High growth potential of transiently 0.2-μm-filterable bacteria with extracellular protease activity in coastal seawater

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Abstract: Bacteria that pass through a 0.2-μm pore-size filter (0.2-μm-filterable bacteria) have been reported from various aquatic environments. It has been recognized that some 0.2-μm-filterable bacteria are “transiently” small/thin cells, which are considered as starvation forms of heterotrophic bacteria, especially in oligotrophic environments. However, whether these “transiently 0.2-μm-filterable bacteria” have roles in organic matter processing in aquatic environments has not yet been well described. Here we found high potential of the transiently 0.2-μm-filterable bacteria with extracellular protease activity in their natural nutritional condition. We conducted microcosm experiments using 0.2-μm-filtered coastal seawater (FSW) without any nutritional amendment, and monitored changes of the cell numbers trapped on a 0.2-μm filter (not 0.2-μm-filterable cells anymore) and their community structure in the FSW microcosms, with extracellular protease activities as indicators of heterotrophic microbial activity. We observed a rapid increase in the 0.2-μm-trapped cell number in the FSW microcosms that originally included only 0.2-μm-filterable microbes. The regenerating 0.2-μm-trapped cells were typical marine bacteria (Alphaproteobacteria, Gammaproteobacteria, Flavobacteria). Extracellular aminopeptidase activities increased with increasing numbers of 0.2-μm-trapped cells. These results suggest that transiently 0.2-μm-filterable-form bacteria in the original coastal seawater recovered their size and were producing extracellular proteases, which might catalyze organic matter processing in seawater. Since organic nutrients were not added, the size increase might be caused by the reduction in competition from larger bacteria and/or the absence of grazing pressure. Our results demonstrate the growth potential and extracellular protease activity of “transiently 0.2-μm-filterable” bacteria in seawater, usually obscured due to the coexistence of grazers and other bacteria.

Key words: coastal seawater, extracellular enzyme activity, grazing pressure, growth rate, 0.2-μm-filterable bacteria

Introduction

The importance of heterotrophic bacteria in marine aquatic ecosystems has been recognized in terms of microbial food webs and the carbon cycle (Azam & Malfatti 2007). In addition to bacterial abundance and community structure in marine ecosystems, bacterial growth and mortality rates, heterotrophic bacterial production, respiration, and activities of extracellular hydrolytic enzymes in seawater have been investigated in an effort to quantify microbiological processes in aquatic environments (e.g., Alonso-Sáez et al. 2008). The abundance of bacteria in the environment is generally determined by the balance between the growth rate and the mortality rate. The growth rate is usually thought to be controlled by nutritional availability and temperature, while the mortality rate is thought to be determined by the grazing of bacterivorous heterotrophs (mostly protistan microzooplankton) and viral lysis. However, it is difficult to estimate the in situ bacterial growth rate and mortality rate separately.

To investigate the abundance and community structure of bacteria, water samples are typically filtered using 0.2–0.45-μm pore-size filters, because most bacterial cells are trapped on filters with pores of this size; however, microbes in 0.2-μm filtrate have been reported from aquatic environments (e.g., MacDonell & Hood 1982, Hahn et al. 2003, Miteva & Brenchley 2005, Maejima et al. 2018). As 0.2-μm-filterable microbes, two types of ultrasmall cells have been reported (Torrella & Morita 1981, Duda et al. 2012): ones that have a larger cell size when incubated with adequate nutrients (Torrella & Morita 1981, Vybiral et al.
have been conducted using a culture-independent method (bacteria and archaea) in natural aquatic environments (Torrella & Morita 1981, Schut et al. 1997, Rappé et al. 2002, Hahn et al. 2003, Nakai et al. 2016). The former group is thought to consist of transiently small/thin or flexible bacteria, which have been considered to represent the starvation forms or morphological adaptive cells of heterotrophic bacteria for oligotrophic growth (Elsaied et al. 2001), especially in natural oligotrophic aquatic habitats.

Phylogenetic analyses of 0.2-µm-filterable prokaryotes (bacteria and archaea) in natural aquatic environments have been conducted using a culture-independent method for 0.1-µm-trapped but 0.2-µm-filterable microbes (Haller et al. 1999, Miyoshi et al. 2005, Naganuma et al. 2007, Nakai et al. 2011, 2013). Known species were identified although novel species are also included (e.g., Nakai et al. 2013). The results support the idea that some of the 0.2 µm-filterable cells in natural aquatic habitats are starvation forms of ordinary heterotrophic bacteria. For example, Haller et al. (1999) compared the 0.1–0.2-µm fraction community and the >0.2-µm fraction community using denaturing gradient gel electrophoresis (DGGE) and sequencing of 16S rRNA gene fragments; they concluded that most of the 0.2-µm-filterable bacteria from the western Mediterranean Sea were starvation forms of typical marine bacteria, rather than obligate ultramicrobacteria. These DNA analyses have been increasing knowledge about community structure of the ultrasmall cells in aquatic environments. However, the kinetics of community changes and their roles in biogeochemical cycles are still unclear.

It is reasonable to think that the transiently ultrasmall cells recover their size and metabolic activities when organic nutrition is supplemented. However, how do transiently small bacteria recover at natural nutritional levels in aquatic environments? We hypothesize that "transiently 0.2-µm-filterable" heterotrophic bacteria would change, even in natural environments, because they are not forms that are dying.

In this study, to clarify the metabolic potential of transiently small/thin or flexible bacteria in marine ecosystems, we prepared microcosms using 0.2-µm-filtered coastal seawater (FSW) without any nutritional amendment, and monitored the changes in abundance of prokaryotes trapped on a 0.2-µm filter (not 0.2-µm-filterable cells anymore), their community structure (described by DGGE), and extracellular protease activities as an indicator of heterotrophic microbial activity over time in the FSW microcosms. We found a rapid increase in the number of 0.2-µm-trapped cells in the microcosm that originally included only 0.2-µm-filterable microbes, indicating that there were transiently small/thin bacteria in the original coastal seawater. The microcosm experiment also demonstrated the growth potential and heterotrophic activity of bacteria that are usually obscured due to the coexistence of grazers in seawater.

Materials and Methods

"0.2-µm-filterable" microbes and "transiently-0.2-µm-filterable" microbes

Wang et al. (2007, 2008) reported that not only cell size but also cell shape and flexibility affect filterability. Therefore, "0.2-µm-filterable microbes" may include longer but thin cells and larger but flexible cells that can pass through the pores of the filter. In this study, we defined the "0.2-µm-filterable microbes" as the microbes that were contained in 0.2-µm filtrates after one-time filtration of natural seawater using a 0.2-µm pore-size filter. Moreover, after short-term incubation of the 0.2-µm filtrates including the "0.2-µm-filterable microbes," the microbes trapped on a 0.2-µm filter were considered to be "transiently 0.2-µm-filterable" microbes because their 0.2-µm-filterability changed. The "transiently 0.2-µm-filterable" community should consist of transiently small/thin or flexible microbes such as heterotrophic bacteria that were under starvation conditions or were in the small-cell phase in their growth cycle when they were filtered.

Microcosm experiments with filtered seawater (FSW) and unfiltered seawater (UNF)

Microcosm experiments were performed two times; Experiment I (Exp I) and Experiment II (Exp II) were conducted in summer (September 2008) and in winter (February 2009), respectively, with surface seawater collected along the coast of Matsuyama in the Seto Inland Sea, Ehime Prefecture, Japan. The Seto Inland Sea is a highly-productive, semi-enclosed coastal sea (reviewed in Takeoka 2002) with an estimated annual primary production of 218 g C m⁻² yr⁻¹ in the water column (Tada et al. 1998). Wada & Suzuki (2011) measured the dissolved organic carbon (DOC) concentration of surface water as 1.13 mg C L⁻¹ at the same site as in this study in September, and as 1.93 mg C L⁻¹ in the eastern part of the Seto Inland Sea (Osaka) in February. From these levels of productivity and DOC concentration, we can consider that our sampling site was not very oligotrophic and also not in a hypereutrophic condition, being a natural aquatic environment for heterotrophic bacteria in both September and February.

Seawater for the experiments was collected using a plastic bucket and was then transferred to polycarbonate bottles through a 150-µm nylon mesh. A subset of collected seawater was gently filtered (<0.01 MPa) through 0.2-µm pore-size polycarbonate Nuclepore filters (Whatman) and used as 0.2-µm-filtered seawater. Microcosms were prepared with unfiltered seawater (UNF) or 0.2-µm-filtered seawater (FSW) in 1-L polycarbonate bottles. Nothing was supplemented to the bottles. Microcosm incubation was conducted at the original seawater temperature (Exp I, 25°C; Exp II, 12°C) and at a lower temperature (6°C) in the dark, to obtain information about microbial growth and the
processing of organic matter by determining the changes between incubations at the original temperature and the lower (refrigerator) temperature. As a control, autoclaved seawater was also incubated at each temperature.

Samples were withdrawn from each incubation bottle on Days 1, 2, 3, 6, and 10 for Exp I, and on Days 0, 1, 3, 5, 8, 13, and 19 for Exp II. For Exp I, the Day 0 sample was taken from prepared UNF water and FSW.

Counts of 0.2-µm-trapped cells

For the cell counts, sample waters from Days 0, 1, 3, and 10 for Exp I and Days 0, 3, 8, and 19 for Exp II were fixed with neutralized formalin (final concentration 2%) and stained with 4,6-diamidino-2-phenylindole (DAPI). To enumerate 0.2-µm-trapped cells, 2- or 3-mL fixed samples were filtered onto 0.2-µm black Nuclepore filters, and counts were performed under an epifluorescence microscope (×1000, Olympus BX51) with excitation/emission wavelengths of 405/440 nm several times with about 1-h intervals (t0, t1, t2, t3). Counts of 0.2-µm-trapped cells, 2- or 3-mL fixed samples were filtered onto 0.2-µm Nuclepore filters and kept at −80°C until DNA extraction. For DNA extraction, the filter was cut into small pieces, immersed in 700 µL of extraction buffer [mixture of 652 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 40 µL of 10% SDS, 4 µL of proteinase K (20 mg/mL)] in a 2-mL tube and incubated at 37°C for 1 h, followed by the addition of 100 µL of 10% CTAB/0.7 M NaCl solution and incubation at 65°C for 10 min. Then, an equal volume (800 µL) of PCI [phenol:chloroform:isoamyl alcohol=25:24:1 (v:v:v)] was added into the tube. After mixing and centrifugation (21,600×g, 4°C, 5 min), the upper aqueous layer was carefully transferred to a fresh 2-mL tube. An equal volume (approximately 800 µL) of C1 [chloroform:isoamyl alcohol=24:1 (v:v)] was added to the tube, and the tube was inverted for mixing. After centrifugation (21,600×g, 4°C, 5 min), the upper layer was transferred to a fresh 1.5-mL tube, and 0.1 volumes of 3 M sodium acetate and 0.6 volumes of isopropanol were added. The sample tubes were then mixed by inversion and stored at 4°C for 1 h until the DNA precipitated. The precipitated DNA was then recovered by centrifugation (21,600×g, 4°C, 20 min). Pellets were washed with cold 70% ethanol, vacuum-dried, and dissolved in 30 µL of sterile ultra-pure water.

Extracellular enzyme activities

Since heterotrophic prokaryotes utilize proteinaceous substrates, we measured extracellular protease activities as an index of heterotrophic activity. Potential protease activities in each freshly withdrawn sample water were assayed using the following peptide analog 4-methyl-coumaryl-7-amide (MCA) substrates (Peptide Institute): two for aminopeptidase (Leu-MCA, Ala-MCA), two for trypsin (Boc-Phe-Ser-Arg-MCA, Boc-Leu-Ser-Thr-Arg-MCA), two for chymotrypsin (Suc-Ala-Ala-Pro-Phe-MCA, Suc-Leu-Leu-Val-Tyr-MCA), and two for aminopeptidase (Leu-MCA, Ala-MCA), two for trypsin (Boc-Phe-Ser-Arg-MCA, Boc-Leu-Ser-Thr-Arg-MCA), two for chymotrypsin (Suc-Ala-Ala-Pro-Phe-MCA, Suc-Leu-Leu-Val-Tyr-MCA). Aliquots of samples were filtered through 0.2-µm-trapped bacteria was counted under a microscope. The sample tubes were then mixed by inversion and stored at 4°C for 1 h until the DNA precipitated. The precipitated DNA was then recovered by centrifugation (21,600×g, 4°C, 20 min). Pellets were washed with cold 70% ethanol, vacuum-dried, and dissolved in 30 µL of sterile ultra-pure water.

PCR-DGGE

Bacterial 16S rRNA gene fragments were amplified by PCR in 100-µL reaction mixtures containing 0.25 µM each of forward primer, 341f-GC-clamp (5′-CGC CCC TTG AGC AGC-3′), and reverse primer, 519r (5′-CCG GCG TTA CGG AGC CAG-3′), and primer, 907r (5′-CCG TTA ATT CMT TTT GAT TT-3′), 0.2 mM dNTP mixture (Takara), 1.5 U of AmpliTaq DNA polymerase (Applied Biosystems), 1×PCR buffer II and 2 mM MgCl2 (Applied Biosystems), 0.05 mg mL−1 bovine serum albumin (Takara), and 3 µL of extracted DNA as the template. PCR was carried out with a GeneAmp9700 (Applied Biosystems) as follows: initial denaturation at 94°C for 5 min; followed by 20 cycles of denaturation at 94°C for 1 min, annealing at 65–55°C (~0.5°C/cycle) for 1 min, and extension at 72°C for 1 min; 15 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min; and a final extension step at 72°C for 7 min. The PCR products were confirmed on a 1.5% agarose gel stained with ethidium bromide.
DGGE analysis was performed as described by Takasu et al. (2011). For each sample, 20 μL of PCR product was applied to each well of an 8% polyacrylamide gel with a denaturing gradient of 25% to 65%. Electrophoresis was performed at a constant voltage of 80 V at 60°C for 15 h in 0.5× TAE buffer. After electrophoresis, gels were stained with SYBR Gold (Molecular Probes) to visualize the bands.

To express the relative similarity of the DGGE profiles among samples in each experiment, nonmetric multidimensional scaling (NMDS) was conducted. The data used for NMDS were presence/absence of DGGE band (52 bands and 50 bands for Exp I and Exp II, respectively) visualized on a DGGE gel. From these data, a Bray–Curtis similarity matrix was calculated for the NMDS analysis.

Identification of bacteria

Bands on DGGE gels were excised on a UV transilluminator and DNA was extracted for sequencing. To extract DNA, excised bands were immersed in 100 μL of TE buffer in 1.5-mL tubes with slow rotation overnight at room temperature. Extracted DNA was re-amplified by PCR with the same protocol given above, except that the reaction mixture volume was 50 μL with 1 μL of DNA template. The PCR products were run on DGGE to confirm that a single DGGE band was amplified, and the PCR products were purified using the QIAquick PCR purification kit (QIAGEN), in accordance with the manufacturer’s instructions. Purified PCR products were sequenced on an ABI Genetic Analyzer 3130 (Applied Biosystems) with BigDye Terminator. Primer 907r was used for sequencing. Some bands that did not have enough purity or quality were not included in further analyses.

Obtained sequences were aligned and compared with known bacterial sequences in the DNA database of the National Center for Biotechnology Information using the Basic Local Alignment Search Tool by Clustal W. The distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). Phylogenetic relationships were inferred using the neighbor-joining method (Saitou & Nei 1987) with 500 bootstrap replications by MEGA7 (Kumar et al. 2016). Archaeoglobus veneficus Huber et al. 1998 SNP6 (NR_102885) was included as an outgroup.

The nucleotide sequences obtained in this study were deposited in the nucleotide sequence database, DNA Data Bank of Japan (DDBJ), under accession numbers LC496396 to LC496447.

Results

Counts of 0.2-μm-trapped cells

At the beginning of both experiments (Day 0), 0.2-μm-trapped cell counts in FSW were very low, as expected, because the waters had just been filtered through a 0.2-μm filter to prepare the FSW prior to making the filter for microscopic cell counting. In all FSW bottles, the numbers of 0.2-μm-trapped cells increased markedly during the course of incubation, although the timing and rate of increase differed among the different temperatures (Fig. 1): At 25°C (Exp I) and at 12 °C (Exp II), the numbers of 0.2-μm-trapped cells in the FSW bottle increased rapidly to a level comparable to the level in natural seawater, while cell counts in the lower-temperature incubation (6°C) increased after Day 3 in both Exp I and Exp II.

Community structure

Changes in community structure were analyzed by PCR-DGGE targeting the 16S rRNA gene fragment. In Exp II, PCR was conducted for all samples. PCR products were detected for all samples from UNF water bottles, but products were not detected on 1.5% agarose gel from the FSW bottles on Days 0 and 1 (Fig. 2). On Day 3 from FSW incubated at 12°C, the numbers of 0.2-μm-trapped cells in the FSW bottle increased rapidly to a level comparable to the level in natural seawater, while cell counts in the lower-temperature incubation (6°C) increased after Day 3 in both Exp I and Exp II.
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Extracellular protease activities

As an index of metabolic activity, extracellular proteolytic activities in UNF and FSW bottles incubated at the original seawater temperature and 6°C were assayed (Figs. 7 and 8). In both Exp I and Exp II, UNF bottles incubated at the original seawater temperature (Figs. 7a and 8a) showed slight increases in the leucine- and alanine-aminopeptidase activities in the UNF bottles at Day 1, followed by a gradual decrease, while trypsin- and chymotrypsin-type activities showed decreases over the entire period, except for a small second peak on Day 3 of Exp I. In the UNF bottles at the lower temperature (Figs. 7e and 8e), aminopeptidase activities increased, while trypsin- and chymotrypsin-type activities decreased. Aminopeptidase in the UNF bottles was mainly attributed to the >0.2-µm fraction, namely, 0.2-µm-trapped aminopeptidase (ectoenzyme of 0.2-µm-trapped cells).

In the FSW bottles, aminopeptidase activities increased coincidentally with increasing of the number of 0.2 µm-
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trapped cells in both experiments at both temperatures (Figs. 1, 7c, 7g, 8c, 8g). The size-fractionated activities shown in Figs. 7d, 7h, 8d, 8h indicated that the 0.2-μm-trapped aminopeptidase coincidently increased with the number of 0.2-μm-trapped cells in the FSW, similar to the case in the UNF bottles. For the aminopeptidase activity in the FSW bottles in Exp II, maximum bulk activity during the incubation period was almost at the same level as in the UNF bottles, although the dates at which the maximum activity occurred differed (Day 1 in UNF and Day 5 in FSW at 12°C, Day 3 in UNF and Day 19 in FSW at 6°C); however, the contribution of the <0.2-μm fraction to the aminopeptidase activity was greater in the FSW bottles than in the UNF bottles (Figs. 8b, 8d, 8f, 8h). Trypsin- and chymotrypsin-type activities decreased in all FSW bottles, with a slower decrease at the lower temperature than at the original seawater temperature. In autoclaved seawater as a control, all protease activities were below the detection limit during the entire experimental period in both Exp I and Exp II (data not shown).

Discussion

In natural aquatic environments, the community of “0.2-μm-filterable bacteria” is thought to consist of obligate ultramicrobacteria and “transiently 0.2-μm-filterable” bacteria (Hahn et al. 2003). In this study, we confirmed the presence of “transiently 0.2-μm-filterable” bacteria in temperate coastal seawater. During the incubation of the FSW bottles in our experiment, a rapid increase in the number of cells that were trapped on 0.2-μm filters was observed (Fig. 1), indicating that at least some of the 0.2-μm-filterable prokaryotes in the original seawater recovered their size to be 0.2-μm-trapped cells. These transiently 0.2-μm-filterable prokaryotes might consist of archaea, not only bacteria. In this study we did not investigate the archaean community, however, at least the identified 0.2-μm-trapped bacteria from the FSW bottles were typical marine bacteria (Alphaproteobacteria, Gammaproteobacteria, and Flavobacteriia; Figs. 5 and 6), suggesting that transiently filterable forms (small/thin or flexible enough to pass through a 0.2-μm filter) of these typical marine bacteria were present in the original coastal seawater both in sum-
mer and in winter. The regenerating 0.2-µm-trapped bacterial communities in the FSW bottles were not the same as those in the UNF bottles based on the DGGE profiles (Figs. 3 and 4), and some bands that appeared in FSW bottles were not seen in the original seawater or UNF bottles. Considering the fact that DGGE profiles usually do not reflect rather minor members of the community, the bacteria that were major members of the regenerating community in FSW bottles might also be in the UNF bottles as minor members at low numbers. Transiently small/thin bacterial communities in aquatic ecosystems are considered to be starvation forms of typical marine bacteria. Our results implied that most extracellular aminopeptidases in the UNF bottles were prokaryotic cell-associated enzymes (ectoenzymes). This result is consistent with our previous findings (Obayashi & Suzuki 2008a). In addition, in the FSW bottles, aminopeptidase activities in the >0.2-µm fraction of the sample were elevated when the bulk aminopeptidase activities were elevated (Figs. 7b, 7f, 8b, 8f), indicating that most extracellular aminopeptidases in the UNF bottles were prokaryotic cell-associated enzymes (ectoenzymes). This result is consistent with our previous findings (Obayashi & Suzuki 2008a). In addition, in the FSW bottles, aminopeptidase activities in the >0.2-µm fraction of the sample were elevated when the bulk aminopeptidase activities were elevated (Figs. 7b, 7f, 8b, 8f), indicating that most extracellular aminopeptidases in the UNF bottles were prokaryotic cell-associated enzymes (ectoenzymes). This result is consistent with our previous findings (Obayashi & Suzuki 2008a).
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Fig. 7. Extracellular protease activities in microcosm bottles in Exp I. Protease activities were measured using two substrates for aminopeptidase (Leu-MCA, Ala-MCA), two for trypsin-type enzymes (Boc-Phe-Ser-Arg-MCA, Boc-Leu-Ser-Thr-Arg-MCA), and two for chymotrypsin-type enzymes (Suc-Ala-Ala-Pro-Phe-MCA, Suc-Leu-Leu-Val-Tyr-MCA). (a) Activities in the bulk (unfiltered) sample in the UNF25C bottle, (b) size-fractionated assay of each activity in the UNF25C bottle, (c) activities in the bulk sample in the FSW25C bottle, (d) size-fractionated assay of each activity in the FSW25C bottle, (e) and (f) are the same as (a) and (b) but for the UNF6C bottle, and (g) and (h) are the same as (c) and (d) but for the FSW6C bottle. Activities in the >0.2-μm fraction were calculated as differences of the activities measured in the unfiltered sample and those in the <0.2-μm filtrate fraction of the sample. *: Extracellular proteolytic activity on Day 2 in UNF bottles and on Days 0, 1, 2, and 3 in FSW bottles was only estimated in bulk samples without size fractionation.

Fig. 8. Same as Fig. 7 but for Exp II. (a) Activities in the bulk (unfiltered) sample in the UNF12C bottle, (b) size-fractionated assay of each activity in the UNF12C bottle, (c) activities in the bulk sample in the FSW12C bottle, (d) size-fractionated assay of each activity in the FSW12C bottle, (e) and (f) are the same as (a) and (b) but for the UNF6C bottle, and (g) and (h) are the same as (c) and (d) but for the FSW6C bottle.
and ectoenzyme activity of 0.2-μm-filterable living cells. Thus, we can suppose two possible processes that elevate the aminopeptidase activities in the <0.2-μm fraction; one is an increase in the cell-free enzymes released from the >0.2-μm cells or from dead cells that have burst due to viral lysis (Arnosti 2011), and the other is an increase in the ectoenzymes of 0.2-μm-filterable living cells. Considering the higher contributions of the <0.2-μm fraction to the aminopeptidase activity in the FSW bottles than those in the UNF bottles throughout the incubation period, at least some of the elevated aminopeptidase activity in the <0.2-μm fraction is thought to involve the contribution of the ectoenzyme activity of 0.2-μm-filterable cells. From these results, we can suppose that 0.2-μm-filterable cells were still present and active even after increasing the number of 0.2-μm-trapped prokaryotes in the FSW bottles, although we did not determine the number of 0.2-μm-filterable cells during the experiment.

In contrast to the aminopeptidase activity, extracellular trypsin-type and chymotrypsin-type activities were not elevated with an increase in the prokaryotic cell number in each experimental bottle. These activities were highest at Day 0, namely, in freshly collected natural seawater, and decreased during the course of the incubation in all bottles (Figs. 7 and 8). This suggests that trypsin- and chymotrypsin-type enzymes should have been present in each type of seawater at the start of the experiment, but were degraded during the incubation period. Although these enzymes might be produced by microbes in the bottles during incubation, degradation surpassed production. At a higher temperature, trypsin-type activity decreased more rapidly, with the decrease of activity being faster in UNF bottles than in FSW bottles. These results imply that extracellular trypsin-type enzymes in the incubation bottles might have been degraded enzymatically because the enzymes themselves are proteins. On the other hand, extracellular trypsin- and chymotrypsin-type activity is usually detected at a high level in natural coastal seawater, with a high contribution of activities in the “dissolved” fraction (Obayashi & Suzuki 2005, 2008a). Producers of “dissolved” trypsin- and chymotrypsin-type enzymes other than prokaryotes might thus be present in natural aquatic ecosystems. Thao et al. (2015) reported that, besides prokaryotes, ciliates could also be a source of extracellular proteases in seawater. Mohapatra & Fukami (2004) also mentioned the contribution of heterotrophic nanoflagellates to the enzyme pool in marine environments while selectively grazing on bacteria. The results of the present study might also be an indirect indication of the partial contribution of organisms other than heterotrophic bacteria to the pool of dissolved extracellular proteases in natural coastal seawater.

From the assay with size fractionation, extracellular protease activities in the FSW bottles on Day 0 unexpectedly appeared to have been contributed to, to a certain extent, by the >0.2-μm fraction (Figs. 7d and 8h), despite the fact that the FSW bottles on Day 0 contained very few 0.2-μm-trapped microbes and particles (Fig. 1b). The activities in the >0.2-μm fraction in Figs. 7 and 8 were calculated by subtraction of the activities measured in the <0.2-μm filtrate of the sample from the measured activities in the unfiltered sample. Therefore, some of the calculated activities in the >0.2-μm fraction might have been caused by underestimation of the <0.2-μm activity because of removal of the dissolved enzymes from the original seawater during the filtration process, possibly due to adsorption of the enzymes onto the filter or other apparatus for size fractionation (Obayashi & Suzuki 2008b). In the FSW on Day 0, there was a rather high apparent contribution of the >0.2-μm fraction to the trypsin- and chymotrypsin-type activity, compared with that in the aminopeptidase activity. Those high apparent contributions of the >0.2-μm fraction activities may reflect the reported finding that dissolved trypsin- and chymotrypsin-type enzyme activities in seawater are readily affected by adsorption during filtration and preservation (Obayashi & Suzuki 2008b, Obayashi et al. 2017).

In aquatic microbial ecology studies, dilution experiments (Landry & Hassett 1982) have been used to estimate the growth rate of phytoplankton or bacterioplankton, and the associated rate of microzooplankton grazing (e.g., Teixeira et al. 2011). For typical dilution experiments, unfiltered seawater and filtered seawater prepared by filtration
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using 0.2-μm filters are mixed at several levels, although more recent studies have prepared filtered seawater by ultrafiltration (30 kDa) for “modified” dilution experiments (Evans et al. 2003, Tsai et al. 2015). From our experiment, it was clarified that, at 3 days after filtration, 0.2-μm-filtered seawater was no longer “particle-free,” even if kept in a refrigerator (6°C). At the in situ temperature (25°C for Exp I and 12°C for Exp II), bacteria in the filtered seawater increased rapidly. This observation should be taken into consideration when conducting dilution experiments with incubation durations of more than several hours at in situ temperatures, especially for studies that include estimates of bacterial growth and mortality rates.

We conclude that transiently 0.2-μm-filterable cells in coastal seawater consisted of small/thin or flexible starvation forms of typical marine bacteria. These bacteria have high growth potential and extracellular protease activity, suggesting that they contribute to biogeochemical material cycles. Formation and regeneration of the 0.2-μm-filterable cells in aquatic ecosystems should be coupled with grazing effects and competition. The starvation forms of bacteria appear to always be ready for growth in changeable coastal aquatic ecosystems.

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