Gene 576 (2016) 650–656

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Active populations of rare microbes in oceanic environments as revealed by bromodeoxyuridine incorporation and 454 tag sequencing

Koji Hamasaki [a,*], Akito Taniguchi [b], Yuya Tada [c], Ryo Kaneko [a], Takeshi Miki [d,e]

[a] Atmosphere and Ocean Research Institute, The University of Tokyo, Chiba 277-8564, Japan
[b] Faculty of Agriculture, Kinki University, Japan
[c] Faculty of Environmental Earth Science, Hokkaido University, Japan
[d] Institute of Oceanography, National Taiwan University, Taiwan
[e] Research Center for Environmental Changes, Academia Sinica, Taiwan

ARTICLE INFO

Available online 22 October 2015

Keywords:
Rare biosphere
Ocean
Bacteria
Bromodeoxyuridine
454 pyrosequencing

ABSTRACT

The "rare biosphere" consisting of thousands of low-abundance microbial taxa is important as a seed bank or a gene pool to maintain microbial functional redundancy and robustness of the ecosystem. Here we investigated contemporaneous growth of diverse microbial taxa including rare taxa and determined their variability in environmentally distinctive locations along a north–south transect in the Pacific Ocean in order to assess which taxa were actively growing and how environmental factors influenced bacterial community structures. A bromodeoxyuridine–labeling technique in combination with PCR amplicon pyrosequencing of 16S rRNA genes gave 215–793 OTUs from 1200 to 3500 unique sequences in the total communities and 175–299 OTUs nearly 860 to 1800 sequences in the active communities. Unexpectedly, many of the active OTUs were not detected in the total fractions. Among these active but rare OTUs, some taxa (2–4% of rare OTUs) showed much higher abundance (>0.10% of total reads) in the active fraction than in the total fraction, suggesting that their contribution to bacterial community productivity or growth was much larger than that expected from their standing stocks at each location. An ordination plot by the principal component analysis presented that bacterial community compositions among 4 sampling locations and between total and active fractions were distinctive with each other. A redundancy analysis revealed that the variability of community compositions significantly correlated to seawater temperature and dissolved oxygen concentration. Also, a variation partitioning analysis showed that the environmental factors explained 49% of the variability of community compositions and the distance only explained 4.0% of its variability. These results implied very dynamic change of community structures due to environmental filtering. The active bacterial populations are more diverse and spread further in rare biosphere than we have ever seen. This study implied that rare microbes are important as an active part of microbial communities functioning ecosystems.

1. Introduction

Microbial activity and community structures are the keys to determine biogeochemical cycles in the ocean, which influence the structures and functions of oceanic food chains and also the efficacy of both biological and microbial carbon pumps for natural CO_2 sequestration into the ocean from the atmosphere (Azam, 1998; Jiao et al., 2010). Therefore, spatio-temporal variability of bacterial diversity and community structures in the ocean are one of the most fundamental research themes in the biological oceanography. In order to have more comprehensive views of microbial diversity in both planktonic and benthic ecosystems in marine environments, the International Census of Marine Microbes (ICoMM) conducted the global survey of microbial diversity with the use of massively parallel sequencing of 16S rRNA hypervariable regions (http://icomm.mbl.edu). Environmental DNA and RNA samples representing various types of ecosystems such as estuaries, oceans, deep-water environments including vents and seeps, seamounts, corals, sponges, microbial mats and biofilms, and polar regimes were collected from all major oceanic regions (Amaral-Zettler et al., 2010). The analyses revealed local diversity patterns of each ecosystem (e.g. Kirchman et al., 2010; Lloyd et al., 2010, Sunagawa et al., 2013, Galand et al., 2009). Also, the synthetic study of globally distributed samples across different ecosystems showed large-scale patterns in microbial communities (Zinger et al., 2011).

The pilot study of the ICoMM project revealed unprecedented depth of prokaryotic diversity from thousands of low-abundance populations that had never been detectable by conventional sequencing efforts (Sogin et al., 2006). This "rare biosphere" contained highly diverse microbial taxa and showed long-tail distributions in their rank-

* Corresponding author at: Associate Professor Microbiology Lab., Dept. of Marine Ecosystem Dynamics Atmosphere and Ocean Research Institute, The University of Tokyo, 5-1-5, Kashiwanoha, Kashiwa, Chiba 277-8564, Japan.
E-mail address: hamasaki@aori.u-tokyo.ac.jp (K. Hamasaki).

http://dx.doi.org/10.1016/j.gene.2015.10.016

abundance curves. They can be important as a seed bank or a gene pool to maintain functional redundancy and robustness of the ecosystem (Pedrós-Alió, 2006). Recent works deeply sequencing both 16S rRNA and 16S rRNA genes (rDNA) in natural microbial communities revealed an activity and potential growth of rare taxa (Campbell et al., 2011; Gaidos et al., 2011; Campbell and Kirchman, 2013; Wilhelm et al., 2014). Positive correlation between 16S rRNA and rDNA frequencies of most individual bacterial taxa was reported in a case of the 3-y monthly monitoring of surface waters off the Delaware coast, suggesting that the abundance was followed by activities (Campbell et al., 2011). However, such correlation was not always observed in other works, suggesting variable growth response of individual taxa or frequent transition between active and dormant in highly fluctuating environment (Campbell and Kirchman, 2013; Wilhelm et al., 2014). Some taxa obviously cycle between abundant and rare (Campbell et al., 2011). Many rare taxa are disproportionately active and contribute to maintain the high levels of microbial diversity observed in natural ecosystems (Jones and Lennon, 2010; Wilhelm et al., 2014).

Here we investigated contemporaneous growth of diverse taxa including rare microbes and determined its variability in environmentally distinctive locations along a north–south transect in the Pacific Ocean in order to assess the dynamic nature of "rare biosphere". We used bromodeoxyuridine (BrdU), halogenated nucleoside that can serve as a thymidine analogue, to label bacteria with *de novo* DNA synthesis in our seawater samples (Urbach et al., 1999, Hamasaki et al., 2007). After the extraction, BrdU-labeled DNA was immunocaptured from total DNA and its PCR amplicons of 16S rDNA were employed for 454 pyrosequencing to read the V6 hypervariable region. In this way, we compared total bacterial communities with DNA-synthesizing (presumably actively growing) bacterial communities including less-abundance or rare phylotypes to assess which taxa were actively growing and whether rare taxa were active or not. We also explored how environmental factors influenced bacterial community structures including highly diverse rare taxa.

2. Materials and methods

2.1. Sampling and BrdU labeling

Surface seawater samples (one of each station) were taken with a clean plastic bucket at four stations along a north–south transect in the South Pacific during the KH-04-5 cruise of R/V Hakuho-maru (Table 1). All samples were pre-filtered through a 200-μm mesh to remove most zooplankton, and performed further procedure within 1 h. Eleven-liters of the pre-filtered samples with BrdU (20 nM in final concentration; Sigma-Aldrich, St. Louis, MO) were incubated in dark bottles at in situ water temperature for 10 h. After incubation, bacterial cells were collected with 0.22-μm-pore-size Sterivex cartridge filters (Millipore, Billerica, MA) with a peristaltic pump. Immediately after filtration, the Sterivex filters were stored at −20 °C until further analysis.

2.2. Environmental parameters

Salinity and temperature of each site were obtained by Sea-Bird's CTD SBE9plus profiler (Sea-Bird Electronics, Inc., Washington, USA). Chlorophyll *a* concentration was measured using Turner Design fluorometer after filterating on GF/F filter and extracting pigments with N, N-dimethylformamide (Holm-Hansen et al., 1965; Suzuki and Ishimaru, 1990). Bacterial abundance was enumerated by direct counting with the use of epifluorescence microscopy (Olympus BX-51, Olympus Corp., Tokyo, Japan) after staining with 4′,6-diamidino-2-phenylindole (DAPI, 1.0 μg mL^{-1} in final concentration; Molecular Probes, Eugene, OR) (Porter and Feig, 1980). Nitrate + nitrite and silicate were measured by continuous flow system (BRAN + LUEBBE; AACS-II).

2.3. DNA extraction

DNA was extracted by using the xanthogenate-sodium dodecyl sulfate (XS) DNA extraction protocol described by Tillett and Neilan (2000). Briefly, each Sterivex filter was added about 2 mL of freshly made XS buffer (1% potassium ethyl xanthogenate [Fluka, Buchs, Switzerland], 100 mM Tris–HCl [pH 7.4], 20 mM EDTA [pH 8.0], 1% sodium dodecyl sulfate, 800 mM ammonium acetate) and the filter was inverted several times to mix. The filter was incubated at 70 °C for 120 min. Following incubation, the tube was vortexed for 10 s and then immediately placed on ice for 30 min. To recover the most bacterial DNA, these procedures were performed twice and then the filters were washed with 1 mL of XS buffer. Cell debris was pelleted by centrifugation at 22,000 *g* for 15 min at 4 °C. The supernatant was transferred to several clean 2.0-mL tubes and mixed with as equal volume of 100% isopropanol. After incubation at room temperature for 10 min, the precipitated DNA was pelleted by centrifugation at 22,000 *g* for 20 min. The DNA pellet was washed twice with 500 μL of 70% ethanol, air dried, and resuspended in 30 μL of TE buffer. The DNA concentration was measured by Nanodrop ND-1000 fluorescence spectrometer (Nanodrop Technologies). The DNA was stored at −80 °C.

2.4. Immunocapture of BrdU-labeled DNA

Immunocapture was performed as described by Urbach et al. (1999) with slight modification (Hamasaki et al. 2007). In the procedures described below, all preparations were incubated at room temperature. Herring sperm DNA (1.25 mg mL^{-1} in PBS; Trevigen Inc., Gaithersburg, MD) was boiled for 1 min, quickly frozen in dry-ice ethanol, and thawed. The solution was mixed (9:1) with anti-BrdU monoclonal antibody (diluted 1:10 in PBS; Sigma-Aldrich, St. Louis, MO) and incubated for 40 min. Extracted DNA samples (1000 or 500 ng) were boiled for 1 min, quickly frozen in dry-ice ethanol, and thawed. Each denatured sample solution was mixed with 10 μL of the herring sperm DNA-antibody mixture and incubated for 30 min. Paramagnetic beads coated with goat anti-mouse immunoglobulin G (Dynabeads; Dynal Biotech, Oslo, Norway) were washed with PBS containing acetylated bovine serum albumin (BSA) (1 mg mL^{-1}; Nacalai Tesque, Kyoto, Japan), using a magnetic concentrator. The beads were resuspended in PBS-BSA at their initial concentration. The bead suspension (25 μL) was mixed with each sample (20 μL). After incubation for 30 min with constant agitation, the beads were washed seven times with 0.5 mL PBS-BSA. BrdU-labeled DNA captured by the beads was eluted by adding 100 μL of 1.7 mM BrdU (in PBS-BSA) and incubating the preparation

Table 1
Environmental factors, sequencing information and diversity estimates for bacteria.

Station ID	Latitude	Temp	Salinity	$NO_2 + NO_3$	SiO_2	PO_4	Chl *a*	Bac no.	No. of reads (bacteria)		Unique tags (bacteria)		Bac OTUs (~3%) SLP/PW-AL	
		°C	PSU	μM	μM	μM	μg L^{-1}	10^5 cells mL^{-1}	Total	Active	Total	Active	Total	Active
SX27	0°	28.7	36.37	2.91	nd	0.38	0.253	9.75	23,744	12,677	3662	900	793	299
SX22	20.0°S	28.6	35.13	nd	nd	0.09	0.095	6.88	22,334	20,340	3226	1832	649	263
SX14	50.0°S	11.3	34.35	10.7	nd	0.81	0.275	10.4	25,433	20,214	2998	1770	355	190
SX11	64.5°S	0.3	33.83	25.8	45.6	1.5	0.854	3.42	23,120	14,801	1163	860	215	175