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The gill chamber epibiosis of deep-sea shrimp *Rimicaris exoculata*: an in-depth metagenomic investigation and discovery of *Zetaproteobacteria*

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Summary

The gill chamber of deep-sea hydrothermal vent shrimp *Rimicaris exoculata* hosts a dense community of epibiotic bacteria dominated by filamentous *Epsilonproteobacteria* and *Gammaproteobacteria*. Using metagenomics on shrimp from the Rainbow hydrothermal vent field, we showed that both epibiont groups have the potential to grow autotrophically and oxidize reduced sulfur compounds or hydrogen with oxygen or nitrate. For carbon fixation, the *Epsilonproteobacteria* use the reductive tricarboxylic acid cycle, whereas the *Gammaproteobacteria* use the Calvin–Benson–Bassham cycle. Only the epsilonproteobacterial epibionts had the genes necessary for producing ammonium. This ability likely minimizes direct competition between epibionts and also broadens the spectrum of environmental conditions that the shrimp may successfully inhabit. We identified genes likely to be involved in shrimp–epibiont interactions, as well as genes for nutritional and detoxification processes that might benefit the host. Shrimp epibionts at Rainbow are often coated with iron oxyhydroxides, whose origin is intensely debated. We identified 16S rRNA sequences and functional genes affiliated with iron-oxidizing *Zetaproteobacteria*, which indicates that biological iron oxidation might play a role in forming these deposits. Fluorescence *in situ* hybridizations confirmed the presence of active *Zetaproteobacteria* in the *R. exoculata* gill chamber, thus providing the first evidence for a *Zetaproteobacteria*–invertebrate association.

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Introduction

The shrimp *Rimicaris exoculata* (Williams and Rona, 1986) dominates the macrofauna at many hydrothermal vent sites along the Mid-Atlantic Ridge (MAR). *Rimicaris exoculata* swim along chimney walls in the gradient between hydrothermal fluids and cold oxygenated ambient seawater, in a temperature range between 3°C and 25°C (Schmidt *et al.*, 2008). Adult *R. exoculata* have a hypertrophied cephalothorax that hosts a dense epibiotic bacterial community. These bacteria colonize the mouthparts and inner faces of the shrimp carapace (Zbinden *et al.*, 2004; Zbinden *et al.*, 2008 and references therein) and are morphologically diverse, including at least six filament types and bacilli (Zbinden *et al.*, 2004; 2008; Corbari *et al.*, 2008a). Molecular studies have revealed two dominant phylotypes belonging to the *Epsilonproteobacteria* and *Gammaproteobacteria*, and lower abundances of *Alphaproteobacteria, Deltaproteobacteria* and *Betaproteobacteria* (Zbinden *et al.*, 2008; Petersen *et al.*, 2010; Hügler *et al.*, 2011; Guri *et al.*, 2012). Based on functional gene surveys of the epibiont community, it was suggested that epibiont chemolithoautotrophy could be powered by a variety of electron sources, including reduced sulfur compounds, hydrogen and methane (Zbinden *et al.*, 2008; Hügler *et al.*, 2011; Petersen *et al.*, 2011; Guri *et al.*, 2012). Furthermore, based on polymerase chain reaction (PCR) amplification of two genes of the reductive tricarboxylic acid (rTCA) cycle and one of the Calvin–Benson–Bassham

(CBB) cycle, it was hypothesized that the dominant epsilonproteobacterial and gammaproteobacterial epibionts could be autotrophic and use different carbon fixation pathways (Hügler *et al*., 2011). In support of this, CO_2 fixation activity has been demonstrated by both *in vitro* and *in vivo* experiments (Polz *et al*., 1998; Ponsard *et al*., 2013), although, only functional gene fragments have been amplified and sequenced from these complex epibiont communities so far, and these fragments could only be putatively assigned to specific epibionts. Hence, the sources of energy and CO_2 fixation pathways of the dominant symbionts remain to be resolved.

Shrimps from the Rainbow hydrothermal vent field of the MAR have a conspicuous rusty colour due to iron oxyhydroxide particle accumulation in their gill chambers. Close association of epibionts with these chemically inert iron oxyhydroxides led to the suggestion that some epibionts might oxidize iron (Zbinden *et al*., 2004; 2008; Corbari *et al*., 2008b; Schmidt *et al*., 2008). However, no study has yet revealed the presence of iron-oxidizing bacteria in the gill chamber or provided evidence that previously identified epibionts can oxidize iron. Since the dominant epibionts have not yet been successfully cultivated, we undertook a metagenomic approach to (i) study the microbial diversity of the *R. exoculata* gill chamber community (while avoiding some of the pitfalls of traditional methods based on PCR amplification of 16S rRNA or functional genes), (ii) determine which energy sources could potentially power this symbiosis and (iii) pinpoint the differences between the epsilonproteobacterial and gammaproteobacterial epibionts.

Results and discussion

Metagenome characteristics and microbial diversity

Taxonomic classification of the metagenome at the level of class and above revealed that a large number of sequences could be affiliated with *Bacteria* (84%), but also with *Eukarya* (14%, probably *R. exoculata*) and *Archaea* (2%) (Fig. 1A). Most of the bacterial sequences were affiliated with *Proteobacteria* (78%), including *Epsilonproteobacteria* (39%), *Gammaproteobacteria*

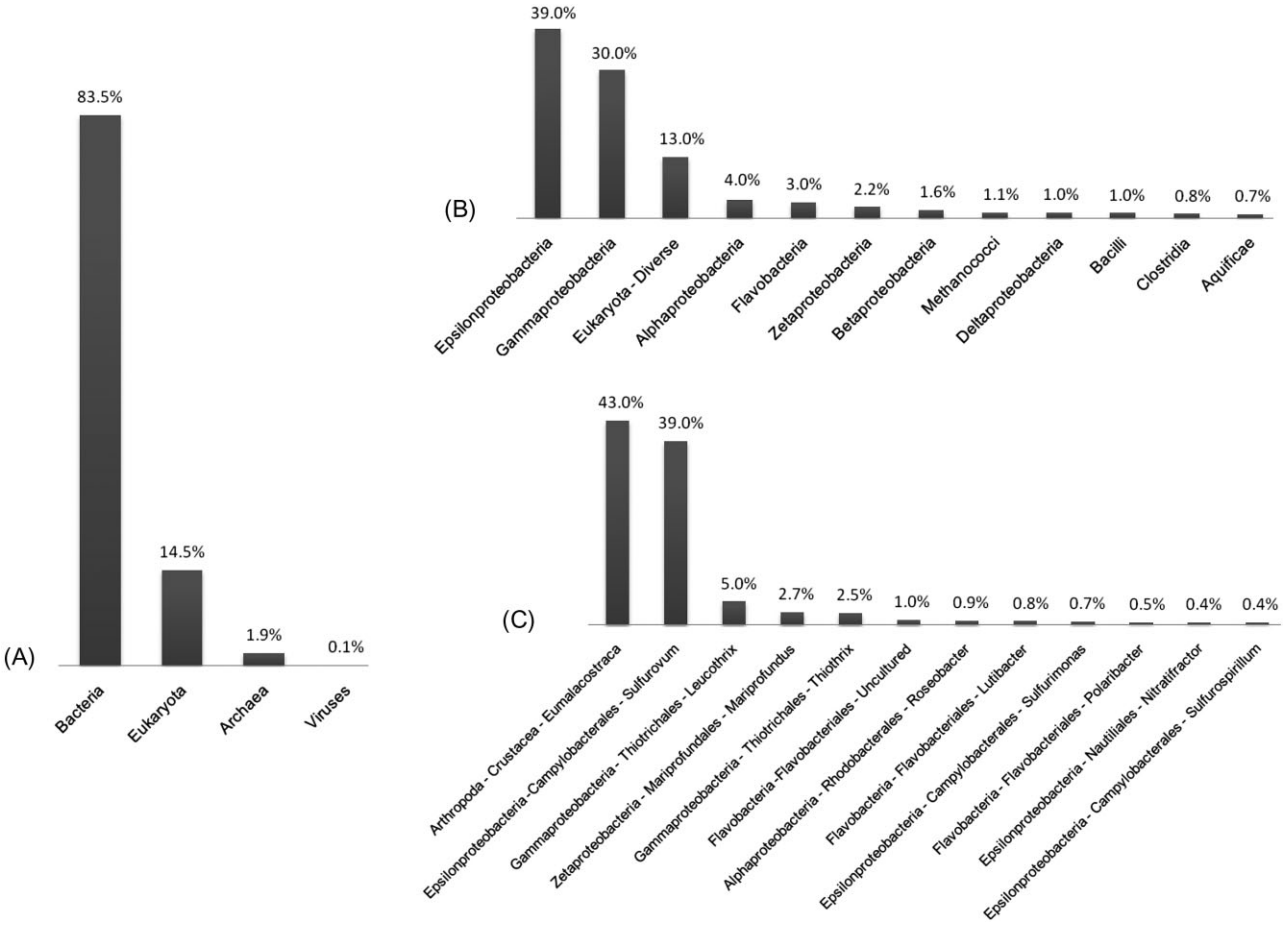

Fig. 1. Distribution of (A) superkingdom and (B) class affiliations of the assembled metagenomic sequences, and (C) distribution of domain, class and genus affiliations of metagenomic 16S and 18S rRNA reads.

(30%), *Alphaproteobacteria* (4%), *Zetaproteobacteria* (2.2%), *Betaproteobacteria* (1.6%) and *Deltaproteobacteria* (1.0%) (Fig. 1B). The metagenome harboured 1029 partial small subunit (SSU) rRNA gene sequences of sufficient quality for taxonomic assignment (Fig. 1C). These SSU sequences affiliated with *Eukarya*/*Eumalacostraca* (44%), *Epsilonproteobacteria*/*Sulfurovum* (38%), *Gammaproteobacteria*/*Leucothrix* (5%) and *Zetaproteobacteria*/*Mariprofundus* (3%). The difference in *R. exoculata* sequence abundance between the 14% taxonomically classified metagenome sequences and 44% taxonomically classified partial SSU sequence can likely be attributed to the high rRNA gene copy numbers of eukaryotic cells, which can number in thousands (Zhu *et al*., 2005), whereas 10 is considered a high number for prokaryotes.

Like other techniques, metagenomics does not necessarily reflect microbial diversity accurately, since biases affect DNA extraction and library preparation before sequencing. Such effects might explain the lack of 16S rRNA sequences affiliating with the methanotrophic gammaproteobacterial epibionts as described previously (Zbinden *et al*., 2008; Guri *et al*., 2012).

For functional analysis, we focused on taxonomically coherent bins of sequences (hereafter referred to as 'taxobins') affiliating with the dominating *Epsilonproteobacteria* and *Gammaproteobacteria*, and with the *Zetaproteobacteria* as potential sources of the iron oxyhydroxides. Previous studies indicated that the main epibiotic community is comprised of several closely related epsilonproteobacterial and gammaproteobacterial phylotypes (Zbinden *et al*., 2004; 2008; Petersen *et al*., 2010; Hügler *et al*., 2011; Guri *et al*., 2012). This was also evident in phylogenetic tree of the epsilonproteobacterial phylotypes that we reconstructed from metagenomic partial 16S rRNA sequences (Supporting Information Fig. S1), although this analysis might overestimate the strain diversity for a number of reasons. Firstly, many partial sequences were too short for it to be possible to confidently determine their phylogeny within the epsilonproteobacterial group. Secondly, many of the distinct phylotypes shown in Supporting Information Fig. S1 were only represented by single reads and may constitute sequencing errors. Finally, as bacterial genomes can harbour multiple copies of slightly different 16S rRNA genes, it is unclear how much of the detected 16S rRNA gene sequence microdiversity was due to inter-strain or intra-strain level variation. It was not possible to discriminate sequences from distinct phylotypes in the metagenome. Hence, the analysed taxobins must be considered as submetagenomes, and all inferred metabolic reconstructions may represent the combined metabolic potentials of a number of phylotypes, albeit of phylotypes that are so closely related that they are unlikely to show any major differences.

The epsilonproteobacterial taxobin consisted of 2103 contigs (2.89 Mbp) (Table 1). Members of the *Epsilonproteobacteria* often constitute the dominant bacterial group in hydrothermal habitats (Campbell *et al*., 2006; Dubilier *et al*., 2008). Several related epsilonproteobacterial isolates have recently been characterized, such as the deep-sea strains *Sulfurovum* sp. NBC37-1 and *Nitratiruptor* sp. SB155-2 (Nakagawa *et al*., 2007), and the coastal strain *Sulfurimonas denitrificans* DSM1251 (Sievert *et al*., 2008a).

The gammaproteobacterial taxobin consisted of 2441 contigs (3.43 Mbp) (Table 1). The filamentous chemolithoheterotrophic sulfur oxidizer *Leucothrix mucor* (Grabovich *et al*., 1999) represents the closest cultured gammaproteobacterial epibiont relative (90.6% 16S rRNA sequence identity). Taxonomic classification attributed 0.47 Mbp (2.2%) of the metagenome to the class *Zetaproteobacteria* and thus allowed identification of zetaproteobacterial genes (Supporting Information Table S2). The class *Zetaproteobacteria* so far contains just a single cultivated species, the neutrophilic iron

Table 1. General features of the epsilonproteobacterial, gammaproteobacterial and zetaproteobacterial sequences of the binned metagenomic dataset from the symbiotic community of the *R. exoculata* gill chamber. *The numbers in brackets correspond to the number of identified 16S rRNA fragments in the entire metagenome, including taxobins and unclassified reads.

	Gill chamber metagenome – *Epsilonproteobacteria*	Gill chamber metagenome – *Gammaproteobacteria*	Gill chamber metagenome – *Zetaproteobacteria*
Total contigs	2 106	2 441	558
Assembly size (bp)	2 887 614	3 427 427	468 699
Min size (bp)	100	100	109
Max size (bp)	31 453	14 111	6 412
GC content	34.3%	38.8%	44.5%
Coding density	87%	84%	88%
Number of tRNAs	71	32	0
Number of rRNAs*	7 (373)	1 (71)	0 (25)
Total ORFs	9 728	11 094	1 809
Hypothetical proteins	1 167	2 054	269
Conserved hypothetical proteins	855	689	106

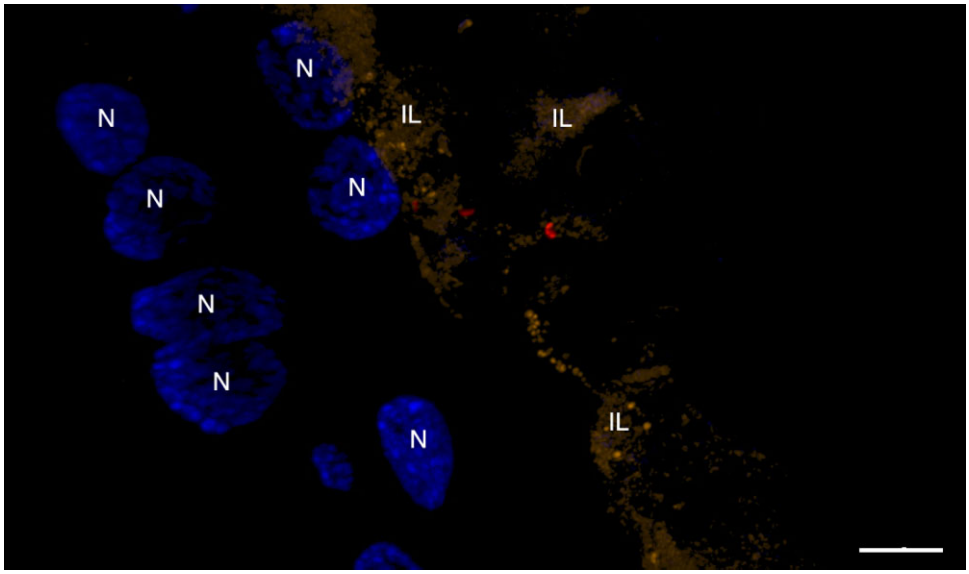

Fig. 2. FISH observation of Rainbow *R. exoculata* specimen gill branchiostegite epibionts. Transverse view of a branchiostegite from a Rainbow vent shrimp with epibionts. *Zetaproteobacteria* (red) were hybridized with the ZETA123 probe. All cells were labelled with DAPI (blue). N: eukaryotic nuclei. *Zetaproteobacteria* were located along the shrimp membrane. *Zetaproteobacteria* were located inside a thick layer of iron oxides (IL).

oxidizer *Mariprofundus ferrooxydans* (Emerson *et al*., 2007; Singer *et al*., 2011). Fluorescence in situ hybridization (FISH) experiments on cephalothorax sections of three shrimps revealed *Zetaproteobacteria* on each specimen (Fig. 2). The *Zetaproteobacteria* were low in abundance and located on the surface of the branchiostegite membrane (inner face of the gill chamber) closely associated with the iron oxyhydroxide layer that accumulates between the shrimp's molts (Corbari *et al*., 2008a,b). This distinct association of close relatives of cultivated neutrophilic iron oxidizers and iron oxyhydroxides deposits in the gill chamber supports the hypothesis that *R. exoculata* associates with iron-oxidizing epibionts (Zbinden *et al*., 2004; 2008; Corbari *et al*., 2008b; Guri *et al*., 2012). *Zetaproteobacteria* were found on all sampled *R. exoculata* individuals, indicating that *Zetaproteobacteria* might be regular members of the *R. exoculata* gill chamber community at the Rainbow hydrothermal vent site.

Metabolism

Carbon fixation. Based on functional gene surveys, it has been postulated that chemoautotrophy at deep-sea hydrothermal vents is mediated by at least two pathways, the rTCA and CBB cycles (Campbell and Cary, 2004; Hügler *et al*., 2011). Our results could corroborate these hypotheses. The epsilonproteobacterial taxobin harboured a complete set of genes for the rTCA cycle (Fig. 3 sequences: HG799104 to HG799148), which is widespread in microaerophilic bacteria. In contrast, the gammaproteobacterial epibiont taxobin harboured genes for the complete CBB cycle (Fig. 4 sequences: HG799149 to HG799155), which is quantitatively the most important mechanism of autotrophic CO_2 fixation on Earth. The coexistence of both CBB and rTCA cycles was reported for the endosymbiont of the deep-sea tube worm *Riftia pachyptila* (Markert *et al*., 2007), switching between these cycles according to environmental conditions. This endosymbiont preferentially uses the CBB cycle in high-energy high-sulfide conditions, and the rTCA in low-energy low-sulfide conditions (Markert *et al*., 2007). Furthermore, the reversibility of the rTCA cycle may provide the *Riftia* endosymbiont with additional metabolic flexibility. Such metabolic adaptation strategies provide a selective advantage under varying environmental conditions (Berg, 2011). In the case of the *Rimicaris* symbiosis, the same advantage may be achieved by coexistence of epibionts with distinct carbon fixation pathways.

The zetaproteobacterial taxobin also contained genes for the CBB cycle, including the *cbbM* gene encoding a form II RuBisCO (Supporting Information Table S2 sequences: HG799156 to HG799158, and HG799163). This indicates that the zetaproteobacterial epibionts have the potential for autotrophic growth. The RuBisCO gene that we found in the gammaproteobacterial taxobin also encodes form II RuBisCO. This form is adapted to low oxygen and medium to high carbon dioxide concentrations (Badger and Bek, 2008) – conditions that likely prevail at hydrothermal vent sites, and particularly in the gill chamber of *R. exoculata* (Zbinden *et al*., 2004). Both RuBisCO form II and the rTCA cycle are often found in

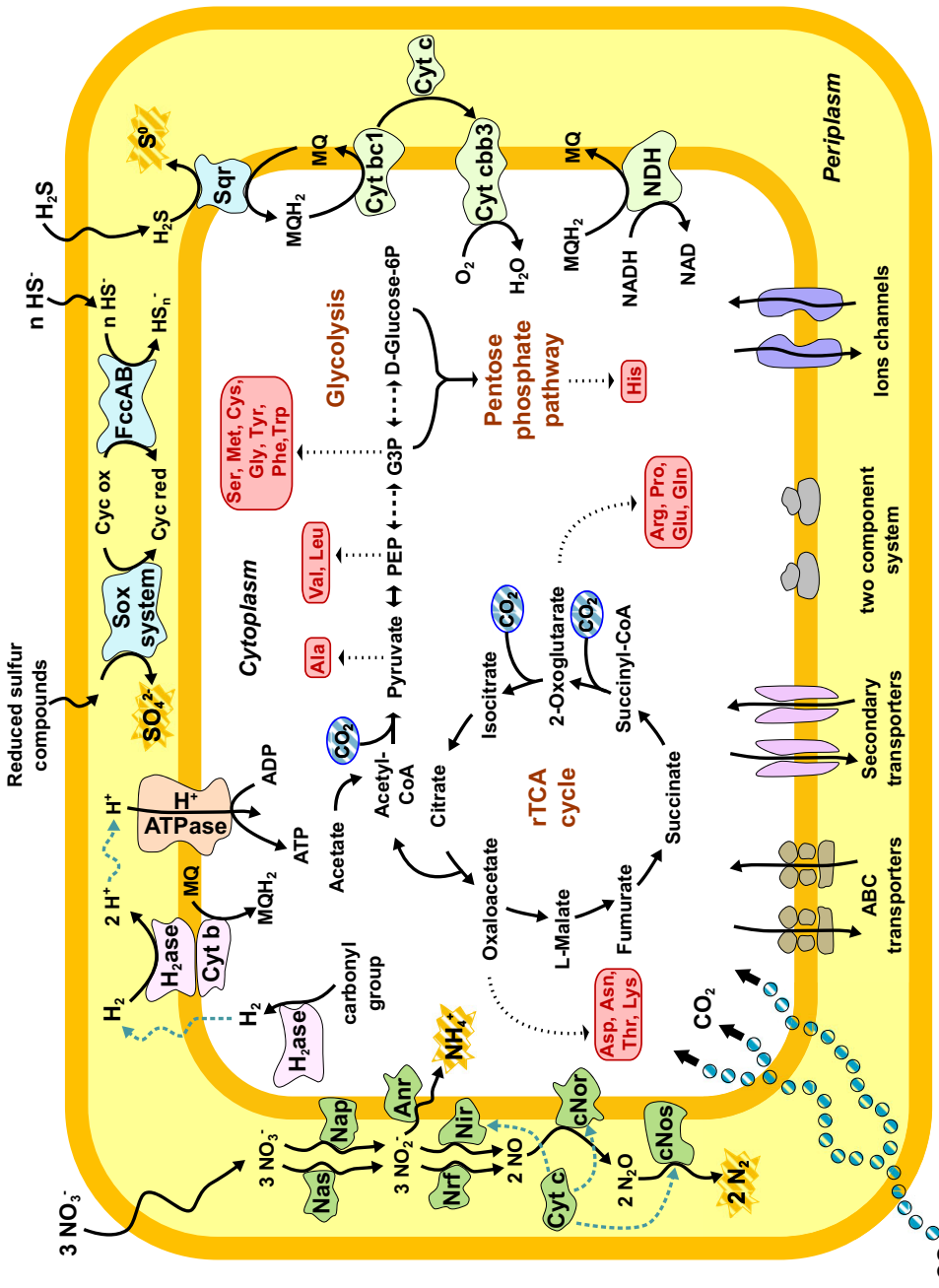

Fig. 3. Central metabolism and solute transport in the epsilonproteobacterial epibionts. Cyt: cytochrome; H_2ase: hydrogenase; Sqr: sulfide-quinone oxidoreductase; Nap: periplasmic nitrate reductase; Nas: cytoplasmic nitrate reductase; Anr: ammonia-forming siroheme nitrite reductase; Nir: cytochrome *cd*$_1$-dependent nitrite reductase; Nrf: cytochrome c nitrite reductase; cNor: nitric oxide reductase; cNos: nitrous oxide reductase. Amino acids are indicated in red on a pink background. Blue striped ovals correspond to diffusing CO_2 particles that are used for the rTCA cycle. Yellow striped stars correspond to final products likely excretion products. Dotted arrows correspond to indirect synthesis (for amino acids), enzymatic action (for cytochrome c) or diffusion (for H_2 and H^+).

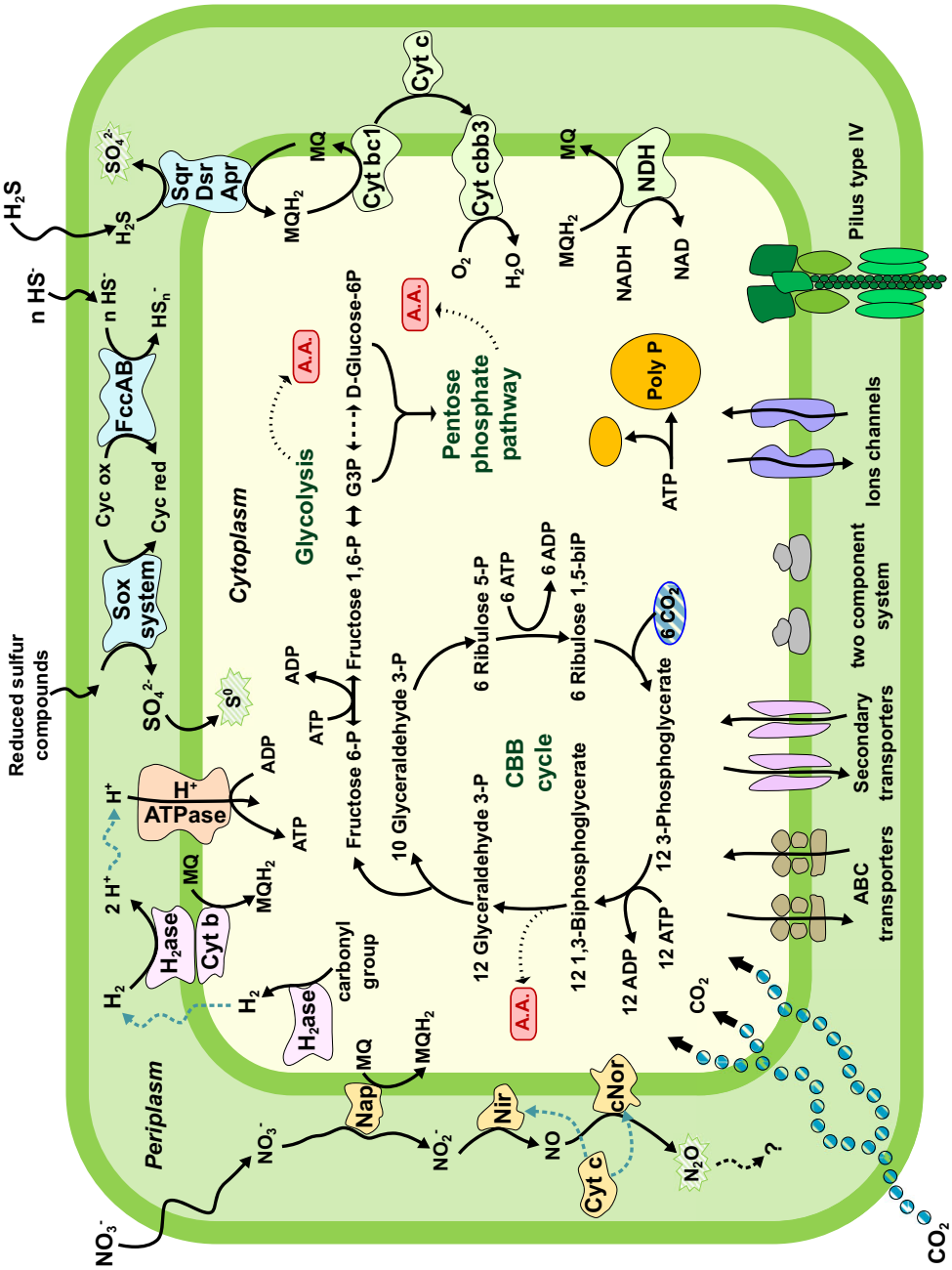

Fig. 4. Central metabolism and solute transport in the gammaproteobacterial epibionts. Cyt: cytochrome; H_2ase: hydrogenase; Sqr: sulfide-quinone oxidoreductase; Nap: periplasmic nitrate reductase; Nir: cytochrome *cd*-dependent nitrite reductase; cNor: nitric oxide reductase; PolyP: polyphosphates. Amino acids are shown in red on a pink background. Blue striped ovals correspond to diffusing CO_2 particles used for the rTCA cycle. Yellow striped stars correspond to final products likely excretion products. Dotted arrows correspond to indirect synthesis (for amino acids), enzymatic action (for cytochrome c) or diffusion (for H_2 and H^+).

autotrophic bacteria living under micro-oxic conditions (Campbell and Cary, 2004) (see Supporting Information Appendix S1).

Sulfur metabolism. Most free-living vent *Epsilonproteobacteria* are sulfur oxidizers (Inagaki *et al*., 2003; Nakagawa *et al*., 2005; Campbell *et al*., 2006; Takai *et al*., 2006; Sievert *et al*., 2008b). So far, the only evidence that the epsilonproteobacterial *R. exoculata* epibionts can oxidize sulfur has come from the amplification and sequencing of *soxB* genes from the epibiont community, which are similar to those of cultivated *Epsilonproteobacteria* (Hügler *et al*., 2011). We identified numerous *sox* genes in the epsilonproteobacterial taxobin (Fig. 3) in eight different contigs (Supporting Information Table S2 sequences: HG799164 to HG799177). Two clusters contained *soxYZA*, one cluster contained *soxYD* and another contained *soxAB. SoxD* and *soxX* also occurred as isolated genes. In facultative chemoautotrophs such as *Paracoccus pantotrophus* GB17 (Friedrich *et al*., 2001), the *sox* genes are organized in a single gene cluster. In contrast, in obligate chemoautotrophs closely related to the *R. exoculata* epsilonproteobacterial epibiont, such as *Sulfurovum* sp. NBC37-1 (Yamamoto *et al*., 2010) and *S. denitrificans* DSM1251 (Sievert *et al*., 2008a), *sox* genes are found in separate clusters spread throughout the genome. As in *S. denitrificans* DSM1251, four copies of the sulfide-quinone reductase (SQR) gene were found (sequences: HG799183 to HG799185), all of which closely affiliated with SQR-encoding genes from *Sulfurovum* sp. NBC37-1. SQR could catalyse the oxidation of sulfide to extracellular elemental sulfur and thereby contribute to the formation of filamentous sulfur (Nakagawa *et al*., 2007). It was hypothesized that sulfur-oxidizing bacteria may use SQR to switch from the complete oxidation of sulfide to sulfate to incomplete oxidation to sulfur, in order to prevent toxic acidification of their environment (Ferrer *et al*., 2011). Similarly, SQR not only could play a role in preventing harmful acidification inside the gill chamber, but also could constitute a way to store biochemical energy in the form of sulfur when sulfide concentrations are high.

Previous molecular studies on sulfur oxidation genes in the epibiotic community identified *aprA* genes that were related to *aprA* genes of *Gammaproteobacteria* (Zbinden *et al*., 2008; Hügler *et al*., 2011). In the gammaproteobacterial taxobin, in addition to the *aprA* gene, we found six *sox* genes in one cluster (*soxAZYX*) and we found other *sox* genes as single genes on separate contigs (*soxB*, *soxW*) (sequences: HG799187 to HG799193). A single SQR gene was found, but no *sox*CD (Fig. 4). It was unclear if these genes were missed due to the incompleteness of the metagenome or whether they are indeed absent from these gammaproteobacterial epibionts. Sulfur-oxidizing bacteria that lack *soxCD*, such as the anoxygenic phototrophic purple sulfur bacterium *Allochromatium vinosum*, produce elemental sulfur as an intermediate in sulfide oxidation (Hensen *et al*., 2006). S*oxCD* genes were required for complete oxidation to sulfate (Friedrich *et al*., 2001). The Sox system without SoxCD can oxidize sulfite and the sulfone group (SO_3^-) in thiosulfate, but cannot oxidize sulfide or elemental sulfur to thiosulfate (Friedrich *et al*., 2001). Thus, the gammaproteobacterial epibiont might oxidize sulfide to elemental sulfur for storage using flavocytochrome *c* sulfide dehydrogenase. Genes were found for both the SSU A (*fccA*) and the large subunit B (*fccB*) (sequences: HG799212 to HG799214). The best-studied enzyme system for the oxidation of stored elemental sulfur is the reversely operating dissimilatory sulfate reductase (*dsr*) of *A. vinosum* encoded by *dsrABEFHCMKLJOPNRS* genes (Grimm *et al*., 2010). We found *dsr* genes in the gammaproteobacterial taxobin in several clusters (*dsrAB*, *dsrFHCMK, dsrLJOP*) and as isolated genes (*dsrA*, *dsrE*, *dsrS*). Intracellular sulfur granules have been identified in the thin filamentous gammaproteobacterial epibionts (Zbinden *et al*., 2008; Petersen *et al*., 2010). *Dsr* might in this case be used to tap stores of intracellular elemental sulfur to compensate for the lower environmental sulfide concentrations that the epibionts encounter when their shrimp hosts swim outside of the hydrothermal stream. Deep-sea hydrothermal vent *Gammaproteobacteria* seem to possess two different sulfur-oxidization pathways: (i) the reverse sulfate reduction using Dsr and Apr and (ii) the Sox system without SoxCD (Yamamoto and Takai, 2011). In case of sulfur-oxidizing symbionts at hydrothermal vents, the supply of reduced sulfur compounds and oxygen for these pathways depends on the movements of the invertebrate host in the mixing zones of deep-sea hydrothermal vent fluids (Yamamoto and Takai, 2011).

In addition to the oxidation of reduced sulfur compounds, the epsilonproteobacterial epibionts, and possibly also the gammaproteobacterial epibionts, may have the potential to use oxidized sulfur compounds as electron acceptors. This hypothesis was supported by the presence of three genes that encode a polysulfide reductase NrfD-related membrane anchor (4Fe-4S ferredoxin iron-sulfur binding), Fe/S proteins (4Fe-4S ferredoxin) and molybdopterin oxidoreductase. These genes were arranged in one cluster in the epsilonproteobacterial taxobin and on several contigs in the gammaproteobacterial taxobin. These three proteins form a periplasmic-facing, membrane-bound complex presumably involved in the reduction of sulfur compounds including elemental sulfur, thiosulfate and polysulfide in *Sulfurovum* sp. NBC37-1. *Sulfurovum* sp.

NBC37-1 has been shown to grow using hydrogen as an electron donor and elemental sulfur as an electron acceptor (Yamamoto *et al*., 2010). The epsilonproteobacterial epibionts therefore might also be capable of anaerobic growth on these substrates, which are both present at the Rainbow site (Charlou *et al*., 2002; 2010).

Hydrogenases. Hydrogen use by chemosynthetic symbionts was recently discovered in the sulfur-oxidizing symbionts of hydrothermal *Bathymodiolus* mussels (Petersen *et al*., 2011). These authors also amplified the key gene for hydrogen oxidation, a [Ni-Fe] hydrogenase, from the epibionts of *R. exoculata*, as did Hügler and colleagues (2011). Because these genes were amplified from a mixed community, H_2-oxidizing potential could not be assigned to a particular epibiont, but phylogenetic analysis suggested that the hydrogenases originated from *Epsilonproteobacteria* and *Deltaproteobacteria* (Hügler *et al*., 2011). Hydrogenases catalyse the reversible oxidation of hydrogen, and can be classified into four groups depending on their physiological function. We found hydrogenases belonging to H_2-uptake and H_2-sensing hydrogenases, in the epsilonproteobacterial taxobin (sequences: HG799450, HG799456, HG799457). Surprisingly, we also found hydrogenases in the gammaproteobacterial taxobin (sequences: HG799425 to HG799433, HG799436, HG799437, HG799441 and HG799444), suggesting that both dominant epibionts have the potential to use hydrogen as an energy source. In addition, H_2-evolving hydrogenases were found in the epsilonproteobacterial taxobin (sequence HG799459). We found 17 hits for *Epsilonproteobacteria* (sequences: HG799449, HG799451 to HG799455, HG799458, HG799460) and 14 hits for *Gammaproteobacteria* (sequences: HG799422 to HG799424, HG799434, HG799435, HG799438, HG799440, HG799442, HG799443, HG799445 to HG799448) to hydrogenase expression, formation and maturation proteins, which are required for functional hydrogenase expression (Vignais, 2008). Two distinct genes encoding H_2-uptake hydrogenases were identified in each epibiont taxobin, indicating the potential importance of H_2 as an electron donor for the symbiosis (Vignais and Colbeau, 2004). This is consistent with the high concentrations of hydrogen measured in Rainbow hydrothermal fluids (Charlou *et al*., 2002). H_2-sensing hydrogenase genes were found in both epibionts. These cytoplasmic enzymes regulate respiratory [Ni-Fe] hydrogenase expression depending on the environmental H_2 concentration (Vignais and Colbeau, 2004). For *Epsilonproteobacteria*, it has also been hypothesized that cytoplasmic hydrogenases could supply the rTCA cycle with low-potential electrons, increasing growth efficiency (Sievert *et al*., 2008a). Three sequences for H_2-evolving hydrogenases were identified in the epsilonproteobacterial taxobin. Genes for hydrogenases belonging to multiple hydrogenase functional groups have been reported in the genomes of other deep-sea hydrothermal vent *Epsilonproteobacteria*. For example, *Sulfurovum* sp. NBC37-1 has four different hydrogenases (two H_2-uptake type, one H_2-sensing type and one H_2-evolving type) and *Nitratiruptor* sp. SB155-2 has three hydrogenases (one of each of these types) (Nakagawa *et al*., 2007). The capacity to use hydrogen is known in many free-living deep-sea *Epsilonproteobacteria* such as *Hydrogenimonas thermophila* (Takai *et al*., 2004), *Sulfurovum lithotrophicum* (Inagaki *et al*., 2004) or *Sulfurimonas paralvinellae* (Takai *et al*., 2005; 2006). This knowledge, together with our data and other recent findings (Petersen *et al*., 2011), strongly suggest that hydrogen is commonly used as energy source in free-living and host-associated microorganisms in hydrothermal ecosystems.

Nitrogen assimilation and nitrate reduction. Two pathways for nitrate reduction were identified in the epsilonproteobacterial taxobin: denitrification with two types of nitrate reductase (Nap and Nas) and dissimilatory nitrate reduction to ammonium via the Nrf system (Fig. 3, sequences: HG799223 to HG799252). The presence of both cytoplasmic (*nas*) and periplasmic (*nap*) nitrate reductase genes indicate the putative ability to adjust the mode of nitrate reduction to fluctuating nitrate concentrations. The presence of periplasmic dissimilatory nitrate reductase (*nap*), cytoplasmic assimilatory nitrate reductase (*nas*) and ammonia-forming nitrite reductase (*nrf*) genes indicate that the epsilonproteobacterial epibionts have the potential to produce ammonium. Consequently, they would not depend on environmental ammonium uptake, unlike the free-living *S. denitrificans*, which lacks *nrf* genes (Sievert *et al*., 2008a). It is intriguing that the host-associated *Epsilonproteobacteria*, including the *R. exoculata* epibiont and bacteria of the genera *Campylobacter* and *Helicobacter*, have the potential to produce ammonium for assimilation, whereas free-living *Epsilonproteobacteria*, such as *S. denitrificans*, rely on ammonium uptake from the environment. Most aquatic animals excrete ammonium, which should be available to their associated microorganisms (Wright, 1995). Accordingly, ammonium oxidation was recently detected in biofilms of marine invertebrates (Heisterkamp *et al*., 2012). For the *R. exoculata* epsilonproteobacterial epibionts, the ability to produce ammonium as a nitrogen source might provide a competitive advantage during its free-living stage. Furthermore, in the gill chamber, this would prevent competition with co-occurring epibionts for ammonium. In the gammaproteobacterial taxobin, one Nap/Nir denitrification system was found, which would

enable dissimilatory nitrate reduction to N_2O (Fig. 4, sequences: HG799257 to HG799272).

A more detailed description of nitrogen assimilation and nitrate reduction pathways in the epsilonproteobacterial taxobin is presented in the Supporting Information Appendix S1.

Oxygen respiration. The epsilonproteobacterial taxobin contained genes for a proton pumping cbb3-type cytochrome c oxidase (*cooN, cooO, cooP*) (sequences: HG799280 to HG799292), whereas genes for the subunit CooQ and the CooGHIS complex were not retrieved. Even though the absence of certain genes from a metagenome might simply be due to the fact that they were not sequenced, it is noteworthy that the *cooGHIS* genes are also lacking in the complete genome of the closest cultured relative of the *R. exoculata* epsilonproteobacterial epibionts, the marine epsilonproteobacterium *S. denitrificans* DSM1251. The CooGHIS complex is involved in assembly and maturation of the cbb3-type cytochrome c oxidase (Pitcher and Watmough, 2004), and the consequences of its absence in *S. denitrificans* DSM1251 are unclear (Sievert *et al*., 2008a). Cbb3-type cytochrome c oxidase genes were also identified in the gammaproteobacterial taxobin (*cooP, cooN* and *cooG*) (sequences: HG799309 to HG799317). In *Proteobacteria*, cbb3-type cytochrome c oxidases are involved in micro-aerobic respiration (Kulajta *et al*., 2006). The presence of these genes, especially *cooG*, indicates the ability to use oxygen under micro-oxic conditions, which is consistent with modelling studies that predict micro-oxic conditions in the gill chamber (Schmidt *et al*., 2009). This is also supported by measurements from within shrimp aggregates, where levels of oxygen measured were half of those in the surrounding seawater (Zbinden *et al*., 2004; Schmidt *et al*., 2009). Moreover, the epibionts are located in the upper part of the gill chamber where the water flow is outgoing and thus, depleted in oxygen and enriched in carbon oxide due to shrimp respiration (Zbinden *et al*., 2004). The *Sulfurimonas gotlandica* GD-1 genome (Grote *et al*., 2012) also features cbb3-type cytochrome c oxidase genes, but growth experiments showed that this strain could not grow with oxygen as the sole electron acceptor. It has been suggested that the cbb3-type cytochrome c oxidase genes might be used for protection from oxidative stress rather than for respiration (Sievert *et al*., 2008a; Grote *et al*., 2012).

Other genes encoding oxygen-reducing terminal oxidases such as cytochrome d ubiquinol oxidases were retrieved in the epsilonproteobacterial (two hits) and gammaproteobacterial (four hits) taxobins. These cytochrome d ubiquinol oxidases are typically found in organisms living in low-oxygen environment which is probably the case for the epibionts.

Insights into the zetaproteobacterial epibiont metabolism. All the characterized *Zetaproteobacteria* are microaerophilic iron oxidizers. In the zetaproteobacterial taxobin, a molybdopterin oxidoreductase Fe_4S_4 region and ferredoxin encoding genes (five hits) were identified (sequences: HG799404 to HG799409). Homologues of these genes are expressed during iron oxidation by *A. ferrooxidans* (Yarzábal *et al*., 2002). One of the ferredoxin genes was located in a cluster with a polysulfide reductase, as identified in the iron oxidizers *M. ferrooxydans*, *Sideroxydans lithotrophicus* and *Gallionella capsiferriformans* (Singer *et al*., 2011). It is therefore likely that the *Zetaproteobacteria* in the *R. exoculata* gill chamber are also iron oxidizers.

The zetaproteobacterial taxobin also contained a number of genes for the biosynthesis of energy storage compounds, including glycogen and polyphosphate (polyP) (nine hits with five polyP kinase sequences: HG799414 to HG799417). Such genes were also found in the gammaproteobacterial taxobin: polyP kinase (two hits, sequences HG799419 and HG799420), dinucleoside polyP hydrolase (one hit, sequence HG799418) and exopolyphosphatase (one hit, sequence HG799421) (Fig. 4). Thus, both the zetaproteobacterial and gammaproteobacterial epibionts might produce polyphosphate. Previous TEM (Transmission Electronic Microscopy) observations and X-ray analyses corroborate these results by showing phosphate-rich granules in filamentous *R. exoculata* epibionts and in *M. ferrooxydans* cells (Zbinden *et al*., 2008; Singer *et al*., 2011). The polyphosphate granules serve as phosphorus and energy storage and may also have a detoxifying function by chelation of harmful heavy metal ions (Kornberg, 1995).

Symbiont–host interactions

The provision of organic compounds for nutrition is one of the potential benefits that animal host's derive from symbiotic associations with chemoautotrophic bacteria. The symbionts in such associations typically oxidize reduced sulfur compounds, methane or hydrogen with inorganic electron acceptors and couple this process to the synthesis of organic molecules from single-carbon compounds such as carbon dioxide or methane (Cavanaugh *et al*., 2006; Dubilier *et al*., 2008). Additional benefits for the host include detoxification of potent metabolic inhibitors such as iron and sulfide, to less toxic compounds such as elemental sulfur via oxidation (Fig. 5).

Role of the epibionts. Recently, it was demonstrated that *R. exoculata* shrimp can take up dissolved organic

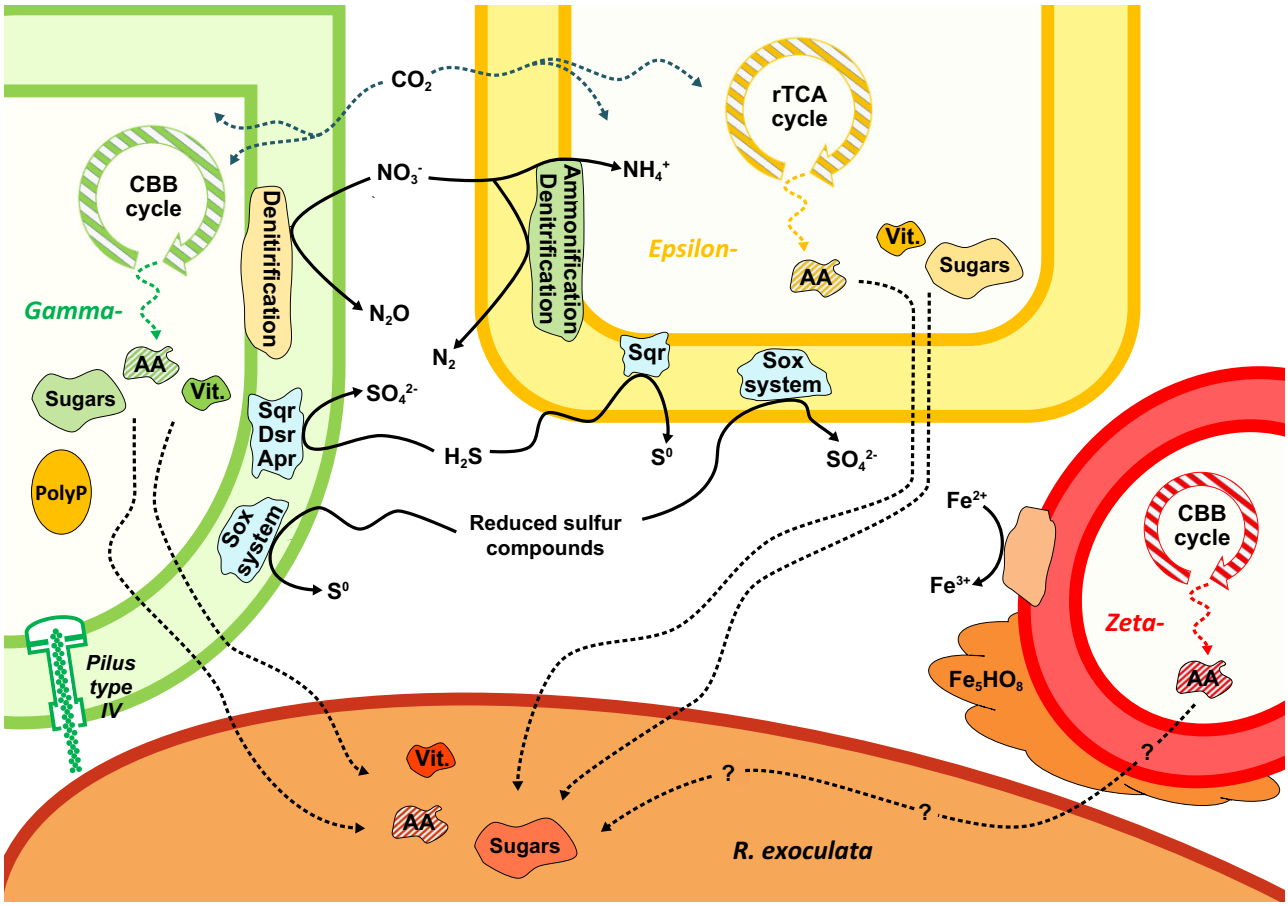

Fig. 5. Scheme of the proposed functioning of the *R. exoculata* gill chamber symbiosis. AA: amino acids; Sqr: sulfide-quinone oxidoreductase; Vit.: vitamins; PolyP: polyphosphates. Dotted arrows indicate indirect synthesis (for amino acids), diffusion (for CO_2) or transfer (for amino acids, sugars and vitamins).

molecules from their gill epibionts, proving that the shrimp gain a nutritional benefit from their epibionts (Ponsard *et al.*, 2013). Amino acids, sugars and a variety of vitamins (such as vitamin B6 or pyridoxal 5'-phosphate) synthesized by the gammaproteobacterial and epsilonproteobacterial epibionts could be suitable compounds for transfer to the *R. exoculata* host (Fig. 5). Amino acids are likely candidates, since lysine uptake has been demonstrated (Ponsard *et al.*, 2013), and various amino acid exporters were identified in the epibiont taxobins (Supporting Information Table S2).

An additional likely benefit for the *R. exoculata* shrimp is the contribution of its epibionts to the detoxification of abundant harmful compounds at Rainbow, such as heavy metals, sulfide, nitrite and Fe(II) (Charlou *et al.*, 2002; 2010). The epsilonproteobacterial and gammaproteobacterial epibionts can convert H_2S to elemental sulfur, the epsilonproteobacterial epibionts can convert nitrite to dinitrogen, and the zetaproteobacterial epibionts can convert Fe(II) to insoluble iron oxides (Fig. 3). These processes are not only the key energy-generating pathways of the epibionts, but also have the beneficial side-effects that their products are less toxic for the shrimp than their reactants. For example, H_2S and sulfide have toxic effects on the aerobic respiration of hydrothermal fauna (Powell and Somero, 1986). Moreover, *Gammaproteobacteria* and *Zetaproteobacteria* have the potential to form polyphosphate granules, which – as discussed above – may chelate toxic heavy metal ions.

Responses to oxidative and thermal stress. Rimicaris exoculata live in steep gradients between oxygenated seawater and anoxic hydrothermal fluids. Consequently, epibionts of the *R. exoculata* gill chamber microbes are continuously subjected to oxidative stress and fluctuating physicochemical conditions as the shrimp move in and out of the vent fluid-seawater mixing zone. Genes related to oxidative stress responses were also found here in epsilonproteobacterial taxobin (see Supporting Information Appendix S1 for details). Hydrothermal fluids can reach up to 350°C and the temperatures in the mixing zones can reach up to 35°C (Ravaux *et al.*, 2003). Heat-

Table 2. Comparison of genes from the epsilonproteobacterial taxobin with two related reference genomes.

Response to environment		Gill chamber *Epsilonproteobacteria* 2.9 Mbp	*Sulfurovum* sp. NBC37-1 2.6 Mbp	*Nitratiruptor* sp. SB155-2 1.9 Mbp
Sensory systems	Sensor histidine protein kinases (HPK) genes	26	16	26
	Transcriptional factor genes	58	34	26
	Chemotaxis proteins	2	0	12
	Methyl-accepting chemotaxis genes	5	0	6
	Proteins with PAS/GGDEF domains genes	17	23	36
Heavy metal transport		20 (Ni, Fe, Zn, Cd, Co, Mg, pb, Mo)	22 (Ni, Fe, Zn, Mn, Co, Cd, Cu, As, Mo, Hg, Ag)	17 (Ni, Fe, Zn, Mn, Co, Cd, Cu, As, Mo, Hg, Ag)
Oxidative stress	Catalase	–	+	–
	Superoxide dismutase	+	–	–
	Cytochrome c peroxidase	+	+	+
	Thiol peroxidase	+	+	+
Virulence factors	Virulence factor MviN	+	+	+
	Hemolysin	+	+	+
	Fibronectin- and fibrinogen-binding protein	+	+	+
	N-linked glycosylation pathway	+	+	+
	Invasion antigen CiaB	+	+	+
	Lytic murein transglycosylase	+	+	+

inducible stress proteins (HSP70 family) were identified in *R. exoculata* (Ravaux *et al*., 2003; 2007). Genes encoding heat shock proteins were also identified in the metagenome for epsilonproteobacterial, gammaproteobacterial and zetaproteobacterial epibionts (Supporting Information Table S2, sequences: HG799339 to HG799381, and HG799461), indicating their ability to cope with the fluctuating conditions of the hydrothermal habitat.

Virulence gene homologues. Many *Epsilonproteobacteria*, such as *Campylobacter jejuni* or *Helicobacter pylori*, are well-known pathogens with characteristic virulence genes. Even non-pathogenic species, such as the deep-sea vent *Epsilonproteobacteria Sulfurovum* sp. and *Nitratiruptor* sp., share many virulence genes with pathogenic species (Nakagawa *et al*., 2007). Homologues of these virulence factors were found in the *R. exoculata* epibiont metagenome (Table 2, sequences: HG799382 to HG799402). N-linked glycosylation (NLG) long believed to occur only in eukaryotes was then discovered in the epsilonproteobacterium *Campylobacter jejuni* (Szymanski *et al*., 1999) and now is recognized as a feature that occurs in members of all domains of life (Dell *et al*., 2010). In eukaryotes, glycosylated proteins are involved in cell–cell and cell–matrix recognition. These proteins can also play a role in host–bacteria interactions, for example allowing *C. jejuni* to evade the human immune system when causing enteric infections (Szymanski and Wren, 2005). The NLG in deep-sea *Epsilonproteobacteria* might have emerged during the evolutionary transition from free-living to symbiotic association with hydrothermal vent invertebrates (Nakagawa *et al*., 2007). It is possible that the NLG in these epsilonproteobacterial epibionts is involved in selective recognition between the epibionts and the shrimp. No such virulence genes were detected in the gammaproteobacterial taxobin.

Surface attachment. The specific colonization of animals by bacterial symbionts is mediated by recognition at the molecular level. Different mechanisms are well understood for some of the model associations, such as the squid *Euprymna scolopes* and its bioluminescent symbiont *Vibrio fischeri* (Troll *et al*., 2009), but are still unknown for the majority of marine symbioses. The *R. exoculata* epibiont metagenome contained several genes coding for well-known cell surface structures and extracellular attachment components, including type IV pili, mannose-sensitive haemagglutinin pili and O-antigens (capsular polysaccharides), which were found here in the epsilonproteobacterial or gammaproteobacterial taxobins (Supporting Information Table S2). These taxobins also contained genes that may be involved in extracellular biofilm matrix formation, such as type II and III secretion systems, MscS family mechanosensitive channels, von Willebrand factor type A domain proteins and genes for exopolysaccharide biosynthesis (one of which had a best BLASTp hit to the epibiont of the deep-sea worm *Alvinella pompejana*) (Danese *et al*., 2000; Whittaker and Hynes, 2002; Durand *et al*., 2003; Thomas *et al*., 2008). These data indicate that the epibionts might produce various extracellular structures as well as matrix polysaccharides

allowing surface adhesion. The latter has been observed using TEM (Corbari *et al*., 2008b; Zbinden *et al*., 2008).

Conclusions

Two distinct sulfur oxidizers dominate the *R. exoculata* gill chamber epibiosis. On all the microscopic observations, the *Epsilonproteobacteria* and *Gammaproteobacteria* occupy the same zones and are present on the whole surface of the branchiostegite. These co-occurring epibionts carry out similar processes such as sulfur and hydrogen oxidation and carbon dioxide fixation. This seeming functional redundancy raises the question of why the epibiont with the greater fitness does not prevail in a Darwinian extinction by the process of competition. We propose that even subtle differences between co-occurring epibionts, such as the use of different CO_2 fixation pathways or the possible independence of the *Epsilonproteobacteria* from ammonia, provide sufficient niche differentiation between the epibionts, which prevent direct competition and allow their stable co-occurrence. Similar associations with multiple distinct sulfur-oxidizing symbionts have also been described for bivalves (Duperron *et al*., 2007; 2008; Raggi *et al*., 2013).

Furthermore, Prosser (2012) proposed that even if two strains appear to have almost identical genetic characteristics, they will probably respond differently to environmental changes. Here, environmental drivers of niche differentiation might include oxygen or sulfide availability, as has been demonstrated for closely associated sulfur-oxidizing *Gammaproteobacteria* and *Epsilonproteobacteria* in a terrestrial cave (Macalady *et al*., 2008).

Thus, the metabolic plasticity of the epibiotic community associated with *R. exoculata* probably confers an adaptive advantage for the shrimp in the highly dynamic hydrothermal mixing zone they colonize, and could help to explain their large success in colonizing many geochemically and physically contrasting hydrothermal vents along the MAR.

Experimental procedures

Shrimp collection and DNA extraction

Rimicaris exoculata specimens were sampled during the 2007 MoMARDREAM-Naut oceanographic cruise at the Rainbow hydrothermal site (36°14'N – 33°54'W, 2320 m depth) on the MAR. Shrimps were collected using the Ifremer research vessel *Pourquoi Pas?* and the submersible *Nautile*. Once aboard, some *R. exoculata* specimens were immediately frozen at −80°C. In the laboratory, three of these specimens were dissected to separate the mouthparts and the inner face of the gill chamber (branchiostegite). These parts were immediately used to extract DNA using a phenol, chloroform and isoamyl alcohol extraction (Zbinden and Cambon-Bonavita, 2003). The quality and size of the extracted genomic DNA was assessed by 0.6% agarose gel electrophoresis. Aliquots of extracted genomic DNA, fosmid control DNA (40 kb size, Epicentre Biotechnologies, Madison, WI, USA), and BAC-Tracker Supercoiled DNA Ladder (Epicentre Biotechnologies) were loaded in parallel for size estimation.

DNA sequencing and assembly

DNA sequencing was performed with a Roche 454 GS FLX Ti sequencer (454 Life Sciences, Branford, CT, USA) at the Centre for Genomic Research of the University of Liverpool with 1.5 picotiter plates (1 011 151 reads amounting to 318 Mbp). Assembly was carried out with a Roche 454 Newbler v.2.3 assembler using default parameters, resulting in 24 529 contigs (831 contigs ≥ 2.5 kbp) of 15.3 Mbp in total (11.1 Mbp ≥ 500 bp). For further details, see Supporting Information Table S1.

Taxonomic classification and annotation

A consensus from five individual taxonomic prediction tools was used to infer the taxonomic affiliation of the metagenome sequences as previously described (Ferrer *et al*., 2012), with modifications as follows. Competitive read recruitment was run with 339 genomes of marine microbes plus three bacterial genomes that, based on 16S rRNA analyses, are currently closest to the main epibionts and zetaproteobacterial epibiont: the *gammaproteobacterium L. mucor* (unpublished), the *epsilonproteobacterium Sulfurovum* sp. NBC37-1 (NCBI RefSeq NC_009663.1) and the *zetaproteobacterium M. ferrooxydans* PV-1 (NCBI RefSeq NZ_AATS0100 000000.1). A rank-based approach was used to consolidate taxonomic predictions of the five tools into a consensus using a weighted assessment on all 27 existing ranks of the NCBI taxonomy from superkingdom to species. As a result, a substantial fraction of the sequences, from the *R. exoculata* gill microbiota metagenome could be classified [superkingdom: 9.9 Mbp (64%); phylum: 9.6 Mbp (63%); class: 9.0 Mbp (59%); genus: 6.6 Mbp (44%)].

Annotation was done using a modified GenDB v2.2.1 annotation system (Meyer *et al*., 2003) as described previously (Ferrer *et al*., 2012). The JCoast software (Richter *et al*., 2008) was used for manual annotation and data mining. Annotations were compared with annotations obtained by RAST (Aziz *et al*., 2008) for epsilonproteobacterial, gammaproteobacterial and zetaproteobacterial taxonomic sequence bins (taxobins) individually. The sequence data reported in this study are available at the European Bioinformatics Institute (study number ERP001477; http://www.ebi.ac.uk/ena/data/view/ERP001477).

FISH

FISH was performed on 0.6 μm transverse sections of *R. exoculata* branchiostegite (Guri *et al*., 2012) as previously described (Durand *et al*., 2010), except for two main modifications: the hybridization and washing temperatures were 35°C and 37°C, respectively, and the reaction mix contained 55% formamide hybridization buffer. Branchiostegite sections

were hybridized using probes Eub338 (Amann *et al*., 1990) and ZETA123 (Kato *et al*., 2009). Observations and imaging were performed using an Apotome Axio Imager Z2 with a COLIBRI system (Carl Zeiss Microimaging GmbH, Göttingen, Germany).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Description of metabolic pathways – carbon assimilation and nitrogen assimilation – metabolic response to environment – monitoring proteins and oxidative stress for epsilonproteobacterial epibionts, cell–cell communication, CRISPRs and transposases.

Fig. S1. Phylogenetic tree showing the affiliation of partial 16S rRNA sequences from the *R. exoculata* gill chamber metagenome that fell into the epsilonproteobacterial epibiont group. The basis tree (only full-length 16S rRNA sequences, > 1300 nt) was calculated with PhyML implemented in ARB, using a positional variability filter for Bacteria. After trimming the first 10 bases and quality filtering to Q28, the 16S rRNA reads were very short (most shorter than 250 bp). We therefore only included sequences that aligned to highly variable regions in the 16S rRNA (these were identified in the alignment by eye). These sequences, together with the 16S rRNA gene fragment sequences from the Epsilon taxobin were added to the basis tree using the ARB

'quick add' tool. Bootstrap values are from 100 replicates. The outgroup (not shown) was *Flavobacterium psychrolimnae* (AB455260, AJ585428). Sequences in purple are 16S tags from the metagenome, sequences in green are from the epsilonproteobacterial taxobin, and sequences in red are isolates. It was not possible to do this analysis with the gammaproteobacterial 16S reads, as previously identified strains of gammaproteobacterial epibionts are more closely related that those from the Epsilonproteobacteria, and such short reads (< 250 bp) did not provide enough resolution for strain analysis in this group.

Table S1. General features of the metagenomic dataset from the symbiotic community of the gill chamber of *R. exoculata*.

Table S2. Annotation of genes relevant to the principal metabolic pathways and specific genes in epsilonproteobacterial, gammaproteobacterial and zetaproteobacterial epibionts.