Ecological Characterization of Anoxygenic Photosynthetic Bacteria in Eutrophicated Marine Environment and Their Application to Aquaculture

（富栄養化海洋環境における酸素非発生型光合成細菌の生態とその養殖分野への応用に関する研究）

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ABSTRACT

Diversity of the photosynthetic bacterial communities in highly eutrophicated Yamagawa Bay sediments

Yamagawa Bay, located in Ibusuki, Kagoshima Prefecture, Japan, is a geographically enclosed coastal marine inlet, and its deteriorating seabed sediments are under an anoxic, reductive, sulfide-rich condition. In order to gain insight into diversity of anoxygenic photosynthetic bacteria (AnPBs) and their ecophysiological roles in the sediments, three approaches were adopted: isolation of AnPBs, PCR-DGGE of 16S rDNA, and PCR-DGGE of *pufM*. Among the bacterial isolates, relatives of *Rhodobacter sphaeroides* were most dominant, suggesting their potential transformation of organic pollutants in the sediments. Abundance of *Chlorobium phaeobacteroides* BS1 was suggested by 16S rDNA PCR-DGGE. It could reflect intensive stratification and resultant formation of the anoxic, sulfide-rich layer in addition to extreme low-light adaptation of this strain. Diverse purple non-sulfur or sulfur bacteria as well as aerobic anoxygenic photoheterotrophs were also detected by *pufM* PCR-DGGE, which could be associated with organic or inorganic sulfur cycling. The outcome of the present study highlights ecophysiologically important roles of AnPBs in the organically polluted marine sediments.

Use of purple non-sulfur photosynthetic bacterium (*Rhodobacter sphaeroides*) for promoting growth of ciliated protozoa

Ciliated protozoa were enriched from the marine environments including seawater and sediment collected from Kuwano-ura Bay, Kamikoshiki Island, Kagoshima, Japan using media containing fish meal with radish leaves (MI and MII) or a medium for microalgal cultivation (MIII). Cultivation in MIII produced the highest number of ciliates, suggesting proliferation of microalgae originated from the sediment sample supported growth of the protozoa. Analyses of 18S ribosomal

RNA genes with PCR-DGGE were applied to tentatively identify large-size ciliates as *Euplotes minuta* and *Cyclidium varibonneti*. An anoxygenic photosynthetic bacterium (AnPB), isolated from a sediment sample of Yamagawa Bay, Kagoshima, Japan, was applied for prey of the ciliated protozoa. Subsequent growth of the ciliates with bacterial predation indicates that AnPBs can be used to promote the ciliate growth.

INTRODUCTION

Community structure of aquatic microbiota altered dramatically over time due to changes in their surrounding environment (Danza et al., 2018; Figueroa et al., 2016; Grubisic et al., 2012; Lindh et al., 2015; Teira et al., 2009; Traving et al., 2017; Zhang et al., 2018). Bacterial assemblages and dominant bacterial groups are highly correlated to the environmental conditions. Thus, thorough understanding of the microbial ecosystems enables us to properly control microbial activities to maximize utilization of available resources in economically and environmentally friendly ways (Bossier et al., 2016; Santander-de Leon et al., 2016; Zhang et al., 2014).

Distinctive features of anoxygenic photosynthetic bacteria (AnPBs) in bioremediation, disease control, biomass production and livestock feeding, as well as their versatile adaptability to diverse environmental conditions, made them attractive resources in low-cost, eco-friendly aquaculture practice, for instance (Hargreaves, 2006; Kuo et al., 2012; Lu et al., 2010; Nayak, 2010; Qi et al., 2009; Suzer et al., 2008; Wang et al., 2008a; Zhang et al., 2014). The phototrophs are Gram-negative prokaryotes, performing anoxygenic photosynthesis using the photo-capturing pigments (bacteriochlorophylls and carotenoids), in which light energy is converted into chemical energy, enabling them to grow photoautotrophically with carbon dioxide as a sole carbon source (Kuo et al., 2012; Pfennig, 1969; Wei et al., 2016). Major groups of AnPBs are purple non-sulfur bacteria, green sulfur bacteria, green non-sulfur bacteria and heliobacteria (Koblížek et al., 2006). Among them, purple non-sulfur phototrophs are ubiquitous in diverse environments due to their high efficiency to utilize a wide range of nutrients (Alloul et al., 2019; Imhoff et al., 2015; Kantachote et al., 2005; Kim et al., 2004; Wei et al., 2016). In addition to their potential to survive in unfavorable environmental conditions (Chae et al., 2006; Kosamu and Obst, 2009; Yegani et al., 2005), they are endowed with ability to change their metabolic

systems among photoautotrophic, photoheterotrophic and chemoheterotrophic growth in response to energy sources available in their habitat (Kim et al., 2004; Kuo et al., 2012). Green sulfur bacteria are characterized by their high ability to harvest light and photosynthesize in dependence on reduced sulfur compounds commonly present in anoxic, highly eutrophicated aquatic environments (Frigaard and Bryant, 2004; Gerardi and Lytle, 2015). Aerobic anoxygenic phototrophic bacteria may form a large part of aquatic bacterioplankton communities with different salinity environments. They grow and perform photosynthesis under aerobic condition without generating oxygen (Eiler, 2006; Lami et al., 2007; Shi et al., 2010).

Marine environments, especially sediments, tend to be contaminated with organic pollutants and may suffer from eutrophication and depletion of oxygen, which makes them suitable for growth of anaerobic microorganisms including AnPBs (Alfiansah et al., 2018; Hou et al., 2017; Moncada et al., 2019; Santander-de Leon et al., 2017). Coastal aquatic systems are highly susceptible and can be severely affected directly or indirectly by adjacent terrestrial ecosystems, anthropogenic activities (*e.g.*, eutrophication) and climate change (Andersson et al., 2015; Kallenborn et al., 2012; Rodríguez et al., 2018). Yamagawa Bay is a basin located in the Kirishima Volcanic Belt of Kagoshima, Japan with a central depth of 50 m and a shallow entrance of 8 m. The geographic features reduce a rate of water exchange and make the water body stagnant for a long time, allowing deposition of organic pollutants and resulting in low dissolved oxygen (Kawaichi et al., 2013; Onoue and Nozawa, 1989).

In aquaculture systems, feed for larval rearing is primarily important in terms of reducing their initial mortality and improving production efficiency of the aquacultured fish. Nutritional value of phyto- and zoo-plankton as fish feed affects feeding efficiency and viability of fish larvae (Das et al., 2012; Wikfors, 2004). Ciliated protozoa are known to be an integral component in the aquatic food web (Beaver and Crisman, 1989; Gates, 1984; Pace, 1986),

transferring energy from the primary producers (phototrophic microalgae) and the secondary producers (bacteria consuming dissolved organic matters) to small fish including fish larvae. Thus, the ciliates can be an ideal candidate of larval prey. In addition, nourishment of ciliates will be a determinant of their mass culturing and nutritive enrichment. Distinctive characteristics of AnPBs observed in their application to bioremediation, probiotics and disease control made them one of the suitable supplements of the ciliates. However, their utilization as feeds for marine organisms has been limited to AnPBs of freshwater or terrestrial origins (Banerjee et al., 2000; Loo et al., 2013), and few have been reported with regard to marine phototrophs so far.

Aims of the present study are as follows: i) gaining insight into diversity of AnPB communities in highly organically polluted and eutrophicated marine sediment environments by selecting Yamagawa Bay, Kagoshima Japan as a representative; ii) establishing enrichment procedures to obtain ciliated protists from marine environments; and iii) evaluating effectiveness of marine-originated AnPBs for feeding ciliates.

CHAPTER 1. DIVERSITY OF THE PHOTOSYNTHETIC BACTERIAL COMMUNITIES IN HIGHLY EUTROPHICATED YAMAGAWA BAY SEDIMENTS

1.1 Introduction

Coastal aquatic systems are highly susceptible and can be directly or indirectly affected by adjacent terrestrial ecosystems, anthropogenic activities and climate change. Yamagawa Bay is a coastal basin located in Ibusuki, Kagoshima Prefecture, Japan with a central depth of up to 50 m with a shallow entrance of 8 m. The geographical feature gives rise to reduction of the water exchange and makes the water stagnant for a long time, allowing deposition of organic matters and resulting in bottom-water hypoxia and sulfide accumulation in the sediment (Ide, 2012).

Aquatic microbiota varies spatio-temporarily due to changes of their surrounding environment, and composition and dominancy of the bacterial assemblages are highly correlated to the environmental conditions; thus, elucidation of the community structure enable us to understand physicochemical status of the environments, especially of the sediments.

Anoxygenic photosynthetic bacteria (AnPBs) are Gram-negative prokaryotes, performing anoxygenic photosynthesis with pigments such as bacteriochlorophylls (Bchl) and carotenoids. They convert light energy into chemical energy and grow autotrophically by using carbon dioxide as a sole source of carbon. Major groups are purple non-sulfur bacteria, purple sulfur bacteria, green sulfur bacteria and green non-sulfur bacteria. Aerobic anoxygenic phototrophic bacteria, accounting for up to 10% of bacterial communities in the marine euphotic zones (Yutin et al., 2007), also produce Bchl *a* and complement their energy requirements by harvesting light under an aerobic condition. Habitats of AnPBs are restricted by availability of light and electron

donors including reduced sulfur or organic compounds for their phototrophic growth as well as redox potential (Guyoneaud et al., 1996; van Gemerden and Mas, 1995). Therefore, community structure of the phototrophs will be a good bioindicator reflecting their ambient pollution levels, especially in the organically polluted marine sediments, resulting in eutrophication, oxygen depletion and high sulfide concentration.

Denaturing gradient gel electrophoresis (DGGE) and next-generation sequencing (NGS) are powerful techniques that can be utilized to profile and compare microbial populations by targeting 16S ribosomal RNA genes present in all the bacteria. The DGGE method was chosen since it is relatively cheaper than NGS and microbial community structures are easily compared as band profiles immediately after electrophoresis (Hanning and Ricke, 2011; Subasinghe et al., 2019).

The aim of this study is to gain insight into diversity of AnPBs in the Yamagawa Bay sediments. Three approaches were adopted: isolation and identification of pigmented anaerobic microorganisms; polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of 16S ribosomal RNA (16S rRNA) genes (16S rDNA); and PCR-DGGE of *pufM* encoding the M subunit of the reaction center complex.

1.2 Materials and Methods

Sample collection

Sediment samples were collected from five stations of Yamagawa Bay, Kagoshima, Japan (Fig. 1-1) from May to November 2016 and May 2017 with a G.S. type core sampler (Ashura). Surface sediments within a depth of 10 mm were used for bacterial isolation and environmental DNA preparation. Water quality parameters of the sampling sites were measured at 1 m above the sea bottom using the multiparameter data sonde Hydrolab DS5 (OTT HydroMet, Loveland, CO, USA), are shown in Table 1-1.

Enrichment and isolation of photosynthetic bacteria

Portions of the collected sediments were transferred into tightly-sealed test tubes filled with 30 mL of Basic I medium (Hoshino and Kitamura, 1984) and cultivated at 20˚C under 12:12 light:dark cycling condition in order to enrich photosynthetic bacteria. Composition of the medium is as follows (concentrations are given as grams per liter except as otherwise noted): KH_2PO_4, 0.5; K_2HPO_4, 0.6; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.2; $CaCl_2 \cdot 2H_2O$, 0.05; $Na_2S_2O_3 \cdot 5H_2O$, 0.1; yeast extract, 0.1; malate, 0.5. The following supplements were also added into the medium of 1 L: the growth factor solution (thiamin-HCl, 0.05; nicotinic acid, 0.05; *p*-aminobenzoic acid, 0.03; vitamin B_{12}, 0.01; pyridoxine-HCl, 0.01; D-biotin, 0.005; shown as grams per 100 mL), 1 mL; the trace element solution (EDTA·2Na, 2.0; $FeSO_4 \cdot 7H_2O$, 2.0; H_3BO_3, 0.1; $CoCl_2 \cdot 6H_2O$, 0.1; $ZnCl_2$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.1; $Na_2MoO_4 \cdot 2H_2O$, 0.02; shown as grams per 100 mL), 1mL.

For AnPB isolation, a double layer agar technique was used by spreading the enriched cultures on Basic I plates solidified with 1.5% of agar and then covering the plates with 1.2% agar. The agar plates were incubated anaerobically with the Anaeropack Kenki system (Mitsubishi Gas Chemical, Tokyo, Japan) under the same condition as above. Pure isolates were obtained by sequential isolation from colonies with different morphology and maintained in Basic I liquid or agar plate media for further application.

Determination of the 16S rDNA nucleotide sequences

Liquid cultures of the isolates were centrifuged to obtain cell pellets, from which the bacterial DNA were extracted with DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Genes of 16S rRNA were amplified by PCR using a universal primer set PrSSU.1F and PrSSU.1R (Table 1-2). A reaction mixture of PCR consisted of 1 x *ExTaq* Buffer (Takara Bio, Otsu, Japan), 100 μM dNTP Mixture (Takara Bio, Otsu, Japan), 0.5 μM primers and 0.025 units·μL^{-1} *ExTaq*

DNA Polymerase (Hot Start Version, Takara Bio, Otsu, Japan), and 5 μL of the bacterial DNA solutions were added to 100 μL of the mixture. Thermal cycling was conducted at 94˚C for 1 min, followed by 25 cycles of denaturation at 94˚C for 30 s, annealing at 58˚C for 30 s and extension at 72˚C for 90 s, and final extension at 72˚C was performed for 7 min. Specific amplification of the target gene was confirmed by subjecting the PCR products to 1.5% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, pH 8.3, 1 mM ethylenediaminetetraacetic acid).

The amplified 16S rDNA fragments were cleaned up with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA), and their nucleotide sequences were determined using a set of universal primers, PrSSU.1F, PrSSU.2F, PrSSU.1R and PrSSU.3R (Table 1-2), with ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA). The obtained sequences were assembled with the program GENETYX-MAC Ver. 19 (Genetyx, Tokyo, Japan). Their most homologous sequences were retrieved from the GenBank DNA database with the program of Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990).

Bacterial community analyses by 16S rDNA PCR-DGGE

Microbial DNAs in the sediments collected from the station 4 (Fig. 1-1) were extracted using PowerSoil DNA Isolation Kit (MOBio, Carlsbad, CA, USA). Amplification of their16S rDNA was conducted by PCR with the primers 341F-GC and 907R (Table 1-2); composition of the reaction mixtures was the same as above. After initial denaturation at 95˚C for 1 min, thermal cycling was performed as follows: 20 cycles of denaturation at 95˚C for 1 min, 62˚C for 1 min with a decrement of 0.8˚C at every cycle and extension at 72˚C for 1 min, followed by 10 cycles of 95˚C for 1min, 52˚C for 1min and 72˚C for 1 min with final extension at 72˚C for 10 min.

The 16S rDNA amplicons were applied to DGGE with DCode System (Bio-Rad, Hercules, CA, USA). The reaction mixtures were applied onto 6% polyacrylamide gel with 25–55% denaturant, in which 100% denaturant contained 40% deionized formamide and 7 M urea. Bands were visualized on a blue light transilluminator Safe Imager 2.0 (Thermo Fisher Scientific, Waltham, MA, USA) by staining the gel with SYBR Gold (Thermo Fisher Scientific, Waltham, MA, USA). Representative bands were excised using 1 mL pipette tips. The gel pieces were suspended in 100 μL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid), and frozen and thawed. The eluted DNA fragments were re-amplified and subjected to nucleotide sequencing with the primer 907R (Table 1-2) and homology searches as mentioned above.

Bacterial community analysis by *pufM* PCR-DGGE

The *pufM* genes were amplified from the sediment DNAs by nested PCR. The first-round amplification was done with an outer primer pair pufM557F and pufM.1R (Table 1-2), whose thermal cycling was as follows: 30 cycles of 94˚C for 30 s, 56˚C for 30 s and 72˚C for 30 s. The amplified products were further subjected to the second run with inner primer sets pufM557FGC and pufM750R (Table 1-2). A primer pufM557F without a GC clamp was also applied in order to obtain higher resolution of the DGGE bands (Achenbach et al., 2001). The same thermal setting as above was adopted, except for the primer set with a GC clamp: annealing temperature and cycle numbers were 58–63˚C and 10–12cycles, respectively.

The amplified products were applied to DGGE as shown above, except for 10% of polyacrylamide and 30–60% or 40–60% of the denaturant. Representative bands were excised, re-amplified with the same primers, and subjected to DGGE. This procedure was repeated until homogeneous sequences were obtained; bands whose sequences were still heterogeneous were excluded for further analyses. Nucleotide sequences were determined using the primer

pufM557F (Table 1-2) and their homology searches were performed.

1.3 Results

Isolation and identification of photosynthetic bacteria

Enrichment of AnPBs from the Yamagawa Bay sediments in the Basic I liquid medium under semi-anaerobic or anaerobic condition showed growth of microbial consortia with the color of green, pink or yellow (Fig. 1-2). Totally 36 bacterial isolates were obtained on the agar plates (*e.g.*, the isolate PSBYam1607St2-5; Fig. 1-3) and their 16S rDNA sequences were determined. They showed identities to known species with 99% or above with one exception (Table 1-3). *Rhodobacter sphaeroides*, an anoxygenic, photosynthetic purple non-sulfur bacterium with a freshwater origin (Pfennig and Trüper, 1971), was most prevalent (16 isolates) in the sediments. The remaining bacterial isolates were primarily chemoorganotrophic and showed no close relationship to AnPBs. Eight isolates possessed homologous sequences to plastidal 16S rDNA of a chlorophyte whose genus was *Chlorella*.

Bacterial community analyzed by 16S rDNA and *pufM* PCR-DGGE

Environmental DNAs extracted from microbial communities in the sediments of the station 4, Yamagawa Bay were applied to 16S rDNA PCR-DGGE. Resultant band profiles were shown in Fig. 1-4. One major band prevailing among all the sediment was observed with some faint bands, whose profiles were indistinguishable among the sampling periods. The nucleotide sequences were identical to *Chlorobium phaeobacteroides*, an anoxygenic photosynthetic green sulfur bacterium with a freshwater origin (Table 1-4; Pfennig, 1968; Pfennig and Overmann, 2001).

Band profiles of *pufM* DGGE were similar among the sampling periods and the triplicated samples with some differences (Fig. 1-5). In order to determine their nucleotide sequences,

representative bands were collected, re-amplified, and electrophoresed on DGGE gel (Figs 1-6 and 1-7). Bands whose sequences had been still heterogeneous were excluded from further analyses. All the bands showed close relationships to known AnPBs or potential Bchl *a*-producing AnPBs, including *Rhodopseudomonas lichen*, *Rhodovibrio sodomensis*, *Porphyrobacter tepidarius*, *Thiorhodococcus bheemlicus*, *Dinoroseobacter shibae*, *Sulfitobacter gutiformis* and *Roseobacter litoralis* (Table 1-5). The band A1 was also homologous to *Sphingomonas lacus*: this species is not possibly a phototroph, since Bchl *a* was not detected, although harboring structural genes, *pufL* and *pufM*, of the type II photosynthetic reaction center (Kim et al., 2015).

Band profiles of *pufM* DGGE with the GC clamp-added primer also showed almost identical patterns with slight variation (Fig. 1-8). All the sequences obtained from the bands 1–4, commonly detected in all the sediments, showed 92% of identities to *Thiocapsa marina* and *Allochromatium phaeobacterium* (Gammaproteobacteria – Chromatiales – Chromatiaceae), both of which are purple sulfur bacteria.

1.4 Discussion

In order to elucidate compositions of bacterial populations in the organically polluted, anoxic, reductive sediments in the geographically enclosed coastal marine inlet, approaches of AnPB isolation and PCR-DGGE of 16S rRNA and *pufM* genes were adopted.

Basic I medium employing the AnPB isolation contains a low concentration of yeast extract as a carbon source and lacks sulfides. Under an anaerobic condition, the medium is selective for purple non-sulfur bacteria among AnPBs; therefore, green sulfur bacteria, which dominantly detected in 16S rDNA PCR-DGGE (mentioned below), were not detected, due to their requirement of sulfur as an electron donor. Among the AnPB isolates from the Yamagawa Bay sediments, relatives of *Rhodob. sphaeroides* were most dominant. This species is known

as an anoxygenic purple non-sulfur bacterium (Pfennig and Trüper, 1971) with a freshwater origin. In view of their diverse metabolic systems and multiple uses (e.g., Lu et al., 2011; Subudhi et al., 2016), the *Rhodob. sphaeroides* isolates may be associated with its rapid growth and the possibility of using a variety of organic pollutant substances as their nutrients.

One isolate, PSBYam1608St4-1 belonged to the same family Rhodobacteraceae as *Rhodob. sphaeroides*. However, the most relative species *Thioclava pacifica* is not phototrophic: it grows chemoautotrophically with thiosulfate, heterotrophically with simple organic compounds, or methylotrophically utilizing hydrogen as an electron donor (Sorokin et al., 2005). The remaining bacterial clones showed no close relationship to AnPBs: all of them are heterotrophic, and no photoautotrophic growth has been reported. An eukaryotic green microalga *Chlorella sorokiniana* was also found in the anaerobically enriched culture, attributable to its capacity of anaerobic growth with photosynthesis and internally produced oxygen (Qiao et al., 2009).

The band profiles of PCR-DGGE were similar throughout the sampling period with slight differences in faint bands, regardless of the primer sets used. Due to its high water depth, the water column of Yamagawa Bay was stratified throughout the year, resulting in poor vertical mixing. Consequently, with loading and accumulation of organic pollutants, the sedimentary environments were continuously in a deleterious condition: poor dissolved oxygen (Table 1-1) and high sulfide content in the pore water (Ide, 2012). The persistence of the microbial community structures during the experimental period could account for such temporal stability of the benthic environments.

The primers universal to the Domain I of 16S rDNA including the hypervariable regions V1–V4 produced one major band in DGGE whose nucleotide sequence was identical to a green sulfur bacterium *Chlorob. phaeobacteroides* BS1. The continual dominance of this bacterial species, which is a strictly anaerobic photolithotroph oxidizing reduced sulfur (Overmann,

2001; Frigaard et al., 2003), was also reported in a brackish lake with oyster aquaculture (Santander-de Leon et al., 2013), reflecting the reductive condition observed in the seabed as mentioned above. However, it should be noted that the strain BS1 was phylogenetically reclassified into the genus *Prosthecochloris* (Imhoff and Thiel, 2010), suggesting its diverse characteristics from *Chlorob. phaeobacteroides*. In fact, existence of *Chlorobium* sp. BS1 was also reported in the Black Sea (Manske et al., 2005) whose water body is characterized by oxic-anoxic transition zone and sulfidic chemocline. The bacterium was inhabited at the depth of upper limit of sulfide-containing water layers with lower limit of downwelling irradiance for photosynthetic carbon fixation. The bacterial assemblage was deposited at the flocculent surface layer of the sea bottom and could survive under the extreme low-light conditions (Marschall et al., 2010). Predominance of the green sulfur bacterium in the Yamagawa Bay sediments could be explained by the similar process to the Black Sea, although its microbial ecophysiology should be elucidated.

A protein PufM, a gene product of *pufM*, is a component of the type II (pheophytin-quinone type) photosynthetic reaction center (RC; Cardona, 2015). The protein is known as an accurate tool for assessing phylogeny and diversity of bacteria employing the photosystem II in nature (Imhoff et al., 2018). Distribution of *pufM* is limited to purple bacteria including aerobic anoxygenic photosynthetic bacteria, and green sulfur bacteria including the genus *Chlorobium* lack the *pufM* gene since their photoenergy capturing depends on the type I (iron-sulfur type) RC with a protein PscA (Cardona, 2015). Thus, PCR-DGGE with the *pufM* primer sets was carried out to clarify temporal diversity of the type II RC-harboring bacteria, eliminating preferred amplification from a limited number of predominant populations, such as *Chlorob. phaeobacteroides* BS1, in 16S rDNA-targeted PCR. As a result, three out of eleven bands sequenced showed high identity to a *pufM* gene of *Rhodop. lichen*; however, its bacterial characteristics have not been reported. Diverse comparable to photosynthetic sulfur oxidizers

were also found: *Rhodov. sodomensis*, *Thiorh. bheemlicus*, *Thioc. marina* and *A. phaeobacterium* are members of purple sulfur bacteria, oxidizing sulfide, thiosulfate or elemental sulfur as electron donors for photolithotrophic growth under an anoxic condition (Imhoff et al., 1998; Caumette et al., 2004; Kumar et al. 2007; Srinivas et al., 2009). This finding is supported by the study of Mukkata et al. (2016), in which high concentration of H_2S was assumed by the detection of anaerobic purple sulfur bacteria *Allochromatium* sp. in shrimp pond. Further, Imhoff et al. (2018) clearly recognized anaerobic purple non-sulfur bacteria *Rhodovibrio* sp. with PufLM sequences. The existence of *Rhodovibrio* sp. with *Allochromatium* sp. refers to the ability of *Rhodovibrio* to tolerate high levels of sulfides.

It is noticeable that *pufM* sequences closely related to aerobic anoxygenic photoheterotrophs were also detected, such as *Ros. litoralis* and *D. shibae*. Both the species were originally isolated from micro- and macroalgal phycosphere (Shiba, 1991; Biebl et al., 2005). The former species belongs to the *Roseobacter* clade based on the 16S rDNA phylogeny, and the latter species is one of the closest sister taxa (Biebl et al., 2005). This lineage is one of the major marine bacterial groups, representing diverse marine habitats from coastal to open oceans as well as of sediments (Buchan et al., 2005). Moreover, several *Roseobacter* isolates harbor abilities to transform inorganic sulfur compounds (González et al., 1999; Buchan et al., 2005). Lenk et al. (2012) also revealed abundance of *Roseobacter* clade bacteria in marine surface sediments of tidal flats. They reported up to 9.6% of relative bacterial abundance of this clade and succeeded in its own enrichment under an anoxic, sulfidic condition. They also detected gene components of the SOX and reverse dissimilatory sulfite reductase (rDSR) pathways for inorganic sulfur oxidation. Taken together, *Roseobacter* is likely to be one of the sulfur oxidizers in highly eutrophicated marine coastal sediments, including Yamagawa Bay.

The present study suggested prevalence of AnPBs under the deteriorating sediment condition. The sulfide-rich reductive environments considered; colonization of the

photosynthetic sulfur oxidizers is very relevant. It is also conceivable that purple non-sulfur phototrophs have a potential role of degrading the organic pollutants. However, distribution and metabolism of the AnPBs must be regulated by the environmental factors in their sedimental habitat, such as oxygen availability, redox potential, types and concentrations of sulfur compounds, and solar illuminance. Hence, more detailed research on relationship between physicochemical factors of the sediments and population dynamics and biological activity of the AnPB communities is expected to improve our understanding of how the AnPBs contribute to cycling of the organic pollutants and their organic or inorganic sulfur derivatives.

Table 1-1. Physicochemical characteristics of Yamagawa Bay

Stations	Depth (m)	Temperature (°C)	Salinity (psu)	DO[a] (mg·L^{-1})	Chl *a*[b] (μg·L^{-1})	pH
Surface layers						
1		30.32±0.01	30.80±0.01	8.85±0.00	1.15±0.10	8.10±0.00
2		30.36±0.00	31.05±0.01	8.75±0.00	0.91±0.07	8.13±0.00
3	≃ 7	30.10±0.01	31.06±0.00	8.60±0.00	1.47±0.22	8.14±0.01
4		30.01±0.00	30.99±0.03	8.23±0.01	1.91±0.09	8.08±0.01
5		29.64±0.01	30.67±0.02	8.00±0.00	0.54±0.03	7.99±0.00
Bottom layers (one meter above the sea bottom)						
1	37.81±0.04	15.05±0.03	34.08±0.10	0.00±0.00	0.81±0.12	7.28±0.01
2	41.09±0.08	14.82±0.02	34.09±0.07	0.00±0.00	0.50±0.04	7.21±0.00
3	43.20±0.07	14.77±0.01	34.10±0.03	0.00±0.00	0.42±0.03	7.17±0.04
4	49.55±0.20	14.72±0.02	34.10±0.05	0.00±0.00	0.37±0.20	7.13±0.01
5	8.31±0.02	28.91±0.01	30.67±0.02	8.16±0.01	0.6±0.07	7.97±0.00

Values are means ± standard errors of the sampling period, May to November 2016.

[a]DO, concentration of dissolved oxygen.

[b]Chl *a*, concentration of chlorophyll *a*.

Table 1-2. Oligonucleotide primers used for PCR amplification, PCR-DGGE and nucleotide sequencing.

Primers	Application	Target genes	Target organisms	Nucleotide sequences (5' –> 3')[ab]	References
PrSSU.1F (27F)	PCR, sequencing	16S rDNA	*Bacteria*	AGAGTTTGATCCTGGCTCAG	Lane, 1991
PrSSU.1R (1525R)	PCR, sequencing	16S rDNA	*Bacteria*	AAAGGAGGTGATCCAGCC	Lane, 1991
PrSSU.2F (355F)	Sequencing	16S rDNA	*Bacteria*	TCCTACGGGAGGCAGCA	This study
PrSSU.3R (531R)	Sequencing	16S rDNA	*Bacteria*	TACCGCGGCTGCTGGCA	Hsiao et al., 2008
341F-GC	PCR-DGGE	16S rDNA	*Bacteria*	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGG CCTACGGGAGGCAGCAG	Muyzer et al., 1993
907R	PCR-DGGE, sequencing	16S rDNA	*Bacteria*	CCGTCAATTCCTTTGAGTTT	Muyzer et al., 1993
pufM557F	PCR-DGGE, sequencing	*pufM*	Purple photosynthetic bacteria	CGCACCTGGACTGGAC	Achenbach et al., 2001
pufM557FGC	PCR-DGGE	*pufM*	Purple photosynthetic bacteria	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGG GCACGGGGGGA CGCACCTGGACTGGAC	Achenbach et al., 2001
pufM.1R	PCR-DGGE	*pufM*	Aerobic anoxygenic phototrophic bacteria	GCRAACCACCAAGCCCA	Schwalbach and Fuhrman, 2005
pufM750R	PCR-DGGE	*pufM*	Purple photosynthetic bacteria	CCCATGGTCCAGCGCCAGAA	Achenbach et al., 2001
pufMR	PCR-DGGE	*pufM*	Aerobic anoxygenic phototrophic bacteria	CCATSGTCCAGCGCCAGAA	Béjà et al., 2002

[a] GC clamps added to 5' termini of the PCR-DGGE primers are underlined.

[b] The International Union of Pure and Applied Chemistry (IUPAC) codes are used for mixed nucleotides.

Table 1-3. BLAST homology searches of 16S rDNA sequences of the bacterial isolates obtained from the Yamagawa Bay sediments

PSB isolates	Isolation years/months	Stations isolated	Sequence length (nts)[a]	Most homologous relatives	Taxonomic groups	Sequence identity (%)
PSBYam1606St1-1	2016/June	1	1412	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	100
PSBYam1606St3-1-1	2016/June	3	787[b]	*Chlorella sorokiniana/variabilis/thermophila*[d]	Trebouxiophyceae - Chlorellales - Chlorellaceae	99
PSBYam1606St3-1-2	2016/June	3	798[b]	*Chlorella sorokiniana/variabilis/thermophila*[d]	Trebouxiophyceae - Chlorellales - Chlorellaceae	99
PSBYam1606St3-1-3	2016/June	3	817[b]	*Chlorella sorokiniana/variabilis/thermophila*[d]	Trebouxiophyceae - Chlorellales - Chlorellaceae	99
PSBYam1606St3-1-4	2016/June	3	829[b]	*Chlorella sorokiniana/variabilis/thermophila*[d]	Trebouxiophyceae - Chlorellales - Chlorellaceae	99
PSBYam1606St3-1-5	2016/June	3	799[b]	*Chlorella sorokiniana/variabilis/thermophila*[d]	Trebouxiophyceae - Chlorellales - Chlorellaceae	99
PSBYam1606St3-1-6	2016/June	3	830[b]	*Chlorella sorokiniana/variabilis/thermophila*[d]	Trebouxiophyceae - Chlorellales - Chlorellaceae	99
PSBYam1606St3-3-1	2016/June	3	898[b]	*Acinetobacter venetianus*	Gammaproteobacteria - Pseudomonadales - Moraxellaceae	100
PSBYam1606St3-5	2016/June	3	1405	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	100
PSBYam1606St3-7-1	2016/June	3	857[b]	*Pseudomonas putida/pseudoalcaligenes*	Gammaproteobacteria - Pseudomonadales - Pseudomonadaceae	100
PSBYam1606St3-9	2016/June	3	1414	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1606St5-1	2016/June	5	1412	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	100
PSBYam1606St5-3	2016/June	5	1389	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1607St1-1	2016/July	1	1426	*Bosea vestrisii/eneae/thiooxidans*	Alphaproteobacteria - Rhizobiales - Bradyrhizobiaceae	99
PSBYam1607St1-5	2016/July	1	1401	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1607St1-7	2016/July	1	1414	*Rhodobacter sphaeroides/johrii*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1607St1-8	2016/July	1	1405	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1607St2-4	2016/July	2	1404	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	100
PSBYam1607St2-5	2016/July	2	1398	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	100

PSBYam1607St2-7	2016/July	2	1404	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1607St3-1	2016/July	3	1414	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1607St3-3	2016/July	3	1413	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1607St3-5	2016/July	3	1405	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1607St4-2	2016/July	4	1340	*Mycoplana ramosa*	Alphaproteobacteria - Rhizobiales - Brucellaceae	98
PSBYam1608St1-1	2016/August	1	1402	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1608St2-1	2016/August	2	1407	*Rhodobacter sphaeroides*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1608St4-1	2016/August	4	756[b]	*Thioclava pacifica*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	99
PSBYam1705St.2-4-A1	2017/May	2	701[c]	*Acidovorax delafieldii*	Betaproteobacteria - Burkholderiales - Comamonadaceae	99
PSBYam1705St.2-4-A2	2017/May	2	703[c]	*Pseudomonas knackmussii/nitroreducens*	Gammaproteobacteria - Pseudomonadales - Pseudomonadaceae	100
PSBYam1705St.2-4-A3	2017/May	2	700[c]	*Pseudomonas knackmussii/nitroreducens*	Gammaproteobacteria - Pseudomonadales - Pseudomonadaceae	99
PSBYam1705St.3-1-A4	2017/May	3	700[c]	*Pseudomonas knackmussii/nitroreducens*	Gammaproteobacteria - Pseudomonadales - Pseudomonadaceae	100
PSBYam1705St.3-2-A8	2017/May	3	703[c]	*Pseudomonas knackmussii/nitroreducens*	Gammaproteobacteria - Pseudomonadales - Pseudomonadaceae	100
PSBYam1705St.3-3-A13	2017/May	3	671[c]	*Chlorella sorokiniana/variabilis/thermophila*[d]	Trebouxiophyceae - Chlorellales - Chlorellaceae	99
PSBYam1705St.4-1-A14	2017/May	4	701[c]	*Acidovorax delafieldii*	Betaproteobacteria - Burkholderiales - Comamonadaceae	99
PSBYam1705St.4-1-A16	2017/May	4	703[c]	*Pseudomonas knackmussii/nitroreducens*	Gammaproteobacteria - Pseudomonadales - Pseudomonadaceae	100
PSBYam1705St.4-2-A18	2017/May	4	666[c]	*Chlorella sorokiniana*	Trebouxiophyceae - Chlorellales - Chlorellaceae	99

[a]Lengths of the nucleotide sequences subjected to BLASTN searches were shown.

[b]A sequencing primer PrSSU.2F was used for sequencing.

[c]Sequencing primers PrSSU.2F and PrSSU.3R were used for sequencing.

[d]Identity to 16S rDNA encoded in their plastidal genomes.

Table 1-4. BLAST homology searches of the 16S rDNA DGGE bands obtained from the Yamagawa Bay sediments

Sampling years/months	Sample serial numbers	Most homologous relatives	Taxonomic groups	Sequence Identity (%)
2016/May	1	*Chlorobium phaeobacteroides* BS1[a]	Chlorobia - Chlorobiales - Chlorobiaceae	100
2016/May	2	*Chlorobium phaeobacteroides* BS1	Chlorobia - Chlorobiales - Chlorobiaceae	100
2016/May	3	*Chlorobium phaeobacteroides* BS1	Chlorobia - Chlorobiales - Chlorobiaceae	100
2016/June	1	*Chlorobium phaeobacteroides* BS1	Chlorobia - Chlorobiales - Chlorobiaceae	100
2016/June	2	*Chlorobium phaeobacteroides* BS1	Chlorobia - Chlorobiales - Chlorobiaceae	100
2016/June	3	*Chlorobium phaeobacteroides* BS1	Chlorobia - Chlorobiales - Chlorobiaceae	100
2016/July	1	*Chlorobium phaeobacteroides* BS1	Chlorobia - Chlorobiales - Chlorobiaceae	100
2016/November	1	*Chlorobium phaeobacteroides* BS1	Chlorobia - Chlorobiales - Chlorobiaceae	100
2016/November	2	*Chlorobium phaeobacteroides* BS1	Chlorobia - Chlorobiales - Chlorobiaceae	100
2016/November	3	*Chlorobium phaeobacteroides* BS1	Chlorobia - Chlorobiales - Chlorobiaceae	100

[a]The strain BS1 was reclassified into the genus *Prosthecochloris* (Imhoff and Thiel, 2010).

Table 1-5. BLAST homology searches of the *pufM* DGGE bands obtained from the Yamagawa Bay sediments

Sampling years/months	Bands (first round DGGE)	Bands (second round DGGE)[a]	Bands (third round DGGE)[b]	Most homologous relatives	Taxonomic groups	Sequence Identity (%)
2016/May	A12	10	11	*Thiorhodococcus bheemlicus*	Gammaproteobacteria - Chromatiales - Chromatiaceae	89
2016/June	A8	8	–	*Dinoroseobacter shibae*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	84
2016/June	A8	8	10	*Rhodovibrio sodomensis*	Alphaproteobacteria - Rhodospirillales - Rhodospirillaceae	80
2016/August	A2	2	–	*Rhodopseudomonas lichen*	Alphaproteobacteria - Rhizobiales - Bradyrhizobiaceae	84
2016/August	A2	2	2	*Rhodopseudomonas lichen*	Alphaproteobacteria - Rhizobiales - Bradyrhizobiaceae	84
2016/August	A10	9	–	*Rhodovibrio sodomensis*	Alphaproteobacteria - Rhodospirillales - Rhodospirillaceae	81
2016/November	A1	1	1	*Sphingomonas lacus*	Alphaproteobacteria - Sphingomonadales - Sphingomonadaceae	82
2016/November	A3	3	4	*Sulfitobacter guttiformis* *Rhodopseudomonas lichen*[c]	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae Alphaproteobacteria - Rhizobiales - Bradyrhizobiaceae	83
2016/November	A3	6	8	*Roseobacter litoralis*	Alphaproteobacteria - Rhodobacterales - Rhodobacteraceae	92
2016/November	A4	4	–	*Porphyrobacter tepidarius*	Alphaproteobacteria - Sphingomonadales - Erythrobacteraceae	85
2016/November	A4	4	5	*Porphyrobacter tepidarius*	Alphaproteobacteria - Sphingomonadales - Erythrobacteraceae	85

[a]DNA fragments which formed the bands excised from the first round DGGE (Fig. 1-5) were re-amplified.
[b]DNA fragments which formed the bands excised from the second round DGGE (Fig. 1-6) were re-amplified.
[c]The nucleotide sequence showed the identical identity value between the two bacterial species.

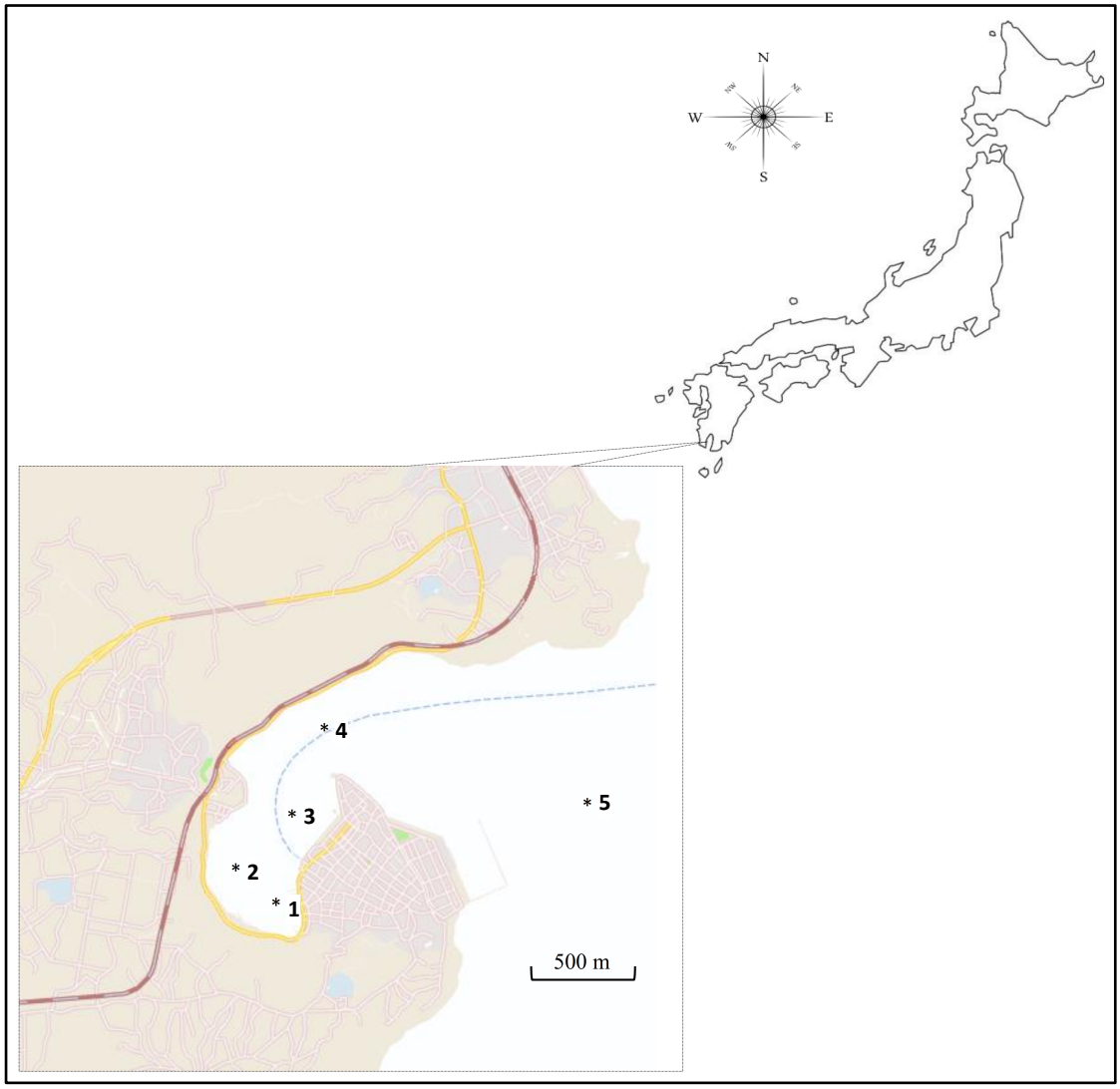

Fig. 1-1. Sampling sites in Yamagawa Bay, Kagoshima, Japan. Geographical coordinates are as follows: 1, 31.2028N/130.6323E; 2, 31.2042N/130.6310E; 3, 31.2072N/130.6335E; 4, 31.2120N/130.6385E; 5, 31.2117N/130.6591E).

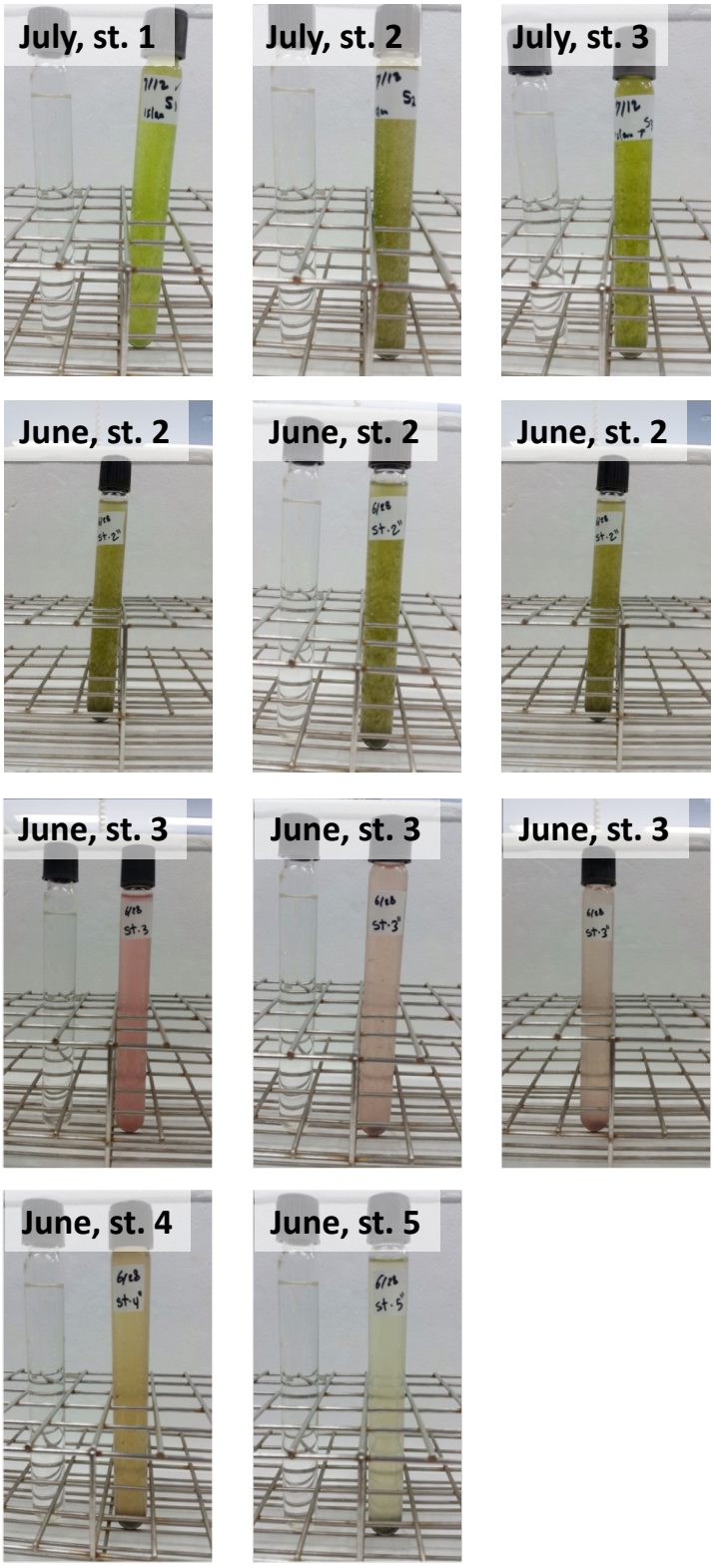

Fig. 1-2. Enrichment cultures of photosynthetic bacteria. The Yamagawa bay sediments were inoculated into the Basic I liquid medium and cultivated. The sediments collected in June and July 2016 are shown as representatives.

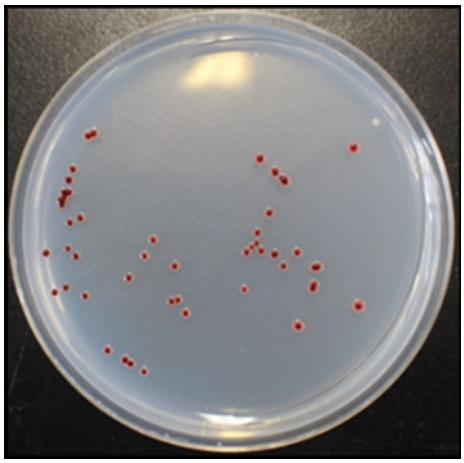

Fig. 1-3. Colonies of the isolated photosynthetic bacterium, PSBYam1607St2-5. Colonies appeared after 3–5 days incubation on the Basic I agar plate.

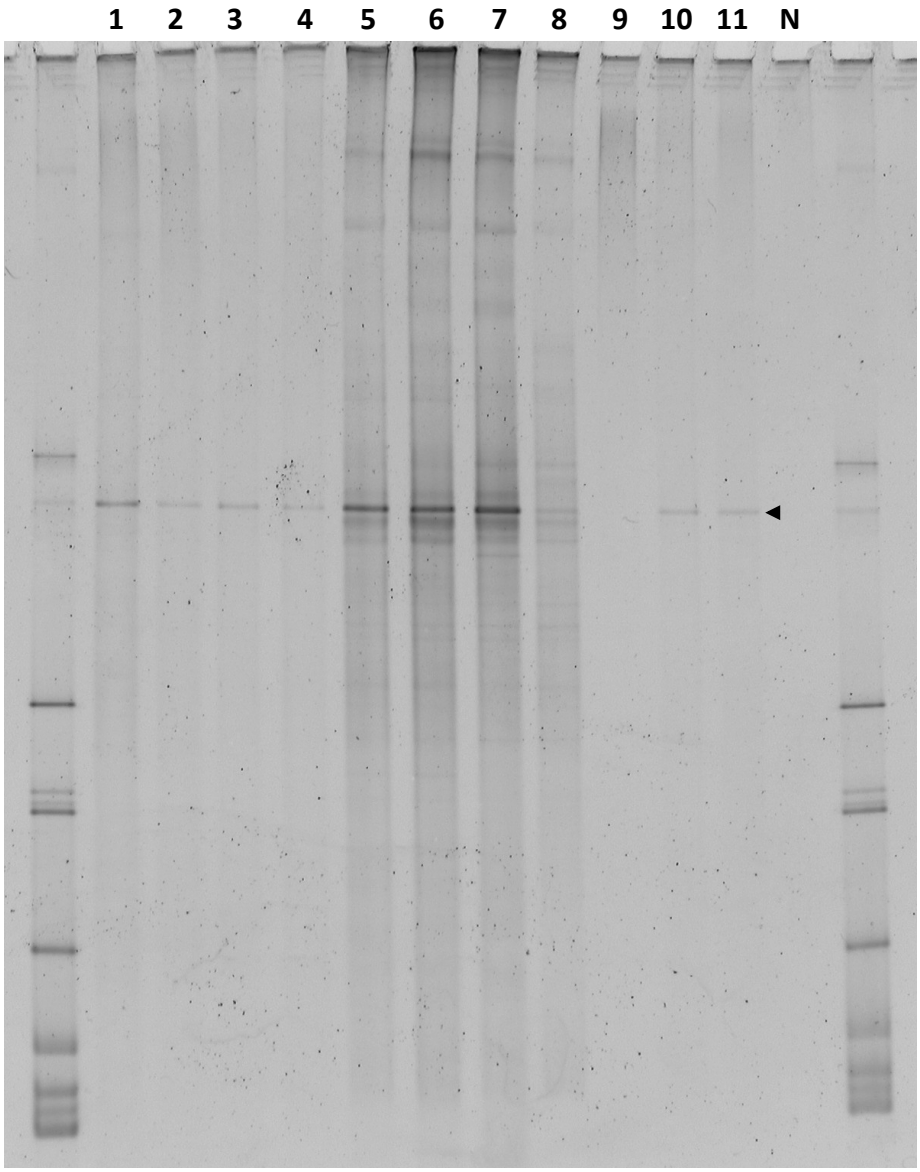

Fig. 1-4. Band profiles of 16S rDNA PCR-DGGE. Amplicons of 16S rDNA amplified from environmental DNAs of Yamagawa Bay sediments were applied. Bands subjected to nucleotide sequencing are shown as an arrowhead. Years/months of sediment sampling and serial numbers (#) of the samples are as follows: lane 1, 2016/May, #1; lane 2, 2016/May, #2; lane 3, 2016/May, #3; lane 4, 2016/June, #1; lane 5, 2016/June, #2; lane 6, 2016/June, #3; lane 7, 2016/July, #1; lane 8, 2016/August, #1; lane 9, 2016/November, #1; lane 10, 2016/November, #2; lane 11, 2016/November, #3. Lane N, no-template control.

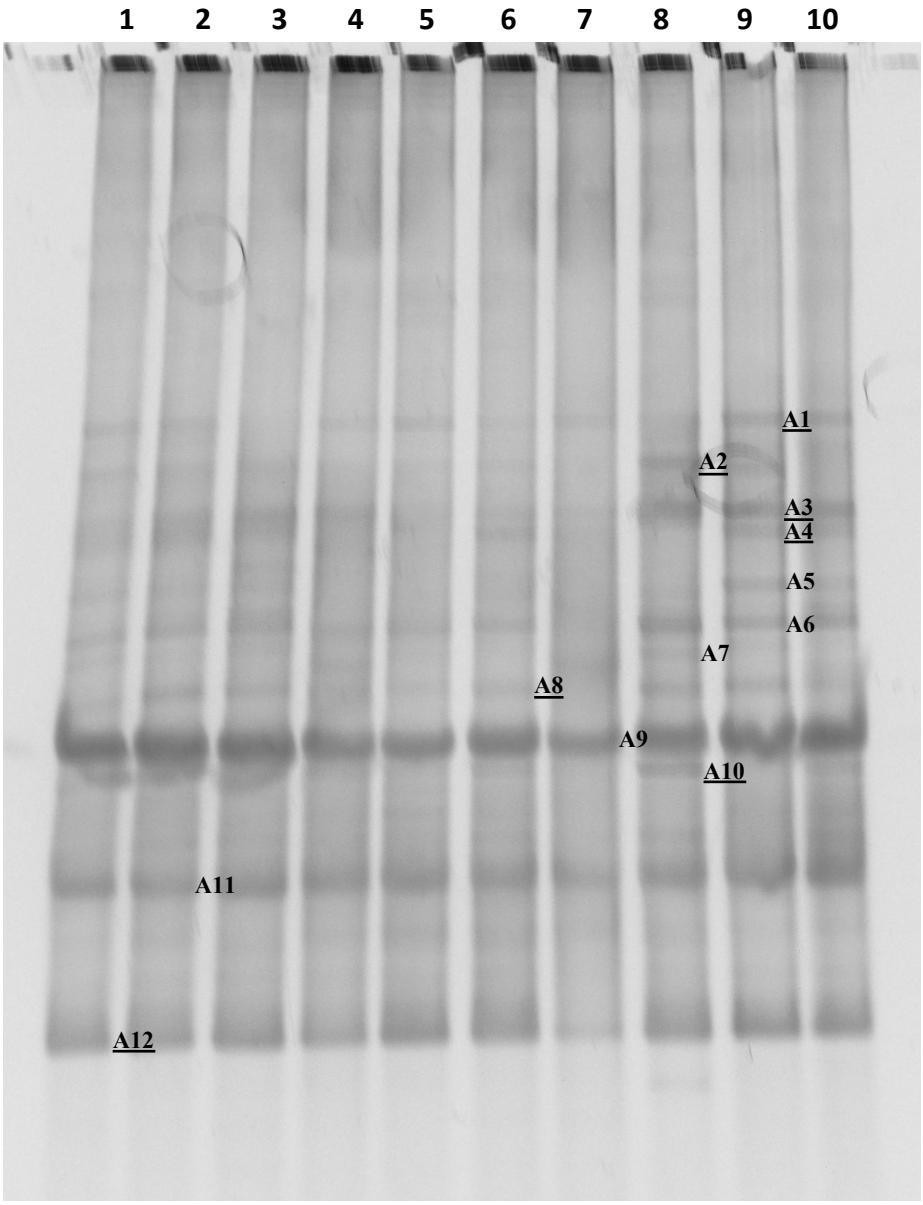

Fig. 1-5. Band profiles of *pufM* PCR-DGGE. Amplicons of *pufM*, amplified from environmental DNAs of Yamagawa Bay sediments with secondary primers pufM557F/pufM750R, were applied. Bands excised for further analyses are shown (A1–A12), and the bands whose nucleotide sequences were determined are underlined. Years/months of sediment sampling and serial numbers (#) of the samples are as follows: lane 1, 2016/May, #1; lane 2, 2016/May, #2; lane 3, 2016/May, #3; lane 4, 2016/June, #1; lane 5, 2016/June, #2; lane 6, 2016/June, #3; lane 7, 2016/July, #1; lane 8, 2016/August, #1; lane 9, 2016/November, #2; lane 10, 2016/November, #3.

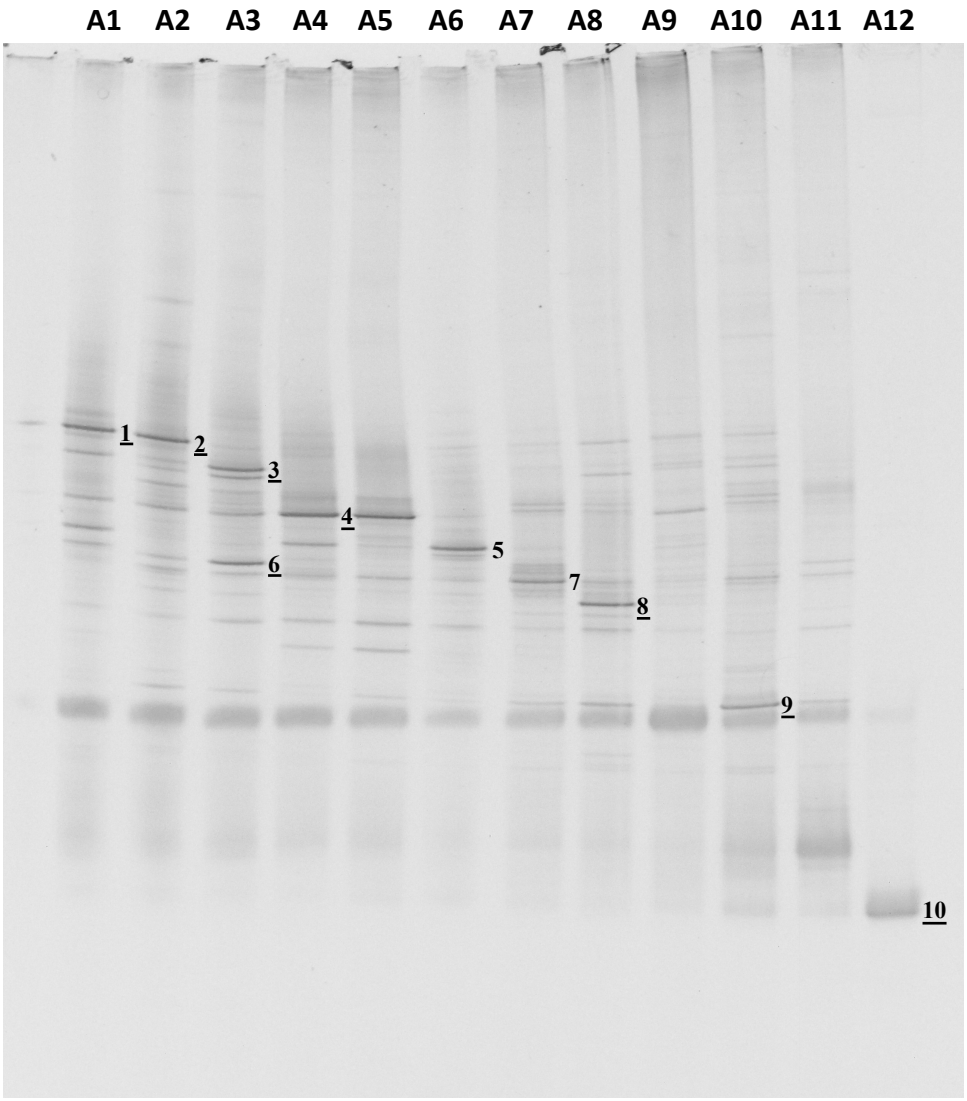

Fig. 1-6. Band profiles of *pufM* PCR-DGGE. Fragments of DNA eluted from excised DGGE bands A1–A12 (Fig. 1-5) were re-amplified by PCR and applied. Bands excised for further analyses are shown (1–10), and the bands whose nucleotide sequences were determined are underlined.

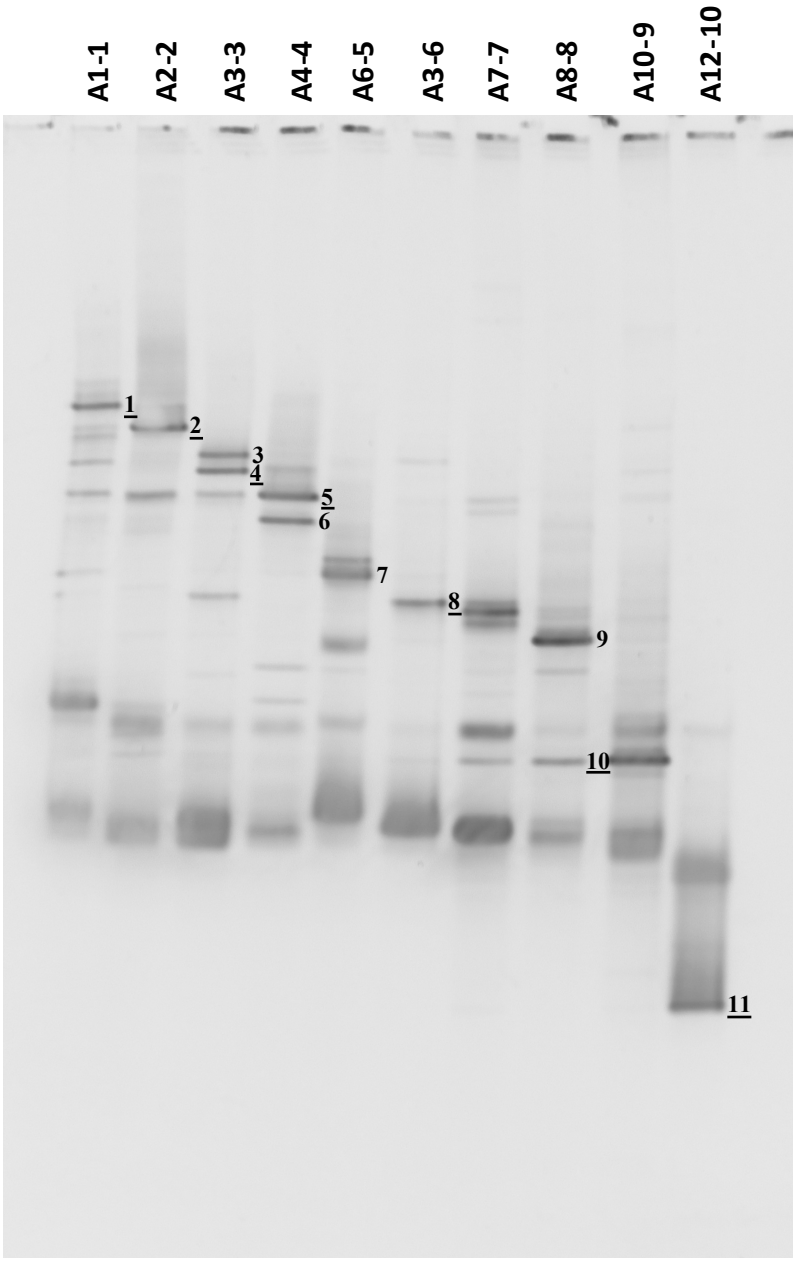

Fig. 1-7. Band profiles of *pufM* PCR-DGGE. Fragments of DNA eluted from excised DGGE bands 1–10 (Fig. 1-6) were re-amplified by PCR and applied. Bands excised for further analyses are shown (1–11), and the bands whose nucleotide sequences were determined are underlined.

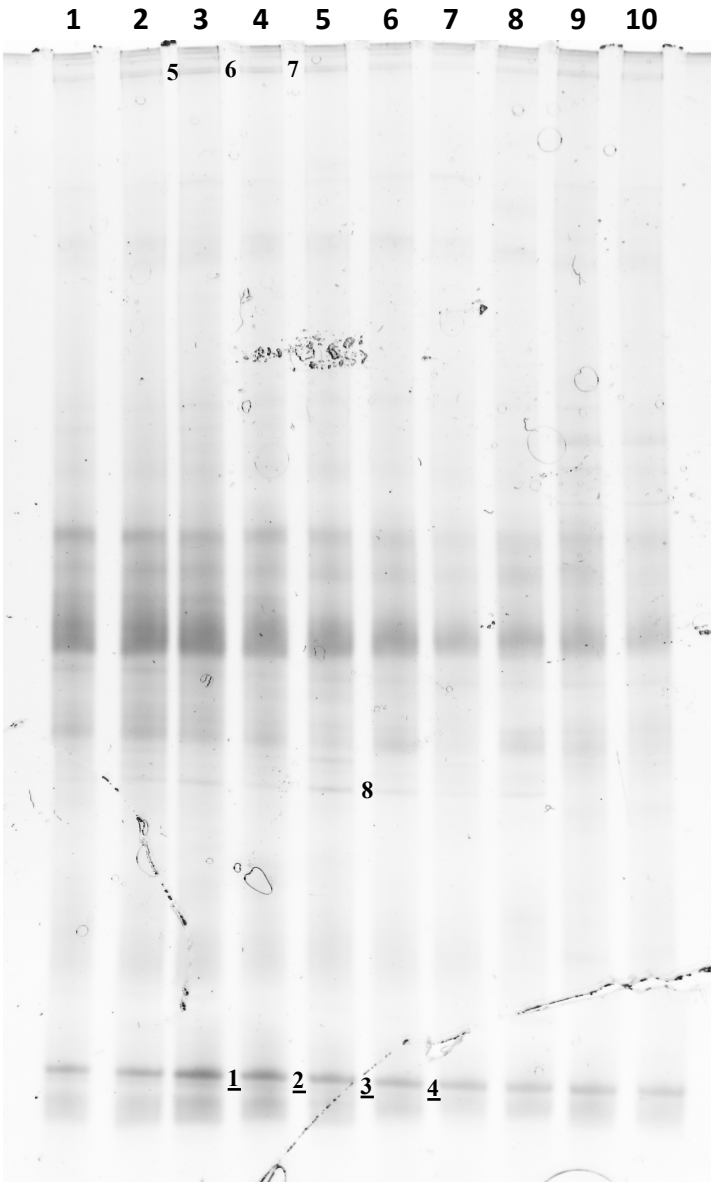

Fig. 1-8. Band profiles of *pufM* PCR-DGGE. Amplicons of *pufM*, amplified from environmental DNAs of Yamagawa Bay sediments with secondary primers pufM557FGC/pufM750R, were applied. Bands excised for further analyses are shown (1–8), and the bands whose nucleotide sequences were determined are underlined. Years/months of sediment sampling and serial numbers (#) of the samples are as follows: lanes 1, 4, 5, 8, 2016/May, #1; lanes 2,6, 9, 2016/May, #2; lane 3, 7, 10, 2016/May, #3. Annealing temperatures/thermal cycling to obtain the *pufM* amplicons with the secondary primers were 60˚C/10 cycles (lanes 1–4), 58˚C/12 cycles (lanes 5–7), and 63˚C/12 cycles (lanes 8–10).

CHAPTER 2. USE OF PURPLE NON-SULFUR PHOTOSYNTETIC BACTERIUM (*RHODOBACTER SPHAEROIDES*) FOR PROMOTING GROWTH OF CILIATED PROTOZOA

2.1 Introduction

Demand for sustainable human food sources with high-quality protein has been growing with a population explosion in recent years. Aquaculture is considered to be one of the viable options for reducing a gap between food production and human consumption (FAO, 2014; United Nations, Department of Economic and Social Affairs, Population Division, 2015). Indeed, aquaculture has contributed significantly to the production of marine food resources since the 1970s; however, the aquaculture industry faces a series of urgent issues (Bentzon-Tilia et al., 2016). Intensive exploitation of aquaculture fields leads to unfavorable impacts on the aquacultured fish as well as on the marine environments, resulting in larval death, fish disease propagation and marine pollution (EL-Haroun et al., 2006; Wang et al., 2008a, b).

Successful development of aquaculture depends on a number of factors correlating conditions of the seedling production as well as the aquatic environments. Fish larval production is often hindered by their high mortality caused by fish diseases or malnutrition (Bricknell and Dalmo, 2005; Conceição and Tandler, 2018). Starvation is considered to be a primary cause of mass mortality of newly hatched fish larvae, up to 99%, owing to insufficient nutritional effect of their prey including brine shrimps, copepods and rotifers (China and Holzman, 2014). Feeding of zooplankton and/or phytoplankton with high nutritional values is primarily important in terms of improvement of the nourishing efficiency of the live feed (Das et al., 2012; Wikfors, 2004). Among the prey organisms, ciliated protozoa are mostly applied to larval feeding, since they occupy important trophic status of the aquatic ecosystem, which is

an integral component of the planktonic food web (Beaver and Crisman, 1989; Gates, 1984; Pace, 1986).

Bacterioplankton and phytoplankton form a basis of the natural food chain in the aquatic ecosystems and are grazed by zooplankton including ciliated protozoa as a main source for their nutritional requirements (Bengtson, 2007; Bentzon-Tilia et al., 2016; Evjemo et al., 2003; Hahn and Höfle, 2001; Pernthaler et al., 2001). Grazing capacity of ciliates depends on characteristics of their prey microorganisms as well as their own feeding behavior: some bacteria are more attractive to the grazer than the others (Ayo et al., 2001; Gruber et al., 2009). Among marine bacterial groups, anoxygenic photosynthetic bacteria (AnPBs) possess distinctive characteristics applicable to fish larval feeding, bioremediation, probiotics and fish disease control, which make them one of the main eco-friendly tools in the aquaculture system (Dawood et al., 2018; Kuo et al., 2012; Lu et al., 2010; Nayak, 2010; Qi et al., 2009; Suzer et al., 2008; Wang et al., 2008a; Zhang et al., 2014).

The aims of this study are: (i) to establish acquiring and cultivating methods of ciliates inhabiting in natural marine environments by comparing their efficiencies of different cultivating methods; and (ii) to test ingestion of an AnPB isolate as feed for the ciliates. The present study will contribute to provide basic knowledge on nourishing approaches for zooplankton and improvement of nutritional values of the ciliates as aquacultured fish feed.

2.2 Materials and Methods

Photosynthetic bacterial isolate

An AnPB isolate PSBYam1607St2-5, whose nucleotide sequence of the 16S ribosomal RNA gene is identical to *Rhodobacter sphaeroides* (Table 1-3, CHAPTER 1), was chosen as prey for ciliates.

Cell morphology of the phototrophic isolate was observed microscopically with 4',6-

diamidino-2-phenylindole (DAPI) staining. The isolate was cultured in tightly sealed test tubes filled with autoclaved Basic I liquid medium (see CHAPTER 1 for its composition) semi-anaerobically under a light-dark cycle of 12 h:12 h at 20˚C for 14 d. The bacterial cells were harvested at the end of their logarithmic growth phase by centrifugation at 9,300 x *g* for 15 min. The cell pellets were resuspended in 10 mL of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) to adjust the turbidity to 1.7±0.2 in absorbance at 660 nm. Staining of the AnPB cells with DAPI was performed according to Porter and Feig (1980) and Nakano et al. (2008). Briefly, 0.5 mL of the liquid culture medium was mixed with 50 µL of 20% PBS-buffered formaldehyde as a cell fixative and 25 µL of 5 $\mu g \cdot mL^{-1}$ DAPI. The stained bacterial cells were placed on a black Nuclepore polycarbonate filter (pore size, 0.2 µm; Whatman Ltd., Kent, UK) under a dark condition. After 20 min incubation, the suspension was filtered, and the filter was rinsed with sterile distilled water. The bacterial cells trapped on the filter were observed with an epifluorescent microscope (BX53, Olympus Co. Ltd., Tokyo, Japan) under UV excitation.

Sample collection and cultivation of ciliated protozoa

For ciliate experiments, sediment and seawater samples were collected from an established station in Kuwano-ura Bay, Kamikoshiki Island, Kagoshima, Japan (Fig. 2-1). Three types of culturing mixtures were tested for ciliate enrichment at 23˚C under continuous light irradiation for 2 wks. The first mixture (MI) followed the composition used by Mukai et al. (2016), in which 0.1 g of anchovy fish meal (*iriko* in Japanese) and 0.1 g of radish (*Brassica rapa*) leaves were added into 5 mL of the seawater sample size-fractionated (15–80 µm) by filtration with polycarbonate filters. The second mixture (MII) consisted of 0.1 g of anchovy fish meal, 0.1 g of radish leaves, and 0.1 g of the sediment sample in 5 mL of autoclaved seawater. In the third mixture (MIII), 0.1 g of the sampled sediment was added into 5 mL of Daigo's IMK medium

(for marine microalgae; Fujifilm Wako Pure Chemical Co., Osaka, Japan). During the culturing period, subsamples were fixed with 2% neutralized formaldehyde and the number of ciliates was counted microscopically using a ruled plankton-counting plate (Rigo, Tokyo, Japan).

The grown ciliates were size fractionated by filtration. All the ciliate-culturing mixtures, MI, MII and MIII, were mixed to ensure presence of all the grown ciliates and passed serially through polycarbonate filters with pore sizes of 84, 30 and 5 μm. The ciliates in the size fractions of 30–84 μm ("large size") and 5–30 μm ("small size") were collected, and their morphology were observed microscopically.

Community structure analyses of the ciliates in the enrichment cultures

The ciliate community structures in the enrichment mixtures were analyzed using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 18S ribosomal RNA genes (18S rDNA). Deoxyribonucleic acids of the ciliates with different sizes, "large size" and "small size", were obtained using DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). Amplification of the 18S rDNA with a universal primer set 1427F-GC (5′-cgcccgccgcgccccgcgcccggcccgccgcccccgcccc-tctgtgatgcccttagatgttctggg-3′, a GC-clamp is underlined; van Hannen et al., 1998; Leão et al., 2012) and 1616R (5′-gcggtgtgtacaaagggcaggg-3′; van Hannen et al., 1998; Leão et al., 2012) was conducted in PCR reaction mixtures of 20 μL, consisting of 1 x *ExTaq* Buffer (Takara Bio, Otsu, Japan), 100 μM dNTP Mixture (Takara Bio, Otsu, Japan), 0.5 μM primers, 0.025 units·μL^{-1} *ExTaq* DNA Polymerase (Hot Start Version, Takara Bio, Otsu, Japan), and 1 μL of ten-times diluted ciliate DNA solutions. A touchdown PCR (Don et al., 1991) was adopted with T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with 95˚C as an initial denaturation for 5 min, followed by 19 cycles of 95˚C for 1 min, 62˚C for 1 min with 0.8˚C decrement per cycle, and 72˚C for 1min; 9 cycles of 95˚C for 1 min, 52˚C for 1 min, and 72˚C for 1 min with final extension at 72˚C for 9 min.

The PCR products were loaded onto 6% polyacrylamide gel, in which 100% denaturant contained 40% deionized formamide and 7 M urea. Electrophoresis was carried out in 0.5 x TAE buffer (40 mM Tris-acetate, pH 8.3, 1 mM ethylenediaminetetraacetic acid) for 16 h under a condition of constant voltage at 60 V and buffer temperature at 60˚C, using DCode System (Bio-Rad, Hercules, CA, USA). The gels were stained with SYBR Gold (Thermo Fisher Scientific, Waltham, MA, USA) and band presence was checked on the blue light transilluminator Safe Imager 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). To obtain nucleotide sequences, the DGGE bands were excised from the gels using 1-mL pipette tips, the gel pieces were suspended in 100 μL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid), and the suspension were frozen and thawed to elute the amplified DNA. Sequencing reaction was done using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA), and the closest relatives of the sequenced 18S rDNA were determined by the basic local alignment search tool (BLAST, Altschul et al., 1990).

Grazing of ciliates on AnPBs

The ciliates with different sizes, “large size” and “small size”, were kept in sterilized seawater for 6 h to empty their food vacuoles. Ten milliliter of the AnPB suspensions, whose cell densities were adjusted to 0.78±0.02 in absorbance at 660 nm, were transferred into 3 polycarbonate bottles (40 mL) containing 30 ml of sterilized seawater-suspended “large” and “small” ciliates with *ca.* 440 individuals·mL^{-1} and 480 individuals·mL^{-1}, respectively. The ciliate suspensions were substituted with sterile seawater in a control group. During the grazing experiment, reduction in number of AnPB was estimated by bacterial turbidity changes at 660 nm (Wei et al., 2016), while the ciliates were counted by a ruled plankton-counting plate (Rigo, Tokyo, Japan) under an optical microscope.

In order to observe ingestion of AnPB by ciliates, the bacterial isolate PSBYam1607St2-5 was fluorescently labeled with 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF) according to the method of Sherr et al. (1987). The fluorochrome was added into the bacterial suspension at a final concentration of 2 $\mu g \cdot mL^{-1}$ and the cells were stained for 2 h at 60˚C. The fluorescently labeled bacteria (FLB) was rinsed by centrifugation and suspending with sterilized seawater four times. The FLB was offered to the ciliates in filtered seawater and observed 24 h after fixation with 2% neutralized formaldehyde with an epifluorescent microscope with blue excitation.

Data analyses

The experiments of the ciliate enrichment and AnPB grazing were carried out in triplicate. Statistical analyses (mean ± standard error) was performed with the analysis of variance (one-way ANOVA) followed by post-hoc Duncan's test using Statistical Analysis System (SAS) version 8.02 for Windows. Differences in the data were considered to be significant at the level of $P \leq 0.05$.

2.3 Results

Cell morphology of the photosynthetic bacterial isolate

Epifluorescence-microscopic observation of the AnPB isolate PSBYam1607St2-5 showed short rod cells (1–2 μm length) with round ends (Fig. 2-2), which is a typical characteristic of *Rhodobacter sphaeroides*, to which the isolate showed identical nucleotide sequences of 16S rDNA. Accordingly, the isolate was tentatively identified as *Rhodob. sphaeroides*.

Cultivation of the ciliated protozoa

Figure 2-3 shows changes in ciliate densities in the culturing mixtures, MI, MII and MIII, to which the seawater and sediment samples were added. Growth of the ciliates were observed in all the cultures and increase in number of the ciliates was notable from the third day. The cell

densities peaked on the seventh or eighth day, reaching 1424±54, 1509±61, and 2081±65 individuals·mL^{-1} in MI, MII, and MIII, respectively. Use of the mixture MIII supplemented with the medium for marine microalgae produced the highest number of ciliates.

Community structure analyses of the ciliates in the enrichment cultures

The ciliates of "large size" (30–84 μm) and "small size" (5–30 μm) in the enrichment mixtures, whose cell morphology were shown in Fig. 2-4, were analyzed by 18S rDNA PCR-DGGE (results are shown in Fig. 2-5). Two unique bands were obtained from the large-size ciliates, whose nucleotide sequences were identical to *Euplotes minuta* (100.00% identity of 162 nucleotides) and *Cyclidium varibonneti* (99.28% identity of 139 nucleotides), respectively. The small-size protozoa provided a single band, and the same nucleotide sequence as the one of *Exuviaella pusilla* was obtained with the identity of 86.05%.

Bacterivory of ciliates on the photosynthetic bacterial isolate

Figure 2-6 shows changes in abundance of the AnPB isolate and the ciliates with large or small sizes during the grazing experiment. The abundance of the bacterial isolate in the control group did not alter significantly until 96 h, then dropped dramatically at 108 h, that was considered as an endpoint of the observation. Cell densities of the ciliates reached at their peak at 2,180–2,220 individuals·mL^{-1} for large-size ciliates and at 1,800–1,940 individuals·mL^{-1} for small-size ciliates in 92–96 h. Concomitantly, decreasing of the bacterial abundance was observed after 24 h but with insignificant difference between the co-cultivation with large-size and small-size ciliates. From 36 to 72 h, decrease in the bacterial number with the large-size ciliates was significantly higher than the one with the small-size ciliates. Feeding of the ciliates on FLB was observed under an epifluorescence microscope with intense DATF fluorescence in possible food vacuoles (Fig. 2-7), confirming the decline of the bacterium in the grazing experiment was a result of ciliate grazing.

2.4 Discussion

As mentioned in CHAPTER 1, presence of the AnPBs, tentatively identified as *Rhodob. sphaeroides*, in all the studied stations of Yamagawa Bay indicates their widespread distribution in the eutrophicated marine sediments. Previous studies including the preceding chapter indicated that AnPBs widely inhabited in diverse environments (Koblížek et al., 2006; Lu et al., 2010; Merugu et al., 2014; Okubo et al., 2006; Subudhi et al., 2016; Zhang et al., 2009). Their widespread appearance can be attributed to their high versatility to utilize a wide range of nutrients (Alloul et al., 2019; Imhoff et al., 2015; Kantachote et al., 2005; Kim et al., 2004; Wei et al., 2016) and their ability to survive in difficult environmental conditions (Chae et al., 2006; Kosamu and Obst, 2009; Yegani et al., 2005).

Importance of ciliates in aquaculture systems as well as in aquatic environments has been noted in several studies (Ajeagah Aghaindum and Foto Menbohan, 2012; Ayo et al., 2001; Ayo et al., 2009; Chen et al., 2012; Dopheide et al., 2011; Gruber et al., 2009; Jiang et al., 2013; Kar et al., 2017; Mukai et al., 2016; Pfister et al., 2002; Posch et al., 2001). The ciliates are one of the preferable nutritious live feed to sustain aquacultured organisms, especially at early growth stages, owing to their rapid proliferation, small sizes, soft bodies, protein and lipid contents of high quality as well as to their adaptability to a wide range of environmental conditions (Corliss, 2002; Das et al., 2012; Jiang et al., 2007, Jiang et al., 2013; Kar et al., 2017; Madoni and Braghiroli, 2007; Montagnes et al., 2010; Mukai et al., 2016; Shil et al., 2013; Velasco-Santamaría and Corredor-Santamaría, 2011). Ciliates are able to cultivate with dramatically high multiplication rates: The present study showed rapid growth during the enrichment cultivation (MI, MII and MIII) from the third to seventh days, which is consistent with the result obtained by Mukai et al. (2016). Das et al. (2012) also observed ciliate increase after 4–5 d of enrichment. Density of the cultured ciliates attained 1,424–2,081 individuals·mL^{-1}, which was higher than that obtained by Mukai et al. (2016; 500-1,000 individuals·mL^{-1}) and almost

identical to Côrtes et al. (2013; 2,500 individuals·mL^{-1}). The difference can be attributable to use of different ciliate species and/or different culture conditions. The highest number of ciliates was obtained from the enrichment culture supplemented with the medium for marine microalgae (MIII), suggesting growth of microalgae originated from the sediment sample and their importance of the ciliate growth as previously reported by Pfister et al. (2002).

The enriched protozoa were composed of at least three different taxa based on the results of 18S rDNA PCR-DGGE: two of the bands obtained from the size fraction of ≥30 μm showed completely or highly identical to *Eupl. minuta* and *Cyclidium varibonneti*, both of which belong to the subphylum Intramacronucleata in the phylum Ciliophora, resulting in their tentative identifications. Another band possessed homology with *Ex. pusilla* with low homology. This species is a member of the class Dinophyceae, taxonomically distant from any ciliates. Its cell morphology, however, did not reflect characteristics of the genus *Exuviaella*, suggesting an artifact in the PCR-DGGE process; taxonomic and phylogenetic positions of this protist should be re-examined in association with its phenotypic characteristics.

Most of the ciliated protozoa feed on bacteria at varying grazing rates, which are dependent on sizes, morphology, surface properties, motility and biochemical compositions of the bacterial cells as well as on sizes and feeding history of predators (Ayo et al., 2009; Battin et al., 2003; Chen et al., 2012; Dopheide et al., 2011; Gruber et al., 2009; Matz and Jürgens, 2005; Posch et al., 2001; Sakaguchi et al., 2001; Shannon et al., 2007). The phototrophic isolate used in this study was extensively grazed, reflecting their high quality as prey. The bacterial size, the length of 1–2 μm, was best fitted to the optimum predator:prey size ratio of about 8:1 proposed by Jonsson (1986). In this context, as Ayo et al. (2001) pointed out, the size of ciliates was positively associated with the grazing rates: the large size ciliate had a higher grazing rate than the small size one as shown in Fig. 2-6.

Fig. 2-1. Sampling station in Kuwano-ura Bay, Kamikoshiki Island, Kagoshima, Japan. The geographical coordinate is 31.84633N/129.937E.

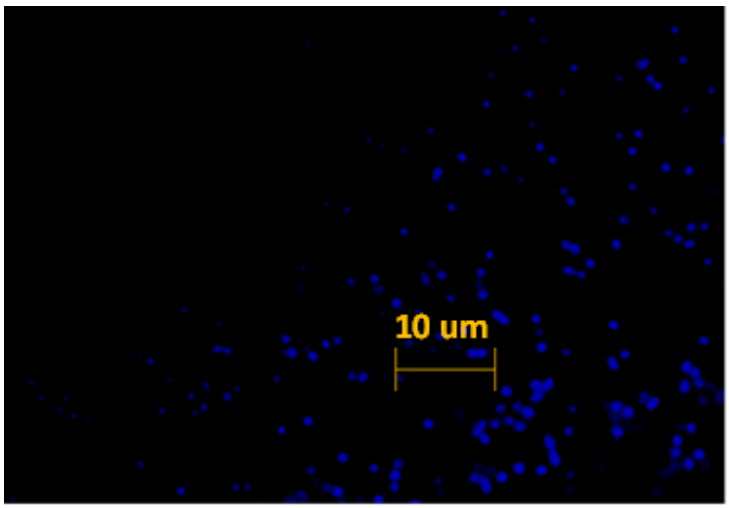

Fig. 2-2. Epifluorescence-microscopic observation of the photosynthetic bacterium PSBYam1607St2-5. The bacterial cells were stained with 4',6-diamidino-2-phenylindole (DAPI) and observed under ultraviolet excitation.

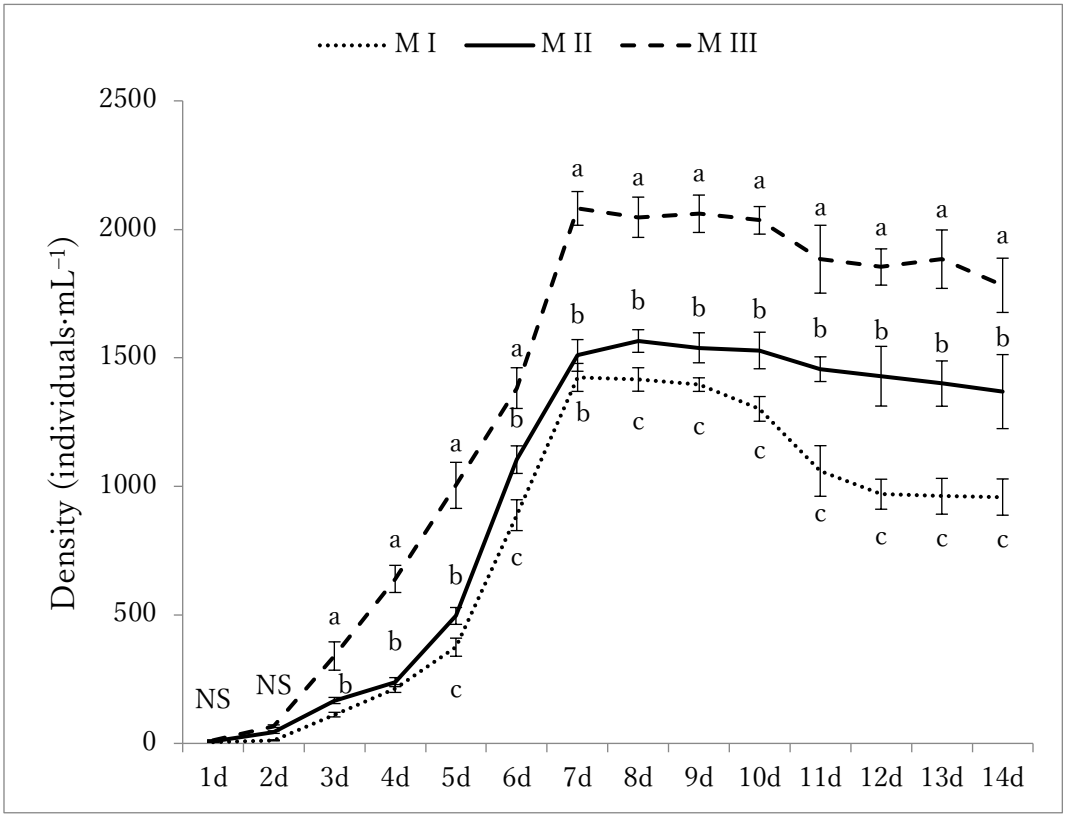

Fig. 2-3. Growth of the ciliates in different culturing mixtures. Marine seawater or sediment samples were added into the mixtures MI, MII and MIII (see "2.2 Materials and Methods") and cultivated. Error bars, standard deviation; lowercase letters, significant difference between the mixtures (ANOVA with post-hoc Duncan's test, $P \leq 0.05$); NS, not significant differences.

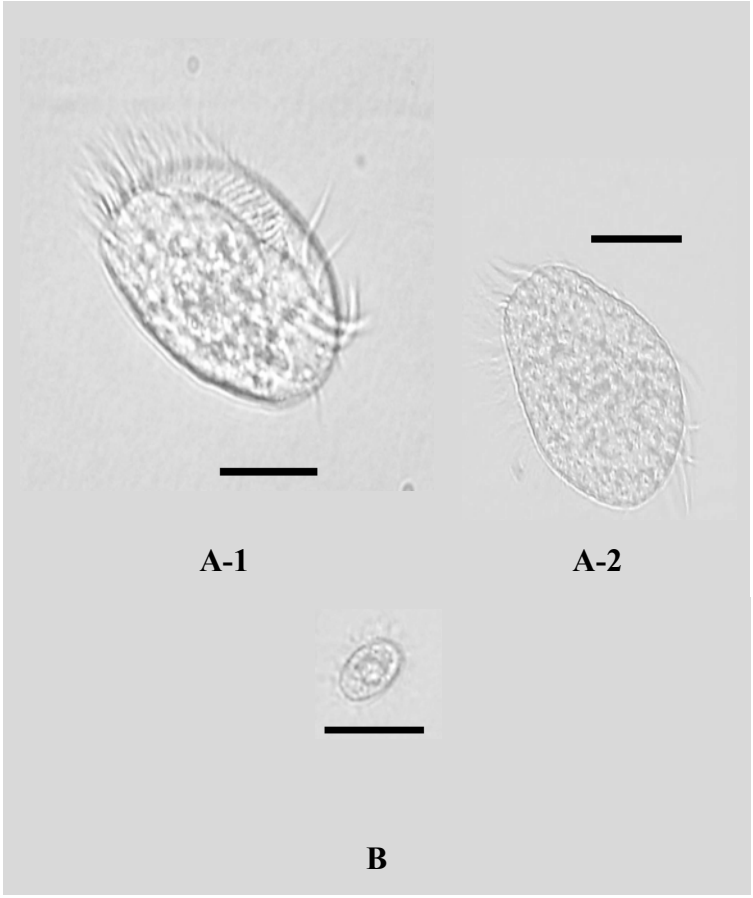

Fig. 2-4. Cell morphology of the size-fractionated ciliates in the enrichment mixtures. Micrographs of the large-size (A-1 and A-2) and small-size (B) ciliates are shown. Scale bars, 10 μm. Tentative identifications according to nucleotide sequences of the DGGE bands A-1 and A-2 (Fig. 2-5) were *Euplotes minuta* and *Cyclidium varibonneti*, respectively.

Fig. 2-5. Band profiles of the 18S rDNA PCR-DGGE. A, large-size ciliates; B, small-size ciliates. Numbers with dots represent the bands subjected to nucleotide sequencing and homology search analyses.

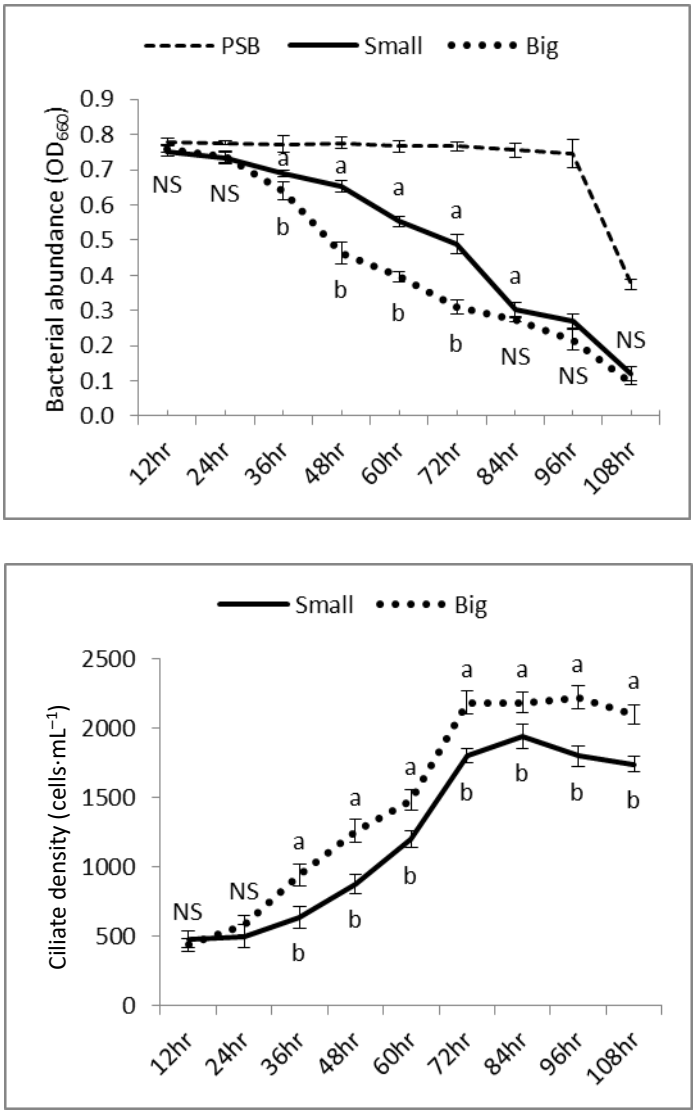

Fig. 2-6. Abundance of the photosynthetic bacterial isolate and ciliates in the grazing experiment. Upper panel, bacterial abundances; lower panel, ciliate abundances. Error bars, standard deviation; lowercase letters, significant difference between the mixtures (ANOVA with post-hoc Duncan's test, $P \leq 0.05$); NS, not significant differences.

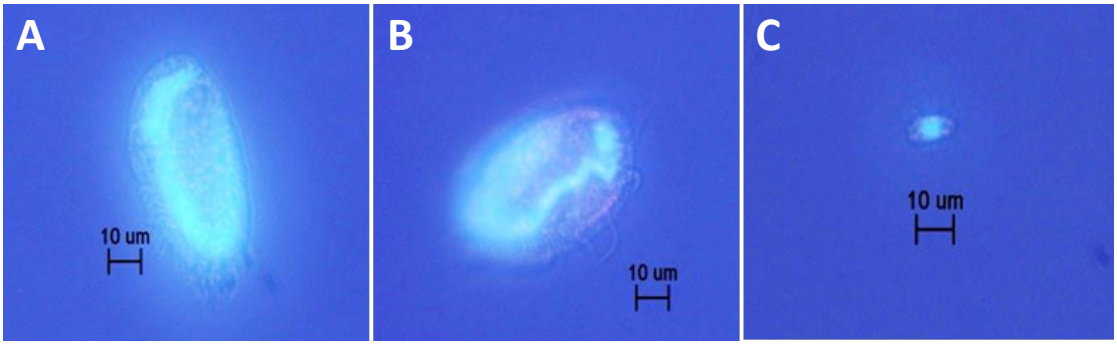

Fig. 2-7. Epifluorescent micrographs of ciliates fed on the fluorescently labeled photosynthetic bacterial isolate. The ciliates and isolate were co-incubated for 24 h and observed under blue excitation after cell fixation with formaldehyde. A, *Euplotes minuta*; B, *Cyclidium varibonneti*; C, the small-size ciliate.

GENERAL CONCLUSION

Considering the multiple benefits of anoxygenic photosynthetic bacteria (AnPBs) in various ecosystems, this study focused on gaining insight into diversity of AnPB community structures in highly eutrophicated marine coastal sediments, particularly in Yamagawa Bay (Kagoshima, Japan) using three approaches: i) isolation of AnPBs; ii) PCR-denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal RNA genes (16S rDNA); and iii) PCR-DGGE of *pufM*. The results suggested prevalence of AnPBs under the deteriorating sediment condition. Among the bacterial isolates, relatives of *Rhodobacter sphaeroides* were most dominant, since the medium used is selective for purple non-sulfur bacteria. Abundance of *Chlorobium phaeobacteroides* BS1 was also suggested by 16S rDNA PCR-DGGE. Diverse purple non-sulfur or sulfur bacteria as well as aerobic anoxygenic photoheterotrophs were detected by *pufM* PCR-DGGE, which could be associated with organic or inorganic sulfur cycling. The results confirmed the dominance of AnPBs in the eutrophicated marine sediments and their important role of metabolizing organic matters and sulfides under the anoxic condition.

Furthermore, ciliates were enriched from marine environments, and practicability of using AnPBs to promote growth of the ciliated protozoa was investigated. The AnPB isolate tentatively identified as *Rhodob. sphaeroides* was tested as feed for large-size (*Euplotes minuta* and *Cyclidium varibonneti*) ciliates as well as for a possible ciliate with small size. All the protists grazed extensively on the bacterium, resulting in their cell density reached maxima of 1,800–2,220 individuals·mL^{-1} in 96 h co-cultivation. These findings suggested that AnPBs could be used to promote ciliate growth.

Ciliates are prospective primary food sources for fish larvae in natural aquatic environments (Nakagawa et al., 2007) and in aquaculture systems (Nagano et al., 2000a; Nagano et al., 2000b). Ingestion of ciliates by mollusks is also reported (Le Gall et al., 1997).

However, proliferation of zooplankton relies on morphology, mobility and chemical composition of prey (Gulati and Demott, 1997). In addition, nutritional values of their predators are influenced by their prey organisms: microalgae and algivorous ciliates showed higher reproduction than bacterivorous ciliates did, although it was the case of feeding of a copepod (Ederington et al., 1995). Further studies on ciliate feeding with AnPBs will clarify their effect on nourishment of the protists and provide alternative of currently used larval feed such as microalgae, rotifer and copepods.

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