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Discovery of a novel sulfur-oxidizing endosymbiont (Ca. Vesicomyosocius atacamensis) associated with a newly described Archivesica species from the Atacama Trench.

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Abstract

Here we report the microbiome composition and lipid (molecular and isotopic) profile of gills from *Archivesica* sp. Atacama., a new species of deep-sea bivalve family Vesicomyidae collected at 2839 m depth on the eastern slope of the Atacama Trench. Metabarcoding unveiled that 99.44% of the microbial ASVs (Amplicon Sequence Variant) obtained from this bivalve's gills belonged to *Ca. Vesicomyosocius* sp. atacamensis, a bacterium closely related to symbionts of other vesicomycoids based on the 16S rRNA phylogeny (a putative chemoautotrophic sulfide-oxidizing bacterium Form I RubisCO). Additional ASVs included microbes from taxa known for their ability to oxidize sulfur. Consistent with the microbiome composition, the analysis of lipid biomarkers in the gills revealed a high abundance of $C_{16:1\omega7}$ and $C_{18:1\omega7}$ fatty acids, well-known markers of sulfide-oxidizing (thiotrophic) bacterial metabolisms. The $\delta^{13}C$ values of the bivalve's bulk gills (-35.5‰) and of individual fatty acids (-40.0 to -46.5 ‰) were typical of bivalves hosting thiotrophic endosymbionts utilizing form I RubisCO for carbon fixation. In addition, nearby sediments showed a significant presence of terminal branched (*iso*/*anteiso* C_{13}-C_{17}), mid branched (10Me-16:0 and 10Me-18:0) and cyclopropyl (Cy_{17} and Cy_{19}) fatty acids, coherent with sulfate-reducing bacterial (SRB) communities found by metabarcoding. These findings confirm that thiotrophic symbiosis provides energy for the new deep-sea *Archivesica* bivalve reported here.

Introduction

The Atacama Trench, located in the South Pacific Ocean by the coast of Chile, was created by the subduction of the Nazca Plate beneath the South American Plate[1]. With an approximate length of 5900 km, and a mean width of about 65 km, it has a maximum depth of 8065 m in front of the Mejillones Peninsula[2]. Chemosymbiotic invertebrates (mussels, clams, tube worms, etc.) inhabit different types of deep-sea cold seeps, hydrothermal vents, and other chemosynthetic habitats around the world and obtain their energy through symbiotic relationships with chemosynthetic bacteria[3-7]. These symbionts use reduced compounds such as hydrogen sulfide, thiosulfate, hydrogen, or methane as electron donors, with oxygen generally serving as the terminal electron acceptor, and supply their host with fixed carbon[8]. In return, the host provides the bacterial symbionts with a protected environment, as well as access to necessary electron donors and acceptors[8]. The first discovery of bivalves (mussels) harboring thiotrophic symbionts was reported at a hydrothermal vent site in the Galápagos Rift zone[9], with subsequent findings at other chemosynthetic habitats revealing the presence of abundant intracellular methanotrophic bacteria in mussel gills[3]. In the case of vesicomyid clams the main symbionts associated are thiotrophic bacteria which oxidize sulfide, providing essential nutrients to their hosts in nutrient-poor environments[3]. Across marine invertebrates, the phylogenetic diversity of chemoautotrophic bacterial symbionts is influenced by host species, environmental characteristics, and geographic location[9-15], with the associations with their hosts studied through electron microscopy, enzymatic assays, NGS (Next Generation Sequencing), molecular and compound-specific isotopic analysis of lipid biosignatures[16]. Lipid biomarkers are valuable tools for characterizing symbiotic bacteria in host tissues, with the analysis of their stable-carbon isotopic composition being especially effective for investigating nutritional sources in vent and seep ecosystems, as different carbon fixation pathways leave distinct isotopic signatures[17,18]. Unlike other cold-seep ecosystems based on

the presence of methane or hydrogen, here we report the microbiome and the molecular and isotopic fingerprint of lipids from a symbiotic system based on sulfur-oxidizing bacteria sheltered by a new species of the *Archivesica* genus of deep-sea bivalves found at the eastern slope of the Atacama Trench.

Results and discussion

Between 24 May and 6 June of 2024, an international team conducted an oceanographic expedition off the coast of Antofagasta onboard the SOI (Schmidt Ocean Institute) vessel (R/V) Falkor (too) (Fig. 1A). The main goal of the FKt240524 expedition "*Unveiling the Living-Fossil Ecosystems of the Atacama Trench*" was to investigate the deep-sea ecosystems inhabiting the eastern slope of the Atacama Trench with modern technologies (including SuBastian, a deep-sea rover onboard SOI vessel able to reach depths up to 4.5 km).

At one of the surveyed sites (Site 9.2; Fig. 1A), we observed several large bivalves, approximately 20 cm in length, distributed in distinct patches across the study area (Fig. 1B-D). Morphological analyses indicate that these specimens represent a new species within the genus *Archivesica* (family Vesicomyidae) (Fig. 2). The genus *Archivesica* currently comprises 18 species widely distributed in deep-sea environments, typically associated with hydrothermal vents and cold seeps. Among these, 12 species exhibit ovate or semi-ovate shells, in which shell length is equal to or less than twice the shell height. Two species, *A. extenta*[19] and *A. tsubasa*[20], are markedly elongated, with shell length exceeding three times the height. Three additional species (*A. garuda*[21], *A. magnocultellu*[22], and *A. similaris*[23]), display intermediate elongation, with length between two and three times the height.

The new species described here falls within this intermediate "elongated" group, with a height-to-length (H/L) ratio of approximately 0.35–0.38. Although a similar ratio is observed *in A.*

similaris, the overall shell morphology differs: the posterior end in the new species is only slightly acute, whereas it is broadly rounded in *A. similaris*.

Diagnosis. Shell large (up to 20.9 cm in length), robust, with a rounded anterior end and a slightly acute posterior end. Sculpture consists of fine concentric rings. Ventral cardinal tooth large and rounded; subumbonal cardinal tooth not basally fused. Pallial sinus short.

Description. Shell large (19.7–20.9 cm), elongated, with H/L ratio ≈ 0.35, and subovate in outline. Posterior region lower than anterior; ventral margin concave. Shell chalky white with fine concentric sculpture and a posterior radial line (Fig. 2A); periostracum grey-brown. Distance from umbo to anterior margin approximately 0.2 of total length; posterior lamellar ligament extending to about 0.8 of shell length; fibrous ligament about 0.2 of total length. Umbones prosogyrous and not in contact (Fig. 2B, C).

The internal shell surface is white, with glossy muscle scar margins and radial striations (Fig. 2D). The pallial line is slightly impressed anteriorly, extending from the posterior margin of the anterior adductor muscle scar to the region of the posterior adductor scar, and includes a shallow pallial sinus with irregular margins. The anterior adductor scar is ovate with a triangular dorsal portion and a more strongly marked posterior margin; secondary pallial attachment scars are faint. The posterior adductor scar is larger, subcircular, and well defined. The anterior pedal retractor scar is deeply impressed and fused with the adductor scar. The nymph is low, approximately one-fifth of total shell length.

Dentition. In the right valve, the cardinal tooth is strong and semi-spherical posteriorly, with a narrow triangular posterior ramus that is not fused to the cardinal tooth (Fig. 2E). In the left valve, the subumbonal cardinal tooth bears two subequal, unfused rami; the anterior ramus is slightly smaller and thinner than the posterior, forming an angle of approximately 50° between their inner margins (Fig. 2A, B, F).

Maximum-likelihood phylogenetic analyses based on mitochondrial (COI, 16S rRNA) and nuclear (28S rRNA) gene markers confirm that this taxon represents a distinct new species within the genus Archivesica (Fig. 3) (The formal taxonomy of the clam is to be presented in a dedicated taxonomic publication "Guzmán et al., manuscript in preparation")

Metabarcoding using primers targeting bacterial, archaeal, and eukaryotic 16S and 18S rRNA gene sequences from inside the bivalve gills revealed that 99.44% of the 241,294 detected ASVs corresponded to a single ASV. Phylogenetic analysis of a 1,499 bp fragment of the 16S rRNA gene identified this dominant ASV as a novel species, *Ca. Vesicomyosocius* atacamensis sp. *nov*. (Fig. 4), belonging to the recently proposed genus Vesicomyosocius[24]. *Vesicomyosocius* (Gammaproteobacteria), is a genus of chemoautotrophic sulfide oxidizing bacteria (SOB) having the smallest reported genome (1.02 Mbp) of autotrophic bacteria[24]. Found as an intracellular symbiont in the gills of deep-sea bivalves such as *Archivesica*, *Calyptogena*, *Ectenagena*, and *Phreagena*, the reported *Vesicomyosocius'* genome[24] contains the genes for sulfide-quinone oxidoreductase (*sqr*), the dissimilatory siroheme sulfite reductase complex (*dsr*), the adenosine 5'-phosphosulfate reductase (*apr*), the ATP sulfurylase (*sat*), and a partial sulfur oxidizing multienzyme SOX system. The SOX gene cluster includes soxXYZA and soxB, but lacks soxCD, an operon required for the complete oxidation of thiosulfate to sulfate. This genomic configuration suggests that *Ca. Vesicomyosocius* sp. atacamensis has a similar sulfur-based energy metabolism.

Consistent with this finding, *Ca. Vesicomyosocius* sp. atacamensis is part of a clade of endosymbionts found inside *Archivesica* bivalves, whose closest species, *Candidatus Vesicomyosocius marissinica*, is an endosymbiont of *Archivesica marissinica*[25]. *Candidatus Vesicomyosocius marissinica* encodes the complete pathways necessary for chemoautotrophic metabolism (SOX and Dsr systems, carbon fixation, and biosynthesis for 20 common amino

acids), thus providing an important input for the host's nutrition[25]. Nevertheless, this endosymbiont lost most regulatory genes for amino acid biosynthesis, indicating its reliance on the host for transcriptional control[25].

In addition, the remaining ASVs (<1%; Fig. S1) comprised a diverse range of bacteria, including several ASVs affiliated with bacterial genera known for their sulfur-oxidizing capabilities (*Sulfurovum*, *Sulfurimonas*, and *Thioprofundum*) although it remains unclear whether these taxa represent true symbionts or bacteria filtered from the surrounding sediments. *Sulfurovum* is a mesophilic chemolithoautotrophic bacterium found in a hydrothermal system in the mid-Okinawa Trough, Japan, which couples the oxidation of sulfur compounds to the reduction of nitrate or molecular oxygen[26]. *Sulfurimonas* is a chemolithoautotrophic Campylobacterota found in deep-sea vents and marine sediments, commonly observed in symbiosis with other organisms and able to use a wide variety of electron donors for growth including sulfide, sulfur, thiosulfate, and sulfite[27,28]. Lastly, *Thioprofundum* is an obligately chemolithoautotrophic, sulfur-oxidizing gammaproteobacterium which uses thiosulfate, sulfur, or tetrathionate as electron donors, and has been found in a hydrothermal field of Suiyo Seamount on the Izu-Bonin Arc in the Western Pacific Ocean[29].

Two ASVs phylogenetically close to sulfate-reducing bacteria (SRB) were also present in the bivalve's gills, likely derived from the surrounding sediments: *Desulfobacula*, a mesophilic, sulfate-reducing bacterium isolated from coastal sediments in the Baltic Sea is able to use sulfate, thiosulfate and sulfite as electron acceptors[30]. The other ASV was a member of the Desulfobulbaceae family (of the phyla Thermodesulfobacteriota), which is largely comprised of anaerobic bacteria that reduces sulfates to sulfides to obtain energy[30,31]. Known as "cable bacteria", they have been reported to transport electrons from sulfide-rich sediments to the oxygen rich sediment in contact with overlying seawater[32,33].

A number of other less-abundant bacterial ASVs in the bivalve's gills sample are worth mentioning, as they have been reported in deep sea environments across the world; *Oscillibacter* sp. (Oscillospiraceae), found as part of the microbiome of Japanese *Corbicula* bivalves[34]; *Euzebya* sp. (Euzebyaceae) part of the microbiome of the sea cucumber *Holothuria edulis*[35]; *Arcobacter* (Arcobacteraceae), a bacterial genus containing species like *A. sulfidicus*, an obligate microaerophile that oxidizes and producer of filamentous sulfur[36]; *Micrococcus* (Micrococcaceae) a genus with species found in deep-sea sediments at 4,448 m deep in the Mariana Trench[37]; *Spongiibacter* (Spongiibacteraceae) obtained from a sulfur-rich benthic habitat in the South China Sea[38]; *Psychrobacter* (Moraxellaceae), typically found in deep sea environments[39], and *Alkanindiges* (Moraxellaceae), found in sulfur-rich benthic habitats and brine pools[40].

As part of the less abundant ASVs, there is also a variety of bacteria found as gut microbiota in a range of other animals, including species from genera such as *Mailhella* (Desulfovibrionaceae)[41], *Butyricimonas* (Odoribacteraceae)[42], *Megamonas* (Selenomonadaceae)[43] and *Tidjanibacter* (Rikenellaceae)[44], to mention a few. It remains to be determined whether all these sequences correspond to intracellular chemosynthetic symbionts, or instead to environmental microbial species from a sulfur rich environment acquired as water passes over the gills during respiration. Nevertheless, the predominance of taxa related to sulfur oxidation indicates that sulfur metabolism likely plays a central role in this microbial assemblage.

The mineral composition of the surface sediments inhabited by this bivalve has recently been reported by us[45]. Briefly, the sediments are dominated by silicates, including quartz, plagioclase (albite), amphibole (actinolite) and micas (chlorite, phlogopite), all common constituents of igneous and sedimentary rocks of the nearby coastal areas. In addition,

scanning electron microscopy (SEM) has revealed the presence of small crystals of carbonates (dolomite, calcite) and sulfides (pyrite) that were found to be authigenic, suggesting an intense cycling of inorganic carbon and sulfur in the inspected sediments coherent with the clam and its symbiont here reported. In contrast to the bulk sediments, inside the bivalve's gills we only found elemental sulfur in the form of hexasulfur-S6, heptasulfur-S7, and octasulfur-S8 (Fig.5), as found by GC-MS analysis, again suggesting the presence of an active sulfur cycle inside the bivalve's gills.

The molecular and isotopic analysis of lipid biosignatures was consistent with the cohort of SOB and SRB found by DNA metabarcoding in the bivalve's gills. The total concentration of fatty acids in the bivalve's tissue was two orders of magnitude higher (2981.3 $\mu g \cdot g^{-1}$ dw) than in the surrounding sediments (13.6 $\mu g \cdot g^{1}$ dw) (Table S1). The fatty acids profile was different in the bivalve versus the sediments (Fig. S2). In the sediments, the range of linear and saturated (*normal*, *n*) fatty acids was greater ($C_{12:0}$ to $C_{28:0}$) than in the bivalve's gills ($C_{14:0}$ to $C_{20:0}$). In both samples, the predominant fatty acid was $C_{16:0}$, followed by different fatty acids in the bivalve ($C_{14:0}$) or the sediments ($C_{18:0}$, $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$) (Fig S2). Terminal (*i*/*a* C_{13}, C_{14}, C_{15}, C_{16} and C_{17}) or mid chain (10Me-16:0 and 10Me-18:0) branched fatty acids, as well as cyclopropyl fatty acids (Cy_{17} and Cy_{19}) were only in the sediment sample (Table S1).

The profile of unsaturated fatty acids was also different in the bivalve and sediment samples. In the sediments only mono-unsaturated fatty acids (MUFAs) of 16,18 and 20 carbons were found, with the most abundant being $C_{16:1\omega7}$ (1.655 $\mu g \cdot g^{-1}$ dw) and $C_{18:1\ \omega9}$ (0.845 $\mu g \cdot g^{-1}$ dw). In contrast, in the bivalve's gills a wide variety of MUFA and polyunsaturated fatty acids (PUFA) of 14, 16, 18, 20, and 22 carbons were found (Table S1), including $C_{16:1\omega7}$ (739.1 $\mu g \cdot g^{-1}$ dw) and $C_{20:1\omega7}$ (385.7 $\mu g \cdot g^{-1}$ dw) as the most abundant (Fig S2). No PUFA were detected in the sediment sample (Fig. S2, Fig. S3).

The organic matter of the bivalve's gills was isotopically depleted (i.e. lower delta values) relative to the surface sediments where it was found (Fig. 6). Both the bulk organic carbon ($\delta^{13}C_{TOC}$) and total nitrogen ($\delta^{15}N$) showed lower values (i.e. more depleted in heavier isotopes ^{13}C and ^{15}N respectively) in the bivalve ($\delta^{13}C_{TOC}$ of -35.5 ‰ and $\delta^{15}N$ of 2.9 ‰) than in the sediments ($\delta^{13}C_{TOC}$ of -22.0 ‰ and $\delta^{15}N$ of 11.9 ‰) (Table S2). Furthermore, the individual fatty acids showed more negative $\delta^{13}C$ values in the bivalve (-40.0 to -46.5 ‰) than in the sediments (-25.2 to -38.6‰) (Table S2), statistically significant at $p<0.0001$. Overall, the sedimentary organic matter was enriched in ^{13}C by ~13‰ relative to the bivalve (Fig. 6).

Compound-specific isotope analysis (CSIA) of clam fatty acids revealed distinct isotopic signatures that discriminate between symbiont- and host-derived lipids. The most $\delta^{13}C$-depleted fatty acids ($C_{14:0}$, $C_{16:1\omega7}$, and $C_{18:1\omega7}$) are consistent with a symbiotic bacterial origin, whereas the more $\delta^{13}C$-enriched fatty acids ($C_{16:0}$, $C_{18:0}$, and $C_{18:1\omega9}$) are attributed to host biosynthesis. Sulfide-oxidizing bacteria are characterized by lipid biomarkers dominated by $C_{16:1\omega7}$ and $C_{18:1\omega7}$[46,47,48], which are abundant in the bivalve gills (24.8% and 3.26%, respectively). The prevalence of these biomarkers provides strong evidence for a substantial contribution of thiotrophic symbionts to the gill lipid pool, consistent with observations in other chemosymbiotic deep-sea mussels[49,50]. Other fatty acids (C_{20} and C_{22}) characteristic of algal biomass are most likely taken up and stored in the tissues of the bivalve after heterotrophic consumption[47]. $\delta^{13}C_{TOC}$ values found in the bivalve tissue (-35.5‰) are typical of deep-sea bivalves containing sulfide-oxidizing symbionts[47,51]. Interestingly, tissues of Vesicomyid bivalves show similar values of $\delta^{13}C$ around the world[47,52,53], suggesting that this family of deep-sea bivalves uses the same inorganic carbon source (DIC) at different locations, with little isotopic variations. The *Archivesica* bivalve reported here uses dissolved inorganic carbon (DIC) for calcification, as the carbon isotopic composition in seawater has a consistent $\delta^{13}C$ composition around 0 ‰[54]. Using a basic mixing model, we estimate the symbiont derived

carbon in the clam, using three different values of the symbiont carbon fraction (0.3, 0.5 and 0.8) (Table S3). The isotope mixing model indicates that low assumed symbiont contributions (*fsym* = 0.3–0.5) yield highly depleted $\delta^{13}C$ values for the symbiont carbon source (−67‰ to −49‰), which fall outside our isotopic ranges. In contrast, a higher contribution (*fsym* ≈ 0.8) produces a more plausible estimate (≈ −39‰), closer to our carbon values. These results suggest that symbionts provide the dominant carbon input to host clam lipids, and that lower contribution scenarios are unlikely. Overall, the model supports a strong reliance of the host on symbiont-derived carbon. In addition, we plot the $\delta^{13}C$ of the bulk clam tissue against the $\delta^{13}C$ bacterial fatty acids ($C_{14:0}$, $C_{16:1\omega7}$ and $C_{18:1\omega7}$). Plotting $\delta^{13}C$ of bulk tissue against $\delta^{13}C$ of bacterial fatty acids revealed a strong positive relationship (R^2 = 0.9855), indicating that variation in host tissue isotopic composition is almost entirely explained by changes in symbiont-derived carbon (Fig S4). This tight correlation supports the mixing model results, confirming that symbionts are the dominant carbon source for the host clam. The close coupling between bulk tissue and bacterial fatty acids suggests efficient transfer of symbiont-fixed carbon into host lipids, with minimal influence from alternative carbon sources. Overall, both the regression and mixing model consistently point to a high reliance on symbiont-derived carbon.

Thioautotrophic and methanotrophic nourishment have been identified as the dominant nutritional strategies at deep-sea hydrocarbon seeps, leading to distinct isotopic signatures of sulfide-derived sulfur and methane-derived carbon[55-57]. Because the gills of deep-sea bivalves house the symbiotic bacteria, their isotopic composition (typically from -51 to -37 ‰) similar to values in this study, reflects a combined signature of both the host and its symbionts[58-60].

Furthermore, the metabolic fingerprint of chemoautotrophic symbionts in deep-sea environments (i.e., methanotrophic or thiotrophic) can be clearly distinguished based on their isotopic signatures in bulk organic matter (i.e. $\delta^{13}C$ of total organic carbon versus $\delta^{15}N$ of total

nitrogen; Fig. 6). The chemoautotrophic metabolisms that sustain the symbiotic systems of different deep-sea environments[55,56], including the Atacama Trench, cluster separately and differ from the isotopic fingerprint of the organic matter in the surrounding sediments, which receives biomass debris falling from the overlying water column (Fig. 6). Also, a clear separation was detected between different types of chemotrophic symbionts in seep environments, gills tissues of the bivalve, the nearby sediment samples and other data reported elsewhere[62] (Fig. 6). Higher $\delta^{13}C$ isotopic fractionation values (>50 ‰) have been reported in the organic matter of bivalves symbiotically associated with methanotrophic bacteria in comparison to that of bivalves associated with thiotrophic bacteria (typically >25‰)[55].

The nitrogen ($\delta^{15}N$) values in the bivalve's gills from the Atacama Trench was similar (±3‰) to values reported in another study[62], which suggests that the bivalves are using a local source of nitrogen (e.g. ammonium assimilation) rather than particulate organic nitrogen coming from the water column. This is in good agreement with the detection of ammonium in pore waters of the sediments hosting the bivalve. Although ammonium assimilation enzyme activity has been identified in the hydrothermal vent bivalve *Turneroconcha magnifica*[61], the understanding of nitrogen uptake and utilization in the thiotrophic symbiosis of the *Archivesica* bivalve reported here remains poorly understood.

In this study, the detection of sulfur compounds in the bivalve's gills is in agreement with previous reports from bivalves with thiotrophic endosymbionts that store elemental sulfur within their gills cells[63]. The $\delta^{13}C$ compound-specific isotopic analysis of fatty acids (Fig. S5) can be used to elucidate different carbon fixation pathways operating in seep environments[64]. Due to the distinct isotopic fractionation associated with various carbon fixation pathways, stable isotope signatures have proven particularly valuable for identifying the nutritional strategies of organisms in hydrothermal vent and sulfur-rich benthic habitat environments[56,61]. Highly negative carbon isotope values (e.g., $\delta^{13}C < -50$‰) in deep-sea bivalve tissues are

typically indicative of methane-derived carbon sources, as methane itself often exhibits $\delta^{13}C$ values ranging from -60‰ to -70‰ or lower[56,61,65]. On the other hand, carbon fixation driven by sulfide oxidation can be done using the Calvin-Benson-Cycle (CBB), other bacteria can used the rTCA pathway[66]. The first pathway results in $\delta^{13}C$ values between -27‰ and -37‰[16], whereas rTCA pathway can produce significantly heavier $\delta^{13}C$ signatures, typically between -9‰ and -16‰[67,68]. The depleted values found for the $\delta^{13}C$ bulk gills of the clam, suggest Calvin-Benson-Cycle (CBB) pathway for autotrophy. The symbiotic bacterial fatty acids (mean -45‰) are consistent with the values reported for SOB in seep-dwelling bivalves in the South China Sea[69], where a range of values from -40‰ to -51‰ was recorded for *Archivesica marissinica* and a range of -37‰ to -50‰ was obtained for *Bathymodiolus aduloides*, evidencing the symbiosis with thiotrophic bacteria.

The absence of other types of energy sources, such as methanotrophic nutrition or methanogenic activity in *Archivesica* sp. Atacama is suggested by several lines of evidence. First, no methanotrophic bacteria were found with NGS metagenomics of the bivalve's gills. Second, the lower bulk carbon isotopic values found in the bivalve biomass ($\delta^{13}C_{TOC}$ of -35‰) are greater than the very depleted values normally found in methane cold-seeps ($\delta^{13}C$< -50‰) with host methanotrophic symbionts[16]. Third, the carbon isotopic composition of fatty acids in the bivalve's gills is heavier than what was reported in sulfur-rich benthic habitat bivalves living with methanotrophic bacteria ($\delta^{13}C$ >-60 or -70‰)[47]. Fourth, no lipids biomarkers specific for methanogenic archaea (2,6,10,15,19-pentametylicosane or PMI, archaeol, crocetane) were found in either the bivalve or surrounding sediments.

Sediments in seep environments reflect the products of *in situ* microbial metabolic processes. In the Atacama Trench site 9.2, the detection of relevant amounts of terminal branched (*i*/*a* C_{13} to C_{17}), mid chain branched (10Me-C_{18} and 10MeC_{18}) and cyclopropyl (Cy_{17} and Cy_{19}) fatty acids in the sediments where the bivalve was collected suggest the presence of SRB. In

particular, the detection of the fatty acids 10Me-C_{16} and Cy_{17} has been described in families containing known SRB representatives such as the Desulfobacteraceae, Desulfovibrionaceae, and Desulfobulbaceae[70-72]. In addition, the fatty acids *i*/*a*-C_{15} and 10Me-C_{18} are abundant in the Desulfohalobiaceae family[73,74]. All these SRB biosignatures reflect a carbon isotopic composition (ca. -30‰; Table S2) characteristic of the use of Rubisco type I (i.e. Calvin-Benson cycle). Since the SRB that perform autotrophy produce biomass that is relatively depleted in ^{13}C compared to the Calvin cycle (i.e. $\delta^{13}C$ from -30‰ to -44‰), the compound-specific $\delta^{13}C$ measured in the sediments for *i*/*a*-C_{15}, Cy_{17} and 10Me-C_{18} (-30.1 to -30.8 ‰) point rather to heterotrophic SRB reflecting the Calvin isotopic signature of their substrate.

In anaerobic sediments, sulfate reducers use organic debris of algae and phytoplankton falling from the water column and on small molecules left over from the decomposition of organic biosignatures by other types of heterotrophic bacteria. In addition to this heterotrophic SRB bacterial, some potential autotrophic SRB using the Wood-Ljungdahl or rTCA pathway could be present. Our recently reported metatranscriptomic analyses of the surrounding sediments further confirm the absence of methane-driven metabolisms[75], in which we show the high abundance of the SOX operon[75], the dissimilatory sulfate reduction and the assimilatory sulfate reduction pathways, among others[75].

The presence of sulfide-oxidizing bacteria found by the microbiome analysis is also evident due to the presence of $C_{16:1\omega7}$ and $C_{18:1\omega7}$ in the bivalve's gills. In the sediments, additional inputs from allochthonous sources, such as phytoplankton from the upper water column and terrestrial plants from the nearby continent, can be deduced from the presence of high molecular-weight fatty acids (C_{24}, C_{26} and C_{28}) widely reported in higher plants[76,77], and the different carbon isotopic fingerprint of the sediments (i.e. overall less depleted than that in the bivalve's gills) characteristic of the Calvin-Benson carbon pathways used by cyanobacteria, algae and land plants[67,78].

The presence of a novel species of the genus *Vesicomyosocius* as the main endosymbiotic bacterium provides clear evidence for a symbiotic relationship of the *Archivesica* bivalve reported here with sulfide-oxidizing (thiotrophic) bacteria. In turn, we detected significant abundance of $C_{16:1\omega7}$ and $C_{18:1\omega7}$ fatty acids, well-established biomarkers for thiotrophs, along with $\delta^{13}C$ values characteristic of thiotrophic symbioses using the CBB cycle for carbon fixation. The absence of biomarkers (e.g. archaeol, crocetane, or PMI) and ^{13}C-depleted isotopic signatures ($\delta^{13}C < -50‰$) associated with methanotrophic archaea and methane-derived carbon rules out methanotrophic nutrition in these bivalves. Additionally, the lipid profile in the sediments supports active microbial sulfate reduction, indicated by the presence of methylated (terminal, and mid-chain branched), and cyclopropyl fatty acids typical of SRB. While $C_{16:1\omega7}$ and $C_{18:1\omega7}$ could originate from various microbial or eukaryotic sources, their context, isotopic composition, and abundance in gills tissue point to a symbiont origin. Finally, the detection of high molecular weight *n*-fatty acids with $\delta^{13}C$ signatures (-25‰ to -27‰) relatively higher than the rest of fatty acids suggests some contribution of allochthonous terrestrial input to the sediment carbon pool.

Overall, the data presented here (Fig. 7) suggests that the thiotrophic symbiont *Ca.Vesicomyosocius* sp. atacamensis is the main source of energy for the newly discovered *Archivesica* bivalve. This is evidenced by the bulk $\delta^{13}C$ of the bivalve's gills and the carbon isotopic composition of its fatty acids, typical of bivalves hosting thiotrophic endosymbionts[47,57]. In turn, the bulk organic carbon $\delta^{13}C$ of the sediments where the bivalve lives, and the SRB lipid biomarkers, are coherent with a sulfur cycle between the bivalve, its endosymbiont (SOB), and the sulfate-rich sediments where the bivalve lives.

We hypothesize that the SRB present in the sediments reduces sulfate and produce hydrogen sulfide (H_2S), which is then incorporated by the highly vascularized bivalve foot, and distributed to the bivalve tissues, included the gills where *Ca. Vesicomyosocius sp.* atacamensis

uses it as energy source to fix carbon and provide energy for the bivalve, leaving behind different species of sulfur in the bivalve tissues.

Materials and methods

Collection and processing of samples prior to analysis

Sampling took place during the Schmidt Ocean Institute's FK240524 expedition aboard the R/V Falkor (too), utilizing the Remotely Operated Vehicle (ROV) SuBastian (details of sampling area and methodological setup can be seen in references[45,75]). Four bivalves (two for molecular and biosignatures analyses and two for morphological analyses) were collected from the site 9.2 at 2839 meters below sea level with the robotic arm from the SuBastian ROV. Push cores (20 cm in length) of seafloor sediments were collected using ROV SuBastian's push-coring system. The sediments from the push-cores were extracted using a specific extruding system and segmented into 6 cm intervals, and only the surface layer (0-6 cm deep) was considered for the present study and stored in 50 ml Falcon tubes. All samples were frozen at -20 °C, then shipped to the Centro de Astrobiología (CAB) in Madrid (Spain) for downstream analysis (DNA and lipids from the bivalve tissue; lipids from surface sediments).

DNA Extraction from bivalve's gills

DNA from the inside the bivalve's gills (one individual) was obtained in Extremophiles Laboratory of the Centro de Astrobiología using the DNeasy Blood and Tissue DNA Isolation Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions, using a FastPrep-24 5G homogenizer (MP Biomedicals, Irvine, USA).

Amplification of COI, 16S and 28S rRNA gene sequence from Clam DNA

COI: To confirm the taxonomic identity of the clam, we amplified the mitochondrial cytochrome c oxidase subunit I (COI) gene, the standard marker for DNA barcoding in animals. Amplification was performed using the universal primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). PCR reactions were conducted under the following conditions: an initial denaturation at 95 °C for 1 min; 35 cycles of 95 °C for 10 s, 47 °C for 30 s, and 72 °C for 60 s; followed by a final extension at 72 °C for 2 min.

16S rRNA: Similarly, *Archivesica* sp. Atacama mitochondrial 16S rRNA mitochondrial gene amplification was performed using the combination of primers pairs a) 16SarL (5'CGCCTGTTTATCAAAAACAT3')-16SarH (5'CCGGTCTGAACTCAGATCACGT3') (initial denaturation at 95°C for 2 min; 35 cycles of 95°C for 20s, 50°C for 25s, and 72°C for 45s; followed by a final extension at 72 °C for 5 min), b) G16S5F (5'GAAATCTGTTTAAAGTTAGTATTGTGAAAGAGTAG3')-AtClam5R (5'CTTTGTACAGTTATTCACTGCAGCCG3') (initial denaturation at 95°C for 2 min; 35 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 1min; followed by a final extension at 72 °C for 5 min), and c) AtClam3F (5'TGATTAAGGGAAAGGTTTGCGACCTC3')-G16S3R (5'AAAAAAGTAAAACATTGCCAACCGATCCTTTCG3'), using the same amplification protocol used in b). The resulting sequences obtained were assembled using the SeqMan tool of the Lasergene suite (DNASTAR, Inc., Madison, WI, USA).

The 28S rRNA gene sequence was amplified by using primers D1F (5'ACCCGCTGAATTTAAGCAT3') and D1R (5'TCGGAAGGAACCAGCTACTA3'). PCR reactions were conducted under the following conditions: an initial denaturation at 95 °C

for 2 min; 35 cycles of 95 °C for 20 s, 45 °C for 20 s, and 72 °C for 45 s; followed by a final extension at 72 °C for 5 min.

COI, 16S and 28S rRNA PCR products were checked for quality using the BioEdit software (Ibis Therapeutics, Carlsbad, USA) and end-trimmed before using the Megablast option for highly similar sequences of the BLASTN algorithm against the National Centre for Biotechnology Information nonredundant database (www.ncbi.nlm.nih.gov) to search for the closest species of each of the isolates obtained.

The resulting sequences of the new *Archivesica* species are available in GenBank under accession number PX551125 (COI), PX530364 (16SrRNA), and PZ377403 (28S rRNA).

Amplification of 16S rRNA sequence from the Clam Symbiont *Vesicomyosocius* sp. Atacamensis

The 16S rRNA *Vesicomyosocius* sp. Atacamensis sequence obtained from the corresponding ASV NGS data, used an anchor to extend its 5' and 3' ends. Primers pairs used were: a) Vesi6S5F (5'GAACTGAAGAGTTTGATCCTG3')-At5R (5'TATTGCTGGATCAGGCTTGCGCCC3') (initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1min; followed by a final extension at 72 °C for 5 min, b) At3F (5'GACACTGAGGTACGAAAGCGTGGG3')-Vesi16S3R (5'TAAGGAGGTGATCCAGCCTC3'), (with same amplification protocol as a), c) 16SarL (5'CGCCTGTTTATCAAAAACAT3')-Arch16SR1 (5'TACCTTTGTACAGTTATTCACT3'), d) 16SarL (5'CGCCTGTTTATCAAAAACAT3')-Arch16SR2 (5'TTTGTACAGTTATTCACTGCAG3'), e) Arch16SF1 (5'TATACCAGCTTTGTACGAAAG3')-16SbrH

(5'CCGGTCTGAACTCAGATCACGT3'), f) Arch16SF2 (5'AAAGGATCGGTTGGCAATGTTT3')-16SbrH (5'CCGGTCTGAACTCAGATCACGT3'), with b, c, d, e and f with the amplification protocol: initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1min; followed by a final extension at 72 °C for 5 min.

The resulting 16S rRNA gene sequence of the new *Vesicomyosocius* species is available in GenBank under accession number PZ315358.

Phylogenetic analyses of COI, 16S, and 28S rRNA sequences

Phylogenetic analyses of all gene sequences *Archivesica* sp. Atacama and *Vesicomyosocius* sp. atacamensis, were independently aligned with the closest corresponding sequences available in GenBank using Multiple Sequence Comparison by Log-Expectation (MUSCLE)[79]. Phylogenetic analyses were subsequently conducted using jModelTest[80] and Maximum Likelihood inference with 1,000 bootstrap replicates, implemented in the freely available Bosque phylogenetic analysis software (version 1.7.152)[81].

Illumina NGS-Based Metabarcoding Sequencing and Analyzes

Sequencing was performed at the facilities of Macrogen Inc (Seoul, South Korea). Libraries were prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the bacterial and archaeal 16S rRNA genes, using the primer pairs (5′-CCTACGGGNGGCWGCAG-3′)/(5′-GACTACHVGGGTATCTAATCC-3′) targeting the V3-V4 region and 21F (5′-TCCGGTTGATCCYGCCGG-3′) / 516R (5′-GGTDTTACCGCGGCKGCTG-3′), respectively. For eukaryotic 18S rRNA genes, the V4 region was amplified using primers (5′-CCAGCAGCCGCGGTAATTCC-3′) / (5′-ACTTTCGTTCTTGATTAA-3′). The input gDNA 2 ng was PCR amplified with 5x reaction

buffer, 1mM of dNTP mix, 500nM each of the universal F/R PCR primer, and Herculase II fusion DNA polymerase (Agilent Technologies, Santa Clara, CA). Cycle conditions for the 1st' PCR were; 3 min at 95°C for heat activation, and 25 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C, followed by a 5-min final extension at 72°C. The universal primer pair with Illumina adapter overhang sequences used for the first amplifications were as follows: 1st PCR products were purified with AMPure beads (Agencourt Bioscience, Beverly, MA). Following purification, 2 µl of 1st PCR products were PCR amplified for final library construction containing the index using NexteraXT Indexed Primer. The cycle conditions for the 2nd PCR were same as the 1st PCR condition except that 10 fewer amplications cycles less were used. PCR products were purified with AMPure beads. The final purified products were then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and their quality checked using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany), and then sequenced using the MiSeq™ platform (Illumina, San Diego, USA).

The resulting raw sequences were processed in MOTHUR software (v.1.43.0, https://mothur.org/) using a custom script based upon MiSeq SOP that maximizes sequence accuracy by restrictive quality thresholds at several steps. All ASV's have been deposited in GenBank, with accession numbers SAMN59684884, SAMN59684885 and SAMN59684886.

Sequence analysis

Adapters/primers of raw reads were trimmed with Cutadapt v4.2[82]. Subsequent processing: quality filtering, dereplication, chimera removal, and inference of amplicon sequence variants (ASVs), was performed with DADA2 v1.22.0[83], implemented in an *ad hoc* R v4.3.1 script. Forward and reverse reads were truncated at 280 and 260 nucleotides, respectively, and merged only when a minimum overlap of 20 nucleotides was achieved. Taxonomic classification was

assigned against the SILVA 138.2 reference database[84] using the naïve Bayesian classifier available in QIIME 2 v2024.10[85], with a confidence threshold of 0.8. Relative abundances were calculated from ASV counts and aggregated at the genus level for downstream visualization in R. In addition, ASVs sequences were then manually checked using the Megablast option for highly similar sequences of the BLASTN algorithm against the National Centre for Biotechnology Information nonredundant database (www.ncbi.nlm.nih.gov).

Extraction, fractionation, and molecular analysis of lipid biomarkers

Lipid compounds were analyzed both in bivalve's gills (1 clam) and the surface sediment where the bivalve thrived. In the Biogeochemistry Laboratory at CAB, the gills of the bivalve were extracted and freeze-dried, along with the sample of surface sediments (0-6 cm of the push core). Once dried, they were ground using a mortar and pestle for biogeochemical analysis.

Subsamples of the surface sediments (ca. 5 g) and the bivalve's gills (0.2 g) were extracted with a mixture of dichloromethane (DCM) and methanol (MeOH) in 3:1 proportion (v:v), using ultrasound sonication (3 cycles of 20 min), after addition of a deuterated fatty acid (tetradecanoic acid, $C_{14}D_{27}$) as internal standard. For each sample, the concentrated organic extract was hydrolyzed overnight with a mixture of methanolic KOH (6% MeOH w/w) at room temperature, and then the acidic fraction was separated from the neutral one with *n*-hexane, following a protocol described elsewhere[86,87]. The acidic fraction needed to be derivatized with methanolic BF_3 to transform the fatty acids into methyl ester derivatives (FAME) prior to their analysis[87,88]. The molecular analysis was accomplished on an 8860 GC system coupled to a 5977B MSD with a single-axis detector (Agilent Technologies), operating with electron ionization at 70 eV and scanning from m/z 50 to 650. Analytical details can be found elsewhere[89]. The molecular identity of the extracted compounds was determined based on the comparison of their mass spectra with those of reference materials (commercial fatty acids),

while their concentration was estimated using external calibration curves of FAMEs of even carbons from C_8 to C_{24}. All chemicals and standards were supplied by Sigma Aldrich (San Louis, Missouri, USA). A procedural blank (*i.e.* only solvents) dopped with the internal standard was analyzed in parallel to the samples, to check for intrinsic contamination during the extraction procedure.

Compound-specific isotope analysis (CSIA) of the extracted compounds

The stable-carbon isotopic analysis of the individual fatty acids from the bivalve's gills and sediment sample were determined by coupling a gas chromatograph-mass spectrometer (Trace GC 1310 ultra/ ISQ QD MS) with an isotope-ratio mass spectrometry system (MAT 253 IRMS, Thermo Fisher Scientific). For the GC analysis, the oven temperature program was set to gradually increase from 70 ºC to 130 ºC at 20 ºC·min^{-1}, and then to 300 ºC at 10 ºC·min^{-1} (held for 15 min). The parameters for IRMS analysis were: electron ionization at 100 eV, Faraday cup collectors m/z 44, 45, and 46, and temperature of the CuO/NiO combustion interface at 1000 ºC. All samples were injected in a PTV injector in splitless mode, with an inlet temperature of 50 ºC, and then temperature was increased 2.5 ºC·sec^{-1} until 320 ºC (held 2.5 min), using helium as carrier gas at a constant flow of 1.1 mL·min^{-1}. The carbon isotopic ratio of the individual lipids (*i.e.* $\delta^{13}C$) separated by GC was measured using carbon dioxide spikes of known isotopic composition, introduced directly into the MS source, three times at the beginning and end of every run. Reference mixture (Indiana University, United States) of known isotopic composition of fatty acids (F-8) was run every three samples to check the accuracy of the isotopic ratio determined by the GCMS-IRMS. The $\delta^{13}C$ data were calculated from the FAMEs values, correcting them for the one carbon atom added in the methanolysis derivatization[90]. The stable-carbon isotopic composition is expressed in per mil notation (‰) and relative to the Pee Dee Belemite (PDB) standard.

Bulk organic composition

The bulk isotopic composition of organic carbon ($\delta^{13}C$) and total nitrogen ($\delta^{15}N$) was measured on the bulk samples of the bivalve's gills and surface sediments using isotope ratio mass spectrometry (IRMS), following USGS methods[91]. Briefly, ground and homogenized subsamples (0.3-0.5 mg) were decarbonated with concentrated HCl (37%) and, after equilibration for 24 h, the pH was adjusted to a neutral value with ultrapure water. The residue was then dried in an oven (50°C) until constant weight (after ca. 72 h), and finally analyzed for isotopic composition in the IRMS (MAT 253, Thermo Fisher Scientific). The $\delta^{13}C$ and $\delta^{15}N$ ratios were reported in the standard per mil notation (‰) using three certified standards (USGS41, IAEA-600, and USGS40) with an analytical precision of 0.1‰. In parallel, the content of total organic carbon (TOC %) and total nitrogen (TN %) was measured with an elemental analyzer (HT Flash, Thermo Fisher Scientific) during the stable isotope measurements.

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Competing Interests

The authors declare no competing interests.

Data Availability

GenBank accession numbers for *Archivesica* sp. Atacama are PX551125 (COX1), PZ377403 (28S), and PX530364 (16S). In the case of Ca. *Vesicomyosocius* sp. atacamensis, GenBank accession numbers are PZ315358 (16S). NGS clam data has GenBank accession number PRJNA1299367.

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Figures and tables

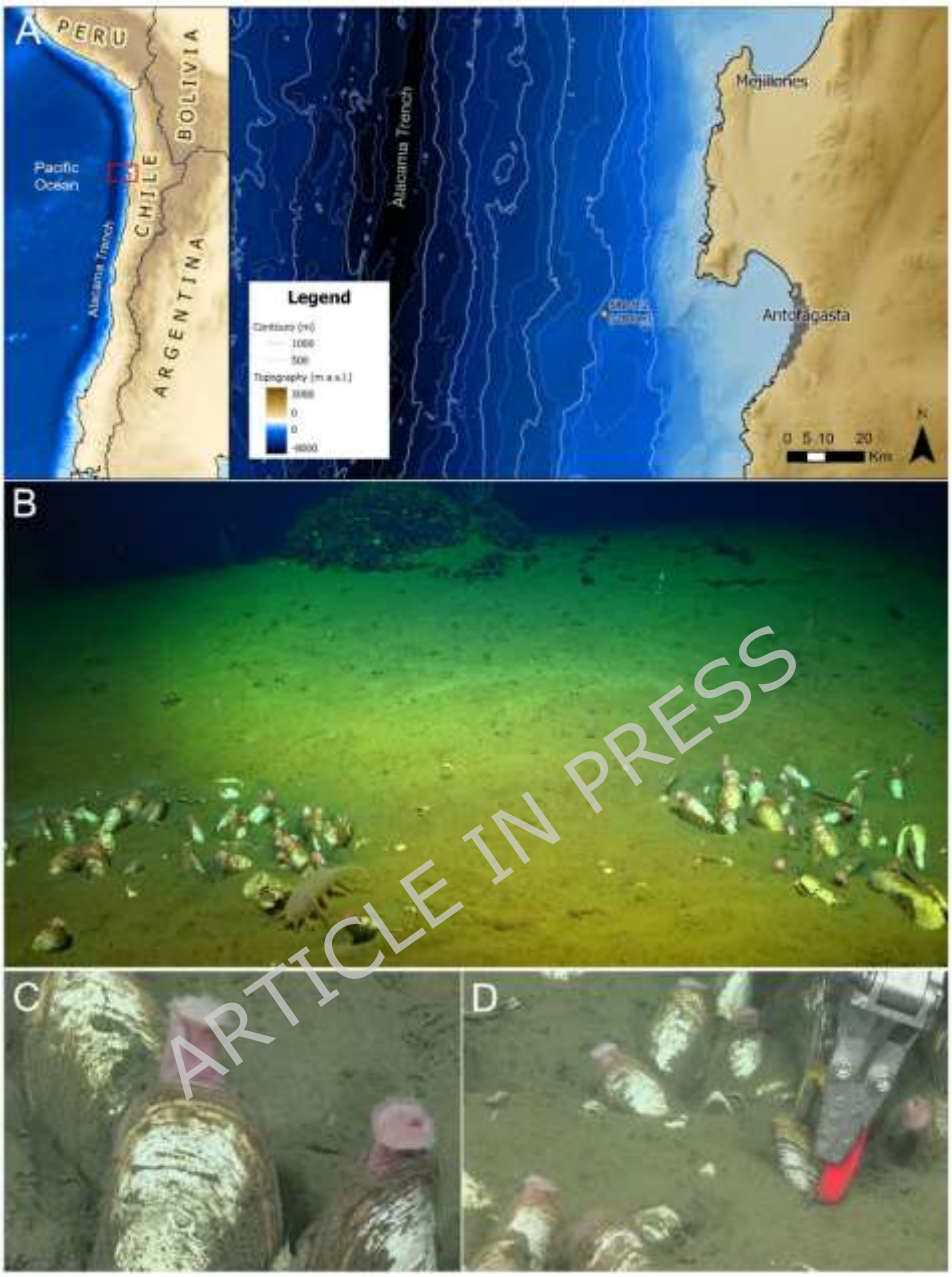

Figure 1. Location of the study site and surrounding environmental views where Archivesica sp. from the Atacama region inhabits. A, Location of site 9.2, one of six sites inspected in the FKt240524 expedition. B, two patches of *Archivesica* bivalves. C, Detail of the bivalve siphons. D, collection of a bivalve with the robotic arm of the remotely operated vehicle SuBastian. The map was generated using ArcGIS Pro 3.5 (Esri Inc.) and using topography data from GEBCO (2024)[92] and geoBoundaries[93].

Figure 2. *Archivesica* sp. Atacama. A) Lateral view right valve. B) Dorsal view. C) Anterior view. D) inner side of the right valve. E) Cardinal teeth of the right valve. F) Cardinal teeth of the left valve. l: right ventral cardinal tooth. 3b: subumbonal cardinal tooth. 2a and 2b: central cardinal tooth anterior ramus (2a) and posterior one (2b).

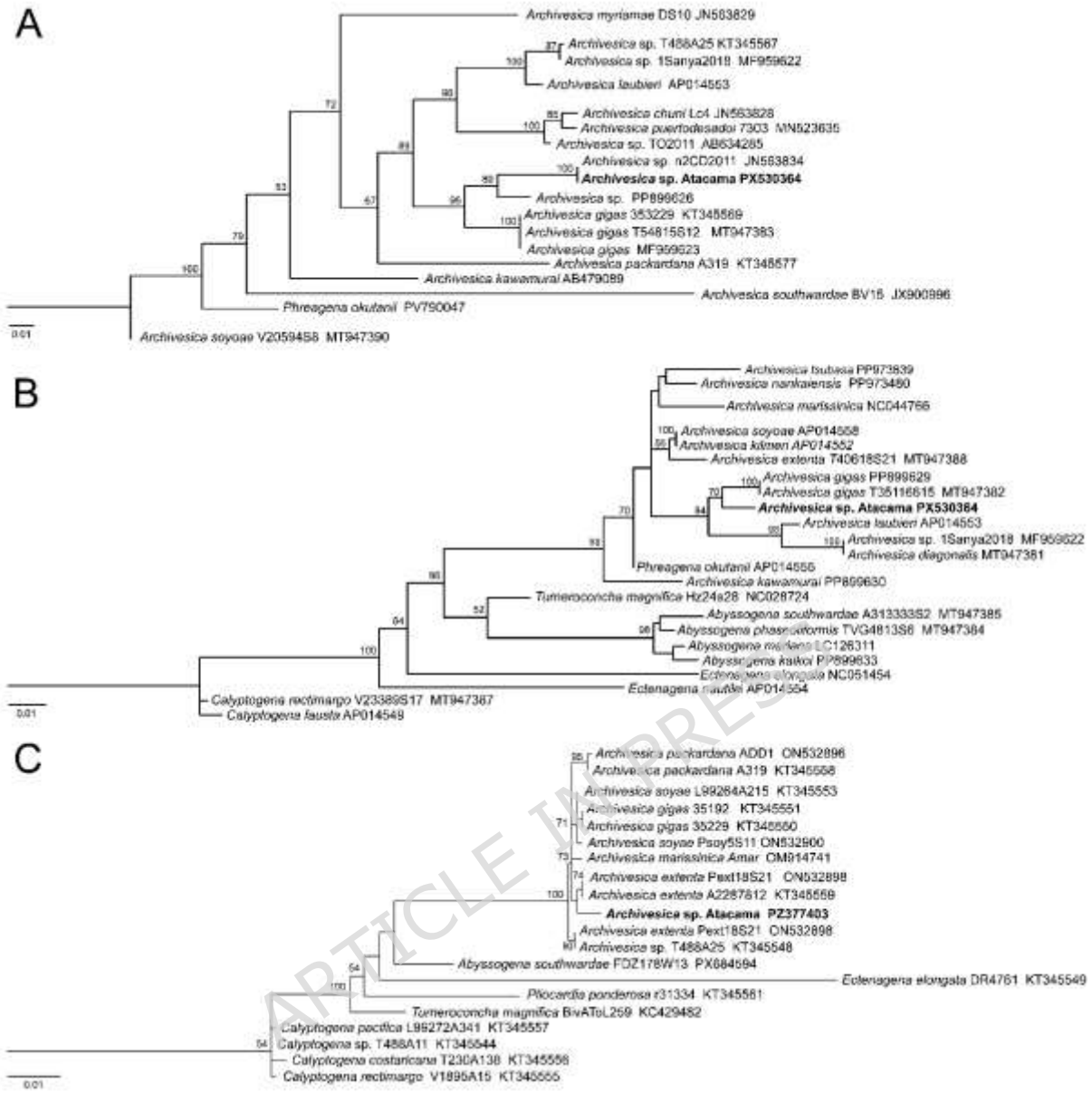

Figure 3. Maximum-likelihood phylogenetic tree of *Archivesica* sp. Atacama and related vesicomyid clams. Phylogenetic relationships were inferred using a maximum-likelihood approach based on mitochondrial COI sequences (A), as well as nuclear 16S rRNA (B) and 28S rRNA (C) gene sequences. Numbers at nodes indicate bootstrap support values (%) from 1000 replicates; only values ≥50% are shown. The scale bar represents 0.01 substitutions per site.

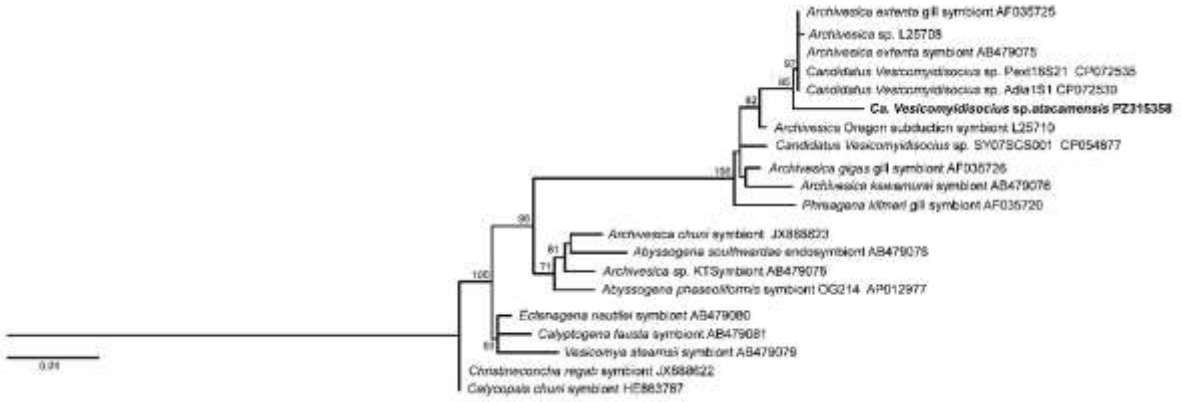

Figure 4. Maximum likelihood (ML) phylogenetic tree obtained from the aligned 16S rRNA gene sequences of Ca. *Vesicomyosocius* sp. atacamensis. Numbers on the nodes represent bootstrap values with 1000 replicates. Only values ≥50% are shown. The scale bar represents 0.01 substitutions per site. Note that *atacamensis* is a provisional identifier referring to the Atacama Trench lineage, and does not constitute a formal taxonomic proposal.

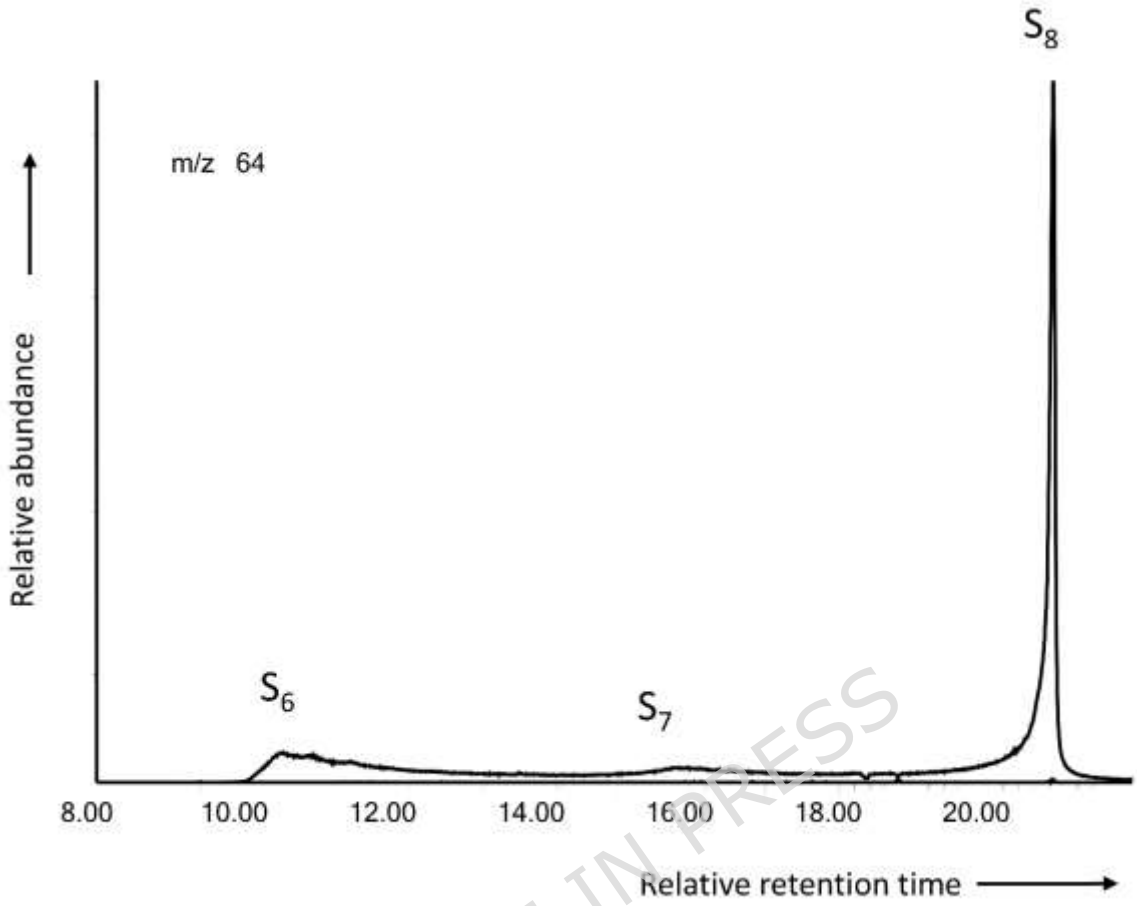

Figure 5. Fragment ion chromatogram (*m/z* 64) showing the different sulfur compounds found in the organic extract from the bivalve's gills.

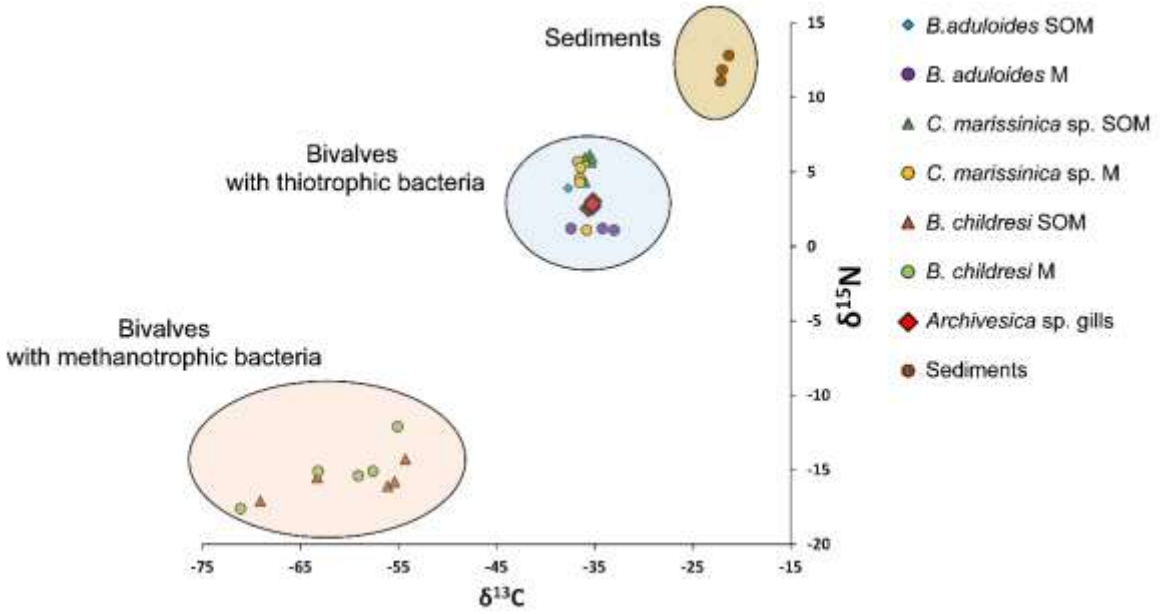

Figure 6. Binary diagram of the carbon and nitrogen isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) of deep-sea bivalves, from this study (i.e. *Archivesica* sp. Atacama, red diamond) and others reporting on other symbiotic bivalves (methanotrophs in *Bathymodiolus childressi*, and thiotrophs in *Bathymodiolus aduloides* and *Calyptogena marissinica*)[52]. In our study, the isotopic ratios were measured on the bulk organic matter of the bivalve's gills. In reference 52, the isotopic ratios are reported on shell organic matrix (SOM), or mantle (M). Data from our study also include isotopic data from the surface sediments where the bivalve Archivesica sp. was collected (brown circles).

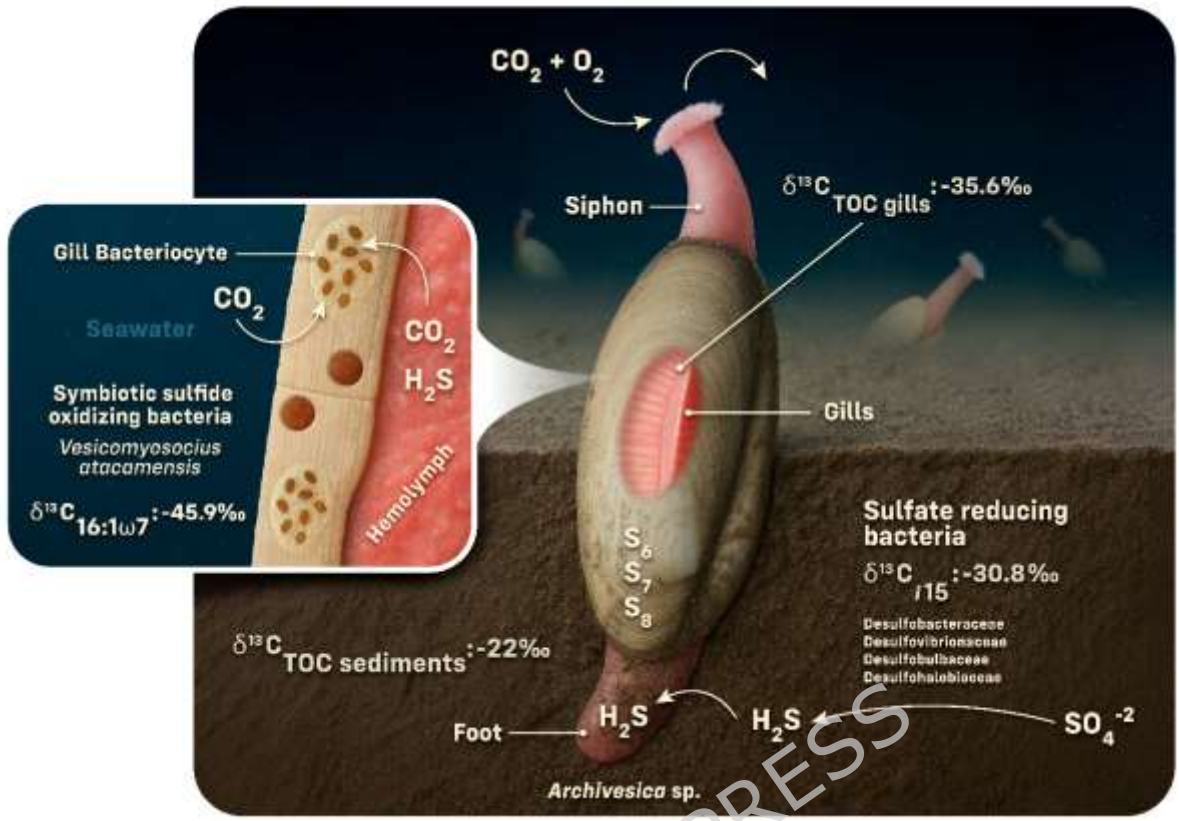

Figure 7. Conceptual model of the thiotrophic symbiosis associated with Ca. *Vesicomyosocius* sp. atacamensis in the deep-sea vesicomyid bivalve *Archivesica* sp. Atacama. The model is based on well-established mechanisms described for vesicomyid bivalves and other thiotrophic symbioses sulfur-rich benthic habitats, in which sulfate-reducing bacteria (SRB) in surrounding sediments generate hydrogen sulfide (H_2S) through sulfate reduction. Hydrogen sulfide is hypothesized to be taken up through the highly vascularized foot of the bivalve and transported to bacteriocytes in the gills, where sulfur-oxidizing bacterial symbionts (SOB) use it as an energy source to fix carbon for the host. Although sulfide uptake and sulfur cycling were not directly demonstrated here, the proposed pathway is supported by geochemical and biomarker evidence and is consistent with mechanisms described in related vesicomyid systems. Stable carbon isotopic compositions ($\delta^{13}C$) of characteristic biomarkers associated with SRB (iC15) and SOB (C16:1ω7), together with bulk sedimentary $\delta^{13}C$ values (total organic carbon), are shown for sediments inhabited by the bivalves. This figure summarizes the hypothesized sulfur-based trophic interactions inferred for the *Archivesica*-symbiont association found in the Atacama trench.