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Reduced chemosymbiont genome in the methane seep thyasirid and the cooperated metabolisms in the holobiont under anaerobic sediment

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Abstract

Previous studies have deciphered the genomic basis of host-symbiont metabolic complementarity in vestimentiferans, bathymodioline mussels, vesicomyid clams and *Alviniconcha* snails, yet little is known about the chemosynthetic symbiosis in Thyasiridae—a family of Bivalvia regarded as an excellent model in chemosymbiosis research due to their wide distribution in both deep-sea and shallow-water habitats. We report the first circular thyasirid symbiont genome, named *Candidatus* Ruthturnera sp. Tsphm01, with a size of 1.53 Mb, 1521 coding genes and 100% completeness. Compared to its free-living relatives, *Ca.* Ruthturnera sp. Tsphm01 genome is reduced, lacking components for chemotaxis, citric acid cycle and de novo biosynthesis of small molecules (e.g. amino acids and cofactors), indicating it is likely an obligate intracellular symbiont. Nevertheless, the symbiont retains complete genomic components of sulphur oxidation and assimilation of inorganic carbon, and these systems were highly and actively expressed. Moreover, the symbiont appears well-adapted to anoxic environment, including capable of anaerobic respiration (i.e. reductions of DMSO and nitrate) and possession of a low oxygen-adapted type of cytochrome *c* oxidase. Analysis of the host transcriptome revealed its metabolic complementarity to the incomplete metabolic pathways of the symbiont and the acquisition of nutrients from the symbiont via phagocytosis and exosome. By providing the first complete genome of reduced size in a thyasirid symbiont, this study enhances our understanding of the diversity of symbiosis that has enabled bivalves to thrive in chemosynthetic habitats. The resources will be widely used in phylogenetic, geographic and evolutionary studies of chemosynthetic bacteria and bivalves.

KEYWORDS
anaerobic respiration, complete genome, interactions, metatranscriptomics, sulphide-oxidizing bacteria

Yunlong Li and Xing He equally contributed to this work.

1 | INTRODUCTION

Allying with chemoautotrophic bacteria, chemosymbiosis is considered a key strategy in the adaptation to harsh marine environments, such as hydrothermal vents and hydrocarbon seeps (Sogin et al., 2021). Fuelled by the nutrient produced by the symbionts, many invertebrates flourish in deep-sea chemosynthetic habitats. They include many molluscs, including bivalves and gastropods, which harbour either thiotrophic and/or methanotrophic bacteria either inside (endosymbiont) or outside (episymbiont) of the gills or other internal organs (Dubilier et al., 2008).

Members of the class Bivalvia have acquired chemosynthetic symbiosis multiple times, with eight families (i.e. Basterotiidae, Nucinellidae, Lucinidae, Mytilidae, Solemyidae, Teredinidae, Thyasiridae and Vesicomyidae) having been reported to host methanotrophic and/or thioautotrophic bacteria (Ip et al., 2021; Oliver & Taylor, 2012; Sun et al., 2017). Among them, mytilid mussels, lucinid clams and vesicomyid clams have been extensively studied concerning symbiont chemoautotrophic capacity (Petersen et al., 2016), molecular cross-talk between symbiont and host (Ip et al., 2021; Sun et al., 2017), mode of symbiont transition (Ikuta et al., 2016) and symbiont strain diversity (Ansorge et al., 2019) but to appreciate the diversity of symbiosis in Bivalvia and to understand their evolution, it is urgent to study the symbiosis in the other families of symbiotic bivalves.

Thyasirid bivalves have also been suggested to be an excellent model for studying the evolution of symbiosis (Dufour, 2005). They are distributed in typical deep-sea chemosynthetic habitats such as hydrothermal vents, hydrocarbon seeps and organic falls, and organic-rich sediments from shallow-water mangroves and sea grass beds to hadal trenches (Dubilier et al., 2008; Katsunori et al., 1999). Thyasirids exhibit diverse modes of association with bacteria, from not hosting bacterial symbiont to having episymbiotic bacteria among the gill microvilli or in a space delimited by the microvilli and cytoplasm, or hosting endosymbiotic bacteria (Dufour, 2005). Most thyasirids host one bacterial symbiont but some host two symbionts (Yoshihiro et al., 2001). The diversity of symbiosis modes in Thyasiridae provides a unique opportunity to test the evolutionary novelties and adaptation to particular ecological niches (Taylor & Glover, 2021).

There have been very few genome sequencing efforts in thyasirids and their symbionts. Among the 130 recognized species of Thyasiridae (WoRMS, 2023), only two species (i.e. *Thyasira* cf. *gouldii* and *Conchocele bisecta*) with the metabolic potentials of the host/symbiont have been studied using the metagenomic approach (Guo et al., 2023; McCuaig et al., 2020). There are only 15 bacterial 16S rRNA gene sequences from the potential thyasirid symbionts on GenBank (last access Jan 2023) and one metagenome-assembled genome of *C. bisecta* symbiont (Guo et al., 2023). Here, using an integrative long-read sequencing and metatranscriptomics approach, we present the complete symbiont genome of a thyasirid, characterized its chemoautotrophic capacity, and we discuss the molecular interactions between the host and symbiont.

2 | MATERIALS AND METHODS

2.1 | Sample collection

A *Thyasira* sp. specimen was collected from the a site on the Haima seeps (110°28.4693′E, 16°54.0143′N, depth 1433 m) using a dip-net by ROV Pioneer during R/V *Xiangyanghong01*'s cruise XYH01-2022-06 in September 2022. The specimen was found in the surface sediments collected from a "clam bed" with empty shells of *Archivesica marissinica* (Chen et al., 2018; Feng et al., 2018). The H_2S and O_2 concentrations were measured from the sediment samples collected by a MBARI push-core using Unisense H_2S and O_2 microsensors attached to a micromanipulator. Upon arrival on the desk, the sediment was sieved and the clam picked up, fixed in RNAlater (Invitrogen) and stored at −80°C in a deep freezer.

2.2 | Genome sequencing, assembly, binning and SNP calling

Genomic DNA was extracted using the SDS method (Pereira et al., 2011) and submitted to Novogene (Beijing) for sequencing using two strategies: Illumina sequencing with 150 bp paired-end mode and PacBio high-fidelity sequencing (HiFi). The short reads were trimmed using Trimmomatic version 0.39 (TruSeq3-PE-2.fa:2:30:10:8:true SLIDINGWINDOW:5:20 LEADING:3 TRAILING:3 MINLEN:75) and then assembled using MEGAHIT version 1.2.9 (--k-list 75,95,115,135) (Bolger et al., 2014; Li et al., 2015). The symbiont genome was binned using maxbin2 version 2.2.7 (Wu et al., 2015) under the default parameters and visualized in blobtools version 1.1.1 (Laetsch & Blaxter, 2017; Figure S1). The genomic completeness and contamination were assessed using CheckM version 1.2.1 Field (Parks et al., 2015) and CheckM2 version 0.1.3 (Chklovski et al., 2022). To obtain the complete and circular-level genome, HiFi sequencing was also applied using the PacBio Sequel II platform in a CCS mode. HiFi reads were extracted by extracthifi version 1.0.0, and then adapters were removed using HiFiAdapterFilt version 2.0 (Sim et al., 2022). The clean HiFi reads were assembled using hifiasm version 0.16.1-r375 (Cheng et al., 2021). The complete genome was manually selected using minimap2 version 2.24-r1122 (Li, 2018) and blobtools version 1.1.1 (Laetsch & Blaxter, 2017). The general information of the symbiont genome was visualized using Proksee (Grant et al., 2023). Single-nucleotide polymorphisms (SNPs) in the symbiont were detected using a pipeline published by Lan et al. (2022), using the following criteria: QD < 2.0 || MQ < 40.0 || SOR > 4.0 || FS > 60.0 || MQRankSum < −12.5 || ReadPosRankSum < −8.0.

2.3 | Phylogenetic analyses in the host and symbiont

Four phylogenetic trees were constructed to determine the relationship between *Thyasira* sp. collected from Haima seeps and other

thyasirids using the concatenated sequences of three genes and each single gene: COI, 18S rRNA gene, and 28S rRNA gene (Figure 1). Sequences from other thyasirids mainly reported in two studies (Fukasawa et al., 2017; Taylor et al., 2007) were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank; Table S1). *Myrtea spinifera* (Bivalvia, Lucinidae) was used as the outgroup. Sequence alignments were performed using Muscle version 5.0.1 under the default settings (Edgar, 2021). TrimAl version 1.4.1 (Capella-Gutiérrez et al., 2009) was used to trim the alignment under the "-automated1" option. Phylogenetic trees were constructed with IQ-TREE version 2.1.3 (Minh et al., 2020) using the maximum likelihood (ML) method under the "-B 1000 -alrt 1000 -m MFP" options (Figure 1). The best-fit models of COI, 18S and 28S were TIM+F+Γ4, TNe+R2 and TN+F+R2, respectively. In addition, three phylogenetic trees based on a single gene were also constructed (Figure S2).

The symbiont 16S rRNA gene sequence was compared in three public databases to obtain sequences with at least >90% identity, including SILVA SSU, NCBI rRNA gene and NCBI nt. Using the obtained sequences, an ML tree based on the 16S rRNA gene sequences was constructed in MEGA 11 (Tamura et al., 2021), implementing the HKY+Γ model and 100 bootstraps. The 16S rRNA gene (accession: LC682250.1) from *Escherichia coli* JCM 16946 was used as the outgroup.

Forty-seven published genomes of chemosynthetic sulphide-oxidizing bacteria were retrieved from GenBank (Table S3). The taxonomy of the symbiont at the genomic level was assigned by GTDB-Tk version 2.1.1 (Chaumeil et al., 2019) based on the tree topology and average nucleotide identity (ANI) comparison with published bacterial genomes. The bacterial proteins, predicted using Prokka version 1.14.6 in this study, were also used to construct a phylogenomic tree (Kocot et al., 2017; Liu et al., 2022). Briefly, the orthologue of proteins was predicted using OrthoFinder version 2.5.4 (Emms & Kelly, 2019) with DIAMOND blastp and then aligned using MAFFT version 7.508 (Katoh & Standley, 2013). Orthologues were discarded if less than 35 species or 20 amino acids were aligned. PhyloPyPruner version 1.2.6 (Thalen, 2018) and FastTree version 2.1.11 (Price et al., 2010) were employed to compute the putative orthologue sequences, resulting in a matrix containing 772 partitions. The ML tree was constructed in IQ-TREE version 2.1.3 (Minh et al., 2020) with the "-MFP" model and 1000 ultra-bootstraps. Endosymbiotic methane-oxidizing bacteria (MOB) from *Bathymodiolus azoricus* was used as the outgroup.

2.4 | Metatranscriptome sequencing and bioinformatic analysis

Total RNA was extracted using the TRIZol reagents (Thermo Fisher Scientific). A meta-transcriptomic library was prepared by removing prokaryotic and eukaryotic ribosome RNA. The leftover RNA

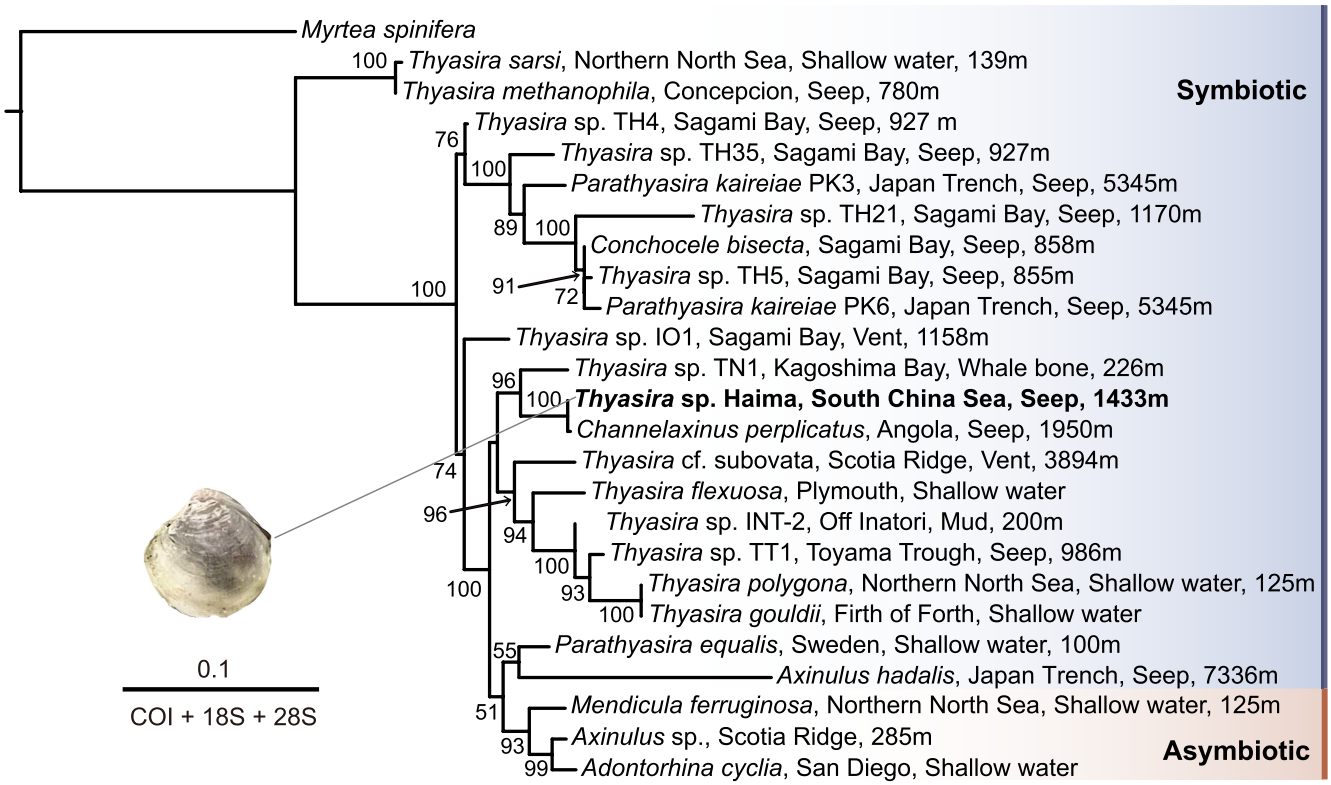

FIGURE 1 Phylogenetic relationships of Thyasiradae based on the concatenated sequences of three genes (COI sequences, 28S rRNA gene sequences and 18S rRNA gene sequences). *Myrtea spinifera* (Bivalvia, Lucinidae) was used as the outgroup species. Only the bootstrap values ≥50 were displayed. *Thyasira* sp. Haima was labeled in bold. Blue and yellow blocks indicate the species with chemosymbiont or without, respectively. The scale bar indicates 0.1 nucleotide substitutions per site.

(containing mRNA mainly) sequences were reverse-transcribed to cDNA and then sequenced on the Illumina NovaSeq platform with paired-end mode and a read length of 150 bp, with the output of approximately 10 Gb. The raw Illumina reads were trimmed using Trimmomatic version 0.39 (Bolger et al., 2014). All the short reads were mapped into the symbiont genome using minimap2 version 2.24-r1122 (Li, 2018) and bowtie2 version 2.4.5 (Langmead & Salzberg, 2012) to obtain the symbiont-derived reads and symbiont-free reads (mainly from the host and potentially some contaminated reads from other species), respectively.

The symbiont genome was analysed using Prokka version 1.14.6 (Seemann, 2014) to determine the coding genes, rRNAs, tRNAs, repeat regions and clustered regularly interspaced short palindromic repeats (CRISPR) elements. The coding genes were annotated using several public databases, that is KEGG ortholog by BlastKOALA (Kanehisa et al., 2016), gene ontology (GO) by Blast2GO version 5-basic against NCBI non-redundant protein database (nr), Cluster of Orthologous Groups of proteins (COG) by eggnog-mapper version 2.1.9 (Cantalapiedra et al., 2021) against eggnog DB version 5.0.2 (Huerta-Cepas et al., 2018). The symbiont-derived reads were mapped to the genome using STAR version 2.7.10a (Dobin et al., 2012) and then quantified using Stringtie version 2.1.1 (Pertea et al., 2015). Transcripts per million (TPM) was adopted to evaluate the gene expression, with genes having a TPM value of >300 defined as highly expressed genes (HEGs). ClusterProfiler version 4.2.0 (Wu et al., 2021) was employed to check the functional categories among the HEGs. *Escherichia coli* K-12 MG1655 (GCA_904425475.1) is a bacterium with the full capability to de novo biosynthesize all amino acids (Dong et al., 2009), which is employed to predict the metabolic potential of *Ca*. Ruthturnera sp. Tsphm01.

The symbiont-free reads were used for de novo assembly using Trinity version 2.13.2 (Grabherr et al., 2011). To reduce the redundancy of assembled genes, only the longest isoform of genes with coding potential, predicted using Transdecoder version 5.5.0 (Haas, 2015), was selected for downstream analyses. In addition, the contaminant sequences from bacteria, viruses, and artificial vectors were identified by searching against NCBI nt database by blastn version 2.13.0 (Camacho et al., 2009); meanwhile, the potential coding sequences were checked using DIAMOND blastx version 2.0.15.153 (Buchfink et al., 2021) against nr and MEGAN version 6.21.7 against megan-map-Feb2022 (Gautam et al., 2022). After removing non-animal transcripts, a decontaminated and non-redundant expressional profile in the gill of *Thyasira* sp. Haima was generated, whose completeness was assessed using BUSCO version 5.2.2 against metazoa_odb10 (Manni et al., 2021). Blastkoala was employed to receive the KEGG orthologs, with the restriction of genus level within the Mollusca. Among them, pathways belonging to ko09160 (human diseases) were manually removed from the dataset. Gene ontology was determined using Blast2GO version 5-basic (Conesa et al., 2005), based on the result of the DIAMOND blastp search against nr with the very-sensitive mode. The gene expression level was quantified using a pipeline in Trinity, that is align_and_estimate_abundance.pl incorporating bowtie2 mapping and RSEM version 1.3.3 (Li & Dewey, 2011). Gene expression in the host was evaluated the same way for the symbiont but the threshold of highly expressed genes (HEGs) was set as higher than 100, resulting in 1216 genes. Functional enrichment of HEGs was conducted using ClusterProfiler version 4.2.0.

3 | RESULTS AND DISCUSSION

3.1 | Host characterization based on morphological and molecular evidence

The shells had two prominent posterior folds, a well-developed and deep posterior sulcus, a distinctly demarcated escutcheon, a projecting auricle (about ½ the length of escutcheon), and a strong sinus of the posterior shell margin. The ctenidium was large and thick, consisting of two demibranchs with numerous tightly spaced filaments. These morphological and anatomical features are consistent with the diagnosis of the genus *Thyasira* (Kamenev, 2023; Oliver & Holmes, 2006; Oliver & Killeen, 2002), and thus the studied thyasirid was identified as *Thyasira* sp. Unfortunately, the fragile shells of the collected specimen were damaged, which prevented their identification at the species level.

The ML concatenated tree based on three genes (i.e. COI, 18S and 28S) showed that the specimen sampled was sister to *Channelaxinus perplicatus* (Figure 1). Specifically, the 18S and 28S rRNA gene sequences of the *Thyasira* sp. Haima showed 100% and 99.80% similarity to the corresponding sequence of *C. perplicatus*, respectively (Figure S2b,c; Taylor et al., 2007). However, these two genes are too conserved for species delimitation in Mollusca (Kocot et al., 2011). The type locality of *C. perplicatus* is the Atlantic Ocean, covering from Southern Iberian Peninsula to Angola (Oliver & Frey, 2014; Salas, 1996; Taylor et al., 2007). In contrast, the COI gene shows a relatively low sequence identity (87.1%) to another *Thyasira* sp. TN1 that was collected in Kagoshima Bay, off Japan (Figure S2a). The similarity of *Thyasira* sp. Haima and *Thyasira* sp. TN1 is 98.4% for the 18S gene, suggesting that it is more closely related to *C. perplicatus*. However, the COI gene sequence of *C. perplicatus* is missing (Table S1). Considering the identification based on morphology, the specimen was tentatively named as *Thyasira* sp. and thereafter referred to as *Thyasira* sp. Haima in this study. Further morphological and molecular studies within this clade shall be performed to better delimit them.

Lucinidae and Thyasiridae were found to be grouped together in a previous study that involved dense transcriptome sampling, although it is possible that they are paraphyletic (Lemer et al., 2019). Chemoautotrophic bacteria have been identified in all clams belonging to Lucinidae and some of those in Thyasiridae (Taylor & Glover, 2021). It is worth noting that *Thyasira* sp. Haima was consistently grouped with other thyasirids with symbionts in this study, which suggests that this species may harbour symbionts from a phylogenetic perspective. Additionally, the asymbiotic group of thyasirids forms a separate monophyletic clade in the tree (Figure 1). This indicates that the transition from a symbiotic to an asymbiotic

state occurred only once in Thyasiridae. However, more sampling is required to support this assumption.

3.2 | Overview of the symbiont genome and its taxonomy

We assembled a complete symbiont genome of *Thyasira* sp. Haima with a size of 1.52 Mb and a GC content of 36.9% (Table 1 and Figure 2). It encodes the whole conserved gene set of Gammaproteobacteria, as indicated by 100% completeness in CheckM and CheckM2 analyses (Table 1). Furthermore, no contamination was undetectable in CheckM2 analysis (Table 1). The symbiont genome comprises 1521 coding genes, 3 rRNA, 36 tRNA, and 1 tmRNA genes, and 1 repeat region (Figure 2). The coding genes were well-annotated, with 1492 of them having significant match (*e*-value <1*e*-5) in public databases (Figure S4a), which encodes key proteins in chemoautotrophic bacteria, such as those with the capability to assimilate inorganic carbon and perform other basic biological processes.

There was no good match (>90% similarity) of the symbiont 16S rRNA gene sequence in SILVA and NCBI rRNA databases. It had 10 hits with over 99% similarity when blasted against the NCBI nt database. In the 16S rRNA gene phylogenetic tree, the newly discovered symbiont in *Thyasira* sp. Haima is clustered with the symbiont of *Thyasira vulcolutre* and placed in a clade with symbionts of vesicomyid clams but far away from a clade containing the symbionts of most other thyasirids (Figure S3). The GTDB-Tk analysis and the phylogenomic tree further support this result. The symbiont of *Thyasira* sp. Haima is clustered with the one clade of pliocardiinae symbionts (genus *Ruthia* in GTDB-Tk and *Candidatus* Ruthturnera in NCBI taxonomy) (Figure 3). Referred to the rule of NCBI and the sampling site (Figures 2 and 3), this bacterium is thus named *Candidatus* Ruthturnera sp. Tsphm01 (accession: GCA_030142985.1). A shared characteristic of *Ca.* Ruthia is that they are thioautotrophic bacteria with reduced genomes ranging from 1.0 to 1.6 Mb (Table S3). Like other sulphide-oxidizing bacteria, *Ca.* Ruthturnera sp. Tsphm01 processes the conserved thioautotrophic pathways for oxidizing reduced sulphur compounds, assimilating inorganic carbon and denitrification.

Compared to the *Thyasira* cf. *gouldii* symbiont (more than 3 Mb by prediction in genus *Sedimenticola*), the other two thyasirid symbionts lack components of flagella, sugar transporters, hydrogen oxidation and the TCA cycle (discussed below). Though comparable genome size and similar functions, *C. bisecta* symbiont (1.33 Mb) and *Thyasira* sp. Haima symbiont (*Ca.* Ruthturnera sp. Tsphm01) are classified into the genus *Thiodubliliella* (family Thioglobaceae) (Guo et al., 2023) and genus *Ruthia* (family Thioglobaceae), respectively. The lack of genomic resources and TEM observation in thyasirid symbionts hinder comprehensive comparison to decipher the evolution of *Thyasira* symbiosis.

TABLE 1 The assembly statistics of *Candidatus* Ruthturnera sp. Tsphm01.

Assembly metric	
Number of contigs	1
Genome size (bp)	1,523,273
GC content (%)	36.9
Number of coding genes	1521
Number of rRNA	3
Number of repeat_region	1
Number of tRNA	36
Number of tmRNA	1
Completeness in CheckM (%)	100
Contamination in CheckM (%)	0.65
Completeness in CheckM2 (%)	100
Contamination in CheckM2 (%)	0
Illumina coverage (X)	1596
HiFi coverage (X)	850

3.3 | Diverse modes of symbioses and their phylogenetic relationships in thyasirid symbionts

There were three symbiotic modes in Thyasiridae, including (1) bacteria present among the microvilli, (2) bacteria in a space delimited by the bacteriocyte membrane or microvilli, and (3) vacuoles within epithelial cells (Dufour, 2005). In this study, three clades in Thyasiridae symbionts were classified based on their molecular phylogeny (Figure 3). *Thyasira* cf. *gouldii* symbiont is observed as type 2, which is classified into the genus *Sedimenticola* (family Sedimenticolaceae) based on 16S rRNA gene sequence (McCuaig et al., 2020). The newly released output about *C. bisecta* clearly demonstrated that these symbionts were aggregated in the apical vesicles (type 3) and might have pathways connecting to environment (Guo et al., 2023), which is consistent with the earlier identification (a more advanced state) by Dufour (2005). Also, it is also consistent with the classification of Bathymodioline symbionts (genus *Thiodubiliella*) at the phylogenomic relationship (Figure 3) and their symbiotic mode (Halary et al., 2008; Ikuta et al., 2021). Previous studies showed symbionts in Pliocardiinae clams are always detected as intracellular endosymbionts within bacteriocytes (Ip et al., 2021; Newton et al., 2007; Perez et al., 2022). Therefore, we make a hypothesis that *Ca.* Ruthturnera sp. Tsphm01 is likely to be Type 3 (endosymbionts), which is shown as a simplified model in Figure 5b. However, we cannot exclude the possibility of other symbiotic modes, which will be investigated in the future with more specimens. Meanwhile, the diverse modes of Thyasiridae symbionts might promote the understanding of the evolution of chemosymbiosis in bivalves, compared to the obligated endosymbionts in Pliocardiinae clams and Bathymodioline mussels.

3.4 | Overview of the gill transcriptome

A total of 48,200,374 clean reads were identified as symbiont-free reads, which were further assembled into 468,519 transcripts. The

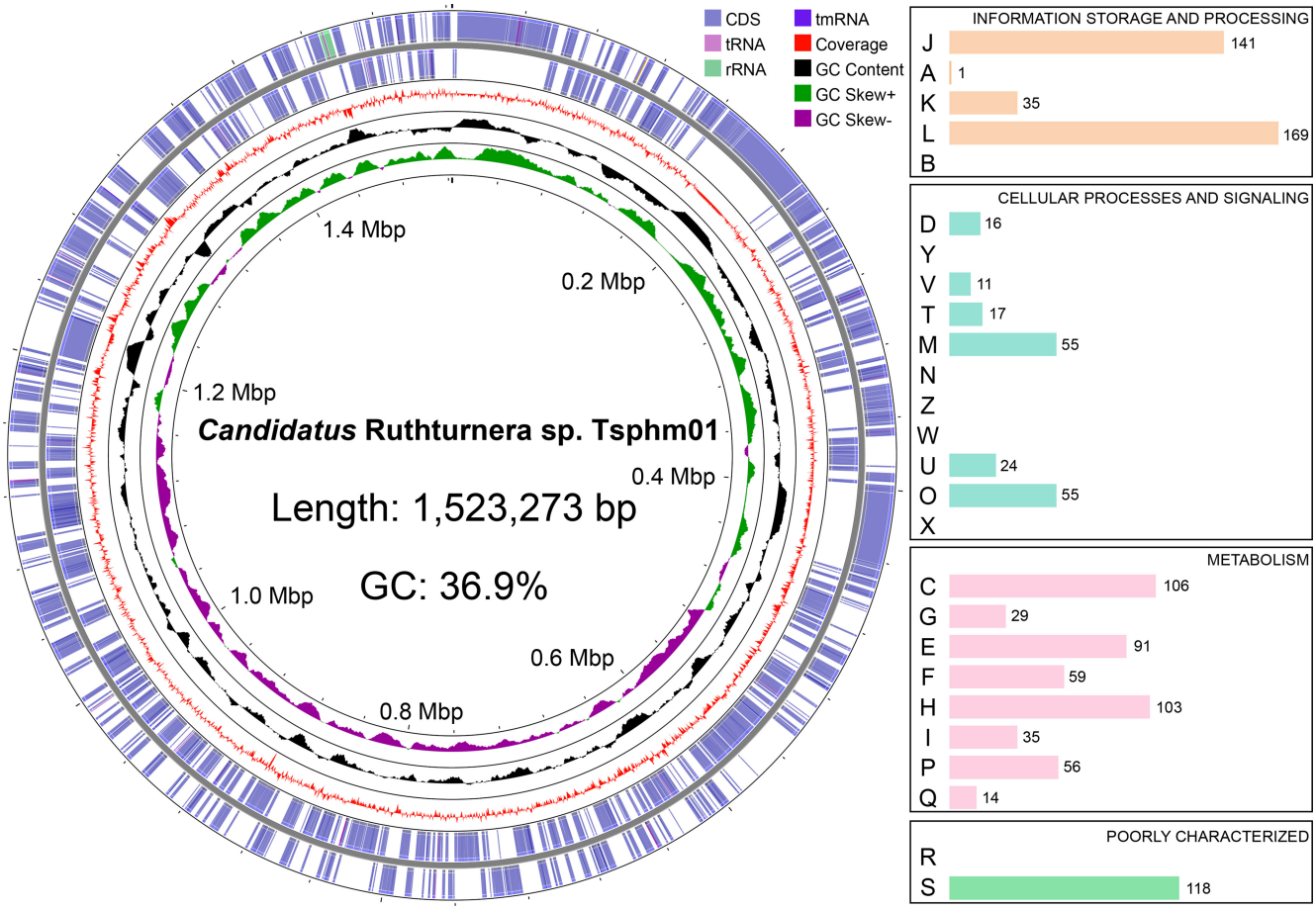

FIGURE 2 An overview of the symbiont genome *Candidatus* Ruthturnera sp. Tsphm01. The left circular view comprises the prediction results by Prokka, the coverage estimated by short-reads, GC content and GC skew. The right bar graph shows the COG annotation of genes in *Candidatus* Ruthturnera sp. Tsphm01. The functional category in COG was shown as the abbreviation. [A] RNA processing and modification; [B] chromatin structure and dynamics; [C] energy production and conversion; [D] cell cycle control, cell division, chromosome partitioning; [E] amino acid transport and metabolism; [F] nucleotide transport and metabolism; [G] carbohydrate transport and metabolism; [H] coenzyme transport and metabolism; [I] lipid transport and metabolism; [J] translation, ribosomal structure and biogenesis; [K] transcription; [L] replication, recombination and repair; [M] Cell wall/membrane/envelope biogenesis; [N] cell motility; [O] posttranslational modification, protein turnover, chaperones; [P] inorganic ion transport and metabolism; [Q] secondary metabolites biosynthesis, transport and catabolism; [R] general function prediction only; [S] function unknown; [T] signal transduction mechanisms; [U] intracellular trafficking, secretion and vesicular transport; [V] defence mechanisms; [W] extracellular structures; [X] mobilome: prophages, transposons; [Y] nuclear structure; [Z] cytoskeleton.

highly redundant transcripts were removed, resulting in 31,020 contigs totaling 34,188,862 bp with an N50 value of 1533 bp. Among them, 20,883 proteins had at least one significant match (*e*-value <1*e*-5) in the nr database. There were 15,026 and 7687 proteins annotated as GO terms and KEGG orthologs, respectively (Figure S4b and Table S7). The host transcripts encapsulate 67.2% completeness of metazoan BUSCOs (Table S2).

3.5 | Chemoautotrophic capacity of the symbiont

Despite having a reduced genome, *Ca*. Ruthturnera sp. Tsphm01 encodes all the core genes and pathways required for oxidizing reduced substances and assimilating CO_2 to biomass. Specifically, the symbiont is capable of using dissimilatory sulphate reduction and oxidation, which catalyses sulphide to sulphate or the other way, which includes: (1) reversible dissimilatory sulphite reductase (*dsrAB*) between sulphide and sulphite; (2) reversible adenylylsulphate reductase (*aprAB*) between sulphite and adenylylphosphosulfate (APS); (3) reversible sulphate adenylyltransferase (*sat*) for the transformation between APS and sulphate. In addition, it actively utilizes the oxidation from thiosulfate to sulphate via the thiosulfate-oxidizing SOX pathway, with the presence of *soxXYZAB* complex and the absence of *soxCD*. Similar to other gammaproteobacterial chemosymbionts, the oxidization of reduced sulphur compounds may provide this thyasirid symbiont sufficient energy for the synthesis of carbohydrates and other organic matters (Breusing et al., 2020; Ip et al., 2021; Lan et al., 2019). The environment settings also suggest a reduced environment appropriate for the growth of anaerobic thioautotrophic bacteria. The O_2 in the sediments was low: decreasing

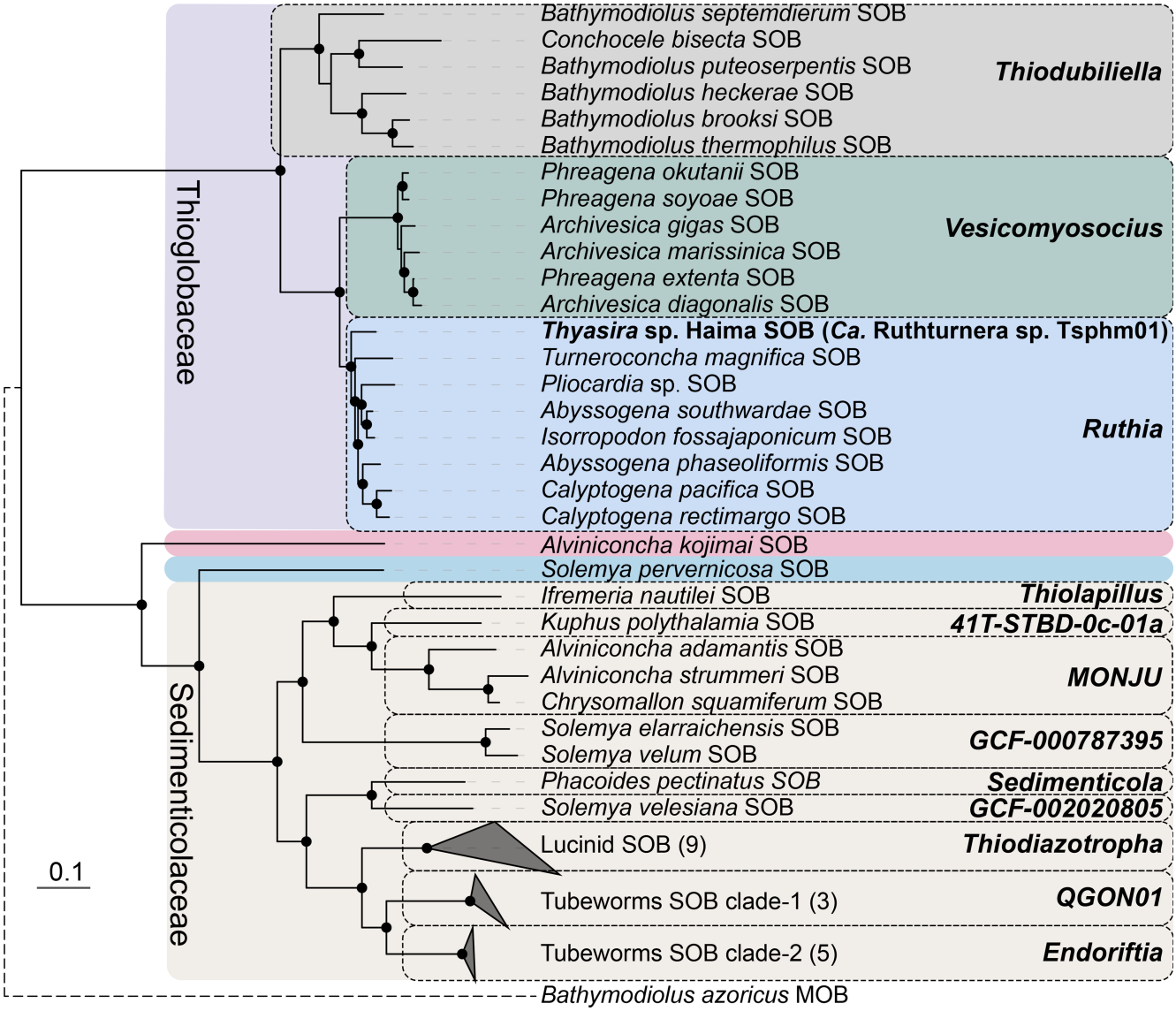

FIGURE 3 The phylogeny of chemosynthetic *Gammaproteobacteria* inhibating in Mollusca, based on the proteins predicted from their genomes. The dark dots on nodes represent the full support from ultrafast bootstrapping in the maximum likelihood test (IQ-TREE2 -m MFP). The scale bar indicates 0.1 amino acids substitutions per sites. The symbionts in dash frame represents that they are from the same genus that GTDB-Tk assigned. The black dot indicates the full support (bootstrap value = 100).

from ~100 μM at the sediment interface to undetectable (<0.3 μM) within several mm. High H_2S concentration (700–1400 μM) was also detected within 3–5 cm.

Analysis of the metatranscriptome in the symbiont showed the active expressions of the carbon fixation pathway (bold and italic labels in Figure 5a, details shown in Table S8). The symbiont genome encodes a complete Calvin-Benson-Bassham (CBB) cycle, and 8 core genes of this cycle were highly transcribed (Figure 4 and Table S8): 3-phosphate dehydrogenase (*GAPDH*, 10,447 TPM) and form II ribulose-bisphosphate carboxylase (RuBisCO, 9014 TPM), transketolase (*tk*, 3240 TPM), fructose-bisphosphate aldolase (*FBA*, 2530 TPM), phosphoglycerate kinase (*PGK*, 2335 TPM), ribulose-phosphate 3-epimerase (*rpe*, 1875 TPM), phosphoribulokinase (*prk*, 1564 TPM) and triosephosphate isomerase (*TPI*, 603 TPM). Powered by the energy from sulphide oxidation, the CBB cycle not only produces glycerate 3-phosphate (3PG) that supports downstream metabolisms (e.g. pyruvate, Acetyl-CoA, valine and leucine biosynthesis) but also is the most critical step in the assimilation of inorganic carbon into biomass which can be further utilized by the host (Figure 5).

SOBs belonging to the family Thiglobaceae with a reduced genome do not possess a complete citric acid cycle (TCA cycle). Consistently, *Ca*. Ruthturnera sp. Tsphm01 in *Thyasira* sp. Haima lacks the genes in two reactions, (1) between 2-oxoglutarate and succinyl-CoA, 2) between malate and oxaloacetate, suggesting the deficiency in the oxidation of organic carbon (Wood et al., 2004). Therefore, like the symbiont of *A. marissinica* (Ip et al., 2021), it may tentatively be defined as an obligate autotrophic species. Nonetheless, the incomplete citrate cycle functions in providing the intermediate or substrates, which are utilized in the biosynthesis of amino acids,

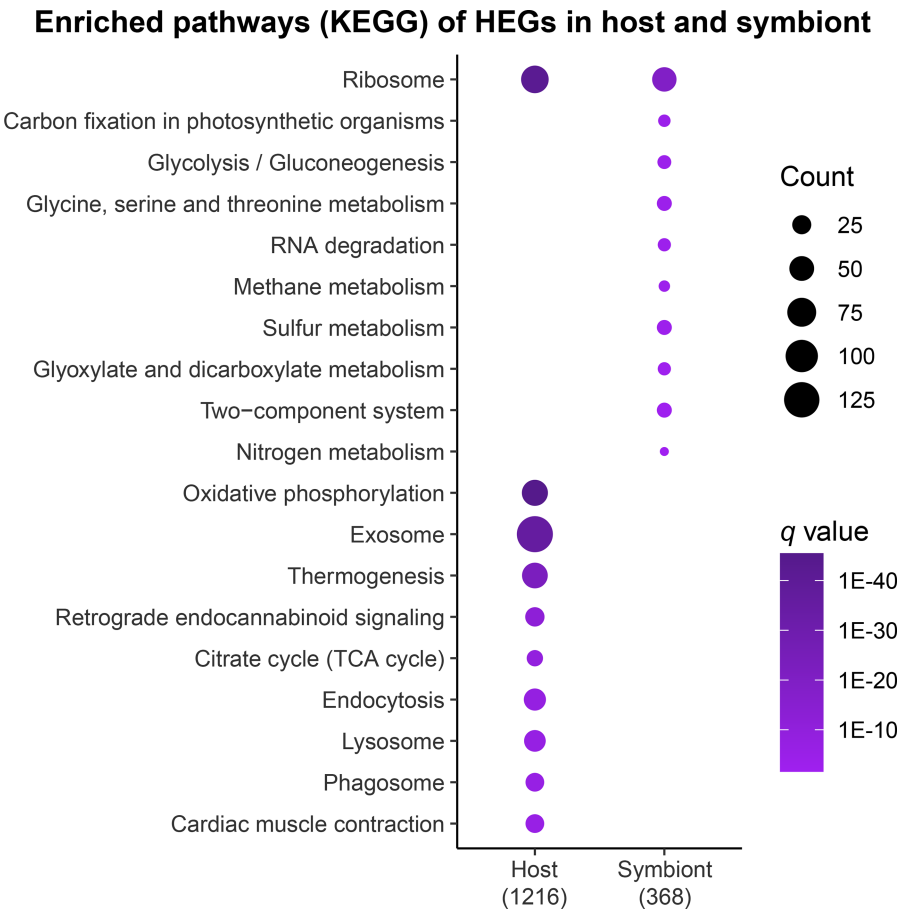

FIGURE 4 Functional enrichment of highly expressed genes (HEGs) in the gill of host and symbiont, based on KEGG pathway.

fatty-acids and cofactors (Kwong et al., 2017). In this study, other genes of the citric acid cycle in *Ca*. Ruthturnera sp. Tsphm01were transcribed, and citrate synthase (*CS*) was detected with relatively high expression (545 TPM). For example, 2-oxoglutarate could be involved in synthesizing Glutamate (Figure 5a).

3.6 | Adaptation to the hypoxia in the symbiont

In the majority of organisms, oxygen serves as the primary electron receptor. Accordingly, the lowest limit of oxygen in sediment for the infaunal macroinvertebrate (>1 mm) community was estimated as 75 μM (Kanaya et al., 2018). In this study, *Thyasira* sp. Haima was found in an anaerobic environment with extremely low oxygen and ample H_2S, which is far below the threshold. The expressional profile uncovered that both the host and symbiont have adapted to survive in such an area by enhancing the binding potential to oxygen and utilizing other substances as electron acceptors. Haemoglobin is one of the most important proteins that transport oxygen in invertebrates, and it also adapts to bind and transport sulphides in vestimentiferan tubeworms (Flores et al., 2005). There was a high hemoglobin expression in the host's gill, up to 8238 TPM and ranking the 5th highest expression in our result, similar to the patterns in *Phreagena okutanii* and *A. marissinica* (Lan et al., 2019), indicating that *Thyasira* sp. Haima aims to trap oxygen as much as possible under hypoxic or anaerobic sediment.

Symbionts could utilize two other sources of electron acceptors to compensate for the deficiency of O_2, and the genes responsible for them were highly expressed in *Ca*. Ruthturnera sp. Tsphm01. Nitrate reduction is reported in chemosymbionts, which produces ammonium, participates in the downstream amino acid syntheses, and function under anaerobic circumstance (Girguis et al., 2000; Hentschel et al., 1993; Sogin et al., 2021). *Candidatus* Ruthturnera sp. Tsphm01 could process the reduction from nitrate to nitrite via nitrate reductases (i.e. alpha, beta, gamma subunits, named *narGHI*) and from nitrite to ammonia via nitrite reductase (*nasBD*). Notably, three genes were annotated as the nitrate/nitrite transporter (*Nrt*), and two of them were actively expressed, that is 2702 and 762 TPM. Apart from that, *Ca*. Ruthturnera sp. Tsphm01 could efficiently reduce dimethyl sulfoxide (DMSO) to methyl thioether (DMS) via anaerobic dimethyl sulfoxide reductase (Paredes et al., 2021), with 3825 TPM in subunit A (*dmsA*) and 5340 TPM in subunit B (*dmsB*), called DMSO respiration. Before the newly assembled genome in this study, *dmsAB*-dependent DMSO respiration was rarely detected in symbionts under the Family Thioglobaceae but commonly found in those under Family Sedimenticolaceae (Table S5). The only exception is *Bathymodiolus thermophilus* symbionts, while only *dmsB* is observed. The DMSO

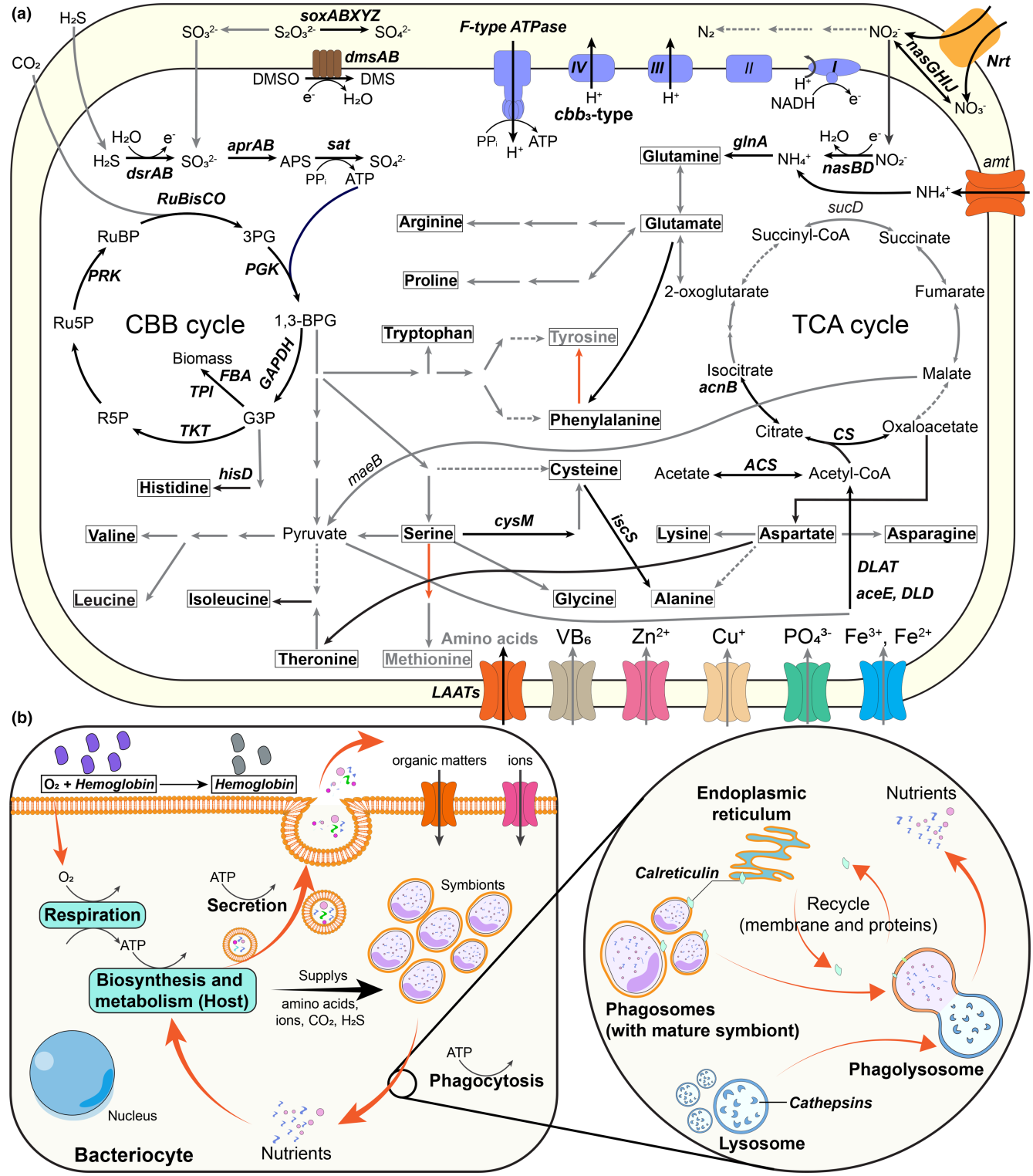

FIGURE 5 (a) Overview of metabolic pathways in *Candidatus* Ruthturnera sp. Tsphm01. The full arrow indicates the presence of the enzymes in transformations or reactions, whereas the dash arrow represents the absence of enzymes. The highly expressed genes in reactions were shown as dark and italic font, with the dark arrow. All the amino acids are shown in bold font with rectangle around and coloured by black. The arrow in orange indicate the complementary reactions from host. (b) Simplified model of host-symbiont interaction in *Thyasira* sp. Haima.

reductase and its active expression in *Ca*. Ruthturnera sp. Tsphm01 implies the adaption to their anaerobic surroundings. Both nitrate- and DMSO-mediated respiration have been documented as the two main strategies in bacteria to cope with anaerobic conditions, such as hypersaline sediment (Matsuzaki et al., 2006; Solchaga et al., 2022).

Cytochrome *c* oxidases are an essential part of the respiratory chain in prokaryotes and eukaryotes, which transfer electrons to O_2 and contribute to the H^+ gradient (Esposti, 2020). Two types of cytochrome c oxidases are present in nearly all sulphide-oxidizing bacteria: the mitochondria-like caa_3-type and cbb_3-type. Only the cbb_3-type cytochrome *c* oxidase in the Complex IV was detected in *Ca*. Ruthturnera sp. Tsphm01, consisting of Subunit I, II and IV (*cooN*, *cooO*, *cooP*). Notably, all three subunits were highly transcribed with 749, 1359 and 1549 TPM. Similarly, the loss of caa_3-type was observed in symbionts from *Solemya velesiana* and *C. bisecta* (Table S5). Previous studies reported that cbb_3-type oxidase has a high affinity for oxygen and could maintain the growth of bacteria under an anaerobic or hypoxic environment (Ekici et al., 2012; Hamada et al., 2014; Pitcher et al., 2002). For example, *Pseudomonas aeruginosa*, a pathogenic bacteria in hypoxic environments, could produce 16 different cbb_3-types, contributing to the robust growth in the hypoxic growth (Hirai et al., 2016). The different structure in the redox-driven pumping mechanism from mitochondria-like caa_3-type enables cbb_3-type to keep catalytic activity at low oxygen concentrations (Buschmann et al., 2010). By contrast, the acquisition of caa_3-types cytochrome *c* oxidase in *Sulfurimonas pluma* was proposed as one of the adaptive strategies in the aerobic chemolithotrophic metabolism (Molari et al., 2023). Therefore, the loss of caa_3-type might be a signature of the thyasirid holobionts from Haima hydrocarbon seep undergoing an anaerobic metabolism for an extended period.

3.7 | Host-symbiont interactions

Our study revealed that *Ca*. Ruthturnera sp. Tsphm01 in *Thyasira* sp. Haima has lost some genes responsible for the biosynthesis of amino acids. It can produce 18 of the 20 standard amino acids, including alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan and valine (shown with complete lines in Figure 5a, details in Table S6). *Candidatus* Ruthturnera sp. Tsphm01 expresses the S-sulfo-L-cysteine synthase (*cysM*, 1338 TPM) and glutamine synthetase (*glnA*, 8094 TPM) to convert serine to cysteine and transform ammonia (NH_4^+) to glutamine, respectively. Luckily, there are missing pathways of the rest two amino acids in the symbiont, which could be compensated by its host or some intermediates supplemented. Specifically, *Thyasira* sp. Haima encodes phenylalanine-4-hydroxylase (*phhA*) to transform phenylalanine to tyrosine, and cysteine-S-conjugate beta-lyase converts cystathionine to homocysteine. It was shown that *Ca*. Ruthturnera sp. Tsphm01 can import these two amino acids (i.e. Tyrosine and Methionine) from the host via its active amino acid transporter, which includes three general L-amino acid transport system substrate-binding proteins (16,429 TPM in *appJ*, 1166 TPM in *appP* and 442 TPM in *appQ*). Recent structural research reports that L-amino acid transporters in human are asymmetric exchangers with a higher affinity on the extracellular side, indicating the net influx of amino acids (Errasti-Murugarren et al., 2019). In contrast, *Thyasira* sp. Haima might rely heavily on its symbiont to meet the requirement of amino acids due to the more broken pathways in its transcriptome compared to the symbiont. It has to be recognized that there is incomplete transcriptomic data (only 67% of metazoan BUSCOs were found), and more data are needed to determine whether the host is indeed reliant on the symbiont to fill the gaps in its amino acid biosynthesis pathways. The host has the potential to synthesize alanine from pyruvate and process some alternative pathways in some amino acids, as revealed by the transcriptomic results in the gill. In summary, the incorporated pathways coupling the host with its symbiont comprise the intact synthesis of 20 amino acids.

Similar to the synthesis of amino acids, there is a deficiency in cofactors in either symbiont *Ca*. Ruthturnera sp. Tsphm01 or its host *Thyasira* sp. Haima when checked individually, whereas it is intact as for the holobiont (Table S6). Both of them are able to process all the reactions of folate, flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), haem, and molybdenum. Concerning vitamin B6 (VB_6), it is absent in the genome of *Ca*. Ruthturnera sp. Tsphm01 but present in the gill transcriptome of *Thyasira* sp. Haima. Also, there are opposite profiles in terms of biotin and coenzyme A (CoA). Besides nitrate/nitrite and amino acids transporter in *Ca*. Ruthturnera sp. Tsphm01, there were some specific ion transports for copper, zinc, iron and phosphate, which are responsible for the uptake of necessary elements in bacteria (Figure 5a). For example, cbb_3-type cytochrome *c* oxidases would be dysfunctional without Cu^+ (Pitcher et al., 2002). Additionally, *Ca*. Ruthturnera sp. Tsphm01 is able to intake ammonium from the host via ammonium transporter (*amt*) and utilize it as the substrate of Glutamine, helping to reduce the NH_4^+ content in the host (Xiang et al., 2020).

It has been reported that most hosts seem to directly digest their symbionts in the chemosymbiosis, called farming mode (Sogin et al., 2020). Therefore, host could utilize the whole profile of the nutrients from the symbiont after digestion. Otherwise, the host must depend on the secretion entirely by the symbiont if the milking mode. Similarly, the functional enrichment of HEGs in the gill illustrated the active phagocytosis (Figure 4 and Table S9), including phagosome (ko04145), lysosome (ko04142) and endocytosis (ko04144), which might support the farming mode of symbiont indirectly. We detected the high calreticulin expression in gill (1653 TPM), the conserved molecule in the endoplasmic reticulum. It was more studied in humans, serving as an "eat me" signal and recruiting the lysosome to the phagocytosis (Schcolnik-Cabrera et al., 2019). Given that, calreticulin might be the initial signal of phagocytosis in *Thyasira* sp. Haima. The active V-type H^+-transporting ATPase would create an acid micro-environment in lysosome for the digestion (Marshansky & Futai, 2008; Moggioli et al., 2023), with 10 of 13 subunits expressing more than 100 TPM. Cathepsins, the critical enzyme for digestion in the lysosome, were highly expressed in the host gill tissue, including Cathepsin B, C, D, F, L and X, ranging from 153 to 2393 TPM. However, the initiation of phagosome forming or the communication between the host and symbionts remains unclear and needs further investigation. The active phagocytosis in the gill might also be involved

in maintaining the homeostasis of symbionts by eliminating other invaded bacteria. Although *Ca*. Ruthturnera sp. Tsphm01 is able to process the general secretory pathway, and some of the genes expressed actively (up to 591 TPM), it might not satisfy the need for nutrients in the host. The secretory pathway might play a role in communicating with the host or other symbionts.

There were 133 HEGs participating in exosome (ko04147), which is responsible for allocating nutrients and communicating among cells in the host. Due to the vestigial digestive tract, chemosymbiotic bivalves, especially those in the deep sea, might solely rely on symbionts in the gill for nutrition. The metatranscriptome of symbionts showed activated biosynthesis and metabolism in bacteriocytes. The exosome could transport the nutrients to other cells without symbionts for maintaining cellular activities, like biomineralization in the mantle. All the above biological processes (e.g. exosome, phagosome and biosynthesis of amino acids) are energy-consuming and are powered by the high expression of genes in oxidative respiration (Figures 4 and 5b).

3.8 | Vertical transmission of *Ca*. Ruthturnera sp. Tsphm01 inferred from its genomic structure

Like other symbionts with reduced genomes, *Ca*. Ruthturnera sp. Tsphm01 lacks genes encoding flagellar and chemotaxis proteins, indicating that these symbionts have lost mobility [supported by KEGG (Table S4) and COG (Figure 3)]. As previously discussed, *Ca*. Ruthturnera sp. Tsphm01 is tentatively classified as an obligate autotrophic bacterium due to the absence of an intact citrate cycle and transporters for glucose. In addition, it cannot synthesize all 20 amino acids and VB_6, suggesting it relies on exogenous sources of amino acids or intermediate synthesis products. *Candidatus* Ruthturnera sp. Tsphm01 could not utilize hydrogen due to the absence of hydrogenases, implying that *Ca*. Ruthturnera sp. Tsphm01 is an exclusively thioautotrophic organism relying on reduced sulphur compounds. Therefore, the symbiont *Ca*. Ruthturnera sp. Tsphm01 in *Thyasira* sp. Haima may be unable to survive in the ambient environment for extended periods.

It is worth noting that symbionts from the Pliocardiinae clams are exclusively found in the genera *Ruthia* and *Vesicomyosocius* (Johnson et al., 2017; Ozawa et al., 2017), with congruent relationships between these clams and their symbionts, as indicated by the phylogenies of their mitochondrial and symbiotic genes (Perez et al., 2022). Some incongruent relationships between them might result from occasional ancient horizontal acquisition at the species level (Ozawa et al., 2017) or horizontal recombination within the populations (Russell et al., 2020). Accordingly, *Ca*. Ruthturnera sp. Tsphm01s in *Thyasira* sp. Haima may also be transmitted vertically. This hypothesis is supported by the phylogenomic tree, which places *Ca*. Ruthturnera sp. Tsphm01 in a different clade from *Ca*. Ruthia marisinica in *A. marissinica*, although these bivalves inhabit the same site (Figure 3). Besides, the undetectable high-quality SNPs from up to 1500 times sequencing coverage indicated the symbiotic population in our sample typically had extremely low genetic diversity, which could be indirect evidence of vertical transmission. Comparatively, the SNP densities in horizontally transmitted SOBs are higher. For example, there are usually higher than 4 SNPs per kilo bases in horizontally transmitted deep-sea mussel symbionts (Ansorge et al., 2019) and 0.01 to 4 SNPs per kilo bases in vertically transmitted Scaly-foot Snail symbionts (Lan et al., 2022). Nonetheless, the actual mode of transmission of *Ca*. Ruthturnera sp. Tsphm01 requires more in-depth study, including analysis of the genetic variation at the population level and fluorescence in situ hybridization (FISH) of its gonad or larva.

4 | CONCLUSION

We report the symbiosis of the newly discovered chemosynthetic bivalve *Thyasira* sp. from Haima seeps in the South China Sea. We assembled the circular genome, and analysed the metabolic pathways of the symbiont *Ca*. Ruthturnera sp. Tsphm01 of the clam. *Candidatus* Ruthturnera sp. Tsphm01 generates energy source from oxidation of reduced sulfur compounds, which fuel the production of organic carbohydrates. This symbiont has a reduced genome and encodes an incomplete pathway in the TCA cycle, chemotaxis and the biosynthesis of small molecules. The results of gill metatranscriptome shed insight into the interactions between the symbiont and host, complementary metabolic pathways between them, and active phagocytosis by the host. In addition, *Ca*. Ruthturnera sp. Tsphm01 may have evolved with the host and have adapted to hypoxia. Overall, *Thyasira* sp. Haima could be an excellent model to study the evolution of chemosymbiosis.

AUTHOR CONTRIBUTIONS

JS conceived the project. XH and YiL collected the samples. GMK identified the species. YuLin performed the environmental factor analysis, YuL performed the phylogenetic analysis in symbionts and the bioinformatics work. XH performed the phylogenetic analysis in thyasirids. YuL, JS and XH wrote the manuscript. YiL, JL and J-WQ edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The raw reads and assemblies were deposited in the NCBI Bioproject database, with the accession numbers PRJNA949122 for the symbiont genome (accession: GCA_030142985.1) and PRJNA951071 for the metatranscriptome of holobiont. In addition, the assemblies and their annotations, commands used in genome assembly, gene expression, and phylogenetic relationships are provided at the GitHub (https://github.com/ylify/Chemosymbiosis-in-the-thyasirid-from-Haima-hydrocarbon-seep).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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