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Study of Sediment Cleanup Using Polychaetes

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Abstract—Polychaetes live in sedimentary organic matter with different origins, such as terrestrial plant residue, phytoplankton, or fish faeces, and their organic matter metabolism is considered to be adapted to each environment.

We investigated functions of organic metabolism in two polychaete species, *Capitella* sp. I and *Perinereis nuntia brevicirris*, living in different coastal habitats. The protease activity of *Capitella* sp. I (89.66 μg/mg), which we found inhabiting sediment under a fish farm, was about 10 times that of *P. nuntia brevicirris* (7.95 μg/mg), which inhabits estuaries. High cellulase (endo-β-1,4-glucanase) activity was detected in *P. nuntia brevicirris* (3.24 μg/mg), whereas scarcely any was detected in *Capitella* sp. I. We isolated cDNA clones of protease (*cCS*) mRNA from *Capitella* sp. I and of cellulase (*pnbEG*) mRNA from *P. nuntia brevicirris*. In *Capitella* sp. I, *cCS* gene expression was observed in the epithelium of the body wall and in the luminal epithelium of the intestine. In *P. nuntia brevicirris*, strong *pnbEG* expression was observed in the intestinal epithelium. The enzyme activity of each polychaete was adapted to its habitat. For efficient bioremediation, it is important to utilize a suitable polychaete by understanding the differences among enzyme revitalizations in polychaeta.

Keywords: *Capitella* sp. I, cellulase, estuary, fish farm, *Perinereis nuntia brevicirris*, protease

INTRODUCTION

Estuaries and coastal zones with fish farms are often polluted by organic contaminants (Hall *et al*., 1992; Jorcin, 2000). In estuaries, sedimentary organic matter generally consists of a mixture of terrestrial plant residues, phytoplankton, and macro- and microalgae; plants transported by rivers also contribute to organic matter in estuaries (Hu *et al*., 2009). In contrast, in some coastal areas, large amounts of unconsumed fish food and faeces are discharged from fish farms to bottom marine sediments, where they have a serious negative effect on the

benthic environment (Tsutsumi and Kikuchi, 1983).

Annelid worms of the Class Polychaeta play an important role in nutrient cycling and in maintaining and sustaining the benthic environment (Henriksen *et al.*, 1983; Hutchings, 1998). The head-down deep deposit-feeding polychaetes are known for having strong effects on bioturbation and nutrient mineralization both by sediment reworking during non-selective feeding and by burrow irrigation (Papaspyrou *et al.*, 2007).

In this study, we investigated enzyme activities in polychaetes inhabiting sediments containing different organic pollutants to ascertain whether their organic matter metabolism was adapted to their inhabited environment.

MATERIALS AND METHODS

Animals

The polychaetes *Capitella* sp. I and *Perinereis nuntia brevicirris* were sampled from the coast of Ainan Town, Ehime, Japan. *Capitella* sp. I was collected from the bottom sediments under fish farms in Fukaura Bay, and *P. nuntia brevicirris* was collected from sediments in the Souzu River estuary, Misho Bay. These collected specimens were kept in sediment from their respective habitats at –20°C until use.

Preparation of crude enzyme

The whole body of the worm was homogenized on ice in 10 mM sodium phosphate buffer (PBS; pH 7.5). The homogenate was centrifuged for 30 min at 10,000 rpm at 4°C, and then the supernatant was used for the determination of enzyme activity.

Determination of protease activity

Protease activity was measured using milk casein as a substrate. Twenty microlitres of the crude enzyme solution extracted from the whole polychaete body was combined with 40 μl of 100 mM PBS (pH 7.0) and 40 μl of 2% milk casein, and incubated at 37°C for 1 h. The reaction was terminated by the addition of 100 μl trichloroacetic acid. The tube was then incubated at 25°C for 30 min, and centrifuged for 15 min at 10,000 rpm. Then, 100 μl of supernatant was mixed with 500 μl of sodium carbonate and 100 μl of 50% phenol reagent solution and incubated at 25°C for 20 min. Activities were determined by measuring the absorbance at 660 nm.

Determination of cellulase activity

Cellulase activity was measured at 37°C for 24 h in 10 mM PBS (pH 7.5) with 0.8% carboxymethyl cellulose (CMC) as the substrate. Reducing sugars produced by the reaction were determined by the dinitrosalicylic reagent method (Miller, 1959).

Table 1. Protease activities (μg/mg) in the whole body of the polychaetes *Capitella* sp. I and *Perinereis nuntia brevicirris*, and in their habitat soils.

Capitella sp.		*P. nuntia brevicirris*	
Whole body	Habitat soil	Whole body	Habitat soil
89.66 ± 4.87	2.24	7.95 ± 2.78	1.16

Cloning of protease and cellulase cDNAs

Total RNA was extracted from the worm tissue with Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). Poly(A)$^+$ RNA was subsequently isolated from total RNA with Oligotex-dT30 (Takara, Otsu, Japan). The cDNA was synthesized from mRNA by using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

We initially performed a polymerase chain reaction (PCR) with two degenerate primer pairs which we designed according to a consensus sequence from protease and cellulase genes, respectively, of other organisms. The amplified PCR products were cloned into the pGEM-T Easy Vector (Promega Corp., Madison, WI, USA) and sequenced.

PCR for 3′-rapid amplification of cDNA ends (RACE) and 5′-RACE of protease and cellulase was performed with a BD Smart RACE cDNA Amplification Kit (BD Biosciences, Palo Alto, CA, USA) with nested primers. The 3′-RACE and 5′-RACE PCR products were cloned into the pGEM-T Easy Vector (Promega) and sequenced.

A homology search of the amino acid sequence deduced from the obtained full-length cDNA was carried out by a FASTA Sequence Similarity Search protein query (Genome Net, Kyoto, Japan). Multiple-sequence alignments and phylogenetic trees were generated with CLUSTAL W software (Thompson *et al.*, 1994).

In situ hybridization

Fresh tissue from *Capitella* sp. I or *P. nuntia brevicirris* was fixed overnight in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 and embedded in OCT compound (Sakura Finetek USA Inc., Torrance, CA, USA). Frozen tissue sections (7 μm thick) were prepared at –20°C on a cryostat microtome. *In situ* hybridization was conducted with a Fisher Biotech staining station following the manufacturer's protocol with minor modifications. The hybridization was carried out at 55°C for 35 h using a DIG-labelled RNA probe, and staining was performed by the nitro-blue tetrazolium chloride (NBT)—5-Bromo-4-Chloro-3′-Indolylphosphatase *p*-Toluidine salt (BCIP) method. Slides were analysed under a light microscope.

Table 2. Cellulase activities (μg/mg) in the whole body of the polychaetes *Capitella* sp. I and *Perinereis nuntia brevicirris*, and in their habitat soils.

Capitella sp.		*P. nuntia brevicirris*	
Whole body	Habitat soil	Whole body	Habitat soil
0.20 ± 0.18	0.27	3.24 ± 0.04	0.02

RESULTS

Protease activity

Protease activity was detected in the whole body of both polychaete taxa, *Capitella* sp. I and *P. nuntia brevicirris*, but was hardly detected in either habitat soil (Table 1). The activity in *Capitella* sp. I (89.66 μg/mg whole body) was about 10 times that in *P. nuntia brevicirris* (7.95 μg/mg whole body).

Cellulase activity

Cellulase activity against CMC was detected in the whole body of *P. nuntia brevicirris* (3.24 μg/mg whole body) (Table 2), whereas scarcely any cellulase activity was detected in *Capitella* sp. I or in the habitat soils.

Cloning and sequence analysis of Capitella sp. I protease and P. nuntia brevicirris cellulase

We used RT-PCR with degenerate primers and the 5′- and 3′-RACE method to isolate cDNA clones of protease from *Capitella* sp. I and of cellulase (endo-β-1,4-glucanase) from *P. nuntia brevicirris*. A FASTA homology search revealed that the amplified protease cDNA from *Capitella* sp. I was most similar to chymotrypsin genes of other species. The *Capitella* sp. I protease gene (*cCS*; GenBank accession number AB558291) comprised 831 nucleotides and contained a single ORF; it encoded a protease 242 amino acids in length with a calculated molecular weight of 24.6 kDa.

The amino acid sequence of *cCS* conserved a histidine and a serine site motif of trypsin family serine proteases.

The full-length sequence of the cloned *P. nuntia brevicirris* cellulase (endo-β-1,4-glucanase) (*pnbEG*) transcript comprised 1481 nucleotides. The *pnbEG* cDNA included a single ORF of 1332 bp and corresponded to a polypeptide of 444 amino acid residues with a calculated molecular weight of 48.6 kDa. The GenBank accession number for the *pnbEG* sequence is AB558290.

Histological localization of the cCS and pnbEG genes

To determine the histological localization of *cCS* and *pnbEG* gene expression, *in situ* hybridization was performed using sections of each polychaete (Fig. 1). In

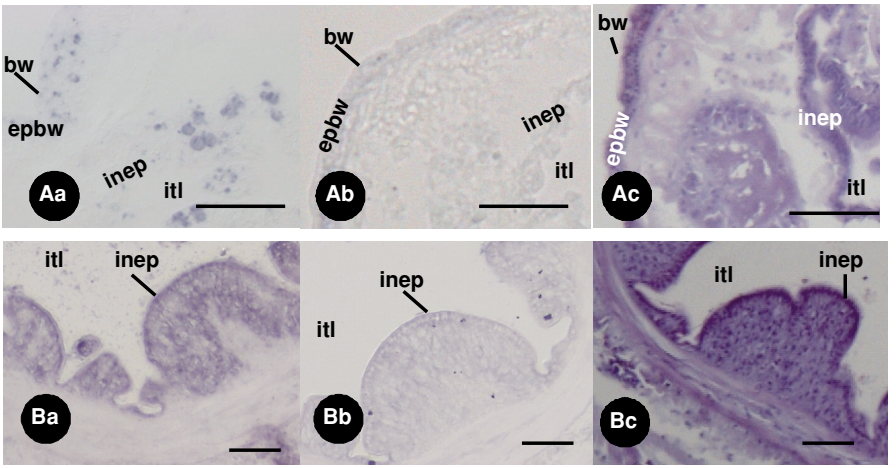

Fig. 1. *In situ* hybridization results for *cCS* mRNA and *pnbEG* mRNA in sections of *Capitella* sp. I and *P. nuntia brevicirris*. Hybridization was performed with digoxigenin (DIG)-labelled antisense and sense probes. Expression of *cCS* mRNA in the section of *Capitella* sp. I (Aa) obtained with anti-sense probe. (Ab) Negative control for the body wall, corresponding to sense probe hybridization. (Ac) Haematoxylin and eosin staining. (Ba) Expression of *pnbEG* mRNA in a body wall section of *P. nuntia brevicirris*. (Bb) Negative control using the sense probe. (Bc) Haematoxylin and eosin staining. (bw, body wall; epbw, epithelium of body wall; inep, intestinal epithelium; itl: intestinal lumen). Bars, 50 μm.

Capitella sp. I, *cCS* expression signals were observed in the epithelium of the body wall (Fig. 1Aa) and in the luminal epithelium of the intestine (Fig. 1Aa), using the antisense probe. In *P. nuntia brevicirris*, strong *pnbEG* hybridization signals were observed in the intestinal epithelium (Fig. 1Ba), using the antisense probe. In the sense probe hybridization, *cCS* and *pnbEG* mRNAs were not detected in any of the tissues (Figs. 1Ab and Bb).

DISCUSSION

In this study, we obtained the novel annelid chymotrypsin-like serine protease gene *cCS* from the polychaete *Capitella* sp. I, which expressed a higher protease activity than that of *P. nuntia brevicirris*. *cCS* mRNA was strongly expressed in intestinal cells, indicating that *cCS* is an endogenous polychaete gene that does not belong to contaminating microorganisms or eukaryotic protists, and that it contributes to proteolysis in the gut tract of *Capitella* sp. I.

Organic matter in the fish-farm sediments is derived mainly from feed waste and faecal matter (Yokoyama *et al*., 2006). Vezzulli *et al*. (2002) suggested that similar protein concentrations between sites can be attributed to a high protein turnover in sediment beneath the fish cages, as indicated by the higher aminopeptidase enzymatic activities found there. Extracellular proteases are secreted by various bacteria in sediments. For bioremediation, mass-cultured

Capitella sp. I colonies have been introduced into sediments at the bottom of a fish farm (Tsutsumi and Montani, 1993). As a result, the biological activities of *Capitella* sp. I markedly enhanced bacterial growth, and subsequently the bacteria decomposed the organic matter within the sediment (Kunihiro *et al.*, 2008). In this study, *Capitella* sp. I collected from bottom sediment under a fish farm had notably higher protease activity than *P. nuntia brevicirris*. This result suggests that *Capitella* sp. I may consume protein-rich organic material as its main food resource using not only enzymes originated from symbiotic bacteria and bacteria whose growth is enhanced by the worms, but also from its own secreted protease.

Terrestrial plant residues, microalgae, and phytoplankton are composed of cellulose. In an estuary, molluscs consuming organic matter had positive cellulase activities (Antonio *et al.*, 2010). In our study, high cellulase activity was detected in *P. nuntia brevicirris* collected from estuarine sediments, but scarcely any such activity was detected in *Capitella* sp. I from the fish-farm sediments. The high cellulase activity of *P. nuntia brevicirris* suggests that it consumes organic matter from both terrestrial and marine environments.

Johnston *et al.* (2005) reported that in abalone, the enzyme activity differs according to the food type given. In our study, we collected two polychaete species from different sites likely to be contaminated by different organic pollutants. We conclude that *Capitella* sp. I, in order to adapt to its habitat in bottom sediment of a fish farm, requires high protease activity, whereas *P. nuntia brevicirris* requires high cellulase activity to adapt to living in an estuary. In addition, these polychaetes produce these enzymes themselves. Hence, each polychaete has an enzyme activity suited to its habitat.

Bioremediation that efficiently utilizes a suitable polychaete can be an effective tool in understanding the differences among enzyme revitalizations in polychaete worm possible.

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