Shallow-water hydrothermal vent system as an extreme proxy for discovery of microbiome significance in a crustacean holobiont

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The shallow-water hydrothermal vent (HV) system off Kueishan Island lies at the end of the Okinawa Trough to the northeast of Taiwan. Near its submarine vent openings, aperiodic vent discharges generate a dynamic acidic (pH 5.5-8.1) and sulfidic (9-3000 µM) ecosystem. The dominant metazoan in this unique environment is the brachyuran vent crab, *Xenograpsus testudinatus*, which has developed robust metabolic strategies and highly adaptive acid-base regulatory mechanisms to maintain its physiological homeostasis. *X. testudinatus* is considered a holobiont, but the symbiotic mechanisms underlying acid and sulfur tolerance in the host-microbe system remain largely unclear. In this study, we used LoopSeq long-read sequencing of the full-length 16S rRNA gene to identify the bacterial communities present in the gills and carapace surface of *X. testudinatus*. The alpha diversity analysis, Venn diagram, and principal coordinate analysis (PCoA) indicated that the gills and carapace surface exhibit different bacterial constituents. Further measurements of relative abundance, coupled with functional predictions and fluorescence *in situ* hybridization (FISH), revealed a predominance of *Sulfurovum* sp. NBC37-1, a key bacterium that can perform sulfur and hydrogen oxidation to support denitrification processes. Consequently, our findings suggest that the symbiotic bacteria may play a critical role in conferring the extraordinary acid and sulfur tolerances of *X. testudinatus*, allowing the crustacean holobiont to thrive in its ecological niche within one of the most extreme marine habitats on Earth.

Introduction

Hydrothermal vent (HV) systems are widely recognized as being among the world's most extreme marine environments. Many characteristics of these systems arise due to underwater volcanic activities, and the first HV system was discovered on the Galapagos rift in 1977 (Lonsdale, 1977; Glowka, 2003). The chemical-rich fluids gushing out of the vent opening create a highly acidic and megathermal environment (Elderfield and Schultz, 1996; Fisher et al., 2007), which is home to microbial populations that exhibit some of the highest diversity on the planet (Van Dover and Trask, 2000; Glowka, 2003; Van Dover, 2014). The diverse microorganisms in HVs build a biological bridge between the extreme environment and chemoautotrophic animals, serving as the primary biomass sources for food webs that support endemic crustacean, polychaete, echinoderm, coelenterate and mollusk species (Little and Vrijenhoek, 2003). Moreover, HV ecosystems are typically dominated by benthic invertebrate taxa that also host symbiotic and chemoautotrophic microorganisms (Dahms et al., 2018; Alfaro-Lucas et al., 2020). The chemoautotrophic microbes acquire electron donors (e.g., sulfide, sulfur, and hydrogen) in vent fluids, electron acceptors (e.g., nitrate and oxygen) in seawater, and inorganic carbon (e.g., CO_2 or CH_4) in vent fluids or seawater (Kuo et al., 2001; Takai et al., 2009).

HV fluids are typically enriched in inorganic carbon, ammonium, methane, sulfur, and hydrogen sulfide (H_2S; Butterfield et al., 1990; Chen et al., 2005). HV dissolved gases or gas bubbles are enriched in carbon dioxide (CO_2) and sulfur dioxide (SO_2; Butterfield et al., 2011; Lin et al., 2019). The high sulfur concentrations in the HV fluids are significant in part because microorganisms that can perform sulfur/sulfide oxidation reactions may utilize the sulfur and sulfide as an energy source (Tunnicliffe, 1991; Tarasov et al., 2005; Akerman et al., 2013). Several prominent sulfur-oxidizing bacteria (SOB) have been identified in shallow-water HV areas, including Vulcano (Eolian Islands, Italy), Milos Island (Greece), Espalamaca (Faial, Azores), and El Hierro (Canary Islands, Spain; Gugliandolo and Maugeri, 1993; Giovannelli et al., 2013; Rajasabapathy et al., 2014; Gonzalez et al., 2020). In addition, chemoautotrophic bacteria have been isolated from crustaceans endemic to deep-sea HVs, such as the vent shrimp *Rimicaris exoculate*, yeti crab *Kiwa hirsute* and galatheid crab *Shinkaia crosnieri* (Goffredi et al., 2008; Zbinden et al., 2008; Tsuchida et al., 2011). These symbiotic bacteria play important roles in primary production and life-sustaining detoxification processes, especially within the gills of HV animals (Fialamedioni et al., 1986; Jouin and Gaill, 1990; Compere et al., 2002; Ponsard et al., 2013; Jan et al., 2014).

At the rifting end of the Okinawa Trough, the shallow-water HV system off Kueishan Island lies just northeast of Taiwan (Hu et al., 2012). This HV system consists of several smokers that discharge highly acidic (pH 1.75-4.60), sulfidic and hot fluids (65-116°C), resulting in relatively low pH (5.4-7.3) and dissolved oxygen (3.9-4.9 mg/L) values compared to the non-vent seawater (Jeng et al., 2004; Han et al., 2014; Chan et al., 2016). The shallow-water HV plumes cause several physicochemical parameters such as temperature, pH, and redox status to vary over an extreme range of values (Peng et al., 2011; Dahms et al., 2018; Pang et al., 2019; Misuraca et al., 2021). As such, vent-associated biological communities must exhibit adaptive behavioral, physiological, morphological and symbiotic features to sustain their lives (Adams et al., 2011; Hu et al., 2016; Allen et al., 2021). For example, previous reports have detailed unique biochemical mechanisms for sulfide detoxification (Wang et al., 2021), behavioral and molecular responses to high temperatures (Gurunathan et al., 2021), and the presence of specialized transepithelial ionocytes to cope with low-pH conditions (Hu et al., 2016). The shallow-water submarine vent field off the southeastern shore of Kueishan Island provides an accessible natural laboratory to study the effects of HV environments on local organisms (Han et al., 2014; Chan et al., 2016; Yang et al., 2016; Pang et al., 2019). *Xenograpsus testudinatus*, a brachyuran crab species, was originally reported to thrive in this extreme shallow-water HV system. It has also been found in shallow-water HV waters at Showa Iwo-jima in Kagoshima and Shikine-jima Island in the Izu archipelago of Japan (Jeng et al., 2004; Ng et al., 2014; Miyake et al., 2019; Yang et al., 2022). Since *X. testudinatus* is a vent-endemic species, it is expected to have strong ion and acid-base regulatory abilities that allow it to cope with the dynamic challenges presented by the environment. A previous study revealed the stimulation of mRNA expression, protein abundance and enzyme activity for basolateral Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase (VHA) in the gills of normal pH-acclimated *X. testudinatus* (pH 8.0) after exposure to acidified seawater (pH 6.5; Hu et al., 2016). The study showed that the subcellular localization of VHA was mainly within the cytosol and along with the apical membrane of the gill lamella epithelium, supporting the idea that VHA is involved in proton secretion as a mechanism of acid excretion (Hu et al., 2016). Additionally, similarly to vertebrate blood, crab hemolymph serves as a major transport medium for materials between cells and all the internal tissues in the body, such as hormones, waste materials, and nutrients, which have also been well documented (Allen et al., 2020; Allen et al., 2021). In this way, the physiochemical parameters in the hemolymph of crabs can be used to describe the physiological responses and adaptive characteristics.

In addition to adaptive mechanisms in the cells of the endemic macroorganisms, symbiotic microorganisms also support the survival of their hosts in extreme HV environments. The first step in understanding the interaction is to identify the microbial compositions in essential tissues, such as the gills and carapace, of the multicellular hosts. The two tissues directly contact the environment, and the carapace also

serves as a shield for decapod crustaceans against the predators like fish (Farre et al., 2021). A white biofilm exists on the carapace surface of wild *X. testudinatus* (as we observed in Figure 1E), which may be a layer of the bacterial mat (Wang et al., 2014). Indeed, a previous study showed that the microbial community on the carapace of fiddler crabs (*Uca panacea*) was closely related to the community in the surrounding sediments (Cuellar-Gempeler and Leibold, 2019). Furthermore, the microbial epibiotic communities' significance on the carapaces of deep-sea galatheid squat lobsters (*Munidopsis alvisca*) was interpreted as a mutualistic host-microbe relationship for sulfide detoxification (Leinberger et al., 2022). Up to now, only two studies have probed *X. testudinatus* tissues to examine the importance and potential contributions of symbiosis-associated bacteria (Ho et al., 2015; Yang et al., 2016); both studies showed diverse bacterial constitutions in different tissues of *X. testudinatus*. However, the two studies examined partial

regions of the bacterial 16S rRNA gene, which usually identifies bacterial compositions only on the taxonomic levels above species or genus.

In the present study, the gills and carapace surface of HV crab *X. testudinatus* were examined in terms of the resident bacterial communities, in order to better understand how the microorganism mediates interactions between the HV environment and endemic animals. To gain in-depth knowledge about *X. testudinatus*-associated bacterial taxa, we used multiple approaches, including LoopSeq full-length 16S rRNA gene sequencing. Such full-length sequencing provides a higher data resolution. We also performed functional annotation of prokaryotic taxa (FAPROTAX) based on the taxonomic profiling results to predict the functions of major resident bacteria. Finally, we used fluorescence *in situ* hybridization (FISH) to profile the localizations of dominant gill-associated bacteria.

Materials and methods

Sample collection

From August 2020 to April 2022, water samples were collected every 1 or 3 months (independent sample number = 10) by SCUBA divers in the vent area (24°50'02.6"N, 121°57'42.8"E; offshore the southeastern tip; 15-17 m depth) and non-vent area (24°50'29.9"N, 121°56'17.1"E; offshore the northwestern tip; 8-10 m depth) of Kueishan Island, Taiwan (Figure 1A). The data from each independently collected water sample was averaged from triplicate samples collected at each sampling time surrounding the hydrothermal vent crabs, but not directly above the vent opening. The crabs *X. testudinatus* were collected from the vent area (Figures 1B–E). Crabs were randomly selected for hemolymph collection (independent sample number = 9) as well from August 2020 to April 2022, and the hemolymph was carefully drawn from the coxa membrane of the 2nd walking legs (the 3rd pereopods) using a disposable 1 mL syringe with 24G X 1 Inch (0.55x25 mm) (Terumo Medical Corporation, Tokyo, Japan). The hemolymph was immediately used for pH measurements. To collect the bacterial community from *X. testudinatus*, the 5th gills (N = 29) were sampled, and the carapace surface was applied using cotton swabs (N = 19). The gill tissues and cotton swabs were separately placed into 1.5 mL tubes and stored in liquid nitrogen on the fisheries craft. Upon arriving at the laboratory, samples were frozen at -80°C until DNA extraction.

FIGURE 1
Landscape of the shallow-water hydrothermal vent system off Kueishan Island and representative images of hydrothermal vent crabs. Map shows Kueishan Island, which is located off the northeastern coast of Taiwan (**A**, upper left). Relative positions, latitude and longitude coordinates of the sample collection sites (vent and non-vent areas) (**A**). The photos show the southeastern tip of Kueishan Island, where white sulfur plumes are visible on the sea surface (**B**), which is emanated from the subsurface hydrothermal vent system (**C**). A large number of endemic crabs (*Xenograpsus testudinatus*) inhabit the vent area (**D**). A white biofilm can be observed on the carapace surface of the animals (**E**).

Measurements of water quality and crab hemolymph parameters

The water quality was assessed according to temperature, pH, dissolved oxygen (DO), alkalinity, sulfide content and
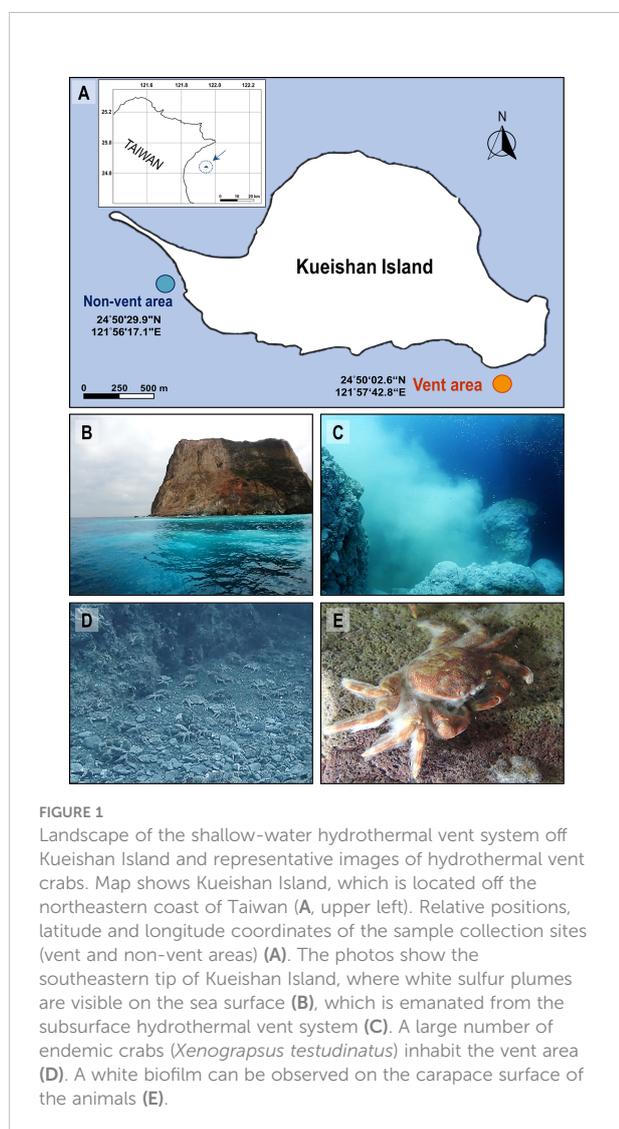
bicarbonate (HCO_3^-) content. The pH, sulfide and HCO_3^- content of crab hemolymph were compared with the corresponding measurements from water samples. The temperatures and pH values of the water samples were measured using a multi-parameter portable meter (Multiline® Multi 3620 IDS, WTW, Weilheim, Germany) equipped with an IDS pH electrode (SenTix® 940, WTW) on the fisheries craft. The crab hemolymph pH was determined using a portable pH meter (Profiline® pH 3310, WTW) equipped with a glass-body micro combination pH electrode (InLab® Micro, Mettler Toledo, Leicester, UK) immediately after hemolymph collection. The water alkalinity was spectrophotometrically assayed following a previously published method (Sarazin et al., 1999). Sulfide content was determined by a spectrophotometric method modified from (Cline, 1969), using a sulfide-sulfur specific mixed diamine reagent containing N, N-Dimethyl-p-phenylenediamine sulfate and ferric chloride in 50% hydrochloric acid. The dissolved inorganic carbon (DIC) content of water samples was analyzed in triplicate using a DIC analyzer (AS-C3, Apollo SciTech, Newark, DE, USA) with nitrogen as an inert gas, followed by quantification *via* a non-dispersive infra-red (NDIR) Li-7000 CO_2/HCO_3^- analyzer (LI-COR, Lincoln, NE, USA). The hemolymph DIC content was measured following a method described previously (Allen et al., 2021). The HCO_3^- concentration of water samples was calculated according to DIC, pH, and temperature using a CO2SYS module undergone within Microsoft Excel (Pierrot et al., 2006). Dissociation constants were as provided and adjusted by two methodological studies (Mehrbach et al., 1973; Dickson and Millero, 1987). Alternatively, the hemolymph HCO_3^- concentration was calculated based on DIC and pH using the Henderson–Hasselbalch equation, with dissociation constants and solubility coefficients as previously described (Truchot, 1976).

DNA extraction

DNA from the crab gills was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), and DNA from the carapace surface (cotton part) was extracted using the QIAamp DNA Microbiome Kit (Qiagen, Hilden, Germany). Both extractions were performed in accordance with the manufacturers' instructions. The quantity and quality of extracted DNA were verified using a NanoDrop 2000™ spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Afterward, the DNA samples were stored at -20°C before use.

Synthetic long-read 16S rRNA gene sequencing

LoopSeq™ 16S Long Read Kit (Loop Genomics, San Jose, CA, USA) was used to construct the bacterial full-length 16S rRNA gene libraries (V1-V9 region). The constructed DNA libraries were

sequenced in a pair-end (2 × 150 bp) configuration on the NextSeq 550 platform (Illumina, San Diego, CA, USA) in accordance with the manufacturer's instructions.

Sequence processing and *in silico* analysis

A Loop Genomics cloud platform (Microsoft Azure) was used to trim Illumina sequencing index and demultiplex the pair-end raw sequences for synthetic long-read reconstruction. The poor-quality reads and chimeric sequences were discarded. The remaining qualified sequences were uploaded to the CLC Genomic Workbench 20.0.1 (CLC Bio, QIAGEN, Aarhus, Denmark) for taxonomic profiling and abundance analysis based on a microbial genome database (2019-08-30, provided by CLC workbench). The total reads number of 16S rRNA gene sequences in each sequenced gill and carapace sample was shown in Table S1 (gill) and Table S2 (carapace). Taxonomic profiling was used to generate the abundance table based on the reads of the 16S rRNA sequence for each taxon. For each taxon, the number of reads in the sample was displayed along with the confidence score. The information was displayed using stacked bar charts. The Alpha diversity indices (Chao 1, Shannon and Simpson) were calculated to assess the heterogeneity of the bacterial community (species). Principal coordinate analysis (PCoA) was performed to determine the dissimilarity of bacterial compositions between the gill and carapace samples, based on the Bray-Curtis distance.

In order to predict the functions of bacteria collected from the gills and carapace surface of *X. testudinatus*, the taxonomic abundance table was used to retrieve functional annotations of identified prokaryotic taxa (FAPROTAX 1.2.5). FAPROTAX provides an informative database that can be used to sort prokaryotic taxa (e.g., classes, genera, or species) into presumptive functions based on studies of culture representatives (Louca et al., 2016). On the basis of the initial output, predicted functions were further classified into incorporative groups and represented as proportions of total predicted functions for each sample.

Fluorescence *in situ* hybridization

FISH was used to detect and locate the bacteria in the gills of *X. testudinatus*. The crab gills were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) solution and temporarily stored in a box filled with ice on the fishing craft. Afterward, the fixed gills were embedded in paraffin wax in the laboratory, and the wax sections (8 µm) were mounted on the slide and stored at 4°C. To perform FISH, dewaxing was performed with Histo-Clear (National Diagnostics, Atlanta, GA), and Histo-Clear was removed using an ethanol series

(100%, 75%, 50% and 25%). After rehydrating the section with 20 mM Tris-HCl, it was treated with proteinase K (5 µg/mL) and lysozyme (5 mg/mL) in Tris-HCl to permeabilize the bacterial walls. The gill sections were hybridized with probe *Sulfurovum*-target EPI653 (5'-ATCTTCCCCTCCCASACTCT-3') (Watsuji et al., 2010), universal bacteria probe EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') (Amann et al., 1990), and a customized probe NBC371 (5'-CCAGTTAGCTTCCCCGCTT-3') designed for *Sulfurovum* NBC37-1 and based on the 16S rRNA gene sequence retrieved from NCBI (AP009179). Fluorescence images were obtained with an Olympus FV3000 confocal laser scanning microscope (Olympus Corporation, Tokyo, Japan).

Statistical analysis

Statistical analyses of relative bacteria abundance (species) and predicted function proportions were performed with GraphPad Prism 9.1.2 (GraphPad Software, San Diego, CA, USA), using Welch's *t*-test to compare the gill and carapace groups. Statistical analyses of alpha and beta diversity were performed with CLC Genomic Workbench 20.0.1 (CLC Bio, QIAGEN). The difference in alpha diversity indices (Chao 1, Shannon, and Simpson) between the gill and carapace groups was determined using the Kruskal-Wallis test. The Bray-Curtis-based permutational analysis of variance (PERMANOVA) was used to compare the difference in bacterial species compositions between gill and carapace groups, which was visualized by PCoA. The level of significance was set at $p < 0.05$.

Results

Environmental water quality and hemolymph parameters of hydrothermal vent crabs

Kueishan Island is located at the end of the Okinawa Trough and possesses a shallow-water HV system on its southeastern tip (vent area; Figures 1A, B). The water around the northwestern tip of Kueishan Island is a non-vent area that contains a coral reef ecosystem and exhibits relatively high biodiversity (Chan et al., 2016). According to our measurements, the environmental parameters of the vent area were more variable than those of the non-vent area (without vent openings). Parameters of water quality were measured in water samples collected from the vent and non-vent areas off Kueishan Island. The temperatures in both areas varied with the season. In the vent areas, water temperatures ranged from 18.6 to 28.3°C, while non-vent areas ranged from 20.2 to 27.9°C (Figure 2A). The dissolved oxygen (DO) ranged from 5.68 to 8.47 mg/L and from 7.16 to 8.68 mg/L in the vent and non-vent areas, respectively (Figure 2B). The alkalinity ranged from 1.56 to 2.43 mM and from 2.16 to 2.51

mM in the vent and non-vent areas, respectively (Figure 2C). Additionally, the pH, sulfide and HCO_3^- contents of environmental water were compared to those of crab hemolymph. The pH ranged from 6.19 to 8.12 in the vent area, and from 7.87 to 8.28 in the non-vent area. However, the crabs inhabiting the vent area seemed to maintain their hemolymph pH in a narrow range of 7.49 to 7.78, in spite of environmental pH fluctuations (Figure 2D). The vent and non-ven sulfide contents ranged from 9 to 3480 µM and from 0 to 23 µM, respectively (Figure 2E). Thus, the vent area had a large variation and much elevated sulfide content. In the crab hemolymph, sulfide was detected at a range of 102 to 747 µM (Figure 2E). As shown in Figure 2F, the HCO_3^- concentrations in the vent and non-vent areas respectively ranged from 0.76 to 2.03 mM and 1.90 to 2.21 mM. Notably, the hemolymph of crab exhibited a considerably large range of HCO_3^- (9.30 to 16.40 mM). An extremely high HCO_3^- concentration in the hemolymph is in line with the idea that HCO_3^- may be held in reserve by the vent crabs for coping with pH drops in the vent area.

Bacterial composition and diversity of the gill and carapace surface

The bacterial compositions were profiled and represented as bar charts in Figure 3A and Table S1, S2. The analysis showed that the gills and carapace surface were both dominated by four classes, including Epsilonproteobacteria, Gammaproteobacteria, Flavobacteriia, and Alphaproteobacteria. Despite sharing overall similar bacterial compositions, there were clear differences in the relative abundances of classes between the gills and carapace. The five most abundant classes in the gills were Epsilonproteobacteria (73.8%), Gammaproteobacteria (9.9%), Flavobacteriia (9.3%), Alphaproteobacteria (3.0%), and Mollicutes (1.5%); the five most abundant classes on the carapace surface were Epsilonproteobacteria (42.2%), Alphaproteobacteria (16.9%), Gammaproteobacteria, Flavobacteriia (10.6%), and Saprospiria (4.7%; Figure S1A). At the genus level, the three most abundant genera in the gills were *Sulfurovum* (67.0%), *Leucothrix* (3.1%), and *Leeuwenhoekiella* (2.7%), while the five most abundant genera on the carapace surface were *Sulfurovum* (28.1%), *Leucothrix* (9.5%), *Polaribacter* (3.5%), *Ruegeria* (2.6%), and *Arcobacter* (2.5%; Figure S1B).

Within the five most abundant classes, a few representative species were evaluated in terms of relative abundance. The box and whisker plot shows the mean and variation of each bacterial species (Figure 3A). The relative abundances of *Sulfurovum* sp. NBC37-1, *Leeuwuwenhoekiella* sp. MAR_2009_32, and *Ornithobacterium rhinotracheale* were significantly higher in the gills (67.0%, 2.7% and 1.3%, respectively) than on the carapace surface (28.0%, 0.1% and 0.3%, respectively; Figures 3A, S2). In contrast, on the carapace, *Nitratifactor salsuginis* (2.9%), *Arcobacter butzleri* (2.1%), *Leucothrix mucor* (9.5%), *Hydrogenvibrio* sp. Milos-T1 (1.2%), *Polaribacter* sp.
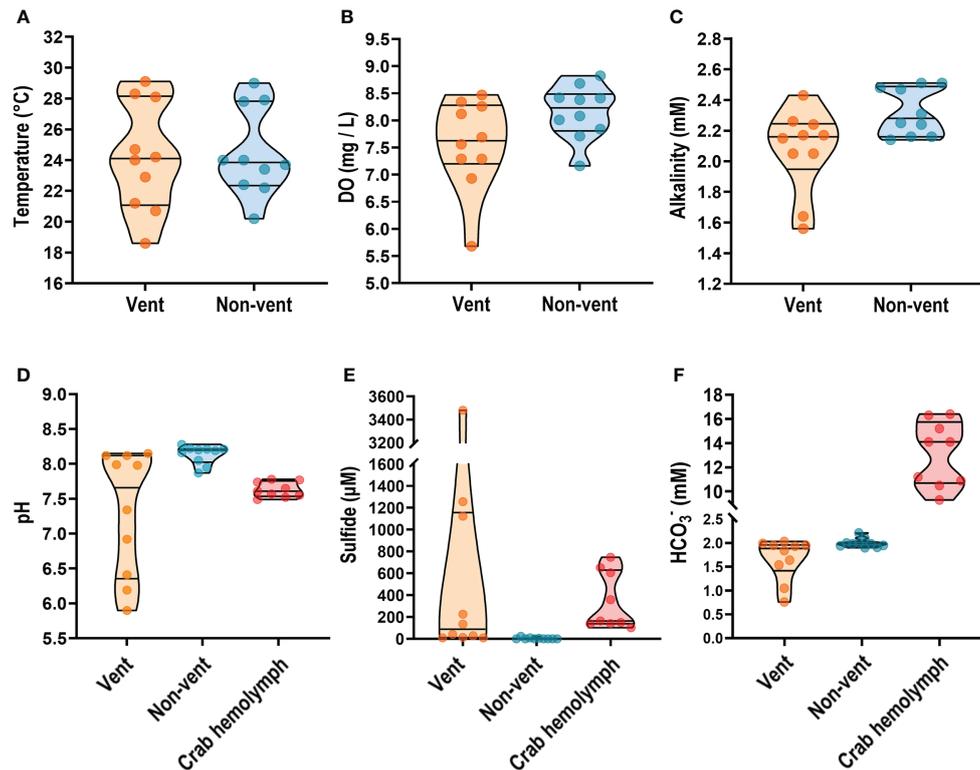
FIGURE 2
On-site measurement of physicochemical parameters from seawater and vent crab hemolymph. Temperature, dissolved oxygen (DO), and alkalinity of seawater samples collected from the vent and non-vent areas **(A–C)** were examined. The pH value, sulfide and HCO_3^- concentrations were estimated both in seawater and crab hemolymph samples **(D–F)**. Parameters are presented as truncated violin plots with individual values also shown.

Hel1_33_49 (1.7%), *Ruegeria* sp. TM1040 (1.6%), and *Pseudovibrio* sp. FO-BEG1 (2.5%) were more abundant than in the gills (respectively 0.7%, 0.4%, 3.1%, 0.1%, 0.2%, 0.2% and 0.1%; Figure 3A; Figure S2).

Indices of alpha diversity, including Chao 1, Shannon, and Simpson, were obtained to characterize the richness and evenness of gill and carapace samples at the species level. The results showed that the carapace surface had more species (Chao 1) and higher diversity (Shannon and Simpson) compared to the gills (Figure 3B). Since the gills were profoundly dominated by *Sulfurovum* sp. NBC37-1 (67%), it is entirely reasonable that the bacterial diversity in the gills would be lower than that on the carapace surface. Additionally, according to the PCoA based on the Bray-Curtis distance, the bacterial species compositions of the gills and the carapace surface were significantly different (PERMANOVA, pseudo-F = 31.2942, p = 0.0001; Figure 4A). In the gills of *X. testudinatus*, a total of 80 genera and 91 species were identified (≥ 0.1% abundance). On the carapace surface, a total of 136 genera and 167 species were identified (≥ 0.1% abundance; Figures 4B, C). The Venn diagram analysis showed that the gills and carapace surface shared 58 genera and 64 species. There were 22 and 78 genera

specific to the gills and carapace surface, respectively (Figure 4B). In the gills, the five most abundant specific species were *Candidatus* Hepatoplasma, *Thiofilum*, *Lacinutrix*, *Chryseobacterium*, and *Thiolinea*. On the carapace surface, they were *Deinococcus*, *Cytophga*, *Truepera*, *Rhodovulum*, and *Rhodopirellula*. In addition, 27 and 103 species were specific to the gills and carapace surface, respectively (Figure 4C). The five most abundant specific species in the gills were *Candidatus* Hepatoplasma crinochetorum, *Thiofilum flexile*, *Lacinutrix* sp. 5H-3-7-4, *Thiolinea disciformis*, and *Methylomicrobium buryatense*. On the carapace surface, *Deinococcus peraridilitoris*, *Flavobacteria bacterium* MS024-2A, *Rhodovulum sulfidophilum*, *Deinococcus deserti*, and *Rubinisphaera brasiliensis* were the five most abundant species (Figure 4C).

Putative functions of bacteria found in the gills and carapace surface

According to FAPROTAX analysis, several functional groups were predicted and identified based on the bacterial taxa profiles
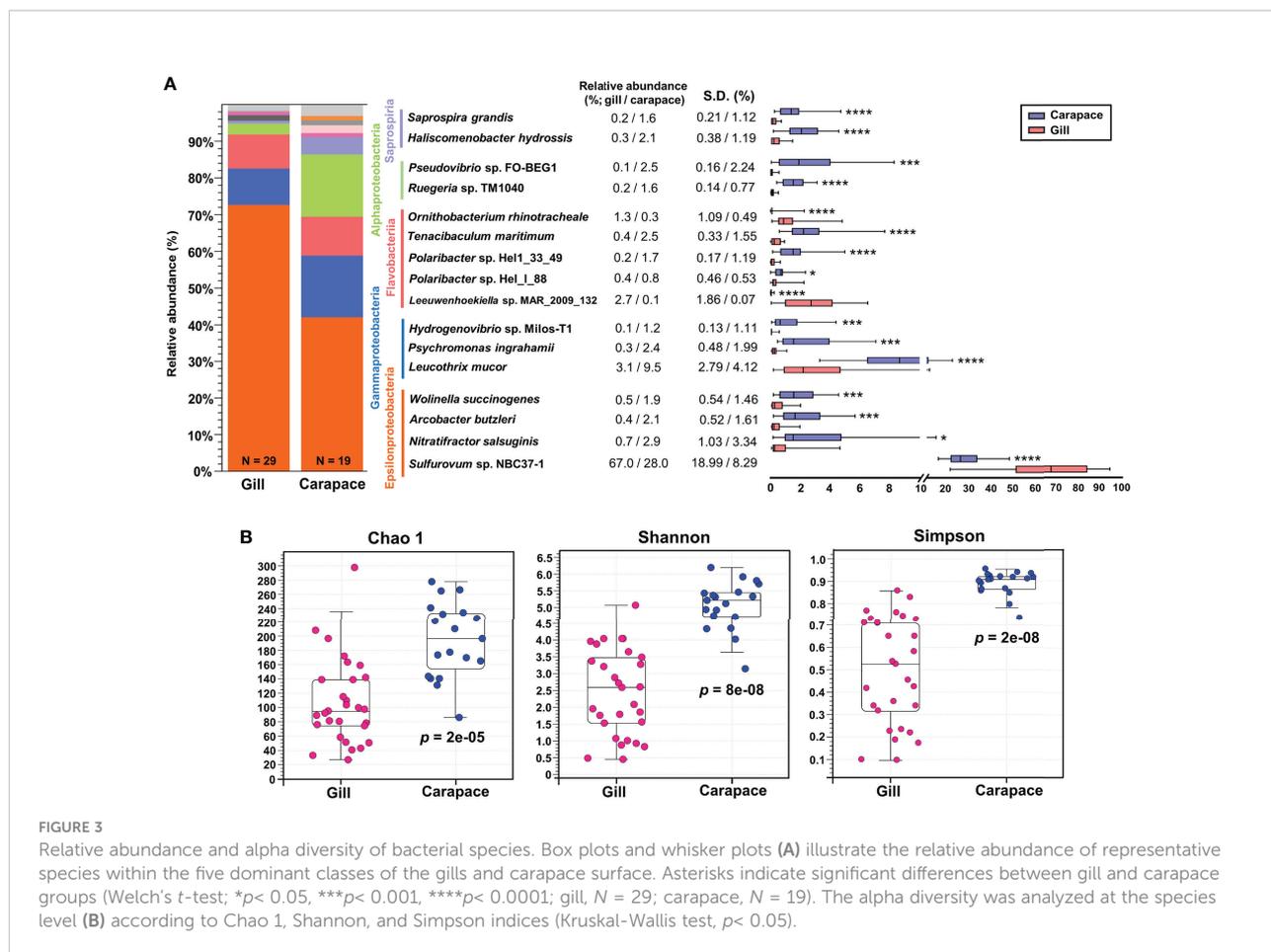
FIGURE 3
Relative abundance and alpha diversity of bacterial species. Box plots and whisker plots **(A)** illustrate the relative abundance of representative species within the five dominant classes of the gills and carapace surface. Asterisks indicate significant differences between gill and carapace groups (Welch's *t*-test; *p< 0.05, ***p< 0.001, ****p< 0.0001; gill, *N* = 29; carapace, *N* = 19). The alpha diversity was analyzed at the species level **(B)** according to Chao 1, Shannon, and Simpson indices (Kruskal-Wallis test, p< 0.05).

(Figure 5). Only two functional groups, methylotrophy (p = 0.3287) and hydrocarbon degradation (p = 0.3401), were not significantly different between the gill and the carapace samples. In the gills, there were higher proportions of bacteria involved in sulfur oxidation, hydrogen oxidation, and denitrification. On the other hand, the community on the carapace surface had higher functional proportions of sulfur reduction, nitrogen fixation, ammonification, chemoheterotrophy, phototrophy, cellulolysis, iron respiration, fumarate respiration, fermentation, ureolysis, and parasites, pathogens or symbionts. The different bacterial functions present in the gills and the surface of the carapace provide critical information for understanding how bacterial biochemical processes may be involved with the response of the vent crab to the HV environment.

The localization of *Sulfurovum* sp. NBC37-1 in the gill of *X. testudinatus*

Using FISH, it was possible to spatially identify *Sulfurovum*-related bacteria in the gills of *X. testudinatus* (Figures 6, S3). The signals from *Sulfurovum*-targeting probe EPI653 almost

completely overlapped with those from universal bacteria probe EUB338, indicating that the signals were from bacteria and further supporting our finding that the most abundant genus in gill samples is *Sulfurovum* (Figure S3). Furthermore, the bacteria labeled with a customized probe designed for *Sulfurovum* sp. NBC37-1 were widely distributed in the afferent vessels of vent crab gills, and these signals also overlapped with EPI653 signals (Figure 6). Moreover, our microscopy analysis revealed that the *Sulfurovum*-related bacteria almost occupied the cells within the afferent vessel (Figures 6C–E), and fluorescence signals were also observed in the epithelial principal cells and pilaster cells on the lamella (Figures 6A, B, S3).

Discussion

The fluids emitted by the shallow-water HVs off Kueishan Island contain high concentrations of sulfide (Fig. 2E), reduced gases and heavy metals (Peng et al., 2011). In addition to affecting the diversity, abundance, biology and ecology of phytoplankton and zooplankton (Tarasov et al., 2005; Mantha
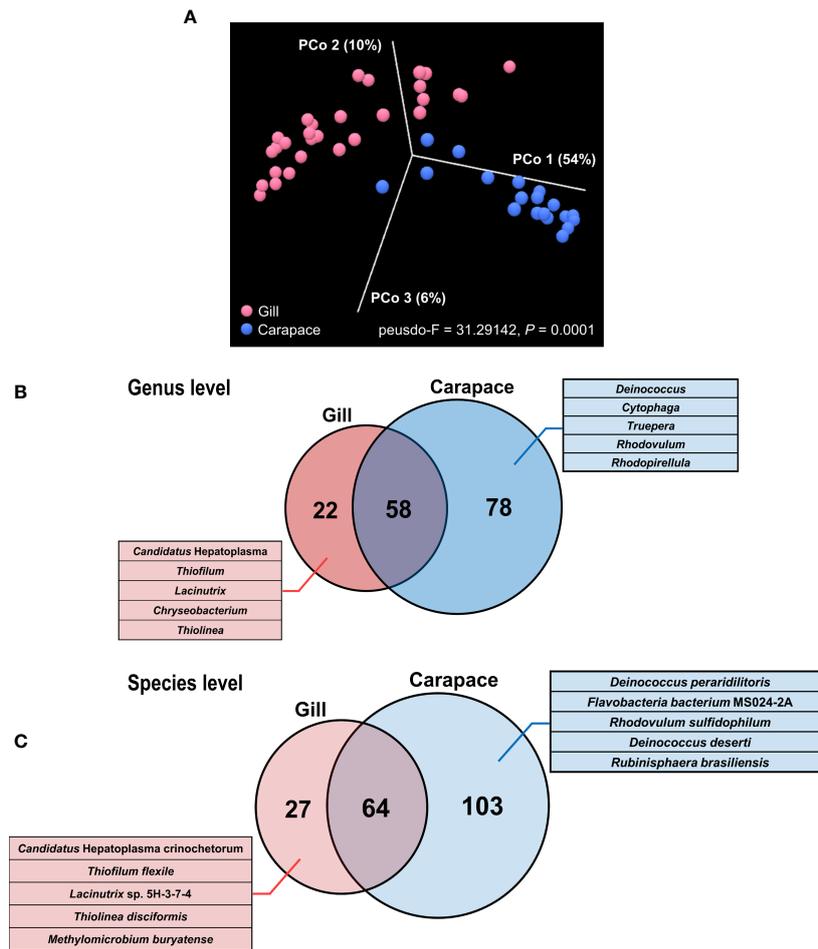
FIGURE 4
Principal coordinate analysis (PCoA) and Venn diagram of gill and carapace bacterial communities. PCoA was performed at the species level based on the Bray-Curtis distance, revealing the relative dissimilarity in bacterial community structures between the gill and carapace surface **(A)**. The Venn diagram shows the distributions of bacterial genera **(B)** and species **(C)**. Numbers of identified shared or specific groups are shown. For gills and carapace, the five most abundant specific genera and species are listed.

et al., 2013), the hydrothermal vent fluids are toxic to most organisms (Dahms et al., 2017; Lebrato et al., 2019). Thus, Kueishan Island HV provides unprecedented opportunities to study responses and adaptations at different levels of biotic integration (biosphere, ecosystem, community, population, cellular, omics-related) in a completely natural extreme environment (Dahms et al., 2018). In the present study, we continually monitored the water quality of the environment for over one year and found that the HV area located off the southeastern tip of the Kueishan Island is a highly dynamic acidic and sulfur-rich environment compared to the northwestern region (the non-vent area). Furthermore, the occurrence of photosynthesis in shallow-water HV systems may result in additional primary productivity, and higher

species diversity than that found in deep-sea HV systems (Tarasov et al., 2005). Algae are one of the common species found in the Kueishan Island shallow-water HV system, which may also serve as an important energy source, followed by bacterial chemosynthesis (Chan et al., 2016; Wang et al., 2022). Additionally, 13 species of mollusks inhabit the vent area, the majority of which are grazers that feed on algae and biofilms (Chen et al., 2018a). While a number of studies have suggested that extreme and fluctuating high sulfide environments may limit the species richness of macrofauna in deep-sea environments (Goffredi and Barry, 2002; Sahling et al., 2002); this is not the case for shallow-water HV systems of Kueishan Island. In this regard, sulfur oxidation has been proposed as a major mechanism of sulfide detoxification in
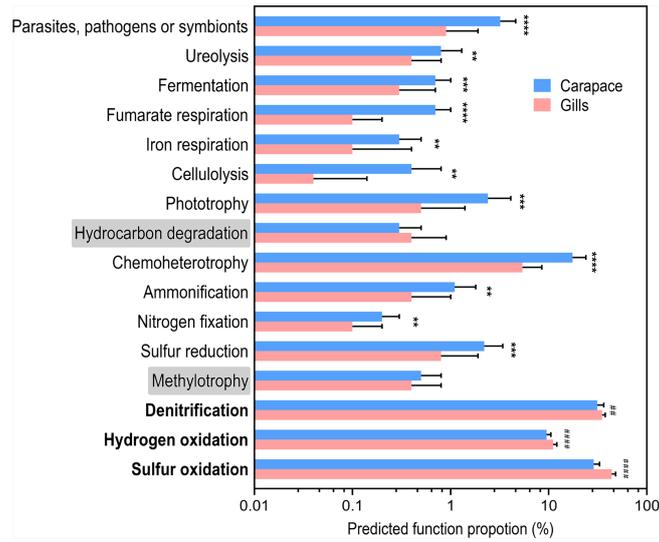
FIGURE 5
Predicted function proportions based on the functional annotation of prokaryotic taxa (FAPROTAX). The proportions of predicted bacterial functions are shown in the barplot. Values are mean + SD. Asterisks indicate groups that are significantly higher in the carapace than the gill. The hash signs indicate groups that are significantly higher in the gill than in the carapace. (Welch's t-test; **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ##$p < 0.01$, ####$p < 0.0001$; gill, $N = 29$; carapace, $N = 19$).

species either with or without bacterial symbionts (Zierenberg et al., 2000). Considering the extremely variable conditions, including the gradient of chemical and physical perturbations emanating from the vent to adjacent regions, the symbiosis of macro- and micro-organisms may provide an important platform for understanding the responsiveness of organisms to such gradients underlying interactions between the environment and endemic organisms (Chan et al., 2016).
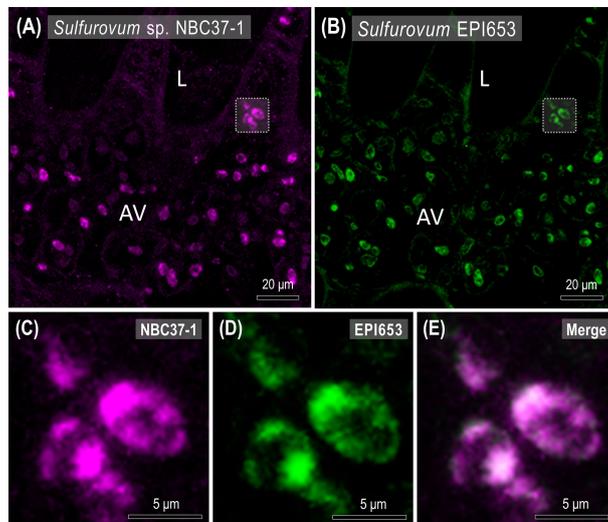
FIGURE 6
Spatial distribution of *Sulfurovum* sp. NBC37-1 in the gills of *X. testudinatus*. Fluorescence *in situ* hybridization (FISH) images show representative Cy5 signals (far-red, *Sulfurovum* sp. NBC37-1) and Alexa 488 signals (green, *Sulfurovum*-target probe EPI653) in the transverse sections of gill filaments. Scale bars = 20 μm **(A, B)** and 5 μm **(C–E)**, respectively. L, lamella; AV, afferent vessel.

Epsilonproteobacteria are dominant in the gills and carapace surface of *X. testudinatus*

We analyzed the bacterial compositions in the gills and carapace surface of shallow-water HV crab *X. testudinatus*. Notably, we identified Epsilonproteobacteria as the dominant class in both gills (73.8%) and carapace (42.2%). According to culture-based characterizations and genetic analyses, Epsilonproteobacteria are associated with the utilization of reduced sulfur, essentially as chemolithoautotrophs (Takai et al., 2003; Inagaki et al., 2004; Takai et al., 2005). Epsilonproteobacteria are also known to be a major class of symbiotic bacteria in other HV endemic creatures, such as yeti crabs (*Kiwa hirsute*), galatheid crabs (*Shinkaia crosnieri*) and vent gastropods (*Cyathermia naticoide*) (Goffredi et al., 2008; Tsuchida et al., 2011; Zbinden et al., 2015). In aquatic animals, the gills serve as an interface between the external environment and internal body fluid, playing primary roles in respiration, osmolyte transport, nitrogenous waste excretion and acid-base regulation (Henry and Wheatly, 1992; Evans et al., 2005). Despite this broad range of functions, few studies have investigated the physiological interactions between animal hosts and symbiotic bacteria in the gills. *Sulfurovum*, a genus of Epsilonproteobacteria, was especially well represented in the bacterial communities of the gills (67%) and the carapace surface (28%). Furthermore, *Sulfurovum* were abundantly labeled in the gills of *X. testudinatus* by FISH. As a SOB, *Sulfurovum* is likely to oxidize reduced sulfur (sulfide, S^{2-}) into non-toxic forms (elemental sulfur S, sulfite SO_3^{2-}, and sulfate SO_4^{2-}), mitigating the toxicity of hydrogen sulfide (H_2S) in hydrothermal vent fluids (Nelson et al., 1995; Akerman et al., 2013).

We identified a few bacterial classes in *X. testudinatus* that are also present in the ambient HV environment. These HV-associated classes include Epsilonproteobacteria, Gammaproteobacteria and Alphaproteobacteria, which have been identified in the environmental waters or fluids of either deep-sea (Adams et al., 2011; Yamamoto and Takai, 2011) or shallow-water HV systems (Giovannelli et al., 2013; Yang et al., 2016). In the sulfur-rich environment, the most abundant class was Epsilonproteobacteria, a biologically and geologically significant bacterial class (Campbell et al., 2006). In line with these findings, Epsilonproteobacteria were also found both in the surrounding water and sediments of the shallow-water HV system located off the coast of Panarea Island in Italy (Maugeri et al., 2009). Phylogenic analysis also revealed a large *Sulfurovum*-related cluster in the shallow-water HV fields of Milos Island in Greece (Giovannelli et al., 2013). Similar results were reported for water and sediment samples collected from the Kueishan Island vent area, which were dominated by *Sulfurovum* (Wang et al., 2015; Yang et al., 2016; Wang et al., 2017). According to stable isotope analysis, the bacteria samples from the vent crab's carapace and the surrounding water did not differ in ^{13}C and ^{15}N

levels, which might be potentially cognate (Wang et al., 2022). Additionally, plenty of white-colored biofilms are growing on the biotic (crab carapace, gastropod shell, algae) and abiotic surfaces (sediment, rock, or artificial fragment) in the underwater environments of Kueishan Island, which are usually fed by HV metazoans, including *X. testudinatus*. Since the vent crab is a benthic crustacean, its bacterial community on the carapace surface could be decided by the environments, particularly sulfur-depositing sediments. The Kueishan Island HV system is a vent area exposed to the intermittent discharge of vent fluids. We found extremely high concentrations of sulfide in the vent area, with dynamically dropping DO concentrations and a great accumulation of sulfide in the vent crab's hemolymph, which is also observed in marine soft sediments-inhabited priapulid worms (*Halicryptus spinulosus*) under anoxic conditions (Oeschger and Vetter, 1992). Thus, it is reasonable that a facultative symbiont of Epsilonproteobacteria-related chemoautotrophic SOB *Sulfurovum* and a sulfur-resistant species *X. testudinatus* would be found in such sulfidic extreme environment.

Biochemical processes of symbiotic bacteria species in HV crabs

In our surveys, we identified the dominant bacteria species as *Sulfurovum* sp. NBC37-1 in both the gills and carapace samples. This strain was previously isolated from the Iheya North field and had 99.1% similarity (16S rRNA) with a strict SOB (*Sulfurovum lithotrophicum* 42BKT) that was also isolated from the Okinawa Trough (Nakagawa et al., 2005a; Nakagawa et al., 2007). The high functional proportions of sulfur oxidation in our study were mainly attributable to the predominance of *Sulfurovum* sp. NBC37-1. According to our FISH results, the presence of *Sulfurovum* sp. NBC37-1 within the afferent vessel, epithelial principal cells and pilaster cells in the gills suggests an endosymbiont relationship between the SOB and the vent crab. The widespread occurrence of the SOB in *X. testudinatus* gills is reminiscent of findings made in the gills of vesicomyid clams (Cruaud et al., 2019), mytilid mussels (Halary et al., 2008; Szafranski et al., 2015; Duperron et al., 2016), and bresiliid shrimps (Tokuda et al., 2008) from hydrothermal vent habitats. Since the gills serve as a boundary between an aquatic animal's internal organs and the external environment, these tissues appear to be an enclave for SOBs to carry out sulfide detoxification and organic production for the hosts (Felbeck and Somero, 1982). Likewise, the high functional proportion of hydrogen (H_2) oxidation in the gills was probably due to *Sulfurovum* sp. NBC37-1 with hydrogenase (Nakagawa et al., 2007). It was previously reported that H_2 can also serve as an energy source in hydrothermal vent systems by powering chemosynthetic symbioses (Petersen et al., 2011). In the denitrification process, *Sulfurovum* may use nitrate (NO_3^-) as

an electron acceptor during sulfur oxidation. Then, *Nitratifractor salsuginis* can transfer electrons from H_2 into NO_3^- to generate nitrogen (N_2) (Inagaki et al., 2004; Nakagawa et al., 2005b; Vetriani et al., 2014). Intriguingly, the amino acid sequences of nitrate reductase (NapA) retrieved from a diffuse-flow HV area on the East Pacific Rise were related to *Sulfurovum* sp. NBC37-1 (44%), *Arcobacter butzleri* (14%) and *N. salsuginis* (7%), suggesting that these species may be involved in denitrification or ammonification (Smith et al., 2007; Vetriani et al., 2014). Notably, sulfur reduction and fumarate respiration were more active on the vent crab carapace surface, which had higher relative abundances of *Wolinella succinogenes* (another Epsilonproteobacterium). Previous studies demonstrated that *W. succinogenes* could oxidize H_2S or bisulfide (HS^-) coupled with fumarate respiration to form polysulfide, which is then reduced by polysulfide reductase in a periplasmic sulfur reduction process (Macy et al., 1986; Krafft et al., 1995; Hedderich et al., 1998; Kroger et al., 2002). Moreover, *W. succinogenes* contains a periplasmic NapA and a cytochrome c3 that are respectively involved in nitrate and nitrite reduction as an ammonification strategy, the functional proportion of which was higher on the carapace surface of the vent crab (Bokranz et al., 1983; Blackmore et al., 1986; Lovley et al., 1999; Simon, 2002; Simon et al., 2003; Strohm et al., 2007).

Among Gammaproteobacteria, *Leucothrix mucor* was the dominant species, as it had a higher relative abundance on the carapace surface than in the gills. Previous studies have shown that the presence of *L. mucor* on the surfaces of marine algae, fish eggs and aquatic invertebrates may occur as an infestation (Johnson et al., 1971; Payne et al., 2007). This filamentous heterotrophic bacterium is also known to be a sulfur oxidizer that utilizes reduced sulfur compounds as energy sources (Grabovich et al., 1999). The Gammaproteobacteria-related symbionts of vent shrimps (*R. exoculate*) were also dominated by *L. mucor* (Petersen et al., 2010). Another Gammaproteobacterium, *Psychromonas ingrahamii* was also more abundant on the vent crab carapace. Genomics analysis of *P. ingrahamii* revealed several reductases for nitrate, nitrite, sulfite, fumarate and fermentation-related enzymes, while culture studies further demonstrated that *P. ingrahamii* is a psychrophilic extremophile with the ability to perform nitrate reduction, fumarate utilization and fermentation (Auman et al., 2006; Riley et al., 2008; Markowitz et al., 2012). However, *P. ingrahamii* have mostly been isolated or identified from low-temperature marine environments, such as sea ice and polar sediments (Breezee et al., 2004; Auman et al., 2006; Canion et al., 2013). To the best of our knowledge, this is the first study to identify *P. ingrahamii* in a hydrothermal HV system. The *Hydrogenvibrio* sp. Milos-T1, which also had a higher abundance on the carapace, was previously isolated from a shallow-water HV system near the island of Milos in Greece and was closely related to *Hydrogenovibrio marinus*, an extremophilic hydrogen-oxidizing bacterium (Nishihara et al.,

1997; Brinkhoff et al., 1999). In addition to hydrogen, *H. marinus* utilizes reduced sulfur compounds as electron donors, and it uses ammonium and urea as sole nitrogen sources, but not N_2, nitrate or nitrite (Nishihara et al., 1991). The fact that ureolysis is performed much more actively on the carapace may be associated with a higher abundance of *Hydrogenvibrio* sp. Milos-T1.

Among Alphaproteobacteria, two species were identified. *Pseudovibrio* sp. FO-BEG1 and *Ruegeria* sp. TM1040, both of which were more abundant on the carapace. It was precisely the same that *Pseudovibrio* sp. FO-BEG1 was shown to be capable of sulfur oxidation, denitrification and fermentation as *Pseudovibrio denitrificans*, a representative species of the genus (Shieh et al., 2004; Romano, 2018). There is evidence that *Psedovibrio*-related bacteria may exhibit symbiotic relationships with several invertebrates, such as corals, sponges and tunicates (Fukunaga et al., 2006; Riesenfeld et al., 2008; Rypien et al., 2010; Santos et al., 2010; Penesyan et al., 2011). Nevertheless, this appears to be the first study to identify *Psedovibrio* in a crustacean. In the case of *Ruegeria* sp. TM1040 (formerly *Silicibacter* sp.), which is typically found in the phycosphere or on the surface of dinoflagellates, genomic analysis showed no relevant genes for sulfur oxidation, denitrification or ammonification, but dimethulsulfonioproponate (DMSP) demethylase was shown to metabolize DMSP which is produced by dinoflagellates and comprises a major source of organic sulfur in the ocean (Moran et al., 2007; Belas et al., 2009).

Flavobacteriia are known as polymer degraders and are found in a wide range of environments, including seawater and sediments (Alonso et al., 2007; Kappelmann et al., 2019). The relative abundances of *Leeuwenhoekiella* sp. MAR_2009_132 and *Ornithobacterium rhinotracheale* were both higher in the gills of *X. testudinatus* than on the carapace surface. Among these two constituents, *Leeuwenhoekiella* sp. MAR_2009_132 was previously isolated near the Island Sylt in the North Sea and was closely related to *Leeuwenhoekiella aequorea* (Hahnke and Harder, 2013; Chen et al., 2018b). Surprisingly, *O. rhinotracheale* is generally known as a species that causes respiratory disease in poultry (Barbosa et al., 2019). In our literature searches, we found no studies on this species related to marine organisms. Only two studies described the detection of *O. rhinotracheale* in seawater samples, but each provided limited information (Valenzuela-Gonzalez et al., 2016; Porchas-Cornejo et al., 2017). On the other hand, *Polaribacter* spp. and *Tenacibaculum maritimum* were more abundant on vent crab's carapace. *Polaribacter* sp. Hel_33_49 and Hel_I_88 (also named Hel1_88) were first isolated from the North Sea during a spring bloom and were reported to feed on polysaccharides and proteins with carbohydrate-active enzymes (CAZymes) and peptidases (Zerillo et al., 2013; Xing et al., 2015). In the case of *T. maritimum*, a prominent pathogen without known host specificity, genomic analysis revealed similar metabolism preferences (saccharide and protein degradation) to those seen in other Flavobacteriia-related bacteria, such as *Polaribacter* spp. (Fukunaga et al., 2006; Perez-Pascual et al.,

2017). The general consensus is that Flavobacteriia subsist on abundant organic matter in the epipelagic zone, which may explain why deep-sea HV systems usually show no or very low proportions of Flavobacteriia-related bacteria in contrast to the shallow-water HV system of Kueishan Island (Goffredi, 2010; Salazar et al., 2016; Cerqueira et al., 2017; Leinberger et al., 2022). Nevertheless, Flavobacteriia were not abundant in other shallow-water HV systems (Rajasabapathy et al., 2018). Since Kueishan Island lies to the southern rifting end of the Okinawa Trough (a back-arc depression of the Ryukyu arc-trench system), the active volcanic features cause unstable hydrothermal activities to occur in the geologically "young" vent system (Konstantinou et al., 2013; Deffontaines et al., 2022). The fluids spilling out of the vents around this young volcanic island aperiodically alter the surrounding water chemistry beneath the sea surface. In addition, frequent weather and current disturbances of typhoons, monsoons and the Kuroshio may cause strong turbulent mixing in Kueishan Island HV system (Liang et al., 2003; Lebrato et al., 2019). These climate features further contribute to the establishment of the marine ecosystem near Kueishan Island, off the northeastern coast of Taiwan. The combination of climate, status as a neritic zone (providing sufficient organic matters) and presence of an extreme HV system (emitting minerals, sulfur compounds, high temperature and low pH) make Kueishan Island a truly unique natural laboratory.

Conclusions

HV systems have taken a place in many critical biogeochemical processes that occur in the modern ocean (Tarasov et al., 2005) and were demonstrated to be an oasis of life in the deep sea, as the area is nourished by chemoautotrophic bacteria. Several known marine chemoautotrophic bacteria utilize sulfide and other reduced sulfur compounds for carbon fixation in food-limited and sulfide-rich deep-sea HV systems. Until recently, most studies on the sulfur detoxification mechanisms in marine species have been focused on those species inhabiting deep-sea HV systems of the mid-ocean ridge (Zierenberg et al., 2000; Zhang et al., 2017). Due to the limited accessibility and difficulty of keeping deep-sea organisms, such mechanisms on a holobiont cannot be practically studied in the deep-sea HV system. Thus, *X. testudinatus* crustacean holobiont residing in the shallow-water HV system at the south end of the Okinawa Trough, Kueishan Island serves as a unique experimental proxy.

HV areas are comprised of submarine fissures at the planet's surface from which geothermally heated water is expelled. These areas are often characterized by variable and extreme physicochemical conditions, as well as the presence of microorganisms that can utilize and supply energy through sulfur-oxidizing processes. Due to the relatively low cost and ease of access to this shallow-water HV system, we are able to carry out a long-term monitoring with frequent observations rather than relying on snapshot estimates. Moreover, our current datasets reveal the bacterial community structure in the gills and the carapace surface of the vent crab *X. testudinatus*. These datasets are derived from the combination of field-based experiments and omic-based approaches, revealing the chemolithotrophic SOB *Sulfurovum* sp. NBC37-1 (an Epsilonproteobacterium) as the most dominant bacteria. Additionally, symbiotic processes involving hydrogen oxidation are also expected to occur in the wake of substrate respiration and reduction (*e.g.*, fumarate and nitrogen oxides), thus supplying energy to metazoans in the sulfidic environments. This study provides profound insights into the mechanisms of bacteria-crustacean symbiosis from the vent ecosystem and demonstrates the significance of bacteria in marine holobionts with implications for the evolution of inter-kingdom symbiosis in Kueishan Island shallow-water HV system, one of the planet's most extreme environments.

Data availability statement

The datasets in this study are deposited in the NCBI Sequence Read Archive (SRA). The 16S rRNA metagenome sequences from each tissue sample can be found according to accession number PRJNA858606 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA858606).

Authors contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer BC declared a shared affiliation with the authors LC and M-CW to the handling editor at the time of review

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.976255/full#supplementary-material

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