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Molecular investigations of the stalked barnacle *Vulcanolepas osheai* and the epibiotic bacteria from the Brothers Caldera, Kermadec Arc, New Zealand

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The hydrothermal-vent barnacle Vulcanolepas osheai *of the subfamily Neolepadinae is one of the most conspicuous organisms at the Brothers Caldera, south Kermadec Arc, New Zealand. Like a neolepad species found in the Lau Basin,* V. osheai *harbours filamentous bacteria on its elongated cirral setae. To define the phylogenetic affiliation of the epibiotic bacteria and the nutrition of the barnacle host, we conducted molecular phylogenetic and isotopic analyses. Analysis of 16S rRNA gene sequences of microbial communities on the cirral setae showed that among 91 bacterial sequences investigated, 28 sequences were related to the ε-proteobacterial endosymbiont of* Alviniconcha *aff.* hessleri*; 11 sequences were related to the epibiont of the bresiliid shrimp* Rimicaris *exoculata. Fluorescence* in situ *hybridization showed that in contrary to results from the 16S rRNA gene-sequence library, approximately 80% of the filamentous bacteria hybridized with a probe targeting the sequences related to the epibiont of the bresiliid shrimp* R. exoculata. *The fatty-acid profiles of the filamentous bacteria and the host barnacle both contained high levels of monounsaturated C_{16} and C_{18} fatty acids, and the carbon isotopic compositions of the biomass and monounsaturated C_{16} and C_{18} fatty acids of both the bacteria and barnacle were nearly identical. This would suggest that the nutrition of the barnacle is highly dependent on bacteria thriving around the barnacle, including the epibiotic bacteria.*

Keywords: molecular investigations, *Vulcanolepas osheai*, epibiotic bacteria, Brothers Caldera, south Kermadec Arc, New Zealand

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INTRODUCTION

External associations between metazoans and filamentous bacteria are widespread in aquatic habitats (Polz & Cavanaugh, 1995). In the deep-sea, such associations have been long known for vent-obligate invertebrates, such as the polychaetous annelid *Alvinella pompejana* (Gail *et al.*, 1987) and the bresiliid shrimp *Rimicaris exoculata* (Van Dover *et al.*, 1988; Gebruk *et al.*, 1993; Segonzac *et al.*, 1993). More recently, filamentous bacteria have been found on the cirral setae of a neolepadine barnacle from the Lau Basin (Southward & Newman, 1998), and filamentous bacteria have been observed on the dermal iron sulphide sclerites of a newly discovered gastropod from the Central Indian Ridge (Goffredi *et al.*, 2004).

Phylogenetic analyses based on 16S rRNA gene sequences reveal that the filamentous epibionts of *Rimicaris exoculata* consist of a single bacterial species belonging to the epsilon subdivision of the Proteobacteria (ε-Proteobacteria) (Polz & Cavanaugh, 1995), while *Alvinella pompejana* harbours filamentous epibionts of several lineages of the ε-Protoebacteria (Haddad *et al.*, 1995). These epibionts can fix CO_2 (Desbruyères *et al.*, 1998; Polz *et al.*, 1998), and genomic analysis has suggested that the epibionts of *Alvinella pompejana* use the reductive tricarboxylic acid (rTCA) cycle for conversion of CO_2 into organic molecules (Campbell *et al.*, 2003). Similarly to marine invertebrates harbouring sulphur-oxidizing chemoautotrophic bacterial endosymbionts (Distel, 1998; Suzuki *et al.*, 2005a, 2005b, 2005c, 2006), it has been hypothesized that *Rimicaris exoculata* and *Alvinella pompejana* have established nutritionally mutualisitc symbioses with the ε-proteobacterial epibionts, and that the host animals have adapted to the ectosymbiosis by specializing the symbiont-housing body part (e.g. the modified setae of an expanded branchial chamber of *Rimicaris exoculata* and the expansions of the epidermis of *Alvinella pompejana*). However, these hypotheses remain speculative.

To expand our understanding of the nature and evolutionary aspects of the intimate relationships between marine

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invertebrates and filamentous bacteria, we conducted molecular phylogenetic analyses of epibotic bacteria associated with the deep-sea hydrothermal vent barnacle *Vulcanolepas osheai* of the subfamily Neolepadinae from the south Kermadec Arc off New Zealand. The fatty acid profiles and carbon isotopic compositions of the host tissue and the epibiont cells were also analysed to better understand the barnacle nutrition.

MATERIALS AND METHODS

Barnacle specimens and sampling site

Barnacles were collected in November 2004 from the north slope of Brothers Caldera, south Kermadec Arc, New Zealand, at a depth of 1313 m (34°52.7′ S 179°4.3′ E; Figure 1A), by means of the manned submersible 'Shinkai 6500'. The site has moderately high temperature venting (67°C), rich in hydrogen sulphide (~4 mM). A dense colony of *Vulcanolepas osheai* was observed (Figure 1B) with co-occurrence of the hydrothermal-vent shrimps *Alvinocaris* spp. and *Lebbeus* sp. (Figure 1B). A typical *Vulcanolepas osheai* specimen from the site is shown in Figure 1C.

Light microscopy

Specimens were dissected and the cirri mounted on slides in corn syrup for examination under transmitted light and by phase contrast.

DNA analysis

Genomic DNA was extracted from the cirral setae clothed with filamentous bacteria using a DNEasy kit (QIAGEN, Valencia, CA) and magnetically purified using a MagExtractor Kit (TOYOBO, Osaka, Japan), in accordance with the manufacturers' instructions. The 16S rRNA gene sequences were amplified through the polymerase chain reaction (PCR) using LA Taq polymerase (TaKaRa, Tokyo, Japan) with the oligonucleotide primers Bac349F and Bac806R (Takai & Horikoshi, 2000). Thermal cycling was performed using a GeneAmp 9700 Thermal Cycler, with 27 cycles of denaturation at 96°C for 20 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 120 seconds. The amplified 16S rRNA gene-sequence products were cloned using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA). Cloned 16S rRNA gene sequences were partially sequenced with an ABI 3100 Capillary Sequencer and a dRhodamine Sequencing Kit according to the manufacturer's recommendations (Perkin Elmer/Applied Biosystems, Foster City, CA). The sequence similarity among all of the partial sequences, which were 500 nucleotides long, was analysed using the FASTA program equipped with the DNASIS software (Hitachi Software, Tokyo, Japan). Partial 16S rRNA gene sequences with more than 97% similarity were grouped and represented by one 16S rRNA gene sequence type (phylotype). In order to validate that the partial 16S rRNA gene sequences obtained in this study covered the diversity of the epibiotic bacteria, a rarefaction analysis was conducted using the analytical approximation algorithm of Hurlbelt (Hurlbelt, 1971) available on the website http://www2.biology.ualberta.ca/jbrzusto/rarefact.php.

In order to obtain sequences from the nearly full region of the 16S rRNA gene, a clone library was constructed using the

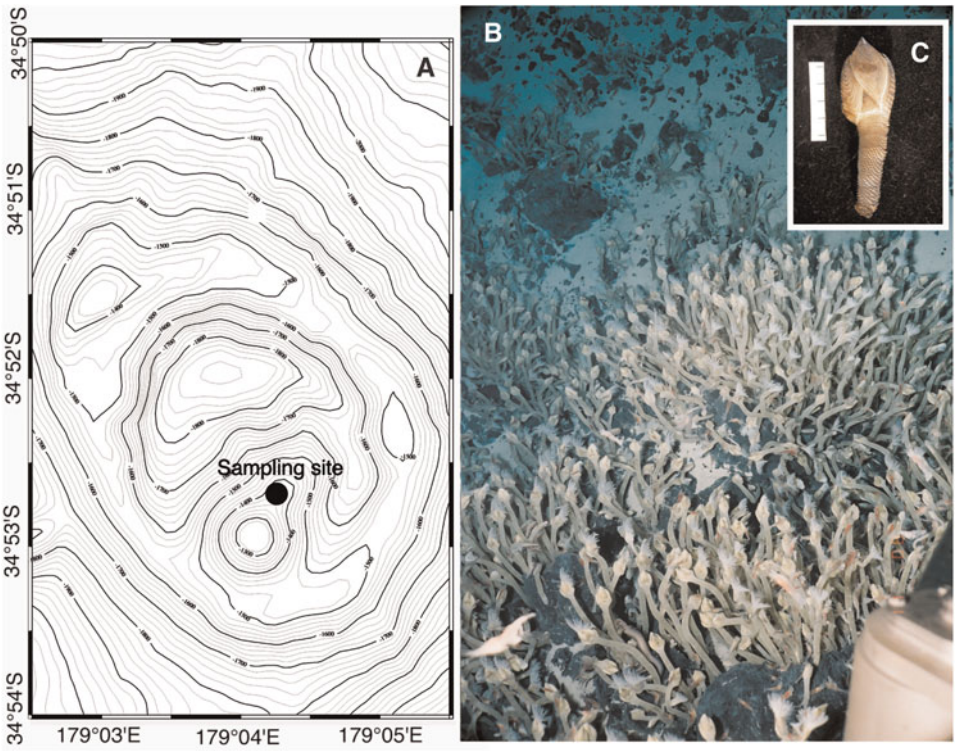

Fig. 1. (**A**) Bathymetric map of the Brothers Caldera, southern Kermadec Arc, New Zealand; (**B**) dense colony of *Vulcanolepas osheai* at the sampling site shown in Figure 1A; (**C**) a typical *i* specimen of *Vulcanolepas osheai* from the Brothers Caldera. Scale, 1 cm per bold unit.

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same method described above, except for the oligonucleotide primers Bac27F and Uni1492R (Lane, 1991). The representative sequences were extended and manually aligned according to the secondary structures using ARB (a software environment for sequence data (Ludwig *et al.*, 2004)). Evolutionary analysis was performed by the neighbour-joining method using PAUP (Swofford, 1999) based on 1393 nucleotide positions (56–1447, *Escherichia coli* numbering).

The accession numbers for the bacterial 16S rRNA gene sequences from the cirral setae are available at DDBJ under the accession numbers AB239758–AB239762.

Fluorescence *in situ* hybridization (FISH) analysis

An rRNA-targeted oligonucleotide probe previously designed for the ε-proteobacterial endosymbiont of *Alvinoconcha* aff. *hessleri* from the Indian Ocean (Suzuki *et al.*, 2005b) was used to detect microbial cells with the most dominant 16S rRNA gene sequence type. Previously designed rRNA-targeted oligonucleotide probes for the epibiont of *Rimicaris exoculata* from a Mid-Atlantic Ridge hydrothermal vent (Polz & Cavanaugh, 1995) and the dominant members of the ε-Proteobacteria (Takai *et al.*, 2004) were modified in the present study. In addition, we used general probes such as EUB338, GAM42a and CF319 (Table 1). These DNA probes were labelled at the 5′ end with either Cy-3 or fluorescein.

For whole-cell hybridization, dissected cirral setae clothed with filamentous bacteria from three individuals were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for two hours and dehydrated in an ethanol series (50, 75, and 100%, v/v). Hybridization was carried out at 46°C in a solution containing 20 mM Tris–HCl (pH 7.4), 0.9 M NaCl, 0.1% sodium dodecyl sulphate, 30% formamide and 50 ng/μl of two of the DNA probes described above. 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. The slides were examined using either an Olympus BX51 microscope or an Olympus FV5000 confocal laser-scanning microscope. A negative control probe for Rim656, in which two-base mismatches were introduced in the middle (5′-CTTCCCCTAACAGACTC-3′), and a negative control probe for EP404 with one-base mismatch (5′-AAAKG YGTCTTCCTCCA-3′) were used for testing unspecific labelling.

Bulk carbon isotopic analysis

Three barnacle individuals were dissected into the cirral setae clothed with filamentous bacteria and the remaining soft-body part, and the two sets of tissues were lyophilized. A small portion of each lyophilized tissue was powdered and then acid-fumed for 6 hours (53). The rest of the untreated lyophilized tissue was stored at −80°C for fatty-acid extraction. The carbon isotopic compositions of the barnacle tissues were analysed by a Thermo Electron DELTA^{plus} Advantage mass spectrometer connected to an elemental analyser (EA1112) through a ConFlo III interface.

Analysis of the fatty-acid methyl-ester (FAME) profiles

For the extraction of cellular fatty acids, a method described by Komagata & Suzuki (1987) was used. Approximately 20 mg of the barnacle tissues were incubated in 1 ml of anhydrous methanolic hydrochloric acid at 100°C for 3 hours. After the addition of 1 ml of deionized, distilled water (DDW) to the cooled aliquots, the fatty-acid methyl-esters (FAMEs) were extracted three times with 3 ml of n-hexane. The n-hexane fractions were washed with an equal volume of DDW and dehydrated with anhydrous Na_2SO_4. The concentrated FAMEs were stored at −20°C for subsequent carbon isotopic analyses.

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 minutes and then increased to 250°C at a rate of 4°C/minute with He at a constant flow of 1.1 ml/minute through a DB-5MS column (30 m × 0.25 μm × 0.25 mm; J&W Scientific). The double-bond positions of the monounsaturated FAME were determined by analysing their dimethyl disulphide adducts (Nichols *et al.*, 1986). The standard nomenclature for fatty acids is used: fatty acids are designated *X*:*Y*Δ*Z*, where *X* is the number of carbon atoms, *Y* is the number of double bonds, and *Z* is the position of the double bond from the carboxyl end.

Compound-specific carbon isotopic analysis

The $\delta^{13}C$ values of the FAMEs were determined by the GC–carbon-isotope ratio MS using a Thermo Electron DELTA^{plus} 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 minutes and then increased to 300°C at a rate of 4°C/minutes with He at a constant flow of 1.1 ml/minute through a HP-5 column (30 m × 0.25 μm × 0.25 mm; Agilent). The isotopic compositions of the FAMEs were measured with an internal isotopic standard (19:0,

Table 1. The oligonucleotide probes used in the present study.

Probe	Sequence (5′–3′)	Target site (*Escherichia coli* positions)	Target group	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S (338–355)	Bacteria	(Giovannoni *et al.*, 1988)
EP404	AAA(G/T)G(C/T)GTCATCCTCCA	16S (404–422)	ε-Proteobacteria	(Takai *et al.*, 2004)
GAM42a	GCCTTCCCACATCGTTT	23S (1027–1043)	γ-Proteobacteria	(Manz *et al.*, 1992)
CF319	TGGTCCGTGTCTCAGTAC	16S (319–336)	Bacteroidetes	(Manz *et al.*, 1996)
EPF93	TCCGCCACTTAGCTGAC	16S (93–109)	NZ-BA-2	(Suzuki *et al.*, 2005b)
EPF656	CTTCCCCTCCCAGACTC	16S (656–674)	NZ-BA-1	(Polz & Cavanaugh, 1995)

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$\delta^{13}C = -29.80$), and correction made for the additional carbon atom from the methanol-derivatizing reagent ($\delta^{13}C = -39.04$). The internal isotopic standard produced measurement errors within 1 for all isotopic analyses.

RESULTS AND DISCUSSION

Phylogenetic affiliations of filamentous bacterial epibionts of *V. osheai*

The phylogenetic affiliations of the filamentous epibionts on the cirral setae of *Vulcanolepas osheai* were determined from the 16S rRNA gene sequences. From the cirral setae of a single barnacle, 91 sequences of the partial region of the 16S rRNA gene were obtained and grouped into 25 phylotypes based on 97% similarity. Rarefaction analysis was conducted to check whether the 25 phylotypes are representative of the diversity of microorganisms associated with the cirral setae. Ninety-one sequences were deemed adequate to cover the epibiont diversity once it was found that 10 more sequences revealed but one additional phylotype. Among 91 sequences, 46 were clustered within the ε-Proteobacteria, of which 11 were related to the epibiont of *Rimicaris exoculata* (the sequence type NZ-BA-1; Figure 2) and 28 sequences were related to the endosymbiont of the hydrothermal-vent gastropod *Alviniconcha* aff. *hessleri* from the Central Indian Ridge (the sequence type NZ-BA-2; Figure 2). Five and 30 sequences were placed within the α-Proteobacteria and the γ-Proteobacteria, respectively. All five α-proteobacterial sequences were closely related to *Loktanella koreensis* (97% similarity). Among the γ-proteobacterial sequences, 18 sequences were related to *Leucothrix mucor* (91% similarity, the sequence type NZ-BA-7). Five sequences were affiliated to the phylum Bacteroides (the sequence types NZ-BA-3 and NZ-BA-8). Two sequences were related to Actinobacteria, and one was related either to the β-Proteobacteria, the δ-Proteobacteria or Firmicutes.

By using the different primer set, one that amplifies the nearly full region of 16S rRNA gene, a clone library was constructed as described above. Examination of 30 sequences showed that among the 22 ε-proteobacterial sequences, two sequences were related to the epibiont of *Rimicaris exoculata* (the sequence type NZ-BA-1), and 20 sequences were related to the endosymbiont of *Alviniconcha* aff. *hessleri* (the sequence type NZ-BA-2). Five and three sequences were placed within the phylum Bacteroides (the sequence types NZ-BA-3 and NZ-BA-8) and the γ-Proteobacteria (the sequence type NZ-BA-7), respectively.

To reveal the phylogenetic affiliations of the epibiont cells on the cirral setae, we conducted FISH analysis. Based on 1393-nucleotide long sequences that nearly cover the 16S rRNA gene, FISH probes were chosen from previous studies and modified. Filamentous cells, which were stained with DAPI, hybridized with the probe EUB338, indicated that the filamentous cells are all bacteria (Figure 3A, B). Despite the dominance of the 16S rRNA gene sequence type NZ-BA-2, the filamentous bacterial epibionts did not hybridize with the probe EPF93 targeting to the sequence type NZBA-2 related to the endosymbiont of *A.* aff. *hessleri*. Instead, approximately 80% of the bacterial filaments hybridized with the probe EPF656 targeting the minor 16S rRNA gene sequence type NZ-BA-1 related the epibiont of *R. exoculata* (Figure 3C). FISH analyses with the probe EPF656 and the probe EP404 specific to the ε-Proteobacteria showed that the filamentous ε-proteobacterial epibionts detected on the cirral setae were all affiliated with the 16S rRNA gene sequence type NZ-BA-1. The discrepancy in results obtained from the 16S rRNA gene-sequence library and FISH analyses might be explained by the difference in DNA copy number between the filamentous ε-proteobacterial epibiont and the ε-proteobacterial species dominantly found in the library. As neither the group-specific probes GAM42a nor CF319 hybridized with the filamentous epibionts (data not shown), it is not clear that the rest of the filamentous bacterial epibionts belong to either the γ-Proteobacteria or the phylum Bacteroides.

Light microscopy observations

Observations of the slide-mounted cirri show that the bacterial filaments on the setae of *Vulcanolepas oshaei* are slightly less numerous than those on the cirral setae of the neolepad species A from the Lau Basin, described by Southward & Newman (1998). At least two morphological forms are visible: some are long and very narrow; others are shorter and wider, showing a 'cellular' structure. These morphs may be related to the DNA differences.

Nutrition of *V. osheai* inferred from fatty-acid profiles and carbon isotopic compositions

There appear to be four potential food sources for setose-feeding barnacles in the deep-sea hydrothermal environment: (1) organic matter of photosynthetic origin (Enright *et al.*, 1981);

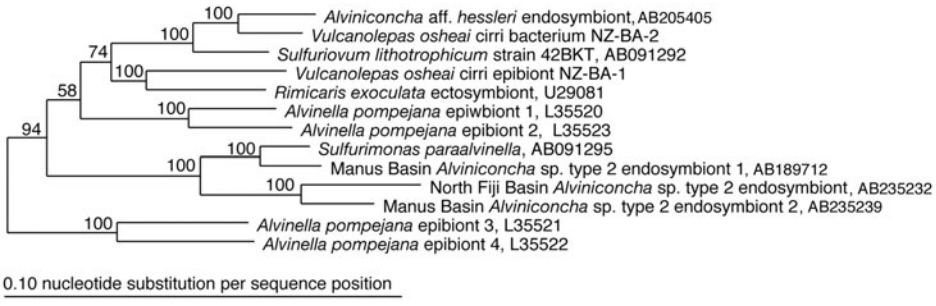

Fig. 2. Neighbour-joining tree of the members of the ε-Proteobacteria, including the *Vulcanolepas* epibiont based on near-complete 16S rRNA gene sequences (1393 nucleotides). Bootstrap values (in per cent) are based on 1000 replicates, and are shown for branches with bootstrap support >50%.

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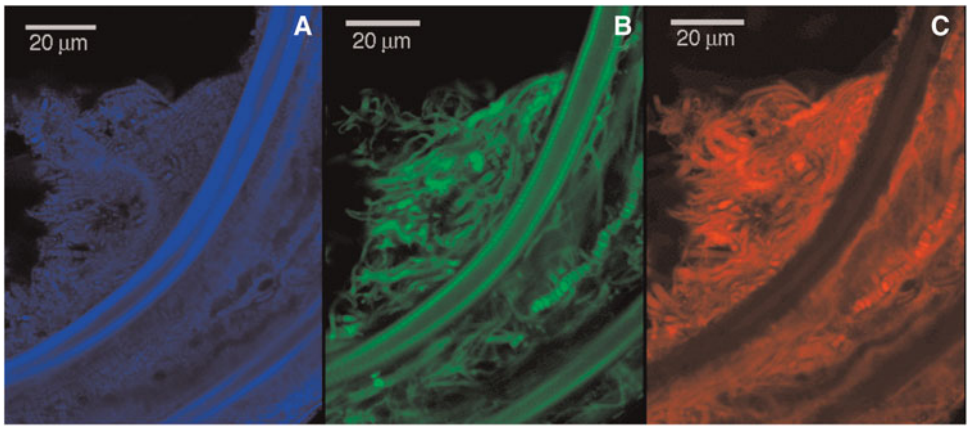

Fig. 3. Epifluorescence micrographs of the filamentous bacteria associated with the cirral setae of *Vulcanolepas osheai* from the Kermadec Arc. (**A**) DNA staining of the cirri with 4′,6-diamidino-2-phenylindole (DAPI); (**B**) fluorescence *in situ* hybridization (FISH) performed with the fluorescein-labelled EPF656 probe (same microscopic field as that of Figure 3A); (**C**) FISH performed with the Cy-3-labelled EUB338 probe.

(2) detritus and free-living bacteria of vent origin (Newman, 1979); (3) eggs, larvae and debris of vent animals; and (4) bacterial epibionts (Southward & Newman, 1998).

Hydrothermal-vent barnacles are known to have cirri and mouth parts specialized for feeding on finer particles than non-vent barnacles (Newman, 1979; Jones, 1993; Newman & Yamaguchi, 1995; Southward *et al.*, 1997; Yamaguchi & Newman, 1997; Southward & Newman, 1998). The epibiont-harbouring neolepadine barnacle, *Vulcanolepas osheai* from the Kermadec Arc, as well as that from the Lau Basin, have considerably elongated cirri with exceptionally long setae, relative to shallow-water as well as other deep-sea barnacles, and their mouth parts appear to be further modified to comb bacterial epibionts from the anterior cirri (Southward & Newman, 1998).

The FISH analysis, reported above, excludes the possibility that *Vulcanolepas osheai* derives its nutrition from endosymbiotic bacteria. It is well established that fatty acids of marine organisms are similar to those of the organisms they feed on (Gardner & Riley, 1972; Ackman & Hooper, 1973; Pranal *et al.*, 1997). Analysis of the FAME profiles from the barnacle tissues showed high levels of the saturated C_{16} and C_{18} fatty acids and the monounsaturated fatty acids 16:1Δ9, 18:1Δ11 and 20:1Δ13, and polysaturated C_{18} fatty acids were also abundant (Figure 4). As the symbiont-free tissue contains a high level of the monounsaturated fatty acids 16:1Δ9 and 18:1Δ11 that are characteristic of those of sulphur-oxidizing bacteria in H_2S-rich marine habitats (Conway & Capuzzo, 1991; Conway *et al.*, 1992; Pranal *et al.*, 1996, 1997; Suzuki *et al.*, 2005b; Zhang *et al.*, 2005) and are depleted in polysaturated C_{20} and C_{22} fatty acids that are enriched in marine organisms deriving their nutrition from photosynthetic food sources (Gardner & Riley, 1972; Ackman & Hooper, 1973; Pranal *et al.*, 1997), it appears that the barnacle is mainly feeding on sulphur-oxidizing bacteria or possibly planktonic larvae that feed on sulphur-oxidizing bacteria. However, some eukaryotes have been shown to require photosynthetic input (Fullarton *et al.*, 1995; Pond *et al.*, 1998, 2000), for essential nutrients not supplied by deep-sea chemosynthetic systems. Thus, a small but essential contribution of photosynthetically derived food to the barnacle's nutrition is quite likely.

In addition to fatty-acid profiles, the carbon isotopic compositions of the tissues and fatty acids of vent animals are

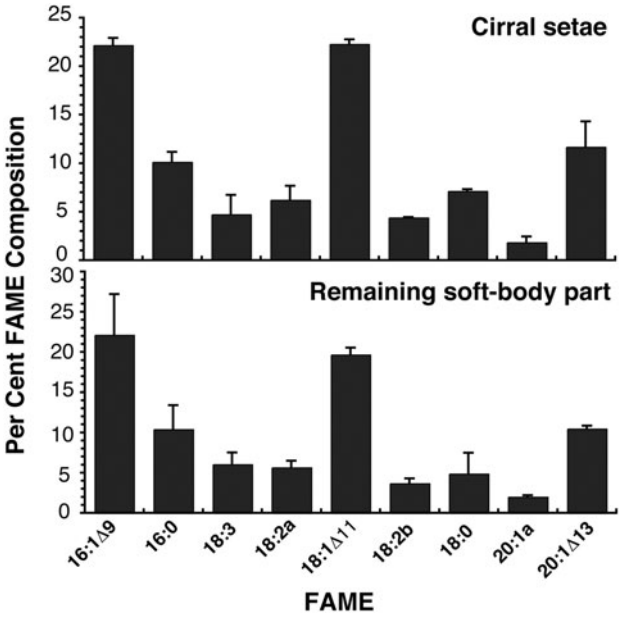

Fig. 4. Fatty acid profiles of the cirral setae clothed with bacteria and the remaining soft-body part from three individual barnacles. The values are means. SDs are based on at least two parallel measurements of tissues of each of the three individuals.

similar to those of their food sources (Conway & Capuzzo, 1991; Conway *et al.*, 1992; Pond *et al.*, 1998, 2000; Suzuki *et al.*, 2005a, 2005b). The barnacle tissues we studied had a $\delta^{13}C$ range from −12.0 to −12.3‰ as shown in Table 1. The carbon isotopic compositions of the symbiont-free tissue are nearly identical to those of the cirral setae with bacterial filaments, indicating that the epibiont biomass is as ^{13}C-depleted as the barnacle host tissue. The carbon isotopic compositions of some FAMEs from the barnacle tissues were measured; the $\delta^{13}C$ 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 analysed in this study were nearly identical in isotope composition to the total biomass, except for the monounsaturated C_{20} fatty acid, which was ^{13}C-depleted by >7.6‰ relative to the biomass (Table 2). The similar carbon isotopic compositions of the biomass

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Table 2. Carbon isotopic compositions of the total biomass and FAME of the barnacle tissues. Carbon isotopic compositions are reported as $\delta^{13}C$ values (‰).

Species tissue	Total biomass	FAME				
		16:1	16:0	18:1	18:0	20:1
Vulcanolepas osheai from the Kermadec Arc						
Cirral setae	−12.3 ± 0.2[a]	−11.7 ± 0.5[b]	−13.6 ± 0.5	−11.7 ± 1.7	−14.0 ± 0.9	−20.4 ± 2.5
Remaining soft body	−12.0 ± 0.1	−12.6 ± 1.9	−14.6 ± 2.6	−11.4 ± 4.8	−14.1 ± 2.4	−19.6 ± 2.4

[a]mean ± SD. At least duplicate measurements were conducted for each of the tissue parts. Three gastropod individuals were analysed;
[b]the isotopic compositions of the total FAMEs were calculated on the basis of the FAME compositions.

and fatty acids of the bacterial epibionts and the barnacle host leads us to suggest that the host barnacle derives most of its nutrition from the bacterial epibionts. However, other nutritional sources within the vent ecosystem cannot be completely excluded. For example, free-living sulphur-oxidizing bacteria and planktonic larvae of vent animals captured by the cirri and digested by the barnacle might provide similar tissue isotope ratios. If feeding on free-living bacteria or planktonic larvae, one would expect a wider range of such organisms to be utilized, and consequently there would be less exact agreement in the FAME profiles. Such possibilities could be investigated by long-term monitoring of free-living microorganisms and planktonic larvae in the barnacle habitat.

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