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Sulphur-isotopic composition of the deep-sea mussel *Bathymodiolus marisindicus* from currently active hydrothermal vents in the Indian Ocean

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Sulphur-isotopic composition of soft tissues from *Bathymodiolus marisindicus* collected from hydrothermal vents in the Indian Ocean was reported. The $\delta^{34}\text{S}$ values of the soft tissues (+3~+5‰ vs Cañon Diablo troilite) were nearly identical to those from the associated hydrothermal fluid and chimney sulphides (+5 to +8‰), but were significantly different from that of the common seawater sulphate (+21‰), which suggested that the endosymbiotic bacteria used sulphide in the fluid as an energy source. Transmission electron microscopic observation of the endosymbionts also suggested that the symbiont is a thioautotroph. *Bathymodiolus* species, which depend on either sulphide or methane oxidation, or both, have a worldwide distribution. *Bathymodiolus marisindicus* from the Indian Ocean has a close relationship with congeners in the Pacific Ocean as evidenced by form of symbiosis. Biogeography and migration of the genus *Bathymodiolus* based on the relevant data are briefly discussed.

INTRODUCTION

The Indian Ocean was the most recent exploration target for the discovery of active hydrothermal vents and associated biological communities, which had previously been reported only in the Pacific and Atlantic oceans. The Indian Ocean is regarded as a key area that links the Atlantic and Pacific oceans and information on Indian Ocean communities will contribute to understanding the biogeographical distribution and migration of animals unique to areas of hydrothermal activity (Tunnicliffe & Fowler, 1996; Tunnicliffe et al., 1998). A currently active hydrothermal vent field, named the Kairei Field, with an associated dense animal community was first identified on the first segment of the Central Indian Ridge, north of the Rodriguez Triple Junction during the RV 'Kairei' and ROV 'Kaiko' cruise in the summer of 2000 (Hashimoto et al., 2001) and has been the subject of intense interest (Van Dover et al., 2001; Van Dover, 2002).

During this cruise, several benthic animals and associated hydrothermal fluids were successfully collected (Hashimoto et al., 2001). A deep-sea mussel, *Bathymodiolus marisindicus* was one of the dominant species of the unique biological community (Hashimoto, 2001). At least 13 species of the genus *Bathymodiolus* have been described from hydrothermal vents and cold-seeps in the Pacific

and Atlantic Basins (Kenk & Wilson, 1985; Hessler & Lonsdale, 1991; Cosel et al., 1994, 1999; Hashimoto & Okutani, 1994; Paull et al., 1995; Cosel & Olu, 1998; Dubilier et al., 1998; Gustafson et al., 1998; Tunnicliffe et al., 1998). Whenever a new habitat for deep-sea mussels or a new species has been discovered, their strategy for energy acquisition, namely, the form of symbiosis has been discussed. Deep-sea mussels have been reported to harbour thioautotrophs and/or methanotrophs as endosymbionts in their gills (Nelson & Fisher, 1995). Several species of *Bathymodiolus* with dual symbiosis have been identified from the Atlantic Basins (Cavanaugh et al., 1992; Fiala-Médioni et al., 1996). Deep-sea mussel species with thioautotrophs have been reported from the western and eastern Pacific and those with methanotrophs from the western Pacific and the Gulf of Mexico (Nelson & Fisher, 1995; Fujiwara et al., 2000).

For elucidation of their energy acquisition, several biochemical methods have been developed: phylogenetic analysis of 16S ribosomal RNA gene sequence (Distel et al., 1988), direct morphological observation of symbionts using transmission electron microscopy (Nelson & Fisher, 1995) and enzymatic assay for methane and sulphide oxidation (Nelson & Fisher, 1995). These methods provide qualitative or semi-quantitative rather than quantitative information on aspects of energy acquisition of the overall

life activity. Another possible and valid indicator is the sulphur-isotope signature (Yamanaka et al., 2000a). Among four species of *Bathymodiolus* from Japanese waters, it has been reported that sulphur-isotopic compositions of the soft tissues from mussels harbouring methanotrophs were close to that of seawater sulphate-sulphur, whereas those harbouring thioautotrophs showed a comparable range with that of the sulphide-sulphur in the ambient venting fluid, reflecting the differences in the major sulphur source in their nutrition (Yamanaka et al., 2000b). In the case of dual symbiosis, the sulphur-isotopic composition shows intermediate values between those of seawater sulphate-sulphur and sulphide-sulphur (Brooks et al., 1987; Cary et al., 1989). The value should provide the degree of dependence for thioautotrophy.

Here we report the sulphur-isotopic compositions of the soft tissues of *Bathymodiolus marisindicus* from the Indian Ocean. The sulphur-isotopic signatures were compared to those of sulphides in the hydrothermal fluid and chimney in order to identify the type of endosymbiotic bacteria. The morphology of endosymbiotic bacteria in the gill tissues of *B. marisindicus* was observed using a transmission electron microscope (TEM) for characterization of the bacteria. Furthermore, the worldwide distribution of the genus *Bathymodiolus* is discussed from the point of view of the mode of energy acquisition.

MATERIALS AND METHODS

Sample collection

Bathymodiolus marisindicus were collected using a rake sampler during dives of the ROV 'Kaiko' (Serial dive

nos. 168 and 169) at 2420 to 2450 m in depth (25°19.17'S 70°02.40'E) (Figure 1). This species was observed attached to the foot of a large chimney complex. Maximum temperature of the venting fluid was approximately 360°C. Hydrothermal fluid (black smoker) and chimney fragments were sampled during the dives. Chemistry of the fluid samples was reported by Gamo et al. (2001).

The *Bathymodiolus* samples were frozen immediately at -80°C for sulphur-isotope analysis or transferred to fresh, chilled (~4°C) seawater for TEM observation after recovery on board and brought into the laboratory.

Sulphur-isotopic measurement

Soft tissues of the specimens were dissected and separated into gill, foot, mantle, and adductor muscle after thawing. Three relatively small specimens were not dissected and used for measurement of the entire sulphur-isotopic compositions. Prior to sulphur-isotopic measurement, pretreatment for removal of excess seawater sulphate and subsequent preparation of pure BaSO₄ separates were undertaken following the procedure described by Mizota et al. (1999) and Yamanaka et al. (2000a,b).

Dissolved hydrogen sulphide in the fluid was fixed on board as ZnS precipitate by adding excess zinc-acetate solution. The metal sulphides from the vent chimneys were recovered by treatment with warm hydrogen peroxide solution to generate sulphate ion, which was finally converted into BaSO₄. Recovered BaSO₄ was converted into SO₂ gas for mass spectrometry according to the procedure of Yanagisawa & Sakai (1983). Sulphur-isotopic measurements were conducted at the Institute for Study of the Earth's Interior, Okayama University, using a

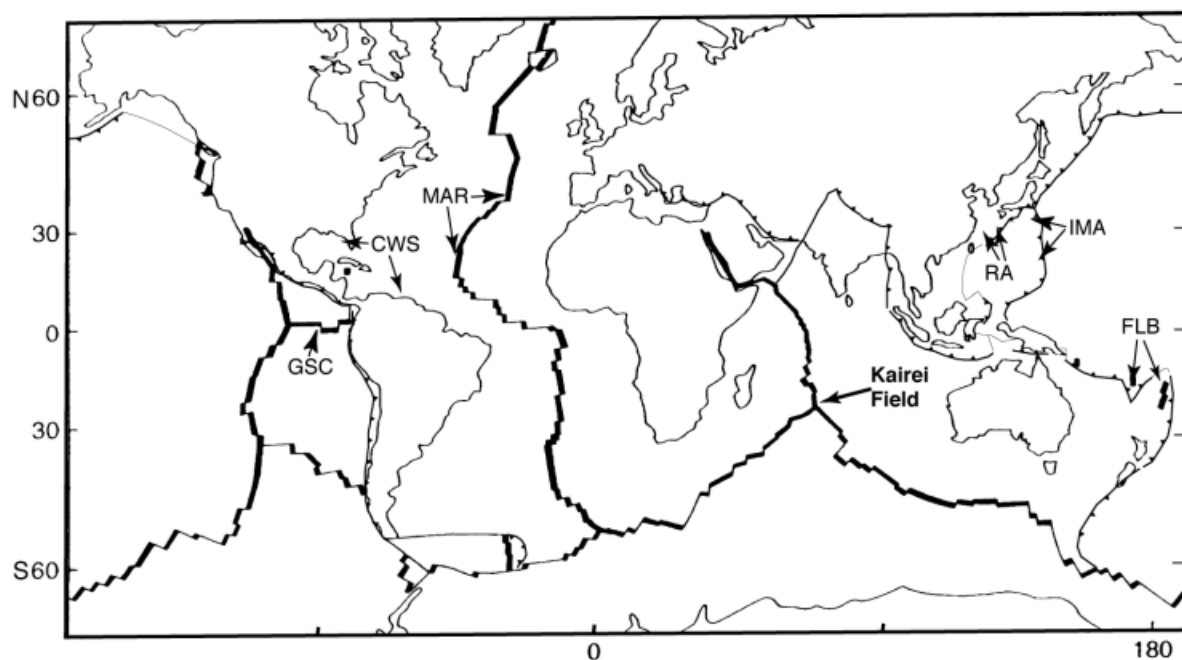


Figure 1. Plate boundaries and reported habitats of the genus *Bathymodiolus* summarized in Table 2. Present study area is the Kairei Field in the Indian Ocean. Area names: GSC, Galápagos spreading centre; CWS, cold-water seepages in western Atlantic Ocean; MAR, Mid-Atlantic Ridge; RA, Ryukyu-arc and back arc basin (Okinawa Trough); IMA, Izu-Ogasawara-Mariana Arcs; FLB, North Fiji and Lau Basins.

Table 1. Sulphur-isotope composition of soft tissues from a *Bathymodiolus marisindicus* and associated sulphides from the Indian Ocean.

Sample	Type	Location	Depth (m)	Sulphur		Kaiko dive no.	
				Concentration [#]	$\delta^{34}\text{S}$ vs CDT		
<i>Bathymodiolus marisindicus</i>	Whole of an individual	25°19.16'S	2432	1.26	+5.4	168	
	Whole of an individual	70°02.40'E		1.38	+5.4	168	
	Whole of an individual			0.99	+4.9	168	
<i>Bathymodiolus marisindicus</i>	Gill*	25°19.16'S,	2442	3.63	+5.6	169	
	Mantle*	70°02.40'E		1.01	+4.3	169	
	Foot*			0.88	+5.7	169	
	Muscle*			0.89	+3.4	169	
	Viscera*			2.22	+5.6	169	
Vent fluid	Dissolved hydrogen sulphide	25°19.14'S, 70°02.40'E	2449		+6.8 to +7.0**	Hot vent fluids (360°C) were collected using the Alvin type titanium syringe sampler. Mixing of ambient seawater during sampling was estimated less than a few percent (Gamo et al., 2001)	168, 169
Chimney	Metal sulphides	25°19.14'S, 70°02.40'E	2449		+4.4 to +7.5	Each mineral between centre and rim of a chimney round section	168, 169
	Sulphate minerals	25°19.14'S, 70°02.40'E	2449		+19.1 to +20.2		

*, Composite sample separate from three individuals; **, unpublished data from Hitoshi Chiba (Okayama University, Japan); #, weight % of the dry soft tissues.

VG Isogas SIRA 10 mass spectrometer with a dual inlet system. Sulphur-isotope composition ($^{34}\text{S}/^{32}\text{S}$) was expressed as $\delta^{34}\text{S}$, a per mil deviation relative to the international sulphur standard, Cañon Diablo troilite (CDT). Overall analytical reproducibility is within $\pm 0.1\%$.

Treatment for transmission electron microscopic observations

Small pieces of the gill tissue were fixed with 2.5% glutaraldehyde in filtered seawater for 24 h at 4°C. Blocks were rinsed ten times with 0.05 M phosphate buffer (pH 7.8) for 10 min at room temperature and post fixed in 1% OsO_4 in 0.05 M phosphate buffer (pH 7.8) at 4°C for two hours. Then, tissues were dehydrated and embedded in EPON 812 resin (TAAB, Aldermaston, UK). Ultra-thin sections of the specimens were stained with uranyl acetate and lead citrate, and were observed by a JEOL JEM-1210 transmission electron microscope at an acceleration voltage of 80 kV.

ANALYTICAL RESULTS

Sulphur-isotope compositions of the *Bathymodiolus* specimens and associated fluid and chimney sulphide fragments are shown in Table 1. The $\delta^{34}\text{S}$ values of the soft tissues from three whole specimens and composite dissected organs from three specimens ranged from +3.4 to +5.7‰. These values were within a comparable range with those obtained from the associated sulphides in the fluids and

chimney fragments (+5 to +8‰). This result indicates that the sulphur nutrition of this species is the hydrothermal sulphides in origin.

Numerous bacteria were observed in the epithelial cells of gill tissue from the *Bathymodiolus* specimens using TEM (Figure 2). The symbionts were contained in vacuoles within the host cells and most of them were localized in the apical region of the cells just below the surface. The

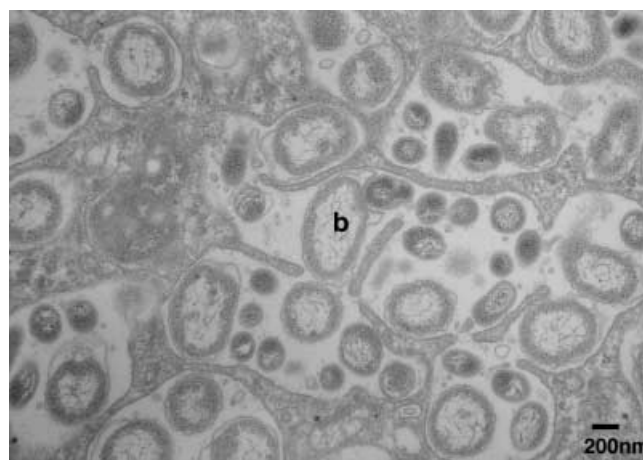


Figure 2. *Bathymodiolus marisindicus*. Transmission electron micrograph of intracellular bacterial symbionts within bacteriocytes in the epithelium of the host's gill. The symbionts were gram-negative and contained no stacked internal membranes, characteristic of methanotrophic bacteria. b, bacteria.

Table 2. An energy requisition of *Bathymodiolus* spp. with bacterial gill symbionts as revealed by different methodology.

Substrate for gill symbiont as evidenced by											
Location		Geochemistry of soft tissues				Microbiological and biochemical gill observations					
Basin	Species	Name of site	Map	Symbiotic type	$\delta^{34}\text{S}(\text{‰})$	$\delta^{13}\text{C}(\text{‰})$	Biomarker fatty acids (FAs)	Ultrastructure	Phylogeny	Enzymology	References
Pacific	<i>B. septemdiarium</i>	Myojin Knoll Caldera	IMA	T	+1.9 to +4.3	–	–	Sulphide ¹	Sulphide ¹	–	Yamanaka et al. (2000b)
		Suiyo Seamount	IMA		–0.4 to +5.5						
Pacific	<i>Bathymodiolus</i> sp.	Kaikata Caldera	IMA	T	–25 to –22	–	–	–	–	–	Kim et al. (1989)
Pacific	<i>Bathymodiolus</i> sp.	Alice Spring	IMA	T	–	–35 to –33 ²	–	–	–	–	Van Dover & Fry (1989)
Pacific	<i>B. platifrons</i>	Off Hatsushima Island*	IMA	M	+17.8 to +19.8	–	–	–	–	–	Yamanaka et al. (2000b)
		North Knoll of the Iheya Ridge	RA	M	+12.7 to +15.6	–	–	Methane ¹	Methane ¹	–	Yamanaka et al. (2000b)
Pacific	<i>B. japonicus</i>	North Knoll of the Iheya Ridge	RA	M	+14.5 to 14.9	–	–	Methane ¹	Methane ¹	–	Yamanaka et al. (2000b)
Pacific	<i>B. aduloides</i>	Off Kikajima Island*	RA	T	–15.4 to –12.4	–	–	–	–	–	Yamanaka et al. (2000b)
Pacific	<i>B. thermophilus</i>	Rose Garden	GSC	T	–	–37 to –31 ³	–	–	Sulphide ⁴	Sulphide ⁵	Kenk & Wilson (1985)
Pacific	<i>B. brevior</i>	North Fiji Basin	FLB		–	–35.8 to –25.7	–	Sulphide	–	–	Dubilier et al. (1998)
		Lau Basin, North Fiji Basin	FLB	T	–	–	Sulphide	–	–	–	Pranal et al. (1997)
Pacific	<i>B. elongatus</i>	North Fiji Basin	FLB	T	–	–	Sulphide	–	–	–	Pranal et al. (1997)
Pacific	<i>B. sp. affinis brevior</i>	North Fiji Basin	FLB	T	–	–	–	Sulphide	–	–	Dubilier et al. (1998)

Table 2. *Continued.*

Atlantic	<i>B. puteoserpentis</i>	Snakepit	MAR	T & M	-	-37 to 33 ³	-	[Dual] ⁵	Dual ³	Methane ³	Cosel et al. (1994)
Atlantic	<i>B. azoricus</i>	Lucky Strike	MAR	T & M	-	-31 to -21 ⁶	-	Dual ⁶	-	-	Cosel et al. (1999)
Atlantic	<i>B. boomerang</i>	Menez Gwen	MAR	T & M	-	-24.9 to -34.9 ^{7†}	Dual ⁷	Dual ⁶	-	-	Cosel & Olu (1998)
Atlantic	<i>Bathymodiolus</i> sp.	South Barbados*	CWS	T & M	-	-	-	Dual ⁸	-	-	Cosel & Olu (1998)
Atlantic	<i>B. heckerae</i>	Florida Escarpment*	CWS	M	-	-	-	Methane ^{8,9}	-	-	Cosel & Olu (1998)
Atlantic	<i>B. childressi</i>	Bush Hill*	CWS	T & M	+8.2, +9.6 ^{#,10}	-74 ¹¹	-	Dual ³	Methane ¹²	Methane	Gustafson et al. (1998)
Atlantic	<i>B. brooksi</i>	Alaminas Canyon*	CWS	T & M	+7.5, +13.4 ^{#,11}	-58 to -40 ¹¹	-	Methane ³	Methane ³	Both positive	Gustafson et al. (1998)
Indian	<i>B. marisindicus</i> [§]	Kairei Field	CWS	T & M	-	-56 to -40 ³	-	Dual ¹³	-	Both positive ¹³	Gustafson et al. (1998)
						-31.3 ± 0.7	-	-	-	-	Van Dover et al. (2001)

T, thioautotrophic; M, methanotrophic bacteria endosymbionts. Numbers (1-13) indicate the source literatures of data following: 1, Fujiwara et al. (2000); 2, Van Dover & Fry (1989); 3, Nelson & Fisher (1995); 4, Distel et al. (1988, 1995) and Distel & Cavanaugh (1994); 5, Distel et al. (1995); 6, Trask & Van Dover (1999); 7, Pond et al. (1998); 8, Cosel & Olu (1998); 9, Olu et al. (1994); 10, Cary et al. (1989); 11, Brooks et al. (1987); 12, Cavanaugh et al. (1987); 13, Fisher et al. (1993). *, Cold seep community; #, analysed samples include seawater sulphate; †, δ³³S values provided only from fatty acids; §, Van Dover et al. (2001) reported as *B. aff. brevior* on the literature.

bacteria were small cocci or short rods that averaged $0.76\ \mu\text{m}$ ($\text{SD}=0.16$, $N=20$) along the major axis, with trilamellar cell envelopes typical of gram-negative bacteria, and did not contain membranes or other distinctive structures in their cytoplasm (Figure 2). No morphological polymorphism was observed in the bacteria except in external form (i.e. cocci or short rod). These features coincided with those of other thioautotrophic symbionts found in marine invertebrates (Fisher, 1990; Fiala-Médioni & Felbeck, 1990). The stacked internal membranes (Fisher, 1990; Fiala-Médioni & Felbeck, 1990), distinct morphologies of the methanotrophic bacteria could not be observed. Divisional stages of the bacteria were observed (data not shown), indicating active reproduction. Secondary lysosomes containing bacteria in intermediate stages of digestion were also observed (data not shown). These lysosomes were concentrated near the basal portion of the host epithelial cells.

DISCUSSION

The available data set on the energy acquisition of *Bathymodiolus* species associated with chemosynthetic-based communities are summarized in Table 2. Few sulphur-isotopic compositions of the soft tissues of the genus *Bathymodiolus* have been reported. *Bathymodiolus platifrons* and *B. japonicus*, harbouring methanotrophic gill symbionts, in Japanese waters had higher $\delta^{34}\text{S}$, reflecting positive use of dissolved marine sulphate ($\delta^{34}\text{S}=+21\text{‰}$) with limited kinetic sulphur-isotopic fractionation (Yamanaka et al., 2000b). *Bathymodiolus septemdirum* and *B. aduloides*, harbouring thioautotrophic endosymbionts, have comparable signatures to those of local sulphides around their habitats (Yamanaka et al., 2000b). These results were fairly consistent with those obtained from the molecular and TEM examinations for gill symbiont (Fujiwara et al., 2000). An undescribed species of *Bathymodiolus* from Kaikata Caldera, Ogasawara, the western Pacific had distinctly lower $\delta^{34}\text{S}$ values (-25 to -22‰) with associated sedimentary sulphides. It was an evidence of the thiotrophic species (Kim et al., 1989). These low $\delta^{34}\text{S}$ values indicate that the sulphides were not derived from magmatic activity but dissimilatory reduction of seawater sulphate by sulphate-reducing bacteria. The present sulphur-isotopic characterization in combination with the TEM observation strongly suggests that *Bathymodiolus marisindicus* harbours a thioautotrophic endosymbiont in the gill.

Limited analytical data on the carbon-isotopic composition of whole soft tissues, selected organs and/or specific fatty acids (biomarkers) from *Bathymodiolus* spp. are also shown (Table 2). It is expected that there may be significant differences in the carbon-isotopic composition between the soft tissues of *Bathymodiolus* spp. with methanotrophs (use of methane as a carbon source) and thioautotrophs (use of dissolved carbon dioxide as a carbon source). Several species of *Bathymodiolus* from the cold-seepage in Gulf of Mexico showed distinctly lower $\delta^{13}\text{C}$ values (-74 to -38‰) (Table 2). These low values may result from the positive use of microbial and organic-matter derived methane with a lower carbon-isotopic signature (Trask & Van Dover, 1999).

The soft-tissue carbon-isotopic compositions of *Bathymodiolus marisindicus* were recently reported by Van Dover et al. (2001) ($\delta^{13}\text{C}$ values; $-31.3\pm 0.7\text{‰}$ vs PDB, an international standard for stable carbon isotope), being clearly comparable to those of thiotrophic mussels (range of $\delta^{13}\text{C}$ values; -37.0 to -28.9‰ , Kennicutt II et al., 1992). The carbon-isotopic result seemingly suggests the absence of methanotrophic symbiont in the species. Nevertheless, as shown in Table 2, the correlation between soft-tissue carbon-isotopic composition and mode of nutrition seems to be questionable. For example, *B. brooksi* and *B. childressi* have similar carbon-isotopic composition (-56 to -40‰), although the former has both thiotrophic and methanotrophic symbionts while the latter has only methanotrophic symbionts. Values obtained for *B. azoricus* (-24.9 to -34.9‰) were similar to those of *B. marisindicus* (-31.3‰). *Bathymodiolus azoricus*, however, has dual modes of nutrition, while the latter is a thiotroph as suggested by the authors. Using soft-tissue carbon-isotopic composition to identify the mode of nutrition, therefore, seems to be unreliable. Using TEM micrographs, Trask & Van Dover, 1999 found significant correlation between the relative abundance of the two types of endosymbionts in *B. azoricus* gill tissues (i.e. higher thiotrophs relative to methanotrophs) and soft-tissue carbon-isotopic composition from the Lucky Strike hydrothermal vent field, Mid-Atlantic Ridge. Such an examination will be needed for sample from the other locations. Although it is clear that thioautotrophic symbionts are present in *B. marisindicus*, the present carbon-isotopic composition cannot eliminate the possibility of dual modes of nutrition. The discussion on the reason interpreting why *B. marisindicus* occurring in Central Indian Ocean Ridge exhibit thiotrophic mode of nutrition based on the carbon-isotopic composition should be made with caution.

Dual symbiosis (i.e. use of both methane and sulphide as energy sources) is widespread in the Atlantic Basin (Table 2). *Bathymodiolus* species that harbour only methanotrophic symbionts have been reported from the north-western Pacific and western Atlantic oceans, while species harbouring only thioautotrophic symbionts occur throughout the Pacific Ocean. Hence, the energy acquisition of *B. marisindicus* from the Indian Ocean was evidently close to those from the Pacific Ocean.

A hypothesis has been proposed that the vent faunas migrate between the Pacific and Atlantic oceans via active ridges in the Indian Ocean (Tunnicliffe et al., 1998). The distributions of *Bathymodiolus* that harbour only thioautotrophic symbionts are likely to be limited to along the active ridges in the Pacific Ocean and the active submarine volcanic chains of island arcs from North Fiji to Izu-Ogasawara (Figure 1). At several hydrothermal vents along the Mid-Atlantic Ridge, however, *Bathymodiolus* species with only thioautotrophic symbionts have yet to be reported. Although the hydrothermal vent fluids are rich both in sulphide and methane, the methane concentration in the fluids may be insufficient to sustain these species of *Bathymodiolus* (Fujiwara et al., 2000). Emission of hydrogen sulphide might depend on hydrothermal activity and vent frequency, both of which are strongly related to the spreading rate of the ridge (Baker et al., 1996). The East Pacific Rise is a super fast spreading

axis, while the south-western Indian Ridge is considered to be one of the slowest spreading ridges in the world oceans (DeMets et al., 1990). The spreading rate of the south-east Indian Ridge is higher than that of the south-western Indian Ridge (DeMets et al., 1990). Hence, the south-western Indian Ridge may be a possible barrier for migration and interaction of the genus *Bathymodiolus* with thiotrophs between the Pacific and Atlantic Basins, while the thiotrophic Pacific species can migrate to the Central Indian Ridge. One possible strategy for crossing the barrier may be a dual symbiosis, i.e. a host mussel harbouring both methanotrophic and thioautotrophic symbionts in their gills. Due to its higher potential of dual symbiosis for adaptation in diverse environments (Van Dover, 2000), only dual symbiotic species may survive throughout the very low-rate spreading axis. On the other hand, the geographical distributions of *Bathymodiolus* species harbouring methanotrophs were strongly controlled by methane concentration (Fujiwara et al., 2000). They could adapt only to methane-rich environments, i.e. cold-water and hydrocarbon seepages, and sediment-hosted hydrothermal systems, where significantly higher concentrations of methane derived from organic matter decomposition are emitted for an extended period of time.

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