 3.0) which makes the resulting reads together into contiguous segments (contigs) based on overlapping regions between each reads. Genome annotation was accomplished by using the Integrated Microbial Genomes Expert Review (IMG-ER) (Markowitz *et al*., 2009), KEGG pathway database (http://www.genome.jp/kegg) and RAST server (http://rast.nmpdr.org). The average nucleotide identity (ANI) was calculated in EzGenome server (http://www.ezbiocloud.net/ezgenome/ani). For comparative genomes, clustering protein based on sequence similarity was determined by GET_HOMOLOGUES program (Contreras-Moreira & Vinuesa, 2013). Clusters of orthologous groups (COGs) were analyzed by Integrated Microbial Genomes (IMG) database release 4.1 (Markowitz *et al*. 2012). Comparative genomic analysis with other bacterial lineages was also performed using IMG database. Orthologous proteins were

defined the reciprocal as best-hit proteins following to a minimum to 70 % of the length of either protein and of 50 % identity calculated by the BLAST algorithm.

COG functional analyses

In specific breakdown of numbers of the genes assigned to COG functional categories, I tried to screen particular genes for characterizing deep-sea bacteria using two criteria. First, a gene that deep-sea bacteria shows at least 1.5 folds more abundance in the numbers of the gene, compared to their surface relatives. Second, this is confirmed in at least 3 deep-sea strains.

Metagenomic data comparison

Screened genes were compared to comparative metagenomic data between a deep and surface layer (Konstantinidis *et al*., 2009). Based on the metagenomic dataset (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2725473/bin/supp_75_16_5345__index.html), abundance S/D (abundance in surface library/ abundance in deep library) of the screened genes were calculated.

Results

Table 5-1. Genes of *Rubritalea* sp. SAORIC-165, *Rubritalea marina* DSM 177716 and *Coraliomargarita akajimensis* DSM 45221.

strain	Size (bp)	GC content	Contigs	Protein coding genes (CDSs)	Proteins assigned to COG
SAORIC-165	4.17	49.4	4	3844	1681
DSM 177716	3.01	51.6	32	2670	1680
DSM 45221	3.75	53.6	1	3126	2035

Table 5-2. Genes of *Oceanibulbus* sp. SAORIC-263, *Oceanibulbus indolifex* HEL-45 and *Sulfitobacter dubius* DSM16472.

Strain	Size (bp)	GC content	Contigs	Protein coding genes (CDSs)	Proteins assigned to COG
SAORIC-263	3.94	61.3	37	3085	2825
HEL-45	3.54	60.3	15	3429	2603
DSM16472	3.67	60.2	21	3558	2724

Table 5-3. Genes of *Sulfitobacter* sp. SAORIC-395, *Sulfitobacter* sp. CB2047 and *Sulfitobacter* sp. EE-36.

Strain	Size (bp)	GC content	Contigs	Protein coding genes (CDSs)	Proteins assigned to COG
SAORIC-395	3.72	60.5	36	2948	2683
CB2047	3.77	60.3	12	2985	2698
EE-36	3.23	60.3	15	2579	2493

Table 5-4. Genes of *Limnobacter* sp. SAORIC-580, *Limnobacter* sp. SAORIC-690 and *Limnobacter* sp. MED105.

Strain	Size (bp)	GC content	Contigs	Protein coding genes (CDSs)	Proteins assigned to COG
SAORIC-580	3.29	52.5	10	2467	2219
SAORIC-690	3.49	51.4	4	2649	2188
MED105	3.39	52.2	46	3109	2411

Table 5-5. Genes of *Erythrobacter* sp. SAORIC-644, *Erythrobacter* sp. SD-21 and *Erythrobacter nanhaisediminis* CGMCC 1.7715

strain	Size (bp)	GC content	Contigs	Protein coding genes (CDSs)	Proteins assigned to COG
SAORIC-644	3.54	60.5	24	3386	2289
SD-21	3.26	62.9	19	2916	1991
CGMCC 1.7715	2.90	62.0	12	2846	1879

Table 5-6. Genes of *Roseobacter* sp. SAORIC-651, *Roseobacter litoralis* Och 149 and *Roseobacter denitrificans* Och 119.

Strain	Size (bp)	GC content	Contigs	Protein coding genes (CDSs)	Proteins assigned to COG
SAORIC-651	4.84	54.8	43	5007	3335
Och 149	4.74	57.2	4	4862	3372
Och 119	4.33	58.9	5	4276	3250

Table 5-7. Numbers of particular genes among *Rubritalea* sp. SAORIC-165 and the relatives, *R. marina* DSM 177716^{T} and *Coraliomargarita akajimensis* DSM 45221^{T}.

COG	Gene	Function & reference	Numbers of COG		
			SAORIC-165	DSM 177716	DSM 45221

Red indicates genes that showed the unique presence and more than 1.5 folds abundance in other two more strains, compared to their surface-derived relatives.

Classification of the COGs by functional categories. One-letter abbreviations for the functional categories: J, translation, including ribosome structure and biogenesis; L, replication, recombination and repair; K, transcription; O, molecular chaperones and related functions; M, cell wall structure and biogenesis and outer membrane; N, secretion, motility and chemotaxis; T, signal transduction; P, inorganic ion transport and metabolism; C, energy production and conversion; G, carbohydrate metabolism and transport; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; H, coenzyme metabolism; I, lipid metabolism; D, cell division and chromosome partitioning; R, general functional prediction only; S, no functional prediction; V, Defense mechanisms; W, Extracellular structures; Y, Nuclear structure; Z, Cytoskeleton; X, Mobilome: prophages, transposons; Q, Secondary metabolites biosynthesis, transport and catabolism.

Table 5-8. Numbers of particular genes among *Oceanibulbus* sp. SAORIC-263 and the relatives, *Oceanibulbus indolifex* HEL-45 and *Sulfitobacter dubius* DSM 16472.

COG	Gene	Function & reference	Number of COG		
			SAORIC-263	DSM16472	HEL-45

Table 5-9. Numbers of particular genes among *Sulfitobacter* sp. SAORIC-395 and the relatives, *Sulfitobacter* sp. CB2047 and EE-36.

COG	Gene	Function & reference	Number of COG		
			SAORIC-395	CB2047	EE-36

Table 5-10. Numbers of particular genes among *Limnobacter* sp. SAORIC-580, *Limnobacter* sp. SAORIC-690 and *Limnobacter* sp. MED105

COG	Gene	Function & reference	Number of COG		
			SAORIC-580	SAORIC-690	MED105

Table 5-11. Numbers of particular genes among *Erythrobacter* sp. SAORIC-644 and the relatives, *Erythrobacter* sp. SD-21 and *E. nanhaisediminis* CGMCC 1.7715.

COG	Gene	Function & reference	Number of COG		
			SAORIC-644	SD-21	CGMCC 1.7715

Table 5-12. Numbers of particular genes among *Roseobacter* sp. SAORIC-651 and the relatives, *R*. *litoralis* Och 149 and *R*. *denitrificans* Och 119.

COG	Gene	Function & reference	Number of COG		
			SAORIC-651	Och 149	Och 119

Table 5-13. Number of genes related with falagella, secretion system and adhersion proteins among deep-s

	Rubritalea SAORIC-165	*Erythrobacter* SAORIC-644	*Oceanibulbus* SAORIC-263	*Sulfitobacter* SAORIC-395	*Limnobacter* SAORIC-580	*Roseob* SAORI

*; predicted lifestyle based on relative abundance in Chapter 3

Table 5-14. Genes more abundantly represent among deep-sea strains.

COG catagory	Gene	Predicted roles	Abundance S/D*

*, data from Konstantinidis *et al*. (2009).

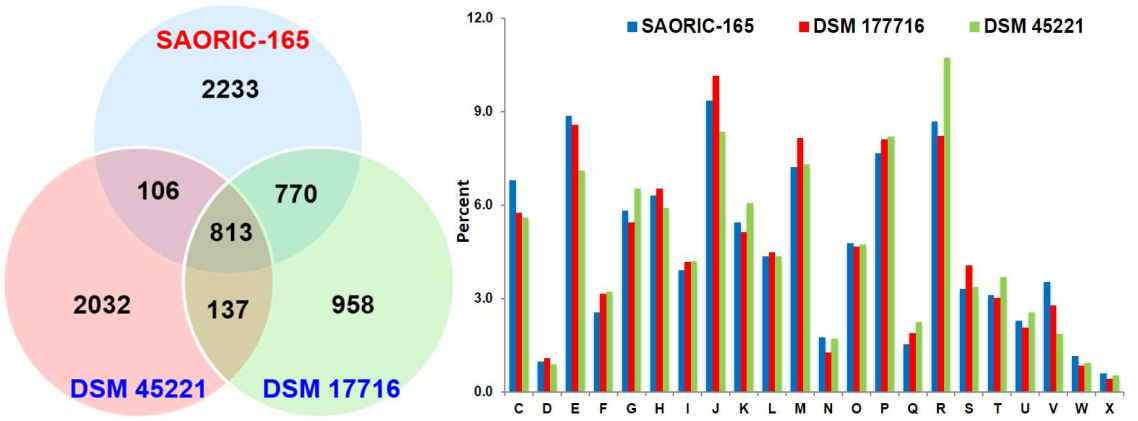

Figure 5-1. Venn diagram of shared and specific CDS genes, and Percentages of COG categories of *Rubritalea* sp. SAORIC-165, *Rubritalea marina* DSM 177716 and *Coraliomargarita akajimensis* DSM 45221.

Classification of the COGs by functional categories. One-letter abbreviations for the functional categories: J, translation, including ribosome structure and biogenesis; L, replication, recombination and repair; K, transcription; O, molecular chaperones and related functions; M, cell wall structure and biogenesis and outer membrane; N, secretion, motility and chemotaxis; T, signal transduction; P, inorganic ion transport and metabolism; C, energy production and conversion; G, carbohydrate metabolism and transport; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; H, coenzyme metabolism; I, lipid metabolism; D, cell division and chromosome partitioning; R, general functional prediction only; S, no functional prediction; V, Defense mechanisms; W, Extracellular structures; Y, Nuclear structure; Z, Cytoskeleton; X, Mobilome: prophages, transposons

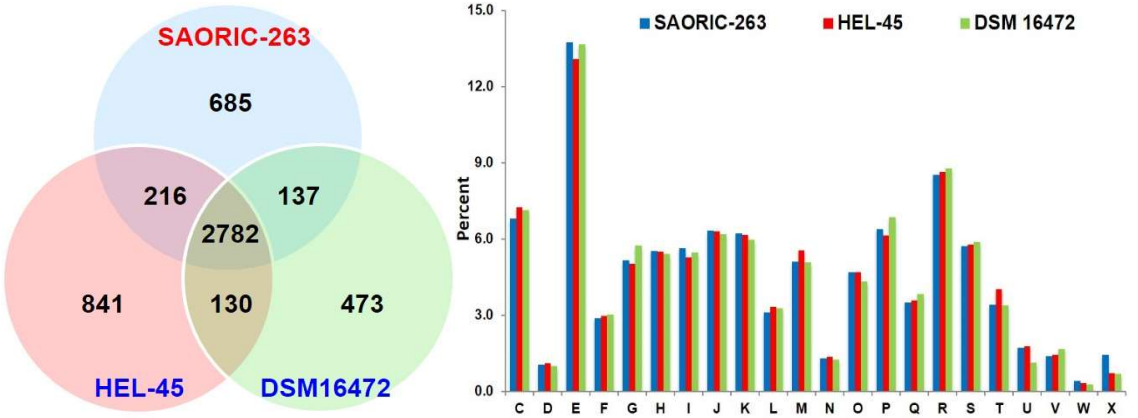

Figure 5-2. Venn diagram of shared and specific CDS genes, and Percentages of COG categories of *Oceanibulbus* sp. SAORIC-263, *Oceanibulbus indolifex* HEL-45 and *Sulfitobacter* sp. DSM 16472.

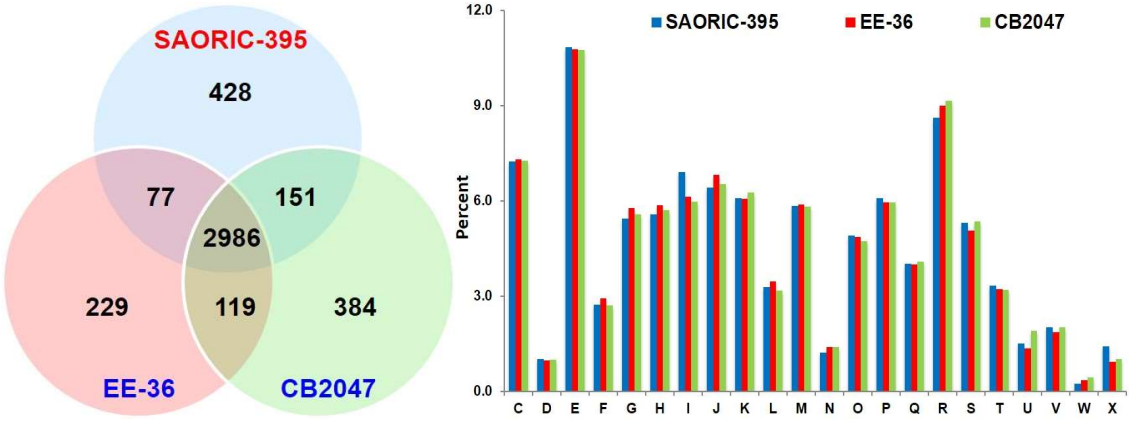

Figure 5-3. Venn diagram of shared and specific CDS genes, and Percentages of COG categories of *Sulfitobacter* sp. SAORIC-395, *Sulfitobacter* sp. EE-36 and *Sulfitobacter* sp. CB2047

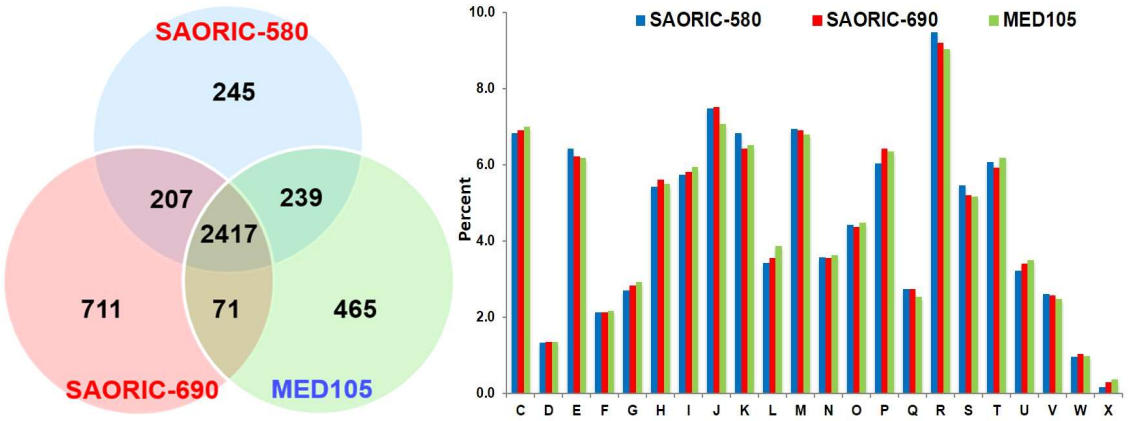

Figure 5-4. Venn diagram of shared and specific CDS genes, and Percentages of COG categories of *Limnobacter* sp. SAORIC-580, *Limnobacter* sp. SAORIC-690 and *Limnobacter* sp. MED105

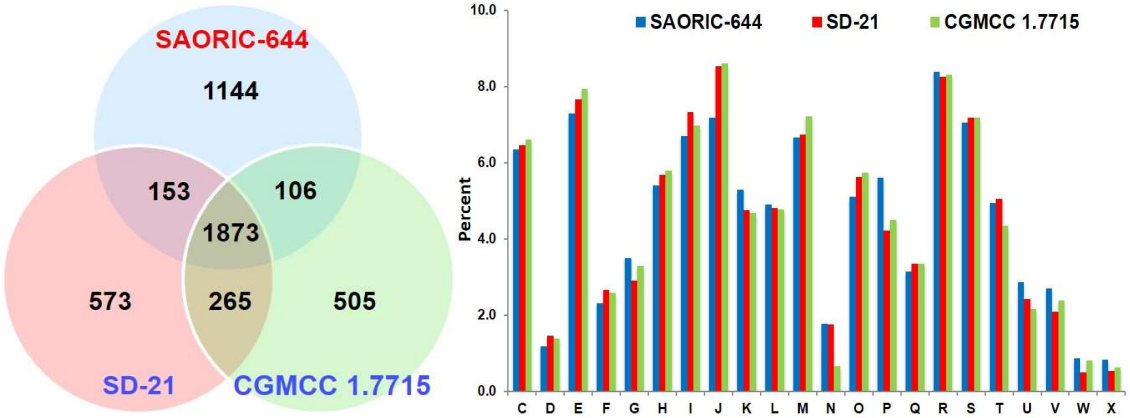

Figure 5-5. Venn diagram of shared and specific CDS genes, and Percentages of COG categories of *Erythrobacter* sp. SAORIC-644, *Erythrobacter* sp. SD-21 and *Erythrobacter* sp. CGMCC 1.7715.

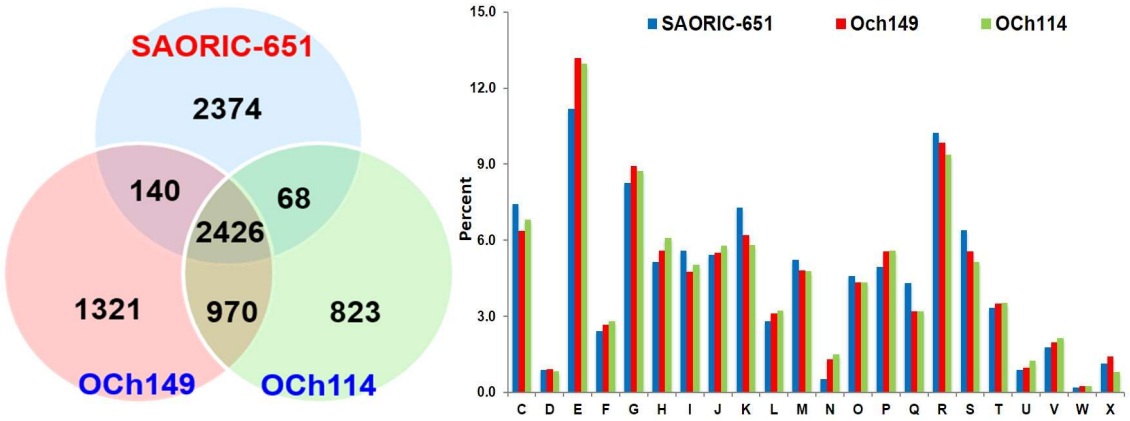

Figure 5-6. Venn diagram of shared and specific CDS genes and percentages of COG categories of *Roseobacter* sp. SAORIC-651, *Roseobacter litoralis* Och 149 and *Roseobacter denitrificans* Och 119.

Figure 5-7. Cartoon depicting cellular processes related with transporters in deep-sea bacteria important fo

Figure 5-8. Cartoon depicting cellular processes related with stress and transcription in deep-sea bacteria important for adaptation to the deep sea.

CHAPTER 6.

General discussion and conclusions

The deep sea has been regarded as an extreme environment due to high pressure, low temperature, low nutrients and no light. Nevertheless, even at the deepest part of the ocean, 10^3 to 10^4 prokaryotic microorganisms are present in one mL of seawater. As for their life, many questions remains, e.g., what kind of microbial communities are formed, what kind of factors control their biomass and community structure, what kind of physiological characteristics they have, what kind of physiological mechanisms make them adapt to deep-sea environments, and so on. Currently, however, our knowledge is quite limited because of the following reasons; first, it is not easy to obtain deep-sea materials including seawater and sediment. Second, it is not easy to duplicate the environmental conditions in laboratory, especially the pressure. Therefore, the interpretation of any data obtained in "normal" laboratory conditions should be carefully made. Third, only a limited group of microorganisms are cultured by ordinary culture techniques. They cannot be the representatives of deep-sea microorganisms. The purpose of this thesis was to clarify the physiological and genetic characteristics of deep-sea bacteria. Those characteristics should be important to understand how deep-sea bacteria adapt to such harsh environmental conditions and how they grow or maintain their populations. In order to overcome the current limitation, I took a couple of new approach. First, I tried to isolate new strains that have never been recovered from the deep sea before. I applied low-nutrient culture media to isolate broad phylogenetic groups. I also tried to describe some new species. Second, I took an approach to combine culture-dependent and culture-independent methods. Third, I tried to see the difference in the distribution and characteristics between PA and FL. Findings in my research are described below. Fourth, I tried to compare the physiological and genetic characteristics between deep-sea isolates and their close relatives isolated from surface layers. For genetic analyses, whole genome

information was obtained for 7 deep-sea isolates. I believe that this is the most efficient approach currently available for characterization of deep-sea bacteria.

Briefly, in chapter 2, I applied newly designed media, of which carbon content was lower than the conventionally used MA or 1/2 MA, to obtain deep-sea prokaryotes. First, my deep-sea isolates belonged to the domain *Bacteria* and none to the domain *Archaea*. Second, over 50 % of the bacterial isolates belonged to the phylum *Proteobacteria* of the domain *Bacteria*. Other isolates were assigned to phyla *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia* and *Lentisphaerae*. *Rubrivirga marina* SAORIC-28^T, *Rubrivirga profundi* SAORIC-476^T, *Aurantivirga profunda* SAORIC-234^T, and *Lentisphaera profundi* SAORIC-696^T were validated and reported as novel deep-sea species. Third, approximately 90 % of the identified isolates have phylogenetically close relatives from surface layer or other environments. Fourth, among three culture methods applied, there were differences in the phylogenetic groups of the isolates. Fifth, at the station MR11-05-S1, there were some vertical differences in the phylogenetic groups. The pattern is, however, not consistent among other stations.

In chapter 3, the vertical community structures of bacteria in two water columns were investigated using pyroseqeuncing technique for clarifying the vertical distribution of the prokaryotes and also for differentiating their particle associated (PA) and free living (FL) state. First, among the phylotypes affiliated with the deep-sea isolates, *Erythrobacter* phylotypes were detected in all depths. *Sulfitobacter*, *Paracoccus*, *Sphinogomonas*, *Colwellia*, *Alcanivorax*, *Marinobacter*, *Alteromonas*, *Moritella* and *Rubritalea*-like phylotypes were more retrieved from the deeper layers than the surface layer. Second, most of the phylotypes affiliated with the deep-sea isolates showed preference toward PA state. Finally, SAR11 and *Sphingomonadales* of *Alphaproteobacteria*, *Oceanospirillales*

and *Alteromonadales* of *Gammaproteobacteria* and *Flavobacteriales* of *Bacteriodetes* were found in all layers. *Deltaproteobacteria*, *Deferribactere*, *Planctomycetes*, *Actinobacteria* and *Nitrospirae* were specific to the deeper layers.

In Chapter 4, physiological characteristics of several deep-sea isolates were investigated together with their close relatives. Growth characteristics, cellular membrane composition and hydrolytic enzymes of eight strains affiliated with phyla *Proteobacteria*, *Verrucomicrobia* and *Bacteroidetes* were tested. First, the growth of the deep-sea isolates decreased with increasing pressure, indicating that they are non-piezophiles. Second, *Rubritalea* sp. SARIC-165 within *Verrucomicrobia* showed optimum growth at 10°C and no growth above 20°C, indicating that the strain is psychrophilic. Third, *Erythrobacter* sp. SAORIC-644 within the class *Alphaproteobacteria* and *Limnobacter* sp. SAORIC-580 within the class *Betaproteobacteria* showed optimum NaCl concentration at 1 and 0 %, respectively. Finally, the deep-sea strains were distinguished by 1-2 more numbers of different types of phospholipids than their surface relatives.

In chapter 5, whole genome of 7 deep-sea strains within phyla *Proteobacteria* and *Verrucomicrobia* were sequenced and their genetic features were identified in comparison with those of close relatives obtained from surface layers. First, the deep-sea isolates, which prefer particle-associated state, contain either secretion systems and flagella, or adhesion proteins. Second, by selecting the genes appeared in more than 3 deep-sea strains, and more than 1.5 folds abundance compared to the genes of the close relatives, 51 genes were listed. The genes were involved in respiration, stresses response, cellular structure, in- and organic substrates metabolism, replication, and transcription. Although the selection criteria were arbitral, these genes were generally in good agreements with those appeared in literatures and metagenomics database. Fourth, by this selection, some

genes that have never been reported before were listed.

There are three methodological points to discuss. First, for the culture works, 1/5 marine agar (MA), 1/10 R2A agar and natural seawater liquid media (NSLM) were used. I succeeded to obtain some new strains. Because I didn't conduct intensive comparable examinations for the media, it is not clear to what extent my new media contributed to the isolation of novel strains. In spite of many new strains, it was failed to isolate some strains like SAR11, the dominant bacterium in the ocean, and *Archaea*. The diluted agar media (1/5 and 1/10) were chosen because of practical reason, i.e., the lower organic concentration. There were more chances to isolate previously unknown strains, however, the media are supposed to be inappropriate for most of deep-sea bacteria. As for NSLM, alghough the method by Connon & Giovannoni (2002) was slightly modified, there may be still possibilities to isolate more unknown strains by the modification of culture methods. In this study, the incubation was performed at 10˚C for 4 weeks in polystyrene microtiter plates. Stingl and colleagues (2007) suggested that the use of Teflon plates cleaned with metal-free HCl yield new SAR11 isolates. Song and colleagues (2009) suggested that long term incubation at low temperatures improve the culturability of the SAR11.

Second, as for genetic analyses, the criteria for comparative works (presence at least 3 genomes, 1.5 fold difference) is arbitral and the number of genomes treated was not enough as statistically reliable results. Furthermore, the genomes belonged to particular phylogenetic groups that make up a small portion of deep-sea bacterial populations. Therefore, the obtained genes that may be important for deep-sea life style should be regarded as preliminary. Nevertheless, the results showed good agreement with metagenomics data and former works for deep-sea bacteria (Konstantinidis *et al*., 2009;

Qin *et al*., 2010; Eloe *et al*., 2011a). In addition, this analysis revealed the possible functions of deep-sea bacteria such as ornithine to glutamate metabolism, detoxification of methylglyoxal and osmotic regulation. Further examinations of these newly recognized functions with other metagenomics database may confirm the presence of unique functional genes in deep-sea environments.

Third, for this thesis work, both culture-dependent (Chapter 2) and culture-independent (Chapter 3) approaches were used. In addition, multiple genetic analyses, i.e, 16S rRNA gene sequencing (Chapter 2), whole genome analyses, and comparision with metagenomics database (Chapter 5) were combined. These combinations made it possible to evaluate the distribution or ecological characteristics of my isolates. For instance, *Erythrobacter* and *Limnobacter* like deep-sea isolates commonly have relatives from surface layer or even terrestrial environments. These groups might be cosmopolitan and/or entered into the deep-sea environments rather "recently". On the other hand, it seems difficult to find relatives of *Rubritalea* like deep-sea isolates in surface layers, suggesting that this group had adapted to the deep-sea, probably after spending long time there. Although further works are required, physiological (abundant phospholipids) and genetic (pili, flagella, adhesion proteins and abundant 51 genes) characteristics of the deep-sea isolates appear to support growth and survival in the-deep sea environment (Figure 6-1).

The findings in this thesis have two implications for studying the prokaryotes in deep-sea. First, this thesis reinforces that cultivation of prokaryotes from the deep sea is important for furthering our understanding of deep-sea prokaryotes. Once we get an isolate without any former record, we are now able to obtain whole genome information or any physiological characteristics rather easily. It expand our view on those

microorganisms in extreme environments. Although it is now possible to clarify the genomic information from one cell (Eloe *et al*., 2011a; Kaster *et al*., 2014), it does not necessarily tell us actual physiological characteristics. In this thesis work, psychrophilic strain of the phylum *Verrucomicrobia* was for the first time isolated from deep-sea. Also, the deep-sea isolates of the family *Burkhoderiaceae*, which sometimes accounts for 10-15 % of deep-sea bacteria population, (Martín-Cuadrado *et al*., 2007; Eloe *et al*., 2010), were first obtained. Furthermore, 22 isolates are considered as novel deep-sea species and are waiting for to be described and reported. Deep-sea microbiology is at the stage of increasing our knowledge on their physiological characteristics and on the strategies to adapt to the deep-sea environments and to continue multiplications by increasing the cultures. Further efforts to improve the cultivation methods will be required.

Second, to my knowledge, this doctoral thesis revealed ecological aspects of some deep-sea bacteria. By separately analyzing PA and FL populations, it was clarified that deep-sea isolates affiliated with genera *Erythrobacter*, *Rubritalea*, *Moritella* and *Colwellia* prefer PA state in the deep sea. On the other hand, *Xanthomonadales* and *Chromatiales* of *Gammaproteobacteria* and *Deltaproteobacteria* prefer to both PA and FL states. Although the method for the differentiation between PA and FL is rather simple and the results does not completely reflect the actual life style in the environments, it should be worth pointed out that the obtained results showed good agreement with genetic data. Further extension to ecological research may confirm the present results.

The purpose of this doctoral thesis appears to be accomplished in that new information was obtained with newly isolated strains and combination of physiological and genetic investigations. Nevertheless, the research raised further questions and future research directions. There may be at least three scientific issues to be investigated. First,

the effort to culture more strains from deep-sea environments should be continued. In my research, SAR11 group of the domain *Bacteria* or members of the domain *Achaea* was not obtained. Because these two share the considerable part of deep-sea environments (Eloe *et al*., 2010), more efforts are definitely required. The improvements of the culture techniques is the key.

The second way is more application of modern molecular techniques, like transcriptomics or proteomics. In this study, particular genes were found to be unique to deep-sea bacterial life. In order to confirm their actual functions, further transcriptomics and proteomics should be followed under the condition mimicing deep-sea conditions.

The third way is through taxonomical study. Taxonomy is the basic research field of biology and has been the basis for any further works (Cho *et al*., 2004; Spring *et al*., 2009; Tamaki *et al*., 2011). In Chapter 2, 22 putative novel deep-sea species were addressed. Among them, *Rubrivirga marina* SAORIC-28^T (Park *et al*., 2013), *Rubrivirga profundi* SAORIC-476^T (Song *et al*., 2015), *Aurantivirga profunda* SAORIC-234^T (Song *et al*., 2015), and *Lentisphaera profundi* SAORIC-696^T (Choi *et al*., 2015) were validated after taxonomical investigations and reported as novel deep-sea species. Further taxonomical study will allow the characterizing and validating of putative novel species and provide more opportunities to investigate bacterial speciation in the deep sea.

In conclusion, diverse bacteria including new phylogenetic groups that had never been isolated were obtained from the deep sea. Some of them were investigated taxonomically, physiologically and genetically by recent molecular techniques. In addition, their preference to either PA or FL life style was investigated. Their physiological and genetic characteristics allowed to consider their ecology and evolutionary processes as well. Further investigation on the isolation and characterization

of more deep-sea bacteria will offer clues to better understand the nature of the deep-sea prokaryotes.

Figure 6-1. Conceptual diagram of factors that are controlling the deep-sea bacterial communities.

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