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Detection of UVBR-sensitive and -tolerant bacteria in surface waters of the western North Pacific

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Abstract: In order to evaluate the effects of solar ultraviolet radiation (UVR) on eubacterial community composition, we examined the tolerance of eubacterial phylotypes to solar UV radiation in surface waters of the western North Pacific during September 2005. Bromodeoxyuridine (BrdU), a halogenated thymine analogue, was used for labeling newly synthesized DNA in proliferating cells. Thymine dimers (TD), which are specifically formed in DNA by biologically harmful ultraviolet B radiation (UVBR; 280–315 nm), were also applied to detect UVB damaged genomes selectively. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) on the labeled samples revealed that UVBR-resistant cells showing active synthesis of DNA without accumulating TD, varied among phylotypes. In addition, UVBR-sensitive band positions with TD indicated inter-specific variations in sensitivity to UVBR. Phylogenetic analysis revealed that 12 DNA sequences were classified into 8 phylogenetic groups: 3 *Roseobacter*, 1 *Sphingomonas*, 2 *Gammaproteobacteria*, 1 *Actinobacteria*, 1 *Synechococcus*, 2 *Prochlorococcus*, 1 plastid and 1 another group. A UVBR-resistant phylotype was affiliated to *Erythrobacter* sp. (previously designated as *Sphingomonas* sp.), which was distributed in warmer waters from the south of Oyashio to Kuroshio regions. A UVBR-sensitive phylotype was affiliated to *Pseudoalteromonas* sp. in *Gammaproteobacteria*. Dominant heterotrophic eubacteria were composed of both sensitive and resistant phylotypes. This is the first report on TD accumulated eubacterial phylotypes in oceanic surface waters.

KEY WORDS: UVBR · Thymine dimer · Phylotype · DGGE · Immunocapture · Marine bacteria

1. Introduction

Recent studies have shown that solar UVB radiation (UVBR: 280 to 315 nm) could negatively influence many aquatic organisms. Exposure to solar UV radiation (UVR) can reduce biological productivity, affect reproduction, and increase mutation rate in microorganisms, macroalgae, eggs, larval stage of fish, and other aquatic animals [1]. Aquatic bacteria, which are a major component of aquatic ecosystems and play a key role in biogeochemical processes such as nutrient cycling [2], are also sensitive to solar UVR, especially biologically harmful UVBR because of their lack of pigments, small cell size and short generation time [3,4].

Although DNA shows peak absorbance at around 260 nm, the absorption spectrum of DNA covers the wavelength of UVBR. As a result, UVBR induces DNA damage and causes dimerizations between adjacent pyrimidine bases (i.e. so-called thymine dimers; [5,6]). The photoproducts interfere with DNA replication and transcription by RNA, since DNA polymerase and reverse transcriptase are stalled or physically blocked by the structural changes in the DNA molecules. In field studies, DNA damage in marine bacteria was greater in those found in surface waters than those in deeper waters [7-9]. Furthermore, higher amounts of DNA photoproducts were found during daytime than at night, indicating that the accumulation of DNA damage occurred under daylight, and its removal took place under dark conditions. UVBR inhibited about 45% of tritiated thymidine (TdR) incorporation, which is widely used as a marker for DNA synthesis by labeling the DNA inside proliferating cells [10,11] in bacterial communities [3,12].

Although the effects of UVR, especially UVBR, on bulk bacterial communities have been studied in the past two decades, little information is available about the impact of UVR on bacterial community composition. Some previous studies used bacterial isolates originating from seawater, and showed interspecific variability not only in the accumulation of thymine dimers [13], but also in the subsequent repair of DNA

damages [14,15]. However, such isolates may be only minor components of natural bacterial populations [16]; because most bacteria are not cultivable in the laboratory, it is still uncertain whether the results of these studies represent the natural bacterial community.

Recent developments in molecular techniques have contributed to revealing the existence of uncultivable bacterial phylotypes. Culture-independent finger printing techniques such as PCR-denaturing gradient gel electrophoresis (DGGE) are useful tools for detecting major phylotypes in the natural bacterial community [17,18]. Such molecular techniques have been applied to the studies of UVR effects in aquatic organisms. For instance, using PCR-DGGE analysis based on 16S rDNA, Winter et al. [19] found a few UVR sensitive phylotypes in UVR-transparent mesocosms. They also found differential phylotypes in active bacterial communities using PCR-DGGE analysis based on double-stranded cDNA synthesized from mRNA of 16S rRNA gene. Recently, using microautoradiography combined with a catalyzed reporter deposition-fluorescence in situ hybridization (MAR-CARD-FISH) technique, Alonso-Sáez et al. [20] found that UVR effects on bacterial activity differed among the group levels in the coastal region of the NW Mediterranean sea. An immunological technique for detecting DNA damage of marine microorganisms has been developed by Buma et al. [21] and has been widely used to quantify thymine dimers [22]. Immunocapturing techniques have also been developed for the detection and enrichment of selected bacteria [23]. By using an immunocapturing technique in which magnetic beads conjugated with a secondary antibody, it is possible to separate DNA photoproducts from normal DNA fractions [6]. However, this technique has never been applied to natural bacterial communities. Similarly, an immunocapturing technique using bromodeoxyuridine (BrdU) and anti-BrdU antibody conjugated with magnetic beads allows the separation of DNA fragments of actively growing bacteria from the total DNA [24,25]. This technique has recently been used for quantification

of DNA-synthesizing bacteria in seawater [26-31]. PCR-DGGE combined with the immunocapturing method succeeded in distinguishing actively growing bacteria from the bacterial community in the ocean [32-34].

In this study, we applied PCR-DGGE combined with immunocapturing techniques to detect actively growing and UV-damaged eubacteria in the northwest Pacific using anti-BrdU and anti-thymine-dimers, respectively. At the same time, the total eubacterial community composition was also assessed by PCR-DGGE targeted to 16S rRNA gene. This is the first report on UVBR-sensitive or -resistant eubacterial phylotypes in the oceanic environment.

2. Materials and Methods

2.1. Sampling and hydrography

Water samples were collected along the "*A-line*" during September 14–18 in 2005 on board the FR/V *Wakataka Maru* (Fisheries Research Agency of Japan). Seawater samples were collected from 0 m depth with an acid-cleaned plastic bucket. The water samples were divided into acid-cleaned polycarbonate bottles. To detect total (non-immunocapturing) eubacterial populations and thymine dimers, subsamples (1 L) were filtered through 0.2 μm pore size polycarbonate Nuclepore filters (Whatman) with gentle vacuum pressure (<150 mmHg) under dim light within 60 minutes of sampling under dim light. The filters were immediately frozen in liquid nitrogen and stored at –80˚C until analysis. The other subsamples were used for the enumeration of heterotrophic bacteria and BrdU-labeling on-deck incubation experiments (see below). Hydrographic data (seawater temperature, salinity, and nutrient concentrations) were obtained from the cruise database. Stations were grouped into three water masses: the Oyashio: <5˚C and <33.6 in salinity at 100 m depth [35], the Kuroshio: >14˚C at 200 m depth [36], and the other stations were treated as the Kuroshio-Oyashio transition

region.

2.2. Measurement of solar radiation

During the sampling period, solar UVB (280–315 nm) radiation was measured with a broadband UVB radiometer (MS-212W, Eko instruments, Co. Ltd.) and UVA (315–400 nm) with a broadband UVA radiometer (MS-212A, Eko instruments, Co. Ltd.). Photosynthetically active radiation (PAR; 400–700 nm) was also measured with a photon sensor (ML-020, Eko instruments, Co. Ltd.). All sensors were attached to the compass deck, and were open to the air.

2.3. Cell density of heterotrophic bacteria

Subsamples (2 ml) were fixed with glutaraldehyde (2% (w/v) final concentration), stored at 4ºC, and analyzed on land after 30 days from the each sampling. All of the analyses were conducted under dim light conditions. The fixed samples (1–1.5 ml) were mixed with SYBR Gold (Molecular Probes Inc.) at a 1/10,000 dilution rate for 15 min to stain the DNA in the bacterial cells. Stained subsamples were filtered through black polycarbonate Nuclepore filters (25 mm in diameter, 0.2 μm in pore size, Whatman) with gentle vacuum pressure (<150 mmHg). Each filter was washed twice with 2 ml of 0.2-μm-filtered seawater and then dried in a desiccator thereafter. The filter was placed onto a glass slide, one drop of non-fluorescing immersion oil (Olympus Co.) was dropped onto the glass, and then the filter was covered with a coverslip. Bacterial cells were counted at a magnification of × 1,000 on an Olympus epifluorescence microscope (model BX 60) equipped with a 100 W mercury lamp. The excitation, dichroic, and emission wavelengths were 360–370 nm (BP360–370), 400 nm (DM400), and 420–460 nm (BA420–460), respectively. Bacterial cells with green fluorescence of SYBR Gold intercalated into DNA were counted, where there were at least 200 cells in a scope field. Twenty scope fields, selected randomly, were

examined in this study.

2.4. BrdU labeling

Bromodeoxyuridine (BrdU) was added to triplicates of subsamples (50 ml) at a final concentration of 20 μM as soon as possible after sampling, and samples were incubated in the dark for 60 min at in situ temperature. After incubation, the seawater samples were filtered through polycarbonate Nuclepore membrane filters (0.2 μm pore size, Whatman). The filters were immediately frozen in liquid nitrogen and stored in a deep freezer (–80ºC) until analysis on land.

2.5. DNA extraction

DNA extraction was performed after Katano et al. [37] as follows. The filters were ground in a lysis solution (30 μl 10% v/v sodium dodecyl sulfate, 500 μl Tris-EDTA (TE) buffer, and 3 μl 10 mg ml^{-1} proteinase K) with autoclaved plastic pestles and incubated at 37ºC for 1 h. Cell lysates were frozen and thawed three times. To remove polysaccharides, a mixture of cetyltrimethlammonium bromide (CTAB, Wako) and 0.7 M NaCl solution was added, and the solution was incubated at 65ºC for 10 min. Polysaccharides and proteins were removed with chloroform-isoamyl alcohol (24:1) and then eliminated with phenol-chloroform-isoamyl alcohol (25:24:1). DNA precipitated with ice-cold isopropanol was rinsed with ice-cold 70% ethanol and resuspended in 100 μl TE buffer. Then the DNA in solution was precipitated with ice-cold 100% ethanol, rinsed with ice-cold 70% ethanol, and resuspended in 47 μl TE buffer. The molecular weight of DNA in each extract was determined by electrophoresis of 5 μl of extract on a 1.5% agarose gel with a 100 bp DNA ladder (New England Biolabs, Inc.). The concentration of DNA in the extracts was quantified by a spectrophotometer (GeneQuant™, GE Healthcare) and adjusted to 1 ng μl^{-1} in TE

buffer.

2.6. Immunocapturing using anti-BrdU antibody

Immunoglobulin-coated paramagnetic beads were used to selectively isolate BrdU-containing DNA from labeled environmental samples. BrdU magnetic bead immunocapture was performed after Urbach et al. [25]. Herring sperm DNA (1.25 mg ml^{-1} in PBS), which had been denatured at 95ºC for 5 min and quickly frozen at –20ºC was mixed at 49:1 ratio with monoclonal anti-BrdU antibody (diluted 1:49 in PBS), and incubated for 30 min. DNA samples denatured at 95ºC for 5 min, and frozen on ice were mixed with herring sperm DNA and anti-BrdU antibody mixture (hereafter referred to as complex-1) at 37ºC for 45 min to immunologically conjugate the BrdU incorporated in DNA of bacterial cells with anti-BrdU antibody. A sheep anti-Mouse IgG secondary antibody coated with paramagnetic beads (Dynal Biotech) was pre-washed five times in PBS containing 1 mg of bovine serum albumin (Sigma) per ml (PBS-BSA), gathered with a magnetic particle concentrator (Dynal Biotech), and resuspended in PBS-BSA with the an initial concentration of $6–7 \times 10^8$ beads ml^{-1}. A 3.13 µl aliquot of antibody with beads was added to each sample and subsequently incubated at 37ºC for 30 min with constant agitation to form complex-1-beads complex (complex-2), then complex-2 was washed seven times with 100 µl of PBS-BSA each time. A fraction of sample DNA containing BrdU was eluted into supernatants by adding 1.7 mM BrdU (in PBS-BSA) and incubated at 37ºC for 30 min with constant agitation. The supernatant was collected in a new tube, and 2 µl of glycogen (20 mg ml^{-1}, Wako) was added to the sample for DNA precipitation. Each immunocaptured DNA sample was finally resuspended in 20 µl of TE buffer.

2.7. Immunocapturing using anti-thymine dimer antibody

TD-containing DNA was also selectively isolated from environmental

samples using immunoglobulin-coated paramagnetic beads through the partially modified protocol of BrdU-immunocapturing. Briefly, denatured herring sperm DNA (1.25 mg ml^{-1} in PBS) was mixed at 49:1 ratio with monoclonal anti-TD antibody (BM12, Kyowa), and incubated for 30 min. Denatured DNA samples were mixed with herring sperm DNA and anti-TD antibody mixture (complex-1) at 37ºC for 45 min to combine the anti-TD and TD included in sample DNA. An aliquot of washed sheep anti-Mouse IgG-coated paramagnetic beads (Dynal Biotech) was added to each sample and subsequently incubated. TD containing the sample DNA fraction was eluted into supernatants by heat shock at 95ºC for 5 min and quickly frozen on ice, then collected in a new tube for isopropanol precipitation. Immunocaptured sample DNA was finally resuspended into 20 µl of TE buffer.

2.8. PCR for DGGE

We PCR-amplified 550 bp of the eubacterial 16S rRNA gene using a universal primer complementary to positions 907–926 (*Escherichia coli* numbering; (5'-CCGTCAATTCMTTTGAGTTT-3') and complementary to positions 341–357 plus a GC clamp (5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-CCTA CGGGAGGCAGCAG-3'; [17,38]). PCR mixtures were prepared in 20 µl total volume containing 1 × Ex Taq™ Buffer (TaKaRa), 2.2 mM $MgCl_2$, 0.5 mM dNTP, 0.25 µM of each primer, 2.5 units of Taq DNA polymerase (Ex Taq™; TaKaRa) and approximately 2 pg of template DNA. PCR conditions followed those of Muyzer et al. [17] with the exception of initial denaturation at 95°C for 5 min. PCR amplification consisting of 35 cycles was performed using a thermal cycler (Eppendorf Mastercycler Personal). PCR products (2 µl) were loaded on a 1.5% agarose gel and electrophoresed with a 100 bp DNA ladder marker (New England Biolabs, Inc.). The agarose gel was stained with ethidium bromide (Et-Br) and photographed as a digital file. Then 5–8 targeted amplicons were mixed in order to avoid PCR bias, and the mixture was concentrated by

ethanol precipitation. The amount of DNA was estimated from its relative intensity compared with the DNA ladder marker on the agarose gel electrophoresis. Band density was determined using Gel-pro Analyzer® version 4.5 (Media Cybernetics).

PCR products (ca. 100 ng of DNA) were loaded onto a 16 × 16 cm, 1 mm thick, 6% polyacrylamide (acrylamide:bis = 37.5:1) gel with a denaturing gradient of 25–70% from top to bottom (7 M urea and 40% formamide were considered as 100% denaturant). Electrophoresis was performed with a Dcode system (Bio-Rad Laboratories) using 1 × TAE running buffer (20 mM Tris, 10 mM acetic acid, 0.5 mM EDTA, pH 8.0) at 60°C for 16 h at 85 V. The gel was stained for 30 min in 1:10,000 diluted SYBR Gold (Molecular Probes, Inc.) and destained for 20 min in distilled water. The DGGE gel was visualized using an Invitrogen Safe Imager and photographed using a Canon digital camera with a high resolution mode.

2.9. Analysis of DGGE patterns

DGGE images were analyzed with a one-dimensional gel analysis tool in the Gel-Pro Analyzer® software (ver. 4.5, Media Cybernetics, Inc). To detect DGGE bands, a density profile was obtained for estimating the ratio of each band's intensity to the total intensity. Bands with relative intensity of ≥0.4% were considered significant in this study. A binary matrix was constructed, taking into account the presence (1) or absence (0) of bands in each lane [39]. The dissimilarity in band-pattern between pairs of samples on the same gel was calculated as a Dice coefficient (S_{Dice}):

$$S_{\mathrm{Dice}} = 2N_{\mathrm{AB}}/(N_{\mathrm{A}} + N_{\mathrm{B}})$$

where N_{AB} is the number of bands found in both patterns, and N_{A} and N_{B} represent the total number of bands in samples A and B, respectively. A distance matrix was analyzed with unweighted pairwise grouping with mathematical averages (UPGMA) to cluster

samples using an average pairwise algorithm. Statistical calculations were performed with the software R (ver. 2.7.2).

2.10. Sequencing analysis

To obtain sequence information from DGGE bands, all visualized bands were excised using a clean razor blade. The cut bands were then washed twice using 2 ml of sterilized Milli-Q water and frozen until re-amplification with PCR. About 1 mm^3 of each band was directly re-amplified. PCR was conducted according to Muyzer et al. [17], with modifications: except initial template denaturing at 95°C for 5 min, followed by 20 cycles of denaturation (1 min at 95°C), annealing [1 min at 65–55°C (touchdown –1°C $cycle^{-1}$)], and extension (3 min at 72°C), followed by 7 cycles of denaturing (1 min at 94°C), annealing (1 min at 55°C), and extension (3 min 72°C), with a final extension (7 min at 72°C). After re-amplified bands were examined with DGGE to confirm their band position and non-contamination, PCR products were purified using the polyethylene glycol (PEG) precipitation method. A 15 µl subsample of the amplified 16S rDNA solution was diluted with 35 µl of distilled water and mixed with 30 µl of PEG solution [20% PEG 6000 (Wako) and 2.5 M NaCl], incubated on ice for 1 h, and centrifuged at 15,000 rpm for 15 min. Precipitates were rinsed with ice-cold 70% ethanol and resuspended in 20 µl of distilled water. The purified amplified products were sequenced on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) with both the 907–926 and 341–357 primers without the GC-clamp [17] using a BigDye v1.0 Terminator cycle sequencing kit (Applied Biosystems). Duplicate samples were analyzed to reduce reading errors by the genetic analyzer. Then 16S rDNA sequences were analyzed for each sample.

2.11. Phylogenetic analysis

Sequences obtained in this study were examined for chimeras, which could be

generated artificially by co-amplification of homologous genes during PCR reactions, using the Ribosomal Database Project's CHECK-CHIMERA program [40]. Chimeras were also checked with the basic local alignment search tool BLAST [41] for short sequences. Non-chimeric sequences were compared to sequences deposited in the bacterial nucleotide sequence databases (i.e., ddbjbct and ddbjenv) of the DNA Data Bank of Japan (DDBJ). Acquired 16S rRNA gene sequences were assigned to phylogenetic groups based on the most similar sequence found in the DDBJ. Bacterial sequences were aligned using CLUSTAL W, and corrected manually to delete ambiguous and non-informative bases with Mega 3 [42]. A distance tree was constructed using the neighbor-joining (NJ) method based on [43] distance. Bootstrap resampling was performed 10,000 times. The nucleotide sequences of the 16S rDNA gene obtained in this study have been deposited in the DDBJ nucleotide sequence database under accession numbers AB449917–AB449930.

3. Results

3.1. Hydrography

Sampling stations were classified into three regions: Oyashio, Kuroshio-Oyashio transition, and Kuroshio regions (Table 1). Sea surface temperature (SST) increased from north to south along the transect, the lowest was 18.4ºC at Station A04 and the highest was 25.5ºC at Station A21. A similar latitudinal gradient pattern was found in chlorophyll *a* (Chl *a*) concentrations (Table 1). Nutrient concentrations were generally low, especially in the southern stations (A13, A17, and A21). The cell density of heterotrophic bacteria ranged from 2.72×10^5 cells ml^{-1} to 6.74×10^5 cells ml^{-1}, and the lowest value was observed at Station A21 in the Kuroshio region.

3.2. UVBR and PAR

Ambient solar UVR and PAR varied among the stations (Fig. 1, Table 2) mainly due to changes in weather conditions. During observations at Stations A09, A13, and A21, weather conditions were generally favorable and the daily UVBR ranged from 8.75 kJ m^{-2} to 12.89 kJ m^{-2}, while those at Stations A04 and A17 were lower (1.99 and 5.28 kJ m^{-2}, respectively) because of cloud covers. At four of five stations, Stations A09, A13, A17, and A21, UVBR during the sampling period accounted for >70% of each daily value. The ratio of UVBR to UVAR (0.99%) at the northernmost Station A04 was lower than that (1.39 to 1.51%) of the other stations. Similarly, daily PAR ranged from 33 mol photons m^{-2} to 43 mol photons m^{-2} at Stations A09, A13, and A21, while those at Stations A04 and A17 were lower (3 and 11 mol m^{-2}, respectively).

3.3. PCR-DGGE

BrdU-immunocapturing combined with DGGE analysis produced banding patterns of BrdU incorporating the community in the study area (Fig. 2). Similarly, TD-immunocapturing combined with DGGE also showed banding patterns of a TD-accumulating community (Fig. 2). These two bacterial community profiles were compared with total bacterial populations (Fig. 2). The clustering analysis of DGGE profiles distinguished total bacterial community composition at northern stations in the Oyashio region (i.e. cluster A; Station A04) from those at southern Oyashio and Kuroshio-Oyashio transition regions (i.e. cluster B; Stations A09, A13, and A17) (Fig. 3). The Kuroshio bacterial community at Station A21 was distinctively clustered (Fig. 3).

To identify UVB-resistant phylotypes, DGGE bands between total and thymine dimmers (TD)-immunocaptured 16S rDNA fractions were compared with each other. DGGE band positions appeared in BrdU lanes, but not in TD lanes, indicating the phylotypes that were actively growing without accumulating TD (hereafter the

phylotypes are referred to AT), that is, those that were resistant to UVBR. Similarly, DGGE band positions appeared in TD lanes, but not in BrdU lanes indicating the phylotypes, which were inactive with accumulating TD (hereafter the phylotypes are referred to IT), that is, those that were sensitive to UVBR. The number of DGGE bands indicating UVBR-resistant phylotypes ranged from 0–2 at each station and the maximum was observed at Stations A04 and A13 (Table 3). The number of UVBR-sensitive phylotypes ranged from 2–6, and the maximum was observed at Station A13.

To estimate the ratios of actively growing or DNA-damaged bacteria to the total number of bacteria, common DGGE bands between total and immunocaptured 16S rDNA fragments were counted and then divided by the band numbers total band numbers detected from the 3 lanes. The former ranged from 11–29% (21% on average) and the latter was from 21–43% (34% on average) (Table 3). The common bands between BrdU and TD lanes were counted as having intermediate tolerance to UVBR (hereafter the phylotypes are referred to as IM). The ratio of IM bands to all band positions including total and immunocaptured bands ranged from 11 to 31% (21% on average).

3.4. Phylogenetic analysis of 16S rRNA gene fragments

We obtained 14 DNA sequences from 45 band positions (Table 4). Six band positions indicated IT bands; band positions 01 at Station A21, 03 at Station A13, 07 at Station A13 and A17, 08 at Stations A04 and A09, 11 at Station A17, and 12 at Stations A21, respectively (Fig. 2). Three band positions represented AT bands; band position 02 at Stations A09 and A13, 09 at Stations A04 and A17, and 10 at Stations A04, respectively (Fig. 2). Four bands (04, 06, 13, and 15 in Fig. 2) were characterized as IM bands, and band position 13 was common from Station A04 to A21. Two

phylotypes (NO_1, band position 05; and NO_2, band positions 14) appeared only in a single lane at the stations.

Of the 15 sequences obtained from the study area, 11 sequences had more than 97% similarity with the closest relatives deposited in the DDBJ and 2 sequences (sequence names AT_3 and IT_6) were less than 97% in homogeneity. The lengths of the oligonucleotide sequences for IT_4 and NO_2 were so short that further phylogenetic analysis was, unfortunately, not possible.

In the neighbor-joining (NJ) tree (Fig. 4) using 521 bp sequences using the archaeon *Sulfolobus solfataricus* as an out-group, 40 sequences were clustered into 8 groups: *Roseobacter*, *Sphingomonas*, *Gammaproteobacteria*, *Actinobacteria*, *Synechococcus*, *Prochlorococcus*, plastids and others. Affiliations of the two unknown phylotypes (AT_3 and IT_6) were revealed by the NJ tree: the sequences of AT_3 and IT_6 were classified into as plastid, and *Gammaproteobacteria*, respectively.

4. Discussion

We identified eubacterial phylotypes with TD in the open ocean. In previous studies carried out in coastal waters, Alonso-Sáez et al. [20] showed that the *Gammaproteobacteria* and *Cytophaga-Flavobacteria-Bacteroides* (CFB) groups possessed higher resistance to UVBR in the northwestern Mediterranean, compared to *Alphaproteobacteria*. In the North Sea, Winter et al. [19], using the PCR-DGGE method based on mRNA fragments of 16S rRNA gene, showed that UVBR differentially affected bacterial activity even among phylotypes in CFB group: some phylotypes were sensitive to UVB, while the others were resistant. Our immunocapture-DGGE analysis provided more detailed information about the tolerance of eubacteria to UVBR in phylotype level. In Oyashio and the transition regions, the

composition of BrdU-incorporating bacterial communities was different from total bacterial communities (Fig. 3). The percentage of overlapping bands for the BrdU-labeled and total bacterial community was 21% on an average (Table 3). Namely, 80% of growing bacterial phylotypes were not detected in the total bacterial community. Considering that PCR-DGGE bands indicate the predominant community members [18] and that the BrdU-immunocapture technique can detect actively growing bacteria specifically [25], the difference between the total and the BrdU-immunocaptured bacterial community indicated that more than half of the growing bacterial phylotypes were minor populations. The results obtained in this study are similar to those of Hamasaki et al. [33] who used the same technique. The low abundance of actively growing bacteria could be caused by the differences in phylotype-specific mortality by viral infection, selective ingestion of actively growing bacteria by heterotrophic nanoflagellates [44,45], and/or UVBR [13,15,46].

For UVBR tolerance, 21–43% (34% on average) of the bands showed overlapping between the TD and total lanes. This higher percentage supports the previous results that bacteria tended to accumulate DNA damage [8,13,47]. Joux et al. [13] showed that five species out of six isolated bacteria easily accumulated thymine dimmers under approximately 1 kJ m^{-2} of UVBR. During sampling, UVB dose were 1.04–10.95 kJ m^{-2}, which reached to 52–87% of the daily UVBR (Table 2), indicating that the UVBR level was sufficient for the surface bacteria to accumulate TD. It is well known that the amount of TD changes diurnally, that is, TD accumulates from noon to early evening with decreasing TdR incorporation [7,8]. In the Kuroshio-Oyashio transition area of the western North Pacific, more than 35% of phylotypes of the total bacterial community experienced TD accumulation (Table 3). Repair of TD would become a key strategy for their survival. In addition, the difference in the UVBR tolerance of bacteria (Table 3) could be related to the intensity of UVBR in the environments they inhabited [46, 48].

It should be noted that, in the Oyashio region, the ratio of overlapping bands of the TD-accumulating phylotypes to the total was the smallest at Station A04 (31%), where UVBR was the lowest and the ratio of UVAR to UVBR was the highest among the stations (Table 2). Since UVAR is important for the photorepair of TD [7,49], low TD accumulation could be due to the high UVAR/UVBR ratio. Another possibility is that low UVBR could lead to low TD accumulation.

Cluster analysis revealed that the total bacterial community composition in the Oyashio region (cluster A) differed from the others (Fig. 3). This was caused by the large number of IM bands that were characterized in the Oyashio region (Table 3). Relatively high concentrations of nutrients and chlorophyll *a* at Stations A04 and A09 indicate higher photosynthetic activity of phytoplankton and the accumulation of labile dissolved organic matter (DOM) derived from phytoplankton. It is well known that photodegradation of DOM by solar UVR forms low molecular weight DOM [50-54], which is subsequently utilized by heterotrophic bacteria [55,56]. In addition, phytoplankton could serve as a sunscreen for bacteria due to absorption and scattering of UVR by the phytoplankton. Thus, the productive Oyashio water might moderate UV stress for bacteria. The two Oyashio-specific phylotypes, *Gammaproteobacteria* (AT_4) and unknown bacteria (IM_5), might adapt to such conditions.

The phylotypes of the *Roseobacter* clade were categorized as IT, IM, and NO types (IT_5, IM_2 and, NO_1) and were commonly found among the sampling stations, although the environmental conditions (i.e. concentrations of chlorophyll *a* and nutrients, and water temperature) were variable (Table 1). Several past studies revealed that *Roseobacter* clade appeared inconsistently in oceanic waters. For example, González et al. [57] found high abundance of *Roseobacter* clade during phytoplankton blooms in the North Atlantic. On the other hand, Suzuki et al. [58] could not find any correlation between the distribution of *Roseobacter* clade and environmental factors (i.e. temperature, nutrients, and chlorophyll *a*) in the North

Pacific off California. Van Mooy et al. [59] showed a significant correlation between the *Roseobacter* clade numbers and light intensity, while no correlation was found between the bacterial abundance and DOM. The appearance of IM phylotypes at all of our sampling stations also showed the ubiquity of the *Roseobacter* clade.

A photosynthetic bacteria *Erythrobacter* sp. (previously called as *Sphingomonas* sp.) was observed in DGGE bands (AT_1), and was classified as a UVB resistant phylotype. Their preferential distribution from the southern Oyashio to the Kuroshio regions coincided with the warm water tolerance of the genus *Erythrobacter citrus*, with a growing range of 4–35ºC and optimum of 25–30ºC [60]. In addition, Joux et al. [13] showed that the *Sphingomonas* sp. strain RB2256 formed colonies without accumulating TD even under high UVB (2.0 kJ m^{-2}), indicating a high level of UVB resistance, and that the *Erythrobacter* sp. might repair DNA damage efficiently under solar radiation. Therefore, photosynthetic bacteria with high UVBR tolerance could proliferate in surface waters. As described above, two phylotypes related to *Alphaproteobacteria* were found. However, TD accumulation was different between the phylotypes, indicating that UV tolerance differs among phylotypes in *Alphaproteobacteria*, even though their phylogenetic positions based on 16S rDNA gene were close to each other.

We also found *Gammaproteobacteria* (IT_1; *Pseudoalteromonas* sp.) to be a UVBR-sensitive phylotype that occurred at Station A13 and A17, although it was also present as an intermediate-UVBR-tolerant phylotype at Station A09. At Stations A09 and A13, the environmental conditions such as UVBR, SST, Chl *a*, and nutrient concentrations were very similar (Tables 1 and 2). Although no clear relationship was found between environmental factors and BrdU incorporation or TD accumulation, UVBR sensitivity in bacteria might vary not only with phylotypes but also with environmental and/or physiological conditions. Alonso-Sáez et al. [20] showed variable UVBR sensitivity of *Gammaproteobacteria* between summer and spring in

NW Mediterranean coastal waters. Furthermore, UVBR sensitivity of *Pseudoalteromonas* sp. differed between sampling days, suggesting changes in their physiological conditions on a day-time scale. Recently, inter-specific variation in recovery from UV damage was also investigated using 90 marine bacterial isolates [14], and showed that most of the *Gammaproteobacteria* had high recovery rates. Simultaneous detections of BrdU incorporation and TD accumulation with physiological parameters such as robustness of ectoenzymes [47] could give more information about bacterial UV tolerance.

In the present study, we showed that about 35% of phylotypes of the total bacterial community accumulated DNA damage while 20% were actively growing. The results indicated that proliferating bacteria were also stressed by UVBR. Stratospheric ozone depletion, which increases the ratio of UVBR to UVAR and UVBR to PAR, may shift essential metabolic energy to repair DNA damage for survival. As observed in the present study, the tolerance to UVBR was different among phylotypes. Such information is essential for the prediction of changes in ecosystems and biogeochemical cycles in the ocean.

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Figure legends

Fig. 1. Daily variation of solar UV radiation (W m^{-2}) on each sampling day. Arrows represent sampling time.

Fig. 2. DGGE image of 16S rRNA genes amplified from total (To), BrdU-incorporating (Br), and thymine-dimer-accumulating (TD) community obtained at each sampling station. Triangles represent excised and sequenced bands. Lane M was used as a marker lane.

Fig. 3. Relationship between total, BrdU-incorporating, and thymine-dimer-accumulating communities from each sampling station. The dissimilarity tree was constructed from the binary matrix translated from a DGGE image (Fig. 2).

Fig. 4. A neighbor-joining tree of partial 16S rDNA sequences obtained from DGGE bands (see Fig. 2). The scale bar represents 0.05 substitutions per nucleotide position.

Table 1
Location, heterotrophic bacterial cell density ($\times 10^5$ cells ml^{-1}), sea surfacewater temperature (SST; ºC), chlorophyll *a* concentration (Chl-a; µg l^{-1}), phosphate (PO_4; µM) and nitrate plus nitrite ($NO_2 + NO_3$; µM) at the 5 m depth in which samples were collected.

Station	Latitude [ºN]	Longititude [ºE]	Water mass	Bacterial cell density [$\times 10^5$ cells ml^{-1}]	SST [ºC]	Chl-a [µg l^{-1}]	PO_4 [µM]	NO_2 [µM]	NO_3 [µM]	$NO_2 + NO_3$ [µM]
A04	42.25	145.13	Oyashio	5.04	18.4	0.57	0.271	0.081	0.697	0.778
A09	41.00	145.75	Oyashio	6.74	19.2	0.55	0.294	0.092	0.177	0.269
A13	40.00	146.25	Oyashio	6.66	20.9	0.38	0.135	0.119	0.000	0.119
A17	39.00	146.75	Transition	5.29	25.1	0.29	0.009	0.114	0.000	0.114
A21	38.00	147.25	Kuroshio	2.72	25.5	0.14	0.012	0.085	0.042	0.127

Table 2

Solar UVR and PAR condition at sampling stations. Daily dose, accumulated dose during sampling (UVR; kJ m^{-2}, PAR; mol m^{-2}) and maximum radiation (UVR; W m^{-2}, PAR; μmol s^{-1} m^{-2}) of UVA and UVB, and ratio of accumulated UVBR to UVAR during sampling (%).

		UVBR				UVAR				UVB ratio	PAR dose			
Station	Sampling time	Daily [kJ m^{-2}]	Sampling period [kJ m^{-2}]	%*	Maximum radiation [W m^{-2}]	Daily [kJ m^{-2}]	Sampling period [kJ m^{-2}]	%*	Maximum radiation [W m^{-2}]	UVB/UVA (%)	Daily [mol m^{-2}]	Sampling period [mol m^{-2}]	%*	Maximum radiation [μmol s^{-1} m^{-2}]
A04	11:40	1.99	1.04	52	0.18	208	105	51	12.00	0.99	6	3	49	397
A09	12:00	8.75	5.91	68	0.56	622	399	64	35.10	1.48	24	16	65	1500
A13	13:00	12.89	10.95	85	0.69	990	790	80	42.70	1.39	43	33	76	1806
A17	13:00	5.28	4.58	87	0.34	398	318	80	20.70	1.44	13	11	81	771
A21	12:50	11.74	9.32	79	0.77	833	615	74	44.10	1.51	33	24	74	1929

* Parcentage of UV dose during sampling to daily dose.

Table 3
Number of DGGE bands* on the total, BrdU-incorporating and thymine-dimer-accumulating DNA and their comparisons.

Station	Number of bands								Number of common bands between			
	Within a lane			Within 3 lanes	AT** (Active without thymine dimer)	IT *** (Inactive with thymine dimer)	IM**** (Intermediate type)	%	To and Br	%	To and Td	%
	To	Br	TD									
A04	14	16	15	26	2	4	8	31	6	23	8	31
A09	8	17	11	21	1	2	6	29	6	29	7	38
A13	13	12	10	21	2	6	3	24	5	24	9	43
A17	10	8	9	16	1	4	2	19	3	19	6	38
A21	14	7	6	19	0	2	4	11	2	11	4	21
Average	11.8	12.0	10.2	20.6	1.2	3.6	4.6	21		21		34

* To, total community without immunocaputring selection; Br, BrdU-incorporating community; TD, thymine-dimer-accumulating community.
** AT, BrdU-incorporating without thymine-diemr-accumulating community in major one.
*** IT, thymine-dimer-accumulating with BrdU-incorporating community in major one.
**** IM, BrdU-incorporating and thymine-dimer-accumulating community, irrespective of major or miner community.

Table 4
Affiliation of excited DGGE band sequences with other seauences depositted in DDBJ.

Sequence name	DGGE band position	Group*	Closest relative's form name (Accession No.)	Sequence length	Similarity (%)
Active without thymine dimer (AT)					
AT_1	09	α, Sphi	*Erythrobacter* sp. MED365 (EU253576)	508	97.7
AT_2	02	Bac	clone SPOTSAPR01_5m159 (DQ009089)	541	100.0
AT_3	10	γ	clone ALN_al06 (AB364989)	545	96.1
Inactive with thymine dimer (IT)					
IT_1	07	γ, Pseu	*Pseudoalteromonas* sp. 36D8 (EF033416)	545	99.5
IT_2	11	Cya	clone 20162U93 (EU237472)	527	100.0
IT_3	12	Cya	*Prochlorococcus marinus* TAK9803-2 (AF311220)	526	99.8
IT_4	01	α, Cand	clone 20162U66 (EU237450)	506	87.4
IT_5	08	α, Rose	Uncultured *Roseobacter* sp. clone EF100- 65C12 (AY627371)	523	100.0
IT_6	03	plastid	DGGE gel band LUR7 (AY960282)	528	95.8
Intermediate type (IM)					
IM_1	15	Actino	*Propionibacterium acnes* isolate Asn14 (DQ672261)	531	100.0
IM_2	06	α, Rose	clone S25_1451 (EF575107)	520	100.0
IM_3	04	Bac	clone OCS182 (AF001660)	528	100.0
IM_4	13	Cya	*Synechococcus* sp. WH 8109 (AY172836)	527	100.0
Not characterized					
NO_1	05	α, Rose	clone CZII_q2 (AB355769)	523	100.0
NO_2	14	Bac	Uncultured *Oceanospirillales* clone ESP200-K15-10 (DQ810547)	526	89.0

* α, γ, Bac, Cya, Cand and Actino indicate *Alpha-*, *Gammaproteobacteria*, unknown group of bacteria, *Cyanobacteria, Candidus* and *Actinobacteria*, respectively; Sphi, *Sphingomonas* spp.; Pseu, *Pseudoalteromonas* spp.; Rose, *Roseobacter* spp.

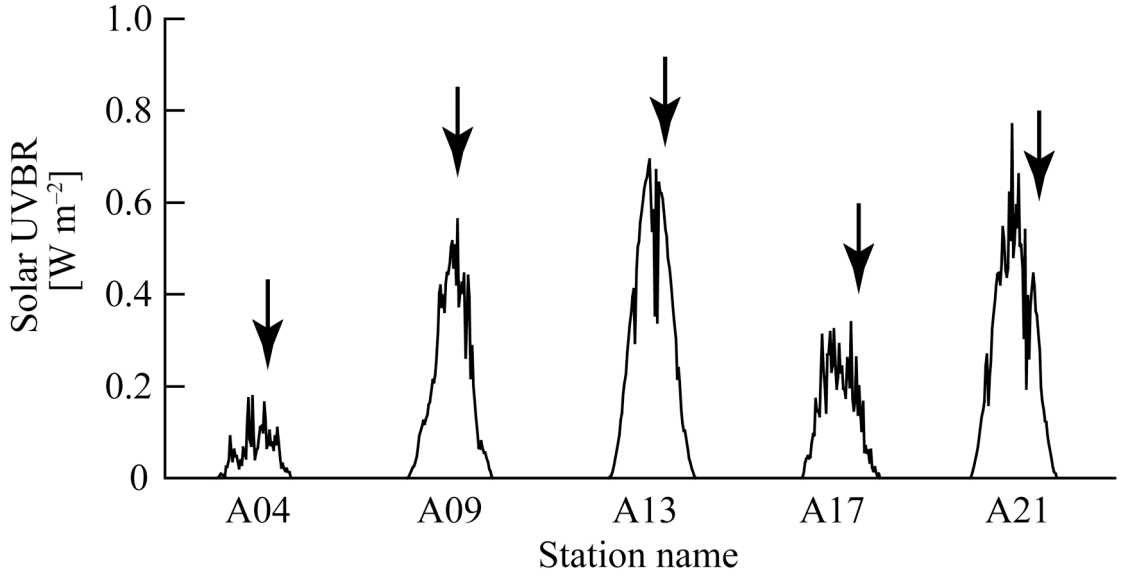

Fig. 1 Kataoka et al.

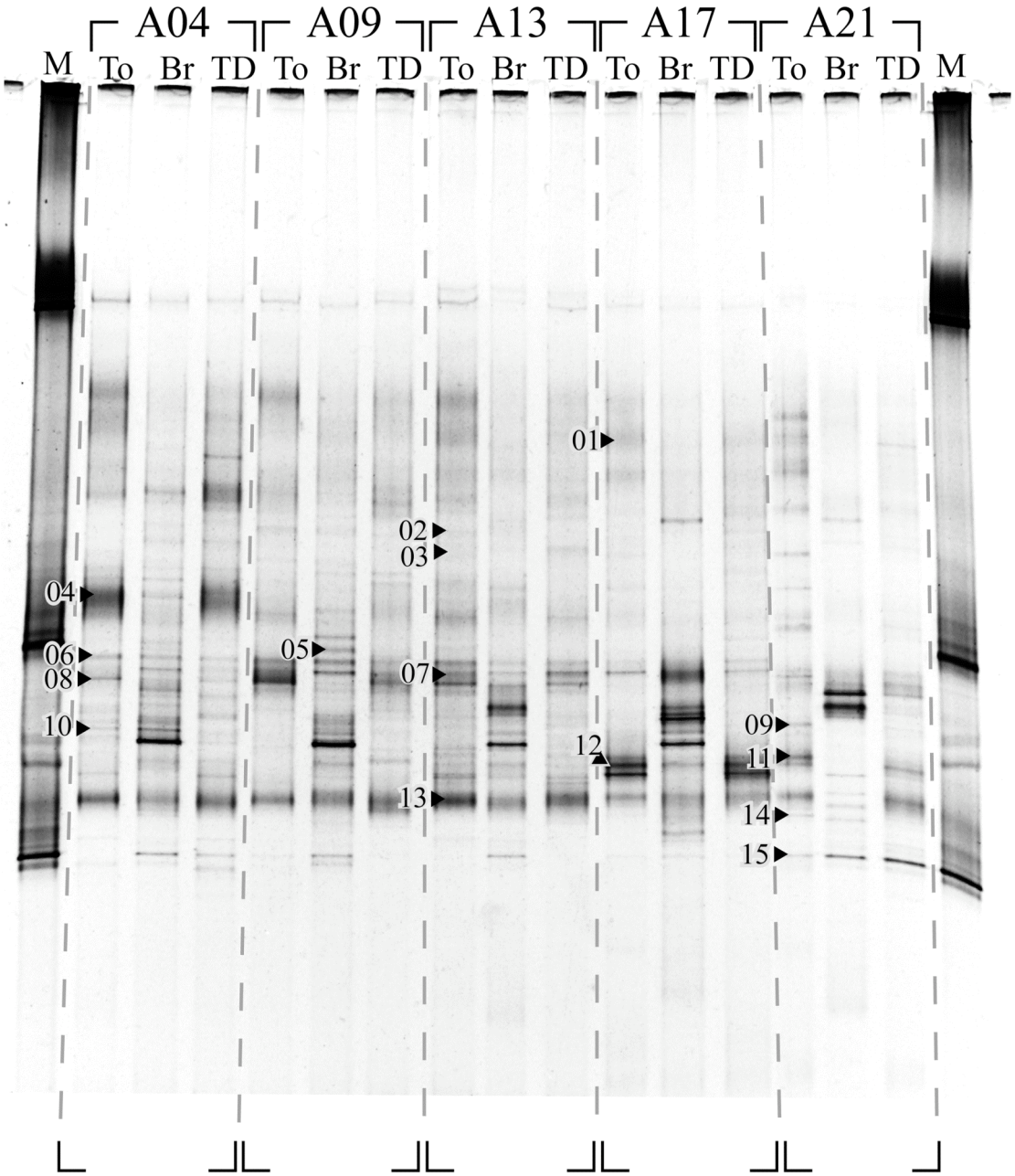

Fig. 2 Kataoka et al.

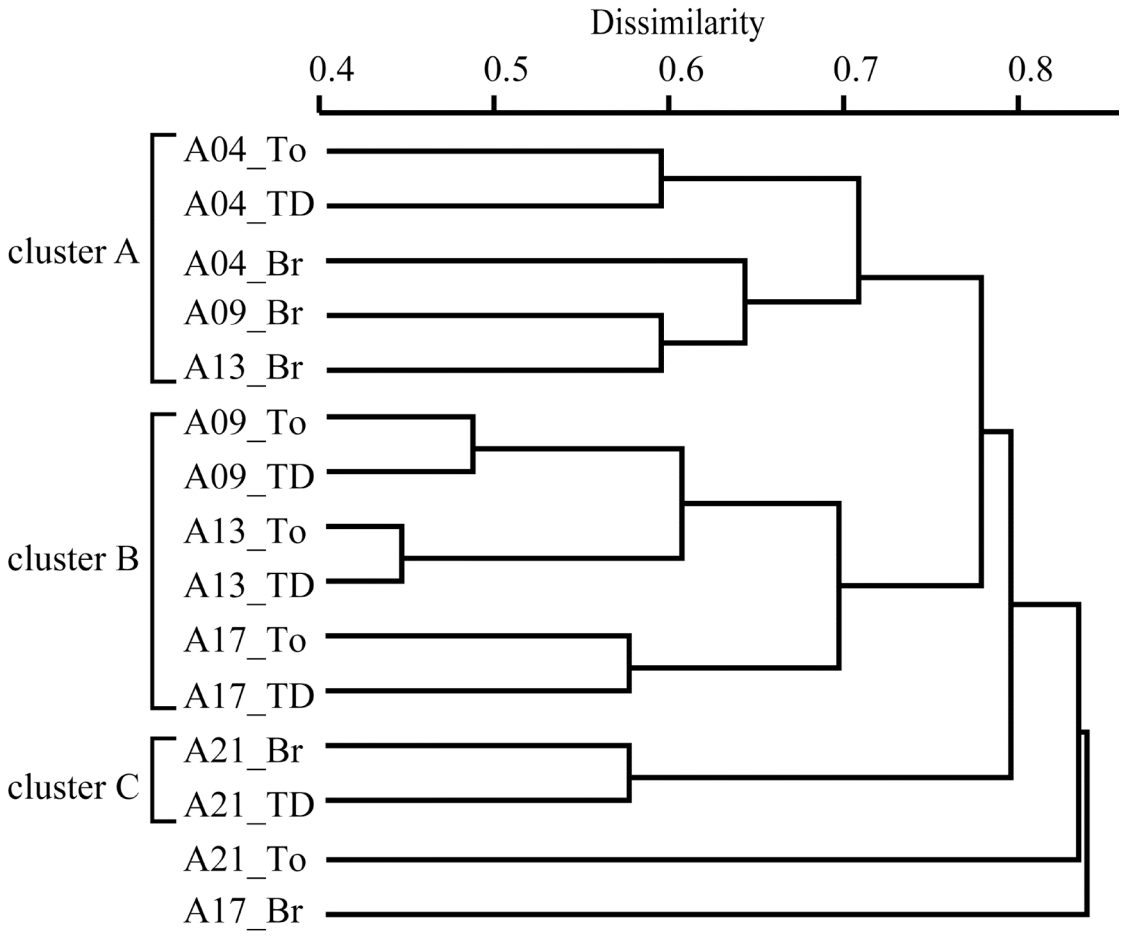

Fig. 3 Kataoka et al.

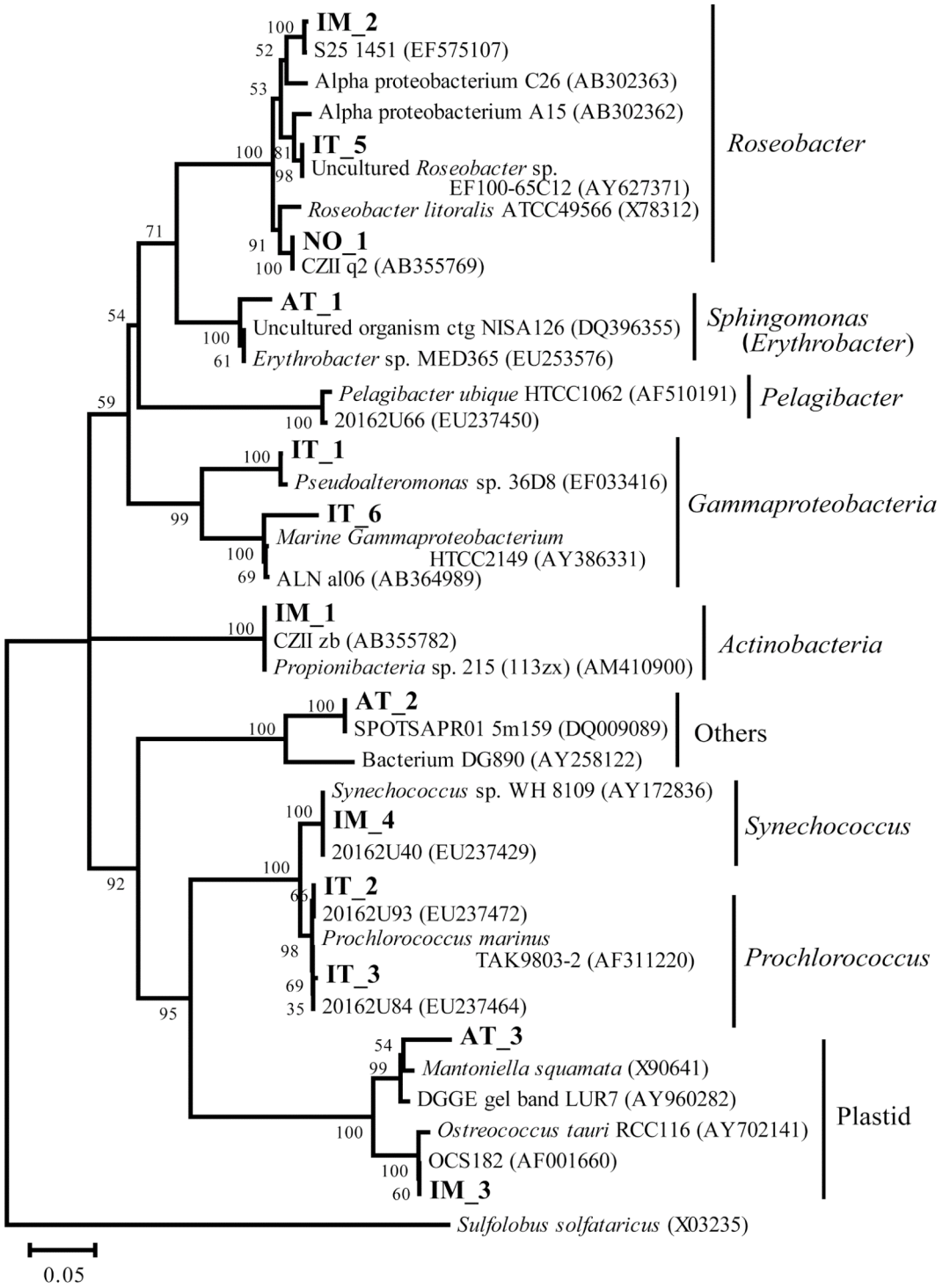

Fig. 4 Kataoka et al.