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粒子捕獲能力を持つ海洋細菌の動態

－The Dynamics of Marine Bacteria with Particle Capturing Activity－

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Introduction

It has been recognized that the concentration of particles in the ocean increase with the decrease of their particle sizes. Recent estimates of oceanic dissolved organic carbon (DOC), which passes through Whatman GF/F filter (pore size 0.7μm), are about 700Gt (Hedges 1992). This fraction, however, contains various particles. In 1990, it was reported that the number of so called sub-micrometer particles (SMPs) is about 10^7 particles ml^{-1}, which is about one order of magnitude larger than those of bacterial cells (Koike et al 1990). Also bulk chemical measurements have confirmed that the "colloidal fraction" (size, 0.001-1 μ m) represent 10 to 50% fraction of total DOC in sea water (Ogawa et al 1992, Benner et al 1992, Gau et al 1994). Although chemical composition and exact origins of SMPs are not clear yet, SMPs were seemed to be composed largely of organic materials (Koike et al 1990, Yamazaki et al 1998).

Most organic particles in the ocean should serve as food source for bacteria. Some bacteria attach to surface of large particles such as marine snow and sea weeds. Marie bacteria recognize the surface chemical compositions of organic particles, synthesize and exclude the enzyme, degrade them into small size (around 100Da), and incorporate them. This process has been investigated by many workers, whereas virtually no work has been accomplished for the interaction with SMPs and bacteria. How does bacterium recognize chemical composition of SMP which has various chemical compositions? What kind of enzyme do the bacteria synthesize and how much enzyme do they exclude out of their cell? Recently, Seo proposed a concept that bacteria have the ability of "particle capturing (PC)" (Seo et al 2007). This means, cells retain the submicron particles on their surfaces prior to utilizations as carbon sources. Seo et al developed new method for isolation of particle captured bacteria using paramagnetic particles as model particles. Also they stated that 10% of total bacteria possess particle capturing activity in the coastal sea water, and their community composition differed depending on the size and/or composition of the particles. On this thesis, the bacteria with PC activity are treated as PC bacteria.

The aim of this study is to clarify the significance of particle capturing process in the ocean. I focused on the abundance, distribution, community structure, enzyme activity of PC bacteria, and the number of SMPs on the cells. In the experiment, PC bacteria were isolated by magnetic separation method described in Seo et al (2007). This is the only method which enables us to isolate PC bacteria so far. For analysis of community structures, I chose Denaturing Gradient Gel Electrophoresis (DGGE). This method allows us to clarify the community structure even though the target bacterial community includes unknown sequences, because gene sequences of the small subunit ribosomal RNA were used for identification of microorganisms. SMPs were observed and enumerated by Atomic Force Microscopy (AFM) which can detect and quantify nano size

samples easily. In addition, AFM gives the information with minimum sample treatments. Thus I could count SMPs with AFM even if they were on the cells.

Chapter 1 Abundances and Spacio Temporal Variability

1.1 Introduction

To clarify the dynamics of PC bacteria, it is necessary to know the basic information such as abundance and distribution. The goal of this chapter is to survey the abundance and distribution of PC bacteria both in coastal and oceanic sea water. This information leads us to discuss the characteristics of PC bacteria.

1.2 Materials and Methods

Study sites and sampling

Sea water samples were collected at three sampling stations along a near shore-off shore transect from the Tokyo Bay to off the Izu Islands, Japan, on the RV *Tansei Maru* (Ocean Research Institute, the University of Tokyo and Japan Agency for Marine-Earth Science and Technology) cruise (KT-06-31) during 6-10 December in 2006 (Sta.T, P,.S, Figure 1). For observation of seasonal abundance of PC bacteria, additional monthly samples were collected from January to November at Sta.M in Sagami Bay (Figure 1). Samples were collected with a 9-liter Niskin CTD rosette sampler (RV *Tansei Maru* Cruise) or with 6-liter Niskin bottle (Sta.M) and sterilely pored into polycarbonate bottles.

PC bacterial concentration

Biodegradable paramagnetic particles (micromod 500nm) polymerized with dextran in aqueous suspension were applied as model particles. They were suspended in phosphate buffered saline (PBS, pH 7.5) and stored at 4℃ until use. The magnetic separation method of PC bacteria by Seo et al (2007) was used with some modifications (Fig 2). Briefly, after filtration through 3μm pore size filter (Nuclepore) to separate free-living bacteria from attached ones, two replicates of 1ml subsamples were incubated at room temperature with 20μl of the particles suspension (ca. 9.5×10^{10} particles ml^{-1}) on a sample mixer (Dynal) at 10 rpm. After 1h incubation, the paramagnetic particles were collected with a 12 tube magnet (Qiagen) for 5 min and the supernatants were removed carefully. The particles were rinsed twice with PBS and suspended in 1ml of PBS to fix with praformaldehyde (2% v/v, final concentration). Samples were stored at 4℃ (Sta.M) or -80℃ (RV Tansei Maru) until enumeration.

Subsamples were stained with 4',6-diamidino-2-phenylindole (DAPI; 1μg mL^{-1} Molecular Probes) and filtered onto black, 0.2 μ m pore size membrane filters (Nuclepore). Bacterial cells in more than 10 randomly selected fields were counted at a magnification of 1000× under an epifluorescence microscope (Olympus model BX51). At least 200 cells were counted on each sample filter.

Environmental parameters

To identify major factors which control PC bacterial abundance and its percentage to total bacteria, 10 parameters were measured (temperature, salinity, chlorophyll a, POC, PON, total bacterial abundance, virus, number of submicron particles, free-living bacterial carbon production, and attached bacterial carbon production).

Temperature, salinity, and Chl *a* were measured by CTD (Sta.T, P, S) or by thermometer, salinometer and fluorometer (Sta.M). Salinity at Sta.M was measured by salinometer (Watabe-Keiki factory No.963 4th model) according to the manufacture's protocol. Chl *a* concentrations at Sta.M was analyzed using procedures described in Welschmeyer (1985). In brief, 500ml of duplicate subsamples were filtered through organic-free glass fiber filters (Whatman GF/F) and Chl *a* were extracted with *N,N*-dimethyl formamide (Wako), then analyzed by a Turner Designs fluorometer (10-AU-005-CE Turner Designs).

Particulate organic matter (POM) was collected by filtering seawater through organic-free GF/F filter (Whatman) and kept at -20℃ until analysis. The frozen filter samples were dried in a vacuum desiccator and exposed to HCl vapor for over night to vaporize inorganic carbonates. After removing HCl, filters were dried in the desiccator again and packed into tablet with tin foil. Amount of organic carbon and nitrogen captured on GF/F were measured with CHN analyzer (Fisons NA1500) using acetanilide (Thermo) as a standard.

Bacteria and virus counting

The free-living and attached bacteria were separated by filtration through 3 μ m pore size filter (Nuclepore). The bacterial cells in the filtrate were defined as free-living bacteria and those retained on the filter were as attached bacteria. Total bacteria were collected from unfiltered sea water and PC bacteria were isolated from free-living bacterial fraction. Each sample was fixed with paraformaldehyde (2% v/v, final concentration) immediately for 20 min to soak into bacteria and stored at 4℃. After stained with DAPI, sample water was filtered through 0.2 μ m black filters (Nuclepore). Bacterial cells in more than 10 randomly selected fields were counted at a magnification of 1000× under an epifluorescence microscope (Olympus model BX51). At least 200 cells were counted on each sample filter.

For measurement of virus, fraction of unfiltered (Sta.T,P,S) or free-living (Sta.M) sea water was fixed with paraformaldehyde (2% v/v, final concentration) for 20 min and stored at -80℃ deep freezer until analysis. Viruses were counted according to the procedure by Shibata et al (2006). Briefly, the samples were filtered through 0.02 μ m pore size Anodisc filter (Whatman) and stained with SYBRGold (Molecular Probes). Virus on the filters were counted with epifluorescence microscopy (Zeiss) a microscopic magnification of 2000×. More than 10 fields were randomly chosen and at least 200 particles per field were enumerated.

Bacterial Production

Bacterial production was estimated by the incorporation of L-[U-^{14}C] Leucine (GE Healthcare, USA) and [methl-^{3}H]Thymidine (GE Healthcare, USA) into the bacterial biomass by the filtration method as described by Kirchman (2001) and Furman et al (1982) respectively. L-[U-^{14}C] Leucine and [methl-^{3}H]Thymidine were added to 10mL of duplicate samples to a final concentration of 20nM. Duplicate control samples were immediately inactivated with 5% of trichloroacetic acid (TCA Wako). All the samples were then incubated for 1~2.5h to allow for the label to be incorporated into the bacterial biomass. After incubation, samples were filtered through 3.0 μ m membrane filter (for attached bacterial observation) followed by 0.22 μ m filtration (for free-living bacterial observation) and fixed with 5% ice cold-TCA. Followed by deposition of protein on the filter, sample filters were rinsed twice with 80% ethanol. After Dried, filters were dissolved into 0.5mL ethyl acetic acid at 4℃ over night and added 5mL scintillation cocktail (Ultima Gold,Packerd). Radioactivities of the samples were counted by scintillation counter (LSC-5100: AIOKA). A conversion factor of 2.65×10^{18} cells mol^{-1} (for Thymidine) and 3.1kg (for Leucine) was applied to the incorporation ratio to carbon production.

SMP counting

SMPs were counted by atomic force microscopy (AFM Shimadzu model SPM-9500 J2) because of high magnification and easiness of the preparation. A portion of 5 ml of sea water was fixed with paraformaldehyde (2% v/v, final concentration) and filtered through 0.2 μ m pore size filter (Nuclepore). The submicron particles were observed and counted directly by AFM by using dynamic mode. The instrument was equipped with a microfabricated and oxide-sharpened Si_3N_4 cantilever (Olympus AFM OMCL-AC160TS-C1) with a pyramidal tip and a force of 42Nm^{-1}. The visible particles ranging from 0.2 to 0.5 μ m and from 0.5 to 1 μ m diameter (Fig 2) were regarded as SMPs and counted separately. They were differentiated from bacteria on the bases of their size, shape and cross-section (Nishino et al. 2004). At least 200 particles from more than 5 fields chosen randomly were counted. As for samples collected at Sta. M (0, 40, 100m) on October 19, 2007, 25ml of sweater samples were filtered through 0.2μm pore size filter (Isopore filter, Millipore) and observed by AFM with larger magnifications (Fig 3). SMPs on at least 100 bacterial cells were counted.

Statistical analysis

Statistical analyses were performed with multiple regression in order to determine the parameter's contributions for the PC bacterial abundance and PC bacterial percentage of total bacteria. Thirteen parameters (Temperature, Salinity, Chlorophyll a, POC, PON, Total bacterial abundance, Virus, >0.5 μ m SMPs, <0.5 μ m SMPs, Leucine incorporation ratio of free-living bacteria, Leucine incorporation ratio of attached bacteria, Thymidine incorporation ratio of

free-living bacteria, and Thymidine incorporation ratio of attached bacteria) were analyzed with PC bacterial abundance. Following analysis of distribution of each data (Shapiro-Wilk test), they were transformed to logarithm if needed. Then, multiple regression using forward stepwise variable selection was performed with JMP (version 6.0.3).

1.3 Results

All data are shown in Table 1. At Sta.M, phytoplankton bloom was seen in March 2006. The thermocline appeared from June to November (Table 1). Range of PC bacterial abundance in all data was from 10^3 to 10^5 cells ml^{-1} and the average was 4.3×10^4 cells ml^{-1} which was one order magnitude smaller than total bacteria.

Fig 5 and 6 show vertical and horizontal distributions and seasonal distributions respectively. Both figures indicate PC bacterial abundance (A) and relative number of PC bacteria to total bacteria as % (B). PC bacterial number tended to decrease with depth at all stations (Fig 5-A). The relative number of PC bacteria to total bacteria showed remarkable difference between coastal (Sta.M, T) and oceanic stations (Sta.P, S) which had the average of 4.4% and 29.5%, respectively (Fig 5-B). From the seasonal profile, low PC bacterial abundance was seen from February to May 2007, and high from June to November 2007 and January 2006 (Fig 6-A). The relative number of PC bacteria were generally higher in deeper layer than shallower layer (Fig 6-B).

However the correlation between PC bacterial number/percentage and other parameters were not clear one by one, these abundance and percentage were formulated using other parameters as below by the analysis of multiple regression.

$$\begin{aligned}\mathrm{Ln(PCbacteria)} = {} & 34.15(\pm 26.69) + 0.15(\pm 0.10)\mathrm{Ln(PON)} - 0.13(\pm 0.10)\mathrm{Ln}(>0.5\,\mu\mathrm{m\,Particles}) \\ & - 8.09(\pm 7.42)\mathrm{Ln(Salinity)} + 0.72(\pm 0.60)\mathrm{Ln\ (Temperature)} \\ & + 0.04(\pm 0.06)\mathrm{Ln(Free\text{-}living\ Bacterial\ Production)}\end{aligned}$$

$$\begin{aligned}\mathrm{Ln(PCbacterial\ \%\ of\ total\ bacteria)} = {} & -4.24(\pm 39.68) + 0.19(\pm 0.15)\mathrm{Ln(PON)} - 0.06(0.15)\mathrm{Ln}(>0.5 \\ & \mu\mathrm{m\,Particles}) + 1.38(\pm 11.05)\mathrm{Ln(Salinity)} - 1.04(\pm 0.90)\mathrm{Ln\ (Temperature)} \\ & - 0.18(\pm 0.09)\mathrm{Ln(Free\text{-}living\ Bacterial\ Production)}\end{aligned}$$

In this model, R^2 was to 0.58 or 0.35, F was 8.45 or 3.12, and p was <0.0001 or <0.0197, respectively. Figure 4 indicates the contributions of the parameters to the PC abundance and percentage in this model. PC abundance was negatively affected by salinity, and positively affected by PON, >0.5 μm particles , temperature and free-living bacterial carbon production, whereas

temperature, >0.5 μ m particles, and free-living bacterial carbon production affected negatively in PC % model.

The actual presence and numbers of SMP on bacterial cells in the environments were directly investigated by AFM. Forty seven to 64% of total bacteria had some SMPs on their surface. The average number of particles on a PC cell was 2.8 (0.9 of >0.5μm particle, 1.9 of <0.5μm particles) regardless of the depth. Figure 8 showed the number of SMP in natural sea water and on the cells separated with <0.5μm diameter particles and >0.5μm diameter particles. Thirty to 79% of total particles counted by AFM in sea water exist on the surface of the bacteria. Under 0.5μm diameter particles were existed more on the cells than sea water. SMP numbers on the cells were not decreased with depth whereas SMPs in sea water decreased with depth.

1.4 Discussion

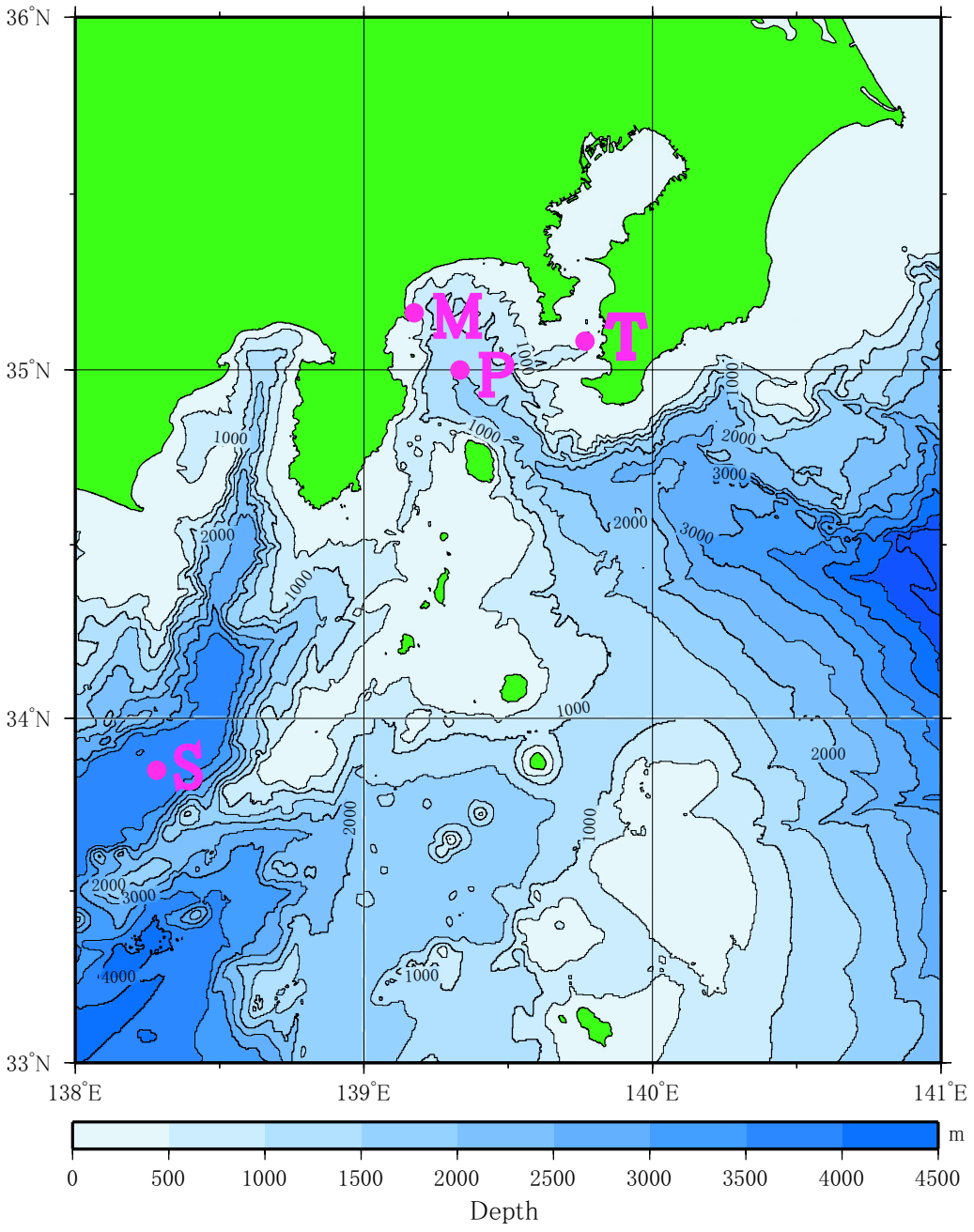
In this chapter, the abundance and distribution of PC bacteria were investigated in both coastal and offshore environments. The possible factor controlling PC bacterial distribution was analyzed with physico-chemical and biological parameters. In addition, the presence of SMPs on cells was directly observed and counted by AFM. Because the concept of PC is new, this is virtually the first environmental work on the distribution.

The magnetic separation method has been proposed and applied to natural seawater samples by Seo et al. (2007). The bacteria thus concentrated are supposed to have captured at least certain number of added paramagnetic particles so that cells are drawn by the magnet. Although it is simple and reproducible, this method detects the cells with potential PC activity, and it does not offer information on the presence of cells actually possessing SMPs on their surface （Fig. 8）. On the other hand, AFM images cannot be the confirmation of PC activity because AFM provides with only morphological characteristics and not chemical nature. It is also difficult to judge whether the particles had been captured, coincidently present near the cells during the preparation step or conversely been excreted from the cells. Nevertheless, it is noteworthy that no such work has ever been done except the one by Seo et al. (2007). In this work, the magnetic separation method and AFM were simultaneously applied and both supported the concept of bacterial PC activity. It was clarified that a considerable number of bacteria are associated with SMPs and that the abundance of PC bacteria vary depending on environmental factors.

The number and relative abundance of PC bacteria to total bacteria may depend on various factors. The hypothetical advantage of PC activity is, first, the cell can recognize the chemical component of the particle by direct contact. Second, the cell is able to synthesize particular enzyme while the particle is still on the surface. Third, the minimum number of enzyme molecules can be synthesized because the substrates are closely associating on the cell surface.

Finally, degraded materials can be taken up by the cells with minimum loss into the surrounding seawater. This scenario, if it's true, lead to the following hypothesis. First, PC activity primarily depends on the status of organic material in seawater. If DOC concentration is high this advantage will diminish. Therefore, the concentration of PC bacteria may not be high in very polluted area. On the other hand, PC activity may play a more important role in oligotrophic area. This was actually the case in this work (Fig. 7). Second, the retention time of the particles on the surface may depend on several factors. Any factor that retards the degradation may increase the apparent numbers. For instance, in cold environments, the rate of degradation should be much slower than at warmer environments and thus more particles may be retained. The relative higher number of particles on the cell surface in deeper layer may be a reflection of longer retention time mainly due to low temperature. Third, the possession of PC activity may result in higher growth efficiency because the activity makes the cells possible to synthesize minimum amount of degradation enzymes and also to take up degraded compounds with minimum loss.

The reason of higher PC bacterial number in summer season at Sta. M is not very clear at the present. However, a typhoon occurred in September (before the sampling day) could explain parts of the reason of high PC bacterial number. It could be considered that change of salinity could enhance the particle capturing activities or mixture of sea water brings something to heighten the PC activity although other month in summer had high abundance of PC bacteria. Thermocline usually develops during this season. Also higher PC bacterial number in association with POC with high C/N ratios needs some more information to explain. To understand the process of both grazing food chain and microbial loop and also the transformation and fate of organic compounds in the environment will be important.



	Sampling	Depth (m)
Sta.M	Monthly (January to November 2007)	0, 10, 20, 30, 40, 60, 100
Sta.T	RV Tansei Maru KT-06-31 (December 2006)	0, 50, 75
Sta.P		0, 50, 100, 500, 1000
Sta.S		0, 50, 100, 500, 1000, 1500

Fig 1 Sampling sites and depth

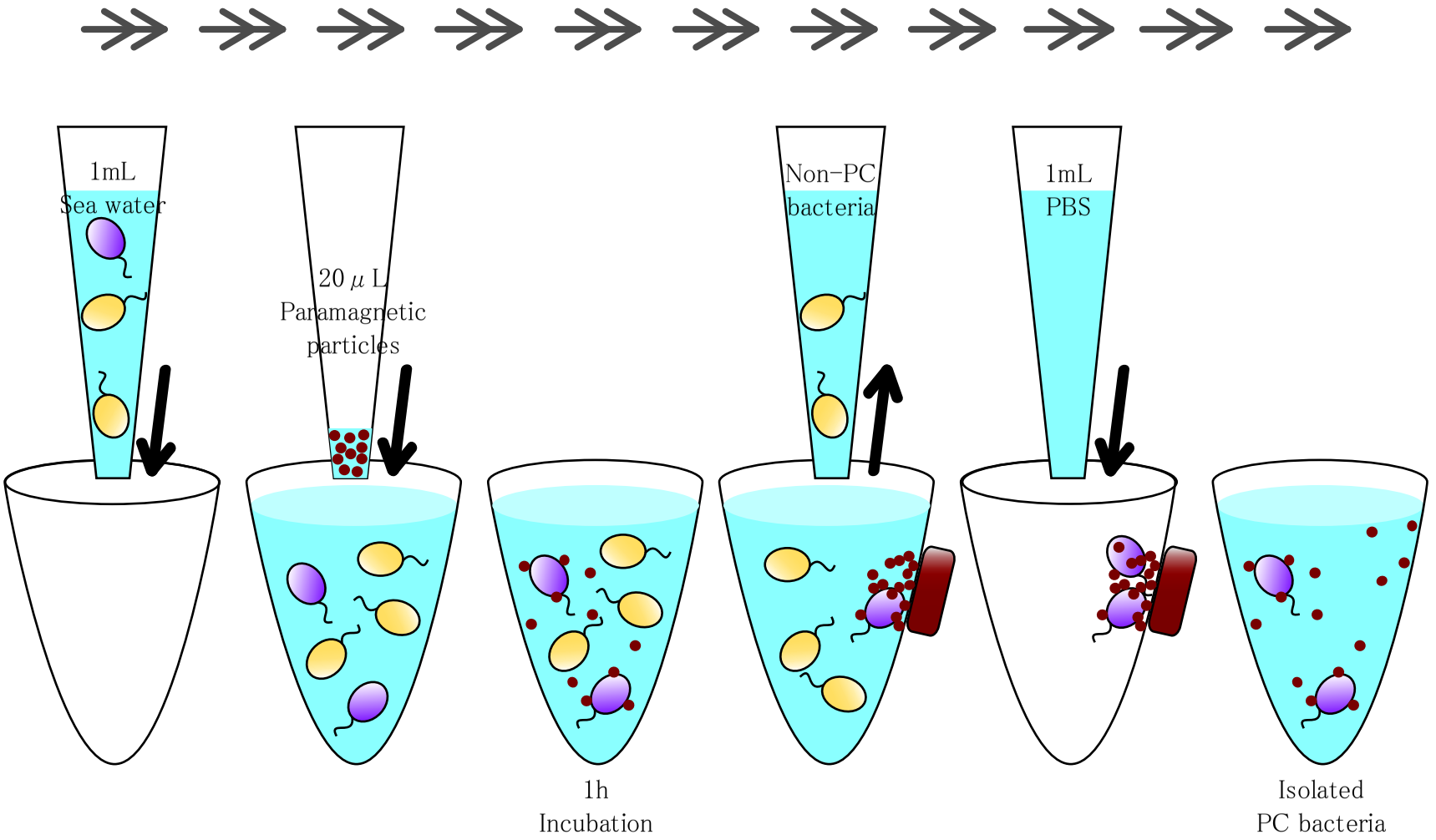


Fig 2 Method of Isoration of bcteria which possess particle capturing activities

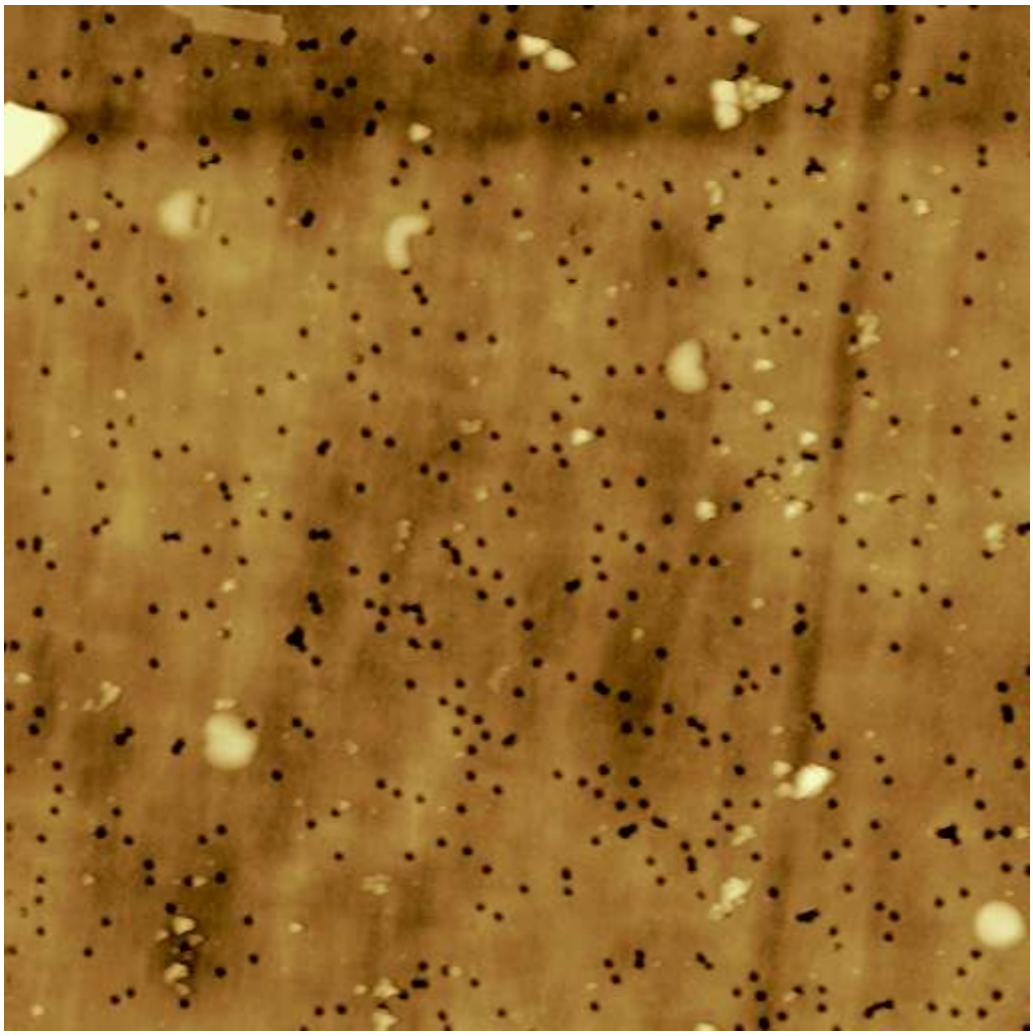


Fig 3 Atomic force microscopy images of natural submicron particles collected at Sta.M on June 14th 2007 100m. 5ml of fixed sea water was filtered through 0.2 μ m nuclepore filter.

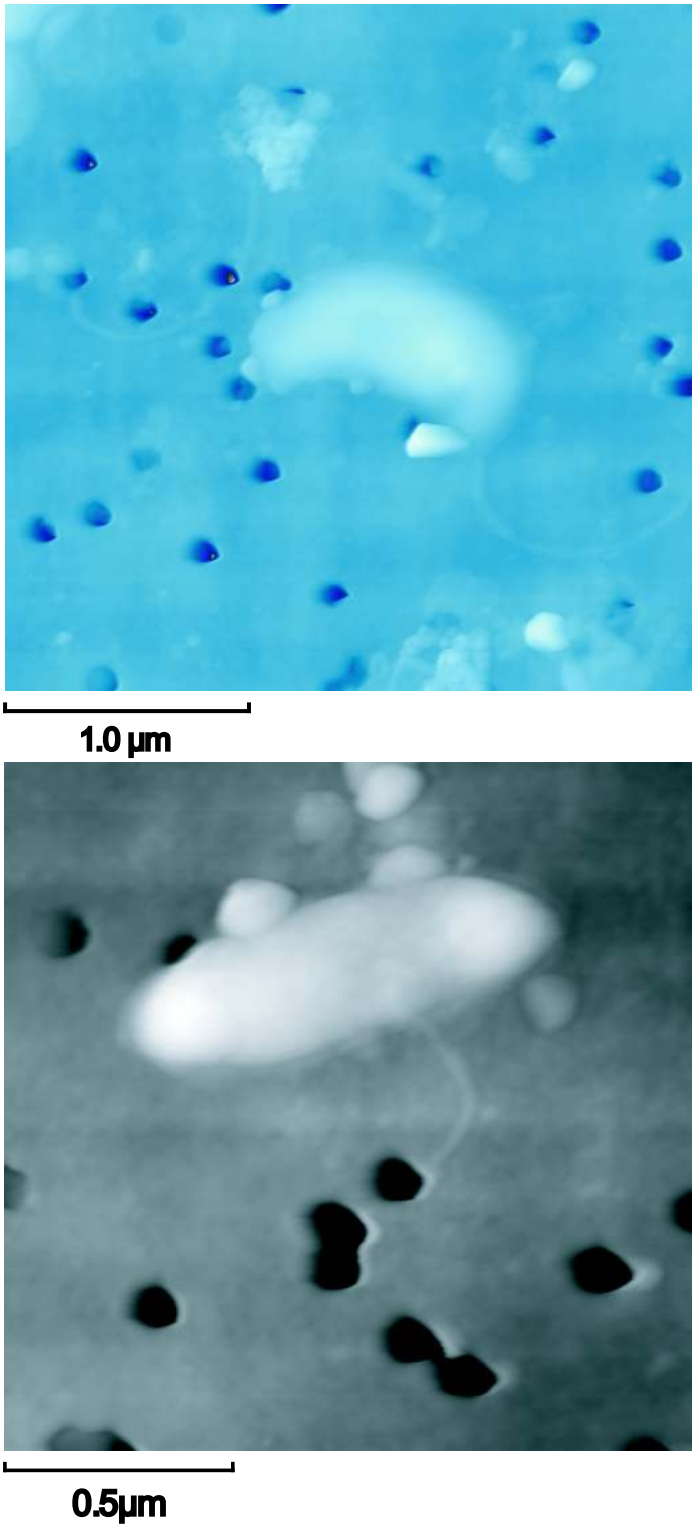


Fig 4 Atomic force microscopy images of natural bacteria filterd through 0.2 μ m filter. Samples were collected 10m of Suruga Bay from Tansei maru at 8th December 2006 during KT-06-31 cruise.

Table 1-1 Summary of all data

								Bacteria			SMPs		Production			
													Attached Bacteria		Free-livivng Bacteria	
Sta.	Date	Depth (m)	Temp (℃)	Sal (PSU)	Chl a (mg m^{-3})	PON (mg m^{-3})	POC (mg m^{-3})	Total ($\times 10^5$ cells ml^{-1})	PC ($\times 10^4$ cells ml^{-1})	Virus ($\times 10^6$ PV ml^{-1})	>0.5 μm ($\times 10^6$ P ml^{-1})	<0.5 μm ($\times 10^6$ P ml^{-1})	C Production (μgC L^{-1} d^{-1})	Dubling Time (10^8cells L^{-1} d^{-1})	C Production (μgC L^{-1} d^{-1})	Dubling Time (10^8cells L^{-1} d^{-1})
T	9 Dec 2006	0	18.1	34.337	0.2	10.6	52.4	5.4±0.5	3.2±0.2	1.1±0.2	0.4±0.3	5.1±1.2	ND	ND	ND	ND
		50	18.0	34.298	0.2	5.5	28.2	6.2±0.7	2.6±0.0	2.0±0.4	0.7±0.4	5.1±2.0	ND	ND	ND	ND
		75	18.0	34.303	0.1	6.4	32.8	6.0±0.7	2.2±0.6	2.1±0.4	0.7±0.5	7.6±2.3	ND	ND	ND	ND
P	8 Dec 2006	0	17.9	34.217	0.2	11.8	63.4	3.5±0.7	19.5±7.0	2.8±0.6	0.9±0.4	5.6±1.0	ND	ND	ND	ND
		50	17.9	34.268	0.2	6.6	34.8	4.1±0.9	15.7±0.3	1.4±0.3	0.6±0.4	8.8±1.5	ND	ND	ND	ND
		100	14.7	34.562	0.0	2.0	13.7	1.3±0.3	8.0±3.7	0.5±0.1	0.7±0.2	10.7±1.8	ND	ND	ND	ND
		500	5.3	34.301	0.0	1.2	1.2	2.0±0.2	3.5±1.1	0.2±0.2	1.7±0.6	6.2±3.2	ND	ND	ND	ND
		1000	3.1	34.488	0.0	1.5	13.6	1.1±0.2	2.7	0.2±0.1	ND	ND	ND	ND	ND	ND
S	7 Dec 2006	0	23.7	34.619	0.1	7.1	41.3	2.5±0.6	8.8±0.6	2.8±0.4	ND	ND	ND	ND	ND	ND
		50	23.7	34.619	0.2	5.1	30.9	2.5±0.4	7.8±0.2	1.7±0.3	1.2±0.7	9.7±2.5	ND	ND	ND	ND
		100	23.7	34.620	0.1	3.6	25.6	3.1±0.5	4.9±0.9	1.5±0.3	1.2±0.4	6.1±2.6	ND	ND	ND	ND
		500	9.2	34.385	0.0	2.2	26.7	1.1±0.1	1.9±0.9	0.4±0.0	0.6±0.3	10.6±3.1	ND	ND	ND	ND
		1000	3.7	34.464	0.0	1.4	13.2	0.8±0.2	0.5	0.3±0.1	ND	ND	ND	ND	ND	ND
		1500	2.6	34.596	0.0	1.0	13.1	0.7±0.2	1.6	0.3±0.1	ND	ND	ND	ND	ND	ND
M	18 Jan 2007	0	15.0	33.908	0.62	5.5	105.5	6.0±1.5	6.0	2.3±0.3	ND	ND	0.6±0.1	ND	1.4±0.3	ND
		10	15.0	34.324	0.87	16.6	183.5	5.9±1.9	4.6±0.0	3.0±0.8	ND	ND	0.3±0.0	ND	1.0±0.1	ND
		20	15.0	34.315	0.93	7.3	124.1	5.6±2.0	4.3±0.0	2.1±0.6	ND	ND	0.3±0.0	ND	1.2±0.3	ND
		30	15.0	34.303	0.95	6.4	108.6	5.3±1.5	3.3±0.0	2.2±0.2	ND	ND	0.7±0.0	ND	1.3±0.1	ND
		40	15.0	34.287	1.01	9.4	134.5	6.1±1.0	3.2±0.7	2.0±0.4	ND	ND	0.5±0.2	ND	0.8±0.2	ND
		60	15.6	34.293	0.95	6.1	116.7	7.1±0.9	5.2±1.3	1.9±0.5	ND	ND	0.9±0.1	ND	1.7±0.1	ND
		100	14.8	34.414	0.20	7.4	121.6	3.3±0.8	2.0±0.1	2.0±0.3	ND	ND	0.1±0.0	ND	0.2±0.0	ND
	23 Feb 2007	0	14.8	34.434	5.50	18.5	175.6	14.6±1.2	3.4±0.4	3.6±0.3	ND	ND	8.0±0.0	3.4±0.2	4.6±3.2	10.3±0.8
		10	15.0	34.497	5.17	21.6	203.0	16.2±1.6	3.9±0.6	3.3±0.4	ND	ND	7.2±0.2	3.2	10.4±0.4	11.6
		20	15.2	34.577	5.03	20.1	192.6	13.5±1.7	2.8±0.2	3.7±0.5	ND	ND	5.9±0.2	1.4±0.1	7.1±0.1	3.2±0.1
		30	15.0	34.563	5.15	17.5	173.5	13.9±1.5	1.9±0.6	3.3±0.6	ND	ND	5.4±0.3	1.5±0.0	5.2±0.2	5.1±0.7
		40	14.7	34.495	4.94	22.7	210.0	13.7±1.8	2.1±0.1	2.8±0.6	ND	ND	4.9±0.3	0.3±0.0	3.2±0.2	0.2
		60	15.2	34.560	4.52	15.6	172.2	13.1±2.2	1.6±0.3	3.0±0.3	ND	ND	5.3±0.6	2.5±0.6	5.3±0.1	4.2±0.3
		100	15.1	34.536	2.42	13.3	138.2	10.9±1.6	1.9±0.7	2.6±0.6	ND	ND	2.0±0.3	0.1±0.0	1.2±0.0	0.3±0.0
	16 Mar 2007	0	15.5	34.552	11.43	37.4	359.1	8.9±1.2	2.2±0.1	7.5±1.0	ND	ND	16.6±0.6	2.9±0.3	9.9	3.4±0.7
		10	15.5	34.543	11.89	31.3	290.5	8.4±0.8	1.3±0.1	6.4±0.9	ND	ND	20.3±1.3	1.0±0.0	5.6	2.0±0.1
		20	15.2	34.510	11.86	32.7	245.5	8.2±0.5	1.4±0.1	6.7±1.2	ND	ND	14.8±0.0	1.4±0.1	5.8	1.8±0.2
		30	15.0	34.509	11.05	28.6	273.5	8.1±1.0	1.5±0.1	5.7±1.0	ND	ND	10.2±0.1	1.7±0.2	2.6	0.4±0.0
		40	15.1	34.513	10.78	26.4	239.1	8.2±0.9	1.8±0.4	6.6±0.8	ND	ND	8.9±0.3	1.9±0.1	3.5	2.2±0.1
		60	15.1	34.522	8.40	21.6	207.6	7.2±0.9	1.7±0.5	6.6±0.9	ND	ND	11.1±0.3	1.0±0.1	1.7	0.2±0.0
		100	14.4	34.445	0.52	6.6	112.6	5.7±0.8	0.5	4.7±0.8	ND	ND	0.3±0.0	0.0±0.0	0.6	0.1
	11 Apr 2007	0	15.7	34.228	7.23	34.3	304.1	16.8±2.7	1.9	3.2±0.5	ND	ND	68.3±1.2	16.9±0.1	53.3±19.5	56.2±1.8
		10	15.7	34.392	7.30	38.1	306.6	14.2±2.4	2.0±0.5	2.9±0.3	ND	ND	31.9±1.2	3.7±0.1	56.3±17.9	4.5±0.3
		20	15.7	34.402	5.40	28.6	245.5	14.6±2.2	2.5±0.1	3.2±0.6	ND	ND	19.1±0.9	2.5±0.3	17.5±0.5	3.7±0.0
		30	15.8	34.412	4.84	19.6	198.1	10.3±1.5	2.1±0.5	3.0±0.7	ND	ND	9.1±0.0	0.6±0.1	10.0±0.3	0.5±0.1
		40	15.7	34.415	4.47	14.8	168.5	9.2±1.1	1.7±0.0	ND	ND	ND	11.5±0.1	4.7±0.3	10.7±0.2	6.0±0.0
		60	15.5	34.476	1.48	14.1	146.9	6.4±1.1	1.5±0.2	4.2±1.1	ND	ND	4.9±0.1	1.7±0.0	4.0±0.3	2.0±0.0
		100	14.7	34.459	0.48	9.0	121.6	4.6±0.7	1.1±0.2	2.9±0.6	ND	ND	1.4±0.0	0.1±0.0	0.8±0.1	0.2
	14 May 2007	0	18.8	34.423	5.61	35.5	353.1	21.0±3.2	0.8±0.2	2.1±0.3	ND	ND	41.1±0.0	14.3±2.1	46.1±5.8	32.6±0.2
		10	19.0	34.479	5.04	32.4	300.8	13.7±3.4	1.2±0.1	2.0±0.4	ND	ND	48.0±1.2	11.8±0.5	22.9±2.3	18.3±2.0
		20	19.0	34.508	4.49	35.9	324.9	11.5±1.8	1.5±0.2	1.6±0.5	ND	ND	27.5±2.1	5.7±0.8	38.7±3.4	6.9
		30	19.0	34.501	3.81	25.8	302.1	13.7±3.1	1.3±0.2	2.1±0.3	ND	ND	20.4±0.2	3.8±0.0	29.1±7.1	5.7±1.3
		40	18.8	34.481	3.32	26.9	244.8	10.6±2.3	0.9±0.1	2.3±0.3	ND	ND	10.3±0.5	1.2±0.1	4.5±0.0	1.5±0.1
		60	18.5	34.485	1.98	24.0	194.1	10.5±2.7	1.1	2.7±0.6	ND	ND	11.5±0.4	2.1±0.1	13.8±1.0	2.6±0.2
		100	18.0	34.412	0.58	12.5	149.4	5.8±1.3	2.0±1.0	2.5±0.5	ND	ND	5.3±0.0	1.8±0.4	16.4	3.4±0.1

ND, no data

Table 1-2 Summary of all data

								Bacteria			SMPs		Production: Attached Bacteria		Production: Free-livivng Bacteria	
Sta.	Date	Depth (m)	Temp (℃)	Sal (PSU)	Chl a (mg m^{-3})	PON (mg m^{-3})	POC (mg m^{-3})	Total ($\times 10^5$ cells ml^{-1})	PC ($\times 10^4$ cells ml^{-1})	Virus ($\times 10^6$ PV ml^{-1})	>0.5 μm ($\times 10^6$ P ml^{-1})	<0.5 μm ($\times 10^6$ P ml^{-1})	C Production (μgC L^{-1} d^{-1})	Dubling Time (10^8cells L^{-1} d^{-1})	C Production (μgC L^{-1} d^{-1})	Dubling Time (10^8cells L^{-1} d^{-1})
M	14 Jun 2007	0	21.5	33.730	4.56	48.7	429.7	9.9±2.4	4.4±0.1	10.2±1.4	1.7±0.4	2.4±1.4	63.7±4.2	14.4±1.6	101.5±6.7	50.3±3.6
		10	21.0	34.028	3.07	32.8	275.6	9.9±1.2	3.7±0.5	18.4±3.9	1.9±0.6	4.0±1.6	5.9±0.0	1.0±0.1	6.3±0.0	1.1±0.2
		20	20.9	34.333	1.95	19.1	196.2	10.4±3.5	2.8±0.2	11.5±2.7	1.5±0.4	4.7±1.5	9.4±0.3	2.3±0.1	14.9±0.5	2.6±0.0
		30	20.5	34.275	1.91	18.6	186.7	9.6±1.9	2.8±0.5	16.7±2.9	1.6±0.5	5.6±1.3	9.6±0.3	4.4±0.2	14.0±0.1	2.9±0.1
		40	19.0	34.242	1.52	14.6	157.1	9.3±1.4	2.6±0.3	5.3±1.2	2.3±0.3	5.0±1.0	3.7±0.0	0.9±0.1	5.2±0.2	1.2±0.0
		60	16.5	34.477	0.21	7.9	138.0	2.6±0.7	2.1±0.2	3.7±0.8	0.9±0.3	3.7±2.5	3.4±0.3	0.5±0.0	4.9±0.0	0.9±0.0
		100	15.0	34.463	0.07	6.7	159.9	2.5±0.3	1.5±0.1	2.3±0.4	1.3±0.4	7.4±2.3	1.9±0.1	0.2±0.0	1.8±0.0	0.4±0.1
	9 Jul 2007	0	22.5	33.106	6.04	77.5	731.4	25.2±3.9	4.7	6.1±1.0	6.2±2.3	13.4±3.7	96.2±3.3	31.9±0.6	99.8±8.8	38.9±2.2
		10	22.2	33.511	3.57	44.3	378.3	24.6±4.9	3.8±0.2	6.8±0.9	2.8±1.0	14.0±3.1	91.9±6.2	41.0±5.8	133.7±4.5	32.4±0.7
		20	21.5	34.057	2.71	29.2	273.8	12.9±1.9	4.3±0.0	1.9±0.4	2.3±0.7	17.9±6.6	28.4±0.3	9.3±0.6	42.3±0.7	13.2±0.0
		30	18.6	34.391	0.76	13.6	161.1	5.9±0.9	3.8	16±0.3	3.6±1.3	17.9±3.1	5.5±0.2	0.9±0.2	4.6±0.3	0.3±0.0
		40	17.1	34.491	0.43	9.9	117.7	4.9±0.8	3.4±0.1	0.9±0.1	3.9±1.4	12.8±1.8	2.3±0.1	0.3±0.0	0.6±0.0	0.3±0.0
		60	16.2	34.488	0.34	6.2	105.9	3.6±0.5	2.3±0.2	0.7±0.2	1.0±0.6	10.3±4.0	1.6±0.0	0.2±0.0	1.2±0.1	0.2±0.1
		100	14.9	34.481	1.00	6.6	105.3	2.3±0.6	3.0±0.1	0.6±0.1	2.4±0.8	13.9±2.2	1.1±0.0	0.2±0.00	0.9±0.1	0.5±0.0
	15 Aug 2007	0	28.5	32.174	5.74	74.6	589.2	45.8±10.3	16.4±2.9	42.1±6.1	3.4±0.4	3.6±1.6	340.2±12.9	44.6±3.2	229.7±11.8	24.7±1.5
		10	26.8	33.487	1.58	26.5	254.6	20.7±2.2	6.6±0.3	34.5±5.2	3.0±0.9	4.6±0.9	98.8±5.3	21.9±0.6	75.7±6.1	19.3±2.2
		20	24.8	33.938	0.69	12.7	147.6	13.6±3.8	3.5±0.5	17.2±2.6	1.8±0.5	8.1±1.9	21.3±0.1	6.2±0.1	21.6±1.8	6.1±0.9
		30	22.6	34.238	0.66	11.8	139.9	13.0±2.2	2.4±0.2	11.3±1.5	2.0±0.7	6.1±1.2	7.6±0.0	2.0±0.0	5.6±0.6	1.5±0.1
		40	21.1	34.345	0.51	10.6	126.1	8.3±1.4	1.7±0.1	7.3±0.8	2.6±1.1	6.9±1.5	3.7±0.2	1.1±0.1	3.9±0.4	0.5±0.0
		60	18.0	34.509	0.10	5.1	97.1	3.6±0.9	1.8±0.8	3.9±0.7	2.0±0.5	7.6±1.2	1.4±0.0	0.1±0.1	0.8±0.1	0.2
		100	16.1	34.504	0.07	5.3	106.5	3.4±0.6	1.1±0.2	1.9±0.2	1.7±0.6	3.2±1.2	1.0±0.0	0.1±0.0	0.2±0.0	0.1
	12 Sep 2007	0	24.5	21.275	5.31	10.0	121.8	24.0±8.2	58.0	13.6±2.1	7.7±2.5	7.9±2.2	66.7±5.2	13.7±0.5	149.9±14.5	55.2±0.2
		10	25.2	32.857	2.50	7.8	104.5	10.1±1.9	27.2±19.7	10.7±1.3	5.4±0.6	4.9±0.7	15.0±0.4	3.4±0.5	27.2±0.9	4.3±0.7
		20	24.0	33.905	1.46	42.0	105.4	7.8±1.4	5.6±0.4	5.3±1.1	2.0±0.4	2.9±0.8	2.1±0.0	0.6±0.0	5.2±0.1	0.6±0.1
		30	22.9	34.086	0.42	6.5	96.6	5.0±0.6	3.8±0.5	7.9±0.9	1.3±0.5	2.2±0.9	2.4±0.3	1.4±0.2	4.5±0.4	1.6±0.2
		40	20.5	34.443	0.19	11.2	113.3	5.0±1.1	4.0±0.9	3.0±0.7	1.0±0.4	2.2±0.7	0.4±0.0	0.1±0.0	1.4	0.2±0.1
		60	17.0	34.523	0.12	11.5	118.7	2.4±0.7	3.0±0.4	1.3±0.2	0.6±0.3	2.6±0.4	0.2±0.1	0.1±0.0	0.6±0.1	0.6
		100	14.0	34.456	0.06	9.0	121.9	2.2±0.5	3.0±0.3	1.4±0.4	0.6±0.3	0.9±0.2	16.3±0.0	3.3±0.0	5.0	1.0±0.1
	19 Oct 2007	0	20.5	33.527	3.74	65.8	446.3	21.2±5.1	2.3±0.4	17.4±3.9	2.8±0.8	1.2±0.7	40.4±3.6	4.6±0.1	85.7±29.3	6.5±2.0
		10	20.5	33.524	3.86	14.7	121.4	19.5±3.1	3.3±0.2	19.7±4.3	2.6±0.5	2.5±1.1	28.8±9.4	3.1±0.3	52.3±5.5	2.3±0.3
		20	20.3	33.533	3.92	13.7	137.8	16.3±3.7	3.0±0.2	17.7±2.2	2.5±0.4	2.6±0.8	27.2±2.5	3.6±0.2	29.5±3.1	3.6±0.3
		30	20.3	33.561	3.30	8.1	153.8	11.8±1.7	2.5±0.1	13.0±1.3	1.5±0.5	2.1±0.5	26.9±0.8	7.4±0.4	29.1±2.4	8.6±0.5
		40	20.5	33.558	3.10	1.9	92.7	12.9±2.1	3.8	12.5±3.0	1.8±0.7	2.2±0.7	15.1±0.6	3.2±0.7	19.5±1.3	2.5±1.0
		60	20.3	33.849	0.67	2.2	97.7	5.5±1.3	2.0	8.0±1.6	0.5±0.3	0.8±0.4	2.4±0.2	0.4±0.1	2.8±0.4	0.7±0.1
		100	17.5	34.431	0.11	1.8	94.0	3.3±0.9	0.6±0.2	1.9±0.5	0.4±0.2	0.8±0.4	0.6±0.0	0.1±0.0	0.3±0.1	0.1
	15 Nov 2007	0	20.5	33.649	1.88	12.0	141.3	3.6±0.7	1.8±0.0	12.3±1.8	0.6±0.2	2.3±0.4	2.1±0.0	0.5±0.0	1.4	1.8±0.1
		10	20.2	33.654	1.64	11.3	125.6	5.4±1.0	2.3±0.2	11.3±2.2	ND	ND	1.9±0.2	0.2±0.0	9.1±4.6	0.8±0.0
		20	20.3	33.629	1.56	9.9	114.8	3.5±0.6	3.0±0.5	13.1±2.7	ND	ND	2.3±0.2	0.7±0.0	3.0±0.2	0.9±0.2
		30	20.4	33.695	1.13	9.5	112.0	3.3±0.5	2.3±0.0	13.4±2.0	ND	ND	1.3±0.2	0.2±0.0	1.8±0.1	0.6±0.0
		40	20.2	33.701	1.07	8.2	115.6	3.7±0.8	2.1±0.3	12.2±1.4	1.0±0.5	1.7±0.9	0.9±0.1	0.1±0.0	1.3±0.0	0.3±0.1
		60	20.2	33.733	0.70	10.6	111.2	3.2±0.8	2.4±0.1	10.5±1.3	ND	ND	0.7±0.0	0.1±0.0	1.2±0.1	0.5±0.0
		100	19.8	34.153	0.14	6.2	100.8	3.2±0.5	2.0±0.2	5.8±1.3	0.5±0.1	1.3±1.1	0.5±0.2	0.1±0.0	1.1±0.4	0.1±0.0

ND, no data

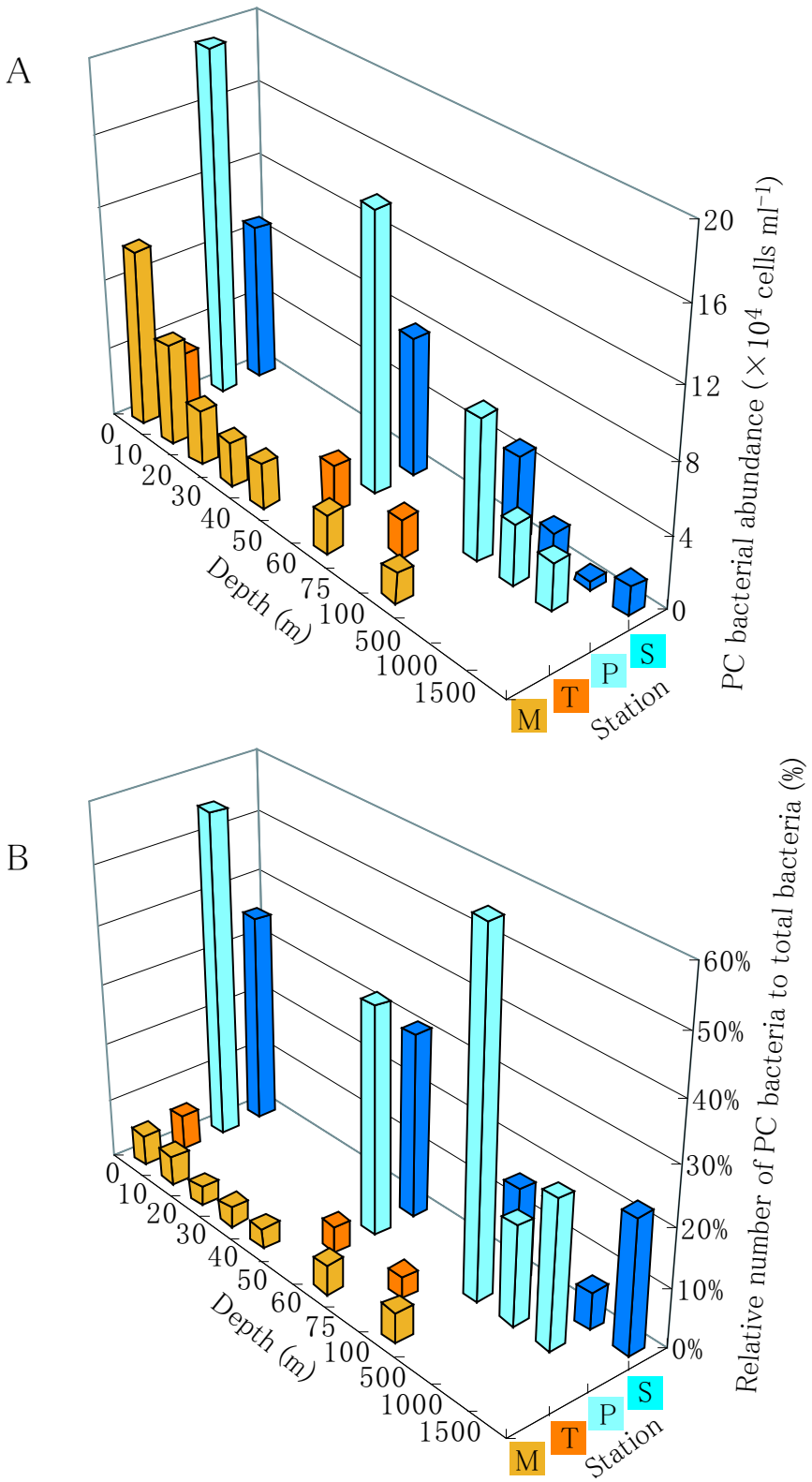


Fig 5 V ertical and holizontal abundance of particle captured bcteria (A) and its percentege of total bacteria (B) isolated from magnetical method at every stations (Sta.M indicate the average of all sampling at Sta.M)

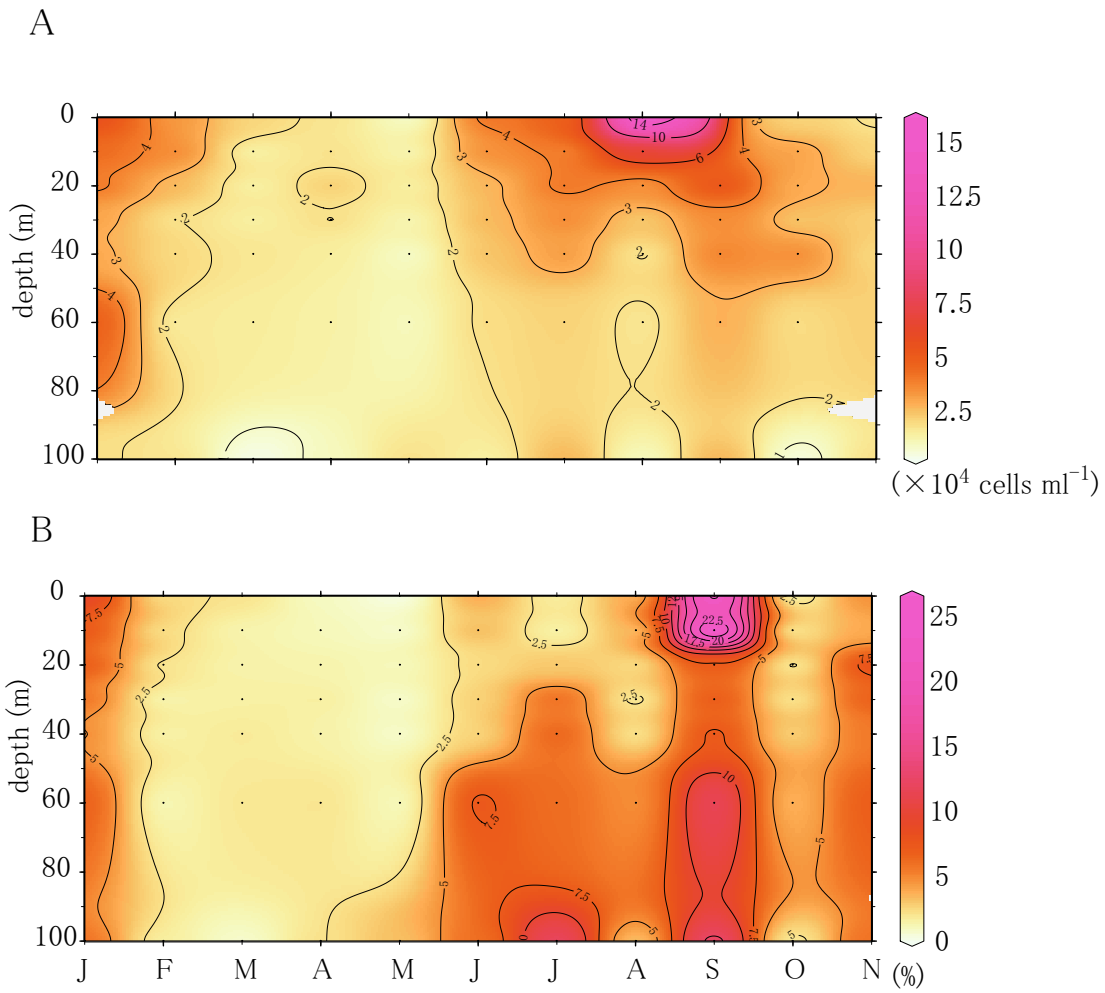
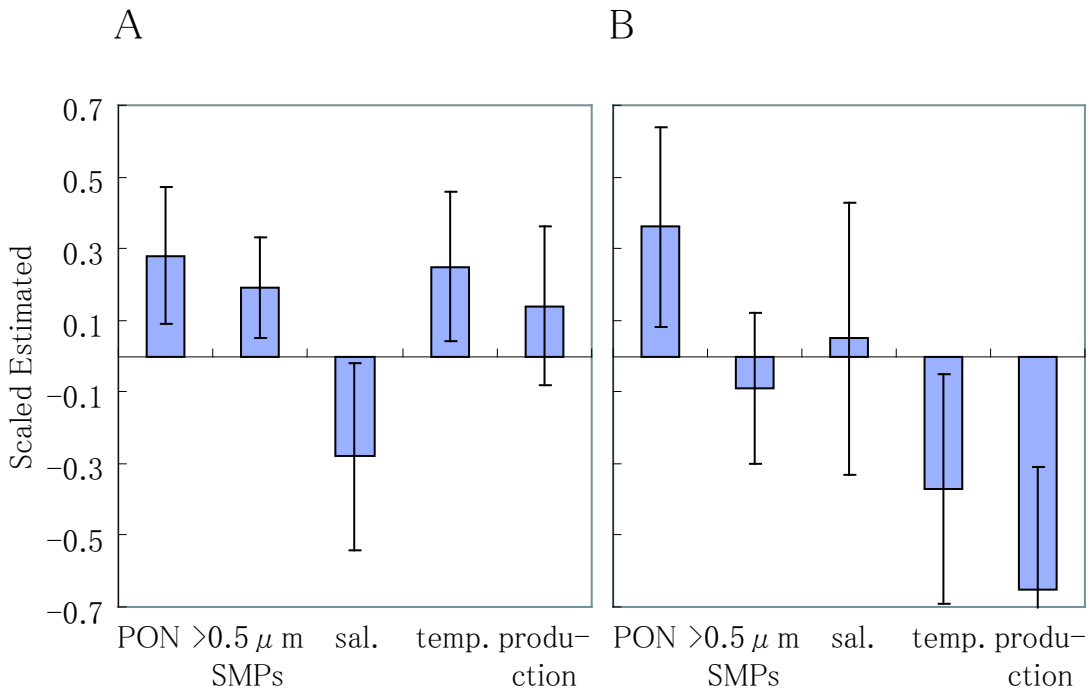


Fig 6 Seasonal abundance of particle captured bcteria (A) and relative number of PC bacteria to total bacteria (B) isolated from magnetical method at Sta.M



*production: carbon production of free-living bacteria

Fig 7 Contribution to multiple regression model for PC bacterial abundance (A) and PC bacterial % of total bacteria (B) analyzed by JMP

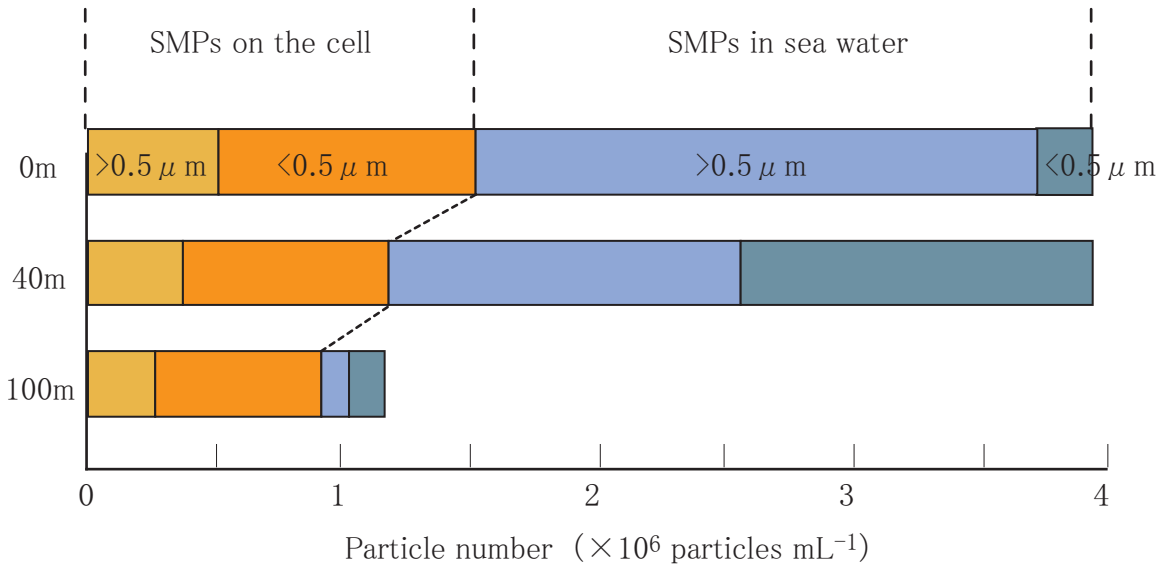


Fig 8 Total SMP number in sea water and SMP numbers on the bacterial cell surface orange showed over 0.5 μ m diameter SMPs and blue showed under 0.5 μ m. Count were conducted by AFM image in 0, 40, 100m of Ougast 14th at Sta.M.

Chapter 2 Community Structures of Particle Capturing Bacteria

2.1 Introduction

In the previous chapter, the wide distribution of bacteria with PC activity was clarified in both coastal and offshore environments. It is also evident that a considerable number of bacteria possess PC activities. However, no information on the bacterial taxonomical group that has PC activity was obtained. Whether PC activity was shared by various types of bacteria or rather restricted to certain group is a critical question when considering the ecological role of each phylogenetic group in the ocean. Seo et al. (2007) analyze the community structure of PC bacteria collected by paramagnetic model beads. They reported that the structure was dependent on both particle size and chemical nature of the particles and the former was more critical as factor. However, there was so far no work on systematic comparison between PC bacteria and total bacterial populations. In this chapter, in order to clarify the interaction between the two bacterial populations, the succession of community structure during course of monthly samplings in Sagami Bay was analyzed.

2.2 Materials and Methods

DNA extraction and PCR amplification

For the analysis of PC bacterial seasonal community structure, monthly samplings from October 2006 to November 2007 (0, 40, 100m) at Sta.M were made. Isolation method of PC bacteria was same as described in Chapter 1, but samples were not suspended with PBS after rinse twice with PBS. For the comparison of PC and free-living bacterial community structure, 1ml of sea water samples were also collected (October, November 2006 and June, July, August, October, November 2007) and centrifuged 15000 rpm for 15 min, then stored at -80℃ after supernatant was carefully removed. DNA from magnetically separated (for PC bacteria) and centrifuged samples (for free-living fraction) was obtained with a InstaGene Matrix（BIO-RAD）in accordance with the manufacturer's instructions. PCR amplification was performed with a GeneAmp 9700 PCR System (PE Applied Biosystems) using *Z-taq* (TaKaRa) and the primers of GC341f (Muyzer et al. 1993) and 907r (Muyzer et al. 1998). Initial denaturation was at 94℃ for 5 min, followed by 80℃ 5 min to reduce unexpected priming, annealing for 10 sec at 65℃, and extension for 20 sec at 72℃. Thermal cycling programs were as follows: denaturation for 2 sec at 98℃; annealing for 10 sec at an initial 65℃, then decreasing by 1℃every 2 cycles to a final 55℃ to reduce unexpected priming; and extension for 20 sec at 72℃. The total number of cycles was adjusted between 25 and 40 depending on the DNA concentrations of the templates to avoid PCR artifacts checked by electrophoresis with a molecular mass standard (EzLoad DNA Mass Ruler).

Denaturing Gradient Gel Electrophoresis (DGGE)

Two hundred ng of PCR product was analyzed by DGGE as described in Yoshida et al (2006). Bands in the DGGE profiles were detected with ImageGauge 4.0 software (Fuji Photo Film); a band was judged as being present if the ratio of its peak height to the total peak height was > 1% in each lane. The DGGE banding pattern was converted into a binary matrix indicating the presence (1) or absence (0) of each band in all lanes. The similarities of bacterial community structures between the samples were visualized from an MDS map based on a distance matrix calculated from the binary matrices. The MDS analysis was conducted with the software package SPSS (version 10.1 for windows).

2.3 Results

The banding patterns on the DGGE showed that dominant PC bacterial members and free-living bacterial members were distinct from each other over the sampling period, even though total bands numbers of PC bacteria (ca. average 44.4 bands) and free-living bacteria (ca. average 41.7 bands) did not differ much. In Fig 9A, 10A and 11A, the arrows indicate the ones present only among PC bacteria. Some of the bands of PC bacteria existed in all months.

MDS analysis clarified dominant PC bacterial members and free-living bacterial members were distinct from each other and the community structures were gradually shifted over the sampling period. Another result is that the extent of diversity of PC bacterial community was larger than that of free-living bacteria. (Fig. 9B, 10B and 11B).

2.4 Discussion

Whether specific bacterial groups show PC activity or the ability is widespread among various phylogenetic groups is an important question. The results indicate that there are at least some specific species that possess the PC activity in the ocean. Certain specific bands were present in all seasons.

The MDS map of PC bacteria tended to show two clusters in all depths; one is from October 2006 to May 2007, and another is from June to November 2007. This could be because thirmocline was present from June and the watermass was separated into upper and deeper layers. The chemical characteristics and retention time of organic matter are usually different between the two layers. For instance, freshly produced organic materials are present in the upper layer whereas metabolized compounds such as detrital particles, faecal pellets or marine snow may be more abundant in the deeper layer. The two clusters could be explained by the difference in such nutritional conditions and also bacterial responses to those nutrient status.

In conclusion, the community structures of PC bacteria and free-living bacteria were not always

same. The seasonal variation of the structures of PC bacteria seems to be larger than those of free-living bacteria. This strongly suggests the varieties of bacteria are involved in PC processes.

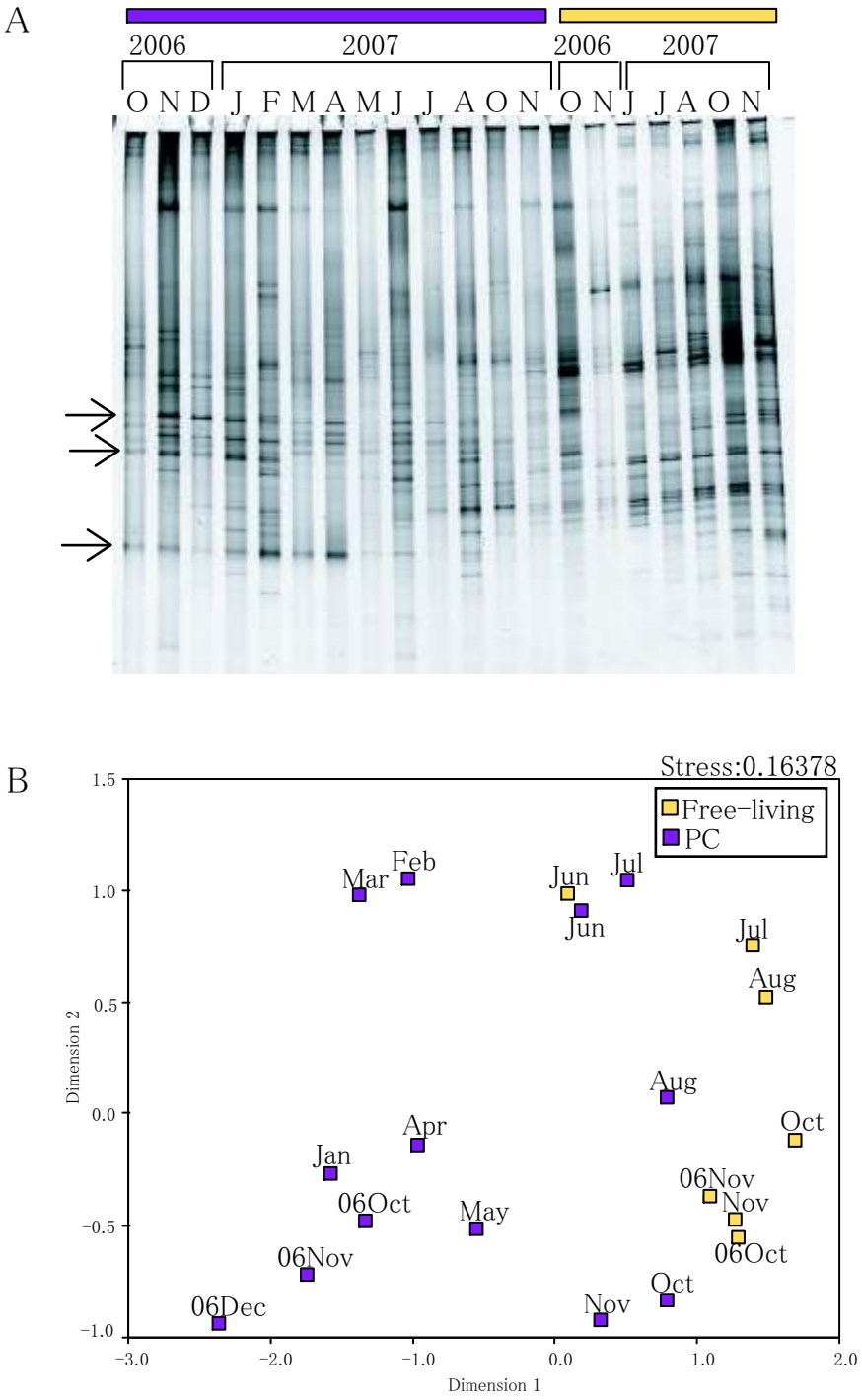


Fig 9 Seasonal DGGE banding profiles (A) and MDS map (B) of free-living and PC bacteria iolated by 0.5 μ m paramagnetic particles separation method at Sta.M 0m from October 2006 to December 2007

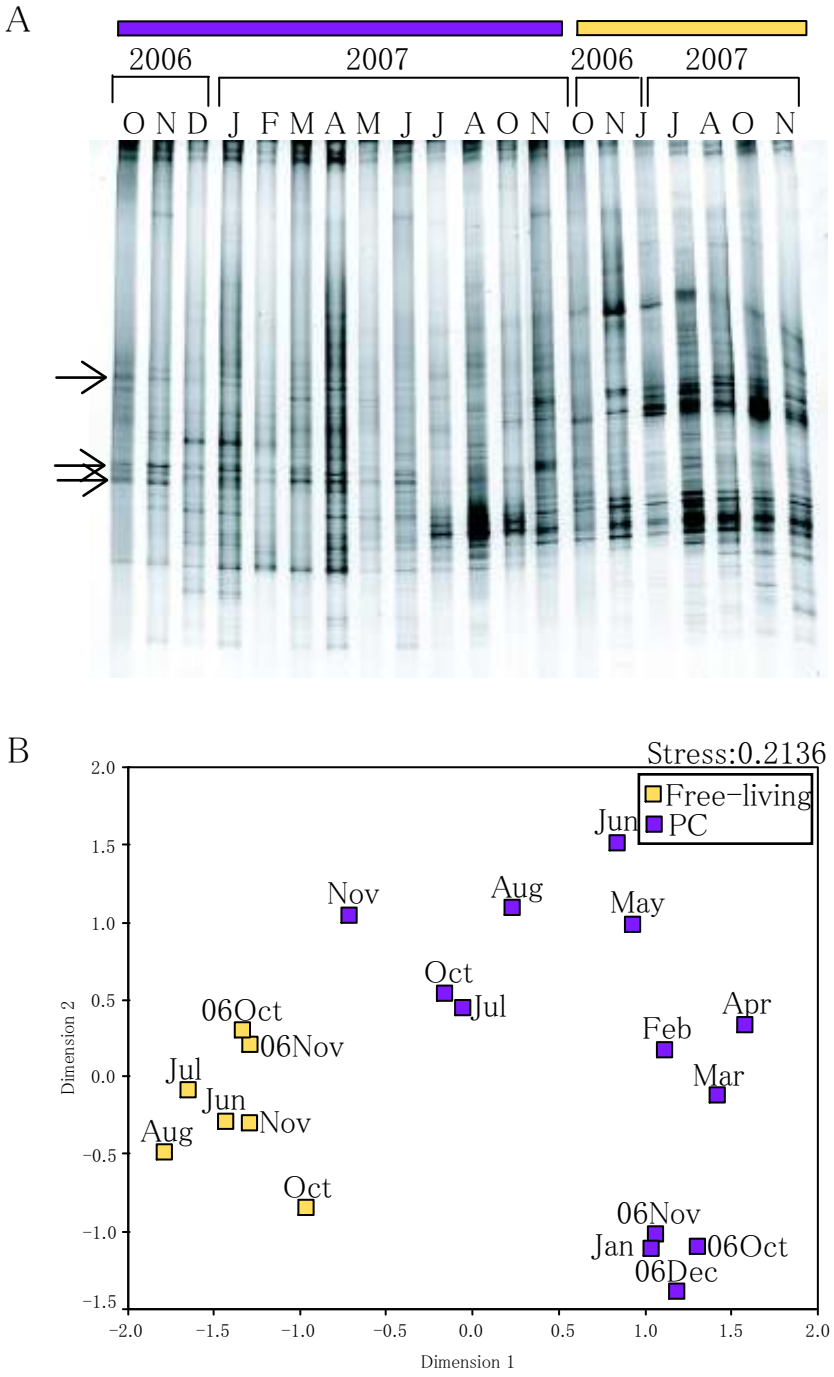


Fig 10 Seasonal DGGE banding profiles (A) and MDS map (B) of free-living and PC bacteria iolated by 0.5 μ m paramagnetic particles separation method at Sta.M 40m from October 2006 to December 2007

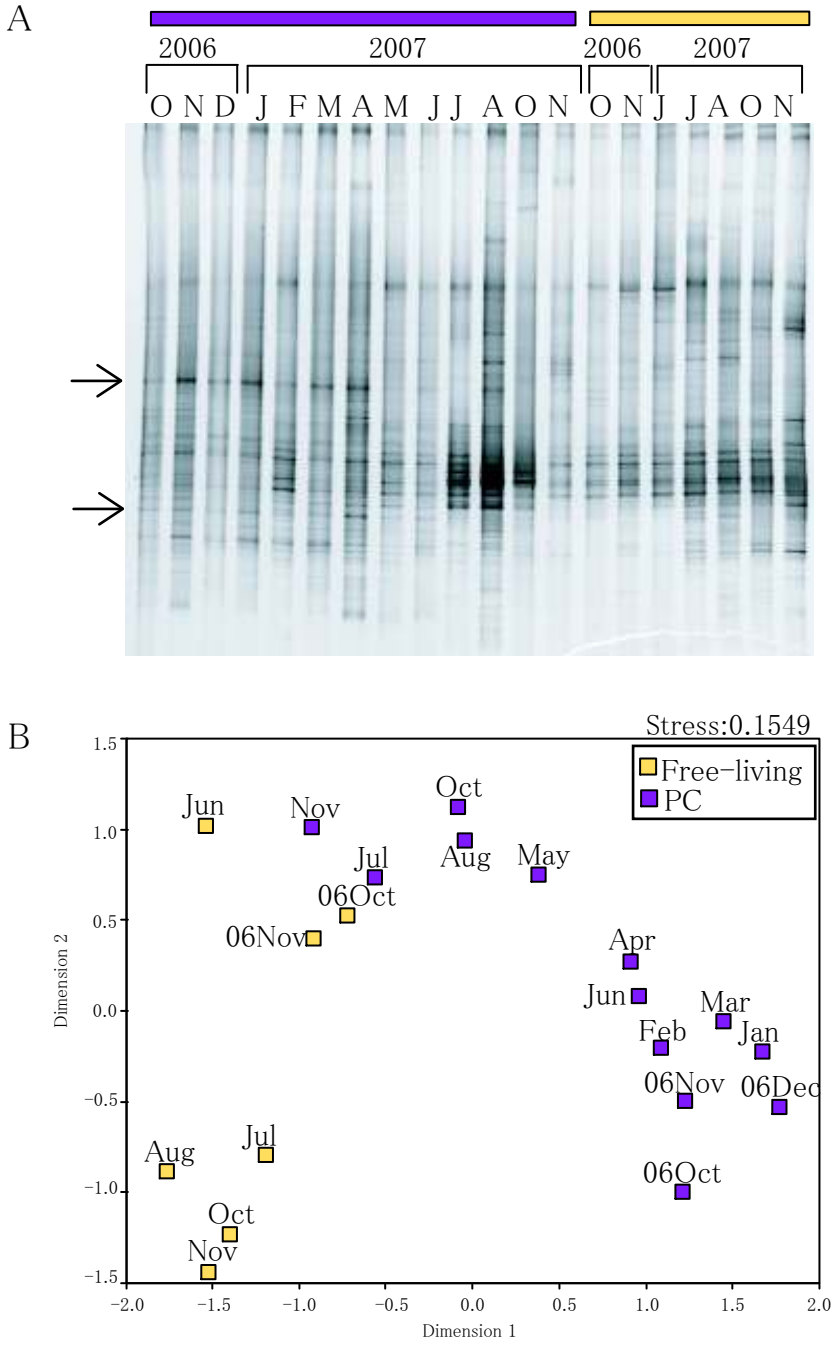


Fig 11 Seasonal DGGE banding profiles (A) and MDS map (B) of free-living and PC bacteria iolated by 0.5 μ m paramagnetic particles separation method at Sta.M 100m from October 2006 to December 2007

Chapter 3 Enzyme Activity of Particle Capturing Bacteria

3.1 Introduction

The previous chapters clarified the wide distribution of PC bacteria in the ocean and their community structure. It was strongly suggested that variety of bacteria have particle capturing activity. However, there is no data whether PC activity is associated with higher metabolic activities. The original hypothesis of PC activity may be meaningful only when the capturing activity is coupled with the efficient degradation and subsequent uptake. Therefore, it is crucial to know whether PC bacteria have higher enzymatic activities.

The purpose of this chapter is to examine the enzymatic activity of both PC bacterial and free-living bacterial cells. As the model substrate, fluorogenic peptide analog substrate was applied to natural samples.

3.2 Materials and Methods

For detection of enzymatic activity, PC bacteria were collected at Sta.M four times (15 August 0, 40, 100m, 19 October 0m, 20 December 0m). Method for the isolation of PC bacteria was same as described in chapter 2 except for using additional type of paramagnetic particles (casein-coated 0.17μm chemicell). Twenty μl of 0.17μm particles suspension (ca. 5.6×10^{14} particles ml^{-1}) was added to 1ml sea water and treated as same as 0.5μm. Because enzyme activity exist in < 0.2μm fraction in the ocean, water samples were divided into four parts (Total, attach, free-living, dissolved) by filtration through 3μm and 0.2μm filter (Nuclepore) in order to measure only PC bacterial enzyme activity. Free-living bacteria were separated into PC and non-PC bacterial fraction by magnetic isolation method. To estimate ectoenzymatic activity of PC bacteria, the activity of non-PC bacteria were measured and subtracted from free-living bacterial activity. This is because isolated PC bacteria could not be separated from paramagnetic particles which interrupt proper measurement of fluorescence peak. In addition, activities in the five parts of water sample (unfiltered sea water, <3μm sea water, <0.2μm sea water, 1h incubated <3μm sea water and 1h incubated <0.2μm sea water) were examined to remove the biases of enzymatic interaction with beads during incubation or attachment of the wall of tubes. Enzyme activities were assayed using fluorogenic peptide analog substrate (Leu-MCA, Peptide Institute) as described by Obayashi and Suzuki (2005) with some modifications. Briefly, sample and substrate solution were dispensed into disposable cuvette and well mixed. The final volume of each mixture was 2.5 ml and the final substrate concentration was 100 μmol L^{-1}. Mixtures of water sample and substrate were incubated at 25 for 1 to 5 h. Before and after the incubation, the fluorescence of the hydrolytic product, 7-amino-4-methylcoumarin (AMC), was measured with a spectrofluorometer (Shimadzu RF-1500)

at an excitation/emission wavelength of 380/460 nm. After subtracting the blank fluorescence intensity of each sample (which was measured without an added substrate), the fluorescence was calibrated using the slope of a calibration curve obtained from several concentrations of AMC. As a control, autoclaved filtered seawater was assayed using the same method to determine the non-enzymatic produced AMC, then it was confirmed that AMC produced non-enzymatically was negligible in this study. The hydrolysis rate of substrate, namely the enzymatic activity in each water sample, was estimated from the change in the concentration of AMC in the cuvette during incubation. After determining the enzymatic activity of each water sample, the ectoenzymatic activity of PC bacteria was calculated for nanomoles of substrate per cell per hour using estimated activities and cell count data.

In all stations, samples for community structure analysis were collected and analyzed by DGGE simultaneously with enzymatic activity. The procedure of DGGE was same as described in chapter 2.

3.3 Results

In all stations, PC bacteria had higher activities (ave. 5.8 times higher) than free-living bacteria. The activities also differed when different size of paramagnetic particles were used for the separation. The specific enzymatic activities of PC bacteria collected with 0.5μm and 0.17μm had 7.2 and 4.4 times higher than those of free-living bacteria, respectively (Fig 13).

DGGE data showed different community structure between 0.5μm PC, 0.17μm PC. However, the difference of community structure was more affected by depth rather than size of the particles.

3.4 Discussion

PC bacteria had higher enzymatic activities than free-living bacterial activity in all experiments even though PC bacterial community structures were different in each sampling time. The results strongly suggest that PC bacteria have generally high activity in the ocean. This also indicates that PC bacteria actually degrade the particles on their cell surface.

Slightly higher enzymatic activity was confirmed when larger particles were used. This reason is not clear from the present work. In general, attached bacteria have higher activity than free-living bacteria (Karner 1992, Azam and Smith 1991, Grossart 2007). The present result is somewhat similar, suggesting a common mechanism between PC bacteria and attached bacteria. The bacteria that can retain larger particles may be able to harbor more enzymes on their cell surface and/or their cellular size may be larger. Further works needs to be done with different sizes of particles under different condition to confirm the factor involved. In addition, only one type of

substrate was used in this work. It is expected that depending on the enzyme in question, the apparent results may vary. It is also noteworthy that bacteria collected by the paramagnetic particle methods are surrounded by many those particles when assayed. The possibility of some biases in the apparent activity is not completely rejected.

In conclusion, bacteria with PC activity have higher enzymatic activity per cell. This strongly indicates that PC activity is coupled with enzymatic degradation processes that should proceed on the cell surface. Further study with variety of model substrates and observation of subsequent uptake process should further clarify the significance of PC activity in the degradation processes.

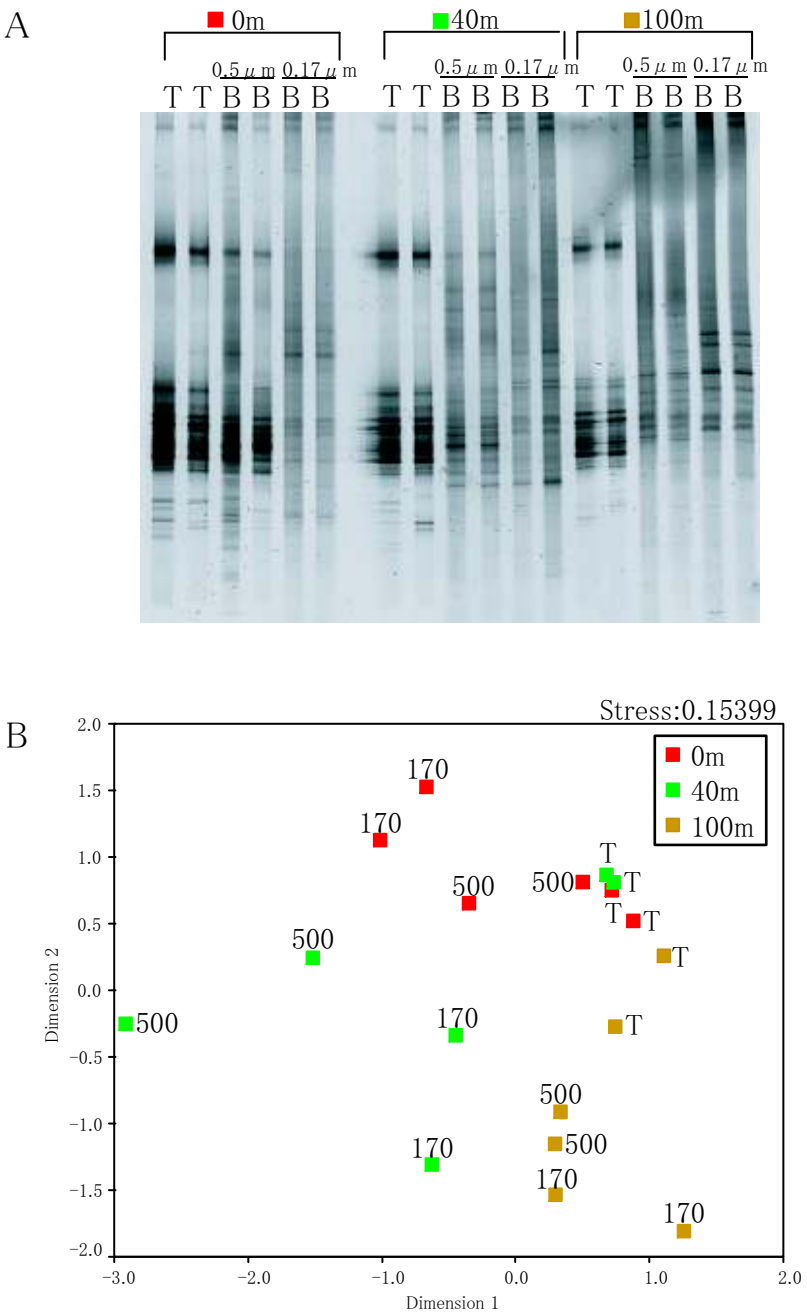


Fig 12 DGGE banding profiles (A) and MDS map (B) of total bacteria and particle captured bacteria recovered from 0, 40, 100m at Sta.M in October 19 by paramagnetic particles of 0.5 μ m and 0.17 μ m

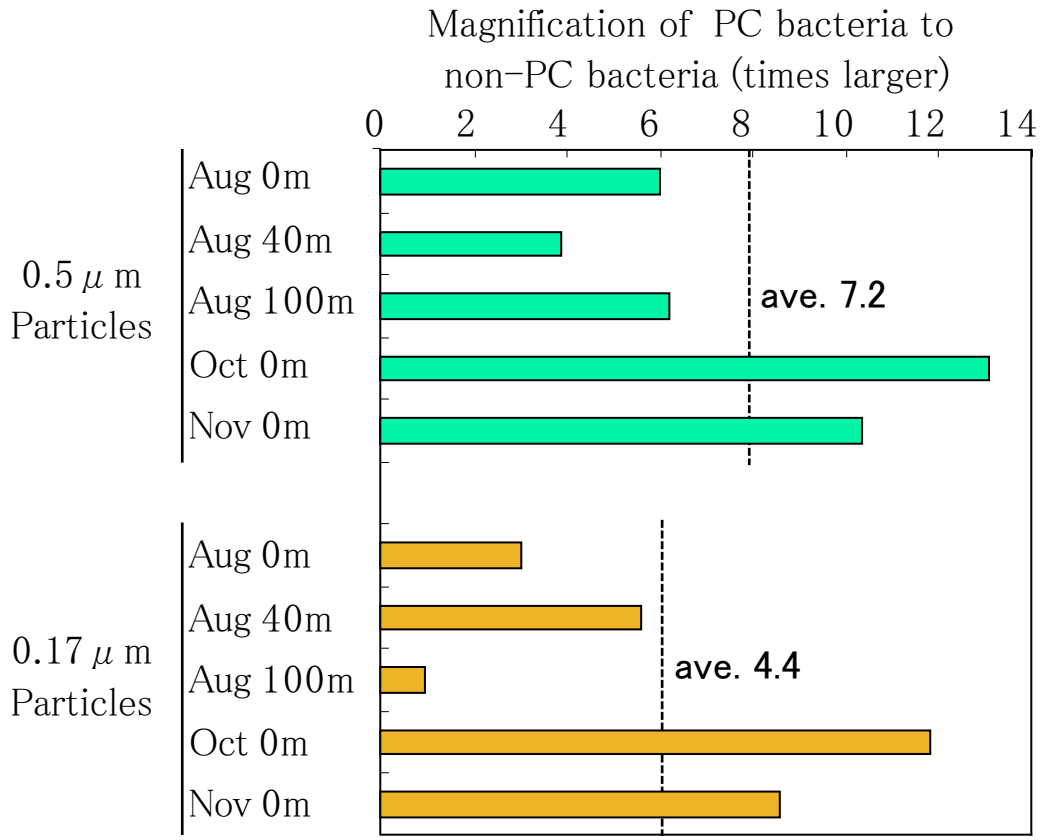


Fig 13 Magnification of PC bacterial enzymatic activities to non-PC bacterial enzymatic activities. Samples were collected in Aug (0, 40, 100m), Oct (0m), and November (0m) using 0.5 and 0.17 μ m diameter paramagnetic particles at Sta.M.

Discussion

In this research, distribution, community structure, enzymatic activities of PC bacteria were investigated for coastal and offshore environments. In addition, the presence of submicron particles on the cell surface was directly confirmed by AFM.

The Paramagnetic separation methods proved to be very useful for the isolation of the bacteria which possess the particle capturing activity. This method enables us not only to measure the abundance and community composition, but also to measure the enzyme activity of PC bacteria. Further information on cellular activity and physiological characteristics should be available by this method. However, the cells collected are regarded as those quickly capturing added model particles. It is not clear whether those cells also possess high capturing activity for naturally occurring particles. It is also not clear whether cells with PC activity can be all recovered by this method. Another drawback is that the cells thus recovered are surrounded by full of those artificial particles and this may cause problems when measuring any activity or parameters. Therefore, development of other method to collect the cells will be desired. Also development of a method that enables us to measure the capturing activity and rate of naturally occurring particles will greatly enhance the knowledge on the PC activities.

The average numbers of PC bacteria were 3.8×10^4 cells ml^{-1} (coastal area) and 6.8×10^4 cells ml^{-1} (oceanic area) in all sampling points when 0.5μm diameter particles was used. These numbers correspond to 4.4% (coastal area) and 29.5% (oceanic area) of total bacteria. Therefore, bacteria with PC activity are quite widespread in the marine environments. This was confirmed by the direct observation using AFM. Currently, very few scientists are applying AFM to natural bacterial cells. In addition, virtually nobody has ever seriously looked at the particle-bacterial interactions. The observation of those particles by electron microscopy should be difficult because of tedious sample pretreatments and possible change in the numbers of particles on cell surface. To my knowledge, this is the first investigation on the submicron particles associated with bacterial cells. It is surprising at relatively high numbers of particles were present on the cell surface. This strongly indicates that bacterial particle capturing activity may play significant role in the degradation and turnover of organic material in the ocean, especially in oligotrophic area and deep sea.

The number or relative number of PC bacteria to total bacteria may vary with various environmental factors. The nutrient status should be most important factor. Figure 14 showed the image of particles in coastal and oceanic water. In the coastal eutrophicated area, the concentration of dissolved organic matter and extracellular enzyme activities may be high. Bacteria may be able to take up dissolved organic material easily and the capturing activity is not required. In the oceanic

environments, on the other hand, the concentration of truly dissolved organic materials those are directly available for bacteria should be much low and bacteria need devices to seek for and gain organic materials efficiently. From the point of concentration, it is expected that PC activity is of considerable importance in geochemical cycles in the ocean. The high enzyme activities of PC bacteria (ca. ave 7 times higher than free-living bacteria) in all experiments support this general view.

The AFM observation clarified that natural bacterial cells have about 3 particles on their surface (Chapter 3). Assuming that the carbon contents of colloidal organic carbon (COC) as 8.6μM C (Yamazaki 1998), total carbon amount of SMPs counted by AFM in the ocean as 5.19Gt., total carbon of SMPs on the bacterial surface accounts for 2.66Gt which is 51.1% of total SMPs, free-living bacterial carbon productions measured by Leucine incorporation rate is 20.8μg C L^{-1} d^{-1}, PC bacterial carbon productions were same as those of free-living bacteria, PC bacterial carbon productions reach 6.23μg C L^{-1} d^{-1}. This means that PC bacteria can degrade SMPs on their cells within 7.5h.

In conclusion, the presence, distribution and significance of bacteria with PC activities were clarified for the first time. As this concept is quite new, there remains many question, such as the physicochemical mechanism of the PC activity, the involvement of cell surface structure, similarity and dissimilarity with attachment process, the rate and significance of material cycles coupled with PC activity, the production and fate of organic particles in the ocean and the contribution of PC bacteria to those geochemical cycles. In future, various works should be done to further establish the concept of PC activity and answer the question raised above.

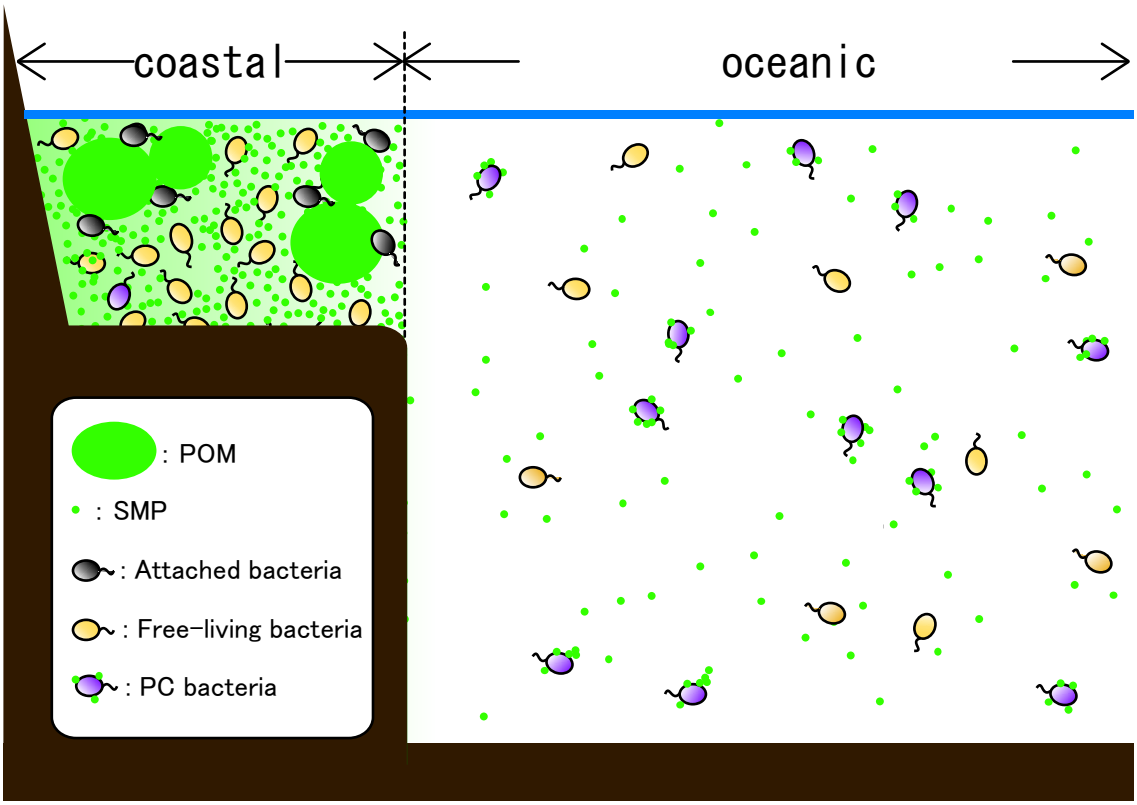


Fig 14 Image of coastal and oceanic sea water

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粒子捕獲能力を持つ海洋細菌の動態

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キーワード;海洋細菌、サブミクロン粒子、粒子捕獲

1. はじめに

海水中には様々な粒子が存在する。1990 年に、いわゆる溶存態とされてきた画分に約 10^7 個 mL^{-1} のサブミクロン粒子が存在することが報告され(Koike et al. 1990)、海水中の粒子はそのサイズが小さいほど数が多いことが明かになった。サブミクロン粒子とは、0.38ー1.0μm のサイズで、主に生物起源と考えられる。一方、海洋細菌は海水 1mL に約 10^6 細胞程度存在するので、微細なコロイド粒子等を含めれば、海洋細菌はその 10ー100 倍量の微小粒子に囲まれていることになる。

細菌は、あらかじめ菌体外で有機物を酵素によって分解し、分子量数百程度以下にまでに小さくした後、菌体内に取り込む。細菌がデトリタスやマリンスノーなどの大きな有機物粒子に付着している場合、その化学組成を認識し、適切な酵素を合成、分泌して分解することができる。では、菌体より小さな微小粒子の場合、細菌はそれらの存在をどのように認識し、どのような酵素を生産してどのように分解するのだろうか。従来の付着細菌のシナリオではこの説明ができない。Seo et al.（2007）は、微小な粒子を菌体表面に保持する能力のある“捕獲細菌”の概念を提案した。磁性有機物粒子をモデル粒子として用い、海水中の細菌群集の約 1 割に粒子捕獲能力があること、これらの捕獲細菌は固有の分類群からなることを示した。しかし、実際に細菌が天然海水中の微小粒子を捕獲しているのか、捕獲がより効率のよい分解に繋がっているかは不明である。

そこで本研究では、サブミクロン粒子を捕獲する海洋細菌の自然環境下での動態を明らかにすることを目的に、沿岸から外洋域の海水中の捕獲細菌数、その群集構造、酵素活性を調べるとともに、高解像度の顕微鏡を用いて、実際に粒子を保持している細菌数、それらの粒子数などを調べた。

2. 試料及び方法

2006 年 12 月の淡青丸の航海で得られた Sta.T、 P、 S の 3 測点と、相模湾西部真鶴沖の観測定点 Sta.M において 2006 年 10 月から 2007 年 11 月まで毎月採取した海水を試水として用いた。デキストランベースの 0.5μm の磁性ビーズをモデル粒子として海水 1mL に加え、1 時間ゆっくりと振とうさせた後、磁石で回収される細菌を粒子捕獲細菌とした。分離した捕獲細菌の菌数・群集構造・活性を、また自然海水中の細菌の観察から、実際に粒子を持つ細菌数とその粒子数を測定した。さらに捕獲細菌の変動が何によって影響されているのかを知るために環境パラメーターを測定し解析した。以下に測定項目と、その方法をまとめた。

①捕獲細菌数:0.5μm のモデル粒子で分離した細菌を DAPI 染色後、蛍光顕微鏡計数

②捕獲細菌の変動要因:海水の環境データから捕獲細菌の数や割合の予測式を、解析ソフト JMP を用いて重回帰分析

③群集構造:0.5μm のモデル粒子で分離した細菌の DNA を抽出し、Denaturing Gradient Gel Electrophoresis（DGGE）法により群集構造解析

④有機物分解活性:0.5μm と 0.17μm のモデル粒子で分離した細菌を使って蛍光標識基質を用いてロイシンアミノペプチダーゼ活性の測定

⑤粒子を持つ細菌数とその粒子数:自然海水を孔径 0.2μm のフィルターで濾過し、原子間力顕

微鏡(AFM)で観察することで菌体上に粒子をもつ細菌を 100 細胞計数し、そこに存在する 0.2～0.5μm および 0.5～1μm の範囲にあたるサブミクロン粒子を計数

環境データとしては、温度、塩分、クロロフィル濃度、懸濁態有機物濃度(POC、PON)、全菌数、ウィルス数、直径 0.2～0.5μm のサブミクロン粒子数、直径 0.5～1μm のサブミクロン粒子数、浮遊細菌、付着細菌の生産速度をそれぞれ測定した。

3. 結果及び考察

得られた結果とその考察を以下にまとめる。

①磁性モデルビーズを用いて求めた捕獲細菌数は一般にいずれの海域でも約 10^3～10^5cells mL^{-1} で、全菌数の 0.4～29.5%を占めた。沿岸域の Sta.M、T では捕獲細菌の全菌に対する割合が低く、1000m 以深の外洋域、Sta.P、S では高くなった。このことより、捕獲細菌は特定の海域や全菌数に依存することなく広く分布することが確認された。

②捕獲細菌の全菌数に対する割合に及ぼす変動要因は、浮遊細菌の生産速度、懸濁態窒素量、直径 0.5μm 以上の粒子数、水温、塩分であった。浮遊細菌の生産速度は負の大きな影響を及ぼすことから、貧栄養的な環境下で捕獲能力がより重要になることを示している。

③Sta.M における群集組成の解析から、捕獲細菌の群集構造は浮遊細菌全体のそれとは違うことがわかった。また捕獲細菌群集の方が浮遊細菌群集より季節変動が大きいことがわかった。この結果は、限定的なグループの細菌が粒子捕獲を行っているのではなく、その環境条件に応じて様々な細菌がそれを行っていることを示している。

④捕獲細菌の単位細胞あたりの有機物分解(ロイシンアミノペプチダーゼ)活性は、粒子を捕獲しなかった浮遊細菌の平均 5.8 倍高い値を示した。また、直径 0.5μm の粒子を捕獲する細菌のほうが、0.17μm の粒子捕獲細菌よりも高い活性を示した(0.5μm で 7.2 倍、0.17μm で 4.4 倍)。この結果は、粒子の捕獲が有機物の酵素分解能とカップルしていることを示す。

⑤自然環境中の細菌を高解像度の原子間顕微鏡で観察したところ、全細菌のうち 47～64%はその表面に粒子を持っていた。また 1 細胞あたりの粒子数を平均すると 2.8 個であった。さらに、海水中の粒子を同様の方法で計数した結果と比較すると、海洋の 30～79%の粒子は、細菌上にあることが明らかになった。この結果はモデルビーズで得られた結果を支持するとともに、粒状有機物の代謝過程が菌体上で進むことを強く示唆している。

以上の結果から、磁性モデルビーズおよび原子間力顕微鏡を使った方法により細菌の粒子捕獲能力の存在を確認するとともに、この能力が酵素活性とカップルしていること、より貧栄養海域での栄養獲得に有利に働くことを示すことができた。また全海洋の粒子のうち、かなりの量が細菌の菌体上にあることを初めて明らかにした。これらの結果はいずれも初めての知見であり、細菌の新たな分解様式として細菌学に大きなインパクトを与えるとともに、海洋の粒子状有機物の分解プロセスに新たな概念を加えることになると考える。

4. 引用文献

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The Dynamics of Marine Bacteria with Particle Capturing Activity

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Keywords; Marine bacteria, Sub-micrometer particles, Particle capture

1. Introduction

It has been recognized that the concentration of particles in the ocean increase with the decrease of their particle sizes. In 1990, Koike et al (1990) reported the presence of so called sub-micrometer particles (SMPs). The number of SMPs is about 10^7 particles ml^{-1}, which is about one order of magnitude larger than those of bacterial cells. This means that one bacterium is surrounded by 10-100 particles including colloidal particles.

When bacteria degrade organic compounds, they synthesize and exclude the enzyme to degrade into small size (around 100Da), before taking up into the cells. This scenario fits well when bacteria degrade large organic material such as marine snow and has been investigated by many workers, whereas virtually no work has been accomplished for small particles. Recently, Seo proposed a concept that bacteria have the ability of "particle capturing (PC)" (Seo et al 2007). This means, cells retain the submicron particles on their surfaces prior to utilizations as nutrient sources. Seo reported that 10% of total bacteria possessed particle capturing activity in the coastal sea water, and the community structure of those PC bacteria differed from that of free-living bacteria or attached bacteria. However, Seo used model paramagnetic beads to collect PC bacteria and it was not clear whether natural marine bacteria actually possess particles, or the particle capturing process links to efficient hydrolysis-uptake.

The aim of this study is to clarify the dynamics of PC bacteria in the ocean. I focused on the abundance, distribution, community structure and enzyme activity of PC bacteria. In addition, the presence and number of SMPs on the cells were directly observed and quantified under the atomic force microscopy (AFM).

2. Sampling and Methods

Sea water samples were collected at three sampling stations (Sta.T, P, S) during the KT-06-31 cruise of RV Tansei Maru cruise (Ocean Research Institute, the University of Tokyo and JAMSTEC) from 6 to 10, December in 2006. For observation of seasonal dynamics of PC bacteria, monthly samplings were made from October 2006 to November 2007 at Sta.M in Sagami Bay. To isolate PC bacteria, dextran-based paramagnetic particles (0.5μm diameter) were used as model particles. One ml of subsamples were incubated with the particles for 1h. The bacteria collected with paramagnetic particles together by a magnet were defined as PC bacteria. I analyzed 5 parameters below using PC bacteria and natural sea water;

①Number: PC bacteria were stained with DAPI and counted with epifluorescence microscopy

②Community composition: DNA of PC bacteria were extracted and Denaturing Gradient Gel Electrophoresis (DGGE) were carried out

③Enzyme activity: PC bacterial enzymatic activities were assayed using fluorogenic peptide analog substrate (Leu-MCA)

④Particle number on the cell: Natural sea water were filtered through 0.2μm filter and SMPs were observed by Atomic Force Microscopy (AFM) and the particles were counted from the image

⑤Environmental factors which varied PC bacterial abundance/percentage: Environmental parameters and PC bacterial numbers/percentages were examined with Multiple regression

Water temperature, salinity, chlorophyll *a* concentration, particular organic carbon (POC), particular organic nitrogen (PON), total bacterial number, virus number, 0.2-0.5μm SMPs number, 0.5-1μm SMPs number, and bacterial production rates were measured.

3. Results and Discussion

①The numbers of PC bacteria were 10^3-10^5 cells ml^{-1} which was 0.4-29.5% of total bacteria. The relative number of PC bacteria to total bacteria increased from coastal towards oceanic area.

②Environmental factors which varied PC bacterial abundance/percentage were bacterial production rates, PON, 0.5-1μm SMPs, temperature and salinity. The percentage of PC bacteria increased with the decrease of bacterial production rate. This indicates that particle capturing activity play more important roles in low nutrient condition.

③Dominant PC bacterial members and free-living bacterial members were distinct from each other and gradually shifted over the sampling period. MDS analysis clarified that the extent of diversity of PC bacterial community was larger than that of free-living bacteria. This means that varieties of bacteria are involved in PC processes.

④The specific enzymatic activities of PC bacteria were 5.8 times higher than those of non-PC bacteria. The activity collected by 0.5μm model particle was higher than that by 0.17μm particle (0.5μm PC bacteria had 7.2 times higher, 0.17μm PC had 4.4 times higher).

⑤From the observation by AFM, 47-64% of marine bacteria have particles on the cell. The average number of particles on a PC cell was 2.8. This result represent that 30-79% of total particles in sea water are on the surface of bacterial cell.

From these results, the presence of bacteria possessing PC activity was confirmed by both paramagnetic particle method and direct observation by AFM. PC activity was coupled with enzymatic activity and SMPs on the cell account for about half of total SMPs in the ocean. The PC activity is expected to play important roles in biogeochemical cycle of particulate organic material in the ocean, especially in oligotrophic environments.

4. References

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