Diversity and Ecology of Chemosynthetic Symbioses in Deep-Sea Invertebrates

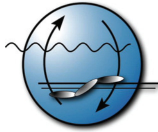
Dissertation
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
- Dr. rer. nat. -

dem Fachbereich Biologie/Chemie der
Universität Bremen
vorgelegt von

Jillian M. Petersen

Bremen
July 2009

Die vorliegende Arbeit wurde in der Zeit von April 2006 bis Juli 2009 in der Symbiose-Gruppe am Max-Planck-Institut für marine Mikrobiologie in Bremen angefertigt.



1. Gutachterin: Dr. Nicole Dubilier
2. Gutachterin: Prof. Dr. Barbara Reinhold

Tag des Promotionskolloquiums: 15. September 2009

Auf dem Deckblatt: *Rimicaris exoculata* Garnelen und *Bathymodiolus puteoserpentis* Muscheln auf einem schwarzen Raucher, Logatchev Hydrothermalfeld, Mittel-Atlantischer Rücken. Copyright Woods Hole Oceanographic Institution.

‘Finish each day and be done with it. You have done what you could. Some blunders and absurdities no doubt crept in, forget them as soon as you can. Tomorrow is a new day, you shall begin it well and serenely...’

- *Ralph Waldo Emerson*

Summary

The energy sources that drive biological processes at deep-sea hydrothermal vents and cold seeps include methane and reduced inorganic compounds such as sulfide and hydrogen. These compounds are unavailable to metazoan life, but can be used by chemoautotrophic or methanotrophic bacteria to fuel chemosynthetic primary production. In these habitats, symbioses between invertebrates and chemoautotrophic or methanotrophic bacteria form the basis of ecosystems that thrive in the absence of sunlight.

This thesis is made up of two thematic parts. In the first part, two reviews on methane-oxidizing symbionts are presented. Methanotrophic symbiotic bacteria have so far been found in invertebrate animals at hydrothermal vents and cold seeps in the deep sea, and in a plant host in a terrestrial wetland. No methane-oxidizing symbiont has yet been cultured. Therefore, the evidence for methanotrophy in these bacteria has come from ultrastructural, enzymatic, physiological, stable isotope, and molecular biological studies of the symbiotic host tissues. Despite being found in a wide range of hosts, all marine methanotrophic symbionts for which 16S rRNA sequences are available belong to a single lineage within the Gammaproteobacteria. Methane-oxidizing symbionts in the terrestrial habitat belong to the Alphaproteobacteria. However, many of these symbionts have not yet been characterized with molecular methods, and may be more diverse than currently recognized. These reviews summarize the current knowledge of methane-based symbioses, and identify topics for future research.

In the second part of this thesis, I present two studies on the ectosymbiosis of *Rimicaris exoculata*, an alvinocaridid shrimp from hydrothermal vents on the Mid-Atlantic Ridge (MAR). These shrimp have filamentous ectosymbionts on modified appendages and the inner surfaces of the gill chamber. The symbionts were previously assumed to be epsilonproteobacterial sulfur oxidizers. Using the full-cycle 16S rRNA approach, I identified a second filamentous ectosymbiont that belongs to a novel symbiotic lineage within the Gammaproteobacteria. To investigate the biogeography of the symbiosis, I compared the 16S rRNA gene sequences of ectosymbionts from four MAR vent fields, Rainbow, TAG, Logatchev, and South MAR, which are separated by up to 8500 km along the MAR. Differences in the 16S rRNA gene for both the gammaproteobacterial and epsilonproteobacterial symbionts were correlated with geographic distance. In contrast, the phylogeny of the free-living relatives of the epsilonproteobacterial symbiont showed no obvious geographic trend. Host-symbiont recognition could explain the observed symbiont distribution.

The metabolism of the symbionts from the Rainbow vent field was investigated by thermodynamic modelling, sequencing of key metabolic genes, and immunohistochemical labelling of proteins in single epibiont cells. Key genes for carbon fixation by the reductive TCA and CBB cycles indicate that both symbionts can fix CO_2. Key genes for hydrogen, methane, and sulfur oxidation indicate that the epibionts have the potential to use these three energy sources. In addition, immunohistochemistry showed that particulate methane monooxygenase is expressed by the gammaproteobacterial symbiont, although it does not belong to any of the currently known methanotrophic lineages. These studies show that the phylogenetic and functional diversity of the shrimp epibiosis is greater than previously recognized.

Zusammenfassung

An Hydrothermalquellen und Cold-Seeps ermöglichen Methan und reduzierte anorganische Verbindungen wie Sulfid und Schwefelwasserstoff biologische Prozesse. Diese Verbindungen sind für höhere Organismen nicht verfügbar, koennen aber von chemoautotrophen und methanotrophen Mikroorganismen für chemosynthetische Primärproduktion genutzt werden. In diesen Habitaten sind Symbiosen zwischen Invertebraten und chemoautotrophen und methanotrophen Bakterien die Grundlage für Licht-unabhängiges Leben.

Diese Dissertation besteht aus zwei Teilen. Im ersten Teil werden zwei Review-Artikel ueber Methanotrophe Symbionten vorgestellt. Methanotrophe Symbionten wurden bislang in Invertebraten an Hydrothermalquellen und Cold-Seeps in der Tiefsee und in einer Moospflanze eines terrestrischen Habitats gefunden. Bislang wurde kein Methan-oxidierender Symbiont im Labor kultiviert. Beweise fuer die Methanotophie diesen Bakterien stammen daher von Untersuchungen zu Ultrastruktur, Enzymatik, Physiologie, Isotopie, und Molekular-Biologie. Trotz ihrer weiten Verbreitung in vielen Wirtsorganismen gehören alle marinen methanotrophen Symbionten mit bekannter 16S rRNA Gensequenz zu einer einzigen Gruppe innerhalb der Gammaproteobakterien. Terrestrische, Methan-oxidierende Symbionten gehören dagegen zu den Alphaproteobakterien. Viele dieser Symbionten sind jedoch noch nicht molekularbiologisch charakterisiert und sind möglicherweise diverser als bislang bekannt. Diese Review-Artikel fassen den aktuellen Wissensstand Methan-basierter Symbiosen zusammen und geben Ausblick auf zukünftige Forschungsschwerpunkte.

Im zweiten Teil dieser Dissertation stelle ich zwei Untersuchungen zur Ektosymbiose von *Rimicaris exoculata*, einer alvinocarididen Garnele von Hydrothermalquellen des Mittel-Atlantischen Rückens (MAR), vor. Modifizerte Gliedmaße und innere Oberflächen der Kiemenhöhlen der Wirte sind von filamentösen Ektosymbionten besiedelt, welche früher als schwefeloxidierende und ausschliesslich epsilonproteobakterielle Organismen beschrieben wurden. Anhand des 16S rRNA Ansatzes habe ich einen zweiten filamentösen Ektosymbionten identifiziert, welcher zu einer neuen Gruppe von symbiontischen Gammaproteobakterien gehört. Die Biogeographie der Ektosymbionten von den vier Hydrothermal-Feldern Rainbow, TAG, Logatchev und South MAR habe ich basierend auf 16S rRNA Gensequenzen vergleichend untersucht. Diese Untersuchungsgebiete sind bis zu 8500 km von einander entfernt. Unterschiede zwischen den 16S rRNA Gensequenzen korrelierten mit der geographischen Verteilung sowohl der gammaproteobakteriellen als auch der epsilonproteobakteriellen Symbionten. Im Gegensatz dazu zeigten die freilebenden Verwandten der epsilonproteobakteriellen Symbionten keine eindeutige Korrelation mit der Geographie. Die spezifische Erkennung von Wirt und Symbiont könnte das Verteilungsmuster erklären.

Abschliessend wurden die Stoffwechsel-Kapazitäten der Symbionten des Rainbow Hydrothermal-Feld anhand thermodynamischer Modellierung, Sequenzierung von Genen zentraler Stoffwechselfunktionen und immunohistochemischer Markierung von Proteinen direkt in Symbionten-Zellen untersucht. Der Nachweis von Schlüsselgenen des reduktiven TCA-Zyklus und des CBB-Zyklus deuten darauf hin, dass beide Symbionten CO_2 fixieren können. Detektierte Gene zur Oxidation von Wasserstoff, Methan und Schwefel zeigen die mögliche Fähigkeit diese Verbindungen als Energiequelle zu nutzen. Darüber hinaus wurde immunohistochemisch die Expression der partikulären Methan Monooxygenase im gammaproteobakteriellen Symbionten gezeigt, wobei dieser Organismus phylogenetisch nicht zu den bislang bekannten Methanotrophen gehört. Diese Untersuchungen legen nahe, dass die phylogenetische und physiologische Diversität dieser Symbiosen größer ist als bislang angenommen.

Contents

I Introduction 13

1 Chemosynthesis 14
1.1 Energy sources for chemosynthetic microorganisms 14
1.2 Carbon sources for chemosynthetic microorganisms 15
1.2.1 CO_2 fixation pathways 17
1.2.2 CH_4 incorporation 19
1.3 Chemosynthetic animals? 21

2 Diversity of habitats 22
2.1 Hydrothermal vents 23
2.1.1 The geological setting of hydrothermal vents 23
2.1.2 Hydrothermal circulation and the chemical and physical properties of vent fluids 28
2.1.3 Heterogeneity of vent fluid composition on the Mid-Atlantic Ridge 32
2.2 Cold Seeps 33
2.2.1 The geological setting of cold seeps 33
2.3 Analogous habitats 34
2.3.1 Shallow-water marine sediments 34
2.3.2 Whale falls 35
2.3.3 Sunken wood and shipwrecks 35

3 Diversity of hosts 36
3.1 Marine invertebrate hosts with endosymbionts 42
3.2 Marine invertebrate hosts with ectosymbionts 46

4 Diversity of microbes 49
4.1 Sulfur-oxidizing symbionts 50

4.2 Methane-oxidizing symbionts 52
4.3 Multiple co-occurring symbionts 52
4.4 Genomics and proteomics of chemosynthetic symbionts 53

5 *Rimicaris exoculata* 55
5.1 Distribution 57
5.2 Reproduction and dispersal 57
5.3 Symbiosis in *Rimicaris* shrimp 59
5.3.1 Role of the symbionts 59
5.3.2 Metabolism of the symbionts 59
5.4 Phylogeny of the *R. exoculata* epibionts 62

6 Microbial biogeography at hydrothermal vents 63

7 Aims 65

II List of Publications 67

III Manuscripts 71

IV Concluding remarks 165

8 Summary and Conclusions 166

9 Outlook 170

Bibliography 174

Acknowledgements 205

V Appendix 206

List of Figures

1.1 The five pathways of CO_2 fixation. 16
1.2 Metabolic pathways in methanotrophs 20
2.1 The global system of mid-ocean ridges 24
2.2 Ridge morphology 28
2.3 Global distribution of hydrothermal vents 29
2.4 The hydrothermal system 31
2.5 Sulfide production at whale falls 36
3.1 Examples of eukaryotic hosts with chemosynthetic symbionts . 38
5.1 The habitat of *Rimicaris exoculata* 56
5.2 Phylogeny of the Alvinocarididae 56
5.3 Distribution of *R. exoculata* 57
5.4 Morphology of the *R. exoculata* symbiosis 60

List of Tables

1.1 Redox reactions that fuel chemosynthesis 15
2.1 Classification of mid-ocean ridges 26
2.2 Chemical composition of hydrothermal fluids 30
3.1 Eukaryotic hosts of chemosynthetic symbionts 39

3-HP 3-hydroxypropionate

3-HP/4-HB 3-hydroxypropionate/4-hydroxybutyrate

ACL ATP citrate lyase

APS adenosine-5'-phosphosulfate

ATP adenosine triphosphate

CBB Calvin-Benson-Bassham

COI cytochrome oxidase subunit I

CODH carbon monoxide dehydrogenase

DNA deoxyribonucleic acid

EPR East Pacific Rise

et al. and others

FISH fluorescence in situ hybridization

FRD fumarate reductase

ICM intracellular cytoplasmic membranes

ITS internal transcribed spacer

MAR Mid-Atlantic Ridge

MOR mid-ocean ridge

Mya million years ago

OOR 2-oxoglutarate:ferredoxin oxidoreductase

PCR polymerase chain reaction

pMMO particulate methane monooxygenase

reductive acetyl CoA reductive acetyl coenzyme A

RNA ribonucleic acid

rTCA reductive tricarboxylic acid

RuBisCO ribulose-1,5-bisphosphate carboxylase/oxygenase

RuMP ribulose monophosphate

TAG Trans-Atlantic Geotransverse

TEM transmission electron microscopy

Part I

Introduction

Chapter 1

Chemosynthesis

Until the late nineteenth century, all life on Earth was thought to derive from the energy of the sun. Photosynthesis by plants was understood to be the basis of all ecosystems, the only process where organic matter was created from its inorganic components. Chemosynthesis first came on the scene in 1890 when the Russian microbiologist Sergei Winogradsky (1856-1953) published the idea that energy could also enter the 'cycle of life' through a novel process independent of sunlight, which he originally termed 'anorgoxydantism' [1]. Chemosynthesis is the process by which microorganisms harness the energy in chemical bonds to convert one-carbon molecules such as carbon dioxide and methane into organic matter. This is in contrast to photosynthesis, where the energy for carbon fixation comes from sunlight. Photosynthesis can be carried out by prokaryotes, single-celled eukaryotes such as diatoms, and by plants, but chemosynthesis is carried out exclusively by prokaryotes belonging to the Bacteria and Archaea. Almost 100 years after chemosynthesis was described, the first ecosystem based entirely on this process was discovered, in the cold, dark depths of the Pacific Ocean. Geologists diving in the manned submersible *Alvin* on the Galapagos Rift in 1977 were expecting to find hydrothermal vents; what they did not expect was the teeming oasis of life that greeted them, giant tubeworms with blood-red plumes, vast beds of mussels and clams, all seemingly living on, and from, tectonic features on the sea floor [2]. The discovery of hydrothermal vents in the deep sea challenged our understanding of the limits of life on Earth [3].

1.1 Energy sources for chemosynthetic microorganisms

Chemosynthesis is powered by redox reactions, in which one compound (the electron donor) becomes oxidized, and another compound (the electron acceptor) becomes reduced. A number of these reactions are listed in (Table 1.1).

Table 1.1: Some energetically favourable redox reactions for chemosynthetic symbiotic and free-living microorganisms in deep-sea hydrothermal vent habitats

Metabolism	e_d^- [a]	e_{acc}^- [b]	Redox reaction	$\Delta G^{0'}$ /rxn [c]	$\Delta G^{0'}$ /e^- [d]	Symbiotic bacteria [e]
Aerobic						
Sulfide oxidation	HS^-	O_2	$HS^- + 2O_2 \rightarrow SO_4^{2-} + H^+$	−750	−94	✓
Methane oxidation	CH_4	O_2	$CH_4 + 2O_2 \rightarrow HCO_3^- + H^+ + H_2O$	−750	−94	✓
Hydrogen oxidation	H_2	O_2	$H_2 + 0.5O_2 \rightarrow H_2O$	−230	−115	✓
Iron oxidation	Fe^{2+}	O_2	$Fe^{2+} + 0.5O_2 + H^+ \rightarrow Fe^{3+} + 0.5H_2O$	−65	−65	–
Manganese oxidation	Mn^{2-}	O_2	$Mn^{2+}0.5O_2 + H_2O \rightarrow MnO_2 + 2H^+$	−50	−25	–
Nitrification	NH_4^+	O_2	$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$	−275	−27.5	–
Anaerobic						
Sulfide oxidation/denitrification	HS^-	NO_3^-	$5HS^- + 8NO_3^- + 3H^+ \rightarrow 5SO_4^{2-} + 4N_2 + 4H_2O$	−3722	−124	✓
Methanogenesis	H_2	CO_2	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	−131	−16	–
Sulfate reduction	H_2	SO_4^{2-}	$SO_4^{2-} + H^+ + 4H_2 \rightarrow HS^- + 4H_2O$	−170	−21	–

[a] electron donor
[b] electron acceptor
[c] energy available ($\Delta G^{0'}$) in kJ per mole per reaction
[d] energy available ($\Delta G^{0'}$) in kJ per mole per electron transferred
[e] ✓ indicates that this metabolism has been identified in symbiotic bacteria

Chemosynthetic microorganisms can be either chemolithoautotrophs that use inorganic chemicals as an electron donor and CO_2 as a carbon source, or chemoorganoheterotrophs that use CH_4 as both electron donor and carbon source.

1.2 Carbon sources for chemosynthetic microorganisms

Chemosynthetic microorganisms that fix carbon dioxide are called chemolithoautotrophs. Methanotrophs are not autotrophic, as their carbon source, methane, is generally classified as an organic molecule. They are however capable of synthesizing organic material from one-carbon compounds, and thus, can play a similar role to autotrophs in chemosynthetic ecosystems. This section covers carbon fixation pathways in chemosynthetic microbes that use CO_2 or CH_4 as a carbon source.

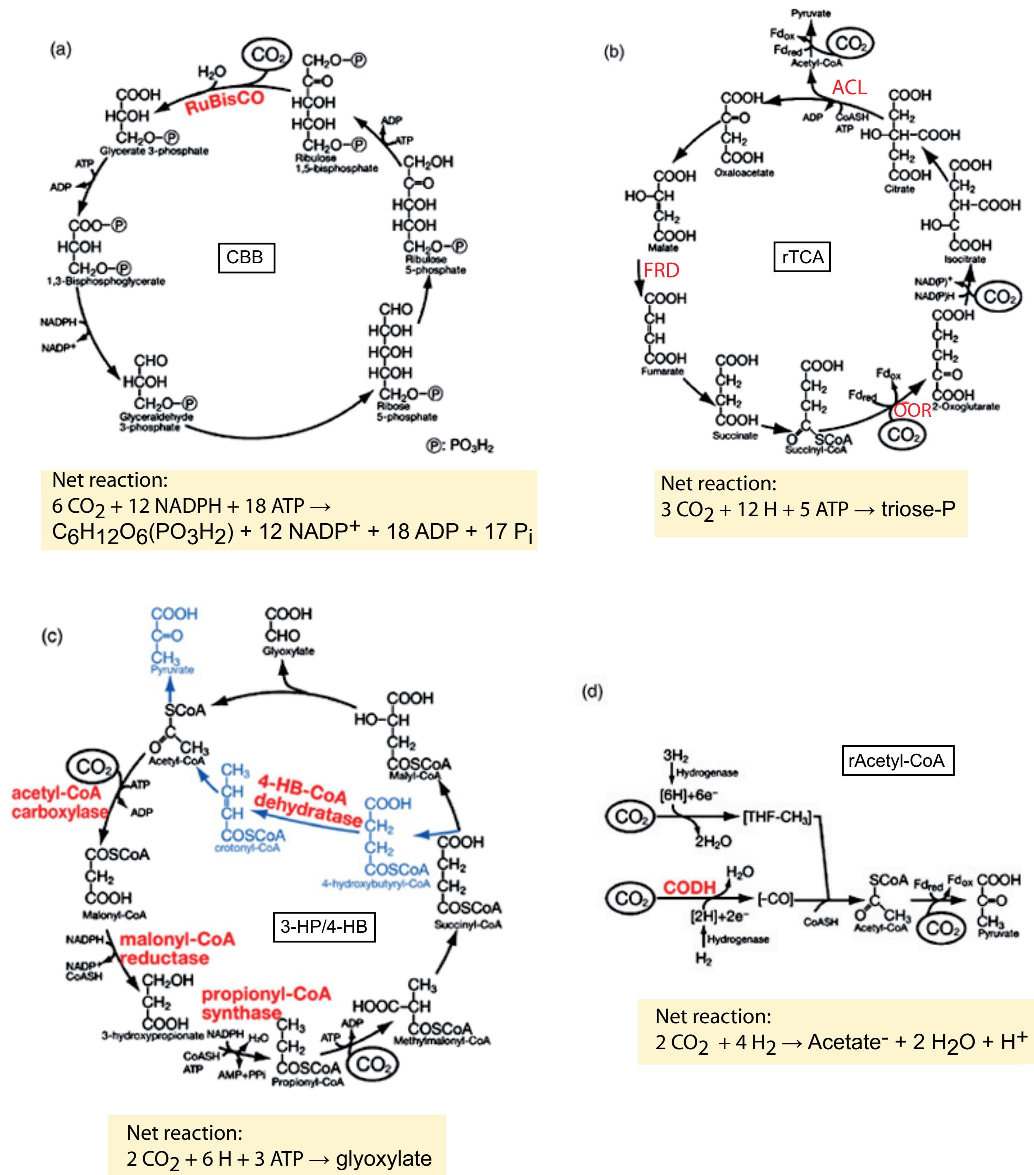


Figure 1.1: **The five pathways of CO_2 fixation.** Key enzymes are shown in red. (a) CBB cycle, (b) rTCA cycle, (c) 3-HP and 3-HP/4-HB (shown in blue) cycle, (d) reductive acetyl-CoA pathway. Modified from [4].

1.2.1 CO_2 fixation pathways

There are currently 5 known pathways of CO_2 fixation (Figure 1.1) [4]: the Calvin-Benson-Bassham (CBB) cycle, reductive tricarboxylic acid (rTCA) cycle, the 3-hydroxypropionate (3-HP) cycle, the reductive acetyl-CoA pathway, and the 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) pathway, which is a variant of the 3-HP cycle [5]. Each pathway has one or more key enzymes, and the presence of genes encoding for these key enzymes is often used daignostically to determine which CO_2 fixation pathway is used. The five pathways differ in several ways, such as energy demand (ATP), the use of different reducing equivalents, the requirement for metals such as Fe, Co, Ni, and Mo in the catalytic centers, the usage of coenzymes, and the oxygen sensitivity of the enzymes [5]. It is hypothesized that the distribution of these pathways in different organisms is determined by these criteria, in addition to organismal phylogeny and evolution [5]. For example, the rTCA cycle and reductive acetyl CoA pathway contain enzymes sensitive to oxygen, and are found in organisms that occupy anoxic or microoxic niches.

The Calvin-Benson-Bassham cycle

The CBB cycle is the most widespread CO_2 fixation pathway on Earth. It is used by green plants and most prokaryotic photoautotrophs, including *Rhodobacter*, *Chromatium*, and Cyanobacteria. It is also present in many free-living (e.g. *Thiobacillus*) and symbiotic chemoautotrophs (e.g. the endosymbionts of *Bathymodiolus* mussels [6] and *Riftia pachyptila* tubeworms, although the tubeworm symbionts can also use the rTCA cycle [7]). The key enzyme of the CBB cycle is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Figure 1.1). There are four different RuBisCO types, forms I-IV. Forms I, II, and III catalyze the carboxylation and oxygenation of ribulose 1,5-bisphosphate, but form IV does not catalyze either of these reactions, and is therefore called a RuBisCO-like protein [8]. Form I is the most prevalent in nature, and has been found in green plants. Forms I and II are present in photo-, and chemoautotrophic bacteria, and RuBisCO form III has so far only been found in Archaea [8,9].

The reductive TCA cycle

The rTCA cycle is essentially a reversal of the oxidative tricarboxylic acid cycle used by most aerobic heterotrophs to gain energy from organic matter [4]. It was originally discovered in the phototrophic green sulfur bacteria [10], and has since been identified in diverse chemoautotrophs including members of the *Aquificales* [11], the Epsilonproteobacteria [12, 13], and some Archaea [14].The presence of this pathway in Archaea and in the deep-branching *Aquificales* indicates that this might have been an early evolutionary form of autotrophy [15]. There are three key enzymes of the rTCA cycle: ATP citrate lyase (ACL), 2-oxoglutarate:ferredoxin oxidoreductase (OOR), and fumarate reductase (FRD) (Figure 1.1). In contrast to organisms using the CBB cycle, hydrothermal vent microorganisms using the rTCA cycle inhabit more reducing zones closer to the vent fluids, supposedly due to the oxygen sensitivity of many of the enzymes involved in the rTCA cycle [4]. Recently, this pathway of carbon fixation was found in the gammaproteobacterial symbiont of the giant tube worm *Riftia pachyptila* [7, 16], see Section 3.1.

3-hydroxypropionate and 3-hydroxypropionate/4-hydroxybutyrate pathways

The first description of the 3-HP cycle was in the anoxygenic photoautotroph *Chloroflexus*. [17]. This cycle has also been found in some hyperthermophilic Archaea [14], and may also be present in the Gamma 1 sulfur-oxidizing endosymbionts of gutless oligochaete worms (M. Kleiner, J. Zarzycki, G. Fuchs, N. C. Verberkmoes, H. Teeling, M. Hecker, T. Schweder, and N. Dubilier, unpublished results). The 3-HP cycle also has three key enzymes: acetyl-CoA/propionyl-CoA carboxylase, malonyl-CoA reductase, and propionyl-CoA synthase (Figure 1.1). The 3-HP/4-HB cycle is a recently-described, novel CO_2 fixation pathway in Archaea [5]. It is a variant of the 3-HP cycle, and has the key enzyme 4-hydroxybutyryl-CoA dehydratase (Figure 1.1) [4, 5]. Most of the organisms using the 3-HP/4-HB cycle to fix CO_2 are faculatative autotrophs that can also grow heterotrophically [4].

The reductive Acetyl-CoA pathway

Unlike the other CO_2 fixation pathways, which are cyclic, and involve the regeneration of a carbon dioxide acceptor molecule, the reductive acetyl CoA pathway is noncyclic [4]. It has so far only been found in chemoautotrophic sulfate-reducing bacteria, acetogens, and methanogenic archaea [4, 15, 18]. The key enzyme of the reductive acetyl CoA pathway is carbon monoxide dehydrogenase (CODH) (Figure 1.1). Organisms using this pathway are strict anaerobes, as it involves enzymes highly sensitive to oxygen [4]. The only hydrothermal vent organisms known to use this pathway are the methanogens [4].

1.2.2 CH_4 incorporation

Methane-oxidizing bacteria use CH_4 as both an electron donor and a carbon source. CH_4 is oxidized to formaldehyde in a series of dissimilatory reactions, and carbon is incorporated into cell components at the level of formaldehyde [19]. There are two pathways of formaldehyde incorporation in methanotrophic bacteria, the serine pathway and the ribulose monophosphate (RuMP) pathway (Figure 1.2) [20]. The organisms that use these different pathways are morphologically, physiologically, and taxonomically distinct [19, 20]. The gammaproteobacterial type I and type X methanotrophs use the RuMP pathway for formaldehyde assimilation. In the RuMP cycle, three formaldehyde molecules are used to make the 3-carbon intermediate glyceraldehyde-3-phosphate, which is then incorporated into cell material [20]. The key enzymes of the RuMP pathway are listed in Figure 1.2. The type II methanotrophs use the serine pathway for formaldehyde assimilation. In this pathway, two formaldehyde molecules are combined with one CO_2 to produce 2-phosphoglycerate, also a 3-carbon intermediate which is incorporated into cell material in these organisms. The serine pathway has four key enzymes, which are listed in Figure 1.2.

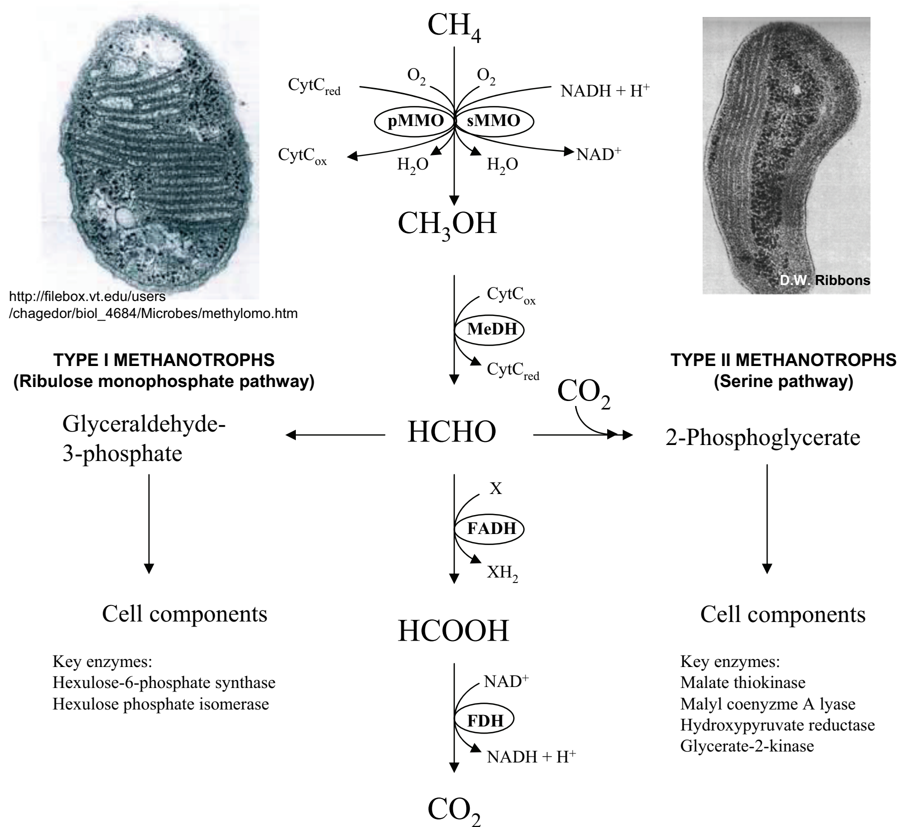


Figure 1.2: **Metabolic pathways in methanotrophic bacteria.** The type I gammaproteobacterial methanotrophs incorporate formaldehyde via the RuMP pathway, and typically have intracellular cytoplasmic membranes (ICM) arranged as bundles throughout the cell, while the alphaproteobacterial methanotrophs incorporate formaldehyde via the serine pathway, and have ICM arranged at the periphery of the cell. The pathway of formaldehyde oxidation to CO_2 is the same in both types. Methane is oxidized to methanol either by a particulate or soluble methane monooxygenase, pMMO and sMMO, respectively. Copper is required for pMMO expression and activity, and sMMO is expressed only under low copper conditions. Most methanotrophs have pMMO, but not all have sMMO [21]. CytC = cytochrome c, MeDH = methanol dehydrogenase, FADH = formaldehyde dehydrogenase, FDH = formate dehydrogenase. Figure inspired by [20, 22].

As mentioned above, type II methanotrophs incorporate one CO_2 for every 2-phosphoglycerate produced. A significant proportion of cellular carbon in these organisms therefore comes from CO_2. In addition, type I, type

X, and type II methanotrophs all gain a certain amount of carbon from CO_2 during anapleurotic CO_2 fixation [21]. Some type X methanotrophs are also capable of autotrophic CO_2 using the enzymes of the CBB pathway. *Methylococcus capsulatus* (Bath), a type X methanotroph, can grow autotrophically on solid media with CO_2 as the sole carbon source and H_2 as energy source [23]. The presence of pathways for autotrophic CO_2 fixation in obligate methanotrophs suggests a common origin for C_1 metabolism in methanotrophic and autotrophic organisms [21].

Recently, a novel lineage of thermoacidophilic methanotrophic bacteria was discovered in geothermal areas in New Zealand, Italy and Russia [24–26], reviewed in [27]. They belong to the phylum Verrucomicrobia, which is in contrast to all previously known methanotrophs that group within the Gamma- or Alphaproteobacteria. Intracellular membrane structures are present in the verrucomicrobial methanotrophs, but they are unlike the stacked membranes of type I and type II methanotrophs. Instead, they contain polyhedral inclusions that appear similar to carboxysomes, intracellular structures in cyanobacteria and chemoautotrophs that contain RuBisCO [25]. This is an intriguing observation, as the whole genome sequence of the verrucomicrobial methanotroph *Candidatus* Methylacidiphilum infernorum contained only some of the key enzymes of the serine pathway, but did have all genes for the CBB cycle [28], and its growth rate was dependent on CO_2 concentration [24]. There was also no homologue of methanol dehydrogenase found, although this enzyme catalyzes the key step of methanol oxidation to formaldehyde (Figure 1.2). These organisms therefore appear to have unique adaptations to growth on methane, some of which are not yet understood.

1.3 Chemosynthetic animals?

Deep-sea hydrothermal vents are some of the most productive animal communities on Earth [29], and their presence, so far from the photic zone, initially puzzled researchers, who asked themselves: what are these animals eating? The only sources of energy at vents are inorganic chemicals such as

sulfide and methane provided by the hot, reduced vent fluids, but until this point, no animal was known to be capable of living autotrophically. Some of the first physiological studies of the giant tubeworm *Riftia pachyptila* showed activity of enzymes involved in the CBB cycle of autotrophic CO_2 fixation and sulfide oxidation [30], leading to the conclusion that the first autotrophic animal had been discovered. However, researchers quickly realized that these animals contained sulfur-oxidizing prokaryotic cells in their tissues [31], that were using the inorganic energy sources at the vents to fix CO_2, providing nutrition for themselves and for their hosts (see boxed text Defining Symbiosis). Many more chemosynthetic symbioses have been described in the intervening 27 years since the first description of sulfur-oxidizing symbionts in *R. pachyptila*, and chemosynthetic symbioses are now known to occur in animals from at least seven different phyla (Table 3.1), with undoubtedly many more waiting to be discovered [32]. It has also become clear that chemosynthetic symbioses are ubiquitous, wherever there is a habitat where reduced and oxidized conditions come into contact, from deep-sea hydrothermal vents, to sewage-outfalls and organic-rich mudflats [32]. Teaming up with chemosynthetic bacterial symbionts is therefore a hugely successful strategy for animals to access new substrates for growth, and to colonize new, and sometimes hostile habitats. The following sections provide an overview of the amazing diversity of habitats, hosts and microbes involved in chemosynthetic symbioses.

Chapter 2

Diversity of habitats

Wherever the anoxic and oxic worlds meet in the marine environment, chemosythetic symbioses have been found. These habitats include the deep-sea hydrothermal vents and cold seeps, and coral reef sediments, sea grass sedi-

Defining Symbiosis. Symbiosis is an intimate association between two different organisms. The term 'symbiosis' (from the Greek *'sym'* = with, *'bio'* = living) has its roots in the late 1800s. Its original use is often attributed to the German biologist Anton de Bary, but it was also coined independently by the German botanist Albert Bernhard Frank at about the same time [33]. De Bary originally used the term in its broadest sense, to include all associations where two unlike organisms lived in or on each other for a substantial part of their life cycles [34]. Later uses of the term included a judgement about the nature of the association, and an alternative definition of the term emerged, one in which 'symbiosis' only referred to mutually beneficial associations, parasitism to associations where one organism benefits to the detriment of another, and commensalism to associations where the partners have no obvious benefit. More recently, de Bary's original, broader definition has become the most widely accepted, as it is becoming clear that the boundaries between 'beneficial' and 'detrimental' associations can be blurred, that the same organism can be beneficial to one host and parasitic to another [35], and as more and more associations are being described for which the role of the partners is unknown. In addition, many of the molecular interactions that are crucial in pathogenesis are being recognized to play a role in beneficial interactions [36, 37]. Symbiosis is used here sensu lato as originally defined by de Bary and Frank.

ments, mangrove peat, and marsh and intertidal sediments in shallow waters [29, 32, 38]. The geochemical and biological processes responsible for the production of the reduced compounds are diverse, as are the physical processes that bring reduced fluids in contact with oxidized seawater. The following sections describe these habitats and processes, focussing on hydrothermal vents and cold seeps.

2.1 Hydrothermal vents

2.1.1 The geological setting of hydrothermal vents

The bathymetry of the ocean floor reveals a number of distinct morphological features, which are the surface indications of geological processes within the Earth. These dynamic processes provide the energy for the thriv-

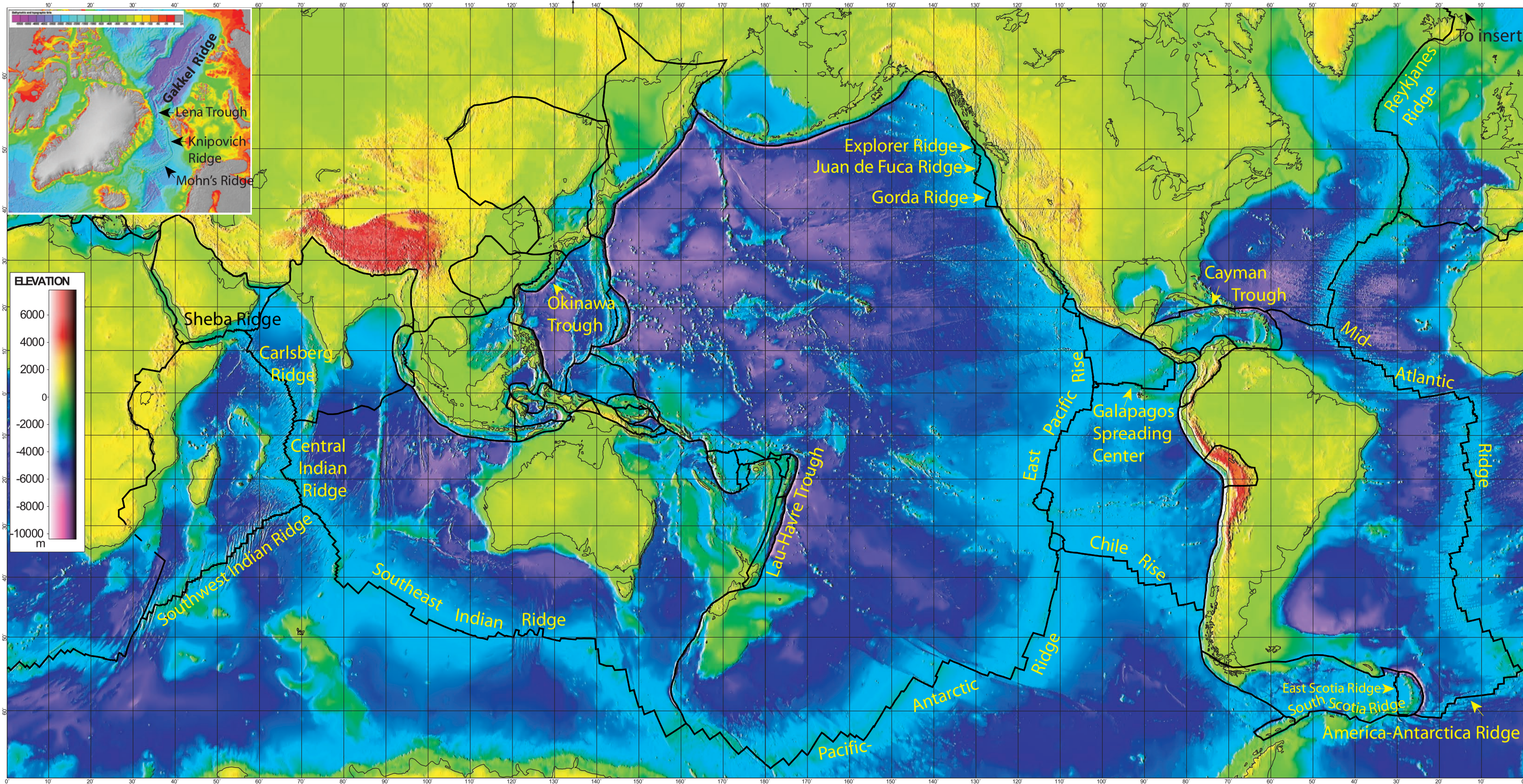


Figure 2.1: **The global system of mid-ocean ridges.** The boundaries of the Earth's tectonic plates (black lines) make up the global system of mid-ocean ridges. The bathymetry of the ocean floor shows that they form giant undersea mountain chains (elevation key on the left-hand side). Modified from [39] with input from [40]. *Inset:* northern extension of the Mid-Atlantic Ridge beyond Iceland, showing the ultraslow-spreading Gakkel, Mohn's and Knipovich Ridges. From [41].

ing chemosynthetic ecosystems found at hydrothermal vents and cold seeps. There are perhaps no other ecosystems on Earth where the link between volcanism and life is more evident [42]. Knowledge of the geological processes responsible is therefore crucial to understanding the biology of hydrothermal vent organisms [29].

Plate tectonics and mid-ocean ridges

Mid-ocean ridges (MORs) are the most conspicuous tectonic features of the seafloor (Figure 2.1). MORs are found at the boundaries between the plates that make up the Earth's crust. As tectonic forces cause the plates to move apart, new oceanic crust is formed as magma from deep in the Earth's mantle rises, erupting to fill the gaps formed by seafloor spreading [43]. The vast majority of the global system of MORs forms a single, 60 000 km-long volcanic undersea mountain range that encircles the globe, and which lies at depths between 2000 and 5000 m below the sea surface [29]. This undersea mountain range is broken up into segments along the ridge axis that are interspersed by transform faults, where tectonic plates slide past each other to accommodate the spreading of a linear ridge system on the spherical Earth. Transform faulting causes the typical zig-zag pattern of the MOR, and can have a dramatic effect on MOR topography [43, 44]. The Mid-Atlantic Ridge, for example, is bisected into a northern and southern section by the Romanche fracture zone, a large and deep transform fault 935 km long and 4 km high [29].

Spreading rates and the different types of mid-ocean ridges

The rate at which tectonic plates move apart is not globally uniform. Spreading rates range from ultraslow at less that 20 mm yr^{-1}, to superfast at up to 170 mm yr^{-1}. Some examples are shown in Table 2.1. The spreading rate affects the morphology of the ridge axis [45]. A cross-section of the ridge axis relief shows the unique features of fast-, intermediate-, and slow-spreading ridges Figure 2.2. If we could drain the water out of the oceans and stand in the centre of a slow-spreading ridge such as the Mid-Atlantic Ridge (MAR),

we would be standing in a rift valley, 20 to 30 km wide, whose steep inner walls tower up to 3 km above the valley floor [43]. Beyond the walls of the central rift, the flanks fall gently away toward the abyssal plains, 5 - 6 km below sea level. In contrast, at an intermediate-spreading ridge such as the East Pacific Rise (EPR) at 20°N, the central rift is only 50 - 200 m deep, superimposed onto a gently rising central morphological high. At spreading rates higher than 90 mm yr^{-1}, no central rift zone is formed, and only a high axial swell is present, such as on the EPR at 3°S. Because there is no deep rift valley formed at fast-spreading ridges, they are typically about 1 km shallower than slow-spreading ridges [43].

Table 2.1: **Classification of mid-ocean ridges according to spreading rate[a][b]**

Ridge category	Spreading rate	Examples[c]
Ultraslow-spreading	20 mm yr^{-1}	Gakkel Ridge, Knipovich Ridge, Lena Trough, Mohns Ridge, Southwest Indian Ridge
Slow-spreading	20-50 mm yr^{-1}	Central Indian Ridge, Mid-Atlantic Ridge
Intermediate-spreading	50-90 mm yr^{-1}	Galapagos Spreading Center, Gorda Ridge, East Pacific Rise 21°N, Explorer Ridge, Juan de Fuca Ridge, Lau Basin Back-Arc Spreading Center, Southeast Indian Ridge
Fast-spreading	90-130 mm yr^{-1}	East Pacific Rise 8-13°N
Superfast-spreading	130-170 mm yr^{-1}	East Pacific Rise 27-32°S

[a] Classification according to [46]
[b] Idea for this table comes from [40]
[c] Examples taken from [29, 46–48]

Hydrothermalism is thought to be mainly volcanically-driven at fast-spreading ridges, and tectonically-driven at slow-spreading ridges [42]. This affects the incidence and longevity of hydrothermal vents along each type of MOR. The incidence of hydrothermal plumes along a ridge axis, is an indicator of hydrothermal venting, and is proportional to the spreading rate, with plume incidence increasing with increasing spreading rate. This model is fairly robust for fast-spreading ridges, and can also be applied to slow-spreading ridges, although these show more variability. The spatial frequency

of hydrothermal vents on the slow-spreading Mid-Atlantic Ridge is estimated to be one vent site every 100 to 350 km of ridge axis, whereas vents on the fast-spreading East Pacific Rise can be found as frequently as one every 5 km of ridge axis. On fast-spreading ridges, only fairly recent hydrothermal activity has been found to date, whereas slow-spreading ridges can co-host fossil and recent activity [29, 49].

Recent investigations of the Gakkel and Southwest Indian Ridges, which have spreading rates below 20 mm yr^{-1}, have identified characteristics of these ultraslow-spreading ridges that set them apart from all other spreading centres (reviewed in [48]). Ultraslow-spreading ridges have a much thinner seismic crust, as thin as 1 km, compared to slow-, intermediate-, fast-, and ultrafast-spreading ridges, which all have a crustal thickness between 6 and 7 km [48]. An additional feature unique to ultraslow-spreading ridges is the presence of amagmatic segments, which results in an unusual ridge geometry [50]. These amagmatic segments at MORs are expected to shed light on processes of relevance not only to seafloor spreading at all spreading rates, but also to processes of continental breakup [48].

Back-arc basins

Back-arc basins are diverse geological settings that involve both divergent plate boundaries, where new seafloor is created, and convergent plate boundaries, where oceanic crust is consumed. Back-arc basins are created when high pressure and heat flow, generated when one plate subducts under another, cause the breakup of the overriding plate, forming a new spreading centre in the vicinity of the arc volcanic front. The development of back-arc basins appears to be episodic, in contrast to MOR spreading centres. Back-arc basins may have alternating periods of activity and extinction, each lasting for millions of years, but the causes for this are poorly understood [29,52].

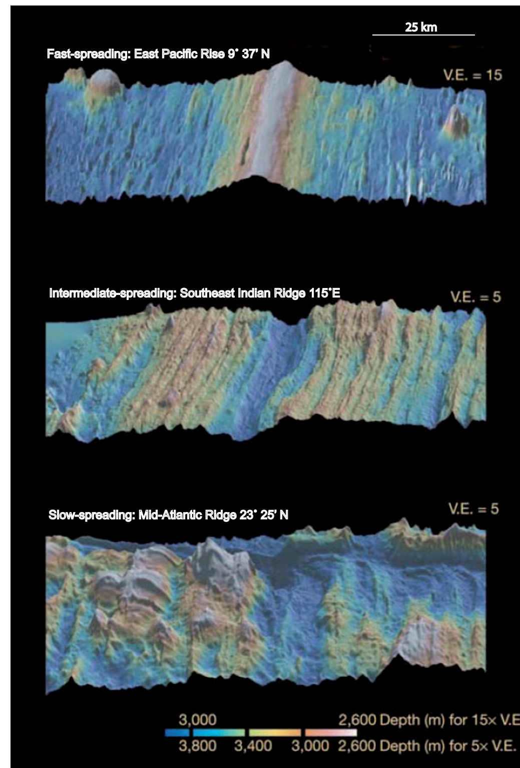


Figure 2.2: **Mid-ocean ridge morphology.** Comparison of ridge morphology between the fast-spreading East Pacific Rise (top), the intermediate-spreading Southeast Indian Ridge (centre), and the slow-spreading Mid-Atlantic Ridge (bottom). The vertical exaggeration (V. E.) is three times greater in the top image. From [51].

2.1.2 Hydrothermal circulation and the chemical and physical properties of vent fluids

The discovery of hydrothermal vents at oceanic spreading centres was in part the result of hypothesis-driven research [53]. Measurements of heat flow along ridge crests showed values that were inconsistent with convective cooling alone of the newly formed oceanic crust. It was hypothesized that the missing element of the heat budget could be explained by convective transport of heat by fluids near ridge crests (for example, [54]). The eventual discovery of high-temperature fluid discharge and the associated metal deposits was therefore anticipated, but was expected to be rare [53]. A look

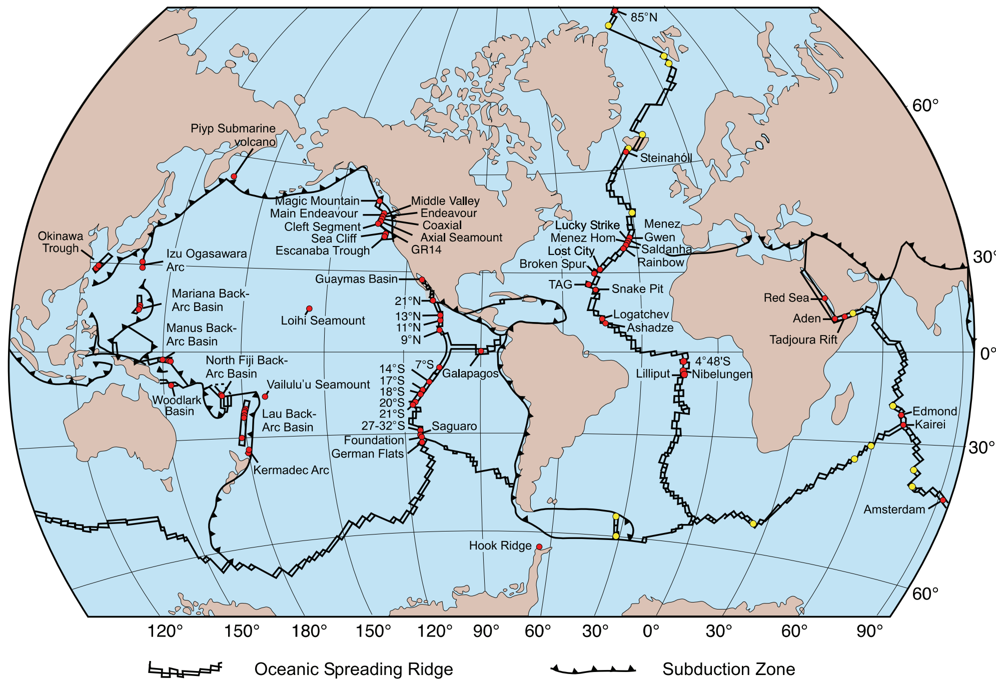


Figure 2.3: **Global distribution of hydrothermal venting.** Known sites are shown in red, sites where hydrothermal venting is indicated by mid-water chemical anomalies are shown in yellow. EPR = East Pacific Rise, TAG = Trans-Atlantic Geotransverse, MEF = Main Endeavour Field. From [53].

at the currently known global distribution of hydrothermal vent sites shows just how common hydrothermal venting is along MORs (Figure 2.3). Hydrothermal venting has been found just about everywhere we have looked; in all ocean basins, and at all spreading rates, from ultraslow to superfast.

The chemical composition of hydrothermal fluids reflects water-rock interactions occurring in the subsurface, and is determined by the geologic and tectonic setting of the vents. The nature of the heat source, the host rock composition, the input of magmatic gases, the permeability of the substrate, and the temperatures and pressures at which the reactions occur, all influence fluid characteristics such as temperature and pH, and the chemical composition [53, 55]. While most hydrothermal fluids have common features that set them apart from seawater, such as high temperature (above 350℃), low pH, enriched sulfide and metal content, and the absence of sulfate and

Table 2.2: **Range of chemical compositions of hydrothermal endmember fluids venting in different settings**[a]

		Basalt	Ultramafic		Back-Arc	Sediment	SW
			High-T	Lost City			
T	℃	≤ 407	350-365	≤ 91	278-334	100-315	2
pH	(at 25℃)	2.8-4.5	2.8-3.9	9-11	< 1-5.0	5.1-5.9	8
H_2	mmol kg^{-1}	0.0005-38	13-19	< 1-15	0.035-0.5	-	-
H_2S	mmol kg^{-1}	0-19.5	1-2.5	< 0.064	1.3-13.1	1.1-5.98	-
CH_4	mmol kg^{-1}	0.007-2.58	0.13-3.5	1-2	0.005-0.06	-	-
NH_3	mmol kg^{-1}	< 0.65	-	-	-	5.6-15.6	-
CO_2	mmol kg^{-1}	3.56-39.9	-	bdl	14.4-200	-	2.36
Fe	mmol kg^{-1}	0.007-18.7	2.41-24	-	0.013-2.5	0-0.18	-
Mn	mmol kg^{-1}	0.059-3.3	2.25	-	0.012-7.1	0.01-0.236	-
Na	mmol kg^{-1}	10.6-983	438-553	479-485	210-590	315-560	464
Ca	mmol kg^{-1}	4.02-109	28-67	< 30	6.5-89	160-257	10.2
K	mmol kg^{-1}	1.17-58.7	20-24	-	10.5-79	13.5-49.2	10.1
Cl	mmol kg^{-1}	30.5-1245	515-750	548	255-790	412-668	545
SO_4^{2-}	mmol kg^{-1}	0	0-1.3	1-4	0	0	28
Mg	mmol kg^{-1}	0	0	< 1	0	0	53

High-T = high-temperature, SW = Seawater (background) values, bdl = below detection limit

[a] Table adapted from [40, 53], data from [56–67]

magnesium, the composition varies from site to site (Table 2.1).

A number of prerequisites are necessary for the generation of seafloor hydrothermal fluids. Seawater can circulate through the oceanic crust at spreading centres because of the presence of a heat source, in the form of a magma chamber or newly solidified rock, and a permeable medium, which is provided by cracks and fissures in the ocean crust. Hydrothermal circulation can be divided into three parts (Figure 2.4) [53, 55]:

1. Recharge zone
2. Reaction zone
3. Upflow zone

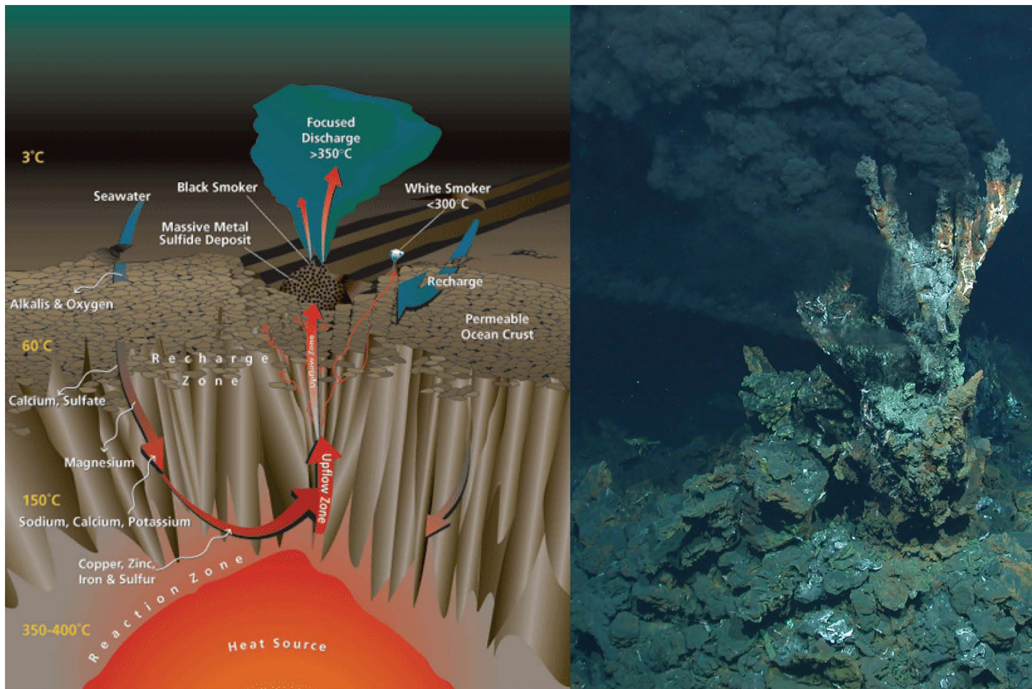


Figure 2.4: **The hydrothermal system**. A. The hydrothermal system can be divided into three parts: the recharge zone, the reaction zone, and the upflow zone. The fluids undergo a series of chemical reactions with subsurface rocks at various temperatures, eventually producing the hydrothermal fluids that exit at seafloor 'black smokers'. Image by E. Paul Oberlander, from [55]. B. Image of a black smoker at the Logatchev hydrothermal vent field, photograph copyright Marum, University of Bremen.

In the recharge zone, seawater enters the crust and percolates downward. The top 300 m of the ocean crust is composed of highly porous volcanic rocks broken apart by cooling cracks and tectonic features. Seawater can enter this layer readily. In this zone, fluid-rock interactions take place at temperatures of up to 60℃. The seawater partially oxidizes the rock, and the fluids become anoxic. Interactions with rock minerals cause the seawater to lose alkali elements such as potassium, rubidium, and cesium. Below this upper layer, the host rock becomes less permeable, which means that below about 300 m, large fractures and fissures become the main conduits for fluid flow. As the fluids move deeper into the crust, the temperature rises. Above 150℃, clay minerals and chlorite precipitate out of the fluid, and essentially

all of the magnesium from the original seawater is removed. Hydroxyl ions are removed from the fluid, resulting in a decrease in fluid pH. Interactions of the acidic fluid then cause calcium, sodium, and potassium to leach out of the rock into the fluid. In addition, the mineral anhydrite (calcium sulfate) is formed, removing about two-thirds of the original seawater sulfate. At temperatures above 250℃, the remaining sulfate is removed from the fluid as metal sulfide minerals are formed [53, 55].

The reaction zone is where high-temperature water-rock reactions occur, at temperatures between 350℃ and 400℃, near the heat source driving the circulation. The reactions that take place in this zone determine the final composition of the seafloor hydrothermal fluids. Metals such as copper, iron, zinc, as well as sulfur, are leached from the rock by the acidic fluids. Hydrogen sulfide for chemosynthesis is produced here [53, 55].

The upflow zone is the area where buoyancy causes the heated fluids to rise to the seafloor. Upflow can be focused along a fissure or crack, or it can move through the more permeable upper layers to exit the seafloor as diffuse flow. When a focused flow of hot (350℃ to 400℃) fluids reaches the seafloor and mixes with the surrounding cold, oxidized seawater, metal sulfides precipitate, giving the appearance of black smoke gushing from chimney structures on the seafloor, which are fittingly called black smokers (Figure 2.4) [53, 55]. Diffuse fluids are generally cooler, with temperatures up to 100℃, and have lost most of their minerals as they precipitate while mixing with seawater along the path of fluid flow below the seafloor. Approximately half of all hydrothermal fluids are believed to be discharged through black smokers, while an equal amount is discharged as diffuse fluid [68, 69].

2.1.3 Heterogeneity of vent fluid composition on the Mid-Atlantic Ridge

Within a single MOR such as the slow-spreading Mid-Atlantic Ridge (MAR), differences in the rock composition in the reaction zone can drastically influence hydrothermal vent fluid composition. Two major types of hydrothermal vents are known on the MAR: basalt- and ultramafic-hosted. Most hy-

drothermal fluids are produced by interactions with the basaltic rocks that make up the ocean's crust. However, a number of unique geological settings have been discovered on the MAR, where mantle rocks such as peridotite form a significant part of the upper oceanic crust, and these are termed ultramafic-hosted. Hydrothermal fluids emitted at these sites bear the unique signature of peridotite-water interactions [57–59,70]. Hydrothermal fluids in ultramafic settings typically have high concentrations of dissolved hydrogen due to serpentinization reactions, and methane due to abiogenic methanogenesis from CO_2 and H_2 [71, 72].

2.2 Cold Seeps

2.2.1 The geological setting of cold seeps

Cold seeps, like hydrothermal vents, are also seafloor expressions of geologic processes within the Earth. Cold seeps are found on continental margins, both on active margins such as the Peruvian Margin, which are in close proximity to plate boundaries, and on passive margins such as the Gulf of Mexico, which are not related to plate boundaries. At active margins, an oceanic plate is subducted beneath a continental plate, pushing the sediment load that has accumulated on the oceanic crust into the hot mantle. When this sediment is compressed and heated, it becomes geothermally altered, and sediment pore fluids and gases are expelled. Where the fluids reach the seafloor, they can form gas-hydrate deposits, pockmarks, gas chimneys, mud volcanoes, brine ponds, and oil and asphalt seeps [73]. The processes occurring on passive margins can also be complex, and depend on the regional geological setting. In the Gulf of Mexico, for example, sediment loading on salt deposits causes their deformation, providing conduits for the escape of gas and hydrocarbons from deeply-buried deposits [74].

Fluids at cold seeps are delivered to the seafloor at velocities of a few tens of centimeters to a few meters per year [75–77]. Cold seep fluids have a much lower temperature than hydrothermal vent fluids, and may be only slightly above ambient seawater (for example [76, 78, 79]). Seep fluids are usually rich in methane and sulfide, and may also contain hydrocarbons other than

methane, depending on the fluid source [73, 74].

2.3 Analogous habitats

Conditions where electron donors and electron acceptors coexist are not only found at hydrothermal vents and cold seeps, but these are the only two habitats where complex communities are entirely reliant on chemosynthetic primary production. Other marine habitats can support chemosynthetic symbioses, but in contrast to hydrothermal vents and cold seeps, these ecosystems rely on the input of organic matter derived from photosynthetic primary production. These include shallow marine sediments, sunken whale carcasses (whale falls), and sunken wood.

2.3.1 Shallow-water marine sediments

Shallow-water is commonly defined as the top 200 m of the ocean. The range of shallow-water habitats where chemosynthetic symbioses have been found includes sewage outfalls, sea grass beds, mangrove muds, marsh and intertidal sediments and coral reef sediments [32]. Some of the host animals common in shallow-water habitats can also be found in deeper waters on continental margins, such as gutless oligochaetes in sediments off the coast of Peru in 300 m water depth [80,81]. In contrast to the deep sea, where each m^2 of sediment receives on average only 1 g of carbon per year from photosynthetic primary production in surface waters [82], shallow marine sediments are generally organic-rich. Under anoxic conditions in shallow marine sediments, organic matter is oxidized by sulfate-reducing bacteria that produce sulfide. In the deeper layers of the sediment, where sulfate is depleted, CO_2 can be used as an electron acceptor by methanogenic archaea that produce methane, but so far, no host with methane-oxidizing symbionts has been described in these habitats. It is possible that the migration distance between the oxidized layers and those containing methane is too great for animals to be able to rely on a methane-based symbiosis for nutrition [32]. Since sulfide and oxygen generally do not co-occur in these habitats, hosts containing chemosynthetic symbioses have evolved a number of behavioral, anatomical, and physiological

strategies to bridge the gap between the oxic and anoxic worlds [38].

2.3.2 Whale falls

In 1989, Smith and colleagues reported the first discovery of a whale carcass, resting on the seabed in 1240 m water depth, which was colonized by fauna, some of which were similar to those found at vents in the East Pacific [83]. Since then, many more natural whale falls have been investigated , and the temporal development of the whale-fall ecosystem has been studied by the intentional sinking of whale carcasses, with sampling at regular intervals afterwards [84]. In addition, similar communities have been shown to colonize cow bones placed deliberately on the ocean floor [85]. The source of organic carbon for sulfate-reducing bacteria at whale falls comes from the degradation of lipids (Figure 2.5), which can make up more than 60% of the whale bone weight [86]. Methane is known to be produced in whale-fall habitats, and aerobic methane-oxidizing bacteria are part of the free-living community at whale falls [87]. Despite this, no methanotrophic symbiosis has yet been discovered in whale-fall fauna.

2.3.3 Sunken wood and shipwrecks

Sunken wood can also provide a habitat for hosts with chemosynthetic symbionts. Many of the animals found on sunken woods rely on the wood itself as a source of nutrition, and live in partnership with heterotrophic symbionts that can degrade cellulose [88]. These symbionts can often also fix inorganic nitrogen [89], supplementing their needs and those of the host since a wood-diet is nitrogen-poor. Sulfide is also produced by sulfate-reducing bacteria that break down the wood, providing a habitat for animals with chemosynthetic sulfide-oxidizing symbionts. Some of the most unusual deep-sea habitats for chemosynthetic symbioses are shipwrecks with their associated organic cargoes. For example, *Lamellibrachia* tube worms have been found in a shipwreck lying at 2800 m water depth in the Mediterranean on decomposing paper in the ship's mailroom [90], and on rotting beans in a shipwreck at 1100 m water depth, 30 km off the coast of Spain [91].

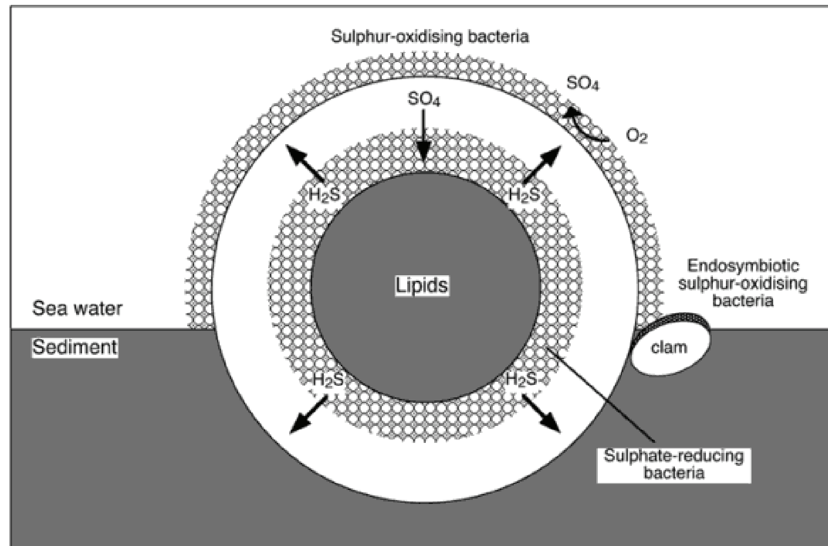


Figure 2.5: **Sulfide production at whale falls.** Schematic cross-section of a whale bone resting on ocean-floor sediment. Sulfate from seawater diffuses into the whale bone, where it is used as an electron donor by lipid-degrading, sulfate-reducing bacteria, which produce sulfide. The sulfide diffuses out of the bone, where it can be oxidized by free-living or symbiotic chemoautotrophic bacteria, providing an energy source for inorganic carbon fixation. Figure from [84].

Chapter 3

Diversity of hosts

At least seven animal phyla are known to host chemosynthetic symbiotic microbes (Table 3.1), from single-celled ciliates, to sponges, worms, snails, and mussels. Hundreds of host species have been described, and there are undoubtedly many more still to be discovered [32]. Many examples of morphological, behavioral, and physical adaptations to the symbiotic bacteria are evident among chemosynthetic hosts, depicting the evolutionary innovations that can emerge when animals and bacteria cooperate. The symbionts can be housed as endosymbionts within the tissues of the host, either intra- or extracellularly. In some cases, novel organs such as the tubeworm tropho-

some have developed to house the symbiotic bacteria. Alternatively, the symbionts can be found attached to the outside surfaces of the host, and these are termed ectosymbionts. Ectosymbioses are often considered to be more primitive than endosymbioses [92, 93], an early stage in the progression from free-living microorganism, to fully integrated endosymbiont, the most evolved of these exemplified by the organelles of eukaryotic cells. However, the limited diversity of some ectosymbioses indicates that these associations have evolved highly specific recognition systems [94–99], and might simply represent an alternative strategy to an endosymbiotic association. This astounding variety of morphological adaptations attests to the adaptive flexibility of both animals and bacteria [32].

The nutritional benefits of the symbioses are often reflected in the host morphology. Many hosts, such as the shallow-water solemyid clams and deep-sea bathymodiolin mussels, have reduced digestive systems compared with close relatives that do not have symbionts [38]. Some, such as the vestimentiferan and pogonophoran tube worms do not have a functional gut at all in the adult stage [38]. The role of ectosymbiotic bacteria in host nutrition is difficult to demonstrate based on their morphology, as there is no host known that has ectosymbionts and a reduced digestive system.

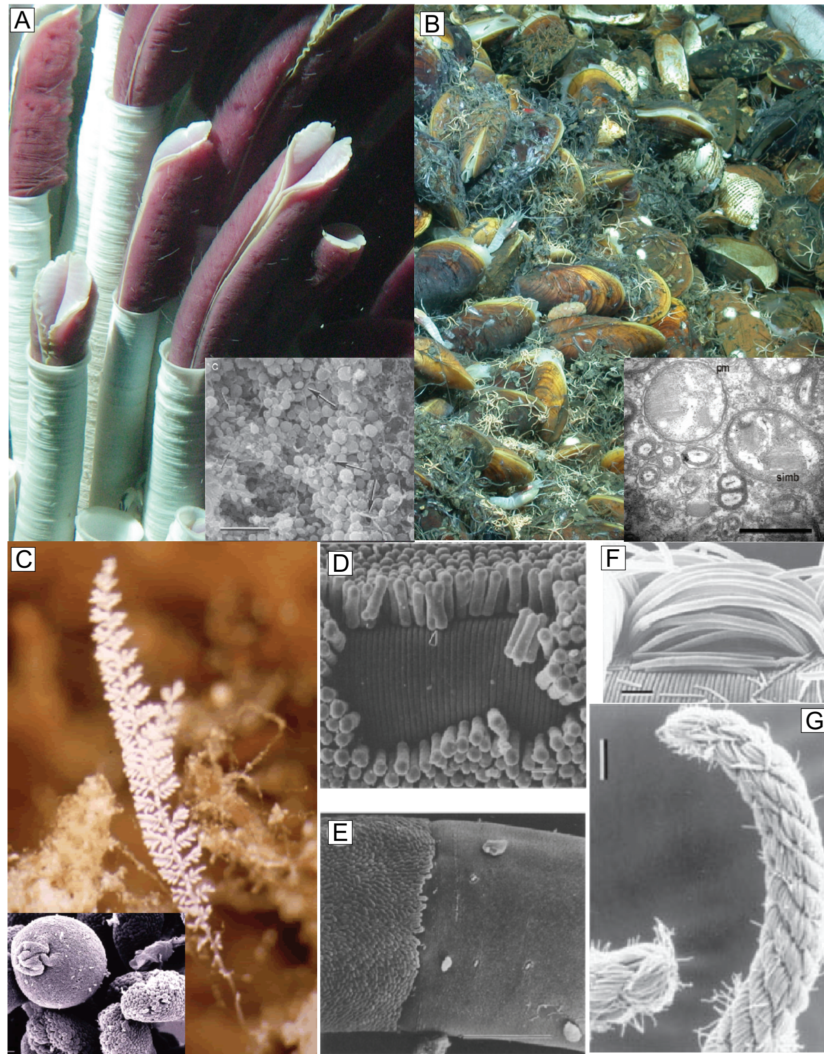


Figure 3.1: **Examples of eukaryote hosts of chemosynthetic bacterial symbionts.** A. The giant tubeworm *Riftia pachyptila* from the East Pacific Rise (http://www.unbsj.ca/sase/biology/huntlab/tubewormclose.JPG) hosts sulfur-oxidizing symbionts *(insert)* in its trophosome [100]. B. The mussel *Bathymodiolus puteoserpentis* from the Logatchev hydrothermal vent field on the MAR (photograph coyright Marum, University of Bremen) hosts methane-oxidizing (*insert*, large morphotype) and sulfur-oxidizing (*insert*, small morphotype) symbionts in the gills [101]. C. Cells of the colonial ciliate *Zoothamnium niveum* are coated with sulfur-oxidizing ectosymbionts (M. Bright, http://www.hydrothermalvent.com) D.-G. Examples of the ectosymbiont coat found on the stilbonematinid worms *Laxus oneistus* (D., [102]), *Catanema* sp. (E., [103]), and *Eubostrichus* cf. *parasitiferus* (F. & G., [103]).

Table 3.1: **Eukaryotic hosts of chemosynthetic symbionts**[a]

Phylum	**Subgroups**[b]	**Host Genus/Genera**[c]	**Common name**	**Type**[d]	**Location of symbionts**	**Habitat**[e]	**Symbiont metabolism**[f]	**Symbiont phylogeny**[g]	**Refs**
Ciliophora	Oligohymenophora Peritrichida	*Zoothamnium*	Colonial ciliate	Epi	Cell surface	Sh	SOX	Gamma	[95]
Ciliophora	Polyhymenophora Heterotrichida	*Folliculinopsis*	Blue-mat ciliate	Epi & Endo	Cell surface & Cytoplasm	V	?	?	[104]
Ciliophora	Karyorelictea	*Kentrophoros*	Free-living ciliate	Epi & Endo	Cell surface & Cytoplasm	Sh	?	?	[105, 106]
Porifera	Demospongiae Cladorhizidae	*Cladorhiza*	Sponge	Endo	Mesohyl, intracellular and extracellular	S	MOX	?	[107, 108]
Platyhelminthes	Catenulida Retronectidae	*Paracatenula*	Mouthless flatworm	Endo	Trophosome, intracellular	Sh	SOX		[109, 110]
Nematoda	Desmodorida Stilbonematinae	*Stilbonema Laxus*	Nematode worm	Epi	Cuticle	Sh	SOX	Gamma	[98, 111]
Nematoda	Monhysterida Siphonolaimidae	*Astomonema*	Mouthless nematode	Endo	Gut lumen	Sh	SOX	Gamma	[111, 112]
Mollusca	Aplacophora Simrothiellidae	*Helicoradomenia*	Worm mollusc	Epi & Endo	Sclerites and mantle cavity	V	?	?	[113]
Mollusca	Bivalvia Solemyidae	*Solemya Acharax*	Awning clam	Endo	Gill, intracellular	V, S, Wo, Sh	SOX	Gamma	[38, 114–116]
Mollusca	Bivalvia Lucinidae	*Lucina Codakia*	Clam	Endo	Gill, intracellular	V, S, Sh	SOX	Gamma	[117–120]
Mollusca	Bivalvia Thyasiridae	*Thyasira Maorithyas*	Clam	Endo	Gill, extra- and intracellular,	V, S, Wh, Sh	SOX	Gamma	[121–123]

Continued on next page. . .

Table 3.1 – continued

Phylum	Subgroups	Host Genus/Genera	Common name	Type	Location of symbionts	Habitat	Symbiont metabolism	Symbiont phylogeny	Refs
Mollusca	Bivalvia Vesicomyidae	*Calyptogena Vesicomya*	Clam	Endo	Gill, intracellular	V, S, Wh	SOX	Gamma	[124–127]
Mollusca	Bivalvia Bathymodiolinae	*Bathymodiolus Idas*	Mussel	Epi & Endo	Gill, extra- and intracellular	V, S, Wh, Wo	SOX & MOX	Gamma	[92, 101, 114, 128, 129]
Mollusca	Gastropoda Provannidae	*Alviniconcha Ifremeria*	Snail	Endo	Gill, intracellular	V	SOX & MOX	Gamma & Alpha & Epsilon	[130–134]
Mollusca	Gastropoda Lepetodrilinae	*Lepetodrilus*	Limpet	Epi	Gill	V	SOX	Gamma	[135]
Mollusca	Gastropoda Peltospiridae	*Crysomallon*	Scaly snail	Epi	Dermal sclerites	V	?	Epsilon, Gamma	[136]
				Endo	Oesophageal gland, intracellular		?	Gamma	
Annelida	Polychaeta Terebellida	*Alvinella*	Pompeii worm	Epi	Integument	V	SOX	Epsilon	[93, 137–139]
Annelida	Polychaeta Vestimentifera	*Riftia Lamellibrachia Escarpia*	Tubeworm	Endo	Trophosome, intracellular	V, S, Wh, Wo	SOX	Gamma	[7, 16, 93, 140–142]
Annelida	Polychaeta Monilifera	*Sclerolinum*	Tubeworm	Endo	Trophosome, intracellular	V, S, Wo	SOX	Gamma	[93, 141, 143, 144]
Annelida	Polychaeta Frenulata	*Siboglinum Oligobrachia*	Beard worm	Endo	Trophosome, intracellular	V, S, Wo, Sh	SOX & MOX	Gamma	[93, 141, 143, 145, 146]

Continued on next page. . .

Table 3.1 – continued

Phylum	Subgroups	Host Genus/Genera	Common name	Type	Location of symbionts	Habitat	Symbiont metabolism	Symbiont phylogeny	Refs
Annelida	Polychaeta *incertae sedis*	*Osedax*	Bone-eating worm	Endo	Root ovisac, intracellular	Wh	HET	Gamma	[147–149]
Annelida	Clitellata Phallodrilinae	*Olavius Inanidrilus*	Gutless oligochaete	Endo	Subcuticular, extracellular	Sh	SOX & SRB	Gamma and Delta	[150–153]
Annelida	Clitellata Tubificinae	*Tubificoides*	Sludge worm	Epi		Sh	SOX	Gamma & Epsilon	[93, 154]
Arthropoda	Decapoda Alvinocarididae	*Rimicaris*	Shrimp	Epi	Gill chamber	V	SOX	Gamma & Epsilon	[155–157]
Arthropoda	Decapoda Galatheoidea	*Kiwa*	Yeti crab	Epi	Bacteriophore setae	V	SOX	Gamma & Epsilon	[158]
Arthropoda	Cirripedia Eolepadidae	*Vulcanolepas*	Barnacle	Epi	Cirral setae	V	?	Gamma & Epsilon	[159, 160]

[a]Table modified from [32] [b]The taxonomy of many of these hosts is still under debate, therefore, the term subgroup is used here [c]These are some examples [d]Episymbiotic on the outside of the host or Endosymbiotic in the host tissues [e]Sh = shallow-water, V = hydrothermal vent, S = cold seep, Wh = whale fall, Wo = wood fall [f]SOX = sulfur-oxidizing symbiont, MOX = methane oxidizing symbiont, SRB = sulfate-reducing bacteria, HET = heterotrophic symbiont (although not strictly chemosynthetic, the heterotrophic symbionts of *Osedax* worms are included here, as these hosts are closely related to hosts that have chemosynthetic symbionts), ? = symbiont metabolism currently unknown [g]Alpha = alphaproteobacteria, Gamma = gammaproteobacteria, Delta = deltaproteobacteria, Epsilon = epsilonproteobacteria

3.1 Marine invertebrate hosts with endosymbionts

Different organs and tissues have evolved to house chemosynthetic endosymbionts in the various host phyla (Table 3.1). Siboglinid tube worms, a group that includes the hydrothermal vent and cold seep vestimentiferans, house their symbionts in a specialized organ called the trophosome [32, 38, 93]. In gutless oligochaetes, the symbiotic bacteria form a thick layer just below the cuticle [151]. In contrast, symbiotic bacteria are found in the gills of all adult bivalve hosts described to date. The symbionts of provannid snails from hydrothermal vents in the Pacific are also housed within the gills [161], but the scaly foot snail from vents in the Indian Ocean shows a novel structural morphology, housing its symbionts in an enlarged esophageal gland [136]. I will use two examples of well-studied symbioses to illustrate the very different ways in which these symbionts and hosts have adapted to the chemosynthetic lifestyle.

Riftia pachyptila

R. pachyptila is one of the dominant and most spectacular members of hydrothermal vent communities on the East Pacific Rise. It can grow up to 2 m in length, and has no mouth or gut. It was also the first animal in which chemosynthetic symbioses were discovered [30, 31]. These worms rely entirely on sulfur-oxidizing gammaproteobacterial symbionts that reside densely packed in the trophosome of the host, a specialized organ that extends throughout the entire length of the worm's body, sometimes making up to 16% of the host's total wet weight [93, 162]. The trophosome is not directly exposed to the outside environment. To provide the symbionts with their nutritional needs, *Riftia* takes up O_2, ${NO_3}^{2-}$ and HS^- from seawater and hydrothermal fluids through its plume, and transported, bound to a modified hemoglobin, via the vascular system to the trophosome (reviewed in [32, 38]). Inorganic carbon is also delivered to the symbionts, dissolved as bicarbonate ions in the slightly alkaline host blood. The passive uptake of inorganic carbon from fluids that bathe the plume is driven by a concentration gradient from externally high CO_2 to internally low CO_2, since at

the external pH of about 6, most inorganic carbon is in the form of CO_2, and in the slightly alkaline host blood, is mostly in the form of bicarbonate [163, 164]. Carbonic anhydrase re-converts the bicarbonate in the blood to CO_2 in the trophosome tissue, feeding the symbiotic bacteria with their source of carbon [165–167].

A recent study by Nussbaumer et al. refuted two of the long-held assumptions about the *R. pachyptila* symbiosis [142]. The first assumption was that juvenile worms take up their symbionts horizontally from the environment by feeding (see boxed text Symbiont Transmission). This was thought to be the case, because juvenile worms have no symbionts, but do have a functional mouth and gut [168]. The second assumption was that the trophosome develops from gut tissue after the symbionts have been taken up [169]. Nussbaumer et al. collected juvenile *R. pachyptila* in varying stages of development by placing colonization devices near adult worms. They showed that instead of entering through the mouth and gut, the symbionts colonize juvenile worms through the skin. After colonizing the worm, the symbionts migrate to the mesodermal tissue which develops into the trophosome [142]. This study was a major breakthrough in understanding host-symbiont interactions and colonization processes in a symbiosis for which no experimental models are available for laboratory manipulation.

Symbiont transmission. To maintain a symbiotic association, the symbionts must be transferred from one host generation to the next, termed transmission. Transmission can be either vertical, from parent to offspring, or horizontal, where aposymbiotic larvae or juveniles take up the symbionts from the surrounding environment, or from co-occurring hosts. Vertical transmission can be shown by the presence of the symbionts in host reproductive tissues, such as the oocytes of vesicomyid clams [170, 171], or the genital pads of gutless oligochaetes [172]. The mode of transmission affects the evolution of the symbiosis, and symbiont genomes and phylogenies can bear the mark of their mode of transmission. Congruence of host and symbiont phylogenies indicates that they have a shared evolutionary history (co-speciation), providing evidence for a vertical mode of transmission (for example, [173, 174]). By the same logic, incongruence of host and symbiont phylogenies indicates that host switching events have occurred, providing evidence for a horizontal mode of transmission (for example, [175, 176]). There are exceptions to this such as the 'leaky' vertical transmission of vesicomyid symbionts, where the host cytochrome oxidase I (COI) and symbiont 16S rRNA phylogenies are largely congruent, but show evidence for rare host-switching events [177]. In addition, horizontally transmitted symbioses are known that do show symbiont-host cospeciation, such as the squid-bioluminescent *Vibrio* symbiosis [178].

Vertically transmitted symbionts undergo their entire life history within a host, and are disconnected from free-living populations. In addition, they also experience population bottlenecks as only a few symbiont cells are passed from one generation to the next [179]. The result of this is the fixation of slightly deleterious mutations and gene loss, which cannot be reversed by recombination with wild-types. Vertically transmitted symbionts therefore have typically reduced genomes, and a higher mutation rate at non-neutral sites, than their free-living relatives [38, 180–183]. They can, in effect, 'streamline' their genomes, as the genes required for survival in the environment are no longer necessary, and can be eliminated.

Horizontal transmission has been termed 'risky', since juvenile hosts rely on coming into contact with their symbionts, which effectively limits the habitats they can colonize to those where the free-living stages of their symbionts are also found [38, 184]. However, horizontal transmission might have the advantage of limiting the degradation of symbiont genomes since there is a large environmental population that can freely recombine, giving them access to a larger gene pool. In addition, it might allow hosts to take up locally adapted symbionts, which has been hypothesized for hydrothermal vent symbioses [184, 185].

***Bathymodiolus* mussels**

Bathymodiolus spp. mussels dominate the biomass at hydrothermal vents and cold seeps world-wide. The first species investigated was *Bathymodiolus thermophilus* from the East Pacific Rise, which harbors chemoautotrophic sulfur-oxidizing symbionts [186]. The first methanotrophic symbiosis was later found in *B. brooksi* from cold seeps in the Gulf of Mexico [187,188], and this was followed by the discovery of mussels hosting both methane-oxidizing and sulfur-oxidizing symbionts [189, 190]. Such a dual symbiosis, where two distinct symbiont types co-occur in host cells was previously unknown to occur in animal-bacteria interactions (see Section 4.3).

The symbionts are housed in specialized gill bacteriocytes in all species described to date except one (see below). Unlike the *Riftia* tubeworms, there are no known carrier proteins in the mussel hemolymph for transporting sulfide or methane. The symbionts are therefore reliant on the diffusion of electron donors and acceptors across host and symbiont membranes [191]. Their location is probably a reflection of this, as the gill is an organ specialized for gas exchange, with a large surface area exposed to the outside environment. The nutritional reliance of *Bathymodiolus* mussels on their chemosynthetic symbionts is reflected in their anatomy. They have a highly reduced gut [192], and cannot filter-feed as effectively as related mussels that do not have symbionts [193]. In addition, mussels held in aquaria that have lost their endosymbionts appear sickly [194], and mussels removed from their hydrothermal energy sources lose most of their symbionts after a period of 10 days, and have thin, watery gills (Unpublished results, Dennis Fink, Christian Borowski, Nicole Dubilier, Symbiosis Group, MPI Bremen). These observations indicate that the symbiosis is likely obligate for the host. The symbionts, however, most probably have a free-living stage outside of the host, as genetic and morphological evidence suggests that they are horizontaly transmitted [185, 194, 195].

The intracellular location of the symbiotic bacteria appears to be conserved in all *Bathymodiolus* species except one, *Bathymodiolus* sp. from the Juan de Fuca Ridge hydrothermal vent field. This species might host its

chemoautotrophic symbionts as epibionts attached to the gill filaments, but this needs to be confirmed as the original material investigated by transmission electron microscopy (TEM) was not well preserved [196]. In a recent molecular analysis of bathymodiolin phylogeny, the Juan de Fuca species appears basal to all other bathymodiolin host species [92]. This could indication that ectosymbioses are basal in this host group, and might represent a primitive state, a precursor to a more intimate endosymbiosis. However, other recent analyses show that epibiotic symbiont location is not basal in either host or symbiont phylogenies in this and other host groups, arguing against a more primitive status for ectosymbioses in the bathymodiolin hosts [32].

3.2 Marine invertebrate hosts with ectosymbionts

Chemosynthetic ectosymbionts have been found on ciliates at hydrothermal vents and in shallow marine environments, on nematode and annelid worms in shallow marine sediments, and at hydrothermal vents on the scaly snail *Crysomallon squamiferum*, the shrimp *Rimicaris exoculata*, the Yeti crab *Kiwa hirsuta*, the Pompeii worm *Alvinella pompejana*, and *Vulcanolepas* barnacles (Table 3.1). Unlike animals that host chemosynthetic endosymbiotic bacteria, the role of these ectosymbionts has been difficult to demonstrate. In most cases, a nutritional role has been presumed by analogy with the chemosynthetic endosymbioses, but direct evidence supporting this is often lacking. Alternative roles have been proposed, such as sulfide detoxification [197]. Although the role of the symbionts is unclear, it is likely that the hosts derive some benefit by asoociating with ecotsymbiotic bacteria, as animals that host ectosymionts also show morphological and behavioral adaptations to the symbiosis that are as varied as those found in endosymbioses (reviewed in [111]). In the following sections, I will illustrate these adaptations with two examples, the colonial ciliate *Zoothamnium niveum*, and the stilbonematinid worms.

Zoothamnium niveum

The peritrich ciliate *Zoothamnium niveum* forms sedentary feather-shaped colonies that contain up to 3000 single ciliate cells called microzooids [111]. Colonies grow attached to surfaces in sea grass beds or mangrove peat where sulfide is formed from the breakdown of organic matter. Each microzooid is covered with a single layer of epibiotic sulfide-oxidizing bacteria [95,198,199]. The ciliate can be cultured in the lab, and its life cycle has been followed over several generations. In addition, aposymbiotic *Z. niveum* can be cultured, but symbiont-free colonies reach a maximum of 10% of the size of colonies with symbionts, indicating a substantial contribution to host nutrition [200]. *Z. niveum* symbionts are vertically transmitted. Swarmer cells, the ciliate dispersal stages, already have a coating of ectosymbionts when they leave an established colony [198].

Ott et al. suggested that *Z. niveum* has developed a behavioral adaptation to provide its symbionts with both sulfide and oxygen. According to their hypothesis, colonies regularly contract towards the attachment surface, into the sulfide-rich diffusive boundary layer, then extend slowly outwards again into the oxygenated seawater [200]. However, a recent study showed that this is probably not the case [201]. These contractions actually result in fluid movement towards the base of the colony, bathing the entire colony in fully oxygenated seawater. These authors therefore modelled the sulfide budget of the symbiosis, and determined that the sulfide flux from the ambient seawater surrounding the colonies would be enough to provide the symbiosis with energy, even at sulfide concentrations of a few µM. Hydrodynamic modelling showed that the unique shape of *Z. niveum* (Figure 3.1) enhances the current flowing through the colony, which is created by the movement of host cilia. This is a nice example of cooperation between host and symbiont. The morphology and behavior of the ciliate host is optimized to provide their symbionts with sulfide and oxygen. The bacterial symbionts also benefit from the association. Sulfide flux to the ciliate-associated bacteria is estimated to be 100 times greater than to free-living bacteria attached to a solid surface in the same environment [201]. The bacteria can therefore afford to 'feed'

their host by passing on chemosynthetically fixed organic carbon.

Stilbonematinid worms

Stilbonematinid nematodes are distributed worldwide, and are found in shallow porous sediments where an oxidized layer of sediment overlies a reduced, sulfidic layer [111]. The worms gain their nutrition by grazing on epibionts that form a coat on the outside surface of the cuticle [103, 202]. They provide the chemoautotrophic ectosymbionts with alternating access to sulfide and oxygen as they migrate through the sediment between the reduced and oxidized layers [203, 204]. The life cycle of the worms includes a number of moulting stages, during which they lose their bacterial coat. There is no evidence for vertical transmission of the symbionts of stilbonematinid worms. Therefore, to maintain the symbiosis, the host must be able to take up its symbiont from an environmental pool of free-living symbionts after hatching, and again after each moult. Since aposymbiotic worms are rarely found in field collections, the recolonization of the cuticle after moulting must be extremely rapid [111]. The highly specific nature of these associations, where sometimes only a single symbiont phylotype is present on the cuticle surface, indicates that the host and symbiont can recognise each other. The recognition and attachment mechanism has been shown to be due to carbohydrate-binding proteins called lectins expressed by the host (see boxed text on lectins) [96,97]. These can recognize specific carbohydrate conformations on the bacterial cell surface in the extracellular matrix.

Lectins. The name lectin is derived from the latin '*legere*', which means 'to select'. Lectins are carbohydrate-binding proteins that recognise and bind specific sugar conformations with high specificity and affinity (for reviews see [205–207]). They are present in all living organisms, and even some viruses [208]. Lectins play a crucial role in cell-cell interactions, and are well-studied in pathogenesis. In some interactions, the lectin is expressed on the pathogen surface, and recognises carbohydrates on target cells, such as in *Helicobacter pylori* infections in the human gastrointestinal tract [209], and in others, the lectin is expressed by the host cells, such as the invasion of human dendritic cells by the human immunodeficiency virus (HIV) [210]. The role of lectins in beneficial associations was demonstrated in plants as early as the 1980s [211], but its role in marine invertebrate symbioses has only recently been recognised. Nussbaumer et al. showed that a mannose-binding lectin is involved in ectosymbiont attachment in two species of stilbonematinid worms, and hypothesized that this could be an important mechanism mediating the specificity of the association [97]. Bulgheresi et al. continued this work, showing that a c-type lectin was expressed in the mucous that is excreted onto the posterior end of the worm, where the ectosymbionts attach. Mucous is also secreted onto the worm's anterior end, but here, the lectin is not present, and the symbionts do not bind to this region. In addition, a recombinant form of this lectin could bind the symbionts in a seawater medium, inducing their aggregation [96]. Although there are possibly more factors involved in host symbiont recognition, it seems likely that lectins play a role in determining the specificity of symbiotic associations, as carbohydrates have an immense potential for structural diversity, which exceeds that of linear nucleic acids or proteins. This would allow hosts to recognise their symbiont out of a myriad of diverse free-living microorganisms.

Chapter 4

Diversity of microbes

To this day, all attempts to isolate chemosynthetic symbionts in pure culture have failed. Culture-independent methods for microbial ecology, pioneered in the 1980s [212, 213], have been invaluable in describing the diversity of

chemosynthetic microbes. Early molecular studies of chemosynthetic symbioses showed that they were extremely specific, identifying a single symbiont species within each host species [214]. However, as more hosts were investigated, and the sinking costs of sequencing allowed more clones to be sequenced from individual hosts, a greater symbiont diversity was revealed [32]. Most chemosynthetic symbionts described to date belong to the Gammaproteobacteria, and have been classified into two functional groups, the sulfur-oxidizing and methane-oxidizing symbionts. Sulfur-oxidizing symbionts from the epsilonproteobacteria have also been described. The following sections describe the phylogenetic diversity within these two functional groups.

4.1 Sulfur-oxidizing symbionts

Analysis of the >100 currently available 16S ribosomal RNA sequences from the gammaproteobacterial sulfur-oxidizing symbionts shows that they fall into at least nine distinct clades, interspersed by sequences from free-living bacteria [32]. Symbiotic associations between marine invertebrates and sulfur-oxidizing gammaproteobacteria have therefore been established multiple times and in multiple bacterial groups. For example, the Gamma 1 endosymbionts of gutless oligochaetes, the endosymbionts of *Astomonema*, and the ectosymbionts of *Laxus* and *Robbea*, share a common ancestor with the free-living anoxygenic phototrophic sulfur oxidizers *Allochromatium vinosum* and *Thiocapsa roseopersicina*. The endosymbionts of the vent snail *Alvinichoncha* sp. type 1 and the cold seep clam *Maorithyas hadalis*, along with free-living sediment clones, form a sister clade to the free-living sulfur-oxidizing *Thiomicrospira* spp.. There is no obvious grouping of gammaproteobacterial sulfur-oxidizing symbionts within each clade based on either host phylum, symbiont location (endo- or ectosymbiotic), habitat type (hydrothermal vent, cold seep, whale fall, or shallow sediment), depth, or geographic location. This indicates that the evolution of chemoautotrophic symbioses is highly complex and is probably influenced by many differnt factors [32, 38].

Epsilonproteobacterial symbionts have also been described from a number of different host phyla. Like many of the gammaproteobacterial sym-

bionts, a number of epsilonproteobacterial symbionts are hypothesized to be sulfur-oxidizers based on phylogenetic relatedness to cultured sulfur-oxidizing strains (for example, [134, 157]). However, in most cases, there is no direct evidence for a sulfur-oxidizing metabolism. Indeed, the Epsilonproteobacteria is a relatively poorly characterized group, which contains few cultured strains [215]. Ectosymbiotic epsilonproteobacteria associate with the a number of hydrothermal vent animals including the shrimp *Rimicaris exoculata* (see Section 5.3), the polychaete worm *Alvinella pompejana*, the snail *Crysomallon squamiferum*, the crab *Kiwa hirsuta*, and the barnacle *Vulcanolepas osheai* (Table 3.1). Recently, the first shallow-water epsilonproteobacterial symbionts were found, on the sludge worm *Tubificoides benedii* from tidal mudflats in the Wadden Sea [154]. The only currently described endosymbiotic epsilonproteobacteria are found in the *Alviniconcha* snails from hydrothermal vents in Pacific back-arc basins [134, 216] and the Central Indian Ridge [132].

Many hosts harbor only a single symbiont 16S rRNA phylotype. Some symbiont 16S rRNA sequences are shared by closely related hosts. Examples include the vestimentiferan tube worms [175, 176], some lucinid clams [117, 217, 218], and *B. azoricus* and *B. puteoserpentis* from the Northern Mid-Atlantic Ridge [101]. This does not necessarily indicate that identical symbionts are shared by these different host species, as the 16S rRNA gene might be highly conserved in these symbiont groups, and analysis of faster-evolving markers or whole genomes might identify differences that are not apparent at the level of the 16S rRNA gene. Indeed, analyses of internal transcribed spacer (ITS) sequences from the sulfur-oxidizing symbionts of *Bathmodiolus* mussels from the Northern MAR showed differences at this level of resolution that were correlated with host geography [185, 195]. In contrast, fingerprinting of the vestimentiferan symbionts by PCR amplification of repetitive elements revealed differences that were correlated with host species [219].

4.2 Methane-oxidizing symbionts

In contrast to the sulfur-oxidizing symbionts, all methane-oxidizing symbionts from marine invertebrates form a monophyletic group [22,32,38]. This group is a sister clade to gammaproteobacterial type I methanotrophs of the genera *Methylomonas*, *Methylobacter*, and *Methylomicrobium*. This is consistent with morphological evidence from TEM studies, which shows the presence of bacteria with internal membrane structures typical of type I methanotrophs in these animals. However, the only 16S rRNA sequences currently published are from the methanotrophic symbionts of *Bathymodiolus* mussels. It is possible that additional lineages of symbiotic methane-oxidizing bacteria will be discovered as more symbioses are investigated with molecular methods.

4.3 Multiple co-occurring symbionts

Multiple co-occurring symbionts were first discovered in *Bathymodiolus* mussels from cold seeps in the Gulf of Mexico and vents on the Mid-Atlantic Ridge. In these mussels, both sulfur-oxidizing and methane-oxidizing symbionts coexist in single host cells, which was previously unknown to occur in any metazoan host [189, 220]. Prior to this discovery, the absence of multiple symbioses was explained by the negative effects of competition between symbionts for space and resources within the host. However, by associating with metabolically diverse symbionts, these mussels have evolved to take advantage of multiple sources of energy, thus partitioning resources among distinct symbiont populations. Since this first discovery, even more diverse associations have been identified. The cold seep mussel *Bathymodiolus heckerae* from the Gulf of Mexico hosts four distinct symbiont phylotypes, two related to sulfur-oxidizing symbionts, one to methane-oxidizing symbionts, and one related to free-living methylotrophs [221]. Six bacterial phylotypes were found in *Idas* mussels from a cold seep in the Mediterranean, four from the same phylogenetic groups as the *B. heckerae* symbionts, one related to heterotrophic free-living Bacteroidetes, and one from a novel gammaproteobac-

terial lineage with no other symbiotic members [128]. Gutless oligochaetes host a symbiotic consortium, with sulfide-oxidizing and sulfate-reducing bacteria [222]. These are involved in an internal sulfur cycle, which has allowed the host to colonize habitats with extremely low sulfide concentrations, in the nanomolar range. In addition, alphaproteobacterial and spirochaete phylotypes have been detected in these hosts, but their functional role is still unknown [151].

4.4 Genomics and proteomics of chemosynthetic symbionts

The recent application of genomics and metagenomics to chemosynthetic symbiotic bacteria has already provided many new insights into symbiont metabolism, host-symbiont interactions, and symbiont evolution. The first chemosynthetic symbiont genomes were from a metagenomic study of the gutless oligochaete *Olavius algarvensis* symbiont consortium [153]. This study identified the chemoautotrophic metabolism of the four symbionts that make up this consortium, showing that the Delta 1 and Delta 4 symbionts are sulfate reducers with the genetic potential for both heterotrophy and autotrophy, and the Gamma 1 and Gamma 3 symbionts are chemoautotrophic sulfide oxidizers. The sulfate reducers and sulfide oxidizers have a syntrophic relationship, exchanging reduced and oxidized sulfur compounds. The ability of all four symbionts to fix CO_2 autotrophically provides the host with multiple sources of carbon. One unique feature of *O. algarvensis* compared to other annelid worms is its lack of nephridia, the organs responsible for removing nitrogenous waste compounds [151]. The metagenome study revealed how this organ loss was made possible, as the symbiont genomes contain a number of genes involved in uptake and breakdown of waste products from host metabolism. The symbionts have therefore taken over the role that was previously played by these organs.

Recent metagenomic and metaproteomic studies of the endosymbiont of *R. pachyptila*, called *Candidatus* Endoriftia persephone, have revealed the use of a previously unsuspected carbon fixation pathway that contributes to the nutrition of the symbiosis under energy-limiting conditions [7, 16]. The

reductive TCA cycle was assumed to be present exclusively in members of the Epsilonproteobacteria, and some members of the Archaea and Aquificales (Section 1.2). The expression of the key enzymes of this pathway was therefore one of the surprising results of the proteomic study of *E. persephone*. Proteomics can provide quantitative information about protein expression, and intriguingly, the enzymes involved in the rTCA cycle were much more abundant in the symbionts that had less energy available in the form of stored internal sulfur. Because the rTCA cycle requires less energy than the CBB cycle, these authors hypothesized that the symbionts can regulate their carbon fixation pathway based on energy availability. The metabolic flexability of *E. persephone* was shown by the presence of genes for nitrate respiration, which is consistent with earlier findings from physiological studies that the *R. pachyptila* hemoglobin can also bind nitrate [223].

Genomic analyses of the endosymbionts from four vesicomyid clam species from hydrothermal vents and colds seeps showed how these symbionts have adapted to an intracellular lifestyle, and how the environment of the host can play a role in symbiont evolution. Newton et al. recently published a comparison of four endosymbiont genomes from hydrothermal vent and cold seep vesicomyid clams [224]. Two of these were whole genome sequences, and the gene content of a further two symbionts was investigated by microarray hybridization. Consistent with findings from genomic studies of vertically-transmitted insect symbionts [182], the vesicomyid chemoautotrophic symbionts have small genomes with an AT-bias, and have lost genes involved in DNA recombination and repair. A number of adaptations to the intracellular lifestyle are apparent in the two genome sequences, such as the loss of genes for FtsZ-based cell division [225]. The *C. okutanii* symbiont even appears to have lost the genes necessary for peptidoglycan synthesis [125]. While the *C. okutanii* symbiont has all genes necessary for nitrate respiration (encoded in the *nar* operon, most of these have been lost in *Ruthia magnifica*. Findings such as these give us invaluable insights into the selective pressures experienced by chemosynthetic symbionts in different habitats. The benefit to *R. magnifica* of being able to respire nitrate apparently does not outweigh the costs of maintaining the *nar* operon.

Chapter 5

Rimicaris exoculata

The discovery of the first hydrothermal vent the Atlantic ocean in 1985 revealed animal communities unlike anything that had been explored untill then [226]. Vent communites in the Eastern Pacific, discovered less than 10 years earlier, were dominated by sessile fauna such as giant tubeworms, vesicomyid clams, and mytilid mussels. In contrast, the TAG vent field on the Mid-Atlantic Ridge was dominated by giant swarms of highly motile shrimp. These shrimp were named *Rimicaris exoculata*, reflecting their habitat on the MAR (‘*rima*’ = rift, crack, or fissure), and their unique morphological characters (from the Latin ‘*exoculo*’ = to deprive of eyes) [227]. The first researchers to descibe this novel species saw that the normal shrimp eyes and eystalks were completely absent [227]. Instead, *R. exoculata* has a pair of large anterior organs (Figure 5.3) that contain a visual pigment, and are connected to the brain by a nerve trunk [228]. They lack image-forming optics such as a lens structure; the shrimp therefore cannot ‘see’ with these highly-modified eyes. Van Dover et al. proposed that this might be an adaptation to the dark vent environment, and the rhodopsin-like pigment could be used by the shrimp to detect thermal radiation emitted by the hot vents, directing them to their source of food and energy [228]. However, light produced by a black smoker is mostly in the range of 750 to 1050 nm [229], and the maximum absorbtion of the *R. exoculata* rhodopsin peaks at 500 nm [228]. The role of the modified eyes of *R. exoculata* is therefore still a matter of debate.

R. exoculata belongs to the vent- and seep-endemic family Alvinocarididae [230] (Figure 5.2). The Alvinocarididae is thought to have diverged not earlier than 20 Mya based on a molecular clock approach using divergence rates estimated for other shrimp species [231]. This group therefore represents the most recent vent and seep divergence event [231]. Older vent- and

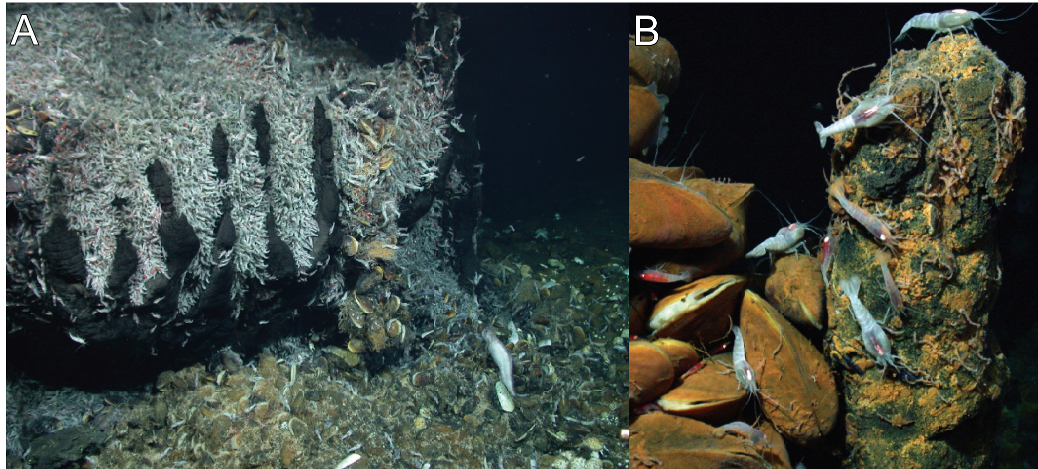


Figure 5.1: **The habitat of *Rimicaris exoculata*.** A. Swarm of shrimp on the 'Irina II' chimney structure at the Logatchev hydrothermal vent field. B. A closer view of the shrimp, with the co-occurring mussel *Bathymodiolus puteoserpentis*. Photographs copyright Woods Hole Oceanographic Institution.

seep-endemic groups include, for example, the vestimentiferan tube worms (100 Mya, [232]), the vesicomyid clams (50 Mya, [233]), and the bathymodiolin mussels (40 Mya, [234]).

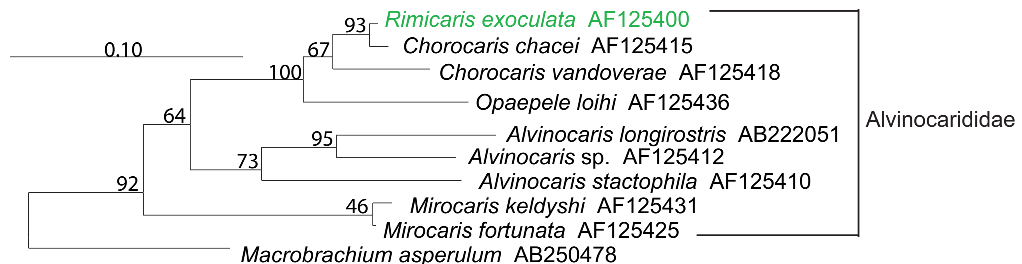


Figure 5.2: **Cytochrome oxidase I phylogeny of the Alvinocarididae.** The closest relative of *Rimicaris exoculata* (green) is the vent shrimp *Chorocaris chacei*. This relationship is confirmed by morphological taxonomy [230]. Tree was calculated by maximum likelihood in ARB [235], with 100 bootstrap resamplings.

5.1 Distribution

Since the discovery of *R. exoculata* at TAG, shrimp belonging to this species have been found at five other vent fields on the Mid-Atlantic Ridge, at Rainbow, Broken Spur, Snake Pit, Logatchev, and South MAR (Figure 5.3), at depths between 2300 m and 3900 m (reviewed in [155]). *R. exoculata* might also be found at the Lucky Strike vent field at 1700 m depth [231], but some reports dispute this. A related species, *R. kairei* is found at two vent fields on the Central Indian Ridge [236–238].

Figure 5.3: **Distribution of *Rimicaris exoculata* on the Mid-Atlantic Ridge.** This species has been found at two ultramafic-hosted vent fields, Rainbow and Logatchev (yellow dots), four basalt-hosted vent fields, Broken Spur, TAG, Snake Pit, and South MAR (red dots), and may also be a part of the faunal community at the ultramafic-hosted Lucky Strike vent field.

5.2 Reproduction and dispersal

The ability to disperse large distances is essential for the survival of hydrothermal vent fauna [239]. Their habitats are 'chemical islands' dispersed

along the vast system of oceanic spreading ridges, and can be short-lived over evolutionary time. The presence of *R. exoculata* shrimp at distant vent fields on the MAR led researchers to conclude that it has a high dispersal capability. This was supported by the work of Creasey et al., who compared allozyme data from *R. exoculata* populations from TAG and Broken Spur, concluding that shrimp from these two sites are conspecific, and that the geographic distance between them (380 km) does not prevent gene flow [240]. Morphological characteristics seem to confirm these observations for populations from the TAG, Broken Spur, and Logatchev vent fields [241], although morphological plasticity is known for a number of vent faunas including the alvinocaridid shrimp, and has often led to confusion about the taxonomy of these organisms (for example, [232, 242–245]).

Although *R. exoculata* has an actively motile adult stage, larval stages are thought to be the major means of dispersal for this species. *R. exoculata* postlarvae have been collected in plankton trawls between 200 - 1000 m above the sea floor around Broken Spur [246, 247]. These contain orange-pigmented lipid reserves rich in wax esters, which is characteristic of marine zooplankton that experience fluctuations in food availability [248]. Lipid reserves allow these organisms to survive long periods without feeding, and the presence of such reserves in *R. exoculata* larval stages suggests that they can survive a long planktonic existence away from the vent environment, which would allow them to disperse to distant sites. Remarkably, these shrimp larvae also contain fatty acids that are typically produced by phytoplankton, indicating that the early life history stages are planktotrophic [249]. Carbon stable isotopic compositions of *R. exoculata* across a wide size range also indicate a change in trophic strategy, with the smaller size classes reliant on photosynthetically-fixed organic matter, and larger adults showing stable isotope signatures indicative of a chemosynthetic food source [250]. There are therefore a number of indications that *R. exoculata* can disperse long distances. Why its distribution, based on current knowledge, is restricted to a single ocean basin is still unknown. This might represent the geographical limit of its dispersal capabilities.

5.3 Symbiosis in *Rimicaris* shrimp

5.3.1 Role of the symbionts

Researchers were quick to realize that the shrimp were different to many of the major vent fauna known at the time of their discovery. Analysis of the shrimp morphology showed that their digestive system was not obviously reduced like the vent fauna that rely on endosymbiotic bacteria for nutrition [251]. Assays for RuBisCO in the hepatopancreas and abdominal muscle were negative, as were assays for bacterial cell wall components (lipopolysaccharide, LPS) in all tissues assayed, except for the stomach, which contained an estimated 10^9 bacterial cells ml^{-1} [251]. These authors therefore concluded that the shrimp are heterotrophic grazers. They also reported that the shrimp have a dense coating of filamentous epibionts on the setae of mouthparts within the expanded gill chamber, particularly on the exopodite of Maxilliped I and scaphognathite of Maxilla II (Figure 5.4), but could not explain the role of these epibionts. A nutritional role for these epibionts has been proposed by numerous authors [252–255], but direct evidence confirming this remains elusive. In addition, it is unclear how energy could be transferred from the epibionts to the host. Three strategies have been proposed to explain this:

1. Scraping of epibionts with modified feeding appendages, and transfer to the mouth [253]

2. Ingesting exuvae after moulting [255]

3. Transfer of small organic compounds from epibiont to host through the carapace and skin [252].

5.3.2 Metabolism of the symbionts

The metabolism of the shrimp epibionts has not been clearly identified in almost 25 years of research since these animals were discovered. Autotro-

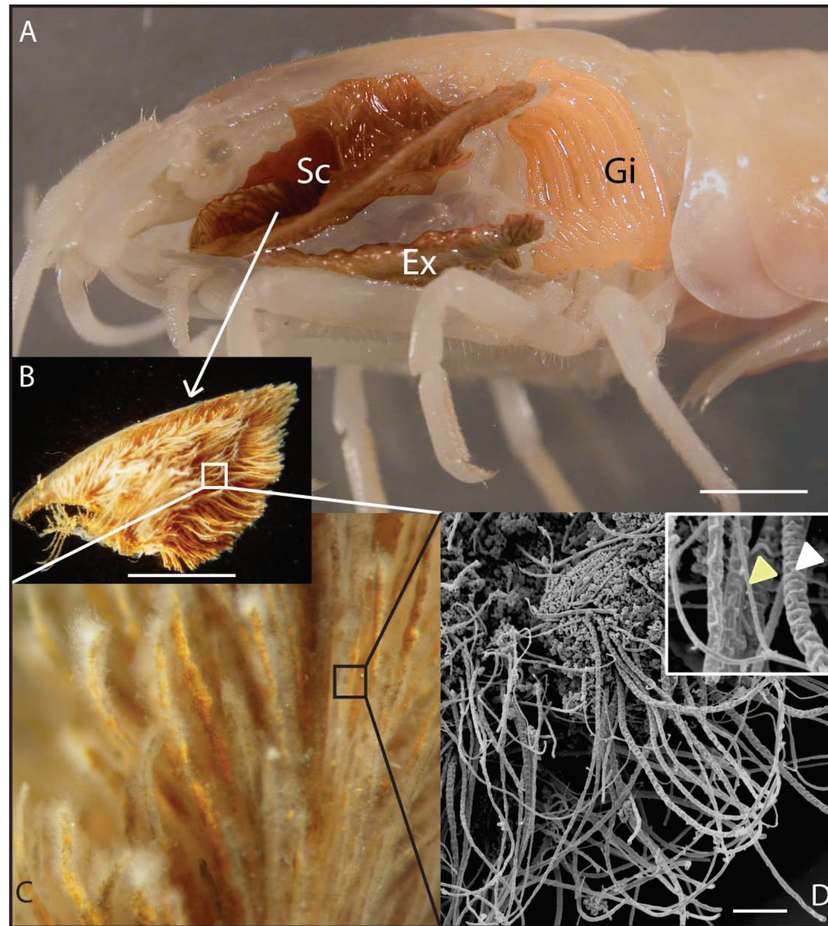


Figure 5.4: **Morphology of the *Rimicaris exoculata* symbiosis.** A. Internal morphology of the gill chamber with the carapace covering removed. The epibionts are attached to the scaphognathite (Sc) and exopodite (Ex). The gills (Gi) can also be seen in this photograph. Scale bar = 5 mm. B. Photograph of the scaphognathite dissected out of the shrimp. Scale bar = 5 mm. C. Closer view of the scaphognathite. The filamentous epibionts can be seen as a white fuzzy material. D. Scanning electron micrograph showing the filamentous epibionts. Scale bar = 10 µm. *Insert:* thin filaments are indicated by a yellow arrowhead, thick filaments by a white arrowhead.

phy has been demonstrated by enzyme assays of RuBisCO [250, 256, 257], and incorporation of inorganic carbon in incubations with radioactively labelled CO_2 [250, 258, 259]. Reduced sulfur compounds have been assumed to fuel the symbiosis [157, 253], but recently, iron oxidation was proposed

as a source of energy for the epibionts on shrimp from the Rainbow vent field [260–262]. Athough free-living iron oxidizing bacteria have been found at hydrothermal vents [263, 264], this would be the first known chemosynthetic symbiosis that uses iron as an energy source. The iron hypothesis is based on a number of observations, mineralogical measurements, and thermodynamic considerations. The epibionts on shrimp from the Rainbow site are closely associated with iron oxide minerals [260]. Spectroscopy revealed that the mineral composition is an amorphous 2-line ferrihydrite, containing mixed valence iron [265]. A direct role of the bacteria in mineral formation is suggested by the close association of the minerals with bacterial cells, but none of the signature structures of iron-oxidizing bacteria, such as iron oxide sheaths or stalks, has been found on the shrimp. Although free-living iron-oxidizing bacteria that produce amorphous minerals are known [266], these minerals can also form on the surfaces of cells that are not using iron as an energy source, as the chemical properties of the cell surface can cause the chemical catalysis of iron oxidation [267]. In a recent study of dual cultures containing one iron-oxidizing strain, it was found that only the organism that cannot grow using iron as an energy source was encrusted with iron oxides under the conditions tested, as it presumably had no mechanisms to avoid encrustation [266]. It has therefore been difficult to prove or disprove that the shrimp epibionts can oxidize the reduced iron from the vent fluids to gain energy for autotrophic carbon fixation.

In addition to dealing with the problem of a highly insoluble end-product (Fe^{2+}), neutrophilic iron oxidizers must also be able to compete with the relatively rapid chemical oxidation of Fe^{2+} by oxygen. Many neutrophilic iron-oxidizing bacteria are therefore restricted to microaerophilic habitats, where oxygen limitation results in a longer half-life of Fe^{2+} in solution, allowing the bacteria to compete effectively with chemical oxidation [268]. Chemical oxidation is also restricted at low temperatures. The half-life of reduced iron in a fully oxygenated seawater solution at 25℃ is only a few minutes, but at 4℃ and reduced oxygen concentrations in the shrimp habitat (approximately 2/3 oxygen saturation), the half-life is extended to 35 hours [260].

Under standard conditions at neutral pH, iron oxidation is thermodynam-

ically less favourable than sulfide, hydrogen, or methane oxidation (Table 1.1). However, the free energy change of a reaction can be influenced by the concentrations of products and reactants. Preliminary calculations by Zbinden et al. [260] suggested that the energy available from oxidation of Fe^{2+} with oxygen was between 25 and 100 times greater than from oxidation of sulfide in the shrimp habitat. This was confirmed by modelling of the energy budget for the shrimp habitat at Rainbow [262]. The potential for a chemosynthetic symbiosis based on iron oxidation has therefore been postulated for the *R. exoculata* epibionts at the Rainbow vent field, but has not been directly proven, and attempts to cultivate the epibionts have been so far unsuccessful [260].

5.4 Phylogeny of the *R. exoculata* epibionts

Before I began this thesis work, there was only a single molecular study investigating the *R. exoculata* epibionts. Polz and Cavanaugh looked at *R. exoculata* from the Snake Pit hydrothermal vent field on the MAR [157]. These authors used the full-cycle 16S rRNA approach, identifying the filamentous epibionts as a single epsilonproteobacterial phylotype. The 26 clones they sequenced were identical, and an oligonucleotide probe designed to specifically target this sequence bound all three epibiont morphotypes found on the shrimp: thick filaments, thin filaments, and rod-shaped bacteria. In addition, slot-blot hybridization was used to quantify the epsilonproteobacterial epibiont contribution to total bacterial ribosomal RNA (rRNA) extracted from material scraped from the shrimp carapace. By this method, the authors showed that 97.6% of total bacterial rRNA associated with the shrimp could be attributed to the epibiont phylotype. Applying the same slot-blot hybridization method to sulfide surfaces sampled from around the shrimp swarms, they showed that the epibiont phylotype also contributed more than 50% of total bacterial rRNA from the surface-associated community. This was surprising, considering what was known about the high diversity of microorganisms in other marine environmental settings that had been revealed by culture-independent methods (reviewed in [269, 270]).

In 2008, Zbinden et al. [156] published the results of a molecular study of

the *R. exoculata* epibionts from the Rainbow vent field on the MAR. They showed that in addition to the epsilonproteobacterial phylotype previously described by Polz and Cavanaugh [157], 16S rRNA sequences from various other bacterial phyla could be amplified and sequenced from the scaphognathite and carapace of a single *R. exoculata* individual. This study showed that shrimp from the Rainbow vent field potentially associate with a wider diversity of bacterial epibionts. However, they did not use fluorescence in situ hybridization to distinguish true ectosymbionts from casually associated bacteria. We would expect these to be present in the gill chamber, which is directly exposed to water from the environment due to the host pumping water through across the gills.

Chapter 6

Microbial biogeography at hydrothermal vents

Biogeography is the study of the distribution of biodiversity over space and time. By studying biogeography, we can gain insights into evolutionary processes such as speciation, extinction, dispersal and species interactions, the mechanisms responsible for creating and maintaining diversity [271]. Biogeography has long been an integral part of ecological studies of macroflora and macrofauna. Despite this, microbial ecologists had not even considered that geography would play a role in microbial evolution until recently. The paradigm that ruled the thinking of microbial ecologists was summarized by the Dutch microbiologist Baas-Becking in his statement 'everything is everywhere, but, the environment selects' [272]. This statement reflects two long-held assumptions about microbial populations: (1) microbes (prokayotes) have extremely large population sizes, and (2) they can disperse freely to all parts of the globe [273]. However, both of these assumptions have recently been disputed [271], and a number of studies published in the last

few years have shown that at least some microbes, both free-living [274–276] and host-associated [195, 277], do indeed have biogeographic patterns.

Why has the role of geography in microbial evolution and ecology only recently been realized? One particular difficulty that hampered such studies in the past was the lack of suitable methods for describing microbial diversity. Molecular methods have revolutionized our understanding of microbial diversity, allowing us to look at the more than 99% of microbes that have not yet been cultured in the lab, and also allowing us to characterize microbes based on their phylogeny rather than morphology and physiology, giving an evolutionary framework to the study of microbial ecology.

In the three decades since their discovery, much research has focussed on the biogeography of hydrothermal vent fauna (reviewed in [3, 46]). Studies of vent faunal distribution have revealed some of the physical, geological, chemical and biotic factors that limit dispersal and colonization in the vent habitat, but so far, the biogeography of vent microbes has received very little attention. There are no published studies on the biogeography of free-living vent microbes, and only a single study has investigated the biogeography of symbiotic microbes at vents. DeChaine et al. showed that the distribution of the chemoautotrophic symbionts of bathymodiolin mussels from vent fields in the Northern Atlantic is correlated with geography, and not, as expected, with host species [195]. It is surprising that there are currently so few studies on the biogeography of vent microbes; hydrothermal vents, as heterogenous chemical 'islands' in the deep sea that are often separated from each other by large geographic distances, provide an ideal system for studying bacterial dispersal, diversification and adaptation to the environment.

Chapter 7

Aims

Methanotrophic symbioses

As a part of this thesis, I wrote two review articles on methanotrophic symbioses. Symbioses with methane-oxidizing bacteria are widespread, and have been known for almost as long as thiotrophic symbioses. Despite this, studies on methane-oxidizing symbionts are still in their infancy. There is evidence for methane-oxidizing symbionts in four different marine animal phyla, and in one terrestrial wetland plant. However, most of these associations have not yet been characterized by molecular methods, and in some cases, the only evidence for a methane-based symbiosis is provided by studies of symbiont ultrastructure. In the first review, my aim was to provide a comprehensive overview of all hosts in which methanotrophic symbioses have been found, and to analyze the evidence used in the past to identify methane-based symbioses. The second review gives an overview of the habitats and hosts where methanotrophic symbionts have been found in the marine environment, and uses the bathymodiolin mussels to illustrate how molecular methods can be used to investigate the diversity and metabolism of symbiotic methane-oxidizing bacteria.

Ectosymbiosis of *R. exoculata*

Before I began this thesis work, the *R. exoculata* epibiosis was considered to be monospecific; a single host associates with a single symbiont [157]. This was based on a molecular study of shrimp from the Snake Pit vent field, which were shown to associate with a single sulfur-oxidizing symbiont that belongs to the Epsilonproteobacteria. Based on the high morphological diversity of the epibionts, and presence of iron oxides on shrimp from the Rainbow vent field that could indicate an association with iron-oxidizing bacteria [260], I

aimed to re-investigate epibiont diversity on shrimp from this hydrothermal vent field using comparative 16S rRNA analysis and fluorescence in situ hybridization. Obtaining samples from hydrothermal vents is a technical and logistical challenge that requires international cooperation. This PhD thesis was made possible by a collaboration with Marie-Anne Cambon-Bonavita (Ifremer) and Magali Zbinden (University of Paris), who kindly provided samples of *Rimicaris exoculata* from the Rainbow and TAG vent fields, and invited me to their laboratories to show me the molecular, electron microscopic, and cultivation techniques they use to study the shrimp symbiosis.

Within the framework of the German Research Foundation's priority program on hydrothermal vents (SPP 1144 'From Mantle to Ocean: Energy-, Material- and Life-cycles at Spreading Axes'), I was able to take part in research cruises to the Logatchev and South MAR hydrothermal vent fields (Figure 5.3). Thus, I had the unique opportunity to investigate the biogeography of the *R. exoculata* epibiosis, by comparing epibionts from four different vent fields on the Mid-Atlantic Ridge: Rainbow, TAG, Logatchev, and South MAR. These sites cover the known distribution range of *R. exoculata* and include ultramafic- and basalt-hosted systems that differ in their availability of energy sources known to fuel chemosynthesis.

Based on the results of my first study that showed a higher phylogenetic diversity of symbionts than previously recongized, I investigated the epibiont functional diversity, focussing on pathways of carbon fixation, and on energy generation from three different energy sources: reduced sulfur compounds, methane, and hydrogen.

Part II

List of Publications

Contributions to the publications and manuscripts presented in this thesis:

1. **Jillian M. Petersen and Nicole Dubilier**. (in press). Symbiotic methane oxidizers. In: Handbook of Hydrocarbon and Lipid Microbiology. Timmis, K. (Ed). Berlin: Springer-Verlag.
   J.M.P.: conceived and wrote the manuscript N.D.: conceived and edited the manuscript

2. **Jillian M. Petersen and Nicole Dubilier**. (2009). Methanotrophic symbioses in marine invertebrates. *Environmental Microbiology Reports* **1**:319-335
   J.M.P.: conceived and wrote the manuscript N.D.: conceived and edited the manuscript

3. **Jillian M. Petersen, Alban Ramette, Christian Lott, Marie-Anne Cambon-Bonavita, Magali Zbinden, and Nicole Dubilier**. Dual symbiosis of the vent shrimp *Rimicaris exoculata* with filamentous gamma- and epsilonproteobacteria at four Mid-Atlantic Ridge hydrothermal vent fields.
   Manuscript accepted for publication in *Environmental Microbiology.*
   J.M.P.: developed the concept, did the 16S rRNA sequencing and analysis, designed FISH probes and performed the FISH experiments, concieved and wrote the manuscript A.R.: helped with the statistical analysis C. L.: did the scanning electron microscopy M.-A.C.-B.: provided samples from the Rainbow and TAG hydrothermal vent fields, and information on the DNA extraction procedure M.Z.: provided samples from the Rainbow and TAG hydrothermal vent fields N.D.: developed the concept with J.M.P., conceived and edited the manuscript

4. **Jillian M. Petersen, Wolfgang Bach, Michael Hügler, Caroline Rühland, Stefan Sievert, Marie-Anne Cambon-Bonavita, Magali Zbinden, and Nicole Dubilier**. Functional aspects of the *Rimicaris exoculata* epibiosis: carbon and energy sources for the filamentous gamma- and epsilonproteobacterial epibionts.

Manuscript in preparation.
J.M.P.: Developed the concept, summarized chemical data for the thermodynamic modelling, amplified and sequenced all functional genes except aclB *, phylogeny of all functional genes except* aprA *performed immunohistochemistry, concieved and wrote the manuscript W.B.: did the thermodynamic modelling M.H.: amplified and sequenced the* aclB *gene C.R.: did the phylogenetic analysis of the* aprA *gene S.S.: supported M.H. M.-A.C.-B. and M.Z.: provided the shrimp samples N.D.: developed the concept with J.M.P., conceived and edited the manuscript*

5. **Frank U. Zielinski, Jillian M. Petersen, Silke Wetzel, Thomas Pape, Richard Seifert, Frank Wenzhöfer, and Nicole Dubilier**. The sulfur-oxidizing endosymbiont of the hydrothermal vent mussel *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae) uses hydrogen as an energy source.
Manuscript in preparation.
F.U.Z.: developed the concept, experimental design, sampling and incubation experiments, data analysis J.M.P.: experimental design, PCR-amplification, sequencing, and phylogeny of hynL, *sampling and incubation experiments S.W.: sampling and incubation experiments T.P.: hydrogen measurements, experimental design R.S.: hydrogen measurements F.W.: helped to develop the concept N.D.: developed the concept, experimental design, conceived and edited the manuscript*
This manuscript is included in the Appendix.

Part III

Manuscripts

Manuscript I:
Symbiotic methane oxidizers

Jillian M. Petersen and Nicole Dubilier

In: K.Timmis (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer: Berlin Heidelberg, pp. 1977-1996.

28 Symbiotic Methane Oxidizers

J. M. Petersen · N. Dubilier
Symbiosis Group, Max Planck Institute for Marine Microbiology, Bremen, Germany
jmpeters@mpi-bremen.de
ndubilie@mpi-bremen.de

1 Introduction 1978

2 Diversity of Hosts and Habitats 1978
2.1 Methanotrophic Symbioses in Marine Habitats 1979
2.1.1 Bathymodiolin Mussels 1982
2.1.2 Provannid Snails 1983
2.1.3 Siboglinid Tubeworms 1983
2.1.4 *Cladorhiza methanophila* 1984
2.2 Methanotrophic Symbioses in Wetland Plants 1984
2.2.1 *Sphagnum cuspidatum 1984*

3 Diversity of Symbionts – Phylogeny 1985

4 Physiology – Evidence for Methanotrophy 1986
4.1 Enzyme Activity 1987
4.2 Incubations with Labeled and Unlabeled Methane 1987
4.2.1 Unlabeled Methane 1987
4.2.2 Radioactively Labeled Methane 1988
4.2.3 Stable Isotope-Enriched Substrates 1988
4.3 Natural Stable Isotope Abundance 1989
4.4 Molecular Characterization 1991

5 Outlook 1992

K. N. Timmis (ed.), *Handbook of Hydrocarbon and Lipid Microbiology*, DOI 10.1007/978-3-540-77587-4_145,
© Springer-Verlag Berlin Heidelberg, 2010

Abstract: Symbiotic methane-oxidizing bacteria are found at hydrothermal vents and cold seeps in the deep sea and in terrestrial wetlands. These very different habitats share a common feature: They provide access to both oxic, electron acceptor-rich fluids, and anoxic, methane-rich fluids. By teaming up with bacteria that can gain energy from the oxidation of CH_4 with O_2, the plant or animal host is indirectly able to live from an energy source that is otherwise only available to methanotrophic microorganisms. Methane is both an energy and carbon source for the bacteria, which contribute organic compounds to their host as a source of nutrition. The host, in turn, provides a stable environment for the bacteria, and provides access to both electron donors and acceptors. As no symbiotic methane oxidizer is available in pure culture, all evidence to date for these symbiotic associations comes from ultrastructural, enzymatic, physiological, stable isotope, and molecular biological studies of the symbiotic host tissues. We present an overview of the range of hosts and habitats in which symbiotic methane oxidizers are found, summarize the ways in which methanotrophic metabolism in the symbiotic bacteria has been confirmed in the past, and identify directions for future research and the methodological developments that will help us understand how symbiotic methane oxidizers interact with their hosts, and how both are influenced by their environment.

1 Introduction

Symbiosis, the “living together of differently-named organisms” (De Bary, 1879) is a driving force in the evolution of life on Earth; it allows the establishment of novel functions when two or more different organisms cooperate. The symbiotic associations between methane-oxidizing bacteria and eukaryotes are an excellent example for the benefits of such a cooperation: No known plant or animal can use methane as an energy or carbon source, but through their symbiotic associations with methane-oxidizing bacteria these eukaryotes can indirectly grow on methane (for reviews see Cavanaugh et al., 2006; DeChaine and Cavanaugh, 2005; Dubilier et al., 2008). The symbiotic methanotrophs oxidize methane aerobically to produce organic compounds that provide the plant or animal host with a source of nutrition. In return, the host provides the symbiotic bacteria with access to methane, the electron donor, and oxygen, the electron acceptor by positioning themselves in the mixing zones between reduced and oxidized fluids. Methanotrophic symbioses are therefore found where the oxic and anoxic worlds meet, at hydrothermal vents and cold seeps where reduced fluids mix with the surrounding oxidized seawater, or in aquatic peat bogs, where oxygen from the atmosphere diffuses into anoxic pools. Sulfide is also commonly present at vents and seeps, and here, chemoautotrophic sulfur-oxidizing bacteria sometimes co-occur with methane-oxidizers in the same host in a dual symbiosis.

To date, symbiotic methane oxidizers have resisted all attempts at cultivation. At least some methane-oxidizing symbionts should, however, be cultivable, as there is evidence that they may occur in a free-living stage (see Section 3). While morphological, physiological, and stable isotope analyses have been the predominant methods for investigating methanotrophic symbioses in the past, molecular methods for the study of uncultured microorganisms are increasingly used to investigate the diversity and metabolism of these symbiotic bacteria.

2 Diversity of Hosts and Habitats

The first methane-based symbiosis was discovered in the deep-sea mussel *Bathymodiolus childressi* from cold seeps in the Gulf of Mexico (Childress et al., 1986). Symbiotic methane-oxidizing

bacteria have since been described in deep-sea bathymodiolin mussels of the genera *Bathymodiolus* and *Idas*, a hydrothermal vent snail (*Ifremeria nautilei*), siboglinid tubeworms, and a marine sponge (*Cladorhiza methanophila*), representing a diversity of animal hosts that spans the groups Porifera, Mollusca, and Annelida, and includes four different metazoan classes and families. They are, however, not limited to associations with animals, or to marine habitats, but have also been described from a wetland plant (summarized in *Table 1*). The ecological niches in which methanotrophic symbioses have been found are limited in marine environments to hydrothermal vents, cold seeps, and mud volcanoes, and in terrestrial habitats to a peatbog in Holland (*Table 1*).

2.1 Methanotrophic Symbioses in Marine Habitats

Marine habitats where methane-oxidizing symbionts have been found include deep-sea hydrothermal vents, cold seeps and mud volcanoes. The processes responsible for methane production, and the physico-chemical nature of these habitats are covered in Section A of this volume (Chapter 5–17, Vol. 1, Part 2 and 3). Hydrothermal vent fauna hosting methane-oxidizing symbionts are found in mixing zones between the pure vent fluids, which can reach temperatures in excess of 300°C and have methane concentrations up to 3.5 mM but no oxygen (Charlou et al., 2002), and ambient seawater, which is about 2°C at 3,000 m depth, and has no measurable methane but is well oxygenated (Van Dover, 2000). At cold seeps and mud volcanoes, the symbiotic fauna are found at or just below the sediment surface where methane-containing, anoxic fluids seep into the overlying oxic seawater (Van Dover, 2000). The methane concentration varies over three orders of magnitude in water samples taken from these habitats and measured on board by gas chromatography, from 0 to a maximum of 985 mM in the Gulf of Mexico (Heeschen et al., 2007).

The minimum methane concentration needed to sustain methanotrophic symbiosis is unknown. For hosts that have only methane oxidizers and must gain most or all of their nutrition from them, methane oxidation rates must be high enough to sustain the metabolic needs of both the symbionts and their host. Furthermore, given the lack of known host carrier proteins for methane, methanotrophic symbionts are dependent on diffusive flux of methane through host membranes, in contrast to free-living methanotrophs that are directly exposed to environmental methane. Methane transport to the symbionts can, however, be greatly enhanced through advective flux, for example, through water currents produced by the ciliary activity of the symbiont-containing filaments of mussel gill tissues. An additional factor that will affect the amount of methane needed to sustain a symbiosis is the efficiency of bacterial methane assimilation, i.e., how much methane is incorporated into cell material and how much is respired to CO_2 (see Section 4.2). Experimental data for the minimum methane concentration needed by a methanotrophic symbiosis is limited to a single study by Kochevar et al. (1992) on symbiotic *Bathymodiolus* mussels from the Gulf of Mexico. Using flow-through pressure respirometry, these authors showed that methane concentrations between 25 and 60 μM were needed for net growth of the mussels (Kochevar et al., 1992). In addition to concentration, methane flux may also be critical to the symbiosis; even very low methane concentrations may be sufficient to at least sustain methanotrophic symbioses if methane flux is high enough. Kochevar et al. (1992) showed *Bathymodiolus* mussels can assimilate methane down to very low concentrations below reliable detection limits of <5 μM.

Table 1

Summary of organisms hosting symbiotic methane-oxidizing bacteria

Group	Common name	Species name	Evidence for methanotrophy[a]								Habitat	Phylogenetic position (if known)[c]	References
			TEM	MeDH	UI	LI	SI	16S	FISH	*pmoA*			
Porifera													
Class Demospongiae													
Family Cladorhizidae	Sponge	*Cladorhiza methanophila*	x	x			x				Mud volcano		Vacelet et al. (1995)
Mollusca													
Class Bivalvia													
Family Mytilidae	Mussel	*Idas* sp.						x	x	x	Seep	γ	Duperron et al. (2008)
	Mussel	*Bathymodiolus japonicus*	x					x			Vent & seep	γ	Fujiwara et al. (2000)
		B. platifrons	x	x			x	x			Vent & seep	γ	Fujiwara et al. (2000); Barry et al. (2002)
		B. childressi	x		x	x	x	x	x	x	Seep	γ	Childress et al. (1986); Duperron et al. (2007); Cary et al. (1988); Kochevar et al. (1992)
		B. boomerang	x								Seep		von Cosel and Olu (1998)
		B. brooksi	x	x			x	x	x	x	Seep	γ	Duperron et al. (2007); Fisher et al. (1993); Cavanaugh et al. (1987)
		B. heckerae	x	x			x	x	x	x	Seep	γ	Duperron et al. (2007)
		B. mauritanicus	x					x	x		Seep	γ	Duperron et al. (2005)
		B. azoricus	x				x	x	x	x	Vent	γ	Trask and Van Dover (1999); Duperron et al. (2006); Spiridonova et al. (2006)

		B. puteoserpentis	x					x	x		Vent	γ	Duperron et al. (2006)
Class Gastropoda													
Family Provannidae	Snail	*Ifremeria nautilei*	x	x		x	x				Vent	γ	Galchenko et al. (1992); Borowski et al. (2002); Beck and Sobjinski (1999); C. Borowski and N. Dubilier unpublished results
	Snail	*Alviniconcha hessleri*	x								Vent		Endow and Ohta (1989)
Annelida													
Class Polychaeta													
Family Siboglinidae	Tubeworm	*Sclerolinum contortum*[b]	x			x					Mud volcano		Pimenov et al. (1999); Pimenov et al. (2000)
	Tubeworm	*Siboglinum poseidoni*	x	x		x	x				Seep		Schmaljohann and Flügel (1987); Schmaljohann et al. (1990)
Bryophyta													
Class Sphagnopsida													
Family Sphagnaceae	Moss	*Sphagnum cuspidatum*				x	x	x	x		Wetlands	α	Raghoebarsing et al. (2005)

[a] *TEM* transmission electron microscopy; *MeDH* methanol dehydrogenase activity; *UI* incubation with unlabeled CH_4; *LI* incubation with $^{13}CH_4$ or $^{14}CH_4$; *SI* natural abundance stable carbon isotope analysis; *16S* 16S rRNA gene sequenced; *FISH* fluorescence in situ hybridization; *pmoA* gene for the active site subunit of particulate methane monooxygenase sequenced

[b] In a recent study, only sulfur-oxidizing (but no methane-oxidizing) symbionts were found in *Sclerolinum contortum* (Lösekann et al., 2008)

[c] *γ Gammaproteobacteria*; *α Alphaproteobacteria*

2.1.1 Bathymodiolin Mussels

Bathymodiolin mussels have characteristically enlarged gills that house their symbiotic bacteria. Their digestive systems are reduced (Gustafson et al., 1998), indicating their dependence on the symbiotic bacteria for nutrition. Two genera of bathymodiolin mussels harbor methanotrophic symbiotic bacteria, *Bathymodiolus* and *Idas*. Mussels of the genus *Bathymodiolus* dominate the biomass at hydrothermal vents and cold seeps worldwide. Their symbiotic bacteria are housed in specialized cells of their gill tissues, called bacteriocytes. *Bathymodiolus* mussels were first discovered at vents on the Galapagos Rift, in association with sulfur-oxidizing endosymbiotic bacteria (Cavanaugh, 1983). The first description of methane-oxidizing symbionts in *B. childressi* (Childress et al., 1986) was followed by the discovery of *Bathymodiolus* mussels that host a dual symbiosis, with both sulfide- and methane-oxidizing bacteria co-existing within single host cells (Fig. 1), which was previously unknown to occur in animal-bacterial symbioses (Distal et al., 1995; Fisher et al., 1993). Ten species of *Bathymodiolus* have been shown to harbor methane-oxidizing bacteria. Of these, three host only methanotrophic bacteria (*B. childressi, B. japonicus,* and *B. platifrons*), six have a dual symbiosis with sulfur- and methane-oxidizing bacteria (*B. boomerang, B. brooksi, B. puteoserpentis, B. azoricus, B.* sp. Wideawake, and *B.* sp. Gabon Margin), and one, *B. heckerae*, can host

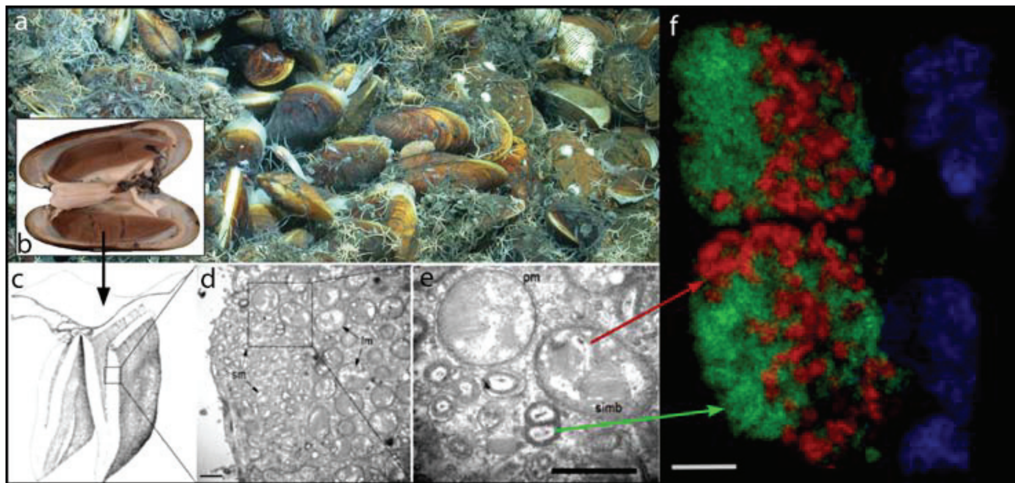


Figure 1

The mussel *Bathymodiolus puteoserpentis* lives in a dual symbiosis with methane- and sulfur-oxidizing bacteria. (a) Image of *B. puteoserpentis* at the Logatchev vent field (Marum, Bremen). (b) Open individual showing the brown, fleshy gills that house the symbiotic bacteria (Christian Borowski, MPI Bremen). (c) Diagram showing one of the two gills. The gill consists of rows of many filaments that house symbiotic bacteria (from Le Pennec and Hily, 1984). (d) Transmission electron microscopy (TEM) image of a cross-section of one gill filament, showing the symbiotic bacteria (from Duperron et al., 2005). (e) TEM showing the two bacterial morphotypes. The large morphotype with stacked internal membranes is the methane oxidizer, the small morphotype is the sulfur oxidizer. (f) Fluorescence in situ hybridization showing two gill cells with co-occurring methane-oxidizing (red) and sulfur-oxidizing symbionts (green) and the DAPI-stained host nuclei (blue) (Dennis Fink, MPI Bremen).

up to four distinct symbiotic phylotypes, two sulfur-oxidizing, one methane-oxidizing, and one related to free-living methylotrophic bacteria (Duperron et al., 2007). Intriguingly, the methylotrophic and methanotrophic symbionts in *B. heckerae* co-occur tightly, suggesting metabolic interactions between these. Methanol, which is produced as an intermediate during methane oxidation by the activity of the methane monooxygenase enzymes (MMO) (Hanson and Hanson, 1996), is a non-polar compound and can easily leak out of the cell membrane of methanotrophs. Its consumption by the symbiotic methylotroph would allow the use of a carbon source that would otherwise be lost to the symbiosis, but this is yet to be proven. An additional benefit of the methylotrophic symbiont may be the removal of toxic metabolites at high methane consumption rates, when intermediary products such as methanol, formaldehyde, and formate may become inhibitory (see Section 4.2.1).

Mussels of the genus *Idas* are close relatives of *Bathymodiolus* species and have been found worldwide at cold seeps (Duperron et al., 2008) and sunken organic debris such as whale and wood falls (Braby et al., 2007). Most *Idas* species appear to have sulfur-oxidizing symbionts (Deming et al., 1997; Southward, 2008). To date, the only known occurrence of symbiotic methane-oxidizing bacteria in *Idas* mussels is from cold seeps in the eastern Mediterranean (Duperron et al., 2008). As in *Bathymodiolus* hosts, the bacteria are found within bacteriocytes of the gill tissue.

2.1.2 Provannid Snails

The provannid snails *Ifremeria nautilei* and *Alviniconcha hessleri* occur in high abundance at hydrothermal vents in back-arc basins of the Western Pacific. Like the bathymodiolin mussels, they have enlarged gills housing the symbiotic bacteria, and a reduced digestive system (Beck and Sobjinski, 1999). Methane-oxidizing symbionts have been detected in *I. nautilei* from the Manus Basin (Beck and Sobjinski, 1999; Galchenko et al., 1992) and North Fiji Basin (Borowski et al., 2002), and in *A. hessleri* from the Manus (Beck and Sobjinski, 1999) and Mariana (Endow and Ohta, 1989) Basins. In both host species, the methanotrophic symbiont is less abundant than co-occurring sulfur-oxidizing symbiont (Beck and Sobjinski, 1999). Its presence is therefore sometimes missed, particularly with methods such as transmission electron microscopy, where often only a very small part of the symbiotic tissue is investigated. Despite the low abundance of methane-oxidizing symbionts, methane might still be a significant source of nutrition, as evidenced by methane incorporation into *I. nautilei* gill tissue in radioactive incubation experiments (Galchenko et al., 1992).

2.1.3 Siboglinid Tubeworms

Siboglinid tubeworms are a group of marine annelids that lack a digestive system and rely on their endosymbionts for nutrition (Felbeck, 1981; Southward et al., 1986). The symbiotic methane-oxidizers are housed in a specialized organ, the trophosome, which extends throughout the body of the worm. There is evidence for methane-oxidizing symbionts in two siboglinid species, *Siboglinum poseidoni* from a methane seep in the Skagerrak (Schmaljohann and Flügel, 1987), and *Sclerolinum contortum* from the Haakon Mosby Mud Volcano (HMMV) in the Barents Sea (Pimenov et al., 1999). However, in a recent molecular analysis of *S. contortum* from HMMV only evidence for sulfur-oxidizing symbionts was found (Lösekann et al., 2008).

The contradictory results for *S. contortum* could be due to the presence of morphologically similar co-occurring host species of which only one hosts methanotrophs, highlighting the need to analyze larger sample populations.

There is a third siboglinid tubeworm species, *Oligobrachia mashikoi,* in which bacterial symbionts were hypothesized to be methanotrophs based on their 16S rRNA phylogeny, although genes characteristic for methantrophy were not found (Kimura et al., 2003). Studies in our group show that the *O. mashikoi* symbionts were more closely related to the sulfur-oxidizing symbiont of O. *haakonmosbiensis* than to known symbiotic and free-living methane oxidizers (Lösekann et al., 2008). This shows the importance of demonstrating methanotrophy in symbiotic bacteria by multiple methods (see Section 4).

2.1.4 *Cladorhiza methanophila*

Marine sponges are commonly found in shallow water environments where they are associated with a metabolically and phylogenetically diverse array of symbiotic bacteria (reviewed in Hentschel et al., 2006; Taylor et al., 2007). Sponges are rarely dominant members of chemosynthetic ecosystems. *C. methanophila* is the exception, as it occurs in large aggregations of hundreds of individuals at a mud volcano in the Barbados Trench (Vacelet et al., 1995). Marine sponges are generally filter-feeders, but the cladorhizid sponges have developed carnivory as an adaptation to the nutrient-poor deep sea (Vacelet and Bouryesnault, 1995). In addition to carnivory, *C. methanophila* also gains nutrition from methane-oxidizing symbiotic bacteria, which might explain their high biomass in the Barbados Trench, as cladorhizids without methanotrophic symbionts, which rely solely on carnivory for nutrition, occur only in small clumps of low density (Vacelet et al., 1996). Symbiotic bacteria are found in the extracellular matrix of the sponge body, and are also abundant in all cell types found in the sponge mesohyl. Methanotrophic symbionts are also found in *C. methanophila* eggs, indicating that the symbiotic bacteria are passed directly from one generation to the next, termed vertical transmission (Vacelet et al., 1996).

2.2 Methanotrophic Symbioses in Wetland Plants

Peatlands cover 3.5% of the Earth's land surface, and are estimated to store one third of the world's terrestrial carbon (Gorham, 1991). Methane is produced biogenically in peatland habitats due to the activity of anaerobic methanogens. While the lower layers of peat bog pools remain anoxic, oxygen from the atmosphere can diffuse into the top layers, creating an interface where both oxygen and methane are available and aerobic methanotrophs can thrive (See Chapter 51, Vol. 4, Part 6).

2.2.1 *Sphagnum cuspidatum*

Sphagnum mosses are aquatic wetland plants that grow submerged in peat bog pools. The genus *Sphagnum* consists of approximately 300 different species, and is distributed worldwide (Opelt et al., 2007), but only one species, *S. cuspidatum* from a peat bog in the Netherlands, is known to harbor methanotrophic endosymbionts (Raghoebarsing et al., 2005). The

methanotrophic symbiont dominates the plant's bacterial community and is found in hyaline cells of the plant and on stem leaves. Incubations with ^{13}C-enriched methane showed that the symbionts provide their plant host with a relatively small proportion of its total carbon (see Section 4.2.3). Their potential role in providing a source of fixed nitrogen in an ecosystem known to be N-limited, was not investigated, but is a possibility as many free-living methanotrophs can fix nitrogen (Dedysh et al., 2004). It remains to be determined whether the association with endosymbiotic methanotrophic bacteria is widespread in wetland ecosystems.

3 Diversity of Symbionts – Phylogeny

All known methanotrophic symbionts belong to either the *Gamma*- or the *Alphaproteobacteria*. Methanotrophic symbionts from marine environments cluster in a monophyletic clade within the *Gammaproteobacteria*, with free-living methane oxidizers in the genera *Methylomonas*, *Methylobacter*, and *Methylomicrobium* as sister groups (*Fig. 2*). In contrast, the *Sphagnum* methane-oxidizing symbiont from a terrestrial peatbog belongs to the *Alphaproteobacteria*, and is related to free-living methane-oxidizing bacteria from the genera *Methylocystis*, *Methylosinus*, and *Methylocella*.

Despite evidence for methane-oxidizing symbionts in several marine host groups, to date there are only 16S rRNA sequences available from the methane-oxidizing symbionts of *Bathymodiolus* and *Idas* mussels. These symbiont sequences are closely related to each other (96.6–99.7%) and fall in a monophyletic cluster within the *Gammaproteobacteria*. This suggests that all marine methanotrophic symbioses evolved from only a single bacterial lineage.

Methanotrophic symbionts of mussels are generally host-specific at the 16S rRNA level. This means that each host species contains a single methanotrophic 16S rRNA phylotype that is distinct from the methane-oxidizing symbionts of other host species. There is one exception, where two mussel species from vents on the Mid-Atlantic Ridge host an identical symbiont 16S rRNA phylotype (Duperron et al., 2006). Analyses at the strain level, based on the internal transcribed spacer of the ribosomal operon, indicate that multiple strains of methanotrophic symbionts can co-occur within these host species (unpublished data from S. Hallam, cited in Won et al., 2003).

The 16S rRNA phylogeny of the mussel symbionts can be useful for assessing how they are transmitted. There are two ways in which hosts can acquire their symbionts, termed vertical and horizontal transmission. In vertical transmission, symbionts are transferred directly from the parent to the egg or embryo. In contrast, horizontal transmission is independent of host reproduction, and the symbionts are taken up from the environment, or from co-occurring hosts. The mode of transmission influences symbiont phylogeny, where vertically transmitted symbionts and their hosts generally show parallel or congruent branching patterns as a result of a tightly shared evolutionary history. Horizontal transmission can decouple the host and symbiont phylogenies. Although 16S rRNA symbiont sequences are not available for all *Bathymodiolus* species, and the phylogenetic resolution of the 16S rRNA gene is limited for *Bathymodiolus* methanotrophic symbionts, our preliminary analysis indicates that host and symbiont phylogenies are incongruent for this group, suggesting horizontal transmission of the methane-oxidizing symbionts. This corresponds well with morphological and phylogenetic analyses of the sulfur-oxidizing symbionts of bathymodiolin mussels indicating that these are also acquired horizontally from the environment (Kadar et al., 2005; Won et al., 2003, 2008).

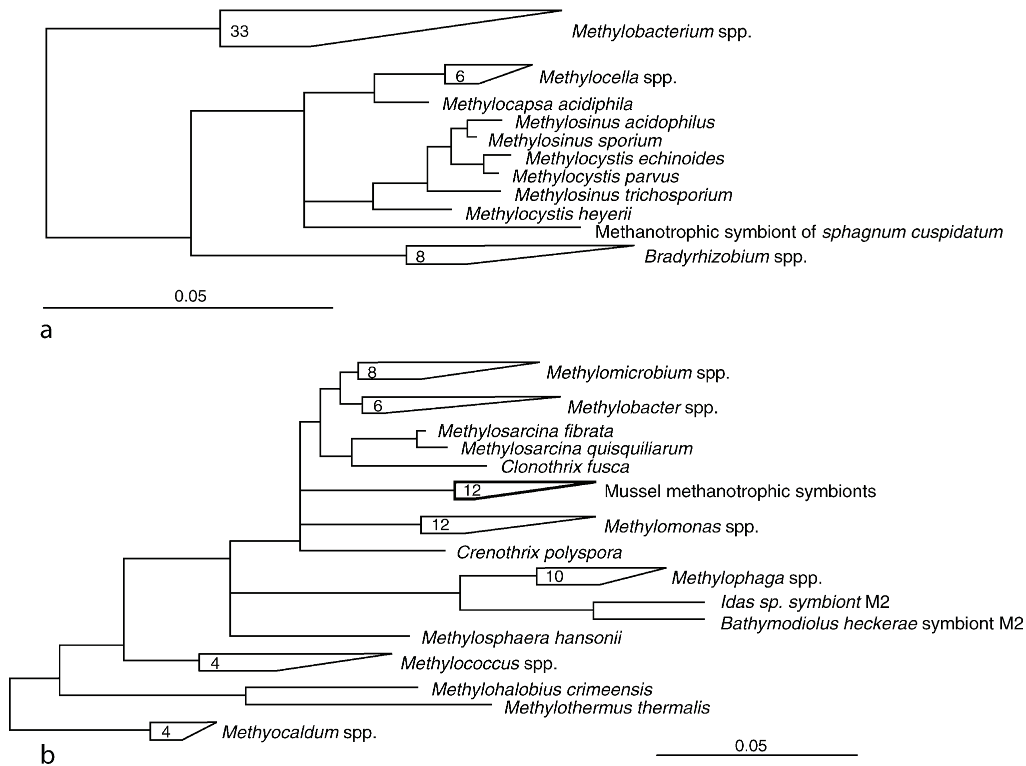


Figure 2

Phylogenetic analyses of the 16S rRNA gene show (a) that the symbiont of the moss *Sphagnum cuspidatum* groups with free-living alphaproteobacterial methane-oxidizers in the genera *Methylocystis* and *Methylosinus*. (b) The marine methanotrophic symbionts from bathymodiolin mussels (nine *Bathymodiolus* and one *Idas* species) form a single, monophyletic clade with *Methylomicrobium*, *Methylobacter*, and *Methylomonas* as sister groups. Two bathymodiolin species also have symbionts related to free-living methylotrophic *Methylophaga*: *Idas* sp. and *B. heckerae*. Both a and b show consensus trees calculated by parsimony, maximum likelihood, and neighbour-joining methods using a 40% positional variability filter with the SILVA (Pruesse et al., 2007) alignment in the ARB package (Ludwig et al., 2004). Bars indicate 5% sequence divergence. Outgroups (not shown) were (a) *Rhodospirillum rubrum*, (b) *Allochromatium vinosum*.

4 Physiology – Evidence for Methanotrophy

In many cases, the evidence for methanotrophy in symbiotic bacteria is circumstantial, based only on ultrastructural observations of stacked intracytoplasmic membranes typical of gammaproteobacterial methanotrophs, or relatedness to known methane-oxidizing bacteria. Methanotrophic metabolism has been indicated for uncultured symbiotic bacteria by a number of methods, which fall into four categories: enzymatic, physiological (incubation with either labeled or unlabeled methane), stable isotope analysis, and molecular biological analysis. Most studies have focused on the symbionts of *Bathymodiolus* mussels, the group in which methanotrophic symbionts were first described.

4.1 Enzyme Activity

Enzyme assays in symbiotic tissues can provide insights into energy generation and carbon fixation pathways in the symbiosis, by measuring the activity of the enzymes unique to these pathways. The oxidation of methane to methanol is the first step in both fixation of carbon into cell material, and oxidation of methane to CO_2 for energy generation. It is catalyzed by a particulate (membrane-bound) or soluble methane monooxygenase, pMMO and sMMO, respectively. Almost all aerobic methanotrophs express pMMO, but difficulties in working with this membrane-bound protein make measuring its activity problematic (Hakemian and Rosenzweig, 2007; Cavanaugh, 1992). The activity of methanol dehydrogenase (MeDH), which catalyzes the further oxidation of methanol to formaldehyde, is therefore often used as an indicator of methanotrophy in symbiont-containing host tissues. MeDH activity has been measured in a number of *Bathymodiolus* species, as well as the sponge, snail, and tubeworm symbiotic tissues (see *Table 1*).

Free-living aerobic methane oxidizers use two alternative pathways for formaldehyde fixation: gammaproteobacterial type I methanotrophs use the ribulose monophosphate (RuMP) pathway, while alphaproteobacterial type II methane oxidizers use the serine pathway (Hanson and Hanson, 1996). As all marine methane-oxidizing symbionts belong to the *Gammaproteobacteria* it is likely that they use the RuMP pathway. The only study of formaldehyde fixation pathways in marine symbiotic methanotrophs is from the tubeworm *S. poseidoni*, where hexulose-6-phosphate synthase activity was measured in the symbiotic trophosome tissue, indicating that these bacteria use the RuMP pathway (Schmaljohann et al., 1990). Indirect evidence for the use of the serine pathway in the alphaproteobacterial methane-oxidizing symbiont of the moss *Sphagnum cuspidatum* was derived from stable carbon isotope values of a hopanoid lipid biomarker in the range typical for serine-cycle methanotrophic bacteria (Raghoebarsing et al., 2005).

4.2 Incubations with Labeled and Unlabeled Methane

4.2.1 Unlabeled Methane

In the first study to show that an animal can grow with methane as its sole carbon and energy source, *Bathymodiolus* mussels from hydrocarbon seeps in the Gulf of Mexico with methanotrophic symbionts in their gills grew when incubated with only methane and seawater (Cary et al., 1988). Physiological incubation experiments can also be carried out in gas-tight chambers, where the consumption of methane and oxygen, and the production of carbon dioxide, is measured by gas chromatography. Kochevar et al. (1992) used this technique to investigate the physiology of the methanotrophic symbiosis in a Gulf of Mexico cold seep mussel, demonstrating very high methane consumption rates as long as oxygen concentrations were high enough (250–300 μM). Methane consumption was inhibited at concentrations above 300 μM CH_4 and the authors speculated that intermediary products of methane oxidation such as formaldehyde and formate could reach inhibitory levels at high consumption rates (Kochevar et al., 1992). Methanol was not considered, but would also become toxic at high concentrations.

4.2.2 Radioactively Labeled Methane

Oxidation of $^{14}CH_4$ and incorporation into tissues containing bacterial symbionts provides strong evidence that methane is used as a carbon and energy source (see Table 1 for a summary of symbiotic associations where methanotrophy has been confirmed in this way). Experiments with mussels incubated with $^{14}CH_4$ showed that transfer of labeled carbon from the symbiotic methanotrophs to the host was very slow (1–5 days) (Fisher and Childress, 1992), in contrast to symbioses with chemoautotrophic sulfur oxidizers in which $^{14}CO_2$ is incorporated into host tissues within hours (reviewed in Cavanaugh et al., 2006). Slow incorporation rates suggest that organic carbon is transferred to the host through the relatively slow digestion of the symbionts rather than the rapid translocation of small organic compounds from symbionts to the host.

Methane oxidation for catabolic (oxidation to carbon dioxide) and anabolic (incorporation into cell material) reactions can be measured in freshly sampled symbiont-containing tissues by incubation with $^{14}CH_4$, and allows quantification of the activity and productivity of the symbiosis. Anabolism is measured by the incorporation of the radioactive label into acid stable compounds, and catabolism by measuring the activity in CO_2 after incubation. Growth efficiency of the symbiotic bacteria can also be estimated with this technique, by comparing the amount of methane oxidized to CO_2 with the amount incorporated. Incubations of *B. brooksi* gill tissue showed that twice as much methane was oxidized to CO_2 as incorporated into biomass (Cavanaugh, 1992). A much higher efficiency of methane assimilation was observed in *Bathymodiolus* mussels from seeps in the Gulf of Mexico with only 30% of the methane consumed released as carbon dioxide (Kochevar et al., 1992). The ratio of catabolic to anabolic methane oxidation in the gill tissues of the vent snail *Ifremeria nautilei* was shown to be ~1:1 (Galchenko et al., 1992). It is unclear if these large differences in carbon conversion efficiency are real or due to experimental variability (e.g., the use of whole animals, homogenized tissues, or symbiont-enriched fractions). Furthermore, these comparisons could be confounded in a dual symbiosis, if sharing of carbon occurs between methanotrophs producing CO_2, and chemoautotrophic symbionts, which fix CO_2 (Fisher, 1996). Finally, methane oxidation rates measured at atmospheric pressure after bringing the animals up from the deep sea may not reflect the true activity of the symbiotic association in situ.

4.2.3 Stable Isotope-Enriched Substrates

Substrates enriched in a rare stable isotope such as ^{13}C are an alternative to radioactive incubations, and do not require specialized handling. Raghoebarsing et al. (2005) were able to show incorporation of ^{13}C-labeled methane into methanotrophic bacterial lipids, with further incorporation into plant-specific sterols, demonstrating that methane contributes to plant carbon, facilitated by symbiotic methane-oxidizing bacteria. It was even possible to quantify this contribution at 10–15% of total carbon assimilation by the plant.

Recent developments in methods for elemental and isotopic imaging at a scale of ~50 nm (nanoscale secondary ion mass spectrometry, or nanoSIMS) offer the exciting possibility to quantify processes at the level of a single bacterial cell after incubation with an isotopically-labeled substrate. (Lechene et al., 2007) used this method to quantify nitrogen fixation in symbiotic bacteria of a wood-eating marine bivalve, and to show transfer of fixed nitrogen to the host. Measuring the activity of individual cells in a symbiotic population will give us insights into host-bacteria interactions at a scale not previously possible.

4.3 Natural Stable Isotope Abundance

The carbon stable isotope composition of symbiotic tissues has been used to demonstrate the significance of methane as a carbon source for the symbiosis (see Table 1 for a summary of organisms for which stable isotope measurements have been published). Stable isotope composition is expressed as $\delta^{13}C$, which is the ratio of the heavy ^{13}C isotope to the light ^{12}C isotope in the sample, compared to the ratio in a standard. Enzymes catalyzing metabolic reactions prefer the lighter isotope (^{12}C), which results in the products of biological processes being "lighter" than reactants, i.e., having a higher ratio of ^{12}C to ^{13}C. This fractionation, expressed in per mille (‰) results in a shift in the stable isotopic composition of products to lighter $\delta^{13}C$ values compared to reactants.

Significant differences have been found in the $\delta^{13}C$ values of symbiotic animals containing methane-oxidizing symbionts (Fig. 3). In theory, it is possible to use $\delta^{13}C$ values to examine the relative contribution of different energy sources to host nutrition, if the carbon stable isotopic compositions of the sources are known, and the extent of fractionation by the symbiotic bacteria. In practice, however, it can be difficult to interpret $\delta^{13}C$ values for animals containing symbiotic methane-oxidizers, as their tissues rarely have $\delta^{13}C$ values that reflect theoretical values calculated from the shift in $\delta^{13}C$ methane values through fractionation. Fractionation factors of symbiotic methane-oxidizing bacteria are not known, and those estimated from studies on free-living bacteria may not be representative (Jahnke et al., 1999; Summons et al., 1994; Templeton et al., 2006). Factors such as the presence of co-occurring chemoautotrophic sulfur-oxidizing symbionts and filter-feeding by the host will also influence the $\delta^{13}C$ of symbiotic animals. In addition, methane-oxidizing bacteria can gain a significant proportion of carbon in cell biomass from CO_2, as much as 15% in Type I and 50% in Type II methanotrophs (Trotsenko and Murrell, 2008), and this could also influence $\delta^{13}C$ values. Another problem is that sampling of carbon sources and specimens from the same site is often not coordinated, and in many studies the $\delta^{13}C$ values of carbon sources are inferred from other studies that may or may not have been close to the site at which the specimens were sampled.

Some of the first measurements in *Bathymodiolus* mussels containing only chemoautotrophic sulfur-oxidizing symbionts showed that these had $\delta^{13}C$ values of −23 to −34‰ (Rau and Hedges, 1979; Spiro et al., 1986), reflecting the fractionation of marine dissolved inorganic carbon ($\delta^{13}C$ around 0‰) by the RuBisCO form I enzyme (Fig. 3). Much lighter $\delta^{13}C$ values (−74‰) were then measured in mussels from a cold seep in the Gulf of Mexico, and this mussel was shown to contain methane-oxidizing symbionts (Cavanaugh et al., 1987). *S. poseidoni* and *C. methanophila* also have such light $\delta^{13}C$ values, −78.3‰, (Schmaljohann et al., 1990) and −48.8‰, (Vacelet et al., 1995), respectively, confirming a trend towards lighter values in symbioses with methanotrophic bacteria in hosts from seeps and mud volcanoes where methane is characteristically light. However, light $\delta^{13}C$ values are not always characteristic of methanotrophic symbioses. Two tubeworm species from the Haakon Mosby mud volcano (HMMV) have $\delta^{13}C$ values of −47.9‰ and −66.7‰ but are only associated with sulfur-oxidizing symbionts (Lösekann et al., 2008). The authors hypothesized that the unusually light values could be due to autotrophic fixation of isotopically light CO_2 from the sediments at the HMMV, or the assimilation of isotopically light dissolved organic carbon (Lösekann et al., 2008). When interpreting carbon stable isotopic compositions, it is therefore important to consider the carbon sources as well as the fractionation.

Methane at hydrothermal vents has a characteristically heavy $\delta^{13}C$ signature. This has been measured at vents in the Pacific (−13‰, Welhan and Craig, 1983), Atlantic

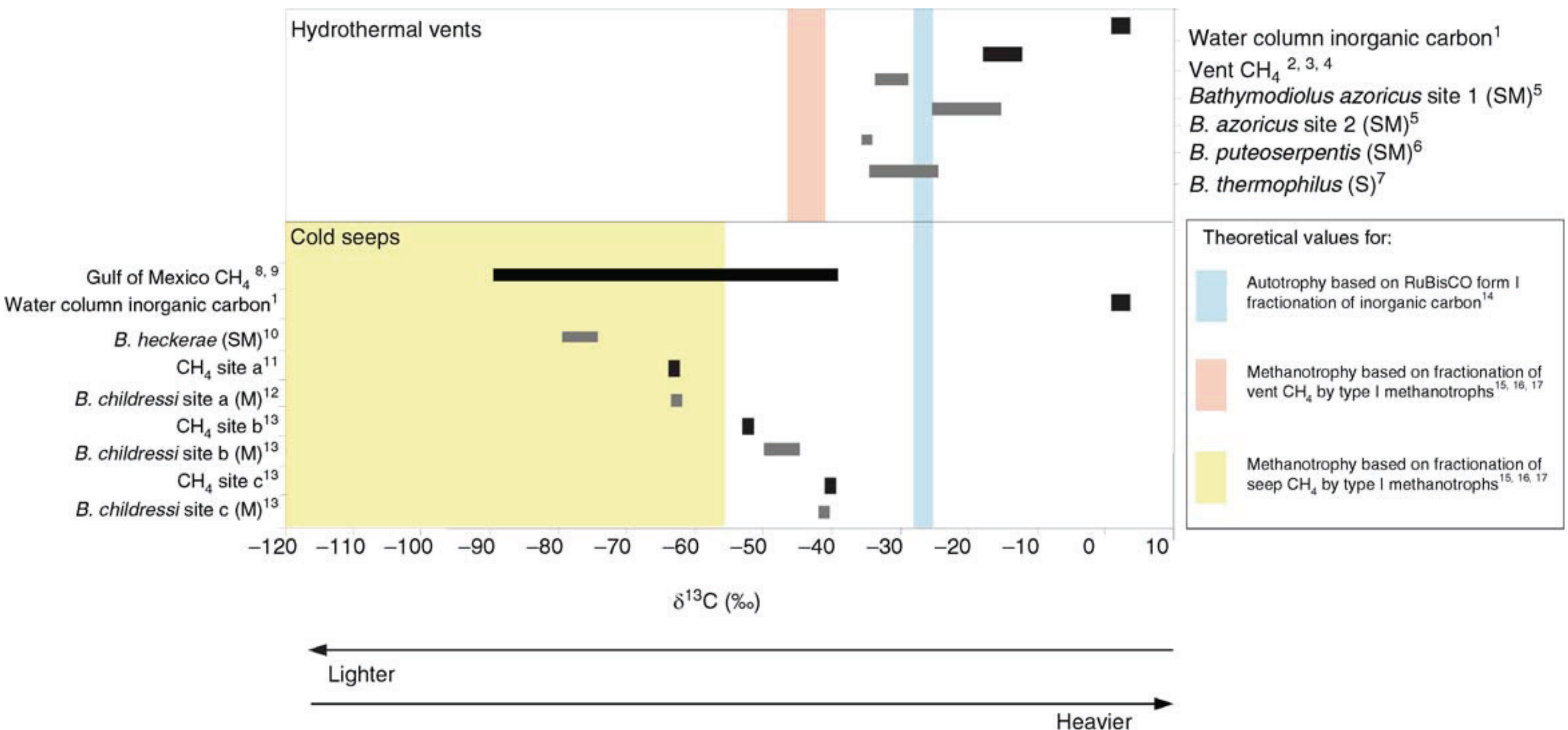


Figure 3

Bathymodiolus mussels from cold seeps generally have lighter $\delta^{13}C$ values than vent species. The blue box indicates the expected theoretical $\delta^{13}C$ values of biomass based on chemoautotrophy (24.4‰ fractionation by the form I RuBisCO enzyme; Robinson et al., 2003) the red and yellow boxes for methanotrophy (16–30‰ fractionation, the maximum value for cultures of methane-oxidizing bacteria; Summons et al., 1994; Jahnke et al., 1999; Templeton et al., 2006). Black bars show the stable isotope signatures of the source carbon, grey bars the measured values for symbiotic mussel gill tissues. The relative contribution of the two carbon sources (CO_2 and CH_4) for mussels with both sulfur-oxidizing (S) and methane-oxidizing (M) symbionts is difficult to quantify because both chemoautotrophy and methanotrophy contribute to biomass. The differences observed for *B. azoricus*

(−16‰, Radford-Knoery et al., 1998) and Indian (−18.5 to −13.8‰, Takai et al., 2004) oceans. It is therefore difficult to estimate the significance of methane to the nutrition of vent animals by measuring the carbon stable isotopic signature, particularly in the vent mussels from the Mid-Atlantic Ridge, which to date have all been shown to host a dual symbiosis with methanotrophic and chemoautotrophic symbionts. Their stable isotope signatures are also influenced by the fixation of inorganic carbon by the co-occurring chemoautotrophic symbionts; furthermore when the chemoautotrophs use CO_2 respired by the methanotrophs, symbiotic tissues will have unusually light $\delta^{13}C$ signatures (Fisher, 1996).

4.4 Molecular Characterization

The gene encoding the subunit containing the active site of particulate methane monooxygenase (pMMO), *pmoA*, is a functional marker for aerobic methane oxidation in bacteria (see McDonald et al., 2008 for a review). Fragments of the *pmoA* gene have been amplified and sequenced from symbiotic gill tissues of five *Bathymodiolus* species. Each appears to have a single *pmoA* gene sequence, except for *B. childressi*, from which five distinct sequences were amplified, four of which group with other *pmoA* sequences from *Bathymodiolus* endosymbionts. The fifth has 91.7% sequence identity to an environmental clone sequence from a hydrothermal vent on the Mid-Atlantic Ridge (Duperron et al., 2007). One study has gone beyond the genetic potential for methanotrophy, and demonstrated the expression of the *pmoA* gene in endosymbionts of *B. azoricus* by simultaneous in situ hybridization of messenger RNA and ribosomal RNA (Pernthaler and Amann, 2004). This study confirms the expression of the *pmoA* gene in the methanotrophic symbiont in a mussel that hosts a dual symbiosis.

In addition to *pmoA*, the gene encoding the large subunit of 1,5-ribulose bisphosphate carboxylase/oxygenase (RuBisCO), *cbbL*, has been amplified and shown to be expressed in methanotrophic symbionts of a *Bathymodiolus* mussel from the western Pacific (Elsaied et al., 2006). Although low level RuBisCO expression and CO_2 fixation activity are known for *Methylococcus capsulatus* Bath (Baxter et al., 2002), a free-living gammaproteobacterial

---

from two sites may be due to differences in the relative contribution of methanotrophy and chemoautotrophy to mussel nutrition, or the relative input of photosynthetically fixed carbon, as these mussels can also filter-feed (Page et al., 1990). In addition, the stable isotope signatures may also reflect differences in the chemical and physical environment of the mussel, which affects the extent of fractionation. *B. childressi* occurs at a number of sites in the Gulf of Mexico, and only has methane-oxidizing symbionts. The measured $\delta^{13}C$ values for *B. childressi* from three different sites (a, b, c) are heavier than their theoretically predicted values, indicating that their methanotrophic symbionts do not fractionate to the extent observed in pure cultures, or that they gain a significant proportion of carbon from sources other than methane, such as filter-feeding, or CO_2 incorporation (Trotsenko and Murrell, 2008). [1]Gruber et al., 1999; [2]Welhan and Craig, 1983; [3]Radford-Knoery et al., 1998; [4]Takai et al., 2004; [5]Trask and Van Dover, 1999; [6]Van Dover and Fry, 1989 [7]Fisher et al., 1988; [8]Lanoil et al., 2001; [9]Sassen et al., 1999; [10]Duperron et al., 2007; [11]Kennicutt et al., 1992; [12]Macavoy et al., 2008; [13]Brooks et al., 1987; [14]Robinson et al., 2003; [15]Summons et al., 1994; [16]Jahnke et al., 1999; [17]Templeton et al., 2006.

methane oxidizer, this gene was not previously known from symbiotic methanotrophs, and the significance of CO_2 as a carbon source for methane-oxidizing symbionts is yet to be investigated. The genome sequence of *M. capsulatus* revealed the potential for an astounding array of additional metabolic capabilities such as hydrogen and sulfide oxidation (Ward et al., 2004; see also Chapter 36, Vol. 2, Part 6), although many of the capabilities suggested by these authors have not yet been demonstrated experimentally (Kelly et al., 2005). Genomic studies of symbiotic methane oxidizers may also reveal genetic potential for the use of additional energy sources.

5 Outlook

Many tools are already in place for investigating symbiotic bacteria, as described in this chapter, but more studies using existing methods as well as the development of new techniques are needed for gaining a better understanding of the symbioses between aerobic methane-oxidizing bacteria and eukaryotes. Molecular methods have been invaluable for describing symbiotic methane-oxidizers, but 16S rRNA sequences from symbionts of other hosts besides bathymodiolin mussels are needed to better understand the diversity, biogeography, and evolution of methanotrophic associations. Genomic analyses of the metabolic potential of symbiotic methanotrophs are underway with the sequencing of symbiont genomes from the hydrothermal vent mussel *B. puteoserpentis* at Genoscope, France in our joint collaboration with Francoise Gaill and Sebastien Duperron (CNRS, France). Post-genomic methods will allow us to quantify the activity and productivity of symbiotic bacteria at the level of the transcriptome and proteome, but in situ fixation techniques are needed to ensure that the long transport times of several hours from the deep sea to the research vessel do not cause metabolic artefacts. Technology for in situ incubations with labeled isotopes and respiration measurements (benthic chambers) has been developed for microbial studies (e.g., within the EU project Exocet/D), but have not yet been deployed on methanotrophic symbioses. Finally, measuring methane concentrations and flux at scales relevant to the distribution of the symbiotic hosts, for example, with reliable sensors for methane, would reveal how spatial and temporal variability influence the biomass and productivity of methanotrophic symbioses. This variability both within a given habitat as well as between different methane-rich environments determines the diversity, abundance, and distribution of methanotrophic symbioses, but understanding the intricate details of this relationship remains a challenge for future research.

Acknowledgments

This work was supported by the Max Planck Society, the Priority Program 1144 (From Mantle to Ocean: Energy- Material- and Life-Cycles at Spreading Axes; contribution number 31) of the German Research Foundation (DFG), the DFG Cluster of Excellence at MARUM, Bremen, and a Marie Curie research training network (MOMARnet) scholarship to JMP. We are grateful to the Census of Marine Life working group ChEss for their support of research on the biogeography of chemosynthetic ecosystems. We would also like to thank two anonymous reviewers for their insightful comments that helped us to improve this chapter.

References

Barry JP, Buck KR, Kochevar RK, Nelson DC, Fujiwara Y, Goffredi SK, Hashimoto J (2002) Methane-based symbiosis in a mussel, *Bathymodiolus platifrons*, from cold seeps in Sagami Bay, Japan. Invertebr Biol 121: 47–54.

Baxter NJ, Hirt RP, Bodrossy L, Kovacs KL, Embley TM, Prosser JI, Murrell JC (2002) The ribulose-1,5-bisphosphate carboxylase/oxygenase gene cluster of *Methylococcus capsulatus* (Bath). Arch Microbiol 177: 279–289.

Beck LA, Sobjinski K (1999) Zur Systematik und Phylogenie hydrothermaler Tiefseemullusken. Courier Forschungsinstitut Senckenberg 215: 31–41.

Borowski C, Giere O, Krieger J, Amann R, Dubilier N (2002) New aspects of the symbiosis in the provannid snail *Ifremeria nautilei* from the North Fiji Back-Arc Basin. Cah Biol Mar 43: 321–324.

Braby CE, Rouse GW, Johnson SB, Jones WJ, Vrijenhoek RC (2007) Bathymetric and temporal variation among *Osedax* boneworms and associated megafauna on whale-falls in Monterey Bay, California. Deep Sea Res A 54: 1773–1791.

Brooks JM, Kennicutt MC, Fisher CR, Macko SA, Cole K, Childress JJ, Bidigare RR, Vetter RD (1987) Deep-sea hydrocarbon seep communities – evidence for energy and nutritional carbon sources. Science 238: 1138–1142.

Cary SC, Fisher CR, Felbeck H (1988) Mussel growth supported by methane as a sole carbon and energy source. Science 240: 79–80.

Cavanaugh CM (1983) Symbiotic chemoautotrophic bacteria in marine invertebrates from sulfide-rich habitats. Nature 302: 58–61.

Cavanaugh CM (1992) Methanotroph-invertebrate symbioses in the marine envrionment: ultrastructural, biochemical and molecular studies. In Microbial Growth on C1 Compounds. JC Murrell and DP Kelly (eds.). Andover, UK: Intercept, pp. 315–328.

Cavanaugh CM, Levering PR, Maki JS, Mitchell R, Lidstrom ME (1987) Symbiosis of methylotrophic bacteria and deep-sea mussels. Nature 325: 346–347.

Cavanaugh CM, McKiness ZP, Newton ILG, Stewart FJ (2006) Marine chemosynthetic symbioses. In The Prokaryotes: An Evolving Electronic Resource for the Microbial Community. M Dworkin, SI Falkow, E Rosenberg, K-H and Schleifer, E (eds.). Stackebrandt New York: Springer.

Charlou JL, Donval JP, Fouquet Y, Jean-Baptiste P, Holm N (2002) Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36° 14′ N, MAR). Chem Geol 191: 345–359.

Childress JJ, Fisher CR, Brooks JM, Kennicutt II MC, Bidigare R, Anderson AE (1986) A methanotrophic marine molluscan (Bivalvia, Mytilidae) symbiosis: Mussels fueled by gas. Science 233: 1306–1308.

De Bary A (1879) 'Die Erscheinung der Symbiose', Vortrag auf der Versammlung der Deutschen Naturforscher und Aerzte zu Cassel. Strassburg: Verlag von Karl J Trubner.

DeChaine EG, Cavanaugh CM (2005) Symbioses of methanotrophs and deep-sea mussels (Mytilidae: Bathymodiolinae). In Molecular Basis of Symbiosis. J Overmann (ed.). Berlin: Springer, pp. 227–250.

Dedysh SN, Ricke P, Liesack W (2004) NifH and NifD phylogenies: An evolutionary basis for understanding nitrogen fixation capabilities of methanotrophic bacteria. Microbiology 150: 1301–1313.

Deming JW, Reysenbach AL, Macko SA, Smith CR (1997) Evidence for the microbial basis of a chemoautotrophic invertebrate community at a whale fall on the deep seafloor: Bone-colonizing bacteria and invertebrate endosymbionts. Microsc Res Tech 37: 162–170.

Distal DL, Lee HKW, Cavanaugh CM (1995) Intracellular coexistence of methanotrophic and thioautotrophic bacteria in a hydrothermal vent mussel. Proc Natl Acad Sci USA 92: 9598–9602.

Dubilier N, Bergin C, Lott C (2008) Symbiotic diversity in marine animals: The art of harnessing chemosynthesis. Nat Rev Microbiol 6: 725–739.

Duperron S, Bergin C, Zielinski F, Blazejak A, Pernthaler A, McKiness ZP, DeChaine E, Cavanaugh CM, Dubilier N (2006) A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge. Environ Microbiol 8: 1441–1447.

Duperron S, Halary S, Lorion J, Sibuet M, Gaill F (2008) Unexpected co-occurrence of six bacterial symbionts in the gills of the cold seep mussel *Idas* sp. (Bivalvia: Mytilidae). Environ Microbiol 10: 433–445.

Duperron S, Nadalig T, Caprais J-C, Sibuet M, Fiala-Medioni A, Amann R, Dubilier N (2005) Dual symbiosis in a *Bathymodiolus* sp. mussel from a methane seep on the Gabon continental margin (southeast Atlantic): 16S rRNA phylogeny and distribution of the symbionts in the gills. Appl Environ Microbiol 71: 1694–1700.

Duperron S, Sibuet M, MacGregor BJ, Kuypers MMM, Fisher CR, Dubilier N (2007) Diversity, relative abundance and metabolic potential of bacterial endosymbionts in three *Bathymodiolus* mussel

species from cold seeps in the Gulf of Mexico. Environ Microbiol 9: 1423–1438.

Elsaied HE, Kaneko R, Naganuma T (2006) Molecular characterization of a deep-sea methanotrophic mussel symbiont that carries a RuBisCO gene. J Mar Biotechnol 8: 511–520.

Endow K, Ohta S (1989) The symbiotic relationship between bacteria and a mesogastropod snail, *Alviniconcha hessleri*, collected from hydothermal vents of the Mariana Back-Arc Basin. Bull Jap Soc Microbiol Ecol 3: 73–82.

Felbeck H (1981) Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia pachyptila* Jones (Vestimentifera). Science 213: 336–338.

Fisher CR (1996) Ecophysiology of primary production at deep-sea vents and seeps. Biosystematics and Ecology Series 11: 313–336.

Fisher CR, Brooks JM, Vodenichar JS, Zande JM, Childress JJ, Burke RA, Jr. (1993) The co-occurrence of methanotrophic and chemoautotrophic sulfur oxidising bacterial symbionts in a deep-sea mussel. Mar Ecol 14: 277–289.

Fisher CR, Childress JJ (1992) Organic carbon transfer from methanotrophic symbionts to the host hydrocarbon-seep mussel. Symbiosis 12: 221–235.

Fisher CR, Childress JJ, Arp AJ, Brooks JM, Distel D, Favuzzi JA, Felbeck H, Hessler R, Johnson KS, Kennicutt MC, Macko SA, Newton A, Powell MA, Somero GN, Soto T (1988) Microhabitat variation in the hydrothermal vent mussel, *Bathymodiolus thermophilus*, at the Rose Garden vent on the Galapagos Rift. Deep Sea Res 35: 1769–1791.

Fujiwara Y, Takai K, Uematsu K, Tsuchida S, Hunt JC, Hashimoto J (2000) Phylogenetic characterisation of endosymbionts in three hydrothermal vent mussels: Influence on host distributions. Mar Ecol Prog Ser 103: 147–155.

Galchenko VF, Pimenov NV, Lein AY, Galkin SV, Miller YM, Ivanov MV (1992) Mixotrophic feeding of *Olgaconcha tufari* Beck (Gastropoda: Prosobrachia) from the active hydrothermal field of the Manus Basin (Bismarck Sea). Dokl Akad Nauk SSSR 323: 776–781.

Gorham E (1991) Northern peatlands – role in the carbon cycle and probable responses to climatic warming. J Appl Ecol 1: 182–195.

Gruber N, Keeling CD, Bacastow RB, Guenther PR, Lueker TJ, Wahlen M, Meijer HAJ, Mook WG, Stocker TF (1999) Spatiotemporal patterns of carbon-13 in the global surface oceans and the oceanic Suess effect. Global Biogeochem Cycles 13: 307–335.

Gustafson RG, Turner RD, Lutz RA, Vrijenhoek RC (1998) A new genus and five new species of mussels (Bivalvia, Mytilidae) from deep-sea sulfide/hydrocarbon seeps in the Gulf of Mexico. Malacologia 40: 63–112.

Hakemian AS, Rosenzweig AC (2007) The biochemistry of methane oxidation. Annu Rev Biochem 76: 223–241.

Hanson RS, Hanson TE (1996) Methanotrophic bacteria. Microbiol Rev 60: 439–47.

Heeschen KU, Hohnberg HJ, Haeckel M, Abegg F, Drews M, Bohrmann G (2007) In situ hydrocarbon concentrations from pressurized cores in surface sediments, Northern Gulf of Mexico. Mar Chem 107: 498–515.

Hentschel U, Usher KM, Taylor MW (2006) Marine sponges as microbial fermenters. FEMS Microbiol Ecol 55: 167–177.

Jahnke LL, Summons RE, Hope JM, Des Marais DJ (1999) Carbon isotopic fractionation in lipids from methanotrophic bacteria II: The effects of physiology and environmental parameters on the biosynthesis and isotopic signatures of biomarkers. Geochim Cosmochim Acta 63: 79–93.

Kadar E, Bettencourt R, Costa V, Santos RS, Lobo-Da-Cunha A, Dando P (2005) Experimentally induced endosymbiont loss and re-acquirement in the hydrothermal vent bivalve *Bathymodiolus azoricus.* J Exp Mar Bio Ecol 318: 99–110.

Kelly DP, Anthony C, Murrell JC (2005) Insights into the obligate methanotroph *Methylococcus capsulatus.* Trends Microbiol 13: 195–198.

Kennicutt II MC, Burke RA Jr, MacDonald IR, Brooks JM, Denoux GJ, Macko SA (1992) Stable isotope partitioning in seep and vent organisms: chemical and ecological significance. Chem Geol 101: 293–310.

Kimura H, Sato M, Sasayama Y, Naganuma T (2003) Molecular characterization and in situ localization of endosymbiotic 16S ribosomal RNA and RuBisCO genes in the pogonophoran tissue. J Mar Biotechnol 5: 261–269.

Kochevar RE, Childress JJ, Fisher CR, Minnich E (1992) The methane mussel: Roles of symbiont and host in the metabolic utilization of methane. Mar Biol 112: 389–401.

Lanoil BD, Sassen R, La Duc MT, Sweet ST, Nealson KH (2001) Bacteria and Archaea physically associated with Gulf of Mexico gas hydrates. Appl Environ Microbiol 67: 5143–5153.

Le Pennec M, Hily A (1984) Anatomie, structure et ultrastructure de la branchie d'un Mytilidae des sites hydrothermeaux du Pacifique oriental. Oceanologica Acta 7: 517–523.

Lechene CP, Luyten Y, McMahon G, Distel DL (2007) Quantitative imaging of nitrogen fixation by individual bacteria within animal cells. Science 317: 1563–1566.

Lösekann T, Robador A, Niemann H, Knittel K, Boetius A, Dubilier N (2008) Endosymbioses between bacteria and deep-sea siboglinid tubeworms from an Arctic Cold Seep (Haakon Mosby Mud Volcano, Barents Sea). Environ Microbiol. 10: 3237–3254.

Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar, Buchner A, Lai T, Steppi S, Jobb G, Forster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, Konig A, Liss T, Lussmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH (2004) ARB: A software environment for sequence data. Nucleic Acids Res 32: 1363–1371.

Macavoy SE, Carney RS, Morgan E, Macko SA (2008) Stable isotope variation among the mussel *Bathymodiolus childressi* and associated heterotrophic fauna at four cold-seep communities in the Gulf of Mexico. J Shellfish Res 27: 147–151.

McDonald IR, Bodrossy L, Chen Y, Murrell JC (2008) Molecular ecology techniques for the study of aerobic methanotrophs. Appl Environ Microbiol 74: 1305–1315.

Opelt K, Chobot V, Hadacek F, Schonmann S, Eberl L, Berg G (2007) Investigations of the structure and function of bacterial communities associated with Sphagnum mosses. Environ Microbiol 9: 2795–2809.

Page HM, Fisher CR, Childress JJ (1990) Role of filter-feeding in the nutritional biology of a deep-sea mussel with methanotrophic symbionts. Mar Biol 104: 251–257.

Pernthaler A, Amann R (2004) Simultaneous fluorescence in situ hybridization of mRNA and rRNA in environmental bacteria. Appl Environ Microbiol 70: 5426–5433.

Pimenov N, Savvichev A, Rusanov I, Lein A, Egorov A, Gebruk A, Moskalev L, Vogt P (1999) Microbial processes of carbon cycle as the base of food chain of Haakon Mosby Mud Volcano benthic community. Geo Marine Letters 19: 89–96.

Pimenov NV, Savvichev AS, Rusanov II, Lein AY, Ivanov MV (2000) Microbiological processes of the carbon and sulfur cycles at cold methane seeps of the North Atlantic. Microbiology 69: 709–720.

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig WG, Peplies J, Glockner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35: 7188–7196.

Radford-Knoery J, Charlou JL, Donval JP, Aballea M, Fouquet Y, Ondreas H (1998) Distribution of dissolved sulfide, methane, and manganese near the seafloor at the Lucky Strike (37 ° 17′ N) and Menez Gwen (37° 50′ N) hydrothermal vent sites on the mid-Atlantic Ridge. Deep Sea Res 45: 367–386.

Raghoebarsing AA, Smolders AJP, Schmid MC, Rijpstra WIC, Wolters-Arts M, Derksen J, Jetten MSM, Schouten S, Damste JSS, Lamers LPM, Roelofs JGM, den Camp HJMO, Strous M (2005) Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. Nature 436: 1153–1156.

Rau GH, Hedges JI (1979) C-13 depletion in a hydrothermal vent mussel – suggestion of a chemosynthetic food source. Science 203: 648–649.

Robinson JJ, Scott KM, Swanson ST, O'Leary MH, Horken K, Tabita FR, Cavanaugh CM (2003) Kinetic isotope effect and characterization of form II RubisCO from the chemoautotrophic endosymbionts of the hydrothermal vent tubeworm *Riftia pachyptila*. Limnol Oceanogr 48: 48–54.

Sassen R, Joye S, Sweet ST, DeFreitas DA, Milkov AV, MacDonald IR (1999) Thermogenic gas hydrates and hydrocarbon gases in complex chemosynthetic communities, Gulf of Mexico continental slope. Org Geochem 30: 485–497.

Schmaljohann R, Faber E, Whiticar MJ, Dando PR (1990) Coexistence of methane-based and sulfur-based endosymbioses between bacteria and invertebrates at a site in the Skagerrak. Mar Ecol Prog Ser 61: 119–124.

Schmaljohann R, Flügel HJ (1987) Methane-oxidizing bacteria in Pogonophora. Sarsia 71: 91–98.

Southward EC (2008) The morphology of bacterial symbioses in the gills of mussels of the genera *Adipicola* and *Idas* (Bivalvia: Mytilidae). J Shellfish Res 27: 139–146.

Southward AJ, Southward EC, Dando PR, Barrett RL, Ling R (1986) Chemoautotrophic function of bacterial symbionts in small Pogonophora. J Mar Biol Assoc U.K. 66: 415–437.

Spiridonova EM, Kuznetsov BB, Pimenov NV, Tourova TP (2006) Phylogenetic characterization of endosymbionts of the hydrothermal vent mussel *Bathymodiolus azoricus* by analysis of the 16S rRNA, *cbbL*, and *pmoA* genes. Microbiology 75: 694–701.

Spiro B, Greenwood PB, Southward AJ, Dando PR (1986) C-13/C-12 ratios in marine invertebrates from reducing sediments – confirmation of nutritional importance of chemoautotrophic endosymbiotic bacteria. Mar Ecol Prog Ser 28: 233–240.

Summons RE, Jahnke LL, Roksandic Z (1994) Carbon isotopic fractionation in lipids from methanotrophic bacteria: Relevance for interpretation of the geochemical record of biomarkers. Geochim Cosmochim Acta 58: 2853–2863.

Takai K, Gamo T, Tsunogai U, Nakayama N, Hirayama H, Nealson KH, Horikoshi K (2004) Geochemical and microbiological evidence for a hydrogen-based, hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) beneath an active

deep-sea hydrothermal field. Extremophiles 8: 269–282.

Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: Evolution, ecology, and biotechnological potential. Microbiol Mol Biol Rev 71: 295–347.

Templeton AS, Chu KH, Alvarez-Cohen L, Conrad ME (2006) Variable carbon isotope fractionation expressed by aerobic CH_4-oxidizing bacteria. Geochim Cosmochim Acta 70: 1739–1752.

Trask JL, Van Dover CL (1999) Site-specific and ontogenetic variations in nutrition of mussels (*Bathymodiolus* sp.) from the Lucky Strike hydrothermal vent field, Mid-Atlantic Ridge. Limnol Oceanogr 44: 334–343.

Trotsenko YA, Murrell JC (2008) Metabolic sspects of aerobic obligate methanotrophy. Adv Appl Microbiol 63: 183–229.

Vacelet J, Bouryesnault N (1995) Carnivorous sponges. Nature 373: 333–335.

Vacelet J, Bouryesnault N, Fiala-Medioni A, Fisher CR (1995) A methanotrophic carnivorous sponge. Nature 377: 296–296.

Vacelet J, Fiala-Medioni A, Fisher CR, Bouryesnault N (1996) Symbiosis between methane-oxidizing bacteria and a deep-sea carnivorous cladorhizid sponge. Mar Ecol Prog Ser 145: 77–85.

Van Dover CL (2000) The Ecology of Deep-Sea Hydrothermal Vents. Princeton, NJ: Princeton University Press.

Van Dover CL, Fry B (1989) Stable isotopic compositions of hydrothermal vent organisms. Mar Biol 102: 257–263.

von Cosel R, Olu K (1998) Gigantism in Mytilidae. A new *Bathymodiolus* from cold seep areas on the Barbados accretionary Prism. C R Acad Sci III 321: 655–663.

Ward N, Larsen O, Sakwa J, Bruseth L, Khouri H, Durkin AS, Dimitrov G, Jiang LX, Scanlan D, Kang KH, Lewis M, Nelson KE, Methe B, Wu M, Heidelberg JF, Paulsen IT, Fouts D, Ravel J, Tettelin H, Ren QH, Read T, DeBoy RT, Seshadri R, Salzberg SL, Jensen HB, Birkeland NK, Nelson WC, Dodson RJ, Grindhaug SH, Holt I, Eidhammer I, Jonasen I, Vanaken S, Utterback T, Feldblyum TV, Fraser CM, Lillehaug JR, Eisen JA (2004) Genomic insights into methanotrophy: The complete genome sequence of *Methylococcus capsulatus* (Bath). PLOS Biology 2: 1616–1628.

Welhan JA, Craig H (1983) Methane, hydrogen and helium in hydrothermal fluids at 21° N on the East Pacific Rise. In Hydrothermal Processes at Seafloor Spreading Centers. PA Rona, K Boström, L Laubier, K Smith (eds.). New York: Plenum, pp. 391–410.

Won YJ, Hallam SJ, O'Mullan GD, Pan IL, Buck KR, Vrijenhoek RC (2003) Environmental acquisition of thiotrophic endosymbionts by deep-sea mussels of the genus *Bathymodiolus.* Appl Environ Microbiol 69: 6785–6792.

Won YJ, Jones WJ, Vrijenhoek RC (2008) Absence of cospeciation between deep-sea mytilids and their thiotrophic endosymbionts. J Shellfish Res 27: 129–138.

Manuscript II: Methanotrophic symbioses in marine invertebrates

Jillian M. Petersen and Nicole Dubilier

Environmental Microbiology Reports **1**:319-335

Environmental Microbiology Reports (2009) **1**(5), 319–335 doi:10.1111/j.1758-2229.2009.00081.x

Minireview

Methanotrophic symbioses in marine invertebrates

Jillian M. Petersen and Nicole Dubilier*
Symbiosis Group, Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359, Bremen, Germany.

Summary

Symbioses between marine animals and aerobic methane-oxidizing bacteria are found at hydrothermal vents and cold seeps in the deep sea where reduced, methane-rich fluids mix with the surrounding oxidized seawater. These habitats are 'oases' in the otherwise nutrient-poor deep sea, where entire ecosystems are fueled by microbial chemosynthesis. By associating with bacteria that gain energy from the oxidation of CH_4 with O_2, the animal host is indirectly able to gain nutrition from methane, an energy source that is otherwise only available to methanotrophic microorganisms. The host, in turn, provides its symbionts with continuous access to both electron acceptors and donors that are only available at a narrow oxic – anoxic interface for free-living methanotrophs. Symbiotic methane oxidizers have resisted all attempts at cultivation, so that all evidence for these symbiotic associations comes from ultrastructural, enzymatic, physiological, stable isotope and molecular biological studies of the symbiotic host tissues. In this review, we present an overview of the habitats and invertebrate hosts in which symbiotic methane oxidizers have been found, and the methods used to investigate these symbioses, focusing on the symbioses of bathymodiolin mussels that have received the most attention among methanotrophic associations.

Introduction

Symbiosis has been a major driving force in the evolution of life on Earth (Margulis and Fester, 1991). When two different organisms cooperate, combining their metabolic and behavioral capabilities, novel trophic strategies can emerge. Methanotrophic symbioses are an excellent example: no animal is known that can live from methane. However, by forming symbiotic associations with aerobic methane-oxidizing bacteria, some marine invertebrates are able to live indirectly from methane (for reviews see DeChaine and Cavanaugh, 2005; Cavanaugh *et al.*, 2006; Dubilier *et al.*, 2008). The evolutionary success of these associations is visible in the large communities they form at deep-sea hydrothermal vents and cold seeps. By teaming up with symbiotic methane oxidizers, the animals can dominate the biomass thanks to the primary productivity of their chemosynthetic symbionts (Van Dover, 2000).

The symbionts use methane as both an electron donor and a carbon source, with oxygen as the electron acceptor, to provide the animal hosts with nutrition. Methanotrophic symbioses are therefore limited to environments where methane co-occurs with oxygen, and have only been found at deep-sea hydrothermal vents and cold seeps. The hosts provide the symbionts with simultaneous access to methane and oxygen, by positioning themselves in the mixing zones between the reducing methane-rich fluids or sediments and the oxidized ambient seawater. They provide the bacteria with a stable environment, helping to buffer them against the temporal and spatial variability in the supply of electron donors and acceptors characteristic of the dynamic vent and seep environment.

All currently known aerobic methanotrophs belong to the *Gammaproteobacteria*, *Alphaproteobacteria* or *Verrucomicrobia* (for recent reviews, see Semrau *et al.*, 2008; Trotsenko and Murrell, 2008). So far, all 16S rRNA sequences of symbiotic methane oxidizers from marine invertebrates belong to a single lineage within the *Gammaproteobacteria*, and are related to type I methanotrophs. Type II methanotrophs belonging to the *Alphaproteobacteria* have not been found as symbionts in marine invertebrates (or in any other animals to our knowledge). However, they are known to form a symbiotic association with a wetland plant (Raghoebarsing *et al.*, 2005). The absence of type II methanotrophs in animals could be due to their lower energy efficiency over type I methanotrophs. For a given amount of methane, the serine pathway used by type II methanotrophs for carbon assimilation requires more energy and is therefore less efficient

Received 12 May, 2009; accepted 16 August, 2009. *For correspondence. E-mail ndubilie@mpi-bremen.de; Tel. (+49) 421 2028 932; Fax (+49) 421 2028 580.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd

than the ribulose monophosphate pathway used by type I methanotrophs (Leak *et al.*, 1985). Given that marine invertebrates rely on their symbionts for most or all of their carbon and energy needs, it may be more advantageous for these to associate with type I than type II methanotrophs. In contrast, a plant host can provide most of its own carbon and energy needs through photoautotrophy, and at least in the wetland plant, their type II methantrophic symbionts provide only 10–15% of their cellular carbon (Raghoebarsing *et al.*, 2005). However, there are still many associations between animals and methanotrophic bacteria that have not yet been characterized by molecular methods. Additional lineages of symbiotic methanotrophic bacteria might still be discovered among these.

To date, no one has succeeded in cultivating symbiotic methane oxidizers. At least some methane-oxidizing symbionts should, however, be cultivable, as there are indications that they may occur in a free-living stage (unpublished data from S. Hallam, cited in Won *et al.*, 2003). In this review, we summarize the morphological, physiological and stable isotope analyses that have been the predominant methods for investigating methanotrophic symbioses in the past. We discuss how molecular methods for the study of uncultured microorganisms can be used to investigate the diversity and metabolism of these symbiotic bacteria, illustrating this with the example of the well-studied symbioses in bathymodiolin mussels.

Marine habitats where methanotrophic symbioses have been found

At hydrothermal vents and cold seeps, animals hosting symbiotic bacteria flourish in dense communities and provide a stark contrast to the rest of the deep sea where the low input of photosynthetically fixed carbon limits biological productivity (Van Dover, 2000). These habitats are shaped by geological and biological processes that bring reduced, methane-rich fluids into contact with the oxygenated bottom seawater, which creates a redox gradient that chemosynthetic microbes can harness for energy to fuel primary production. Depending on the geological setting, methane can be produced biogenically by anaerobic microorganisms belonging to the *Archaea*, abiotically through water-rock interactions in hydrothermal systems, or thermogenically through geothermal alteration of buried organic matter.

Substantial differences in methane concentrations have been measured in environments where methanotrophic symbioses are found, with μM to low mM methane concentrations common at hydrothermal vents and higher mM concentrations dominating at cold seeps (see below). Since the solubility of gaseous substrates such as methane increases with pressure, water depth greatly influences how much methane is available in deep-sea habitats. For example, the solubility of methane in 20°C seawater at atmospheric pressure is 1.25 mM, but would be 250 mM at this temperature 2000 m below the sea surface. All available data for methane concentrations at vents and seeps comes from *ex situ* measurements in which samples are collected in the deep sea and methane measured on board ship. Depressurization and degassing of methane in these samples is therefore a problem for obtaining precise data on methane concentrations in the deep sea.

Hydrothermal vents

While the first chemosynthetic symbioses discovered at hydrothermal vents were those between marine invertebrates and sulfur-oxidizing bacteria (Cavanaugh *et al.*, 1981; Felbeck, 1981), a number of hosts with methane-oxidizing symbionts are now known from vents around the world (DeChaine and Cavanaugh, 2005; Cavanaugh *et al.*, 2006; Dubilier *et al.*, 2008). Hydrothermal vents are found at tectonic plate boundaries, and typically form along mid-ocean ridges where two plates diverge and new oceanic crust is formed. Methane at hydrothermal vents is predominantly produced abiotically in water-rock interactions by inorganic synthesis (for example, Fisher-Tropsch type reactions), where oxidized carbon is reduced to CH_4 and other low molecular weight hydrocarbons under conditions of excess H_2 (reviewed in McCollom and Seewald, 2007). These conditions are found in serpentinite-hosted hydrothermal systems such as the Rainbow and Logatchev vent fields on the Mid-Atlantic Ridge (Charlou *et al.*, 2002). Methane in end-member fluids can reach concentrations as high as 3500 μM at serpentinite-hosted sites (Charlou *et al.*, 2002), while the methane concentration of fluids venting in other geological settings, such as basalt-hosted sites, varies between 7.5 and 2150 μM (McCollom and Seewald, 2007). Despite the typically lower methane concentrations at basalt-hosted vents, these can also host methanotrophic symbioses (DeChaine and Cavanaugh, 2005). At vents covered by sediments, such as the Guaymas Basin, both thermogenic methane, produced by pyrolysis of organic material, as well as biogenic methane, produced by methanogenic archaea can contribute significantly to fluid methane contents (Welhan, 1988). It is currently not known to what extent biogenic methane might also contribute to the fluids of non-sedimented hydrothermal vents, but subsurface methanogenic archaea are clearly present at such vents based on geochemical and microbiological studies (Takai *et al.*, 2004).

Cold seeps

The first methanotrophic symbiosis was discovered in bathymodiolin mussels from cold seeps in the Gulf of

Mexico (Childress *et al.*, 1986). Cold seeps are the result of geological processes at continental margins and plate boundaries, where fluids and gases from buried hydrocarbon deposits reach the seafloor. The surface expressions of these processes include mud volcanoes, pockmarks, brine pools, and oil and asphalt seeps (Jørgensen and Boetius, 2007). Methane at cold seeps is produced thermogenically or biogenically. The methane concentration varies over at least six orders of magnitude in these habitats from below detection to a maximum of 985 mM in Gulf of Mexico gas-hydrate-bearing sediments (Heeschen *et al.*, 2007).

How much methane is needed to support methanotrophic symbiosis?

For hosts that must gain most or all of their nutrition from methanotrophic symbionts, methane oxidation rates must be high enough to meet the metabolic needs of both the symbionts and their host. In contrast to free-living methanotrophs that are directly exposed to environmental methane, all known methanotrophic associations are endosymbiotic with the bacteria occurring within their hosts. These are therefore dependent on diffusive flux of methane through host membranes, as there are no known host carrier proteins for methane (Nelson and Fisher, 1995). Methane transport to the symbionts can be, however, greatly enhanced through advective flux, for example, through water currents produced by the host along the symbiont-containing tissues such as the gills of bathymodiolin mussels. Experimental data for the minimum methane concentration needed by a methanotrophic symbiosis are only available from a single study (Kochevar *et al.*, 1992). Using flow-through pressure respirometry, Kochevar and colleagues (1992) showed that methane concentrations between 25 and 60 μM were required for net growth of *Bathymodiolus* mussels with methanotrophic symbionts. Not only methane concentrations but also methane flux may be critical to the symbiosis. *Bathymodiolus* mussels can assimilate methane down to very low concentrations below reliable detection limits of < 5 μM (Kochevar *et al.*, 1992). This suggests that even very low methane concentrations may be sufficient to at least sustain methanotrophic symbioses if methane flux is high enough.

Too much methane may also limit methanotrophic symbioses. In the study just cited above, Kochevar and colleagues (1992) showed that methane consumption was inhibited at concentrations above 300 μM CH_4. Inhibition could be caused by the build-up of toxic intermediary products of methane oxidation such as methanol, formaldehyde and formate.

Oxygen concentrations in the animal's environment may also limit the ability of the symbionts to sustain a methanotrophic metabolism. Kochevar and colleagues (1992) showed that methane consumption of symbiotic mussels dropped sharply at oxygen concentrations below 50 μM. In general, oxygen concentrations measured around seep and vent animals are higher than this, and can reach up to 200 μM (Mullaugh *et al.*, 2008), but measurements as low as 0 μM have been reported, indicating that the animals are exposed to periods of anoxia (Fisher *et al.*, 1988; Lösekann *et al.*, 2008; Nees *et al.*, 2008).

To date, symbioses with methane-oxidizing bacteria have not been found in chemosynthetic environments such as whale falls and wood falls, although sulfur-oxidizing symbionts can occur here (Dubilier *et al.*, 2008). Goffredi and colleagues (2008) measured up to 4.2 μM CH_4 in sediments at a whale fall in Monterey Canyon, which is within the range reported from seawater just above seep mussels containing methanotrophic symbionts (Duperron *et al.*, 2005). It is possible that a methanotrophic symbiosis is yet to be discovered at wood and whale falls.

Methods for demonstrating methanotrophy in uncultured symbiotic bacteria

In many cases, there is little direct evidence that symbiotic bacteria found in marine invertebrates are able to use methane as an energy source. Evidence is often limited to ultrastructural observations or 16S rRNA analyses showing relatedness to known methanotrophic bacteria. A number of methods have been used to examine if symbiotic bacteria found in marine invertebrates are methanotrophs (Table 1).

Ultrastructure

Methanotrophs are a unique group of bacteria, highly specialized to use methane, and their metabolic specialization can be reflected in their ultrastructure. Intracytoplasmic membranes (ICMs) are found in all currently known aerobic methanotrophs (reviewed in Hanson and Hanson, 1996; Trotsenko and Murrell, 2008), with the exception of the alphaproteobacterial *Methylocella* species and verrucomicobial methanotrophs (Dunfield *et al.*, 2007). The particulate methane monooxygenase (pMMO, see below) is usually localized in the ICMs, and correspondingly these membranes are not present when some strains are grown under conditions where pMMO is not expressed (Hanson and Hanson, 1996). Gammaproteobacterial type I methanotrophs contain characteristic ICM bundles in their cytoplasm in contrast to alphaproteobacterial type II methanotrophs, in which the ICMs are arranged as paired layers at the cell periphery. Transmission electron microscopy of symbiotic tissues is commonly used to look for the presence of ICMs as an

Table 1. Summary of animals hosting methane-oxidizing symbionts.

Group	Common name	Species name	Evidence for methanotrophy[a]									Habitat[b]	References
			TEM[d]	MeDH	UI	LI	SI	16S	FISH	*pmoA*	*mxaF*		
Porifera													
Class *Demospongiae*													
Family *Cladorhizidae*	Sponge	*Cladorhiza methanophila*	x	x			x					S	1
Mollusca													
Class *Bivalvia*													
Family *Mytilidae*	Mussel	*Idas* sp.						x	x	x		S	2
	Mussel	*Bathymodiolus japonicus*	x					x				V&S	3
		B. platifrons	x	x			x	x				V&S	3, 4
		B. childressi	x		x	x	x	x	x	x		S	5, 6, 7, 8
		B. boomerang	x									S	9
		B. brooksi	x	x			x	x	x	x		S	6, 10, 11
		B. heckerae	x	x			x	x	x	x		S	6
		B. mauritanicus	x					x	x			S	12
		B. azoricus	x				x	x	x	x	x	V	13, 14, 15, 16
		B. puteoserpentis	x					x	x		x	V	14, 16
Class *Gastropoda*													
Family *Provannidae*	Snail	*Ifremeria nautilei*	x	x		x	x					V	17, 18, 19, 20
	Snail	*Alviniconcha hessleri*	x									V	21
Annelida													
Class *Polychaeta*													
Family *Siboglinidae*	Tubeworm	*Sclerolinum contortum*[c]	x			x						S	22, 23
	Tubeworm	*Siboglinum poseidoni*	x	x		x	x					S	24, 25

a. TEM, transmission electron microscopy; MeDH, methanol dehydrogenase activity; UI, incubation with unlabelled CH_4; LI, incubation with $^{14}CH_4$; SI, natural abundance stable carbon isotope analysis; 16S, 16S rRNA gene sequenced; FISH, fluorescence *in situ* hybridization; *pmoA*, gene for the active site subunit of particulate methane monooxygenase sequenced; *mxaF*, PCR product was obtained with primers targeting the *mxaF* gene, which encodes the large subunit of MeDH.
b. S = seep, V = vent.
c. In a recent study, only sulfur-oxidizing (but no methane-oxidizing) symbionts were found in *Sclerolinum contortum* (Lösekann *et al.*, 2008).
d. Intracytoplasmic membranes typical of type I methanotrophs were found in all of these animals.
References: 1, Vacelet *et al.* (1995); 2, Duperron *et al.*, 2008; 3, Fujiwara *et al.* (2000); 4, Barry *et al.* (2002); 5, Childress *et al.* (1986); 6, Duperron *et al.* (2007); 7, Cary *et al.* (1988); 8, Kochevar *et al.* (1992); 9, von Cosel and Olu (1998); 10, Fisher *et al.* (1993); 11, Cavanaugh *et al.* (1987); 12, Duperron *et al.* (2005); 13, Trask and Van Dover (1999); 14, Duperron *et al.* (2006); 15, Spiridonova *et al.* (2006); 16, Pimenov *et al.* (2002); 17, Galchenko *et al.* (1992); 18, Borowski *et al.* (2002); 19, Beck and Sobjinski (1999); 20, C. Borowski and N. Dubilier, unpublished results; 21, Endow and Ohta (1989); 22, Pimenov *et al.* (1999); 23, Pimenov *et al.* (2000); 24, Schmaljohann and Flügel (1987); and 25, Schmaljohann *et al.* (1990).

indicator of methanotrophic symbionts. To date, only bacteria with ICM bundles typical of type I methanotrophs have been found in marine invertebrates (Table 1), confirming 16S rRNA analyses that have identified marine methanotrophic symbionts as type I *Gammaproteobacteria* (see below).

Enzyme activity

Enzyme assays were among the first methods used to show that symbiotic bacteria were methane oxidizers (Cavanaugh *et al.*, 1987). The oxidation of methane to methanol is the first step in both the fixation of carbon into cell material, and the oxidation of methane to CO_2 for energy generation. It is catalysed by a particulate (membrane-bound) or soluble methane monooxygenase, pMMO and sMMO respectively (Hanson and Hanson, 1996; Trotsenko and Murrell, 2008). Almost all aerobic methanotrophs express pMMO, but difficulties in working with this membrane-bound protein make measuring its activity problematic (Cavanaugh, 1992; Hakemian and Rosenzweig, 2007). Therefore, the activity of methanol dehydrogenase (MeDH), which catalyses the further oxidation of methanol to formaldehyde, is often used as an indicator of methanotrophy in symbiont-containing host tissues. However, MeDH is not limited to methanotrophs. This enzyme is also present in methylotrophic bacteria that cannot oxidize methane. The presence of MeDH activity is therefore not necessarily an indicator of methanotrophic symbionts, particularly given the recent observation that methylotrophic bacteria distinct from methane oxidizers can occur as symbionts in marine invertebrates (Duperron *et al.*, 2007).

Physiological experiments

The activity and productivity of methane-oxidizing symbionts can be assessed by incubating freshly collected whole animals or symbiont-containing tissues with labelled or unlabelled methane. The first experiments to show growth of an animal with methane as the sole carbon and energy source were done by incubating the

animals in gas-tight chambers, where the consumption of methane and oxygen, and the production of carbon dioxide were measured by gas chromatography (Cary *et al.*, 1988). Methane oxidation for catabolic (oxidation to carbon dioxide) and anabolic (incorporation into cell material) reactions can be measured by incubation with $^{14}CH_4$. Catabolism is measured by the amount of radioactive label in CO_2 after incubation. Anabolism is measured by the incorporation of the radioactive label into acid-stable compounds. Oxidation of $^{14}CH_4$ and incorporation into symbiotic tissues provides strong evidence that methane is used as a carbon and energy source. Not all symbiotic methanotrophs show the same carbon incorporation efficiency, which can be estimated by the ratio of catabolic to anabolic methane oxidation (see under *Bathymodiolin mussels*).

Natural stable isotope abundance

The carbon stable isotope composition of symbiotic tissues has been used to demonstrate the significance of methane as a carbon source for the symbiosis (see Table 1 and Fig. 1). Stable carbon isotope composition is expressed as $\delta^{13}C$, which is the ratio of the heavy ^{13}C isotope to the light ^{12}C isotope in the sample, compared with the ratio in a standard. Because the enzymes that catalyse metabolic reactions prefer the lighter (^{12}C) to the heavier (^{13}C) isotope, the products of biological processes are 'lighter' than reactants, so they have a higher ratio of ^{12}C to ^{13}C. This fractionation, expressed in per mille (‰) results in a shift in the stable isotopic composition of products to lighter $\delta^{13}C$ values compared with reactants. The $\delta^{13}C$ of animals with methane-oxidizing symbionts

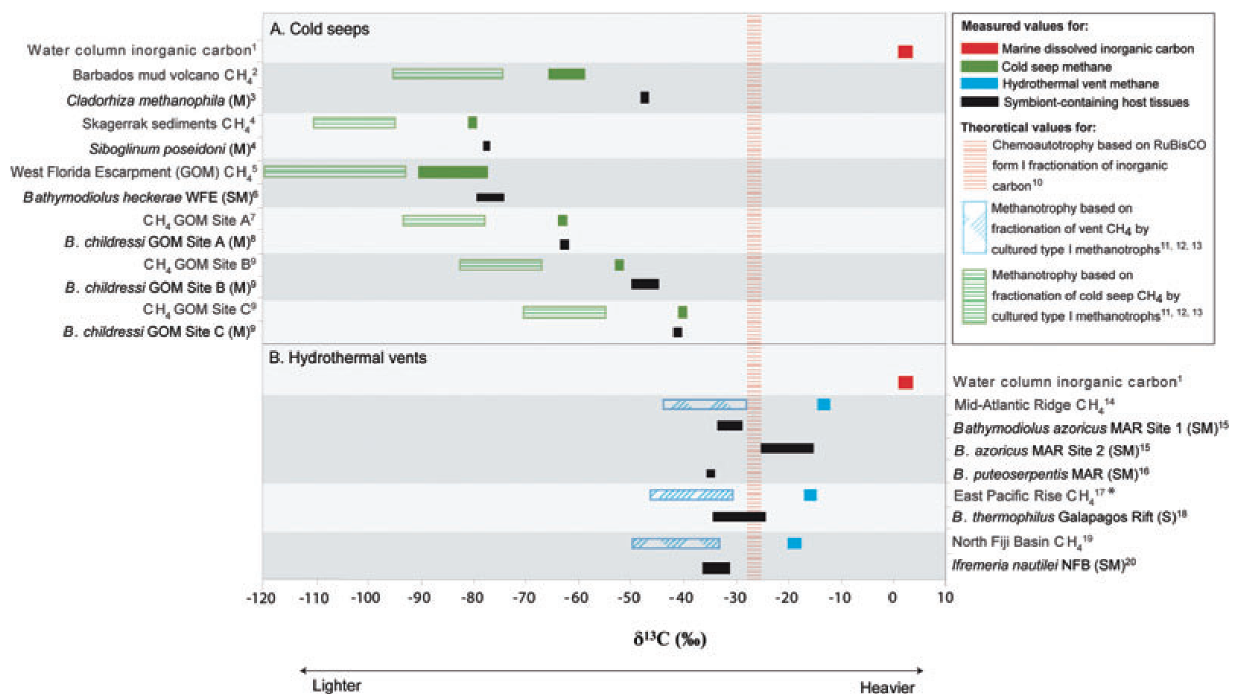


Fig. 1. Stable carbon isotope values of cold seep (A) and hydrothermal vent (B) animals hosting methane-oxidizing symbionts.
The $\delta^{13}C$ signatures of methane at cold seeps are shown in green, and at hydrothermal vents in blue. The $\delta^{13}C$ signature of inorganic carbon is shown in red. The striped boxes show the theoretically predicted values that a methanotroph or a chemoautotroph would have if it only used the carbon source with the $\delta^{13}C$ value shown in the bar to its right. The black bars show the actual values measured in symbiotic tissues. The $\delta^{13}C$ values of most hosts are heavier than their predicted values. Explanations for this discrepancy are described in the section *Natural stable isotope abundance*. The theoretically predicted values for methanotrophic bacteria are based on fractionation values of 16–30‰ measured in bulk cells of cultured methanotrophs (Summons *et al.*, 1994; Jahnke *et al.*, 1999; Templeton *et al.*, 2006), the theoretically predicted values for chemoautotrophic bacteria are for those that use form I RuBisCO to fix CO_2, such as the sulfur-oxidizing symbionts of *Bathymodiolus* mussels (based on fractionation of 24.4‰ by form I RuBisCO; Scott *et al.*, 2004).
S, host has sulfur-oxidizing symbionts; M, host has methane-oxidizing symbionts; SM, host has co-occurring sulfur- and methane-oxidizing symbionts; EPR, East Pacific Rise; GOM, Gulf of Mexico; MAR, Mid-Atlantic Ridge; NFB, North Fiji Basin; WFE, West Florida Escarpment. The asterisk '*' indicates no published measurements for methane at the Galapagos Rift were available.
References: 1, Gruber *et al.* (1999); 2, Vacelet *et al.* (1996); 3, Vacelet *et al.* (1995); 4, Schmaljohann *et al.* (1990); 5, Martens *et al.* (1991); 6, Duperron *et al.* (2007a); 7, Kennicutt *et al.* (1990); 8, Macavoy *et al.* (2008); 9, Brooks *et al.* (1987); 10, Scott *et al.* (2004); 11, Summons *et al.* (1994); 12, Jahnke *et al.* (1999); 13, Templeton *et al.* (2006); 14, Radford-Knoery *et al.* (1998); 15, Trask and Van Dover (1999); 16, Van Dover and Fry (1989); 17, Welhan and Craig (1983); 18, Fisher *et al.* (1988); 19, Ishibashi *et al.* (1994); and 20, Borowski *et al.* (2002).

generally reflects the isotopic composition of the source methane, with a shift towards lighter $\delta^{13}C$ values caused by the symbiont's fractionation of methane. Both biogenic and thermogenic methane have characteristically light $\delta^{13}C$ values (Schoell, 1988), compared with abiotic methane, which has heavier $\delta^{13}C$ values (Welhan, 1988). The contribution of biogenic or thermogenic methane to host nutrition in cold seep fauna with methane-oxidizing symbionts is reflected in their relatively light $\delta^{13}C$ values (Fig. 1). The relatively heavy $\delta^{13}C$ values of vent fauna with methane-oxidizing symbionts could reflect the signature of an abiotic methane source, but will be influenced by other factors such as CO_2 fixation by co-occurring sulfur-oxidizing symbionts.

In theory, it is possible to use $\delta^{13}C$ values to examine the relative contribution of different energy sources to host nutrition, if both the carbon stable isotopic compositions of the carbon sources, and the extent of fractionation by the symbiotic bacteria are known. In practice, however, the $\delta^{13}C$ values for animals containing symbiotic methane oxidizers can be difficult to interpret, as their tissues rarely have $\delta^{13}C$ values that would be expected through fractionation of the source methane or inorganic carbon (Petersen and Dubilier, 2009). Most hosts can use alternative sources of nutrition such as carnivory in sponges, the uptake of dissolved organic material in tubeworms or filter-feeding in mussels (see sections below for each host), which will also influence their $\delta^{13}C$ values. Fractionation factors of symbiotic methane-oxidizing bacteria are not known, and those estimated from studies on free-living bacteria show a range of values between 16‰ and 30‰ and may not be relevant for symbiotic methanotrophs (Summons *et al.*, 1994; Jahnke *et al.*, 1999; Templeton *et al.*, 2006). Also, if the symbiotic bacteria are methane-limited, they will not fractionate to the extent seen in pure cultures. A further problem is that methane-oxidizing bacteria gain a significant proportion of carbon from CO_2 through anapleurotic reactions, as much as 15% in type I methanotrophs (Trotsenko and Murrell, 2008), and this could also influence $\delta^{13}C$ values. Finally, the sampling of carbon sources and specimens is often not closely coordinated and the $\delta^{13}C$ values of carbon sources are inferred from other studies that may or may not have sampled methane and CO_2 close to the site at which the specimens were sampled.

Molecular characterization

The gene typically used as a functional marker for aerobic methane oxidizers is *pmoA*, encoding the active site subunit of particulate methane monooxygenase (pMMO) (see McDonald *et al.*, 2008 for a review). The presence of *pmoA* in symbiont-containing tissues, as shown by PCR amplification and sequencing, provides an indication that symbiotic bacteria can oxidize methane (Table 1). Primers targeting the genes for subunits of sMMO (*mmoX*) and MeDH (*mxaF*) can also be used to investigate methanotrophic and methylotrophic potential in uncultivated symbionts (McDonald *et al.*, 2008). There is only one report of the successful amplification of the *mxaF* gene, by Pimenov and colleagues (2002), who obtained a PCR product of the expected size using primers specific to the *mxaF* gene, from hydrothermal vent mussels with methanotrophic symbionts (see Table 1). As the authors did not describe a sequence for the obtained PCR product, there is currently no *mxaF* gene from a methanotrophic symbiont in the database. The *mmoX* gene has not been amplified or sequenced from symbiotic methanotrophs, indicating that these might not have a soluble methane monooxygenase.

Marine invertebrate hosts of methanotrophic symbionts

Symbiotic methane-oxidizing bacteria have been described in such diverse marine invertebrates as the sponge *Cladorhiza methanophila*, tubeworms of the *Siboglinum* genus, the hydrothermal vent snails *Ifremeria nautilei* and *Alviniconcha hessleri*, and deep-sea bathymodiolin mussels of two genera, *Bathymodiolus* and *Idas*. This diversity of hosts spans four different metazoan classes and families, and includes animals from the groups *Porifera*, *Mollusca* and *Annelida* (Table 1).

Cladorhizid sponges

Although marine sponges are commonly associated with a highly diverse array of symbiotic bacteria (reviewed in Hentschel *et al.*, 2006; Taylor *et al.*, 2007), methane-oxidizing bacteria have only been described in a single species, *Cladorhiza methanophila*. Like the other cladorhizid sponges, *C. methanophila* has developed carnivory as a unique adaptation to the nutrient-poor deep sea, and cannot filter-feed (Vacelet *et al.*, 1995). Sponges are rarely dominant members of chemosynthetic ecosystems, but *C. methanophila* is the exception. It occurs in large aggregations of hundreds of individuals at a mud volcano in the Barbados Trench (Vacelet *et al.*, 1995). Transmission electron microscopy showed that symbiotic bacteria with ICMs typical of type I methanotrophs occurred inside the sponge body, both extracellularly in the sponge mesohyl, as well as intracellularly, in all mesohyl cell types (Vacelet *et al.*, 1995). Methanol dehydrogenase activity in the symbiont-containing sponge tissues and very negative $\delta^{13}C$ values (from –48.4‰ to –48.8‰, Fig. 1) also indicated methylotrophic metabolism and contribution of methane to sponge nutrition. Bacteria with an ultrastructure typical of methane-oxidizing bacte-

ria were also found in *C. methanophila* eggs (Vacelet *et al.*, 1996), indicating that these bacteria are passed directly from one generation to the next, termed vertical transmission. Given that cladorhizid sponges without methanotrophic symbionts commonly occur in the deep-sea in only small clumps of low density due to nutrient limitation, it is likely that the relatively large biomass of *C. methanophila* in the Barbados Trench is due to nutritional benefits provided by its methane-oxidizing symbionts (Vacelet *et al.*, 1996).

Siboglinid tubeworms

Siboglinids are a group of marine annelid tubeworms with a greatly reduced digestive system. They were originally thought to live heterotrophically, as some of the very thin and long siboglinid species were shown to take up dissolved organic matter through the epidermis (Southward *et al.*, 1979). However, all currently known siboglinid worms have an obligate association with endosymbiotic bacteria that contribute to host nutrition (Bright and Giere, 2005).

Most hydrothermal vent and cold seep siboglinids investigated to date are associated with sulfur-oxidizing symbionts; however, methane-oxidizing symbionts have been described in two siboglinid species, *Siboglinum poseidoni* from a methane seep in the Skagerrak (Schmaljohann and Flügel, 1987), and *Sclerolinum contortum* from the Haakon Mosby Mud Volcano (HMMV) in the Barents Sea (Pimenov *et al.*, 1999). Both species harboured endosymbiotic bacteria with type I ICMs (Schmaljohann and Flügel, 1987; Pimenov *et al.*, 2000), and $^{14}CH_4$ oxidation could also be measured in both (Schmaljohann and Flügel, 1987; Pimenov *et al.*, 1999). However, a recent molecular analysis of *S. contortum* from HMMV provided evidence that these tubeworms host only sulfur-oxidizing symbionts (Lösekann *et al.*, 2008). The presence of morphologically similar co-occurring host species at HMMV of which one hosts methanotrophs and the other thiotrophs could explain these contradictory results for *S. contortum*. While Lösekann and colleagues (2008) found no evidence for such cryptic speciation based on the analysis of two host genes, the small number of only three individuals analysed in this study may not have been sufficient to reveal genetic differences within the HMMV *S. contortum* population.

Siboglinum poseidoni is found in an area of methane seepage in the Skagerrak, north of Denmark. The tubeworms are only present in steeply sloping areas where mudslides expose the deeper sediment layers, bringing methane-saturated sediment to within 20 cm of the sea floor (Dando *et al.*, 1994). *Siboglinum poseidoni* reaches a maximum length of 19 cm (Flügel and Callsencencic, 1992), and shows no mechanism for the active uptake of methane, and therefore appears to be restricted to areas with high methane concentrations, close to the sediment surface. Activity of the key enzyme of the ribulose monophosphate pathway of C_1 assimilation, hexulose 6-phosphate synthase, was shown in *S. poseidoni* (Schmaljohann *et al.*, 1990), indicating that its symbionts may be related to type I methanotrophs. However, no phylogenetic analysis has been done to confirm this.

In a third siboglinid tubeworm species, *Oligobrachia mashikoi,* bacterial symbionts were hypothesized to be methanotrophs based on their 16S rRNA gene phylogeny, but genes characteristic for methantrophy were not found despite multiple attempts to amplify these (Kimura *et al.*, 2003). Studies in our group showed that the *O. mashikoi* symbints were more closely related to the sulfur-oxidizing symbiont of *O. haakonmosbiensis* than to known symbiotic and free-living methane oxidizers (Lösekann *et al.*, 2008).

Provannid snails

Hydrothermal vents in back-arc basins of the Western Pacific are often dominated by provannid snails belonging to two genera, *Ifremeria* and *Alviniconcha* (Endow and Ohta, 1989; Galchenko *et al.*, 1992; Beck and Sobjinski, 1999). These hosts have enlarged gills housing the symbiotic bacteria, and a reduced digestive system (Beck and Sobjinski, 1999), indicating their dependence on the symbiotic bacteria for nutrition. Provannids are often characterized as having only chemoautotrophic sulfur-oxidizing symbionts (Windoffer and Giere, 1997; Suzuki *et al.*, 2006a,b). However, there is evidence for methane-oxidizing symbionts in *A. hessleri,* based on transmission electron microscopy, and in *I. nautilei* based on ultrastructural, enzymatic, stable isotope and physiological studies (Table 1). In both host species, the methanotrophic symbiont is less abundant than the co-occurring sulfur-oxidizing symbiont (Beck and Sobjinski, 1999). With methods such as transmission electron microscopy, where only a very small part of the symbiotic tissue can be investigated, its presence can therefore often be missed. Physiological experiments that showed incorporation of radioactively labelled methane into *I. nautilei* gill tissue showed that methane might still be a significant source of nutrition for the snail, despite their low abundance (Galchenko *et al.*, 1992). Indeed, the activity of the symbiotic bacteria and their relative contribution to host nutrition need not be reflected in their abundance, but incubations quantifying the relative activity of co-occurring methane- and sulfur-oxidizing symbionts that would indicate the relative contribution of sulfur and methane as energy sources for the host are lacking.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd,

Bathymodiolin mussels

Bathymodiolin mussels were the first animals in which symbiotic methane-oxidizing bacteria were found (Childress *et al.*, 1986), and most of what we know about methanotrophic symbioses is based on studies of these mussels. Like the provannid snails, the mussels have reduced digestive systems (Gustafson *et al.*, 1998) and characteristically enlarged gills in which the symbionts are housed in specialized gill bacteriocytes (Fig. 2). All bathymodiolin mussels have symbiotic bacteria, but most host species harbour sulfur-oxidizing symbionts, while only some harbour methane-oxidizing symbionts (Duperron *et al.*, 2009).

Mussels of the genus Idas*. Idas* mussels have been found worldwide at cold seeps and sunken organic debris such as whale and wood falls (Braby *et al.*, 2007; Duperron *et al.*, 2009). Most *Idas* species appear to have sulfur-oxidizing symbionts (Deming *et al.*, 1997; Southward, 2008). Only a single species of *Idas,* found at a cold seep in the Mediterranean, has been shown to harbour symbiotic methane oxidizers based on 16S rRNA analysis and the presence of the *pmoA* gene in its symbiotic gill tissues (Duperron *et al.*, 2008). In addition to its methanotrophic symbiont, five further symbiont 16S rRNA phylotypes were found in this mussel (Duperron *et al.*, 2008).

Mussels of the genus Bathymodiolus*.* Mussels of the genus *Bathymodiolus* dominate the biomass at many hydrothermal vents and cold seeps throughout the world. They were first discovered at vents on the Galapagos Rift, in association with sulfur-oxidizing endosymbiotic bacteria (Cavanaugh, 1983), but only 3 years later, the first description of methane-oxidizing symbionts followed in *B. childressi* from cold seeps in the Gulf of Mexico (Childress *et al.*, 1986).

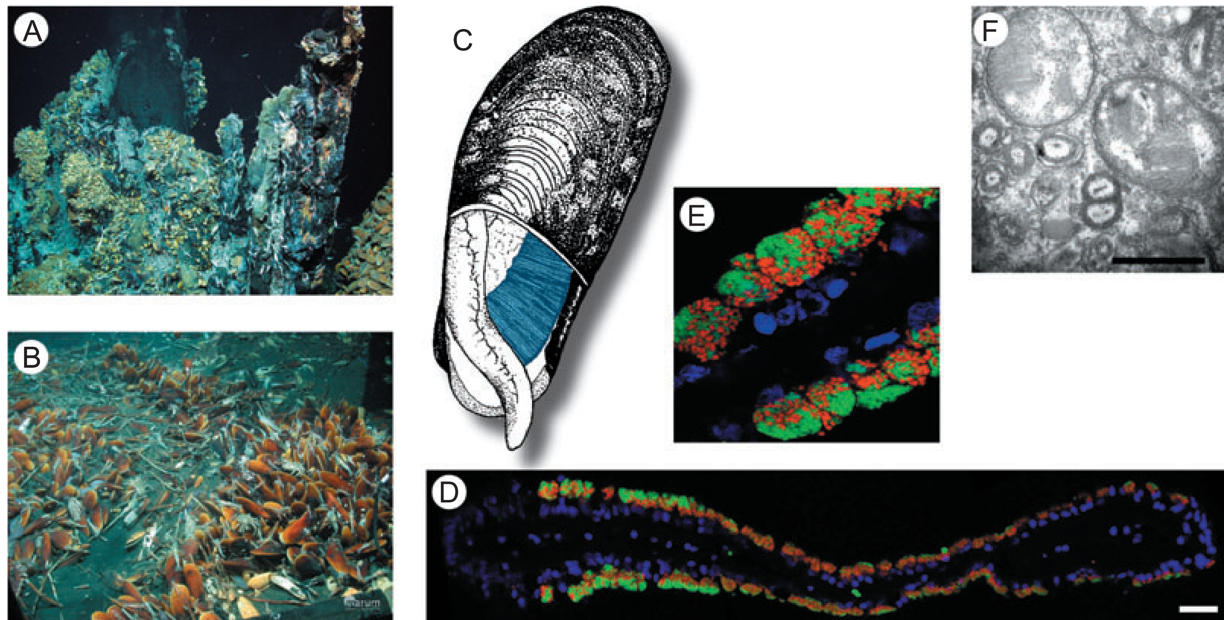


Fig. 2. Bathymodiolin mussel symbiosis.
A. The vent habitat. *Bathymodiolus puteoserpentis* mussels covering a sulfide chimney at the Logatchev hydrothermal vent field on the Mid-Atlantic Ridge (MARUM; University Bremen, Expedition HYDROMAR II M64/2). Mussels can be seen with co-occurring shrimp, *Rimicaris exoculata*.
B. The seep habitat. *Bathymodiolus* sp. from a cold seep off the West African coast cover the sedimented seafloor (MARUM; University Bremen, Expedition GUINECO M76/3b).
C. Sketch of a *Bathymodiolus* mussel with a part of the shell removed to show the gill (in blue), which houses the symbiotic bacteria (Christian Lott, MPI Bremen/HYDRA). The gill is composed of rows of many filaments.
D. Fluorescence *in situ* hybridization of a cross-section through a single filament of the symbiont-containing gill tissue of *B. puteoserpentis*, showing the host nuclei in blue (DAPI staining), the methane-oxidizing symbionts in red (hybridized with a specific probe for the *B. puteoserpentis* methanotroph 16S rRNA sequence), and the sulfur-oxidizing symbionts in green (hybridized with a specific probe for the 16S rRNA sequence of the *B. puteoserpentis* sulfur oxidizer, image from Dennis Fink, MPI Bremen, FISH probes from Duperron *et al.*, 2006). The ciliated edge (on the left) is directly exposed to the fluid flowing across the gills and the abundance of symbionts is highest here. Scale bar = 10 μm.
E. Enlarged view of (D). The methane-oxidizing and sulfur-oxidizing symbionts co-occur in the host bacteriocytes. Scale bar = 5 μm.
F. Transmission electron micrograph of a *Bathymodiolus* mussel that harbours a dual symbiosis (from Duperron *et al.*, 2005). The small morphotype is the sulfur oxidizer, the large morphotype is the methane oxidizer, which has intracytoplasmic membranes typical of type I methanotrophs. Scale bar = 1 μm.

Single, dual and multiple symbioses

In the first bathymodolin hosts to be studied, each host was associated with only a single type of symbiont, *B. thermophilus* from the Galapagos Rift with a sulfur oxidizer (Cavanaugh, 1983) and *B. childressi* from the Gulf of Mexico with a methane oxidizer (Childress *et al.*, 1986). Dual symbioses in which two bacteria co-exist within a single cell were unknown to occur in animal-bacterial symbioses. These were first discovered in *Bathymodiolus* mussels that host both sulfide- and methane-oxidizing bacteria that co-occur in the gill bacteriocytes (Fig. 2) (Fisher *et al.*, 1993; Distel *et al.*, 1995). To date, 10 species of *Bathymodiolus* have been shown to harbour methane-oxidizing bacteria (summarized in Table 1). Of these, three host only methanotrophic bacteria (*B. childressi, B. japonicus* and *B. platifrons*), and six have a dual symbiosis with sulfur- and methane-oxidizing bacteria (*B. boomerang, B. brooksi, B. puteoserpentis, B. azoricus, B.* sp. Wideawake and *B.* sp. Gabon Margin). One species, *B. heckerae*, from the Gulf of Mexico, can host up to four distinct symbiotic phylotypes, two sulfur oxidizers, one methane oxidizer, and one not previously described from any marine invertebrate symbiosis that is related to free-living methylotrophic bacteria (Duperron *et al.*, 2007). Intriguingly, studies of the distribution of each symbiont type in *B. heckerae* with fluorescence *in situ* hybridization showed that the methylotrophic and methanotrophic symbionts co-occurred tightly in this mussel species, suggesting metabolic interactions between them. Methanol, which is produced as an intermediate during methane oxidation by the activity of the methane monooxygenase enzymes (MMO) (Hanson and Hanson, 1996), is a non-polar compound and can easily leak out of the cell membrane of methanotrophs before it can be further oxidized to formaldehyde. Methanol consumption by the co-occurring methylotroph would allow the use of a carbon source that would otherwise be lost to the symbiosis, but this is yet to be proven. At high methane consumption rates, toxic intermediary metabolites such as methanol, formaldehyde and formate may reach inhibitory concentrations (see the section How much methane is needed to support methanotrophic symbiosis above). The methylotrophic symbiont would provide the additional benefit of removing these toxic compounds, perhaps allowing higher methane turnover in this species.

Transfer of carbon from symbiont to host

Organic carbon can be passed from symbiont to host either by the active or passive transfer of small organic molecules from the symbiont cells, by host digestion of the symbionts, or a combination of both (Cavanaugh *et al.*, 2006). In *Bathymodiolus* mussels incubated with $^{14}CH_4$, labelled organic carbon only appeared in the host after 1–5 days, indicating that the transfer of organic carbon from the symbiotic methanotrophs to the host occurs by the digestion of the symbionts (Fisher and Childress, 1992), rather than the translocation of small organic compounds, which is rapid and occurs within seconds in the hydrothermal vent tubeworm *Riftia pachyptila* (Felbeck and Turner, 1995). The occurrence of degenerate stages of the methanotrophic symbionts in transmission electron micrographs of *Bathymodiolus* mussels is also consistent with digestion as a means of carbon transfer from symbiotic methane oxidizers to their hosts (Barry *et al.*, 2002).

Stable isotope composition

Significant differences have been found in the $\delta^{13}C$ values of *Bathmodiolus* mussels containing methane-oxidizing symbionts (Fig. 1). Mussels with only methane-oxidizing symbionts typically have lighter $\delta^{13}C$ values than those with both methane- and sulfur-oxidizing symbionts. In the latter, both CO_2 from autotrophy and CH_4 from methanotrophy contribute to biomass, and it has been hypothesized that the relative abundance of sulfur- and methane-oxidizing symbionts in mussel gill tissues will affect their $\delta^{13}C$ values (Trask and Van Dover, 1999; Colaco *et al.*, 2002). For example, in *B. azoricus* from the Mid-Atlantic Ridge which hosts a dual symbiosis, mussels from Site 1 had lighter values than at Site 2 (Fig. 1), and this was attributed to the higher abundance of methanotrophs in mussels from Site 1 (Trask and Van Dover, 1999). However, as in other dual symbioses, the relative contribution of the sulfur- and methane-oxidizing symbionts to host biomass is difficult to quantify and has not yet been characterized in detail. A further problem in the interpretation of carbon stable isotope values of hosts with dual symbionts arises if the sulfur-oxidizing symbionts incorporate CO_2 produced by the co-occurring methanotrophs, as this will be isotopically lighter than seawater CO_2 (Fisher, 1996).

Filter-feeding by the host can also influence $\delta^{13}C$ values. A number of *ex situ* incubation studies have shown that *Bathymodiolus* species can filter-feed, but they do this less effectively than shallow-water mussels that rely on filter-feeding for all of their nutrition (Page *et al.*, 1990; Page *et al.*, 1991; Pile and Young, 1999). The *in situ* contribution of filter-feeding to host nutrition is still unknown, but a recent attempt to model the relative contribution of symbiosis and filter-feeding in *B. azoricus* indicated that small mussels are heavily dependant on filter-feeding while larger mussels rely mainly on symbiosis for their nutrition (Martins *et al.*, 2008).

Efficiency of methane assimilation

While the amount of methane needed to sustain a symbiosis has only been investigated experimentally for one

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd,

Bathymodiolus species (see the section *How much methane is needed to support methanotrophic symbiosis?* above), the efficiency of bacterial methane assimilation, the amount of methane incorporated into cell material compared with the amount respired to CO_2, has been investigated in a number of mussel species. In *B. brooksi* nearly 70% of the methane taken up by the symbionts was oxidized to CO_2 (Cavanaugh, 1992). A much higher efficiency of methane assimilation was observed in *B. childressi* from a methane seep in the Gulf of Mexico with only 30% of the methane consumed released as carbon dioxide (Kochevar *et al*., 1992). Pimenov and colleagues (2002) showed a range of efficiencies from 17.5% to 49% methane oxidized to CO_2 in *B. azoricus* and *B. puetoserpentis*, while Robinson and colleagues (1998) showed that an average of 40% of methane carbon was oxidized to CO_2 in *B. puteoserpentis*. The reasons for these large differences in carbon conversion efficiency between methanotrophic symbionts that are so closely related are not known. Furthermore, as described above, these comparisons could be confounded in a dual symbiosis, if sharing of carbon occurs between methanotrophs producing CO_2, and chemoautotrophic symbionts, which fix CO_2 (Fisher, 1996). Finally, methane oxidation rates measured at atmospheric pressure after bringing the animals up from the deep sea may not reflect the true activity of the symbiotic association *in situ*. We are unaware of any study where methane oxidation in symbiotic animals was measured in pressure vessels that would better simulate conditions in their natural habitat.

Molecular characterization

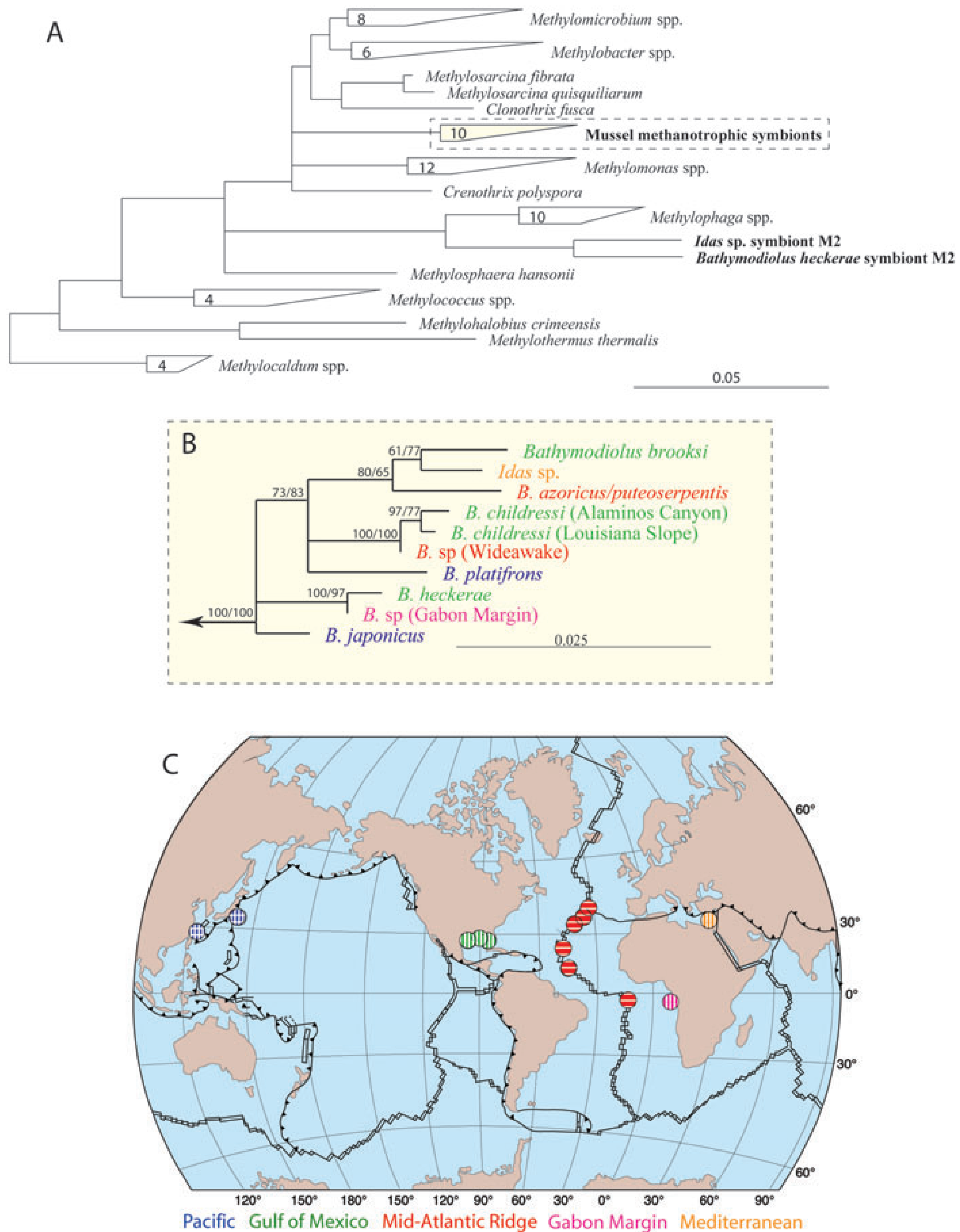
Fragments of the *pmoA* gene have been amplified and sequenced from symbiotic gill tissues of five *Bathymodiolus* and one *Idas* species (Table 1). Only a single *pmoA* gene sequence was retrieved from each host species, except for *B. childressi*, from which five phylogenetically distinct sequences were amplified. This result was surprising because only a single 16S rRNA symbiont phylotype was found in *B. childressi* (Duperron *et al*., 2007), and no known free-living methanotroph has more than three copies of the *pmoA* gene (Dunfield *et al*., 2007). Four of the *B. childressi pmoA* sequences were closely related to each other and grouped with *pmoA* sequences from other *Bathymodiolus* hosts, but the fifth was phylogenetically distinct from all known *Bathymodiolus* symbiont *pmoA* sequences and had 91.7% identity to an environmental clone sequence from a hydrothermal vent on the Mid-Atlantic Ridge (Duperron *et al*., 2007). We are currently examining if these five *pmoA* sequences originated from additional methanotrophic symbionts that were not detected in the Duperron and colleagues' (2007) study, or if there is indeed only a single methanotrophic symbiont in *B. childressi* with an unusually high copy number of *pmoA* genes.

One study has demonstrated the expression of the *pmoA* gene in symbiotic methane-oxidizers. With simultaneous *in situ* hybridization of messenger RNA and ribosomal RNA, Pernthaler and Amann (2004) showed that the *pmoA* gene is expressed in single cells of the methanotrophic symbiont in *B. azoricus*, which also hosts co-occurring sulfur-oxidizing symbionts.

Phylogeny of the bathymodiolin mussel symbionts

Analysis of the 16S rRNA sequences of the bathymodiolin mussel symbionts show that they are closely related to each other (96.6–99.7% identity), and cluster in a monophyletic clade within the *Gammaproteobacteria*, suggesting that they evolved from only a single bacterial lineage (Fig. 3). Free-living type I methane oxidizers in the genera *Methylomonas*, *Methylobacter* and *Methylomicrobium* are sister groups to the symbiotic methane oxidizers (Fig. 3). With only one exception, each host species contains a single methanotrophic 16S rRNA phylotype, which means that methanotrophic symbionts of mussels are generally host-specific at the 16S rRNA level. *Bathymodiolus azoricus* and *B. puteoserpentis* from vents on the Mid-Atlantic Ridge, however, share an identical symbiont 16S rRNA

Fig. 3. Phylogeny and biogeography of the bathymodiolin mussel symbionts.
A. Phylogenetic analyses of the 16S rRNA gene show that the marine methanotrophic symbionts from bathymodiolin mussels (nine *Bathymodiolus* and one *Idas* species) form a single, monophyletic clade with *Methylomicrobium*, *Methylobacter* and *Methylomonas* as sister groups. Two bathymodiolin species also have symbionts related to free-living methylotrophic *Methylophaga*: *Idas* sp. and *B. heckerae*. This is a consensus tree calculated by parsimony, maximum likelihood and neighbour-joining methods using a 40% positional variability filter with the SILVA (Pruesse *et al*., 2007) alignment in the ARB package (Ludwig *et al*., 2004). Bar indicates 5% sequence divergence. Outgroup (not shown) was *Allochromatium vinosum*.
B. There is no obvious geographic trend in the phylogeny of methane-oxidizing symbionts from bathymodiolin mussels. Symbionts from distant geographic locations can be more closely related (for example *B. heckerae* from the Gulf of Mexico and *B*. sp. from the Gabon Margin), and symbionts from different host species in the same geographic region can be more distantly related (for example, *B. heckerae*, *B. brooksi* and *B. childressi*, all found in the Gulf of Mexico). The tree was calculated as in (A), except that a 50% positional variability filter was used. Bootstrap values (maximum likelihood/parsimony, 100 replicates) indicate the robustness of each node. Bar indicates 2.5% sequence divergence. Outgroup (not shown) was *Methylomicrobium album*.
C. Map showing the habitat locations of the mussel species. Vents = horizontal stripes, Cold seeps = vertical stripes. *Bathymodiolus japonicus* and *B. platifrons* from the West Pacific are found at both vents and seeps.



phylotype (Duperron *et al.*, 2006). Analyses at the strain level, based on the internal transcribed spacer of the ribosomal operon, indicate that multiple strains of methanotrophic symbionts can co-occur within these host species (unpublished data from S. Hallam, cited in Won *et al.*, 2003).

Transmission

There have been no experimental studies on the transmission of methane-oxidizing symbionts, but the 16S rRNA phylogeny of methanotrophic symbionts can be used to assess their mode of transmission. There are two

ways in which hosts can acquire their symbionts, termed vertical and horizontal transmission (reviewed in Bright and Bulgheresi, 2009). In vertical transmission, symbionts are transferred directly from the parent to the egg or embryo. If symbiont transmission is vertical, hosts and symbionts have a parallel or congruent phylogeny as a result of their tightly shared evolutionary history (for example in vesicomyid clams as described in Peek *et al.*, 1998; Goffredi *et al.*, 2003; Hurtado *et al.*, 2003, but also see Stewart *et al.*, 2008 for evidence for lateral symbiont acquisition). In contrast, horizontal transmission is independent of host reproduction, and the symbionts are taken up from the environment, or from co-occurring hosts. Horizontal transmission can decouple host and symbiont phylogenies (for example in tubeworms as described in Nelson and Fisher, 2000; Vrijenhoek *et al.*, 2007). Although 16S rRNA symbiont sequences are currently only available for a limited number of *Bathymodiolus* species, and the phylogenetic resolution of their 16S rRNA gene is limited, our preliminary analysis indicates that host and symbiont phylogenies are incongruent for this group (J.M. Petersen and N. Dubilier, unpublished data). This corresponds well with morphological and phylogenetic analyses of the sulfur-oxidizing symbionts of bathymodiolin mussels, indicating that these are also acquired horizontally from the environment (Won *et al.*, 2003; Kadar *et al.*, 2005; DeChaine *et al.*, 2006; Won *et al.*, 2008).

Biogeography

The phylogeny of the symbiotic bacteria can also provide insights into evolutionary processes such as dispersal and colonization that affect their diversity and distribution. Bathymodiolin mussels occur worldwide at geographically isolated vent and seep sites. It was assumed that geography would play a major role in determining the distribution of vent biota (Van Dover *et al.*, 2002), but the phylogeny of methanotrophic symbionts shows no obvious geographic trend (Fig. 3). One intriguing observation is that symbionts from the Eastern Atlantic (*B.* sp. Gabon Margin) are more closely related to those of *B. heckerae* from the Gulf of Mexico than to the *B. puteosperpentis* symbionts from the Mid-Atlantic Ridge, which is much closer geographically. Deep ocean currents crossing the Atlantic from east to west have been proposed to explain this observation (see the Census of Marine Life/ChEss website on the Atlantic equatorial belt hypothesis: http://www.noc.soton.ac.uk/chess/science/equatorial_belt.html). This hypothesis is supported by phylogenetic analyses showing a close relationship between the bathymodolin mussel hosts from seeps in the Gulf of Mexico and Barbados with species from seeps in the East Atlantic (Cordes *et al.*, 2007; Roy *et al.*, 2007).

Concluding remarks

Molecular methods have been invaluable in understanding symbioses between marine invertebrates and methane-oxidizing bacteria, but there are still many methane-oxidizing symbionts, identified by the methods listed above, for which 16S rRNA gene sequences are not available. Further studies on the phylogeny of methane-oxidizing symbionts from hosts other than the bathymodiolin mussels will help us to understand how these highly successful partnerships have evolved. These are of particular interest for examining if all methane-oxidizing symbionts continue to belong to only a single lineage, in contrast to sulfur-oxidizing symbionts, where sequences are available from many different host groups and show that the symbionts belong to at least nine distinct phylogenetic clades (Dubilier *et al.*, 2008). In addition, investigating the current distribution of methane-oxidizing symbionts with high resolution markers such as the internal transcribed spacer will provide insights into evolutionary processes, such as dispersal, colonization and adaptation to new habitats, overcoming the challenges of observing these processes in the remote deep sea.

Many tools are already in place for measuring the *ex situ* activity of the symbiotic bacteria, as described in this review, but advances in deep-sea technology for incubations at the sea floor will help us to understand the productivity of these symbioses. Also important are reliable methods for measuring *in situ* methane concentrations as all previous measurements were made *ex situ*. Detailed *in situ* measurements of methane at time and space scales relevant to the symbiotic bacteria will reveal how habitat variability affects the diversity, distribution and abundance of symbiotic methane oxidizers.

Genomic studies are currently underway with the sequencing of symbiont genomes from the hydrothermal vent mussel *B. puteoserpentis* at Genoscope, France in our joint collaboration with Francoise Gaill and Sebastien Duperron (CNRS, France). It may be possible to identify adaptations of symbiotic bacteria to their invertebrate hosts by comparing the genomes of symbiotic methanotrophs to those of free-living methane oxidizers (Ward *et al.*, 2004; Hou *et al.*, 2008; others in progress, see http://genomesonline.org). However, genetic adaptation to a symbiotic lifestyle need not be complex, particularly if the symbionts have a free-living stage, as assumed for methanotrophic symbionts. A recent study showed that the acquisition of only a single regulatory gene (involved in inducing a mediator of biofilm formation) may have been sufficient to enable free-living *Vibrio fisheri* to establish a symbiotic relationship with their invertebrate hosts (Mandel *et al.*, 2009). Indeed, changes at the genetic level might not even be required, as epigenetic mechanisms are also known to cause phenotypic heterogeneity in

clonal bacterial populations (reviewed in Veening *et al*., 2008).

Post-genomic methods will allow us to quantify the activity and productivity of symbiotic bacteria at the level of the transcriptome and proteome. However, *in situ* fixation techniques on the sea floor, which are also in development at our institute, are needed to prevent metabolic artifacts due to long transport times of several hours from the deep sea to the research vessel. Future studies that integrate deep-sea technology, biogeochemistry and molecular ecology will help us better understand the intricate relationships and interactions between symbiotic methane oxidizers, their hosts and their environment.

Acknowledgements

This work was supported by the Max Planck Society, the Priority Program 1144 (From Mantle to Ocean: Energy-Material- and Life-Cycles at Spreading Axes; contribution number 42) of the German Research Foundation (DFG), the DFG Cluster of Excellence at MARUM, Bremen and a Marie Curie research training network (MOMARnet) scholarship to J.M.P. We are grateful to the Census of Marine Life working group ChEss for their support of research on the biogeography of chemosynthetic ecosystems. Many thanks to Christian Lott for help in preparing the figures, and to three anonymous reviewers for their insightful comments that helped us to improve the manuscript.

References

Barry, J.P., Buck, K.R., Kochevar, R.K., Nelson, D.C., Fujiwara, Y., Goffredi, S.K., and Hashimoto, J. (2002) Methane-based symbiosis in a mussel, *Bathymodiolus platifrons*, from cold seeps in Sagami bay, Japan. *Invertebr Biol* **121:** 47–54.

Beck, L.A., and Sobjinski, K. (1999) Zur Systematik und Phylogenie hydrothermaler Tiefseemullusken. *Courier Forschungsinstitut Senckenberg* **215:** 31–41.

Borowski, C., Giere, O., Krieger, J., Amann, R., and Dubilier, N. (2002) New aspects of the symbiosis in the provannid snail *Ifremeria nautilei* from the North Fiji Back Arc Basin. *Cah Biol Mar* **43:** 321–324.

Braby, C.E., Rouse, G.W., Johnson, S.B., Jones, W.J., and Vrijenhoek, R.C. (2007) Bathymetric and temporal variation among *Osedax* boneworms and associated megafauna on whale-falls in Monterey Bay, California. *Deep Sea Res* **54:** 1773–1791.

Bright, M., and Bulgheresi, S. (2009) Microbial symbiont transmission. *Nat Rev Microbiol* (in press).

Bright, M., and Giere, O. (2005) Microbial symbiosis in *Annelida*. *Symbiosis* **38:** 1–45.

Brooks, J.M., Kennicutt, M.C., Fisher, C.R., Macko, S.A., Cole, K., Childress, J.J., *et al.* (1987) Deep sea hydrocarbon seep communities – evidence for energy and nutritional carbon sources. *Science* **238:** 1138–1142.

Cary, S.C., Fisher, C.R., and Felbeck, H. (1988) Mussel growth supported by methane as sole carbon and energy source. *Science* **240:** 78–80.

Cavanaugh, C.M. (1983) Symbiotic chemoautotrophic bacteria in marine invertebrates from sulfide-rich habitats. *Nature* **302:** 58–61.

Cavanaugh, C.M. (1992) Methanotroph-invertebrate symbioses in the marine envrionment: ultrastructural, biochemical and molecular studies. In *Microbial Growth on C1 Compounds*. Murrell, J.C., and Kelly, D.P. (eds). Andover, UK: Intercept, pp. 315–328.

Cavanaugh, C.M., Gardiner, S.L., Jones, M.L., Jannasch, H.W., and Waterbury, J.B. (1981) Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila* Jones: possible chemoautotrophic symbionts. *Science* **213:** 340–342.

Cavanaugh, C.M., Levering, P.R., Maki, J.S., Mitchell, R., and Lidstrom, M.E. (1987) Symbiosis of methylotrophic bacteria and deep-sea mussels. *Nature* **325:** 346–347.

Cavanaugh, C.M., McKiness, Z.P., Newton, I.L.G., and Stewart, F.J. (2006) Marine chemosynthetic symbioses. In *The Prokaryotes: An Evolving Electronic Resource for the Microbial Community*. Dworkin, M., Falkow, S.I., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds). New York, USA: Springer.

Charlou, J.L., Donval, J.P., Fouquet, Y., Jean-Baptiste, P., and Holm, N. (2002) Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36°14′N, MAR). *Chem Geol* **191:** 345–359.

Childress, J.J., Fisher, C.R., Brooks, J.M., Kennicutt, M.C., II, Bidigare, R., and Anderson, A.E. (1986) A methanotrophic marine molluscan (*Bivalvia*, *Mytilidae*) symbiosis: mussels fueled by gas. *Science* **233:** 1306–1308.

Colaco, A., Dehairs, F., Desbruyeres, D., Le Bris, N., and Sarradin, P.M. (2002) $\delta^{13}C$ signature of hydrothermal mussels is related with the end-member fluid concentrations of H_2S and CH_4 at the Mid-Atlantic Ridge hydrothermal vent fields. *Cah Biol Mar* **43:** 259–262.

Cordes, E.E., Carney, S.L., Hourdez, S., Carney, R.S., Brooks, J.M., and Fisher, C.R. (2007) Cold seeps of the deep Gulf of Mexico: community structure and biogeographic comparisons to Atlantic equatorial belt seep communities. *Deep Sea Res* **54:** 637–653.

von Cosel, R., and Olu, K. (1998) Gigantism in *Mytilidae*. A new *Bathymodiolus* from cold seep areas on the Barbados Accretionary Prism. *C R Acad Sci Biol* **321:** 655–663.

Dando, P.R., Bussmann, I., Niven, S.J., Ohara, S.C.M., Schmaljohann, R., and Taylor, L.J. (1994) A methane seep area in the Skagerrak, the habitat of the pogonophore *Siboglinum poseidoni* and the bivalve mollusk *Thyasira sarsi*. *Mar Ecol Prog Ser* **107:** 157–167.

DeChaine, E.G., and Cavanaugh, C.M. (2005) Symbioses of methanotrophs and deep-sea mussels (*Mytilidae*: *Bathymodiolinae*). In *Molecular Basis of Symbiosis*. Overmann, J. (ed.). Berlin, Germany: Springer, pp. 227–250.

DeChaine, E.G., Bates, A.E., Shank, T.M., and Cavanaugh, C.M. (2006) Off-axis symbiosis found: characterization and biogeography of bacterial symbionts of *Bathymodiolus* mussels from Lost City hydrothermal vents. *Environ Microbiol* **8:** 1902–1912.

Deming, J.W., Reysenbach, A.L., Macko, S.A., and Smith, C.R. (1997) Evidence for the microbial basis of a chemoautotrophic invertebrate community at a whale fall on the deep seafloor: bone-colonizing bacteria and invertebrate endosymbionts. *Microsc Res Tech* **37:** 162–170.

Distel, D.L., Lee, H.K.W., and Cavanaugh, C.M. (1995) Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel. *Proc Natl Acad Sci USA* **92:** 9598–9602.
Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6:** 725–739.
Dunfield, P.F., Yuryev, A., Senin, P., Smirnova, A.V., Stott, M.B., Hou, S.B., *et al.* (2007) Methane oxidation by an extremely acidophilic bacterium of the phylum *Verrucomicrobia*. *Nature* **450:** 879–882.
Duperron, S., Nadalig, T., Caprais, J.-C., Sibuet, M., Fiala-Médioni, A., Amann, R., and Dubilier, N. (2005) Dual symbiosis in a *Bathymodiolus* sp. mussel from a methane seep on the Gabon continental margin (Southeast Atlantic): 16S rRNA phylogeny and distribution of the symbionts in gills. *Appl Environ Microbiol* **71:** 1694–1700.
Duperron, S., Bergin, C., Zielinski, F., Blazejak, A., Pernthaler, A., McKiness, Z.P., *et al.* (2006) A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (*Bivalvia*: *Mytilidae*), from hydrothermal vents along the northern Mid-Atlantic Ridge. *Environ Microbiol* **8:** 1441–1447.
Duperron, S., Sibuet, M., MacGregor, B.J., Kuypers, M.M.M., Fisher, C.R., and Dubilier, N. (2007) Diversity, relative abundance and metabolic potential of bacterial endosymbionts in three *Bathymodiolus* mussel species from cold seeps in the Gulf of Mexico. *Environ Microbiol* **9:** 1423–1438.
Duperron, S., Halary, S., Lorion, J., Sibuet, M., and Gaill, F. (2008) Unexpected co-occurrence of six bacterial symbionts in the gills of the cold seep mussel *Idas* sp. (*Bivalvia*: *Mytilidae*). *Environ Microbiol* **10:** 433–445.
Duperron, S., Lorion, J., Lopez, P., Samadi, S., Gros, O., and Gaill, F. (2009) Symbioses between deep-sea mussels (*Mytilidae*: *Bathymodiolinae*) and chemosynthetic bacteria: diversity, function and evolution. *C R Acad Sci Biol* **332:** 298–310.
Endow, K., and Ohta, S. (1989) The symbiotic relationship between bacteria and a mesogastropod snail, *Alviniconcha hessleri*, collected from hydothermal vents of the Mariana Back-Arc Basin. *Bull Jpn Soc Microbiol Ecol* **3:** 73–82.
Felbeck, H. (1981) Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia pachyptila* Jones (*Vestimentifera*). *Science* **213:** 336–338.
Felbeck, H., and Turner, P.J. (1995) CO_2 transport in catheterized hydrothermal vent tubeworms, *Riftia pachyptila* (*Vestimentifera*). *J Exp Zool* **272:** 95–102.
Fisher, C.R. (1996) Ecophysiology of primary production at deep-sea vents and seeps. *Biosystematics and Ecology Series* **11:** 313–336.
Fisher, C.R., and Childress, J.J. (1992) Organic carbon transfer from methanotrophic symbionts to the host hydrocarbon-seep mussel. *Symbiosis* **12:** 221–235.
Fisher, C.R., Childress, J.J., Arp, A.J., Brooks, J.M., Distel, D., Favuzzi, J.A., *et al.* (1988) Microhabitat variation in the hydrothermal vent mussel, *Bathymodiolus thermophilus*, at the Rose Garden vent on the Galapagos Rift. *Deep Sea Res* **35:** 1769–1791.
Fisher, C.R., Brooks, J.M., Vodenichar, J.S., Zande, J.M., Childress, J.J., and Burke, R.A., Jr (1993) The co-occurrence of methanotrophic and chemoautotrophic sulfur oxidising bacterial symbionts in a deep-sea mussel. *Mar Ecol* **14:** 277–289.
Flügel, H.J., and Callsencencic, P. (1992) New observations on the biology of *Siboglinum poseidoni* Flügel and Langhof (*Pogonophora*) from the Skagerrak. *Sarsia* **77:** 287–290.
Fujiwara, Y., Takai, K., Uematsu, K., Tsuchida, S., Hunt, J.C., and Hashimoto, J. (2000) Phylogenetic characterisation of endosymbionts in three hydrothermal vent mussels: influence on host distributions. *Mar Ecol Prog Ser* **103:** 147–155.
Galchenko, V.F., Pimenov, N.V., Lein, A.Y., Galkin, S.V., Miller, Y.M., and Ivanov, M.V. (1992) Mixotrophic feeding of *Olgaconcha tufari* Beck (*Gastropoda*: *Prosobrachia*) from the active hydrothermal field of the Manus Basin (Bismarck Sea). *Doklady Akademii nauk SSSR* **323:** 776–781.
Goffredi, S.K., Hurtado, L.A., Hallam, S., and Vrijenhoek, R.C. (2003) Evolutionary relationships of deep-sea vent and cold seep clams (Mollusca : Vesicomyidae) of the 'pacifica/lepta' species complex. *Mar Biol* **142:** 311–320.
Goffredi, S.K., Wilpiszeski, R., Lee, R., and Orphan, V.J. (2008) Temporal evolution of methane cycling and phylogenetic diversity of archaea in sediments from a deep-sea whale-fall in Monterey Canyon, California. *ISME J* **2:** 204–220.
Gruber, N., Keeling, C.D., Bacastow, R.B., Guenther, P.R., Lueker, T.J., Wahlen, M., *et al.* (1999) Spatiotemporal patterns of carbon-**13** in the global surface oceans and the oceanic Suess effect. *Global Biogeochem Cycles* **13:** 307–335.
Gustafson, R.G., Turner, R.D., Lutz, R.A., and Vrijenhoek, R.C. (1998) A new genus and five new species of mussels (*Bivalvia*, *Mytilidae*) from deep-sea sulfide/hydrocarbon seeps in the Gulf of Mexico. *Malacologia* **40:** 63–112.
Hakemian, A.S., and Rosenzweig, A.C. (2007) The biochemistry of methane oxidation. *Annu Rev Biochem* **76:** 223–241.
Hanson, R.S., and Hanson, T.E. (1996) Methanotrophic bacteria. *Microbiol Rev* **60:** 439–471.
Heeschen, K.U., Hohnberg, H.J., Haeckel, M., Abegg, F., Drews, M., and Bohrmann, G. (2007) *In situ* hydrocarbon concentrations from pressurized cores in surface sediments, northern Gulf of Mexico. *Mar Chem* **107:** 498–515.
Hentschel, U., Usher, K.M., and Taylor, M.W. (2006) Marine sponges as microbial fermenters. *FEMS Microbiol Ecol* **55:** 167–177.
Hou, S., Makarova, K.S., Saw, J.H.W., Senin, P., Ly, B.V., Zhou, Z., *et al.* (2008) Complete genome sequence of the extremely acidophilic methanotroph isolate V4, *Methylacidiphilum infernorum*, a representative of the bacterial phylum *Verrucomicrobia. Biol Direct* **3:** 26.
Hurtado, L.A., Mateos, M., Lutz, R.A., and Vrijenhoek, R.C. (2003) Coupling of bacterial endosymbiont and host mitochondrial genomes in the hydrothermal vent clam *Calyptogena magnifica*. *Appl Environ Microbiol* **69:** 2058–2064.
Ishibashi, J.I., Wakita, H., Nojiri, Y., Grimaud, D., Jean-baptiste, P., Gamo, T., *et al.* (1994) Helium and carbon geochemistry of hydrothermal fluids from the North Fiji Basin Spreading Ridge (Southwest Pacific). *Earth Planet Sci Lett* **128:** 183–197.
Jahnke, L.L., Summons, R.E., Hope, J.M., and des Marais, D.J. (1999) Carbon isotopic fractionation in lipids from methanotrophic bacteria II: the effects of physiology and

environmental parameters on the biosynthesis and isotopic signatures of biomarkers. *Geochim Cosmochim Acta* **63:** 79–93.

Jørgensen, B.B., and Boetius, A. (2007) Feast and famine – microbial life in the deep-sea bed. *Nat Rev Microbiol* **5:** 770–781.

Kadar, E., Bettencourt, R., Costa, V., Santos, R.S., Lobo-Da-Cunha, A., and Dando, P. (2005) Experimentally induced endosymbiont loss and re-acquirement in the hydrothermal vent bivalve *Bathymodiolus azoricus*. *J Exp Mar Biol Ecol* **318:** 99–110.

Kennicutt, M.C., Burke, R.A., Macdonald, I.R., Brooks, J.M., Denoux, G.J., and Macko, S.A. (1990) Stable isotope partitioning in seep and vent organisms – chemical and ecological significance. *Chem Geol* **101:** 293–310.

Kimura, H., Sato, M., Sasayama, Y., and Naganuma, T. (2003) Molecular characterization and *in situ* localization of endosymbiotic 16S ribosomal RNA and RuBisCO genes in the pogonophoran tissue. *Mar Biotechnol* **5:** 261–269.

Kochevar, R.E., Childress, J.J., Fisher, C.R., and Minnich, E. (1992) The methane mussel – roles of symbiont and host in the metabolic utilization of methane. *Mar Biol* **112:** 389–401.

Leak, D.J., Stanley, S.H., and Dalton, H. (1985) Implication of the nature of methane monooxygenase on carbon assimilation in methanotrophs. In *Microbial Gas Metabolism, Mechanistic, Metabolic and Biotechnological Aspects*. Poole, R.K., and Dow, C.S. (eds). London, UK: Academic Press, pp. 201–208.

Lösekann, T., Robador, A., Niemann, H., Knittel, K., Boetius, A., and Dubilier, N. (2008) Endosymbioses between bacteria and deep-sea siboglinid tubeworms from an Arctic Cold Seep (Haakon Mosby Mud Volcano, Barents Sea). *Environ Microbiol* **10:** 3237–3254.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363–1371.

Macavoy, S.E., Carney, R.S., Morgan, E., and Macko, S.A. (2008) Stable isotope variation among the mussel *Bathymodiolus childressi* and associated heterotrophic fauna at four cold-seep communities in the Gulf of Mexico. *J Shellfish Res* **27:** 147–151.

McCollom, T.M., and Seewald, J.S. (2007) Abiotic synthesis of organic compounds in deep-sea hydrothermal environments. *Chem Rev* **107:** 382–401.

McDonald, I.R., Bodrossy, L., Chen, Y., and Murrell, J.C. (2008) Molecular ecology techniques for the study of aerobic methanotrophs. *Appl Environ Microbiol* **74:** 1305–1315.

Mandel, M.J., Wollenberg, M.S., Stabb, E.V., Visick, K.L., and Ruby, E.G. (2009) A single regulatory gene is sufficient to alter bacterial host range. *Nature* **458:** 215–218.

Margulis, L., and Fester, R. (1991) *Symbiosis as a Source of Evolutionary Innovation*. Cambridge, MA, USA: MIT Press.

Martens, C.S., Chanton, J.P., and Paull, C.K. (1991) Biogenic methane from abyssal brine seeps at the base of the Florida Escarpment. *Geology* **19:** 851–854.

Martins, I., Colaco, A., Dando, P.R., Martins, I., Desbruyeres, D., Sarradin, P.M., *et al.* (2008) Size-dependent variations on the nutritional pathway of *Bathymodiolus azoricus* demonstrated by a C-flux model. *Ecol Modell* **217:** 59–71.

Mullaugh, K.M., Luther, G.W., Ma, S., Moore, T.S., Yucel, M., Becker, E.L., *et al.* (2008) Voltammetric (micro)electrodes for the *in situ* study of Fe^{2+} oxidation kinetics in hot springs and $S_2O_3^{2-}$ production at hydrothermal vents. *Electroanalysis* **20:** 280–290.

Nees, H.A., Moore, T.S., Mullaugh, K.M., Holyoke, R.R., Janzen, C.P., Ma, S., *et al.* (2008) Hydrothermal vent mussel habitat chemistry, pre- and post-eruption at 9°50′ North on the East Pacific Rise. *J Shellfish Res* **27:** 169–175.

Nelson, D.C., and Fisher, C.R. (1995) Chemoautotrophic and methanotrophic endosymbiotic bacteria at deep-sea vents and seeps. In *The Microbiology of Deep-Sea Hydrothermal Vents*. Karl, D.M. (ed.). Boca Raton, FL, USA: CRC Press, pp. 125–167.

Nelson, K., and Fisher, C.R. (2000) Absence of cospeciation in deep-sea vestimentiferan tube worms and their bacterial endosymbionts. *Symbiosis* **28:** 1–15.

Page, H.M., Fisher, C.R., and Childress, J.J. (1990) Role of filter-feeding in the nutritional biology of a deep-sea mussel with methanotrophic symbionts. *Mar Biol* **104:** 251–257.

Page, H.M., Fiala-medioni, A., Fisher, C.R., and Childress, J.J. (1991) Experimental evidence for filter-feeding by the hydrothermal vent mussel, *Bathymodiolus thermophilus*. *Deep Sea Res* **38:** 1455–1461.

Peek, A.S., Feldman, R.A., Lutz, R.A., and Vrijenhoek, R.C. (1998) Cospeciation of chemoautotrophic bacteria and deep sea clams. *Proc Natl Acad Sci USA* **95:** 9962–9966.

Pernthaler, A., and Amann, R. (2004) Simultaneous fluorescence *in situ* hybridization of mRNA and rRNA in environmental bacteria. *Appl Environ Microbiol* **70:** 5426–5433.

Petersen, J.M., and Dubilier, N. (2009) Symbiotic methane oxidizers. In *Handbook of Hydrocarbon and Lipid Microbiology*. Timmis, K. (ed.). Berlin, Germany: Springer-Verlag, (In press).

Pile, A.J., and Young, C.M. (1999) Plankton availability and retention efficiencies of cold-seep symbiotic mussels. *Limnol Oceanogr* **44:** 1833–1839.

Pimenov, N., Savvichev, A., Rusanov, I., Lein, A., Egorov, A., Gebruk, A., *et al.* (1999) Microbial processes of carbon cycle as the base of food chain of Hakon Mosby Mud Volcano benthic community. *Geo-Mar Lett* **19:** 89–96.

Pimenov, N.V., Savvichev, A.S., Rusanov, I.I., Lein, A.Y., and Ivanov, M.V. (2000) Microbiological processes of the carbon and sulfur cycles at cold methane seeps of the North Atlantic. *Microbiology* **69:** 709–720.

Pimenov, N.V., Kalyuzhnaya, M.G., Khmelenina, V.N., Mityushina, L.L., and Trotsenko, Y.A. (2002) Utilization of methane and carbon dioxide by symbiotrophic bacteria in gills of *Mytilidae* (*Bathymodiolus*) from the Rainbow and Logachev hydrothermal fields on the Mid-Atlantic Ridge. *Microbiology* **71:** 587–594.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W.G., Peplies, J., and Glockner, F.O. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35:** 7188–7196.

Radford-Knoery, J., Charlou, J.L., Donval, J.P., Aballea, M., Fouquet, Y., and Ondreas, H. (1998) Distribution of dissolved sulfide, methane, and manganese near the seafloor

at the Lucky Strike (37°17′N) and Menez Gwen (37°50′N) hydrothermal vent sites on the mid-Atlantic Ridge. *Deep Sea Res* **45:** 367–386.

Raghoebarsing, A.A., Smolders, A.J.P., Schmid, M.C., Rijpstra, W.I.C., Wolters-Arts, M., Derksen, J., *et al.* (2005) Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. *Nature* **436:** 1153–1156.

Robinson, J.J., Polz, M.F., Fiala-Medioni, A., and Cavanaugh, C.M. (1998) Physiological and immunological evidence for two distinct C1-utilizing pathways in *Bathymodiolus puteoserpentis* (*Bivalvia*: *Mytilidae*), a dual endosymbiotic mussel from the Mid-Atlantic Ridge. *Mar Biol* **132:** 625–633.

Roy, K.O.L., von Cosel, R., Hourdez, S., Carney, S.L., and Jollivet, D. (2007) Amphi-Atlantic cold-seep *Bathymodiolus* species complexes across the equatorial belt. *Deep Sea Res* **54:** 1890–1911.

Schmaljohann, R., and Flügel, H.J. (1987) Methane-oxidizing bacteria in *Pogonophora*. *Sarsia* **72:** 91–99.

Schmaljohann, R., Faber, E., Whiticar, M.J., and Dando, P.R. (1990) Coexistence of methane based and sulfur-based endosymbioses between bacteria and invertebrates at a site in the Skagerrak. *Mar Ecol Prog Ser* **61:** 119–124.

Schoell, M. (1988) Multiple origins of methane in the Earth. *Chem Geol* **71:** 1–10.

Scott, K.M., Schwedock, J., Schrag, D.P., and Cavanaugh, C.M. (2004) Influence of form IA RubisCO and environmental dissolved inorganic carbon on the δ^{13}C of the clam-chemoautotroph symbiosis *Solemya velum*. *Environ Microbiol* **6:** 1210–1219.

Semrau, J.D., DiSpirito, A.A., and Murrell, J.C. (2008) Life in the extreme: thermoacidophilic methanotrophy. *Trends Microbiol* **16:** 190–193.

Southward, A.J., Southward, E.C., Brattegard, T., and Bakke, T. (1979) Further experiments on the value of dissolved organic matter as food for *Siboglinum fiordicum* (*Pogonophora*). *J Mar Biol Assoc UK* **59:** 133–148.

Southward, E.C. (2008) The morphology of bacterial symbioses in the gills of mussels of the genera *Adipicola* and *Idas* (Bivalvia : Mytilidae). *J Shellfish Res* **27:** 139–146.

Spiridonova, E.M., Kuznetsov, B.B., Pimenov, N.V., and Tourova, T.P. (2006) Phylogenetic characterization of endosymbionts of the hydrothermal vent mussel *Bathymodiolus azoricus* by analysis of the 16S rRNA, *cbbL*, and *pmoA* genes. *Microbiology* **75:** 694–701.

Stewart, F.J., Young, C.R., and Cavanaugh, C.M. (2008) Lateral symbiont acquisition in a maternally transmitted chemosynthetic clam endosymbiosis. *Mol Biol Evol* **25:** 673–687.

Summons, R.E., Jahnke, L.L., and Roksandic, Z. (1994) Carbon isotopic fractionation in lipids from methanotrophic bacteria – relevance for interpretation of the geochemical record of biomarkers. *Geochim Cosmochim Acta* **58:** 2853–2863.

Suzuki, Y., Kojima, S., Watanabe, H., Suzuki, M., Tsuchida, S., Nunoura, T., *et al.* (2006a) Single host and symbiont lineages of hydrothermalvent gastropods *Ifremeria nautilei* (*Provannidae*): biogeography and evolution. *Mar Ecol Prog Ser* **315:** 167–175.

Suzuki, Y., Kojima, S., Sasaki, T., Suzuki, M., Utsumi, T., Watanabe, H., *et al.* (2006b) Host-symbiont relationships in hydrothermal vent gastropods of the genus *Alviniconcha* from the Southwest Pacific. *Appl Environ Microbiol* **72:** 1388–1393.

Takai, K., Gamo, T., Tsunogai, U., Nakayama, N., Hirayama, H., Nealson, K.H., and Horikoshi, K. (2004) Geochemical and microbiological evidence for a hydrogen-based, hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) beneath an active deep-sea hydrothermal field. *Extremophiles* **8:** 269–282.

Taylor, M.W., Radax, R., Steger, D., and Wagner, M. (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiol Mol Biol Rev* **71:** 295–347.

Templeton, A.S., Chu, K.H., Alvarez-Cohen, L., and Conrad, M.E. (2006) Variable carbon isotope fractionation expressed by aerobic CH_4-oxidizing bacteria. *Geochim Cosmochim Acta* **70:** 1739–1752.

Trask, J.L., and Van Dover, C.L. (1999) Site-specific and ontogenetic variations in nutrition of mussels (*Bathymodiolus* sp.) from the Lucky Strike hydrothermal vent field, Mid-Atlantic Ridge. *Limnol Oceanogr* **44:** 334–343.

Trotsenko, Y.A., and Murrell, J.C. (2008) Metabolic aspects of aerobic obligate methanotrophy. *Adv Appl Microbiol* **63:** 183–229.

Vacelet, J., Bouryesnault, N., Fiala-Medioni, A., and Fisher, C.R. (1995) A methanotrophic carnivorous sponge. *Nature* **377:** 296–296.

Vacelet, J., FialaMedioni, A., Fisher, C.R., and Boury-Esnault, N. (1996) Symbiosis between methane-oxidizing bacteria and a deep-sea carnivorous cladorhizid sponge. *Mar Ecol Prog Ser* **145:** 77–85.

Van Dover, C.L. (2000) *The Ecology of Deep-Sea Hydrothermal Vents*. Princeton, NJ, USA: Princeton University Press.

Van Dover, C.L., and Fry, B. (1989) Stable isotopic compositions of hydrothermal vent organisms. *Mar Biol* **102:** 257–263.

Van Dover, C.L., German, C.R., Speer, K.G., Parson, L.M., and Vrijenhoek, R.C. (2002) Marine biology – evolution and biogeography of deep-sea vent and seep invertebrates. *Science* **295:** 1253–1257.

Veening, J.W., Smits, W.K., and Kuipers, O.P. (2008) Bistability, epigenetics, and bet-hedging in bacteria. *Annu Rev Microbiol* **62:** 193–210.

Vrijenhoek, R.C., Duhaime, M., and Jones, W.J. (2007) Subtype variation among bacterial endosymbionts of tubeworms (*Annelida*: *Siboglinidae*) from the Gulf of California. *Biol Bull* **212:** 180–184.

Ward, N., Larsen, Ø., Sakwa, J., Bruseth, L., Khouri, H., Scott Durkin, A., *et al.* (2004) Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). *PLoS Biol* **2:** e303.

Welhan, J.A. (1988) Origins of methane in hydrothermal systems. *Chem Geol* **71:** 183–198.

Welhan, J.A., and Craig, H. (1983) Methane, hydrogen and helium in hydrothermal fluids at 21°N on the East Pacific Rise. In *Hydrothermal Processes at Seafloor Spreading Centers*. Rona, P.A., Boström, K., Laubier, L., and Smith, K. (eds). New York, USA: Plenum, pp. 391–410.

Windoffer, R., and Giere, O. (1997) Symbiosis of the hydro-

thermal vent gastropod *Ifremeria nautilei* (*Provannidae*) with endobacteria – structural analyses and ecological considerations. *Biol Bull* **193:** 381–392.

Won, Y.J., Hallam, S.J., O'Mullan, G.D., Pan, I.L., Buck, K.R., and Vrijenhoek, R.C. (2003) Environmental acquisition of thiotrophic endosymbionts by deep-sea mussels of the genus *Bathymodiolus*. *Appl Environ Microbiol* **69:** 6785–6792.

Won, Y.J., Jones, W.J., and Vrijenhoek, R.C. (2008) Absence of cospeciation between deep-sea Mytilids and their thiotrophic endosymbionts. *J Shellfish Res* **27:** 129–138.

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

Jillian M. Petersen, Alban Ramette, Christian Lott, Marie-Anne Cambon-Bonavita, Magali Zbinden, and Nicole Dubilier

In press, *Environmental Microbiology*

environmental microbiology

society for applied microbiology

Environmental Microbiology (2009) doi:10.1111/j.1462-2920.2009.02129.x

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

Jillian M. Petersen,[1] Alban Ramette,[1] Christian Lott,[1,2] Marie-Anne Cambon-Bonavita,[3] Magali Zbinden[4] and Nicole Dubilier[1]*

[1]*Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359, Bremen, Germany.*

[2]*HYDRA Institute for Marine Sciences, Elba Field Station, Fetovaia, Via del Forno 80, 57034 Campo nell'Elba, Italy.*

[3]*Laboratoire de Microbiologie des Environnements Extrêmes, Département d'Etude des Ecosystèmes profonds, Ifremer, UMR 6197 Ifremer/CNRS/UBO, Centre de Brest, BP 70, 29280 Plouzané, France.*

[4]*UMR SAE 7138, Adaptations aux milieux extrêmes, Université Pierre et Marie Curie, 7, quai Saint Bernard, 75252 Paris Cedex 05, France.*

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*.

Received 12 May, 2009; accepted 4 November, 2009. *For correspondence. E-mail ndubilie@mpi-bremen.de; Tel. (+49) 421 2028 932; Fax (+49) 421 2028 580.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd

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

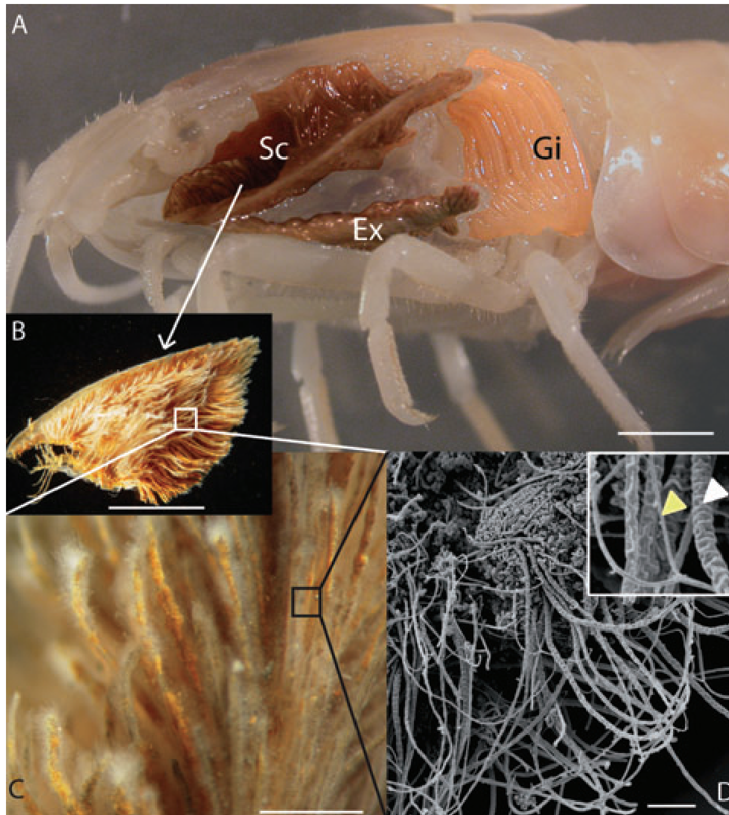


Fig. 1. Morphology of the *Rimicaris exoculata* symbiosis.
A. The *R. exoculata* gill chamber with carapace removed, showing the mouthparts (Sc and Ex) to which the shrimp epibionts are attached. Sc, scaphognathite; Ex, exopodite; Gi, gill. Scale bar = 5 mm.
B. Scaphognathite dissected out of the shrimp. 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

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

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

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

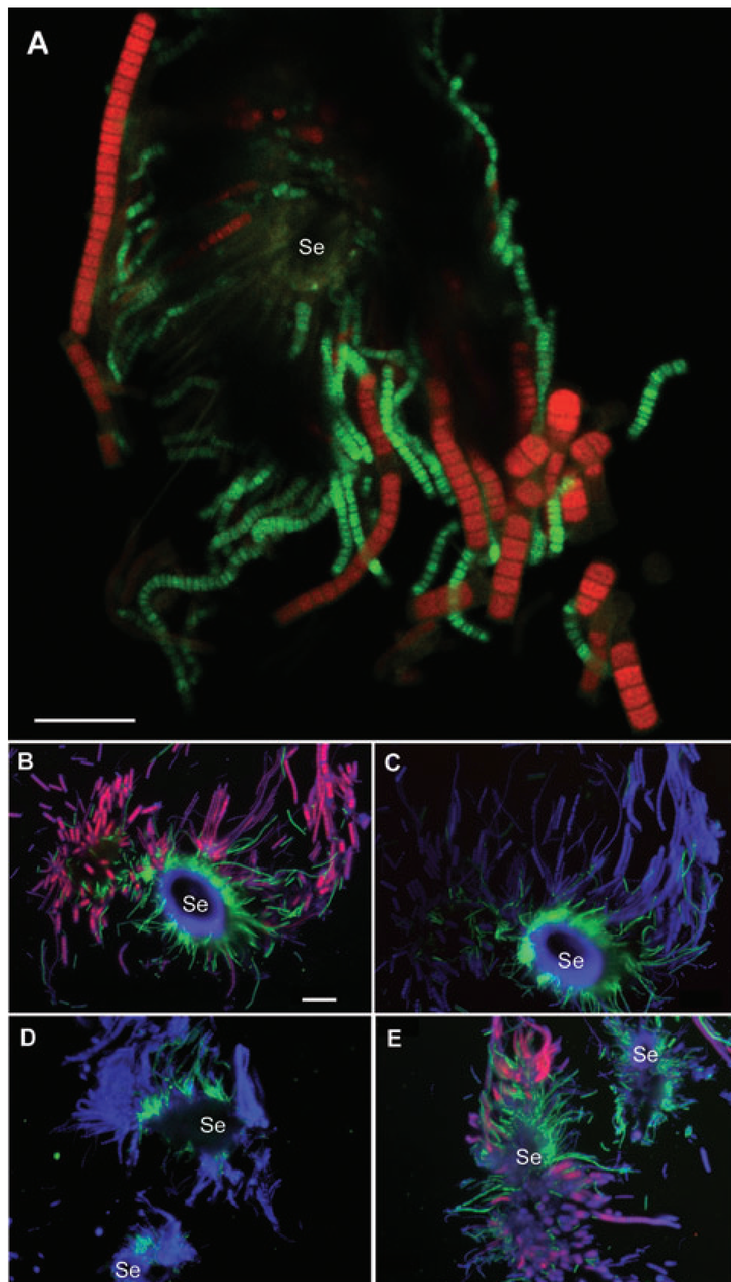


Fig. 2. Fluorescence *in situ* hybridization.
A. Confocal laser scanning micrograph of a cross-section through a *R. exoculata* scaphognathite from Individual 1 from the Rainbow vent site, showing the two symbionts attached to the scaphognathite seta (Se). The Gamma symbiont (green) was hybridized with 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

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

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 ($r = 0.43$, $P < 0.001$).

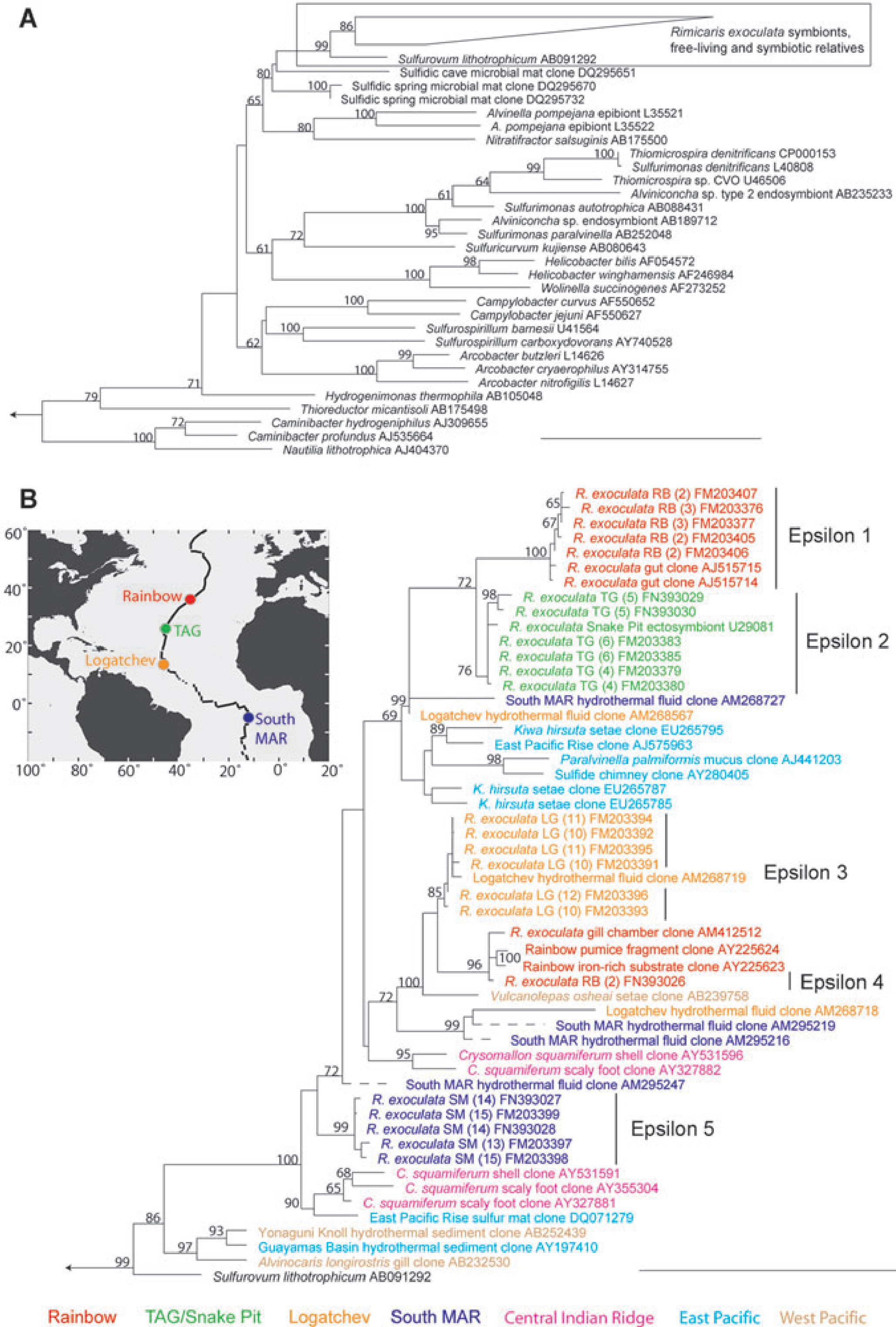
Unlike the Epsilon symbionts, the Gamma symbiont 16S rRNA sequences did not differ sufficiently to resolve geographic clades. The Gamma 2 symbiont sequences from the South MAR vent did, however, form a separate cluster to sequences from the northern MAR vent fields Rainbow, TAG and Logatchev (Gamma 1) 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. 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

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*



© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

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.

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, Rex-oepsLG86, 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).

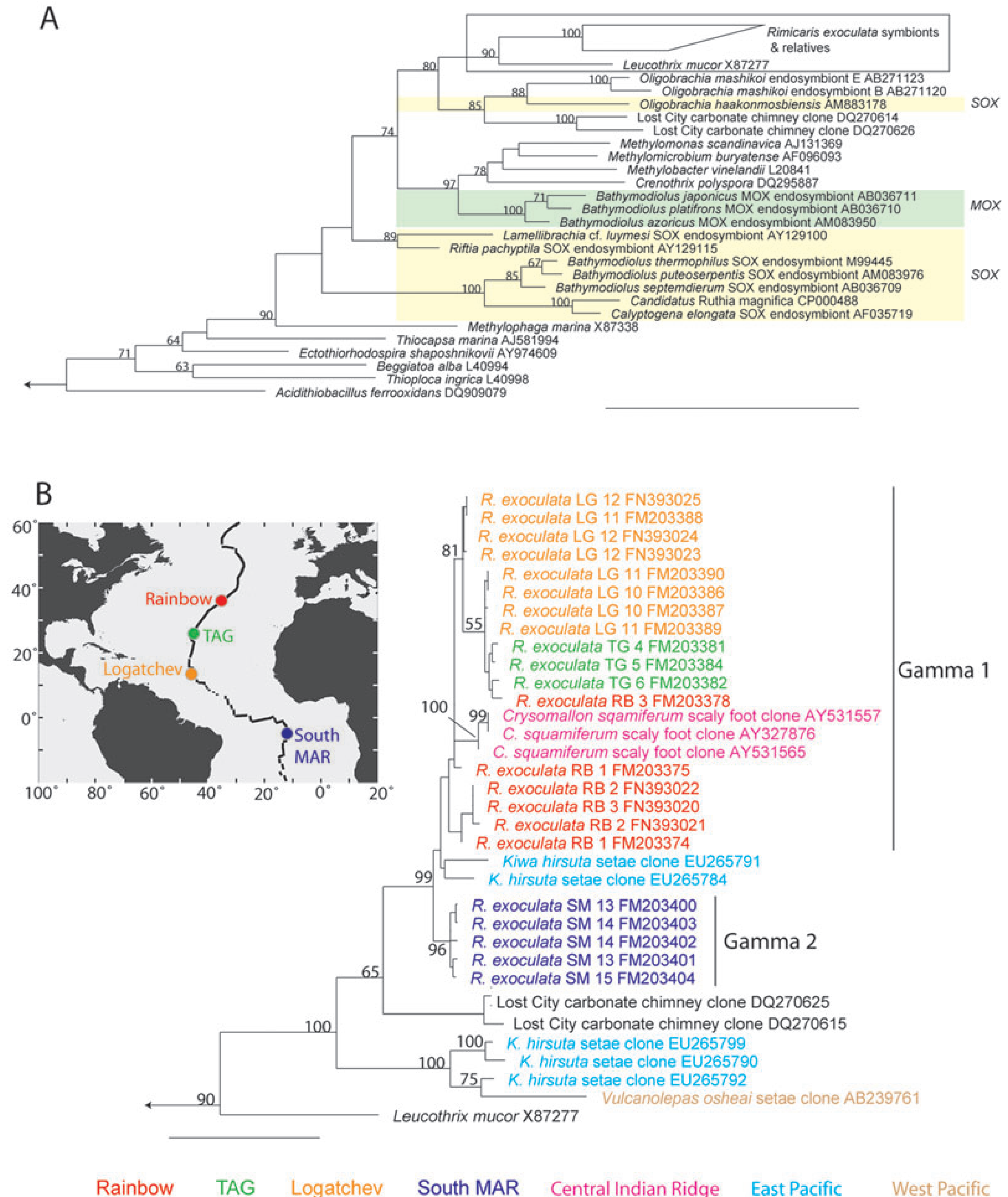
Both the Gamma and Epsilon symbionts were present on all shrimp from all four vent sites. Their relative abundance varied considerably, even among individuals collected at the same time from the same site. Given the high motility of the shrimp it is unlikely that this variability reflects vent fluid 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

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*



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* sym-

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.

bionts (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,

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

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

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

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,

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

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).

Acknowledgements

We would like to thank the captains and crews of the research vessels and ROVs that contributed to the sampling effort (Table S1), and Valérie Cueff-Gauchard (Exomar 2005), Olav Giere (M64/1) and Frank Zielinski (M64/2) for their help with the on-board work. Many thanks to Dennis Fink (MPI Bremen) for his expertise in Adobe Photoshop, to Claudia Bergin (MPI Bremen) for an introduction to confocal microscopy, and to Sandra Schoettner (MPI Bremen) and Erwan Roussel (Ifremer) for help in the lab. This work was supported by the Max Planck Society, the Priority Program 1144 (From Mantle to Ocean: Energy- Material- and Life-Cycles at Spreading Axes; contribution number 44) of the German Research Foundation (DFG), the DFG Cluster of Excellence at MARUM, Bremen, and a Marie Curie research training network (MOMARnet) scholarship to J.M.P. We are grateful to the Census of Marine Life working group ChEss for their support of research on the biogeography of chemosynthetic ecosystems.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25:** 3389–3402.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990) Combination of 16S ribosomal RNA-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56:** 1919–1925.

Brock, T.D. (1966) The habitat of *Leucothrix mucor*, a widespread marine microorganism. *Limnol Oceanogr* **11:** 303–307.

Bulgheresi, S., Schabussova, I., Chen, T., Mullin, N.P., Maizels, R.M., and Ott, J.A. (2006) A new C-type lectin similar to the human immunoreceptor DC-SIGN mediates symbiont acquisition by a marine nematode. *Appl Environ Microbiol* **72:** 2950–2956.

Casanova, B., Brunet, M., and Segonzac, M. (1993) L'impact d'une épibiose bactérienne sur la morphologie fonctionelle de crevettes associées à l'hydrothermalisme médio-atlantique. *Cah Biol Mar* **34:** 573–588.

Cavanaugh, C.M., and Robinson, J.J. (1996) CO_2 fixation in chemoautotrophic-invertebrate symbioses: expression of form I and form II RubisCO. In *Microbial Growth on C1 Compounds*. Lidstrom, M.E., and Tabita, F.R. (eds). Dordrecht, the Netherlands: Kluwer, pp. 285–292.

Cavanaugh, C.M., McKiness, Z.P., Newton, I.L.G., and Stewart, F.J. (2006) Marine chemosynthetic symbioses. In *The Prokaryotes: An Evolving Electronic Resource for the Microbial Community*. Dworkin, M., Falkow, S.I., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds). New York, USA: Springer, pp. 475–507.

Charlou, J.L., Donval, J.P., Fouquet, Y., Jean-Baptiste, P., and Holm, N. (2002) Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36°14′N, MAR). *Chem Geol* **191:** 345–359.

Chu, K.H., Li, C.P., and Ho, H.Y. (2001) The first internal transcribed spacer (ITS-1) of ribosomal DNA as a molecular marker for phylogenetic and population analyses in *Crustacea*. *Mar Biotechnol* **3:** 355–361.

Corbari, L., Zbinden, M., Cambon-Bonavita, M.A., Gaill, F., and Compere, P. (2007) Bacterial symbionts and mineral deposits in the branchial chamber of the hydrothermal vent shrimp *Rimicaris exoculata*: relationship to moult cycle. *Aquat Biol* **1:** 225–238.

Creasey, S., Rogers, A.D., and Tyler, P.A. (1996) Genetic comparison of two populations of the deep-sea vent shrimp *Rimicaris exoculata* (*Decapoda*: *Bresiliidae*) from the Mid-Atlantic Ridge. *Mar Biol* **125:** 473–482.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22:** 434–444.

DeChaine, E.G., Bates, A.E., Shank, T.M., and Cavanaugh, C.M. (2006) Off-axis symbiosis found: characterization and biogeography of bacterial symbionts of *Bathymodiolus* mussels from Lost City hydrothermal vents. *Environ Microbiol* **8:** 1902–1912.

Diaz, C.L., Melchers, L.S., Hooykaas, P.J.J., Lugtenberg, B.J.J., and Kijne, J.W. (1989) Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis. *Nature* **338:** 579–581.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6:** 725–739.

Duperron, S., Bergin, C., Zielinski, F., Blazejak, A., Pernthaler, A., McKiness, Z.P., *et al.* (2006) A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (*Bivalvia*: *Mytilidae*), from hydrothermal vents along the northern Mid-Atlantic Ridge. *Environ Microbiol* **8:** 1441–1447.

Folmer, O., Black, M.B., Hoeh, W.R., Lutz, R.A., and Vrijenhoek, R.C. (1994) DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from metazoan invertebrates. *Mol Mar Biol Biotechnol* **3:** 294–299.

Galchenko, V.F., Pimenov, N.V., Lein, A.I., Galkin, S.V., Moskalev, L.I., and Ivanov, M.V. (1989) Autotrophic CO_2 assimilation in tissues of prawn *Rimicaris exoculata* from the Mid-Atlantic Ridge hydrothermal area. *Doklady Akademii Nauk SSSR* **308:** 1478–1481.

Gebruk, A.V., Pimenov, N.V., and Savvichev, A.S. (1993) Feeding specialization of bresiliid shrimps in the TAG site hydrothermal community. *Mar Ecol Prog Ser* **98:** 247–253.

Gebruk, A.V., Southward, E.C., Kennedy, H., and Southward, A.J. (2000) Food sources, behaviour, and distribution of hydrothermal vent shrimps at the Mid-Atlantic Ridge. *J Mar Biol Assoc UK* **80:** 485–499.

Gloter, A., Zbinden, M., Guyot, F., Gaill, F., and Colliex, C. (2004) TEM-EELS study of natural ferrihydrite from geological-biological interactions in hydrothermal systems. *Earth Planet Sci Lett* **222:** 947–957.

Goffredi, S.K., Waren, A., Orphan, V.J., Van Dover, C.L., and Vrijenhoek, R.C. (2004) Novel forms of structural integration between microbes and a hydrothermal vent gastropod from the Indian Ocean. *Appl Environ Microbiol* **70:** 3082–3090.

Goffredi, S.K., Jones, W.J., Erhlich, H., Springer, A., and Vrijenhoek, R.C. (2008) Epibiotic bacteria associated with the recently discovered Yeti crab, *Kiwa hirsuta*. *Environ Microbiol* **10:** 2623–2634.

Gourdine, J.P., Markiv, A., and Smith-Ravin, J. (2007) The three-dimensional structure of codakine and related marine C-type lectins. *Fish Shellfish Immunol* **23:** 831–839.

Grabovich, M.Y., Muntyan, M.S., Lebedeva, V.Y., Ustiyan, V.S., and Dubinina, G.A. (1999) Lithoheterotrophic growth and electron transfer chain components of the filamentous gliding bacterium *Leucothrix mucor* DSM 2157 during oxidation of sulfur compounds. *FEMS Microbiol Lett* **178:** 155–161.

Gros, O., Liberge, M., Heddi, A., Khatchadourian, C., and Felbeck, H. (2003) Detection of the free-living forms of sulfide-oxidizing gill endosymbionts in the lucinid habitat (*Thalassia testudinum* environment). *Appl Environ Microbiol* **69:** 6264–6267.

Hall, T. (1997–2001) *BioEdit*. [WWW document]. URL http://www.mbio.ncsu.edu/BioEdit/bioedit.html

Harmer, T.L., Rotjan, R.D., Nussbaumer, A.D., Bright, M., Ng, A.W., DeChaine, E.G., and Cavanaugh, C.M. (2008) Free-living tube worm endosymbionts found at deep-sea vents. *Appl Environ Microbiol* **74:** 3895–3898.

Harrison, J.S. (2001) Phylogeny, biogeography, and speciation in the genus *Austinixa* and related genera (*Crustacea*: *Brachyura*: *Pinnotheridae*). PhD Thesis. College Station, TX, USA: Texas A&M University.

Haselkorn, T.S., Markow, T.A., and Moran, N.A. (2009) Multiple introductions of the *Spiroplasma* bacterial endosymbiont into *Drosophila*. *Mol Ecol* **18:** 1294–1305.

Hunter, R.L., Webb, M.S., Iliffe, T.M., and Bremer, J.R.A. (2008) Phylogeny and historical biogeography of the cave-adapted shrimp genus *Typhlatya* (Atyidae) in the Caribbean Sea and Western Atlantic. *J Biogeogr* **35:** 65–75.

Inagaki, F., Takai, K., Nealson, K.H., and Horikoshi, K. (2004) *Sulfurovum lithotrophicum* gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the *Epsilonproteobacteria* isolated from Okinawa Trough hydrothermal sediments. *Int J Syst Evol Microbiol* **54:** 1477–1482.

Jannasch, H.W., Wirsen, C.O., and Molyneaux, S.J. (1991) Chemosynthetic microbial activity at the 23° and 26°N Mid-Atlantic Ridge vent sites. *Ridge Events* 19.

Kimura, H., Sato, M., Sasayama, Y., and Naganuma, T. (2003) Molecular characterization and *in situ* localization of endosymbiotic 16S ribosomal RNA and RuBisCO genes in the pogonophoran tissue. *Mar Biotechnol* **5:** 261–269.

Komai, T., and Segonzac, M. (2008) Taxonomic review of the hydrothermal vent shrimp genera *Rimicaris* Williams & Rona and *Chorocaris* Martin & Hessler (*Crustacea*: *Decapoda*: *Caridea*: *Alvinocarididae*). *J Shellfish Res* **27:** 21–41.

Lee, K.H., and Ruby, E.G. (1994) Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. *Appl Environ Microbiol* **60:** 1565–1571.

Lösekann, T., Robador, A., Niemann, H., Knittel, K., Boetius, A., and Dubilier, N. (2008) Endosymbioses between bacteria and deep-sea siboglinid tubeworms from an Arctic Cold Seep (Haakon Mosby Mud Volcano, Barents Sea). *Environ Microbiol* **10:** 3237–3254.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, *et al.* (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363–1371.

Mandel, M.J., Wollenberg, M.S., Stabb, E.V., Visick, K.L., and Ruby, E.G. (2009) A single regulatory gene is sufficient to alter bacterial host range. *Nature* **458:** 215–217.

Meng, X.H., Wang, Q.Y., Jang, I.K., Liu, P., and Kong, J. (2009) Genetic differentiation in seven geographic populations of the fleshy shrimp *Penaeus (Fenneropenaeus) chinensis* based on microsatellite DNA. *Aquaculture* **287:** 46–51.

Miethling, R., Wieland, G., Backhaus, H., and Tebbe, C.C. (2000) Variation of microbial rhizosphere communities in response to crop species, soil origin, and inoculation with *Sinorhizobium meliloti* L33. *Microb Ecol* **40:** 43–56.

Muyzer, G., Teske, A., Wirsen, C.O., and Jannash, H.W. (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164:** 165–172.

Nyholm, S.V., and McFall-Ngai, M.J. (2004) The winnowing: establishing the squid-*Vibrio* symbiosis. *Nat Rev Microbiol* **2:** 632–642.

Page, T.J., Short, J.W., Humphrey, C.L., Hillyer, M.J., and Hughes, J.M. (2008) Molecular systematics of the Kakaducarididae (Crustacea: Decapoda: Caridea). *Mol Phylogenet Evol* **46:** 1003–1014.

Perner, M., Kuever, J., Seifert, R., Pape, T., Koschinsky, A., Schmidt, K., *et al.* (2007) The influence of ultramafic rocks

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

on microbial communities at the Logatchev hydrothermal field, located 15°N on the Mid-Atlantic Ridge. *FEMS Microbiol Ecol* **61:** 97–109.

Pimenov, N.V., Kalyuzhnaya, M.G., Khmelenina, V.N., Mityushina, L.L., and Trotsenko, Y.A. (2002) Utilization of methane and carbon dioxide by symbiotrophic bacteria in gills of *Mytilidae* (*Bathymodiolus*) from the Rainbow and Logachev hydrothermal fields on the Mid-Atlantic Ridge. *Microbiol* **71:** 587–594.

Polz, M.F., and Cavanaugh, C.M. (1995) Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site. *Proc Natl Acad Sci USA* **92:** 7232–7236.

Polz, M.F., Robinson, J.J., Cavanaugh, C.M., and Van Dover, C.L. (1998) Trophic ecology of massive shrimp aggregations at a Mid-Atlantic Ridge hydrothermal vent site. *Limnol Oceanogr* **43:** 1631–1638.

Pond, D., Dixon, D., and Sargent, J. (1997) Wax-ester reserves facilitate dispersal of hydrothermal vent shrimps. *Mar Ecol Prog Ser* **146:** 289–290.

Pond, D.W., Gebruk, A., Southward, E.C., Southward, A.J., Fallick, A.E., Bell, M.V., and Sargent, J.R. (2000) Unusual fatty acid composition of storage lipids in the bresilioid shrimp *Rimicaris exoculata* couples the photic zone with MAR hydrothermal vent sites. *Mar Ecol Prog Ser* **198:** 171–179.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W.G., Peplies, J., and Glockner, F.O. (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35:** 7188–7196.

Rieley, G., Van Dover, C.L., Hedrick, D.B., and Eglinton, G. (1999) Trophic ecology of *Rimicaris exoculata*: a combined lipid abundance stable isotope approach. *Mar Biol* **133:** 495–499.

Robinson, J.J., Polz, M.F., Fiala-Medioni, A., and Cavanaugh, C.M. (1998) Physiological and immunological evidence for two distinct C_1-utilizing pathways in *Bathymodiolus puteoserpentis* (*Bivalvia*: *Mytilidae*), a dual endosymbiotic mussel from the Mid-Atlantic Ridge. *Mar Biol* **132:** 625–633.

Ruby, E.G. (2008) Symbiotic conversations are revealed under genetic interrogation. *Nat Rev Microbiol* **6:** 752–762.

Schmidt, K., Koschinsky, A., Garbe-Schonberg, D., de Carvalho, L.M., and Seifert, R. (2007) Geochemistry of hydrothermal fluids from the ultramafic-hosted Logatchev hydrothermal field, 15°N on the Mid-Atlantic Ridge: temporal and spatial investigation. *Chem Geol* **242:** 1–21.

Schmidt, C., Le Bris, N., and Gaill, F. (2008a) Interactions of deep-sea vent invertebrates with their environment: the case of *Rimicaris exoculata*. *J Shellfish Res* **27:** 79–90.

Schmidt, C., Vuillemin, R., Le Gall, C., Gaill, F., and Le Bris, N. (2008b) Geochemical energy sources for microbial primary production in the environment of hydrothermal vent shrimps. *Mar Chem* **108:** 18–31.

Segonzac, M., Desaintlaurent, M., and Casanova, B. (1993) Enigma of the trophic adaptation of the shrimp *Alvinocarididae* in hydrothermal areas along the Mid-Atlantic Ridge. *Cah Biol Mar* **34:** 535–571.

Shank, T.M., Lutz, R.A., and Vrijenhoek, R.C. (1998) Molecular systematics of shrimp (Decapoda: Bresiliidae) from deep-sea hydrothermal vents, I: enigmatic 'small orange' shrimp from the Mid-Atlantic Ridge are juvenile *Rimicaris exoculata*. *Mol Mar Biol Biotechnol* **7:** 88–96.

Southward, A.J., and Newman, W.A. (1998) Ectosymbiosis between filamentous sulphur bacteria and a stalked barnacle (Scalpellomorpha, Neolepadinae) from the Lau Back Arc Basin, Tonga. *Cah Biol Mar* **39:** 259–262.

Steedman, H.F. (1957) Polyester wax – new ribboning embedding medium for histology. *Nature* **179:** 1345–1345.

Suzuki, Y., Suzuki, M., Tsuchida, S., Takai, K., Horikoshi, K., Southward, A.J., *et al.* (2009) Molecular investigations of the stalked barnacle *Vulcanolepas osheai* and the epibiotic bacteria from the Brothers Caldera, Kermadec Arc, New Zealand. *J Mar Biol Assoc UK* **89:** 727–733.

Teske, P.R., Froneman, P.W., Barker, N.P., and McQuaid, C.D. (2007) Phylogeographic structure of the caridean shrimp *Palaemon peringueyi* in South Africa: further evidence for intraspecific genetic units associated with marine biogeographic provinces. *Afr J Mar Sci* **29:** 253–258.

Tyler, P.A., and Young, C.M. (1999) Reproduction and dispersal at vents and cold seeps. *J Mar Biol Assoc UK* **79:** 193–208.

Tyler, P.A., and Young, C.M. (2003) Dispersal at hydrothermal vents: a summary of recent progress. *Hydrobiologia* **503:** 9–19.

Van Dover, C.L. (2000) *The Ecology of Deep-Sea Hydrothermal Vents*. Princeton, NJ, USA: Princeton University Press.

Van Dover, C.L., Fry, B., Grassle, J.F., Humphris, S., and Rona, P.A. (1988) Feeding biology of the shrimp *Rimicaris exoculata* at hydrothermal vents on the Mid-Atlantic Ridge. *Mar Biol* **98:** 209–216.

Van Dover, C.L., German, C.R., Speer, K.G., Parson, L.M., and Vrijenhoek, R.C. (2002) Marine biology – evolution and biogeography of deep-sea vent and seep invertebrates. *Science* **295:** 1253–1257.

Vrijenhoek, R.C. (1997) Gene flow and genetic diversity in naturally fragmented metapopulations of deep-sea hydrothermal vent animals. *J Hered* **88:** 285–293.

Williams, A.B., and Rona, P.A. (1986) Two new caridean shrimps (*Bresiliidae*) from a hydrothermal field on the Mid-Atlantic Ridge. *J Crustacean Biol* **6:** 446–462.

Wirsen, C.O., Jannasch, H.W., and Molyneaux, S.J. (1993) Chemosynthetic microbial sctivity at Mid-Atlantic Ridge hydrothermal vent sites. *J Geophys Res* **98:** 9693–9703.

Zbinden, M., and Cambon-Bonavita, M.A. (2003) Occurrence of *Deferribacterales* and *Entomoplasmatales* in the deep-sea alvinocarid shrimp *Rimicaris exoculata* gut. *FEMS Microbiol Ecol* **46:** 23–30.

Zbinden, M., Le Bris, N., Gaill, F., and Compere, P. (2004) Distribution of bacteria and associated minerals in the gill chamber of the vent shrimp *Rimicaris exoculata* and related biogeochemical processes. *Mar Ecol Prog Ser* **284:** 237–251.

Zbinden, M., Shillito, B., Le Bris, N., de Montlaur, C.D., Roussel, E., Guyot, F., *et al.* (2008) New insigths on the metabolic diversity among the epibiotic microbial communitiy of the hydrothermal shrimp *Rimicaris exoculata*. *J Exp Mar Biol Ecol* **359:** 131–140.

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.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, *Environmental Microbiology*

Manuscript IV: Functional aspects of the *Rimicaris exoculata* epibiosis: carbon and energy sources for the filamentous gamma- and epsilonproteobacterial epibionts

Jillian M. Petersen, Wolfgang Bach, Michael Hügler, Caroline Rühland, Stefan Sievert, Marie-Anne Cambon-Bonavita, Magali Zbinden, and Nicole Dubilier

In preparation

Functional aspects of the *Rimicaris exoculata* epibiosis: carbon and energy sources for the filamentous gamma- and epsilonproteobacterial epibionts.

Jillian M. Petersen[1], Wolfgang Bach[2], Michael Hügler[3], Caroline Rühland[1], Stefan Sievert[4], Marie-Anne Cambon-Bonavita[5], Magali Zbinden[6], Nicole Dubilier[1]

[1]Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359, Bremen, Germany

[2]Department of Geosciences, University of Bremen, Germany

[3]Technologiezentrum Wasser, Karlsruhe, Germany

[4]Department of Biology, Woods Hole Oceanographic Institution, MA, USA

[5]Laboratoire de Microbiologie et de Biotechnologie des Extrêmophiles, Département de Valorisation des Produits, IFREMER, Centre de Brest, BP 70, 29280 Plouzané, France

[6]UMR SAE 7138, Adaptations aux milieux extrêmes, Université Pierre et Marie Curie, 7, quai Saint Bernard, 75252 Paris Cédex 05, France

Keywords: hydrothermal vent, symbiosis, chemosynthesis, methane, oxygen, sulfide

Running head: Carbon and energy sources for *R. exoculata* epibionts

Corresponding author: Nicole Dubilier

Max Planck Institute for Marine Microbiology

Celsiusstr. 1

28359 Bremen

Germany

Phone: +49 421 2028 932

Email: ndubilie@mpi-bremen.de

Summary

Hydrothermal vents on the Mid-Atlantic Ridge (MAR) are dominated by giant swarms of the shrimp *Rimicaris exoculata*. These shrimp have a dual symbiosis with filamentous gamma- and epsilonproteobacteria, which cover specialized appendages and surfaces within the gill chamber. The role of the epibionts is unclear, and although they are considered to be sulfur-oxidizing chemolithoautotrophs, there is little direct evidence for this. We amplified and sequenced functional genes for carbon fixation via the Calvin-Benson-Bassham (CBB) cycle, typically found in gammaproteobacteria, and the reductive tricarboxylic acid (rTCA) cycle, typically found in epsilonproteobacteria, showing that both symbionts have the potential to fix CO_2. To investigate the sources of energy for the *R. exoculata* epibionts, we modeled the energy budget of the *R. exoculata* habitat at the Rainbow vent field (MAR) for a number of possible microbial metabolisms, and showed that most energy can be gained from hydrogen, methane, and iron oxidation with oxygen. Based on these results, we amplified and sequenced key genes for hydrogen and methane oxidation, and also for sulfur oxidation. We hypothesize that both the gamma- and the epsilonproteobacterial symbiont can oxidize hydrogen and sulfur. In addition, we suggest that the gammaproteobacterial symbiont can oxidize methane based on immunohistochemistry showing the expression of particulate methane monooxygenase (pMMO). This is remarkable, because the gammaproteobacterial symbiont belongs to a lineage previously unknown to contain methane oxidizers. The metabolic flexibility of the *R. exoculata* epibionts might explain their evolutionary success in the unstable hydrothermal vent environment.

Introduction

The input of organic matter from the photic zone to the seafloor is extremely low in the deep sea where most hydrothermal vents are found (Van Dover, 2000). However, hydrothermal vents provide ideal conditions for chemosynthetic microbes. Vent fluids laden with reduced inorganic compounds such as H_2S and CH_4 exit the seafloor where they mix with cold, oxygenated seawater. This creates a mixing zone where oxidized and reduced compounds coexist. Chemosynthetic microbes that can harness the energy released by redox reactions to create organic matter from single carbon compounds such as CO_2 and CH_4 are the primary producers at deep sea hydrothermal vents, and many hydrothermal vent faunas have evolved symbiotic associations with chemosynthetic bacteria, which provide them with a source of nutrition (reviewed in Cavanaugh et al., 2006; Dubilier et al., 2008). The animal host provides a stable environment for the bacteria, and simultaneous access to electron donors and acceptors by positioning themselves where vent fluids mix with seawater.

Hydrothermal vents on the slow-spreading Mid-Atlantic Ridge (MAR) are dominated by *Rimicaris exoculata*, an alvinocaridid shrimp that is only found at MAR hydrothermal vents (reviewed by Schmidt et al., 2008). This species forms giant swarms of up to 3000 individuals/m^2 (Gebruk et al., 2000) on chimney structures at six MAR vent fields (Fig. 1). *R. exoculata* has a dense bacterial epibiosis on modified mouthparts and on the inner surfaces of the gill chamber. The role of the epibionts is unclear, and although they have been hypothesized to contribute to host nutrition, the transfer of energy from the epibionts to the host has not yet been directly demonstrated (Gebruk et al., 1993). Alternative food sources for the shrimp could include grazing of free-living chemoautotrophic bacteria from chimney surfaces, or a chemoautotrophic gut community (Van Dover et al., 1988; Polz et al., 1998).

The metabolism of the epibionts has not been clearly identified. Autotrophic activity was demonstrated by RuBisCO activity in epibionts from the TAG and Snake Pit vent fields (Wirsen et al., 1993; Cavanaugh and Robinson, 1996; Polz et al., 1998), and epibionts from both of these vent fields have been shown to incorporate radioactively labeled inorganic carbon (Galchenko et al., 1989; Jannasch et al., 1991; Polz et al., 1998). Internal sulfur globules have been observed in the shrimp epibionts from the TAG vent field, indicating that they are chemoautotrophic sulfur-oxidizers (Gebruk et al., 1993).

A novel metabolic strategy, iron oxidation, has been proposed to fuel the *R. exoculata* symbiosis at the Rainbow vent field (Zbinden et al., 2004; Corbari et al., 2008). The Rainbow end-member fluids have unusually high concentrations of reduced iron, and *R. exoculata* from Rainbow has a typical orange colour due to the presence of iron oxyhydroxide precipitates in the gill chamber (Charlou et al., 2002). A close association has been shown between the mineral precipitates and the epibotic bacteria, and iron oxide particles have been observed attached to epibiont cells (Gloter et al., 2004; Zbinden et al., 2004; Anderson et al., 2008). Gloter et al. (2004) investigated the iron oxide minerals with spectroscopic methods, but could not determine the role of the bacteria in their precipitation based on the structure and composition of the minerals. The oxidation of ferrous iron by bacteria for energy conservation is notoriously difficult to demonstrate. The pathways of iron oxidation are currently unknown, and although cytochromes have been implicated in the mechanism of iron oxidation, no key gene for this metabolism has yet been identified (Croal et al., 2007; Jiao and Newman, 2007). The presence of iron minerals does not provide unequivocal evidence for an iron-oxidizing metabolism, as iron oxides can form on bacterial cell surfaces as a result of passive Fe sorption to bacterial cell walls (Fortin and Langley, 2005). At circumneutral pH, the chemical oxidation of Fe^{2+} to

Fe^{3+} is extremely rapid. Ferric iron is highly insoluble under these conditions and precipitates as iron hydroxide (FeOOH), which is positively charged, and can therefore bind to the negatively charged bacterial cell surfaces. Iron hydroxides have also been shown to be autocatalytic, that is, they can catalyze the chemical oxidation of Fe^{2+} (Park and Dempsey, 2005; Rentz et al., 2007). Iron-oxidizing bacteria must therefore not only avoid encrustation by iron oxides, they must be able to compete with their own metabolic by-products for reduced iron.

Recently, we investigated the phylogeny of the epibiotic bacteria associated with *R. exoculata* from four Mid-Atlantic Ridge vent fields, covering its distribution range of 8500 km along the MAR (Petersen et al., submitted). Although a previous study had identified only a single filamentous, epsilonproteobacterial symbiont phylotype on shrimp from the Snake Pit vent field (Polz and Cavanaugh, 1995), we showed that *R. exoculata* at all four vent fields host filamentous gammaproteobacterial symbionts. Our study showed that the filamentous Gamma and Epsilon symbionts dominated the epibiosis on all shrimp investigated, independent of differences in the chemical composition of the habitat. Neither of the filamentous ectosymbionts are closely related to known iron-oxidizing bacteria. The closest cultured relative of the Epsilon symbiont is the chemolithoautotrophic sulfur oxidizer *Sulfurovum lithotrophicum* (Inagaki et al., 2004). The novel Gamma symbiont is related to the filamentous marine bacterium *Leucothrix mucor*, which has been described as a sulfur-oxidizing chemolithoheterotroph (Grabovich et al., 1999).

The purpose of this study was to reexamine the energy sources that could be fuelling the *R. exoculata* symbiosis at the Rainbow vent field (Fig. 1). The results of earlier studies of the symbiont metabolism were often interpreted with the assumption that the *R. exoculata* epibiosis consists of a single epsilonproteobacterial phylotype. We aimed to identify pathways of carbon fixation and energy generation in the

epibionts by targeting key genes for these processes in known chemolithoautotrophic epsilon- and gammaproteobacteria.

Table 1. Reactions tested in the thermodynamic model, and the change in free energy of each reaction under standard conditions

Metabolism	**Reaction**	**ΔG^0 (kJ/Mol)**
Aerobic		
Sulfide oxidation	$H_2S_{(aq)} + 2\ O_{2(aq)} \Rightarrow SO_4^{-2} + 2\ H^+$	-750
Methane oxidation	$CH_{4(aq)} + 2\ O_{2(aq)} \Rightarrow HCO_3^- + H^+ + H_2O$	-823
Hydrogen oxidation	$2H_{2(aq)} + O_{2(aq)} \Rightarrow 2\ H_2O$	-526
Iron oxidation	$4\ Fe^{2+} + O_{2(aq)} + 10\ H_2O \Rightarrow 4\ Fe(OH)_{3(s)} + 8\ H^+$	-115
Anaerobic		
Methanogenesis	$4\ H_{2(aq)} + HCO_3^- + H^+ \Rightarrow CH_{4(aq)} + 3\ H_2O$	-230
Sulfate reduction	$4\ H_{2(aq)} + SO_4^{2-} + 2\ H^+ \Rightarrow H_2S_{(aq)} + 4\ H_2O$	-303
Anaerobic methane oxidation	$CH_{4(aq)} + SO_4^{2-} + H^+ \Rightarrow H_2S(aq) + HCO_3^- + H_2O$	-73

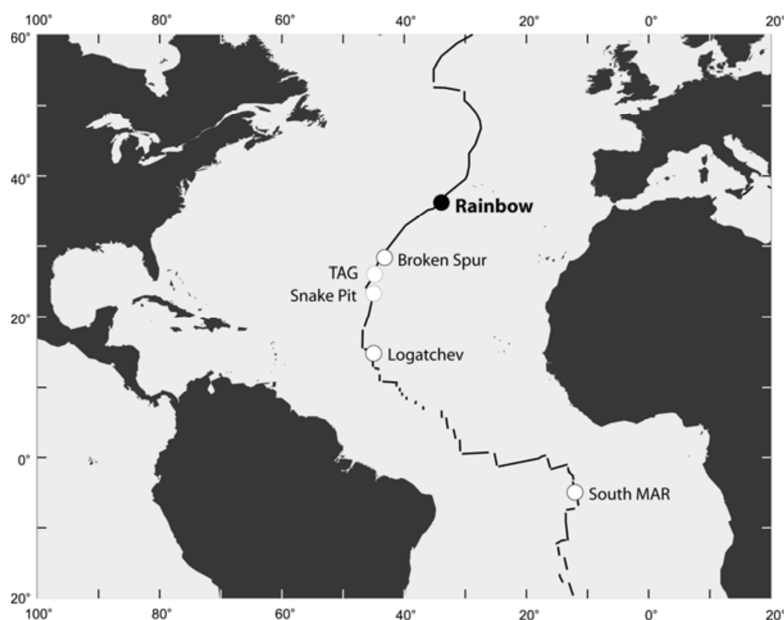


Figure 1. Map showing the locations of hydrothermal vent fields on the Mid-Atlantic Ridge (MAR) where *R. exoculata* has been found, according to (Schmidt et al., 2008). The Rainbow vent field is indicated in bold (black circle).

Results and Discussion

Geochemical modelling

The amount of energy that microbes gain by catalyzing certain redox reactions (Table 1) depends on the difference in reduction potential between oxidants (electron acceptors) and reductants (electron donors), and on the availability (concentrations) of oxidants and reactants. The *Rimicaris exoculata* habitat is a mixing zone between the hot, reduced hydrothermal vent fluids rich in electron donors, and the surrounding seawater, which is oxygen-saturated. We modelled the chemical conditions in the shrimp habitat at the Rainbow vent field, based on the mixing of pure vent fluids and

seawater, to determine the potential energy to be gained from certain redox reactions along a mixing gradient representing the shrimp's habitat (Table 1). Two extreme conditions were tested: (1) mineral precipitation suppressed, and (2) mineral precipitation proceeding to saturation. The actual conditions will lie between these two extremes. We therefore present both models. Under both conditions tested, the most energy could be gained by hydrogen oxidation with oxygen, followed by methane oxidation with oxygen (Fig. 2). Fe^{2+} oxidation with oxygen could also provide energy for microbial chemosynthesis in the shrimp's habitat, reflecting the availability of reduced iron at the Rainbow vent field, where the iron concentration in the hydrothermal fluids is unusually high (Charlou et al., 2002). Sulfide oxidation with oxygen could still provide energy for chemosynthesis in the shrimp's habitat, but the energy available from sulfide oxidation is much less than from hydrogen, methane, or iron oxidation. Because of the high Fe:H_2S ratios of Rainbow vent fluids, there is very little sulfide left in the second model (mineral precipitation allowed) (Fig. 2B). Mapping of the thermal range of the shrimp habitat (Schmidt et al., 2008) onto the thermodynamic model shows that the shrimp position themselves within a narrow range that exactly corresponds to the predicted range where the highest potential energy can be gained from hydrogen, methane, and iron oxidation (Fig. 2).

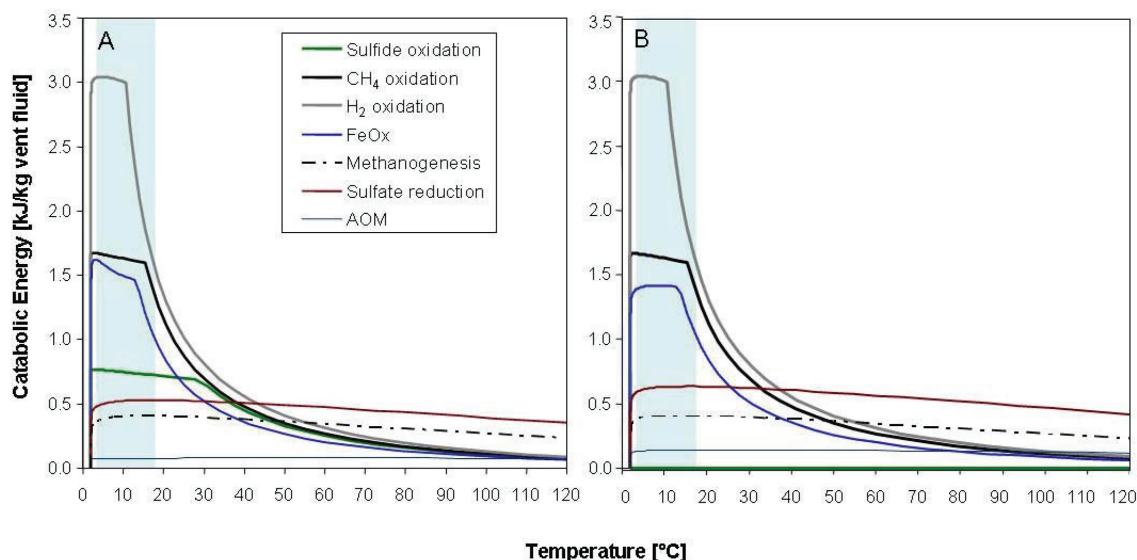


Figure 2. Energy budget modelling. Theoretical energy budget of microbial metabolisms modelled for a mixing zone between ambient seawater and the Rainbow end-member vent fluids, up to 120°C. Energy yield is presented per mol reaction (see Table 1). Blue shaded areas represent the range of temperatures measured in *R. exoculata* swarms (Schmidt et al., 2008). A) Mineral precipitation suppressed, B) Mineral precipitation proceeding to saturation.

Epibiont energy metabolism and carbon fixation

The shrimp epibionts were previously hypothesized to be chemoautotrophic sulfur oxidizers. Our model predicted that hydrogen and methane could fuel the shrimp symbiosis at the Rainbow vent field, and also predicts that sulfur oxidation is less favorable than hydrogen or methane oxidation. To investigate the metabolic potential of the shrimp symbionts, we tested for the presence of indicator genes for carbon fixation and energy generation in the epibiotic community on three *Rimicaris exoculata* individuals collected at the Rainbow vent field. By PCR we amplified key

genes for two pathways of autotrophic carbon fixation, *cbbM* for the Calvin-Benson-Bassham (CBB) cycle, and *aclB* for the reverse tricarboxylic acid (rTCA) cycle, and for 3 different modes of energy generation, *pmoA* for methane oxidation (MOX), *aprA* for sulfur oxidation (SOX), and *hynL* for hydrogen oxidation (HOX). All five genes could be amplified from DNA extracted from the shrimp epibionts, and for each gene, at least one sequence was found that was identical in clone libraries from all three individuals, indicating that these sequences could be from the symbionts, and not from contaminants from the environment or casually associated bacteria.

Calvin-Benson-Bassham cycle

Genes for the form I RuBisCO, *cbbL*, could not be amplified from any of the specimens in this study, using a number of different primer sets. The gene encoding the form II RuBisCO, *cbbM*, however, could be amplified from the epibionts of all three shrimp. All *cbbM* sequences from individuals 1 (46 clones sequenced) and 2 (4 clones sequenced) were identical, and this sequence was also found in 5 out of the 9 clones sequenced from individual 3 (Table 2). The most closely related sequence is an environmental clone, amplified from cold seep sediments in the Japan Trench (92.4% amino acid identity) (Elsaied and Naganuma, 2001), and groups together with the *cbbM* sequences from the phototrophic alphaproteobacteria *Rhodopseudomonas palustris* and *Rhodobacter sphaeroides* (Fig. 3A). To date, genes encoding for RubisCO form II have only been found in free-living and endosymbiotic gammaproteobacteria, and in free-living alpha- and betaproteobacteria, but so far have never been found in epsilonproteobacteria (Tabita, 1999; Cavanaugh et al., 2006; Scott et al., 2006). In addition, the phylogeny of RubisCO II is incongruent with 16S rRNA phylogeny. It is therefore most likely that the *cbbM* identified in this study is from the Gamma symbiont, indicating that this symbiont can use the CBB cycle for

autotrophic growth, and could contribute to host nutrition by providing a source of organic carbon.

Table 2. Functional gene clone library analysis

		c16	c18	c20		
		shared (total)	shared (total)	shared (total)	Protein name	Characteristic for:*
CO_2 fixation	***aclB -1***	1 (9)	11 (23)	18 (31)	ATP citrate lyase	rTCA
	aclB -2	5 (9)	11 (23)	13 (31)	"	"
	cbbM	46 (46)	4 (4)	5 (9)	RubisCO form II	CBB
Energy generation	***pmoA***	34 (34)	43 (43)	4 (4)	Particulate methane monooxygenase	MOX
	aprA	39 (41)	12 (12)	24 (24)	APS reductase	SOX
	hynL - 1	57 (67)	10 (10)	26 (29)	Membrane-bound NiFe hydrogenase	HOX
	hynL - 2	3 (67)		1 (29)	"	"
	hynL - 3	2 (67)		1 (29)	"	"

* rTCA = reductive tricarboxylic acid cycle, CBB = Calvin-Benson-Bassham cycle, MOX = methane oxidation, SOX = sulfur oxidation, HOX = hydrogen oxidation

Reductive TCA cycle

The reductive TCA cycle is essentially a reversal of the well-known oxidative tricarboxylic acid (TCA) cycle. One of the key steps of this cycle is the ATP- and Coenzyme-A-dependent cleavage of citrate to acetyl-coA and oxaloacetate, catalysed by the enzyme ATP citrate lyase (ACL). The *aclB* gene encodes the beta subunit of ACL, and is a diagnostic marker for the rTCA cycle (Campbell et al., 2003; Hugler et al., 2007). We found 2 *aclB* sequences that differed by 2% in all 3 clone libraries,

indicating that *R. exoculata* epibionts use the reductive TCA cycle for CO_2 fixation (Table 2). Both grouped with other AclB sequences from epsilonproteobacteria (Fig. 3B). One sequence fell in a group with *Candidatus* Arcobacter sulfidicus, the second did not group with any currently available sequences, and is most closely related to the AclB of *Sulfurimonas autotrophica* (98% amino acid sequence identity, Fig. 3B). So far, use of the rTCA cycle for CO_2 fixation has been found in members of the Epsilonproteobacteria, the *Aquificales*, in some species of Chlorobia, and in the gammaproteobacterial sulfur-oxidizing symbiont of the hydrothermal vent tubeworm *Riftia pachyptila*. However, the *R. pachyptila* symbiont (*Candidatus* Endoriftia Persephone) and members of the Aquificaceae do not have an ATP citrate lyase, but use an alternative mechanism to cleave citrate to acetyl-CoA and oxaloacetate (Hugler et al., 2005; Hugler et al., 2007; Markert et al., 2007; Robidart et al., 2008). Based on our results, we could not determine which of the two sequences is from the *R. exoculata* epsilonproteobacterial symbiont. Results from our lab showed the presence of a second epsilonproteobacterial 16S rRNA phylotype in a clone library from Individual 1, and an oligonucleotide probe designed for this phylotype bound to rod-shaped bacteria that were present at low abundance on three *R. exoculata* individuals from the Rainbow vent field (J.M. Petersen, unpublished observations). It is possible that one of the *aclB* sequences identified in this study is from this epsilon phylotype.

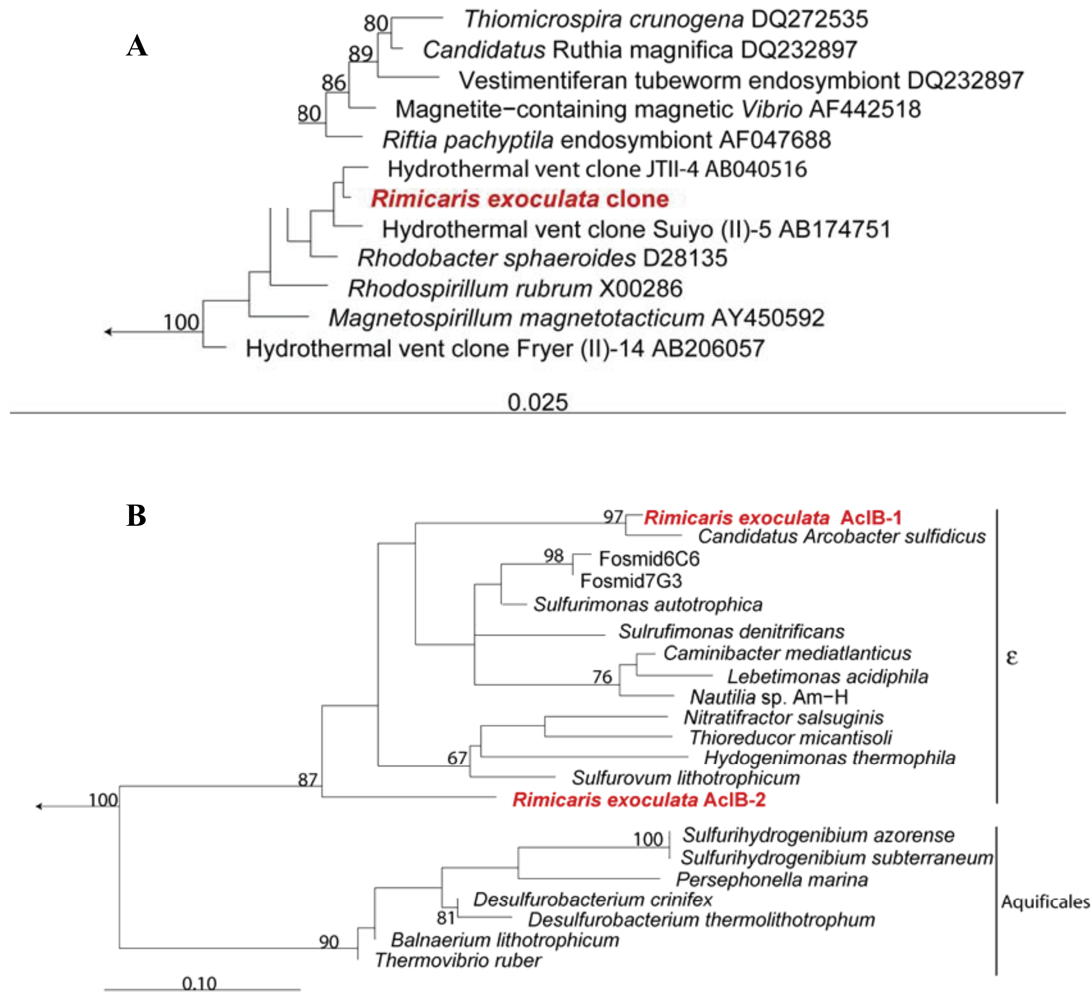


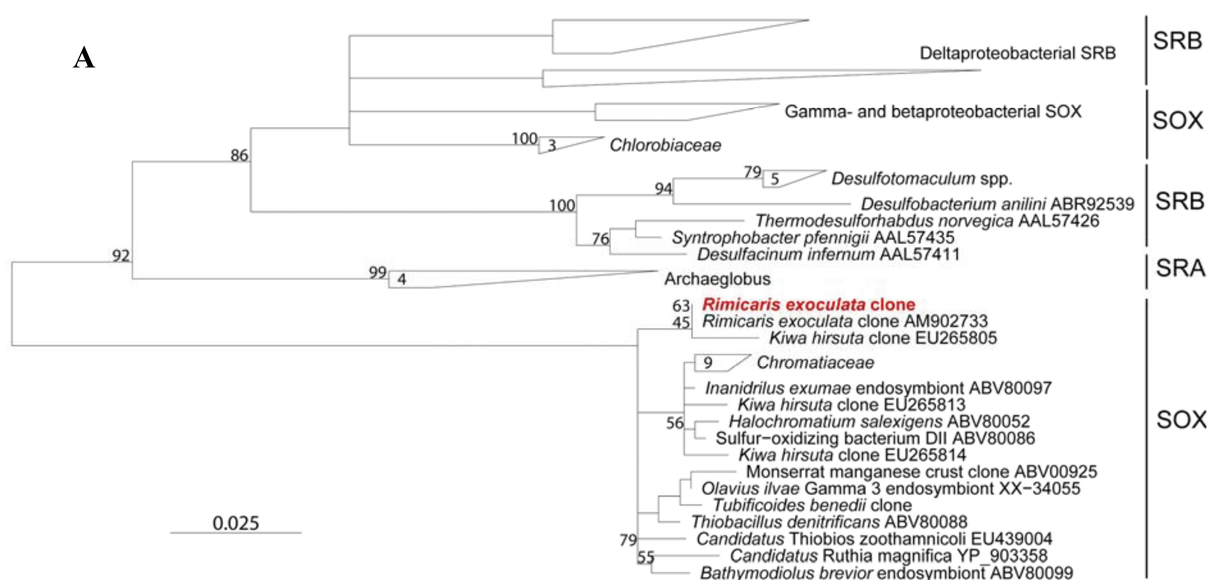
Figure 3. Phylogeny of partial amino acid sequences of RuBisCO and ATP citrate lyase. A) Maximum likelihood phylogeny of RuBisCO form II amino acid sequences. Outgroup (not shown) consisted of RuBisCO form I amino acid sequences.
B) Maximum likelihood phylogeny of partial ATP citrate lyase beta subunit amino acid sequences. Outgroup (not shown) consisted of the ATP citrate lyase amino acid sequences of *Chlorobium* spp.. ε = epsilonproteobacteria. Sequences from this study are shown in bold red. Bootstrap values (from 100 replicates) greater than 60 are shown.

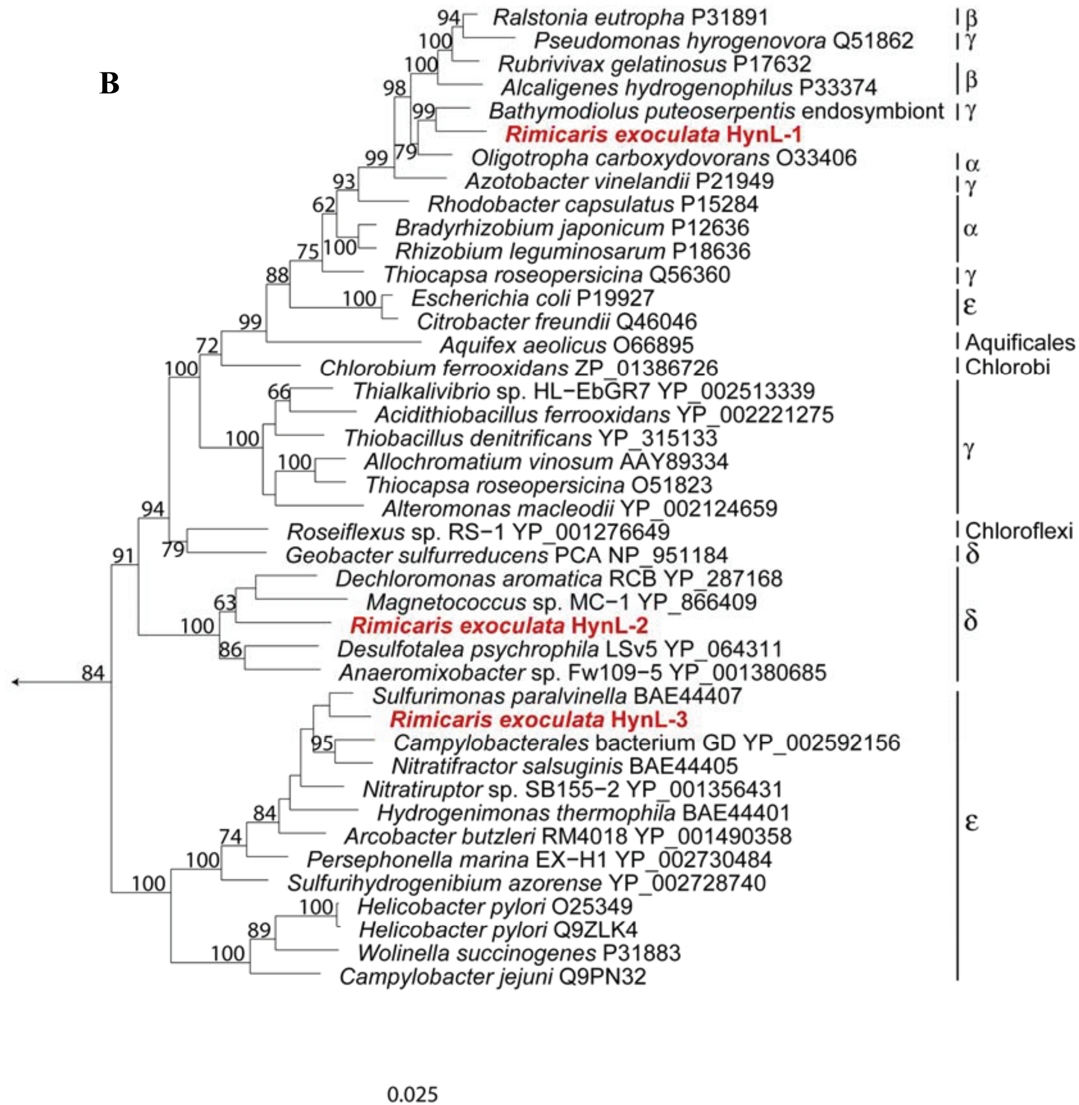
Sulfur oxidation (SOX)

Our thermodynamic model predicted that there is less energy to be gained from sulfide oxidation in the shrimp habitat at Rainbow, than from other potential energy-yielding reactions (Fig. 2). Nevertheless, *Bathymodiolus azoricus* mussels, which co-occur with the shrimp at Rainbow, have been shown to harbor sulfur-oxidizing symbionts (Duperron et al., 2006). In addition, gamma- and epsilonproteobacterial symbionts dominate the *R. exoculata* epibiosis at other MAR vent fields that have high sulfide concentrations (Petersen et al., submitted). We therefore also investigated the sulfur-oxidizing potential of the epibionts.

Adenosine-5'-phosphosulfate (APS) reductase oxidizes sulfite to APS in sulfur-oxidizing prokaryotes. The oxidation of sulfite to sulfate via this pathway generates ATP through substrate-level phosphorylation, and is thus, an energy conserving process (Sanchez et al., 2001). The a*prA* gene, encoding for the alpha subunit of APS reductase, has been found in many sulfur-oxidizing microorganisms (Meyer and Kuever, 2007a, b), and we were able to amplify this gene from the epibionts of all three *R. exoculata* individuals investigated. All *aprA* sequences from individuals 2 (12 sequences) and 3 (24 sequences) were identical, and this sequence type was also found in individual 1, represented by 39 out of 41 clones sequenced (Table 2). An identical sequence was found in a previous clone library from a single *R. exoculata* individual from the Rainbow vent field (Zbinden et al., 2008). The *R. exoculata* APS reductase sequence grouped with those of gammaproteobacterial sulfur-oxidizing bacteria (Fig. 4A), and fell in a clade with an APS reductase amplified from the epibionts of the Yeti crab *Kiwa hirsuta* from a hydrothermal vent on the Pacific Antarctic Ridge (Goffredi et al., 2008). 16S rRNA sequences that are closely related to the *R. exoculata* Gamma epibionts have been found in a clone library from *K. hirsuta* (Goffredi et al., 2008). The APS pathway for sulfur oxidation is not known to

occur in epsilonproteobacterial sulfur oxidizers, which instead, oxidize sulfide or thiosulfate via the Sox system (Grzymski et al., 2008; Sievert et al., 2008). A recent metagenomic study of the epsilonproteobacterial epibionts of the vent polychaete *Alvinella pompejana* reported the identification of genes for APS reductase and APS sulfurylase (Grzymski et al., 2008). However, based on BLAST results, the gene annotated as APS reductase is more similar to genes for assimilatory than dissimilatory sulfur metabolism, and a BLASTx search of the *A. pompejana* metagenome sequences with the *aprA* sequence from various sulfur-oxidizing bacteria returned no significant hits (J. M. Petersen, unpublished results). It is therefore most likely that the *R. exoculata aprA* sequence is from the Gamma symbiont, indicating that it has the potential to use reduced sulfur compounds to fuel chemolithoautotrophic growth.




B
Ralstonia eutropha P31891
Pseudomonas hyrogenovora Q51862
Rubrivivax gelatinosus P17632
Alcaligenes hydrogenophilus P33374
Bathymodiolus puteoserpentis endosymbiont
Rimicaris exoculata HynL-1
Oligotropha carboxydovorans O33406
Azotobacter vinelandii P21949
Rhodobacter capsulatus P15284
Bradyrhizobium japonicum P12636
Rhizobium leguminosarum P18636
Thiocapsa roseopersicina Q56360
Escherichia coli P19927
Citrobacter freundii Q46046
Aquifex aeolicus O66895
Chlorobium ferrooxidans ZP_01386726
Thialkalivibrio sp. HL-EbGR7 YP_002513339
Acidithiobacillus ferrooxidans YP_002221275
Thiobacillus denitrificans YP_315133
Allochromatium vinosum AAY89334
Thiocapsa roseopersicina O51823
Alteromonas macleodii YP_002124659
Roseiflexus sp. RS-1 YP_001276649
Geobacter sulfurreducens PCA NP_951184
Dechloromonas aromatica RCB YP_287168
Magnetococcus sp. MC-1 YP_866409
Rimicaris exoculata HynL-2
Desulfotalea psychrophila LSv5 YP_064311
Anaeromixobacter sp. Fw109-5 YP_001380685
Sulfurimonas paralvinella BAE44407
Rimicaris exoculata HynL-3
Campylobacterales bacterium GD YP_002592156
Nitratifractor salsuginis BAE44405
Nitratiruptor sp. SB155-2 YP_001356431
Hydrogenimonas thermophila BAE44401
Arcobacter butzleri RM4018 YP_001490358
Persephonella marina EX-H1 YP_002730484
Sulfurihydrogenibium azorense YP_002728740
Helicobacter pylori O25349
Helicobacter pylori Q9ZLK4
Wolinella succinogenes P31883
Campylobacter jejuni Q9PN32
β
γ
β
γ
α
γ
α
γ
ε
Aquificales
Chlorobi
γ
Chloroflexi
δ
δ
ε
0.025

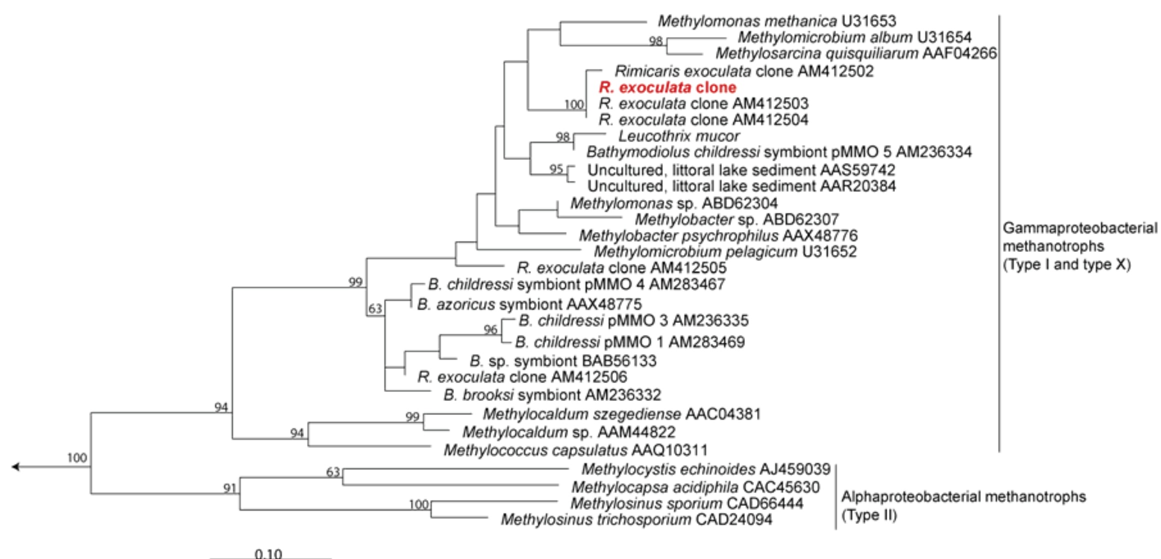


Figure 4. Phylogeny of partial amino acid sequences of APS reductase, NiFe group 1 hydrogenases, and particulate methane monooxygenase. A) Maximum likelihood phylogeny of partial sequences of the alpha subunit of APS reductase, key gene of sulfur oxidation and sulfate reduction via the APS pathway. SRB = sulfate-reducing bacteria, SRA = sulfate-reducing archaea, SOX = sulfur-oxidizing bacteria. B) Maximum likelihood phylogeny of the large subunit of NiFe group 1 hydrogenases. Outgroups (not shown) were the NiFe hydrogenases belonging to groups 2, 3, and 4 (Vignais et al., 2001). α = alphaproteobacteria, β = betaproteobacteria, γ = gammaproteobacteria, δ = deltaproteobacteria, ε = epsilonproteobacteria. C) Maximum likelihood phylogeny of partial sequences of the beta subunit of particulate methane monooxygenase. Outgroup (not shown) consisted of *Crenothrix polysphora* pMMO sequences.

Sequences from this study are shown in bold red. Bootstrap values (from 100 replicates) greater than 60 are shown.

Hydrogen oxidation (HOX)

Group 1 iron-nickel hydrogenases (according to the Vignais et al. (2001) nomenclature) are membrane-bound proteins directly involved in hydrogen oxidation for energy generation via oxidative phosphorylation. These uptake hydrogenases transfer 2 electrons from molecular hydrogen (H_2) to a transmembrane cytochrome b subunit (reviewed in Vignais and Billoud, 2007).

Three sequence groups were identified in clone libraries of the gene for the NiFe uptake hydrogenase, designated *hynL-1*, *hynL-2*, and *hynL-3* (Table 2). *HynL-1* was the most abundant sequence group, and was present in clone libraries from all three individuals (Table 2). *HynL-2* and *hynL-3* were also included in the phylogenetic analysis, even though they were only found in Individuals 1 and 3, as they could have been missed due to the low number of *hynL* sequences obtained from Individual 2 (Table 2). HynL-1 was closely related to the uptake hydrogenase sequence of the gammaproteobacterial chemoautotrophic endosymbiont of the mussel *Bathymodiolus puteoserpentis* from the Logatchev vent field on the MAR (87.3% amino acid sequence identity). The most closely related sequence from a cultured organism is the hydrogenase of *Oligotropha carboxydovorans* (84.4% amino acid identity), a chemolithoautotrophic alphaproteobacterium (Fig. 4B). No 16S rRNA sequences closely related to the *B. puteoserpentis* endosymbionts have been found in *R. exoculata* clone libraries (Petersen et al., submitted, J. M. Petersen, unpublished observations). The HynL-1 sequence fell in a clade with hydrogenases from other proteobacteria, but excluded the sequences from epsilonproteobacteria. We therefore hypothesize that the *hynL-1* gene is from the *R. exoculata* Gamma symbiont.

HynL-2 was affiliated with a group containing the uptake hydrogenases of deltaproteobacteria (Fig. 4B). Deltaproteobacterial 16S rRNA sequences were found in a clone library of *R. exoculata* epibionts from the Rainbow vent field (Zbinden et

al., 2008), but their presence and abundance has not yet been investigated with FISH. Many sulfate-reducing bacteria use hydrogen as an electron donor, reducing sulfate to sulfide. Our thermodynamic model predicted that this metabolism would yield energy in the shrimp habitat (Fig. 2). If sulfate-reducing bacteria are indeed present as epibionts on *R. exoculata*, they could provide the sulfide-oxidizing symbionts with an additional source of reducing power, allowing for internal sulfur cycling. So far, such an internal sulfur cycle in a symbiotic consortium has only been shown in the gutless oligochaete worm *Olavius algarvensis*, which lives in shallow sediments where the concentration of sulfide can be extremely low, in the nanomolar range (Dubilier et al., 2001). The presence of sulfate-reducing symbionts on shrimp at some vent fields might compensate for low sulfide concentrations in the hydrothermal fluids, as is the case at Rainbow (Charlou et al., 2002).

The third sequence group, HynL-3, grouped with hydrogenase sequences from epsilonproteobacteria (Fig. 4B). The epsilonproteobacterial HynL sequences formed a separate cluster to all other group 1 NiFe hydrogenases, with 100% bootstrap support. The presence of three different hydrogenase sequence groups indicates that hydrogen can be used as an energy source by multiple symbionts. This is consistent with our thermodynamic model, which predicts that hydrogen oxidation could potentially provide the most energy for chemolithoautotrophic growth in the shrimp habitat. Hydrogen oxidation is widespread in members of the epsilonproteobacteria that inhabit hydrothermal vents, both free-living (Takai et al., 2005; Campbell et al., 2006) and ectosymbiotic (Grzymski et al., 2008). Hydrogen has only recently been identified as an energy source for the gammaproteobacterial sulfur-oxidizing symbionts of the hydrothermal vent mussel *Bathymodiolus puteoserpentis* (Zielinski et al., in prep). While the role of hydrogen as an energy source for chemosynthetic symbioses has been long overlooked, the recent study by Zielinski and colleagues,

and the results presented here, confirm that its use is more widespread than previously thought.

Methane oxidation (MOX)

The oxidation of methane to methanol is the first step in both methane oxidation to CO_2 for energy generation, and oxidation to formaldehyde for incorporation into cell biomass. This reaction is catalysed by a membrane-bound particulate or a cytoplasmic soluble methane monooxygenase (pMMO and sMMO, respectively). Only some methanotrophic bacteria possess sMMO, but all methanotrophs investigated to date, except for *Methylocella* species, have the particulate form (reviewed in Hanson and Hanson, 1996; Trotsenko and Murrell, 2008).

We were able to amplify the *pmoA* gene from all three *R. exoculata* individuals, indicating that the epibiotic bacteria have the potential to oxidize methane. The *pmoA* clones from all 3 individuals were identical (Table 2). This sequence was also obtained from the single *R. exoculata* individual investigated by (Zbinden et al., 2008). The *R. exoculata* epibiont pMMO sequence grouped with the gammaproteobacterial pMMO sequences, and formed a sister clade to pMMO sequences from free-living gammaproteobacterial methanotrophs of the genera *Methylomonas*, *Methylomicrobium*, and *Methylosarcina* (Fig. 4C).

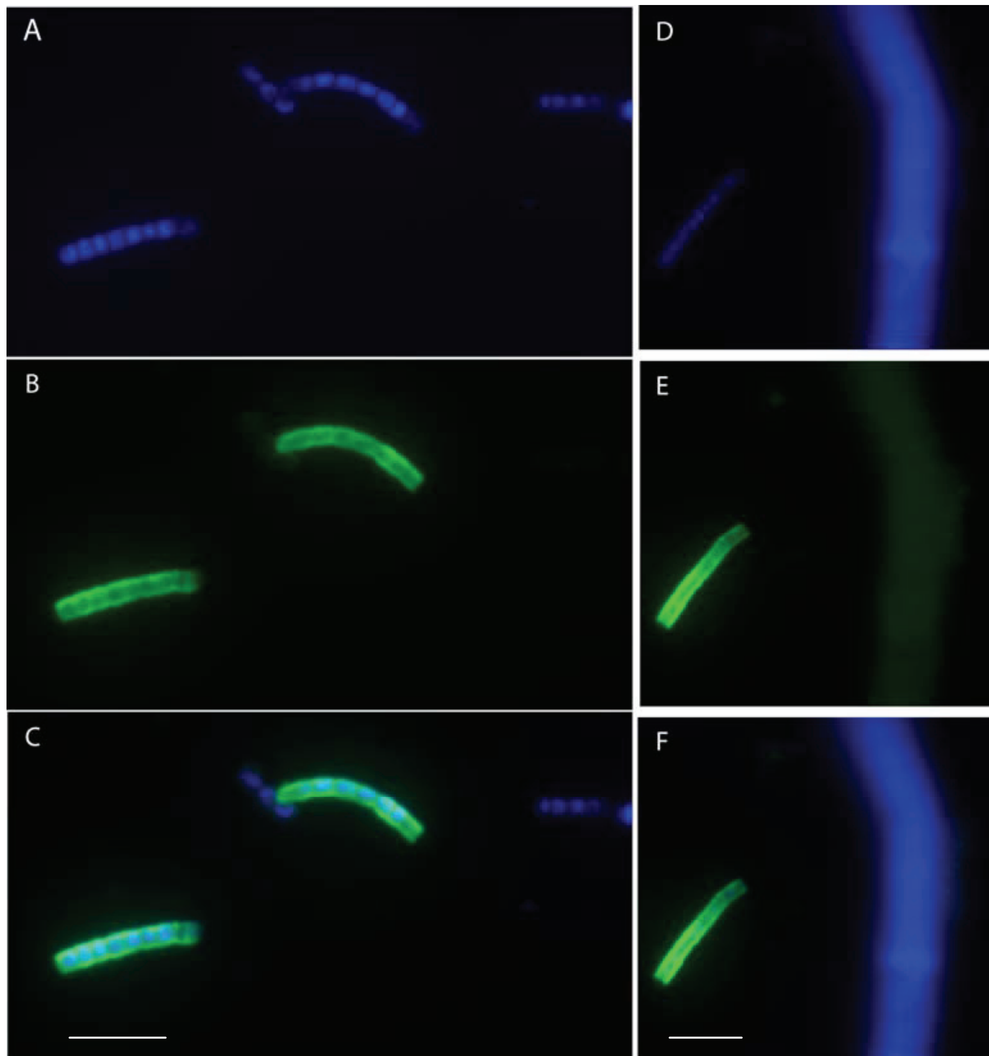


Figure 5. Immunohistochemical staining of particulate methane monooxygenase

A) and D) Epifluorescence micrograph of DAPI-stained filamentous epibionts. The thin Gamma symbionts can be seen in A) and D), the thick Epsilon symbiont is shown on the right-hand-side in D). B) and E) pMMO signal in the same field as A) and D). C) and F) overlay of the two channels. The pMMO signal is only seen in filaments with the Gamma symbiont morphology. Scale bars = 5 µm.

There are currently three known lineages containing methanotrophic bacteria. The first belongs to the Alphaproteobacteria, the second belongs to the Gammaproteobacteria, and the third belongs to the Verrucomicrobia (reviewed in Hanson and Hanson, 1996; Semrau et al., 2008; Trotsenko and Murrell, 2008). No members of any of these three lineages have yet been identified in multiple molecular studies of the bacterial epibionts of *Rimicaris* shrimp (Polz and Cavanaugh, 1995; Zbinden et al., 2008; Petersen et al., submitted). It was therefore unclear which symbiont contains the *pmoA* gene and thus, has the potential to oxidize methane. To investigate this, we used immunohistochemistry to demonstrate the expression of pMMO in single epibiont cells.

Immunohistochemical staining of pMMO

We used an anti-pMMO antiserum for immunohistochemistry of the *R. exoculata* epibionts (Brantner et al., 2002). We saw signals corresponding to the Gamma symbiont morphology (Petersen et al., submitted), indicating that they express a particulate methane monooxygenase (Fig. 5). No signals were seen in the large filaments, which correspond to the Epsilon symbiont morphology (Fig. 5). This provides strong evidence that the *R. exoculata* Gamma symbiont can oxidize methane.

There is currently no known sulfur-oxidizer that can also oxidize methane. Likewise, no methanotroph is known to use sulfur as an energy source. The genome sequence of *M. capsulatus* (Bath) revealed the genetic potential for the oxidation of reduced compounds other than methane. It contains both membrane-bound and soluble uptake hydrogenases, and the potential for growth on reduced sulfur compounds was suggested by the presence of genes from the Sox pathway. However, this last observation remains controversial, as the key gene of this pathway, *soxB*, was

not found (Ward et al., 2004; Kelly et al., 2005). Therefore, although this genome sequence shows the potential for metabolic flexibility, most functional studies to date on cultured strains show that methanotrophs are highly specialized to grow on methane as a single carbon and energy source. There are no known methane-oxidizing bacteria in the *R. exoculata* Gamma symbiont lineage, but recent studies suggest that the ability to oxidize methane is more widespread among bacteria than previously assumed, and our results confirm this (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008). In addition to methane, we hypothesize that the Gamma symbiont of *R. exoculata* has the potential to oxidize reduced sulfur compounds and hydrogen, showing unprecedented metabolic flexibility in a methane-oxidizing bacterium.

Metabolic flexibility and adaptation to the vent habitat

Metabolic flexibility in a nutritional symbiosis can be achieved in at least two different ways. Firstly, a host can associate with multiple symbiont types with distinct metabolic functions, such as the Mid-Atlantic Ridge hydrothermal vent mussels *Bathymodiolus* that have a dual symbiosis with methane-oxidizing and sulfur-oxidizing bacteria, allowing them to successfully colonize all known Mid-Atlantic Ridge vent fields despite varying availabilities of sulfide and methane at these sites. Associations with multiple closely related bacterial phylotypes that are inferred to have the same basic role in the symbiosis were unknown until recently. *Olavius algarvensis*, a gutless marine worm, has been shown to associate with two distinct sulfur-oxidizing symbionts, and also with two distinct sulfate-reducers (Woyke et al., 2006). The cold seep mussel *Bathymodiolus heckerae* from the Gulf of Mexico associates with two closely-related sulfur oxidizers, a methane oxidizer, and a phylotype related to free-living methylotrophs (Duperron et al., 2007). The advantage

to the host of harboring metabolically distinct symbionts is clear in environments where the availability of energy sources fluctuates, but the advantage of harboring two sulfur-oxidizing symbionts in the case of *B. heckerae*, or the *R. exoculata* epibiosis, where it appears that both the Gamma and Epsilon symbionts can both oxidize hydrogen and reduced sulfur compounds, requires explanation. It is possible that niche partitioning in symbiosis goes beyond the use of either methane or reduced sulfur compounds. Duperron et al. (2007) hypothesized that the two thiotrophic symbiont types might be specialized to use different reduced sulfur compounds, such as H_2S or thiosulfate, both of which are present in the fluids around mussel beds (Waite et al., 2008) .

An alternative strategy is for a host to associate with metabolically flexible symbionts that can use a number of energy sources. Symbiont niches can be temporally partitioned, with each symbiont able to fix CO_2 under a certain set of conditions. *Rimicaris* shrimp are highly motile, and move through extremely sharp gradients between anoxic vent fluids containing the electron donors, and fully oxygenated seawater. It is therefore likely that the ectosymbionts are regularly exposed to varying concentrations of electron donors and acceptors, although long periods of anoxia are unlikely because of the host's requirement for oxygen. In addition, there may be a successional pattern of symbiont colonization, and different developmental stages of the epibiosis during the shrimp's moulting cycle (Corbari et al., 2008). We have observed that the Epsilon symbionts tend to dominate the epibiosis in freshly moulted individuals, whereas the Gamma symbionts dominate on individuals that are close to ecdysis (J. M. Petersen, unpublished observations). Further studies on the metabolic potential of the symbionts, their abundance, and activity under various environmental conditions will help to explain how resources are shared by the symbionts, and how *R. exoculata* benefits from the association.

Experimental procedures

Sampling and storage. *Rimicaris exoculata* was collected at the Rainbow vent field (36°14.0' N, Mid-Atlantic Ridge, 2300 m depth) during the Exomar cruise (chief scientist A. Godfroy) in 2005 with the research vessel *L'Atalante* and the remotely operated vehicle *Victor*. Samples were processed on board immediately after retrieval. They were either frozen for DNA or fixed for immunohistochemistry (IHC) analysis. Samples for IHC were fixed at 4°C for 4 – 10 h in 2% formaldehyde in 0.2 µm filtered seawater. After fixation, samples were washed 3 times at 4 °C for 30 min in 0.2µm filtered seawater, then stored at −20°C in a 50% ethanol 50% filtered seawater solution.

DNA extraction. Genomic DNA was extracted separately from scaphognathite, exopodite, and carapace tissue with the FastDNA SPIN kit for soil (Qbiogene, Carlsbad, California). DNA was stored in aliquots at −20 °C.

Amplification of functional marker genes

All of the following genes were amplified from pooled scaphognathite, exopodite, and carapace DNA:

cbbM The gene encoding the ribulose-1,5-bisphosphate-carboxylase/-oxygenase (RuBisCO) form II large subunit (*cbbM*) was amplified using the forward primer cbbM1-Els and the reverse primer cbbM2-b (Elsaied and Naganuma, 2001). PCR cycling conditions were: 4 min at 92°C followed by 35 cycles of 1 min at 92°C, 1 min 30 s at 62°C, 1 min at 72°C, then 1 cycle of 9 min at 92°C.

aprA The gene encoding adenosine 5'-phosphosulfate reductase (APS) subunit A (*aprA*) was amplified as in (Blazejak et al., 2006), using the primers AprA-1-FW and AprA-5-RV from (Meyer and Kuever, 2007a).

hynL The gene encoding the large subunit of the NiFe hydgrogenase was amplified with the primers HUPLX1 and HUPLW2 (Csaki et al., 2001). PCR conditions were as described in (Zielinski et al., In prep).

pmoA The gene encoding the particulate methane monooxygenase subunit B (*pmoA*) was amplified using the primers A189F and MB661R from Duperron et al. (2007). PCR cycling conditions were as follows: 4 min at 92°C followed by 33 cycles of 1 min at 92°C, 1 min 30 s at 55°C, 1 min at 72°C, then 1 cycle of 9 min at 92°C. Purified PCR products of *cbbM*, *pmoA*, *aprA,* and *hynL* were cloned and sequenced according to (Petersen et al., submitted).

aclB Fragments of the beta subunit of the ATP citrate lyase gene (*aclB*) were amplified using the primer set 892F/1204R, using the conditions as described before (Campbell et al., 2003). For each functional gene fragment five parallels were amplified and pooled. PCR products were gel-purified using the QIAGEN QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned into pCR4-TOPO plasmid vectors with the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. An environmental clone library for each gene was constructed. From each library 96 clones were randomly chosen and analyzed for the insert-containing plasmid by direct PCR with the vector primers M13F and M13R followed by gel electrophoreses of the amplified products. PCR products of the correct size were sequenced using the M13 primer set. Sequencing was performed using the

BigDye Terminator v1.1 sequencing kit in a 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA) as specified by the manufacturer.

Functional gene phylogenies

Functional gene sequences were checked against GenBank using tBLASTx. Sequence data was analyzed using the ARB package (www.arb-home.de, Ludwig et al., 2004). Phylogenetic trees were generated from amino acid sequences using maximum likelihood analysis. Bootstrap values were calculated with 100 resamplings. Only values greater than 60 are shown.

Immunohistochemistry

We were able to obtain a polyclonal rabbit anti-pMMO antibody from Dr. M. L. Collins, University of Wisconsin, Milwaukee (Brantner et al., 2002). With this antibody, we developed a protocol for immunohistochemistry on the *R. exoculata* epibionts. Formaldehyde-fixed scaphognathites (Petersen et al., submitted) were embedded in paraffin wax and 6 µm sections were 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. The sections were washed once for 2 min in 96% ethanol. Sections were rehydrated by 2 min at RT in 80% ethanol followed by 2 min at RT in 70% ethanol. The epitope recovery procedure used was heat-induced epitope retrieval (HIER). HIER was done by placing the sections in a glass staining dish, then covering the sections with10 mM sodium citrate buffer, pH 6.0. The glass dish was then placed in a glass container filled with 300 ml tap water, and this was microwaved in a kitchen microwave at full power for 4 min. The tap water was replaced, and the glass container was microwaved again at full power for 4 min. The sections were

allowed to sit for a further 10 min in the hot buffer after microwaving. The sections were dipped in distilled water, then blocked in 1 X phosphate-buffered saline (PBS), pH 8.0, 1% blocking reagent (Roche), 1% bovine serum albumin (BSA) at RT for 30 min. The freshly-prepared primary antibody mix (1X PBS, 1% BR, 3% BSA) was pipetted onto the sections, and the slides were left in a humidified chamber at RT for 2 h. Slides were washed in 1X PBS, 1% BR, 1% BSA at RT for 15 min, then in 1X PBS at RT for 15 min. A freshly-prepared secondary antibody mix (1X PBS, 1% BR, 3% BSA) was pipetted onto the sections, then left in a humidified chamber at RT for 1 h. Slides were washed twice in 1X PBS for 15 min at RT. The secondary antibody was detected by catalyzed reporter deposition (CARD) by incubation with $Alexa_{488}$-labeled tyramide in amplification buffer (PBS [pH 7.6], 0.1% [wt/vol] blocking reagent, 10% [wt/vol] dextran sulfate, 2 M NaCl, freshly added 0.0015% [vol/vol] H_2O_2) for 10 min at 37°C, then washed in 1X PBS at 46°C for 15 min. Sections were dipped twice in MilliQ water, then once in 96% ethanol. For fluorescence microscopy, sections were embedded in 4’,6’-diamidino-2-phenylindole (DAPI)-amended mounting solution and evaluated on an Axioplan II microscope (Carl Zeiss, Jena, Germany).

Thermodynamic modeling

Thermodynamic calculations of free energies ($\Delta_r G$) of catabolic reactions were conducted using the relation $\Delta_r G = R \cdot T \cdot 2.303 \cdot (\log Q - \log K)$, where Log Q is the sum of log activities of reaction products subtracted by the sum of log activities of the reactants, and log K is the equilibrium constant of reactions for a range of temperatures and a pressure of 30 MPa. Log K values were calculated using SUPCRT92 and a database that has all updates for inorganic aqueous species up to November 2007 (see OBIGT database for detailed documentation:

http://affinity.berkeley.edu/predcent/download/obigt). The extended Debye–Hückel equation was used to calculate activity coefficients with B–dot extended parameters and hard core diameters from Wolery (2004). Dissolved neutral species were assigned an activity coefficient of one, except non–polar species for which CO_2 activity coefficients were used (Drummond, 1981). Geochemical mixing calculations were carried out using the program REACT (GWB 7.0.2) and a thermodynamic database calculated by SUPCRT92 (Johnson et al., 1992) for 50 MPa and temperatures from 0 to 400°C. The higher pressure was chosen, because the speciation calculations of the hydrothermal endmember fluid yield unreliable results close to the critical point of seawater (405°C, 30 MPa). In the mixing calculations, all redox reactions were suppressed. Mineral precipitation was allowed in a subset of the calculations. The mixing calculations provide estimates of the quantities of free energy available in the areas where high-temperature hydrothermal vent fluid and cold seawater mix. To determine the relative hierarchy of energy source, calculated energies where normalized to kg of endmember vent fluid (see McCollom, 2007).

Acknowledgements

We would like to thank the captains and crews of the RV *L'Atalante* and ROV *Victor* for help with the sampling effort during the 2005 Exomar cruise (chief scientist A. Godfroy), and also to Valérie Cueff-Gauchard who helped with the processing on board. Many thanks to Silke Wetzel and Nicole Rödiger, from the Max Planck Institute in Bremen, for technical support. This work was supported by the Max Planck Society, the Priority Program 1144 (From Mantle to Ocean: Energy- Material- and Life-Cycles at Spreading Axes; contribution number xx) of the German Research Foundation (DFG), the DFG Cluster of Excellence at MARUM, Bremen, and a Marie Curie research training network (MOMARnet) scholarship to JMP.

References

Anderson, L., Halary, S., Lechaire, J.P., Boudier, T., Frebourg, G., Marco, S. et al. (2008) Tomography of bacteria-mineral associations within the deep-sea hydrothermal vent shrimp *Rimicaris exoculata*. *Comptes Rendus Chimie* **11**: 268-280.

Blazejak, A., Kuever, J., Erseus, C., Amann, R., and Dubilier, N. (2006) Phylogeny of 16S rRNA, ribulose 1,5-bisphosphate carboxylase/oxygenase, and adenosine 5 '-phosphosulfate reductase genes from gamma- and alphaproteobacterial symbionts in gutless marine worms (Oligochaeta) from Bermuda and the Bahamas. *Appl Environ Microbiol* **72**: 5527-5536.

Brantner, C.A., Remsen, C.C., Owen, H.A., Buchholz, L.A., and Collins, M.L.P. (2002) Intracellular localization of the particulate methane monooxygenase and methanol dehydrogenase in *Methylomicrobium album* BGS. *Arch Microbiol* **178**: 59-64.

Campbell, B.J., Stein, J.L., and Cary, S.C. (2003) Evidence of chemolithoautotrophy in the bacterial community associated with *Alvinella pompejana*, a hydrothermal vent polychaete. *Appl Environ Microbiol* **69**: 5070-5078.

Campbell, B.J., Engel, A.S., Porter, M.L., and Takai, K. (2006) The versatile epsilon-proteobacteria: key players in sulphidic habitats. *Nat Rev Microbiol* **4**: 458-468.

Cavanaugh, C., and Robinson, J.J. (1996) CO_2 fixation in chemoautotrophic-invertebrate symbioses: Expression of form I and form II RubisCO. In *Microbial Growth on C1 Compounds*. Lidstrom, M.E., and Tabita, F.R. (eds): Kluwer.

Cavanaugh, C.M., McKiness, Z.P., Newton, I.L.G., and Stewart, F.J. (2006) Marine chemosynthetic symbioses. In *The Prokaryotes: An Evolving Electronic Resource for the Microbial Community*. Dworkin, M., Falkow, S.I., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds). New York: Springer.

Charlou, J.L., Donval, J.P., Fouquet, Y., Jean-Baptiste, P., and Holm, N. (2002) Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36° 14' N, MAR). *Chem Geol* **191**: 345-359.

Corbari, L., Zbinden, M., Cambon-Bonavita, M.A., Gaill, F., and Compere, P. (2008) Bacterial symbionts and mineral deposits in the branchial chamber of the hydrothermal vent shrimp *Rimicaris exoculata*: relationship to moult cycle. *Aquatic Biology* **1**: 225-238.

Croal, L.R., Jiao, Y.Q., and Newman, D.K. (2007) The *fox* operon from Rhodobacter strain SW2 promotes phototrophic fe(II) oxidation in *Rhodobacter capsulatus* SB1003. *J Bacteriol* **189**: 1774-1782.
Csaki, R., Hanczar, T., Bodrossy, L., Murrell, J.C., and Kovacs, K.L. (2001) Molecular characterization of structural genes coding for a membrane bound hydrogenase in *Methylococcus capsulatus* (Bath). *FEMS Microbiol Lett* **205**: 203-207.
Drummond, S. E. Jr. (1981) Boiling and mixing of hydrothermal fluids: chemical effects on mineral precipitation. PhD Thesis, The Pennsylvania State University.
Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6**: 725-739.
Dubilier, N., Mülders, C., Ferdelman, T., de Beer, D., Pernthaler, A., Klein, M. et al. (2001) Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. *Nature* **411**: 298-302.
Dunfield, P.F., Yuryev, A., Senin, P., Smirnova, A.V., Stott, M.B., Hou, S.B. et al. (2007) Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia. *Nature* **450**: 879-U818.
Duperron, S., Sibuet, M., MacGregor, B.J., Kuypers, M.M.M., Fisher, C.R., and Dubilier, N. (2007) Diversity, relative abundance and metabolic potential of bacterial endosymbionts in three *Bathymodiolus* mussel species from cold seeps in the Gulf of Mexico. *Environ Microbiol* **9**: 1423-1438.
Duperron, S., Bergin, C., Zielinski, F., Blazejak, A., Pernthaler, A., McKiness, Z.P. et al. (2006) A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (Bivalvia : Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge. *Environ Microbiol* **8**: 1441-1447.
Elsaied, H., and Naganuma, T. (2001) Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* **67**: 1751-1765.
Fortin, D., and Langley, S. (2005) Formation and occurrence of biogenic iron-rich minerals. *Earth-Sci Rev* **72**: 1-19.
Galchenko, V.F., Pimenov, N.V., Lein, A.I., Galkin, S.V., Moskalev, L.I., and Ivanov, M.V. (1989) Autotrophic CO_2 assimilation in tissues of prawn *Rimicaris exoculata* from the Mid-Atlantic Ridge hydrothermal area. *Doklady Akademii Nauk SSSR* **308**: 1478-1481.
Gebruk, A.V., Pimenov, N.V., and Savvichev, A.S. (1993) Feeding specialization of Bresiliid shrimps in the TAG site hydrothermal community. *Mar Ecol Prog Ser* **98**: 247-253.

Gebruk, A.V., Southward, E.C., Kennedy, H., and Southward, A.J. (2000) Food sources, behaviour, and distribution of hydrothermal vent shrimps at the Mid-Atlantic Ridge. *J Mar Biol Assoc UK* **80**: 485-499.

Gloter, A., Zbinden, M., Guyot, F., Gaill, F., and Colliex, C. (2004) TEM-EELS study of natural ferrihydrite from geological-biological interactions in hydrothermal systems. *Earth Planet Sci Lett* **222**: 947-957.

Goffredi, S.K., Jones, W.J., Erhlich, H., Springer, A., and Vrijenhoek, R.C. (2008) Epibiotic bacteria associated with the recently discovered Yeti crab, *Kiwa hirsuta*. *Environ Microbiol* **10**: 2623-2634.

Grabovich, M.Y., Muntyan, M.S., Lebedeva, V.Y., Ustiyan, V.S., and Dubinina, G.A. (1999) Lithoheterotrophic growth and electron transfer chain components of the filamentous gliding bacterium *Leucothrix mucor* DSM 2157 during oxidation of sulfur compounds. *FEMS Microbiol Lett* **178**: 155-161.

Grzymski, J.J., Murray, A.E., Campbell, B.J., Kaplarevic, M., Gao, G.R., Lee, C. et al. (2008) Metagenome analysis of an extreme microbial symbiosis reveals eurythermal adaptation and metabolic flexibility. *Proc Natl Acad Sci U S A* **105**: 17516-17521.

Hanson, R.S., and Hanson, T.E. (1996) Methanotrophic bacteria. *Microbiol Rev* **60**: 439-471.

Hugler, M., Wirsen, C.O., Fuchs, G., Taylor, C.D., and Sievert, S.M. (2005) Evidence for autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle by members of the epsilon subdivision of proteobacteria. *J Bacteriol* **187**: 3020-3027.

Hugler, M., Huber, H., Molyneaux, S.J., Vetriani, C., and Sievert, S.M. (2007) Autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle in different lineages within the phylum Aquificae: evidence for two ways of citrate cleavage. *Environ Microbiol* **9**: 81-92.

Inagaki, F., Takai, K., Nealson, K.H., and Horikoshi, K. (2004) *Sulfurovum lithotrophicum* gen. nov., sp nov., a novel sulfur-oxidizing chemolithoautotroph within the Epsilonproteobacteria isolated from Okinawa Trough hydrothermal sediments. *Int J Syst Evol Microbiol* **54**: 1477-1482.

Islam, T., Jensen, S., Reigstad, L.J., Larsen, O., and Birkeland, N.K. (2008) Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the Verrucomicrobia phylum. *Proc Natl Acad Sci U S A* **105**: 300-304.

Jannasch, H.W., Wirsen, C.O., and Molyneaux, S.J. (1991) Chemosynthetic microbial activity at the 23° and 26°N Mid-Atlantic Ridge vent sites. *Ridge Events* **19**.

Jiao, Y., and Newman, D.K. (2007) The *pio* operon is essential for phototrophic Fe(II) oxidation in *Rhodopseudomonas palustris* TIE-1. *J Bacteriol* **189**: 1765-1773.

Johnson, J. W., Oelkers, E. H., and Helgeson, H. C. (1992) SUPCRT92: a software package for calculating the standard molal thermodynamic properties of minerals, gases, aqueous species, and reactions from 1-5000 bars and 0-1000°C. *Comput Geosci* **18**: 899-947.

Kelly, D.P., Anthony, C., and Murrell, J.C. (2005) Insights into the obligate methanotroph *Methylococcus capsulatus*. *Trends Microbiol* **13**: 195-198.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363-1371.

Markert, S., Arndt, C., Felbeck, H., Becher, D., Sievert, S.M., Hugler, M. et al. (2007) Physiological proteomics of the uncultured endosymbiont of *Riftia pachyptila*. *Science* **315**: 247-250.

Meyer, B., and Kuever, J. (2007a) Molecular analysis of the diversity of sulfate-reducing and sulfur-oxidizing prokaryotes in the environment, using *aprA* as functional marker gene. *Appl Environ Microbiol* **73**: 7664-7679.

Meyer, B., and Kuever, J. (2007b) Molecular analysis of the distribution and phylogeny of dissimilatory adenosine-5'-phosphosulfate reductase-encoding genes (*aprBA*) among sulfuroxidizing prokaryotes. *Microbiol* **153**: 3478-3498.

McCollom, T. M. (2007) Geochemical constraints on sources of metabolic energy for chemolithoautotrophy in ultramafic-hosted deep-sea hydrothermal systems. *Astrobiology* **7**: 933-950.

Park, B., and Dempsey, B.A. (2005) Heterogeneous oxidation of Fe(II) on ferric oxide at neutral pH and a low partial pressure of O_2. *Environ Sci Technol* **39**: 6494-6500.

Petersen, J.M., Ramette, A., Lott, C., Cambon-Bonavita, M.A., Zbinden, M., and Dubilier, N. (submitted) Dual symbiosis of the vent shrimp *Rimicaris exoculata* with filamentous Gamma- and Epsilonproteobacteria at four Mid-Atlantic Ridge hydrothermal vent fields.

Pol, A., Heijmans, K., Harhangi, H.R., Tedesco, D., Jetten, M.S.M., and den Camp, H. (2007) Methanotrophy below pH1 by a new Verrucomicrobia species. *Nature* **450**: 874-U817.

Polz, M.F., and Cavanaugh, C.M. (1995) Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site. *Proc Natl Acad Sci U S A* **92**: 7232-7236.

Polz, M.F., Robinson, J.J., Cavanaugh, C.M., and Van Dover, C.L. (1998) Trophic ecology of massive shrimp aggregations at a Mid-Atlantic Ridge hydrothermal vent site. *Limnol Oceanogr* **43**: 1631-1638.

Rentz, J.A., Kraiya, C., Luther, G.W., and Emerson, D. (2007) Control of ferrous iron oxidation within circumneutral microbial iron mats by cellular activity and autocatalysis. *Environ Sci Technol* **41**: 6084-6089.

Robidart, J.C., Bench, S.R., Feldman, R.A., Novoradovsky, A., Podell, S.B., Gaasterland, T. et al. (2008) Metabolic versatility of the *Riftia pachyptila* endosymbiont revealed through metagenomics. *Environ Microbiol* **10**: 727-737.

Sanchez, O., Ferrera, I., Dahl, C., and Mas, J. (2001) In vivo role of adenosine-5 '-phosphosulfate reductase in the purple sulfur bacterium *Allochromatium vinosum*. *Arch Microbiol* **176**: 301-305.

Schmidt, C., Le Bris, N., and Gaill, F. (2008) Interactions of deep-sea vent invertebrates with their environment: The case of *Rimicaris exoculata*. *J Shellfish Res* **27**: 79-90.

Scott, K.M., Sievert, S.M., Abril, F.N., Ball, L.A., Barrett, C.J., Blake, R.A. et al. (2006) The genome of deep-sea vent chemolithoautotroph *Thiomicrospira crunogena* XCL-2. *Plos Biol* **4**: 2196-2212.

Semrau, J.D., DiSpirito, A.A., and Murrell, J.C. (2008) Life in the extreme: thermoacidophilic methanotrophy. *Trends Microbiol* **16**: 190-193.

Sievert, S.M., Scott, K.A., Klotz, M.G., Chain, P.S.G., Hauser, L.J., Hemp, J. et al. (2008) Genome of the epsilonproteobacterial chemolithoautotroph *Sulfurimonas denitrificans*. *Appl Environ Microbiol* **74**: 1145-1156.

Tabita, F.R. (1999) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A different perspective. *Photosynth Res* **60**: 1-28.

Takai, K., Campbell, B.J., Cary, S.C., Suzuki, M., Oida, H., Nunoura, T. et al. (2005) Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of Epsilonproteobacteria. *Appl Environ Microbiol* **71**: 7310-7320.

Trotsenko, Y.A., and Murrell, J.C. (2008) Metabolic aspects of aerobic obligate methanotrophy. In *Advances in Applied Microbiology, Vol 63*, pp. 183-229.

Van Dover, C.L. (2000) *The Ecology of Deep-Sea Hydrothermal Vents.* Princeton, N. J.: Princeton University Press.

Van Dover, C.L., Fry, B., Grassle, J.F., Humphris, S., and Rona, P.A. (1988) Feeding biology of the shrimp *Rimicaris exoculata* at hydrothermal vents on the Mid-Atlantic Ridge. *Mar Biol* **98**: 209-216.

Vignais, P.M., and Billoud, B. (2007) Occurrence, classification, and biological function of hydrogenases: An overview. *Chem Rev* **107**: 4206-4272.

Vignais, P.M., Billoud, B., and Meyer, J. (2001) Classification and phylogeny of hydrogenases. *FEMS Microbiol Rev* **25**: 455-501.

Waite, T.J., Moore, T.S., Childress, J.J., Hsu-Kim, H., Mullaugh, K.M., Nuzzio, D.B. et al. (2008) Variation in sulfur speciation with shellfish presence at a Lau Basin diffuse flow vent site. *J Shellfish Res* **27**: 163-168.

Ward, N., Larsen, O., Sakwa, J., Bruseth, L., Khouri, H., Durkin, A.S. et al. (2004) Genomic insights into methanotrophy: The complete genome sequence of *Methylococcus capsulatus* (Bath). *Plos Biol* **2**: 1616-1628.

Wirsen, C.O., Jannasch, H.W., and Molyneaux, S.J. (1993) Chemosynthetic microbial sctivity at Mid-Atlantic Ridge hydrothermal vent sites. *Journal of Geophysical Research-Solid Earth* **98**: 9693-9703.

Wolery, T. J. (2004) Qualification of thermodynamic data for geochemical modeling of mineral-water interactions in dilute systems. In *Energy*. U. S. D. o. (ed). Bechtel SIAC Company, LLC.

Woyke, T., Teeling, H., Ivanova, N.N., Huntemann, M., Richter, M., Gloeckner, F.O. et al. (2006) Symbiosis insights through metagenomic analysis of a microbial consortium. *Nature* **443**: 950-955.

Zbinden, M., Le Bris, N., Gaill, F., and Compere, P. (2004) Distribution of bacteria and associated minerals in the gill chamber of the vent shrimp *Rimicaris exoculata* and related biogeochemical processes. *Mar Ecol Prog Ser* **284**: 237-251.

Zbinden, M., Shillito, B., Le Bris, N., de Montlaur, C.D., Roussel, E., Guyot, F. et al. (2008) New insigths on the metabolic diversity among the epibiotic microbial communitiy of the hydrothermal shrimp *Rimicaris exoculata*. *J Exp Mar Biol Ecol* **359**: 131-140.

Zielinski, F.U., Petersen, J.M., Wetzel, S., Pape, T., Seifert, R., Wenzhöfer, F., and Dubilier, N. (In prep) The sulfur-oxidizing endosymbiont of the hydrothermal vent mussel *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae) uses hydrogen as an energy source.

Part IV

Concluding remarks

Chapter 8

Summary and Conclusions

R. exoculata *epibiont diversity*

Before I began this thesis work, our understanding of the diversity of the *R. exoculata* epibiosis was limited to one molecular study from a single vent field, which showed that the epibiosis is a monoculture of filamentous epsilonproteobacteria, despite the presence of various morphotypes [157]. As for the function of the epibiotic bacteria, observations of internal sulfur globules and the phylogenetic relationship of the epibionts to sulfur-oxidizing bacteria provided indications that they use sulfur as an energy source [157, 253]. During the first part of my thesis, I re-examined the *Rimicaris exoculata* epibiont diversity. I had the unique opportunity to examine samples from four different Mid-Atlantic Ridge hydrothermal vent fields that cover the entire 8500 km range of this species. By applying the full-cycle 16S rRNA approach from the epibiotic bacteria, I discovered that the *R. exoculata* epibiosis is dominated by two distinct symbionts, the previously described Epsilon symbiont and a novel Gamma symbiont. Both symbionts were present on all shrimp investigated at all four vent fields, despite the geochemical differences and geographic distances that separate these sites. This attests to the stability of the symbiosis, and indicates that the association is obligate for the host, although the role of the symbionts could not be determined.

The Gamma symbiont belonged to a clade that was not previously known to contain symbiotic bacteria. Sequences related to the *R. exoculata* Gamma symbiont were present in clone libraries from other hydrothermal vent animals that have epibionts, the scaly snail *C. squamiferum*, Yeti crab *K. hirsuta*, and barnacle *V. osheai* [136, 158, 160]. However, the ectosymbiotic nature of this group had not been recognized. The *R. exoculata* Epsilon symbiont also grouped with sequences from epibionts of these three other vent animals. None of these four hosts are closely related. It is therefore likely that

both the Gamma and Epsilon symbiont lineages have been horizontally transmitted between different host lineages. Such horizontal transmission events have occurred in other associations. For example, host-switching events were identified by comparison of the phylogenies of insects and their *Spiroplasma* endosymbionts, which were incongruent. In this case, it was hypothesized that horizontal transmission events could have occurred where the unrelated hosts share a common habitat [278]. There is currently no hydrothermal vent known where the four hosts of the Gamma and Epsilon symbionts co-occur. Further exploration may uncover such a site in the future. However, if the free-living stages of the symbionts are capable of dispersing between vent fields independent of their hosts, then the hosts need not co-occur for such a transmission event to happen. A recent study of the horizontally transmitted squid-*Vibrio fischeri* symbiosis revealed a mechanism that could explain how host-switching events can occur, as introducing a single regulatory gene was sufficient to change the host range of one symbiont strain [279].

Carbon and energy sources for the epibionts

Until recently, only two energy sources were recognized to fuel deep-sea chemosynthetic symbioses: methane and reduced sulfur compounds (H_2S, HS^- , ${S_2O_3}^{2-}$) [32, 38]. During my thesis, I also contributed to work that demonstrated that a third energy source, H_2 , fuels the *Bathymodiolus puteoserpentis* symbiosis (see Zielinski et al., included in the Appendix to this thesis). My results during the first part of this thesis showed that the phylogenetic diversity of the *R. exoculata* epibiosis was greater than previously assumed; I therefore wanted to investigate the use of energy sources other than reduced sulfur compounds by the shrimp symbionts. Thermodynamic modelling, which was done in collaboration with Wolfgang Bach (University of Bremen), showed that the range of energy sources that could fuel chemosynthetic growth in the shrimp habitat at Rainbow included hydrogen, reduced iron, and methane. Surprisingly, although the epibionts had long been assumed to be sulfide oxidizers, this metabolism would theoretically provide less energy than hydrogen, methane, or iron oxidation. Key genes of hydrogen,

methane, and sulfur oxidation could be amplified from the shrimp epibionts, indicating that the metabolic diversity in the epibionts is also greater than previously assumed. The pathways involved in bacterial iron oxidation have not yet been elucidated, therefore, the role of iron as an energy source for the *R. exoculata* symbiosis could not be investigated by molecular methods. A number of recent studies have identified the crucial role that c-type cytochromes play in iron oxidation [280,281]. However, they are not exclusively present in iron-oxidizers, and therefore cannot be used as a diagnostic marker for iron oxidation. It may not be possible to demonstrate unequivocably that the epibionts can grow on reduced iron until they have been shown to do so in pure culture.

Based on the key gene phylogenies, I speculated that the Epsilon symbiont can use hydrogen as an energy source, and fixes CO_2 autotrophically by the reductive TCA cycle. All currently published genomes from epsilonproteobacterial sulfur oxidizers have the Sox pathway for sulfur oxidation [138, 282], and primers for the key gene of this pathway, *soxB*, are available [283], and could be used in the future to investigate the potential for sulfur oxidation by the Epsilon symbiont. Surprisingly, my results indicate that the Gamma symbiont has the potential to oxidize both methane and reduced sulfur. There is currently no organism known to use both methane and reduced sulfur compounds as energy sources. The phylogeny of the *R. exoculata* Gamma symbiont places it in a clade with *Leucothrix mucor*, a sulfur-oxidizing bacterium, and as a sister clade to the sulfur-oxidizing endosymbiont of *Oligobrachia haakonmosbiensis* (Figure 5, Manuscript III). In my analyses, these clades appear to be more closely related to free-living and symbiotic methane-oxidizing gammaproteobacteria than to sulfur-oxidizers. While the expression of the key gene for methane oxidation could be demonstrated in the Gamma symbiont by immunohistochemistry, there was no antibody available to test for expression of key genes for sulfur oxidation. These observations should certainly be followed up in the future. The presence of both genes in single cells of the Gamma symbiont could be confirmed by methods such as single gene FISH, currently in development in the Molecular Ecology department, MPI Bremen (C. Moraru, R. Amann). If future studies

can confirm that the *R. exoculata* Gamma symbiont uses both methane and sulfide, this could imply that the common ancestor of gammaproteobacterial methane and sulfur oxidizers could do both, but two lineages have lost the ability to use one energy source, either methane or sulfide, and specialized on the other. Alternatively, re-examining known sulfur-oxidizers for methane oxidizing activity, and vice versa, might reveal that this ability is widespread.

Biogeography of symbionts and hosts

To investigate the biogeography of the shrimp host, I chose two mitochondrial marker genes that had been used successfully in previous studies to identify geographically separate sub-species populations of aquatic shrimp [284–286]. In my study, these genes did not reveal vent site-specific patterns across the entire 8500 km range of this species. Despite the genetic similarities in the host populations, site-specific differences were seen in the symbiont populations. Comparing the symbiont 16S rRNA sequences from the four distant vent fields revealed a biogeographic pattern. Both symbionts showed a significant correlation between genetic distance on the 16S rRNA gene and geographic distance between sampling sites, which indicates spatial isolation of the symbionts. Motivated by these results, researchers from Ifremer in France have started to look at the population genetic structure of *R. exoculata* from the MAR, using more variable microsatellite markers to identify population differences that could not be seen on the basis of mitochondrial marker genes (S. Arnaud, personal communication).

In contrast to the spatial structuring seen in the symbiont populations, free-living relatives of the Epsilon symbionts did not group according to geography. If the free-living populations do not show spatial structuring, then the observed symbiont distribution could be due to host-symbiont recognition. However, there were very few sequences available from free-living members of the Epsilon symbiont clade, and none from the Gamma symbiont clade. The spatial distribution of free-living symbiont diversity has not yet been investigated for any beneficial symbiosis, even though understanding this diversity is critical in interpreting biogeographic patterns observed in host-associated

bacteria. For example, recently developed models that include variations in the environmental distribution of pathogens more accurately predicted patterns of disease outbreaks than simple host-to-host direct transmission models [287, 288]. The next step in understanding the biogeography of the *R. exoculata* symbiosis will be an in-depth investigation of the free-living symbiont distribution in their habitats along the MAR. This will help us to interpret the symbiont distribution patterns identified in this thesis.

Chapter 9

Outlook

I have observed a number of exciting developments in microbial ecology during my work on this thesis. The first is a conceptual development. Coincident with the 200th anniversary of Darwin's birth this year, and the 150th anniversary of the publication of 'The Origin of Species', there has been a renewed interest in defining a species concept for bacteria, which is less focussed on taxonomic classification, and more on understanding evolutionary processes in prokaryotes (see recent reviews by Fraser et al. in the special section on speciation in Science [289], by Whitaker et al. and Polz et al., among others, in a special issue of the Proceedings of the Royal Society on species and speciation in microorganisms [290, 291]). The species concept has long been a subject of argument among microbiologists; failure to resolve this issue is most likely due to our lack of understanding of how evolutionary forces shape microbial diversity. Molecular methods, in particular, high throughput sequencing of highly variable regions of the 16S rRNA gene have revealed that microorganisms in the environment are extraordinarily diverse (reviewed in [292]), but explaining this diversity is still a challenge. This should be the aim of a bacterial species concept.

In this thesis, I identified divergent populations of ectosymbiotic bacteria

on shrimp hosts at geographically separate and geochemically distinct hydrothermal vent sites. However, their free-living counterparts do not seem to be distributed according to geography. These observations provide the groundwork for future studies on the evolution of the *R. exoculata* symbiosis. If the distribution of free-living symbionts is found to be cosmopolitan at MAR vents and not influenced by geography, then there must be other barriers present that partition the variation in symbiont populations into the discrete lineages observed. An example of such a barrier is niche differentiation, that was shown to cause partitioning of diversity between marine planktonic and particle-associated members of the *Vibrionaceae* in a recent study by Hunt et al. [293]. The Hunt et al. study showed that sequence clusters identified by multilocus sequence analysis are indeed ecologically relevant. It is very likely that interactions with the shrimp host have been important drivers in the evolution of the symbiont populations. The application of population genetics to symbiotic bacteria has already showed that interactions with the host influence their evolution. For example, Peek et al. compared the evolutionary rate at the 16S rRNA locus between sulfur-oxidizing chemolithoautotrophic bacteria with differing life histories. Vertically transmitted symbionts showed an increased evolutionary rate compared to close relatives that are exclusively free-living [294]. This was consistent with other studies of vertically transmitted symbionts, based on the 16S rRNA gene, multiple housekeeping loci, and whole-genome comparisons [181, 183]. Surprisingly, horizontally transmitted symbionts showed even slower evolutionary rates than exclusively free-living relatives, but this observation could not be explained [294]. Applications of such techniques to the *R. exoculata* symbiosis might help to explain the evolutionary dynamics of horizontally transmitted associations.

The second development I have witnessed is technological. In a review article published in 1987 [295], Carl Woese remarked:

> Microbiology is . . . being inundated with sequence information, which accumulates so rapidly that the reading and entering of data are becoming major concerns, while the actual sequencing operations will soon cease to be rate-limiting factors.

A similar 'inundation' is in progress today. According to the Genomes OnLine Database, there are currently 1027 complete published bacterial genomes, with another 2609 ongoing projects listed [296]. This has been made possible by drastic reductions in the cost of sequencing. The first human genome, completed in 2003, cost three hundred million dollars to sequence, and took 13 years. Currently, a single human genome using next generation sequencing technology such as 454 FLX Ti costs one million dollars and can be completed in three days. Next-next generation technologies based on single molecule sequencing, due to enter the market in mid-2010, have the potential to deliver a complete human genome in 15 minutes at a cost of a few hundred dollars [297].

The dynamic nature of microbial genome evolution was already hinted at before comparison of whole genome sequences became feasible. For example, Thompson et al. showed extensive size variation and allelic diversity in a group of *Vibrio splendidus* strains with more than 99% rRNA sequence identity, by extensive multi-locus sequencing and genome size estimation by pulse field gel electrophoresis [298]. Comparative genomics is beginning to confirm these observations. Genome plasticity is reflected in variations in gene content between closely related strains [299], and the variable gene content is considered to have adaptive ecological significance. Antibiotic-resistance genes are a prime example of this. Comparing the genomes of geographically separate *R. exoculata* epibionts will undoubtedly reveal variations in gene content, and these could provide insights into the role of adaptation in symbiont diversification. Recently, a comparison of the sulfur-oxidizing endosymbionts of four species of vesicomyid clams revealed that the symbionts of two host species have genes for nitrate respriation, and two do not [224]. This was interpreted as a reflection of the geochemical ecology of the hosts, as the symbionts that can respire nitrate are found in hosts that spend much of their time buried in sediments where oxygen might be limiting. This is a wonderful example of how comparative genomics can identify ecological factors that play a role in determining microbial diversity, factors that might not have been considered in previous attempts to characterize a microbe's environment.

Ultimately, genomics will help to identify the extent of microbial diversity, and population genetics will provide the tools necessary to interpret this immense diversity. The *Rimicaris exoculata* symbiosis will be an ideal model system for investigating the effects of spatial separation, adaptation to changing environmental conditions, and biotic interactions in microbial evolution. The results from this thesis provide the first steps towards this goal.

Bibliography

[1] L. G. Ackert Jr, "The role of microbes in agriculture: Sergei Vinogradskii's discovery and investigation of chemosynthesis, 1880-1910," *Journal of the History of Biology*, vol. 39, pp. 373–406, 2006.

[2] K. M. Kusek, "Through the porthole 30 years ago," *Oceanography*, vol. 20, pp. 138–141, 2007.

[3] C. L. Van Dover, C. R. German, K. G. Speer, L. M. Parson, and R. C. Vrijenhoek, "Marine biology - evolution and biogeography of deep-sea vent and seep invertebrates," *Science*, vol. 295, pp. 1253–1257, 2002.

[4] S. Nakagawa and K. Takai, "Deep-sea vent chemoautotrophs: diversity, biochemistry and ecological significance," *FEMS Microbiology Ecology*, vol. 65, pp. 1–14, 2008.

[5] I. A. Berg, D. Kockelkorn, W. Buckel, and G. Fuchs, "A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in archaea," *Science*, vol. 318, pp. 1782–1786, 2007.

[6] E. M. Spiridonova, B. B. Kuznetsov, N. V. Pimenov, and T. P. Tourova, "Phylogenetic characterization of endosymbionts of the hydrothermal vent mussel *Bathymodiolus azoricus* by analysis of the 16S rRNA, *cbbL*, and *pmoA* genes," *Microbiology*, vol. 75, pp. 694–701, 2006.

[7] S. Markert, C. Arndt, H. Felbeck, D. Becher, S. M. Sievert, M. Hugler, D. Albrecht, J. Robidart, S. Bench, R. A. Feldman, M. Hecker, and T. Schweder, "Physiological proteomics of the uncultured endosymbiont of *Riftia pachyptila*," *Science*, vol. 315, pp. 247–250, 2007.

[8] F. R. Tabita, S. Satagopan, T. E. Hanson, N. E. Kreel, and S. S. Scott, "Distinct form I, II, III, and IV RuBisCO proteins from the three kingdoms of life provide clues about RuBisCO evolution and structure/function relationships," *Journal of Experimental Botany*, vol. 59, pp. 1515–1524, 2008.

[9] F. R. Tabita, T. E. Hanson, H. Li, S. Satagopan, J. Singh, and S. Chan, "Function, structure, and evolution of the RuBisCO-like proteins and their RuBisCO homologs," *Microbiology and Molecular Biology Reviews*, vol. 71, pp. 576–599, 2007.

[10] M. C. Evans, B. B. Buchanan, and D. I. Arnon, "A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 55, pp. 928–934, 1966.

[11] M. Hugler, H. Huber, S. J. Molyneaux, C. Vetriani, and S. M. Sievert, "Autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle in different lineages within the phylum Aquificae: evidence for two ways of citrate cleavage," *Environmental Microbiology*, vol. 9, pp. 81–92, 2007.

[12] M. Hugler, C. Wirsen, G. Fuchs, C. Taylor, and S. Sievert, "Evidence for autotrophic CO_2 fixation via the reductive tricarboxylic acid cycle by members of the epsilon subdivision of proteobacteria," *Journal of Bacteriology*, vol. 187, pp. 3020–3027, 2005.

[13] K. Takai, B. J. Campbell, S. C. Cary, M. Suzuki, H. Oida, T. Nunoura, H. Hirayama, S. Nakagawa, Y. Suzuki, F. Inagaki, and K. Horikoshi, "Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of Epsilonproteobacteria," *Applied and Environmental Microbiology*, vol. 71, pp. 7310–7320, 2005.

[14] M. Hugler, H. Huber, K. Stetter, and G. Fuchs, "Autotrophic CO_2 fixation pathways in archaea (Crenarchaeota)," *Archives of Microbiology*, vol. 179, pp. 160–173, 2003.

[15] M. T. Madigan and J. M. Martinko, *Brock Biology of Microorganisms.* Upper Saddle River, N. J.: Pearson Prentice Hall, eleventh edition ed., 2006.

[16] J. C. Robidart, S. R. Bench, R. A. Feldman, A. Novoradovsky, S. B. Podell, T. Gaasterland, E. E. Allen, and H. Felbeck, "Metabolic versatility of the *Riftia pachyptila* endosymbiont revealed through metagenomics," *Environmental Microbiology*, vol. 10, pp. 727–737, 2008.

[17] G. Strauss and G. Fuchs, "Enzymes of a novel autotrophic CO_2 fixation pathway in the phototrophic bacterium *Chloroflexus aurantiacus*, the 3-hydroxypropionate cycle," *European Journal of Biochemistry*, vol. 215, pp. 633–643, 1993.

[18] J. Meuer, H. C. Kuettner, J. K. Zhang, R. Hedderich, and W. W. Metcalf, "Genetic analysis of the archaeon *Methanosarcina barkeri* Fusaro reveals a central role for Ech hydrogenase and ferredoxin in methanogenesis and carbon fixation," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 99, pp. 5632–5637, 2002.

[19] M. E. Lidstrom, "Aerobic methylotrophic prokaryotes," in *The Prokaryotes* (M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, eds.), pp. 618–634, New York: Springer, 2006.

[20] R. S. Hanson and T. E. Hanson, "Methanotrophic bacteria," *Microbiological Reviews*, vol. 60, pp. 439–471, 1996.

[21] Y. A. Trotsenko and J. C. Murrell, "Metabolic aspects of aerobic obligate methanotrophy," in *Advances in Applied Microbiology*, vol. 63, pp. 183–229, 2008.

[22] E. G. DeChaine and C. M. Cavanaugh, "Symbioses of methanotrophs and deep-sea mussels (Mytilidae: Bathymodiolinae)," in *Molecular Basis of Symbiosis* (J. Overmann, ed.), pp. 227–250, Berlin: Springer, 2005.

[23] N. J. Baxter, R. P. Hirt, L. Bodrossy, K. L. Kovacs, T. M. Embley, J. I. Prosser, and J. C. Murrell, "The ribulose-1,5-bisphosphate carboxylase/oxygenase gene cluster of *Methylococcus capsulatus* (Bath)," *Archives of Microbiology*, vol. 177, pp. 279–289, 2002.

[24] P. F. Dunfield, A. Yuryev, P. Senin, A. V. Smirnova, M. B. Stott, S. Hou, B. Ly, J. H. Saw, Z. Zhou, Y. Ren, J. Wang, B. W. Mountain, M. A. Crowe, T. M. Weatherby, P. L. E. Bodelier, W. Liesack, L. Feng, L. Wang, and M. Alam, "Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia," *Nature*, vol. 450, pp. 879–882, 2007.

[25] T. Islam, S. Jensen, L. J. Reigstad, O. Larsen, and N.-K. Birkeland, "Methane oxidation at 55 degrees C and pH 2 by a thermoacidophilic bacterium belonging to the Verrucomicrobia phylum," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 105, pp. 300–304, 2008.

[26] A. Pol, K. Heijmans, H. R. Harhangi, D. Tedesco, M. S. M. Jetten, and H. J. M. O. den Camp, "Methanotrophy below pH 1 by a new Verrucomicrobia species," *Nature*, vol. 450, pp. 874–U17, 2007.

[27] J. D. Semrau, A. A. DiSpirito, and J. C. Murrell, "Life in the extreme: thermoacidophilic methanotrophy," *Trends in Microbiology*, vol. 16, pp. 190–193, 2008.

[28] S. Hou, K. S. Makarova, J. H. W. Saw, P. Senin, B. V. Ly, Z. Zhou, Y. Ren, J. Wang, M. Y. Galperin, M. V. Omelchenko, Y. I. Wolf, N. Yutin, E. V. Koonin, M. B. Stott, B. W. Mountain, M. A. Crowe, A. V. Smirnova, P. F. Dunfield, L. Feng, L. Wang, and M. Alam, "Complete genome sequence of the extremely acidophilic methanotroph isolate V4, *Methylacidiphilum infernorum*, a representative of the bacterial phylum Verrucomicrobia," *Biology Direct*, vol. 3, 2008.

[29] C. L. Van Dover, *The Ecology of Deep-Sea Hydrothermal Vents*. Princeton, N. J.: Princeton University Press, 2000.

[30] H. Felbeck, “Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia pachyptila* Jones (Vestimentifera),” *Science*, vol. 213, pp. 336–338, 1981.

[31] C. M. Cavanaugh, S. L. Gardiner, M. L. Jones, H. W. Jannasch, and J. B. Waterbury, “Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila* Jones: possible chemoautotrophic symbionts.,” *Science*, vol. 213, pp. 340–342, 1981.

[32] N. Dubilier, C. Bergin, and C. Lott, “Symbiotic diversity in marine animals: the art of harnessing chemosynthesis,” *Nature Reviews Microbiology*, vol. 6, pp. 725–739, 2008.

[33] A. B. Frank, “Ueber die biologischen Verhaeltnisse des Thallus einiger Krustenflechten,” *Beitraege zur Biologie der Pflanzen*, vol. 2, pp. 123–200, 1877.

[34] A. De Bary, *‘Die Erscheinung der Symbiose’, Vortrag auf der Versammlung der Deutschen Naturforscher und Aerzte zu Cassel.* Strassburg: Verlag von Karl J. Trubner, 1879.

[35] E. G. Ruby, “Symbiotic conversations are revealed under genetic interrogation,” *Nature Reviews Microbiology*, vol. 6, pp. 752–762, 2008.

[36] U. Hentschel, M. Steinert, and J. Hacker, “Common molecular mechanisms of symbiosis and pathogenesis,” *Trends in Microbiology*, vol. 8, pp. 226–231, 2000.

[37] A. C. Silver, Y. Kikuchi, A. A. Fadl, J. Sha, A. K. Chopra, and J. Graf, “Interaction between innate immune cells and a bacterial type III secretion system in mutualistic and pathogenic associations,” *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 104, pp. 9481–9486, 2007.

[38] C. Cavanaugh, Z. McKiness, I. Newton, and F. Stewart, “Marine chemosynthetic symbioses,” in *The Prokaryotes: An Evolving Electronic Resource for the Microbial Community* (M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, eds.), New York: Springer, 2006.

[39] P. Bird, “An updated digital model of plate boundaries,” *Geochemistry Geophysics Geosystems*, vol. 4, 2003.

[40] F. U. Zielinski, *Geobiological Coupling of Hydrothermal Vent Fluids with Endosymbiotic Primary Producers of Bathymodiolus Mussels from Hydrothermal Vents on the Mid-Atlantic Ridge.* PhD thesis, University of Bremen, 2008.

[41] M. Jakobsson, R. Macnab, L. Mayer, R. Anderson, M. Edwards, J. Hatzky, H. W. Schenke, and P. Johnson, "An improved bathymetric portrayal of the Arctic Ocean: implications for ocean modeling and geological, geophysical and oceanographic analyses," *Geophysical Research Letters*, vol. 35, 2008.

[42] D. Kelley, J. Baross, and J. Delaney, "Volcanoes, fluids, and life at mid-ocean ridge spreading centers," *Annual review of earth and planetary sciences*, vol. 30, pp. 385–491, 2002.

[43] H. U. Schmincke, *Volcanism*. Berlin Heidelberg: Springer, 2006.

[44] H. V. Thurman and A. P. Trujillo, *Essentials of Oceanography*. Pearson Prentice Hall, 2002.

[45] K. Macdonald, D. Scheirer, and S. Carbotte, "Mid-ocean ridges - discontinuities, segments and giant cracks," *Science*, vol. 253, pp. 986–994, 1991.

[46] E. Ramirez-Llodra, T. M. Shank, and C. R. German, "Biodiversity and biogeography of hydrothermal vent species: thirty years of discovery and investigations," *Oceanography*, vol. 20, pp. 30–41, 2007.

[47] S. Juniper and V. Tunnicliffe, "Crustal accretion and the hot vent ecosystem," *Philosophical Transactions of the Royal Society A - Mathematical Physical and Engineering Sciences*, vol. 355, pp. 459–474, 1997.

[48] J. E. Snow and H. N. Edmonds, "Ultraslow-spreading ridges: rapid paradigm changes," *Oceanography*, vol. 20, pp. 90–101, 2007.

[49] C. Lalou, J. Reyss, E. Brichet, M. Arnold, G. Thompson, Y. Fouquet, and P. Rona, "New age data for Mid-Atlantic Ridge hydrothermal sites - TAG and Snakepit chronology revisited," *Journal of Geophysical Research*, vol. 98, pp. 9705–9713, 1993.

[50] P. J. Michael, C. H. Langmuir, H. J. B. Dick, J. E. Snow, S. L. Goldstein, D. W. Graham, K. Lehnert, G. Kurras, W. Jokat, R. Muhe, and H. N. Edmonds, "Magmatic and amagmatic seafloor generation at the ultraslow-spreading Gakkel ridge, Arctic Ocean," *Nature*, vol. 423, pp. 956–961, 2003.

[51] W. R. Buck, L. L. Lavier, and A. N. B. Poliakov, "Modes of faulting at mid-ocean ridges," *Nature*, vol. 434, pp. 719–723, 2005.

[52] F. Martinez, K. Okino, Y. Ohara, A.-L. Reysenbach, and S. K. Goffredi, "Back-Arc Basins," *Oceanography*, vol. 20, pp. 116–127, 2007.

[53] M. K. Tivey, "Generation of seafloor hydrothermal vent fluids and associated mineral deposits," *Oceanography*, vol. 20, pp. 50–65, 2007.

[54] M. Talwani, C. Windisch, and M. Langseth, "Reykjanes-Ridge crest - detailed geophysical study," *Journal of Geophysical Research*, vol. 76, pp. 473–517, 1971.

[55] S. E. Humphris and T. McCollom, "The cauldron beneath the seafloor," *Oceanus*, vol. 41, pp. 18–21, 1998.

[56] K. M. Haase, S. Petersen, A. Koschinsky, R. Seifert, C. W. Devey, R. Keir, K. S. Lackschewitz, B. Melchert, M. Perner, O. Schmale, J. Sueling, N. Dubilier, F. Zielinski, S. Fretzdorff, D. Garbe-Schoenberg, U. Westernstroeer, C. R. German, T. M. Shank, D. Yoerger, O. Giere, J. Kuever, H. Marbler, J. Mawick, C. Mertens, U. Stoeber, C. Ostertag-Henning, H. Paulick, M. Peters, H. Strauss, S. Sander, J. Stecher, M. Warmuth, and S. Weber, "Young volcanism and related hydrothermal activity at 5° S on the slow-spreading southern Mid-Atlantic Ridge," *Geochemistry Geophysics Geosystems*, vol. 8, 2007.

[57] J. L. Charlou, J. P. Donval, Y. Fouquet, P. Jean-Baptiste, and N. Holm, "Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36° 14' N, MAR)," *Chemical Geology*, vol. 191, pp. 345–359, 2002.

[58] E. Douville, J. L. Charlou, E. H. Oelkers, P. Bienvenu, C. F. Jove Colon, J. P. Donval, Y. Fouquet, D. Prieur, and P. Appriou, "The Rainbow vent fluids (36° 14' N, MAR): the influence of ultramafic rocks and phase separation on trace metal content in Mid-Atlantic Ridge hydrothermal fluids," *Chemical Geology*, vol. 184, pp. 37–48, 2002.

[59] K. Schmidt, A. Koschinsky, D. Garbe-Schonberg, L. M. de Carvalho, and R. Seifert, "Geochemistry of hydrothermal fluids from the ultramafic-hosted Logatchev hydrothermal field, 15 degrees N on the Mid-Atlantic Ridge: temporal and spatial investigation," *Chemical Geology*, vol. 242, pp. 1–21, 2007.

[60] K. L. Von Damm, J. M. Edmond, B. Grant, C. I. Measures, B. Walden, and R. F. Weiss, "Chemistry of submarine hydrothermal solutions at 21°N, East Pacific Rise," *Geochimica et Cosmochimica Acta*, vol. 49, pp. 2197–2220, 1985.

[61] J. A. Welhan and H. Craig, "Methane, hydrogen and helium in hydrothermal fluids at 21°N on the East Pacific Rise," in *Hydrothermal Processes at Seafloor Spreading Centers* (P. A. Rona, K. Bostrom, L. Laubier, and K. Smith, eds.), pp. 391–410, New York, N. Y.: Plenum, 1983.

[62] C. R. German and K. L. Von Damm, "Hydrothermal processes," in *Treatise on Geochemistry, Volume 6: The Oceans and Marine Geochemistry* (H. D. Holland and K. K. Turekian, eds.), pp. 181–222, Elsevier, London, 2004.

[63] J. Trefry, D. Butterfield, S. Metz, R. Massoth, GJAnd Trocine, and R. Feely, "Trace-metals in hydrothermal solutions from cleft segment on the southern Juan de Fuca Ridge," *Journal of Geophysical Research*, vol. 99, pp. 4925–4935, 1994.

[64] J. I. Ishibashi and T. Urabe, "Hydrothermal activity related to arc-backarc magmatism in the western Pacific," in *Backarc Basins: Tectonics and Magmatism* (B. Taylor, ed.), pp. 451–495, Plenum, New York, N. Y., 1995.

[65] D. Kelley, J. Karson, D. Blackman, G. Fruh-Green, D. Butterfield, M. Lilley, E. Olson, M. Schrenk, K. Roe, G. Lebon, P. Rivizzigno, and AT3-60 Shipboard Party, "An off-axis hydrothermal vent field near the Mid-Atlantic Ridge at 30° N," *Nature*, vol. 412, pp. 145–149, 2001.

[66] D. Kelley, J. Karson, G. Fruh-Green, D. Yoerger, T. Shank, D. Butterfield, J. Hayes, M. Schrenk, E. Olson, G. Proskurowski, M. Jakuba, A. Bradley, B. Larson, K. Ludwig, D. Glickson, K. Buckman, A. Bradley, W. Brazelton, K. Roe, M. Elend, A. Delacour, S. Bernasconi, M. Lilley, J. Baross, R. Summons, and S. Sylva, "A serpentinite-hosted ecosystem: The lost city hydrothermal field," *Science*, vol. 307, pp. 1428–1434, 2005.

[67] G. Proskurowski, M. D. Lilley, D. S. Kelley, and E. J. Olson, "Low temperature volatile production at the Lost City Hydrothermal Field, evidence from a hydrogen stable isotope geothermometer," *Chemical Geology*, vol. 229, pp. 331–343, 2006.

[68] K. L. Von Damm and M. D. Lilley, "Diffuse flow hydrothermal fluids from 9° 50 N East Pacific Rise: Origin, evolution and biogeochemical controls," in *The Subseafloor Biosphere at Mid-Ocean Ridges* (W. S. D. Wilcock, E. F. DeLong, D. S. Kelley, J. A. Baross, and S. C. Cary, eds.), pp. 245–268, American Geophysical Union, Washington, D. C., 2004.

[69] S. Veirs, R. McDuff, and F. Stahr, "Magnitude and variance of near-bottom horizontal heat flux at the Main Endeavour hydrothermal vent field," *Geochemistry Geophysics Geosystems*, vol. 7, 2006.

[70] A. Koschinsky, D. Garbe-Schonberg, S. Sander, K. Schmidt, H. H. Gennerich, and H. Strauss, "Hydrothermal venting at pressure-temperature conditions above the critical point of seawater, 5° S on the Mid-Atlantic Ridge," *Geology*, vol. 36, pp. 615–618, 2008.

[71] T. M. McCollom and J. S. Seewald, "Abiotic synthesis of organic compounds in deep-sea hydrothermal environments," *Chemical Reviews*, vol. 107, pp. 382–401, 2007.

[72] N. Sleep, A. Meibom, T. Fridriksson, R. Coleman, and D. Bird, "H_2-rich fluids from serpentinization: geochemical and biotic implications," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 101, pp. 12818–12823, 2004.

[73] B. B. Jorgensen and A. Boetius, "Feast and famine - microbial life in the deep-sea bed," *Nature Reviews Microbiology*, vol. 5, pp. 770–781, 2007.

[74] C. R. Fisher, H. Roberts, E. E. Cordes, and B. Bernard, "Cold Seeps and Associated Communities of the Gulf of Mexico," *Oceanography*, vol. 20, pp. 118–129, 2007.

[75] M. Tryon, K. Brown, M. Torres, A. Trehu, J. McManus, and R. Collier, "Measurements of transience and downward fluid flow near episodic methane gas vents, Hydrate Ridge, Cascadia," *Geology*, vol. 27, pp. 1075–1078, 1999.

[76] H. Niemann, T. Losekann, D. de Beer, M. Elvert, T. Nadalig, K. Knittel, R. Amann, E. J. Sauter, M. Schluter, M. Klages, J. P. Foucher, and A. Boetius, "Novel microbial communities of the Haakon Mosby mud volcano and their role as a methane sink," *Nature*, vol. 443, pp. 854–858, 2006.

[77] A. Boetius and E. Suess, "Hydrate Ridge: a natural laboratory for the study of microbial life fueled by methane from near-surface gas hydrates," *Chemical Geology*, vol. 205, pp. 291–310, 2004.

[78] M. S. Berger and C. M. Young, "Physiological response of the cold-seep mussel *Bathymodiolus childressi* to acutely elevated temperature," *Marine Biology*, vol. 149, pp. 1397–1402, 2006.

[79] C. K. Paull, B. Hecker, R. Commeau, R. P. Freemanlynde, C. Neumann, W. P. Corso, S. Golubic, J. E. Hook, E. Sikes, and J. Curray, "Biological communities at the florida escarpment resemble hydrothermal vent taxa," *Science*, vol. 226, pp. 965–967, 1984.

[80] A. Blazejak, C. Erseus, R. Amann, and N. Dubilier, "Coexistence of bacterial sulfide oxidizers, sulfate reducers, and spirochetes in a gutless worm (Oligochaeta) from the Peru margin," *Applied and Environmental Microbiology*, vol. 71, pp. 1553–1561, 2005.

[81] N. Finogenova, “6 new species of marine Tubificidae (Oligochaeta) from the continental shelf off Peru,” *Zoologica Scripta*, vol. 15, pp. 45–51, 1986.

[82] R. Jahnke and G. Jackson, “The spatial distribution of sea-floor oxygen consumption in the Atlantic and Pacific Oceans,” in *Deep-Sea Food Chains and the Global Carbon Cycle* (Rowe, GT And Pariente, V, ed.), vol. 360 of *NATO Advanced Science Institutes Series, Series C, Mathematical and Physical Sciences*, pp. 295–307, 1992.

[83] C. R. Smith, K. Kukert, R. A. Wheatcroft, P. A. Jumars, and J. W. Deming, “Vent fauna on whale remains,” *Nature*, vol. 341, pp. 27–28, 1989.

[84] C. R. Smith and A. R. Baco, “Ecology of whale falls at the deep-sea floor,” in *Oceanography and Marine Biology*, vol. 41, pp. 311–354, London: Taylor & Francis Ltd, 2003.

[85] W. J. Jones, S. B. Johnson, G. W. Rouse, and R. C. Vrijenhoek, “Marine worms (genus Osedax) colonize cow bones,” *Proceedings of the Royal Society B - Biological Sciences*, vol. 275, pp. 387–391, 2008.

[86] P. A. Allison, C. R. Smith, H. Kukert, J. W. Deming, and B. A. Bennett, “Deep-water taphonomy of vertebrate carcasses: A whale skeleton in the bathyal Santa Catalina Basin,” *Palaeobiology*, vol. 17, pp. 78–89, 1991.

[87] S. K. Goffredi, R. Wilpiszeski, R. Lee, and V. J. Orphan, “Temporal evolution of methane cycling and phylogenetic diversity of archaea in sediments from a deep-sea whale-fall in Monterey Canyon, California,” *ISME Journal*, vol. 2, pp. 204–220, 2008.

[88] D. L. Distel, W. Morrill, N. MacLaren-Toussaint, D. Franks, and J. Waterbury, “*Teredinibacter turnerae* gen. nov., sp. nov., a dinitrogen-fixing, cellulolytic, endosymbiotic gammaproteobacterium isolated from the gills of wood-boring molluscs (Bivalvia : Teredinidae),” *International Journal of Systematic and Evolutionary Microbiology*, vol. 52, pp. 2261–2269, 2002.

[89] C. P. Lechene, Y. Luyten, G. McMahon, and D. L. Distel, “Quantitative imaging of nitrogen fixation by individual bacteria within animal cells,” *Science*, vol. 317, pp. 1563–1566, 2007.

[90] D. J. Hughes and M. Crawford, “A new record of the vestimentiferan *Lamellibrachia* sp. (Polychaeta: Siboglinidae) from a deep shipwreck in the eastern Mediterranean,” *JMBA2 Biodiversity Records*, vol. 5198, 2006.

[91] P. R. Dando, A. J. Southward, E. C. Southward, D. R. Dixon, A. Crawford, and M. Crawford, "Shipwrecked tube worms," *Nature*, vol. 356, pp. 667–667, 1992.

[92] S. Duperron, J. Lorion, P. Lopez, S. Samadi, O. Gros, and F. Gaill, "Symbioses between deep-sea mussels (Mytilidae: Bathymodiolinae) and chemosynthetic bacteria: diversity, function and evolution," *Comptes Rendus Biologies*, vol. 332, pp. 298–310, 2009.

[93] M. Bright and O. Giere, "Microbial symbiosis in Annelida," *Symbiosis*, vol. 38, pp. 1–45, 2005.

[94] C. Rinke, S. Schmitz-Esser, A. Loy, M. Horn, M. Wagner, and M. Bright, "High genetic similarity between two geographically distinct strains of the sulfur-oxidizing symbiont *Candidatus* Thiobios zoothamnicoli," *FEMS Microbiology Ecology*, vol. 67, pp. 229–241, 2009.

[95] C. Rinke, S. Schmitz-Esser, K. Stoecker, A. Nussbaumer, D. Molnar, K. Vanura, M. Wagner, M. Horn, J. Ott, and M. Bright, ""Candidatus thiobios zoothamnicoli" an ectosymbiotic bacterium covering the giant marine ciliate Zoothamnium niveum," *Applied and Environmental Microbiology*, vol. 72, pp. 2014–2021, 2006.

[96] S. Bulgheresi, I. Schabussova, T. Chen, N. P. Mullin, R. M. Maizels, and J. A. Ott, "A new C-type lectin similar to the human immunoreceptor DC-SIGN mediates symbiont acquisition by a marine nematode," *Applied and Environmental Microbiology*, vol. 72, pp. 2950–2956, 2006.

[97] A. D. Nussbaumer, M. Bright, C. Baranyi, C. J. Beisser, and J. A. Ott, "Attachment mechanism in a highly specific association between ectosymbiotic bacteria and marine nematodes," *Aquatic Microbial Ecology*, vol. 34, pp. 239–246, 2004.

[98] C. Bayer, N. R. Heindl, C. Rinke, S. Luecker, J. A. Ott, and S. Bulgheresi, "Molecular characterization of the symbionts associated with marine nematodes of the genus *Robbea*," *Environmental Microbiology Reports*, vol. 2, pp. 136–144, 2009.

[99] M. Polz, D. Distel, B. Zarda, R. Amann, H. Felbeck, J. Ott, and C. Cavanaugh, "Phylogenetic analysis of a highly specific association between ectosymbiotic, sulfur-oxidizing bacteria and a marine nematode," *Applied and Environmental Microbiology*, vol. 60, pp. 4461–4467, 1994.

[100] C. M. Cavanaugh, "Symbioses of chemoautotrophic bacteria and marine invertebrates from hydrothermal vents and reducing sediments," *Bulletin of the Biological Society of Washington*, vol. 6, pp. 373–388, 1985.

[101] S. Duperron, C. Bergin, F. Zielinski, A. Blazejak, A. Pernthaler, Z. P. McKiness, E. DeChaine, C. M. Cavanaugh, and N. Dubilier, “A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (Bivalvia : Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge,” *Environmental Microbiology*, vol. 8, pp. 1441–1447, 2006.

[102] M. F. Polz, J. A. Ott, M. Bright, and C. M. Cavanaugh, “When bacteria hitch a ride,” *ASM News*, vol. 66, pp. 531–539, 2000.

[103] M. F. Polz, H. Felbeck, R. Novak, M. Nebelsick, and J. A. Ott, “Chemoautotrophic, sulfur-oxidizing symbiotic bacteria on marine nematodes - morphological and biochemical characterization,” *Microbial Ecology*, vol. 24, pp. 313–329, 1992.

[104] A. Kouris, S. K. Juniper, G. Frebourg, and F. Gaill, “Protozoan-bacterial symbiosis in a deep-sea hydrothermal vent folliculinid ciliate (*Folliculinopsis* sp.) from the Juan de Fuca Ridge,” *Marine Ecology*, vol. 28, pp. 63–71, 2007.

[105] T. Fenchel and B. J. Finlay, “Kentrophores - a mouthless ciliate with a symbiotic kitchen garden,” *Ophelia*, vol. 30, pp. 75–93, 1989.

[106] H. D. Goertz, “Symbiotic associations between ciliates and prokaryotes,” in *The Prokaryotes* (M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt, eds.), pp. 364–402, New York: Springer, 2006.

[107] J. Vacelet, N. Bouryesnault, A. Fialamedioni, and C. R. Fisher, “A methanotrophic carnivorous sponge,” *Nature*, vol. 377, pp. 296–296, 1995.

[108] J. Vacelet, A. Fiala-Medioni, C. R. Fisher, and N. Boury-Esnault, “Symbiosis between methane-oxidizing bacteria and a deep-sea carnivorous cladorhizid sponge,” *Marine Ecology Progress Series*, vol. 145, pp. 77–85, 1996.

[109] H. Gruber and J. Ott, “Localization of sulfur in the symbionts of a rectronectid plathelminth by EDX and EFTEM,” *40th European Marine Biology Symposium*, p. 101, 2005.

[110] J. Ott, G. Rieger, R. Rieger, and F. Enderes, “New mouthless interstitial worms from the sulfide system symbiosis with prokaryotes,” *Marine Ecology*, vol. 3, pp. 313–334, 1982.

[111] J. A. Ott, M. Bright, and S. Bulgheresi, “Marine microbial thiotrophic ectosymbioses,” *Oceanography and Marine Biology: an Annual Review*, vol. 42, pp. 95–118, 2004.

[112] N. Musat, O. Giere, A. Gieseke, F. Thiermann, R. Amann, and N. Dubilier, "Molecular and morphological characterization of the association between bacterial endosymbionts and the marine nematode *Astomonema* sp. from the Bahamas," *Environmental Microbiology*, vol. 9, pp. 1345–1353, 2007.

[113] S. Katz, C. M. Cavanaugh, and M. Bright, "Symbiosis of epi- and endocuticular bacteria with *Helicoradomenia* spp. (Mollusca, Aplacophora, Solenogastres) from deep-sea hydrothermal vents," *Marine Ecology Progress Series*, vol. 320, pp. 89–99, 2006.

[114] Y. Fujiwara, M. Kawato, T. Yamamoto, T. Yamanaka, W. Sato-Okoshi, C. Noda, S. Tsuchida, T. Komai, S. S. Cubelio, T. Sasakis, K. Jacobsen, K. Kubokawa, K. Fujikura, T. Maruyama, Y. Furushima, K. Okoshi, H. Miyake, M. Miyazaki, Y. Nogi, A. Yatabe, and T. Okutani, "Three-year investigations into sperm whale-fall ecosystems in Japan," *Marine Ecology*, vol. 28, pp. 219–232, 2007.

[115] D. Krueger, N. Dubilier, and C. Cavanaugh, "Chemoautotrophic symbiosis in the tropical clam *Solemya occidentalis* (Bivalvia: Protobranchia): ultrastructural and phylogenetic analysis," *Marine Biology*, vol. 126, pp. 55–64, 1996.

[116] R. G. B. Reid and F. R. Bernard, "Gutless bivalves," *Science*, vol. 208, pp. 609–610, 1980.

[117] P. Durand, O. Gros, L. Frenkiel, and D. Prieur, "Phylogenetic characterization of sulfur-oxidizing bacterial endosymbionts in three tropical Lucinidae by 16S rDNA sequence analysis," *Molecular Marine Biology and Biotechnology*, vol. 5, pp. 37–42, 1996.

[118] E. A. Glover and J. D. Taylor, "Diversity of chemosymbiotic bivalves on coral reefs: Lucinidae (Mollusca, Bivalvia) of New Caledonia and Lifou," *Zoosystema*, vol. 29, pp. 109–181, 2007.

[119] J. D. Taylor and E. A. Glover, "Lucinidae (Bivalvia) - the most diverse group of chemosymbiotic molluscs," *Zoological Journal of the Linnean Society*, vol. 148, pp. 421–438, 2006.

[120] S. Duperron, A. Fiala-Medioni, J. C. Caprais, K. Olu, and M. Sibuet, "Evidence for chemoautotrophic symbiosis in a Mediterranean cold seep clam (Bivalvia : Lucinidae): comparative sequence analysis of bacterial 16S rRNA, APS reductase and RubisCO genes," *FEMS Microbiology Ecology*, vol. 59, pp. 64–70, 2007.

[121] D. Distel and A. Wood, "Characterization of the gill symbiont of *Thyasira flexuosa* (Thyasiridae: Bivalvia) by use of polymerase chain

reaction and 16S rRNA sequence analysis," *Journal of Bacteriology*, vol. 174, pp. 6317–6320, 1992.

[122] S. C. Dufour, "Gill anatomy and the evolution of symbiosis in the bivalve family Thyasiridae," *Biological Bulletin*, vol. 208, pp. 200–212, 2005.

[123] Y. Fujiwara, C. Kato, N. Masui, K. Fujikura, and S. Kojima, "Dual symbiosis in the cold-seep thyasirid clam *Maorithyas hadalis* from the hadal zone in the Japan Trench, western Pacific," *Marine Ecology Progress Series*, vol. 214, pp. 151–159, 2001.

[124] J. J. Childress, C. R. Fisher, J. A. Favuzzi, and N. K. Sanders, "Sulfide and carbon-dioxide uptake by the hydrothermal vent clam, *Calyptogena magnifica*, and its chemoautotrophic symbionts," *Physiological Zoology*, vol. 64, pp. 1444–1470, 1991.

[125] H. Kuwahara, T. Yoshida, Y. Takaki, S. Shimamura, S. Nishi, M. Harada, K. Matsuyama, K. Takishita, M. Kawato, K. Uematsu, Y. Fujiwara, T. Sato, C. Kato, M. Kitagawa, I. Kato, and T. Maruyama, "Reduced genome of the thioautotrophic intracellular symbiont in a deep-sea clam, *Calyptogena okutanii*," *Current Biology*, vol. 17, pp. 881–886, 2007.

[126] I. L. G. Newton, T. Woyke, T. A. Auchtung, G. F. Dilly, R. J. Dutton, M. C. Fisher, K. M. Fontanez, E. Lau, F. J. Stewart, P. M. Richardson, K. W. Barry, E. Saunders, J. C. Detter, D. Wu, J. A. Eisen, and C. M. Cavanaugh, "The *Calyptogena magnifica* chemoautotrophic symbiont genome," *Science*, vol. 315, pp. 998–1000, 2007.

[127] F. Zal, E. Leize, D. R. Oros, S. Hourdez, A. Van Dorsselaer, and J. J. Childress, "Haemoglobin structure and biochemical characteristics of the sulphide-binding component from the deep-sea clam *Calyptogena magnifica*," *Cahiers de Biologie Marine*, vol. 41, pp. 413–423, 2000.

[128] S. Duperron, S. Halary, J. Lorion, M. Sibuet, and F. Gaill, "Unexpected co-occurrence of six bacterial symbionts in the gills of the cold seep mussel *Idas* sp. (Bivalvia : Mytilidae)," *Environmental Microbiology*, vol. 10, pp. 433–445, 2008.

[129] E. C. Southward, "The morphology of bacterial symbioses in the gills of mussels of the genera *Adipicola* and *Idas* (Bivalvia : Mytilidae)," *Journal of Shellfish Research*, vol. 27, pp. 139–146, 2008.

[130] C. Borowski, O. Giere, J. Krieger, R. Amann, and N. Dubilier, "New aspects of the symbiosis in the provannid snail *Ifremeria nautilei* from the North Fiji Back Arc Basin," *Cahiers de Biologie Marine*, vol. 43, pp. 321–324, 2002.

[131] Y. Suzuki, S. Kojima, H. Watanabe, M. Suzuki, S. Tsuchida, T. Nunoura, H. Hirayama, K. Takai, K. H. Nealson, and K. Horikoshi, "Single host and symbiont lineages of hydrothermalvent gastropods *Ifremeria nautilei* (Provannidae): biogeography and evolution," *Marine Ecology Progress Series*, vol. 315, pp. 167–175, 2006.

[132] Y. Suzuki, T. Sasaki, M. Suzuki, Y. Nogi, T. Miwa, K. Takai, K. H. Nealson, and K. Horikoshi, "Novel chemoautotrophic endosymbiosis between a member of the epsilonproteobacteria and the hydrothermal-vent gastropod *Alviniconcha* aff. *hessleri* (Gastropoda: Provannidae) from the Indian Ocean," *Applied and Environmental Microbiology*, vol. 71, pp. 5440–5450, 2005.

[133] Y. Suzuki, T. Sasaki, M. Suzuki, S. Tsuchida, K. H. Nealson, and K. Horikoshi, "Molecular phylogenetic and isotopic evidence of two lineages of chemoautotrophic endosymbionts distinct at the subdivision level harbored in one host-animal type: the genus *Alviniconcha* (Gastropoda: Provannidae)," *FEMS Microbiology Letters*, vol. 249, pp. 105–112, 2005.

[134] H. Urakawa, N. Dubilier, Y. Fujiwara, D. E. Cunningham, S. Kojima, and D. A. Stahl, "Hydrothermal vent gastropods from the same family (Provannidae) harbour epsilon- and gamma-proteobacterial endosymbionts," *Environmental Microbiology*, vol. 7, pp. 750–754, 2005.

[135] A. E. Bates, "Feeding strategy, morphological specialisation and presence of bacterial episymbionts in lepetodrilid gastropods from hydrothermal vents," *Marine Ecology Progress Series*, vol. 347, pp. 87–99, 2007.

[136] S. K. Goffredi, A. Waren, V. J. Orphan, C. L. Van Dover, and R. C. Vrijenhoek, "Novel forms of structural integration between microbes and a hydrothermal vent gastropod from the Indian Ocean," *Applied and Environmental Microbiology*, vol. 70, pp. 3082–3090, 2004.

[137] S. C. Cary, M. T. Cottrell, J. L. Stein, F. Camacho, and D. Desbruyeres, "Molecular identification and localization of filamentous symbiotic bacteria associated with the hydrothermal vent annelid *Alvinella pompejana*," *Applied and Environmental Microbiology*, vol. 63, pp. 1124–1130, 1997.

[138] J. J. Grzymski, A. E. Murray, B. J. Campbell, M. Kaplarevic, G. R. Gao, C. Lee, R. Daniel, A. Ghadiri, R. A. Feldman, and S. C. Cary, "Metagenome analysis of an extreme microbial symbiosis reveals eurythermal adaptation and metabolic flexibility," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 105, pp. 17516–17521, 2008.

[139] A. Haddad, F. Camacho, P. Durand, and S. C. Cary, "Phylogenetic characterization of the epibiotic bacteria associated with the hydrothermal vent polychaete *Alvinella pompejana*," *Applied and Environmental Microbiology*, vol. 61, pp. 1679–1687, 1995.

[140] R. A. Feldman, T. M. Shank, M. B. Black, A. R. Baco, C. R. Smith, and R. C. Vrijenhoek, "Vestimentiferan on a whale fall," *Biological Bulletin*, vol. 194, pp. 116–119, 1998.

[141] K. M. Halanych, "Molecular phylogeny of siboglinid annelids (a.k.a. pogonophorans): a review," *Hydrobiologia*, vol. 535/536, pp. 297–307, 2005.

[142] A. D. Nussbaumer, C. R. Fisher, and M. Bright, "Horizontal endosymbiont transmission in hydrothermal vent tubeworms," *Nature*, vol. 441, pp. 345–348, 2006.

[143] T. Loesekann, A. Robador, H. Niemann, K. Knittel, A. Boetius, and N. Dubilier, "Endosymbioses between bacteria and deep-sea siboglinid tubeworms from an Arctic Cold Seep (Haakon Mosby Mud Volcano, Barents Sea)," *Environmental Microbiology*, vol. 10, pp. 3237–3254, 2008.

[144] N. V. Pimenov, A. S. Savvichev, I. Rusanov, A. Y. Lein, and M. V. Ivanov, "Microbiological processes of the carbon and sulfur cycles at cold methane seeps of the north atlantic," *Microbiology*, vol. 69, pp. 709–720, 2000.

[145] N. Kubota, M. Kanemori, Y. Sasayama, M. Aida, and Y. Fukumori, "Identification of endosymbionts in *Oligobrachia mashikoi* (Siboglinidae, Annelida)," *Microbes and Environments*, vol. 22, pp. 136–144, 2007.

[146] D. J. Thornhill, A. A. Wiley, A. L. Campbell, F. F. Bartol, A. Teske, and K. M. Halanych, "Endosymbionts of *Siboglinum fiordicum* and the phylogeny of bacterial endosymbionts in Siboglinidae (Annelida)," *Biological Bulletin*, vol. 214, pp. 135–144, 2008.

[147] A. G. Glover, B. Kallstrom, C. R. Smith, and T. G. Dahlgren, "Worldwide whale worms? A new species of *Osedax* from the shallow north Atlantic," *Proceedings of the Royal Society B-Biological Sciences*, vol. 272, pp. 2587–2592, 2005.

[148] S. K. Goffredi, V. J. Orphan, G. W. Rouse, L. Jahnke, T. Embaye, K. Turk, R. Lee, and R. C. Vrijenhoek, "Evolutionary innovation: a bone-eating marine symbiosis," *Environmental Microbiology*, vol. 7, pp. 1369–1378, 2005.

[149] G. Rouse, S. Goffredi, and R. Vrijenhoek, "*Osedax*: bone-eating marine worms with dwarf males," *Science*, vol. 305, pp. 668–671, 2004.

[150] A. Blazejak, J. Kuever, C. Erseus, R. Amann, and N. Dubilier, "Phylogeny of 16S rRNA, ribulose 1,5-bisphosphate carboxylase/oxygenase, and adenosine 5 '-phosphosulfate reductase genes from gamma- and alphaproteobacterial symbionts in gutless marine worms (Oligochaeta) from Bermuda and the Bahamas," *Applied and Environmental Microbiology*, vol. 72, pp. 5527–5536, 2006.

[151] N. Dubilier, A. Blazejak, and C. Ruehland, "Symbioses between bacteria and gutless marine oligochaetes," in *Progress in Molecular and Subcellular Biology*, Progress in Molecular and Subcellular Biology : 41, pp. 251–275, Springer-Verlag Berlin, 2006.

[152] C. Ruehland, A. Blazejak, C. Lott, A. Loy, C. Erseus, and N. Dubilier, "Multiple bacterial symbionts in two species of co-occurring gutless oligochaete worms from Mediterranean sea grass sediments," *Environmental Microbiology*, vol. 10, pp. 3404–3416, 2008.

[153] T. Woyke, H. Teeling, N. N. Ivanova, M. Huntemann, M. Richter, F. O. Gloeckner, D. Boffelli, I. J. Anderson, K. W. Barry, H. J. Shapiro, E. Szeto, N. C. Kyrpides, M. Mussmann, R. Amann, C. Bergin, C. Ruehland, E. M. Rubin, and N. Dubilier, "Symbiosis insights through metagenomic analysis of a microbial consortium," *Nature*, vol. 443, pp. 950–955, 2006.

[154] C. Ruehland, O. Giere, and N. Dubilier, "Epifilamentous bacteria of a Wadden Sea oligochaete are related to gamma- and epsilonproteobacterial deep-sea hydrothermal vent epibionts," In prep.

[155] C. Schmidt, N. Le Bris, and F. Gaill, "Interactions of deep-sea vent invertebrates with their environment: the case of *Rimicaris exoculata*," *Journal of Shellfish Research*, vol. 27, pp. 79–90, 2008.

[156] M. Zbinden, B. Shillito, N. Le Bris, C. D. de Montlaur, E. Roussel, F. Guyot, F. Gaill, and M. A. Cambon-Bonavita, "New insigths on the metabolic diversity among the epibiotic microbial communitiy of the hydrothermal shrimp *Rimicaris exoculata*," *Journal of Experimental Marine Biology and Ecology*, vol. 359, pp. 131–140, 2008.

[157] M. F. Polz and C. M. Cavanaugh, "Dominance of one bacterial phylotype at a Mid-Atlantic Ridge hydrothermal vent site," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 92, pp. 7232–7236, 1995.

[158] S. K. Goffredi, W. J. Jones, H. Erhlich, A. Springer, and R. C. Vrijenhoek, "Epibiotic bacteria associated with the recently discovered Yeti crab, *Kiwa hirsuta*," *Environmental Microbiology*, vol. 10, pp. 2623–2634, 2008.

[159] A. J. Southward and W. A. Newman, "Ectosymbiosis between filamentous sulphur bacteria and a stalked barnacle (Scalpellomorpha, Neolepadinae) from the Lau Back Arc Basin, Tonga," *Cahiers de Biologie Marine*, vol. 39, pp. 259–262, 1998.

[160] Y. Suzuki, M. Suzuki, S. Tsuchida, A. J. Southward, and T. Yamaguchi, "Molecular phylogenetic and isotopic evidence for ectosymbiosis between filamentous bacteria and the stalked barnacle *Vulcanolepas osheai* (Cirripedia, Scalpellomorpha, Eolepadidae, Neolepadinae) from the Brothers Caldera, south Kermadec Arc, New Zealand," In prep.

[161] L. A. Beck and K. Sobjinski, "Zur systematik und phylogenie hydrothermaler tiefseemullusken.," *Courier Forschungsinstitut Senckenberg*, vol. 215, pp. 31–41, 1999.

[162] J. J. Childress, A. J. Arp, and C. R. Fisher, "Metabolic and blood characteristics of the hydrothermal vent tube worm *Riftia pachyptila*," *Marine Biology*, vol. 83, pp. 109–124, 1984.

[163] S. Goffredi, J. Childress, N. Desaulniers, R. Lee, F. Lallier, and D. Hammond, "Inorganic carbon acquisition by the hydrothermal vent tubeworm *Riftia pachyptila* depends upon high external pCO_2 and upon proton-equivalent ion transport by the worm," *Journal of Experimental Biology*, vol. 200, pp. 883–896, 1997.

[164] J. J. Childress, R. W. Lee, N. K. Sanders, H. Felbeck, D. R. Oros, A. Toulmond, D. Desbruyeres, M. C. Kennicutt, and J. Brooks, "Inorganic carbon uptake in hydrothermal vent tubeworms facilitated by high environmental pCO_2," *Nature*, vol. 362, pp. 147–149, 1993.

[165] M. C. De Cian, A. C. Andersen, X. Bailly, and F. H. Lallier, "Expression and localization of carbonic anhydrase and ATPases in the symbiotic tubeworm *Riftia pachyptila*," *Journal of Experimental Biology*, vol. 206, pp. 399–409, 2003.

[166] M. C. De Cian, X. Bailly, J. Morales, J. M. Strub, A. Van Dorsselaer, and F. H. Lallier, "Characterization of carbonic anhydrases from *Riftia pachyptila*, a symbiotic invertebrate from deep-sea hydrothermal vents," *Proteins - Structure Function and Genetics*, vol. 51, pp. 327–339, 2003.

[167] R. Kochevar and J. Childress, "Carbonic anhydrase in deepsea chemoautotrophic symbioses," *Marine Biology*, vol. 125, pp. 375–383, 1996.

[168] M. Jones and S. Gardiner, "Evidence for a transient digestive tract in Vestimentifera," *Proceedings of the Biological Society of Washington*, vol. 101, pp. 423–433, 1988.

[169] E. Southward, "Development of the gut and segmentation of newly settled stages of *Ridgeia* (Vestimentifera) - implications for relationship between Vestimentifera and Pogonophora," *Journal of the Marine Biological Association of the United Kingdom*, vol. 68, pp. 465–487, 1988.

[170] K. Endow and S. Ohta, "Occurence of bacteria in the primary oocytes of vesicomyid clam *Calyptogena soyae*," *Marine Ecology Progress Series*, vol. 64, pp. 309–311, 1990.

[171] S. C. Cary and S. J. Giovannoni, "Transovarial inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 90, pp. 5695–5699, 1993.

[172] O. Giere and C. Langheld, "Structural organization, transfer and biological fate of endosymbiotic bacteria in gutless oligochaetes," *Marine Biology*, vol. 93, pp. 641–650, 1987.

[173] P. H. Degnan, A. B. Lazarus, C. D. Brock, and J. J. Wernegreen, "Host-symbiont stability and fast evolutionary rates in an ant-bacterium association: Cospeciation of *Camponotus* species and their endosymbionts, *Candidatus* Blochmannia," *Systematic Biology*, vol. 53, pp. 95–110, 2004.

[174] M. Thao, N. Moran, P. Abbot, E. Brennan, D. Burckhardt, and P. Baumann, "Cospeciation of psyllids and their primary prokaryotic endosymbionts," *Applied and Environmental Microbiology*, vol. 66, pp. 2898–2905, 2000.

[175] K. Nelson and C. R. Fisher, "Absence of cospeciation in deep-sea vestimentiferan tube worms and their bacterial endosymbionts," *Symbiosis*, vol. 28, pp. 1–15, 2000.

[176] R. C. Vrijenhoek, M. Duhaime, and W. J. Jones, "Subtype variation among bacterial endosymbionts of tubeworms (Annelida : Siboglinidae) from the Gulf of California," *Biological Bulletin*, vol. 212, pp. 180–184, 2007.

[177] F. J. Stewart, C. R. Young, and C. M. Cavanaugh, "Lateral symbiont acquisition in a maternally transmitted chemosynthetic clam endosymbiosis," *Molecular Biology and Evolution*, vol. 25, pp. 673–687, 2008.

[178] B. W. Jones, J. E. Lopez, J. Huttenburg, and M. K. Nishiguchi, "Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis," *Molecular Ecology*, vol. 15, pp. 4317–4329, 2006.

[179] A. Mira and N. Moran, "Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria," *Microbial Ecology*, vol. 44, pp. 137–143, 2002.

[180] N. A. Moran, "Tracing the evolution of gene loss in obligate bacterial symbionts," *Current Opinion in Microbiology*, vol. 6, pp. 512–518, 2003.

[181] N. A. Moran, "Accelerated evolution and muller's rachet in endosymbiotic bacteria," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 93, pp. 2873–2878, 1996.

[182] N. A. Moran, J. P. McCutcheon, and A. Nakabachi, "Genomics and evolution of heritable bacterial symbionts," *Annual Review of Genetics*, vol. 42, pp. 165–190, 2008.

[183] N. A. Moran, H. J. McLaughlin, and R. Sorek, "The dynamics and time scale of ongoing genomic erosion in symbiotic bacteria," *Science*, vol. 323, pp. 379–382, 2009.

[184] T. L. Harmer, R. D. Rotjan, A. D. Nussbaumer, M. Bright, A. W. Ng, E. G. DeChaine, and C. M. Cavanaugh, "Free-living tube worm endosymbionts found at deep-sea vents," *Applied and Environmental Microbiology*, vol. 74, pp. 3895–3898, 2008.

[185] Y. J. Won, S. J. Hallam, G. D. O'Mullan, I. L. Pan, K. R. Buck, and R. C. Vrijenhoek, "Environmental acquisition of thiotrophic endosymbionts by deep-sea mussels of the genus *Bathymodiolus*," *Applied and Environmental Microbiology*, vol. 69, pp. 6785–6792, 2003.

[186] C. M. Cavanaugh, "Symbiotic chemoautotrophic bacteria in marine invertebrates from sulfide-rich habitats.," *Nature*, vol. 302, pp. 58–61, 1983.

[187] J. J. Childress, C. R. Fisher, J. M. Brooks, M. C. Kennicutt II, R. Bidigare, and A. E. Anderson, "A methanotrophic marine molluscan (Bivalvia, Mytilidae) symbiosis: mussels fueled by gas.," *Science*, vol. 233, pp. 1306–1308, 1986.

[188] C. M. Cavanaugh, P. R. Levering, J. S. Maki, R. Mitchell, and M. E. Lidstrom, "Symbiosis of methylotrophic bacteria and deep-sea mussels.," *Nature*, vol. 325, pp. 346–347, 1987.

[189] C. R. Fisher, J. M. Brooks, J. S. Vodenichar, J. M. Zande, J. J. Childress, and J. Burke, R. A., "The co-occurrence of methanotrophic and chemoautotrophic sulfur oxidising bacterial symbionts in a deep-sea mussel.," *Marine Ecology*, vol. 14, pp. 277–289, 1993.

[190] D. L. Distel, H. K. W. Lee, and C. M. Cavanaugh, "Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel.," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 92, pp. 9598–9602, 1995.

[191] C. R. Fisher, "Ecophysiology of primary production at deep-sea vents and seeps," *Biosystematics and Ecology Series*, vol. 11, pp. 313–336, 1996.

[192] R. G. Gustafson, R. D. Turner, R. A. Lutz, and R. C. Vrijenhoek, "A new genus and five new species of mussels (Bivalvia, Mytilidae) from deep-sea sulfide/hydrocarbon seeps in the Gulf of Mexico," *Malacologia*, vol. 40, pp. 63–112, 1998.

[193] H. M. Page, C. R. Fisher, and J. J. Childress, "Role of filter-feeding in the nutritional biology of a deep-sea mussel with methanotrophic symbionts," *Marine Biology*, vol. 104, pp. 251–257, 1990.

[194] E. Kadar, R. Bettencourt, V. Costa, R. S. Santos, A. Lobo-Da-Cunha, and P. Dando, "Experimentally induced endosymbiont loss and reacquirement in the hydrothermal vent bivalve *Bathymodiolus azoricus*," *Journal of Experimental Marine Biology and Ecology*, vol. 318, pp. 99–110, 2005.

[195] E. G. DeChaine, A. E. Bates, T. M. Shank, and C. M. Cavanaugh, "Off-axis symbiosis found: characterization and biogeography of bacterial symbionts of *Bathymodiolus* mussels from Lost City hydrothermal vents," *Environmental Microbiology*, vol. 8, pp. 1902–1912, 2006.

[196] Z. P. McKiness, E. R. McMullin, C. R. Fisher, and C. M. Cavanaugh, "A new bathymodioline mussel symbiosis at the Juan de Fuca hydrothermal vents," *Marine Biology*, vol. 148, pp. 109–116, 2005.

[197] G. N. Somero, J. J. Childress, and A. A. E., "Transport, metabolism and detoxification of hydrogen sulphide in animals from sulphide-rich marine envrionments," *Critical Reviews in Aquatic Sciences*, vol. 1, pp. 591–614, 1989.

[198] M. Bauer-Nebelsick, C. F. Bardele, and J. A. Ott, "Redescription of *Zoothamnium niveum* (Hemprich & Ehrenberg, 1831) Ehrenberg, 1838 (Oligohymenophora, Peritrichida), a ciliate with ectosymbiotic, chemoautotrophic bacteria," *European Journal of Protistology*, vol. 32, pp. 18–30, 1996.

[199] M. Bauer-Nebelsick, C. F. Bardele, and J. A. Ott, "Electron microscopic studies on *Zoothamnium niveum* (Hemprich & Ehrenberg, 1831) Ehrenberg 1838 (Oligohymenophora, Peritrichida), a ciliate with ectosymbiotic, chemoautotrophic bacteria," *European Journal of Protistology*, vol. 32, pp. 202–215, 1996.

[200] J. A. Ott, M. Bright, and F. Schiemer, "The ecology of a novel symbiosis between a marine peritrich ciliate and chemoautotrophic bacteria," *Marine Ecology-Pubblicazioni Della Stazione Zoologica Di Napoli I*, vol. 19, pp. 229–243, 1998.

[201] H. Roy, K. Vopel, M. Huettel, and B. B. Jorgensen, "Sulfide assimilation by ectosymbionts of the sessile ciliate, *Zoothamnium niveum*," *Marine Biology*, vol. 156, pp. 669–677, 2009.

[202] F. Riemann, F. Thiermann, and L. Bock, "*Leptonemella* species (Desmodoridae, Stilbonematinae), benthic marine nematodes with ectosymbiotic bacteria, from littoral sand of the North Sea island of Sylt: taxonomy and ecological aspects," *Helgoland Marine Research*, vol. 57, pp. 118–131, 2003.

[203] J. A. Ott, R. Novak, F. Schiemer, U. Hentschel, M. Nebelsick, and M. Polz, "Tackling the sulfide gradient - a novel strategy involving marine nematodes and chemoautotrophic ectosymbionts," *Marine Ecology-Pubblicazioni Della Stazione Zoologica Di Napoli I*, vol. 12, pp. 261–279, 1991.

[204] F. Schiemer, R. Novak, and J. Ott, "Metabolic studies on thiobiotic free-living nematodes and their symbiotic microorganisms," *Marine Biology*, vol. 106, pp. 129–137, 1990.

[205] D. Kilpatrick, "Animal lectins: a historical introduction and overview," *Biochimica et Biophysica Acta-General Subjects*, vol. 1572, pp. 187–197, 2002.

[206] G. J. Arason, "Lectins as defence molecules in vertebrates and invertebrates," *Fish & Shellfish Immunology*, vol. 6, pp. 277–289, 1996.

[207] P. L. De Hoff, L. M. Brill, and A. M. Hirsch, "Plant lectins: the ties that bind in root symbiosis and plant defense," *Molecular Genetics and Genomics*, vol. 282, pp. 1–15, 2009.

[208] N. Sharon and H. Lis, *Lectins.* Springer, Dordrecht, The Netherlands, 2007.

[209] M. Bergman, G. Del Prete, Y. van Kooyk, and B. Appelmelk, "*Helicobacter pylori* phase variation, immune modulation and gastric autoimmunity," *Nature Reviews Microbiology*, vol. 4, pp. 151–159, 2006.

[210] Y. van Kooyk and T. Geijtenbeek, "DC-sign: escape mechanism for pathogens," *Nature Reviews Immunology*, vol. 3, pp. 697–709, 2003.

[211] C. Diaz, L. Melchers, P. Hooykaas, B. Lugtenberg, and J. Kijne, "Root lectin as a determinant of host-plant specificity in the *Rhizobium*-legume symbiosis," *Nature*, vol. 338, pp. 579–581, 1989.

[212] G. J. Olsen, D. J. Lane, S. J. Giovannoni, N. R. Pace, and D. A. Stahl, "Microbial ecology and evolution - a ribosomal RNA approach," *Annual Review of Microbiology*, vol. 40, pp. 337–365, 1986.

[213] N. R. Pace, D. A. Stahl, D. J. Lane, and G. J. Olsen, "The analysis of natural microbial populations by ribosomal RNA sequences," *Advances in Microbial Ecology*, vol. 9, pp. 1–55, 1986.

[214] D. L. Distel, D. J. Lane, G. J. Olsen, S. J. Giovannoni, B. Pace, N. R. Pace, D. A. Stahl, and H. Felbeck, "Sulfur-oxidizing bacterial endosymbionts: analysis of phylogeny and specificity by 16S rRNA sequences," *Journal of Bacteriology*, vol. 170, pp. 2506–2510, 1988.

[215] B. J. Campbell, A. S. Engel, M. L. Porter, and K. Takai, "The versatile epsilonproteobacteria: key players in sulphidic habitats," *Nature Reviews Microbiology*, vol. 4, pp. 458–468, 2006.

[216] Y. Suzuki, S. Kojima, T. Sasaki, M. Suzuki, T. Utsumi, H. Watanabe, H. Urakawa, S. Tsuchida, T. Nunoura, H. Hirayama, K. Takai, K. Nealson, and K. Horikoshi, "Host-symbiont relationships in hydrothermal vent gastropods of the genus *Alviniconcha* from the Southwest Pacific," *Applied and Environmental Microbiology*, vol. 72, pp. 1388–1393, 2006.

[217] O. Gros, M. Liberge, and H. Felbeck, "Interspecific infection of aposymbiotic juveniles of *Codakia orbicularis* by various tropical lucinid gill endosymbionts," *Marine Biology*, vol. 142, pp. 57–66, 2003.

[218] P. Durand and O. Gros, "Bacterial host specificity of Lucinacea endosymbionts: interspecific variation in 16S rRNA sequences," *FEMS Microbiology Letters*, vol. 140, pp. 193–198, 1996.

[219] C. A. Di Meo, A. E. Wilbur, W. E. Holben, R. A. Feldman, R. C. Vrijenhoek, and S. C. Cary, "Genetic variation among endosymbionts of widely distributed vestimentiferan tubeworms," *Applied and Environmental Microbiology*, vol. 66, pp. 651–658, 2000.

[220] D. Distel, H.-W. Lee, and C. Cavanaugh, "Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel," *Proceedings of the National Academy of Sciences of the U. S. A.*, vol. 92, pp. 9598–9602, 1995.

[221] S. Duperron, M. Sibuet, B. J. MacGregor, M. M. M. Kuypers, C. R. Fisher, and N. Dubilier, "Diversity, relative abundance and metabolic potential of bacterial endosymbionts in three *Bathymodiolus* mussel species from cold seeps in the Gulf of Mexico," *Environmental Microbiology*, vol. 9, pp. 1423–1438, 2007.

[222] N. Dubilier, C. Muelders, T. Ferdelman, D. deBeer, A. Pernthaler, M. Klein, M. Wagner, C. Erseus, F. Thiermann, J. Krieger, O. Geire, and R. Amann, "Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm.," *Nature*, vol. 411, pp. 298–302, 2001.

[223] E. Hahlbeck, M. A. Pospesel, F. Zal, J. J. Childress, and H. Felbeck, "Proposed nitrate binding by hemoglobin in *Riftia pachyptila* blood," *Deep Sea Research*, vol. 52, pp. 1885–1895, 2005.

[224] I. L. G. Newton, P. R. Girguis, and C. M. Cavanaugh, "Comparative genomics of vesicomyid clam (Bivalvia: Mollusca) chemosynthetic symbionts," *BMC Genomics*, vol. 9, pp. 585–598, 2008.

[225] H. Kuwahara, Y. Takaki, T. Yoshida, S. Shimamura, K. Takishita, J. D. Reimer, C. Kato, and T. Maruyama, "Reductive genome evolution in chemoautotrophic intracellular symbionts of deep-sea *Calyptogena* clams," *Extremophiles*, vol. 12, pp. 365–374, 2008.

[226] P. Rona, G. Klinkhammer, T. Nelsen, J. Trefry, and H. Elderfield, "Black smokers, massive sulfides and vent biota at the Mid-Atlantic Ridge," *Nature*, vol. 321, pp. 33–37, 1986.

[227] A. Williams and P. Rona, "2 new caridean shrimps (Bresiliidae) from a hydrothermal field on the Mid-Atlantic ridge," *Journal of Crustacean Biology*, vol. 6, pp. 446–462, 1986.

[228] C. Van Dover, E. Szuts, S. Chamberlain, and J. Cann, "A novel eye in eyeless shrimp from hydrothermal vents of the Mid-Atlantic Ridge," *Nature*, vol. 337, pp. 458–460, 1989.

[229] C. Van Dover, G. Reynolds, A. Chave, and J. Tyson, "Light at deep-sea hydrothermal vents," *Geophysical Research Letters*, vol. 23, pp. 2049–2052, 1996.

[230] T. Komai and M. Segonzac, "Taxonomic review of the hydrothermal vent shrimp genera *Rimicaris* Williams & Rona and *Chorocaris* Martin & Hessler (Crustacea : Decapoda : Caridea : Alvinocarididae)," *Journal of Shellfish Research*, vol. 27, pp. 21–41, 2008.

[231] T. M. Shank, M. B. Black, K. M. Halanych, R. A. Lutz, and R. C. Vrijenhoek, "Miocene radiation of deep-sea hydrothermal vent shrimp (Caridea : Bresiliidae): Evidence from mitochondrial cytochrome oxidase subunit I," *Molecular Phylogenetics and Evolution*, vol. 13, pp. 244–254, 1999.

[232] M. B. Black, K. M. Halanych, P. A. Y. Maas, W. R. Hoeh, J. Hashimoto, D. Desbruyeres, R. A. Lutz, and R. C. Vrijenhoek, "Molecular systematics of vestimentiferan tubeworms from hydrothermal vents and cold-water seeps," *Marine Biology*, vol. 130, pp. 141–149, 1997.

[233] A. S. Peek, R. G. Gustafson, R. A. Lutz, and R. C. Vrijenhoek, "Evolutionary relationships of deep-sea hydrothermal vent and cold-water seep clams (Bivalvia : Vesicomyidae): results from mitochondrial cytochrome oxidase subunit I," *Marine Biology*, vol. 130, pp. 151–161, 1997.

[234] S. Kiel, "New records and species of molluscs from tertiary cold-seep carbonates in Washington State, USA," *Journal of Paleontology*, vol. 80, pp. 121–137, 2006.

[235] W. Ludwig, O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K. H. Schleifer, "Arb: a software environment for sequence data," *Nucleic Acids Research*, vol. 32, pp. 1363–1371, 2004.

[236] C. L. Van Dover, S. E. Humphris, D. Fornari, C. M. Cavanaugh, R. Collier, S. K. Goffredi, J. Hashimoto, M. D. Lilley, A. L. Reysenbach, T. M. Shank, K. L. Von Damm, A. Banta, R. M. Gallant, D. Gotz, D. Green, J. Hall, T. L. Harmer, L. A. Hurtado, P. Johnson, Z. P. McKiness, C. Meredith, E. Olson, I. L. Pan, M. Turnipseed, Y. Won, C. R. Young, and R. C. Vrijenhoek, "Biogeography and ecological setting of Indian Ocean hydrothermal vents," *Science*, vol. 294, pp. 818–823, 2001.

[237] J. Hashimoto, S. Ohta, T. Gamo, H. Chiba, T. Yamaguchi, S. Tsuchida, T. Okudaira, H. Watabe, T. Yamanaka, and M. Kitazawa, "First hydrothermal vent communities from the Indian Ocean discovered," *Zoological Science*, vol. 18, pp. 717–721, 2001.

[238] H. Watabe and J. Hashimoto, "A new species of the genus Rimicaris (Alvinocarididae : Caridea : Decapoda) from the active hydrothermal vent field, "Kairei field," on the Central Indian Ridge, the Indian Ocean," *Zoological Science*, vol. 19, pp. 1167–1174, 2002.

[239] P. A. Tyler and C. M. Young, "Dispersal at hydrothermal vents: a summary of recent progress," *Hydrobiologia*, vol. 503, pp. 9–19, 2003.

[240] S. Creasey, A. D. Rogers, and P. A. Tyler, "Genetic comparison of two populations of the deep-sea vent shrimp *Rimicaris exoculata* (Decapoda: Bresiliidae) from the Mid-Atlantic Ridge," *Marine Biology*, vol. 125, pp. 473–482, 1996.

[241] A. L. Vereshchaka, "Comparative morphological studies on four populations of the shrimp *Rimicaris exoculata* from the Mid-Atlantic Ridge," *Deep Sea Research*, vol. 44, pp. 1905–1921, 1997.

[242] E. C. Southward, V. Tunnicliffe, and M. Black, "Revision of the species of *Ridgeia* from northeast Pacific hydrothermal vents, with a redescription of *Ridgeia piscesae* Jones (Pogonophora, Obturata equals Vestimentifera)," *Canadian Journal of Zoology*, vol. 73, pp. 282–295, 1995.

[243] M. B. Black, R. A. Lutz, and R. C. Vrijenhoek, "Gene flow among vestimentiferan tubeworm (*Riftia pachyptila*) populations from hydrothermal vents of the Eastern Pacific," *Marine Biology*, vol. 120, pp. 33–39, 1994.

[244] M. L. Jones, "On the Vestimentifera, new phylum: six new species, and other taxa, from hydrothermal vents and elsewhere," *Bulletin of the Biological Society of Washington*, vol. 6, pp. 117–158, 1985.

[245] T. M. Shank, R. A. Lutz, and R. C. Vrijenhoek, "Molecular systematics of shrimp (Decapoda : Bresiliidae) from deep-sea hydrothermal vents, I: enigmatic "small orange" shrimp from the Mid-Atlantic ridge are juvenile Rimicaris exoculata," *Molecular Marine Biology and Biotechnology*, vol. 7, pp. 88–96, 1998.

[246] P. Herring and D. Dixon, "Extensive deep-sea dispersal of postlarval shrimp from a hydrothermal vent," *Deep Sea Research*, vol. 45, pp. 2105–2118, 1998.

[247] D. Dixon, L. Dixon, and D. Pond, "Recent advances in our understanding of the life history of bresiliid vent shrimps on the MAR," *Cahiers de Biologie Marine*, vol. 39, pp. 383–386, 1998.

[248] D. Pond, D. Dixon, and J. Sargent, "Wax-ester reserves facilitate dispersal of hydrothermal vent shrimps," *Marine Ecology Progress Series*, vol. 146, pp. 289–290, 1997.

[249] D. W. Pond, D. R. Dixon, M. V. Bell, A. E. Fallick, and J. R. Sargent, "Occurrence of 16:2(n-4) and 18:2(n-4) fatty acids in the lipids of the hydrothermal vent shrimps *Rimicaris exoculata* and *Alvinocaris markensis*: nutritional and trophic implications," *Marine Ecology Progress Series*, vol. 156, pp. 167–174, 1997.

[250] M. F. Polz, J. J. Robinson, C. M. Cavanaugh, and C. L. Van Dover, "Trophic ecology of massive shrimp aggregations at a Mid-Atlantic Ridge hydrothermal vent site," *Limnology and Oceanography*, vol. 43, pp. 1631–1638, 1998.

[251] C. L. Van Dover, B. Fry, J. F. Grassle, S. Humphris, and P. A. Rona, "Feeding biology of the shrimp *Rimicaris exoculata* at hydrothermal vents on the Mid-Atlantic Ridge," *Marine Biology*, vol. 98, pp. 209–216, 1988.

[252] B. Casanova, M. Brunet, and M. Segonzac, "Enigma of the trophic adaptation of the shrimp Alvinocarididae in hydrothermal areas along the Mid-Atlantic Ridge," *Cahiers de Biologie Marine*, vol. 34, pp. 573–588, 1993.

[253] A. V. Gebruk, N. V. Pimenov, and A. S. Savvichev, "Feeding specialization of Bresiliid shrimps in the TAG site hydrothermal community," *Marine Ecology Progress Series*, vol. 98, pp. 247–253, 1993.

[254] A. V. Gebruk, E. C. Southward, H. Kennedy, and A. J. Southward, "Food sources, behaviour, and distribution of hydrothermal vent shrimps at the Mid-Atlantic Ridge," *Journal of the Marine Biological Association of the United Kingdom*, vol. 80, pp. 485–499, 2000.

[255] M. Segonzac, M. Desaintlaurent, and B. Casanova, "Enigma of the trophic adaptation of the shrimp Alvinocarididae in hydrothermal areas along the Mid-Atlantic Ridge," *Cahiers de Biologie Marine*, vol. 34, pp. 535–571, 1993.

[256] C. Cavanaugh and J. J. Robinson, "CO_2 fixation in chemoautotrophic-invertebrate symbioses: Expression of form I and form II RubisCO," in *Microbial growth on C1 compounds* (M. E. Lidstrom and F. R. Tabita, eds.), Kluwer, 1996.

[257] C. O. Wirsen, H. W. Jannasch, and S. J. Molyneaux, "Chemosynthetic microbial activity at Mid-Atlantic Ridge hydrothermal vent sites," *Journal of Geophysical Research*, vol. 98, pp. 9693–9703, 1993.

[258] V. F. Galchenko, N. V. Pimenov, A. I. Lein, S. V. Galkin, L. I. Moskalev, and M. V. Ivanov, "Autotrophic CO_2 assimilation in tissues

of prawn *Rimicaris exoculata* from the Mid-Atlantic Ridge hydrothermal area," *Doklady Akademii Nauk SSSR*, vol. 308, pp. 1478–1481, 1989.

[259] H. W. Jannasch, C. O. Wirsen, and S. J. Molyneaux, "Chemosynthetic microbial activity at the 23° and 26'N Mid-Atlantic Ridge vent sites," *Ridge Events*, vol. 19, 1991.

[260] M. Zbinden, N. Le Bris, F. Gaill, and P. Compere, "Distribution of bacteria and associated minerals in the gill chamber of the vent shrimp *Rimicaris exoculata* and related biogeochemical processes," *Marine Ecology Progress Series*, vol. 284, pp. 237–251, 2004.

[261] L. Corbari, M. Zbinden, M. A. Cambon-Bonavita, F. Gaill, and P. Compere, "Bacterial symbionts and mineral deposits in the branchial chamber of the hydrothermal vent shrimp *Rimicaris exoculata*: relationship to moult cycle," *Aquatic Biology*, vol. 1, pp. 225–238, 2008.

[262] C. Schmidt, R. Vuillemin, C. Le Gall, F. Gaill, and N. Le Bris, "Geochemical energy sources for microbial primary production in the environment of hydrothermal vent shrimps," *Marine Chemistry*, vol. 108, pp. 18–31, 2008.

[263] B. M. Toner, C. M. Santelli, M. A. Marcus, R. Wirth, C. S. Chan, T. McCollom, W. Bach, and K. J. Edwards, "Biogenic iron oxyhydroxide formation at mid-ocean ridge hydrothermal vents: Juan de Fuca Ridge," *Geochimica et Cosmochimica Acta*, vol. 73, pp. 388–403, 2009.

[264] K. J. Edwards, W. Bach, and T. M. McCollom, "Geomicrobiology in oceanography: microbe-mineral interactions at and below the seafloor," *Trends in Microbiology*, vol. 13, pp. 449–456, 2005.

[265] A. Gloter, M. Zbinden, F. Guyot, F. Gaill, and C. Colliex, "TEM-EELS study of natural ferrihydrite from geological-biological interactions in hydrothermal systems," *Earth and Planetary Science Letters*, vol. 222, pp. 947–957, 2004.

[266] S. Schaedler, C. Burkhardt, F. Hegler, K. L. Straub, J. Miot, K. Benzerara, and A. Kappler, "Formation of cell-iron-mineral aggregates by phototrophic and nitrate-reducing anaerobic Fe(II)-oxidizing bacteria," *Geomicrobiology Journal*, vol. 26, pp. 93–103, 2009.

[267] C. S. Chan, G. De Stasio, S. A. Welch, M. Girasole, B. H. Frazer, M. V. Nesterova, S. Fakra, and J. F. Banfield, "Microbial polysaccharides template assembly of nanocrystal fibers," *Science*, vol. 303, pp. 1656–1658, 2004.

[268] G. K. Druschel, D. Emerson, R. Sutka, P. Suchecki, and G. W. Luther, III, "Low-oxygen and chemical kinetic constraints on the geochemical niche of neutrophilic iron(II) oxidizing microorganisms," *Geochimica et Cosmochimica Acta*, vol. 72, pp. 3358–3370, 2008.

[269] J. A. Fuhrman, S. H. Lee, Y. Masuchi, A. A. Davis, and R. M. Wilcox, "Characterization of marine prokaryotic communities via DNA and RNA," *Microbial Ecology*, vol. 28, pp. 133–145, 1994.

[270] D. M. Ward, M. M. Bateson, R. Weller, and A. L. Ruffroberts, "Ribosomal RNA analysis of microorganisms as they occur in nature," *Advances in Microbial Ecology*, vol. 12, pp. 219–286, 1992.

[271] J. B. H. Martiny, B. J. M. Bohannan, J. H. Brown, R. K. Colwell, J. A. Fuhrman, J. L. Green, M. C. Horner-Devine, M. Kane, J. A. Krumins, C. R. Kuske, P. J. Morin, S. Naeem, L. Ovreas, A. L. Reysenbach, V. H. Smith, and J. T. Staley, "Microbial biogeography: putting microorganisms on the map," *Nature Reviews Microbiology*, vol. 4, pp. 102–112, 2006.

[272] L. G. M. Baas-Becking, *Geobiologie of inleiding tot de milieukunde.* Van Stockkum and Zoon, 1934.

[273] R. T. Papke and D. M. Ward, "The importance of physical isolation to microbial diversification," *FEMS Microbiology Ecology*, vol. 48, pp. 293–303, 2004.

[274] N. L. Held and R. J. Whitaker, "Viral biogeography revealed by signatures in *Sulfolobus islandicus* genomes," *Environmental Microbiology*, vol. 11, pp. 457–466, 2009.

[275] R. J. Whitaker, D. W. Grogan, and J. W. Taylor, "Geographic barriers isolate endemic populations of hyperthermophilic archaea," *Science*, vol. 301, pp. 976–978, 2003.

[276] A. C. Martiny, A. P. K. Tai, D. Veneziano, F. Primeau, and S. W. Chisholm, "Taxonomic resolution, ecotypes and the biogeography of *Prochlorococcus*," *Environmental Microbiology*, vol. 11, pp. 823–832, 2009.

[277] D. Falush, T. Wirth, B. Linz, J. K. Pritchard, M. Stephens, M. Kidd, M. J. Blaser, D. Y. Graham, S. Vacher, G. I. Perez-Perez, Y. Yamaoka, F. Megraud, K. Otto, U. Reichard, E. Katzowitsch, X. Y. Wang, M. Achtman, and S. Suerbaum, "Traces of human migrations in *Helicobacter pylori* populations," *Science*, vol. 299, pp. 1582–1585, 2003.

[278] T. S. Haselkorn, T. A. Markow, and N. A. Moran, "Multiple introductions of the *Spiroplasma* bacterial endosymbiont into Drosophila," *Molecular Ecology*, vol. 18, pp. 1294–1305, 2009.

[279] M. J. Mandel, M. S. Wollenberg, E. V. Stabb, K. L. Visick, and E. G. Ruby, "A single regulatory gene is sufficient to alter bacterial host range," *Nature*, vol. 458, pp. 215–U7, 2009.

[280] L. R. Croal, Y. Jiao, and D. K. Newman, "The *fox* operon from *Rhodobacter* strain SW2 promotes phototrophic Fe(ii) oxidation in *Rhodobacter capsulatus* SB1003," *Journal of Bacteriology*, vol. 189, pp. 1774–1782, 2007.

[281] Y. Jiao and D. K. Newman, "The pio operon is essential for phototrophic fe(ii) oxidation in rhodopseudomonas palustris tie-1," *Journal of Bacteriology*, vol. 189, pp. 1765–1773, 2007.

[282] S. M. Sievert, K. A. Scott, M. G. Klotz, P. S. G. Chain, L. J. Hauser, J. Hemp, M. Huegler, M. Land, A. Lapidus, F. W. Larimer, S. Lucas, S. A. Malfatti, F. Meyer, I. T. Paulsen, Q. Ren, J. Simon, and USF Genom Class, "Genome of the epsilonproteobacterial chemolithoautotroph *Sulfurimonas denitrificans*," *Applied and Environmental Microbiology*, vol. 74, pp. 1145–1156, 2008.

[283] B. Meyer, J. F. Imhoff, and J. Kuever, "Molecular analysis of the distribution and phylogeny of the *soxB* gene among sulfur-oxidizing bacteria - evolution of the Sox sulfur oxidation enzyme system," *Environmental Microbiology*, vol. 9, pp. 2957–2977, 2007.

[284] T. J. Page, J. W. Short, C. L. Humphrey, M. J. Hillyer, and J. M. Hughes, "Molecular systematics of the Kakaducarididae (Crustacea : Decapoda : Caridea)," *Molecular Phylogenetics and Evolution*, vol. 46, pp. 1003–1014, 2008.

[285] P. R. Teske, P. W. Froneman, N. P. Barker, and C. D. McQuaid, "Phylogeographic structure of the caridean shrimp *Palaemon peringueyi* in South Africa: further evidence for intraspecific genetic units associated with marine biogeographic provinces," *African Journal of Marine Science*, vol. 29, pp. 253–258, 2007.

[286] R. L. Hunter, M. S. Webb, T. M. Iliffe, and J. R. A. Bremer, "Phylogeny and historical biogeography of the cave-adapted shrimp genus *Typhlatya* (Atyidae) in the Caribbean Sea and western Atlantic," *Journal of Biogeography*, vol. 35, pp. 65–75, 2008.

[287] R. Breban, J. M. Drake, D. E. Stallknecht, and P. Rohani, "The role of environmental transmission in recurrent avian influenza epidemics," *PLOS Computational Biology*, vol. 5, 2009.

[288] P. Rohani, R. Breban, D. E. Stallknecht, and J. M. Drake, "Environmental transmission of low pathogenicity avian influenza viruses and its implications for pathogen invasion," *Proceedings of the National Academy of Sciences of the U. S. A.*, 2009.

[289] C. Fraser, E. J. Alm, M. F. Polz, B. G. Spratt, and W. P. Hanage, "The bacterial species challenge: making sense of genetic and ecological diversity," *Science*, vol. 323, pp. 741–746, 2009.

[290] R. J. Whitaker, "Allopatric origins of microbial species," *Philosophical Transactions of the Royal Society B - Biological Sciences*, vol. 361, pp. 1975–1984, 2006.

[291] M. F. Polz, D. E. Hunt, S. P. Preheim, and D. M. Weinreich, "Patterns and mechanisms of genetic and phenotypic differentiation in marine microbes," *Philosophical Transactions of the Royal Society B - Biological Sciences*, vol. 361, pp. 2009–2021, 2006.

[292] S. M. Huse, L. Dethlefsen, J. A. Huber, D. M. Welch, D. A. Relman, and M. L. Sogin, "Exploring microbial diversity and taxonomy using SSU rRNA hypervariable tag sequencing," *PLOS Genetics*, vol. 4, 2008.

[293] D. E. Hunt, L. A. David, D. Gevers, S. P. Preheim, E. J. Alm, and M. F. Polz, "Resource partitioning and sympatric differentiation among closely related bacterioplankton," *Science*, vol. 320, pp. 1081–1085, 2008.

[294] A. S. Peek, R. C. Vrijenhoek, and B. S. Gaut, "Accelerated evolutionary rate in sulfur-oxidizing endosymbiotic bacteria associated with the mode of symbiont transmission," *Molecular Biology and Evolution*, vol. 15, pp. 1514–1523, 1998.

[295] C. R. Woese, "Bacterial evolution," *Microbiological Reviews*, vol. 51, pp. 221–271, 1987.

[296] K. Liolios, K. Mavromatis, N. Tavernarakis, and N. C. Kyrpides, "The Genomes On Line Database (GOLD) in 2007: status of genomic and metagenomic projects and their associated metadata," *Nucleic Acids Research*, vol. 36, pp. D475–D479, 2008.

[297] P. K. Gupta, "Single-molecule DNA sequencing technologies for future genomics research," *Trends in Biotechnology*, vol. 26, pp. 602–611, 2008.

[298] J. Thompson, S. Pacocha, C. Pharino, V. Klepac-Ceraj, D. Hunt, J. Benoit, R. Sarma-Rupavtarm, D. Distel, and M. Polz, "Genotypic diversity within a natural coastal bacterioplankton population," *Science*, vol. 307, pp. 1311–1313, 2005.

[299] D. Medini, C. Donati, H. Tettelin, V. Masignani, and R. Rappuoli, "The microbial pan-genome," *Current Opinion in Genetics & Development*, vol. 15, pp. 589–594, 2005.

ACKNOWLEDGEMENTS

Thanks to....

Prof. Dr. Rudolf Amman, thank you for taking me into the Molecular Ecology group, and always giving me a chance.

Prof. Dr. Barbara Reinhold-Hurek, thank you for your willingness to review this thesis.

Prof. Dr. Wolfgang Bach, Dr. Alban Ramette, the members of my thesis committee, thanks for the discussions, your ideas, your input.

Prof. Dr. Mathilde Cannat, organizer of the MoMARnet project that provided my funding.

Dr. Marie-Anne Cambon-Bonavita, Dr. Magali Zbinden, for the fruitful cooperation.

Prof. Dr. Chuck Fisher, for inviting me to the Gulf of Mexico for my first 'hands-on' experience of *Bathymodiolus*.

The mollies, the ex-mollies, my office buddies, my lab buddies, my lunch buddies, my coffee and shopping buddies, **The symbionts, the ex-symbionts**.

Some of you don't fit into one simple category, you've all helped me in so many ways: Regina, Claudia, Silke, Patricia, Caro R., Torben, Julia, Christian L., Christian B., Kyoko, Dagmar, Elke, Martha, Frank Z., Melissa, Dennis, Karina.

Bernd Stickfort, my hero in times of bibliographic need, **Christiane Glöckner**, thank you for your help and support, always with a smile.

Marc, for all the coffees, for the steak, the MLs, the acetate talk, for telling me 'she'll be right'.

The family, Mum, Dad - only ever a thought away, with boundless unconditional love, Onsie, Sam, Malte.

Nicole - these might just be the most difficult sentences of the whole thesis. I can never replace the nights, the weekends, the hours in a plane that you spent commenting, criticizing, rewording, rethinking with me. You've given me every opportunity, and always found the time to listen. I've learned so much from working with you. Not only that, it's been a hell of a good time. I would be proud to one day be able to pass on your professionalism, your enthusiasm, your criticism, your praise.

Part V

Appendix

The sulfur-oxidizing endosymbiont of the hydrothermal vent mussel *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae) uses hydrogen as an energy source

Frank U. Zielinski[1,2], Jillian M. Petersen[1], Silke Wetzel[1], Thomas Pape[3‡], Richard Seifert[3], Frank Wenzhöfer[4], Nicole Dubilier[1*]

[1] Max Planck Institute for Marine Microbiology, Department of Molecular Ecology
Celsiusstr. 1, 28359 Bremen, Germany

[2] Helmholtz-Center for Environmental Research – UFZ, Department of Environmental Microbiology, Permoserstraße 15, 04318 Leipzig, Germany

[3] University of Hamburg, Institute for Biogeochemistry and Marine Chemistry, Bundesstr. 55, 20146 Hamburg, Germany

[4] Max Planck Institute for Marine Microbiology, Department of Biogeochemistry
Celsiusstr. 1, 28359 Bremen, Germany

[‡] present adress: University of Bremen, Research Center Ocean Margins, Department of General and Marine Geology, Leobener Str., 28359 Bremen, Germany

* Corresponding author:
Phone: 0049 (0)421 2028 932; Fax: 0049 (0)421 2028 580; Email: ndubilie@mpi-bremen.de

---

Abstract

Hydrothermal vents along the slow-spreading Mid-Atlantic Ridge (MAR) occur in both ultramafic and basaltic settings and differ prominently in their fluid composition. High temperature fluids discharged from the ultramafic-hosted Logatchev vent field at the northern MAR (14°45'N) contain hydrogen up to 19 mM due to a geochemical process known as serpentinization. In contrast, fluids emanating from the basalt-hosted Comfortless Cove field at the southern MAR (4°48'S) hold hydrogen concentrations of only 61 µM. Only nanomolar concentrations are present in the basalt-hosted Lilliput diffuse fluids (9°33'S). Deep-sea hydrothermal vent mussels belonging to the genus *Bathymodiolus* are found at all three vent fields and dominate the invertebrate community. They thrive in areas supplied by diffuse fluids as a result of a dual symbiosis with sulfur- and methane-oxidizing bacterial endosymbionts. To date, reduced sulfur compounds and methane are the only two energy sources known to fuel chemosynthetic symbioses. However, hydrogen is a potential energy source for chemolithotrophic bacteria but has never been shown to be utilized by any of the chemosynthetic symbionts nourishing invertebrates in reducing environments. We incubated endosymbiont containing gill tissues of *B. puteoserpentis* (Logatchev) and *B.* sp. (Comfortless Cove and Lilliput) in the presence of hydrogen and sulfide. Hydrogen was rapidly consumed by *B. puteoserpentis* but only moderately by *B.* sp. However, consumption rates of *B.* sp. increased with elevated hydrogen partial pressures indicating that hydrogen uptake can be stimulated. In contrast, sulfide consumption rates did not differ in these mussels. Both hydrogen and sulfide stimulated carbon fixation in *B. puteoserpentis* gill tissues with similar CO_2 incorporation rates. Furthermore, the *hynL* gene coding for the large subunit of a membrane-bound respiratory hydrogen uptake [NiFe]-hydrogenase and indicative of energy conservation across the bacterial respiratory chain was found in *B. puteoserpentis* and *B.* sp. gill DNA extracts. The *hynL* gene was also amplified from *B.* cf. *thermophilus* which solely hosts chemoautotrophic symbionts whereas methanotrophs are absent. This implies that hydrogen may be an energy source for the sulfur-oxidizing symbiont of bathymodiolin vent mussels. Attempts to amplify this gene from cold-seep bathymodiolin mussels have so far been unsuccessful indicating that hydrogen is not recruited for energy conservation in seep mussels. While hydrogen-based energy

conservation by chemoautotrophic symbionts of bathymodiolin vent mussels is strongly supported for *B. puteoserpentis* by physiological evidence we can not exclude at this point that the methanotrophic endosymbiont may also contribute to hydrogen consumption with or without conserving energy from this process. Concurrent with hydrogen concentrations in high temperature and diffuse fluids our data indicate that chemoautotrophy in bathymodiolin mussels may be sulfur-based at basalt-hosted vent settings and cold seeps but hydrogen-based at ultramafic-hosted vents. Accordingly, the chemoautotrophic strains of Rainbow and Lost City mussels may also rely on hydrogen rather than on sulfide for energy conservation. Meanwhile and at first approximation the Logatchev mussel population may oxidize 12-30 mol hydrogen per hour corresponding to 270-670 liters of hydrogen. Endosymbionts of *B. puteoserpentis* may therefore play an appreciable role in hydrogen removal from diffuse fluids before hydrogen is issued into the open ocean.

Keywords: hydrothermal vents, Mid-Ocean Ridges, Mid Atlantic Ridge, Serpentinization, Logatchev, *Bathymodiolus puteoserpentis*, mussels, bivalvia, endosymbiosis, chemoautotrophic bacteria

Introduction

The Logatchev hydrothermal vent fauna is dominated by the mussel *Bathymodiolus puteoserpentis* that forms dense aggregates in areas of diffuse hydrothermal fluid-flow. Mussel biomass at Logatchev has been estimated to exceed 70 kg m^{-2} (Gebruk *et al.*, 2000). In the absence of light and thus photosynthetic primary production, the abundance of *B. puteoserpentis* is the result of a symbiotic association with chemosynthetic bacteria that reside intracellularly in specialized gill bacteriocytes. *B. puteoserpentis* hosts two types of gammaproteobacterial endosymbionts: one is related to free-living and symbiotic type I methanotrophs and the other to chemoautotrophic symbionts of other *Bathymodiolus* hosts (Duperron *et al.*, 2006). It is generally assumed that bacterial symbionts that fall within the methanotrophic and chemoautotrophic clades of mussel symbionts use methane and reduced sulfur compounds as an energy source. However, even very closely related bacteria may have very different metabolic repertoires as a result of horizontal gene transfer enabling them to occupy very different ecological niches (Suen *et al.*, 2007). Furthermore, for the chemoautotrophic clade of bathymodiolin symbioses, clear evidence for the use of reduced sulfur compounds as an electron donor has only been presented for a single host species, *B. thermophilus*. In this mussel, both sulfide and thiosulfate stimulated CO_2 fixation (Belkin *et al.*, 1986; Nelson *et al.*, 1995).

In *B. puteoserpentis*, evidence for the use of methane as an energy source is based on $^{14}CH_4$ oxidation experiments, immunodetection of methanol dehydrogenase (a key enzyme in methane oxidation), hexulose-phosphate synthase assays (key enzyme of the ribulose monophosphate cycle of formaldehyde assimilation), amplification of genes coding for key enzymes in the oxidation of methane (methane monooxygenase (*pmoA*) and methanol dehydrogenyase (*mxaF*) (Pimenov *et al.*, 2002; Robinson *et al.*, 1998), and the expression of the *pmoA* gene (Pernthaler *et al.*, in prep.).

In contrast, it has been more difficult to prove chemoautotrophy based on reduced sulfur compounds for the second endosymbiont of *B. puteoserpentis*. Both sulfide and thiosulfate did not stimulate $^{14}CO_2$ fixation and enzyme assays with the key enzyme for the assimilation of CO_2 via the Calvin Cycle (RubisCO - ribulose 1,5-bisphosphate carboxylase) were either negative or showed only low activities in two independent studies (Pimenov *et al.*, 2002; Robinson *et al.*, 1998). However, immunodetection indicated the presence of RubisCO in *B. puteoserpentis* gill tissues (Robinson *et al.*, 1998). Recently, (Pernthaler *et al.*, in prep.) showed that the *aprA* gene is expressed in the chemoautotrophic endosymbiont of *B. puteoserpentis*. This gene codes for the alpha subunit of adenosine-5'-phosphosulfate reductase (APS reductase) and is used by sulfur-oxidizing bacteria to oxidize sulfite (Hipp *et*

al., 1997; Sánchez *et al.*, 2001). This suggests that the chemoautotrophic endosymbiont of *B. puteoserpentis* is able to use reduced sulfur compounds as an energy source.

To date methane and reduced sulfur compounds are the only two energy sources known to fuel chemosynthetic symbioses (Cavanaugh *et al.*, 2006; Stewart *et al.*, 2005). In theory, however, other reduced compounds such as hydrogen, ammonium, nitrite, ferrous iron, and manganese could also be used as electron donors (Amend and Shock, 2001; Fisher *et al.*, 2007; Jannasch and Mottl, 1985; Jørgensen and Boetius, 2007). Hydrogen is a particularly favorable energy source as the energy yield from hydrogen oxidation is higher than from methane or sulfur oxidation and much higher than the energy yield from the oxidation of all other potential inorganic electron donors. For example, the standard molal Gibbs free energy (ΔG^0) yielded from the aerobic oxidation of hydrogen at 4°C (-528 kJ mol^{-1} per mol oxygen) exceeds the energy yield from aerobic methane oxidation (-412 kJ mol^{-1}) and aerobic sulfide oxidation at the same conditions (-378 kJ mol^{-1}) (Amend and Shock, 2001).

A wide range of phylogenetically diverse bacteria use hydrogen as an energy source including free-living aerobic methane- and sulfur-oxidizing bacteria. *Acidithiobacillus ferrooxidans* which was initially characterized as a sulfur- and ferrous iron-oxidizing aerobic chemolithoautotroph also grows aerobically on hydrogen (Drobner *et al.*, 1990; Ohmura *et al.*, 2002). Besides, the potential to grow on hydrogen has been inferred from the complete genome sequences of the sulfide-oxidizing chemolithoautotrophs *Thiobacillus denitrificans* and *Thiomicrospira crunogena* (Beller *et al.*, 2006; Scott *et al.*, 2006) and the methanotroph *Methylococcus capsulatus* (Bath) (Ward *et al.*, 2004).

Hydrothermal vent fields along the slow-spreading Mid-Atlantic Ridge (MAR) can be grouped into basalt-hosted and ultramafic-hosted systems. Whereas in basalt-hosted systems the upper oceanic crust is entirely composed of basaltic rocks, in ultramafic-hosted systems the upper oceanic crust is primarily composed of mantle-derived peridotite (one type of ultramafic rocks) (Wetzel and Shock, 2000). Thus, these two systems differ in their fluid composition as a result of basalt-seawater or peridotite-seawater reactions (Wetzel and Shock, 2000). Hydrothermal fluids discharged from ultramafic-hosted vent fields along the MAR such as Logatchev (14°45'N) and Rainbow (36°14'N) contain large quantities of dissolved hydrogen while the hydrogen content of fluids discharged from basaltic-hosted systems is comparably low (Charlou *et al.*, 2002; Charlou *et al.*, 1996; Douville *et al.*, 2002; Schmidt *et al.*, 2007). The wealth of hydrogen in in ultramafic-hosted hydrothermal systems results from a geochemical process known as serpentinization (Allen and Seyfried, 2003, 2004; Bach *et al.*, 2004; Bach *et al.*, 2006; Berndt *et al.*, 1996; Charlou *et al.*, 2002; Paulick *et al.*, 2006; Schmidt *et al.*, 2007; Sleep *et al.*, 2004; Wetzel and Shock, 2000).

In this study, we examined if hydrogen could be used as an energy source by the endosymbionts of the Logatchev hydrothermal vent mussel *B. puteoserpentis*. With 19 mM Logatchev fluids bear the highest endmember amounts of hydrogen ever reported for a hydrothermal system unperturbed by magmatic and eruptive events (Lilley *et al.*, 2003; Schmidt *et al.*, 2007). Two questions were addressed: First, do the symbionts consume hydrogen, and second, does hydrogen consumption result in energy conservation? Furthermore, hydrogen consumption rates of *B. puteoserpentis* symbionts were compared with consumption rates of *B. sp.* symbionts from the basalt-hosted vent fields Comfortless Cove (4°48'S, MAR) and Lilliput (9°33'S, MAR) with hydrogen endmember concentrations ~300 times lower than at Logatchev (Haase *et al.*, 2007a; Haase *et al.*, 2007b). Finally, hydrogen consumption rates were compared to the sulfide consumption rates in these mussels. The importance of hydrogen and sulfide for endosymbionts of bathymodiolin mussels settling in ultramafic- or basalt-hosted vent settings is discussed.

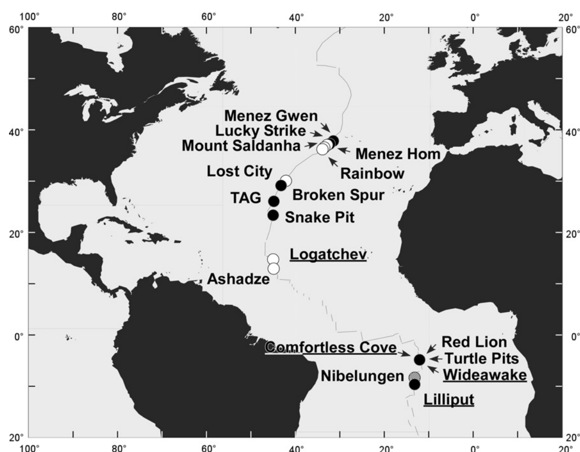


Figure 1. Map showing currently known active hydrothermal vent fields along the Mid-Atlantic Ridge. Fields where hydrogen and /or sulfide consumption experiments were performed are underlined. White and black circles denote ultramafic-hosted and basalt-hosted vent fields, respectively. Fluids of the Nibelungen field support an ultramafic-hosted setting. However, only basaltic were thus far recovered (Koschinsky *et al.*, 2006).

Material and Methods

Sampling sites

Mussels for on board physiological experiments were collected at 4 hydrothermal vent fields on the Mid-Atlantic Ridge (MAR). Specimens of *Bathymodiolus puteoserpentis* were sampled at Logatchev on the northern MAR (14°45'N). Specimens belonging to an undescribed *Bathymodiolus* species (*B. sp.*) were collected at Turtle Pits, Comfortless Cove (4°48'S) and Lilliput (9°33'S) on the southern MAR (Haase *et al.*, 2007a; Haase *et al.*, 2007b) (Figure 1). This species apparently differs from *B. puteoserpentis* (N.N. – personal communication) but has not yet been named. Mussels were sampled using nets (40 cm length, 20 cm diameter opening, mesh size 335 µm [Hydrobios, Kiel, Germany]) handled by the manipulator arm of the ROV Quest (Research Center Ocean Margins, Bremen, Germany) or Jason (Woods Hole Oceanographic Institution, Woods Hole, MA, USA). Tissues from these mussels were also used for molecular purposes. Besides, *B.* cf. *thermophilus* from a hydrothermal vent field on the Pacific-Antarctic Ridge (PAR), *B. brooksi*, *B. heckerae*, and *"B." childressi* from cold seep sites in the Gulf of Mexico (GoM) as well as *B.* cf. *boomerang* from a seep at the Gabon-Congo-Angola Continental Margin (GCM) (Olu-Le Roy *et al.*, 2007a; Olu-Le Roy *et al.*, 2007b) were sampled for molecular purposes only and processed as described previously (Duperron *et al.*, 2005; Duperron *et al.*, 2007; Zielinski *et al.*, 2009). Sampling sites and coordinates are listed in Table 1.

Mussel dissection

For hydrogen and sulfide consumption experiments and $^{14}CO_2$ incorporation incubations tissues from 3 and 2 individuals, respectively were used in each experiment. Mussels were opened with a scalpel by cutting through the posterior and anterior adductor muscles. Viability was tested by prodding the food with a dissection needle and only mussels whose foot contracted were used. The foot and both gills were separated from the remaining tissue using dissection scissors. Tissue pieces of 6 mm in diameter were cut out of the gill and the foot tissues using a steel hole puncher. One tissue piece from each individual was frozen in liquid nitrogen for weight determination in the home laboratory. For negative controls, foot tissue that does not contain endosymbiotic bacteria as well as boiled gill tissue was used.

Table 1. Sampling sites and coordinates of mussel specimens

Ocean / Gulf Field	**Ridge / CM** Site	H_2 at site [µM]	**Cruise name** H_2S at site [µM]	**Vessel** Depth [m]	**Leg** Latitude	**ROV / HOV** Longitude	**month and year** Date	**chief scientist** Dive (sample ID)	Purpose[a]
Atlantic	**Mid-Atlantic Ridge**		**Hydromar II**	**Meteor**	**M64/2**	**Quest**	**May 2005**	**K. S. Lackschewitz**	
			Hydromar III	**Maria S. Merian**	**MSM04/3**	**Jason**	**Feb 2007**	**C. Borowski**	
Logatchev	Irina II	5.9[b]	6[c]	3037	14°45.1629'N[d]	44°58.7478'W[d]	23 May 05	58 (266ROV/7)	H_2, *hynL*
		4.1[e]	nd	3020	14°45.1682'N	44°58.7475'W	30 Jan 07	J2-256 (253ROV/8)	H_2S, ^{14}C
		196.2[e]	< 6[f]	3035	14°45.1791'N	44°58.7627'W	3 Feb 07	J2-259 (263ROV/12)	H_2S, ^{14}C
		nd	nd	3033	14°45.1783'N	44°58.7641'W	4 Feb 07	J2-260 (267ROV/1)	H_2S, ^{14}C
	Quest	nd	nd	3046	14°45.1747'N[d]	44°58.8359'W[d]	22 May 05	57 (263ROV/5)	H_2
		4.2[b]	70[e]	3045			26 May 05	61 (281ROV/3)	H_2, *hynL*
		1178.6[e]	32[f]	3028	14°45.1818'N	44°58.8309'W	5 Feb 07	J2-261 (271ROV/5)	H_2S, ^{14}C
			Marsued II	**Meteor**	**M64/1**	**Quest**	**April 2005**	**K. Haase**	
			Marsued III	**Meteor**	**M68/1**	**Quest**	**May 2006**	**A. Koschinsky**	
Wideawake	-	?	?	2987	4°48.62'S	12°22..35'W	12 Apr 05	39 (125ROV12)	*hynL*
		16.8[g]	59[f]	2987	4°48.64'S	12°22.37'W	10 May 06	86 (3ROV/6)	H_2S
		0.2[g]	115[f]	2986			12 May 06	88 (12ROV/9)	H_2S
Comfortless Cove	Sisters Peak	nd	nd	2996	4°48.19'S	12°22.30'W	14 May 06	89 (20ROV/1)	H_2
	Golden Valley	0.1[g]	nd	2984	4°48.17'S	12°22.27'W	15 May 06	90 (24ROV/5)	H_2
Lilliput	Main-Lilliput	0.02[h]	nd	1495	9°32.86'S	13°12.56'W	25 Apr 05	47 (200ROV/9)	*hynL*
	Limtoc	0.03[g]	nd	1495	9°32.96'S	13°12.53'W	18 May 06	91 (39ROV/6)	H_2, H_2S
		0.1[g]	6[f]	1497			27 May 06	95 (70ROV/8)	H_2, H_2S
East Pacific	**Pacific-Antarctic Ridge**		**Foundation III**	**Sonne**	**SO157**	**-**	**Jun/Jul 01**	**P. Stoffers**	
German Flats		nd	nd	2212	37°47.5'S	110°54.9'W	28 Jun 01	(30GTV-2 & 3)	*hynL*
Atlantic	**Gabon Continental Margin**		**Biozaire 2**	**?**	**?**	**Victor 6000**	**Nov 2001**	**M. Sibuet**	*hynL*
	Regab	nd	nd	3150 m	5°52.8'S	9°37.9'E	?	? (Ang M5)	*hynL*
Gulf of Mexico	**West Florida Escarpment**		**Deep Seeps 11/1**	**?**	**?**	**Alvin**	**Oct 2003**	**C. R. Fisher & B. Carney**	
		nd	nd	3284 m	26°02.0'N	84°55.0'W	?	3915 (GoM M6)	*hynL*
	Lousiana continental slope								
	Atwater	nd	nd	1893	27°34.1'N	88°29.8'W	?	3918 (GoM M24)	*hynL*
	Alaminos	nd	nd	2226	26°21.3'N	94°30.1'W	?	3923 (GoM M29)	*hynL*
	Alaminos	nd	nd	2226	26°21.3'N	94°30.1'W	?	3924 (GoM M34)	*hynL*

[a] Legend: H_2, hydrogen consumption experiments; H_2S, sulfide consumption experiments; ^{14}C, $^{14}CO_2$ incorporation experiments; *hynL*, molecular investigation for membrane-bound respiratory hydrogen uptake [NiFe]-hydrogenases;[b] T. Pape and R. Seifert, personal communication; [c] values from (Schmidt *et al.*, 2007) and A. Koschinsky, personal communication; [d] due to inaccurate positioning during the Hydromar II cruise coordinates given are based on highly accurate data obtained during the Hydromar III cruise (Borowski *et al.*, 2007); [e] R. Keir and O. Schmale, personal communication; [f] A. Koschinsky and Katja Schmidt, personal communication; [g] R. Seifert, personal communication; [h] values from (Haase *et al.*, 2007a); Abbreviations: CM, Continental Margin; nd, not determined

Hydrogen consumption incubations

Experimental setup

For hydrogen partial pressures up to 1000 ppm (in helium) glass serum vials (58°ml) were fully filled with chilled (4°C) sterile-filtered (0.22 µm) bottom seawater retrieved from 3000 m water depth. One piece of gill tissue was placed in the vial, the vial closed with a gas-tight rubber stopper avoiding any inclusion of air bubbles, and crimped with aluminum seals. Control vials contained foot tissue, boiled gill tissue or no tissue at all. 20 ml of hydrogen gas (100 ppm, 250 ppm or 1000 ppm in helium, Linde) were injected through the rubber stopper using a gas-tight syringe with a second syringe allowing pressure compensation through the outflow of seawater. For hydrogen partial pressures above 1000 ppm (in air) glass serum vials were progressed as described above with the exception that the vials were filled with only 38 ml of seawater. Pure hydrogen gas (100%) (Air Liquide) was then injected in the air headspace through the rubber stopper to the desired final concentration (2100 ppm and 3000 ppm) using a gas-tight syringe. All vials were placed upside down to avoid possible gas exchange via the rubber stopper and incubated at 4°C on a slowly rotating table. At given time points a subsample was taken with a gas-tight syringe from the headspace with the pressure decrease compensated through the inflow of chilled sterile-filtered seawater from a second syringe.

Analysis of the headspace hydrogen content

The H_2 concentration in the headspace was determined using a gas chromatograph (Thermo Trace GC ultra) equipped with a packed stainless steel column (Molecular Sieve 5A, carrier gas: He) and a pulse discharge detector (PDD). Recording and calculation of results was performed using a PC operated integration system (Thermo Chrom Card A/D). Analytical procedures were calibrated daily with commercial gas standards (Linde).

Incubation conditions

In incubation vials containing hydrogen up to 1000 ppm in helium as headspace gasses the equilibrium between dissolved and headspace gasses led to oxygen degassing and hydrogen/helium dissolution. The theoretical concentrations of dissolved hydrogen and oxygen as well as the partial pressure of oxygen in the headspace after establishment of this equilibrium were calculated from (Crozier and Yamamoto, 1974) and (Weiss, 1970) under the assumption of Henry's law i.e. that the concentration of a dissolved gas in a solution is directly proportional to the partial pressure of that gas above the solution. The molar volume of an ideal gas (22.414 $l\ mol^{-1}$) was used to convert between the partial pressure of a gas (ppm) and the amount of that gas (moles) in the headspace. Based on these theoretical calculations, oxygen conditions in the incubation vials were sufficient for complete hydrogen oxidation, yet microaerobic. As is typical for mytilid bivalves oxygen consumption rates of Bathymodioline mussels decline with decreasing oxygen concentrations (Kochevar *et al.*, 1992). Therefore, gill oxygen consumption rates are only low under microaerobic conditions (Childress *et al.*, 1986; Kochevar *et al.*, 1992) and do not account for substantial oxygen removal from vials during the time of incubation. Furthermore, the addition of oxygen-saturated seawater for pressure compensation with each subsample taken from the headspace for hydrogen analysis regularly replenished the vials with oxygen. Table 2 lists the calculated dissolved hydrogen and oxygen concentrations under 1 atmosphere of helium or air and different hydrogen partial pressures.

Table 2. Incubation conditions at different H_2 partial pressures in a 58 ml incubation vial filled with 38 ml seawater (4°C, 35 $^o/_{oo}$)

H_2 partial pressure [ppm]	Major headspace gas	O_2 partial pressure [%]	H_2 [nmol]	O_2 [µmol]	O_2/H_2 ratio	Dissolved H_2 [nM]	Dissolved O_2 [µM]	O_2/H_2 ratio in solution
100	helium	1*	89	9	101	77	11	143
250	helium	1*	223	9	40	194	11	57
1000	helium	1*	892	9	10	775	11	14
2100	air	21	1874	187	100	1627	324	199
3000	air	21	2677	187	70	2325	324	139

*Although incubation conditions were microaerobic under a helium headspace mitochondrial respiration likely accounted only for low oxygen consumption rates under these conditions (Childress *et al.*, 1986; Kochevar *et al.*, 1992). Furthermore, oxygen was regularly replenished with the addition of oxygen-saturated seawater each time a subsample was taken from the headspace for hydrogen analysis. Thus, oxygen concentrations were sufficient for hydrogen oxidation. Note that for the complete oxidation of hydrogen only half the amount of oxygen is required ($H_2 + ½ O_2 \rightarrow H_2O$).

Sulfide consumption incubations

For sulfide consumption incubations glass serum vials and tissue pieces were prepared as described for hydrogen consumption incubations with the exception that the seawater to headspace ratio (v/v) was ⅓ and that the headspace always contained air. Control vials contained foot tissue or no tissue at all. A Na_2S stock solution was added to the seawater to a final concentration below 50 µM. The initial dissolved sulfide concentration was determined immediately after sulfide addition as described below and varied between 12 and 41 µM. All vials were incubated at 4°C on a slowly rotating table. Within 3 hours after sulfide addition subsamples were taken and their sulfide content fixed in a zinc acetate solution (2%, w/v). The concentration of the resulting ZnS precipitate was measured spectrophotometrically at 663 nm by means of the methylene blue method according to Pachmayr as described in (Trüper and Schlegel, 1964). The assay volume was adjusted to 10 ml.

Rate calculation

The amount of hydrogen gas in the headspace (in moles) was calculated from the hydrogen concentration (in ppm) using the molar volume of an ideal gas (22.414 l mol^{-1}) for conversion. The rates of hydrogen removal from the headspace and sulfide removal from the seawater (in nmol h^{-1} (piece tissue)$^{-1}$) were calculated for the first 1 to 3 hours after addition of the electron source performing linear regression through the data points obtained during this incubation period and computing the slope. The effect of methodological hydrogen and sulfide removal from the headspace and seawater, respectively through subsampling was considered. The rates at which hydrogen and sulfide vanished from incubation vials containing only seawater but no tissues (chemical oxidation, hydrogen loss by diffusion) were subtracted from the tissue rates. Resulting rates were then normalized to gram wet weight (in nmol h^{-1} (g wet weight)$^{-1}$).

$^{14}CO_2$ fixation incubations

Incubation conditions

For $^{14}CO_2$ incorporation experiments in the presence of hydrogen or sulfide (H_2S / HS^-) gill tissue pieces of 6 mm in diameter were prepared as described for hydrogen incubations and incubated for 30, 60, 90, 120, and 180 minutes. Care was taken to use gill tissues from the same individual for all time points and negative controls. Glass serum vials (58°ml) were filled with seawater, one piece of gill tissue, and closed and crimped as described above with the exception that only 10 ml of seawater was added. Vials were supplemented with hydrogen gas or Na_2S to a final concentration of 20 µM while negative controls were left unsupplemented. Pure hydrogen gas (100%) (Air Liq-

uide) was injected to the air headspace through the rubber stopper to a final concentration of 2.6% using a gas-tight syringe thus resulting in a dissolved H_2 concentration of 20 µM (at 4°C, 35 ‰) (Crozier and Yamamoto, 1974). To facilitate the establishment of the equilibrium between hydrogen in the headspace and hydrogen in solution, a stock solution of dissolved hydrogen gas (775 µM, 1 atm H_2 [100%], 4°C, 35 ‰) was injected to the seawater through the rubber stopper to a final concentration of 20 µM using a gas-tight syringe. All vials were supplemented with $^{14}CO_3^{2-}$ to a final activity of 30 kBq/ml (stock solution 37 MBq/ml; Perkin Elmer). Vials were turned upside down and incubated as described above. CO_2 fixation was stopped by adding formaldehyde to a final concentration of 4%.

Measurements of $^{14}CO_2$ incorporation

After formaldehyde treatment the vials were placed upright without shaking to ensure sedimentation of gill tissue. Vials were then opened and a 2 ml seawater subsample was added to a scintillation cocktail (Ultima Gold, Perkin Elmer) to determine the remaining $^{14}CO_3^{2-}$ activity in the medium. To expel the remaining $^{14}CO_3^{2-}$ from the seawater the pH was adjusted to < 2 by adding 1 M HCl. Acidified vials were placed on a shaker under an exhaust hood and shaken for at least 12 h at maximum speed to ensure complete expulsion of CO_2. To determine the remaining $^{14}CO_2$ activity in the medium after acidification and CO_2 expulsion a seawater subsample was pipetted into a scintillation cocktail. The remaining seawater medium with the gill tissue was poured through a 0.22 µm filter until visually dry. The filter with the gill tissue was covered with a scintillation cocktail and the ^{14}C activity measured with a liquid scintillation counter (Tri-Carb 3100TR, Perkin Elmer).

Amplification of the gene coding for the large subunit (hynL) of a membrane-bound respiratory hydrogen uptake [NiFe]-hydrogenase

DNA extraction, cloning, and sequencing

Total DNA was extracted from gill tissues according to the method described by (Zhou *et al.*, 1996). The previously published primers HUPLX1 and HUPLW2 targeting the highly conserved N- and C-terminal regions of the large subunit of [NiFe] hydrogenases (Csáki *et al.*, 2001) were used to amplify a fragment of approximately 1700 bp length. The reaction mixtures for PCR amplification contained 50 pmol of each primer, 2.5 µmol of each dNTP, 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 amplification program consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 45°C for 1.5 min, and 72°C for 2 min, and a final elongation step at 72°C for 10 min. Due to unspecific amplification, PCR products were separated on a 1% agarose gel. The appropriate band was cut from the gel and purified from gel pieces with the QIAquick PCR purification kit (QIAGEN). Prior to cloning, the PCR product was directly sequenced to confirm specific amplification. Sequences were compared with entries in the public database by using the tBLASTx search algorithm (Altschul *et al.*, 1997). Separated and purified PCR products were ligated at 4°C overnight with the pGEM-T Easy vector (Promega) in the following reaction mixture: 1 × rapid ligation buffer, 50 ng pGEM-T Easy vector, 3 µl PCR purification product, 3 Weiss units T4 DNA ligase, made up to a final volume of 10 µl with sterile water. The ligation product was used for transformation with the TOPO-TA kit (Invitrogen). Clone libraries of 96 clones were constructed for each individual. The insert size was controlled by PCR screening with vector primers M13F and M13R (Yanisch-Perron *et al.*, 1985). 16 partial sequences were obtained for each individual. Partial sequences were

aligned and compared with BioEdit (Hall, 1999) using the Clustal W implementation (Thompson *et al.*, 1994). An internal primer HUPL540F (5'-GAAGCAGATTTAATGGCGGTAGC-3') was designed for full sequencing by manual identification of an appropriate site in the alignment. 2 clones from each individual were chosen for full sequencing. Full sequencing was done using plasmid preps (QIAprep Miniprep kit, QIAGEN) and assembled using Sequencher (www.genecodes.com). Sequencing reactions were performed by using ABI BigDye and an ABI PRISM 3100 genetic analyser (Applied Biosystems).

All *hynL* gene sequences obtained from *B. puteoserpentis* (Logatchev – MAR), *B. sp.* (Wideawake – MAR), *B. sp.* (Lilliput – MAR), and *B.* cf. *thermophilus* (German Flats – PAR) have been deposited at the EMBL database (Kulikova *et al.*, 2007) under accession numbers X00000-X00000, respectively.

Phylogenetic reconstruction

Almost complete *hynL* gene sequences were imported into BioEdit and translated to the corresponding amino acid sequence. Protein sequences used to reconstruct hydrogenase phylogeny in a previous review (Vignais *et al.*, 2001) were imported into BioEdit and aligned with the sequences generated in this study. The alignment generated in BioEdit was imported into ARB (Ludwig *et al.*, 2004), where it was manually corrected. Parsimony and maximum likelihood trees were calculated with the ARB package.

Results

H_2 consumption in B. puteoserpentis (Logatchev) and B. sp. (Comfortless Cove and Lilliput)

Gill tissues of *B. puteoserpentis* collected from the ultramafic-hosted Logatchev vent field with high in situ hydrogen concentrations consumed hydrogen rapidly from the incubation vials. In contrast, gill tissues collected from the basalt-hosted Comfortless Cove and Lilliput vent fields with low in situ hydrogen concentrations consumed hydrogen only moderately. In incubation vials containing the negative controls boiled gill tissue, symbiont-free foot tissue, and 0.22 μm filtered seawater without any tissue hydrogen decreased only marginally (Figure 2). Within nine hours Logatchev gill tissues consumed ~ 72% of the hydrogen gas whereas Comfortless Cove and Lilliput tissues consumed ~ 21% and ~ 16%, respectively. The controls accounted for a loss of only ~ 6%. Due to an unfavorable ratio of headspace to seawater volume hydrogen no longer sufficiently dissolved in the seawater after three measurements (20 hours of incubation) and was thus not fully consumed from the headspace. The hydrogen consumption rates were calculated for the first 1-3 hours and were similar for the two Logatchev sampling sites Irina II and Quest. At 100 ppm hydrogen partial pressure Irina II and Quest gill tissues consumed hydrogen at a rate of 478 ± 197 (n = 3) and 493 ± 221 (n = 3) nmol h^{-1} (g wet weight)$^{-1}$, respectively. Hydrogen consumption by *B. sp.* gill tissues was 6-fold lower in Comfortless Cove mussels and 13-fold lower in Lilliput mussels.

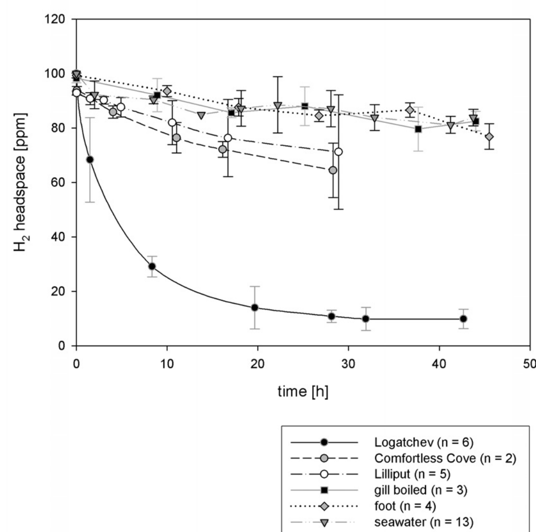


Figure 2. Hydrogen consumption over time in mussel gill tissues. Hydrogen concentrations decreased rapidly in vials with symbiont-containing gill tissues from Logatchev but only moderately in vials with gill tissues from Comfortless Cove and Lilliput. Symbiont-free foot tissue, boiled gill tissue, or seawater barely consumed hydrogen.

Hydrogen consumption rates in vials containing boiled gill tissues, foot tissues, and 0.22 µm filtered seawater without any tissue were only low and in the same range with 0.1 ± 0.1 (n = 3), 0.2 ± 0.1 (n = 4), and 0.2 ± 0.1 nmol h^{-1} (n = 13), respectively (Figure 3).

Table 3 Hydrogen consumption rates at different partial pressures in mussels from vent fields with high (Logatchev) and low (Comfortless Cove and Lilliput) hydrogen concentrations

vent field / control	site	tissue	H_2 headspace [ppm]	Rate (Mean ± SD) [nmol h^{-1} (g wet weight)$^{-1}$]	n	H_2 at site [µM][a]
Logatchev	Irina II, Quest	gill	100	486 ± 187	6	4.2-5.9[b]
	Irina II	gill	100	478 ± 197	3	5.9[b]
	Quest	gill	100	493 ± 221	3	4.2[b]
Comfortless Cove	Sisters Peak	gill	100	77 ± 21	2	nd[c]
	Sisters Peak, Golden Valley	gill	1000	446 ± 223	5	0.1[d]
Lilliput	Limtoc	gill	100	37 ± 45	5	0.03[e]
		gill	250	71 ± 32	4	
		gill	2100	153 ± 38	3	
		gill	3000	377 ± 141	3	
Controls	Irina II	gill boiled	100	0.1 ± 0.1*	3	5.4[b]
	Irina II, Sisters Peak, Limtoc	foot	100	0.2 ± 0.1*	4	-
	-	seawater	100	0.2 ± 0.1*	13	-

[a] as determined by discrete sampling and analysis on board; [b] T. Pape and R. Seifert, personal communication; [c] hydrogen concentrations in Sisters Peaks diffuse fluids were not determined, the endmember concentration at Sisters Peak was 61 µM (Haase *et al.*, 2007b); [d] value refers to the Golden Valley site; [e] values from (Haase *et al.*, 2007a) and A. Koschinsky, personal communication; *rate in nmol h^{-1}, nd – not determined

Effect of H_2 partial pressure on H_2 consumption rate

To investigate the effect of H_2 partial pressure on H_2 consumption rates gill tissues from Comfortless Cove and Lilliput mussels were incubated at partial pressures higher than 100 ppm. At Comfortless Cove a tenfold increase in partial pressure resulted in a 4-fold increase in H_2 consumption. At Lilliput where only little hydrogen was consumed at 100 ppm an increase in partial pressure to 250 ppm stimulated hydrogen consumption 2-fold (Figure 4). At 2100 ppm and 3000 ppm gill tissues showed 4 and 10-fold higher consumption rates (Figure 5). The values for all consumption rates are listed in Table 3.

Hydrogen vs. sulfide consumption rates

For comparison of hydrogen with sulfide consumption rates gill tissues from the ultramafic-hosted Logatchev field and the basalt-hosted vent fields at 4°48’S were also incubated in the presence of sulfide. Sulfide consumption rates of *B. puteoserpentis* (Logatchev) and *B. sp.* (Wideawake and Lilliput) gill tissues were in the same range. *B. puteoserpentis* and *B. sp.* gill tissues from the Logatchev Quest and Wideawake sites consumed sulfide at a rate of 9.2 ± 3.8 (n = 3) and 8.8 ± 1.2 (n = 4) µmol h^{-1} (g wet weight)$^{-1}$, respectively. *B. puteoserpentis* and *B. sp.* gill tissues from the Logatchev Irina II and Lilliput Limtoc sites consumed sulfide at 3 to 4-fold lower rates (Table 4). There was no apparent correlation between the sulfide consumption rates and the total sulfide concentrations at the sampling sites. In general, sulfide consumption rates were higher than hydrogen consumption rates, however, hydrogen con-

sumption experiments were performed at much lower concentrations (0.08-2.3 µM dissolved hydrogen versus 12-41 µM total sulfide). For the Logatchev mussel *B. puteoserpentis* extrapolation to 5 µM dissolved hydrogen which is consistent with minimum in situ concentrations suggests 3 to 9-fold higher hydrogen than sulfide consumption rates (31.3 µmol h^{-1} (g wet weight)$^{-1}$, Figure 6).

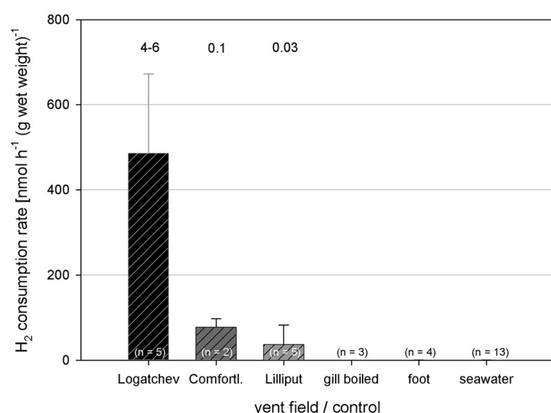


Figure 3. Hydrogen consumption rates in mussels from vent fields with high (Logatchev) and low (Comfortless Cove and Lilliput) hydrogen concentrations. Hydrogen consumption rates were much higher in the gill tissues of mussels from Logatchev than in those from Comfortless Cove and Lilliput Numbers in the upper part of the graph denote the hydrogen concentrations at the sampling sites. Rates for boiled gill, foot and plain seawater are in nmol h^{-1}.

$^{14}CO_2$ Incorporation in the presence of H_2 and H_2S

To investigate if hydrogen consumption is linked to energy conservation and thus CO_2 fixation, gill tissues from Logatchev mussels were incubated with hydrogen in seawater containing $^{14}CO_3^{2-}$ (30 kBq ml^{-1}). Control gill tissues were incubated in the presence of sulfide or without an electron source. Under aerobic conditions (1 atm) and in the presence of hydrogen (2.6% H_2 partial pressure, 20 µM dissolved H_2) or sulfide (20 µM) gill tissues clearly incorporated $^{14}CO_3^{2-}$. After two hours of incubation ^{14}C tissue activities were 3.3 (H_2) and 3.9-fold (H_2S) higher than ^{14}C activities of gill tissues that were incubated without an electron source (Figure 7).

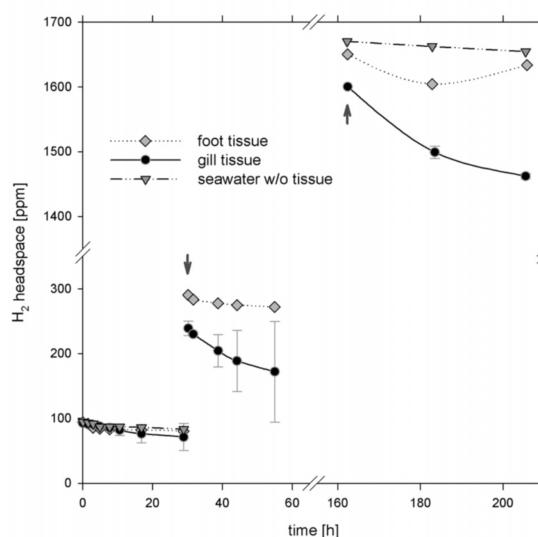


Figure 4. Stimulation of hydrogen consumption in *B. sp.* (Lilliput). At 100 ppm hydrogen was hardly consumed. An increase in partial pressure (arrow) stimulated hydrogen consumption and resulted in an increase in consumption rates. The effect was observed for nearly nine days.

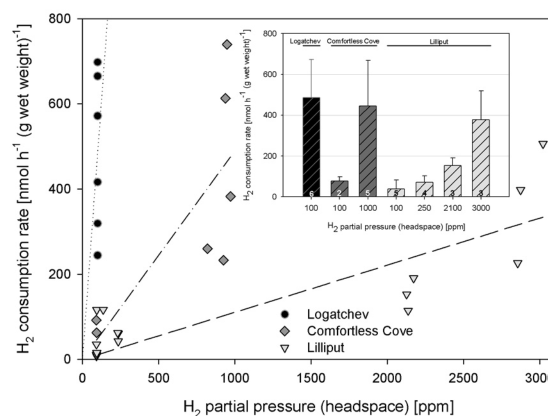


Figure 5. Effect of partial pressure on H_2 consumption rates in *B. sp.* (Comfortless Cove and Lilliput). Consumption rates generally increased with increasing partial pressures. Extrapolation from Logatchev consumption rates at 100 ppm partial pressure (77 nM dissolved H_2) (dotted line) suggests extraordinary high consumption rates at in situ conditions. Inset: Bar chart of the same data with error bars.

Table 4 Sulfide consumption rates in mussels from ultramafic-hosted (Logatchev) and basalt-hosted (Wideawake and Lilliput) hydrothermal vent fields

vent field / control	site	tissue	S^{2-}_{tot} [µM][a]	S^{2-}_{tot} rate (Mean ± SD) [µmol h^{-1} (g wet weight)$^{-1}$]*	n	S^{2-}_{tot} at site [µM][b]
Logatchev	Irina II	gill	12-16	3.3 ± 1.0	10	0.4-6.0[c]
	Quest	gill	20	9.2 ± 3.8	3	32-70[c]
Wideawake	-	gill	34-37	8.8 ± 1.2	4	59-115[d]
Lilliput	Limtoc	gill	18-41	2.2 ± 0.8	6	31-36[d]
Controls	Irina II, Quest	foot	12-20	-	5	
	Wideawake	foot	34-37	0.1 ± 0.1	2	
	Limtoc	foot	18-41	0.3 ± 0.1	2	

[a] sulfide concentrations at which incubations were performed; [b] sulfide concentrations as determined by discrete sampling and analysis on board; [c] values from (Schmidt *et al.*, 2007) and A. Koschinsky, personal communication; [d] A. Koschinsky and H. Strauss, personal communication; * rate of sulfide loss (chemical oxidation) from incubation vials containing only 0.22 µm filtered seawater and no tissue is considered.

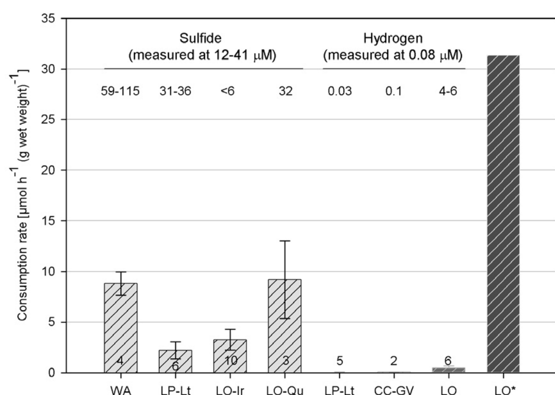


Figure 6. Sulfide and hydrogen consumption rates of *Bathymodiolus* gill tissues from basalt- and ultramafic hosted vent fields. Sulfide consumption rates measured at 12-41 µM (left four bars) were considerably higher than hydrogen consumption rates measured at 0.08 µM (following three bars). However, extrapolation of hydrogen consumption rates of Logatchev mussels to 5 µM, consistent with minimum Logatchev in situ hydrogen concentrations, suggests that hydrogen consumption rates may be 3 to 9-fold higher than sulfide consumption rates at Logatchev (last bar). Numbers in the upper part of the graph denote the sulfide and hydrogen concentrations, respectively at the sampling site as determined by discrete sampling (see Table 3 and Table 4 for references). Numbers at the bottom of the graph denote the number of investigated individuals. Abbreviations: WA – Wideawake; LP-Lt – Lilliput, Limtoc; LO-Ir – Logatchev, Irina II; LO-Qu – Logatchev, Quest; CC-GV – Comfortless Cove, Golden Valley; LO – Logatchev, Irina II and Quest; LO* - Logatchev hydrogen consumption rate extrapolated to 5 µM.

Membrane-bound respiratory hydrogen uptake [NiFe]-hydrogenase

B. puteoserpentis (Logatchev) gill tissues from individuals used for hydrogen consumption experiments were subsequently examined for the presence of bacterial membrane-bound respiratory hydrogen uptake [NiFe]-hydrogenases and the gene coding for the large subunit (*hynL*) of this group of hydrogenases could be amplified. In the light of this result other bathymodiolin mussels both from basalt-hosted vent fields and cold seeps were also investigated for the presence of the *hynL* gene. Whereas the vent mussels *B. sp.* (Lilliput and Wideawake) and *B.* cf. *thermophilus* (German Flats, PAR) yielded *hynL* amplification products attempts to amplify this gene from the seep mussels *B. brooksi*, *B. heckerae*, and *“B.” childressi* from the Gulf of Mexico as well as *B.* cf. *boomerang* from the Gabon-Congo-Angola Continental Margin have so far been unsuccessful. *B. sp.* (Comfortless Cove) was not included in this study. All *hynL* amplification products comprised 1636 bp translating into 545 amino acids and showed >95% sequence similarity at the amino acid level. Phylogenetic analyses based on the HynL amino acid sequence confirmed that *Bathymodiolus* hydrogenases fall within the group of membrane-bound

respiratory hydrogen uptake [NiFe]-hydrogenases (Figure 8). All *Bathymodiolus* hydrogenases form a monophyletic cluster and are most closely related to the hydrogenase from the alphaproteobacterial chemolithoautotroph *Oligotropha carboxidovorans* with a sequence similarity of 78%.

Discussion

Hydrogen is consumed by endosymbionts of *bathymodiolin mussels*

Bathymodiolin mussels along the Mid-Atlantic Ridge (MAR) live in a dual symbiosis with chemosynthetic bacteria which are housed in gill bacteriocytes (DeChaine and Cavanaugh, 2006; Distel *et al.*, 1995; Duperron *et al.*, 2006). Either one of two energy sources has to date been shown to be utilized by these endosymbionts: reduced sulfur compounds and methane (DeChaine and Cavanaugh, 2006). Here we have shown that gill tissues of MAR bathymodiolin mussels consumed hydrogen. Gill tissues of *B. puteoserpentis* from the ultramafic-hosted Logatchev vent field (14°45'N) with high in situ hydrogen concentrations consumed hydrogen rapidly whereas *B. sp.* gill tissues from the basalt-hosted vent fields Comfortless Cove (4°48'S) and Lilliput (9°33'S) consumed hydrogen only moderately. The fact that symbiont-free foot tissues and boiled gill tissues consumed no hydrogen above the seawater controls indicates that hydrogen consumption is mediated by the the chemosynthetic endosymbionts in the gill tissues. Hydrogen consumption by chemosynthetic endosymbionts of hydrothermal vent invertebrates has not been shown previously for any of the deep-sea vent invertebrates.

Hydrogen consumption is coupled to CO_2 incorporation

Candidate hydrogenases for hydrogen consumption

The fact that symbiont-containing gill tissues consumed hydrogen points to the existence of hydrogenases which catalyze the reversible oxidation of molecular hydrogen. Hydrogenases comprise three phylogentically distinct classes, [NiFe]-hydrogenases, [FeFe]-hydrogenases, and iron-sulfur cluster-free [Fe]-hydrogenases (Schwartz and Friedrich, 2006; Vignais *et al.*, 2001; Vignais and Colbeau, 2004; Vignais *et al.*, 2004). The latter class has thus far only been detected in methanogenic archaea (Vignais and Colbeau, 2004). [FeFe]-hydrogenases occur mainly in bacteria but are rather involved in hydrogen production (Vignais *et al.*, 2004). Thus, these two classes of hydrogenases are unlikely candidates for hydrogen consumption by bathymodiolin gill tissues. [FeFe]-hydrogenases have also been found in eukaryotes but appear to be restricted to anaerobic hydrogenosome-possessing protists and chloroplasts of green algae (Vignais *et al.*, 2001; Vignais *et al.*, 2004). Therefore it is highly unlikely that bathymodiolin hydrogen consumption is of eukaryotic origin. The most likely candidate for hydrogen consumption by bathymodiolin gill tissues are [NiFe]-hydrogenases of endosymbiotic origin. First, [NiFe]-hydrogenases tend to be involved in hydrogen consumption, second, they appear to be present only in bacteria and archaea and third, to date they have remained undetected in eukaryotes (Vignais *et al.*, 2001; Vignais *et al.*, 2004).

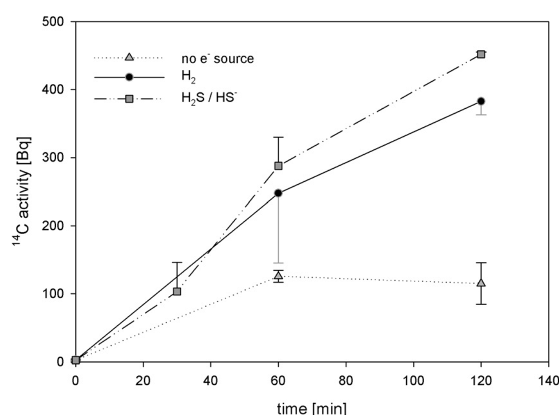


Figure 7. ^{14}C activity in Logatchev gill tissues after incubation with $^{14}CO_3^{2-}$ in the presence of hydrogen or sulfide and without an electron source. CO_2 fixation is clearly stimulated in the presence of hydrogen and sulfide. CO_2 incorporation rates are comparable for both electron sources. Data are from mussels collected from the Quest site. Gill tissues of two individuals each were used in the incubations. Error bars for H_2 and H_2S plots are given in only one direction.

0.10

Rubrivax gelatinosus HupL P17632 β
Ralstonia eutropha HoxG P31891 β
Pseudomonas hydrogenovora HupL Q51862 γ
Alcaligenes hydrogenophilus HupL P33374 β
***Bathymodiolus sp.* symbiont (Wideawake, 4°48'S - MAR) HynL X00000** γ
***Bathymodiolus sp.* symbiont (Lilliput, 9°33'S - MAR) HynL X00000** γ
B. cf. *thermophilus* symbiont (German Flats 37°47'S - PAR) HynL X00000 γ
B. *puteoserpentis* symbiont (Logatchev 14°45'N - MAR) HynL X00000 γ
Oligotropha carboxydovorans HoxL O33406 α
Rhizobium leguminosarum HupLP18636 α
Bradyrhizobium japonicum HupL P12636 α
Rhodobacter sphaeroides HupL O86468 α
Rhodobacter capsulatus HupL P15284 α
Thiocapsa roseopersicina HupL Q56360 γ
Escherichia coli HyaB P19927 γ
Citrobacter freundii HyaB Q46046 γ
Thiocapsa roseopersicina HydL O51823 γ
Azotobacter chroococcum HupL P18191 γ
Azotobacter vinelandii HoxG P21949 γ
Aquifex aeolicus HupL O66895
Helicobacter pylori strain 26695 HynL O25349 ε
Helicobacter pylori strain J99 HynL Q9ZLK4 ε
Wolinella succinogenes HynL P31883 ε
Campylobacter jejuni HynL Q9PN32 ε
Archaeoglobus fulgidus VhtA O28891
Desulfomicrobium baculatus HysA P13065 δ
Desulfitobacterium dehalogenans HynL Q9RPI2
10 ***Group 2 hydrogenases***

98/95; 100/100; 50/69; 84/98; 100/100; 87/95; 100/100; 45/71; 86/ 76; 80/ 76; 100/81; 100/100; 90/99; 100/ 100; 90/100; 100/100; 100/100; 86/59; 100/100; 100/100; 39/100; 100/100; 100/100

Membrane-associated respiratory uptake [NiFe] hydrogenases (Group1)

Figure 8. Maximum likelihood phylogenetic tree of membrane-bround respiratory hydrogen uptake (Group 1) [NiFe]-hydrogenases. The topology of the tree is confirmed by maximum parsimony. Numbers at nodes denote maximum likelihood and parsimony bootstrap values, respectively. The tree is based on the amino acid sequences of the large subunits. *Bathymodiolus* HynL sequences had 508 amino acids and the complete alignment comprised 979 amino acids. Sequences from *Bathymodiolus* endosymbionts are shown in bold. Cytoplasmic heterodimeric (Group 2) [NiFe] hydrogenases were included as an outgroup. The scale bar represents 10% sequence divergence. The phylogenetic position of hydrogenase possessing proteobacteria is indicated on the right hand site (- . Abbreviations: MAR, Mid-Atlantic Ridge; PAR, Pacific-Antarctic Ridge

Candidate [NiFe]-hydrogenases for hydrogen consumption

Two phylogenetically distinct groups of [NiFe]-hydrogenases could in theory account for endosymbiotic hydrogen consumption, a membrane-associated respiratory hydrogen uptake [NiFe]-hydrogenase (Group 1) of the membrane-bound and periplasmically oriented type or a cytoplasmic heteromultimeric reversible [NiFe]-hydrogenase (Group 3) of the bidirectional NAD-linked type (Group 3d) (Vignais *et al.*, 2001). Group 1 [NiFe]-hydrogenases are directly involved in energy conservation along the respiratory chain. They split hydrogen on the periplasmic site of the cell membrane and feed the electrons into the respiratory chain. Thus they contribute to a proton motive force and subsequent ATP production (Vignais *et al.*, 2001; Vignais and Colbeau, 2004; Vignais *et al.*, 2004). Group 3d [NiFe]-hydrogenases couple the oxidation of hydrogen to the reduction of $NAD(P)^+$ or vice versa. Thus they may generate NAD(P)H needed for CO_2 fixation via the Calvin cycle and biosynthetic pathways. However, they are not directly involved in energy conservation. Yet, they may indirectly contribute to a proton motive force if the generated NADH is reoxidized by a respiratory NADH dehydrogenase. Direct coupling of a NAD(P)-dependent bidirectional [NiFe]-hydrogenase with a respiratory NADH dehydrogenase and thus energy conservation has for example been suggested for the cyanobacterium *Synechocystis* to complement the missing NADH binding subunit of its NADH dehydrogenase but has not yet been proven (Vignais *et al.*, 2004).

Hydrogen consumption and energy conservation

Incorporation of $^{14}CO_2$ by gill tissues of *B. puteoserpentis* (Logatchev) in the presence of hydrogen shows that hydrogen stimulates carbon fixation and indicates that hydrogen consumption is linked to energy conservation. Evidence for the latter is supported by the existence of the gene coding for the large subunit of a membrane-bound respiratory hydrogen uptake (Group 1) [NiFe]-hydrogenase. This indicates that an energy-transducing [NiFe]-hydrogenase is present. The occurrence of the *hynL* gene in *B. sp.* (Wideawake and Lilliput - MAR), and *B.* cf. *thermophilus* (German Flats – Pacific Antarctic Ridge) from basalt-hosted vent fields implies that these mussels, too may derive energy from hydrogen consumption.

Which symbiont consumes hydrogen

Theoretical considerations

Since MAR bathymodiolin mussels live in dual symbiosis with both a chemoautotroph and a methanotroph (Duperron *et al.*, 2006) the question arises which of the two endosymbionts accounts for hydrogen consumption. Among free-living bacteria both sulfur-oxidizing chemoautotrophs and methanotrophs have been shown to use hydrogen as an energy source. Growth on hydrogen has for example been shown for the free-living sulfur-oxidizing chemoautotroph *Acidithiobacillus ferrooxidans* (Drobner *et al.*, 1990; Ohmura *et al.*, 2002). Besides, the potential to grow on hydrogen has been inferred from the complete genomes of the sulfur-oxidizing chemolithoautotrophs *Thiobacillus denitrificans* and *Thiomicrospira crunogena* (Beller *et al.*, 2006; Scott *et al.*, 2006). Methanotrophs have long been known to oxidize hydrogen in addition to methane since the enzyme methane monooxygenase which catalyzes the first step in methane oxidation co-oxidizes a variety of other substrates including hydrogen. However, no energy is yielded from this process (DiSpirito *et al.*, 2004). On the other hand, a gene cluster coding for a membrane-bound respiratory uptake [NiFe] hydrogenase has been found in the type X methanotroph *Methylococcus capsulatus* (Bath) suggesting hydrogen-dependent energy conservation in this strain (Csáki *et al.*, 2001; Ward *et al.*, 2004). The respective enzyme has biochemically been characterized revealing a high affinity for hydrogen (Hanczár *et al.*, 2002). Furthermore, a soluble cytoplasmic NAD reducing hydrogenase has also been found in this strain (Hanczár *et al.*, 2002; Ward *et al.*, 2004) which has been shown to provide

NADH needed for the oxidation of methane to methanol by methane monooxygenase (Hanczár *et al.*, 2002). Thus, both the sulfur- and the methane-oxidizing endosymbiont of MAR batyhymodiolin mussels could in theory account for hydrogen consumption and exploit hydrogen as an energy source.

Experimenal facts

Incorporation of $^{14}CO_2$ by gill tissues of *B. puteoserpentis* (Logatchev) in the presence of hydrogen indicates that the chemoautotrophic symbiont is a likely candidate for using hydrogen as an energy source. In all methanotrophs investigated so far CO_2 fixation via the Calvin cycle has only been found in type X methanotrophs (Bowman, 2006). However, the methane-oxidizing symbiont of bathymodiolin mussels is most closely related to type I methanotrophs (Cavanaugh *et al.*, 2006). Nevertheless, we can currently not exclude that the methanotrophic endosymbiont contributes to hydrogen consumption with or without conserving energy from this process.

The assumption that the chemoautotroph uses hydrogen as an energy source is supported by the fact that the *hynL* gene was also found in the vent mussel *B.* cf. *thermophilus* (German Flats – Pacific Antarctic Ridge), a mussel that solely hosts sulfur-oxidizing chemoautotrophic endosymbionts whereas methanotrophs are absent (J. M. Struck and N. Dubilier, unpublished data). This indicates that hydrogen-dependent energy conservation can be attributed to the physiological capabilities of the chemoautotrophic endosymbiont rather than to the methanotrophic symbiont. Thus, our data strongly imply that the sulfur-oxidizing chemoautotrophic endosymbiont of *B. puteoserpentis* uses hydrogen as an energy source.

Hydrogen consumption rates

Hydrogen consumption rates of MAR bathymodiolin gill tissues were considerably higher in *B. puteoserpentis* from the ultramafic-hosted Logatchev vent field than in *B. sp.* from the basalt-hosted Comfortless Cove and Lilliput fields. This is consistent with the high and low hydrogen concentrations at these vent fields (Table 3). At Logatchev where hydrogen consumption experiments were performed with mussels from two distinct sites (Irina II and Quest) consumption rates were in the same range consistent with hydrogen concentrations at the sampling sites which did not vary greatly either (Irina II 5.9 µM, Quest 4.2 µM). The low hydrogen consumption rates of *B. sp.* gill tissues from basalt-hosted vent fields may be explained by transcriptional regulation of the genes coding for the uptake [NiFe]-hydrogenase. In the free living aerobic hydrogen-oxidizing bacteria *Ralstonia eutotropha* and *Rhodobacter capsulatus* which have extensively been investigated for the regulation of the operons coding for respiratory uptake [NiFe]-hydrogenases, expression of the respective genes is inhibited in the absence of hydrogen (Schwartz and Friedrich, 2006). Thus, the lack of hydrogen at basalt-hosted vent fields may inhibit the expression of genes involved in symbiotic hydrogen uptake. Therefore, under experimental conditions the expression of respective genes must first be induced and the [NiFe]-hydrogenase as well as the enzymes involved in electron transport along the respiratory chain be synthesized before hydrogen uptake can even start. It appears reasonable to assume that the experimental conditions (Table 2) may not have favored sufficient expression of hydrogen uptake genes in mussels from low hydrogen basalt-hosted vent fields to fully exploit the hydrogen uptake machinery. Nevertheless, the fact that hydrogen consumption could be stimulated with increasing hydrogen partial pressures in Comfortless Cove and Lilliput mussels indicates that the symbionts may have responded to the increase in hydrogen by switching from sulfur and methane oxidation to hydrogen oxidation. An alternative explanation for the low hydrogen consumption rates of *B. sp.* gill tissues from basalt-hosted vent fields may be the activity of a constitutively expressed cytoplasmic NAD-linked [NiFe]-hydrogenase (Group 3d) as is apparently the case in the type X methanotroph *Methylococcus capsulatus* (Bath) (Hanczár *et al.*, 2002).

Importance of hydrogen over sulfide

Hydrogen versus sulfide consumption rates

Experimental hydrogen consumption rates were lower than sulfide consumption rates, however, hydrogen consumption experiments were performed at much lower concentrations (0.08-2.3 µM dissolved hydrogen versus 12-41 µM total sulfide). The fact that hydrogen consumption rates increased with increasing dissolved hydrogen concentrations suggests considerably higher consumption rates at higher dissolved hydrogen concentrations. Furthermore, a considerable drawback of sulfide consumption experiments using mussel tissues is the problem that endosymbiotic sulfide consumption can not reliably be distinguished from host mitochondrial sulfide consumption. Mitochondrial sulfide oxidation has first been shown for the coastal clam *Solemya reidi* (Powell and Somero, 1986a) which like bathymodiolin mussels hosts chemoautotrophic endosymbionts. Subsequently, this phenomenon has been demonstrated across a wide range of organisms including polychaete worms, clams, fishes and chickens (Grieshaber and Volkel, 1998; Yong and Searcy, 2001). Obviously, mitochondrial sulfide oxidation is a means of sulfide detoxification, however, appears also to be a means of energy conservation (Powell and Somero, 1986b; Yong and Searcy, 2001). Therefore, sulfide consumption of *Bathymodiolus* gill tissues is certainly the sum of endosymbiotic and mitochondrial sulfide oxidation. Even if the sulfide oxidation rate of foot tissue is subtracted from the gill tissue rate the resulting rate may not reliably be attributed to the chemoautotrophic endosymbionts. This is mainly because gill tissue which primarily serves as an oxygen uptake organ (from an evolutionarily point of view) is undeniably more efficient in gas and solute uptake than the structurally, morphologically, and physiologically differing foot tissue. These considerations indicate that hydrogen and sulfide consumption rates may not directly be comparable.

For *B. sp.* mussels from the basalt-hosted Comfortless Cove and Lilliput vent fields comparison of hydrogen and sulfide consumption rates at similar experimental concentrations does not make much sense anyway since hydrogen concentrations at the sampling sites are also only in the nanomolar range, much lower than sulfide concentrations which are in the micromolar range (A. Koschinsky and R. Seifert, personal communication). However, for *B. puteoserpentis* from the ultramafic-hosted Logatchev field direct comparison of hydrogen and sulfide consumption rates is useful since hydrogen and sulfide concentrations at the sampling site are at least in the micromolar range (A. Koschinsky and R. Seifert, personal communication). Extrapolation to 5 µM dissolved hydrogen which is consistent with lower range in situ concentrations at the sampling site suggests higher hydrogen than sulfide consumption rates for *B. puteoserpentis* gill tissues (31 versus 3-9 µmol h^{-1} (g wet weight)$^{-1}$.

Hydrogen and sulfide concentrations in different vent settings

A way to estimate the importance of hydrogen and sulfide at basalt- and ultramafic-hosted vent fields is to compare the ratio between dissolved hydrogen and total dissolved sulfide ($H_2/[S^{2-}_{tot}]$) at the sampling sites. The respective concentrations are usually obtained by discrete sampling followed by analysis on board. However, it should be noted that this approach may result in erroneously low sulfide concentrations due to redox processes during fluid transport or possible sulfide loss during recovery (Schmidt *et al.*, 2007). In situ measurements of hydrogen sulfide in diffuse hydrothermal fluids reveal generally higher sulfide concentrations than is suggested by discrete sampling (Zielinski *et al.*, in prep.). According to discrete sampling the $H_2/[S^{2-}_{tot}]$ ratio at the Comfortless Cove sampling site (Golden valley) was between 0.1 and 0.01 (A. Koschinsky and R. Seifert, personal communication). At the Lilliput sampling site (Limtoc) this ratio was ~ 0.0001 (Haase *et al.*, 2007a). Thus, hydrogen concentrations at these basalt-hosted vent fields are only low compared to sulfide concentrations even

if the sulfide bias due to discrete sampling is ignored. The $H_2/[S^{2-}_{tot}]$ ratio at the Logatchev Irina II and Quest sampling sites were 10 to 1 and ~ 0.1, respectively. Recent measurements suggest a ratio of ~ 30 with hydrogen concentrations exceeding 1 mM (A. Koschinsky and R. Seifert, personal communication). Logatchev endmember concentrations suggest a ratio of 7.6 (Schmidt *et al.*, 2007). Thus, an excess of hydrogen over sulfide is generally apparent in Logatchev diffuse hydrothermal fluids.

Considering experimental data and fluid chemistry indicates that energy conservation by chemoautotrophic endosymbionts of MAR bathymodiolin mussels may be sulfur-based at the basalt-hosted Comfortless Cove and Lilliput vent fields but hydrogen-based at the ultramafic-hosted Logatchev vent field.

General importance of hydrogen as an energy source

Implications for B. azoricus at Rainbow and Lost City

The above outlined hypothesis may apply to basalt- and ultramafic-hosted vent fields in general. Thus, hydrogen-based energy conservation by chemoautotrophic endosymbionts of bathymodiolin mussels may also be considered for the ultramafic-hosted Rainbow and Lost City vent fields with H_2/H_2S endmember ratios of 13 and 4 to 7, respectively (Charlou *et al.*, 2002; Kelley *et al.*, 2001). However, whereas *Bathymodiolus azoricus* is abundant at Rainbow (Desbruyères *et al.*, 2001) only two live specimens have to date been found at Lost City (DeChaine *et al.*, 2006). It has been suggested that the generally low biomass at Lost City is the result of hydrogen-rich fluids poor in the sulfide species that are typically relied on by vent faunal assemblages (Kelley *et al.*, 2005). However, the discovery of a dense aggregation of fragmentary valves belonging *B. azoricus* in the Lost City area (Lost Village) indicates that large populations have existed at Lost City in recent times (Galkin, 2006) and points to the potential of Lost City fluids to support chemosynthesis-based symbioses. Since hydrothermal activity at Lost City has been ongoing for at least 30,000 years (Früh-Green *et al.*, 2003) sulfide concentrations have likely also been low at the time when Lost Village mussels flourished, i.e. decades ago (Galkin, 2006). Thus, the chemoautotrophic symbiont of Lost Village mussels may rather have relied on hydrogen rather than on sulfide until a shutdown of hydrothermal activity may have caused the extinction of this mussel population.

In situ measurements of H_2S concentrations in hydrothermal diffuse fluids at the ultramafic-hosted Logatchev field revealed sufficient sulfide concentrations (~30 μM) to support the chemoautotrophic endosymbiont of *B. puteoserpentis* (Zielinski *et al.*, in prep.). Unfortunately, no in situ sulfide data are available for Rainbow and Lost City. The endmember H_2S concentration at Rainbow is 1.2 mM (Charlou *et al.*, 2002). In contrast, the Lost City sulfide endmember is only 0.064 mM. (Kelley *et al.*, 2001). Either way, since sulfide does not mix conservatively with seawater due to precipitation with metal ions and biological consumption (Johnson *et al.*, 1988) sulfide abundance in the endmember fluid does not necessarily reflect sulfide availability in diffuse fluids. Especially at Rainbow where ferrous iron exceeds H_2S by a factor of 24 (Douville *et al.*, 2002) sulfide may be completely precipitated and not be present as bioavailable H_2S/HS^- in diffuse fluids. Therefore it remains unclear if the Rainbow and Lost City chemoautotrophic endosymbionts could in theory rely on sulfide. The Rainbow and Lost City fluid compositions indicate that endosymbiotic chemoautotrophy may rather be based on hydrogen.

Implications for B. puteoserpentis at Logatchev

At Logatchev where both hydrogen and H_2S concentrations in diffuse fluids are sufficient to support hydrogen and sulfur-based chemoautotrophy, the chemoautotrophic endosymbiont of *B. puteoserpentis* may switch between hydrogen and sulfide depending on the availability of these electron donors or

use both simultaneously. Interestingly, hydrogen oxidation in the free-living sulfur- and ferrous iron oxidizing chemoautotroph *Acidithiobacillus ferrooxidans* appears to be repressed in the presence of sulfur and ferrous iron suggesting preferential growth on these substrates (Drobner *et al.*, 1990). This is surprising given the fact that the energy yield from aerobic hydrogen oxidation is higher than from aerobic sulfide or Fe^{2+} oxidation and that aerobic hydrogen oxidation does not require reverse electron transport in order to generate reducing equivalents. Further work is needed to shed light on this issue.

Attempts to correlate in situ sulfide and methane concentrations with symbiont abundance and activity at ultramafic vent settings must take in situ hydrogen concentrations into consideration as in situ sulfide concentrations may be low (Rainbow, Lost City) but the sulfur-oxidizing chemoautotrophic symbiont nonetheless abundant (DeChaine *et al.*, 2006).

Implications for cold seep bathymodiolin mussels

Hydrogen does apparently not occur in relevant concentrations in cold seep fluids (Jørgensen and Boetius, 2007; Sibuet and Olu-Le Roy, 2002; Sibuet and Olu, 1998). Therefore, cold-seep bathymodiolin mussels may exclusively rely on methane and/or sulfide provided with cold-seep fluids. The fact that the *hynL* gene could not be amplified from any of the investigated seep mussels indicates that the chemoautotrophic strains of seep bathymodiolin mussels may not be equipped with a membrane-bound respiratory hydrogen uptake [NiFe]-hydrogenase.

Implications of the presence / absence of an uptake [NiFe]-hydrogenase in vent and seep mussels, respectively

(Jones and Vrijenhoek, 2006; Jones *et al.*, 2006) have recently resolved the phylogenetic relationship within bathymodiolin mussels. Accordingly, bathymodiolin mussels fall within two clades the "childressi" clade comprising mainly seep mussels (including "*B.*" *childressi*) and the "thermophilus" clade comprising both seep mussels (*B. brooksi* and *B. heckerae*) and vent mussels (including among others *B.* cf. *thermophilus* and *B. puteoserpentis*). One conclusion drawn from the phylogenetic relationship was that *B. brooksi* and *B. heckerae* have independently invaded deep-sea cold seeps. Within the "thermophilus" clade *B. brooksi* constitutes the most basal lineage wheras *B. heckerae* branched off last from its common ancestor with the *B. puteoserpentis* / *B. azoricus* lineage. Regarding the presence or absence of an uptake [NiFe]-hydrogenase in members of the "thermophilus" clade the phylogenetic pattern suggests two scenarios: First, the chemoautotrophic symbiont of the common ancestor of the "thermophilus" clade acquired an uptake [NiFe]-hydrogenase by horizontal gene transfer which was then secondarily lost in *B. brooksi*, and *B. heckerae* as an adaptation to the hydrogen deficient seep environment where an energy transducing hydrogenase is dispensable. Second, the uptake [NiFe]-hydrogenase was acquired by the common ancestor of all other mussels in the "thermophilus" clade including *B.* cf. *thermophilus* and *B. puteoserpentis* and was then secondarily lost only in *B. heckerae*. Either way secondarily loss of the [NiFe]-hydrogenase is a more parsimonious explanation than multiple or independent acquisition of closely related [NiFe]-hydrogenases in "thermophilus" clade vent mussels.

Due to the existence of uptake [NiFe]-hydrogenases in all vent mussels investigated so far and belonging to the "thermophilus" clade we hypothesize that all vent members of the "thermophilus" clade are equipped with uptake [NiFe]-hydrogenases given the fact that hydrogen is generally present in vent diffuse fluids even though concentrations are only low at basalt-hosted vent systems.

Estimation of total hydrogen consumption mediated by B. puteoserpentis endosymbionts

Hydrogen consumption experiments were performed under normal pressure and

at low dissolved hydrogen concentrations for Logatchev mussels (~ 77 nM). Therefore, hydrogen consumption rates of *B. puteoserpentis* endosymbionts may be far from the rates at which hydrogen is oxidized at Logatchev in situ where dissolved hydrogen concentrations of 6 µM may be the minimum in diffuse fluids (T. Pape and R. Seifert, personal communication). This assumption is supported by the fact that an increase in hydrogen partial pressure in experiments with Comfortless Cove and Lilliput mussels resulted in an increase in hydrogen consumption rates. Using data from Comfortless Cove, extrapolation to 5 µM dissolved hydrogen suggests a hydrogen consumption rate of ~ 3.2 µmol h^{-1} (g wet weight)$^{-1}$ for Logatchev mussel symbionts. Higher consumption rates of around 31 µmol h^{-1} (g wet weight)$^{-1}$ are in theory possible when extrapolating from zero through Logatchev rates at 100 ppm (Figure 5, dotted line). Even higher hydrogen consumption rates of ~ 47 µmol h^{-1} (g wet weight)$^{-1}$ were measured in incubation experiments with Logatchev gill tissues in hydrogen-saturated seawater (775µM at 4°C, 35 ‰) using Clark-type H_2 sensors (Frank Wenzhöfer, unpublished data). Either way, neglecting the effect of hydrostatic pressure on hydrogen consumption rates our data allow at first approximation an estimation of hydrogen oxidation mediated by *B. puteoserpentis* endosymbionts under in situ conditions. Neglecting the influence of pressure may be justified since (Belkin *et al.*, 1986) have shown that CO_2 incorporation rates for the sulfide-oxidizing endosymbiont of *B. thermophilus* were not significantly different at 1 atm and under in situ pressure (250 atm). Therefore, assuming a gill weight of around 1 g one single *B. puteoserpentis* specimen may account for the oxidation of 60 µmol hydrogen h^{-1}. At Logatchev, *B. puteoserpentis* accounts for most of the invertebrate biomass and exceeds 70 kg m^{-2} (wet weight with shells) in Irina II mussel beds (Gebruk *et al.*, 2000). Mussel aggregations at Logatchev range from small patches 20-30 cm in diameter to larger aggregations and mussel beds covering an area of 3 m^2 (for example at Quest) and 12 m^2 (for example at Irina II), respectively (Jens Stecher, personal communication). (Gebruk *et al.*, 2000) described the extended mussel beds at Irina II as 20 m in diameter. Based on these data and personal observations we estimate the mussel population at Logatchev to range between 200,000 and half a million individuals. This population may thus remove 12-30 mol hydrogen h^{-1} corresponding to 270-670 liters of pure hydrogen. Mussel patches, aggregations, and beds of *B. puteoserpentis* may therefore play an appreciable role in hydrogen removal from diffuse fluids before hydrogen is issued into the open ocean.

Acknowledgements

This work would not have been possible without the skill and cooperation of the chief scientists, the shipboard scientific parties, the captains and crews of the R/Vs and the engineers and pilots of the ROVs. We especially thank the chief scientists Klas Lackschewitz (M64/2), Andrea Koschinsky (M68/1), and Christian Borowski (MSM04/3), captain Martin Kull and the crew of the R/V Meteor, captain Friedhelm von Staa and the crew of the R/V Maria S. Merian as well as Volker Rathmeyer, Götz Ruhland, Christian Seiter and crew of the ROV Quest and N.N. and crew of the ROV Jason for their best support to collect mussels and perform experiments on board. Sébastien Duperron is acknowledged for providing gill tissues from cold seep bathymodiolin mussels. This work was supported by grants from the priority program 1144 of the German Science Foundation (DFG). This is publication number XY of the priority program 1144 “From Mantle to Ocean: Energy-, Material- and Life cycles a Spreading Axes” of DFG.

References

Allen, D.E., Seyfried, W.E., 2003. Compositional controls on vent fluids from ultramatic-hosted hydrothermal systems at mid-ocean ridges: An experimental study at 400°C, 500 bars. Geochim. Cosmochim. Acta 67 (8), 1531-1542.

Allen, D.E., Seyfried, W.E., 2004. Serpentinization and heat generation: Constraints from Lost City and

Rainbow hydrothermal systems. Geochim. Cosmochim. Acta 68 (6), 1347-1354.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25 (17), 3389-3402.

Amend, J.P., Shock, E.L., 2001. Energetics of overall metabolic reactions of thermophilic and hyperthermophilic Archaea and Bacteria. FEMS Microbiol. Rev. 25 (2), 175-243.

Bach, W., Garrido, C.J., Paulick, H., Harvey, J., Rosner, M., 2004. Seawater-peridotite interactions: First insights from ODP Leg 209, MAR 15°N. Geochem. Geophys. Geosyst. 5, doi: 10.1029 / 2004GC000744.

Bach, W., Paulick, H., Garrido, C.J., Ildefonse, B., Meurer, W.P., Humphris, S.E., 2006. Unraveling the sequence of serpentinization reactions: petrography, mineral chemistry, and petrophysics of serpentinites from MAR 15°N (ODP Leg 209, Site 1274). Geophys. Res. Lett. 33 (13).

Belkin, S., Nelson, D.C., Jannasch, H.W., 1986. Symbiotic assimilation of CO_2 in two hydrothermal vent animals, the mussel *Bathymodiolus thermophilus* and the tube worm *Riftia pachyptila*. Biol. Bull. 170 (1), 110-121.

Beller, H.R., Chain, P.S.G., Letain, T.E., Chakicherla, A., Larimer, F.W., Richardson, P.M., Coleman, M.A., Wood, A.P., Kelly, D.P., 2006. The genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium *Thiobacillus denitfificans*. J. Bacteriol. 188 (4), 1473-1488.

Berndt, M.E., Allen, D.E., Seyfried, W.E., 1996. Reduction of CO_2 during serpentinization of olivine at 300°C and 500 bar. Geology 24 (4), 351-354.

Borowski, C., Asendorf, V., Bürk, D., Jr., A.C., Elder, R., Fabian, M., Forte, P., Fuhrmann, R., Hansen, S., Jost, C.L., Keir, R., Kevis-Stirling, J., Kevis-Stirling, N., Perner, M., Petersen, J.M., Røy, H., Schauer, R., Schmale, O., Schmidt, K., Scott, D., Waters, R., Wefers, P., Wetzel, S., 2007. New coordinates for the hydrothermal structures in the Logatchev vent field at 14°45'N on the Mid-Atlantic Ridge. InterRidge News 16, 13-14.

Bowman, J., 2006. The methanotrophs - the families Methylococcaceae and Methylocystaceae. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E. (Eds.), The Prokaryotes. Springer-Verlag, Berlin, Heidelberg, pp. 266-289.

Cavanaugh, C.M., McKiness, Z.P., Newton, I.L.G., Stewart, F.J., 2006. Marine chemosynthetic symbioses. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E. (Eds.), The Prokaryotes. Springer-Verlag, Berlin, Heidelberg, pp. 473-507.

Charlou, J.L., Donval, J.P., Fouquet, Y., Jean-Baptiste, P., Holm, N., 2002. Geochemistry of high H_2 and CH_4 vent fluids issuing from ultramafic rocks at the Rainbow hydrothermal field (36°14'N, MAR). Chem. Geol. 191 (4), 345-359.

Charlou, J.L., Donval, J.P., Jean-Baptiste, P., Dapoigny, A., 1996. Gases and helium isotopes in high temperature solutions sampled before and after ODP Leg 158 drilling at TAG hydrothermal field (26°N, MAR). Geophys. Res. Lett. 23 (23), 3491-3494.

Childress, J.J., Fisher, C.R., Brooks, J.M., Kennicutt, M.C., Bidigare, R., Anderson, A.E., 1986. A methanotrophic marine molluscan (Bivalvia, Mytilidae) symbiosis: mussels fueled by gas. Science 233 (4770), 1306-1308.

Crozier, T.E., Yamamoto, S., 1974. Solubility of hydrogen in water, seawater, and NaCl solutions. J. Chem. Eng. Data 19 (3), 242-244.

Csáki, R., Hanczár, T., Bodrossy, L., Murrell, J.C., Kovács, K.L., 2001. Molecular characterization of structural genes coding for a membrane bound hydrogenase in *Methylococcus capsulatus* (Bath). FEMS Microbiol. Lett. 205 (2), 203-207.

DeChaine, E.G., Bates, A.E., Shank, T.M., Cavanaugh, C.M., 2006. Off-axis symbiosis found: characterization and biogeography of bacterial symbionts of *Bathymodiolus* mussels from Lost City hydrothermal vents. Environ. Microbiol. 8 (11), 1902-1912.

DeChaine, E.G., Cavanaugh, C.M., 2006. Symbioses of methanotrophs and deep-sea mussels (Mytilidae: Bathymodiolinae). In: Overmann, J. (Ed.), Molecular Basis of Symbiosis. Springer-Verlag, Berlin, Heidelberg, pp. 227-249.

Desbruyères, D., Biscoito, M., Caprais, J.-C., Colaço, A., Comtet, T., Crassous, P., Fouquet, Y., Khripounoff, A., Le Bris, N., Olu, K., Riso, R., Sarradin, P.M., Segonzac, M., Vangriesheim, A., 2001. Variations in deep-sea hydrothermal vent communities on the Mid-Atlantic Ridge near the Azores plateau. Deep-Sea Res. I 48 (5), 1325-1346.

DiSpirito, A.A., Kunz, R.C., Choi, D.-W., Zahn, J.A., 2004. Respiration in methanotrophs. In: Zannoni, D. (Ed.), Respiration in archaea and bacteria. Vol 2: Diversity of prokaryotic respiratory systems. Springer, Dordrecht, The Netherlands, pp. 149-168.

Distel, D.L., Lee, H.K.W., Cavanaugh, C.M., 1995. Intracellular coexistence of methanotrophic and thioautotrophic bacteria in a hydrothermal vent mussel. Proc. Natl. Acad. Sci. USA 92 (21), 9598-9602.

Douville, E., Charlou, J.L., Oelkers, E.H., Bienvenu, P., Colon, C.F.J., Donval, J.P., Fouquet, Y., Prieur, D., Appriou, P., 2002. The rainbow vent fluids (36°14'N, MAR): the influence of ultramafic rocks

and phase separation on trace metal content in Mid-Atlantic Ridge hydrothermal fluids. Chem. Geol. 184 (1-2), 37-48.

Drobner, E., Huber, H., Stetter, K.O., 1990. *Thiobacillus ferrooxidans*, a facultative hydrogen oxidizer. Appl. Environ. Microbiol. 56 (9), 2922-2923.

Duperron, S., Bergin, C., Zielinski, F., Blazejak, A., Pernthaler, A., McKiness, Z.P., DeChaine, E., Cavanaugh, C.M., Dubilier, N., 2006. A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge. Environ. Microbiol. 8 (8), 1441-1447.

Duperron, S., Nadalig, T., Caprais, J.C., Sibuet, M., Fiala-Medioni, A., Amann, R., Dubilier, N., 2005. Dual symbiosis in a *Bathymodiolus* sp. mussel from a methane seep on the Gabon continental margin (southeast Atlantic): 16S rRNA phylogeny and distribution of the symbionts in gills. Appl. Environ. Microbiol. 71 (4), 1694-1700.

Duperron, S., Sibuet, M., MacGregor, B.J., Kuypers, M.M.M., Fisher, C.R., Dubilier, N., 2007. Diversity, relative abundance and metabolic potential of bacterial endosymbionts in three *Bathymodiolus* mussel species from cold seeps in the Gulf of Mexico. Environ. Microbiol. 9 (6), 1423–1438.

Fisher, C.R., Takai, K., Le Bris, N., 2007. Hydrothermal vent ecosystems. Oceanography 20 (1), 14-23.

Früh-Green, G.L., Kelley, D.S., Bernasconi, S.M., Karson, J.A., Ludwig, K.A., Butterfield, D.A., Boschi, C., Proskurowski, G., 2003. 30,000 years of hydrothermal activity at the Lost City vent field. Science 301 (5632), 495-498.

Galkin, S.V., 2006. Lost Village - a "faubourg" of Lost City: benthic studies using Mir submersibles at North Atlantic hydrothermal sites in 2005. InterRidge News 15, 18-24.

Gebruk, A.V., Chevaldonné, P., Shank, T., Lutz, R.A., Vrijenhoek, R.C., 2000. Deep-sea hydrothermal vent communities of the Logatchev area (14°45'N, Mid-Atlantic Ridge): diverse biotopes and high biomass. J. Mar. Biol. Assoc. U.K. 80 (3), 383-393.

Grieshaber, M.K., Volkel, S., 1998. Animal adaptations for tolerance and exploitation of poisonous sulfide. Annu. Rev. Physiol. 60, 33-53.

Haase, K.M., Koschinsky, A., Devey, C.W., Fretzdorff, S., German, C., Lackschewitz, K.S., Melchert, B., Petersen, S., Seifert, R., Stecher, J., Giere, O., Paulick, H., Yoerger, D., M64/1, M68/1, 2007a. Diking, young volcanism and diffuse hydrothermal activity on the southern Mid-Atlantic Ridge. Marine Geology, submitted.

Haase, K.M., Petersen, S., Koschinsky, A., Seifert, R., Devey, C.W., Keir, R., Lackschewitz, K.S., Melchert, B., Perner, M., Schmale, O., Süling, J., Dubilier, N., Zielinski, F., Fretzdorff, S., Garbe-Schönberg, D., Westernströer, U., German, C.R., Shank, T.M., Yoerger, D., Giere, O., Kuever, J., Marbler, H., Mawick, J., Mertens, C., Stöber, U., Ostertag-Henning, C., Paulick, H., Peters, M., Strauss, H., Sander, S., Stecher, J., Warmuth, M., Weber, S., 2007b. Young volcanism and related hydrothermal activity at 5°S on the slow-spreading southern Mid-Atlantic Ridge. Geochem. Geophys. Geosyst. 8 (11), Q11002, doi: 11010.11029 / 12006GC001509.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41 (95-98).

Hanczár, T., Csáki, R., Bodrossy, L., Murrell, J.C., Kovács, K.L., 2002. Detection and localization of two hydrogenases in *Methylococcus capsulatus* (Bath) and their potential role in methane metabolism. Arch. Microbiol. 177 (2), 167-172.

Hipp, W.M., Pott, A.S., ThumSchmitz, N., Faath, I., Dahl, C., Truper, H.G., 1997. Towards the phylogeny of APS reductases and sirohaem sulfite reductases in sulfate-reducing and sulfur-oxidizing prokaryotes. Microbiology 143, 2891-2902.

Jannasch, H.W., Mottl, M.J., 1985. Geomicrobiology of deep-sea hydrothermal vents. Science 229 (4715), 717-725.

Johnson, K.S., Childress, J.J., Hessler, R.R., Sakamoto-Arnold, C.M., Beehler, C.L., 1988. Chemical and biological interactions in the Rose Garden hydrothermal vent field, Galapagos spreading center. Deep-Sea Res. A 35 (10-11), 1723-1744.

Jones, W.J., Vrijenhoek, R.C., 2006. Evolutionary relationships within the *"Bathymodiolus" childressi* group. Cah. Biol. Mar. 47 (4), 403-407.

Jones, W.J., Won, Y.J., Maas, P.A.Y., Smith, P.J., Lutz, R.A., Vrijenhoek, R.C., 2006. Evolution of habitat use by deep-sea mussels. Mar. Biol. 148 (4), 841-851.

Jørgensen, B.B., Boetius, A., 2007. Feast and famine - microbial life in the deep-sea bed. Nat. Rev. Microbiol. 5 (10), 770-781.

Kelley, D.S., Karson, J.A., Blackman, D.K., Früh-Green, G.L., Butterfield, D.A., Lilley, M.D., Olson, E.J., Schrenk, M.O., Roe, K.K., Lebon, G.T., Rivizzigno, P., 2001. An off-axis hydrothermal vent field near the Mid-Atlantic Ridge at 30°N. Nature 412 (6843), 145-149.

Kelley, D.S., Karson, J.A., Früh-Green, G.L., Yoerger, D.R., Shank, T.M., Butterfield, D.A., Hayes, J.M., Schrenk, M.O., Olson, E.J., Proskurowski, G., Jakuba, M., Bradley, A., Larson, B., Ludwig, K., Glickson, D., Buckman, K., Bradley, A.S., Brazelton,

W.J., Roe, K., Elend, M.J., Delacour, A., Bernasconi, S.M., Lilley, M.D., Baross, J.A., Summons, R.T., Sylva, S.P., 2005. A serpentinite-hosted ecosystem: The Lost City hydrothermal field. Science 307 (5714), 1428-1434.

Kochevar, R.E., Childress, J.J., Fisher, C.R., Minnich, E., 1992. The methane mussel: roles of symbiont and host in the metabolic utilization of methane. Mar. Biol. 112 (3), 389-401.

Koschinsky, A., Billings, A., Devey, C.W., Dubilier, N., Duester, A., Edge, D., Garbe-Schönberg, D., German, C.R., Giere, O., Keir, R., Lackschewitz, K.S., Mai, H.A., Marbler, H., Mawick, J., Melchert, B., Mertens, C., Peters, M., Sandler, S., Schmale, O., Schmidt, W., Seifert, R., Seiter, C., Stöber, U., Suck, I., Walter, M., Weber, S., Yoerger, D., Zarrouk, M., Zielinski, F., 2006. Discovery of new hydrothermal vent fields on the Southern Mid-Atlantic Ridge (4°S - 10°S) during Cruise M68/1. InterRidge News 15, 9-15.

Kulikova, T., Akhtar, R., Aldebert, P., Althorpe, N., Andersson, M., Baldwin, A., Bates, K., Bhattacharyya, S., Bower, L., Browne, P., Castro, M., Cochrane, G., Duggan, K., Eberhardt, R., Faruque, N., Hoad, G., Kanz, C., Lee, C., Leinonen, R., Lin, Q., Lombard, V., Lopez, R., Lorenc, D., McWilliam, H., Mukherjee, G., Nardone, F., Pastor, M.P.G., Plaister, S., Sobhany, S., Stoehr, P., Vaughan, R., Wu, D., Zhu, W., Apweiler, R., 2007. EMBL Nucleotide Sequence Database in 2006. Nucl. Acids Res. 35 (suppl_1), D16-D20.

Lilley, M.D., Butterfield, D.A., Lupton, J.E., Olson, E.J., 2003. Magmatic events can produce rapid changes in hydrothermal vent chemistry. Nature 422 (6934), 878-881.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H., 2004. ARB: a software environment for sequence data. Nucleic Acids Res. 32 (4), 1363-1371.

Nelson, D.C., Hagen, K.D., Edwards, D.B., 1995. The gill symbiont of the hydrothermal vent mussel *Bathymodiolus thermophilus* is a psychrophilic, chemoautotrophic, sulfur bacterium. Mar. Biol. 121 (3), 487-495.

Ohmura, N., Sasaki, K., Matsumoto, N., Saiki, H., 2002. Anaerobic respiration using Fe^{3+}, S^0, and H_2 in the chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans*. J. Bacteriol. 184 (8), 2081-2087.

Olu-Le Roy, K., Caprais, J.C., Fifis, A., Fabri, M.C., Galéron, J., Budzinsky, H., Le Ménach, K., Khripounoff, A., Ondréas, H., Sibuet, M., 2007a. Cold-seep assemblages on a giant pockmark off West Africa: spatial patterns and environmental control. Mar. Ecol. 28 (1), 115-130.

Olu-Le Roy, K., von Cosel, R., Hourdez, S., Carney, S.L., Jollivet, D., 2007b. Amphi-Atlantic cold-seep *Bathymodiolus* species complexes across the equatorial belt. Deep-Sea Res. I 54 (11), 1890-1911.

Paulick, H., Bach, W., Godard, M., De Hoog, J.C.M., Suhr, G., Harvey, J., 2006. Geochemistry of abyssal peridotites (Mid-Atlantic Ridge, 15°20'N, ODP Leg 209): Implications for fluid/rock interaction in slow spreading environments. Chem. Geol. 234 (3-4), 179-210.

Pernthaler, A., Ward, J.E., Zielinski, F., Borowski, C., Dubilier, N., in prep. Expression patterns of *pmoA* and *aprA* in symbionts of the hydrothermal vent mussel *Bathymodiolus puteoserpentis*.

Pimenov, N.V., Kalyuzhnaya, M.G., Khmelenina, V.N., Mityushina, L.L., Trotsenko, Y.A., 2002. Utilization of methane and carbon dioxide by symbiotrophic bacteria in gills of Mytilidae (*Bathymodiolus*) from the Rainbow and Logachev hydrothermal fields on the Mid-Atlantic Ridge. Microbiology 71 (5), 587-594.

Powell, M.A., Somero, G.N., 1986a. Adaptations to sulfide by hydrothermal vent animals: sites and mechanisms of detoxification and metabolism. Biol. Bull. 171 (1), 274-290.

Powell, M.A., Somero, G.N., 1986b. Hydrogen sulfide oxidation is coupled to oxidative phosphorylation in mitochondria of *Solemya reidi*. Science 233 (4763), 563-566.

Robinson, J.J., Polz, M.F., Fiala-Medioni, A., Cavanaugh, C.M., 1998. Physiological and immunological evidence for two distinct C_1-utilizing pathways in *Bathymodiolus puteoserpentis* (Bivalvia: Mytilidae), a dual endosymbiotic mussel from the Mid-Atlantic Ridge. Mar. Biol. 132 (4), 625-633.

Sánchez, O., Ferrera, I., Dahl, C., Mas, J., 2001. In vivo role of adenosine-5 '-phosphosulfate reductase in the purple sulfur bacterium Allochromatium vinosum. Arch. Microbiol. 176 (4), 301-305.

Schmidt, K., Koschinsky, A., Garbe-Schönberg, D., de Carvalho, L.M., Seifert, R., 2007. Geochemistry of hydrothermal fluids from the ultramafic-hosted Logatchev hydrothermal field, 15°N on the Mid-Atlantic Ridge: Temporal and spatial investigation. Chem. Geol. 242 (1-2), 1-21.

Schwartz, E., Friedrich, B., 2006. The H_2-metabolizing prokaryotes. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E. (Eds.), The Prokaryotes. Springer-Verlag, Berlin, Heidelberg, pp. 496-563.

Scott, K.M., Sievert, S.M., Abril, F.N., Ball, L.A., Barrett, C.J., Blake, R.A., Boller, A.J., Chain, P.S.G.,

Clark, J.A., Davis, C.R., Detter, C., Do, K.F., Dobrinski, K.P., Faza, B.I., Fitzpatrick, K.A., Freyermuth, S.K., Harmer, T.L., Hauser, L.J., Hügler, M., Kerfeld, C.A., Klotz, M.G., Kong, W.W., Land, M., Lapidus, A., Larimer, F.W., Longo, D.L., Lucas, S., Malfatti, S.A., Massey, S.E., Martin, D.D., McCuddin, Z., Meyer, F., Moore, J.L., Ocampo, L.H., Paul, J.H., Paulsen, I.T., Reep, D.K., Ren, Q.H., Ross, R.L., Sato, P.Y., Thomas, P., Tinkham, L.E., Zeruth, G.T., 2006. The genome of deep-sea vent chemolithoautotroph *Thiomicrospira crunogena* XCL-2. PLoS Biol. 4 (12), 2196-2212.

Sibuet, M., Olu-Le Roy, K., 2002. Cold seep communities on continental margins: Structure and quantitative distribution relative to geological and fluid venting patterns. In: Wefer, G., Billett, D., Hebbeln, D., Jørgensen, B.B., Schlüter, M., Van Weering, T. (Eds.), Ocean Margin Systems. Springer-Verlag, Berlin, Heidelberg, pp. 235-251.

Sibuet, M., Olu, K., 1998. Biogeography, biodiversity and fluid dependence of deep-sea cold-seep communities at active and passive margins. Deep-Sea Res. II 45 (1-3), 517-567.

Sleep, N.H., Meibom, A., Fridriksson, T., Coleman, R.G., Bird, D.K., 2004. H_2-rich fluids from serpentinization: geochemical and biotic implications. Proc. Natl. Acad. Sci. USA 101 (35), 12818-12823.

Stewart, F.J., Newton, I.L.G., Cavanaugh, C.M., 2005. Chemosynthetic endosymbioses: adaptations to oxic-anoxic interfaces. Trends Microbiol. 13 (9), 439-448.

Suen, G., Goldman, B.S., Welch, R.D., 2007. Predicting prokaryotic ecological niches using genome sequence analysis. PLoS ONE 2 (8), e743.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22 (22), 4673-4680.

Trüper, H.G., Schlegel, H.G., 1964. Sulphur metaboilsm in Thiorhodaceae I. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie Van Leeuwenhoek 30 (3), 225-238.

Vignais, P.M., Billoud, B., Meyer, J., 2001. Classification and phylogeny of hydrogenases. FEMS Microbiol. Rev. 25 (4), 455-501.

Vignais, P.M., Colbeau, A., 2004. Molecular biology of microbial hydrogenases. Curr. Issues Mol. Biol. 6, 159-188.

Vignais, P.M., Willison, J.C., Colbeau, A., 2004. Hydrogen Respiration. In: Zannoni, D. (Ed.), Respiration in Archaea and Bacteria. Vol 2: Diversity of Prokaryotic Respiratory Systems. Springer, Dordrecht, The Netherlands, pp. 233-260.

Ward, N., Larsen, O., Sakwa, J., Bruseth, L., Khouri, H., Durkin, A.S., Dimitrov, G., Jiang, L.X., Scanlan, D., Kang, K.H., Lewis, M., Nelson, K.E., Methé, B., Wu, M., Heidelberg, J.F., Paulsen, I.T., Fouts, D., Ravel, J., Tettelin, H., Ren, Q.H., Read, T., DeBoy, R.T., Seshadri, R., Salzberg, S.L., Jensen, H.B., Birkeland, N.K., Nelson, W.C., Dodson, R.J., Grindhaug, S.H., Holt, I., Eidhammer, I., Jonasen, I., Vanaken, S., Utterback, T., Feldblyum, T.V., Fraser, C.M., Lillehaug, J.R., Eisen, J.A., 2004. Genomic insights into methanotrophy: the complete genome sequence of *Methylococcus capsulatus* (Bath). PLoS Biol. 2 (10), 1616-1628.

Weiss, R.F., 1970. Solubility of Nitrogen, Oxygen and Argon in Water and Seawater. Deep-Sea Res. Oceanogr. Abstr. 17 (4), 721-735.

Wetzel, L.R., Shock, E.L., 2000. Distinguishing ultramafic- from basalt-hosted submarine hydrothermal systems by comparing calculated vent fluid compositions. J. Geophys. Res. 105 (B4), 8319-8340.

Yanisch-Perron, C., Vieira, J., Messing, J., 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33 (1), 103-119.

Yong, R., Searcy, D.G., 2001. Sulfide oxidation coupled to ATP synthesis in chicken liver mitochondria. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129 (1), 129-137.

Zhou, J.Z., Bruns, M.A., Tiedje, J.M., 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62 (2), 316-322.

Zielinski, F.U., Borowski, C., Gennerich, H.H., Dubilier, N., Wenzhöfer, F., in preparation. *In situ* measurements of hydrogen sulfide, oxygen, and temperature in diffuse fluids of the ultramafic-hosted Logatchev hydrothermal vent field (Mid-Atlantic Ridge): implications for symbiont-containing *Bathymodiolus* mussels. Intended as article in Deep-Sea Research I.

Zielinski, F.U., Pernthaler, A., Duperron, S., Raggi, L., Giere, O., Borowski, C., Dubilier, N., manuscript II. Widespread occurrence of an intranuclear bacterial parasite in vent and seep mussels. Environ. Microbiol. 11 (5), 1150-1167.