Single host and symbiont lineages of hydrothermal-vent gastropods *Ifremeria nautilae* (Provannidae): biogeography and evolution

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ABSTRACT: Hydrothermal-vent gastropods belonging to the genus *Ifremeria* of the family Provannidae, which derive their nutrition from chemoautotrophic bacterial endosymbionts, constitute an important faunal element in the ecology of deep-sea hydrothermal systems in the SW Pacific. In order to determine phylogenetic relationships between the hosts and endosymbionts of *Ifremeria* gastropods, as well as their fatty-acid profiles and bulk and compound-specific carbon-isotopic signatures, we analyzed *Ifremeria* gastropods from the Manus, North Fiji and Lau Back-Arc Basins in the SW Pacific. Partial sequences of the mitochondrial cytochrome *c* oxidase subunit I gene suggest that *Ifremeria* gastropods from the 3 basins belong to a single species, *I. nautilae*. Based on the 16S rRNA gene sequences and results from fluorescence *in situ* hybridization analysis, the gill endosymbionts of *Ifremeria* gastropods from the 3 basins were grouped in a single lineage of *γ*-Proteobacteria, with sequence similarities of >98.3%. Placement of the endosymbionts within this single lineage was supported by fatty-acid profiles and carbon-isotopic compositions of the *Ifremeria* gastropods. Phylogenetic relationships inferred among gastropod hosts and among their endosymbionts were not congruent, implying that acquisition of endosymbionts might be from the environment rather than through vertical transmission. Differences in geographical distribution and host speciation pattern between the confamilial *Alviniconcha* and *Ifremeria* gastropods might be attributed to the remarkable differences in symbiotic strategy with chemoautotrophic bacteria.

KEY WORDS: Chemoautotrophic bacteria · Endosymbiosis · Provannidae · Gastropod

INTRODUCTION

Unlike hydrothermal-vent communities in the eastern Pacific, where large vestimentiferan tubeworms and bivalve mollusks are the most conspicuous organisms, the hydrothermal-vent sites in the SW Pacific are dominated by gastropods (Desbruyeres et al. 1994, Galkin 1997). In the Manus, North Fiji and Lau Back-Arc Basins, *Alviniconcha* and *Ifremeria* gastropods of the Provannidae family are the dominant primary consumers (Desbruyeres et al. 1994, Galkin 1997). For their nutrition, the 2 provannid gastropods are known to depend on the intracellular chemoautotrophic bacteria found in specialized cells called bacteriocytes in their gill filaments (Stein et al. 1988, Windooffer & Giere 1997, Suzuki et al. 2005a,b, Urakawa et al. 2005). While the distribution of *Ifremeria* gastropods is restricted to the 3 SW Pacific basins (Waren & Bouchet...
Alviniconcha gastropods have a biogeographic distribution that extends from the SW Pacific basins to the Central Indian Ridge of the Indian Ocean (Hashimoto et al. 2001) and the Mariana Trough in the western Pacific (Hesseler & Lonsdale 1991).

Previously, Ifremeria gastropods from the Manus and North Fiji Basins were grouped as a single species, Ifremeria nautilei (Kojima et al. 2000). Urakawa et al. (2005) reported phylogenetic affiliations of endosymbionts in I. nautilei from the Manus Basin. However, the host phylogenies of the Ifremeria gastropods from the Lau Basin and the phylogenies of the endosymbionts from the North Fiji and Lau Basins are currently unknown. We also reported that the Alviniconcha genus consists of 5 lineages (Kojima et al. 2001, 2004, Suzuki et al. 2006), each of which harbors phylogenetically distinct chemosynthetic bacteria belonging to either the γ- or the ε-Proteobacteria (Suzuki et al. 2005a, b, 2006, Urakawa et al. 2005). Given that Alviniconcha and Ifremeria gastropods employ similar nutritional strategies and inhabit similar hydrothermal vent habitats, Ifremeria gastropods may have established endosymbiotic relationships with diverse groups of chemosynthetic bacteria. In the present study, we conducted a molecular survey of the host and endosymbiont lineages of Ifremeria gastropods from the 3 back-arc basins in the SW Pacific. We also conducted fatty acid profile and carbon-isotopic analyses of the gastropod tissues, in order to identify phenotypic traits and determine the carbon metabolism of the bacterial endosymbionts.

MATERIALS AND METHODS

Gastropod specimens. Gastropod specimens were collected from the 3 back-arc basins in the SW Pacific utilizing the manned submersibles ‘Shinkai 2000’ and ‘Shinkai 6500’. The latitude/longitude and depth of the sampling site, the date of the sampling, and the submersible used for the sampling are summarized in Table 1. References for maps of the sampling locations are also listed in Table 1.

<table>
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<th>Species</th>
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Table 1. Ifremeria nautilei. Number of individuals studied in the SW Pacific (n), sample locations and depths, dates, dive numbers (submersible used) and references containing maps of the sample locations.

DNA analysis of mitochondrial cytochrome c oxidase subunit I (COI) gene sequences. Total DNA was extracted from the head-food region of the specimens from the 3 back-arc basins by either (1) grinding, digestion with sodium dodecyl sulfate, and extraction with phenol and chloroform; or (2) by using the DNEasy kit (QIAGEN) and magnetically purifying using a MagExtractor kit (TOYOBO), in accordance with the manufacturer’s instructions. A fragment (about 960 bp) of the mitochondrial gene for COI was amplified by the polymerase chain reaction (PCR) with primers COI-B (Hasegawa et al. 1996) and COI-6 (Shimayama et al. 1990). The conditions for PCR were as follows: 94°C for 60 s; and then 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 obtained fragment was determined using a sequencer (ABI3100 or ABI3130, Applied Biosystems) with the primer COI-3 (Shimayama et al. 1990), in addition to the PCR primers.

Phylogenetic relationships were estimated by the maximum-parsimony (MP) method, using a multiple, equally parsimonious heuristic search with tree bi-section–reconnection and 1000 random addition sequence replicates, and by the maximum-likelihood (ML) method in the PAUP* package (Version 4.0b10) (Swofford 2002), and by the neighbor-joining (NJ) method (Saitoh & Nei 1987) in MEGA 3 (Kumar et al. 2004). In addition, the 7 sequences obtained from the Ifremeria specimen, the endosymbiont of which was analyzed by Urakawa et al. (2005), were used for analysis. As an outgroup, the dominant haplotype of Alviniconcha hessleri from the Mariana Trough (Kojima et al. 2001; AB051791) was also used.

The mitochondrial COI gene sequences of Ifremeria gastropods from the Manus, North Fiji and Lau Basins are available from the DDBJ (DNA databank of Japan) under the accession numbers AB238951 to AB238957.

DNA analysis of endosymbiont 16S rRNA gene sequences. The endosymbiont DNA was extracted from the dissected gill tissues using the DNEasy kit and magnetically purified using MagExtractor, as described above. The endosymbiont 16S rRNA gene sequences were amplified by PCR using LA Taq poly-
merase (TaKaRa) with the oligonucleotide primers Bac27F and Uni1492R (Lane 1991). Thermal cycling was performed using a GeneAmp 9700 thermal cycler, with 27 cycles of denaturation at 96°C for 20 s, annealing at 53°C for 45 s, and extension at 72°C for 120 s. The amplified 16S rRNA gene–sequence products were cloned and then sequenced with an ABI 3100 capillary sequencer and a dRhodamine sequencing kit as per the manufacturer’s recommendations (Perkin Elmer/Applied Biosystems). Bacterial clone libraries were constructed using the original TA cloning kit (Invitrogen).

The sequence similarity among all of the partial 16S rRNA gene sequences, which were 500 nucleotides long, was analyzed using the FASTA program with the DNA-SIS software (Hitachi Software). A single phylogenetic clone type (phyotype) was obtained from the clone-type analysis, and the partial sequence was extended and manually aligned according to the secondary structures using ARB (a software environment for sequence data; Ludwig et al. 2004). Phylogenetic analysis was performed by the NJ, MP, and ML methods using PAUP (Swofford 2002), based on 817 nucleotide positions (60 to 875, Escherichia coli numbering).

The bacterial 16S rRNA gene sequences from the gill endosymbionts of Ifremeria gastropods from the Manus, North Fiji, and Lau Basins are available from DDBJ under the accession numbers AB238958 to AB238964.

Fluorescence in situ hybridization (FISH) analysis. A previously designed rRNA-targeted oligonucleotide probe for the endosymbiont of Ifremeria nautilii from the Manus Basin was modified in the present study (Urakawa et al. 2005). The probe, hereafter referred to as Ifre576, is 17 bases long, which corresponds to Escherichia coli positions 576 to 592 (5'-GACTAAACCGCCTACCC-3'). For whole-cell hybridization, dissected gill filaments from 3 individuals were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 2 h and dehydrated in an ethanol series (50, 75, and 100%, v/v), followed by 3 washes in xylene and infiltration with paraffin wax. The wax-embedded specimens were then sectioned (thickness: ~3 µm) and mounted on 3-aminopropyltriethyloxy-silane (APTS)-coated slides. For the deparaffinized specimens, hybridization was conducted at 46°C in a solution containing 20 mM Tris-HCl (pH 7.4), 0.9 M NaCl, 0.1% sodium dodecyl sulfate, 30% (v/v) formamide, and 50 ng µl⁻¹ of the Ifre576 probe and the ‘universal’ bacterial probe EUB338 (Giovannoni et al. 1988), which were labeled at the 5'-end with Cy-3 and fluorescein, respectively. After hybridization, the slide was washed at 48°C in a solution lacking the probe and formamide at the same stringency, adjusted by NaCl concentration (Lathe 1985), and subsequently stained with 4',6-diamidino-2-phenylindole (DAPI) at 0.4 µg ml⁻¹. The slides were examined using either an Olympus BX51 microscope or an Olympus FV5000 confocal laser-scanning microscope. A negative control probe for Ifre576, in which 2-base mismatches were introduced in the middle (5'-GACTAAACCGCCTACCC-3'), was used for testing unspecific labeling.

Bulk carbon-isotopic analysis. Gastropod individuals were dissected into gill and mantle tissues, and the dissected tissues were lyophilized. A small portion of each lyophilized tissue was powdered and then acid-fummed for 6 h (Van Dover 2002). The rest of the untreated lyophilized tissue was stored at ~80°C for fatty-acid extraction. The carbon-isotopic compositions of the cultures and the gastropod tissues were analyzed by a Thermo Electron DELTAplus Advantage mass spectrometer connected to an elemental analyzer (EA1112) through a ConFlo III interface. The measured isotopic composition was expressed as δ¹³C, which can be defined as follows:

\[ \delta^{13}C = \left( \frac{^{13}C/^{12}C}_{\text{sample}} / \frac{^{13}C/^{12}C}_{\text{standard}} - 1 \right) \times 10^3 \]  

where \( \left( ^{13}C/^{12}C \right)_{\text{sample}} \) is the ¹³C/¹²C abundance ratio for the sample and \( \left( ^{13}C/^{12}C \right)_{\text{standard}} \) is the ¹³C/¹²C abundance ratio for the Pee Dee Belemnite carbonate (PDB) standard. The values of δ¹³C therefore represent the difference, in parts per thousand (per mille, ‰), between the ¹³C/¹²C value of the sample and that of the standard.

Analysis of the fatty-acid methyl-ester (FAME) profiles. For the extraction of cellular fatty acids (FA), a method described in Komagata & Suzuki (1987) was used. Approximately 20 mg of gastropod tissue was incubated in 1 ml of anhydrous methanolic hydrochloric acid at 100°C for 3 h. After the addition of 1 ml of deionized, distilled water (DDW) to the cooled aliquots, FAMEs were extracted 3 times with 3 ml of n-hexane. The n-hexane fractions were washed with an equal volume of DDW and dehydrated with anhydrous Na₂SO₄. The concentrated FAMEs were incubated in 1 ml of anhydrous methanolic hydrochloric acid at 100°C for 3 h. After the addition of 1 ml of deionized, distilled water (DDW) to the cooled aliquots, FAMEs were extracted 3 times with 3 ml of n-hexane. The n-hexane fractions were washed with an equal volume of DDW and dehydrated with anhydrous Na₂SO₄. The concentrated FAMEs were stored at ~20°C for subsequent carbon-isotopic analyses. Although this extraction method degrades the cyclopropyl FAME (Moss et al. 1974), it enables minimum loss of material during extraction.

The identities of the FAMEs were determined by comparison of the retention times and spectra to those of known FAME standards by gas chromatography-mass spectrometry (GC-MS), using a Shimadzu GCQ GC-MS system. The oven temperature was set to 140°C for 3 min and then increased to 250°C at a rate of 4°C min⁻¹, with He at a constant flow of 1.1 ml min⁻¹ through a DB-5MS column (30 m × 0.25 µm × 0.25 mm; J&W Scientific). The double-bond positions of the monounsaturated FAMEs were determined by analyzing their dimethyl disulfide adducts (Nichols et al.
The standard nomenclature for FA was used. FAs are designated \( \text{X:Y}\Delta \text{Z} \), where \( \text{X} \) is the number of carbon atoms, \( \text{Y} \) is the number of double bonds, and \( \text{Z} \) is the position of the double bond from the carboxyl end.

Compound-specific carbon-isotopic analysis. The \( \delta^{13} \text{C} \) values of the FAMEs were determined by the GC-carbon-isotope ratio MS using a Thermo Electron DELTAplus Advantage mass spectrometer connected to a GC (Agilent 6890) through a GC/C/C/III interface. The oven temperature was set to 120°C for 3 min and then increased to 300°C at a rate of 4°C min\(^{-1} \), with He at a constant flow of 1.1 ml min\(^{-1} \) through a HP-5 column (30 m \( \times \) 0.25 \( \mu \)m \( \times \) 0.25 mm; Agilent). The isotopic compositions of the FAMEs were measured with an internal isotopic standard (19:0, \( \delta^{13} \text{C} = -29.80\% \)), and the additional carbon atom from the methanol-derivatizing reagent (\( \delta^{13} \text{C} = -39.04\% \)) was corrected. The measurement errors produced by the internal isotopic standard were within 1\% for all isotopic analyses.

RESULTS

Phylogenetic analysis of \( \text{Ifremeria} \) gastropods

Partial nucleotide sequences (771 base pairs) of the mitochondrial COI gene were obtained from a single specimen from the Lau Basin and 3 specimens from the Manus and North Fiji Basins. All determined sequences formed a clear monophyletic cluster with the \( \text{Ifremeria nautilei} \) sequences determined by Kojima et al. (2000) (data not shown). Therefore, the sequence similarity among the \( \text{Ifremeria} \) gastropods from the 3 SW Pacific basins suggests that they are all affiliated to the single species \( \text{I. nautilei} \).

Phylogenetic analysis revealed that the \( \text{Ifremeria} \) gastropods from 3 basins were divided into 2 clades, namely those from the Manus Basin group and those from the 2 remaining basins (Fig. 1). The monophyly of the latter was supported by high bootstrap value (96/94/91 for NJ/MP/ML methods, respectively), and a sequence of a single specimen from the Lau Basin was identical to 2 of those from the North Fiji Basin. On the other hand, the monophyly of the former was supported by relatively low bootstrap values (66/75/65 for NJ/MP/ML), and additional phylogenetic analyses that included previously analyzed haplotypes suggested that the Manus Basin group is paraphyletic (data not shown).

Phylogenetic analyses of \( \text{Ifremeria} \) endosymbionts

The phylogenetic relationships among gill endosymbionts, the host lineages of which were analyzed as described above, were determined based on 16S rRNA gene sequences. Examination of the 8 clones generated from the 16S rRNA gene–sequence library from the gill filaments of each gastropod showed only 1 phylotype. Phylogenetic analysis placed the phylotypes within the \( \gamma \)-Proteobacteria (Fig. 2). The \( \gamma \)-proteobacterial phylotypes of the \( \text{Ifremeria} \) gastropods are closely related to a previously described endosymbiont of \( \text{I. nautilei} \) from the Manus Basin, and share sequence similarities of >98.3\% (Urakawa et al. 2005). The closest relative of the \( \gamma \)-proteobacterial phylotypes of the \( \text{Ifremeria} \) endosymbionts was the endosymbiont harbored in \( \text{Alviniconcha hessleri} \) from the Mariana Trough in the western Pacific (Suzuki et al. 2005a).

In order to ensure that the phylotypes revealed by 16S rRNA gene–sequence clone-library analysis were...
only those of the endosymbionts in the gill filaments, we conducted FISH analysis using the probe Ifre576 for all *Ifremeria* gastropod specimens examined in the present study. The sections of the gill filaments were hybridized with the EUB338 and Ifre576 probes, followed by DNA staining with DAPI. Representative epifluorescence micrographs of the gill filaments of the *Ifremeria* gastropod from the Lau Basin are shown in Fig. 3. The presence of dense aggregates of bacterial cells, as well as host nuclei (labeled 'N' in Fig. 3A,D), in

Fig. 3. Epifluorescence micrographs of the endosymbiotic bacteria associated with the gill filaments of *Ifremeria nautillei* from the Lau Back-Arc Basin. (A) DNA staining of the section of the gill filaments with 4',6-diamidino-2-phenylindole (DAPI). In addition to the bacterium-like cells, the host nuclei are stained and labeled 'N.' (B) Fluorescence in situ hybridization (FISH) performed with the fluorescein-labeled Ifre576 probe (same microscopic field as that of Panel A). (C) FISH performed with the Cy-3-labeled EUB338 probe. (D) DNA staining of the section of the gill filaments with DAPI. (E) FISH performed with the Cy-3-labeled Ifre576 probe in which 2-base mismatches were introduced (same microscopic field as that of Panel D). (F) FISH performed with the Cy-3-labeled EUB338 probe.
the gill filaments was confirmed by FISH analysis with the EUB338 probe and DAPI staining (Fig. 3A,C,D,F). Hybridization with the Ifre576 probe specific for the γ-proteobacterial phylotype gave a signal pattern almost identical to that of the EUB338 probe (Fig. 3B,C), indicating that most of the endosymbiotic bacterial cells are affiliated to the phylotype. A negative control probe, in which 2-base mismatches were introduced in the middle of the Ifre576 probe, did not hybridize with the bacterial cells under the same conditions used for FISH analysis with the mismatch-free Ifre576 probe, indicating the absence of nonspecific labeling (Fig. 3E,F).

Bulk carbon-isotopic analysis

The δ¹³C values of the gastropod gill and mantle tissues were measured. The gastropod tissues had a δ¹³C range from −28.2 to −33.9‰, as shown in Table 2. Despite the abundance of endosymbiont cells in the gill, the carbon-isotopic composition of the symbiont-free mantle tissue was nearly identical to that of gill tissue, indicating that the endosymbiont biomass was as ¹³C-depleted as the symbiont-free gastropod tissue.

FAME profiles

The analysis of the FAME profiles from the gastropod tissues showed high levels of saturated C₁₆ and C₁₈ fatty acids and of the monounsaturated fatty acids 16:1Δ8 and 16:1Δ9, with 18:1Δ11, and 20:1Δ13 (Fig. 4). In addition, the monosaturated fatty acid 16:1Δ8 was particularly abundant in the gill tissue.

Compound-specific carbon-isotopic analysis

The carbon-isotopic compositions of several FAMEs from the gastropod tissues were measured; the ¹³C values of the FAMEs after correction for the methanol-derivatizing reagent and the total FAMEs calculated on the basis of the FAME compositions are shown in Table 2. The FAMEs analyzed in this study were all ¹³C-depleted relative to the gastropod biomass, by 4.4 to 12.5‰.

DISCUSSION

Single host and endosymbiont lineages

Although the host lineages of Alviniconcha gastropods are diverse (Kojima et al. 2001, 2004, Suzuki et al. 2006), Ifremeria gastropods from the 3 back-arc basins in the SW Pacific are closely related and all affiliated to the single species I. nautilei (Kojima et al. 2000). Ifremeria gastropods examined to date by 16S rRNA gene sequence analysis all harbor chemoautotrophic endosymbionts that phylogenetically fall into the single lineage of the γ-Proteobacteria. The monounsaturated C₁₆ fatty acids 16:1Δ8 and 16:1Δ9 are thought to originate mostly from the γ-proteobacterial endosymbionts, given that they were abundant in the gastropod tissues.
symbiont-bearing gill tissue. These fatty acids were abundant in the gill tissues of all *Ifremeria* gastropods examined in the present study, as well as in specimens previously sampled from the White Lady site in the North Fiji Basin (Pranal et al. 1996).

The carbon-isotopic compositions of the gastropod tissues, as well as the carbon isotope fractionation patterns of the fatty acids relative to whole-gastropod tissues, were similar among *Ifremeria* gastropods from the 3 basins and *Alviniconcha* gastropods that depend upon γ-proteobacterial endosymbionts for nutrition (Table 2). Previous enzymatic analysis has shown that the endosymbionts of *Ifremeria* gastropods from the North Fiji and Lau Basins mediate the Calvin–Benson cycle for CO₂ fixation (Desbruyères et al. 1994). The carbon-isotopic compositions of the *Ifremeria* gastropods examined in the present study are consistent with the chemosynthesis of the Calvin–Benson cycle. These polyphasic results indicate that *Ifremeria* gastropods are nutritionally dependent upon chemosynthetic endosymbionts within the single lineage of the γ-Proteobacteria.

**Host–symbiont relationships in *Ifremeria* gastropods**

Phylogenetic relationships among hosts and their symbionts have provided fundamental insights into the evolutionary histories of deep-sea hydrothermal vent fauna and their endosymbionts, as well as the modes of symbiont acquisition (Distel 1998, Peek et al. 1998, DiMeo et al. 2000, Won et al. 2003). Although the endosymbiont of the *Ifremeria* gastropod from the Lau Basin grouped separately from the endosymbiont taxa from the North Fiji Basin, the gastropod host from the Lau Basin clustered with those from the North Fiji Basin. Similarly, *Ifremeria* hosts and endosymbionts from the Manus Basin previously examined by Urakawa et al. (2005), as well as the *Ifremeria* specimens examined in the present study, exhibited phylogenetic relationships that were incongruent with each other. This lack of congruence between host and endosymbiont phylogenies may have resulted from the absence of coevolution of *Ifremeria* gastropods and their endosymbionts through horizontal transmission of the symbionts from one generation to the next. However, the phylogenetic incongruence might be
ascribed to polymorphic populations of the gastropods with respect to the genotype of symbionts.

**Dispersal capabilities of the 2 provannid gastropods**

Vent animals with planktonic larval stages tend to have considerable dispersal capabilities (Van Dover et al. 2002). Based on larval and egg capsule observations in *Alviniconcha* gastropods, it appears that *Alviniconcha* gastropods undergo planktonic development (Waren & Bouchet 1993), whereas the modes of development in *Iremeria* gastropods are currently unknown. Kojima et al. (2000) implied that the difference in the dispersal potential of the 2 provannid gastropods might be attributed to the non-planktonic development of *Iremeria* gastropods.

*Bathymodiolus* mussels, which are able to form dual endosymbioses with sulfur-oxidizing chemosynthetic and methanotrophic bacteria (Fisher 1990), are almost ubiquitous in deep-sea hydrothermal-vent and cold-seep environments, while most other bivalve hosts harbor a single lineage of sulfur-oxidizing chemosynthetic endosymbionts (Distel et al. 1994). Won et al. (2003) speculated that the global distribution of *Bathymodiolus* mussels is related to this capacity for dual endosymbioses. In the present study, it was determined that in sharp contrast to *Alviniconcha* gastropods, which have endosymbiotic relationships with chemosynthetic bacteria in 6 lineages of both γ- and ε-Proteobacteria (Suzuki et al. 2006), the endosymbionts of *Iremeria* gastropods belonged to a single lineage of γ-Proteobacteria. Taken together, the endosymbiont diversities of provannid gastropods might be correlated with their dispersal capabilities.

**Host evolution possibly affected by chemoautotrophic endosymbiosis**

*Alviniconcha* spp. and *Iremeria nautiliei* thrive in the hydrothermal-vent habitats in the SW Pacific. Although *Alviniconcha* gastropods have diversified to a great extent and have established mutualistic endosymbioses with a wide variety of chemoautotrophic bacteria, *Iremeria* gastropods have endosymbiotic relationships that are restricted to 1 group of the Proteobacteria. While the mechanisms employed by both gastropods for maintaining or changing their endosymbiotic relationships remain unclear, the congruence between the host and endosymbiont diversities might support the inference that the evolution of the 2 hydrothermal-vent-endemic gastropods has been profoundly influenced by their endosymbiotic associations with chemoautotrophic bacteria.

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**LITERATURE CITED**


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