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Research paper

Expression of genes involved in the uptake of inorganic carbon in the gill of a deep-sea vesicomyid clam harboring intracellular thioautotrophic bacteria

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ABSTRACT

Deep-sea vesicomyid clams, including the genus *Phreagena* (formerly *Calyptogena*), harbor thioautotrophic bacterial symbionts in the host symbiosome, which consists of cytoplasmic vacuoles in gill epithelial cells called bacteriocytes. The symbiont requires inorganic carbon (Ci), such as CO_2, HCO_3^-, and CO_3^{2-}, to synthesize organic compounds, which are utilized by the host clam. The dominant Ci in seawater is HCO_3^-, which is impermeable to cell membranes. Within the bacteriocyte, cytoplasmic carbonic anhydrase (CA) from the host, which catalyzes the inter-conversion between CO_2 and HCO_3^-, has been shown to be abundant and is thought to supply intracellular CO_2 to symbionts in the symbiosome. However, the mechanism of Ci uptake by the host gill from seawater is poorly understood. To elucidate the influx pathway of Ci into the bacteriocyte, we isolated the genes related to Ci uptake via the pyrosequencing of cDNA from the gill of *Phreagena okutanii*, and investigated their expression patterns. Using phylogenetic and amino acid sequence analyses, three solute carrier family 4 (SLC4) bicarbonate transporters (*slc4co1*, *slc4co2*, and *slc4co4*) and two membrane-associated CAs (*mcaco1* and *mcaco2*) were identified as candidate genes for Ci uptake. In an *in situ* hybridization analysis of gill sections, the expression of *mcaco1* and *mcaco2* was detected in the bacteriocytes and asymbiotic non-ciliated cells, respectively, and the expression of *slc4co1* and *slc4co2* was detected in the asymbiotic cells, including the intermediate cells of the inner area and the non-ciliated cells of the external area. Although subcellular localizations of the products of these genes have not been fully elucidated, they may play an important role in the uptake of Ci into the bacteriocytes. These findings will improve our understanding of the Ci transport system in the symbiotic relationships of chemosynthetic bivalves.

1. Introduction

To synthesize organic compounds, inorganic carbon (Ci, including CO_2, HCO_3^-, and CO_3^{2-}) is fixed by thioautotrophic bacterial symbionts in the specialized cells (bacteriocyte) of the gill or trophosome in the deep-sea chemosymbiotic animals, such as bivalves and vestimentiferan tubeworms (Van Dover, 2000). These animals have vestigial or no digestive tracts, and thus depend on their symbionts for nutrients (Boss and Turner, 1980; Pennec and Fiala-Medioni, 1988; Jones et al., 1981). The bacterial symbionts are localized in the symbiosome, which is an intracellular vacuole in the bacteriocyte (Fiala-Medioni and Lepennec, 1988; Cavanaugh et al., 1981). Inorganic carbon taken up from seawater and/or transported in the hemolymph from host aerobic respiratory metabolism is utilized by the symbiont for carbon fixation. In general, at pH values between 7 and 8, bicarbonate (HCO_3^-) is the most abundant form of Ci in seawater or hemolymph, but is impermeable to biological membranes.

Abbreviations: Ci, inorganic carbon; CA, carbonic anhydrase; MCACO1 and 2, membrane-associated carbonic anhydrases of *P. okutanii*; SLC4CO1, 2, 3, and 4, four isozymes of solute carrier family 4 in *P. okutanii*; ISH, *in situ* hybridization.

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A deep-sea hydrothermal vent-living giant vestimentiferan tubeworm, *Riftia pachyptila*, which harbors thioautotrophic bacteria (Cavanaugh et al., 1981), has been well studied regarding Ci uptake from seawater. Owing to the abundance of CO_2 in its slightly acidic habitat (pH 6.2 ± 0.14; total Ci, 4.7 ± 0.54 mM; Childress et al., 1993a) and somewhat alkaline pH of their body fluid (Goffredi et al., 1997), CO_2 is taken up into the plume-like gill of the host by diffusion (Goffredi et al., 1997) and accumulates in the body fluid (Childress et al., 1993b) being transported mainly as HCO_3^- (Toulmond et al., 1994). In the gill, carbonic anhydrase (CA), which catalyzes the reversible reaction between CO_2 and HCO_3^-, quickly converts CO_2 to HCO_3^- to enhance the diffusion of Ci from the environment (Goffredi et al., 1997; De Cian et al., 2003).

Unlike *R. pachyptila*, the total Ci concentration (total Ci, 2.3 mmol/kg) in the seep site of vesicomyid clam colonies is approximately half of that measured at the site of *R. pachyptila* (Tsunogai et al., 1996) in a pH of 7.7 (Hongo et al., 2013), very similar to other common deep-sea environments (Tsunogai et al., 1996). Thus, the influx of CO_2 into the bacteriocyte and the symbiosome by diffusion may not be sufficient for the autotrophic metabolism of vesicomyid clams. However, carbon-fixation experiments using whole deep-sea vesicomyid clams have shown that 29%–57.1% of total labeled carbon is localized to the gills (Childress et al., 1993b). Previously, we found that two CAs were abundantly expressed in the cytoplasm of the bacteriocytes in the gill of *Phreagena okutanii*, formerly *Calyptogena okutanii* (Huber, 2015), belonging to the family Vesicomyidae (Hongo et al., 2013). While it is considered that the two cytoplasmic CAs facilitate Ci transport from the cytoplasm to the symbiont in the symbiosome (Hongo et al., 2013), the pathway of Ci uptake from seawater or hemolymph to cytoplasm in the bacteriocytes remains to be elucidated.

Two protein families related to Ci transport are known in animals. The first protein family is the solute carrier family 4 (SLC4), of which the major function is bicarbonate transport (Romero et al., 2013). The SLC4 family contains three major phylogenetic clades associated with unique functions (Romero et al., 2013): (1) Na^+-independent exchanger of Cl^- and HCO_3^- (anion exchanger, AE) group, (2) Na^+-dependent HCO_3^- transporter group including a Na^+/HCO_3^- cotransporter, either electrogenic ("e" suffix, NBCe) or neutral ("n" suffix, NBCn), and Na^+-driven Cl^-/HCO_3^- exchangers (NDCBE), and (3) Na^+-dependent borate transporter, SLC4A11, of which the function has not been conclusively established, but which transports borate instead of HCO_3^- (Romero et al., 2013; Park et al., 2004). Although many studies have been conducted on the expression and activities of SLC4 bicarbonate transporters in vertebrates, only the functions of NBCe and NDCBE have been characterized in invertebrates, using the long fin squid, *Doryteuthis pealeii*, as a model (Virkki et al., 2003; Piermarini et al., 2007). The NBCe of *D. pealeii* is transcribed in the gill and suggested to take up Ci for the regulation of hemolymph pH and ion homeostasis (Piermarini et al., 2007). The second protein family is the membrane-associated CA belonging to the α-CA family, where its membrane topology can be divided into two types: transmembrane (CA IX, −XII, −XIV, and −XV) and glycosyl-phosphatidyl-inositol (GPI)-anchored (CA IV) types (Zhu and Sly, 1990; Whittington et al., 2001). The green alga *Chlamydomonas reinhardtii* and the sea anemone *Anemonia viridis*, which harbors photosymbiotic dinoflagellates, take up Ci from environmental water or seawater for photosynthesis using the membrane-associated CA (Coleman et al., 1984; Moroney et al., 1985; Furla et al., 2000). Based on this, we previously proposed two possible mechanisms of Ci uptake (Hongo et al., 2013): (1) influx of HCO_3^- in the cytoplasm of the bacteriocytes mediated by a SLC4 bicarbonate transporter in the apical membrane of the bacteriocyte, or (2) facilitated CO_2 diffusion through catalyzed interconversion of HCO_3^- and CO_2 by a membrane-associated CA on the apical membrane of the bacteriocyte. However, neither bicarbonate transporter genes nor membrane-associated CA genes have yet to be identified in vesicomyid clams.

In this study, we isolated SLC4 bicarbonate transporter and membrane-associated CA genes, expressed in the gill of *P. okutanii*, by cDNA pyrosequencing. Functions of the candidate gene products were predicted based on phylogenetic and amino acid sequence analyses. Moreover, the localization of cells expressing these genes was investigated by *in situ* hybridization. On the basis of our findings, we discuss the roles of SLC4 bicarbonate transporters and membrane-associated CAs in the mechanism of Ci uptake in *P. okutanii*.

2. Methods

2.1. Animal sampling

Phreagena okutanii was collected from a seep area off Hatsushima (35° 00.9559′ N, 139° 13.3202′ E; 855 m depth), Sagami Bay, Japan with a scoop sampler using the Remotely Operated Vehicles (ROV) *Hyper-Dolphin* and *Kaiko 7000II* from the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) in 2008, 2011, and 2012. After a period of recovery on board, the clams were maintained in aquaria filled with chilled surface seawater (4 °C) for 2–24 h, and individuals that extended their siphons and feet were chosen for dissection. Their gill, foot, and mantle were excised immediately and washed several times with 0.22-μm-filtered seawater. For RNA extraction, each sample was immersed in RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C overnight before storage at −80 °C until use.

For *in situ* hybridization (ISH), small pieces (5 × 5 × 5 mm) of gill were fixed with 4% paraformaldehyde (PFA) in PBS treated with diethyl pyrocarbonate (DEPC) at 4 °C overnight. Subsequently, the fixed gills were dehydrated through an ethanol series (25, 50, 75, and 100% ethanol on ice) and kept in 100% ethanol at −20 °C until use. All animal experiments were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan).

2.2. RNA extraction

The gill, foot, and mantle of *P. okutanii*, preserved in RNAlater (Thermo Fisher Scientific) at −80 °C, were cut into small pieces on ice, and total RNA was extracted using an RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Contaminating DNA in the purified total RNA was digested using Turbo RNase-free DNase (Thermo Fisher Scientific).

2.3. Construction and sequencing of cDNA libraries

Construction and normalization of cDNA libraries were outsourced and performed by DNAFORM (Kanagawa, Japan). The first strand cDNA from gill RNA was synthesized with a *Gsu*I-adapted oligo(dT) primer, 5′-CAAGCAGAAGACGGCATACGACTGGAG$(T)_{16}$VN-3′. After the first strand cDNA synthesis, a full-length cDNA/RNA hybrid was obtained by cap-trapping methods (Carninci et al., 1997; Shibata et al., 2001), and the RNA from the hybrid molecule was digested by alkali treatment. The 5′ end of the single stranded cDNA was ligated to *Gsu*I-adapted GN5 and N6 linkers (Table S1). The second strand cDNA was synthesized from the single strand cDNA as a template with specific primer, 5′-AATGATACGGCGCTGGAGGACAGGTTCAGAGTTC-3′. For cDNA normalization, the cDNA was re-annealed after denaturation, and only double strand DNA was digested with a double-strand specific DNA nuclease, and the normalized single strand DNA recovered. The normalized single stranded DNA was amplified by polymerase chain reaction (PCR) with the primers used for the first and second strand cDNA synthesis.

To obtain as long a sequence as possible, 5′ and shotgun cDNA libraries were constructed from the normalized cDNA samples. The 5′ region of the normalized cDNA was amplified by PCR using a 5′ adaptor primer-appended GS FLX sequence primer and biotin. The cDNA products amplified by this PCR and the normalized cDNA samples were cut into hundreds of base pairs by an acoustic solubilizer (Covaris, Inc., Woburn, MA, USA) to be used for preparation of the 5′ and shotgun cDNA libraries. The 5′ regions of cDNA fragments were recovered by magnetic