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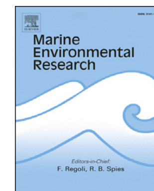

Extracellular proteases are released by ciliates in defined seawater microcosms

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A B S T R A C T

The biodegradation of proteins in seawater requires various proteases which are commonly thought to be mainly derived from heterotrophic bacteria. We, however, found that protists showed a high protease activity and continuously produced trypsin-type enzymes. The free-living marine heterotrophic ciliate *Paranophrys marina* together with an associated bacterium was isolated and used for microcosm incubation with different concentrations of killed bacteria as food for 10 days. The results showed that the coexistence of the ciliate with its associated bacterium produced a significant protease activity in both cell-associated and cell-free fractions while that in the associated bacterium only microcosm was negligible. The protease profiles are different between cell-associated and cell-free fractions, and a trypsin-type enzyme hydrolyzing Boc-Val-Leu-Lys-MCA was detected throughout the period in the presence of ciliates. This suggests that ciliates release proteases into the surrounding environment which could play a role in protein digestion outside cells. It has been previously suggested that bacteria are the major transformers in seawater. We here present additional data which indicates that protists, or at least ciliates with their specific enzymes, are a potential player in organic matter degradation in water columns.
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1. Introduction

Biological decomposition of organic matter in water columns occurs when microbial extracellular enzymes reduce macromolecules derived from all organisms to low molecular weight substances (Hoppe, 1983). This process is required for heterotrophic bacteria because only compounds that have molecular masses <600 Da can be transported across cell membranes (Weiss et al., 1991). Extracellular enzymes, therefore, are undoubtedly major driving forces to achieve this in bacteria. The biological degradation of organic matter is also important for carbon and nutrient cycling in the marine environment. Extracellular enzymes are either bound to cell surfaces, which are called ectoenzymes (Chróst, 1990), or released into the environment by secretion and via cell lysis (Arnosti, 2011; Maire et al., 2012; Sinsabaugh and Follstad Shah, 2012). Oceanic extracellular enzymes investigated so far comprise proteases, glucosidases, chitinase, lipase and phosphatase with proteases being the highest in magnitude (Arnosti, 2011; Hoppe et al., 2002). Consequently, extensive studies on proteases have been reported regarding their occurrence and significance in seawater (Bong et al., 2013; Obayashi and Suzuki, 2008; Steen and Arnosti, 2013).

Most research on extracellular enzymes in marine systems has been aimed at determining the activity of heterotrophic bacterial communities in the context of degradation of complex substrates (Obayashi et al., 2010; Pantoja and Lee, 1999; Tranvik et al., 1993). The traditional approach where only bacteria are taken into account and arbitrarily considered to be enzyme producers is inadequate when only bulk seawater without fractionation has been analyzed (Allison et al., 2012; Arnosti et al., 2012; Arnosti and Steen, 2013; D'Ambrosio et al., 2014). Various microbes in the community can certainly contribute to the bulk enzyme pool and previous research has shown that heterotrophic protists play fundamental and unanticipated roles in the marine food web (Weber et al., 2012) as bacterial grazers (Sherr and Sherr, 2002) and nutrient remineralizers (Nagata, 2000). Therefore, it is prerequisite that enzyme

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sources other than bacteria should also be investigated (Mohapatra and Fukami, 2004; Salerno and Stoecker, 2009; Thao et al., 2014). Our recent work suggests that protists could be an important source of proteases (Thao et al., 2014). Further investigation is required to determine the community strategies – which type of extracellular enzymes are produced by which organism, what benefits from hydrolysis products and the extent to which various organisms collaborate or compete for substrates. Protists are generally believed to contain digestive enzymes in the food vacuole (Muller and Törö, 1962; Nagata and Kirchman, 1992). However, our previous paper (Thao et al., 2014) indicated another possibility that the positively produced or leaked protist proteases are helpful for bacteria to use high molecular matter. Karner et al. (1994) also reported the potential role of nanoflagellates in ectohydrolase production. Allison (2005) reported enzyme producer-cheater model. However, the model does not mention who each player is. We hypothesize here that producer and cheater are protist and bacteria, respectively.

Given previous evidence of the significant role of protists in the organic matter cycle, we investigated the protease production dynamics of marine heterotrophic protists by monitoring aminopeptidase, trypsin-type and chymotrypsin-type activities over 10 days in microcosm experiments using freshly isolated marine protist species and separated ciliate associated bacteria. This is the first study using a defined system to reveal that protists are prominent shareholders in the protease pool of the marine environment. It was known that *Pseudomonas aeruginosa* is ubiquitously found in the ocean (Khan et al., 2007; Kimata et al., 2003) and its membrane porin protein OprP could be detected in seawater (Tanoue et al., 1995; Suzuki et al., 1997). Since the proteins of *P. aeruginosa* could be examined for chasing the protein degradation (Obayashi et al., 2010), we used the *P. aeruginosa* as a model bacterium of this study.

As an enzyme terminology, past reports have used various terminology depend on the enzyme existence as describe above. We here use simple terms; "cell-free" enzymes which are released outside cell, and "cell-associated" enzymes which are present in cytosol or anchored to cytosolic and outer membranes.

2. Materials and methods

2.1. Protist isolation and culture

A ciliate was isolated from seawater collected in coastal Matsuyama, Ehime, Japan and cloned five times using the limiting dilution method in 96-well-tissue-culture plates in Eagle's minimum essential medium supplemented with fetal bovine serum (final concentration, 10%), penicillin (final concentration, 10 IU ml^{-1}), streptomycin (final concentration, 10 $\mu g\ ml^{-1}$), and tetracycline (final concentration, 50 $\mu g\ ml^{-1}$) at 20 °C in the dark. The ciliate was subcultured in 50-μm-filtered-autoclaved seawater (auto-SW) and fed on environmental bacteria and microwave-killed *P. aeruginosa* strain eco3 (Nonaka et al., 2010) at 20 °C in the dark. Antibiotics were used to kill various symbiotic/associated bacteria that the ciliate possesses inside or on the cell surface. However, some still survive and are recognized as antibiotic-resistant "associated bacteria" in this manuscript.

The full length 18S rRNA gene was determined in order to identify isolated ciliates. DNA was extracted from cells using a GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma–Aldrich, Tokyo), and PCR using universal primers (forward: 5′-ACCTGGTTGATCCTGCCAGT-3′, reverse: 5′-TGATCCTTCTGCAGGTTCACCTAC- 3′) (Sogin, 1990) was performed. The amplicon was purified with a MinElute PCR Purification Kit (QIAGEN, Tokyo), and was cloned in pGEM® -T vector (Promega, Tokyo). The plasmid was purified with a Mini Plus™ Plasmid DNA Extraction System (Viogene, Taiwan), and was sent to the Integrated Center for Sciences, Ehime University, Japan for DNA sequencing. The ciliate was finally identified as *Paranophrys marina* (Thompson and Berger, 1965) with identities of 1707/1714 (99%). This ciliate is tentatively assigned to the family Uronematidae (Thompson, 1964) which also includes the genera *Uronema* and *Miamiensis* (Thompson and Moewus, 1964), which are free-living and parasitic ciliates. The abundance and ecological importance for this ciliate in the sea have not been known.

2.2. Separation of ciliate-associated bacteria

The ciliate grows with associated bacteria as mentioned above but it is unclear whether they are commensal or mutualistic. Because it is impossible to completely remove bacteria from ciliates, we separated the associated bacteria from the ciliate and compared the result from the ciliate-microcosm with that from the associated bacteria only microcosm. After antibiotic treatment, the ciliate-associated bacteria were separated by filtration of the ciliate culture through an 0.8 μm cellulose acetate filter (Advantec) to remove ciliates leaving any associated bacteria able to filter through.

2.3. Mirowave-killed P. aeruginosa as ciliate food

P. aeruginosa strain eco3 (Nonaka et al., 2010) was cultured in medium with phosphate-deficient artificial seawater according to the method of Thao et al. (2014). The medium was artificial seawater as described in Yamada et al. (2000) supplemented with 0.1 M sodium N-2-hydroxyethyl piperazine-N′-2-ethanesulfonate (HEPES) (pH 7.0), 7 mM $(NH_4)_2SO_4$, 20 mM sodium succinate (pH 7.0), 0.1% (v/v) trace ion mixture described in Hancock et al. (1981), 0.2 mM sodium phosphate buffer (pH 7.0) and 1% proteose peptone No. 3 (Becton, Dickinson). Cells were incubated at 37 °C with shaking at 85 rpm in the dark to allow the production of porin P (OprP) which can be detected ubiquitously in the ocean (Tanoue et al., 1995). After 24 h the bacteria were killed using a microwave oven (700 W, Panasonic) by boiling for 2 min. The killed bacteria were then centrifuged at 3000 × g at 4 °C for 30 min to harvest and serve as food for the ciliates. The cell pellet was harvested and resuspended in auto-SW to yield 4.96×10^{10} cells ml^{-1} and served as food for the ciliates. The killed-*P. aeruginosa* cells are hereafter referred to as "Pa" to distinguish them from live bacteria. To test whether the bacteria were completely killed, Pa cells were streaked onto marine agar plates and visualized with a LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes) at the same time. No colonies or live cells could be found indicating that all bacteria were killed successfully.

2.4. Design of microcosm and sub-sampling

Before Starting The Experiment, The Ciliate Culture Was Pre-Grown In Auto-Sw supplemented with food bacterial cells (final concentration, 1 $\mu l\ ml^{-1}$). The production of proteases was investigated in two experimental groups. Three microcosms were prepared in triplicate in the first (Exp1) and in duplicate in the second (Exp2) experiments. The microcosm flask names were: "Pa" to which only killed-Pa cells were added, "Pa + B" to which Pa and separated ciliate-associated bacteria were added, and "Pa + CB" to which Pa and ciliates were added. The initial composition is summarized in Table 1. To examine the effect of Pa concentration (food for ciliates) on enzyme activity, different amounts of Pa were added; 1.24×10^{10} and 6.20×10^{10} Pa cells for Exp1 and Exp2, respectively. All flasks were brought to a final volume of 250 ml

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