論文内容の要旨

論文題目 Particle-capture by marine bacteria

(海洋細菌の微粒子捕獲に関する研究)

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Introduction

Major challenge of microbial oceanography is the elucidation of the role of microorganisms at the biogeochemical cycles of marine environments. It is known that about 30% of primary production of the ocean is performed by microorganisms. They participate in the marine carbon cycles by the consumption (degradation and absorption) or remineralization of organic matter. Marine organic matter is divided into particulate organic matter (POM) and dissolved organic matter (DOM) according to the size. Regarding the bacterial strategies in order to use organic matter, mostly two were explained; particle-attachment to POM or free-living to DOM. Interestingly, recent reports revealed that dissolved organic matters consist of various forms including transparent gels, and colloids, particles, and mucus sheets and bundles, which formed by aggregation of transparent gels. Even particles in the ocean are the smaller, the more abundant, and which are absolutely important to biogeochemical cycles in marine environments. Such small organic matters should be tasty food for bacteria and bacteria would degrade or modify them to acquire favorable organic matters for themselves. And it is necessary to define the new strategy of bacteria utilizing specific dissolved organic matter such as submicron particles, which cant be directly transported through membrane.

Through the thesis, I hypothesized that bacteria may possess submicron particles around their surface, which supposed to promote effective consumption of those particles by increasing the density of organic matter around them, and these processes were named "particle-capture", and the bacteria those have the ability of particle capture were named "Particle-capture bacteria (PCB)".

Promising objectives of the thesis are 1) the method evaluation of the separation of PCB using magnetic particles, 2) the direct observation of PCB using atomic force microscopy (AFM), and 3) the characterization of PCB community using molecular approaches (degenerating gradient gel electrophoresis, DGGE).

Materials and Method

Major methods used in this thesis are followings.

Sampling. For basic and methodological examinations, seawater samples collected from Tokyo Bay were used. Seawater samples were also obtained from Tokyo Bay, Sagami Bay, and offshore environments during the KT-05-16 cruise of R/V 'Tansei Maru' (Ocean Research Institute, The University of Tokyo, and Japan Agency for Marine-Earth Science and Technology (JAMSTEC)). Stations S1, S2, and S3 were located in the southern, middle, and northern regions, respectively, of the Kuroshio Current.

Magnetic separation. The magnetic separation method using biodegradable paramagnetic particles was examined. 1-ml seawater samples were transferred to 1.5-ml microcentrifuge tubes, and magnetic separation was initiated immediately by the addition of 20 μl of the particle suspension. The mixtures were incubated at room temperature at 10 rpm for 1 h on a sample mixer. The paramagnetic particles were then collected with a magnet and the supernatants were removed carefully. The particles were rinsed twice with artificial seawater and frozen until analysis.

Community structure analysis. Nucleic acids were extracted by using the commercialized kits. For RNA sample, cDNAs were synthesized by reverse-transcription. PCR was conducted from DNA samples and RNA-derived cDNA samples, with primers those amplify DNA fragments including V3 region of 16S rRNA with GC clamp, GC rich sequences for the DGGE analysis. Amplicons were separated by their sequences by DGGE and the banding pattern was visualized by statistical analysis. Sequencing analysis of some prominent bands excised from DGGE gels was also conducted.

AFM analysis. Bacterial cells collected from Tokyo Bay, Sagami Bay and the offshore stations were observed by AFM. Bacterial cells were differentiated from non-living particles on the basis of their size, shape and cross-section. Cells with particulate materials around them were counted, and the relative number of particle-possessing cells among the total number of bacterial cells was determined.

Results

Evaluation of Magnetic Separation. Magnetic separation method was evaluated in order to collect bacteria possessing particle-capture ability (particle-capture bacteria, PCB). Reliability was confirmed through community structure analysis. First trial to collect PCB from natural seawater was resulted that community structure of triplicate of magnetically separated samples were agreed well and were different from those of natural seawater. Close inspection into the phylotypes of magnetically separated showed α-, β-, and γ-Proteobacteria, Actinobacteria, and CFB group (*Cytophaga–Flavobacterium–Bacteroides*) phyla. Interestingly, several prominent bands of submicron-sized (130 nm) particle samples were closely related to *Roseobacter* isolates. And a band, which belongs to the CFB group, appeared in all magnetically separated samples. It was showed that the abundances of magnetically separated bacterial cells were constant at approximately 10% of total bacteria throughout the investigations.

Secondly, incubation time with model particles were tested whether affect to the community structure of magnetically separated samples. Results showed that community structure and abundance of magnetically separated samples were mostly consistent from 1h to 8h and greatly shift around 24 h incubation suggesting 1h-incubation would be proper for efficient experimental procedures.

Spatial distribution of bacteria possessing submicron sized particles on their surface. Bacterial cells

collected from samples from both coastal and open ocean environments and concentrated on Isopore filters were observed under AFM, and those surrounded by particulate materials were counted. The sizes and numbers of particles on each cell were variable, and it was difficult to distinguish any general trends. The relative numbers were high in the inner part of Tokyo Bay, but few were detected in the surface layers at other stations. Among the offshore stations, the ratios were relatively high in the middle layer (500 to 2000 m) and declined with depth.

Effect of various size and substances of particles. Community structure analysis to see the changes on different types of particles to associated were performed using 8 types paramagnetic particles; 130nm, dextran; 150nm, silica; 250nm, dextran; 300nm, silica; 500nm, dextran; 500nm, silica; 6μm, silica; 6μ, polylactic acid. As consistent with above results, community structures of magnetically separated samples were different from those of natural seawater. And also among magnetically separated samples of different particles, it was clearly shown that community structures were changed size- and substance-dependently. Submicron sized samples (130nm to 300nm) were shown consisting similar community structure, while they were different from large particle (6μm) samples. Regarding on substance of particles, same substance samples were shown consisting similar community structures although size of particles they associated was slight different, 130nm and 250nm of dextran, 150nm and 300nm of silica, respectively. These results were consistent among Yokohama Port and Shinagawa Port samples.

Interesting thing was 500nm particle samples. Patterns of community structure shifts were different between Yokohama Port and Shinagawa Port; at Yokohama Port, 500nm samples were presented middle of other submicron samples and large particle samples, while at Shinagawa Port, they were close to large particle samples.

Effect of metabolic activity. DNA- and RNA-derived DGGE banding patterns were compared between submicron sized (130nm) particle samples and large (1μm) particle samples from the 'total' seawater and the 3μm filtrate, at Sinagawa port, Yokohama port, and Aburatsubo port, respectively. DNA-derived profiles showed consistently clear differences between submicron and large particle samples through 3 different stations. While, RNA-derived profiles varied among stations, showing inconsistent different patterns between submicron and large particle samples; at Yokohama station, 1μm samples and the 130nm sample from total seawater and 1μm samples from 3μm filtrate were plotted closely together, while the 130nm sample from 3μm filtrate seawater were plotted apart. At Sinagawa station, all samples were plotted apart regardless the size of particles and sampling fractions. At Aburatsubo station, except the 1μm sample from total, all samples were plotted close.

Phylotypes assumed as key members who drove above results were suggested from sequencing analysis. Members that only appeared among RNA level were closely related to members of α-proteobacteria; Metholybacter and Xanthobacter and uncultured γ-proteobacteria.

Effect of ionic interaction between PC bacteria and particles. Effect of ionic interaction on PC was investigated performing experiments adding chelating agent, EDTA (ethylenediaminetetraacetic acid). EDTA volume was adjusted from preliminary experiments using famous model marine bacteria, *Vibrio parahaemolyticus*, and added to natural seawater. Increasing the concentration of EDTA, the relative number of PC bacteria decreased subsequently. Additional experiments about the effect of flagella on PC were performed. 3 types of mutants were used; YM4, no flagella; YM18, polar flagella, YM19, lateral flagella.

Relative number of PC was high from YM19. And other 3 strains including wild type was shown similar % of PC to the total abundance.

Discussion

In aquatic environments large numbers of submicron particles are generally present, and their turnover and fate are of considerable ecological importance in biogeochemical cycles in marine environments. From the results of this thesis, it was suggested that some bacterial community would capture submicron sized particulate organic matter such as submicron particles (SMPs). Their abundance was about 10% of total bacterial abundance of surface water and relatively high in the inner part of Tokyo Bay and at depths of 500 to 2000 m in the open ocean. Mesopelagic to bathypelagic is known as the last frontier in the earth and recently several international big projects are conducting in order to investigate biogeochemical process to solve a riddle on the marine carbon cycles. And there are known as sinking particles are abundant and bacterial respiration rate and exoenzymatic activity are high. With high relative abundance of particle-capture bacteria, possibilities were that particle-capture bacterial community might play important role in the biogeochemical cycles around mesopelagic to bathypelagic.

And it was also suggested that particle-capture ability might be the fundamental way for survival of marine bacteria. Particle-capture bacterial community were distributed phylogenetically broadly among α-, β-, γ-proteobacteria, CFB group, Actinomyces, Firmicutes, etc. Close inspection into phylotypes of PCB suggested that particle-capture as a fundamental way for survives. For example, *Roseobacter sp.*, which is the typical free-living bacteria, may be necessary to uptake nutrients from DOM. And also in the case of couple of members of CFB, they live close to the phytoplankton may capture submicron particulate matter leaked from upper trophic. Comparison between DNA and RNA level community structure analysis revealed that metabolic activity should be hardly related with particle-capture by bacteria suggesting other physiochemial factors might affect particle-capture. Then I tested whether ionic interaction between bacteria and SMPs would consist of particle-capture process using chelating agent. Results showed that relative particle-capture bacterial number decreased as chelating agent added, which might suggest ionic interaction between bacteria and SMPs would consist of particle-capture. That is, surface charge of bacteria that might be by nature or affected by surroundings, possibly would be one of important factor for particle-capture. Additional experiments suggested that surface features which bacteria possess by nature, might affect PC such as lateral flagella and ionic status. In summary this thesis proposed that:

1. The method of collecting particle-capture bacteria was developed in this thesis, and suggested the novel concept for bacteria to utilize submicron particles.

2. Direct observation by AFM revealed the presence of particle-capture bacteria, and particle-capture bacteria is dominant around mesopelagic and bathypelagic. In the context of biogeochemical cycles of marine environments, it is suggested that particle-capture bacteria may play an important role in the marine carbon cycles around deep sea.

3. Community structure analysis of particle-capture bacteria from DNA and RNA derived samples suggested physiochemical properties might be the important factors for possessing particle-capture ability. In the context of microbial oceanography, particle-capture may be the ability by nature of bacteria.