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Relation	



**Identification of a Novel Vanadium-binding Protein by EST Analysis on the Most
Vanadium-rich Ascidian, *Ascidia gemmata***

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Abbreviated title: Novel Vanabins from *A. gemmata*

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Abstract Ascidiaceans are known to accumulate extremely high levels of vanadium in their blood cells (up to 350 mM). The branchial sac and the intestine are thought to be the first tissues to contact the outer environment and absorb vanadium ions. The concentration of vanadium in the branchial sac and the intestine of the most vanadium-rich ascidian *A. gemmata* were determined to be 32.4 mM and 11.9 mM, respectively. Using an expressed sequence tag (EST) analysis of a cDNA library from the intestine of *A. gemmata*, we determined 960 ESTs and found 55 clones of metal-related gene orthologs, 6 redox-related orthologs, and 18 membrane transporter orthologs. Among them, 2 genes, exhibited significant similarity to the vanadium-binding proteins of other vanadium-rich ascidian species, were designated AgVanabin1 and AgVanabin2. Immobilized metal ion affinity chromatography revealed that recombinant AgVanabin1 bound to metal ions with an increasing affinity for Cu(II) > Zn(II) > Co(II), and AgVanabin2 bound to metal ions with an increasing affinity for Cu(II) > Fe(III) > V(IV). To examine the use of AgVanabins for a metal absorption system, we constructed *Escherichia coli* strains that expressed AgVanabin1 or AgVanabin2 fused to maltose binding protein and secreted into the periplasmic space. We found that the strain expressing AgVanabin2 accumulated about 13.5 times more Cu(II) ions than the control TB1 strain. Significant accumulation of vanadium was also observed in the AgVanabin2-expressing strain as seen by a 1.5-fold increase.

Keywords : *vanadium, vanabin, metal accumulation, ascidian*

Introduction

Ascidiaceans, also known as tunicates or sea squirts (Chordata, Urochordata, Ascidiaceae),

accumulate extremely high concentrations of vanadium (Michibata et al., 2003, 2007; Ueki and Michibata, 2008). In particular, species belonging to the family Ascidiidae are known to accumulate vanadium in excess of 350 mM, which corresponds to about 10^7 times the concentration of vanadium ions normally dissolved in seawater (Michibata et al., 1986, 1991). Vanadium ions are accumulated in the vacuole of the vanadocytes, which are a type of blood (coelomic) cell (Michibata et al., 1987; Ueki et al., 2002).

We have identified many genes involved in the accumulation and redox of vanadium in ascidians, mainly through biochemical and molecular biological methods. Vanadium-binding proteins were purified from blood cells, coelomic fluid, and intestines of *Ascidia sydneiensis samea*, which contained 12.8 mM vanadium in the blood cells (Michibata et al., 1986). The related proteins were purified by ion exchange chromatography and metal ion affinity chromatography (Ueki et al., 2003a; Yoshihara et al., 2005, 2008; Yoshinaga et al., 2006, 2007). In addition, enzymes in the pentose phosphate pathway that is known to produce two molecules of NADPH per cycle were identified by specific monoclonal antibodies and polymerase chain reaction (PCR) (Uyama et al., 1998a, b, c; Ueki et al., 2000), and produced NADPH was revealed to be involved in redox of vanadium (Kawakami et al., 2009). However, further information is required regarding other genes and proteins besides those mentioned above, not only to understand the fundamental mechanism of hyperaccumulation, but also to identify other proteins with the ability to bind and transport high amounts of heavy metal ions.

An expressed sequence tag (EST) analysis is a powerful tool to investigate cell and tissue functions, as well as gene expression profiles. Gene expression profiles based on EST analysis of fertilized eggs, embryos, and several tissues have been reported for some ascidian species (Makabe et al., 2001; Satou et al., 2002, 2003; Yamaguchi et al., 2002, 2004), including *A.*

sydneiensis samea and *Ciona intestinalis*, which contained 0.6 mM of vanadium in their blood cells (Michibata et al., 1986), and *Halocynthia roretzi*, which contained 0.01 mM of vanadium in their blood cells (Michibata et al., 1986). In our previous study, we successfully identified two novel Vanabins by EST analysis on the blood cells of *A. sydneiensis samea* (Yamaguchi et al., 2002, 2004), as well as from *C. intestinalis* (Trivedi et al., 2003). However, related proteins and genes have not been annotated in the most vanadium-rich ascidian species *A. gemmata*, which contains 350 mM of vanadium in their blood cells (Michibata et al., 1991). This lack of data may be caused by interference from the high levels of vanadium, even though this should be expected during the isolation of proteins and genes involved in hyperaccumulation.

Here we report the results of EST analysis for a cDNA library of the intestine in *A. gemmata*. The intestine was chosen as the target of the analysis because vanadium dissolved in seawater is absorbed into the body of ascidians through the branchial sac connected with the intestine. We sequenced 960 cDNA clones obtained from the cDNA library. A BLAST search was performed against protein sequences, which revealed 55 metal-related proteins, including two types of novel vanadium-binding proteins. Redox-related proteins and membrane transporters were also identified in the EST analysis. The two novel Vanabins, AgVanabin1 and AgVanabin2, were expressed in *E. coli* and assessed for their metal-binding ability. We also tested their ability to absorb metal ions when they are expressed in the periplasm of *E. coli*, and found that AgVanabin2 protein significantly absorbed V(IV) and Cu(II) ions.

Materials and Methods

Metal Determination, RNA Extraction, and Construction of the cDNA Library from *A. gemmata*

Adult specimens of the ascidian *A. gemmata* were collected at Kojima port, Okayama Prefecture, Japan. Branchial sacs and intestines were excised from their bodies (ca. 5 cm in length). For metal extraction, each tissue was weighed and homogenized in 0.1 M nitric acid, and the supernatant was obtained by centrifugation at $10,000 \times g$ for 10 min. After centrifugation, an aliquot of the supernatant was used to measure vanadium concentration by atomic absorption spectroscopy (A-220Z; Varian Inc.). Metal content was expressed as a molar concentration of metal compared to their wet weight prior to homogenization.

Intestines from another individual of *A. gemmata* (ca. 5 cm) was homogenized in a solution containing 4 M guanidine thiocyanate, 0.1% sodium *N*-lauryl sarcosinate, 5 mM EDTA, and 40 mM Tris-HCl at pH 7.0 and further homogenized with an ultrasonicator. The homogenate was added to a solution of 50% cesium trifluoroacetate (CsTFA) and 100 mM EDTA and centrifuged ($100,000 \times g$, 16 h, 15°C) in an ultracentrifuge (model 70P72; Hitachi, Tokyo, Japan). Precipitated RNA was recovered and dissolved in sterilized RNase-free water. Poly(A)⁺ RNA was purified using Oligotex-dT30 (TaKaRa, Kyoto, Japan).

cDNA libraries were constructed using the Uni-Zap cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Resulting cDNAs were ligated into the pBluescript II SK(+) vector predigested with *Eco*RI and *Xho*I (Stratagene), and the plasmids were introduced into XL10-Gold ultracompetent cells (Stratagene).

DNA Sequencing and BLAST Analysis

Transformed *E. coli* cells bearing pBluescript plasmids (Stratagene) were plated on LB/Amp

plates. We randomly selected colonies from the library and grew the cells in liquid LB medium to obtain plasmid DNA. The plasmid DNA was used for DNA sequencing with a M13 reverse primer (TaKaRa). Using BLASTX (Altschul et al., 1990), an about 500-bp DNA sequence from each cDNA clone was compared to sequences in the SwissProt database to identify related proteins. Several cDNA clones of particular interest were sequenced. cDNA with a sequence similarity probability $>1e^{-05}$ was classified as non-similar; sequences with a similarity probability $<1e^{-05}$ were classified as similar. The nucleotide sequence data were registered under the accession numbers FS996288–FS997156 in the DDBJ/EMBL/GenBank databases.

Preparation of Recombinant Protein and Purification of AgVanabins

To construct plasmids for the expression of fusion proteins, cDNA fragments of vanabin orthologs spanning the coding region of the two novel vanabin orthologs, AgVanabin1 and AgVanabin2, were amplified by PCR using specific primer sets to which the following artificial restriction sites had been added. AgVanabin1 forward/reverse primer: 5'-GAATTCAAAAAGATTAGACACGAG-3'/5'-TCTAGATTAGTGGCCCGGTCTACA-3'. AgVanabin2 forward/reverse primer: 5'-GAATTCATGAAAGGGTGCGCCG-3'/5'-GTCGACTTAGGCATCAGGGAAG-3'. The amplified fragments were digested with *EcoRI* and *XbaI* for AgVanabin1 and *EcoRI* and *SalI* for AgVanabin2, and then were ligated into the corresponding site of pMAL-c2x or pMal-p2x (New England BioLabs, Ipswich, MA). The vanabin-coding regions were ligated to both the Tac promoter and the coding region for maltose-binding protein (MBP) to produce a fusion protein. The plasmid was introduced into a primary bacterial host, *E. coli* DH5 α , and then transformed into the TB1 strain for AgVanabin1

and AgVanabin2 protein expression. An overnight culture of non-induced TB1 cells bearing vanabin-expressing plasmids was diluted 1:10 in LB medium containing $50 \mu\text{g}\cdot\text{mL}^{-1}$ ampicillin and 0.25 mM IPTG (Wako Pure Chemical Industries, Tokyo, Japan), and then cultured at 37°C for 6 h. The bacterial cells were collected by centrifugation ($2500 \times g$, 10 min, 4°C) and sonicated in amylose resin column buffer (20 mM Tris-HCl (pH7.4), 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide, 10 mM mercaptoethanol). The fusion protein was purified from this lysate on an amylose resin column according to the manufacturer's protocol (New England BioLabs). The purity of the fusion proteins was confirmed by SDS-PAGE. Protein concentrations were measured with a protein assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin (Pierce, Rockford, IL) as a standard.

To dissociate recombinant AgVanabin2 from the MBP component of the fusion protein, the proteins were digested with 1/200 (w/w) factor Xa (Haematologic Technologies, Essex Junction, VT) for 16 h at 30°C in elution buffer on an amylose resin column. AgVanabin2 was separated by DEAE-sephacel anion exchange chromatography in 50 mM Tris-HCl (pH 7.4). In the case of AgVanabin1, we could not obtain pure and native protein after Factor Xa digestion. Therefore, we used AgVanabin1-MBP fusion protein for metal-binding assays.

Metal Reagents

Vanadyl sulfate ($\text{VO}\text{SO}_4\cdot n\text{H}_2\text{O}$, $n = 3-4$; 99.9%), along with MgCl_2 , CaCl_2 , MnCl_2 , FeCl_3 , CoSO_4 , CuCl_2 , and ZnCl_2 were purchased from Wako Pure Chemical Industries. Each metal was dissolved in ultrapure water. To prevent precipitation under the assay conditions, vanadyl sulfate was dissolved upon use and mixed with iminodiacetic acid (IDA) in a 1:1 molar ratio.

Vanadium Binding Assay with Immobilized Metal Ion Affinity Chromatography (IMAC)

Proteins (50 μg per 250 μL) were incubated with 1 mM EDTA for 1 h at 4°C and then dialyzed against column buffer (20 mM phosphate buffer (pH 7.4), 100 mM NaCl) overnight at 4°C. For IMAC preparation, 100 μL of slurry (Chelating Sepharose Fast Flow; GE Healthcare, Piscataway, NJ) was washed in 1 mL of Milli-Q water (Millipore, Billerica, MA), centrifuged ($600 \times g$, 2 min, 4°C), and then the supernatant was removed. This process was repeated three times. Consecutively, Mg(II), Ca(II), VO(II), Mn(II), Fe(III), Co(II), Cu(II), and Zn(II) were immobilized by adding 400 μL of 100 mM metal solution and shaking the solution for 30 min at room temperature (RT). The resin was washed by Milli-Q water (three times) and column buffer (twice) in the manner described above. Dialyzed proteins were added to the resin and shaken for 30 min at 4°C. After centrifugation, the supernatants were kept as a non-bound fraction and the resin was washed twice by column buffer. For elution, we used 400 μL column buffer at pH 7.4 supplemented with 100 mM NaCl and 25 mM EDTA. Eluted proteins were analyzed by SDS-PAGE using a 14% polyacrylamide gel.

Periplasmic Expression of MBP–AgVanabin1 or MBP–AgVanabin2 Fusion Proteins in *E. coli*

The expression protocol was performed as described by Ueki et al., (2003b). Overnight cultures of *E. coli* TB1 cells possessing pMAL-p2, pMAL-p2-AgVanabin1, or pMAL-p2-AgVanabin2 plasmids in MJS-Amp medium (12.5 mM Tris (pH 7.2), 50 mM NaCl, 20 mM NH_4Cl , 1 mM KCl, 1 mM MgCl_2 , 0.1 mM CaCl_2 , 0.05 mM MnCl_2 , 0.4% (v/v) glycerol, 0.8% (w/v) Casamino

acid, 0.005% thiamine, and $100 \mu\text{g}\cdot\text{mL}^{-1}$ ampicillin) were diluted 1:9 with fresh MJS-Amp medium and cultured at 37°C for 6 h with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG).

A whole-cell sample was prepared by diluting pelleted cells 1:10 (e.g., 1 mL for 10 mL culture) in lysis buffer (10 mM Na_2HPO_4 , 30 mM NaCl, 10 mM EDTA, 10 mM EGTA, 0.25% Tween 20, 10 mM 2-mercaptoethanol (pH7.0)), followed by lysis with an ultrasonicator.

The cytoplasmic and periplasmic fractions were obtained using the cold osmotic shock method (Neu and Heppel, 1965). Cells were harvested by centrifugation and diluted 1:10 in 30 mM Tris-HCl and 20% sucrose (pH 8.0). EDTA was added to the suspended cells at a final concentration of 1 mM. The cells were incubated for 10 min. at RT and harvested by centrifugation at $10,000 \times g$ for 30 s. After removing the supernatant, the cells were suspended in the same volume (e.g., 1 mL for 10 mL culture) of ice-cold 5 mM MgSO_4 and incubated on ice for 10 min. After centrifugation at $10,000 \times g$ for 30 s, the supernatant was used as the periplasm fraction.

Metal Accumulation in *E. coli* Expressing AgVanabin1 or AgVanabin2

The protocol used was described in our previous article (Ueki et al., 2003b). Overnight cultures of *E. coli* TB1 strains with (or without) plasmids in MJS-Amp (or MJS) medium were diluted ten times with fresh MJS-Amp (or MJS) medium containing 0.5 mM IPTG and metal ions at the desired concentration. The initial concentration of each metal was expressed as the amount of metal ions added to the medium. The culture volume was 15 mL, and the culture was performed in 50-mL conical tubes (Falcon type 2070; BD Biosciences) rotated at 200 rpm. The cells were incubated at 37°C for 6 h, harvested by centrifugation at $10,000 \times g$ for 5 min at 4°C , washed

three times with excess MJS medium without metal, and dried at 65°C for 24 h. After measuring the dry weight (dw), the cell pellet was treated overnight with 300 µL of 1 M nitric acid. After centrifugation, an aliquot of the supernatant was used to measure the metal concentration by atomic absorption spectroscopy (A-220Z; Varian Inc.). Metal content was expressed as the weight of metal per weight of the dried cells ($\text{ng}\cdot\text{mg}^{-1}$ dw). Statistical significance was assessed using a Student's two-tailed *t*-test.

Results

EST Sequencing and Categorization of Orthologs

We initially tried to isolate mRNA from the blood cells of *A. gemmata*, but were not successful, which may have been due to high levels of vanadium contained within the blood cells (347 mM) (Michibata et al., 1991). Therefore, we isolated mRNA from the other tissues, namely the branchial sac and intestine for this study. The concentration of vanadium in the branchial sac and the intestine from three individual adult specimens of *A. gemmata* (ca. 5 cm) were 32.4 ± 5.5 mM ($n = 3$) and 11.9 ± 4.0 mM ($n = 3$), respectively. These values were significantly higher than respective tissues in other ascidian species (Michibata et al., 1986, 1991). The intestine was consequently chosen as a target tissue for EST analysis.

mRNA from the intestine in an adult specimen of *A. gemmata* (ca. 5 cm) was purified to construct a cDNA library. About 0.5 g of tissues was used for RNA extraction, and 50 µg of total RNA was purified. Poly(A)⁺ RNA was extracted from 40 µg of total RNA and used for cDNA library construction. As a result, about 6.2×10^4 primary recombinant clones were obtained with

an average cDNA length of 1.2 kbp.

The nucleotide sequences of 960 cDNA clones were randomly selected from the cDNA library and sequenced. Obtained ESTs were compared to known sequences in the SwissProt database. DNA with a sequence similarity probability $>1e^{-05}$ was classified as non-similar, while sequences with a similarity probability $<1e^{-05}$ were classified as similar. Of the 870 clones that yielded significant length (>150 bp) of nucleotide sequence data, the sequences of 539 were similar and 331 were non-similar to characterized proteins. As shown in Table 1, cDNAs with sequence similarity were placed into one or more of the KOG categories (Tatusov et al., 2003). Fifty-five metal-related clones were further subdivided into six groups for metal relationships such as Ca, Mg, V, Fe, Fe/Cu and Zn (Table 2). Redox-related clones and transporters are listed in Tables 3 and 4, respectively. The nucleotide sequence data were registered under the accession numbers FS996288–FS997156 in the DDBJ/EMBL/GenBank databases.

Table 1

Table 2

Table 3

Table 4

In the present EST analysis, we identified four ESTs (AG-1_C05, AG-1_H05, AG-2_F11, and AG-3_G11) for two novel vanabin genes. Novel vanabins, which we have termed AgVanabin1 and AgVanabin2, had 18 conserved cysteines, which is common across vanabins described previously. The interval of cysteine residues is also highly conserved (Fig. 1). Comparison of each sequence of vanabin revealed that AgVanabin1 and AgVanabin2 were similar to each other (bootstrap value of 80.4% from a neighbor-joining analysis), and were also similar to VanabinP of *A. sydneiensis samea* (bootstrap value of 67.7% from a neighbor-joining analysis). However, the composition of amino acid residues in AgVanabin1 and AgVanabin2 was different from those in vanabins of *A. sydneiensis samea*. Vanabins of *A. sydneiensis samea* had a high number of lysine residues in the core region between the 1st and 18th conserved cysteine, which has been suggested by ESR and NMR studies to be the binding site for V(IV) ions (Fukui

Fig. 1

et al., 2003; Hamada et al., 2005). For example, Vanabin2 of *A. sydneiensis samea* contains 13 lysines (15.1%) and 5 arginines (5.8%) within the 86 amino acid core region. AgVanabin1 has 4 lysines (4.1%) and 8 arginines (8.3%) within the 97 amino acid core region, while AgVanabin2 has 4 lysines (4.2%) and 6 arginines (6.3%) within the 95 amino acid core region.

In addition to vanabins, we noticed several metal-related genes in the EST analysis. One is an iron storage protein, ferritin, which was reported to bind V(IV) ions by an ESR study (Grady et al., 2000). The heavy subunit of ferritin was found in two clones (AG-10_B05 and AG-4_E08). The representative sequence is shown in Fig. 2. Ferritin consists of 24 subunits of two types, namely heavy and light subunits, which form a shell-like structure with a hollow interior (Aisen et al., 1999). A histidine residue corresponding to the vanadium-binding site of human ferritin (Grady et al., 2000) is also conserved in the ascidian ferritin heavy subunit. These characteristics are well conserved in ferritin proteins identified from another vanadium-rich ascidian, *A. sydneiensis samea* (Yamaguchi et al., 2002, 2004). This observation suggests that ferritin may play a role in the storage of vanadium *in vivo*.

Fig. 2

Three EST clones (AG-5_E12, AG-6_G08, and AG-10_G05) were found to encode glutathione-S-transferase (GST). GSTs are universal enzymes that utilize glutathione (GSH) to detoxify a wide range of toxic compounds. In our previous studies, GSTs were revealed to be major proteins produced in the intestine of *A. sydneiensis samea* that could bind to V(IV) and V(V) ions (Yoshinaga et al., 2006, 2007). The partially translated sequence of the EST clones was most similar to two GSTs from another vanadium-rich ascidian, *C. intestinalis*, but was not clearly clustered with any class of GSTs in other organisms.

Metal-Binding Assay for Vanabin Orthologs

To examine the metal-binding ability of vanabin orthologs, we performed IMAC for AgVanabin1 and AgVanabin2 (Fig. 3). AgVanabin1 was applied to IMAC as a fusion protein because it could not be digested by Factor Xa. As a result, MBP–AgVanabin1 bound to the resin via Cu(II), Co(II), and Zn(II), respectively. The order of affinity was Cu(II) > Zn(II) > Co(II), as extrapolated from the elution pattern by a high-salt and chelating buffer (Fig. 3A). The MBP–AgVanabin1 did not bind to V(IV)-chelating resin under these conditions, perhaps due either due to the fusion of MBP to AgVanabin1 or the low binding capacity of AgVanabin1. In the case of AgVanabin2, we could apply purified AgVanabin2 to the chelating resin after Factor Xa digestion and found that it could bind to V(IV) and several other metal ions with the affinity order of Cu(II) > Fe(III) > V(IV) (Fig. 3B).

Fig. 3

Expression of MBP–AgVanabin Fusion Proteins in the *E. coli* Periplasm

Plasmids encoding MBP–AgVanabin1 and MBP–AgVanabin2 were constructed and introduced into the *E. coli* TB1 strain. We have termed them TB1/MBP–AgVanabin1 and TB1/MBP–AgVanabin2, respectively. As a control, an empty TB1 strain and a TB1 strain expressing MBP alone (TB1/MBP) were used. To examine the subcellular localization of MBP, MBP–AgVanabin1, and MBP–AgVanabin2, we used the cold osmotic shock method to obtain the periplasmic fraction (Neu and Heppel, 1965). After inducing expression of the fusion proteins in MJS-Amp medium with 0.5 mM IPTG at 37°C for 6 h, intense bands for MBP, MBP–AgVanabin1, and MBP–AgVanabin2 were detected in the periplasmic fraction of TB1/MBP, TB1/MBP–AgVanabin1, and TB1/MBP–AgVanabin2 strains, respectively (Fig. 4).

Fig. 4

Most of the fusion proteins were in the periplasm, as was the control MBP, although some remained in the cytoplasm. MBP–AgVanabin1 and MBP–AgVanabin2 fusion proteins were not seen prior to induction, and MBP itself was present in extremely low concentrations without induction under this condition (data not shown).

Bioaccumulation of Metals

We examined the possibility of whether *E. coli* strains expressing MBP–AgVanabin1 or MBP–AgVanabin2 fusion proteins could accumulate V(IV), Fe(III), Co(II), Ni(II), Cu(II), and Zn(II) ions. The expression of the fusion protein in the cells was induced by 0.5 mM IPTG in MJS medium containing each metal ion at an initial concentration of 10 μ M for 6 h at 37°C. The cells were harvested, washed, and dried. After extracting the metals from the cells treated with nitric acid, the metal content was measured by atomic absorption spectrometry and normalized by their dry weight.

Significant accumulation was found only for Cu(II) and V(IV) ions. The accumulation of Fe(III), Co(II), Ni(II) and Zn(II) was not significantly different from the values of parent strain TB1 for $239,257 \pm 16,521$, 8.23 ± 39.41 , 20.46 ± 17.68 and 145.56 ± 31.64 ng·mg⁻¹ dw, respectively. TB1 cells accumulated Cu(II) ions at a concentration of 173.62 ± 43.06 ng·mg⁻¹ dw. The average copper content increased approximately threefold by the expression of MBP (559.81 ± 64.19 ng·mg⁻¹ dw), and the difference between TB1 and TB1/MBP was significant ($P < 0.05$). The expression of MBP–AgVanabin2 significantly increased the accumulation of Cu(II) ions (2360.91 ± 462.05 ng·mg⁻¹ dw). The enhancement factor was approximately 13.5-fold compared to the TB1 strain ($P < 0.05$). In contrast, Cu(II) ion accumulation by MBP–AgVanabin1 (550.79

$\pm 6.50 \text{ ng}\cdot\text{mg}^{-1} \text{ dw}$) was not significantly different from that of TB1/MBP ($P > 0.05$). These results clearly indicated that AgVanabin2, but not AgVanabin1, enhanced the ability of TB1 strains to accumulate Cu(II) ions. Expression of MBP–AgVanabin2 also enhanced the accumulation of V(IV) ions in the periplasm. The TB1 strain accumulated $7.47 \pm 0.48 \text{ ng}\cdot\text{mg}^{-1} \text{ dw}$ of V(IV) ions, while the TB1/MBP–AgVanabin2 strain accumulated $10.12 \pm 0.64 \text{ ng}\cdot\text{mg}^{-1} \text{ dw}$ of V(IV) ions. The enhancement factor was about 1.5-fold compared to the TB1 strain ($P < 0.05$). TB1/MBP–AgVanabin1 did not accumulate V(IV) ions significantly under this condition.

Discussion

In this study, we performed an EST analysis on the intestine of the most vanadium-rich ascidian *Ascidia gemmata* to obtain molecular information related to its extremely high level of vanadium accumulation. We attempted to analyze blood cells from three ascidian species, *A. gemmata*, *Ascidia ahodori*, and *Phallusia mammillata*, which contain more vanadium than *Ascidia sydneyensis samea* or *Ciona intestinalis*, but were unsuccessful as no RNAs were recovered from the blood cells of these species. The high levels of vanadium contained in the blood cells may inhibit any process of RNA extraction. The intestine was used as the source for EST analysis in this study because vanadium in seawater is absorbed into the body of ascidians through the branchial sac and intestine, and the concentration of vanadium in the intestine reaches 11.9 mM based on the results of our measurements.

We determined the partial nucleotide sequence of 960 cDNA clones selected randomly. We focused on the identification of metal-related genes, and discovered 55 genes, as well as redox-related genes and transporter genes, based on the similarity to their orthologs in other

organisms (Tables 2–4). In particular, we found four ESTs similar to vanabins, which are the vanadium-binding proteins that we initially found in *A. sydneiensis samea* (Ueki et al., 2003a), and we also identified the orthologs in two other species, *C. intestinalis* and *Ciona savignyi* (Trivedi et al., 2003). The novel vanabins, which we named AgVanabin1 and AgVanabin2, have 18 conserved cysteines that are common to previously described vanabins. The interval of the cysteine residues was highly conserved (Fig. 1). Our hypothesis that vanabins are unique to vanadium-rich ascidians is supported in this study.

Using the *in vitro* metal-binding assay by IMAC, AgVanabin2 was revealed to bind V(IV) and several other metal ions with the affinity order of Cu(II) > Fe(III) > V(IV) (Fig. 3B). The affinity order was similar to that of Vanabin2 of *A. sydneiensis samea*, which bound strongly to Cu(II), Fe(III), and V(IV), and weakly to Co(II) and Zn(II) at a pH of 7.4, although the buffer compositions were different (Ueki et al., 2009). The results of this study were also supported by a description that Co (II), Ni (II), and Zn (II) are the group of metals that have a weak holding capacity (Kawakami et al., 2006). In contrast, AgVanabin1 could bind to Cu(II) > Zn(II) > Co(II), but not the V(IV)-chelating resin (Fig. 3A), although it was applied to IMAC as a fusion protein because of the difficulty in obtaining it in its native form. Examining other expression systems in a future study is warranted.

One of our goals is to create a metal biosorption system using ascidian gene resources. There could be two goals; one is to remove hazardous heavy metals from liquid wastes, and the other is to recover valuable heavy metal ions in natural environments. Studies have sought metal-binding peptides with the ability to bind heavy metals in various living organisms to improve the metal-binding abilities of microorganisms via heterogeneous protein expression. We previously constructed a biosorption system in *E. coli* using Vanabin1 and Vanabin2 of *A. sydneiensis*

samea and found that both strains significantly absorbed Cu(II) ions (Ueki et al., 2003b). In this study, we constructed a similar system using AgVanabin1 and AgVanabin2. We found that the biosorption system using AgVanabin2 was effective for both V(IV) and Cu(II) ions. These results are consistent with IMAC analysis (Fig. 3). AgVanabin2 could bind to V(IV) ions and several other metal ions with the affinity order of Cu(II) > Fe(III) > V(IV) (Fig. 3B), and *E. coli* cells expressing MBP–AgVanabin2 successfully accumulated both V(IV) and Cu(II) ions, although the accumulation levels were very different. In contrast, accumulations of V(IV), Co(II), Fe(III), Ni(II), and Zn(II) ions by MBP–AgVanabin1 were not significantly different from MBP alone. This could have been due a low binding ability of AgVanabin1, or possibly due to structural disorders as was suggested by the IMAC experiment.

In conclusion, this study revealed several novel genes including two vanabins that are expressed in the intestine of the most vanadium-rich ascidian *A. gemmata*. This is the first report on genes and proteins that are involved in the accumulation of vanadium in the most vanadium-rich ascidian *A. gemmata*. One of the two vanabins, AgVanabin2, was found to bind V(IV) ions. They were used to construct an *E. coli* biosorption system through heterologous expression of the proteins. The cells that expressed the fusion proteins were examined with V(IV), Cu(II), Fe(III), Co(II), Ni(II), and Zn(II) ions. The results indicated that this biosorption system using MBP–AgVanabin2 was effective for both Cu(II) and V(IV) ions. As the next step, we may be able to improve this biosorption system by comparing Cu(II)- and V(IV)-binding sites in vanabins from these two ascidian species and modifying the amino acid sequences in each vanabins.

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AgFerritin	1	- - - - M A E Q S - S S S G N N A G A W L K T C E E G V N N Q V N L E L Y A S Y M Y M A M G H F F D R	46
AsFerritin	1	- - - - M A E Q A Q S S S G N T G G V W S K A C E D G L S N Q V N L E L Y A S Y M Y M A M G H F F D R	47
CiFerritin	1	M D Q Q Q Q Q S T S S S G N T A G E W A K Q C E D G L N N Q I N L E L Y A S Y V Y M A M G H Y F D R	51
mouse	1	- - - - - M T T A S P S Q V R Q N Y H Q D A E A A I N R Q I N L E L Y A S Y V Y L S M S C Y F D R	44
human	1	- - - - - M T T A S T S Q V R Q N Y H Q D S E A A I N R Q I N L E L Y A S Y V Y L S M S Y Y F D R	44
AgFerritin	47	D D V A L K N V A K Y F L K C S E E E R E H A D K I V K Y H N Q R G G T T T F F Q I K P P G A F D P A	97
AsFerritin	48	D D V A L K N I S K Y F L K S A E E E R E H A N K L V A F H N Q R G G T T A Y F Q I K P P T A F D P A	98
CiFerritin	52	D D V A L K N V S K F F K E C S E E E R E H A N K M V E F H N R R G G N T T Y F P I K S P G P F G P D	102
mouse	45	D D V A L K N F A K Y F L H Q S H E E R E H A E K L M K L Q N Q R G G R I F L Q D I K K P D R D D W E	95
human	45	D D V A L K N F A K Y F L H Q S H E E R E H A E K L M K L Q N Q R G G R I F L Q D I K K P D C D D W E	95
AgFerritin	98	T F S V L K A M Q C A L A L E V N V N K S L L A L H D T G N - - G D P E F Q D F I E A N F L H E Q V E	146
AsFerritin	99	S F N A L K A M Q C A L A L E V N V N K S L L T L H D T A D - - G D P E F Q D F I E A N F L H E Q V E	146
CiFerritin	103	N F N T I K A M K C A L A L E V N V N K S L L A L H E T A N - - G D P E F Q D F I E A N Y L H E Q V E	151
mouse	96	S G - - L N A M E C A L H L E K S V N Q S L L E L H K L A T D K N D P H L C D F I E T Y Y L S E Q V K	144
human	96	S G - - L N A M E C A L H L E K N V N Q S L L E L H K L A T D K N D P H L C D F I E T H Y L N E Q V K	144
AgFerritin	147	A I K T L K D Y I T N L K R V G T - - - G L G E Y M F D K H F L D E - - - -	177
AsFerritin	147	A I K T L K D Y I T N L K R V G T - - - G L G E Y M F D K H F L D E - - - -	177
CiFerritin	152	A I K Q L K D Y I T N L E L V G T - - - G L G E Y M F D K H F K S S - - - -	182
mouse	145	S I K E L G D H V T N L R K M G A P E A G M A E Y L F D K H T L G H G D - E S	182
human	145	A I K E L G D H V T N L R K M G A P E S G L A E Y L F D K H T L G D S D N E S	183

Fig. 2 Amino acid sequence of ferritins. Sequences were obtained from *Ascidia gemmata* (AgFerritin, this study), *Ascidia sydneiensis samea* (AsFerritin; Yamaguchi et al. 2004), *Ciona intestinalis* (CiFerritin, XP_002127679), mice (P09528), and humans (P02794). Identical residues are boxed and putative iron-binding residues are shaded in light gray. Histidine residues (reported to be a vanadium-binding site in the human ferritin heavy subunit) are shaded in dark gray.

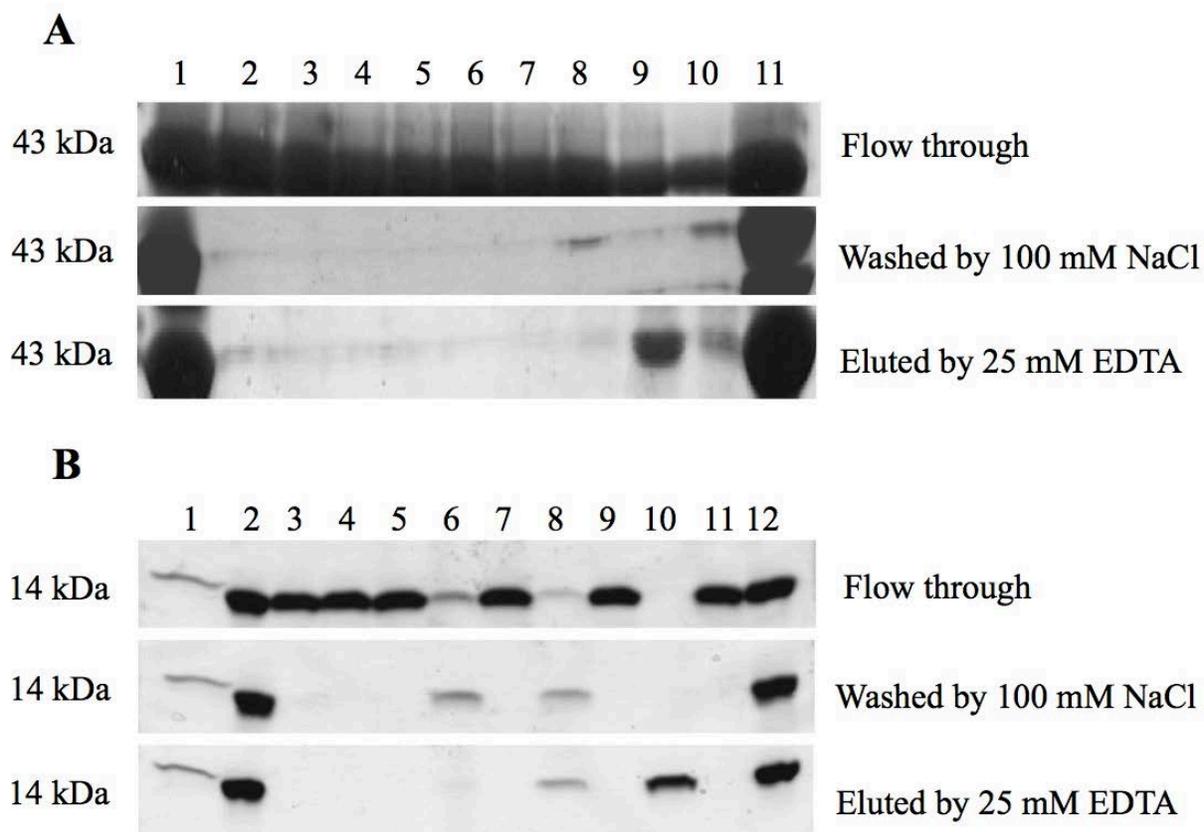


Figure 3.

Fig. 3 Binding of metal ions by MBP–AgVanabins as assayed by immobilized metal ions affinity chromatography (IMAC). **(A)** MBP–AgVanabin1. Lanes 1 and 11 = loading control of MBP–AgVanabin1, lane 2 = no metal, lane 3 = Mg(II), lane 4 = Ca(II), lane 5 = V(IV), lane 6 = Mn(II), lane 7 = Fe(III), lane 8 = Co(II), lane 9 = Cu(II), and lane 10 = Zn(II). **(B)** MBP–AgVanabin2. Lane 1 = LMW marker (14 kDa), lanes 2 and 12 = AgVanabin2, lane 3 = no metal, lane 4 = Mg(II), lane 5 = Ca(II), lane 6 = V(IV), lane 7 = Mn(II), lane 8 = Fe(III), lane 9 = Co(II), lane 10 = Cu(II), and lane 11 = Zn(II).

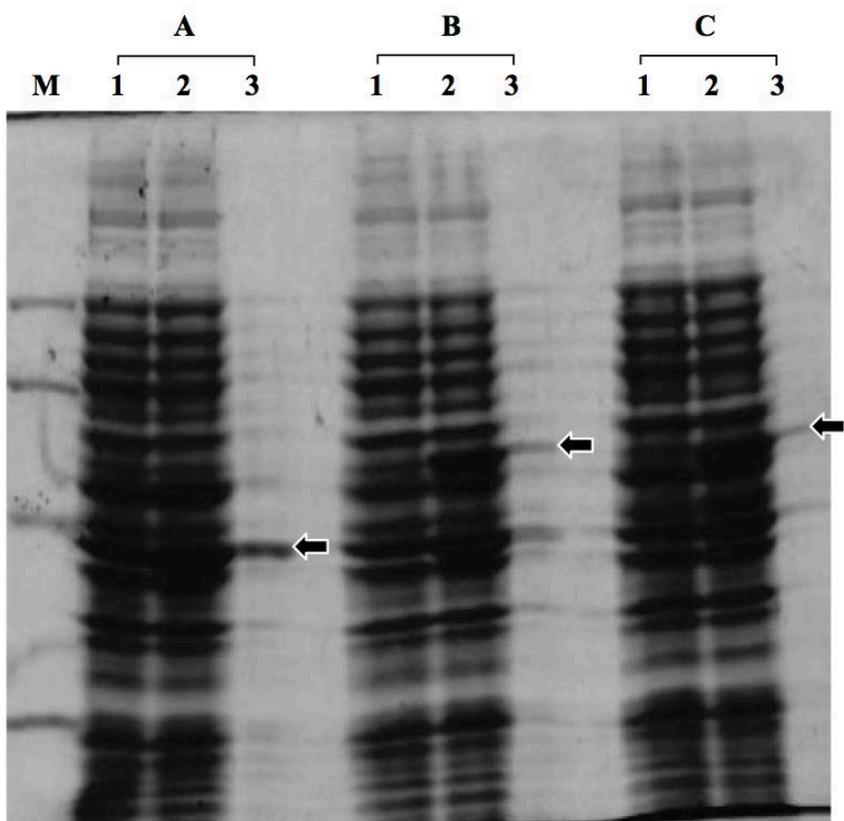


Figure 4.

Fig. 4 Expression and localization of MBP and MBP-AgVanabins. (A) MBP, (B) MBP-AgVanabin1, and (C) MBP-AgVanabin2. M = marker, lane 1 = homogenate from non-induced cells, lane 2 = homogenate from induced cells, and lane 3 = periplasmic fraction from induced cells. Arrows indicate induced protein bands.

Table 1. Classification of ESTs according to KOG.

KOG ID	KOG description	number of ESTs	gene products (number of ESTs if multiple ESTs found)
A	RNA processing and modification	20	Splicing factor, hnRNP, snRNP
B	Chromatin structure and dynamics	5	protamine
C	Energy production and conversion	34	ATP synthase subunit(5), cytochrome c oxydase(16), V-ATPase(2)
D	Cell cycle control, cell division, chromosome partitioning	24	cyclin(8), checkpoint(6)
E	Amino acid transport and metabolism	8	glutamine synthase(2)
F	Nucleotide transport and metabolism	4	nucleoside diphosphokinase(4)
G	Carbohydrate transport and metabolism	14	glucose dehydrogenase, beta-glucosidase
H	Coenzyme transport and metabolism	3	
I	Lipid transport and metabolism	13	
J	Translation	79	ribosomal protein, elongation factor, initiation factor
K	Transcription	14	
L	Replication	8	checkpoint(6)
M	Cell wall/membrane/envelope biogenesis	5	beta glucanase, beta-glucan synthase, extensin
N	Cell motility	1	thymosin beta
O	Posttranslational modification	38	glutathione peroxidase, thioredoxin, glutathione S-transferase(3)
P	Inorganic ion transport and metabolism	33	ferritin(2), transferrin, Vanabin(4), solute carrier protein(11), chloride channel, voltage-dependent anion channel(3), ABC transporter(2), catalase
Q	Secondary metabolites biosynthesis	14	alcohol dehydrogenase(10), cytochrome P450(2)
R	General function prediction only	24	serine protease inhibitor(2)
S	Function unknown	54	F-box/leucine rich(6), zinc finger(6)
T	Signal transduction mechanisms	96	notch/delta/fibropellin (19), vitelline coat proprotein (30),
U	Intracellular trafficking	25	importin(4), translocon(3), secretary(4)
V	Defense mechanisms	4	lectin, serine protease
W	Extracellular structures	0	
X	Not categorized (by NCBI staff)	0	
Y	Nuclear structure	4	importin(4)
Z	Cytoskeleton	53	actin(8), myosin(2), tubulin(41)
others	no hits	331	
sum		908	

Note: Several EST clones were classified into two or more categories. Therefore, the number of total KOD clones (908) is larger than the number of EST clones (870).

Table 2. A list of metal-related ESTs identified in the intestine from *Ascidia gemmata*.

EST-ID	Gene product	KOG description	Metal	Species
AG-1_F11	Agrin(heparin sulfate proteoglycan)	Posttranslational modification, protein turnover	Ca	<i>Bos taurus</i>
AG-7_C05	Annexin	Intracellular trafficking, secretion and vesicular transport	Ca	<i>Schistosoma japonicum</i>
AG-1_A05	Calcium-response factor CaRF	Transcription/Posttranslational	Ca	<i>Strongylocentrotus purpuratus</i>
AG-9_E03	Calmodulin	Signal transduction mechanisms	Ca	
AG-10_G04	Calmodulin	Signal transduction mechanisms	Ca	<i>Pan troglodytes</i>
AG-8_C02	Nucleoside diphosphate kinase	Nucleotide transport and metabolism	Mg	<i>Graphocephala atropunctata</i>
AG-9_A05	Nucleoside diphosphate kinase	Nucleotide transport and metabolism	Mg	<i>Triboleum castaneum</i>
AG-8_H03	Nucleoside diphosphate kinase /abnormal wing disk protein	Nucleotide transport and metabolism	Mg	<i>Anopheles gambiae str. PEST</i>
AG-4_C08	Nucleoside diphosphate kinase Ndpkz4 (NDK)	Nucleotide transport and metabolism	Mg	<i>Danio rerio</i>
AG-1_C05	Vanabin	Inorganic ion transport and metabolism	V	<i>A.sydneyensis samea</i>
AG-1_H05	Vanabin	Inorganic ion transport and metabolism	V	<i>A.sydneyensis samea</i>
AG-2_F11	Vanabin	Inorganic ion transport and metabolism	V	<i>A.sydneyensis samea</i>
AG-3_G11	Vanabin	Inorganic ion transport and metabolism	V	<i>A.sydneyensis samea</i>
AG-6_F09	Catalase	Inorganic ion transport and metabolism	Fe	<i>Gallus gallus</i>
AG-8_D08	Cytochrome b	Energy production and conversion	Fe	<i>Ciona intestinalis</i>
AG-7_F09	Cytochrome P450	Secondary metabolites biosynthesis, transport and catabolism	Fe	<i>Rattus norvegicus</i>
AG-10_B05	Ferritin heavy chain	Inorganic ion transport and metabolism	Fe	<i>Argopecten irradians</i>
AG-4_E08	Ferritin heavy polypeptide 1	Inorganic ion transport and metabolism	Fe	<i>Gallus gallus</i>
AG-3_F06	Thymine dioxygenase (iron/ascorbate family oxidoreductase)	Secondary metabolites biosynthesis, transport and catabolism	Fe	<i>Strongylocentrotus purpuratus</i>
AG-4_D06	Trasferrin	Inorganic ion transport and metabolism	Fe	<i>Halocynthia roretzi</i>
AG-9_E06	Cytochrome c oxidase subunit	Energy production and conversion	Fe Cu	<i>Phallusia mammilata</i>
AG-2_F06	Cytochrome c oxidase subunit I	Energy production and conversion	Fe Cu	<i>Phallusia ingeria</i>
AG-1_B03	Cytochrome c oxidase subunit	Energy production and conversion	Fe Cu	<i>Ciona savignyi</i>

	I			
AG-4_F03	Cytochrome c oxidase subunit I	Energy production and conversion	Fe Cu	<i>Ciona intestinalis</i>
AG-10_D01	Cytochrome c oxidase subunit I	Energy production and conversion	Fe Cu	<i>Ciona intestinalis</i>
AG-8_F05	Cytochrome c oxidase subunit II	Energy production and conversion	Fe Cu	<i>Ciona intestinalis</i>
AG-7_G09	Cytochrome c oxidase subunit II	Energy production and conversion	Fe Cu	<i>Ciona intestinalis</i>
AG-8_D07	Cytochrome c oxidase subunit II	Energy production and conversion	Fe Cu	<i>Ciona savignyi</i>
AG-10_A09	Cytochrome c oxidase subunit II	Energy production and conversion	Fe Cu	<i>Ciona intestinalis</i>
AG-8_F10	Cytochrome c oxidase subunit III	Energy production and conversion	Fe Cu	<i>Ciona savignyi</i>
AG-9_F05	Cytochrome c oxidase subunit III	Energy production and conversion	Fe Cu	<i>Ciona savignyi</i>
AG-9_F11	Cytochrome c oxidase subunit III	Energy production and conversion	Fe Cu	<i>Halocynthia roretzi</i>
AG-10_B08	Cytochrome c oxidase subunit III	Energy production and conversion	Fe Cu	<i>Halocynthia roretzi</i>
AG-8_G09	Cytochrome c oxidase subunit III	Energy production and conversion	Fe Cu	<i>Ciona savignyi</i>
AG-1_F08	Cytochrome oxidase subunit I	Energy production and conversion	Fe Cu	<i>Daphnia pulex</i>
AG-10_A06	Cytochrome P450	Secondary metabolites biosynthesis, transport and catabolism	Fe	<i>Schistosoma japonicum</i>
AG-5_A04	Zinc finger protein	Function unknown	Zn	<i>Ciona intestinalis</i>
AG-8_H12	Zinc finger protein	Function unknown	Zn	<i>Ciona intestinalis</i>
AG-7_B06	Zinc finger protein 85 (HPF4,HTF1)	Function unknown	Zn	<i>Gallus gallus</i>
AG-8_E07	Zinc finger. CCHC domain containing 10	Function unknown	Zn	<i>Gallus gallus</i>
AG-1_C11	Alcohol dehydrogenase	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Chaenorhabditis elegans</i>
AG-1_E03	Alcohol dehydrogenase	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Chaenorhabditis elegans</i>
AG-6_H08	Alcohol dehydrogenase Zn-dependent	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Cenarchaeum symbiosum</i>
AG-8_F04	Alcohol dehydrogenase class 3	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Ciona intestinalis</i>
AG-2_E06	Alcohol dehydrogenase NAD-dependent	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Bradyrhizobium sp. BTAi1</i>
AG-3_D05	Alcohol dehydrogenase NAD-dependent	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Bradyrhizobium sp. BTAi1</i>

AG-5_C08	Alcohol dehydrogenase NAD-dependent	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Bradyrhizobium japonicum</i> USDA 110
AG-6_A12	Alcohol dehydrogenase NAD-dependent	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Chaenorhabditis elegans</i>
AG-4_G12	Alcohol dehydrogenase Zn-dependent	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Cenarchaeum symbiosum</i>
AG-7_G01	Alcohol dehydrogenase Zn-dependent	Secondary metabolites biosynthesis, transport and catabolism	Zn	<i>Cenarchaeum symbiosum</i>
AG-9_H01	Pem4/zinc finger	General function prediction only	Zn	<i>Ciona intestinalis</i>
AG-4_B06	Preimplantation protein/2C4D/phoccin	Cell cycle control, cell division, chromosome partitioning	Zn	<i>Homo sapiens</i>
AG-2_B04	KF-1a (zinc finger/protein interaction)	Posttranslational modification, protein turnover	Zn	<i>Xenopus laevis</i>
¥AG-6_B11	MGC89077/Ype15	Function unknown	Zn	<i>Mus musculus</i>
AG-1_G11	Nephrocystin isoform 2<Zn-finger/SH3>	Function unknown	Zn	<i>Danio rerio</i>

Table 3. A list of redox-related ESTs identified in the intestine from *Ascidia gemmata*.

EST-ID	Gene product	KOG description	Function	Species
AG-8_D02	Glutathione peroxidase	Posttranslational modification, protein turnover, chaperones	Redox	<i>Haemonchus contortus</i>
AG-5_E12	Glutathione S-transferase (GST)	Posttranslational modification, protein turnover, chaperones	Redox	<i>Gallus gallus</i>
AG-6_G08	Glutathione S-transferase / glutathione-requiring prostaglandin D synthase	Posttranslational modification, protein turnover, chaperones	Redox	<i>Ciona intestinalis</i>
AG-10_G05	Glutathione S-transferase / glutathione-requiring prostaglandin D synthase	Posttranslational modification, protein turnover, chaperones	Redox	<i>Ciona intestinalis</i>
AG-5_B08	Thioredoxin (TRX)	Posttranslational modification, protein turnover, chaperones	Redox	<i>Fasciola hepatica</i>
AG-2_A07	Thioredoxin-related transmembrane protein (thioredoxin-like protein)	Posttranslational modification, protein turnover, chaperones		<i>Canis familiaris</i>

Table 4. A list of solute-carrier or channel-related ESTs identified in the intestine from *Ascidia gemmata*.

EST-ID	Gene product	KOG description	Substrate	Species
AG-5_H01	Vacuolar-type H ⁺ -ATPase subunit A	Energy production and conversion	H	<i>A. sydneiensis samea</i>
AG-10_C03	Vacuolar-type H ⁺ -ATPase subunit B	Energy production and conversion	H	<i>A. sydneiensis samea</i>