Extremophiles (2009) 13:895–903
DOI 10.1007/s00792-009-0277-8

ORIGINAL PAPER

Expression of genes for sulfur oxidation in the intracellular chemoautotrophic symbiont of the deep-sea bivalve *Calyptogena okutanii*

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Received: 25 May 2009 / Accepted: 19 August 2009 / Published online: 4 September 2009

Abstract To understand sulfur oxidation in thioautotrophic deep-sea clam symbionts, we analyzed the recently reported genomes of two chemoautotrophic symbionts of *Calyptogena okutanii* (Candidatus *Vesicomyosocius okutanii* strain HA: Vok) and *C. magnifica* (Candidatus *Ruthia magnifica* strain Cm: Rma), and examined the sulfur oxidation gene expressions in the Vok by RT-PCR. Both symbionts have genes for sulfide-quinone oxidoreductase (*sqr*), dissimilatory sulfite reductase (*dsr*), reversible dissimilatory sulfite reductase (*rdsr*), sulfur-oxidizing multienzyme system (*sox*) (*soxXYZA* and *soxB* but lacking *soxCD*), adenosine phosphosulfate reductase (*apr*), and ATP sulfurylase (*sat*). While these genomes share 29 orthologous genes for sulfur oxidation implying that both symbionts possess the same sulfur oxidation pathway, Rma has a rhodanese-related sulfurtransferase putative gene (Rmag0316) that has no corresponding ortholog in Vok, and Vok has one unique *dsrR* (COSY0782). We propose that *Calyptogena* symbionts oxidize sulfide and thiosulfate, and that sulfur oxidation proceeds as follows. Sulfide is oxidized to sulfite by *rdsr*. Sulfite is oxidized to sulfate by *apr* and *sat*. Thiosulfate is oxidized to zero-valence sulfur by *sox*, which is then reduced to sulfide by *dsr*. In addition, thiosulfate may also be oxidized into sulfate by another component of *sox*. The result of the RT-PCR showed that genes (*dsrA, dsrB, dsrC, aprA, aprB, sat, soxB,* and *sqr*) encoding key enzymes catalyzing sulfur oxidation were all equally expressed in the Vok under three different environmental conditions (aerobic, semioxic, and aerobic under high pressure at 9 MPa), indicating that all sulfur oxidation pathways function simultaneously to support intracellular symbiotic life.

Keywords Symbiosis · Chemosynthesis · Sulfur metabolism · Intracellular symbiont

Communicated by A. Driessen.

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Introduction

Vesicomyid clams, including *Calyptogena* spp., form dense communities on the deep-sea floor near hydrothermal vents and seeps (Van Dover 2000; Fisher 1990). These deep-sea clams have vestigial digestive tracts and are nutritionally dependent on chemoautotrophic sulfur-oxidizing symbiotic bacteria, which are harbored within their gill epithelial cells (Fialamedioni et al. 1993). Hydrogen sulfide taken up by the clams through their foot is transported to the symbionts by Zn^{2+} sulfide-binding proteins in the blood (Childress et al. 1993) and oxidized to sulfate by the symbiont in the gill. The released energy is conserved in the form of ATP, and used for inorganic carbon fixation and other symbiotic metabolism.

Oxidation of hydrogen sulfide to sulfate is one of the major chemoautotrophic pathways by chemoautotrophic bacteria at deep-sea vent and seep environments to obtain the energy from chemical sources. In sulfur-oxidizing bacteria, many enzymes have been shown to be involved in sulfur oxidization (Friedrich et al. 2001; Nelson and Fisher 1995). The symbiont of the giant tubeworm, *Riftia pachyptila,* has been reported to have adenosine phosphosulfate (APS) reductase (APR), ATP sulfurylase (SAT), and reversible dissimilatory sulfite reductase (rDSR), indicating that they oxidize sulfide to sulfate via APS (Laue and Nelson 1994; Beynon et al. 2001; Markert et al. 2007). The genome of *Thiomicrospira crunogena* XCL-2, a free-living sulfur-oxidizing bacterium isolated from deep-sea hydrothermal vent, lacks the APS pathway but has two sets of genes for sulfur oxidation: the sulfur-oxidizing multi-enzyme system (SOX), and sulfide-quinone oxidoreductase (SQR) (Scott et al. 2006). Sox is a well-characterized multi-enzyme system for thiosulfate oxidation, and is capable of oxidizing various reduced sulfur compounds (hydrogen sulfide, elemental sulfur, thiosulfate, and sulfite) to sulfate (Friedrich et al. 2001; Rother et al. 2001). Sqr catalyzes the oxidation of sulfide to elemental sulfur, leading to the deposition of sulfur (Schutz et al. 1999).

Calyptogena symbionts are gammaproteobacteria, and phylogenetically divided into two major clades (Peek et al. 1998). Recently, the genomes of two symbionts in *C. okutanii* (Candidatus *Vesicomyosocius okutanii* strain HA: Vok) and *C. magnifica* (Candidatus *Ruthia magnifica* strain Cm: Rma), respectively, belonging to these different *Calyptogena* symbiont clades, were reported (Kuwahara et al. 2007; Newton et al. 2007). *Calyptogena* symbionts are vertically transmitted via eggs, and are thought to have co-evolved with the *Calyptogena* clams (Peek et al. 1998). Comparative genome analysis of vertically transmitted intracellular symbionts provides insights into reductive genome evolution (Kuwahara et al. 2008). Kuwahara et al. (2008) has reported that (1) the Vok and Rma genomes are highly similar to each other in both gene content and order and, (2) many deletions in various sizes (<100 bp to 11 kbp) were detected in both genomes, and (3) reductive genome evolution is currently ongoing towards a smaller size genome in both genomes (Kuwahara et al. 2008). However, no comparative study for sulfur oxidation genes of these symbionts has been reported. Moreover, whether these sulfur-oxidizing pathways are functional in the *Calyptogena* symbiont under any environmental conditions remains unknown. To clarify this, we compared the sulfur oxidation genes between the genomes of Vok and Rma, and examined the expression of those key enzyme genes (*dsrA*, *dsrB*, *dsrC*, *aprA*, *aprB*, *sat*, *soxB,* and *sqr*) of the sulfur oxidation pathways in Vok under various environmental conditions.

Materials and methods

Collection and incubation of *Calyptogena okutanii* clams in different environmental conditions

Calyptogena okutanii clams were collected from a seep off Hatsushima in Sagami Bay, Japan at a depth of 890–1000 m during dives of the submersible "Shinkai 6500" and of the remotely operated vehicle "Hyper-dolphin" of JAMSTEC. Collected *Calyptogena okutanii* clams were maintained at 4°C in, (a) aerated sea-water (aerobic condition) overnight under atmospheric pressure, (b) sea-water without aeration (semioxic condition) under atmospheric pressure for 1 week, and (c) aerated sea-water in a Deep-Aquarium (a pressure-controlled chamber for living organisms (Koyama et al. 2002)) at 9 MPa for a week (aerobic condition under high pressure corresponding to the natural habitat pressure). After incubation under the different conditions, the clams were immediately dissected, and gill tissues containing the symbionts were frozen in liquid nitrogen and stored at −80°C until used.

Identification of *Calyptogena* clam species

In the seep of Sagami-bay, *Calyptogena soyoae* and *Calyptogena okutanii* live in the same colony. Morphologically, these two clams are very similar (Kojima and Ohta 1997). The mitochondrial cytochrome oxidase subunit 1 gene (*coI*) was used to identify the *Calyptogena* species. From the foot tissue, DNA was extracted and purified by using the DNeasy tissue kit (QIAGEN). A set of primers for the PCR amplification of *coI*; Caly-COI-forword primer, 5′-GGCG CTGGAGTTTTAGGGGATAG-3′, and Caly-COI-reverse primer, 5′-ATTACGATCAGTTAATAGTATGGTC-3′, was designed on the basis of the consensus sequence between the COI genes of *Calyptogena okutanii* (AB197907) and *Calyptogena soyoae* (AB110744). The PCR conditions entailed 94°C for 60 s, and 30 cycles of 92°C for 40 s, 50°C for 90 s and 72°C for 90 s, followed by extension at 72°C for 10 min. Because the *coI* from *Calyptogena okutanii* contains one *Xba*I site but *C. soyoae* does not, Amplified DNA was digested with *Xba*I, and then the size of fragment was checked by electrophoresis on 3% agarose gel. We also confirmed the sequences of the amplified DNA. DNA sequencing was performed by the dideoxy cycle sequencing method using a model ABI PRIZM 3100 Genetic Analyzer (Applied Biosystems).

Comparison of sulfur oxidation genes in the *Calypotogena* symbiont genomes

The complete genome sequences with annotations of Vok and Rma (accession numbers: NC009465, NC008610,

respectively) were used. To find the homologous sulfur oxidation genes between Vok and Rma, the amino acid sequences of sulfur oxidation genes in Vok or Rma were compared in the complete genome sequence of Vok or Rma using BLAST program.

RT-PCR analysis of sulfur oxidation genes

After contaminated DNA was digested using Turbo RNase-free DNase (Ambion), total RNA was extracted from the gill tissue using a SV Total RNA Isolation System (Promega). Using total RNA as the template, RT-PCR was carried out using a ThermoScript™ RT-PCR System (Invitrogen) with the specific primer sets for each of the sulfur oxidizing key enzyme genes, which were designed based on the Vok genome sequence (Table 1). The amplification protocol entailed 98°C for 2 min and 30 cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 1 min, followed by extension at 72°C for 10 min. In the negative control samples, the reverse transcriptase was omitted. Following the amplification, electrophoresis of the reaction products was carried out in 3% agarose gel. Finally, the sequence of each RT-PCR product was confirmed by DNA sequencing.

Results and discussion

Genes for sulfur oxidation in the *Calyptogena* symbiont genomes

A variety of genes involved in sulfur oxidation, *sox* (except *soxC* & *D*), *dsr*, *sqr*, *apr* and *sat*, were found in the genomes of both Vok and Rma (Kuwahara et al. 2007; Newton et al. 2007). While these two genomes share 29 orthologous genes for sulfur oxidation, Rma has one unique sulfur oxidation-related gene (Rmag0316, Rhodanese-related sulfur-transferase putative) that has no corresponding ortholog in the Vok genome, and Vok has one unique sulfur oxidation-related gene (COSY0782, *dsrR*) (Table 2; Fig. 1). Moreover, *dsrN* and *dsrR* are separated in the Vok genome, whereas *dsrR* is fused to the 3′ terminal end of *dsrN* in the Rma genome (Fig. 1). This result suggested that deletion in non-coding region between *dsrN* and *dsrR* had occurred during the reductive genome evolution fusing these two genes. No rhodanese gene was found in either genome, but four genes encoding putative rhodanese family proteins (Table 2; Fig. 1), which may function as a rhodanese, oxidizing thiosulfate to sulfite in Vok and/or Rma, were found. Overall, sulfur oxidation genes of Vok and Rma were almost the same, which suggested that gene sets of sulfur oxidation were necessary for the lives of *Calyptogena* symbionts.

We found that in both genomes of Vok and Rma most sulfur oxidizing genes were located in three clusters, *soxXYZA*, *dsrABEFHC1MKLJOPNR* and *aprAMB*/*sat*, though other genes were located separately (Tables 1, 2). Among the *sox* genes, *soxXYZA* formed a cluster, but *soxB* was separated, and *soxCD* was not found. This gene arrangement is similar to that in *Thiomicrospira crunogena* XCL2, where the *sox* genes are localized in three locations: *soxXYZA* cluster, *soxB* and *soxCD* (Scott et al. 2006). In the clam symbionts, fourteen *dsr* genes were found to form a cluster in the order *dsrABEFHCMKLJOPNR*, which is nearly the same as those in *Allochromatium vinosum* and *Thiobacillus denitrificans*, except that *dsrS* is missing from the *Calyptogena* symbionts. The presence of three putative copies of *dsrC* suggests the pathway mediated by DsrC is especially important for the symbiont. In addition, two *sqr* genes (*sqr1* and *sqr2*) were found at separate locations in the symbiont genomes (Fig. 1). The genes encoding ATP sulfurylase and APS reductase formed the *sat*/*aprMBA* cluster.

Sulfur oxidation pathways in *Calyptongena* symbionts

Sulfur oxidation pathways deduced from the set of sulfur oxidation-related genes found in the two *Calyptogena* symbionts are the same which are shown in Fig. 2. We deduced that hydrogen sulfide is oxidized via sox independent pathway [hydrogen sulfide (S^{2-}) → sulfite (SO_3^{2-}) → APS → sulfate (SO_4^{2-})]. The dsrAB functions as a reverse dissimilatory sulfite reductase that catalyzes sulfide to sulfite (Fig. 2). APR and SAT oxidize sulfite to sulfate via APS (Fig. 2).

Calyptogena symbionts have the sox system consisting of *soxXYZAB* but lack soxCD. Thus, thiosulfate is presumably utilized via sox multienzyme system lacking soxCD in two ways, [thiosulfate ($S_2O_3^{2}$) → zero-valence sulfur] and [thiosulfate ($S_2O_3^{2}$) → sulfate (SO_4^{2-})] (Fig. 2). The produced zero-valence sulfur is then reduced to hydrogen sulfide (S^{2-}) by DSR (Fig. 2). Many bacteria with a sox system lacking *soxCD* component have been reported (Friedrich et al. 2005; Beller et al. 2006; Hensen et al. 2006; Grimm et al. 2008). *SoxCD* is homologous to sulfite dehydrogenase, but has been shown to function as a sulfane dehydrogenase (Friedrich et al. 2001). In these bacteria, it is thought that the sox system feeds sulfane derived from thiosulfate into other sulfur oxidation pathways. In some cases, this involves incorporation of the sulfane into distinct zero-valence sulfur localized as periplasmic sulfur globules or extracellular sulfur deposit (Hensen et al. 2006). The symbiont of *Calyptogena* is well known to deposit the sulfur globules in the periplasm (Vetter 1985). Rapid elemental sulfur decrease has been reported in the gill tissue when the *Calyptogena* clam is transferred from sulfide-rich condition to non-sulfide environment (Childress et al. 1993). In

Table 1 List of primers for RT-PCR of sulfur-compound oxidation genes

Genes	Primer sequence	Amplified length (bp)
dsrA (COSY0795)	Forward 5′-GTCTTGTGTCGGTGCTTCACG-3′	114
	Reverse 5′-TTGTAAGGTAGTGCTGGACGATGC-3′	
dsrB (COSY0974)	Forward 5′-GATGGACATGTGCGGTTTAC-3′	136
	Reverse5′-TCATGGTTACTGATGGACCTG-3′	
*dsrC*1 (COSY0790)	Forward 5′-TTAGGCGCAAAAGTAGATGAAGAAG-3′	172
	Reverse 5′-TCTGCAGGTGCAATTTGGTACTC-3′	
*dsrC*2 (COSY0805)	Forward 5′-AACAGGTAATGGCTACTTAGTTGATCC-3′	180
	Reverse 5′-CCAACGACTTTGGCAAAGGTTC-3′	
*dsrC*3 (COSY0575)	Forward 5′-TGTATGGAGAAGAAATGACATTAGCTG-3′	206
	Reverse 5′-CATATTACGTTCATTAGGTTGATTGGC-3′	
aprA (COSY0092)	Forward 5′-TAATCGCATGACGACGGTAG-3′	121
	Reverse5′-CTTACAAGCGGCCTTAGCAG-3′,	
aprB (COSY0091)	Forward 5′-AATTGATGTACGTGGTTATGCAG-3′	150
	Reverse5′-CATGGCTGAGTCGTAATTGG-3′	
sat (COSY0089)	Forward 5′-TTAGATATGCGTTATGCAGGC-3′	119
	Reverse5′-CCATAATAATCATTAACACCGGC-3′	
soxB (COSY0161)	Forward 5′-CTCAAGACAATCGATACGAGG-3′	190
	Reverse5′-CCTGCTTTAACACCAATCTGC-3′	
*sqr*1 (COSY0750)	Forward 5′-ATAGCATCAGGTGGGCGG-3′	356
	Reverse 5′-CGGACCACCACGTATCGC-3′	
*sqr*2 (COSY0953)	Forward 5′-GCTGTTGGTTGGCGTAGTG-3′	284
	Reverse 5′-CAGGACCTAAACCTTCAATCTCG-3′	
Rhodanese 1 (COSY0255)	Forward 5′-TGACAATCAATAATGTTGTATCAGCAGTAG-3′	172
	Reverse 5′-GCCGCTGGACAAAATCCTGTAA-3’	
Rhodanese 3 (COSY0838)	Forward 5′-GCTAATGAGCAAATATTTACAACTATTACG-3′	184
	Reverse 5′-ATCCGACCTTTCTCTCCCTTTC-3′	
Rhodanese 4 (COSY0905)	Forward 5′-ACGGCGCTCAATTAGTTGATGTC-3′	126
	Reverse 5′-CAGCCTTATTACGGTCAATGATTCTATCAG-3′	
Rhodanese 5 (COSY0913)	Forward 5′-GGATAGATATCAAAAATTAGTAGCACAAGC-3′	194
	Reverse 5′-AAACACATGCACTCTCAAGTATACC-3′	
cbbM (COSY0653)	Forward 5′-GAACATAATGCCTAATTTTGGTGG-3′	305
	Reverse 5′-GCTTAGGAACGTGGAAATCAATC-3′	
narG (COSY0649)	Forward 5′-TATCCTAGAACTCGTCCAGACTTG-3′	381
	Reverse 5′-AGAACCAGCTGCATAAGATACC-3′	
16S (COSY0907d)	Forward 5′-TACGGGAGGCAGCAG-3′	516
	Reverse 5′-TCGCCACTAAAGGGTACCCCC-3′	
COI (*C. okutanii*)	Forward 5′-GGCGCTGGAGTTTTAGGGGATAG-3′	513
	Reverse 5′-ATTACGATCAGTTAATAGTATGGTC-3′	
18S (*C. okutanii*)	Forward 5′-CTAATACATGCAACACAGCTCCG-3′	188
	Reverse 5′-TAGGCATAGCACGTACCATCG-3′	

Allocromatium vinosum, which lacks *soxCD*, dsr genes are required for oxidation of stored sulfur (Dahl et al. 2005). In the *Calyptogena* symbiont, the presence of *dsr*, *sqr* and *sox* supports the idea of storage form zero-valence sulfur functioning as a reservoir of reduction potential for sulfur oxidation pathways (Vetter 1985) (Fig. 2).

The biochemical pathway of thiosulfate oxidation by purified reconstituted sox system has been intensively studied in *Paracoccus pantotrophus* (Friedrich et al. 2000). In this bacterium, soxYZ, soxAX and soxB are essential components of thiosulfate oxidation to sulfate with cytochrome C as an electron acceptor (Friedrich et al. 2000). Without soxCD, 2 mol of electron are produced per mol of thiosulfate, and an addition of soxCD increases the yield to approximately 8 mol of electron (Friedrich et al. 2000). In *Calyptogena* clams, symbiont can use either sulfide or

Table 2 List of the sulfur oxidation-related genes found in two *Calyptogena* symbiont genomes

Genes	Gene function	*C. okutanii* locus tag	No. of amino acids	Strand[a]	*C. maginifica* locas tag	No. of amino acids	Strand[a]	Identity[b]
sat	ATP sulfurylase	COSY0089	402	+	Rmag0085	402	+	82% (402)
aprM	Adenylylsulfate reductase membrane anchor	COSY0090	289	+	Rmag0086	289	+	95% (289)
aprB	Adenylylsulfate reductase beta subunit	COSY0091	159	+	Rmag0087	159	+	98% (159)
aprA	Adenylylsulfate reductase alpha subunit	COSY0092	627	+	Rmag0088	627	+	92% (627)
soxB	Sulfur oxidation protein SoxB	COSY0161	629	+	Rmag0156	629	+	86% (629)
–	Rhodanese-related sulfurtransferase putative[c]	COSY0255	67	–	No hit	–	–	–
–	Rhodanese-related sulfurtransferase putative[d]	No hit	–		Rmag0316	137	+	–
dsrC3	Predicted DsrC-like protein	COSY0575	113	+	Rmag0622	108	+	87% (108)
soxA	Sulfur oxidation protein SoxA	COSY0730	271	–	Rmag0805	271	–	81% (271)
soxZ	Sulfur oxidation protein SoxZ	COSY0731	100	–	Rmag0806	100	–	87% (100)
soxY	Sulfur oxidation protein SoxY	COSY0732	147	–	Rmag0807	147	–	72% (147)
soxX	Sulfur oxidation protein SoxX	COSY0733	115	–	Rmag0808	115	–	84% (115)
sqr1	Sulfide-quinone reductase	COSY0750	375	–	Rmag0824	376	–	78% (375)
dsrR	Intracellular sulfur oxidation protein DsrR	COSY0782	113	–	No hit	–		–
dsrN	Intracellular sulfur oxidation protein DsrN	COSY0783	458	–	Rmag0858 [h]	566	–	77% (456)
dsrP	Intracellular sulfur oxidation protein DsrP	COSY0784	400	–	Rmag0859	400	–	71% (399)
dsrO	Intracellular sulfur oxidation protein DsrO	COSY0785	243	–	Rmag0860	243	–	85% (243)
dsrJ	Intracellular sulfur oxidation protein DsrJ	COSY0786	127	–	Rmag0861	127	–	80% (110)
dsrL	Intracellular sulfur oxidation protein DsrL	COSY0787	654	–	Rmag0862	654	–	91% (654)
dsrK	Intracellular sulfur oxidation protein DsrK	COSY0788	521	–	Rmag0863	518	–	86% (517)
dsrM	Intracellular sulfur oxidation protein DsrM	COSY0789	257	–	Rmag0864	257	–	80% (257)
dsrC1	Intracellular sulfur oxidation protein DsrC	COSY0790	107	–	Rmag0865	107	–	94% (107)
dsrH	Intracellular sulfur oxidation protein DsrH	COSY0791	98	–	Rmag0866	98	–	87% (98)
dsrF	Intracellular sulfur oxidation protein DsrF	COSY0792	134	–	Rmag0867	132	–	84% (134)
dsrE	Intracellular sulfur oxidation protein DsrE	COSY0793	133	–	Rmag0868	133	–	93% (133)
dsrB	Intracellular sulfur oxidation protein DsrB	COSY0794	357	–	Rmag0869	357	–	96% (357)
dsrA	Intracellular sulfur oxidation protein DsrA	COSY0795	433	–	Rmag0870	433	–	90% (433)
dsrC2	Intracellular sulfur oxidation protein DsrC	COSY0805	96	+	Rmag0881	96	+	93% (96)
–	Rhodanese family protein[e]	COSY0838	139	+	Rmag0932	139	+	65% (138)
–	Rhodanese family protein[f]	COSY0905	123	+	Rmag1009	95	+	88% (95)
–	Rhodanese family protein[g]	COSY0913	159	–	Rmag1011	161	–	75% (159)
sqr2	Sulfide-quinone oxidoreductase	COSY0953	428	+	Rmag1053	431	+	85% (427)

[a] + and − show the gene coding strand in the genome

[b] Numbers of conserved amino acid residues are shown in the parenthesis

[c] The gene named rhodanese 1 in this paper COSY0255 was annotated as putative rhodanese-related sulfurtransferase. However, re-analysis showed that COSY0255 lacks a rhodanese domain. We conclude that the product of COSY0255 is not a rhodanese-related sulfurtransferase

[d] The gene is named rhodanese 2 in this paper

[e] The gene is named rhodanese 3 in this paper

[f] The gene is named rhodanese 4 in this paper

[g] The gene is named rhodanese 5 in this paper

[h] In the Rma genome, *dsrN* and *dsrE* are fused at the 3′ terminal end of *dsrN*

thiosulfate as a substrate to support the fixation of inorganic carbon (Childress et al. 1991). These findings support that *Calyptogena* symbiont sox system oxidizes thiosulfate to sulfate (Fig. 2).

In the symbiont of the vestimentiferan giant tubeworm, *Riftia pachyptila*, sulfur oxidation proceeds via the rDSR–APR–SAT pathway (Laue and Nelson 1994; Beynon et al. 2001; Markert et al. 2007). On the other hand, in free-living *T. crunogena* XCL2, sulfur oxidation proceeds via the SOX and SQR enzyme systems (Scott et al. 2006). In *Calyptogena* symbionts, reductive genome evolution is ongoing to eliminate genes that are not essential for

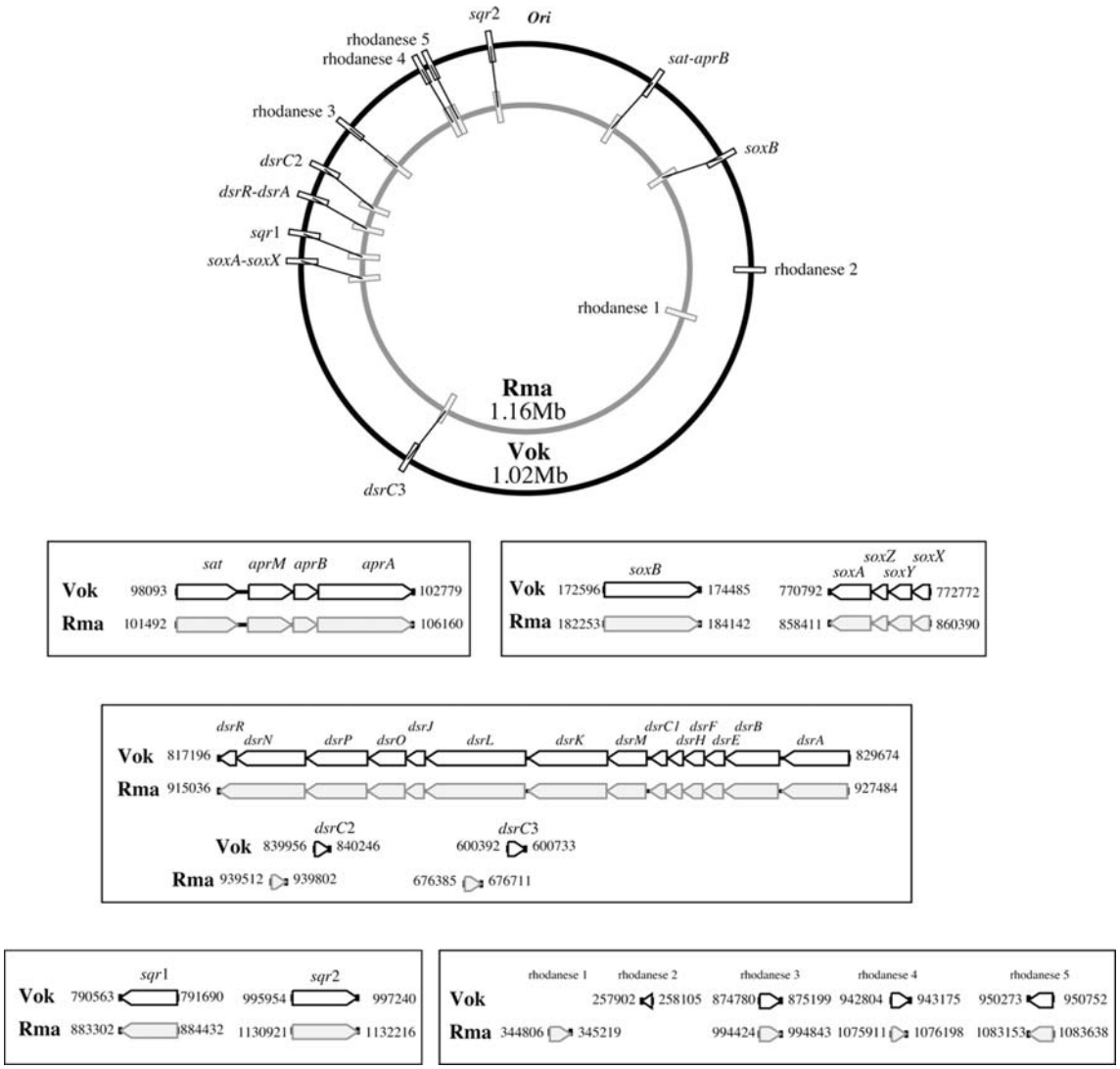

Fig. 1 Maps of the Vok and Rma genomes showing the positions of putative genes for sulfur oxidation. Vok and Rma genomes show the *outer* and *inner circles*, respectively. *Lower boxes* show the gene orders (arrangements) in the gene clusters. *Arrowheads* show the gene coding region. *Black* and *gray* colors denote Vok and Rma, respectively. The numbers at the terminals of the sequences indicate the nucleotide number from the replication origin

intracellular symbiotic life (Kuwahara et al. 2008); nonetheless, they still posses full gene sets for several distinct sulfur oxidation pathways. This suggests that sulfur oxidation pathways play a critical role in these symbionts and raises the question whether these genes are constitutively expressed and whether the expression patterns of sulfur oxidation genes may change under different environmental conditions.

Expressions of essential genes involved in sulfur oxidation in *Calyptogena okutanii*

To obtain gene expressions profiles of Vok's sulfur oxidation pathways and to examine the change of gene expression in response to various environmental conditions, we used reverse transcription-PCR (RT-PCR). Genes encoding eight key enzymes for sulfur oxidation (*dsr*A, *dsr*B, *apr*A, *apr*B, *sat*, *sox*B, *sqr*1 and *sqr*2) were selected for their expressions to be detected. These key enzymes are the major components involved in their respective sulfur oxidation biochemical reactions, i.e., [hydrogen sulfide (S^{2-}) → sulfite (SO_3^{2-}) → APS → sulfate (SO_4^{2-})], [thiosulfate ($S_2O_3^{2}$) → zero-valence sulfur] and [thiosulfate ($S_2O_3^{2}$) → sulfate (SO_4^{2-})] (refer to Fig. 2). The collected *Calyptogena okutanii* clams were incubated at 4°C in, (a) aerated sea-water (aerobic condition) overnight under atmospheric pressure, (b) sea-water without aeration (semioxic condition) under atmospheric pressure for 1 week, and (c) aerated sea-water in a Deep-Aquarium (a pressure-controlled chamber for living organisms (Koyama

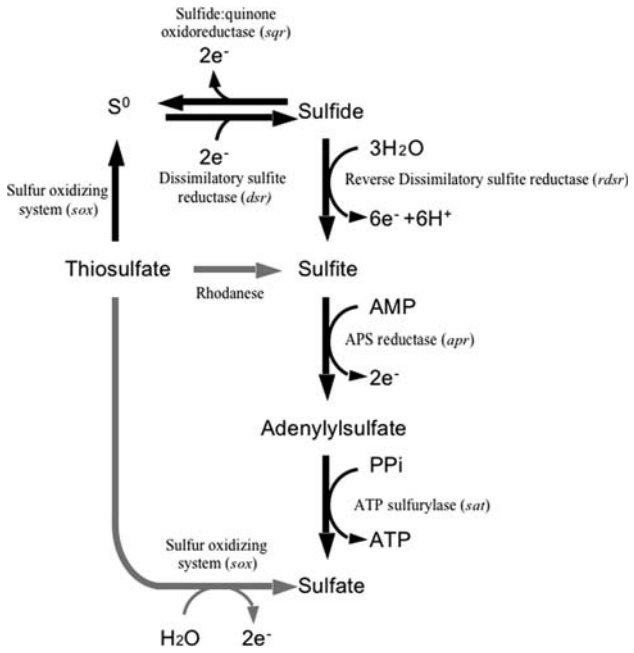

Fig. 2 Sulfur oxidation pathways deduced from the genome sequences of the *Calyptogena* symbionts. *Solid arrows* indicate pathways of sulfur-compound oxidation or reduction in the Vok and Rma. The *gray arrows* represent reactions possibly catalyzed by putative rhodanese, and sox. Liberated electrons pass through the electron transport system to generate a proton motive force, which is employed to drive ATP production. S^0 is zero-valence sulfur

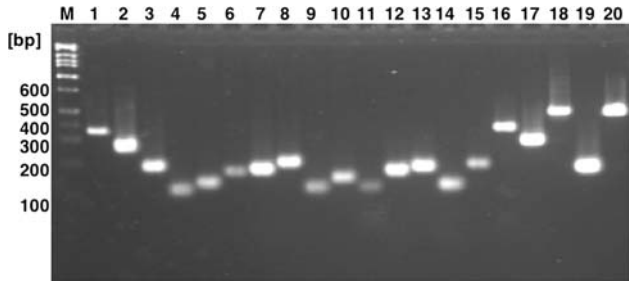

Fig. 3 Electrophoregram of the RT-PCR products of eight key sulfur-oxidation enzyme genes expressed in *Calyptogena okutanii* gill tissue. Fragments of the genes encoding key enzymes for sulfur oxidation were amplified by RT-PCR, after which the products were subjected to 3% agarose gel electrophoresis. All the detected bands showed cDNA amplicons of the expected length, and their DNA sequences were confirmed (data not shown). Negative controls (RT-PCR lacking reverse transcriptase) showed no amplicons (data not shown). Lane M, Hi-Lo DNA markers (50 bp to 10 kbp; Bionexus Inc., Oakland, CA); lane 1, *sqr1*; lane 2, *sqr2*; lane 3, *soxB*; lane 4, *dsrA*; lane 5, *dsrB*; lane 6, *dsrC1*; lane 7, *dsrC2*; lane 8, *dsrC3*; lane 9, *aprA*; lane 10, *aprB*; lane 11, *sat*; lane 12, *rhodanese1* (COSY0255); lane 13, *rhodanese3* (COSY0838); lane 14, *rhodanese4* (COSY0905); lane 15, *rhodanese5* (COSY0913); lane 16, *narG*; lane 17, *rubisco*; lane 18, 16S-rDNA; lane 19, 18S-rDNA; and lane 20, *coI*. Rhodanese 1 (COSY0255) was previously annotated as a putative rhodanese-related sulfurtransferase (7). However, re-analysis showed that rhodanese 1 (COSY0255) lacks a rhodanese domain. We conclude that the product of COSY0255 is not a rhodanese-related sulfurtransferase

et al. 2002)) at 9 MPa for a week (aerobic condition under equivalent pressure of their natural habitat). Expression of four other genes (16S rRNA and Rubisco (*cbbM*) from the symbiont; 18S rRNA and cytochrome oxidase I (*coI*) from the host) served as a control. Expression of all eight sulfur oxidation genes was detected under aerobic condition (Fig. 3), and there was no significant change in their expressions under two other conditions tested (data not shown). Likewise, there was no change in the expression of the gene encoding nitrate reductase gamma subunit, genes encoding putative rhodanese family proteins or the three copies of *dsrC* gene when examined under the same conditions (Fig. 3). In addition, no sequence variation was found in any of the examined RT-PCR products. This result suggested that genetic diversities of both the symbiont and the host populations are low in examined three samples.

Despite the presence of three putative copies of *dsrC* and their constitutive expression suggests the importance of DsrC in the symbiont, the *dsrC* encodes a family of soluble cytoplasmic proteins whose function remains unknown (Pott and Dahl 1998). Whether rhodanese family proteins of *Calyptogena* symbionts possess thiosulfate sulfur-transferase activity that breaks the S–S bond of thiosulfate to generate sulfur and sulfite remains unclear, but the *Calyptogena* symbionts utilize the thiosulfate as an electron donor (Childress et al. 1991). In conclusion, our findings suggest that all sulfur oxidation pathways in Vok are functional, and their expression levels are unaffected by the examined environmental changes.

Calyptogena symbiont oxidizes sulfide and thiosulfate

The reason for why the *Calyptogena* clam symbiont has two different sulfur oxidation pathways may be explained by the environmental conditions of the vesicomyid clam habitats. It has been proposed that vestimentiferan giant tubeworms are sulfide-specialists, mussels are thiosulfate-specialists and vesicomyid clams play an intermediate role of these two (Nelson and Fisher 1995). Increase in the sulfide concentration stimulate CO_2 fixation and O_2 consumption by the *R. pachyptila* symbiont in vitro, whereas exposing the isolated symbiont to 5–3,000 mM thiosulfate did not affect O_2 consumption (Wilmot and Vetter 1990). These findings are in good agreement with the report that the *R. pachyptila* symbiont has no genes for *sox* multienzyme system (Markert et al. 2007). On the other hand, the mussel *Bathymodiolus thermophilus*, which harbors thioautotrophic symbionts, has a greater preference for thiosulfate as a sulfur source than for sulfide (Nelson and Fisher 1995). This likely reflects the fact that *Bathymodiolus* mussels are unable to retain sulfide in their blood due to a lack of a sulfide-binding protein (Nelson and Fisher

1995), and that sulfide is unstable in aerobic environment and spontaneously oxidized to elemental sulfur by oxygen. Thiosulfate is more stable and thus more abundant in a variety of natural environments (Hensen et al. 2006). Because vesicomyid clams live in an intermediate environment between those two, the clams have acquired and retained the genes required for both oxidation pathways of sulfide and thiosulfate.

In the present study, we showed that the *Calyptogena* symbionts Vok and Rma have same set of sulfur oxidation genes, and at least two distinct sulfur oxidation pathways, the *sox*- and *rdsr–apr–sat* pathways for oxidations of thiosulfate and sulfide, respectively. Having sox multienzyme system lacking sox CD enables elemental sulfur to serve as reservoirs of reduced sulfur compounds. Our study also showed that expressions of enzyme genes for all the pathways did not change under three different environmental conditions examined. These findings indicate that two sulfur oxidation pathways are equally functional and active under the examined environmental conditions, and that sulfide, elemental sulfur and thiosulfate are all usable in the intracellular environment of the symbionts. Perhaps because of the simplicity of the gene expression regulatory system in the *Calyptogena* symbionts (Kuwahara et al. 2007), and of the homeostatic intracellular environment of the host cell, all of the genes for sulfur oxidation are constitutively expressed irrespective to the host's environment. This may be advantageous for vesicomyid clam symbionts, enabling them to utilize three kinds of sulfur compounds (sulfide, thiosulfate and elemental sulfur) for energy conversion in the deep-sea environment where sulfide concentrations may rapidly fluctuate.

Acknowledgments We are grateful to the captains and crew of the R/V Natsushima and R/V Yokosuka and to the operation teams of the DSRV Shinkai6500 and ROV Hyperdolphin for sample collection. We thank Drs. K. Takai, T. Nunoura, and Y. Fujiwara at JAMSTEC and H. Kojima at Hokkaido University for their discussion. We also thank M. Kawato at JAMSTEC for the help with DNA sequencing and Dr. P. Kottapalli for helpful comments.

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