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Dual symbiosis of the vent shrimp *Rimicaris exoculata* with filamentous gamma- and epsilonproteobacteria at four Mid-Atlantic Ridge hydrothermal vent fields

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Summary

The shrimp *Rimicaris exoculata* from hydrothermal vents on the Mid-Atlantic Ridge (MAR) harbours bacterial epibionts on specialized appendages and the inner surfaces of its gill chamber. Using comparative 16S rRNA sequence analysis and fluorescence *in situ* hybridization (FISH), we examined the *R. exoculata* epibiosis from four vents sites along the known distribution range of the shrimp on the MAR. Our results show that *R. exoculata* lives in symbiosis with two types of filamentous epibionts. One belongs to the *Epsilonproteobacteria*, and was previously identified as the dominant symbiont of *R. exoculata*. The second is a novel gammaproteobacterial symbiont that belongs to a clade consisting exclusively of sequences from epibiotic bacteria of hydrothermal vent animals, with the filamentous sulfur oxidizer *Leucothrix mucor* as the closest free-living relative. Both the epsilon- and the gammaproteobacterial symbionts dominated the *R. exoculata* epibiosis at all four MAR vent sites despite striking differences between vent fluid chemistry and distances between sites of up to 8500 km, indicating that the symbiosis is highly stable and specific. Phylogenetic analyses of two mitochondrial host genes showed little to no differences between hosts from the four vent sites. In contrast, there was significant spatial structuring of both the gamma- and the epsilonproteobacterial symbiont populations based on their 16S rRNA gene sequences that was correlated with geographic distance along the MAR. We hypothesize that biogeography and host–symbiont selectivity play a role in structuring the epibiosis of *R. exoculata*.

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Introduction

The alvinocaridid shrimp *Rimicaris exoculata* (Williams and Rona, 1986) is endemic to hydothermal vents on the Mid-Atlantic Ridge (MAR) (Schmidt *et al.*, 2008a). Large swarms containing as many as 3000 shrimp per m^2 (Gebruk *et al.*, 2000) aggregate on hydrothermal vent chimneys in the mixing zone between electron donor-rich vent fluids and the surrounding oxidized seawater. The source of nutrition for *R. exoculata* is unclear, but a large chemoautotrophic bacterial biomass would be needed to support such dense swarms at hydrothermal vents in the deep sea where the input of organic matter from photosynthesis is extremely low (Van Dover, 2000).

The stable isotopic composition of adult shrimp indicates a chemosynthetic food source (Van Dover *et al.*, 1988; Rieley *et al.*, 1999). Unlike the bathymodiolin mussels they co-occur with on the MAR, which rely on endosymbiotic methane- and sulfur-oxidizing bacteria for their nutrition (Robinson *et al.*, 1998; Pimenov *et al.*, 2002; Duperron *et al.*, 2006), *R. exoculata* appears to have no endosymbiotic bacteria (Van Dover *et al.*, 1988). Instead, the shrimp host a dense covering of epibiotic bacteria on specialized appendages within the gill chamber (Fig. 1). The morphology of the shrimp shows adaptation to the symbiosis, as the mouthparts within the gill chamber are atypically large and densely covered with setae, to which the ectosymbionts are attached (Van Dover *et al.*, 1988; Casanova *et al.*, 1993; Gebruk *et al.*, 1993; Komai and Segonzac, 2008). A nutritional role has been suggested for the ectosymbionts, but it is unclear how energy could be transferred from the ectosymbionts to the host. *Rimicaris exoculata* does not have a reduced

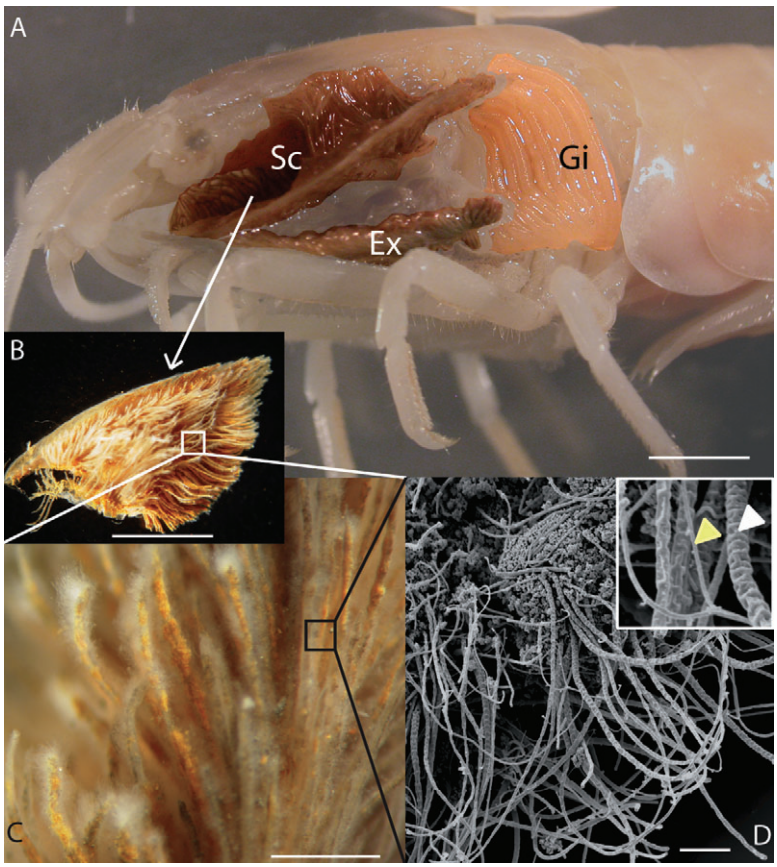


Fig. 1. Morphology of the *Rimicaris exoculata* symbiosis.
A. The *R. exoculata* gill chamber with carapace removed, showing the mouthparts (Sc and Ex) to which the shrimp epibionts are attached. Sc, scaphognathite; Ex, exopodite; Gi, gill. Scale bar = 5 mm.
B. Scaphognathite dissected out of the shrimp. Scale bar = 5 mm.
C. Closer view of the scaphognathite setae. The filamentous epibiotic bacteria can be seen as a white fuzzy material.
D. Scanning electron microscope image showing the filamentous epibionts. Insert: a 'thick' filament is indicated with a white arrowhead, a 'thin' filament with a yellow arrowhead. Scale bar = 10 μm.

gut like many chemosynthetic hosts with endosymbiotic bacteria that rely on their endosymbionts for nutrition (Van Dover *et al.*, 1988). To gain nutrition from the bacterial ectosymbionts, *R. exoculata* could either take up organic compounds from them through trans-epidermal transfer, or ingest them. Two possible ways in which the shrimp could ingest their symbionts are: (i) the shrimp harvest their ectosymbionts using their modified feeding appendages, and transfer them to the mouth (Gebruk *et al.*, 1993), or (ii) the shrimp ingest their exuvae after moulting (Segonzac *et al.*, 1993). While the benefit of the association to the shrimp host remains unclear, chemosynthetic ectosymbionts would represent a rich source of nutrition, enabling the shrimp to live, indirectly, from the inorganic energy sources abundant at hydrothermal vents. The bacteria most likely benefit from the association as the shrimp position themselves in the mixing zone between electron donor-rich vent fluids and the surrounding seawater, allowing the ectosymbionts stable and simultaneous access to electron donors and acceptors.

The metabolism of the ectosymbionts has not been clearly identified. Autotrophy has been demonstrated by RuBisCO activity (Wirsen *et al.*, 1993; Cavanaugh and Robinson, 1996; Polz *et al.*, 1998) and the incorporation of radioactively labelled inorganic carbon (Galchenko *et al.*, 1989; Jannasch *et al.*, 1991; Polz *et al.*, 1998). A number of different electron donors have been suggested to fuel the symbiosis. The observation of internal sulfur globules in shrimp ectosymbionts from the Trans-Atlantic Geotransverse (TAG) vent field led to the conclusion that the ectosymbionts are chemoautotrophic sulfur-oxidizing bacteria (Gebruk *et al.*, 1993). At the Rainbow vent field, the ectosymbionts are associated with iron oxyhydroxide minerals that appear to have been precipitated by a biological rather than chemical process (Gloter *et al.*, 2004), and this led to the hypothesis that the ectosymbionts at this vent field might gain their energy by iron oxidation (Gloter *et al.*, 2004; Zbinden *et al.*, 2004). This would be a novel process, as all currently known chemosynthetic symbioses rely on the oxidation of methane or reduced sulfur compounds (Cavanaugh *et al.*, 2006; Dubilier *et al.*, 2008).

Hydrothermal vents on the MAR can either be ultramafic- or basalt-hosted. In basalt-hosted systems, the end-member vent fluids are enriched in sulfide and depleted in hydrogen and methane. In contrast, fluids in ultramafic-hosted systems are enriched in hydrogen and methane, and depleted in sulfide (Charlou *et al.*, 2002; Schmidt *et al.*, 2007). The geological setting of the vent fields has been hypothesized to influence the diversity of the free-living bacterial community (Perner *et al.*, 2007), but this has not yet been investigated for symbiotic bacteria. Schmidt and co-workers (2008b) modelled energy

Table 1. Clone library results.

	Rainbow (RB)			TAG (TG)			Logatchev (LG)			South MAR (SM)		
Individual No.	1	2	3	4	5	6	10	11	12	13	14	15
Epsilon 1		31 (3)	48 (2)									
Epsilon 2				26 (2)	8 (2)	2 (2)						
Epsilon 3							56 (3)	55 (2)	26 (1)			
Epsilon 4		4 (1)	8									
Epsilon 5										18 (1)	27 (2)	15 (2)
Gamma 1	58 (2)	26 (2)	7 (2)	15 (1)	62 (1)	49 (1)	10 (2)	2 (3)	12 (3)			
Gamma 2										17 (2)	18 (2)	31 (1)
CFB	5	17	3	7	13	3	6	9	9	5	11	21
Other	13	1	3	5		11	10	6	36	25	19	9
Total No. of partial sequences	76	79	69	53	83	65	88	81	90	65	75	82

Number of partial sequences found in each clone library that belonged to the 5 Epsilon symbiont and 2 Gamma symbiont groups. Within each of these 7 groups, sequences shared > 99% identity. Number of full sequences analysed is shown in parentheses. CFB (*Cytophaga-Flavobacteria-Bacteroidetes*) and Other include phylogenetically diverse sequences that were not found in all individuals.

budgets at two MAR vent sites and suggested that the diversity of the *R. exoculata* epibiosis might differ between the ultramafic-hosted Rainbow and basalt-hosted TAG sites, based on thermodynamic predictions of the energy available from the oxidation of different electron donors.

The first molecular studies of the *R. exoculata* epibionts were from shrimp collected at the Snake Pit vent field on the MAR (Polz and Cavanaugh, 1995). These showed that they all belong to a single phylotype within the *Epsilonproteobacteria*, despite the presence of various morphotypes (Polz and Cavanaugh, 1995). A recent study of *R. exoculata* from the Rainbow vent field on the MAR suggested that the epibiont diversity might be higher than previously assumed based on 16S rRNA gene sequences and ultrastructural observations, but fluorescence *in situ* hybridization was not used to distinguish between epibionts and casually associated bacteria or contaminants (Zbinden *et al.*, 2008).

In this study, we analysed the phylogeny of *R. exoculata* and their epibionts from four vent fields, Rainbow, TAG, Logatchev and South MAR. These four vent fields are separated by up to 8500 km along the MAR and span the known distribution range of *R. exoculata*. Two of the vents, Rainbow and Logatchev, are ultramafic-hosted, while the two others, TAG and South MAR, are basalt-hosted (Table S1). Our aim was to re-examine the diversity of the shrimp ectosymbionts based on observations that multiple morphotypes occur on the shrimp (Polz and Cavanaugh, 1995; Zbinden *et al.*, 2004; Zbinden *et al.*, 2008), and that epibiont diversity might differ between ultramafic- and basalt-hosted vent fields.

Results

Host phylogeny

Alignment of the mitochondrial cytochrome oxidase subunit I (COI) genes from 12 *R. exoculata* individuals, three from each of the four vent sites, identified one shared and five non-shared substitutions. The single shared substitution at position 525 is shared by the three TAG shrimp and one Logatchev shrimp. Since the COI gene was so highly conserved in shrimp from geographically distant sampling sites, we analysed an additional mitochondrial marker gene encoding cytochrome b (CytB). Although the CytB gene contained more substitutions than the COI gene in *R. exoculata* populations (four shared, 11 non-shared substitutions), geographic clades also could not be identified based on this gene (Fig. S1).

Diversity of R. exoculata *epibionts*

16S rRNA clone libraries were constructed for the same 12 individuals used for host phylogenetic analyses. Between 53 and 90 clones were partially sequenced for each individual, and clones were assigned to groups with > 99% sequence similarity (Table 1). Seven groups dominated the clone libraries from all four vent fields, of which five belonged to the *Epsilonproteobacteria* (Epsilon 1–5), and two belonged to the *Gammaproteobacteria* (Gamma 1 and 2) (Table 1).

FISH analyses showed that the epsilon- and gammaproteobacterial groups that dominated the clone libraries also dominated the bacterial community on *R. exoculata* from all four vent fields. To show this, we did three-colour hybridizations with probes specific to the epsilon- and gammaproteobacterial groups found in the clone libraries the general bacterial probe EUB I-III (Amann *et al.*, 1990; Daims *et al.*, 1999), and 4′,6-diamidino-2-phenylindole (DAPI) staining (Fig. 2) (see Table S2 for probes used in this study). The specific probes hybridized with filamentous epibionts that could be distinguished from each other based on their morphology (Fig. 2). The probes specific to the gammaproteobacterial sequences hybridized with filaments that had coccoid-shaped cells and a diameter of

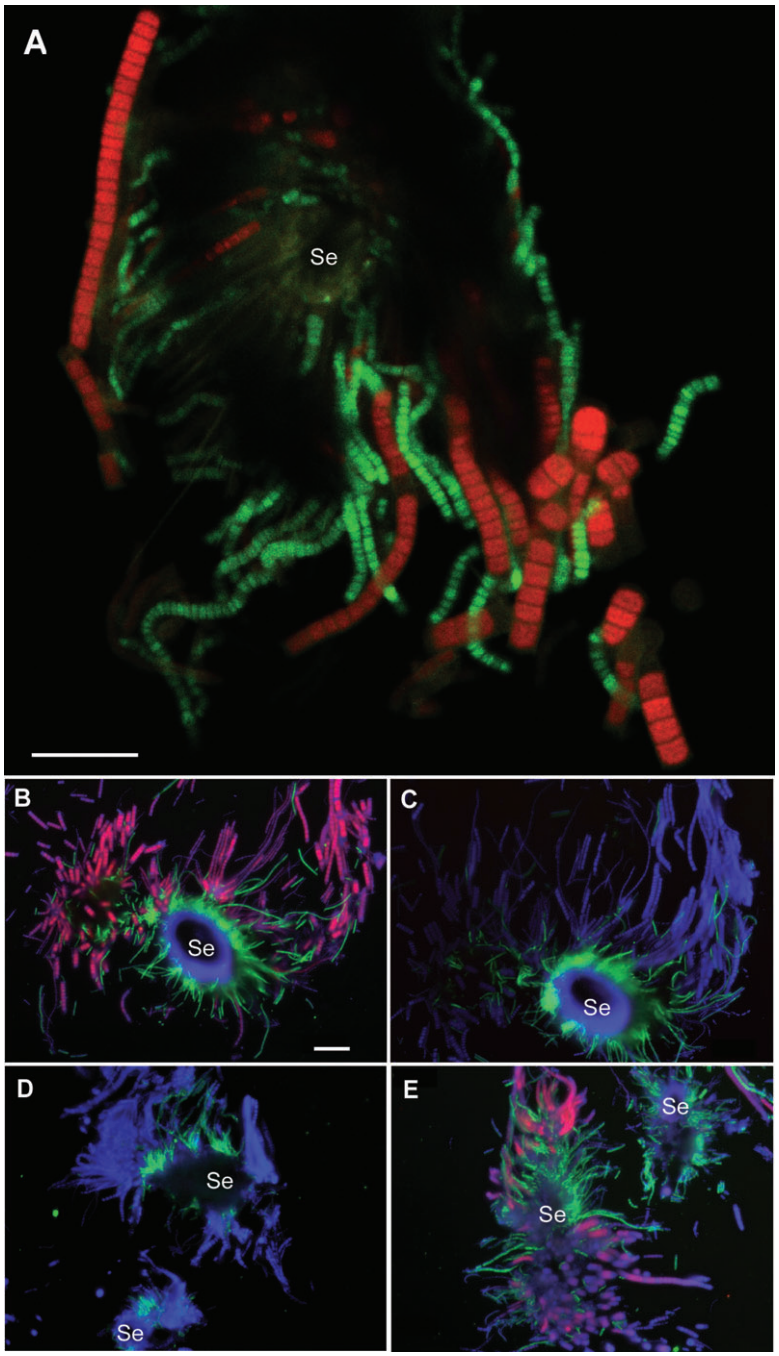


Fig. 2. Fluorescence *in situ* hybridization. A. Confocal laser scanning micrograph of a cross-section through a *R. exoculata* scaphognathite from Individual 1 from the Rainbow vent site, showing the two symbionts attached to the scaphognathite seta (Se). The Gamma symbiont (green) was hybridized with the Rexogam1268RT probe targeting the Rainbow and TAG Gamma symbiont sequences, and the Epsilon symbiont (red) was hybridized with the Epsilon 1 probe targeting the Rainbow Epsilon 1 symbiont. B–E. Epifluorescence micrographs of cross sections through *R. exoculata* scaphognathite setae (Se) showing the specificity of the site-specific probes designed for the Epsilon symbionts. The DAPI stain is shown in blue, probe signals for the Gamma symbionts in light green, and probe signals for the Epsilon symbionts in pink. (B) South MAR *R. exoculata* Individual 17 hybridized with the South MAR Epsilon 5 probe and the Rexogam1268LS Gamma probe. (C) The same South MAR individual as in (B), hybridized with the Rexogam1268LS Gamma probe and the Epsilon 3 probe. The Logatchev Epsilon 3 probe shows no signals when hybridized with the South MAR individual. (D and E) Images from Logatchev Individual 10, hybridized in (D) with the South MAR Epsilon 5 probe and in (E) with the Logatchev Epsilon 4 probe. No signals are seen on the Logatchev individual with the South MAR Epsilon 5 probe. (D) and (E) are also hybridized with the Rexogam1268LS probe for the Gamma symbionts. Scale bars = 20 μm. The scale bar in (B) applies to images (B–E).

approximately 1 μm, while the probes specific to the epsilonproteobacterial sequences hybridized with filaments that were 3 μm in diameter (Fig. 2).

The relative abundance of the 16S rRNA clone sequences from the epsilon- and gammaproteobacterial groups varied considerably both among individuals from the same site and individuals from different sites (Table 1). Both groups were present in 11 of the 12 shrimp examined, and their relative abundance in the clone libraries was consistent with observations of their relative abundance by FISH. Only one individual had no epsilonproteobacterial sequences in its 16S rRNA clone library (Individual 1 in Table 1). However, this symbiont was clearly present on this individual based on FISH, albeit at low abundance.

16S rRNA gene phylogeny of the epsilonproteobacterial symbionts

The epsilonproteobacterial 16S rRNA sequences from the *R. exoculata* clone libraries fell in a clade that included

Fig. 3. 16S rRNA phylogeny of the epsilonproteobacterial symbiont.
Maximum likelihood phylogeny of the *R. exoculata* Epsilon symbiont 16S rRNA genes. The numbers in parentheses refer to the number assigned to each individual. (Table S3). The *Gammaproteobacterium Vibrio cholerae* (accession number AY494843) was used as an outgroup (arrow).
A. Relationship of the *R. exoculata* Epsilon symbionts to other members of the *Epsilonproteobacteria*.
B. Expanded view of boxed area in (A). Phylogeny of the *R. exoculata* Epsilon symbionts and their close relatives, both invertebrate-associated and free-living. Colours indicate the geographic location of the sampling sites. Bars indicate 10% estimated sequence divergence. Only bootstrap values (100 re-samplings) over 60 are shown.

previously published sequences from *R. exoculata* epibionts from the Snake Pit vent field (Polz and Cavanaugh, 1995), and clone sequences from the gut of *R. exoculata* from the Rainbow vent field (Zbinden and Cambon-Bonavita, 2003) (Fig. 3). The closest cultured relative was *Sulfurovum lithotrophicum,* a rod-shaped sulfur-oxidizing chemolithoautotroph isolated from a hydrothermal vent in the Western Pacific (Inagaki *et al*., 2004) (91.9–92.9% sequence identity to the *R. exoculata* Epsilon 1–5 symbionts, Fig. 3). Sequences from bacteria associated with other hydrothermal vent invertebrates, the gastropod *Crysomallon squamiferum*, the barnacle *Vulcanolepas osheai*, the crab *Kiwa hirsuta* and the polychete worms *Alvinella pompejana* and *Paralvinella palmiformis* also fell between the *R. exoculata* Epsilon symbionts (Fig. 3B). A number of sequences from free-living hydrothermal vent bacteria also belonged to this clade (Fig. 3B).

16S rRNA gene phylogeny of the gammaproteobacterial symbionts

The gammaproteobacterial 16S rRNA sequences from the *R. exoculata* clone libraries formed a clade with bacteria associated with the hydrothermal vent animals *C. squamiferum* and *K. hirsuta* (Fig. 4). The closest relatives of this clade were free-living bacteria from a carbonate chimney at Lost City, and a clade of bacteria associated with *V. osheai* and *K. hirsuta*. The closest cultured free-living relative to the *R. exoculata* Gamma symbionts was *Leucothrix mucor*, a filamentous sulfur-oxidizer (Grabovich *et al*., 1999), with 90.2–90.8% sequence identity. The most closely related symbiont sequences were from the endosymbionts of the siboglinid tubeworms *Oligobrachia mashikoi* (88.5–90.0% sequence identity) and *O. haakonmosbiensis* (88.5–89.4% sequence identity). The clade containing the *R. exoculata* Gamma 1 and 2 sequences always grouped with *L. mucor* and the *O. haakonmosbiensis* symbionts in all phylogenetic analyses, but the relationship of this group to the methane- and sulfur-oxidizing endosymbionts of other chemosynthetic invertebrates, and to free-living methane- and sulfur-oxidizing bacteria was not consistent between treeing methods.

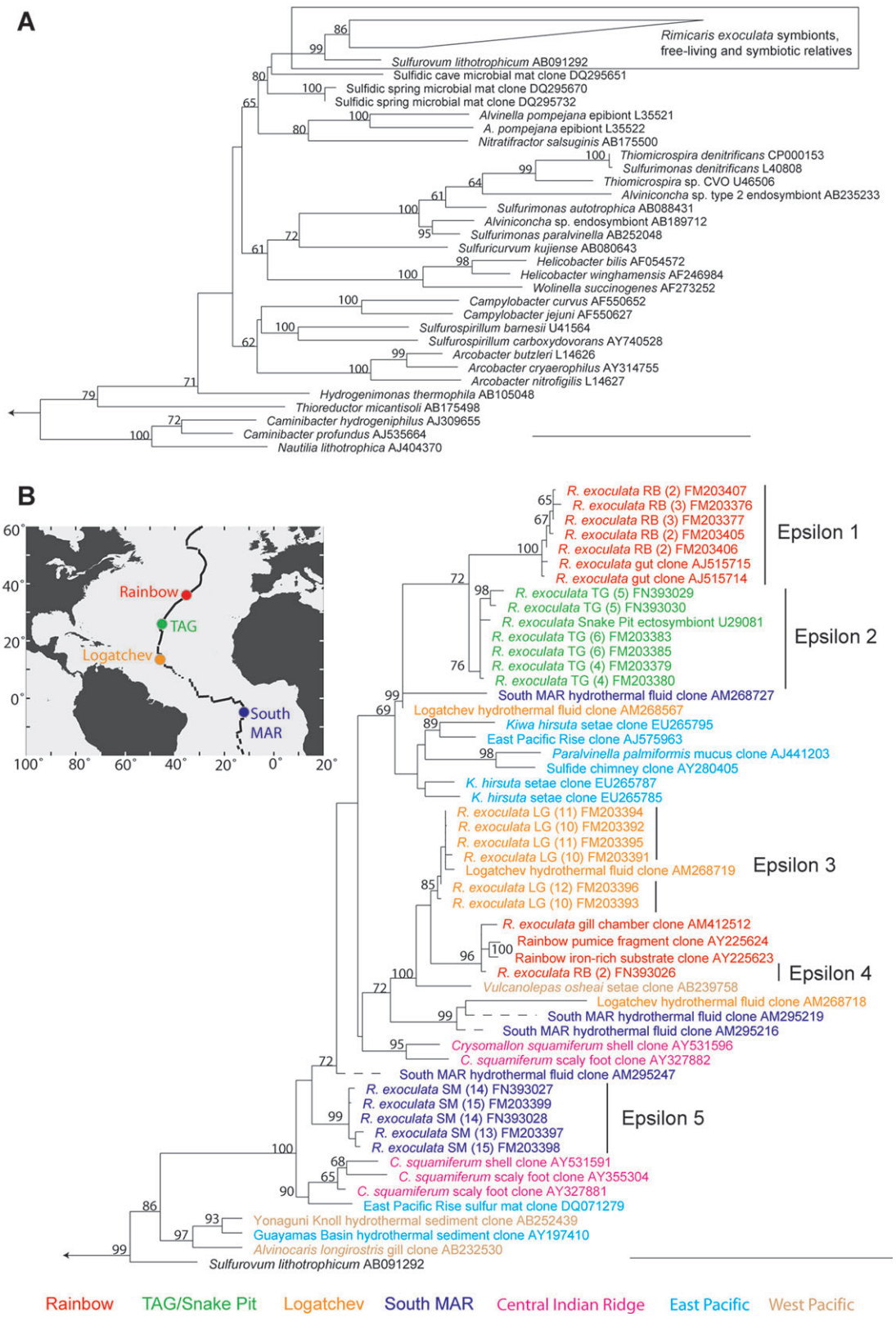
Biogeography of the R. exoculata *symbionts*

The *R. exoculata* Epsilon 1–5 symbionts formed geographic groups based on 16S rRNA analyses that were supported by maximum likelihood, parsimony, and neighbour-joining methods (Fig. 3B). With the exception of Rainbow, shrimp from each vent site harboured only a single group of sequences with $>99\%$ identity. At Rainbow, the shrimp harboured two phylogenetically distinct groups, with $>99\%$ sequence identity within each group: the dominant Epsilon 1 group, and the low-abundance group Epsilon 4. Sequence identities between the Epsilon 1–5 groups varied from 93.5% to 97.5%. Sequences from the ultramafic-hosted sites Rainbow and Logatchev did not group together to the exclusion of sequences from the basalt-hosted sites TAG and South MAR. Statistical analysis of the epibiont sequences showed a significant correlation between genetic distance based on the 16S rRNA gene and geographic distance along the MAR ($r = 0.43$, $P < 0.001$).

Unlike the Epsilon symbionts, the Gamma symbiont 16S rRNA sequences did not differ sufficiently to resolve geographic clades. The Gamma 2 symbiont sequences from the South MAR vent did, however, form a separate cluster to sequences from the northern MAR vent fields Rainbow, TAG and Logatchev (Gamma 1) in all treeing methods (Fig. 4B). Although site-specific geographic clades could not be resolved, a significant correlation was found between genetic and geographic distance for the Gamma symbiont ($r = 0.85$, $P < 0.001$).

To ensure that the site-specific differences in 16S rRNA found in the clone libraries are genuine and not caused by PCR or cloning bias, we designed probes for *in situ* detection of the epibiont groups (summarized in Table S2). For the Gamma symbionts, two probes were designed; one for the symbiont sequences from the northernmost vent fields, Rainbow and TAG, and one for those from the southernmost vent fields, Logatchev and South MAR (Table S2). The probe for the Rainbow and TAG Gamma symbionts bound specifically to all of the thinner filaments (Gamma morphotype) in *R. exoculata* individuals from the two target sites, and no signals were observed with this probe in individuals from either the Logatchev or South MAR vent fields. Conversely, the Logatchev and South MAR probe specifically bound all filaments with the Gamma morphology on *R. exoculata* from the target sites, and showed no signals on Rainbow or TAG individuals.

The Epsilon symbiont sequences were more divergent than the Gamma symbiont sequences, and this allowed us to design site-specific FISH probes for these groups.


A
Rimicaris exoculata symbionts, free-living and symbiotic relatives
Sulfurovum lithotrophicum AB091292
Sulfidic cave microbial mat clone DQ295651
Sulfidic spring microbial mat clone DQ295670
Sulfidic spring microbial mat clone DQ295732
Alvinella pompejana epibiont L35521
A. pompejana epibiont L35522
Nitratifractor salsuginis AB175500
Thiomicrospira denitrificans CP000153
Sulfurimonas denitrificans L40808
Thiomicrospira sp. CVO U46506
Alviniconcha sp. type 2 endosymbiont AB235233
Sulfurimonas autotrophica AB088431
Alviniconcha sp. endosymbiont AB189712
Sulfurimonas paralvinella AB252048
Sulfuricurvum kujiense AB080643
Helicobacter bilis AF054572
Helicobacter winghamensis AF246984
Wolinella succinogenes AF273252
Campylobacter curvus AF550652
Campylobacter jejuni AF550627
Sulfurospirillum barnesii U41564
Sulfurospirillum carboxydovorans AY740528
Arcobacter butzleri L14626
Arcobacter cryaerophilus AY314755
Arcobacter nitrofigilis L14627
Hydrogenimonas thermophila AB105048
Thioreductor micantisoli AB175498
Caminibacter hydrogeniphilus AJ309655
Caminibacter profundus AJ535664
Nautilia lithotrophica AJ404370
B
Rainbow
TAG
Logatchev
South MAR
60° 40° 20° 0°
100° 80° 60° 40° 20° 0° 20°
R. exoculata RB (2) FM203407
R. exoculata RB (3) FM203376
R. exoculata RB (3) FM203377
R. exoculata RB (2) FM203405
R. exoculata RB (2) FM203406
R. exoculata gut clone AJ515715
R. exoculata gut clone AJ515714
Epsilon 1
R. exoculata TG (5) FN393029
R. exoculata TG (5) FN393030
R. exoculata Snake Pit ectosymbiont U29081
R. exoculata TG (6) FM203383
R. exoculata TG (6) FM203385
R. exoculata TG (4) FM203379
R. exoculata TG (4) FM203380
Epsilon 2
South MAR hydrothermal fluid clone AM268727
Logatchev hydrothermal fluid clone AM268567
Kiwa hirsuta setae clone EU265795
East Pacific Rise clone AJ575963
Paralvinella palmiformis mucus clone AJ441203
Sulfide chimney clone AY280405
K. hirsuta setae clone EU265787
K. hirsuta setae clone EU265785
R. exoculata LG (11) FM203394
R. exoculata LG (10) FM203392
R. exoculata LG (11) FM203395
R. exoculata LG (10) FM203391
Logatchev hydrothermal fluid clone AM268719
R. exoculata LG (12) FM203396
R. exoculata LG (10) FM203393
Epsilon 3
R. exoculata gill chamber clone AM412512
Rainbow pumice fragment clone AY225624
Rainbow iron-rich substrate clone AY225623
R. exoculata RB (2) FN393026
Epsilon 4
Vulcanolepas osheai setae clone AB239758
Logatchev hydrothermal fluid clone AM268718
South MAR hydrothermal fluid clone AM295219
South MAR hydrothermal fluid clone AM295216
Crysomallon squamiferum shell clone AY531596
C. squamiferum scaly foot clone AY327882
South MAR hydrothermal fluid clone AM295247
R. exoculata SM (14) FN393027
R. exoculata SM (15) FM203399
R. exoculata SM (14) FN393028
R. exoculata SM (13) FM203397
R. exoculata SM (15) FM203398
Epsilon 5
C. squamiferum shell clone AY531591
C. squamiferum scaly foot clone AY355304
C. squamiferum scaly foot clone AY327881
East Pacific Rise sulfur mat clone DQ071279
Yonaguni Knoll hydrothermal sediment clone AB252439
Guayamas Basin hydrothermal sediment clone AY197410
Alvinocaris longirostris gill clone AB232530
Sulfurovum lithotrophicum AB091292
Rainbow
TAG/Snake Pit
Logatchev
South MAR
Central Indian Ridge
East Pacific
West Pacific

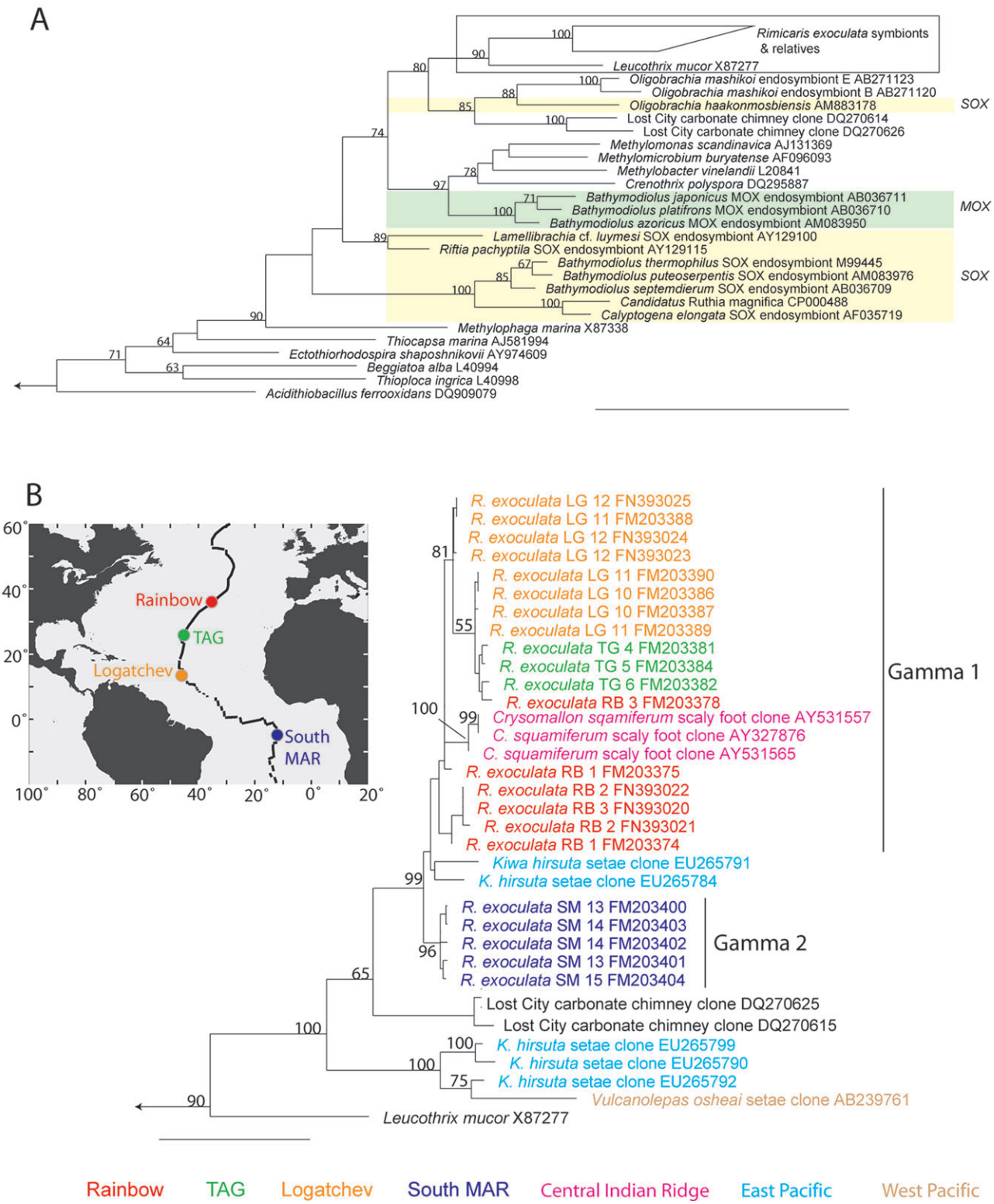

A
Rimicaris exoculata symbionts & relatives
Leucothrix mucor X87277
Oligobrachia mashikoi endosymbiont E AB271123
Oligobrachia mashikoi endosymbiont B AB271120
Oligobrachia haakonmosbiensis AM883178
Lost City carbonate chimney clone DQ270614
Lost City carbonate chimney clone DQ270626
Methylomonas scandinavica AJ131369
Methylomicrobium buryatense AF096093
Methylobacter vinelandii L20841
Crenothrix polyspora DQ295887
Bathymodiolus japonicus MOX endosymbiont AB036711
Bathymodiolus platifrons MOX endosymbiont AB036710
Bathymodiolus azoricus MOX endosymbiont AM083950
Lamellibrachia cf. luymesi SOX endosymbiont AY129100
Riftia pachyptila SOX endosymbiont AY129115
Bathymodiolus thermophilus SOX endosymbiont M99445
Bathymodiolus puteoserpentis SOX endosymbiont AM083976
Bathymodiolus septemdierum SOX endosymbiont AB036709
Candidatus Ruthia magnifica CP000488
Calyptogena elongata SOX endosymbiont AF035719
Methylophaga marina X87338
Thiocapsa marina AJ581994
Ectothiorhodospira shaposhnikovii AY974609
Beggiatoa alba L40994
Thioploca ingrica L40998
Acidithiobacillus ferrooxidans DQ909079
SOX
MOX
SOX
B
Rainbow
TAG
Logatchev
South MAR
60°
40°
20°
0°
100°
80°
60°
40°
20°
0°
20°
R. exoculata LG 12 FN393025
R. exoculata LG 11 FM203388
R. exoculata LG 12 FN393024
R. exoculata LG 12 FN393023
R. exoculata LG 11 FM203390
R. exoculata LG 10 FM203386
R. exoculata LG 10 FM203387
R. exoculata LG 11 FM203389
R. exoculata TG 4 FM203381
R. exoculata TG 5 FM203384
R. exoculata TG 6 FM203382
R. exoculata RB 3 FM203378
Crysomallon sqamiferum scaly foot clone AY531557
C. squamiferum scaly foot clone AY327876
C. squamiferum scaly foot clone AY531565
R. exoculata RB 1 FM203375
R. exoculata RB 2 FN393022
R. exoculata RB 3 FN393020
R. exoculata RB 2 FN393021
R. exoculata RB 1 FM203374
Kiwa hirsuta setae clone EU265791
K. hirsuta setae clone EU265784
R. exoculata SM 13 FM203400
R. exoculata SM 14 FM203403
R. exoculata SM 14 FM203402
R. exoculata SM 13 FM203401
R. exoculata SM 15 FM203404
Lost City carbonate chimney clone DQ270625
Lost City carbonate chimney clone DQ270615
K. hirsuta setae clone EU265799
K. hirsuta setae clone EU265790
K. hirsuta setae clone EU265792
Vulcanolepas osheai setae clone AB239761
Leucothrix mucor X87277
Gamma 1
Gamma 2
Rainbow
TAG
Logatchev
South MAR
Central Indian Ridge
East Pacific
West Pacific

Fig. 4. 16S rRNA phylogeny of the gammaproteobacterial symbiont.
Maximum likelihood phylogeny of the *R. exoculata* Gamma symbiont 16S rRNA genes. The numbers in parentheses refer to the number assigned to each individual (Table S3). The Alphaproteobacterium *Rhodocyclus tenuis* (accession number D16208) was used as an outgroup (arrow).
A. Relationship of the *R. exoculata* Gamma symbionts to other members of the *Gammaproteobacteria*. Methane-oxidizing endosymbionts (MOX) are shaded in green and sulfur-oxidizing endosymbionts in yellow (SOX). Bar indicates 10% estimated sequence divergence.
B. Phylogeny of the *R. exoculata* Gamma symbionts and their close relatives, both invertebrate-associated and free-living. Colours indicate the geographic location of the sampling. Bar indicates 5% estimated sequence divergence. Only bootstrap values (100 re-samplings) over 60 are shown.

The Epsilon probes were used to identify the epibionts on *R. exoculata* from a single vent field, and tested against non-target organisms from the three other vents. For example, the probe targeting the South MAR Epsilon 5 symbionts bound specifically to the epsilonproteobacterial epibionts of shrimp from South MAR, and showed no signals when hybridized with shrimp from the Rainbow, TAG, Logatchev and vents (Fig. 2).

The probe targeting the Logatchev Epsilon 3 symbiont has no mismatches to the Rainbow Epsilon 4 symbiont, and could therefore be used to examine the relative abundance of the Epsilon 1 and 4 symbionts on Rainbow shrimp (Fig. S2). The Epsilon 1 symbionts were more abundant than the Epsilon 4 symbionts, just as the Epsilon 1 sequences were numerically dominant over the Epsilon 4 sequences in the clone libraries (Table 1). To confirm that the signals on the Rainbow shrimp were due to the presence of the Epsilon 4 and not the Logatchev Epsilon 3 symbiont, we also used another probe, RexoepsLG86, which perfectly matches the Logatchev Epsilon 3 symbiont, but has 1 mismatch to the Rainbow Epsilon 4 symbiont (Fig. S2). This probe did not hybridize with bacteria on the Rainbow shrimp.

For the free-living relatives of the Epsilon symbionts, there was no obvious geographic trend in their 16S rRNA phylogeny, or for invertebrate-associated bacteria that fell within the *R. exoculata* epibiont clade (Fig. 3B); for example, environmental sequences from hydrothermal fluids at both the Logatchev and South MAR vent fields fell throughout the tree and did not group according to geography. Thus, there appears to be no genetic structuring of the free-living relatives of the Epsilon symbionts. We could not analyse the distribution of free-living relatives of the Gamma symbionts, as no sequences from free-living bacteria were available that fell within the Gamma symbiont clade.

Discussion

Rimicaris exoculata *has a dual symbiosis with filamentous* Epsilon- *and* Gammaproteobacteria

The *R. exoculata* symbiosis was previously considered to be composed of a single epsilonproteobacterial phylotype based on 16S rRNA sequencing, FISH, and slot-blot hybridization of individuals from the Snake Pit vent site (Polz and Cavanaugh, 1995). A recent study showed that additional bacterial phylotypes were present in clone libraries from shrimp from the Rainbow vent site (Zbinden *et al*., 2008), but they were not investigated with FISH. We identified a second, novel symbiont type, belonging to the *Gammaproteobacteria*, as a dominant member of the *R. exoculata* epibiosis at all four MAR vent sites studied here. We did not have specimens of *R. exoculata* from Snake Pit to re-examine the diversity of the epibionts at this site, but it is likely that the Gamma symbionts are also present on Snake Pit shrimp, and were missed in the original study (Polz and Cavanaugh, 1995).

Both the Gamma and Epsilon symbionts were present on all shrimp from all four vent sites. Their relative abundance varied considerably, even among individuals collected at the same time from the same site. Given the high motility of the shrimp it is unlikely that this variability reflects vent fluid chemistry. An alternative explanation is that there is a sequential colonization pattern of the two symbiont types that is linked to the molting cycle of the shrimp (Corbari *et al*., 2007). We did not have enough individuals from different molting stages to test this hypothesis, but based on our observations, freshly molted shrimp were first colonized by the Epsilon symbionts while later stages were dominated by Gamma symbionts.

The *R. exoculata* Gamma symbionts fell in a clade consisting exclusively of bacteria associated with hydrothermal vent animals (the scaly snail *Crysomallon squamiferum* and the Yeti crab *K. hirsuta*, Fig. 4). The energy sources used by the bacteria associated with these vent animals are not known, but key genes for sulfur oxidation and sulfate reduction could be amplified from *K. hirsuta* epibiotic material (Goffredi *et al*., 2008). The closest relatives of the vent symbiont clade are the endosymbionts of *Oligobrachia* spp. tubeworms, and the free-living, filamentous bacterium *L. mucor*. The endosymbiont of *O. haakonmosbiensis* has been identified as a sulfur oxidizer (Lösekann *et al*., 2008), while the metabolism of the *O. mashikoi* symbiont is unclear (Kimura *et al*., 2003). *Leucothrix mucor* was originally characterized as a chemoorganoheterotroph (Brock, 1966), but was later shown to grow chemolithoheterotrophically, using reduced sulfur compounds as an energy source (Grabovich *et al*., 1999). The close phylogenetic relationship of the *R. exoculata* Gamma symbionts to *L. mucor* and the sulfur-oxidizing

symbiont of *O. haakonmosbiensis* could indicate that the *R. exoculata* Gamma symbionts use reduced sulfur compounds as an energy source. However, both free-living and symbiotic methane-oxidizing bacteria were the sister group to the clade containing the *R. exoculata* Gamma symbionts, *Oligobrachia* spp. symbionts and *L. mucor*, although this branching order received very little bootstrap support (Fig. 4). The metabolic capabilities of the *R. exoculata* Gamma symbionts therefore remain unclear.

Epibionts of hydrothermal vent animals

Close relatives of both the *R. exoculata* Gamma and Epsilon symbionts identified in this study have also been found in clone libraries from three other hydrothermal vent animals: the Yeti crab *K. hirsuta* (Goffredi *et al*., 2008), the scaly snail *C. squamiferum* (Goffredi *et al*., 2004) mentioned above and the barnacle *V. osheai* (Suzuki *et al*., 2009). The presence of close relatives of both symbiont types on such diverse hosts could be due to multiple horizontal transmission events in both bacterial lineages. It was recently shown that a single gene is sufficient to alter the host range of bioluminescent *V. fischeri* symbionts (Mandel *et al*., 2009), which could explain how such events occur. Multiple horizontal transmission events have been proposed for *Spiroplasma* bacterial endosymbionts in phylogenetically unrelated host insects (Haselkorn *et al*., 2009). Unlike the diverse insect hosts of *Spiroplasma* spp. that share common habitats, there is no currently known hydrothermal vent site where the distribution ranges of *R. exoculata*, *K. hirsuta*, *V. osheai* and *C. squamiferum* overlap. It is possible that such a vent site exists, but has not yet been discovered. Alternatively, free-living forms of the symbiotic bacteria that can disperse between vent sites might allow horizontal transmission between unrelated hosts that do not co-occur.

Role of the epibionts

A nutritional role has been proposed for the ectosymbionts of *K. hirsuta*, but this is still unresolved, as the crab host has never been observed to feed off its epibionts (Goffredi *et al*., 2008). 'Lau sp. A', a relative of *V. osheai* that has a morphologically similar ectosymbiosis was also hypothesized to feed off its epibionts by using modified mandibles to comb the filamentous bacteria from their cirral setae (Southward and Newman, 1998). All *R. exoculata* individuals we investigated from four separate MAR vent fields had both Gamma and Epsilon symbionts. While this suggests that the association is obligate for the shrimp host, the role of the symbionts in shrimp nutrition remains unclear. This is a common theme for all currently known ectosymbiotic associations at hydrothermal vents, where a clear contribution to host nutrition has not yet been shown.

Host biogeography

The genes encoding COI and CytB have been used successfully in previous studies of shrimp phylogeography to differentiate geographically separate populations within a species, at geographic scales ranging from tens of kilometers (for example, Page *et al*., 2008) to hundreds of kilometers (for example, Teske *et al*., 2007; Hunter *et al*., 2008). Despite this, neither of these genes could resolve site-specific populations of *R. exoculata* at hydrothermal vents included in this study, which were separated by up to 8500 km along the MAR. This suggests that there is significant migration and therefore gene flow between populations over vast geographic distances (Vrijenhoek, 1997). High migration rates have been hypothesized for *R. exoculata* populations along the North MAR (Creasey *et al*., 1996; Shank *et al*., 1998). In addition, larval properties such as a planktotrophic lifestyle (Tyler and Young, 1999) and the presence of storage compounds of likely photosynthetic origin (Pond *et al*., 1997; Pond *et al*., 2000) indicate a prolonged larval stage capable of long-distance dispersal. Although we could not distinguish geographically separate *R. exoculata* populations based on analysis of mitochondrial genes, more variable markers such as the nuclear internal transcribed spacer (Chu *et al*., 2001) or microsatellites (Meng *et al*., 2009) might identify phylogeographic patterns that are not visible at the level of the COI or CytB gene.

Symbiont biogeography

Although geographically separate host populations could not be identified, the symbiont populations of *R. exoculata* at the four vent sites investigated showed significant spatial structuring, which we verified with FISH. We were only able to investigate a limited number of individuals from each vent site due to sampling limitations; however, for two of the four vent sites, different individuals were used for 16S rRNA gene sequencing and FISH, meaning that a total of six individuals were analysed from each of these two sites, and the results of both methods were always consistent. Mantel tests showed significant spatial structuring of the symbiont populations and indicated that geography affects the diversity of shrimp epibionts, not vent geochemistry.

The *R. exoculata* symbionts are attached to the outside surfaces of the shrimp, and must recolonize the shrimp exoskeleton after each molt. In addition, a free-living form of the Epsilon symbiont has been shown to make up a substantial proportion of the free-living community on sulfides at the Snake Pit vent site (Polz and Cavanaugh, 1995). They are therefore most likely horizontally transmitted. Assuming horizontal transmission of the symbionts, two models could explain the spatial

structuring and biogeographic pattern we saw in the distribution of symbiont populations. In the first model, free-living symbiont populations are genetically isolated due to the existence of barriers to gene flow between vent fields. In this model, dispersal events are rare, and diversification of the symbiont population at a particular site is greater than transport of the symbionts, either free-living forms or host-attached, that would cause the mixing of populations. If this is the case, then the spatial structuring of symbiont populations would be expected to reflect the diversity of free-living forms of the symbionts. For example, only the Epsilon 2 symbiont would be found in the free-living community at the TAG vent site. Biogeography has been hypothesized to be a significant factor in structuring the diversity of hydrothermal vent fauna (Van Dover *et al*., 2002), and was recently shown to be the major factor in structuring populations of endosymbiotic bacteria at vents on the northern MAR (DeChaine *et al*., 2006).

In the second model, we assume that the free-living symbiont populations are not spatially structured along the MAR. For example, free-living forms of the Rainbow symbiont occur at other vent sites, and the TAG, Logatchev and South MAR symbionts occur at the Rainbow site in a free-living form. The structuring of symbiont populations at each vent site would therefore be due to specific colonization of the hosts by their symbionts from the pool of diverse free-living forms. This model requires highly specific recognition mechanisms between the hosts and their symbionts, and indeed these are known to play a critical role in horizontally transmitted animal-bacteria endosymbioses (Ruby, 2008). The association between the squid *Euprymna scolopes* and its luminescent bacterial symbionts *Vibrio fischeri* is one of the best understood model systems for symbiont recognition in horizontally transmitted associations (Nyholm and McFall-Ngai, 2004; Ruby, 2008). Remarkably, only a single regulatory gene is needed for conferring *V. fischeri* strains that can normally not colonize *E. scolopes* with the ability to infect these hosts (Mandel *et al*., 2009). A recent study of the nematode *Laxus oneistus* ectosymbiosis showed the role of host-expressed lectins in symbiont recognition and attachment to the worm cuticle (Bulgheresi *et al*., 2006), and a host-expressed lectin in the clam *Codakia orbicularis* was hypothesized to be involved in the recognition of its sulfur-oxidizing symbionts (Gourdine *et al*., 2007). The introduction of a single lectin gene into a plant that normally hosts nitrogen-fixing *Rhizobium leguminosarum* bv. *viciae* confers the ability to be colonized by *R. leguminosarum* bv. *trifolii* (Diaz *et al*., 1989). These last examples explain how specific recognition factors could be present in the *R. exoculata* populations at each vent field despite the lack of genetic structuring of their mitochondrial marker genes for COI and CytB: it is possible that symbiont specificity is determined by lectins or other genes that we did not analyse in this study.

It is not currently possible to determine which of the two models above best explains the observed geographic structuring of the *R. exoculata* symbionts. Only a very limited number of sequences are currently available from free-living bacteria related to the *R. exoculata* symbionts. These all belong to the *Epsilonproteobacteria* and do not group according to their geography. For example, sequences from free-living bacteria at the South MAR and Logatchev vent sites fell throughout the tree, and did not group exclusively with the symbiont sequences from these sites (Fig. 3B). This limited data set indicates that specific host-symbiont recognition drives the geographic grouping of the *R. exoculata* symbionts, but the number of sequences from free-living relatives of the shrimp symbionts is currently much too limited to provide sufficient support for either model. Extensive analyses of the free-living gamma- and epsilonproteobacterial populations from MAR vent sites are needed to better understand the processes causing the geographic structuring of the *R. exoculata* symbiont populations.

Conclusions and outlook

It is now known that free-living forms of horizontally transmitted symbionts can occur in the environment (Lee and Ruby, 1994; Miethling *et al*., 2000; Gros *et al*., 2003; Harmer *et al*., 2008), and that the abundance of the free-living forms can be correlated with the presence of the host (Lee and Ruby, 1994; Harmer *et al*., 2008). Surprisingly however, no study to date has investigated the spatial or geographic structure of free-living symbiont populations, although understanding their distribution patterns is crucial for determining the factors responsible for this structuring. This study provides the basis for future investigations of free-living forms of the *R. exoculata* epibionts. Comparing the distribution patterns of symbiotic bacteria and their free-living counterparts will provide insights into evolutionary processes such as migration, geographic isolation and symbiont-host interactions, and how these have shaped the diversity of symbiotic bacteria.

Experimental procedures

Sampling and storage

Rimicaris exoculata were collected at four vent fields along the MAR with a slurp gun on remotely operated vehicles (ROVs) (see Table S1 for the cruises, ROV and ship names, and chief scientists involved in the sampling). Samples were processed on board immediately where possible, or a maximum of 12 h after retrieval. They were either frozen for DNA or fixed for FISH analysis. Samples for FISH were fixed

at 4°C for 4–10 h in 2% formaldehyde in 0.2 mm filtered seawater. After fixation, samples were washed 3 times at 4°C for 30 min in 0.2 μm filtered seawater, then stored at –20°C in a 50% ethanol 50% filtered seawater solution.

DNA extraction and PCR amplification

Genomic DNA was extracted separately from scaphognathite, exopodite and carapace tissue (Fig. 1) with the FastDNA SPIN kit for soil (Qbiogene, Carlsbad, CA, USA). DNA was stored in aliquots at –20°C. The 16S rRNA gene was amplified from pooled scaphognathite, exopodite, and carapace DNA of 3 individuals from each sampling site. The universal bacterial primers 8F and 1492R (Muyzer *et al.*, 1995) were used for the amplification step. The reaction mixtures for PCR amplification contained 50 pmol of each primer, 2.5 μmol of each deoxynucleotide triphosphate, 1× Eppendorf buffer, 1 U of Eppendorf Taq polymerase and approximately 200 ng of genomic DNA. The final volume was adjusted to 50 μl with sterile water. The PCR program involved an initial denaturation step at 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 42°C for 1.5 min, and 72°C for 2 min, with a final elongation step at 72°C for 10 min. PCR bias was minimized by using only 25 (Rainbow) or 20 (TAG, Logatchev, South MAR) cycles, and pooling 4 (Rainbow) or 10 (TAG, Logatchev, South MAR) separate PCRs for each individual. PCR products were purified with a QIAquick PCR purification kit (QIAGEN, Hilden).

Cloning and sequencing of 16S rRNA genes

Purified PCR products were ligated at 4°C overnight with the pGEM-T Easy vector (Promega). The ligation product was used for transformation with a TOPO-TA kit (Invitrogen, Carlsbad, CA, USA). Clone libraries of 96 clones per shrimp were constructed. The insert size of white *Escherichia coli* colonies was controlled by PCR screening with vector primers M13F and M13R, using 0.5 μl of an overnight liquid culture as a template in a 20 μl reaction mixture. Partial sequencing of all positive clones was done using ABI BigDye and an ABI PRISM 3100 genetic analyser (Applied Biosystems, Foster City, CA, USA). Sequences were imported into BioEdit (Hall, 1997–2001) and aligned with ClustalW. Sequence groups were identified by visual inspection of the alignment. The phylogenetic affiliation of each group was determined by using BLAST (Altschul *et al.*, 1997) for a few representative sequences from each group. Clones from the identified ectosymbiont groups were randomly chosen for full sequencing. Full sequences were assembled using Sequencher (http://www.genecodes.com).

Phylogenetic analyses

Sequences covering most of the 16S rRNA gene (900–1400 nt) were used for phylogenetic analyses. These were aligned against close relatives in ARB (Ludwig *et al.*, 2004) using the Silva small subunit alignment (http://www.arb-silva.de; Pruesse *et al.*, 2007). The automatic alignment was refined by hand. Maximum likelihood, parsimony and neighbour-joining phylogenies were calculated in ARB using 30% (Epsilon symbiont phylogeny, 1408 columns used for calculation) and 50% (Gamma symbiont phylogeny, 1405 columns used for calculation) positional variability filters. Positional variability filters were calculated using ARB with > 200 sequences across the *Epsilonproteobacteria* (for the Epsilon symbiont phylogeny), or the *Gammaproteobacteria* (for the Gamma symbiont phylogeny). A termini filter was used for all analyses, removing the primer sequences. Bootstrapping was done with the PhyML package in ARB, with 100 re-samplings.

Amplification and sequencing of shrimp mitochondrial genes

The mitochondrial cytochrome oxidase subunit I (COI) gene was amplified with the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) using the following PCR cycling conditions: initial denaturation at 95°C for 5 min, followed by 36 cycles at 95°C for 1 min, 43°C for 1.5 min and 72°C for 2 min, then a final elongation step at 72°C for 10 min. The mitochondrial cytochrome b gene was amplified with primers designed for decapod crustaceans, Cybf and Cybr (Harrison, 2001), using the following cycling conditions: initial denaturation at 95°C for 5 min, followed by 36 cycles at 95°C for 1 min, 50°C for 1.5 min and 72°C for 2 min, then a final elongation step at 72°C for 10 min. PCR products were used for direct sequencing (see above).

Probe design and fluorescence in situ *hybridization (FISH)*

Oligonucleotide probes were designed either with the probe design function in ARB or by visually identifying a suitable target site in the ARB alignment. Vent field-specific probes were designed for the Epsilon symbionts, and were tested by cross-hybridization with samples from all other vent sites included in the study. It was not possible to design vent field-specific probes for the Gamma symbionts, as the 16S rRNA sequences were too closely related. In this case, two probes were designed, one targeting the Logatchev and South MAR sequences, the other targeting the Rainbow and TAG sequences. Probes for the Gamma symbiont sequences had at least two mismatches to all other sequences in the NCBI database.

Whole scaphognathite tissues were embedded in either paraffin or Steedman's wax (Steedman, 1957) and 6 μm thick sections cut with an RM 2165 microtome (Leica, Germany). The sections were collected on Superfrost Plus slides (Roth, Germany). Wax was removed from paraffin sections by washing in Roti-Histol (Roth, Germany) 3 times for 10 min each, and from Steedman's sections by washing in 96% ethanol 3 times for 5 min each. Sections were circled with a wax pen (PAP-pen, Kisker Biotech, Steinfurt, Germany), then rehydrated in an ethanol series consisting of 1 min in 96% ethanol, 1 min in 80% ethanol, then 1 min in 50% ethanol. Sections were hybridized in a buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS, with the appropriate formamide concentration) containing probes at an end concentration of 5 ng μl^{-1}. Sections were hybridized for 3 h at 46°C, then washed for 30 min at 48°C with buffer (0.1 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS, 5 mm EDTA), then rinsed in

distilled water. To stain all DNA, sections were covered in a 1% DAPI solution, left for 3 min, rinsed with distilled water, then dipped in 96% ethanol and air dried. Sections were mounted in a mixture of Citifluor and Vectashield and examined using both a fluorescence microscope (Zeiss Axioskop, Germany) and a confocal laser-scanning microscope (Zeiss CLSM 510, Germany).

Scanning electron microscopy

For scanning electron microscopy, *R. exoculata* specimens were fixed as above. Single filaments of the scaphognathite were dehydrated in an ethanol series (70–100%) and 100% acetone, transferred to hexamethyldisilazane and air dried. Dried specimens were placed on carbon adhesive tabs on an aluminium stub, sputtered with gold and viewed in a Philips XL20 at 15 kV.

Statistical analysis

To test the hypothesis that genetic distances between the epibiont 16S rRNA sequences were correlated with their geographical distances, Mantel tests using 1000 permutations were performed using the R (v.2.8.0) package Vegan (http://www.r-project.org). Geographical distances were estimated using Google Earth v.4.3 (http://earth.google.de). Along-ridge distances were estimated for MAR sites, as the axial valley is hypothesized to create corridors for the dispersal of organisms between vent fields (Tyler and Young, 2003).

Nucleotide sequence accession numbers

The sequences from this study are available through GenBank under the following accession numbers: FM203374–FM203407 and FN393020–FN393030 (symbiont 16S rRNA sequences), FN392996–FN393007 (*R. exoculata* COI gene) and FN393008–FN393019 (*R. exoculata* cytochrome b gene).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Parsimony network of host cytochrome b genes. Parsimony network inferred from 484 positions of the cytochrome b gene of *Rimicaris exoculata* from Rainbow (red), TAG (green), Logatchev (orange) and South MAR (blue). Each circle represents one sequence (individual number listed in circles), except for Individuals 1 and 12, which were identical. Lines connecting genotypes are one nucleotide difference, small white circles represent hypothetical unsampled ancestors. Calculated with the TCS program (Clement *et al*., 2000).
Fig. S2. A. Fluorescence *in situ* hybridization of *R. exoculata* Individual 1 from the Rainbow vent field with the Epsilon 1 probe (pink) showing the abundance of the Epsilon 1 symbiont. The Gamma symbiont is shown in green (hybridized with the probe Rexogam1268RT).
B. FISH of the same individual with the Epsilon 3 probe (pink) showing the abundance of the Epsilon 4 symbiont. The Gamma symbiont is shown as above.
C. FISH of the same individual with the RexoepsLG86 (5′-ctcgtcagccagtgc-3′) with competitor (5′-ctcgtcagccagtac-3′), showing that the signals in (B) are due to the presence of the Epsilon 4 symbiont, and not the Logatchev Epsilon 3. DAPI stain is shown in blue in all 3 images. The scale bar in (C) (20 μm) applies to all images.
Table S1. Summary of sampling sites and cruises.
Table S2. FISH probes designed for this study.
Table S3. Individual numbers used for sampling, and corresponding name used in text and figures.

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