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Research Article

RESEARCH ARTICLE

Analysis of functional gene transcripts suggests active CO_2 assimilation and CO oxidation by diverse bacteria in marine sponges

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One sentence summary: Functional transcripts indicate autotrophic CO2 assimilation through the reductive pentose phosphate cycle, heterotrophic anaplerotic CO2 assimilation, and aerobic CO oxidation by phylogenetically diverse bacteria in three sympatric sponges *T. swinhoei*, *P. simplex* and *Ph. fusca*.

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ABSTRACT

Bacteria are the dominant symbionts in sponges and are regarded as important contributors to ocean nutrient cycling; however, their roles in carbon utilization in sponge holobionts are seldom identified. Here, the *in situ* active bacteria and their CO_2 assimilation and CO oxidation functions in sponges *Theonella swinhoei*, *Plakortis simplex* and *Phakellia fusca* were evaluated using the analysis of functional gene transcripts. Phylogenetically diverse bacteria belonging to 16 phyla were detected by 16S rRNA analysis. Particularly, some of the active bacteria appeared to be sponge-specific or even sponge species-specific. Transcribed autotrophic CO_2 assimilation genes *rbcL* and *rbcM*, anaplerotic CO_2 assimilation gene *accC* and aerobic CO oxidation gene *coxL* were uncovered and assigned to a wide variety of bacterial lineages. Some of these carbon metabolism genes showed specificity to sponge species or different transcriptional activity among the sponge species. This study uncovered the phylogenetic diversity of transcriptionally active bacteria especially with CO_2 assimilation or CO oxidation functions, providing insights into the ecological functions of the sponge-symbiotic bacteria regarding carbon metabolism.

Keywords: sponge holobiont; bacterial symbionts; CO_2 assimilation; CO oxidation; transcriptional activity

INTRODUCTION

The oceans are a major sink for CO_2 and CO (Swinnerton, Linnenbom and Lamontagne 1970; Sabine *et al.* 2004). Oceanic carbon biogeochemical transformation spans a tremendous geographic range at the bathymetric and latitudinal scales and is of considerable importance to the global carbon circulation (Cox *et al.* 2000). Microbes are regarded as the main transformers in carbon cycling and substantial contributors to the energy flow in the ocean (Cotner and Biddanda 2002; Madsen 2011; DeLorenzo *et al.* 2012). CO_2 assimilation by microorganisms in marine environments is one of the most promising solutions to the predicament of CO_2 emission and a fundamental means of generating organic carbon (Hugler and Sievert 2011).

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As an essential component of benthic communities worldwide and active suspension feeders, marine sponges have a significant influence on the nutrient availability in their associate habitats, such as supporting the nutrient needs for coral reefs and other organisms (de Goeij *et al.* 2013; Lesser and Slattery 2013; Pita *et al.* 2018). Marine sponges are complex holobionts that accommodate abundant and diverse microbes, including bacteria, archaea and fungi (Fiore, Jarett and Lesser 2013; Gao *et al.* 2014; He *et al.* 2014; Naim *et al.* 2014; Jasmin, Anas and Nair 2015; Morrow, Fiore and Lesser 2016; Thomas *et al.* 2016; Webster and Thomas 2016), and may play important ecological roles in element cycling (Han *et al.* 2012; de Goeij *et al.* 2013; Feng *et al.* 2016, 2018; Pita *et al.* 2018). The assimilation of dissolved inorganic and organic carbon by the sponge-microbe consortia, along with their filter-feeding abilities, provides sponges with a suite of nutritional capabilities in their oligotrophic habitats (de Goeij *et al.* 2008, 2013). Some symbiotic photoautotrophs may even provide their sponge hosts with a nutritional benefit and enhanced scope for growth under high CO_2 concentrations (Morrow *et al.* 2015).

Microbes can autotrophically assimilate CO_2 via six pathways: the reductive pentose phosphate (rPP) cycle, the reductive tricarboxylic acid (rTCA) cycle, the reductive acetyl coenzyme A (rACA) pathway, the 3-hydroxypropionate (3-HP) bicycle, the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) cycle and the dicarboxylate/4-hydroxybutyrate cycle (Hugler and Sievert 2011). These metabolic pathways include a variety of processes involving alternative energy usage (phototrophic or chemotrophic) under different conditions (Vacelet *et al.* 1996; Hentschel, Usher and Taylor 2006; Taylor *et al.* 2007; Hoffmann *et al.* 2009). Heterotrophic microbes are unable to use CO_2 as their only carbon source; however, they have the ability to assimilate CO_2 in various anaplerotic reactions during biosynthesis (Hesselsoe *et al.* 2008). CO oxidation, which produces CO_2, is carried out by a diverse group of aerobic bacteria (King 2003b). These CO-oxidizing microbes play important roles in the global CO biogeochemistry and contribute to CO scavenging in their niches (Moran *et al.* 2004; Simister *et al.* 2013).

The functional gene based analysis has improved our knowledge of the roles that marine microbes play in the global biogeochemical cycles (Ferrera *et al.* 2015). Genes involved in CO_2 assimilation pathways have been identified in sponge symbionts; for instance, the photoautotrophic rPP pathway in cyanobacteria *Synechococcus spongiarum* and *Myxosarcina* sp. (Burgsdorf *et al.* 2015; Yu *et al.* 2015), the autotrophic 3-HP/4-HB cycle in archaea *Cenarchaeum symbiosum* and *Nitrosopumilus* sp. LS_AOA (Hallam *et al.* 2006), the rACA pathway and the rTCA cycle in *Poribacteria* sp. (Siegl *et al.* 2011; Tian *et al.* 2016). Genome revelation of the sulfur-oxidizing bacterium *Thioalkalivibrio nitratireducens*, identified from the sponge *Haliclona cymaeformis*, has illustrated the chemoautotrophic rPP pathway and versatile heterotrophic metabolic capabilities (Tian *et al.* 2014). The symbionts of the sponge *Neamphius huxleyi* are likely to use two alternative pathways for CO_2 assimilation, i.e. the chemoautotrophic rPP cycle and the rTCA cycle (Li *et al.* 2014). Metagenomic analysis of the sponge *Theonella swinhoei* has uncovered the genes of the symbiotic *Entotheonella* sp. related to the chemoautotrophic life style employing the rPP pathway, sulfur oxidation and reduction, as well as those allowing utilization of organic compounds (e.g. chitin, N-acetylglucosamine), indicating the mixotrophic life style of this bacterial genus (Liu *et al.* 2016). The genes involved in CO oxidation have been found in the uncultured sponge-symbiotic *Poribacteria* sp. (Siegl *et al.* 2011), *Rhodospirillaceae* (Karimi *et al.* 2018) and the metagenome of the sponge *Cymbastela concentrica* (Thomas *et al.* 2010). In addition, uncultured chemosynthetic symbionts, such as ammonia-oxidizing archaea and bacteria, nitrite-oxidizing bacteria and sulfur-oxidizing bacteria, have been detected in different sponges from various geographic locations by using gene-targeted sequencing approaches (Hoffmann *et al.* 2009; Lopez-Legentil *et al.* 2010; Mohamed *et al.* 2010; Nishijima *et al.* 2010; Thomas *et al.* 2010; Han *et al.* 2012; Ribes *et al.* 2012; Radax *et al.* 2012a, 2012b; Han, Li and Zhang 2013; Feng *et al.* 2016; Jensen *et al.* 2016). These investigations indicate the complexity of carbon metabolism with a wide variety of pathways operating in the symbiotic consortium of sponges.

However, most of the present functional evaluation of sponge microbiota is based on gene analysis at the DNA level. DNA-based analyses often allow inference with respect to the metabolic potentials of microbes involved in ecological processes (Wang *et al.* 2013). As a comparison, RNA-based approaches could give further insights into the active functions of the microbiota (Feng *et al.* 2018). For example, transcripts of genes related to photoautotrophic CO_2 assimilation through the rPP cycle or CO oxidation have been explored in the sponges *Stylissa carteri*, *Geodia barretti*, and *Xestospongia muta* by metatranscriptomic analysis (Moitinho-Silva *et al.* 2014). To date, comparatively, there is still a lack of comprehensive knowledge about the phylogenetic diversity of *in situ* active bacterial lineages that are responsible for CO_2 assimilation and CO oxidation in sponge holobionts. In this study, 16S rRNA together with the transcripts of the functional genes essential for CO_2 assimilation and CO oxidation were analyzed to reveal the *in situ* active bacteria with CO_2 assimilation and CO oxidation functions among sympatric sponges in the South China Sea.

MATERIALS AND METHODS

Sampling

The sponges *T. swinhoei* (class Demospongiae, order Tetractinellida, family Theonellidae), *Plakortis simplex* (class Homoscleromorpha, order Homosclerophorida, family Plakinidae) and *Ph. fusca* (class Demospongiae, order Axinellida, family Axinellidae) were collected by scuba diving near Yongxing Island (112° 20′ E, 16° 50′ N) in the South China Sea at approximately 10 m depth from 9:15 to 9:50 am on the 20th of June 2013. These sponge species were selected because they are abundant and coexist in the same reef. Samples were transferred underwater to sealable plastic bags containing seawater, brought to the boat and stored on ice. Three individuals per sponge species were sampled. The sponge samples were sliced into 1 cm^3 pieces with sterile scalpels and were transferred into 10 volumes of RNAfixer stabilization solution (YuanPingHao, Beijing, China) after they had been rinsed three times with sterile artificial seawater (Mohamed *et al.* 2010). In addition, three seawater samples (2 liters per sample) were collected in the proximity (less than 1 min from the sponges), before the sponges were collected, and were filtered through 0.22 μm filters (Millipore, Bedford, USA). The collected sponge and seawater samples were preserved in RNAfixer stabilization solution. The time between sample acquisition and fixation was no longer than 20 min (Ozturk *et al.* 2013). RNAfixer-fixed sponge and filtered seawater samples were stored at −80°C before total RNA and DNA extraction within 2 weeks.

RNA extraction and cDNA synthesis

RNAfixer-fixed sponge and filtered seawater samples were ground in liquid nitrogen with a mortar and pestle. RNA was extracted using the PrepRNA/DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNase-free DNase I (Fermentas, Hanover, USA) was used to digest the residual genomic DNA at 37°C for 60 min. RNA quality and integrity was checked by gel electrophoresis and by examining the A260/A280 ratio (ranging from 1.98–2.02) using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The final RNA concentration and purity were quantified using the Qubit system (Invitrogen, Darmstadt, Germany). First-strand cDNA synthesis was performed using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, USA). Each reaction volume was 10 μl containing 100 ng RNA, 0.5 μl random hexamers primer (50 ng/μl), 5 μl cDNA Synthesis Mix and RNase-free water. This reaction was carried out by incubating at 25°C for 10 min, then at 50°C for 50 min and terminated at 85°C for 5 min. All cDNA aliquots were stored at −80°C before polymerase chain reaction (PCR) amplification.

16S rRNA pyrosequencing and phylogenetic analysis

Using cDNA, bacterial 16S rRNA fragments from sponges *T. swinhoei*, *P. simplex*, *Ph. fusca* and environmental seawater were amplified and barcoded using Titanium adapter sequences A (forward primer) and B (reverse primer) for pyrosequencing. A 10-bp barcode sequence was added to the PCR primers to distinguish the sequences by source. The primer pair U789F&U1068R was used to amplify the hypervariable V5-V6 region of bacterial 16S rRNA sequences (Fiore *et al.* 2015). The PCR mixture contained 0.25 μl of Titanium Taq polymerase (Clontech, Mountain View, USA), 2.5 μl of Titanium Taq buffer, 0.5 μl dNTPs (Promega, Madison, USA, 100 mM solution), 0.2 μl of each barcoded primer (5 μM solution) and 2 μl of cDNA template. The reaction was performed on a Thermocycler (Eppendorf Mastercycler, Wesseling-Berzdorf, Germany) using the following protocol: initial denaturation for 5 min at 95°C, 28 cycles of 95°C for 30 s, 53°C for 30 s and 72°C for 45 s, followed by 10 min at 72°C. The cDNAs of three individuals for each sponge or seawater sample were PCR-amplified, and three technical replicates per individual were used. The PCR products of replicates were pooled together for pyrosequencing to reduce potential bias amplification (Feng *et al.* 2016, 2018) (Fig. S1, Supporting Information). PCR products were assessed by gel electrophoresis (1.5% agarose gel) and purified with MinElute Gel Extraction Kit (Qiagen). The amplicons were then quantified by a Qubit 2.0 Fluorometer using a Qubit dsDNA HS Assay Kit (Invitrogen). A mixture of PCR products was prepared by mixing 200 ng of purified 16S rRNA amplicons from each sponge or seawater sample and then pyrosequenced on the ROCHE 454 FLX Titanium platform (Roche, Basel, Switzerland) at the National Human Genome Centre of China at Shanghai, China, according to the manufacturer's manual.

The Mothur platform v.1.36.0 was used for all analyses according to Schloss *et al.* (2009). Sequences were aligned to the data derived from the full-length SILVA alignment distributed by Mothur. Raw sequences with lengths of less than 200 bp, with mismatches on the primer or barcode, containing a homopolymer longer than 6 nucleotides, or with a mean quality score below 25 were discarded. The resulting alignment was then filtered to remove columns that did not contain any information. Reads were pre-clustered so that any sequences differing by a single nucleotide were considered to be identical. Pre-clustered reads were classified using the Mothur implementation of the RDP Bayesian classifier using a cutoff of 60% bootstrap support over 100 iterations. Sequences that were classified as belonging to mitochondria, chloroplasts or archaea, and sequences that were classified as 'unknown' or 'unclassified' at the phylum level were removed from the dataset. Chimeras were identified using a Mothur-based implementation of Chimera Slayer using a region-specific version of the Gold database. The remaining aligned sequences were used to generate a pairwise distance matrix and clustered into operational taxonomic units (OTUs) using average-linkage clustering at 97% sequence identity. OTUs were classified using the Mothur implementation of the RDP classifier.

Construction of functional gene fragment libraries and phylogenetic analysis

The cDNA template was used for PCR amplification of the CO_2 assimilation genes *rbcL* and *rbcM* (encoding the large chain subunit of RuBisCO and the single subunit RuBisCO, respectively, being essential in the rPP cycle), *aclB* (encoding ATP citrate lyase subunit B, which is active in the rTCA cycle), *cooS* (encoding the catalytic subunit of anaerobic CO dehydrogenase/Acetyl CoA synthase, that takes part in the rACA pathway), *mcrA* (encoding the subunit of malonyl-coenzyme A reductase, that is active in the 3-HP bicycle), *accC* (encoding the carboxylase subunit of the acetyl coenzyme A carboxylase complex, that conducts carboxylation reactions) and CO oxidation gene *coxL* (encoding the large subunit of aerobic CO dehydrogenase) with the primers shown in Table 1. Since no primers were available for the *mcrA* gene of the 3-HP pathway, and that pathway has only been identified from the *Chloroflexus* clade so far (Hugler and Sievert 2011), primers were designed according to the *mcrA* genes of *Chloroflexus aurantiacus* OK-70-fl (AY530019, 3663 bp in full length), *C. aurantiacus* J-10-fl (Caur_2614, 3660 bp in full length) and *Chloroflexus* sp. Y-400-fl (CHY400_RS13755, 3660 bp in full length). Alignment of these three full-length *mcrA* genes, constructed using the Blastn suite-2sequences model (https://blast.ncbi.nlm.nih.gov/Blast.cgi), showed 99% sequence identity and 100% Query Coverage value. The primer pair was designed using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/); its targeting specificity was tested by BLAST search against the NCBI nucleotide database. The designed forward primer McrA1F and reverse primer McrA1R targeted the 1424–1443 region and 1888–1907 region of the *mcrA* gene in *C. aurantiacus* OK-70-fl, respectively, to amplify a 484 bp long fragment.

PCR was carried out in a total volume of 40 μl containing 2 μl cDNA, 0.4 μl of each primer (10 μM solution) and 20 μl TaqMasterMix (CoWin Biotech, Beijing, China). PCR amplifications were performed on a Thermocycler (Eppendorf, Hamburg, Germany) according to the following procedure: 95°C for 5 min, followed by 30 cycles of 95°C for 40 s, 1 min at specific annealing temperature (see Table 1), and 72°C for 1 min and final extension at 72°C for 15 min. For negative control, a similar procedure was carried out using purified RNA in place of the template to ensure that there was no genomic DNA contamination. cDNAs of three individuals for each sponge species and of the seawater samples were PCR-amplified, and three technical replicates per individual were processed. The PCR products of replicates were pooled together for pyrosequencing to reduce potential bias amplification (Feng *et al.* 2016, 2018) (Fig. S1, Supporting Information).

Table 1. Primers for the PCR amplification of key genes in the CO_2 assimilation and CO oxidation pathways.

Primer	Sequence 5′-3′	PCR product length (bp)	Tm (°C)	Reference
CO_2 assimilation gene				
rbcL				
RubIgF	GAYTTCACCAARGAYGAYGA	812	55	(Spiridonova *et al.* 2004)
RubIgR	TCRAACTTGATYTCYTTCCA			(Spiridonova *et al.* 2004)
rbcM				
RuIIF1	GGHAACAACCARGGYATGGGYGA	800	56	(Spiridonova *et al.* 2004)
RuIIR3	CGHAGIGCGTTCATGCCRCC			(Spiridonova *et al.* 2004)
RuIIF2	GGIACVATCATCAARCCVAA			(Spiridonova *et al.* 2004)
RuIIR2	TGRCCIGCICGRTGRTARTGCA	400	58	(Spiridonova *et al.* 2004)
aclB				
aclB892F	TGGACMATGGTDGCYGGKGGT	312	54	(Campbell and Cary 2004)
aclB1204R	ATAGTTKGGSCCACCTCTTC			(Campbell and Cary 2004)
cooS				
cooS805f	AARSCMCARTGTGGTTTTGG	1817	55	(Hunger, Gossner and Drake 2011)
cooS2623r	TTTTSTKMCATCCAYTCTGG			(Hunger, Gossner and Drake 2011)
mcrA				
McrA1F	CCGCTATCGGTCAGCTCATT	484	55	Designed in this study
McrA1R	GCAATGTGGACGCGATCTTC			Designed in this study
accC				
ACAC254f	GCTGATGCTATACATCCWGGWTAY	509	56	(Auguet *et al.* 2008)
ACAC720r	GCTGGAGATGGAGCYTCYTCWATT			(Auguet *et al.* 2008)
CO oxidation gene				
OMP clade *coxL*				
OMPf	GGCGGCTTYGGSAASAAGGT	1272	58	(King 2003a)
O/Br	YTCGAYGATCATCGGRTTGA			(King 2003a)
BMS clade *coxL*				
BMSf	GGCGGCTTYGGSTCSAAGAT	1272	58	(King 2003a)
O/Br	YTCGAYGATCATCGGRTTGA			(King 2003a)

Presence and sizes of these amplification products were estimated by gel electrophoresis (1.5% agarose gel). The PCR products were gel-purified with MinElute Gel Extraction Kit (Qiagen). The purified PCR products were cloned with TA-Cloning Kit (CoWin Biotech) and transformed to *Escherichia coli* DH5α competent cells (CoWin Biotech) according to the standardized instructions. The positive clones were screened by ampicillin resistance and identified by PCR-screening with vector-specific M13 primers. A variable number (24–72) of clones from each clone library were sequenced on an ABI 3100 capillary sequencer (Sangon Corp., Shanghai, China).

All the obtained nucleotide sequences were trimmed manually using ClustalW implemented in MEGA 6 with default settings. BLAST searches were performed in the NCBI nucleotide database using the trimmed sequences. An OTU was defined by 5% dissimilarity threshold using the Mothur package (Schloss *et al.* 2009). This sequence dissimilarity cutoff represented at least one amino acid substitution in the functional gene-deduced peptide sequences. For each gene studied, randomly chosen clones were sequenced and the resulting data was combined to make a rarefaction curve, following the previous categorization strategy (Mohamed *et al.* 2010; Radax *et al.* 2012b). To construct the phylogenetic trees, one representative sequence from each OTU and the closest sequence to that, retrieved from the NCBI nucleotide database, were aligned using ClustalW implemented in the MEGA X (Kumar *et al.* 2018). The evolution models of the sequence collections were evaluated using Akaike information criteria tests implemented by jModelTest 2 (Darriba *et al.* 2012). The Maximum-likelihood trees were constructed in MEGA X with 1000 bootstrap replicates, respectively, based on the best-fit evolution model with a gamma shape parameter derived from jModelTest (Darriba *et al.* 2012). Bootstrap analysis was used to estimate the reliability of the phylogenetic reconstructions (with 1000 replicates).

RESULTS

Phylogenetic diversity of bacteria with transcriptional activity in sponge holobionts

Approximately 40 000 raw pyrosequencing reads of the bacterial 16S rRNA amplicons were obtained from the sponges *T. swinhoei*, *P. simplex*, *Ph. fusca* and seawater samples. After removing the noise and poor-quality reads, 26 141 reads with an average read length of 200 bp were used for subsequent analyses (Fig. S2, Supporting Information). At a confidence threshold of 60%, 25 959 qualified reads could be assigned to known phyla using the RDP classifier. Totally, the OTUs were taxonomically assigned to 16 phyla in *P. simplex*, 14 in *T. swinhoei*, 7 in *Ph. fusca* and 7 in the seawater. Only 6 phyla, i.e. Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria and Proteobacteria, were shared between the sponges and seawater. Some phyla, including Deinococcus-Thermus, Firmicutes, Gemmatimonadetes, Poribacteria, Spirochaetes, Tectomicrobia, Verrucomicrobia, AncK6, PAUC34f, SBR1093 and TM7, were detected only in the sponges, while Planctomycetes was detected only in the seawater. Fig. 1A shows the *in situ* active bacterial phyla with high relative abundance (> 1% relative abundance of the reads in the corresponding dataset). The major active phyla (> 5% relative abundance of the reads in the corresponding dataset) in *T. swinhoei* were Proteobacteria (26.1%), Cyanobacteria (22.2%),

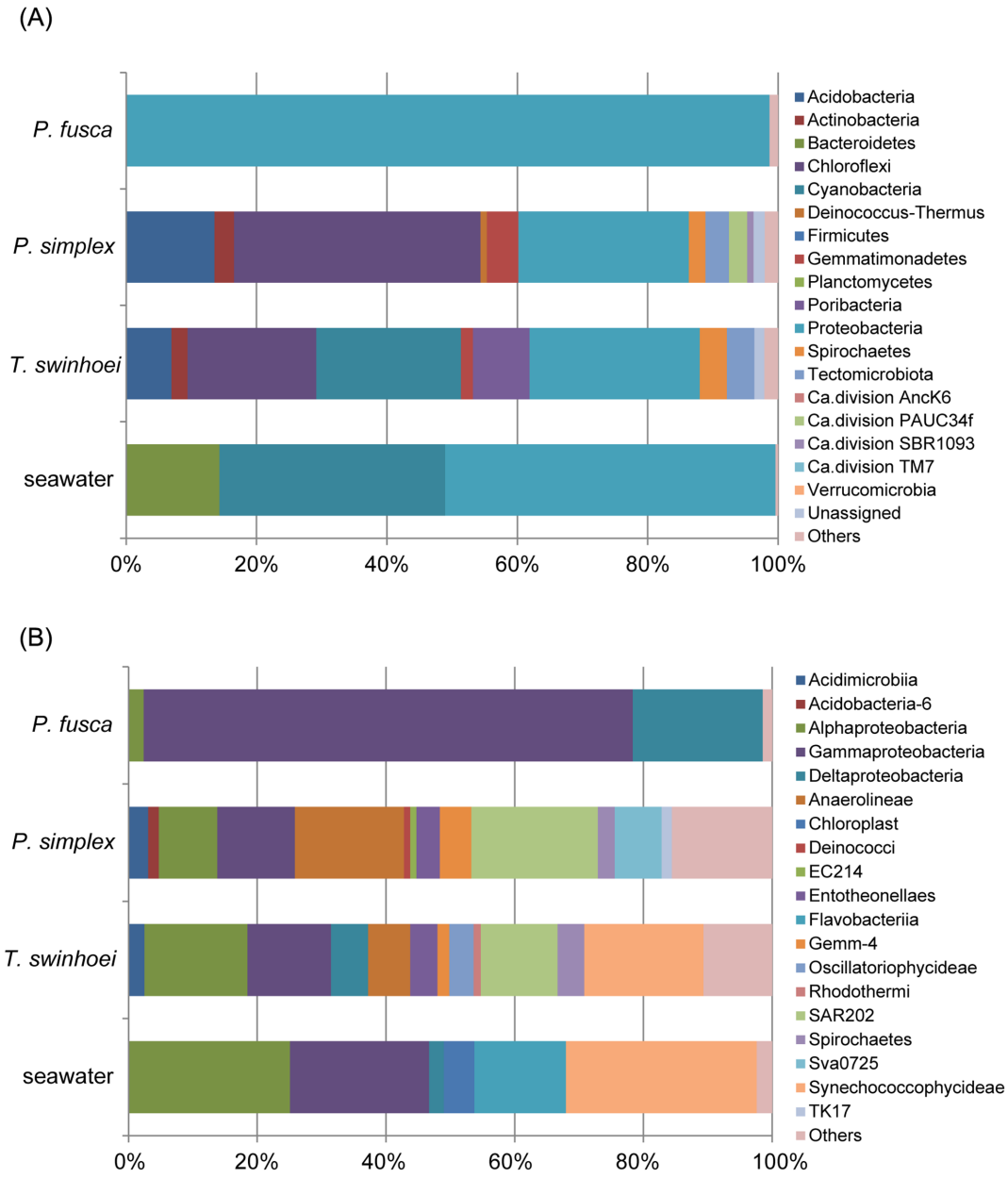

Figure 1. The *in situ* active bacterial communities in sponges *T. swinhoei*, *P. simplex*, *Ph. fusca* and the environmental seawater at phylum **(A)** and class **(B)** levels.

Chloroflexi (19.7%), Poribacteria (8.7%) and Acidobacteria (6.9%). *Plakortis simplex* hosts abundant *in situ* active Chloroflexi (37.8%), Proteobacteria (26.1%) and Acidobacteria (13.5%); the most abundant phylum in *Ph. fusca* was Proteobacteria (98.7%) (Fig. 1A). More than 99% of the reads from the seawater samples were affiliated with three phyla: Proteobacteria (50.6%), Cyanobacteria (34.6%) and Bacteroidetes (14.3%) (Fig. 1A). Classes of high relative abundance (> 1% relative abundance of the reads in the corresponding dataset) are shown in Fig. 1B. Alphaproteobacteria and Gammaproteobacteria were preferentially enriched in seawater (25.1%, 21.6%), *T. swinhoei* (16.0%, 12.9%) and *P. simplex* (9.1%, 12.1%). Synechococcophycideae was particularly dominant in seawater (29.6%) and *T. swinhoei* (18.5%); SAR202 was predominant in *T. swinhoei* (11.9%) and *P. simplex* (19.7%). More than 96% of the reads from *Ph. fusca* were affiliated with Gammaproteobacteria (75.9%) and Deltaproteobacteria (20.2%). Flavobacteriia were prevalent in seawater (14.2%) and Anaerolineae in *P. simplex* (16.9%) (Fig. 1B).

A total of 1156 different 16S rRNA OTUs were detected, including 446 OTUs from seawater, 425 OTUs from *T. swinhoei*, 310 OTUs from *P. simplex* and 150 OTUs from *Ph. fusca* (Fig. S2, Supporting Information). There were 605 OTUs that were present in only one species of sponge and 401 OTUs were found only in the environmental seawater. Meanwhile, 122 OTUs were shared between at least two sponge species and 45 OTUs were shared between the sponges and seawater (Fig. S2, Supporting Information). Among all these OTUs, 34 dominant OTUs (> 2% relative abundance of the reads in corresponding dataset) were identified (Fig. 2A). Thiohalorhabdales_OTU1 (66.6%) and NB1-i_OTU2 (19.9%) were dominant in *Ph. fusca*;

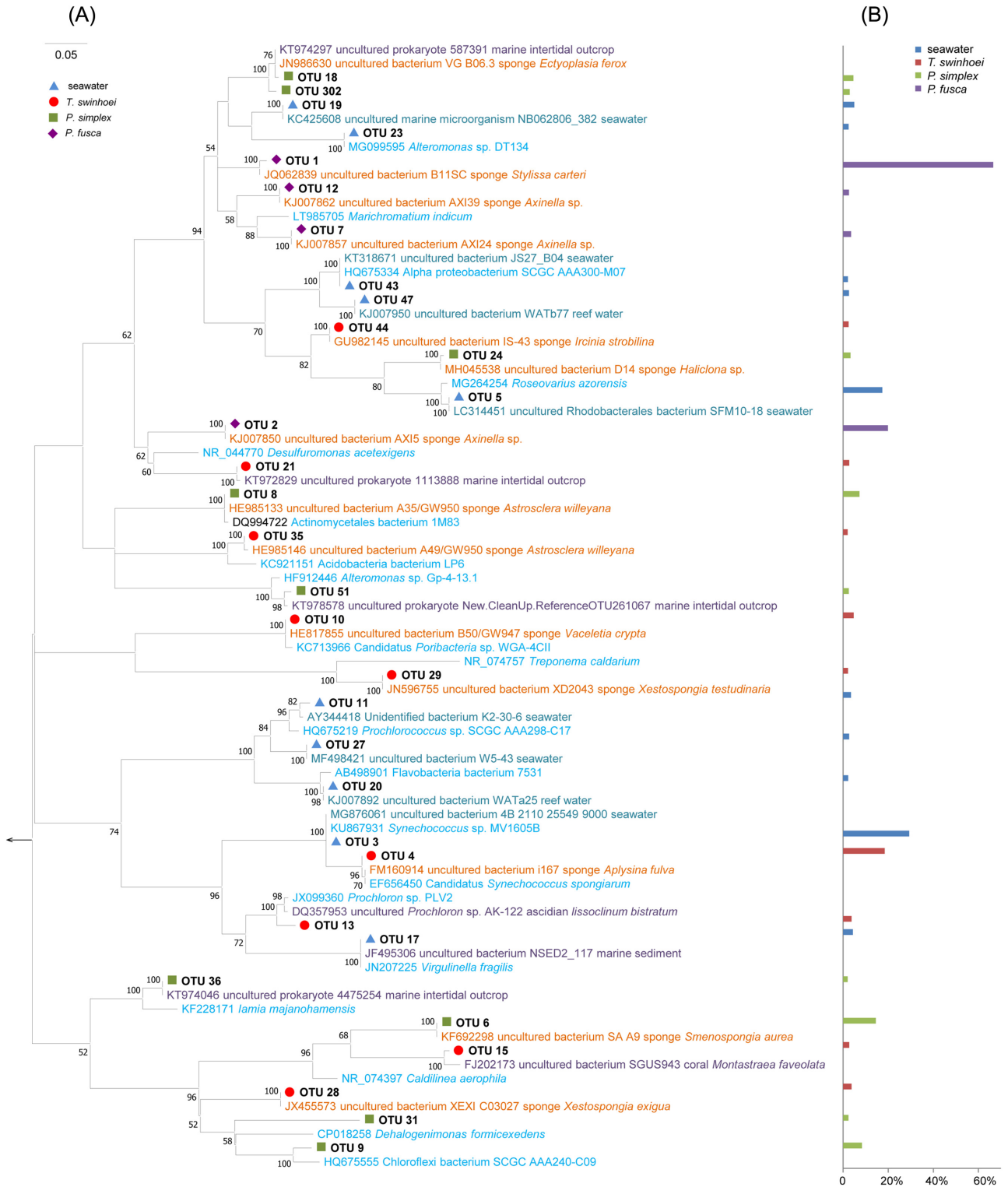

Figure 2. (A) Phylogenetic tree of the top 34 dominant 16S rRNA OTUs from the cDNA libraries of sponges *T. swinhoei*, *P. simplex*, *Ph. fusca* and environmental seawater based on 250 nucleotide sites. A representative of each OTU is highlighted. The scale bar represents 5% sequence divergence per homologous position. Bootstrap values more than 50% of 1000 replicates are shown. The arrow represents the outgroup sequence, i.e. the archaeal 16S rRNA sequence KC357907 of Candidatus *Nitrosoarchaeum limnia* BG20. **(B)** The relative abundance of each OTU in the corresponding clone library.

Caldilineaceae_OTU6 (14.5%) and SAR202_OTU9 (8.3%) were predominant in *P. simplex*; Synechococcus_OTU4 (18.5%) and Poribacteria_OTU10 (4.7%) were enriched in *T. swinhoei*; and Synechococcus_OTU3 (29.4%) and Rhodobacteraceae_OTU5 (17.4%) were dominant in seawater (Fig. 2B). These results, together with those at phylum and class levels, suggest considerable sponge- and sponge species specific diversity of bacteria with transcriptional activity.

Phylogenetic analysis of transcriptionally active CO_2 assimilation and CO oxidation genes in sponge holobionts

The sequences corresponding to *rbcL*, *rbcM*, *cooS*, *aclB*, *mcrA*, *accC* and *coxL* genes were amplified using the primers listed in Table 1. As a result, fragments matching the *rbcL*, *rbcM*, *accC* and *coxL* genes were successfully detected in the cDNA of the sponge and seawater samples, while those of *aclB*, *cooS* and *mcrA* were not detected. Rarefaction analysis at 5% nucleotide cutoff shows that for most of the genes sequencing reached an asymptote, except for *rbcM* in *P. simplex*, *coxL* in *T. swinhoei* and *accC* in the seawater (Table S1, Fig. S3, Supporting Information). Venn analysis of the transcribed *rbcL*, *rbcM*, *accC* and *coxL* genes revealed that few *rbcL*, *rbcM*, *accC* or *coxL* OTUs were shared between different sponges, or between a sponge and the environmental seawater sample (Fig. S4, Supporting Information).

A total of 22 *rbcL* OTUs were detected in the cDNA of sponge and seawater samples, including 15 bacterial OTUs and seven algal OTUs. A total of 11 bacterial OTUs were ascribed to the photoautotrophic Oscillatoriales-, Chroococcales- and Prochlorococcaceae-like clusters. The OTUs belonging to the last two clusters were closely related to the cyanobacterial species, i.e. *Synechococcus* spp. (WP_025781876, AM701776, CP000097, CP000110), *Cyanobium gracile* PCC 6307 (CP003495) and *Prochlorococcus marinus* (CP000576), respectively (Fig. 3A). In addition, the remaining four bacterial OTUs fell into the chemoautotrophic Beta- and Gammaproteobacteria-like clusters (Fig. 3A). Meanwhile, 48 *rbcM* OTUs, including six bacterial OTUs and 42 algal OTUs, were uncovered from the investigated sponges and seawater samples. Phylogenetic analysis showed that the six bacterial OTUs grouped into the chemoautotrophic Proteobacteria-like cluster, while the 42 algal OTUs were affiliated with the photoautotrophic Dinophyceae-like cluster (Fig. 3C). The closest relatives found for these bacterial OTUs were *Sediminimonas qiaohouensis* (WP_026756535), *Phaeospirillum fulvum* (HQ877086), *Leptothrix cholodnii* SP6 (CP001013), *Thioalkalicoccus limnaeus* (HQ877072) and *Thiothrix nivea* (WP_002706799). Therefore, these results suggest that a fraction of phylogenetically diverse Proteobacteria- and Cyanobacteria-like species may assimilate CO_2 via the photo- or chemo-autotrophic strategies involving the rPP pathway.

Amplification of the fragments corresponding to the *accC* gene resulted in sequences representing 27 bacterial OTUs. These OTUs were interspersed among a broad range of bacterial clusters, including Actinobacteria-, Bacteroidetes-, Chlamydiae-, Chloroflexi-, Cyanobacteria-, Firmicutes-, Alpha-, Beta- and Gammaproteobacteria-like clusters (Fig. 4A). Phylogenetic analysis showed three OTUs matching best with the photoautotrophic *Prochlorococcus marinus* str. AS9601 (CP000551), *Crinalium epipsammum* PCC 9333 (CP003620) and chemoautotrophic *Nitrosomonas* sp. AL212 (CP002552), respectively. The remaining 24 OTUs were closely related to a broad range of heterotrophic bacteria among the Actinobacteria-, Bacteroidetes-, Chlamydiae-, Chloroflexi-, Firmicutes-, Alpha- and Gammaproteobacteria-like clusters. Particularly, only accCphf5 (KP056327) was shared among the three sponges. Thus, a fraction of phylogenetically diverse heterotrophic bacteria might contribute to CO_2 assimilation via carboxylation reactions in sponge holobionts.

Fragments corresponding to the CO oxidation related gene *coxL* were successfully amplified from the cDNA of sponge samples and seawater. The detected *coxL* OTUs grouped into the *Oligotropha-Mycobacterium-Pseudomonas* (OMP) clade and the *Burkholderia-Mesorhizobium-Sinorhizobium* (BMS) clade, according to the reported *coxL* phylotype classification (King 2003a) (Fig. 5A). In the BMS clade, 51 OTUs fitted into the Proteobacteria-like cluster; OTUs in this lineage were closely related to preoteobacterial species. The remaining seven OTUs in the BMS clade that fell into the Bacteroidetes-, Gemmatimonadetes-, Firmicutes- and Chloroflexi-like clusters, and matched best with *Phaeodactylibacter xiamenensis* (KGE88473), *Phaeodactylibacter xiamenensis* (KGE88473), *Alicyclobacillus herbarius* (WP_026962862) and *Thermomicrobium roseum* (WP_015922159). In the OMP clade, 16 OTUs fell into the Proteobacteria-like cluster. These OTUs were closely related to preoteobacterial species. Two OTUs were affiliated with Actinobacteria-like cluster and matched best with *Nocardioides* sp. JS614 (CP000509) and *Pseudonocardia dioxanivorans* CB1190 (CP002593). Thus, the phylogeny of *coxL* transcripts suggests that a broad range of phylogenetically diverse bacteria may actively oxidize CO in the investigated sponges.

The proportions of different OTUs, identified by the transcribed *rbcL*, *rbcM*, *accC*, and *coxL* genes, in corresponding clone libraries are shown in Fig. 3B, 3D, 4B, and 5B, respectively. In the case of *rbcL*, the amounts of different sequence variants described are in a comparable range in both sponge samples and seawater. The diversity of *rbcM* OTUs is greater in sponges than that in seawater; however, it is the opposite case for *accC*. For *coxL*, *Ph. fusca* and seawater have less OTUs compared to the other sponges analyzed. These results indicate that the various strategies for CO_2 assimilation and CO oxidation are possibly used in different proportions within the communities in different sponges and seawater.

DISCUSSION

Compared with rDNA-based strategy, which only indicates the metabolism potentials of microbes involved in ecological processes, RNA-based approach provides further insights into the activity of sponge microbiota. In this study, the detection of functional gene transcripts indicates the presence of *in situ* active bacteria with CO_2 assimilation and CO oxidation functions in the investigated sponges and the seawater surrounding them. A fraction of phylogenetically diverse Proteobacteria- and Cyanobacteria-like species may assimilate CO_2 via the photo- or chemoautotrophic strategies through the rPP pathway, and phylogenetically diverse heterotrophic bacteria might contribute to CO_2 assimilation via carboxylation reactions. Although the microbiota in different sympatric sponges varied extensively, the amounts of OTUs with potential abilities of CO_2 assimilation and CO oxidation remained within a comparable range.

The detection of transcribed bacterial *rbcL*/*rbcM* and *accC* genes indicated that bacteria in sponge holobionts could assimilate CO_2 via the autotrophic rPP cycle, and via the anaplerotic CO_2 assimilation pathway, respectively. Active Thaumarchaeota was once suggested to be present in the same investigated

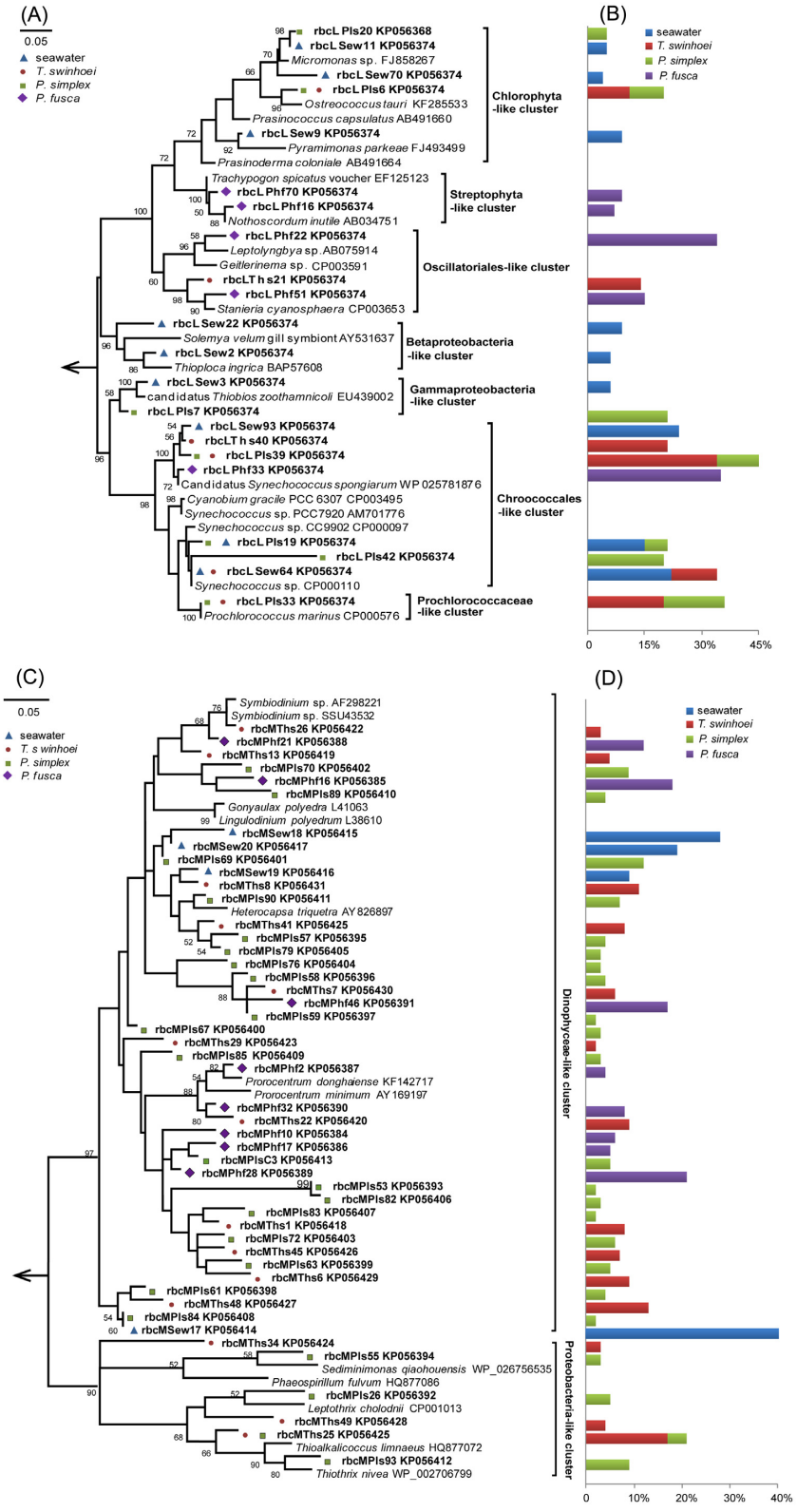

Figure 3. (A) Phylogenetic tree of the transcribed *rbcL* (A) and *rbcM* **(C)** genes from sponges *T. swinhoei*, *P. simplex*, *Ph. fusca* and environmental seawater derived cDNA libraries based on 812 and 400 nucleotide sites, respectively. A representative of each OTU is highlighted. The scale bar represents 5% sequence divergence per homologous position. Bootstrap values more than 50% of 1000 replicates are shown. **(B)** The relative abundance of each *rbcL* (B) and *rbcM* (D) OTU in the corresponding clone library. The arrows in A and C represent the outgroup sequences, i.e. the archaeal *rbcL* sequence L21_1928 of *Methanoculleus chikugoensis* and the archaeal *rbcM* sequence MCON_3086 of *Methanosaeta concilii*, respectively.

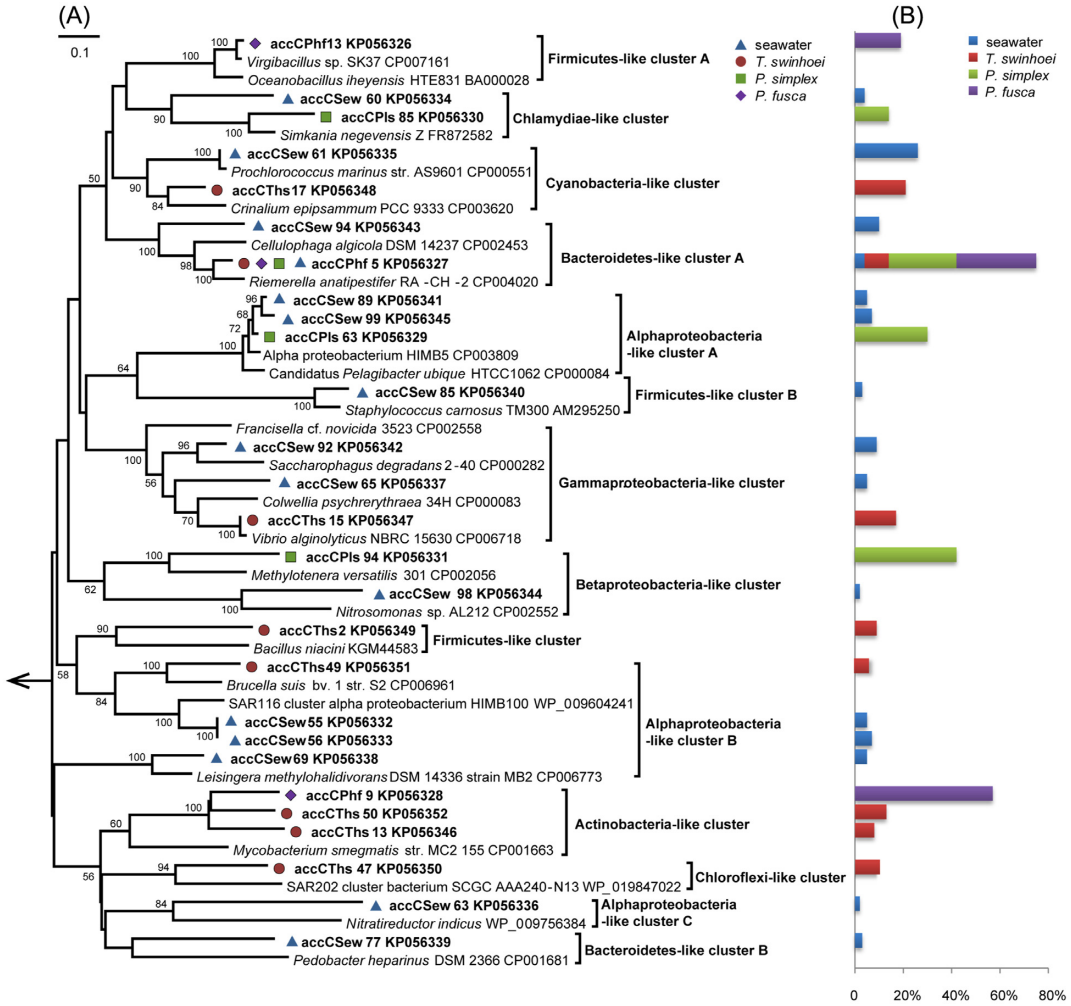

Figure 4. (A) Phylogenetic tree of the transcribed *accC* gene from sponges *T. swinhoei*, *P. simplex*, *Ph. fusca* and environmental seawater derived cDNA libraries based on 509 nucleotide sites. A representative of each OTU is highlighted. The scale bar represents 10% sequence divergence per homologous position. Bootstrap values more than 50% of 1000 replicates are shown. **(B)** The relative abundance of each *accC* OTU in corresponding clone library. The arrow represents the outgroup, the archaeal *coxL* sequence Saci_2117 of *Sulfolobus acidocaldarius*.

sponges and seawater (Feng *et al.* 2018), but the thaumarchaeotal *accC* genes were not detected here, indicating that the bacteria may play an important role in CO_2 assimilation rather than archaea.

Although the rPP cyle is energetically more costly than other strategies for CO_2 assimilation, and may therefore be unfavorable in an energy-limited environment (Purkamo *et al.* 2015), it is the only light-driven autotrophic CO_2 assimilation pathway on the Earth, and is of great importance in the ocean (Scanlan *et al.* 2009; Bowler, Vardi and Allen 2010). The transcriptional activity and diversity of the detected *rbcL* and *rbcM* genes suggest that bacterial assimilation of CO_2 via the rPP pathway plays a significant role in carbon sequestration in a variety of microbes in sponges and seawater. Phylotypes of transcribed *rbcL* and *rbcM* genes were not randomly distributed among the investigated sponge and seawater samples collected from the same habitat, but rather formed distinct clusters, indicating that sponge species can affect the composition of autotrophic CO_2-assimilating communities. Diverse phylotypes of the transcribed *rbcL* and *rbcM* genes show that a fraction of divergent cyanobacteria and microalgae lineages may assimilate CO_2 via the photoautotrophic rPP cycle in the investigated sponges. Cyanobacteria and microalgae are proven to be the main primary producers in the ocean (Scanlan *et al.* 2009; Bowler, Vardi and Allen 2010); they have been detected from a variety of sponges by rRNA-based assays (Granados *et al.* 2008; Weisz *et al.* 2010; He *et al.* 2014; Moitinho-Silva *et al.* 2014; Burgsdorf *et al.* 2015) and metagenomic analysis (Gao *et al.* 2014). Thus, in addition to microalgae, e.g. Chlorophyta-, Streptophyta- and Dinophyceae-like lineages, a portion of the symbiotic bacteria might actively assimilate CO_2 via the photoautotrophic rPP pathway in sponge holobionts.

Additionally, a fraction of the transcribed *rbcL* and *rbcM* fragments falling into the Proteobacteria-like clusters were affiliated with sulfur-oxidizing chemoautotrophs *Thioploca ingrica*, *Candidatus Thiobios zoothamnicoli*, *Thioalkalicoccus limnaeus* and *Thiothrix nivea* (Bryantseva *et al.* 2000; Lapidus *et al.* 2011), and the sulfur-oxidizing symbiotic chemoautotroph within the clam *Solemya velum* (Schwedock, Harmer and Scott 2004). These findings imply that particular bacterial clades might couple the chemoautotrophic rPP pathway of CO_2 assimilation with sulfur metabolism in sponges, which supports the previous discoveries of the chemoautotrophic sulfur-oxidizing bacteria within

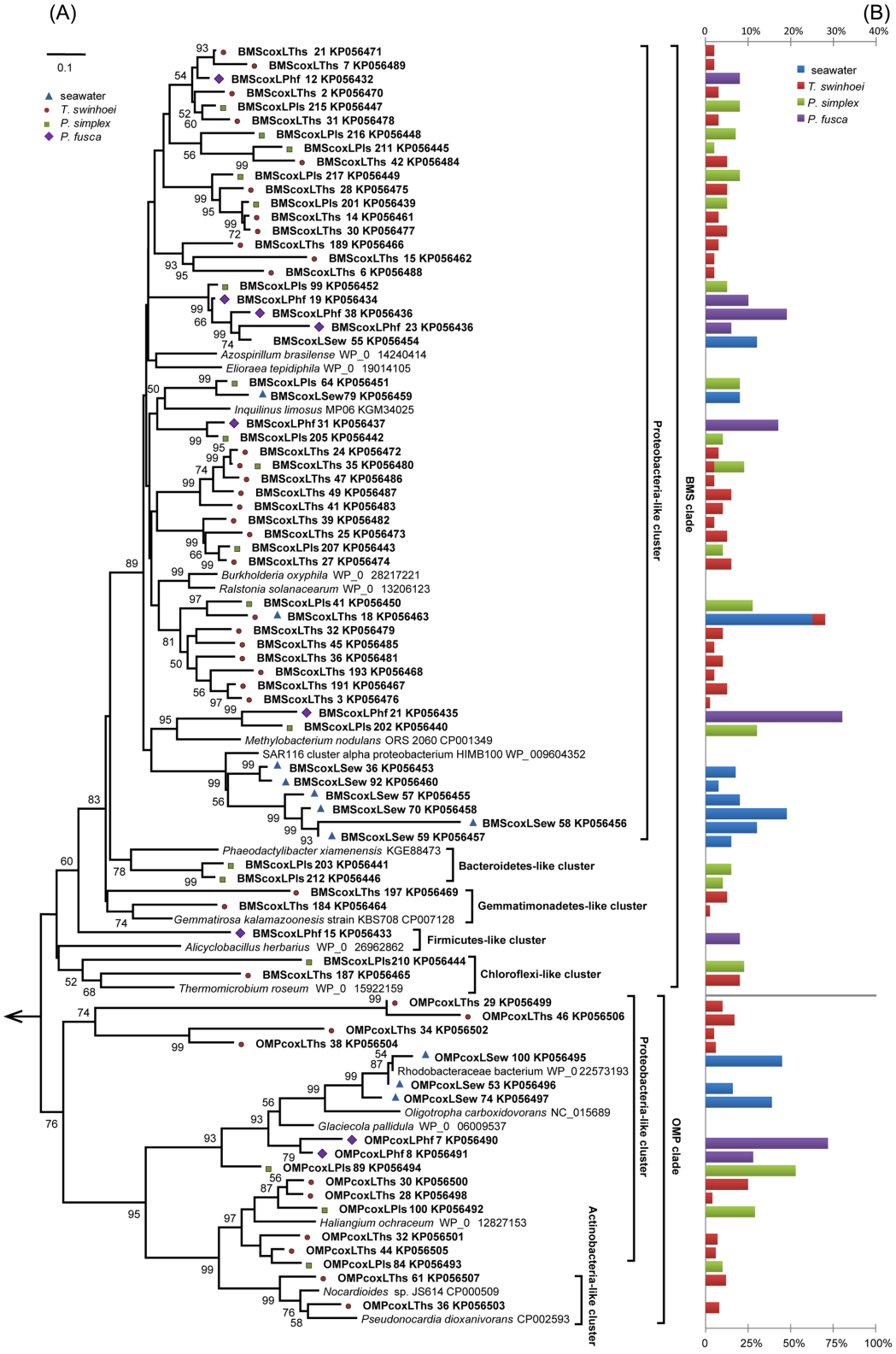

Figure 5. (A) Phylogenetic tree of the transcribed *coxL* gene from sponges *T. swinhoei*, *P. simplex*, *Ph. fusca* and environmental seawater derived cDNA libraries based on 1272 nucleotide sites. A representative of each OTU is highlighted. The scale bar represents 10% sequence divergence per homologous position. Bootstrap values more than 50% of 1000 replicates are shown. **(B)** The relative abundance of each *coxL* OTU in the corresponding clone library. The arrow represents the outgroup, the archaeal *accC* sequence Mbur_2426 of *Methanococcoides burtonii*.

sponges (Meyer and Kuever 2008; Nishijima *et al.* 2010; Tian *et al.* 2016).

Meanwhile, we detected the transcribed *accC* phylotypes affiliated with the homologues from a variety of bacterial lineages, most of which consist of heterotrophs. The heterotrophic demand for CO_2 could arise from multiple cellular processes, e.g. anaplerotic CO_2 assimilation reactions to replenish the TCA cycle intermediates, synthesis of amino acids and precursors of nucleic acids and biosynthesis of fatty acids (Lombard and Moreira 2011). This indicates the carbon incorporation metabolism via anaplerotic CO_2 assimilation pathways in heterotrophic bacteria, which is consistent with the previous surveys performed on the Arctic microplanktons (Alonso-Saez *et al.* 2010), but differs from the surveys done on some lakes, in which both the *accC* genes involved in the heterotrophic anaplerotic pathway and in the autotrophic 3-HP/4-HB pathway were uncovered (Auguet *et al.* 2008; Lliros *et al.* 2011), indicating that prokaryotic community performing carboxylation reaction could be affected by their habitats.

CO is the simplest oxocarbon generated during decomposition of organic compounds. In aquatic environments, significant CO production occurs in the photochemical degradation of dissolved organic matter (Valentine and Zepp 1993). Bacteria are considered as the dominant CO scavengers (Tolli and Taylor 2005). Although *cooS* genes were not detected in this study, the CO consumption potential in sponges by aerobic CO-oxidizing bacteria was suggested by detecting *coxL* transcripts. Furthermore, our molecular survey of *coxL* gene transcripts revealed a phylogenetically diverse group of CO oxidizers, including Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Gemmatimonadetes and Proteobacteria. This revelation was consistent with the discovery of abundant bacterial *coxL* genes from the metagenomes of the sponges *Cymbastela concentrica* and *Neamphius huxleyi* (Li *et al.* 2014; Thomas *et al.* 2010). Thus, a wide range of phylogenetically diverse bacteria in sponge holobionts display the CO-oxidation capacity. Additionally, the communities of the revealed aerobic CO-oxidizers differ among the investigated sponge species and seawater. This difference most likely reflects the various responses of diverse bacterial communities to their sponge hosts, as particular bacteria may have different affinities toward CO (King and Hungria 2002).

Though high-throughput sequencing analysis has been successfully performed to reveal the diversity of certain functional genes, it is not easy to design highly efficient primers for high-throughput sequencing of functional genes (Dowle *et al.* 2015), e.g. for 812-bp *rbcL* gene and 1272-bp *coxL* gene in this study. The metagenomic approach is not very efficient in detailed analysis of functional genes, since it is not target gene directed, allowing such analysis to only some extent. Therefore, traditional PCR-based target gene analysis still has some advantages in conducting environmental studies of microbial functions (Feng *et al.* 2018; Lynn *et al.* 2016). Despite the unavoidable bias inherent in a PCR-based approach, which may hinder unveiling the real diversity of the genes of interest, some of the marker genes used in this study have remained useful for studying microbial CO_2 assimilation and CO oxidation due to their relatively high degree of conservation, widespread distribution, functional significance and an increasing number of sequences published in GenBank.

The autotrophic CO_2 incorporation through the rTCA cycle and the 3-HP pathway were not verified to be present in these sponges, because no *aclB* gene or *mcrA* gene transcripts were detected. Neither could we verify the rACA pathway since no *cooS* genes were detected. One possible reason may be that these genes are not present (at detectable levels) in the analyzed samples, or the sponge microbiota hosts different types of *aclB*, *mcrA* or *cooS* genes that could not be detected with the primers or the PCR procedure used in this study. The primers could be optimized by blasting the primer sequences against sponge metagenomes in the SRA database and editing them accordingly if there are any hits (Horn *et al.* 2016). To compare the difference in gene expression among the transcribed *rbcL*, *rbcM*, *accC* and *coxL* genes, a qPCR assay for each functional gene across samples will be carried out in the future (similarly to Fiore *et al.* 2015). Since a considerable part of the RuBisCO-encoding genes originated from eukaryotic species, phylogenetic diversity of eukaryotes in sponges will be investigated in the future as well. Given that the functional genes may be transferred horizontally between species (especially in bacteria), the whole concept of gene-specific phylogenetic diversity is problematic to some extent. Metatranscriptomics is probably a better approach to reveal the metabolic potential and link it to the phylogenetic diversity (Moitinho-Silva *et al.* 2017). The physiological and biochemical measurements of the bacterial CO_2 assimilation and CO oxidation rates are also necessary to gain further insight in the functions of these communities and will be carried out in the future. Moreover, the presence and diversity of transcribed CO_2 assimilation and CO oxidation genes verified in this study warrant further investigation into their functional and ecological importance in sponges. Altogether, this investigation contributes to better understanding of the carbon metabolism of sponge-symbiotic microbiota.

CONCLUSIONS

Phylogenetically diverse bacteria with transcriptional activity were detected in the sponge holobionts, which showed significant variations among three sympatric sponges *T. swinhoei*, *P. simplex*, *Ph. fusca* and the seawater from their environment. The analysis of transcriptionally active functional genes revealed that the autotrophic CO_2 assimilation through the rPP pathway, heterotrophic anaplerotic CO_2 assimilation and aerobic CO oxidation may be performed by phylogenetically diverse bacteria in sponge holobionts. Though the microbiotas varied extensively in the composition of bacterial species in different sponge holobionts, they could still be functionally equivalent in their bacterial CO_2 assimilation and CO oxidation capabilities.

ACCESSION NUMBERS

Representatives of all the OTUs obtained in this study were deposited in GenBank under the accession numbers: KP056362-KP056383 for the *rbcL* genes, KP056384-KP056431 for the *rbcM* genes, KP056432-KP056507 for the *coxL* genes and KP056326-KP056352 for the *accC* genes. The datasets produced by pyrosequencing were submitted to NCBI SRA database under the accession number SRR5313724.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest. None declared.

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