# Host-Symbiont Relationships in Hydrothermal Vent Gastropods of the Genus *Alviniconcha* from the Southwest Pacific

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Hydrothermal vent gastropods of the genus *Alviniconcha* are unique among metazoans in their ability to derive their nutrition from chemoautotrophic γ- and ε-proteobacterial endosymbionts. Although host-symbiont relationships in *Alviniconcha* gastropods from the Central Indian Ridge in the Indian Ocean and the Mariana Trough in the Western Pacific have been studied extensively, host-symbiont relationships in *Alviniconcha* gastropods from the Southwest Pacific remain largely unknown. Phylogenetic analysis using mitochondrial cytochrome *c* oxidase subunit I gene sequences of host gastropods from the Manus, North Fiji, and Lau Back-Arc Basins in the Southwest Pacific has revealed a new host lineage in a *Alviniconcha* gastropod from the Lau Basin and the occurrence of the host lineage *Alviniconcha* sp. type 2 in the Manus Basin. Based on 16S rRNA gene sequences of bacterial endosymbionts, two γ-proteobacterial lineages and one ε-proteobacterial lineage were identified in the present study. The carbon isotopic compositions of the biomass and fatty acids of the gastropod tissues suggest that the γ- and ε-proteobacterial endosymbionts mediate the Calvin-Benson cycle and the reductive tricarboxylic acid cycle, respectively, for their chemoautotrophic growth. Coupling of the host and symbiont lineages from the three Southwest Pacific basins revealed that each of the *Alviniconcha* lineages harbors different bacterial endosymbionts belonging to either the γ- or ε-*Proteobacteria*. The host specificity exhibited in symbiont selection provides support for the recognition of each of the host lineages as a distinct species. The results from the present study also suggest the possibility that *Alviniconcha* sp. types 1 and 2 separately inhabit hydrothermal vent sites approximately 120 m apart in the North Fiji Basin and 500 m apart in the Manus Basin.

Of the marine invertebrates that derive their nutrition from methane-oxidizing (methanotrophic) and/or sulfur-oxidizing chemoautotrophic (thioautotrophic) bacterial endosymbionts, such as vestimentifera tubeworms and bivalve mollusks, hydrothermal vent gastropods belonging to the genus *Alviniconcha* from the family *Provannidae* differ in that they are capable of harboring primary endosymbionts that do not belong to the γ-*Proteobacteria* (3, 4, 24). The hydrothermal vent gastropods of the genus *Alviniconcha* are currently known to inhabit five tectonically distinct settings, including the Central Indian Ridge in the Indian Ocean, the Mariana Trough in the Western Pacific and the Manus, North Fiji, and Lau Back-Arc Basins in the Southwest Pacific (25).

*Alviniconcha hessleri* from the Mariana Trough harbors a sulfur-oxidizing chemoautotrophic γ-proteobacterial endosymbiont that mediates the Calvin-Benson cycle to fix $CO_2$ (21), whereas *Alviniconcha* aff. *hessleri* from the Central Indian Ridge harbors a chemoautotrophic ε-proteobacterial endosymbiont that mediates the reductive tricarboxylic acid (rTCA) cycle for $CO_2$ fixation (22). Phylogenetic analyses of host gastropods have revealed that two host lineages, referred to as *Alviniconcha* sp. types 1 and 2, inhabit deep-sea hydrothermal fields in the Manus and North Fiji Basins (14). The phylogenetic affiliation of the endosymbionts of *Alviniconcha* sp. types 1 and 2, as well as those of the host and endosymbionts of *Alviniconcha* gastropods from the Lau Basin, remain poorly defined. Considering the diversity of chemoautotrophic metabolism in prokaryotes and the ability of *Alviniconcha* gastropods to form endosymbiotic relationships with two phylogenetically and physiologically distinct chemoautotrophic bacteria, *Alviniconcha* gastropods from the Southwest Pacific might be associated with previously unknown chemoautotrophic endosymbionts.

The principle aim of the present study was to identify and clarify the host-endosymbiont lineages of the *Alviniconcha* gastropods from the Southwest Pacific ocean. In addition, we attempted to determine the nature of the carbon metabolism and the trophic relationships between *Alviniconcha* and their endosymbionts using bulk and compound-specific carbon isotopic analyses.

## MATERIALS AND METHODS

**Gastropod specimens.** Gastropod specimens were collected from the Manus, North Fiji, and Lau Back-Arc Basins in the South West Pacific using the *Shinkai 2000* and *Shinkai 6500* manned submersibles. The geographic coordinates, depth of the sampling sites, date of sampling and the submersible used for the sampling

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TABLE 1. Species of *Alviniconcha* gastropods sampled, number of individuals, sample locations, dive numbers, and references containing maps of the sample locations

| Species | n | Collection site | Latitude/longitude | Depth (m) | Date (yr.mo.day) | Dive | Site reference |
|---|---|---|---|---|---|---|---|
| *Alviniconcha* sp. type 1 | 1 | White Lady site, North Fiji Basin | 16°59.5′S/173°54.9′E | 1,970 | 1991.9.8 | *Shinkai 6500* #78 | 2 |
| *Alviniconcha* sp. type 1 | 1 | White Lady site, North Fiji Basin | 16°59.5′S/173°54.9′E | 1,970 | 1991.9.11 | *Shinkai 6500* #80 | 2 |
| *Alviniconcha* sp. type 2 | 3 | STARMER II site, North Fiji Basin | 16°59.3′S/173°55.0′E | 1,990 | 1991.10.21 | *Shinkai 6500* #92 | 2 |
| *Alviniconcha* sp. type 2 | 2 | Vienna Woods site, Manus Basin | 3°9.8′S/150°16.7′E | 2,510 | 1995.10.20 | *Shinkai 6500* #296 | 6, 9 |
| *Alviniconcha* sp. type 1 | 3 | PACMANUS Field D, Manus Basin | 3°43.7′S/151°40.2′E | 1,630 | 1998.11.8 | *Shinkai 2000* #1067 | 6, 9 |
| *Alviniconcha* sp. type 2 | 1 | PACMANUS Field E, Manus Basin | 3°43.6′S/151°40.3′E | 1,676 | 1998.11.2 | *Shinkai 2000* #1062 | 6, 9 |
| *Alviniconcha* sp. | 1 | Vai Lili site, Lau Basin | 22°12.9′S/176°36.5′W | 1,722 | 2004.10.8 | *Shinkai 6500* #847 | 2 |

are summarized in Table 1. References containing maps of the sampling locations are also listed in Table 1.

**DNA analysis of the mitochondrial cytochrome oxidase I (COI) gene sequences.** Total DNA was extracted from the head-food region of the specimens from the three back-arc basins by grinding, digestion with sodium dodecyl sulfate, and extraction with phenol and chloroform. The downstream region of the COI mitochondrial gene (approximately 690 bp) was amplified by the PCR using primers COI-B (8) and COI-6 (20). The PCR conditions were as follows: 94°C for 60 s followed by 30 to 40 cycles at 92°C for 40 s, 50°C for 60 s, and 72°C for 90 s. The nucleotide sequence of the amplified fragment was determined by using an ABI3100 sequencer and a Prism Big Dye terminator sequencing kit (Applied Biosystems, Inc., Foster City, CA) with primers COI-B, TW-2 (14), COI-3 (20), Gastro-2 (12), Gastro-3 (13) and COI-6.

To better understand the phylogenetic relationships among host lineages, the upstream region of the COI gene was amplified by PCR using primers LCO1490 and HCO2198 (5) for each of the five host lineages: *A. hessleri* (SMR-93-10 in reference 12), *Alviniconcha* sp. type 1 (MB-96-16 in reference 14), *Alviniconcha* sp. type 2 (FB-90-7 in reference 12), *Alviniconcha* aff. *hessleri* (the first specimen in reference 11), and *Alviniconcha* sp. from the Lau Basin (Fig. 1). The primers COI-7 (20) and COI-D (16) were used to amplify the COI fragment of *Ifremeria nautilei*, a member of the Provannidae from the North Fiji (haplotype F4 in reference 13) and the Manus Basins (haplotype M1 in reference 13). *Ifremeria* was used as the outgroup for phylogenetic analysis. The conditions for PCR were 94°C for 60 s, followed by 30 to 40 cycles of 92°C for 40 s, 50°C for 60 s, and 72°C for 90 s.

Phylogenetic relationships were estimated by the maximum-parsimony (MP) method using a multiple, equally parsimonious, heuristic search with tree bisection-reconnection and 1,000 random addition sequence replicates, the maximum-likelihood (ML) method of the PAUP* package (version 4.0b10) (23), and the neighbor-joining (NJ) method (19) of the MEGA package (version 2.19 [16]).

The mitochondrial COI gene sequences from the *Alviniconcha* gastropods are available at the DDBJ under accession numbers AB235211 to AB235227.
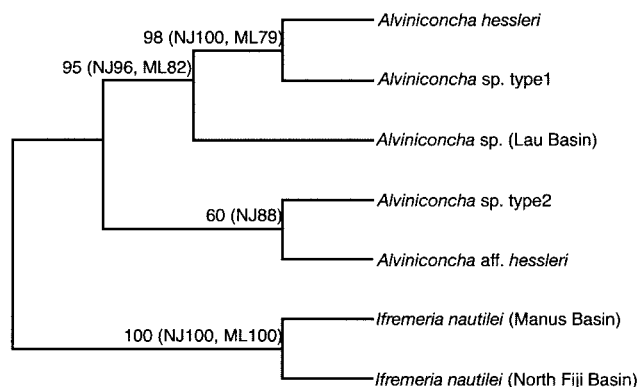


FIG. 1. Phylogenetic relationships among the five *Alviniconcha* lineages known to date. The tree was constructed by using the MP method with two lineages of *Ifremeria nautilei* used as outgroup taxa. Numbers at the branch nodes represent bootstrap values (1,000 replicates) obtained from the MP, NJ, and the ML methods; only values greater than 50% are indicated.

**DNA analysis of the endosymbiont 16S rRNA gene sequences.** Endosymbiont 16S rRNA gene sequences were analyzed as previously described (22), except that the endosymbiont DNA was extracted from the dissected gill tissue by using a DNeasy kit (QIAGEN, Valencia, CA) in accordance with the manufacturer's instructions. Endosymbiont 16S rRNA gene sequences were amplified by PCR using LA *Taq* polymerase (TaKaRa, Shiga, Japan) with Bac27F and Uni1492R oligonucleotide primers (17). The PCR conditions were 96°C for 120 s, followed by 27 to 30 cycles of 96°C for 20 s, 53°C for 45 s, and 72°C for 120 s. The amplified 16S rRNA gene sequence products were cloned by using an Original TA Cloning Kit (Invitrogen, Carlsbad, CA) before being sequenced with an ABI 3100 sequencer and dRhodamine Sequencing Kit according to the manufacturer's recommendations (Perkin-Elmer/Applied Biosystems).

A single phylogenetic clone type (phylotype) was obtained from the clone type analysis. The partial sequence was extended and manually aligned by using the secondary structures with ARB (18). Evolutionary analysis was performed by the NJ and MP methods using PAUP (23) based on 1,160 nucleotide positions (64 to 1417 [*Escherichia coli* numbering]), which have more than 50% identity across the sequences analyzed.

The bacterial 16S rRNA gene sequences from the gill endosymbionts are available at the DDBJ under the accession numbers AB235228 to AB235239.

**Bulk carbon isotopic analysis.** Bulk carbon isotopic analyses were undertaken as described previously (22). The gastropod specimens were dissected into gill and mantle tissues, which were then lyophilized. The carbon isotopic compositions of the cultures and the acid-fumed gastropod tissues were analyzed by using a Thermo Electron DELTA$^{plus}$ Advantage mass spectrometer connected to an elemental analyzer (EA1112) through a ConFlo III interface. The measured isotopic composition was expressed as δ$^{13}$C, which can be defined as follows: $\delta^{13}C = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1] \times 10^3$, where $(^{13}C/^{12}C)_{sample}$ is the $^{13}C/^{12}C$ abundance ratio for the sample and $(^{13}C/^{12}C)_{standard}$ is the $^{13}C/^{12}C$ abundance ratio for the Pee Dee Belemnite carbonate standard. The values of δ$^{13}$C therefore represent the difference, in parts per thousand (per mille [‰]), between the $^{13}C/^{12}C$ value of the sample and that of the standard.

**Compound-specific carbon isotopic analysis.** Cellular fatty acids were extracted by using a previously described method (15). Briefly, approximately 20 mg of the gastropod tissue was incubated in 1 ml of anhydrous methanolic hydrochloric acid at 100°C for 3 h. The fatty acid methyl esters (FAMEs) were then extracted with *n*-hexane. The identities of the FAMEs were subsequently determined by comparison of their retention times and spectra to those of known FAME standards by gas chromatography-mass spectrometry (GCQ; Shimadzu, Tokyo, Japan). The oven temperature was set to 140°C for 3 min before being increased to 250°C at a rate of 4°C/min with He at a constant flow of 1.1 ml/min through a DB-5MS column (30 m by 0.25 μm by 0.25 mm; J&W Scientific, Folsom, CA). Standard nomenclature was used for fatty acids, which were designated in an *X:Y* format, where *X* is the number of carbon atoms and *Y* is the number of double bonds.

The δ$^{13}$C values of the FAMEs were determined using the GC-carbon-isotope ratio MS using a Thermo Electron DELTA$^{plus}$ Advantage mass spectrometer connected to a gas chromatograph (Agilent 6890; Agilent, Mountain View, CA) through a GC/C/III interface as described previously (22). The oven temperature was set to 120°C for 3 min before being increased to 300°C at a rate of 4°C/min with He at a constant flow of 1.1 ml/min through an HP-5 column (30 m by 0.25 μm by 0.25 mm; Agilent). The isotopic compositions of the FAMEs were measured with an internal isotopic standard (19:0, δ$^{13}$C = −29.80‰) with a correction made for the additional carbon atom from the methanol-derivatizing reagent (δ$^{13}$C = −39.04‰). The internal isotopic standard reduced measurement errors to within 1‰ for all isotopic analyses.
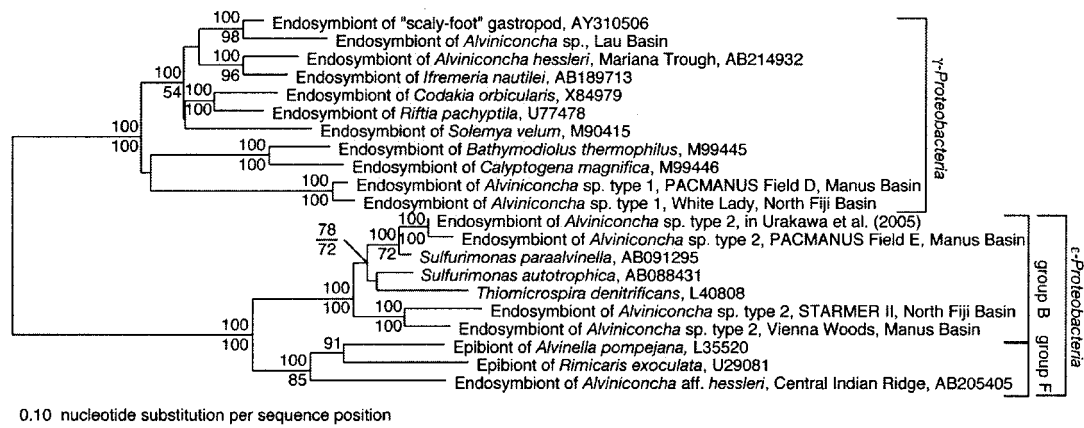
FIG. 2. Distance tree of the members of the γ- and ε-*Proteobacteria*, as well as the *Alviniconcha* endosymbionts based on near-complete 16S rRNA gene sequences (1,160 nucleotides). Bootstrap values (in percent values) are based on 1,000 replicates (NJ and MP) and are shown for branches with more than 50% bootstrap support.

## RESULTS AND DISCUSSION

**Genetic differentiation of the host gastropods.** In order to reveal the host lineages of *Alviniconcha* gastropods and their distribution in the South West Pacific basins, a nucleotide sequence for the downstream region of the COI mitochondrial gene (696 bp) was determined for a single specimen from the Vai Lili site in the Lau Basin, as well as several sites in the Manus and North Fiji Basins. As summarized in Table 1, *Alviniconcha* sp. type 1 inhabited PACMANUS field D in the Manus Basin and the White Lady site in the North Fiji Basin, whereas *Alviniconcha* sp. type 2 occurred in PACMANUS field E and at the Vienna Woods site in the Manus Basin and in the STARMER II site in the North Fiji Basin. Consequently, it appears that the distribution of *Alviniconcha* spp. types 1 and 2 within the fields and sites examined in the present study does not overlap. Instead, despite the relatively close geographic proximity of *Alviniconcha* sp. types 1 and 2—120 m between the White Lady and STARMER II sites in the North Fiji Basin, as well as the 500 m between the PACMANUS fields D and E in the Manus Basin—an allopatric distribution was assumed for the two *Alviniconcha* spp. This is the first report of *Alviniconcha* sp. type 2 in the Manus Basin.

Phylogenetic analysis also revealed that the sequence identities between the host lineage from the Lau Basin specimen and the four other host lineages ranged from 88.3 to 91.6%, which is markedly lower than the identities among these lineages themselves, which ranged from 98.8 to 100%. It is therefore proposed that the gastropod from the Lau Basin represent a new distinct lineage in the genus *Alviniconcha*. The finding of marked genetic differentiation in the Lau Basin specimen is consistent with a previous study in which 20 enzymatic systems in *Alviniconcha* gastropods from the Lau and North Fiji Basins were analyzed (1).

To better understand phylogenetic relationships among the five *Alviniconcha* lineages, the nucleotide sequence from the upstream COI gene region (517 bp) was then determined and combined to that from the downstream COI gene region for the specimen from the Lau Basin, as well as each of the four other *Alviniconcha* lineages, for which the nucleotide sequence

of the downstream region had been determined previously (11–13). On the basis of the combined COI nucleotide sequences (1,231 bp) from the five *Alviniconcha* lineages and two confamilial *Ifremeria* gastropods, it was shown that the three host lineages of *A. hessleri*, *Alviniconcha* sp. type 1, and *Alviniconcha* spp. from the Lau Basin formed a clade with high bootstrap probabilities (>82%). The remaining two lineages, *Alvinoconcha* sp. type 2 and *Alvinoconcha* aff. *hessleri*, were found to cluster together in NJ and MP trees with relatively high (88%) and low (60%) bootstrap probabilities, respectively. With the exception of *Alviniconcha* sp. type 2, the four lineages clustered with a bootstrap probability of 60% on the ML tree.

**Phylogenetic affiliations of the *Alviniconcha* endosymbionts.** To determine the phylogenetic affiliations of gill endosymbionts, the host lineages of which were identified above, were analyzed based on their 16S rRNA gene sequences. The examination of at least eight clones generated from a 16S rRNA gene-sequence library from the gill filaments of each gastropod showed only one 16S rRNA gene sequence. As shown in Fig. 2 and Table 2, phylogenetic analysis placed the endosymbiont phylotypes within either the γ- or ε-*Proteobacteria*.

Specimens of *Alviniconcha* sp. type 1 from the PACMANUS field D in the Manus Basin and the White Lady site in the North Fiji Basin were found to harbor closely related γ-proteobacterial endosymbionts (Fig. 2). Similarly, the sequence identities among γ-proteobacterial endosymbionts within the Manus and North Fiji Basins were greater than 99.3 and 97.8%, respectively. Sequence identities between specimens from the two basins were greater than 97.7%. The endosymbionts of *Alviniconcha* sp. type 1 were related to free-living *Thiomicrospira* spp. with sequence identities of less than 90%.

Conversely, specimens of *Alviniconcha* sp. type 2 from the Vienna Woods site in the Manus Basin and the STARMER II site in the North Fiji Basin both harbor ε-proteobacterial endosymbionts (Fig. 2). The sequence identities of the ε-proteobacterial endosymbionts within the Manus and North Fiji Basins were greater than 99.6%, and the sequence identities between the two basins were greater than 95.0%. The ε-proteo-

TABLE 2. Carbon isotopic compositions of total biomass and FAMEs in gastropod tissues

| Host species and host tissue | Sampling site; symbiont lineage | Mean total biomass ± SD | Mean carbon isotopic composition (‰ $\delta^{13}C$ ± SD) of FAME: | | | | |
|---|---|---|---|---|---|---|---|
| | | | 16:1 | 16:0 | 18:1 | 18:0 | 20:2 |
| *Alviniconcha* sp. type 1, Gill | White Lady site, North Fiji Basin; γ-*Proteobacteria* | −30.7 ± 0.6 | −38.4 ± 0.8 | −38.2 ± 0.7 | −37.4 ± 0.4 | −36.9 ± 0.1 | −37.0 ± 0.4 |
| *Alviniconcha* sp. type 2, | STARMER II site, North Fiji Basin; ε-*Proteobacteria* | | | | | | |
| Gill | | −10.8 ± 0.2 | −4.7 ± 0.2 | −4.7 ± 0.2 | −5.7 ± 0.4 | −4.6 ± 0.2 | −6.3 ± 0.2 |
| Mantle | | −11.4 ± 0.3 | −5.1 ± 0.2 | −5.4 ± 0.1 | −6.0 ± 0.4 | −6.3 ± 0.3 | −5.9 ± 0.9 |
| *Alviniconcha* sp. type 2 | Vienna Woods site, Manus Basin; ε-*Proteobacteria* | | | | | | |
| Gill | | −13.3 ± 0.8 | −6.6 ± 1.4 | −8.9 ± 0.2 | −7.6 ± 0.5 | −10.3 ± 1.2 | −9.3 ± 0.0 |
| Mantle | | −13.4 ± 0.2 | −6.1 ± 0.2 | −8.4 ± 0.9 | −8.2 ± 0.6 | −9.0 ± 1.0 | −9.8 ± 0.7 |
| *Alviniconcha* sp. type 1 | PACMANUS field D, Manus Basin; γ-*Proteobacteria* | | | | | | |
| Gill | | −30.5 ± 0.3 | −37.5 ± 1.0 | −38.0 ± 0.7 | −36.6 ± 0.9 | −36.6 ± 0.0 | −36.8 ± 0.6 |
| Mantle | | −30.0 ± 0.1 | −36.7 ± 0.4 | −37.1 ± 0.2 | −37.6 ± 0.1 | −36.9 ± 0.3 | −37.3 ± 0.1 |
| *Alviniconcha* sp. type 2 | PACMANUS field E, Manus Basin; ε-*Proteobacteria* | | | | | | |
| Gill | | −10.6 | −5.7 | −5.7 | −5.7 | −5.4 | −7.1 |
| Mantle | | −10.3 | −6.4 | −6.5 | −7.3 | −6.3 | −7.4 |
| *Alviniconcha* sp. | Vai Lili site, Lau Basin; γ-*Proteobacteria* | | | | | | |
| Gill | | −30.5 | −36.1 | −36.8 | −36.1 | −35.8 | −36.0 |
| Mantle | | −30.0 | −35.6 | −36.3 | −36.1 | −36.0 | −35.7 |
| *Alviniconcha hessleri* | Alice Springs, Mariana Basin[a]; γ-*Proteobacteria* | | | | | | |
| Gill | | −29.7 ± 0.4 | −38.6 ± 0.9 | −39.5 ± 0.6 | −37.7 ± 0.5 | −36.5 ± 0.7 | −36.9 ± 0.4 |
| Mantle | | −28.4 ± 0.5 | −36.1 ± 0.4 | −36.8 ± 0.1 | −36.4 ± 0.5 | −35.7 ± 0.4 | −36.5 ± 0.9 |
| *Alviniconcha* aff. *hessleri* | Kairei Field, Central Indian Ridge[b]; ε-*Proteobacteria* | | | | | | |
| Gill | | −11.0 ± 0.1 | −5.1 ± 0.9 | −6.5 ± 0.9 | −7.7 ± 0.8[d] | −8.5 ± 1.4 | −7.6 ± 0.8 |
| Mantle | | −10.7 ± 0.1 | ND[c] | −8.3 ± 0.9 | −9.1 ± 1.4[d] | −9.2 ± 0.9 | −8.6 ± 2.0 |

[a] Suzuki et al. (21).
[b] Suzuki et al. (22).
[c] ND, not determined.
[d] This value is for $C_{18:2}$.

bacterial endosymbionts belong to a subgroup of the ε-*Proteobacteria* called group B that includes *Thiomicrospira denitrificans* and *Sulfurimonas* spp. which have been cultured previously. Although Urakawa et al. (24) reported that the *Alviniconcha* sp. type 1 from at the PACMANUS field E harbors an ε-proteobacterial endosymbiont belonging to group B (24), we subsequently determined that the gastropod specimens examined earlier (24) were affiliated with *Alviniconcha* sp. type 2. We also examined one specimen from the PACMANUS field E and determined that it should be regarded as *Alviniconcha* sp. type 2 and that, as shown in Fig. 2, the endosymbiont is closely related to that reported previously (24). Furthermore, the endosymbiont lineage from *Alviniconcha* sp. type 2 from at PACMANUS field E is clearly different from those found at the Vienna Woods site in the Manus Basin and the STARMER II site in the North Fiji Basin (identities of less than 93.5%).

The *Alviniconcha* sp. from the Lau Basin harbors a γ-proteobacterial endosymbiont that is phylogenetically distinct from those found in other *Alviniconcha* lineages (Fig. 2). The γ-proteobacterial endosymbiont is closely related to the endosymbiont of the "scaly-footed" hydrothermal gastropod from the Central Indian Ridge with which it shares a sequence identity of ca. 95%.

**Carbon metabolism in the *Alviniconcha* endosymbionts.** The carbon isotopic compositions of the biomass and fatty acids of an organism are correlated with the pathways involved in $CO_2$ fixation and subsequent acetyl-coenzyme A (acetyl-CoA) synthesis by the organism (10). Previously, enzymatic analysis has revealed that the γ-proteobacterial endosymbiont of *A. hessleri* from the Mariana Trough utilizes the Calvin-Benson cycle and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) for $CO_2$ fixation (21); the ε-proteobacterial endosymbiont of *Alvinoconcha* aff. *hessleri* from the Central Indian Ridge mediates the rTCA cycle for the conversion of $CO_2$ into organic matter (22). The biomass of *A. hessleri* has a $\delta^{13}C$ value of approximately −29‰, and the fatty acids of the gastropod tissues are depleted in $^{13}C$ relative to the gastropod biomass by 6.8 to 9.8‰ (Table 2). Conversely, the biomass of *Alvinoconcha* aff. *hessleri* has a $\delta^{13}C$ value of approximately −11‰, and the fatty acids of the gastropod tissues are enriched in $^{13}C$ relative to the gastropod biomass by 1.5 to 5.9‰ (Table 2). Since the $\delta^{13}C$ values of $CO_2$ in hydrothermal fluids from globally distributed deep-sea hydrothermal fields exhibit relatively small variation (−1 to −7‰ [7]), the carbon isotopic composition of the biomass and fatty acids of the gastropod tissues can be used to elucidate nature of the pathways through which hydrothermal $CO_2$ was fixed, and fatty acids were subsequently synthesized by the bacterial endosymbionts.

The *Alviniconcha* gastropods that harbor γ-proteobacterial endosymbionts have biomass $\delta^{13}C$ values ranging from −30.0 to −30.7‰ with fatty acids depleted of $^{13}C$ relative to the biomass by 5.3 to 7.7‰ (Table 2); these findings are consistent with the chemoautotrophy of the Calvin-Benson cycle and the subsequent synthesis of fatty acids from $^{13}C$-depleted acetyl-CoA (21). As with *Alvinoconcha* aff. *hessleri*, the biomass $\delta^{13}C$ value of other *Alviniconcha* gastropods that harbor ε-proteobacterial endosymbionts have values ranging from −10.3 to −13.4‰ with fatty acids enriched in $^{13}C$ relative to the biomass by 2.9 to 7.3‰ (Table 2). Since the $^{13}C$ enrichment of fatty acids relative to biomass is a characteristic of fatty acid

synthesis from $^{13}C$-enriched acetyl-CoA produced through the rTCA cycle, the observed isotope fractionation patterns are most likely to reflect the chemoautotrophy typical of the rTCA cycle. Although the carbon isotopic composition of hydrothermal $CO_2$ from the sites and enzymatic activity of key enzymes involved in the two chemoautotrophic cycles were not examined in the present study, it seems likely that the γ- and ε-proteobacterial *Alviniconcha* endosymbionts assayed in the present study are chemoautotrophs that convert $CO_2$ into organic matter via the Calvin-Benson and rTCA cycles, respectively.

**Host-symbiont relationships among the *Alviniconcha* gastropods.** In this and previous studies, the phylogenetic relationships of host gastropods and their bacterial endosymbionts were conducted in conjunction with bulk and compound-specific carbon isotopic analyses at a global scale across five tectonic settings. Analysis of fatty acid profiles of gastropod tissues revealed that the symbiont-free mantle tissue contained substantially more monosaturated $C_{16}$ and $C_{18}$ fatty acids and that these were derived from the endosymbionts (21, 22). Furthermore, the carbon isotopic composition of the biomass and fatty acid content of the gill and symbiont-free gastropod tissues examined in the present study were similar (Table 2). This finding suggests that endosymbiont cells are consumed by, and incorporated into, the host gastropods.

Coupling of the host and endosymbiont lineages of *Alviniconcha* gastropods showed that, although *Alviniconcha* sp. type 2 is associated with bacterial endosymbionts from two related ε-proteobacterial lineages, each of the other *Alviniconcha* lineages harbors bacterial endosymbionts from one distinct lineage within either the γ- or ε-*Proteobacteria*. These host-symbiont relationships in *Alviniconcha* gastropods could potentially be used to motivate for the recognition each host lineage as a separate species. Furthermore, since *Alviniconcha* sp. types 1 and 2 harbor γ- and ε-proteobacterial endosymbionts, respectively, and, given that they appear to exclusively inhabit adjacent hydrothermal vents in the Manus and North Fiji Basins, then the possibility that they occupy separate ecological niches should be explored further.

In the present study, the symbiotic relationship in *Alviniconcha* gastropods from the Southwest Pacific was defined more clearly by using molecular phylogenetic analyses and carbon isotopic characterizations. Further ecological and anatomical studies of these uniquely evolved gastropod assemblages might increase our understanding of the gastropod-proteobacterial endosymbioses that have been discovered in hydrothermal vent ecosystems around the globe.

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