

## ORIGINAL ARTICLE

# Allying with armored snails: the complete genome of gammaproteobacterial endosymbiont

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**Deep-sea vents harbor dense populations of various animals that have their specific symbiotic bacteria. Scaly-foot gastropods, which are snails with mineralized scales covering the sides of its foot, have a gammaproteobacterial endosymbiont in their enlarged esophageal glands and diverse epibionts on the surface of their scales. In this study, we report the complete genome sequencing of gammaproteobacterial endosymbiont. The endosymbiont genome displays features consistent with ongoing genome reduction such as large proportions of pseudogenes and insertion elements. The genome encodes functions commonly found in deep-sea vent chemoautotrophs such as sulfur oxidation and carbon fixation. Stable carbon isotope (<sup>13</sup>C)-labeling experiments confirmed the endosymbiont chemoautotrophy. The genome also includes an intact hydrogenase gene cluster that potentially has been horizontally transferred from phylogenetically distant bacteria. Notable findings include the presence and transcription of genes for flagellar assembly, through which proteins are potentially exported from bacterium to the host. Symbionts of snail individuals exhibited extreme genetic homogeneity, showing only two synonymous changes in 19 different genes (13810 positions in total) determined for 32 individual gastropods collected from a single colony at one time. The extremely low genetic individuality in endosymbionts probably reflects that the stringent symbiont selection by host prevents the random genetic drift in the small population of horizontally transmitted symbiont. This study is the first complete genome analysis of gastropod endosymbiont and offers an opportunity to study genome evolution in a recently evolved endosymbiont.**

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## Introduction

Scaly-foot gastropods, tentatively named '*Cryso-mallon squamiferum*', were first described in 2001 (Van Dover *et al.*, 2001). These snails live a sedentary life at the base of a black smoker chimney,

in the Kairei hydrothermal field in the Indian Ocean. Dark-colored sclerites cover the sides of the snails' foot in a roof-tile fashion (Warén *et al.*, 2003; Goffredi *et al.*, 2004). The dominant crystalline mineral phase is pyrite, whereas greigite, the sulfide analog of magnetite, is present in lower proportions and is responsible for the ferrimagnetism (Warén *et al.*, 2003; Suzuki *et al.*, 2006). In addition, the shell of scaly-foot gastropod is fortified by a unique multilayer structure (Yao *et al.*, 2010). These defenses are probably acquired for the protection against environmental threats including grazing (Suzuki *et al.*, 2006). The scaly-foot gastropods,

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however, share the habitat around the base of a black smoker chimney with a different kind of gastropod, *Alviniconcha* sp., which have a bare foot and very thin soft shell.

Many deep-sea vent endemic mollusks harbor specific symbionts, although *Alviniconcha* gastropods exhibit marked diversity in their symbiont ribotypes within and among populations (Suzuki *et al.*, 2005; Beinart *et al.*, 2012). The scaly-foot gastropod houses its gammaproteobacterial symbionts within the cells of its enlarged esophageal gland (Goffredi *et al.*, 2004). The endosymbiont population is highly clonal within and among snail individuals at the 16S rRNA gene sequence level (Goffredi *et al.*, 2004). In addition, *Deltaproteobacteria* and *Epsilonproteobacteria* densely colonize the surface of scales (Goffredi *et al.*, 2004). The gastropod nutritionally depends on the intracellular gammaproteobacterium that synthesizes organics from CO<sub>2</sub> potentially by oxidizing reduced sulfur compounds (Goffredi *et al.*, 2004). Until recently, ecophysiological characteristics of symbiotic *Gammaproteobacteria* in deep-sea vents had remained mostly unknown because of their resistance to cultivation outside of specialized host tissues. However, genome sequences have been determined for endosymbionts of *Riftia pachyptila* (tubeworm) (Markert *et al.*, 2007) and *Calyptogena* species (bivalves endemic in deep-sea vents) (Kuwahara *et al.*, 2007; Newton *et al.*, 2007). Complete genome sequence has also been determined for the sulfur-oxidizing endosymbiont of *Olavius algarvensis* (shallow oligochaete) (Woyke *et al.*, 2006). These genome sequences have provided new insights into symbiont evolution, physiological functions and interactions with their host animals. For example, the marine endosymbionts, including heritable ones, have significantly larger genomes with higher GC contents compared with insect endosymbionts, suggesting their recent association with hosts (Woyke *et al.*, 2006; Kuwahara *et al.*, 2007; Markert *et al.*, 2007; Newton *et al.*, 2007). As expected from the large genome sizes, the marine gammaproteobacterial endosymbionts retain metabolically complete genomes, and even have multiple pathways both for energy metabolism (Woyke *et al.*, 2006; Kuwahara *et al.*, 2007; Newton *et al.*, 2007) and for carbon fixation (Markert *et al.*, 2007). These endosymbiont genomes therefore provide a rare opportunity to study the early stages of genome reduction during symbiont evolution.

In deep-sea vent ecosystems, chemoautotrophic *Epsilonproteobacteria* are dominant and metabolically versatile, suggesting that deep-sea vent chemoautotrophs, even if they are epi- or endosymbionts, experience broad fluctuations in the availabilities (both kind and concentration) of electron donors, electron acceptors and carbon sources (Nakagawa *et al.*, 2005, 2007). Specific ecophysiological differences between gammaproteobacterial and epsilonproteobacterial deep-sea vent chemoautotrophs

have not been clarified yet. Several genetic and biochemical studies demonstrated that the chemoautotrophic *Gammaproteobacteria* and *Epsilonproteobacteria* fix CO<sub>2</sub> via Calvin-Benson-Basham cycle and reductive tricarboxylic acid cycle, respectively (Hügler *et al.*, 2005; Takai *et al.*, 2005; Nakagawa and Takai, 2008). In addition, repertoire of the sulfur-oxidation pathways differs between deep-sea vent *Gammaproteobacteria* and *Epsilonproteobacteria* (Nakagawa and Takai, 2008; Yamamoto and Takai, 2011). However, little is known about how these differences influence their ecological performance.

At present, no genome sequence is available for any gastropod endosymbionts. In this study, we describe the complete genome sequence analysis of gammaproteobacterial endosymbiont found in scaly-foot gastropod. To assess the physiology of gastropod, we also performed the <sup>13</sup>C tracer experiments and hemolymph glycan analysis. We sequenced the symbiont genome specifically as an effort to address (1) genomic differences with other chemoautotrophs, (2) genome evolution in a recently derived symbiont and (3) endosymbiont physiology. This study represents a step toward a better understanding of genome dynamics and evolution of symbiotic bacteria in deep-sea vents, and will lead to more specifically focused biochemical investigations.

## Materials and methods

### *Sampling and DNA/RNA preparation*

Samples used in this study were obtained by means of DSV *Shinkai 6500* at the Kairei hydrothermal field (Van Dover *et al.*, 2001) in the Central Indian Ridge (25° 19.23'S, 70° 02.42'E). All samples were collected from a single colony during dive no. 1169 on 6 November 2009, unless noted otherwise. Although *Alviniconcha* sp. represents the gastropods visually observed from the submersible's window in this hydrothermal field, we discovered a large and dense population of scaly-foot gastropods covered by *Rimicaris* shrimps (water depth = 2420 m) (Supplementary Figure S1). Once onboard the ship, the esophageal gland was dissected from a single snail (Supplementary Figure S1c) and homogenized with glass Teflon homogenizer powered by a standard electric drill in 5 ml of phosphate-buffered saline at pH 7.0 on ice. Debris was pelleted at 800 × g for 5 min, and supernatant was filtered through a 10-μm nylon mesh filter (Millipore, Billerica, MA, USA). The filtrate was further centrifuged at 14 000 r.p.m. for 10 min to recover endosymbiont cells. The cell pellet was washed three times in phosphate-buffered saline and stored at -80 °C until taken to the laboratory. Genomic DNA (~ 500 μg) was extracted manually using the phenol/chloroform method. In addition, cell pellet was washed in RNAlater (Ambion, Austin, TX, USA) and stored at -80 °C.

### Genome sequencing and assembly

The genome was sequenced using 454 Titanium (Roche, Branford, CT, USA) with the paired-end library of 3 kb. Library preparation and sequencing were performed at TaKaRa Bio Inc. (Otsu, Japan). The sequence reads (a total of 188 Mb in 492 255 reads) were assembled using the GS De Novo Assembler (Newbler) version 2.3 (Roche/454 Life Sciences, Branford, CT, USA). Low-sequence quality regions and potential sequence errors were searched and confirmed by Sanger sequencing. Together, all reads provided about  $\times 65$  coverage of the genome.

### Sequence analysis and annotation

Genes were identified using Glimmer (Salzberg *et al.*, 1998) and GeneMarkS (Besemer and Borodovsky, 2005), followed by manual screening. Additional open reading frames that were homologous to genes identified in other organisms were searched from intergenic region. The open reading frames were compared with the nonredundant database of the NCBI, UniProt and Kyoto Encyclopedia of Genes and Genomes GENES database, using BLASTP (Altschul *et al.*, 1997). Protein domains were searched against the PFAM database (Bateman *et al.*, 1999). The annotated functions were classified into clusters of orthologous group categories (Tatusov *et al.*, 2000). The metabolic pathway was reconstructed using the Kyoto Encyclopedia of Genes and Genomes and MetaCyc (Caspi *et al.*, 2008) databases. Signal peptides and membrane-spanning domains were predicted using the SIGNALP (Nielsen *et al.*, 1997), and SOSUI (Hirokawa *et al.*, 1998), respectively. Transfer RNAs were predicted by tRNAscan-SE (Lowe and Eddy, 1997). We defined orthologs using Inparanoid/MultiParanoid (Remm *et al.*, 2001; Alexeyenko *et al.*, 2006), in which one open reading frame is the closet relative of the other and *vice versa* with the BLAST score  $>40$  and over 70% coverage. Pseudogenes were defined as genes with frameshift mutations, insertion sequence elements, other insertions or deletions, premature stop codons or truncations that nevertheless showed high homology to the functional genes.

### Genetic individuality analyses

A total of 19 genes including a pseudogene were sequenced for each symbiont extracted from 32 different individuals. Genes were selected based on PubMLST database (<http://pubmlst.org/>). Primers used in this study were listed in Supplementary Table S1. Genes were PCR amplified with LA Taq polymerase (TaKaRa Bio Inc.) using initial denaturation at 96 °C for 1 min, 30 cycles of 96 °C for 20 s, 60 °C for 45 s and 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR product was cleaned with exonuclease I (Affymetrix, Santa Clara, CA, USA) and shrimp alkaline phosphatase

(GE Healthcare, Piscataway, NJ, USA), and subjected to direct sequencing with an ABI 3130xl sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Likewise, six different gene sequences were determined for host mitochondria (Supplementary Table S1).

### Reverse transcription PCR

Total RNA was extracted from cells stored in RNAlater using High Pure RNA Isolation Kit (Roche), according to the manufacturer's instructions. cDNA was synthesized using transcriptor high-fidelity cDNA synthesis kit and random hexamers (Roche). Primers for nine flagellar genes were constructed (Supplementary Table S1). Amplification protocol was as described above. In the negative control, the reverse transcription was omitted. Sequences were confirmed as described above.

### Incubation with $^{13}\text{C}$ -bicarbonate

Scaly-foot gastropods can be kept alive in a tank for over 3 weeks. Immediately after the collection, gastropods were transferred in 2.5 l plastic jars (BBL Microbiology Systems, Cockeysville, MD, USA) at 4 °C (Supplementary Figure S2a). Each jar was filled with one individual gastropod and 1.5 l of surface seawater containing 3 mM  $\text{NaH}^{13}\text{CO}_3$ . Potential energy substrates were added at final concentrations of 100  $\mu\text{M}$  (sulfide) or 20%  $\text{H}_2$  (v/v). Gastropods killed by freezing (1–2 h at  $-80$  °C) were also incubated as negative control in the presence of sulfide or  $\text{H}_2$ . After 64.5 h incubation in dark, the gastropods were dissected into esophageal glands and feet and kept at  $-80$  °C. At the laboratory, samples were lyophilized, powdered and then acid-fumed for over 10 h. The carbon isotopic compositions were analyzed by a Thermo Electron (Bremen, Germany) DELTA<sup>Plus</sup> Advantage mass spectrometer connected to an elemental analyzer (EA1112) through a ConFlo III interface. When the alanine standard ( $\delta^{13}\text{C}$ ,  $-19.4\%$ ) was measured for 10 times, the s.d. was 0.3‰. Samples of this tracer experiments were prepared and analyzed in 2007.

### Glycan analysis

The hemolymph was collected from snails incubated for 18 h with sulfide,  $\text{H}_2$  (as described above) and thiosulfate (50  $\mu\text{M}$ ) (Supplementary Figure S2b), centrifuged for a few minutes and then stored at  $-80$  °C. N-glycans were released and purified from 20  $\mu\text{l}$  of hemolymph as described previously (Amano *et al.*, 2010) (detailed in Supplementary Methods). The aoWR (aminoxy-functionalized peptide compound)-tagged glycans were crystallized with 2,5-dihydroxybenzoic acid and analyzed by MALDI-TOF MS (4700 Proteomics Analyzer; Applied Biosystems) in reflector, positive ion mode. Data were analyzed by GlycoMod Tool (Cooper *et al.*, 2001)

and glyfinTMS software (<http://www.jamstec.go.jp/software/glyfintms/e/>). Glycans were quantified by comparative analyses between the areas of the mass signals and the internal standard (20 pmol maltohexaose). Oligosaccharides with masses greater than ~1000 Da exhibited similar signal strengths, irrespective of structure (Naven and Harvey, 1996). Two-way analysis of variance was performed with Prism software (GraphPad Software, La Jolla, CA, USA). Less than 1  $\mu$ l of hemolymph was required for this analysis.

#### Nucleotide sequence accession number

Genome sequences of gammaproteobacterial endosymbiont and host mitochondrion have been deposited in DDBJ/EMBL/GenBank databases under the project accession numbers AP012978 and AP013032, respectively.

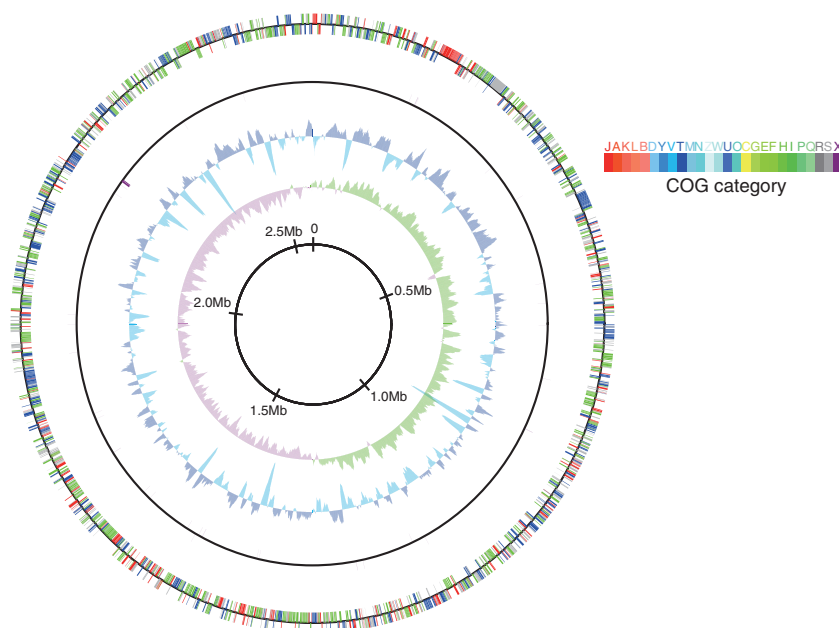
## Results and Discussion

#### General genomic characteristics

Pyrosequence reads were assembled into three large scaffolds, which were assigned to the genome fragments from gastropod (15 611 bp, 2 contigs), mitochondria (7764 bp, 1 contig) and symbiotic bacteria (2 591 967 bp, 53 contigs). The gaps between contigs were closed by primer walking. We could assemble the genome of gammaproteobacterial endosymbiont into a complete circular genome, which is 2 597 759 bp in length, with an average G + C content of 65.1 mol% (Figure 1 and Table 1).

The genome had a 16S rRNA gene sequence identical to that reported in previous clone analysis (Goffredi *et al.*, 2004) (Supplementary Figure S3a). No additional large contig was obtained. The successful assemblage indicated high genetic clonality of endosymbiont within a host gastropod, contrasting to the co-occurrence of divergent endosymbionts in deep-sea vent bivalves (Distel *et al.*, 1995). Nucleotide position 1 of chromosome was assigned to the predicted replication origin (*ori*), flanked on one side by *dnaA*, *dnaN* and *recF* genes and on the other by *rpmH* gene. Cumulative GC skew analysis also suggested that *ori* should have been localized to the same region (Figure 1).

The endosymbiont genome contained a total of 2249 protein-coding DNA sequences, resulting in a coding density of 82.6%. Of the predicted coding DNA sequences, 1677 (74.6%) have been assigned to a putative function using BLASTP searches with an *E*-value cutoff of  $\geq 10^{-5}$  (Table 1). Approximately one-third of the predicted proteins revealed top BLAST hits to tubeworm symbionts (492 proteins to *Tevnia* symbiont and 240 proteins to *Riftia* symbiont). In addition, 586 coding DNA sequences had their top hits to one of the purple sulfur bacteria (*Chromatiaceae/Ectothiorhodospiraceae* group), including *Thioalkalivibrio* (112 hits), *Thiocapsa* (82 hits) and *Allochromatium* (73 hits) (Table 1). Compared with genomes of these free-living relatives (size, 2.7–5.7 Mb; G + C content, 46.2–68.0 mol%; coding density, 86.8–91.7%), the symbiont genome was relatively smaller in size, gene-sparse, but not apparently AT-rich (Table 1).



**Figure 1** Schematic circular diagram of the scaly-foot endosymbiont genome. Outer circle, predicted coding regions on the plus and minus strands, color-coded by functional categories; second circle, RNA genes on the plus and minus strands; third circle, GC content showing deviation from average (65.1%); and fourth circle, GC skew. The GC content, and GC skew were calculated using a sliding window of 5 kb in step of 2.5 kb.



**Table 1** General genomic features of the scaly-foot gastropod endosymbiont and free-living purple sulfur bacteria

Characteristic	Scaly-foot gastropod endosymbiont	<i>Allochrochromatium vinosum</i>	<i>Thioalkalivibrio sulfidophilus</i>	<i>Thioalkalivibrio</i> sp. K90mix
Size (bp)	2 597 759	3 669 074	3 464 554	2 985 056
G + C content (%)	65.1	64.2	65.1	65.5
Number of protein-coding gene	2249	3302	3319	2942
Number of pseudogenes	152	82	36	33
Coding density (%)	82.6	90.6	87.5	89.6
<i>Number of identified RNA genes</i>				
rRNA operons	1	3	3	3
tRNA	40	51	44	48
<i>Number of chemotaxis genes</i>				
<i>che</i>	13	34	8	9
<i>mcp</i>	12	28	14	6

We also assembled the mitochondrial genome into a complete circular genome that was 15 388 bp in length (Supplementary Figure S4).

#### *Pseudogenes and genomic islands*

We discovered 152 endosymbiont sequences that appeared to be pseudogenes (Table 1). In previously reported genomes of free-living purple sulfur bacteria, number of pseudogenes varied ranging from 33 (*Thioalkalivibrio* sp. K90mix) to 82 (*Allochrochromatium vinosum*). Recently evolved pathogens/symbionts generally have many pseudogenes and low coding density, reflecting that gene inactivation outpaces DNA deletion (Burke and Moran, 2011). Pseudogenized genes in the snail symbiont included putative ABC transporters of organics and putative sugar phosphotransferase systems, suggesting the endosymbiont had an ability to grow heterotrophically until recently (Figure 2). Tubeworm symbiont was suggested to have an ability to grow heterotrophically in the free-living state (Markert *et al.*, 2007). At least 39 regions were identified as insertion sequence elements. None of the insertion sequence elements exhibited significant similarities to those of the phylogenetic relatives or gammaproteobacterial symbionts.

#### *Flagellum*

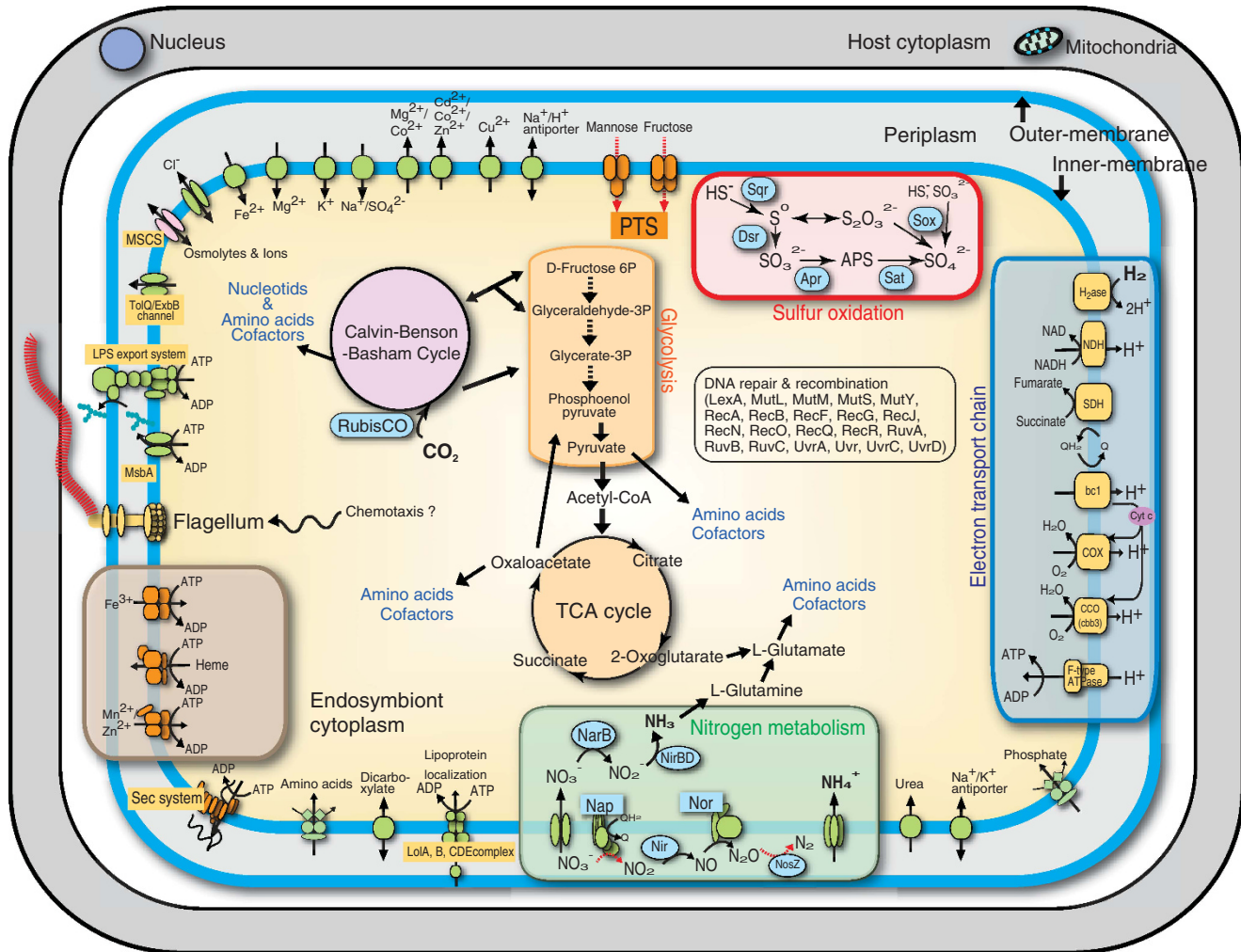
Flagellar motility is essential for the infection of some symbionts, including both facultative and obligate ones (Dulla *et al.*, 2012; Rio *et al.*, 2012). At least 20 genes have been identified to be necessary for bacterial flagellar motility (Pallen and Matzke, 2006). The endosymbiont of scaly-foot gastropod had all but one flagellin gene, and these genes were mostly organized in three regions. Although some hook-associated genes have been pseudogenized, the flagellar genes in the endosymbiont genome mostly remained intact, which included core proteins of the type III secretion

system (Supplementary Table S2). The reverse transcription PCR showed that genes encoding the type III secretion system were all expressed (Supplementary Figure S5), potentially suggesting the flagellar genes have undergone functional divergence. In many endosymbiotic bacteria of insects, for example, flagellar proteins (basal body with or without hook) have been regarded to serve as a protein transporter to maintain the symbiotic system (Maezawa *et al.*, 2006; Toft and Fares, 2008). The actual function of flagellar genes should be confirmed directly by experimental works in the future.

Chemotaxis proteins have important roles in bacterial adaptation to deep-sea vents (Scott *et al.*, 2006; Nakagawa *et al.*, 2007; Takaki *et al.*, 2010; Xie *et al.*, 2011). Even endosymbionts are no exception when in the free-living state. The endosymbiont of scaly-foot gastropod, compared with free-living relatives, has a similar number of chemotaxis proteins (Table 1). The genome included 13 chemotaxis (*che*) genes and 12 methyl-accepting chemotaxis proteins. In addition, the symbiont has a wide array of transport machineries including detoxification systems of heavy metals such as arsenate and copper (Figure 2). Even though the host animal must provide the endosymbiont with a relatively stable habitat, these functions probably reflect the endosymbiont's ability to sense and respond to the surrounding environment in its free-living state.

#### *Sulfur-compound oxidation*

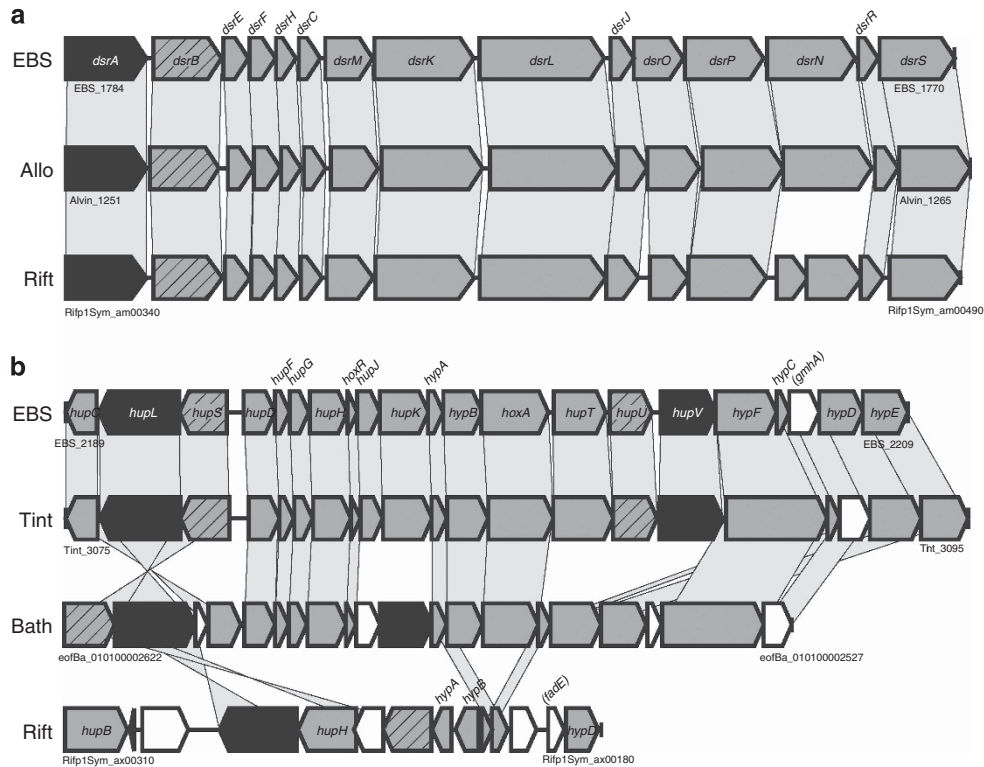
Figure 2 shows the reconstruction of gastropod endosymbiont metabolism. The endosymbiont is a hydrogen/sulfur-oxidizing chemoautotroph, sharing the pathways involved in converting inorganic carbon into organics with other gammaproteobacterial chemoautotrophs. Similar with other sulfur-oxidizing *Gammaproteobacteria*, the endosymbiont has *sqr* (EBS\_1809, EBS\_1831), *dsrAB* (EBS\_1783-1784), *aprAB* (EBS\_2148-2149), *sat* (EBS1086) and



**Figure 2** The major metabolic pathways and solute transport in the scaly-foot gastropod endosymbiont. The Kyoto Encyclopedia of Genes and Genomes database was used for the reconstruction of the metabolic pathways. Red dotted lines are inoperative due to pseudogenes.

*soxXYZA* (EBS\_1635-1638)/*soxB* (EBS\_0922) genes for sulfur oxidation (Figure 2). The order and composition of putative *dsr* gene cluster was almost identical with those of *A. vinosum* (Dahl *et al.*, 2005) and *Riftia* symbiont (Gardebrecht *et al.*, 2012) (Figure 3a). The isolated location of *soxB* relative to other *sox* genes was unusual but previously reported in gammaproteobacterium *Thiomicrospira crunogena* (Scott *et al.*, 2006). *SoxB* of the scaly-foot symbiont and *T. crunogena* share 60% amino acid sequence identity (Supplementary Figure S3b). Incongruence between tree topologies (Supplementary Figures S3a and b) could be explained by horizontal gene transfer (Petersen *et al.*, 2012). For alphaproteobacterial *Paracoccus pantotrophus*, a total of 15 genes comprising a single *sox* gene cluster, have been investigated in detail (Friedrich *et al.*, 2005). Of the *sox* genes, seven genes, *soxXYZABCD*, code for periplasmic proteins responsible for the oxidation of sulfur compounds

(sulfide, elemental sulfur, thiosulfate and sulfite) (Friedrich *et al.*, 2005). The genome of scaly-foot gastropod endosymbiont lacks *soxCD* genes, which encode the sulfur dehydrogenase and mediate a unique oxidative six-electron transfer. To our knowledge, only sulfur-oxidizing *Alphaproteobacteria* and *Epsilonproteobacteria* have the *soxCD* genes (Friedrich *et al.*, 2005; Nakagawa *et al.*, 2007). In addition, chemoautotrophic *Gammaproteobacteria* including scaly-foot gastropod endosymbiont generally lack *SorAB*, which catalyze the oxidation of sulfite to sulfate and is usually present in mesophilic chemoautotrophic *Epsilonproteobacteria* and *Alphaproteobacteria* (Kappler *et al.*, 2000). In contrast, deep-sea vent chemoautotrophic *Epsilonproteobacteria* lack *Dsr/Apr/Sat* (Nakagawa *et al.*, 2007). Although some patterns have been emerging in the repertoire of the sulfur-oxidation pathways in chemoautotrophs, how it affects the ecological performance in deep-sea vents remains to be studied.



**Figure 3** Comparative organization of *dsr* (a) and hydrogenase gene (b) clusters. EBS, scaly-foot gastropod endosymbiont; Allo, *A. vinosum*; Bath, *Bathymodiolus* sp. endosymbiont; Rift, *R. pachyptila* endosymbiont; Tint, *Thiomonas intermedia* K12. Genes are represented by arrows of length proportional to the gene length. Structural genes are represented by hatched arrows (small subunits) or black arrows (large subunits). White arrows without gene names are hypothetical protein genes. Gray bars indicate orthologous genes. Sequence information was retrieved from GenBank.

### Hydrogen oxidation

Hydrogen gas could be the alternative energy source to sulfur compounds for deep-sea vent-dominating chemoautotrophs (Nakagawa *et al.*, 2005). Hydrogenases catalyze the reversible oxidation of  $H_2$  to protons and electrons. The hydrogenases are classified into four distinct groups based on cellular functions and amino acid sequences: group 1, membrane-bound  $H_2$ -uptake hydrogenase; group 2,  $H_2$ -sensing or cyanobacterial hydrogenase; group 3,  $F_{420}$ -reducing, bifunctional hyperthermophilic hydrogenase, methylviologen-reducing hydrogenase and bidirectional NAD-linked hydrogenase; and group 4, membrane-bound  $H_2$ -evolving hydrogenase (Vignais *et al.*, 2001). The hydrogenase genes usually occur as tightly clustered functional units or operons, which consist of hydrogenase subunit genes (structural genes) and accessory or maturation genes encoding proteins necessary for the assembly of functional holoenzyme (for review, see Vignais *et al.*, 2001). The compositions of hydrogenase gene clusters vary among *Gammaproteobacteria* (Vignais *et al.*, 2001). Although heritable endosymbiont of *Calypptogena* clams have no hydrogenase (Kuwahara *et al.*, 2007; Newton *et al.*, 2007), other previously characterized deep-sea vent gammaproteobacterial chemoautotrophs appear to have group 1 hydrogenases (Scott *et al.*, 2006; Markert *et al.*, 2007;

Anantharaman *et al.*, 2013). In the snail endosymbiont genome, we found one of the group 1 hydrogenase (EBS\_2190-2191) and one of the group 2 hydrogenase (EBS\_2203-2204) (Figure 3b and Supplementary Figure S6). Recent studies suggested that not only sulfur compounds but also  $H_2$  was potentially utilized as an energy source by endosymbionts of mussel *Bathymodiolus*, tubeworm *R. pachyptila* and shrimp *Rimicaris exoculata* (Petersen *et al.*, 2011). Sequences and gene order of hydrogenase genes of scaly-foot gastropod endosymbiont showed the highest similarities, not to those of phylogenetic relatives, but to those of *Thiomonas intermedia* K12 (betaproteobacterium) (Arsène-Ploetze *et al.*, 2010) (Figure. 3b). Only 10.3% (235 hits) of the predicted proteins of endosymbiont showed top BLAST hits to members of *Betaproteobacteria*. This may suggest that the hydrogenase gene cluster has been horizontally transferred, although members of the class *Betaproteobacteria* are rare in deep-sea vents (Takai *et al.*, 2006). Recent studies suggested that acquisition of hydrogenase genes via horizontal gene transfer might have a profound role in the metabolic evolution of various deep-sea vent *Gammaproteobacteria* (Kleiner *et al.*, 2012). Deep-sea vent-dominating epsilonproteobacterial chemoautotrophs commonly have the group 4 hydrogenase as



well as groups 1 and 2 hydrogenases (Nakagawa *et al.*, 2007), which is suggestive of the efficient energy metabolism similar to the 'intracellular H<sub>2</sub>-cycling mechanism' of sulfate-reducing bacteria (Odom and Peck Jr, 1981).

#### *Aerobic respiration*

The symbiont genome encodes all the necessary machinery for aerobic respiration, including NADH-quinone oxidoreductase, succinate dehydrogenase and cytochrome *bc*<sub>1</sub>-type ubiquinol oxidoreductase (Figure 2). The composition of terminal oxidases varies among gammaproteobacterial chemoautotrophs; the scaly-foot symbiont has two cytochrome *cbb*<sub>3</sub>-type oxidases and one *bd*-type cytochrome oxidase as *Calyptogena* symbionts do (Kuwahara *et al.*, 2007; Newton *et al.*, 2007) (Figure 2). The *Escherichia coli* *bd*-type cytochrome oxidase has a high affinity for O<sub>2</sub>, and it is expressed under microaerobic conditions (D'mello *et al.*, 1996). The presence of both *cbb*<sub>3</sub>-type and *bd*-type cytochromes may allow the symbionts to survive under a wide range of redox conditions.

#### *Nitrogen metabolism*

Among deep-sea vent-dominating chemoautotrophs, most members of *Epsilonproteobacteria* utilize nitrate both as an electron acceptor and as a nitrogen source (Nakagawa *et al.*, 2005; Nakagawa and Takai, 2008). In contrast, lack of a dissimilatory denitrification pathway (sequential reduction of nitrate to nitrogen gas) is common in previously sequenced deep-sea vent gammaproteobacterial chemoautotrophs (Scott *et al.*, 2006; Markert *et al.*, 2007). Among the genes necessary for the denitrification, *napA* (periplasmic nitrate reductase gene), *napF* (gene necessary for the assembly of the iron-sulfur center of NapA) and *nosZ* (nitrous-oxide reductase gene) have become pseudogenized in the scaly-foot gastropod symbiont (Figure 2). We identified intact genes associated with assimilatory ammonification (sequential reduction of nitrate to ammonium): ferredoxin-nitrate reductase (NarB; EBS\_0633, EBS\_0643) and nitrite reductase (NAD(P)H) (NirBD; EBS\_0631, EBS\_0632).

#### *Central metabolism and carbon fixation*

The gastropod symbiont genome contained nearly all genes needed to reconstruct the complete central pathways, that is, TCA cycle, Embden–Meyerhof–Parnas and pentose phosphate pathways (Figure 2). The complete pathways for the formation of the all amino acids, nucleotides, fatty acids and phospholipids from intermediates of the central metabolism could also be reconstructed (Figure 2). Previous research has suggested that chemoautotrophic deep-sea vent *Gammaproteobacteria* fix CO<sub>2</sub> by way of the Calvin–Benson–Bassham (CBB) cycle (Robinson

*et al.*, 1998; Scott *et al.*, 2006). Ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) is a key enzyme of the CBB cycle and is classified into four different groups, that is forms I to IV. The scaly-foot gastropod symbiont genome encodes genes of both a form IA<sub>q</sub> (EBS\_0695-0696) and a form II (EBS\_0699) RubisCO for CO<sub>2</sub> fixation. These genes are organized in an operon with two posttranscriptional activators (*cbbOQ*; EBS\_0693-0694, EBS\_0697-0698) and a transcriptional regulator (*lysR*; EBS\_0700). No additional form of RubisCO was found. The repertoire of RubisCOs in the endosymbiont genome is unique because previously sequenced chemoautotrophic *Gammaproteobacteria* with both form IA<sub>q</sub> and form II RubisCO include form IA<sub>c</sub> (Badger and Bek, 2008). Form I RubisCO, which has a higher CO<sub>2</sub>/O<sub>2</sub> specificity, is probably more highly expressed under oxic conditions, whereas form II is expressed under reducing conditions (Badger and Bek, 2008). Although the co-occurrence of both CBB and reductive TCA cycles was suggested in the *Riftia* symbiont (Markert *et al.*, 2007), there is no sequence evidence for the alternative carbon-fixation pathways in the genome of scaly-foot gastropod symbiont.

#### *<sup>13</sup>C incorporation*

We incubated gastropods onboard the ship with <sup>13</sup>C-bicarbonate under hydrogen/sulfur-oxidizing conditions. The <sup>13</sup>C incorporation occurred even in the absence of H<sub>2</sub> or sulfide (Table 2), suggesting the presence of stored energy source such as intracellular S<sup>0</sup> observed in marine sulfur-oxidizing *Gammaproteobacteria* (Brock and Schulz-Vogt, 2011). The above-mentioned repertoire of sulfur-oxidation genes in the endosymbiont, that is, the presence of *dsr* genes and the absence of *soxCD* genes, suggests that the bacterium forms sulfur globules (Friedrich *et al.*, 2005). The δ<sup>13</sup>C values fluctuated highly, probably due to individual differences in metabolic status, and thus their availability/preference of energy source provided was unclear from this experiment (Table 2). The δ<sup>13</sup>C values, however, do indicate that all tissue samples, except for those from the killed control are enriched in <sup>13</sup>C, demonstrating symbiont chemoautotrophy (Table 2). Compared with similar tracer experiments (Watsuji *et al.*, 2010, 2012), the degree of <sup>13</sup>C enrichment was apparently modest. Future tracer experiments should be performed at higher temperatures and for longer durations. In addition, the effect of hydrostatic pressure should also be addressed.

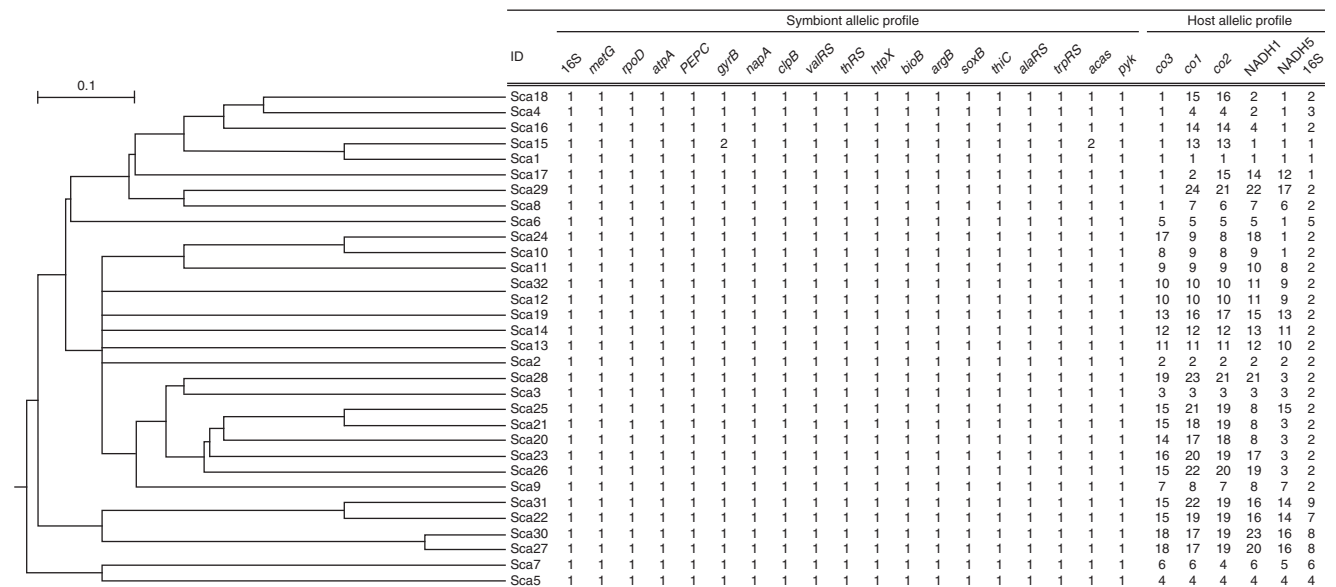
#### *Host hemolymph glycan analysis*

Glycans are highly sensitive biomarkers mirroring the cell/organism status (Varki, 1993; Gagneux and Varki, 1999). We therefore analyzed hemolymph N-glycans of snails incubated in the presence of H<sub>2</sub> and/or sulfur compounds to assess their response to



**Table 2** Stable isotope composition of scaly-foot gastropods incubated with  $^{13}\text{C}$ -bicarbonate

Incubation conditions <sup>a,b</sup>	$\delta^{13}\text{C}$ of tissue (Average $\pm$ s.d.)		Number of samples	Reference
	Esophageal gland	Foot		
SW	+ 6.8	+ 12.9	1	This study
SW + 100 $\mu\text{M}$ sulfide	+ 7.4	+ 6.0	1	This study
SW + 20% $\text{H}_2$ (v/v)	- 11.3	+ 17.6	1	This study
SW + 100 $\mu\text{M}$ sulfide + 20 % (v/v) $\text{H}_2$	+ 12.4 $\pm$ 9.5	- 1.5 $\pm$ 1.3	2	This study
<b>Control</b>				
SW + 100 $\mu\text{M}$ sulfide (dead)	- 20.1	- 17.6	1	This study
SW + 20% $\text{H}_2$ (v/v) (dead)	- 20.5	- 17.9	1	This study
Not incubated	- 20.7 $\pm$ 0.9	- 18.2 $\pm$ 0.6	4	Goffredi <i>et al.</i> , 2004

<sup>a</sup>SW, surface seawater containing 3mM  $^{13}\text{C}$ -bicarbonate.<sup>b</sup>Dead, snails killed by freezing were incubated.**Figure 4** A unweighted pair group method with arithmetic mean dendrogram drawn on the basis of the alleles present at each of the 19 loci shown together with the symbiont and host allelic profiles.

the potential energy sources for symbionts. All of the prominent glycans were not significantly changed during the incubation without potential energy sources (Supplementary Figure S7). In addition, the amounts of two glycans, that is, IDs, 1826 and 2160, remained constant under any incubation conditions. In contrast, other hemolymph *N*-glycan profile changed significantly when snails were incubated with sulfur compounds and  $\text{H}_2$  (Supplementary Figure S7). In particular, the amount of 1664 glycan changed significantly almost in all culture conditions, including the  $\text{H}_2$ -oxidizing condition. Although the glycome data indicated the gastropod cells quickly responded to both  $\text{H}_2$  and sulfur compounds, much remains to be studied, including whether the substrates affect directly or indirectly (symbionts first and then host), and how proteins in hemolymph are changed.

### Genetic polymorphism

Previous studies showed that increased genomic plasticity might confer a competitive advantage enabling deep-sea vent chemoautotrophs to thrive in ever-changing steep physical–chemical gradients (Nakagawa *et al.*, 2005, 2007). We sequenced a total of 19 genes including one pseudogene (*napA*) for each symbiont extracted from 32 different individuals. Unexpectedly, the endosymbiont exhibited extreme genetic homogeneity, showing only two synonymous changes in 19 different genes (13810 positions in total) determined for 32 individual gastropods (Figure 4). In contrast, host mitochondria genes were highly divergent, and 31 haplotypes were identified in 32 individuals (Figure 4). Silent mutations of *gyrB* and *acas* of symbiont were exclusively detected in a single snail (ID, Sca15); however, its host haplotype was not unique,

suggesting the horizontal transmission of endosymbiont (Figure 4). The fate of genetic variations depends both on the fitness effect (selection coefficient) and on the effective population size. Effective population sizes should be small in vertically transmitted symbionts, because the symbionts experience a population bottleneck during transmission from one host generation to the next (Moran, 1996). This host-mediated reduction in population size must have consequences for their evolution, including high levels of random genetic drift (stochastic changes in gene frequency) and a faster rate of substitution at nearly neutral sites (Ohta, 1987, Burke and Moran, 2011). Therefore, extreme homogeneity of gastropod symbiont population again rejected their vertical transmission. Horizontally transmitted symbionts are specifically selected and taken up from the environment anew by each host generation. We consider that the effective symbiont selection by host gastropod overcomes the effect of random genetic drift in the symbiont population. Previous studies also reported slow rates of nucleotide substitution in the 16S rRNA gene of various environmentally acquired symbionts, reflecting efficient purifying selection (Peek *et al.*, 1998). Likewise, highly clonal population has also been reported for the environmentally transmitted, symbiotic sulfur-oxidizing gammaproteobacterium of ciliate *Zoothamnium* (Rinke *et al.*, 2009).

## Conclusions

Our genome analysis of gastropod endosymbiont gives new insights into symbiosis, as well as into an ability to survive in deep-sea vents. Many findings must be further elucidated by experimental studies. As the gastropod can be kept alive at least for several weeks, the availability of the genome sequence will allow the detailed assessment of their ecophysiology when used in combination with high-throughput techniques such as microarray, proteome and metabolome analyses. As additional genome sequences become available, it will be of particular interest to carry out more detailed analyses of the common and specific genes corresponding to the lifestyles, that is, (i) free-living vs symbiotic in deep-sea hydrothermal environments and (ii) gamma- vs epsilon-*Proteobacteria*. This would help to clarify the factors controlling host-symbiont combinations in deep-sea vents.

## Conflict of Interest

The authors declare no conflict of interest.

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