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Minireview

Indigenous ectosymbiotic bacteria associated with diverse hydrothermal vent invertebrates

Shana K. Goffredi*
Biology Department, Occidental College, 1600 Campus Rd, Los Angeles, CA 90041, USA.

Summary

Symbioses involving bacteria and invertebrates contribute to the biological diversity and high productivity of both aquatic and terrestrial environments. Well-known examples from chemosynthetic deep-sea hydrothermal vent environments involve ectosymbiotic microbes associated with the external surfaces of marine invertebrates. Some of these ectosymbioses confer protection or defence from predators or the environment itself, some are nutritional in nature, and many still are of unknown function. Several recently discovered hydrothermal vent invertebrates, including two populations of yeti crab (*Kiwa* spp.), a limpet (*Symmetromphalus* aff. *hageni*), and the scaly-foot snail (as yet undescribed), support a consortium of diverse bacteria. Comparisons of these ectosymbioses to those previously described revealed similarities among the associated microorganisms, suggesting that certain microbes are indigenous to the surfaces of marine invertebrates. In particular, members of the *Thiovulgaceae* (epsilonproteobacteria) and *Thiotrichaceae* (gammaproteobacteria) appear to preferentially form ectosymbioses with vent crustaceans and gastropods. Interactions between specific *Proteobacteria* and the surfaces of many marine invertebrates likely have ecological and evolutionary significance at these chemically challenging habitats.

Introduction

Associations between bacteria and the body surfaces of aquatic invertebrates have been studied for decades. Historical documentation suggests that the prevalence and possible importance of these associations, whether beneficial or detrimental, was realized long ago (Anderson and Stephens, 1969; Johnson *et al.*, 1971; Sochard *et al.*, 1979). Today, there are many well-known ectosymbioses involving hosts within at least three subgroups of the *Ciliophora*, one *Euglenozoan*, two *Nematoda* subfamilies, three *Mollusca* families, two classes of *Annelida*, and many *Crustacea*, including both decapods and cirripeds (Buck *et al.*, 2000; Polz *et al.*, 2000; Dubilier *et al.*, 2008). The vent shrimp *Rimicaris exoculata*, for example, possesses dense bacteria on its carapace and mouthparts (Van Dover *et al.*, 1988). Once thought to be a monoculture of a single, pleomorphic epsilonproteobacterium, it is now known that the bacterial community includes two dominant bacterial types, whatever the prevailing chemical conditions surrounding the animal (Gebruk *et al.*, 1993; Polz and Cavanaugh, 1995; Struck *et al.*, 2008). Similarly, certain polychaetes (ex. *Alvinella* spp.), nematodes (ex. *Laxus* spp.), ciliates (ex. *Zoothamnium niveum*), and even amphipods (within the genus *Niphargus*), to name a few, are colonized by one or a few, sometimes highly ordered bacterial phylotypes, whereas many other crustaceans, the nematode *Eubostrichus*, and certain molluscs, such as the peltospirid scaly-foot snail, appear to associate with relatively diverse bacterial communities (Polz *et al.*, 1994; 1999; Haddad *et al.*, 1995; Goffredi *et al.*, 2004; Rinke *et al.*, 2006; Miyake *et al.*, 2007; Goffredi *et al.*, 2008; Dattagupta *et al.*, 2009).

It has been suggested that ectosymbiosis is the first step towards endosymbiosis and is the least intimate association on a large continuum from external attachment to extracellular to intracellular endosymbionts, with greater integration and coordination of host and symbiont towards the latter (Smith, 1979; Rosati, 2004). Certainly, evidence for this exists, including the phylogenetic similarity between the gammaproteobacterial ectosymbiont of *Laxus* spp. and the ‘Gamma 1’ endosymbiont of gutless oligochaetes within the subfamily *Phallodrilinae* (Dubilier *et al.*, 2008), as well as the sole gammaproteobacterial ectosymbiont on the ciliate *Z. niveum*, which is most closely related to the endosymbiont of the scaly-foot snail

Received 4 October, 2009; accepted 14 December, 2009. *For correspondence. E-mail sgoffredi@oxy.edu; Tel. (+1) 323 259 1470; Fax (+1) 323 341 4974.

(Rinke *et al*., 2006). However, evidence that ectobiota not only exhibit, in many cases, some degree of site preference on the host (Goffredi *et al*., 2004) but also use specific signalling between themselves and their host (Nussbaumer *et al*., 2004) suggests that surface associations are more than simply an incomplete step along the path towards endosymbiotic integration. Arguably, these ectosymbiotic associations, despite lesser morphological integration, can be complex and require significant molecular communication, recognition and coordination between partners, as is observed with the better-studied endosymbioses.

Specific interactions between host and bacteria have been observed for many ectosymbiotic associations, including animal hosts that have soft tissue body surfaces, as in the case of alvinellid polychaetes, as well as those that are covered in an exoskeleton or tough 'non-living' cuticle, such as members of the ecdysozoa (e.g. nematodes and crustaceans). For example, in the marine nematode *Laxus oneistus*, recognition and binding by bacterial ectosymbionts are mediated by a mannose-specific lectin secreted on the host cuticle surface (Nussbaumer *et al*., 2004; Bulgheresi *et al*., 2006). Even the rigid exoskeleton of crustaceans is controlled, in part, by the activities of the host, and could thus exert control over the specific composition and distribution of microbes attaching to these surfaces. A recent report demonstrated that abundant filamentous bacteria on the exoskeleton of a cave amphipod (*Niphargus* sp.) were distinct from the free-living biofilm forming phylotypes found within the environment, suggesting a highly specific association between these microbes and their host (Dattagupta *et al*., 2009). In this particular study, continuous reacquisition of the specific ectosymbiont by the amphipod, despite frequent molting and maintenance in aquaria for long periods, was observed (Dattagupta *et al*., 2009).

The presumed reliance of many of these host animals on their ectobiota suggests that evolutionary mechanisms have ensured the recruitment and maintenance of a particular microbe or microbial community. For example, it is thought that the scaly-foot snail relies on iron sulfide precipitation mediated by its deltaproteobacterial ectobionts, which act to protect the snail from predation (Goffredi *et al*., 2004). Similarly, it is widely known that colonization of embryos and developing crustacean larvae by specific bacteria affords protection against fungi and pathogenic bacteria, as well as from predators (Gil-Turnes *et al*., 1989; Gil-Turnes and Fenical 1992; Lopanik *et al*., 2004). Additionally, aposymbiotic stilbonematid nematodes (*Laxus* spp.) are never found in the wild, suggesting an obligate association, at least for the animal host (Bulgheresi *et al*., 2006). Finally, a diversity of marine hosts appears to use their ectobiota as a source of nutrition (Van Dover *et al*., 1988; Ott *et al*., 1991; Polz *et al*., 1992; 1994; Bauer-Nebelsick *et al*., 1996; Rinke *et al*., 2006; Suzuki *et al*., 2009). Most crustaceans exhibit grooming behaviour and for *Rimicaris*, in particular, ectosymbiont-like phylotypes have been recovered from the gut, suggesting the very likely ingestion of these bacteria as a food source (Zbinden and Cambon-Bonavita, 2003). In the case of the ciliate *Kentrophoros*, the food groove is dramatically reduced and the ciliate is thought to rely exclusively on the phagocytosis of ectosymbionts for nutrition (Fenchel and Finlay, 1989). It should be noted that this particular ectosymbioses is also perpetuated via vertical transmission during binary fission between mother and daughter cells, thus, too, suggesting a specific and stable association at time scales that go beyond the lifetime of an individual host.

The external nature of invertebrate–microbe ectosymbioses raises many interesting questions. For example, are there factors that predispose a particular bacterial group to a successful ectosymbiotic lifestyle? If so, can patterns in the identity and distribution of these bacteria be detected across diverse host species? Conversely, does the host play a role in attracting the correct symbiont and in maintaining the association, despite, in some cases, periodic molting and lack of obvious special structures for bacterial attachment? The purpose of this review was to add to a growing phylogenetic data set of ectosymbiotic bacteria associated with hydrothermal vent invertebrates, with primary emphasis on crustaceans and molluscs distributed broadly, with the intention of identifying possible patterns that may help answer some of these outstanding questions.

Hydrothermal vent ectosymbioses

Ectosymbiotic bacterial assemblages have been previously described for a suite of taxonomically and geographically diverse hydrothermal vent crustacean and gastropod hosts, including the originally described yeti crab *Kiwa hirsuta* from the Pacific Antarctic Ridge and another currently undescribed *Kiwa* species from Costa Rica (Fig. 1A), a similarly hairy crab (*Shinkaia crosnieri*) from the Okinawa Trough, vent barnacles from both the Lau Basin (undescribed sp. A) and Kermadec Arc, New Zealand (*Vulcanolepas osheai*), the Mid-Atlantic Ridge vent shrimp *R. exoculata*, and the 'scaly-foot' snail (currently undescribed) from the Indian Ocean (Polz and Cavanaugh, 1995; Southward and Newman, 1998; Goffredi *et al*., 2004; 2008; Rinke *et al*., 2006; Miyake *et al*., 2007; Struck *et al*., 2008; Petersen *et al*., 2009; Suzuki *et al*., 2009). Additionally, we present here data on the bacteria associated with a new population of limpet *Symmetromphalus* aff. *hageni* (class *Gastropoda*, family

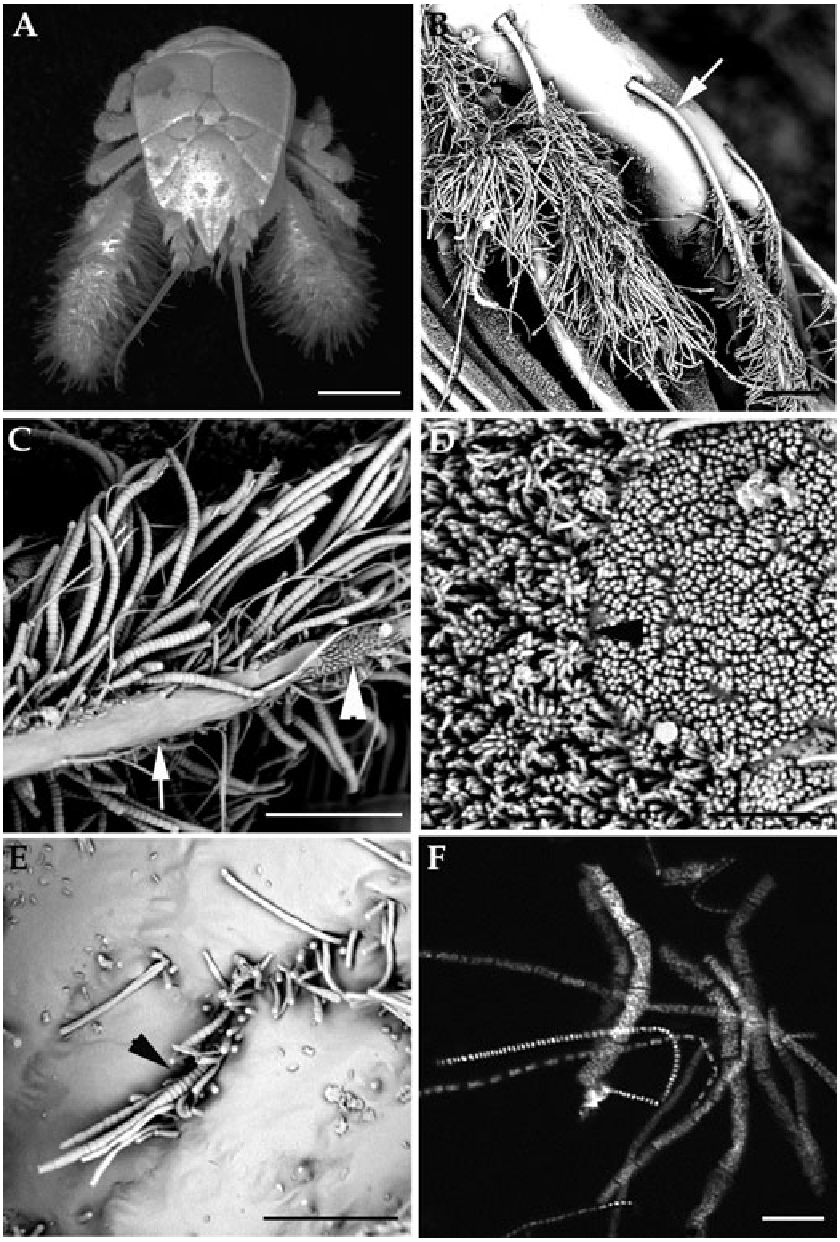


Fig. 1. *Kiwa* n. sp. (Costa Rica).
A. Whole individual. Scale bar, 1 cm.
B. Scanning electron micrograph (SEM) showing numerous setae (arrow) with filamentous bacteria at the distal ends. Scale bar, 25 μm. *Kiwa* specimens for SEM were HMDS (hexamethyldisilizane)-dried, palladium coated, and examined with a FEI Phenom instrument.
C. SEM showing close-up of the filamentous and rod-shaped (arrowhead) bacteria present on the setae (arrow). Scale bar, 10 μm.
D. SEM close-up of rod-shaped bacteria carpeting the abdomen of the crab. Note the abrupt separation between populations of bacteria in different orientations on the crab surface (black arrowhead). Scale bar, 5 μm.
E. SEM close-up of the filamentous and rod-shaped bacteria present on the exoskeleton. There is little evidence for visible exoskeleton degradation, but note the pits possibly made by bacteria (arrowhead). Scale bar, 10 μm.
F. Fluorescence *in situ* hybridization (FISH) microscopy showing a variety of bacterial morphotypes recovered from the setae, hybridized with a general bacterial probe (Eub338) labelled with Cy3. Scale bar, 10 μm. Samples for FISH, initially preserved in paraformaldehyde, were rinsed twice with 1× phosphate-buffered saline (PBS), transferred to 70% ethanol, and stored at −20°C. Hybridization was carried out according to Goffredi and colleagues 2008. Samples were imaged on a Nikon Eclipse E80i fluorescence microscope.

Neomphalidae) collected from the North Fiji Basin in May 2005 (Fig. 2A and C), in comparison to those described above. All limpets (including *Symmetromphalus*, *Lepetodrilus* and *Olgaosolaris* spp.) were collected during the Fiji/Lau expedition (May 2005, R. Vrijenhoek, Chief scientist) with the *R.O.V. Jason* (16°59.4′S, 173°54.9′E, 1980 m depth). *Symmetromphalus* aff. *hageni* occurred in dense clusters on the shell of a larger snail *Ifremeria nautilei*, which was found on the walls of active hydrothermal chimneys. The feeding biology of *S.* aff. *hageni* is not known, but active foraging may be limited based on an observed reduction in radular morphology (Beck, 1992). All N. Fiji Basin individuals of *S.* aff. *hageni* greater than ~3 mm shell length, which varied from 1 to 21 mm, had unusual epipodial papillae that harboured filamentous bacteria (Fig. 2B, D and E). This morphological modification has not been observed in other populations of *Symmetromphalus* (A. Waren, Swedish Museum of Natural History, pers. comm.).

The site of attachment and degree of bacterial coverage on crustacean and molluscan hosts varies, with the crabs (Fig. 1B, C, E and F; Goffredi *et al.*, 2008) and barnacles (Southward and Newman, 1998) possessing setae that, in turn, are covered with clusters of filamentous bacteria, while *Rimicaris* possesses bacteria primarily on the setae of modified feeding appendages and gill

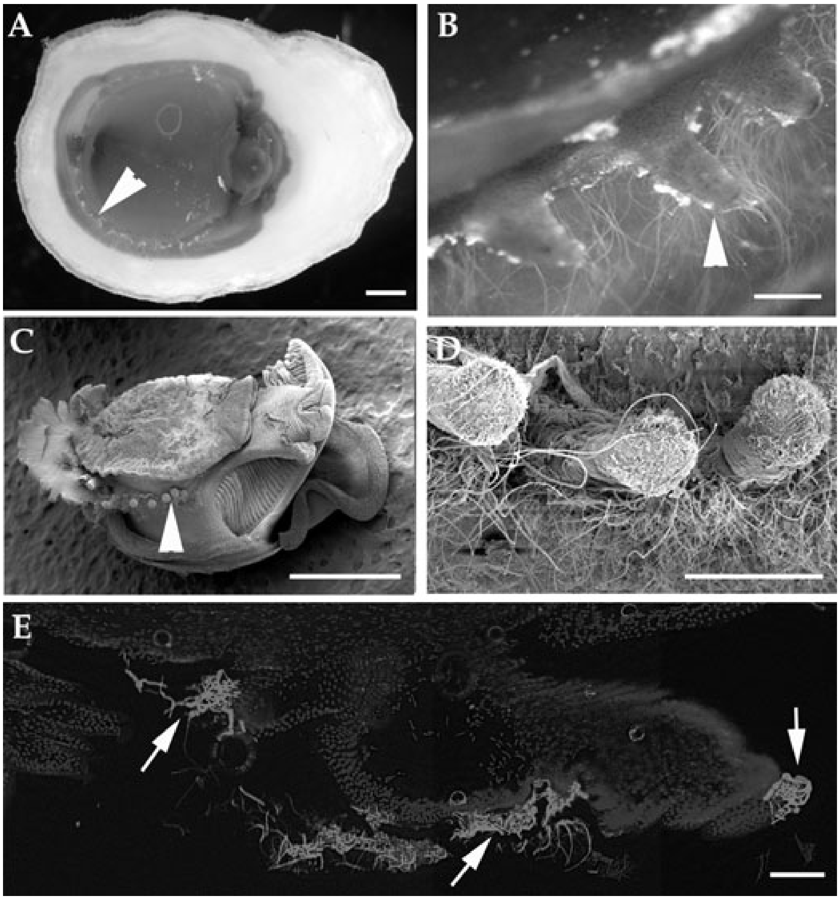
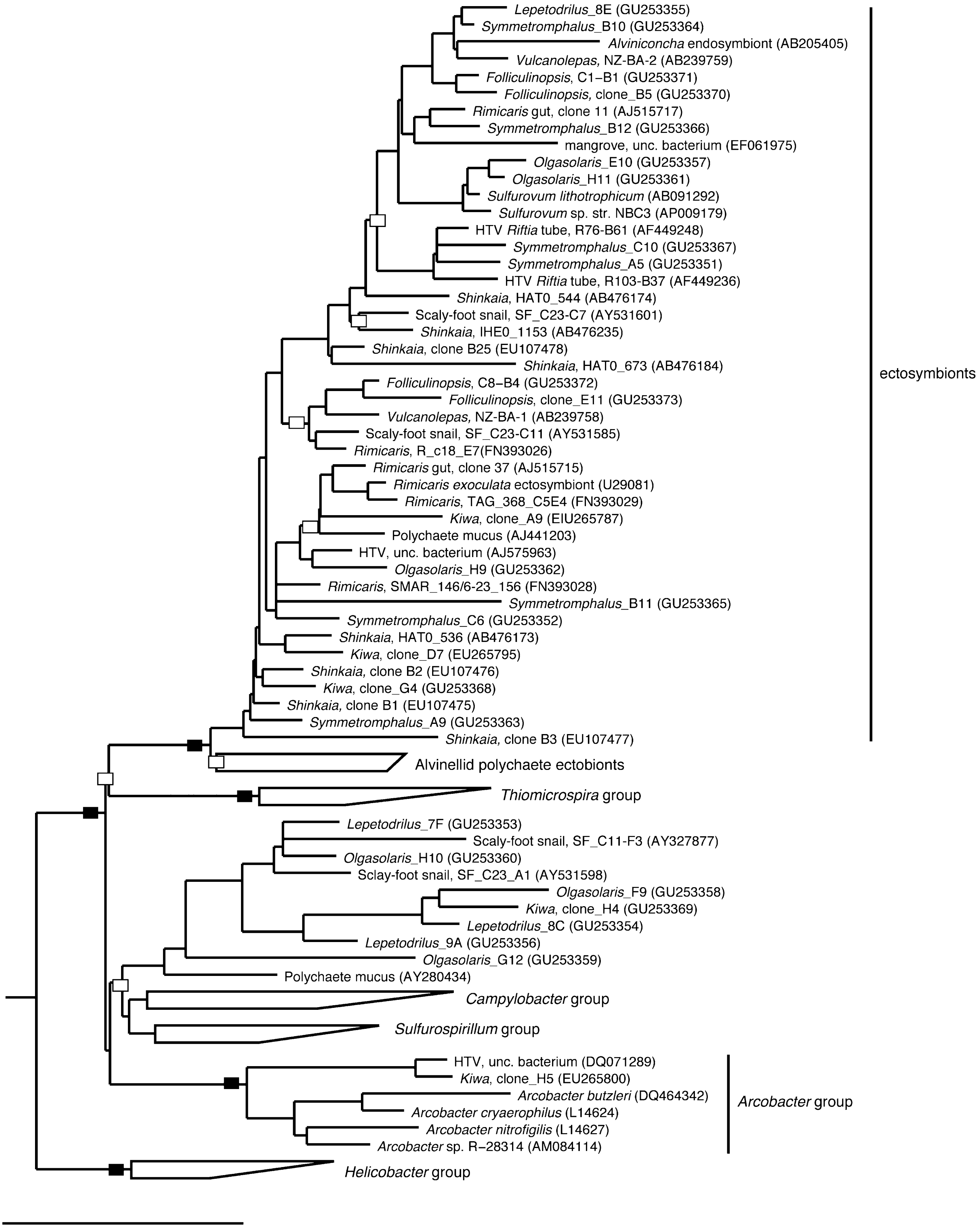


Fig. 2. *Symmetromphalus* aff. *hageni* (North Fiji Basin).
A. Whole individual, showing filamentous bacteria along the foot (arrowhead). Scale bar, 1 mm.
B. Light micrograph showing epipodial papillae (arrowhead) with filamentous white bacteria. Scale bar, 100 μm.
C. SEM of whole individual, showing position of epipodial papillae (arrowhead) along the foot of the animal. Scale bar, 1 mm. *Symmetromphalus* specimens for SEM were critical-point dried, gold coated, and examined with a Hitachi S-4300 field emission instrument.
D. SEM close-up of the filamentous bacteria present on the individual papillae. Scale bar, 100 μm.
E. Fluorescence *in situ* hybridization (FISH) microscopy showing bacteria associated with the papillae (in cross-section), hybridized with a general bacterial probe (Eub338) labelled with Cy3. Scale bar, 10 μm. Preservation, embedding and FISH hybridization of samples was carried out according to Goffredi and colleagues 2008. Samples were imaged with a DeltaVision restoration microscopy system (Applied Precision).

chamber (Van Dover *et al.*, 1988; Gebruk *et al.*, 1993). The scaly-foot snail possesses unusual sclerites that are covered with both filamentous and coccoid bacteria (Goffredi *et al.*, 2004), while unique soft tissue extensions on *S.* aff. *hageni* presumably accommodate bacterial attachment (Fig. 2B, D and E). All are from hydrothermal vents with the exception of the new species of *Kiwa* from a subduction zone cold-seep area off of Costa Rica (Fig. 1A; A. Thurber, Scripps Institution of Oceanography, pers. comm.; S.K. Goffredi, pers. obs.). Specimens of *Kiwa* examined in this study were collected during Costa Rica expedition AT15-44 (February 2009, L. Levin, Chief scientist) with the *D.S.R.V. Alvin* (8°55.9′N, 84°18.7′W, ~1000 m depth).

Despite differences in host taxonomy, nature of the host surface and geographic location among the animal hosts described above, bacterial ectosymbionts recovered from this diverse group of deep-sea invertebrates are closely related (Figs 3 and 4). Compilation of 16S ribosomal RNA sequences revealed that certain microbial groups, namely the *Thiovulgaceae* (a group within the epsilonproteobacteria) and *Thiotrichaceae* (within the gammaproteobacteria), appear to preferentially associate with these invertebrates. Different host species, particularly the two separate *Kiwa* populations, inhabit different ocean basins > 5000 km apart, thus, the striking phylogenetic resemblance in bacterial groups among them yields important information concerning the specificity, coordination and

Fig. 3. Phylogenetic relationships of epsilonproteobacteria associated with hydrothermal vent invertebrates, based on sequence divergence within the 16S rRNA gene. Additional sequences were obtained from GenBank and Greengenes and compiled and aligned with our 16S rRNA sequences using the ARB automated alignment tool with subsequent manual refinements (Ludwig *et al.*, 2004). For near full-length representatives and closest relatives, neighbour-joining analysis was conducted with Olsen distance correction, using 5000 bootstrap replicates to assign confidence levels to nodes. Neighbour-joining trees of partial sequences recovered in our study were made via parsimony insertion of partial sequences (500–600 bp) within the tree of ‘near full length’ sequences (~1465 bp). Greengenes was also used to check for potential chimeras (Desantis *et al.*, 2006). *Leucothrix mucor* (X87277) was used as an outgroup (not shown). Symbols next to nodes correspond to bootstrap values based on neighbour-joining distance and 3000 replicates (open symbol = 50–90%, closed symbol = 90+% bootstrap support). HTV, hydrothermal vent. unc., uncultured. All samples for molecular analysis were extracted, amplified and sequenced according to Goffredi and colleagues (2008). GenBank accession numbers for epsilonproteobacteria sequences obtained in this study are GU253351–GU253373.


Lepetodrilus_8E (GU253355)
Symmetromphalus_B10 (GU253364)
Alviniconcha endosymbiont (AB205405)
Vulcanolepas, NZ-BA-2 (AB239759)
Folliculinopsis, C1–B1 (GU253371)
Folliculinopsis, clone_B5 (GU253370)
Rimicaris gut, clone 11 (AJ515717)
Symmetromphalus_B12 (GU253366)
mangrove, unc. bacterium (EF061975)
Olgasolaris_E10 (GU253357)
Olgasolaris_H11 (GU253361)
Sulfurovum lithotrophicum (AB091292)
Sulfurovum sp. str. NBC3 (AP009179)
HTV Riftia tube, R76-B61 (AF449248)
Symmetromphalus_C10 (GU253367)
Symmetromphalus_A5 (GU253351)
HTV Riftia tube, R103-B37 (AF449236)
Shinkaia, HAT0_544 (AB476174)
Scaly-foot snail, SF_C23-C7 (AY531601)
Shinkaia, IHE0_1153 (AB476235)
Shinkaia, clone B25 (EU107478)
Shinkaia, HAT0_673 (AB476184)
Folliculinopsis, C8–B4 (GU253372)
Folliculinopsis, clone_E11 (GU253373)
Vulcanolepas, NZ-BA-1 (AB239758)
Scaly-foot snail, SF_C23-C11 (AY531585)
Rimicaris, R_c18_E7(FN393026)
Rimicaris gut, clone 37 (AJ515715)
Rimicaris exoculata ectosymbiont (U29081)
Rimicaris, TAG_368_C5E4 (FN393029)
Kiwa, clone_A9 (EIU265787)
Polychaete mucus (AJ441203)
HTV, unc. bacterium (AJ575963)
Olgasolaris_H9 (GU253362)
Rimicaris, SMAR_146/6-23_156 (FN393028)
Symmetromphalus_B11 (GU253365)
Symmetromphalus_C6 (GU253352)
Shinkaia, HAT0_536 (AB476173)
Kiwa, clone_D7 (EU265795)
Shinkaia, clone B2 (EU107476)
Kiwa, clone_G4 (GU253368)
Shinkaia, clone B1 (EU107475)
Symmetromphalus_A9 (GU253363)
Shinkaia, clone B3 (EU107477)
ectosymbionts
Alvinellid polychaete ectobionts
Thiomicrospira group
Lepetodrilus_7F (GU253353)
Scaly-foot snail, SF_C11-F3 (AY327877)
Olgasolaris_H10 (GU253360)
Sclay-foot snail, SF_C23_A1 (AY531598)
Olgasolaris_F9 (GU253358)
Kiwa, clone_H4 (GU253369)
Lepetodrilus_8C (GU253354)
Lepetodrilus_9A (GU253356)
Olgasolaris_G12 (GU253359)
Polychaete mucus (AY280434)
Campylobacter group
Sulfurospirillum group
HTV, unc. bacterium (DQ071289)
Kiwa, clone_H5 (EU265800)
Arcobacter butzleri (DQ464342)
Arcobacter cryaerophilus (L14624)
Arcobacter nitrofigilis (L14627)
Arcobacter sp. R–28314 (AM084114)
Arcobacter group
Helicobacter group

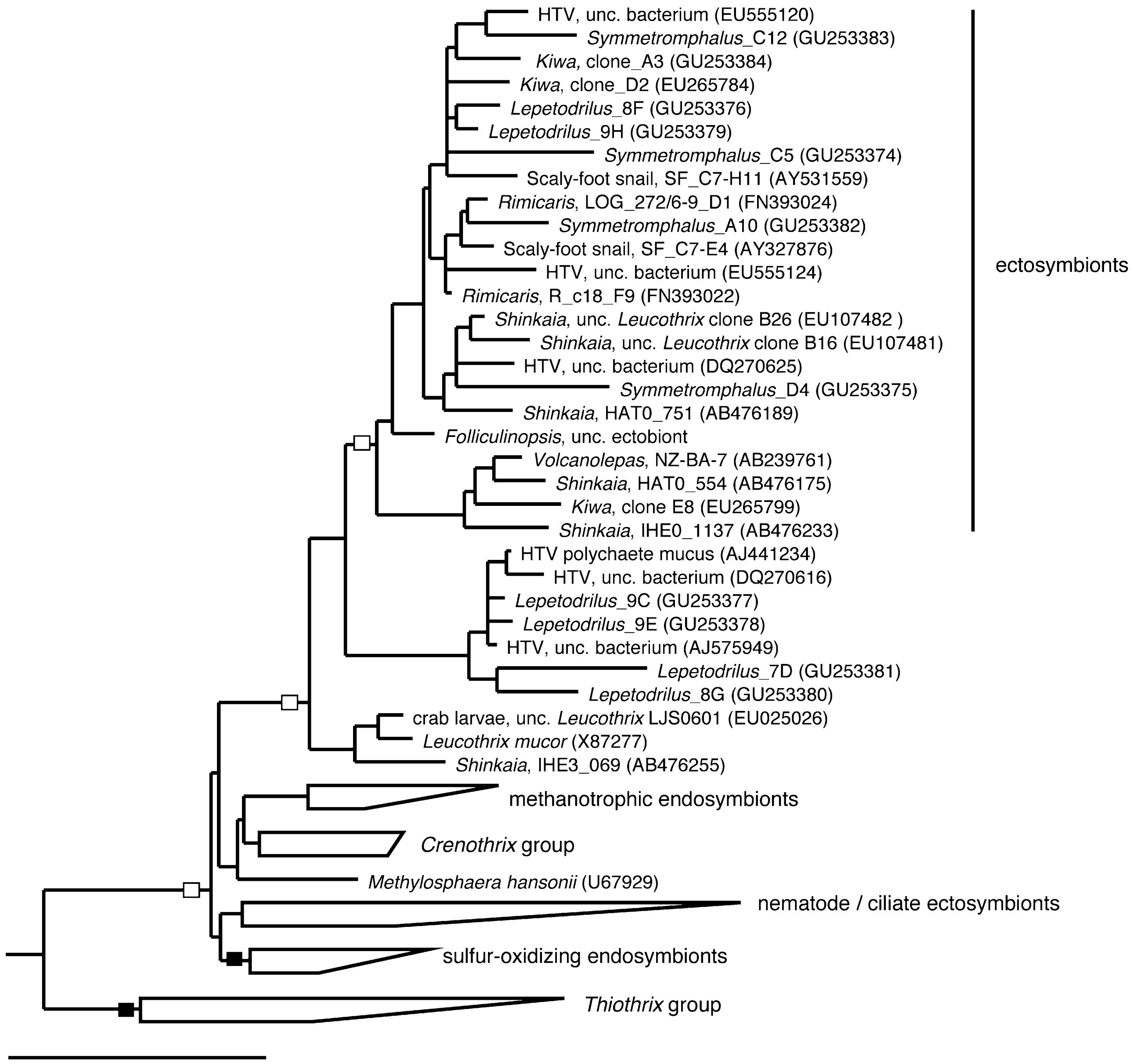


Fig. 4. Phylogenetic relationships of gammaproteobacteria associated with hydrothermal vent invertebrates, relative to selected cultured and environmental sequences in public databases, based on sequence divergence within the 16S rRNA gene. Neighbour-joining tree (Olsen correction) of 16S rRNA genes with, in some cases, parsimony insertion of partial sequences into a backbone tree of a subset of near full-length sequences. *Sulfurospirillum arcachonense* (Y11561) was used as an outgroup (not shown). Symbols next to nodes correspond to bootstrap values based on neighbour-joining distance and 3000 replicates (open symbol = 50–90%, closed symbol = 90+% bootstrap support). HTV, hydrothermal vent. unc., uncultured. Sequences were compiled and aligned as described for Fig. 3. GenBank accession numbers for gammaproteobacteria sequences obtained in this study are GU253374–GU253384.

prevalence of vent and seep invertebrate–bacterial ectosymbioses. Likewise, these phylotypes were recovered from the surfaces of both molluscs and crustaceans, including the setae of the crab *K. hirsuta* and the epipodial papillae of the limpet *S.* aff. *hageni*, further supporting a possible microbial community that characteristically inhabits the body surfaces of hydrothermal vent invertebrates.

Epsilonproteobacteria – *Thiovulgaceae*

Epsilonproteobacteria were most abundant on the epipodial papillae of *S.* aff. *hageni* (60% of the recovered bacterial phylotypes, out of 72 clones), and primarily belonged to the Marine Group 1 (also referred to as 'Group F', Nakagawa *et al.*, 2005), within the newly established family *Thiovulgaceae* (Campbell *et al.*, 2006), with *Sulfurovum lithotrophicum* as the closest cultured relative (93–95% similarity; Inagaki *et al.*, 2004). Previous molecular analyses revealed the setae-associated bacteria of *Kiwa* spp. to also be dominated by this same group of epsilonproteobacteria, comprising 50–82% of the recovered bacterial ribotypes, for the PAR and Costa Rica crab populations respectively (Goffredi *et al.*, 2008; A.C. Gregory and S.K. Goffredi, unpubl. data). Similarly, epsilonproteobacteria were reported to comprise 30–80% of the bacteria associated with the elongated cirral setae of the stalked barnacle *Volcanolepas osheai* (Suzuki *et al.*, 2009). Other hydrothermal vent hosts, including *S. crosnieri*, *R. exoculata*, another vent shrimp *Alvinocaris longirostris*, and the scaly-foot snail have also been observed to possess ectosymbionts within this bacterial group (Fig. 3; Goffredi *et al.*, 2004; 2008; Miyake *et al.*, 2007; Struck *et al.*, 2008; Tokuda *et al.*, 2008; Petersen *et al.*, 2009). Several bacterial phylotypes associated with the heterotrichid ciliate *Folliculinopsis* also cluster within this group (Fig. 3; S.K. Goffredi, unpubl. data). These ectosymbionts are all within 90% similar to each other, based on 16S rRNA, and cluster together within a well-supported clade that also includes several free-living phylotypes recovered from hydrothermal vent areas, as well as *Olgasolaris*, a limpet collected within the vicinity of *Symmetromphalus* and not known to possess ectosymbionts (Fig. 3; S.K. Goffredi, pers. obs.).

Epsilonproteobacteria are an ecologically important group of bacteria, particularly at deep-sea hydrothermal vents (Nakagawa *et al.*, 2005; Campbell *et al.*, 2006). Members of the sulfur-utilizing Marine Group 1 can make up $> 90\%$ of the total rRNA signature at deep-sea vents, including the surfaces of chimneys, metazoans and sediments (Campbell *et al.*, 2006; Nakagawa *et al.*, 2007). *Sulfurovum lithotrophicum*, in particular, the only cultured representative within this group and the bacterium most closely related to the ectosymbionts discussed above, is a strict sulfur oxidizer that can oxidize elemental sulfur or thiosulfate, with oxygen or nitrate as electron acceptors (Inagaki *et al.*, 2004; Nakagawa *et al.*, 2007). Epsilonproteobacteria in general can be rod, vibrio or filamentous shaped; however, *S. lithotrophicum* has been reported as either coccoid or short rods (Inagaki *et al.*, 2004). Interestingly, many of the bacteria observed on the Costa Rican *Kiwa* population were short rods (Fig. 1C and D). It has been suggested that the versatile energy metabolism and genome plasticity of the epsilonproteobacteria, and, in particular, the hydrothermal vent bacterium *Sulfurovum* sp. NBC37-1, explains their endemism to very specific yet diverse ecological niches, such as the external surfaces of animals (Nakagawa *et al.*, 2005; 2007).

Gammaproteobacteria – *Thiotrichaceae*

Bacteria most closely related to the chemolithoheterotroph *Leucothrix* (*Thiotrichaceae*) were common on the epipodial papillae of *Symmetromphalus* aff. *hageni* (~30% of the recovered bacterial phylotypes, out of 72 clones). Similar to the epsilonproteobacteria Marine Group 1, previous molecular analysis also revealed the presence of *Leucothrix*-like gammaproteobacteria on the setae of both *K. hirsuta* (25% of recovered bacterial phylotypes; Goffredi *et al.*, 2008) and *V. osheai* (~20% of recovered bacterial phylotypes; Suzuki *et al.*, 2009), as well as other hydrothermal vent hosts, including *S. crosnieri*, *R. exoculata*, and the scaly-foot snail (Fig. 4; Goffredi *et al.*, 2004; Miyake *et al.*, 2007; Struck *et al.*, 2008; Petersen *et al.*, 2009). Recently, *Leucothrix*-like gammaproteobacteria were also found to be common members of the ectosymbiotic community (~13% of recovered ribotypes) on the shallow water oligochaete *Tubificoides benedii* from eutrophic coastal sediments (Ruehland and Dubilier, 2010). Similarly, a bacterium associated with the ciliate *Folliculinopsis* also belongs to this group (Fig. 4, A. Kouris, University of Illinois at Urbana-Champaign, pers. comm.). These *Leucothrix*-like ectosymbionts are all within 91% similarity of each other, based on 16S rRNA, and remain distinct from gammaproteobacterial ectosymbionts previously recovered from nematodes. They cluster together within a well-supported clade that also includes free-living phylotypes from hydrothermal vent areas (Fig. 4). Notably, *Lepetodrilus*, collected in the area immediately surrounding *Symmetromphalus* in N. Fiji Basin (S.K. Goffredi, pers. obs.), generally possessed bacterial phylotypes that were distinct from the main ectosymbiont clade and more related to those found associated with the mucous of polychaete tubes and vent surfaces (Fig. 4).

Bacteria within the *Thiotrichaceae* are not generally reported as being common members of the free-living bacterial community at hydrothermal vents. They have, however, been previously observed on the appendages and eggs of benthic marine crustaceans (e.g. shrimp, crabs and copepods), although not on planktonic crustaceans (Anderson and Stephens, 1969; Johnson *et al.*, 1971; Carman and Dobbs, 1997). This likely reflects the dispersal capabilities and typical habitat preference of *Leucothrix*-like gammaproteobacteria. *Leucothrix mucor*, in particular, has been described as an ‘infestation’ on algae and invertebrates (Harold and Stanier, 1955; Bland and Brock, 1973; Oeschger and Schmaljohann, 1988). These filamentous heterotrophic marine bacteria are known for aerobic sulfide oxidation (Brock, 2006), and likely gain benefit not only from organic carbon compounds exuded by the host but also from the movement of these host between the oxic–anoxic interfaces of their respective habitats (Polz *et al.*, 2000). The closely related and mostly freshwater chemoautotrophic counterpart *Thiothrix* has also been found to associate with invertebrates, including mayfly larvae and amphipods (both marine and freshwater; Larkin *et al*., 1990; Gillan and Dubilier, 2004; Dattagupta *et al*., 2009).

Conclusions and future questions

Certain microbial groups, including epsilonproteobacteria (*Thiovulgaceae*) and gammaproteobacteria (*Thiotrichaceae*), appear to preferentially form ectosymbioses with hydrothermal vent, and possibly other, invertebrates and even protozoans. In some cases, ectosymbionts preferentially colonize specific body parts, they exist on the animal surface in greater abundance than on non-living substrate, and they can be quite regular in their distribution. Thus, it does not appear that ectosymbionts simply attach to all available substrates, but rather have varying degrees of adaptation to life on an animal surface. This may be especially true for vent crustaceans that, by inhabiting rocky substrate, are not the only available hard substrate suitable for microbial attachment and colonization, unlike other crustaceans that more commonly inhabit soft sediments. The fact that distinct populations of *R. exoculata* from very different chemical and physical regimes possess similar bacterial ectosymbionts (Petersen *et al*., 2009) or that similar ectosymbionts have been recovered from populations of the hairy yeti crab, *Kiwa* spp. from both hydrothermal vent and cold seep habitats (S.K. Goffredi, unpubl. data) suggests that the animal host matters as much, if not more than, specific locale or environmental conditions.

Despite this pattern in the identity and distribution of ectosymbionts from diverse crustaceans and gastropods, many important questions still remain. For example, the exact function of most ectosymbiotic bacteria is not yet known, although there is likely a spectrum of interactions. Evidence suggests a nutritional link for some, including both *K. hirsuta* and *S.* aff. *hageni*; however, isotopic and molecular evidence for nutritional integration can be ambiguous if the host also consumes similar bacteria that dominate non-living surfaces within the environment. Previous studies on non-vent crustaceans have suggested a nutritional role for the ectobiota and cited, as evidence, dramatic reductions in the nutritional well-being of the host upon exposure to antibiotics (Johnson *et al*., 1971). Additionally, the caridean shrimp *Palaemonetes pugio* and the 'hairy' decapod *S. crosnieri* have been observed in aquaria to actively graze their ectobiota (Johnson *et al*., 1971; Miyake *et al*., 2007). Nevertheless, direct evidence for a putative nutritional interaction is generally lacking in most cases, thus, examining net transfer of labelled compounds from symbionts to host, or massive grazing of symbionts, is a necessary next step in symbiosis research.

The mechanism for continued maintenance of these ectosymbiotic associations, despite molting by the host, as in the case of crustaceans, is also not known, although microbial chemotaxis is surely involved. We have personally observed that all young Costa Rican yeti crabs over 2 mm carapace length (adults can achieve ~4 cm carapace length) have ectosymbiotic bacteria, suggesting acquisition at a very young life stage. It is not known, however, whether this occurs all at once or as a succession over time. Dattagupta and colleagues (2009) suggest the possible role of maternal transmission of ectosymbionts during egg brooding in the freshwater amphipod *Niphargus* and cite stable possession of a specific ectobiotic phylotype, despite maintenance in aquaria for long periods, as evidence.

As new ectobiota are discovered in association with aquatic crustaceans and molluscs, it will be interesting to uncover their phylogenetic affiliations and interactions with the host. For example, ectosymbionts have been reported for aplacophorans; however, their identity and prevalence within this group is not currently known (Katz *et al*., 2006). Even though these invertebrate–microbe associations are external by nature, there is a pattern emerging that is driven by as yet unknown influences. In the future, it may be possible to better understand the pervasiveness of crustacean and molluscan–ectosymbiont interactions and implications for survival in challenging oceanic habitats.

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