Extremophiles (2008) 12:735–739
DOI 10.1007/s00792-008-0172-8

NOTE

Molecular characteristics of the tubeworm, *Ridgeia piscesae*, from the deep-sea hydrothermal vent

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Received: 18 December 2007 / Accepted: 30 April 2008 / Published online: 3 June 2008

Abstract *Ridgeia piscesae*, living around the extremely harsh hydrothermal vent in deep sea, is an ideal model for studying the adaptative mechanism to extreme environment. For insights of its molecular characteristics, a cDNA library of *R. piscesae* was constructed. A total of 879 expressed sequence tags (ESTs) were sequenced and 199 genes were identified for the first time. They were found to be involved in basal metabolism, adaptation and defense, or signal transduction. Among them, we found 23 various chitin-binding proteins, which are the major component of the chitinous tube that prevents the tubeworms from predators and surrounding extreme environment. Additionally, high polymorphism also exists in other genes, such as myohemerythrin, lysozyme. The gene-expression profile might help to further understand the molecular basis of tubeworm physiology. It will also lay a good foundation for functional studies on the adaptation to extreme environments.

Communicated by K. Horikoshi.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-008-0172-8) contains supplementary material, which is available to authorized users.

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Keywords Hydrothermal vent · Tubeworm · *Ridgeia piscesae* · cDNA library · Expressed sequence tag

Since their discovery in 1977, deep-sea hydrothermal vents have become one of the most attractive extreme environments for scientists. Besides the high pressure and temperature there, the vent fluids are anoxic and contain toxic hydrogen sulfide, heavy metals such as arsenic, cadmium, radium as well. Although the environmental conditions are remarkably rough, hydrothermal vents provide the habitat for many creatures, including chemosynthetic bacteria, tubeworms, fish, crabs and clams. To get accustomed to the extreme physical and chemical conditions, the creatures living around the hydrothermal vents must be extensively modified (Gaill 1993).

The tubeworm is the major fauna around the hydrothermal vents. These gutless worms rely on their symbiotic bacteria located in the trophosome to get nutrition. The tubeworms supply hydrogen sulfide to the symbiotic bacteria to produce energy and then the bacteria use the energy to convert carbon dioxide into organic carbon to feed the worms. During the process of energy production, the bacteria convert the toxic hydrogen sulfide into less harmful sulfur (Childress and Fisher 1992; Felbeck and Jarchow 1998; Bright et al. 2000). In addition to the help from their symbiotic bacteria, the tubeworms themselves must be evolved to settle down in hydrothermal vent. It has been reported that the tubeworm, *Riftia pachyptila*, exhibits high activity of carbonic anhydrase and the enzyme plays an important role in the carbon concentration (De Cian et al. 2003). The carbonic anhydrase catalyzes the reversible hydration of CO_2 into bicarbonate and a proton, and participates in a broad range of physiological processes such

as acid–base homeostasis, carbon dioxide and ion transport and respiration. It is also involved in the pH regulation in the body fluids of host and CO_2 transport for endosymbioses (Henry 1996). In addition, these tubeworms are covered with chitinous tube, which supports their bodies and protects them from the surrounding extreme environment. The chitinous tube is an excellent first line against predators as well (Gaill 1993; Shillito et al. 1995). However, in comparison with their symbiotic bacteria, little is known of the molecular characteristics of the tubeworms.

The hydrothermal system on the Endeavour segment of the Juan de Fuca Ridge is extremely vigorous and it is an ideal habitat for a variety of vent fauna (Delaney et al. 1992; Butterfield et al. 1994; Tivey et al. 1999). Among all the vent animals, *Ridgeia piscesae*, a tubeworm, is the foundation specie in many Juan de Fuca Ridge hydrothermal vent communities (Tunnicliffe 1991; Urcuyo et al. 2003). They were once hypothesized to be several different species due to their highly variable morphology (Jones 1985; Tunnicliffe 1988). But subsequent analysis of molecular phylogeny and a morphological reassessment have shown that all the morphotypes in fact belong to a single species (Southward et al. 1995; Southward et al. 1996; Black et al. 1998; Carney et al. 2002). Now, it is generally accepted that *R. piscesae* has two marked variations in growth forms, short-fat and long-skinny. Short-fat *R. piscesae* is found attached to sulfide chimneys of relatively high flow vent fluid of ~15–20°C above ambient temperature and high concentrations of sulfide. In contrast, the long-skinny morph is found in low temperature, low concentrations of sulfide and low-flow basalt habitats. The varieties of morphotypes suggest that *R. piscesae* may display a classic case of phenotypic plasticity, where a given genotype can produce multiple phenotypes depending upon different microhabitat conditions (Black et al. 1994; Zhivotovsky et al. 1996). It is also indicated that this species of vestimentiferan can adapt to a wide range of vent conditions (Tunnicliffe and Juniper 1990; Sarrazin et al. 1997).

In this study, a cDNA library of long-skinny *R. piscesae* was constructed for gene expression profile studies in a genomic scale. A total of 879 clones were sequenced and 199 genes were identified. Some identified genes, such as chitin-binding proteins, might be important for the adaptation of *R. piscesae* to hydrothermal vent. This study will contribute to the tubeworm gene index and might help to further understand the molecular basis of the adaptation of tubeworms to the hydrothermal vents.

Long-skinny *R. piscesae* were collected during Alvin dive 4243 on 9 August 2006, at Cathedral vent, Main Endeavor Field of the Juan de Fuca Ridge (47° 56′ N, 129° 05′ W, 2,181 m depth). Six individuals were sampled. They were immediately dissected on ice after collection and parts of their body wall tissues were kept in RNAlater buffer (QIAGEN) respectively. When isolating RNA, these samples were mixed together.

mRNA was isolated with a Oligotex Direct mRNA Midi/Maxi Kit (QIAGEN) according to the manufacturer's instructions. This method can target at only poly-A-tailed mRNA that eukaryotes (hostes) have but prokaryocytes (symbiotic bacteria) do not. Then the cDNA library was constructed using a SMART^{TM} cDNA library construction kit (Clontech) with slight modifications. Briefly, cDNA synthesis, *sfi*I digestion and size fractionation were performed according to the manufacturer's instructions. Afterwards, the obtained cDNAs were cloned into *Sfi*I-linearized pcDNA3.0 vector and then transformed into *Escherichia coli* DH5α by electroporation (Mou et al. 2002).

Inserted cDNA fragments were sequenced using ABI Prism 377 DNA sequencers (Applied Biosystems) with the universal primers T7 (5′-AATACGACTCACTATAG-3′) and SP6 (5′-ATTTAGGTGACACTATAGAA-3′). The sequences of cDNA clones were compared with sequences in National Center for Biotechnology Information (NCBI) using the BlastX network service (Altschul et al. 1997). Sequences that showed significant homology with known genes were categorized according to their functions. Domain search was performed in Pfam (http://www.sanger.ac.uk). Sequence assembly and alignment were performed by DNAMAN and Clustal X software, respectively.

A cDNA library (5.71×10^6 cfu) with average insert size of ~1.0 kb was constructed from the mixed specimens of *R. piscesae*. A total of 879 clones were randomly selected and sequenced. The sequences were processed and compared in GenBank database. Of the 879 clones, 512 expressed sequence tags (ESTs) were identified as orthologs of 199 known genes by Blast searches. The cDNA library was not normalized and the clone abundance should therefore reflect the mRNA profile and gene expression pattern of *R. piscesae*. Several enzymes involved in the intermediary sythesis and the catabolism were identified, including electron transport chain related proteins, synthesis and modification proteins. The basal metabolism is the guarantee of energy supply and synthesis of essential components for its survival and growth. Furthermore, several adaptation and defense related genes were found, such as chitin-binding proteins, lysozyme, chitinase, myohemerythrin. Nine distinct cell proliferation related genes were also found in the cDNA library. Their existence in lower organism indicates that these proteins are evolutionally conserved. Additionally, the sequence of other 367 cDNA clones failed to reveal exact function similar to that previously described in NCBI. Part of these unknown genes might be specifically expressed in *R. piscesae* and

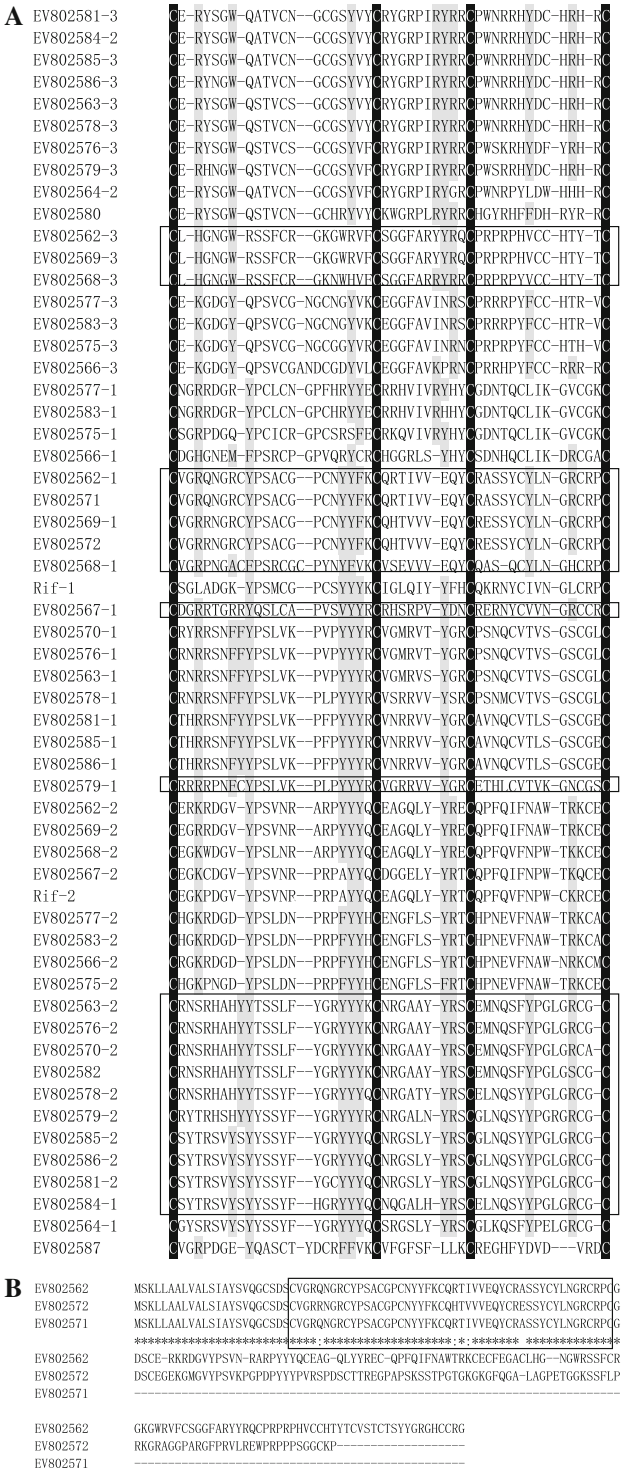

Fig. 1 Alignment of chitin-binding proteins. **a** A multiple sequence alignment of CBDs using Clustal X (Thompson et al. 1994). 1, 2, 3 after the accession No. represents the numberof CBD in the corresponding chitin-binding protein. The cysteines conserved are *white on a black background* and the disulfide bonds are suggested to be the major force to stabilize the folding; the conservation of aromatic residues and charged resides are *shaded gray*, which are likely to be involved in the interaction with chitin (Shen and Jacobs-Lorena 1999). Rif-1 and Rif-2 are from *Riftia pachyptila* (*AAF76890*). **b** Sequence alignment of chitin-binding proteins with novel CBDs. *Asterisks* conserved amino acids; *colon* very similar amino acids. The *parts boxed* are predicted to be the new CBD

play a role in its adaptation to hydrothermal vents. The summary of identified genes was shown in Table S1.

The chitin-binding proteins appeared most frequently in the cDNA library, 23 various types from 226 ESTs. Chitin is the major component of the chitinous tube and chitin-binding proteins play an important role in the formation of chitinous tube. The function of chitin-binding protein ranges from a simple binding, like lectins, to chitinase activity, or even antimicrobial and antifungal properties in case of tachycitin (Kawabata et al. 1996). The cDNA library revealed that *R. piscesae* has high chitin-binding protein polymorphism. Domain search on Pfam revealed that some members have a single or multiple chitin-binding domains (CBDs) close to Pfam type 2 CBD (Fig. 1a). In addition, novel regions (boxed) were found to be similar to CBD and contain several conserved amino acids as well, especially cysteine (Fig. 1a). Furthermore, although the known CBDs could not be identified in *EV802571* and *EV802572*, they share high similarities with the recognized chitin binding protein (*EV802562*), especially the novel region (boxed) (Fig. 1b). Therefore, we propose that the cysteine-rich region we found might be a novel type of CBD or at least a CBD-like domain. The diversity and similarity of the amino acid sequence among these CBDs suggest that they might arise by divergent evolution from a common ancestor and CBD may be the result of the gradual evolution from a non-chitin-binding sequence. Furthermore, the duplication and transposition of this CBD may have contributed to the functional diversification of chitin-binding proteins. The duplication of the CBDs may have been driven by the improved and expanded functions of the genes containing multiple CBDs (Shen and Jacobs-Lorena 1999). The strikingly high content of polymorphism of chitin-binding proteins reflects their importance in adaptation to the harsh environment.

Apart from the physical barriers, several lytic enzymes related to innate immunity were also found in the cDNA library, such as chitinase and lysozyme. Chitinase is a hydrolase, involved in the defense against bacterial and fungal pathogens (Lindsay and Gooday 1985; Manson et al. 1992). Lysozyme is an enzyme responsible for breaking down the bacterial cell wall peptidoglycans and provides protection against bacterial infection. In addition, it is also known to be an opsonin and activate the complement system and phagocytes (Jolles and Jolles 1984; Grinde 1989). Interestingly, the identified lysozyme also contains a conserved domain of destabilase, an endo-epsilon (gamma-Glu)-Lys isopeptidase. It is the guarantee for the flow of humoral circulation (Zavalova et al. 1996).

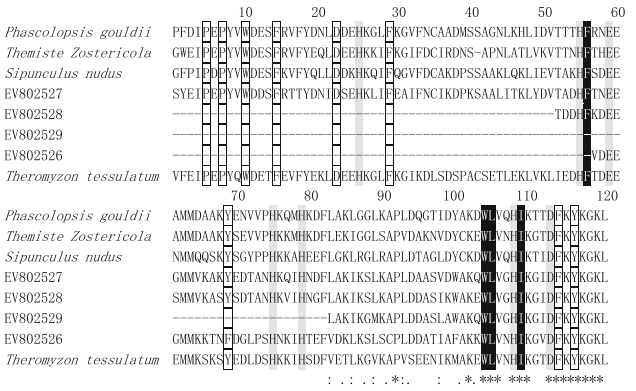

Fig. 2 Amino acid sequence alignment of myohemerythrin with Clustal X. *Asterisks* conserved amino acids; *colon* very similar amino acids; *dots* similar amino acids. The residues involved in iron binding are *shaded gray*; residues constitute O_2-binding pocket are *white on a black background* and *residues boxed* have been indicated to be important for structural maintenance (Xiong et al. 2000; Farmer et al. 2001). Sequences used in the aligment are *Phascolopsis gouldii* (*AAB22826*), *Themiste zostericola* (*1A7D*), *Sipunculus nudus* (*CAG14944*) and *Theromyzon tessulatum* (*AAG01808*)

Fibrinogen was also found in the cDNA library. It is a sticky, fibrous coagulant that plays a key role in blood clotting and involved in tissue repair processes and wound healing (Lord 2007). It might help repair the vulnerable plume of tubeworm.

Hemoglobins are well known for O_2 transport in tubeworms and binding hydrogen sulfide to feed endosymbiotic bacteria as well. Surprisingly, we did not find any hemoglobin by sequencing and instead found some myohemerythrins, another type of oxygen-binding molecule. Myohemerythrin, a cytoplasmic monomeric protein, was reported to be present in the muscles of sipunculans and annelids, and involved in intracellular oxygen transfer and storage (Vanin et al. 2006). Four types of myohemerythrin were found in our cDNA library (Fig. 2). Since the sample of *R. piscesae* was collected from the region in Juan de Fuca Ridge where the concentration of hydrogen sulfide is low, high expression level of hemoglobin might not be necessary for their survival (Carney et al. 2007). Deep-sea hydrothermal vent organisms have previously been shown to possess altered enzyme forms as a result of adaptation to their extreme environment (Felbeck 1981; Hentschel and Felbeck 1993; Lutz et al. 1994; Young et al. 1996; Zal et al. 1998). Expression of myohemerythrin instead of hemoglobin in the tubeworm from Juan de Fuca Ridge further confirms that environmental factors may indeed influence the gene expression.

In summary, 879 ESTs were sequenced and 199 genes were identified in *R. piscesae* for the first time. It provides a global understanding of the mRNA transcripts. Besides, it is interesting to find that polymorphism exist in some genes such as myohemerythrin, chitin binding protein and etc. Our study not only contributes to understand the gene expression pattern in tubeworm but also provides insights to the molecular mechanism underlying the adaptation to extreme environments and innate immune system. It will be interesting to study the exact function of these identified genes in the future.

Acknowledgments The authors would like to express their appreciation to the crews of Alvin. Many thanks to Prof. Huaiyang Zhou, Dr. Brian Marquardt, Dr. Helen White, Mr. Mark Spear, Prof. Debbie Kelley, Prof. Marv Lilley, Prof. Peter R. Girguis and other friendly scientists. Their expertises enabled high quality sample collections. We would also like to thank Prof. Congzhao Zhou for his help in sequences analysis and the anonymous reviewers for their helpful comments. This work was supported by China Ocean Mineral Resources R&D Association (DYXM-115-02-2-16) and oceanographic research grant from Third Institute of Oceanograpy, State Oceanic Administration, China.

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