Bacterial-invertebrate symbioses: from an asphalt cold seep to shallow waters

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To Pablo
to my family

‘La simbiosis, la unión de distintos organismos para formar nuevos colectivos, ha resultado ser la más importante fuerza de cambio sobre la Tierra’

L. Margulis & D. Sagan, 1995

‘La unión hace la fuerza’

- Frase popular

Abstract

Symbiotic associations are complex partnerships that can lead to new metabolic capabilities and the establishment of novel organisms. The diversity of these associations is very broad and there are still many mysteries about the origin and the exact relationship between the organisms that are involved in a symbiosis (host and symbiont). Some of these associations are essential to the hosts, such as the chemosynthetic symbioses occurring in invertebrates of the deep-sea. In others the host probably would rather not be the host, as in the case of parasitic microbes. My PhD research focuses on symbiotic and parasitic associations in chemosynthetic and non-chemosynthetic invertebrates. This thesis describes and discusses three different aspects of associations between bacteria and marine invertebrates. The first aspect focuses on chemosynthetic associations from a unique asphalt seep called Chapopote in the Gulf of Mexico (GoM). Phylogenetic analyses of host genes (cytochrome-c-oxidase subunit I) and bacterial genes (16S rRNA) in two *Bathymodiolus* mussel species and an *Escarpia* tubeworm showed that both the hosts and their chemosynthetic symbionts are very similar to their congeners from the northern GoM. Unexpectedly, a novel symbiont most closely related to hydrocarbon degrading bacteria of the genus *Cycloclasticus* was discovered in *B. heckerae*. Stable carbon isotope values in *B. heckerae* tissues of lipids typical for *Cycloclasticus* spp. were consistently heavier by 2.5‰ than other lipids indicating that the novel symbiont might use isotopically heavy hydrocarbons from the asphalt seep as an energy and carbon source. The discovery of a novel symbiont that may be able to metabolize hydrocarbons is particularly intriguing because until now only methane and reduced sulfur compounds have been identified as energy sources in chemosynthetic symbioses. The large amounts of hydrocarbons available at Chapopote would provide these mussel symbioses with a rich source of nutrition. The second aspect of this thesis deals with bacteria that infect the nuclei of

marine invertebrates and were recently found to be widespread in deep-sea *Bathymodiolus* mussels. Because of their potentially lethal effect on bivalve populations, I looked for the presence of intranuclear bacteria in economically important and commercially available bivalve species, i.e. oysters (*Crassostrea gigas*), razor clams (*Siliqua patula* and *Ensis directus*), blue mussels (*Mytilus edulis*), Manila clams (*Venerupis philippinarum*), and common cockles (*Cerastoderma edule*). Fluorescence in situ hybridization (FISH) revealed the presence of intranuclear bacteria in all investigated bivalves except oysters and blue mussels. Preliminary tests with real-time PCR showed massive amounts of intranuclear bacteria in some of the bivalve species, raising the question if these might affect not only the health of the bivalves but possibly also of the humans that eat them. In the third and final aspect of my thesis, I examined the general diversity of bacteria in the gill tissues of deep-sea and shallow-water mussels and clams. Comparative 16S rRNA sequence analysis and cultivation experiments revealed a much higher diversity than previously recognized. This thesis shows that bivalves are ideal models for studying the microbiota of marine invertebrates because of the high diversity of both highly specific and more generalized symbiotic and parasitic bacteria in their gill tissues.

Zusammenfassung

Symbiotische Assoziationen sind komplexe Partnerschaften, die zu neuen metabolischen Fähigkeiten und der Etablierung neuartiger Organismen führen können. Die Vielfalt dieser Assoziationen ist sehr hoch, und in vielen Fällen bleiben ihr Ursprung und die genaue Beziehung zwischen den in die Symbiose eingebundenen Organismen (Wirt und Symbiont) ungeklärt. Einige dieser Verbindungen sind unverzichtbar für den Wirt, wie etwa die chemosynthetische Symbionten, die bei Invertebraten in der Tiefsee vorkommen. In einigen anderen wäre der Wirt wohl lieber nicht der Wirt, wie im Fall von parasitischen Mikroorganismen. Die Forschung meiner Dissertation konzentriert sich auf symbiotische und parasitische Assoziationen in chemosynthetischen und nicht-chemosynthetischen Wirbellosen.

Die vorliegende Arbeit beschreibt und diskutiert drei verschiedene Aspekte der Assoziationen zwischen Bakterien und marinen Invertebraten. Der erste Aspekt konzentriert sich auf chemosynthetische Assoziationen an einem einzigartigen Asphaltvulkan, dem Chapopote im Golf von Mexico (GoM). Phylogenetische Analysen von Wirtsgenen (Cytochrom-c-Oxidase Untereinheit I) und bakteriellen Genen (16S rRNA) in zwei *Bathymodiolus*-Muschelarten und einem *Escarpia*-Röhrenwurm haben gezeigt, dass sowohl die Wirte als auch ihre chemosynthetischen Symbionten ihren Artverwandten aus dem nördlichen GoM sehr ähnlich sind. Unerwarteterweise wurde in *B. heckerae* ein neuer Symbiont entdeckt, der am nächsten mit den Kohlenwasserstoffe abbauenden Bakterien des Genus *Cycloclasticus* verwandt ist. Die stabilen Kohlenstoffisotope der für *Cycloclasticus* typischen Lipide in den Geweben von *B. heckerae* waren durchgängig um 2.5‰ schwerer als bei anderen Lipiden. Dies deutet darauf hin, dass der neuartige Symbiont isotopenschwere Kohlenwasserstoffe aus dem Asphaltvulkan als Energie- und Kohlenstoffquelle nutzen könnte. Die Entdeckung eines neuartigen Symbionten, der in der Lage sein könnte, Kohlenwasserstoffe zu metabolisieren, ist besonders

faszinierend, da bisher nur Methan und reduzierte Schwefelverbindungen als Energiequelle in chemosynthetischen Symbiosen identifiziert worden sind. Die großen Mengen von Kohlenwasserstoffen, die bei Chapopote verfügbar sind, würden dieser Muschelsymbiose eine reichhaltige Nährstoffquelle zur Verfügung stellen.

Der zweite Aspekt dieser Arbeit beschäftigt sich mit Bakterien, die die Zellkerne von marinen Invertebraten infizieren und vor Kurzem weit verbreitet in *Bathymodiolus*-Muscheln der Tiefsee gefunden wurden. Wegen ihrer potentiell tödlichen Auswirkungen auf Bivalven-Populationen habe ich besonders nach der Präsenz von intranuklearen Bakterien in ökonomisch bedeutsamen und kommerziell erhältlichen Muschelspezies gesucht, d.h. in Austern (*Crassostrea gigas*), Schwertmuscheln (*Siliqua patula* und *Ensis directus*), Miesmuscheln (*Mytilus edulis*), Venusmuscheln (*Venerupis philippinarum*) und Herzmuscheln (*Cerastoderma edule*). Die Fluoreszenz-*in-situ*-Hybridisierung (FISH) brachte intranukleare Bakterien in allen untersuchten Muscheln zum Vorschein, außer in Austern und Miesmuscheln. Vorläufige Tests mit Hilfe der Real-time PCR zeigten hohe Mengen von intranuklearen Bakterien in einigen der Bivalvenspezies, was die Frage aufwirft, ob diese nicht nur die Gesundheit der Muscheln, sondern möglicherweise auch die der sie verzehrenden Menschen beeinträchtigen könnten.

Im dritten und letzten Aspekt meiner Doktorarbeit habe ich die allgemeine Diversität von Bakterien in den Kiemengeweben von Tiefsee- und Flachwassermuscheln untersucht. Vergleichende 16S rRNA-Sequenzanalyse und Kultivierungsexperimente haben eine deutlich höhere Diversität enthüllt, als vorher bekannt war. Diese Dissertation zeigt, dass Bivalvia aufgrund der hohen Diversität von sowohl hochspezifischen als auch generalisierten symbiotischen und parasitischen Bakterien in ihren Kiemengeweben ideale Modellorganismen sind, um die Mikrobiota von marinen Invertebraten zu studieren.

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Preface

About the structure of this thesis

This thesis is composed of four general parts. Part I is the Introduction, where all the concepts on which this thesis is based are summarized. Within this part Chapter 1 describes the main models of symbiosis in a general context. Chapters 2-5 describe the habitats, hosts, symbionts and methods relevant to this thesis. The aims of this thesis are explained in Chapter 6. Part II is the summary and discussion of the results obtained during the PhD period. Three manuscripts are anticipated as a result of this thesis work and they are included in Part III. Part IV is the conclusion of the thesis. Herein I summarize and bring up the outlook of my area of investigation. The main objective of this thesis is accomplished in the moment you reader have fun learning about symbiosis through these pages.

Abbreviations

APR - Dissimilatory adenosine-5'-phosphosulfate reductase
aprA - Gene coding for the alpha-subunit of the APR
APS - Adenosine-5'-phosphosulfate
BLAST - Basic Local Alignment Search Tool
CARD - Catalyzed reporter deposition
CH_4 - Methane
CO_2 - Carbon dioxide
COI - Mitochondrial cytochrome c oxidase subunit I
CTAB - Hexadecyl-trimethyl-ammonium bromide
DAPI - 4'6-diamidino-2-phenylindole
DNA - Deoxyribonucleic acid
EDTA - Ethylendiamintetraacetic acid
FA - Formamide
FISH - Fluorescence *in situ* hybridization
GoM - Gulf of Mexico
H_2S - Hydrogen sulfide
mRNA - Messanger RNA
MTPH - Methyl-toluene-phenol hydroxylase
NIX - Nuclear inclusion X
PCR - Polymerase chain reaction
pmoA - Gene coding for the pMMO active subunit
pMMO - Particulate methane monooxygenase
RNA - Ribonucleic acid
ROV - Remotely Operated Vehicle
rRNA - Ribosomal RNA

SO_4^{2-} - Sulfate
Taq - *Thermus aquaticus*

Part I

Introduction

Chapter 1

Invertebrate-bacteria associations

Symbiotic bacteria are widespread within almost all invertebrate animals. Insects are the most studied group and they overwhelm the pool of described invertebrate species, 1 million species are formally described but it is estimated that there are about 3 to 30 million species (Gaston 1994). Far less is known about the biodiversity of marine species than terrestrial ones but it is estimated that there are 1-10 million species of only deep-sea invertebrates (May 1992), and that marine invertebrates have the greatest phylogenetic diversity among animals (Brusca and Brusca 1990, McFall-Ngai and Ruby 2000). Thus, it is likely that the greatest variety of animal-bacterial symbioses occurs within this group. Marine bacteria-invertebrate associations have been greatly studied in marine annelids like *Riftia pachyptila* (Cavanaugh *et al.* 1981, Di Meo *et al.* 2000, Bright and Sorgo 2003, Bright and Bulgheresi 2010), *Olavius algarvensis* (Dubilier *et al.* 2001, Ruehland *et al.* 2008) or *Escarpia* and *Lamellibrachia* vestimentiferans (reviewed by McMullin *et al.* 2003, Bright and Bulgheresi 2010), in sponges (Vacelet and Donadey 1977, Friedrich *et al.* 1999, Radjasa and Sabdono 2009), and among the mollusks, the squid *Euprymna scolopes* (McFall-Ngai and Kimbell 2001, McFall-Ngai *et al.* 2010), clams (Southward 2009, Fisher 1990, Newton *et al.* 2007), mytilids (Distel1994, Nelson *et al.* 1995, Van Dover and Trask 2000, Duperron *et al.* 2009) and other mussels such as *Lyrodus pedicellatus* (Distel *et al.* 2002). Three types of metabolic interactions have been recognized in symbioses in general, and also in bacteria-marine invertebrates symbioses in particular: 'phototrophic' - where bacteria like cyanobacteria live associated

to sponges, ascidians, or echiuroid worms and gain energy from light (Usher 2008); 'heterotrophic' - where bacteria use organic compounds as carbon source. Examples are sponges (e.g. Friedrich *et al.* 1999) and *Osedax* spp. symbioses (e.g. Rouse *et al.* 2004); and 'chemosynthetic' - where bacteria convert one or more carbon molecules (usually carbon dioxide or methane) and nutrients into organic matter using methane (methanotrophs) or inorganic compounds such as hydrogen sulphide (thiotrophs) as electron donors (for a review on chemosynthesis see Dubilier *et al.* 2008). Chemoautotrophic bacteria (as thiotrophs) would use CO_2 as carbon source. If we track back and observe the symbiotic associations in the whole invertebrate group we find that the insect symbiosis research is the oldest within the symbiology studies (Hertig and Wolbach 1924, Buchner 1965). This is the cutting edge area and I think we should learn about it and discuss general results compared with insect models. Then, we will be able to standardize names and concepts and expand the symbiology studies with a better foundation.

1.1 The different models

This section summarizes some of the most important models of invertebrate symbiosis. They are the most studied models and the most advanced in the sense of information and understanding; therefore they are the most complete. I have chosen examples to include one of each case of symbiosis: heterotrophic, chemoautotrophic, mixed, intracellular, extracellular, obligatory, and facultative (see Glossary for explanation of concepts). The focus of this thesis is on marine symbioses, however I start by introducing a terrestrial model because of its great importance to symbiology studies: the insect-bacteria symbioses. I do not choose only one insect model because I would like to show the symbiosis diversity in insect studies. These diverse associations are not strict and are even dynamic, making their study more difficult and challenging, however concepts are broad and well accepted. For example the 'S' concept, about facultative symbionts, (more details in Section 1.1.1) is a very dynamic concept that leaves the door open to include many different associations and gives importance to the non-obligatory associations. We

find in insect-bacteria associations all the main different symbioses described so far: intracellular or extracellular, obligate or facultative, mutualistic, commensalistic or parasitic. Some of these bacteria have been cultivated, which permits a better understanding about the transmission process, the ecological importance, and the physiological intricacies of the different symbioses. We know now that bacterial symbionts influence many physiological functions of insects. In conclusion, insect studies teach us many biochemical pathways used by the insect-bacteria association, the experimental design used for their study, and the ecological importance that they might have. Perhaps we would find all these functions in bacterial symbionts from marine organisms but the studies are far too few in comparison. Comparisons of marine and terrestrial symbioses should improve our understanding of both. In next section (Section 1.1.2) I go directly to the marine systems and introduce the heterotrophic symbiosis of squid-*Vibrio* bacteria. As this bacterium has also been cultivated, the study at the molecular and physiological level is remarkable, being perhaps the most understood marine symbioses at the molecular level. In Section 1.1.3 I do a synthesis of the gutless worm symbiosis, as this might be one of the most studied models where there is the presence of both chemoautotrophic and non-chemoautotrophic bacteria. This is a very particular symbiosis because it is a well studied extracellular but endogenous marine symbiosis. Finally in section 1.1.4 I introduce vesicomyids clams as they maintain a very well studied chemoautotrophic symbiosis. It is a one-to-one (bynarian) host-symbiont obligatory association and they are a group close-related to the main group of interest in this thesis, the *Bathymodiolus* mussels, which I will be introducing in Section 3.1. Also, they belong to the bivalves, which are the focus of the third manuscript.

1.1.1 Insects

Insects are the largest described group of eukaryotic organisms where symbiotic microorganisms are universally present. It is believed that they have the most diversified symbiotic associations, both inside and outside their bodies (Bourtzis and Miller 2003). Symbionts influence insect nutrition, develop-

ment, reproduction and speciation, immunological responses, and habitat selection (Bourtzis and Miller 2003, Siozios *et al.* 2008, Bourtzis 2008, Buchner 1965), making insects the most versatile organisms on Earth. Insect symbionts are classified under two categories: 'primary' (P) and 'secondary' (S) symbionts, based on characteristic traits that for S-symbionts are complex and therefore difficult to define. P-symbionts are large bacteria hosted in specialized host cells (bacteriocytes), transmitted in a vertical mode (from parents to offspring), and have a coevolutionary history with their hosts. Insects with P-endosymbionts have a nutrient-poor diet, therefore their symbionts are nutritionally important to gain essential amino acids, vitamins, and other cofactors. S-symbionts are a very heterogeneous group because they are usually incidental infections with a highly variable function (Baumann 2005, Bourtzis 2008). Both positive and negative effects on the host have been observed in symbiotic associations involving secondary symbionts. Some of the positive effects are the capacity of infected hosts to survive heat stress, develop resistance to parasitic wasps, or exhibit altered host plant preference (e.g. Montllor *et al.* 2002, Oliver *et al.* 2005, Oliver *et al.* 2003, Scarborough *et al.* 2005, Tsuchida *et al.* 2004). In other cases, the facultative symbionts affect growth, reproduction, and longevity of the host (Chen *et al.* 2000; Min and Benzer 1997, Stouthamer *et al.* 1999). The importance of the S-symbiont is undetermined in part because of the dynamism that a symbiosis can have, e.g. 'replacement' can occur in aphids: an S-symbiont can take over the nutritional role of the disappeared P-endosymbiont (Koga *et al.* 2003). Furthermore, bacteria like *Wolbachia* that are members of the obligate intracellular rickettsiales forge not only parasitic relationships with arthropods, but also mutualistic relationships, primarily with nematodes (Merçot and Poinsot 2009). To date only S-symbionts have been cultivated (e.g. *Burkhordelia* of the broad-headed bug *Riptortus clavatus* (Heteroptera: Alydidae)). Nevertheless, non-cultivating methods like whole genome sequencing let us now gain insight into several species, and even more completely, the genome sequencing of both, the host and symbiotic bacteria. As example we have now the aphid-*Buchnera* association that have been sequenced twice (International Aphid Genomics Consortium 2010, Shigenobu *et al.* 2000).

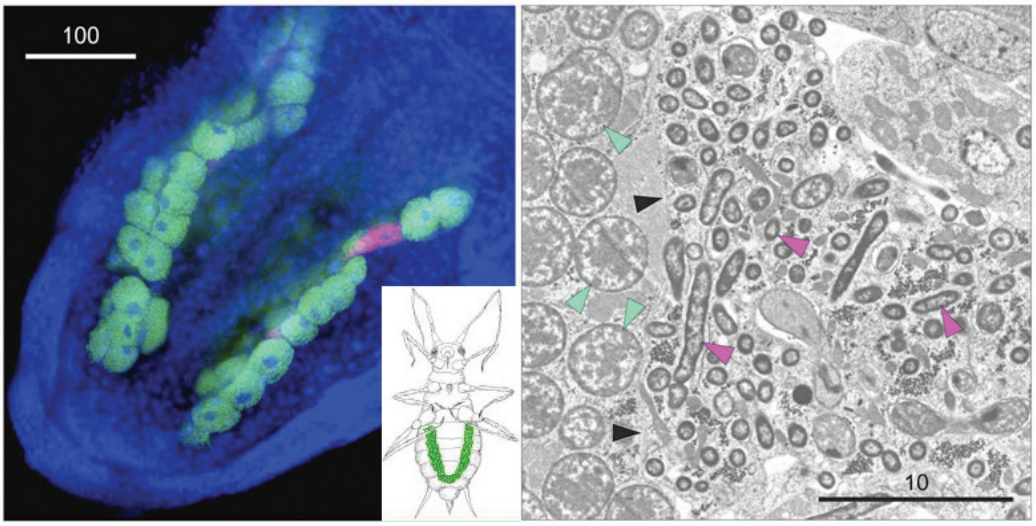

Figure 1.1: Aphid-*Buchnera* symbiosis. In the inset, the aphid scheme showing *Buchnera* in green. Left image showing symbiont-containing bacteriocytes within aphid abdomen revealed by FISH specific probes. Blue is a general DNA stain, highlighting aphid nuclei, in green *Buchnera* bacteria (P-symbionts) and in red *Regiella* (S-symbionts). In the right image, a micrograph showing elongate *Regiella* cells within a bacteriocyte (pink arrows) and nerby bactetiocytes containing *Buchnera* (green arrows). Black arrows indicate the bacteriome cell membrane. Scale bars are in microns. (From IAC 2010)

Buchnera genome analysis uncovered a large number of genes that likely code for amino acid biosynthesis genes and almost none for non-essential amino acids. It also revealed that obligate bacterial endosymbionts of insects have lost many genes and are among the smallest of known bacterial genomes. Another interesting observation is the absence of immunological response elements in the host, as it is the immune deficiency (IMD) pathway, which is present in other non-symbiotic insects and controls the recognition of Gram-negative bacteria. Also, the host lacks peptidoglycan recognition proteins (PGRPs), that detect certain pathogens and trigger immunological responses. In parallel with this genomes analysis there has been also great progress in the study of molecular processes that govern host-bacteria physiology, many genetic studies show the importance and evolution of certain genes. In conclusion the area of the insect symbiology is an important area with most advanced research.

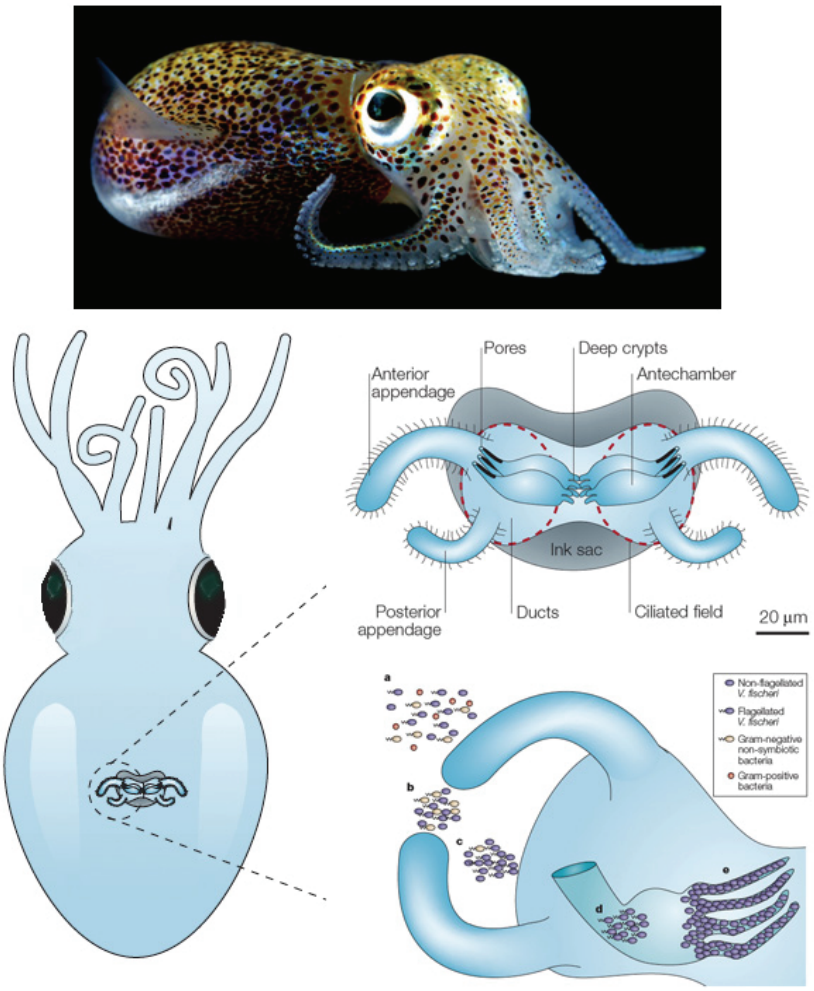

Figure 1.2: The light organ of the *Euprymna scolopes* squid is located in the ventral part of the body. The internal components of the squid light organ at hatching have very well developed appendages to which bacteria are attracted. Appendages regress when *Vibrio fisheri* has successfully colonized the crypt epithelium. The image below-right depicts the progression of the colonization. (a) Mucus is secreted in appendages as a positive feedback response to bacteria peptidoglycans. (b) Only viable Gram-negative bacteria form dense aggregations. (c) Motile or non-motile *V. fischeri* out-compete other bacteria and become dominant in the aggregation. (d) *V. fischeri* are the only bacteria able to migrate through the pores and colonize the host tissue. (e) Symbiotic *V. fischeri* become non-motile and induce host-epithelial cell swelling. Only bioluminescent *V. fischeri* will sustain long-term colonization of the crypt epithelium. (*E. scolopes* photo: E. Roettinger. Schemes from Nyholm and McFall-Ngai 2004)

1.1.2 Squid

The symbiotic association between the Hawaiian bobtail squid *Euprymna scolopes* and the bioluminescent bacterium *Vibrio fischeri* has been utilized as a model system for understanding many symbiologically essential questions, i.e., the effects of beneficial bacteria on animal development, the transmission hypothesis, and the role of the immune system in the acquisition and maintenance of symbiosis. *V. fischeri* is a heterotroph and it is found in free-living stage. When associated to the light-organ crypt (Fig. 1.2), its host provides to the bacteria carbon and nitrogen in the form of peptides and proteins (Graf and Ruby 1998). Over-population of the crypt spaces is controlled by a daily venting event at dawn, involving the expulsion of 95% of the crypt contents via the pores each dawn (Lee and Ruby 1994). The remaining crypt symbionts will then multiply to repopulate the crypts over the following day, completing the day-dawn cycle that the symbiosis has. The light produced by the symbiont is emitted downward, and the squid can manipulate the intensity of the light to match the intensity of down-welling moon and starlight, thus masking its silhouette to evade bottom-dwelling predators (Jones and Nishiguchi 2004). Whole genome sequencing (Ruby *et al.* 2005, Mandel *et al.* 2009) has brought also many insights into the potential functions of symbiosis. *V. fischeri* is an extracellular bacterial symbiont and it is transmitted horizontally (taken newly from the environment in each generation). The acquisition occurs thanks to the activation of the juvenile ciliated special tissue by bacterial peptidoglycans. After hatching, the host tissue enters in contact with many microbe membrane-associated molecules and starts secreting mucus abundantly. This mucus permits adhesion of bacteria, especially Gram negative, but at the end of 2-3 hours the aggregation is dominated by *V. fischeri* (McFall-Ngai *et al.* 2010). From here on, symbionts induce morphological changes in the host tissue and in the behaviour of hemocytes that flow through the host's hemolymph. It is not clear what the function of these hemocytes may be, but it seems they have some type of memory that make them distinguish symbionts from host cells and clear other bacteria by phagocytosis (Nyholm *et al.* 2009, McFall-Ngai *et al.* 2010).

Molecular signalization pathways are still unknown but very active research on the matter is underway.

1.1.3 Gutless oligochaetes

As their name indicates, gutless oligochaetes have no mouth or gut, therefore, these worms (2-50 mm long and 0.1 - 0.3 mm thick) depend obligatorily on symbiotic bacteria for nutrition (Dubilier *et al.* 2008). The symbionts are extracellular but occur endogenously between the cuticule and epidermis. An oligochaete like *Olavius algarvensis* can harbor as many as six co-occurring symbionts that belong to the Gamma-, Delta-, or Alphaproteobacteria, and a spirochaete has also been found (Blazejak *et al.* 2006, Ruehland *et al.* 2008). Enzyme assays, immunohistochemistry, and labeled carbon experiments indicate that at least some of the bacterial symbionts are thiotrophic, using reduced sulfur compounds as electron donors and fixing CO_2 autotrophically to generate organic carbon compounds (Dubilier *et al.* 2006, Ruehland *et al.* 2008). A metagenomic analysis performed in the oligochaete *Olavius algarvensis* showed that most probable the symbionts are engaged in a syntrophic sulfur cycle where Deltaproteobacteria are sulfate reducers and produce the reduced sulfur compounds that thiotrophic gammaproteobacteria oxidize as their primary energy source (Dubilier *et al.* 2001, Woyke *et al.* 2006). It is been proposed that some of the symbionts in these worms have a vertical transmission (Dubilier *et al.* 2006). Furthermore, the genome is not reduced but contains a high number of transposable elements. This may mean that symbionts are vertically transmitted and they are in an early stage of genome reduction (Dubilier *et al.* 2008).

1.1.4 Vesicomyid clams

Large vesicomyid clams (e.g. *Calyptogena* spp., "*Ectenagena*" *extenta*) have only a vestigial digestive tract, thus they depend nutritionally on their intracellular gammaproteobacteria symbionts. Individual bacteria are contained in a membrane-bound vacuole, and these are housed within host bacteriocytes. Symbionts are chemoautotrophs using energy from sulfide oxidation.

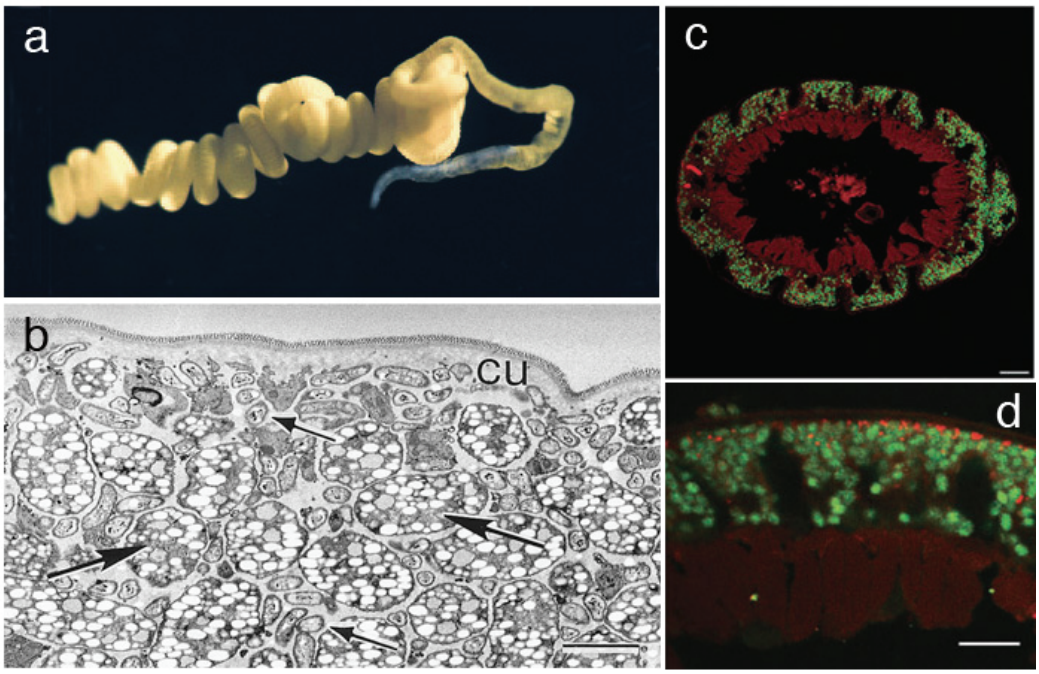

Figure 1.3: Gutless oligochaete symbiosis. As they lack a digestive system, gutless oligochaetes host bacterial symbionts to get their nutrition. (a) One of the model gutless oligochaete *Olavius algarvensis* (Photo: N.Dubilier). (b) Transmission electron micrograph of symbiont-containing region below the worm cuticle (cu). Small and large symbiont morphotypes are shown with smaller and larger arrows, respectively. Scale bar: 2 μm. (From Dubilier *et al.* 1995). (c and d) FISH identification of bacterial symbionts with specific probes. Two of the six contained phylotypes are localized, Gamma 1 (green) and Gamma 3 (red). Scale bars: 20 μm in (c) and 10 μm in (d). (From Ruehland *et al.* 2008)

It seems that vesicomyids synthesize a di-globular, non-heme molecule that runs within the blood serum and binds free sulfide, perhaps via Zn^{2+} residues (Childress *et al.* 1993; Franck *et al.* 2000), to provide their symbionts with the required electron donor. The transfer of nutritional compounds to the host is still not clear, but detection of lysozymes in the gills of the vent bivalve *Calyptogena magnifica* (Fiala-Médioni *et al.* 1994) could be an evidence of the digestion of the symbionts by the host. Symbionts are transmitted vertically between host generations via the egg (Cary and Giovannoni 1993); this model is supported by the phylogenetic coupling of mitochondrial with symbiont genes (Hurtado *et al.* 2003). As symbionts have not been cultured,

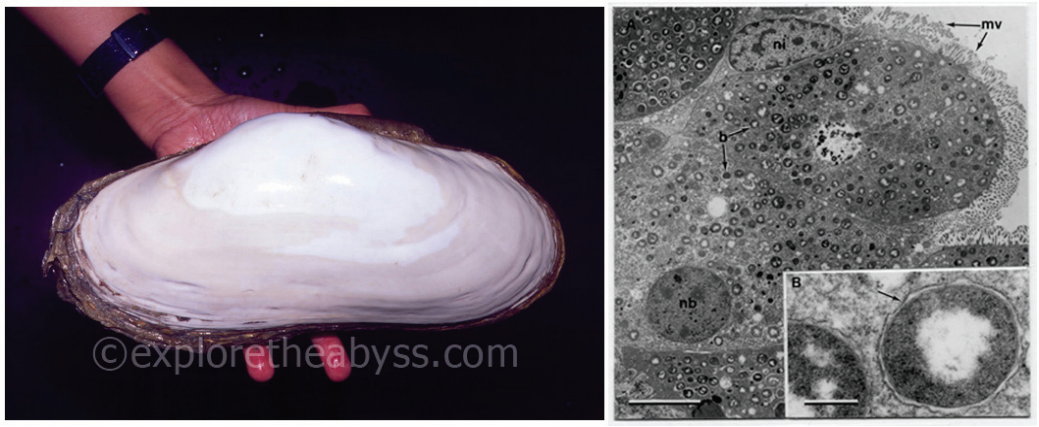

Figure 1.4: *Calyptogena*-thiotrophs symbiosis. A large ∽20 cm *Calyptogena* clam is shown in left image (photo: www.exploretheabyss.com). In the right (A) Transmission electron micrograph of gill filament, showing coccoid-shaped symbiotic bacteria within a bacteriocyte and intercalary cells lacking symbionts; b: bacteria; mv: microvilli (of both cell-types); nb: nucleus of bacteriocyte; ni: nucleus of intercalary cell. (B) Higher magnification. Ultrastructure typical Gram-negative bacteria and peribacterial membrane (arrow). Scale bars: A, 5 μm, B, 0.25 μm. (From Cavanaugh 1985).

metagenomics have been used to sequence the bacterial genomes. Two different whole genome sequencings were performed by Kuwahara *et al.* 2007 and Newton *et al.* 2007: after a small process of tissue homogenization, filtration and host DNA digestion, bacterial cells were separated from the host which permitted submission of the bacterial DNA to a whole genome sequence analysis with little host DNA interference. The symbiont genomes sequencing has shown that these are the smallest genomes within autotrophic bacteria, and also has given the possibility of linking the symbiosis metabolism and transmission hypothesis, with the potential implicated genes, as well as a better overview of the diversification and genomic evolution. One of the latest descriptions shows that vesicomyid symbionts have two different sulfur oxidation pathways, one for thiosulfate and one for sulfide, which could be an adaptation to the resource competition between tubeworms and bivalves (Harada *et al.* 2009).

1.2 Summary: The role of symbioses

Symbiosis is a way to obtain shelter, nutrients (needed compounds), or energy. But this is not the only level of importance that an association between organisms has: it also stamps evolutionary traces on both sides, and sometimes it brings a new organism into play. We know now many of the roles that symbionts have in some of the well studied symbioses, however there are still many unclear pathways and many mysterious processes, such as how nutrients are transmitted or how obligatory an association is. After physiological, stable isotopic, enzymatic, and molecular studies, we know now that essential amino acids, vitamins, and other cofactors are transmitted from symbionts to insects; also that C1-elements are transferred from methanotrophs to mussels, snails, and tubeworms (for a review of methanotrophs see Petersen and Dubilier 2009) in an organic source form, and that new fixed carbon compounds are provided by chemoautotrophic (sulfur oxidizers) symbionts to their hosts that vary from ciliates to arthropods, including nematodes, mollusks, and annelids (see Dubilier *et al.* 2008 for a review).

Box 1. Symbiosis Concept and Symbiology. ***Symbiosis*** is a greek word (συμβιωση) meaning: coexistence. As there are inconsistencies in the use of the term symbiosis, this thesis will refer to the concept as defined here. This word was first introduced as a biological descriptor in the second half of the 19th century. The study of lichens made explicit the need for a term to describe the coexistence of different organisms that result in a '"new"' entity with distinct morphological, genetic and metabolic capabilities. The concept was brought into use by two different lichen biologists, the Swiss, Simon Schwendener (1829-1919) and the German, Anton de Bary (1831-1888), who was likely the first to use the term '"symbiosis"' (de Bary 1878). Sometime before, the German botanist, Albert Bernhard Frank (1839-1900), proposed the term '"symbiotism"' (Frank 1877) but his work was less widely read than that of de Bary's. Around the same time, the terms *mutualism* and *commensalism* were coined by P.J. Van Beneden (1845-1910) referring to a 'benefit to both organisms' or a 'benefit to one of the associated organisms, with no benefit or harm to the other', respectively. *Symbiosis* with a mutualistic concept is used nowadays very often thanks to the historical connection to sociology, economy, politics, philosophy and other non-scientific endevours (Margulis and Fester 1991). However, to strictly define an association between organisms as parasitic, mutualistic, or otherwise, is not an easy matter, as certain associations are not stable and the definition of a benefit is not straightforward. Furthermore, the molecular mechanisms enabling the establishment of a parasitic, mutualistic, or any other association, are often similar (Hentschel *et al.* 2000), thus it is most logical to study these associations as 'symbiosis' *sensu lato*: This thesis shall be considered a *symbiological* study, contributing with novel description and understanding of two different systems: an endosymbiotic and an endonuclear one. Though not commonly used in literature, the term ***symbiology*** is the 'study of the symbioses' (Read 1970). I consider symbiology to refer to and focus on the different symbiotic systems and the mechanisms that govern them. A symbiological study aims to describe the different members of an association, their role, and their relationship, to model the symbiosis on a holistic system level. This contributes to the understanding of the physiology, ecology and evolution of the organisms involved in the association.

Chapter 2

Habitats

In this chapter I will introduce the habitats that are of interest to this thesis, from a general view to the specific habitat. Two main marine ecosystems are reviewed: deep-sea cold seeps and shallow-water coastal zone. These habitats differ greatly, but they both host a stable bivalve community.

2.1 Deep-sea cold seeps

As a general description, a 'cold seep' is a site where there is seepage of hydrocarbons (in gas or liquid state), other gases such as hydrogen sulfide, carbon dioxide, and also brines, which combine to make the environment very energy-rich. The main hydrocarbon gas in most seeps is methane. There is not yet an unequivocal explanation about how the seepage composition in cold seeps is so highly charged in methane, it seems that phase partitioning and fractionation during upward migration of hydrocarbons, and interaction with water, minerals, and catalytically active transition metals in sedimentary basins determine the final gas and oil composition (Seewald 2003). This would be the explanation for the petrogenic origin of methane. Nevertheless, there is active microbial activity in the subsurface that would be responsible for the biogenic methane and sulfide supply. Methanogenic archaea and sulfate reducers, carry out diverse anoxic processes: methane generation, anaerobic methane oxidation (AOM) and sulfide production. These processes determine habitat geochemistry.

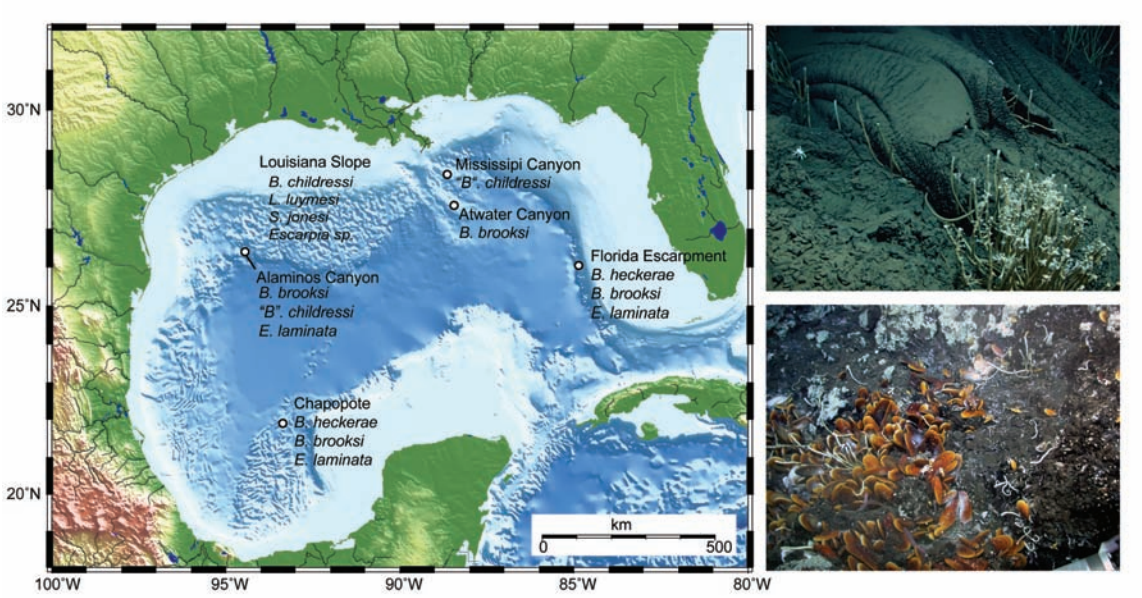

Figure 2.1: Gulf of Mexico. The most studied sites are mapped with their respective *Bathymodiolus* and tubeworm fauna. The two pictures in the right show the megafauna in Chapopote site (MARUM Copyright).

2.1.1 Gulf of Mexico

The Gulf of Mexico (GoM) is the ninth largest body of water in the world, with an oval shape and a diameter of 1500 Km. It connects with the Atlantic Ocean through the Florida Strait between the U.S.A. and Cuba, and with the Caribbean Sea via the Yucatan Channel between Mexico and Cuba. The GoM seafloor is composed principally of evaporates, red sediment, intrusive, and metamorphic rocks. Underneath, a few kilometers below the surface floor, a huge deposit of hydrocarbons is found. These deposits date from the upper Jurassic period and are considered to jointly represent one of the biggest reserves in the world (Nehring 1991). Saline deposits are found towards the surface sediment, creating a very dynamic floor. Diapirism, or saline-density movements, commonly occur throughout the GoM. Cold seeps (methane and hydrocarbon seepage) are widespread in the GoM as a result of its special tectonics and geological history. Here, hydrocarbon seepage and gas hydrates are two of the common settings. Methane and sulfide, chemosynthetic life-sustaining elements, are normally present providing rich

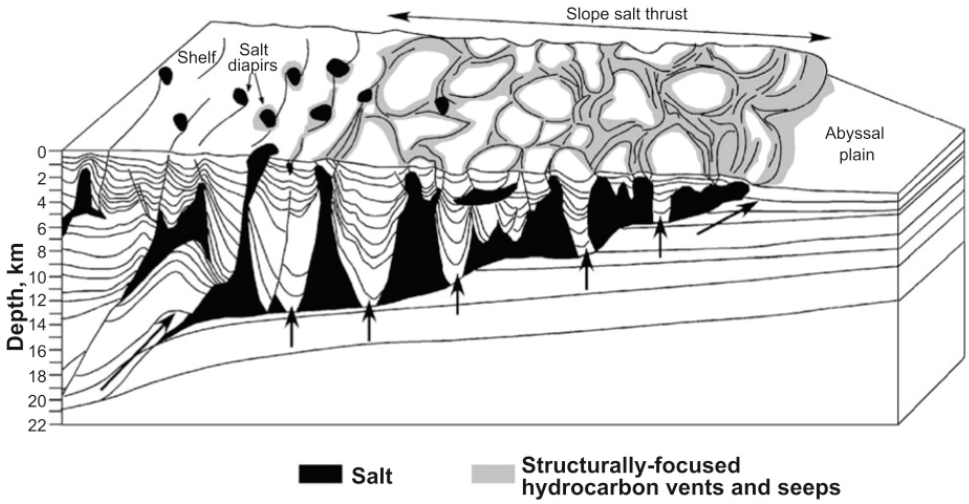

Figure 2.2: Salt domes. Diapirism, or saline-density movements, commonly occur throughout the GoM allowing hydrocarbons to seep to the surface. Source rocks are deeply buried beneath the allochthonous salt. Fluids migrate upward through holes in the salt thrust (arrows). Within basins, salt and related faluts provide conduits for vertical migration of fluids to reservoirs and to seafloor. (From Sassen *et al.* 2004)

energy sources for chemosynthetic bacteria (Lanoil *et al.* 2001, Orcutt *et al.* 2005). The biology of the seeps in the northern Gulf of Mexico is well studied (e.g. Fisher 1993, Cavanaugh *et al.* 1987, Cavanaugh 1993, Carney 1994, Cordes *et al.* 2005, 2007), but in the southern part the studies are scarcer, making interesting a comparison between the symbiotic fauna of the northern and southern sites. In the southern GoM, off the Mexican state of Campeche there is a region called Campeche Knolls with a depth of almost 3000 m. This area has a hummocky (many low ridges present) topography derived from diapirism. Traps or paths of hydrocarbons in this zone are found frequently seeping to the sediment surface and water column (Ewing 1991, Zhao and Lerche 1993). A very unique area of the deep ocean was discovered here in November 2003: among the large deserts of soft sediment a monticule of solidified asphalt was found (MacDonald *et al.* 2004). Lava-like fluids and well-developed metazoan communities living in between the asphalt layers were observed. The locality was called Chapopote ("tar" in Spanish).

2.1.2 Chapopote

Chapopote is a unique cold seep where asphalt, gas hydrates, and hydrocarbons are present all together in a deep-sea environment (2930 meters depth). It seems that the asphalt flows out from time to time, making the habitat very dynamic. It is suggested that shifts in this habitat occur in relatively short time periods (MacDonald *et al.* 2004), consequently the biological community would have to re-structure constantly. Nevertheless, shrimps, tubeworms, bivalves, and other fauna are abundant and coexist in this amazing environment. What makes this site a special habitat with unique parameters is that the oil has more asphaltenes that make it heavier, with a density higher than water. Thus, the oil stays in the seafloor, while in other settings the oil leaks upward to the water surface. And it is not just that it stays in the depths but it stays in an oxic area and hydrocarbons can be aerobically oxidized. Also, this is a system with new substrate for the megafauna to settle, as there are not just carbonates but also solid asphalt formations.

2.2 Shallow-water coastal zone

The "coastal zone" is a transitional area in which terrestrial environments influence marine environments and marine influence terrestrial ones (Carter 1988). This is a zone conformed mainly of shallow water habitats that are characterized depending on their geographic location, and biogeochemical parameters. Important parameters are the depth, grain size (fine-grained or coarse-grained), sedimentology (soft bottom, carbonate concretions), and hydrodynamics. Five trophic guilds are recognized within the shallow water mollusks: suspension-feeders, deposit-feeders, carnivores, woodborers and chemoautotrophs; and these are distributed within six habitat tiers: epifaunal cemented, epifaunal byssate, semi-infaunal, shallow infaunal, deep infaunal and boring (Stanley 1970, Grill and Zuschin 2001) (Fig. 2.3). In contrast to the deep sea, where there is lack of organic matter from photosynthetic primary production, shallow waters are organic rich at the pelagic and benthic level. Additionally, loads of organic matter and nutrients from human practices are deposited in these ecosystems (Andersson *et al.* 2005). For all

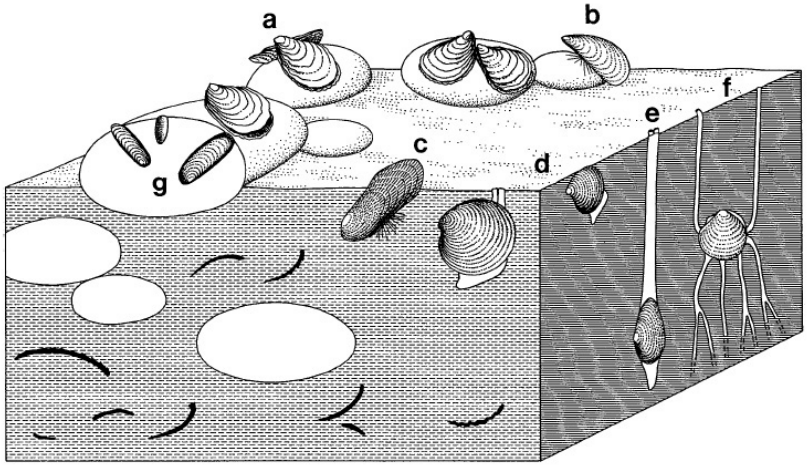

Figure 2.3: Bivalves in their habitat tiers. (a) epifaunal cemented (e.g. oysters), (b) epifaunal byssate (e.g. mytilids), (c) semi-infaunal (e.g. modiolids), (d) shallow infaunal (e.g. venerids), (e) deep infaunal (e.g. mactrids, razor clams), (f) deep infaunal (lucinids) - that have a tube system which is formed with their extendable foot to obtain hydrogen sulfide from underlying sediments, (g) boring (e.g. lithophagins). (From Grill and Zuschin 2001).

of the above reasons, life of bivalves in this ecosystem becomes very dynamic, as they should be constantly adapting to the changing conditions. Yet, they might not succeed and diseases would diminish bivalve populations (see also Section 3.3: shallow-water bivalves).

Chapter 3

Hosts

3.1 Deep-sea *Bathymodiolus* mussels

Deep-sea mytilid mussels of the genus *Bathymodiolus* have been found and studied all around the world. The presence of this genus is limited to hydrothermal vents and cold seeps (Distel *et al.* 2000, Miyazaki *et al.* 2010). Bathymodiolin mussels (*Bathymodiolus* spp. and relatives) rely for their nutrition on endosymbionts harbored in bacteriocytes, specialized cells of the gill tissue. Some bathymodiolin species host thiotrophic symbionts, methanotrophs or both (see Section 4.1 for more details on symbionts). They retain the ability to filter-feed which, in combination with their symbiotic associations, contributes to their broad geographic success (Fisher *et al.* 1987). In the Gulf of Mexico seeps, five bathymodiolin species have been described, three of them belonging to the *Bathymodiolus* genus: *B. childressi*, *B. brooksi*, *B. heckerae* (Gustafson *et al.* 1998). *B. childressi* mussels have been found all along the Louisiana slope including the Alaminos Canyon. *B. brooksi* has been found in the Atwater Canyon and co-existing with *B. childressi* in the Alaminos Canyon, and with *B. heckerae* in the West Florida Escarpment. *B. heckerae* mussels have been reported from the West Florida Escarpment, and also out of the GoM in Blake Ridge, off East Florida (see Figure 2.1 for species location in the GoM). Bathymodiolin mussels harbor different endosymbionts. Whereas *B. childressi* has only methanotrophs (Fisher *et al.* 1987, Distel and Cavanaugh 1994, Duperron *et al.* 2007), *B. brooksi* and *B. heckerae* possess a dual symbiosis of thiotrophic and methanotrophic bacte-

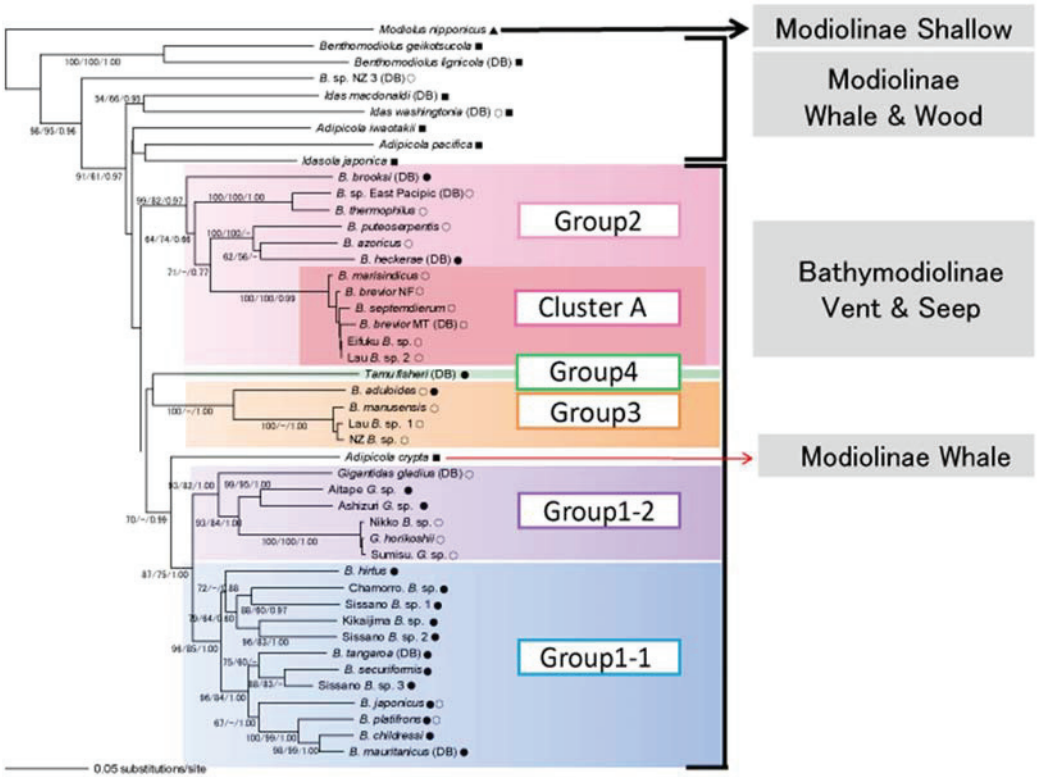

Figure 3.1: Phylogeny of *Bathymodiolus* mussels based on COI and ND4 sequences. The scale bar indicates 0.01 substitutions per site. (empty circles) hydrothermal vent; (full circles) cold-water seep; (squares) wood/whale bone; (triangles) shallow. (From Miyazaki *et al.* 2010).

ria (Cavanaugh *et al.* 1987, Fisher 1993, Duperron *et al.* 2007). Recently, more than two phylotypes of bacteria were observed, namely in *B. heckerae* which harbors four co-ocurring symbionts, a methanotroph, two phylogenetically distinct thiotrophs, and a methylotroph-related one (Duperron *et al.* 2007). To date, phylogeny and distribution of *Bathymodiolus* spp. mussels and their symbionts from the GoM have only been described in species from northern locations (see Table 3.1), and it is not known how mussels and their symbionts from the southern GoM are related to the former ones. Mitochondrial cytochrome c oxidase subunit I (COI) gene has been used to determine the phylogeny within *Bathymodiolus* species (Miyazaki *et al.* 2004, Iwasaki *et al.* 2006, Jones *et al.* 2006) with a good definition. However analysis with several concatenated genes as ND4 and 28S rRNA (e.g. Won *et al.* 2008,

Miyazaki *et al.* 2010) promise to give better phylogenetic histories.

Table 3.1: Distribution of *Bathymodiolus* mussels and their symbionts. T indicates thiotrophic and M methanotrophic and their relative abundance. HV–hydrothermal vent; CS–cold seep. (modified from DeChaine and Cavanaugh 2005 and Duperron *et al.* 2005

Zone	Species	Symb	Hab	Reference
PACIFIC				
East P. Rise	*B. thermophius*	T	HV	Fiala-Médioni *et al.* 1986
North Fiji	*B. brevior*	T	HV	Distel and Cavanaugh 1994, Dubilier *et al.* 1998
Japan	*B. japonicus*	M	HV and CS	Hashimoto and Okutani 1994
	B. platifrons	M	HV and CS	Fujiwara *et al.* 2000, Barry *et al.* 2002
	B. septemdierum	T	HV	Fujiwara *et al.* 2000
	B. sp.	T	HV	McKiness *et al.* 2005
ATLANTIC				
Mid-Atlantic Ridge	*B. azoricus*	T>M	HV	Fiala-Medioni *et al.* 2002
	B. puteoserpentis	T>M	HV	Distel *et al.* 1995
Gulf of Mexico & Blake Ridge	*B. childressi*	M	CS	Fisher *et al.* 1987 Distel and Cavanaugh 1994
	B. heckerae	M>T	CS	Cavanaugh *et al.* 1987, Salerno *et al.* 2005, Duperron *et al.* 2007
	B. brooksi	M>T	CS	Fisher 1993, Duperron *et al.* 2007
Gabon Margin	*Bathymodiolus sp.*	M + T	CS	Duperron *et al.* 2005
Barbados	*B. boomerang*	M + T	CS	von Cosel and Olu 1998

3.2 Deep-sea *Escarpia* tubeworms

Adult vestimentiferan tubeworms lack a digestive tract and depend on their chemoautotrophic symbionts for nourishment. They host their symbionts in a specialized organ, the trophosome, a highly irrigated tissue complexed with bacteriocytes. Tubeworm taxonomy has been intensively investigated

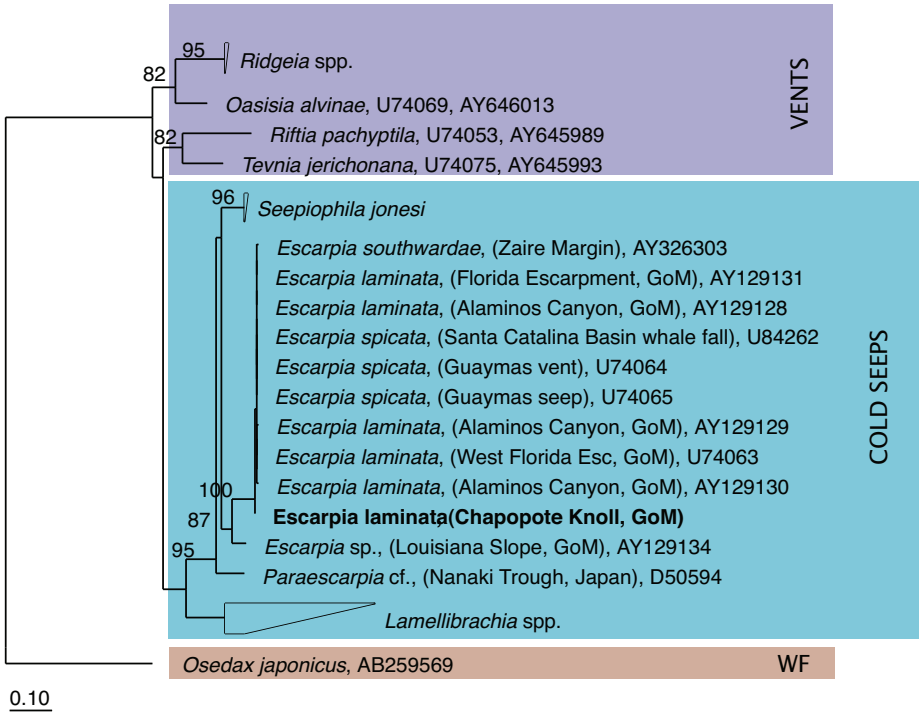

Figure 3.2: Phylogeny of vestimentiferan tubeworms based on COI sequences. Tubeworms from the three different deep-sea habitats are shown: vents (violet), cold seeps (blue) and wood-fall (W - in brown). New specimen from this study appears in bold. The tree was built based on all the sequences publicly available, using RAxML, with 100 bootstrap replicates and rooted on *Osedax japonicus*. Scale bar indicates 10% estimated base substitution.

in recent years (e.g. McHugh 2000, Halanych *et al.* 2001, Rousset *et al.* 2007, McMullin *et al.* 2003). The current classification places all tubeworms inside the vestimentiferan group which belongs to the family *Siboglinidae* (McMullin *et al.* 2003). They have many morphological and molecular features in common, such as no mouth or functional gut, a trophosome tissue full of symbiotic bacteria and closely related COI sequences. Tubeworms are found generally in highly sulfidic habitats on continental margins, hydrothermal vents, and cold seeps, with seeps inhabited mainly by escarpids and lamellibrachids. Both groups are widely distributed in all ocean basins but the Indian. Vestimentiferan tubeworms from the northern Gulf of Mexico have

been well studied, for example by McMullin *et al.* 2003, who made an extensive study of the phylogeny and biogeography of these tubeworms and their symbionts using the 18S rRNA, COI, and 16S rRNA genes. A lot of data was generated from this study which showed that there was no congruence or clear pattern between both host and symbiont phylogeny. Two escarpid species *Escarpia laminata* and *Seepiophila jonesi*, are characteristic in the GoM basin, as is the lamellibrachid *Lamellibrachia luymesi* (Nelson *et al.* 1995, McMullin *et al.* 2003). Therefore, we expect to find these tubeworms or closely related species at Chapopote. No molecular studies, as far as we know, have been performed with species in the southern GoM and thus it is of interest to compare these southern tubeworms and their symbionts from an asphaltic location, with the not too distant northern tubeworms. Vent vestimentiferan symbionts are related and belong to the Gammaproteobacteria group. Seep symbionts are phylogenetically more diverse; nevertheless, lamellibrachid and escarpid symbionts form a cluster with the sulfide-oxidizer symbiont bacteria of the vent vestimentiferans within the Gammaproteobacteria (Fig. 3.2).

Table 3.2: Distribution of *Escarpia* tubeworms and their symbionts. G1, G2 and G3 indicate thiotrophic symbionts belonging to the different groups suggested by McMullin et al 2003. WF–wood fall; HV–hydrothermal vent; CS–cold seep

Zone	**Host sp.**	**Acc**	**Ref**	**Symb**	**Acc**	**Ref**	**Hab**	**Depth**	**Site**
Eastern Pacific	*E. spicata*	U84262	Feldman 1998	G2	U77482	Feldman 1997	WF	1240	Santa Catalina
	E. spicata	U74065	Black 1997	G1	DQ232903	Vrijenhoek et al 2007	CS	1653	Guaymas TF
	E. spicata	U74064	Black 1997	V	AF165908,9	Di Meo et al 2000	V	2020	Guaymas ST
	L. barhami		Black 1997	G1	DQ232902	Vrijenhoek et al 2007	CS	1653	Guaymas TF
	L. barhami	U74055	Black 1997	G1	AY129113	Nelson and Fisher 2000; McMullin et 2003	V	2400	Juan de Fuca, Middle Valley
	L. barhami	AY129137,8	McMullin *et al.* 2003	G2	AY129093,4	McMullin *et al.* 2003	CS	1000	Monterey Canyon
Western Pacific	*P. echinospica*	N/D	Southward 2002	N/D			V	1660-1900	Manus Basin
	P. echinospica	D50594	Kojima 1997	N/D			CS	1200	Nankai Trough
	P. echinospica	D50595	Kojima 1997	N/D			V	680-1000	Okinawa Trough
	New escarpid	D50593	Kojima 1997	N/D			CS	300	Nankai Trough
	L. columna	U74061	Black 1997	G1	U77481	Feldman 1997	V	1890	S. Lau Basin
Gulf of Mexico	*E. laminata*	AY129128-30	McMullin *et al.* 2003	G2	AY129102,8	Nelson and Fisher 2000; McMullin et 2003	CS	2200	Alaminos Canyon

Continued on next page...

Table 3.2 – continued

Zone	Host sp.	Acc	Ref	Symb	Acc	Ref	Hab	Depth	Site
	E. laminata	U74063, AY129131	Black 1997	G1	AY129106,7	Nelson and Fisher 2000; McMullin *et al.* 2003	CS	3300	Florida Escarpment
	Second escarpid	AY129134	McMullin et 2003	G3	AY129089	McMullin et 2003	CS	540-640	Louisiana Slope
	E. laminata		This study	G1		This study	CS	2915	Chapopote
	Lamellibrachia sp.			G1	U77479	Feldman 1997	CS	∽1500	Green Canyon
	L. cf. luymesi			G3	AY129100	Nelson and Fisher 2000	CS	∽1500	Green Canyon
	Lamellibrachia sp.			G3	AY129110	Nelson and Fisher 2000	CS	540 580	Bush Hill
	S. jonesi			G3	AY129092	Nelson and Fisher 2000	CS		Garden Banks
E Atlantic	*E. southwardae*	AY3263034	Andersen 2004	N/D					Zaire Margin

3.3 Shallow-water bivalves

Shallow water bivalves are widespread along coastal habitats. They are filter-feeding animals that draw water in over their gills, extracting organic matter from the water in which they live. An oyster can filter up to five liters of water per hour (Prieur *et al.* 1990). Suspended matter (phytoplankton, zooplankton, algae, and other nutrients and particles) is trapped in the mucus of a gill, and from there is transported to the mouth, where it is eaten, digested, and expelled as feces or pseudofeces. Due to this filter-feeding mechanism, a high quantity of bacteria accumulates in the gill tissue. Bivalves harvested for human consumption are submitted to a depuration process, where water is run through their gills, to reduce the amount of particles and bacteria present on this tissue. Bacterial communities of bivalves have been characterized, but mainly from the human health standpoint of view, biasing research toward the study of the pathogenic bacteria diversity. There are also research efforts in analyzing the potential of bivalve-bacteria associations as producers of metabolites with antimicrobial agents (e.g. Zheng *et al.* 2005, Lemos *et al.* 1985, Ivanova *et al.* 1998, Burgess 1999). Zheng *et al.* (2005) described that more cultivated bacteria associated with invertebrates (20%) have antimicrobial activity than bacteria isolated from seaweed (11%), water (7%), or sediment (5%). These bacteria are not considered 'true symbionts' but only associated bacteria. However, depending on the symbiosis definition used (i.e. the *sensu lato* concept) these bacteria could be considered real symbionts as their nutrition would be based on the vitamins, polysaccharides, and fatty acids from the host tissue; and on the other hand they would be excreting products such as amino acids and toxins, propitious to their host's development (Zheng *et al.* 2005, Armstrong *et al.* 2001).

Box 2. Immunology of bivalves. Molecular studies of bivalve recognition systems for bacteria are limited. Nevertheless, there is already some knowledge about the bivalve immunological system and the responses that take place when confronting bacteria or pathogens. Bivalves possess various levels of defense mechanisms, and in general, they have very effective humoral and cellular defense responses. The first level includes natural physicochemical barriers, such as the exoskeletons, cockles, cuticles and mucus. The second level of defense includes circulating hemocytes and soluble factors in the hemolymph. Antimicrobial peptides secreted by hemocytes have been identified: mytilins, myticins, and defensis (Gestal *et al.* 2007). However, the main activity of the hemocytes is the phagocytosis, as invertebrates lack leukocytes, monocytes, or macrophages. And while they have not evolved a complex immunology (Canesi *et al.* 2002), hemocytes can have a response (chemotaxis or chemokinesis) to molecules or metabolites of bacteria (certain lipopolysaccharides, formylated compounds, peptides, or lectins that are undoubtedly involved in cell recognition by opsonization) and together with the other hemolymph factors, trigger a wide range of defense mechanisms (Canesi *et al.* 2002). After nonself-recognition (by ligand-receptor interactions not characterized to date) the foreign bacterium or the particle is internalized into a primary phagosome. Lysosomal granules fuse with this phagosome to form the secondary phagosome and shortly after integrate to mould a vacuole. Digestive glands provide enzymes to these vacuoles, and these enzymes have been observed, for example, in *Mytilus edulis* to possess N-acetyl-muramyl-hidrolases, lysozymes capable of degrading bacteria cell walls (Birkbeck *et al.* 1987). Different bacterial sensitivities suggest that the role of surface interactions between bacteria and hemolymph components is crucial in determining the fate of the invading microorganism in the tissue (Prieur *et al.* 1990, Rinkevich and Müller 1996).

Chapter 4

Bacterial Symbionts

4.1 Chemosynthetic symbionts

Chemosynthetic symbiosis was discovered almost 30 years ago in marine invertebrates, in particular within the megafauna from the hydrothermal vents and cold seeps where it was observed that primary production is not based on photo- but chemosynthesis (Cavanaugh *et al.* 1981). It has been inferred that these symbioses (thiotrophic and methanotrophic) are based on a mutualistic association where the host provides the substrates to the symbiont and the symbiont pays in return by providing organic carbon. Chemosynthetic symbioses are widespread in marine invertebrates, and the relationship varies depending on the host organism. For example, in shrimps the association is epibiotic (e.g. Segonzac *et al.* 1993, Petersen *et al.* 2010); with clams (e.g. Van Dover and Trask 2000), mussels (e.g. DeChaine and Cavanaugh 2005), and tubeworms (e.g. Cavanaugh 1985) it is intracellular; and finally extracellular symbionts are found in gutless oligochaetes (e.g Dubilier *et al.* 2001, Ruehland *et al.* 2008) and sponges (e.g Vacelet and Donadey 1977, Friedrich *et al.* 1999). Intracellular symbiosis is very specific and few symbiont phylotypes, based on electron microscopy, 16S rRNA sequence analysis, and FISH, are detected in each host, meaning that the diversity is limited and probably species-specific. The relative abundance of each phylotype is also variable. The studies in bathymodiolin mussels where thiotrophic, methanotrophic, or both bacterial symbionts are present with different relative abundances of each phylotype are shown in Table 3.1.

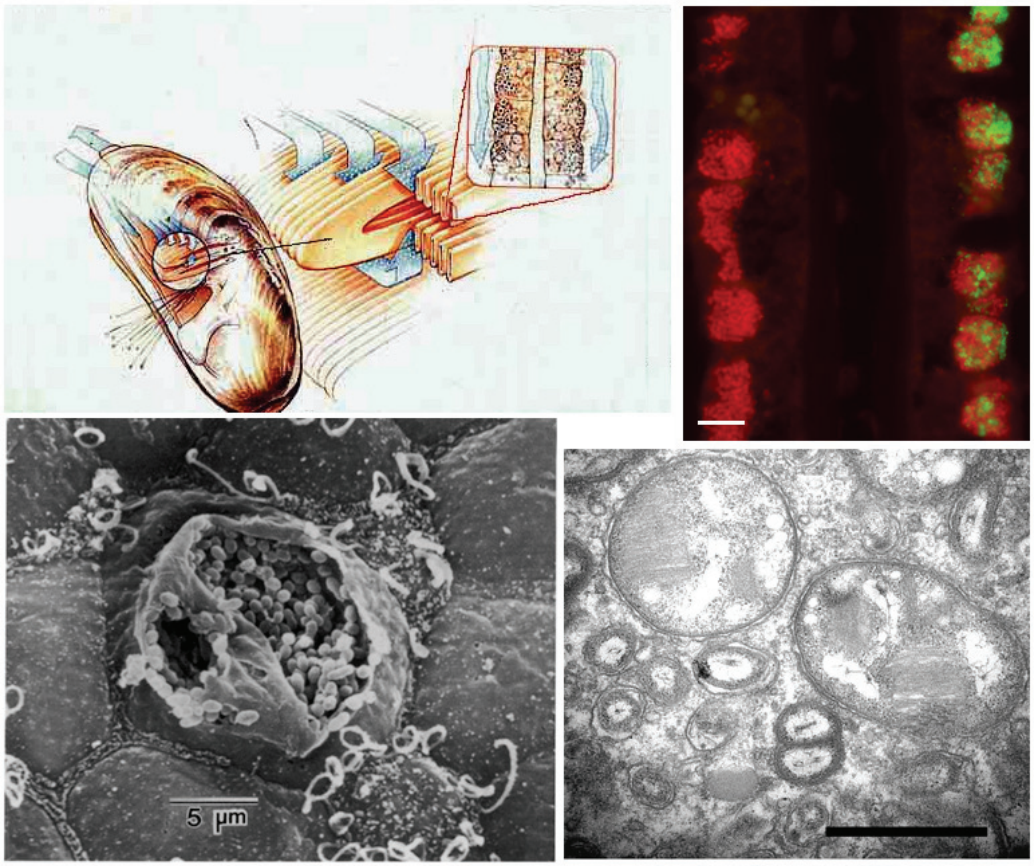

Figure 4.1: Bathymodiolin mussels host in their gills thiotrophic, methanotrophic or both types of bacteria. Top-left image depicts the location of gills, how the water flow through them (blue arrows) and a transversal cut normally used for microscopical preparations (Scheme from: http://homes.bio.psu.edu). In the top-right a transversal cut of a *Bathymodiolus* that harbours a dual symbiosis is hybridized with FISH specific probes for thiotrophic (green) and for methanotrophic bacterial symbionts (red). Scale bar: 10mum. (Photo: L.Raggi). Bottom-left image is a scanning electron micrograph showing an opened bacteriocyte revealing abundant intracellular bacteria. (Photo: Fisher *et al.* 1987). Bottom-right is a transmission electron micrograph showing small morphotype (thitrophs) and large morphotype bacteria (methanotrophs). Scale bar: 1 mum. (From Duperron *et al.* 2005).

4.1.1 Thiotrophic symbionts

Thiotrophic or sulfur oxidizer bacteria (also called chemoautotrophic) are able to get their energy from sulfide or other inorganic sulfur compounds, oxidizing it with oxygen or nitrate. The ATP that is produced fuels autotrophic CO_2 fixation (Figure 4.2). Although different phylotypes are found in each host the majority belong to the Gammaproteobacteria group (Figure 4.4). In the recently sequenced chemosynthetic endosymbiont genomes of the clams *Calyptogena magnifica* (*Candidatus* Ruthia magnifica) and *C. okutanni* (*Candidatus* Vesicomyosocius okutanii), a large number of biosynthetic pathways were present (Newton *et al.* 2007, Kuwahara *et al.* 2007). The sulfur oxidation process has been analyzed by means of genes and their transcripts by Harada and collegues (2009) and it seems oxidation pathways function simultaneously. They proposed that thiotrophic symbionts oxidize sulfide and thiosulfate. Sulfide is oxidized to sulfite by reversible dissimilatory sulfite reductase (*rdsr*). Sulfite is oxidized to sulfate by adenosine 5′-phosphosulfate (APS) reductase (*apr*) and ATP sulfurylase (*sat*). By means of the sulfur-oxidizing multienzyme system (*sox*), thiosulfate is oxidized to elemental sulfur, which is then reduced to sulfide by dissimilatory sulfite reductase (*dsr*). In addition, thiosulfate may also be oxidized into sulfate by another component of *sox* (Figure 4.2). The enzyme APR is present in both the reductive and the oxidative sulfur pathways, catalizing the transformation between APS and sulfite, in both directions. The *aprA* gene encodes for the alpha subunit of this enzyme, and it has become a marker gene to identify the presence of thiotrophic bacteria in a symbiotic system. Thiotrophic bacteria are potentially providing their host with the majority of its nutrition (Newton *et al.* 2007, Harada *et al.* 2009).

4.1.2 Methanotrophic symbionts

Aerobic methanotrophs are bacteria that use methane as both an energy (electrondonor) and a carbon source (for review see Cavanaugh *et al.* 2006, McDonald *et al.* 2008 and Petersen and Dubilier 2009). They are included in the broader class of the methylotrophs, which are defined as oxidizers of

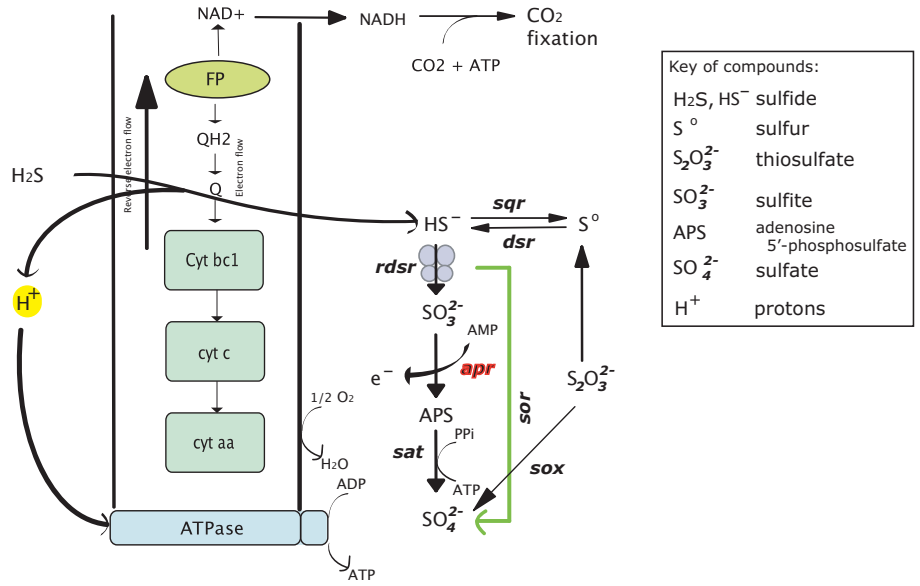

Figure 4.2: Oxidation of reduced sulfur compounds by sulfur chemolitotrophs (thiotrophs). Sulfide is oxidized to sulfite by reversible dissimilatory sulfite reductase *rdsr*. Sulfite is oxidized to sulfate by adenosine 5́-phosphosulfate (APS) reductase *apr* and ATP sulfurylase *sat*. By means of the sulfur-oxidizing multienzyme system *sox*, thiosulfate is oxidized to elemental sulfur, which is then reduced to sulfide by dissimilatory sulfite reductase *dsr*. In addition, thiosulfate may also be oxidized into sulfate by another component of *sox*. Almost all sulfur-oxidation pathways are present in a thiotrophic symbiont except for the sulfite oxidoreductase pathway (green arrow), which does not have sulfite as intermediate compound. From all pathways electrons from sulfur compounds feed into an electron transport chain (through membrane proteins: flavoprotein (FP), quinone (Q) and cytochromes bc1, c, aa) and drive a proton motive force that results in ATP production and a reverse electron flow that produce reducing power (NADH) for C fixation.(Image modified from: Harada *et al.* 2009, Newton *et al.* 2007, Madigan and Martinko 2009).

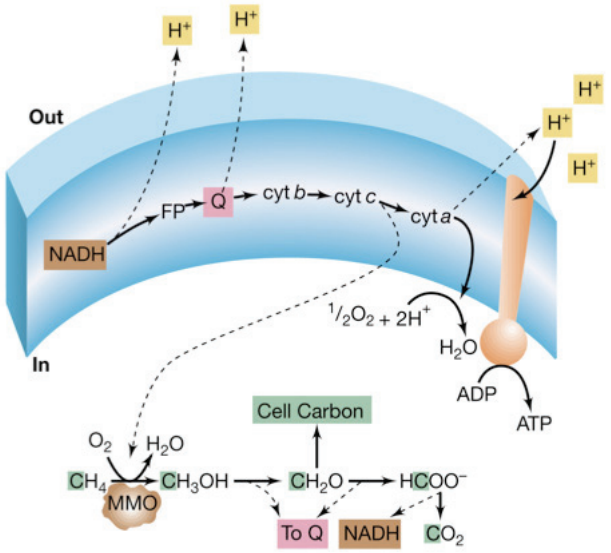

Figure 4.3: Aerobic methane oxidation by methanotrophs. Methane (CH_4) is converted to methanol (CH_3OH) by the enzyme methane monooxygenase. A proton motive force is established from electron flow in the membrane, and this fuels ATPase. Methanotrophs assimilate either all or one-half of their carbon (depending on the pathway used) at the oxidation state of formaldehyde (CH_2O). (Madigan and Martinko 2009).

C1 compounds, such as methanol, formate, and carbon monoxide (Bowman 2006). The gene coding for the active subunit of the particulate methane monooxygenase (*pmoA*) is an indicator of the aerobic methane oxidation pathway. The particulate methane monooxygenase (pMMO) is a membrane bound copper and iron containing enzyme and it is the first enzyme in the aerobic oxidation of methane pathway (Figure 4.3). It has been found in all methane-oxidizing bacteria investigated so far (Elsaied *et al.* 2006, Nercessian *et al.* 2005) except for the genus *Methylocella* (Theisen *et al.* 2005). It catalyzes the transformation of methane into methanol. Methanol is further converted to formaldehyde, and this is easily recognized in the biosynthesis pathways. The symbiont transfers the assimilated carbon rapidly to the host (Fisher *et al.* 1987, Fisher and Childress 1992, Streams *et al.* 1997) and the isotopic signature of the tissue (principally membrane lipids) becomes very negative, close to the values of the biogenic methane (Jahnke *et al.* 1995,

Pond *et al.* 1998, MacAvoy *et al.* 2002).

4.2 Hydrocarbon degraders

No hydrocarbon degrader symbiont has yet been described. However 79 bacterial species have been described that degrade hydrocarbons and use them as the sole carbon and energy source (Prince 2005). Crude oil or petroleum is a complex mixture (perhaps the most complex organic substance on Earth) of more than 17,000 compounds that can be classified into four groups: saturated and aromatic hydrocarbons, and non-hydrocarbon components: resins, and asphaltenes (Head *et al.* 2006). There are two types of isolated bacteria that use hydrocarbons almost exclusively as their carbon source, the ones that use a variety of saturated hydrocarbons: *Alcanivorax* spp., *Oleiphilus* spp., *Oleispira* spp., *Thalassolitus* spp., and *Planomicrobium* spp.; and *Cycloclasticus* spp. that use a range of polycyclic aromatic hydrocarbons (PAH). However, there are a good number of bacteria that degrade PAH but not as their only source belonging to the genus (*Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Beijerinckia*, *Alcaligenes*, *Micrococcus*, *Vibrio*, and *Mycobacterium*). *Cycloclasticus* are thus unique and are commonly found blooming in oil spills (Kasai *et al.* 2002, Maruyama *et al.* 2003). The first *Cycloclasticus* sp. bacterium was isolated in 1995 being *Methylobacter*, *Methylomonas* and the sulfur-oxidizing symbionts isolated from marine invertebrates *Lucinoma aequizonata* and *Thyasira flexuosa* the closest relatives (Dyksterhouse *et al.* 1995). Fatty acid composition of isolated *Cycloclasticus* is not peculiar as their predominant fatty acids are $16\omega7cis$ and 16:0, which are characteristic of general bacteria. However an unidentified fatty acid peak with a carbon length of 11.798 was observed by Dyksterhouse *et al.* (1995). Methane has not been observed to be degraded by *Cycloclasticus* but biphenyl, naphtalene, anthracene, phenanthrene, salicylate, toluene, benzoate, acetate, propionate, and glutamate were degraded and utilized as sole carbon source, after observations both in culture and in the environment (Dyksterhouse *et al.* 1995, Kasai *et al.* 2002, 2003, Demaneche *et al.* 2004).

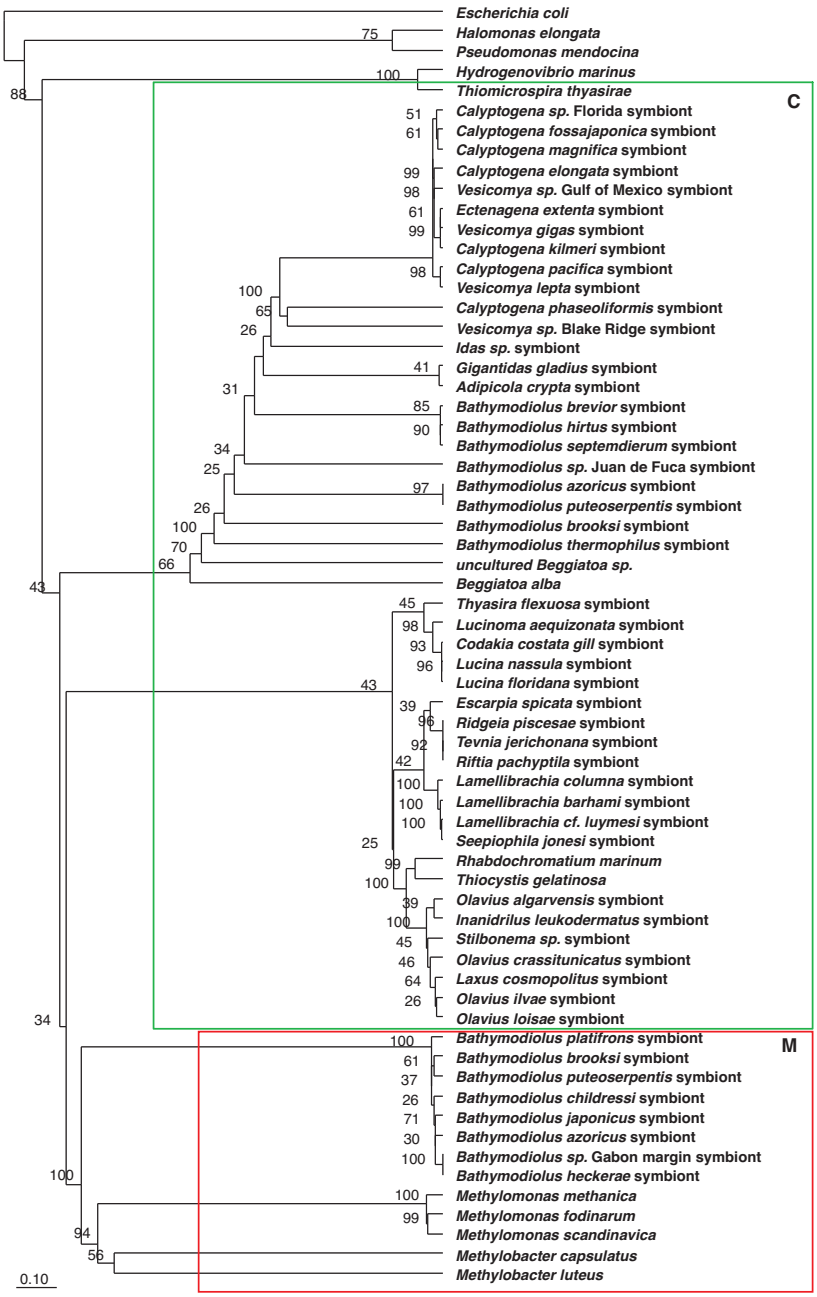

Figure 4.4: Phylogeny of thiotrophic and methanotrophic endosymbionts hosted by marine invertebrates and free-living gammaproteobacteria. Tree inferred from 16S rRNA gene sequences and based on 1000 maximum parsimony replicates. The two usual phylotypes present in *Bathymodiolus* spp. are boxed and lettered (C and M) for chemoautotrophic and methanotrophic respectively. All symbiotic bacteria are labelled 'symbiont' while free-living are designated by taxonomic name alone. (Modified from DeChaine *et al.* 2006).

4.3 Intranuclear parasites

Endonuclear organisms have been observed and studied since the nineteenth century (reviewed by Görtz 1983). The first studied organisms were eukaryotes, such as flagellates present in ciliates nuclei. Nuclear bacteria in ciliates were observed later and enough evidence to corroborate they were bacteria was summarized by Preer (1975). Endonuclear symbiosis commonly occurs in ciliates (Görtz 2006). It was not until 1986 that Elston (1986) described an endonuclear pathogenic bacterium in the gills of the razor clam (*Siliqua patula*) as unprecedented in metazoans, calling it 'nuclear inclusion x' (NIX). Kerk et al. (1992) isolated the RNA of NIX bacteria to analyze the 16S rRNA gene and they concluded it was a novel genus within the Gammaproteobacteria. Zielinski *et al.* (2009) found an intranuclear bacterium in gill tissues of *Bathymodiolus* mussels calling it *Candidatus* Endonucleobacter bathymodiolin. The 16S rRNA gene sequences were not found in a regular clone library with bacterial universal primers but with specific primers. A global distribution study showed the presence of the endonuclear bacteria in *B. puteoserpentis* (from the Mid-Atlantic Ridge), *B. azoricus* (the collection site is not specified), *B. brooksi* (from the northern GoM), *B. heckerae* (from the southern GoM) and other *Bathymodiolus* spp. from the Mid-Atlantic Ridge and Pacific Antarctic Ridge. All 16S rRNA gene sequences group together within the Gammaproteobacteria phylum. Closely related sequences to the ones found in mussels have been found in other invertebrates as sponges, corals, ascidians and sea slugs. It is suggested that this bacterium is host-specific, as only one sequence of 16S rRNA is found in each host species. In Zielinski *et al.* (2009) a name for the mussels phylotype is proposed: *Candidatus* E. bathymodioli. How pathogenic these bacteria are for the mussels is still unknown. Interestingly, the endonuclear bacteria are not found in the bacteriocyte nuclei but exclusively in intercalary cell nuclei, that are free of other symbionts. This suggests that the methanotrophic and thiotrophic symbionts might prevent the infection in bacteriocyte cells and thereby prevent host death. In sponges, an endonuclear bacterium has been clearly observed and its morphology is very similar to the one observed in the *Bathymodiolus*

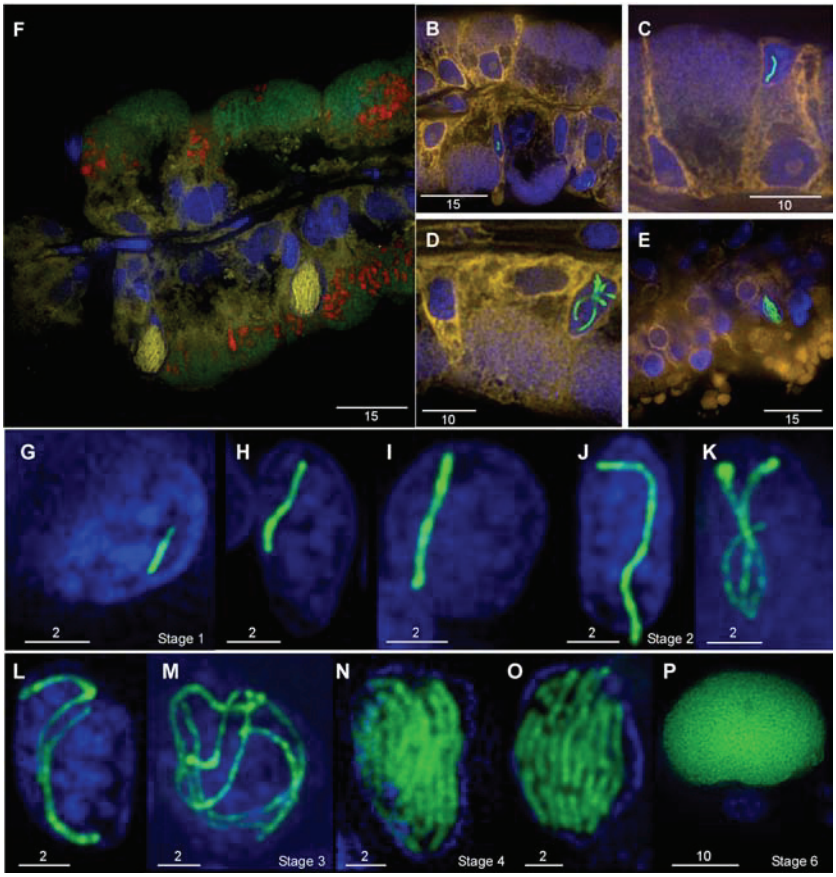

Figure 4.5: 'Ca. E. bathymodioli' in various mussel tissues and developmental stages. BD. Non-ciliated gill tissue with intranuclear bacterium in intercalary cells which alternate with bacteriocytes. E. Gut tissue. In images BE intranuclear bacteria are shown in green and eukaryotic tissue is represented in yellow. Nuclei and bacterial endosymbiotic DNA in bacteriocytes appear in blue. F. Non-ciliated gill tissue with intranuclear bacteria; intranuclear bacteria appear in bright yellow, whereas eukaryotic tissue is represented by a yellowish to brownish colour. Chemoautotrophic and methanotrophic bacterial endosymbionts in bacteriocytes are shown in green and red respectively. Nuclei were stained with DAPI and appear in blue. GP. Developmental stages of Ca. E. bathymodioli in *B. puteoserpentis* gill tissues. The intranuclear bacterium appears in green, the nucleus in blue. Images HM result from projection of a stack of several two-dimensional layers onto one single layer reflecting the overall three-dimensional structure on a two-dimensional plane. GJ. Series showing growth from a single short rod to a single filament in Stages 1 and 2. K. Two overlapping filaments or filament in the process of longitudinal binary fission in transition from Stage 2 to Stage 3. L. Two separate filaments (Stages 23). M. Filamentous assembly consisting of either one single long coiled filament or several filaments (Stage 3). N. Stacks of shorter filaments (Stage 4) resulting from transverse fissions of coiled filaments. O. Long rods resulting from division of Stage 4 filaments. (Zielinski *et al.* 2009).

mussels (Friedrich *et al.* 1999). No further studies about this bacterium in sponges has been done yet. Zielinski et al. (2009) proposed a developmental cycle for these novel bacteria in bathymodiolin mussels (Figure 4.5). It has been reported that mussels inhabiting different habitats might be infested with parasites in different abundances due to differences in their physiological condition (Smith *et al.* 2000, Bergquist *et al.* 2004). Also, massive mortalities have been reported without apparent explanation. Thus, it is of interest to study the distribution and abundance of the endonuclear bacteria in mussels coming from habitats with different environmental factors and with economical importance as are the shallow-water bivalves.

Chapter 5

Methods of study

5.1 Cultivation

Most of what we know about physiology of organisms is based on laboratory cultures. Cultivation is limited because so little is known about the needs for growing a specific organism. Each organism has different needs. A culture medium has to have all the nutrients, metals, and extra organic compounds that the organism to grow requires. There are "selective", "differential", and "enriched" media that define the isolation of a particular species. For the intranuclear bacteria that I attempted to grow in this present work, we deal with bacteria that grow in a high organic content environment: the bivalve tissue. Therefore, enriched media were the preferred ones for the assay to grow bivalve intranuclear bacteria. Three different media were used in solid (with 1.5 % Agar) and liquid presentations: Marine Medium 2216 (Difco), Minimum Medium with and without CTAB (0.1% yeast, 0.01% peptone, 1.5% agar, 100m CTAB, dissolved in sea water), and WL Nutrient Medium (Difco) in 3.5% NaCl. To start the cultures bivalve sample was homogenated with sterile sea water by using a homogenizer and was serially diluted with sterile sea water. Then, an aliquot of each dilution was spread onto the isolation medium plate or in bottles with liquid medium.

5.2 Molecular markers: *16S rRNA*, *aprA*, *pmoA*

The study of symbiosis is not an easy task, especially because the system is usually not separable and should be studied as a whole. Thus, independent-

culture methods are essential to the study of symbioses. Molecular studies of bacterial diversity probably started with the work of Lane *et al.* 1985 where an easy method was proposed to rapidly analyze the 16S rRNA genes of a non-isolated group of bacteria. This method permits the analysis of each organism's molecules separately, giving then the possibility of studying a host and a symbiont simultaneously. The *in situ* hibridization (ISH) method (Giovannoni *et al.* 1988) and then later the fluorescence *in situ* hibridization (FISH) method (Amann *et al.* 1995) completed the analysis (full 16S rRNA cycle analysis), because based on the same molecule is possible to localize specific bacteria, in this case the specific symbiont inside the host (e.g. Dubilier *et al.* 1999, Blazejak *et al.* 2006, Duperron *et al.* 2007).

To investigate more about energy sources, the amplification and sequencing of metabolic marker genes was performed. The already well investigated genes in mussel and tubeworm symbionts are the genes of the active subunit of the particulate methane oxigenase (*pmoA*), marker of the methanotrophy, and of the subunit A of the adenosyl-phosphate reductase (*aprA*) marker of the thiotrophy. In addition in this thesis, assays to amplify metabolic marker genes of hydrocarbon degradation, like mono- and di-oxygenases were performed (see Manuscript I for more detail in material and methods).

Aims

There are still many basic questions to be answered in the microbial symbiology studies, questions inherited from the microbial ecology fundamentals. Who is out there, or better said: which symbionts are out there? Where and how many are there? What is their function? This thesis is a study aimed to find answers to these questions.

The main objective of this thesis is to describe and analyze the symbiont-host diversity in what it seems to be three different symbiosis scenarios: a mutualistic, a parasitic, and a probable commensal. The first case is illustrated by the chemosynthetic *Bathymodiolus* mussels and tubeworms symbioses. The second, by the intranuclear bacteria found in *Bathymodiolus* spp. and shallow-water bivalves. What I call the third symbiosis consists of all gill-associated bacteria found in all the studied bivalve species.

Chemosynthetic Symbiosis

In order to gain greater insight into this broad topic and re-investigate *Bathymodiolus* sp. and cold seep tubeworm symbioses, I had the opportunity to be involved in a collaboration that my supervisor Dr. Nicole Dubilier had forged with Dr. Antje Boetius, and be able to investigate the megafauna and its associated symbiotic microbiota present in a newly discovered cold seep that has the unique characteristic of presenting asphalt flows, giving a series of new parameters that influence the life of the mentioned organisms. These new settings that I have described in Section 2.1.2 are the explanation for the discovery of a new hydrocarbon-degrading symbiont present in *Bathymodiolus heckerae*. The results of this investigation are presented in Chapter 6 and synthesized in the Manuscript I. During the search for this

new symbiont mentioned above in other sites and other *Bathymodiolus* spp., I observed as yet undescribed so far epibiontic Epsilonproteobacteria lying on apical filaments of the *Bathymodiolus childressi* gills. Epsilonproteobacteria have been described as sulfur oxidizing symbionts in invertebrate species (e.g. *Rimicaris exoculata*, *Alvinoconcha hessli*). I have included these results in a review of the bivalve microbiota in the Manuscript III.

Intranuclear bacteria and other associated bacteria in bivalves

Based on the results of the study by Zielinski et al (2009) where I had the opportunity to participate in researching the presence of intranuclear bacteria in *Bathymodiolus* spp. (Manuscript IV) and the state of the art that I described in Section 4.3, I investigated the economically more important shallow-water bivalves, focusing on the distribution of intranuclear bacteria. The objective of this study was to determine whether these bacteria are broadly present in bivalves and to develop a method to screen for these intranuclear bacteria. The results of this investigation are shown in Manuscript II. After microbiological and molecular studies, a high diversity of bacteria was found in the studied bivalves. Some of these bacteria were isolated and many of them were observed with FISH methods in association with the bivalve gill tissue. The results of these investigations are summarized in Manuscript III, giving an overview of past studies and showing how shallow-water bivalves could be used as a model for studying bacteria-invertebrate associations.

Part II

Results and Discussion

Chapter 6

Studies from an asphalt cold seep

Chemosynthetic life was discovered in Chapopote, southern Gulf of Mexico (GoM) associated to lava-like flows of solidified asphalt, oil seeps and gas hydrate deposits were also present (MacDonald et al. 2004). The site is colonized by animals with chemosynthetic symbionts such as vestimentiferan tubeworms, mussels, and clams. Morphological and molecular analyses (COI gene) of 4 mussel individuals and 4 tubeworms, two mussel species are present at Chapopote, *Bathymodiolus heckerae* and *B. brooksi*, and a single *Escarpia* tubeworm species. Comparative 16S rRNA sequence analysis and FISH showed that all three host species harbor intracellular sulfur-oxidizing symbionts that are highly similar or identical to the symbionts found in the same host species from northern GoM sites. The mussels also harbor methane-oxidizing symbionts, and these are identical to their northern GoM conspecifics. Unexpectedly, we discovered a novel symbiont in *B. heckerae*, that is closely related to hydrocarbon degrading bacteria of the genus *Cycloclasticus*. We found in *B. heckerae* the phenol hydroxylase gene and stable carbon isotope analyses of lipids typical for heterotrophic bacteria were consistently heavier in *B. heckerae* by 3 than in *B. brooksi*, indicating that the novel symbiont might use isotopically heavy hydrocarbons from the asphalt seep as an energy and carbon source. The discovery of a novel symbiont that may be able to metabolize hydrocarbons is particularly intriguing because until now only methane and reduced sulfur compounds have been identified as energy sources in chemosynthetic symbioses. The large amounts of hydrocarbons available at Chapopote would provide these mussel sym-

bioses with a rich source of nutrition. In this chapter I present all the results obtained throughout the investigation of this subject.

6.1 Phylogeny of tubeworms and mussels from Chapopote

Phylogenetic resolution with the COI gene worked well for bathymodiolin mussels, integrating the Chapopote individuals into defined groups of *B. heckeare* and *B. brooksi* (Figure 6.2 a) species. However, as observed before (for review see McMullin *et al.* 2003), the resolution of this gene is not sufficient for determining tubeworm species (Figure 6.1 a), especially within the escarpids which have a very similar COI sequence. In spite of that, it gives a good definition of the tubeworm genera, in this case *Escarpia*. The phylogenetic analysis of the tubeworms including other molecular markers (as the 18S and ND4 mitochondrial gene) would be needed to differentiate between *Escarpia* species. However, vestimentiferan tubeworms have a remarkable plasticity (Black *et al.* 1997) and therefore *E. laminata*, *E. southwardae* and *E. spicata* could be the same species. To resolve this, a population genetic study would be required.

6.2 Phylogeny of chemosynthetic *Bathymodiolus* and *Escarpia* symbionts

In the two tubeworm individuals that were analyzed by 16S rRNA, the presence of a single thiotrophic bacterial phylotype falling in the Group 1 (defined by McMullin *et al.* 2003) was found (Figure 6.1b). The *Escarpia* sp. symbiont 16S rRNA sequence of this study was identical to the *E. laminata* from the Florida Escarpment in the northern GoM (McMullin *et al.* 2003), and *E. spicata* from the Guaymas seep (Vrijenhoek *et al.* 2007). Two *Escarpia* tubeworms from two collection sites in Chapopote were analyzed with FISH specific probes designed for this study (see details sites and probes in Manuscript I). Symbionts were localized in only one of the analyzed tubeworms. High abundance through the whole transversal worm tissue could be detected in a patchy distribution (Figure 6.3 c). DAPI signals correlated with the symbiont signals. The explanation for the lack of FISH signals in

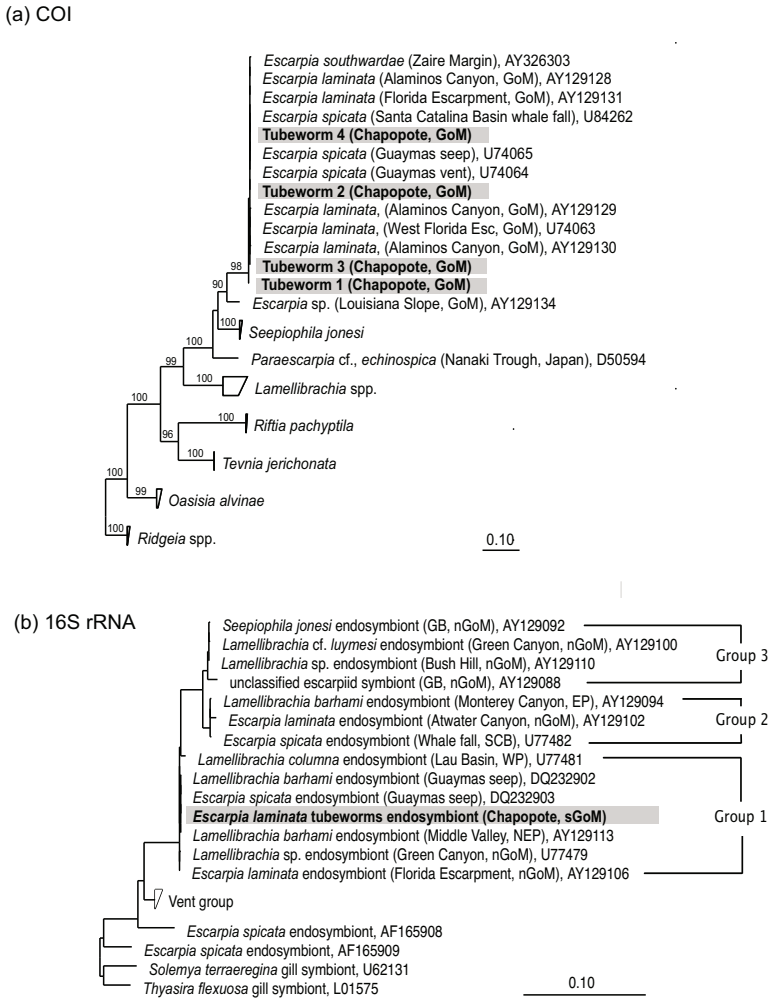

FIG 2. Phylogenetic affiliation of Escarpia tubeworms and their bacterial symbionts. (a) Tree based on COI gene sequences. Maximum-likelihood tree showing vestimentiferan tubeworm species from vent and seep environments including the 4 individuals of this study (sequences highlighted in gray). Only bootstrap values greater than 70 % are shown. (b) Tree based on 16S rRNA gene sequences. Maximum-likelihood tree shows within the gamma-proteobacteria phylum, thiotrophic symbionts of seep and vent vestimentiferans. Only one phylotype was present in the two investigated tubeworms (in bold), and fell in group 1 (McMullin at al 2003) within Escarpia spicata, Escarpia laminata and Lamellibrachia spp.

Figure 6.1: Phylogenetic affiliation of *Escarpia* tubeworms and their bacterial symbionts. (a) Tree based on COI gene sequences. Maximum-likelihood tree showing vestimentiferan tubeworm species from vent and seep environments including the 4 individuals of this study (sequences highlighted in gray). Only bootstrap values greater than 70% are shown. (b) Tree based on 16S rRNA gene sequences. Maximum-likelihood tree shows within the Gammaproteobacteria phylum, thiotrophic symbionts of seep and vent vestimentiferans. Only one phylotype was present in the two investigated tubeworm individuals (in bold), falling in group 1 (McMullin *et al.* 2003) with *Escarpia spicata*, *Escarpia laminata* and *Lamellibrachia* spp. symbionts.

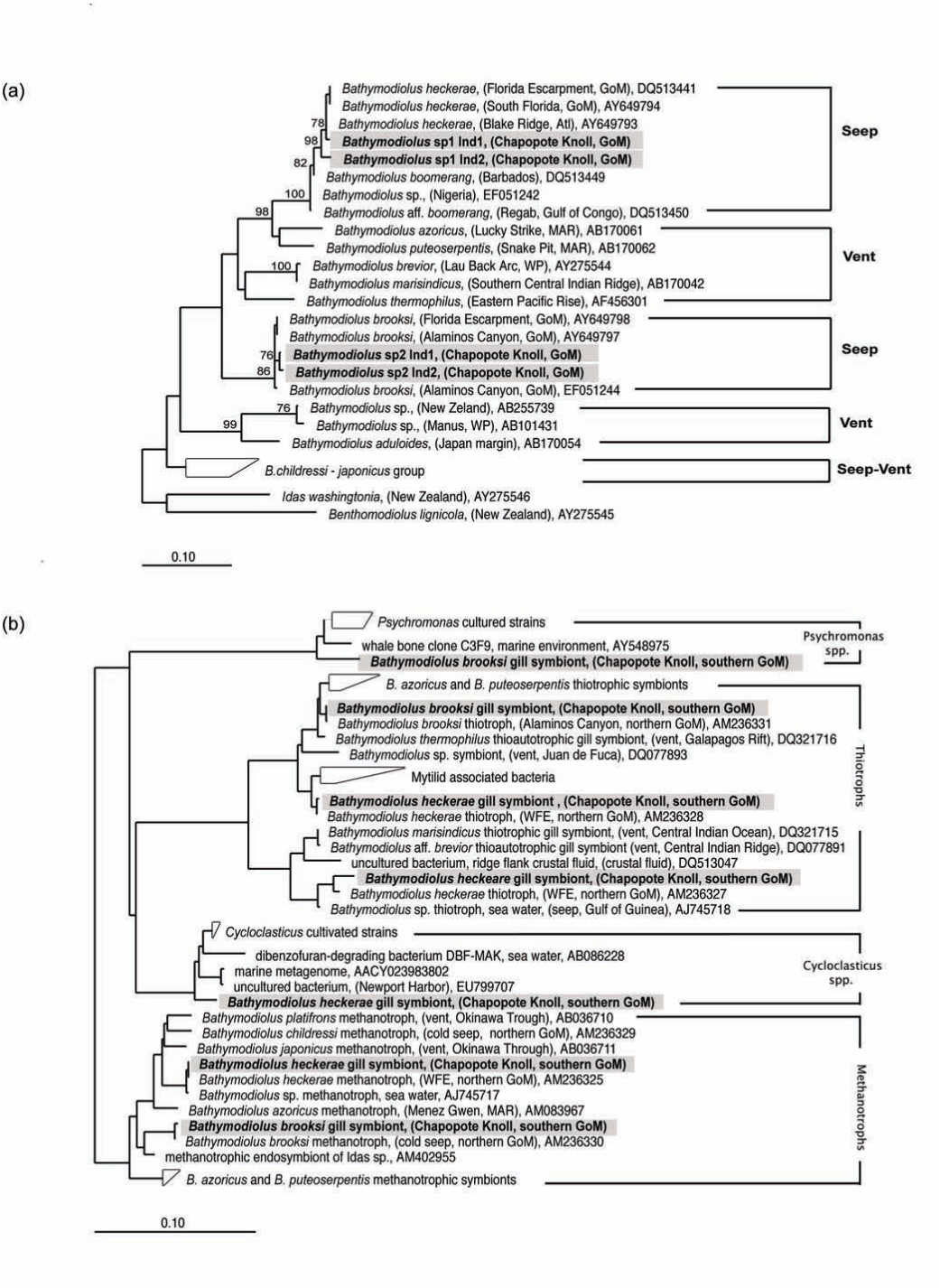

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Figure 6.2: Phylogenetic affiliation of *Bathymodiolus* mussels and their bacterial symbionts. (a) Tree based on COI gene sequences. Maximum likelihood tree showing *Bathymodiolus* spp. from vent and seep environments including the 4 individuals of this study (highlighted in gray). Only bootstrap values greater than 70% are shown. (b) Phylogenetic reconstruction of bacterial symbionts of *Bathymodiolus* mussels based on 16S rRNA gene sequences. Maximum-likelihood tree shows within the Gammaproteobacteria phylum thiotrophic, *Cycloclasticus*-related, *Psychromonas*-related and methanotrophic bacteria. The phylotypes investigated in this study are shown in bold. Note that *B. heckerae* individuals have two different thiotrophic phylotypes, one *Cycloclasticus*-related and one methanotrophic phylotypes, and *B. brooksi* present only one thiotrophic, one *Psychromonas*-related, and one methanotrophic phylotypes.

the second tubeworm analyzed might be because based on ROV images, the sample was coming from a tubeworm community that looked dead, therefore their symbionts might not be very active any more or tubeworms might be loosing them. TEM observations of the sample would be necessary to corroborate the absence of the symbionts. We might even observe many symbionts being digested.

The 16S rRNA analysis of the bathymodiolin mussels showed that in *B. heckerae*, two different thiotrophic bacteria phylotypes (TI and TII) and one methanotrophic (M) were present (Figure 6.2. In *B. brooksi*, only one thiotrophic (TI) and one methanotrophic (M) were recognized. With FISH specific probes, each phylotype of thiotrophs and methanotrophs were localized in both individuals of each species (Figure 6.3). When comparing to the mussels from the northern GoM (Duperron *et al.* 2007) they have more thiotrophic bacteria based on the clone libraries and also with FISH observations. In *B. heckerae* there is a clear dominance of TII symbiont over TI and methanotrophs (Figure 6.3 e-g). This is a very interesting difference with the other Gulf of Mexico mussels investigated to date (Cavanaugh 1993, Fisher 1993, Duperron *et al.* 2007) where the dominant phylotype has always been the methanotrophic one. Although there are not punctual measurements for sulfide, or other sulfur sources, or methane compounds in the collection site, we know that there is absence of sulfide in the water column and there is high methane concentration (A. Boetius, personal comm). In

consequence we suspect that the sulfide might be diffusing with difficulties from below the asphalt and being consumed by the thiotrophic bacteria as soon as it reaches the mussels. The presence of both thiotrophic phylotypes present in one bacteriocytes (Figure 6.3 e and f), supports the idea that each phylotype consumes a different sulfur source, however we can not discard that they could be competing for the same resource and that is why in some bacteriocytes one phylotype seem to dominate. It has been shown a positive correlation between the amount of compounds present in the environment and the quantity of each symbiont in bathymodiolin mussels (Trask and Van Dover 1999, Fiala-Medioni *et al.* 2002, Salerno *et al.* 2005), however it could also depend on the host needs, if the sulfur compounds provided by thiotrophs (i.e. vitamins, amino acids, carbon compounds from CO_2 assimilation, etc) are low, they could stimulate augmentation of thiotrophic bacterial content in the tissue to be able to increase the sulfur-compounds uptake. Nevertheless, it is clear that the total and relative abundance of the different phylotypes depends to a great extent on the biogeochemistry of the environment (Duperron *et al.* 2007).

6.3 Novel symbionts in *Bathymodiolus* mussels

In addition to the symbionts described above, bacterial phylotypes that have far never been found in close association with animals were found. A *Psychromonas*-related species was found in the 16S rRNA clone library of *B. brooksi*. With specific probes for this bacterial genus the presence of *Psychromonas*-related bacteria in the mussel tissue was confirmed (Figure 6.3 k). Like the previously described thiotrophic and methanotrophic bacteria, the *Psychromonas*-related phylotype also appeared to be present within the host tissue, however they do not seem to be intracellular like the first ones (Figure 6.3). They were far less abundant than the thiotrophic and methanotrophic symbionts, and could only be found by FISH in one of the two *B. brooksi* individuals. *Psychromonas* are heterotrophic gammaproteobacteria frequently found in cold-water sediments (Ivanova *et al.* 2004, Xu *et al.* 2003).

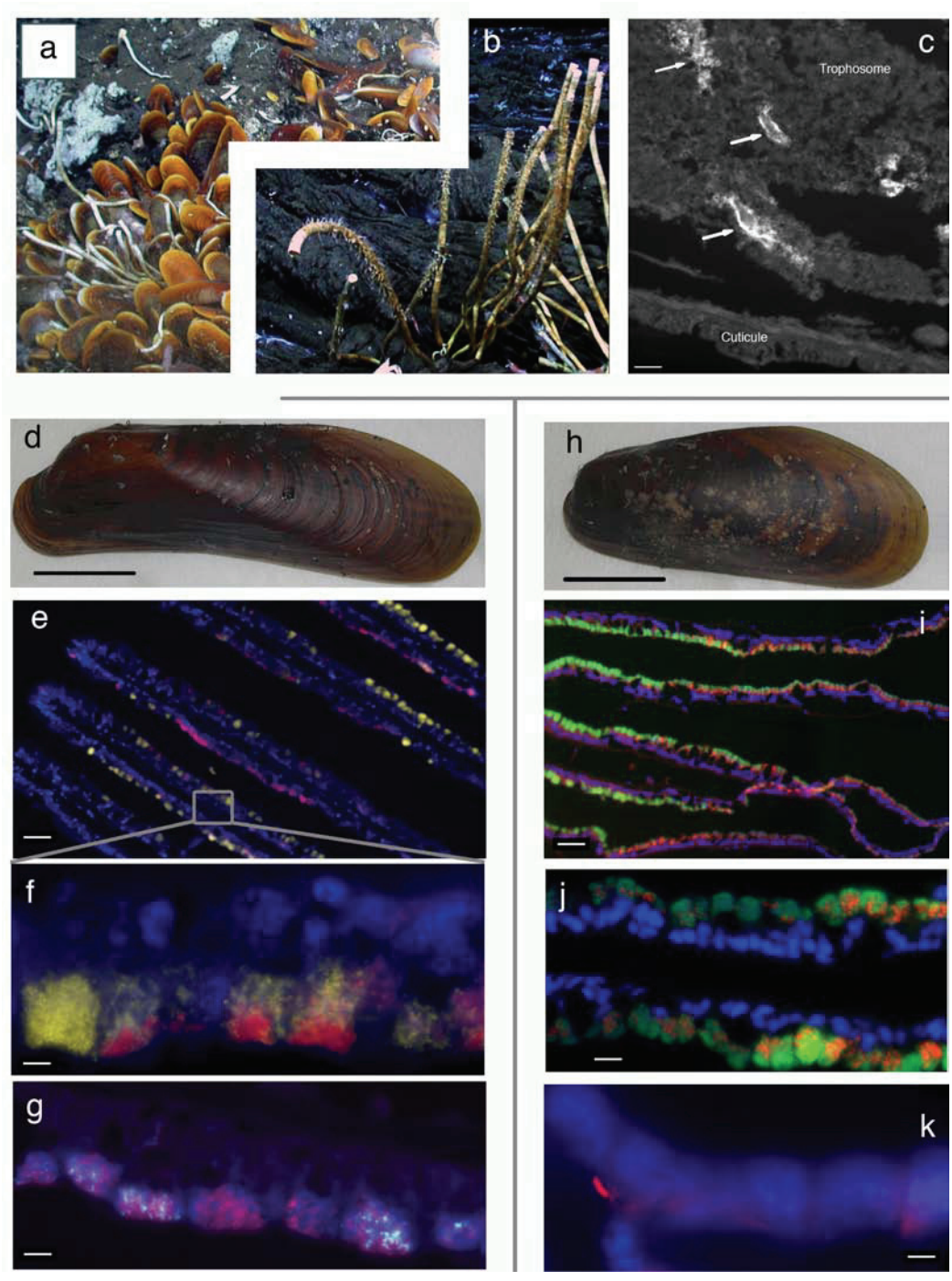

Figure 6.3: Legend on the next page

Figure 6.3: *Bathymodiolus* mussels and *Escarpia* tubeworms from Chapopote. FISH images of bacteriocytes in the mussel gill filaments and in the tubeworm trophosome. (a) *Bathymodiolus brooksi* and *B. heckerae* mussels together with escarpid tubeworms on the asphalt bottom at Chapopote, southern Gulf of Mexico. Each species harbors its own specific bacterial phylotypes. (b) *Escarpia* tubeworms from this study bear chemoautotrophic tubeworm symbionts. (c) Localization of the symbionts (arrows) with a FISH specific probe through a tubeworm cross-section. (d-g) *B. heckerae* mussel and respective FISH images: *B. heckerae* shell has an elongated shape (d); its filamentous gills house methanotrophs (not shown here) and two different chemoautotrophic bacterial phylotypes. The host nuclei are in blue, thiotrophs TI in red, and thiotrophs TII in yellow (e, f). A new hydrocarbon degrader (*Cycloclasticus*-related) symbiont in green, co-exists with the methanotrophic bacteria in blue and the thiotrophs in pink (g). (h-k) *B. brooksi* mussel (h) and respective FISH images: The shape of the *B. brooksi* shell is rounder and it is smaller than *B. heckerae*. *B. brooksi* gill filaments (autofluorescence of the tissue is purple) house a methanotrophic bacterial phylotyope, in red, and a thiotrophic one, in green (i). A detail of (i) shows host nuclei in blue, methanotrophs in red and thiotrophs in green (j). A *Psychromonas*-related bacteria was associated with *B. brooksi* gill tissues (k). Scale bars: (c, e, i) = 50μm; (d, h) = 5 cm; (f, g, k) = 5 μm; (j) = 10 μm.

Only once, another *Psychromonas*-related phylotype has been observed associated with an animal, in the bones of a whale fall (Goffredi *et al.* 2004). The presence of these bacteria seem to be related to a high organic matter content in the environment, which in this case it exists at the Chapopote site and would explain their presence in *B. brooksi* tissue.

A *Cycloclasticus*-related bacterium was found in *B. heckerae* 16S rRNA clone library. With specific FISH probes I observed *Cycloclasticus*-related bacteria co-existing in the same bacteriocytes as the thio- and methanotrophic endosymbionts in both *B. heckerae* individuals (Figure 6.3g). With the PHLIP image software it was calculated that the *Cycloclasticus*-related bacteria make up 6% of the total endosymbiont biovolume (results from D. Fink, MPI Bremen). I suggest that the hydrocarbons in the environment make *Cycloclasticus* sp. presence possible, as they have been found also in oily sediments from shallow and deep waters (see section 4.2. However, this is the first time that *Cycloclasticus* sp. is observed as an intracellular bacterium within animal tissues. The membrane molecules of this bacterium

might be similar to the symbionts to the point that *B. heckerae* mussels let them get inside them. The association might provide this bacterium the advantage of being in an aerobic habitat with available nutrients (as the flow of water through the gills would bring the necessary resources) and for the mussel this would provide a new nutrition source from the degradation of aromatic compounds.

6.4 Host-bacteria specificity

The two different systems that I have been working with, *Escarpia* tubeworms and *Bathymodiolus* mussels, show contrasting patterns in their symbiont specificity. A lack of host-specificity can be observed in *Escarpia* tubeworms: the same symbiont phylotype is broadly distributed within different tubeworm species. And the same tubeworm species from different habitats or geographic locations can host a different bacterial phylotype (see Figure 6.1 b). This might be explained by a horizontal transmission of symbionts, that in other tubeworms like *Ridgeia piscesae* and *Riftia pachyptila* has been corroborated (Bright and Sorgo 2003, Nussbaumer *et al.* 2006). However, a horizontal system could also have a very specific symbiont selection, like in the squid-*Vibrio* symbiosis (see section 1.1.2). For the tubeworms, this could mean that the molecular recognition process is not highly specific, allowing promiscuity of bacterial phylotypes within the different hosts. This is in contrast to the mussels where each mussel species hosts specific bacterial phylotypes (Fig 7.2b). It might certainly be that some symbionts, like the thiotrophs TI and methanotrophs from this study, are transmitted vertically explaining why symbionts are exactly the same (based on 16S rRNA) in mussels from distant places as northern and southern Gulf of Mexico, but others like thiotroph TII (where northern and southern phylotypes are close-related but not identical) are perhaps transmitted environmentally. An alternative explanation to the vertical transmission is a very specific host-symbiont recognition system, where methanotrophs and thiotrophs in each host-species might have particular molecules and they would be recognized and interiorized. Future studies focusing in the study of recognition molecules

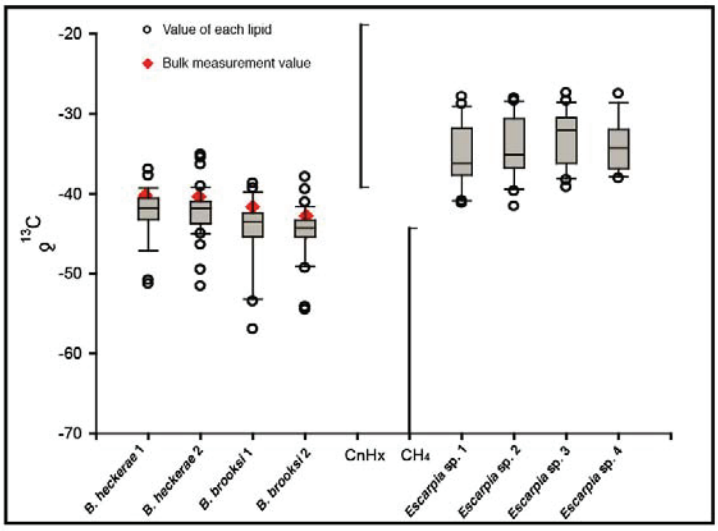

Figure 6.4: Stable carbon isotope measurements of lipids extracted from two *B. heckerae* (heck), two *B. brooksi* (brook), and 4 *Escarpia* sp. (esc) tissue. Carbon isotope values of lipids (circles and bars) and bulk tissue (red diamonds).The values of methane (CH4) and heavier hydrocarbons (CnHx) that are characteristic of the site are shonw.

(e.g.lectins) might reveal the mechanism for the host-symbiont specificity, and might also reveal how novel associations e.g. *B. heckerae-Cyclocasticus* can be established.

6.5 Metabolism of the symbioses

Genes *aprA* (subunit A gene of the adenosyl-phosphate reductase [APR]) and *pmoA* (active subunit gene of the particulate methane monooxygenase [pMMO]) were analyzed. An *aprA* gene sequence was found in each host species (6.5 a). After the 16S rRNA clone library there are two thiotrophic bacterial phylotypes, thus two different *aprA* sequences were expected. Most likely the *aprA* clone library was not screened enough to find the second thiotrophic phylotype, that it is perhaps the low abundant phylotype (TI). Based on 16S rRNA and *aprA* analyses both investigated tubeworms contain only one identical thiotrophic phylotype, and each mussel species also bear an own thiotrophic phylotype. One *pmoA* sequence was also present and unique in each mussel species (6.5 b).

The values of this study for the $\delta^{13}C$ of the fatty acids of the *Escarpia* tubeworms ranged from -27‰ for the short chain fatty acids to -43‰ for the longer or complex lipids. As can be seen in Figure 6.4 the compound specific stable carbon isotopes of the different *Escarpia* species are all very similar between individuals. This is congruent with former studies as isotopic measurements would show us a value close to -29‰ after the fractionation of CO_2 in the chemoautotrophic process (Fang *et al.* 1993). For the mussels we have a bit more complex story because of the dual symbiosis. The close phylogenetic relationship of the thiotrophic- and methanotrophic-related endosymbionts found in this study with the previously described ones (i.e. in the northern GoM and other basins), the presence of the *pmoA* and *aprA* genes, and their lipid isotopic values (see Manuscript I), suggest that these endosymbionts have the potential to oxidize both sulfur and methane. However, electron microscopy, and metabolic assays, like sulfide and methane incorporations are needed to confirm this. The main isotopic values of both mussel species are between -40 and -60‰ (see Figure 6.4). These values have direct correlation to the isotopic value of the carbon source. Methane values for the thermogenic methane in the northern GoM are between -44 and -46‰ and for biogenic methane -64 to -65‰ (Sassen *et al.* 1999).

Specific biomarkers for the novel symbionts (6.3) would have been ideal to track isotopic signatures, however no specific lipid markers are known from cultivated *Psychromonas* spp., or *Cycloclasticus* spp. except for the short length peak of 11.798 (see 4.2, but no short lipids were analyzed. Following the hypothesis that *Cycloclasticus*-related bacteria could also be degrading hydrocarbons, I looked for the genetic presence of an oxygenase. I found the methyl-toluene-phenol hydroxylase (MTPH) gene in *B. heckerae* mussels (Figure 6.5). Furthermore, when analyzing the lipid data (by F. Schubotz and show with details in Manuscript I) we observed that lipids were heavier in *B. heckerae* than in *B. brooksi* (Figure 6.4). In fact, bulk tissue and compound specific stable carbon isotopes showed a mean average enrichment for *B. heckerae* in comparison to *B. brooksi*. According to the fatty acid composition and the phylogenetic analyses it is clear that both mussels host a dual symbioses. If both symbionts would utilize the same carbon sources

we would expect them to have similar lipid isotopic values. Therefore, the average 2.5‰ depletion of *B. brooksi* compared to *B. heckerae* is most likely explained 1) by the presence of the additional hydrocarbon degrading symbiont, or 2) more active bacteria in one of the species. Although I have showed the gene for the MTPH enzyme to be present, both results give us only the clue for a potentially active association. Here we give first evidence for the still incomplete study of the intracellular microbial community of bathymodiolin mussels that seems to be more diverse than previously recognized. In fact different microbial populations could cause the presence of all these different lipids that we find among the hosts. Clearly more physiological data are needed to explain the ecological role of these new mysterious symbioses.

6.6 Summary

This is the first time that *B. brooksi* and *B. heckerae*) presented higher abundance of thiotrophic than methanotrophic bacteria. Relative and total symbiont abundance might depend on environmental geochemistry, and on host and symbiont metabolism. The inhabitation of a new symbiont might be provoked by the characteristic settings that this asphaltic cold seep has, providing this bacterium the advantage of being in an aerobic habitat with available nutrients (as the flow of water through the gills might be bringing the necessary resources) and for the mussel this would provide a new nutrition source coming from the degradation of aromatic compounds.

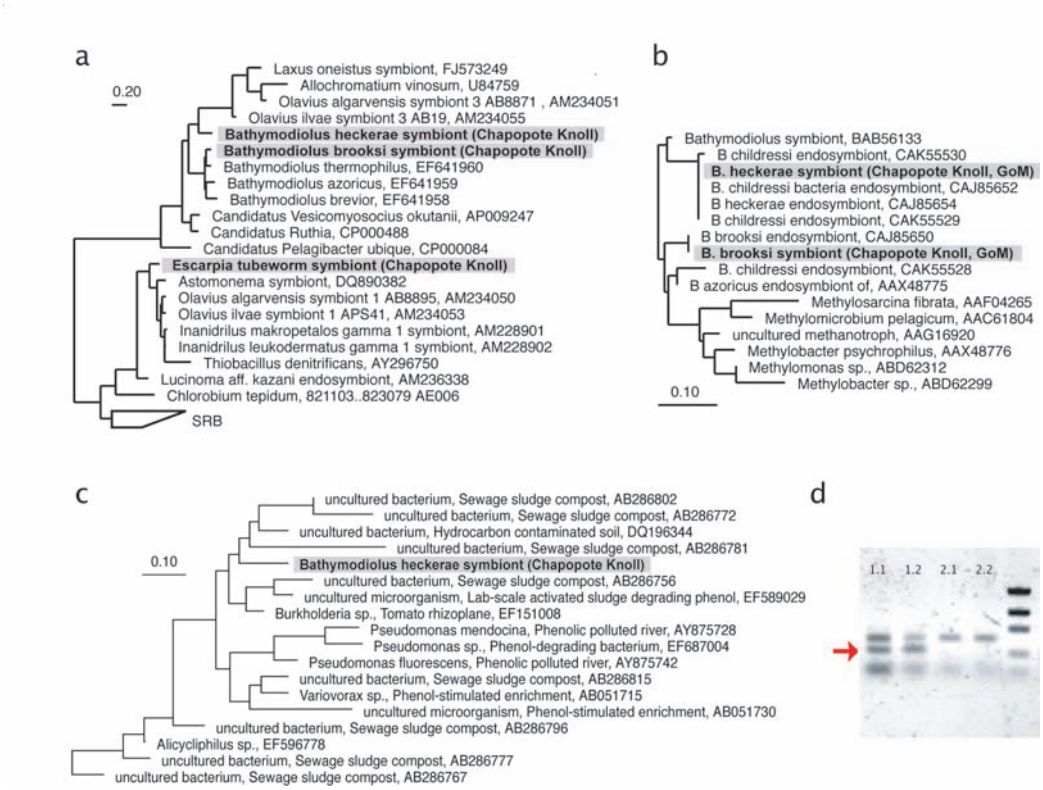

Figure 6.5: Phylogenetic reconstruction of bacterial symbionts based on metabolic marker genes. The three sequences of this study are highlighted in gray. (a) Maximum-likelihood tree based on the alpha subunit of the APS reductase gene (*aprA*) sequences. (b) Maximum-likelihood tree based on the active subunit of the particulate MMO gene (*pmoA*) sequences. This gene was present only in *Bathymodiolus* spp. The sequences of this study (in bold) grouped with former *Bathymodiolus* sequences. (c) Maximum-likelihood tree based on the MTPH gene. (d) MTPH gene was present only in *B. heckerae* (1.1 and 1.2) and not in *B.brooksi* (2.1 and 2.2). The sequence fell within a cluster of sequences from hydrocarbon environments.

Chapter 7

Bacteria associated with bivalves

Bacteria associated with bivalves is a very extensive topic. If we have a quick overview of all the bacteria that have been described as symbionts, pathogens, or simply bacteria occasionally associated to a certain bivalve tissue, many branches of the bacterial kingdom are covered (see Figure 7.1), with a preference for Gram-negative bacteria. It is true that bacteria could be opportunistic and be only in transit, however, it is interesting to note that not all and every group of bacteria are covered, suggesting that certain bacteria do not live well in a bivalve habitat. For example Deltaproteobacteria and Planctomycetes have never been found associated with bivalves. However, these bacteria have been found associated with other invertebrates like sponges and in the case of Deltaproteobacteria even as part of an endosymbiotic association (i.e. as in the oligochaete symbiosis described in Section 1.1.3). In the next sections I discuss the results obtained from molecular analysis presenting the intranuclear bacteria and the general observed diversity.

7.1 Intranuclear bacteria

Bacteria that invade eukaryotic nuclei are commonly found in protists but have rarely been observed in multicellular eukaryotes. Recently, we described intranuclear bacteria in deep-sea hydrothermal vent and cold seep mussels of the genus *Bathymodiolus* (Zielinski *et al.* 2009). Phylogenetic analyses showed that these bacteria belong to a monophyletic clade of Gammaproteobacteria associated with marine animals as diverse as sponges, corals,

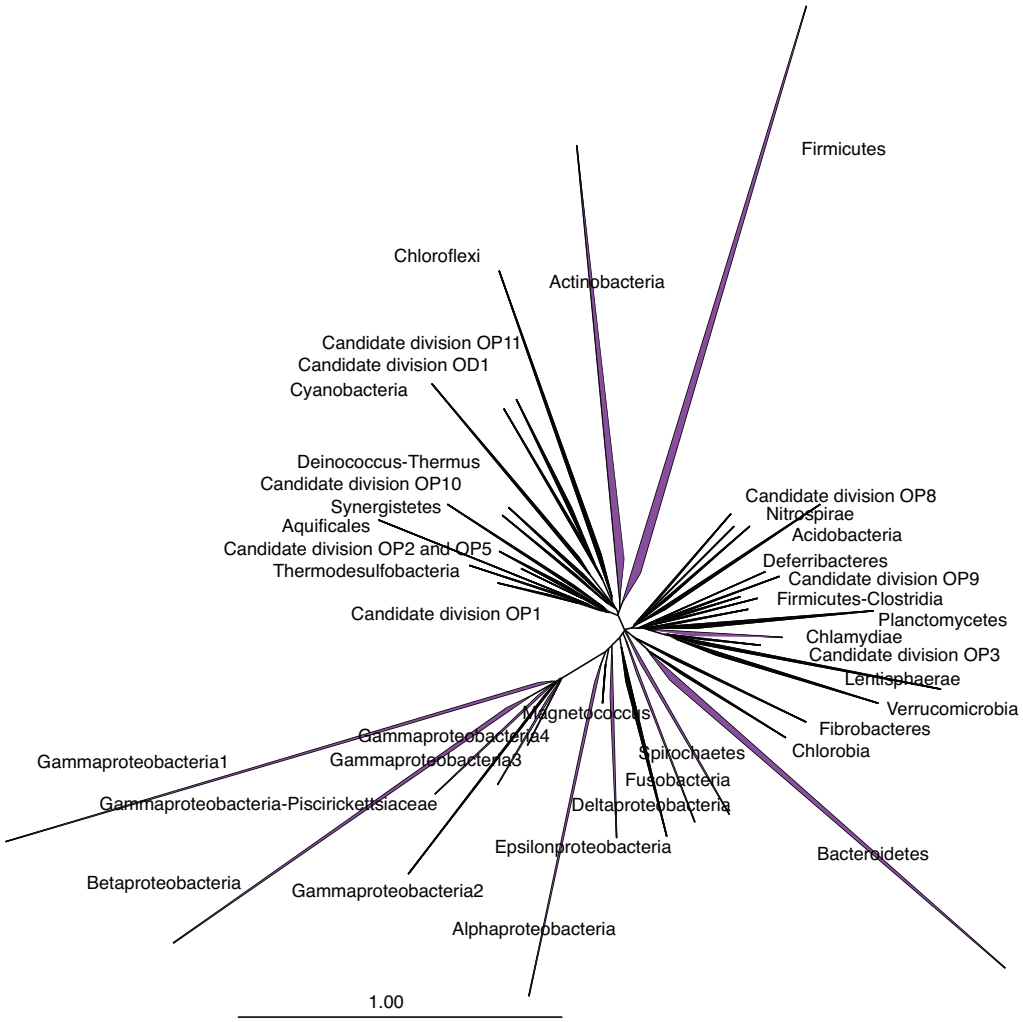

Figure 7.1: Phylogenetic tree of the main bacterial phyla based on 16S rRNA gene. Most of the existing bacteria phyla are visualized here. Branches in purple are the phyla for which bacterial species have been found associated to bivalves.

bivalves, gastropods, echinoderms, ascidians, and fish. However, except for the bathymodiolin mussels and a shallow water bivalve (the Pacific razor clam *Siliqua patula*) none of these metazoa-associated bacteria have been shown to occur inside nuclei. It has been suggested that intranuclear bacteria may cause mass mortalities in bivalves but a causal relationship has never been established. Because of their potentially lethal effect on bivalve populations, I looked for the presence of intranuclear bacteria in economically important and commercially available bivalve species, i.e. oysters (*Crassostrea gigas*), razor clams (*Siliqua patula* and *Ensis directus*), blue mussels (*Mytilus edulis*), manila clams (*Venerupis philippinarum*), and common cockles (*Cerastoderma edule*). Fluorescence *in situ* hybridization (FISH) revealed the presence of intranuclear bacteria in all investigated bivalves except oysters and blue mussels (see details in Manuscript II). A FISH probe targeting all currently known intranuclear gammaproteobacteria was designed for future high-throughput analyses of marine invertebrates. Furthermore, primers were designed to quantify the abundance of intranuclear bacteria with real time PCR. Preliminary tests with these primers showed massive amounts of intranuclear bacteria in some bivalve species, raising the question if these might significantly affect not only the health of the bivalves but possibly also of the humans that eat them.

7.2 Diversity of bacteria associated with bivalves

A high diversity of bacterial phylotypes was found in our 16S rRNA sequence analysis, some are recurrent bacteria that have shown up in previous bivalve studies (Table 7.1). The representation of bacteria herein has a gammaproteobacteria dominance, as has been seen in the previous molecular studies of bivalve-associated bacterial communities (Schulze *et al.* 2006, Cavallo *et al.* 2009). Bivalve sequences fell in clades with bacteria from organic-rich environments: oil spills, bone-falls, feces or invertebrate tissue. Most of them belong to the Gammaproteobacteria group (Figure 7.2) but also there are Alphaproteobacteria (Figure 7.3), Epsilonproteobacteria (Figure 7.3), Bacteroidetes (Figure 7.4), Actinobacteria and Spirochetes (Figure 7.5). It can

be hypothesized that these bacteria are specialized on high-organic matter habitats and that more analysis might show a host species-specific bacterial community.

In the Epsilonproteobacteria group it was observed that there is a particular group that encloses hydrothermal vent sequences (7.3). Other two sequences are from invertebrate tissue, *Paralvinella palmiformis* and *B. platifrons*. This last one is the closest relative to the sequence of *B. childressi* analyzed herein. FISH analysis revealed that Epsilonproteobacteria are attached to cilia-like structures of the mussel gill (see Fig. 2 in Manuscript III). Other Epsilonproteobacteria have been described as invertebrate ectosymbionts (e.g. in *Rimicaris exoculata*, which it is not surprising that the diversity of this association is not limited to certain invertebrates (i.e. crustaceans) but it might extend widely in the whole invertebrate group.

Associated spirochaetes stand out because they seem to be a stable bacterial community in bivalve styles (Noguchi 1921, Bernard 1970, Paster *et al.* 1996, Prieur *et al.* 1990, Margulis and Fester 1991). In my FISH observations (see Fig. 2 in Manuscript III) I see them well established in bivalve gill tissue. Also, a spirochete sequence from DNA gills has been described in a *Lucinoma* sp. cold-seep clam by Duperron *et al.* (2007). It might be worthwhile to do a population genetics study to investigate the variability of these spirochetes species within the different bivalves. It is not clear so far if spirochetes have an ecological role or importance in the association with bivalves, but they might be an ubiquitous microflora within the mollusks group (Prieur *et al.* 1990). Margulis et al. (1991) named as symbionts the studied spirochetes in oysters. We might be able to study them as ubiquitous symbiotic bacteria in bivalves.

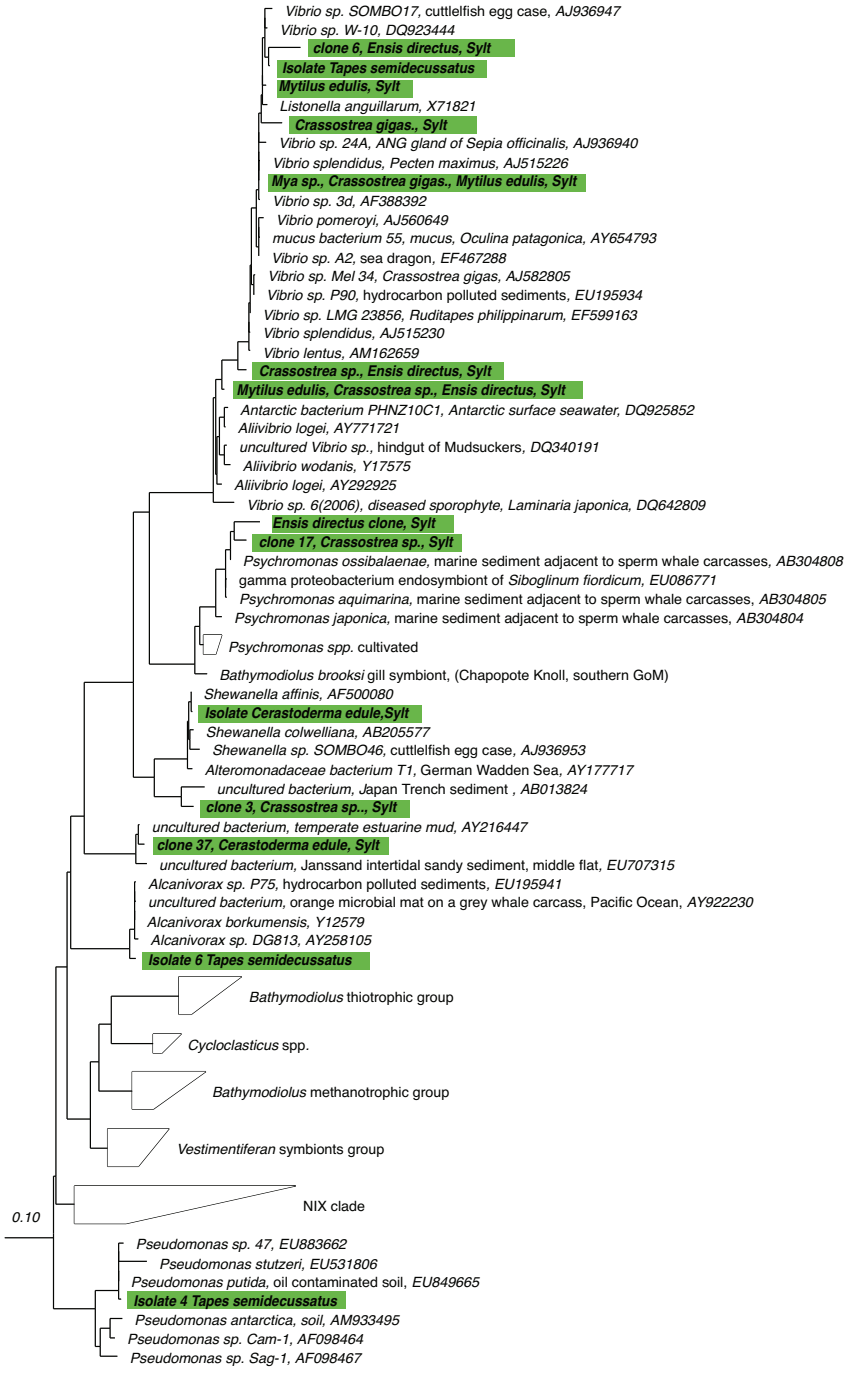

Figure 7.2: Gammaproteobacterial diversity in bivalves. The clones and isolates obtained in this study are highlighted in green. Clones or isolates are shown and named after their host origin.

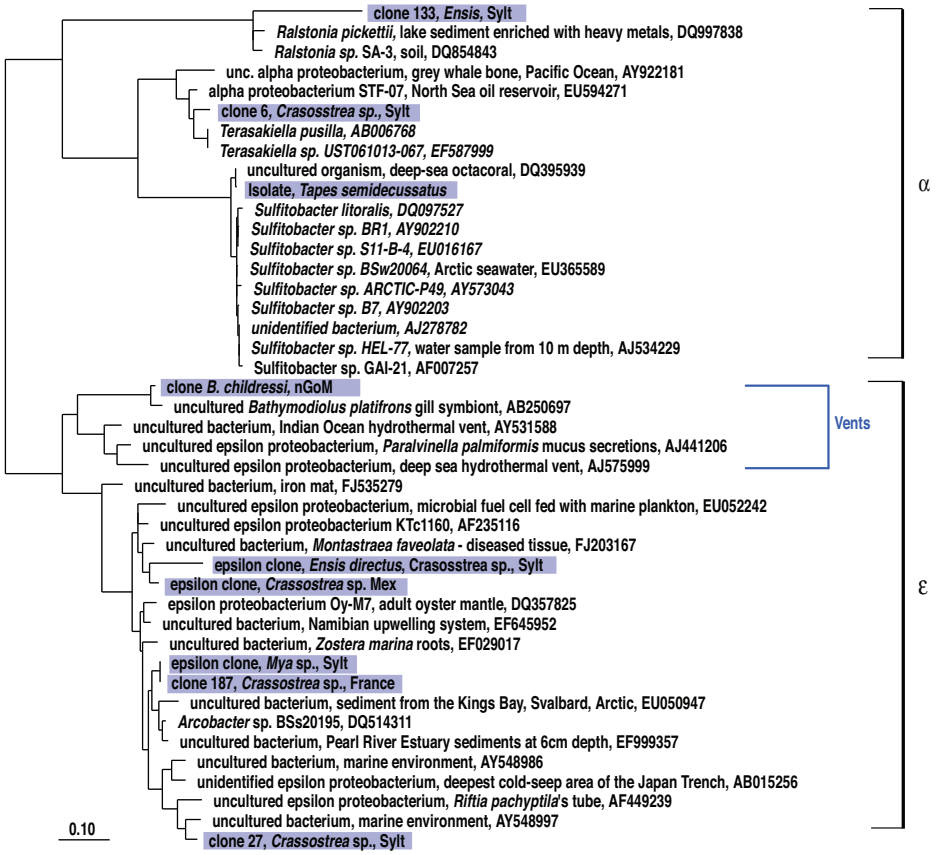

Figure 7.3: Alpha- and Epsilonproteobacterial diversity in bivalves. The clones and isolates obtained in this study are highlighted in blue. Clones from *Ensis directus*, *Crassostrea gigas*, *Tapes semidecussatus*, *B. childressi* and *Mya* sp. 16S rRNA libraries. Hydrothermal vent group is highlighted also.

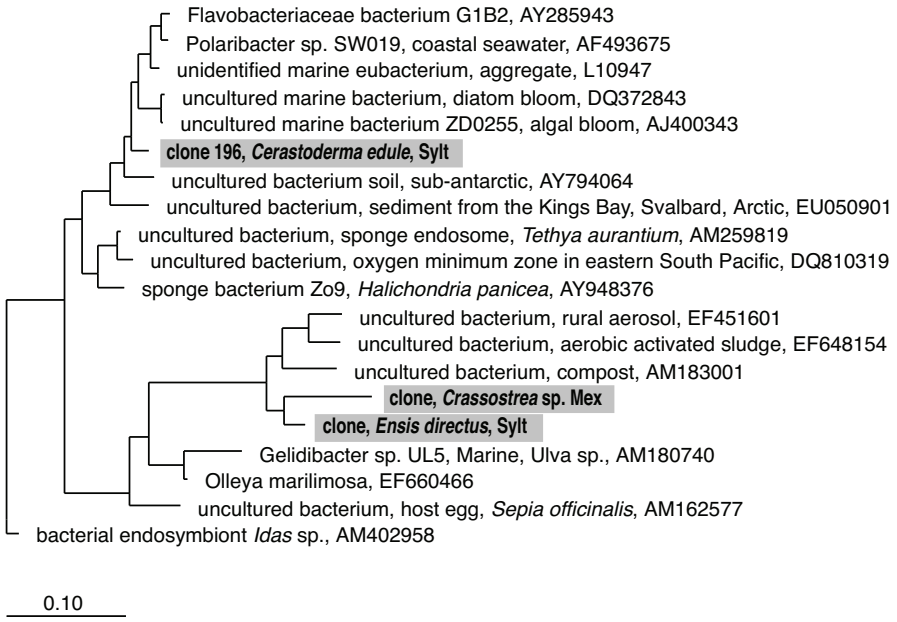

Figure 7.4: Bacteroidetes in bivalves. The clones obtained in this study are highlighted in gray.

Species	Vibrio	Pseudomonas	Achromobacter-Acinetobacter-Shewanella spp.	Enterobacteria	other Gamma	Micrococcus	Spirochetes	Bacteroidetes	Hydrocarbon-degraders other Alpha	Epsilon	Chlamydia	Photobacterium	Proteolytic bact.	Fermentative bact.	Others	References
Oysters																
Ostrea edulis						M										Noguchi 1921
Crassostrea gigas	CSCISCS	C		IS			S		ISS		ISS	ISS		C		C		Prieur 1981, Sugita et al 1981, Schultze et al. 2006, This study
C. virginica	C 25%	C31%				MC		C26%					C 87%					Noguchi 1921, Murchelano & Brown 1968
C. cuculata	C	C	C		C	C	C										*Neisseria, Corynobacterium*	Rajagopalan &Sivalingan 1978
Mussels																
Mytilus edulis	CSCS					S		M				C		C				Noguchi 1921, Prieur 1981, this study
Mercenaria mercenaria																
Mytilus viridis	C	C	C		C	C	C										*Neisseria, Corynobacterium*	Rajagopalan &Sivalingan 1978
Mytilus coruscus	C	C			C		C										*Moraxella*	Sugita et al 1981
Clams																
Cerastoderma eduli						CS			S									this study
Tapes (Venerupis) spp.	CISSC	ISCCS		IS			CS	CS	M	IS	ECS	CS	IS		C		C	*Sulfitobacter*	Noguchi 1921, Prieur 1981, Sugita et al 1981, Schultze et al. 2006, This study
Siliqua patula																
Ensis spp.	CS						S		M	S		S	S					Noguchi 1921
Mya arenaria	CCS	C	C				S		M	C			S				*Corynobacterium, Arthrobacter*	Noguchi 1921, Cundell & Young 1975
Mactra veneriformis	C	C			C			C	M									Noguchi 1921, Sugita et al 1981
Phacosoma japonicum	C	C			C													Sugita et al 1981
Scapharca broughtonii	C	C			C													Sugita et al 1981
Panopea abrupta	IS	IS		IS			IS											Schultze et al. 2006

Table 7.1: Bacterial diversity studies in bivalves. Studies where bacteria have been observed microscopically (M), cultured (C), isolated (I), or their 16S rRNA sequenced (S). Some letters are repeated because more than one study have characterized the species. In red the sequences and cultures from this study.

7.3 Bacterial cultivation

Bacteria belonging to the same monophyletic clade to which intranuclear bacteria belong (called here NIX-clade, Figure 7.6) have recently been isolated from several organisms: a tropical sponge (Nishijima et al *in prep*), a sea slug *Elysia ornata* (Kurahashi and Yokota 2007), and an echinoid *Tripneustes gratilla* (Becker *et al.* 2007). Unluckily, in these studies there has not been microscopical observations (Nishijima and Kurahashi, pers. comm.), and it is not known if bacteria were in the host nuclei. Nevertheless, I decided to try the cultivation of the intranuclear bacteria associated to *Tapes semidecussatus*, *Cerastoderma edule*, *Crasosstrea gigas*, and *Ensis directus* with the media used in the two published studies and an enriched medium. Selected bivalves were the ones that presented NIX-related bacteria in the 16S rRNA analysis and glycerol samples were available.

No NIX-related bacteria were cultivable. However, many other types of bacteria were isolated. Not very surprisingly I could isolate several *Vibrio* spp. from *T. semidecussatus*. *Vibrio* spp. seem to be recurrent in bivalves (see Table 7.1 for all the cultivated and observed bacteria in bivalves) and in fact they are called facultative pathogens (Prieur *et al.* 1990, Beaz-Hidalgo *et al.* 2010). The study of *Vibrio* spp. is very broad because of the human pathogenic strains that associate to and can be transmitted through edible bivalves (e.g. Canesi *et al.* 2005, Paranjpye and Strom 2005). *Vibrio* strains were also obtained from *Tapes semidecussatus*, including in the media with CTAB, that was used before by Plante and coworkers (2008) to isolate surfactant-resistant bacteria with the aim to obtain bacteria potentially usuful for environmental remediation. Already observed in previous works (Rajagopalan and Sivalingam 1978, Sugita *et al.* 1981) I could cultivate bacteria from the Actinobacteria phylum (*Kocuria* sp. and *Dermacoccus* sp.), and Bacillus sp. of the Firmicutes phylum. *Krokinobacter* sp. and *Alcanivorax* sp. isolates are of special interest because the first ones are bacteria that seem to be specialized in the degradation of organic matter (Khan *et al.* 2006) and the second ones are hydrocarbon-as sole source degrading bacteria (Head *et al.* 2006). Many times coastal bivalve have been taken as a biologi-

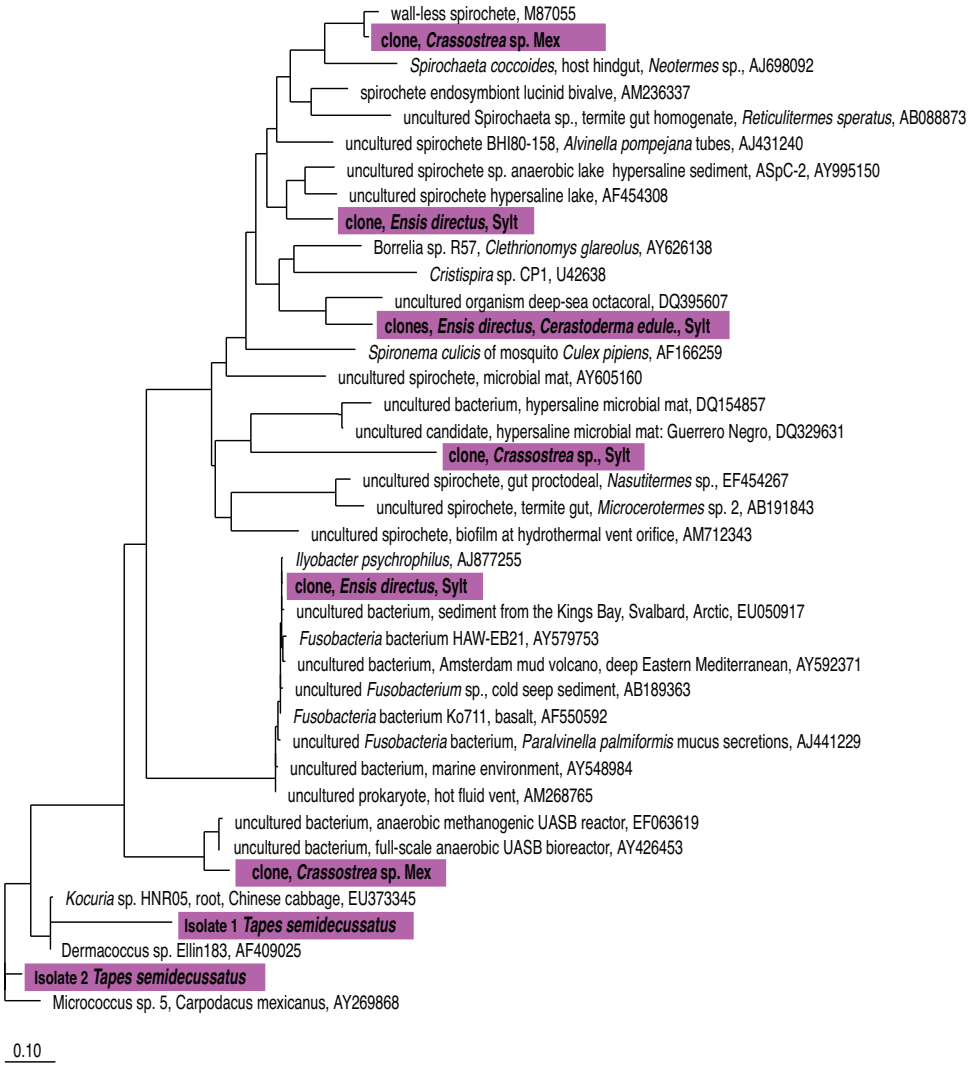

Figure 7.5: Spirochaete and Fusobacteria diversity in bivalves. The clones and isolates obtained in this study are highlighted in violet. Clones or isolates are shown and named after their host origin.

cal marker to assess pollution (e.g. Nishihama *et al.* 1998, Bresler *et al.* 1999, Verlecar *et al.* 2006). The presence of *Alcanivorax* bacteria in clams tissue could indicate a strong oil influence in North Sea beaches, and in fact this can be an easy-to-evaluate biological marker for oil pollution: the presence of *Alcanivorax* spp. in bivalves tissue.

7.4 Summary and Outlook

Bivalve gills provide to bacteria an ideal habitat, with protection from grazers and constant fluid of nutrients. Bacteria might be complementing their host nutrition or contributing to metabolite production. Degradation of bacteria by bivalve enzymes has been observed and it seems that this degradation provides dissolved compounds (Birkbeck and McHenery 1982, Amouroux and Amouroux 1988, McHenery and Birkbeck 1985) and improves bivalve nutrition (Delaunay *et al.* 1992). Bacteria could provide 5% to 10% carbon, and 20% nitrogen from the bivalve requirements (Prieur *et al.* 1990). A stable microbiota could be providing protection to bivalves thanks to competition against other bacteria potentially pathogenic. Also, microbiota could be secreting antimicrobial substances that have been observed to be commun in bacteria isolated from bivalves (Zheng *et al.* 2005).

Ecological studies with molecular techniques are scarce and they could help to disentangle the interaction patterns between the endemic microflora and the invasive one, explaining the benefits and contrarieties that symbiotic or pathogenic bacteria bring along. It is important to understand the distribution of pathogenic bacteria in the marine environments to predict potential health concerns transmitted by seafood. Ecological parameters such as nutrient availability, temperature, and salinity influence the presence and persistence of bacteria. However, I suggest that bacteria like *Vibrio*, *Pseudomonas*, Spirochetes and Epsilonproteobacteria (see Table 7.1 for different bacteria occurrence) present in bivalves are not only randomly there. It is in part the result of the surrounding water community but it might also be the result of a common evolution between host and bacteria that normally associate to invertebrates or high organic matter content habitats. We would

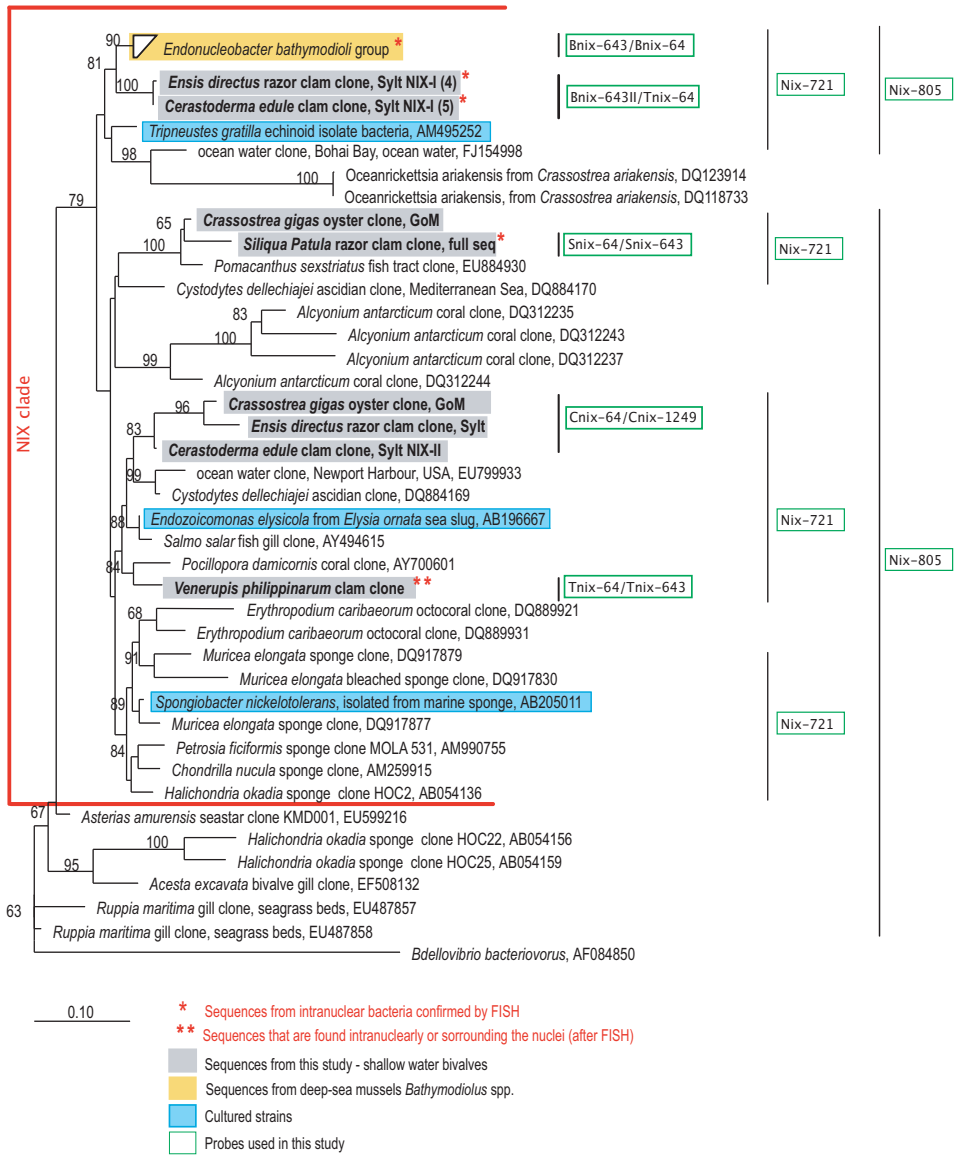

Figure 7.6: 16S rRNA phylogenetic tree based on maximum likelihood (RAxML) analysis. NIX-clade belongs to the Gammaproteobacteria. Sequences from this study (highlighted in grey) and all closely related sequences found in the literature, including the three cultivated strains (in blue) and the Candidatus Endonuclear bathymodioli (in yellow) are shown. Probes (in green square) designed to target each specific host were designed for use in FISH analysis and Nix-721 and Nix-805 for real time PCR. Sequences with a star (*) indicate nuclear phylotypes confirmed by FISH. The T. semidecussatus sequence (with two stars **) was found typically surrounding the nuclei and very rarely inside.

need more studies to be able to say that these bacteria are symbiotic, but from a point of view of the broad symbiosis concept they are, as they appear to be consistent in the bivalves microflora.

Part III

Manuscripts

Resulting manuscripts from this thesis work and contributions:

Manuscript I: **L. Raggi, F. Schubotz, K.-U. Hinrichs, J.M. Petersen, J. Felden and N. Dubilier**. Bacterial symbionts of *Bathymodiolus* mussels and *Escarpia* tubeworms from Chapopote, an asphalt seep in the southern Gulf of Mexico.

Manuscript in preparation.

L.R.: developed the concept together with N.D., did the 16S rRNA sequencing and analyses, designed FISH probes and performed the FISH experiments, helped with the carbon isotope analyses, conceived and wrote the manuscript. F.S.: did the carbon isotope analysis of lipids. K.U.H.: developed the carbon isotope analysis method. J.M.P.: conceived and edited the manuscript with L.R. J.F.: provided samples, and information about the geochemical parameters from the Chapopote site. N.D.: developed the concept with L.R., conceived and edited the manuscript.

Manuscript II: **L.Raggi, D. Fink and N. Dubilier**. An intranuclear bacterial parasite in shallow water bivalves.

Manuscript in preparation.

L.R.: developed the concept together with N.D., did the 16S rRNA sequencing and analysis, designed FISH probes and performed the FISH experiments, conceived and wrote the manuscript. D.F.: performed the qPCR. N.D.: developed the concept with L.R., conceived and edited the manuscript.

Manuscript III: **L.Raggi, and N. Dubilier**. Minireview: Bacterial diversity of shallow-water bivalves.

Manuscript in preparation.

L.R.: developed the concept, did the 16S rRNA sequencing and analyses, performed cultivation experiments, conceived and wrote the manuscript. N.D.: developed the concept with L.R., conceived and edited the manuscript.

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Manuscript I:
Bacterial symbionts of *Bathymodiolus* mussels and *Escarpia* tubeworms from Chapopote, an asphalt seep in the southern Gulf of Mexico.

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In Preparation

Bacterial symbionts of *Bathymodiolus* mussels and *Escarpia* tubeworms from Chapopote, an asphaltic seep in the southern Gulf of Mexico

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Abstract

Chemosynthetic life was recently discovered in the southern Gulf of Mexico (sGoM) where lava-like flows of solidified asphalt cover a large area at 3000 m depth; with oil seeps and gas hydrate deposits also present (MacDonald et al. 2004). Animals with chemosynthetic symbionts such as vestimentiferan tubeworms, mussels, and clams colonize this site called Chapopote. Based on morphological and molecular analyses (COI gene), two mussel species are present at this site, *Bathymodiolus heckera*e and *B. brooksi*, and a single *Escarpia* tubeworm species. Comparative 16S rRNA sequence analysis and FISH showed that all three host species harbor intracellular sulfur-oxidizing symbionts that are highly similar or identical to the symbionts found in the same host species from northern GoM (nGoM) sites. The mussels also harbor methane-oxidizing symbionts, and these are identical to their northern GoM conspecifics. Unexpectedly, we discovered a novel symbiont in *B. heckerae* that is closely related to hydrocarbon degrading bacteria of the genus *Cycloclasticus*. We found in *B. heckerae* the Methyl-toluene-phenol hydroxylase (MTPH) gene and stable carbon isotope analyses of lipids indicative for heterotrophic bacteria were consistently heavier in *B. heckerae* by 3‰ than in *B. brooksi.*, indicating that the novel symbiont might use isotopically heavy hydrocarbons from the asphalt seep as an energy and carbon source. The discovery of a novel symbiont that may be able to metabolize hydrocarbons is particularly intriguing because until now only methane and reduced sulfur compounds have been identified as energy sources in chemosynthetic symbioses. The large amounts of hydrocarbons available at Chapopote would provide these mussel symbioses with a rich source of nutrition.

Introduction

The discovery of cold seeps was a surprising event showing great communities of big sized organisms living at these recondite places (Paull *et al*. 1984). Cold seep ecosystems are some of the most productive on Earth, and it was initially unclear how, with what it seemed to be very little input of organic matter from photosynthesis, these new fauna could even survive down there (Van Dover 2000). Association of these organisms with chemosynthetic bacteria is the answer. More and more different sites and chemosynthetic-based habitats are been discovered, as the underwater tools are each time more equipped to stay longer and deeper in the sea floor. In April 2004 a natural asphalt (bitumen) deposit at 3000 m depth in the Campeche Knolls, southern Gulf of Mexico (sGoM), was discovered and was named Chapopote (MacDonald *et al*. 2004). Cold seeps with methane and hydrocarbon seepage are widespread in the GoM as a result of its unique tectonics and geological history (Macgregor 1993, Bryant *et al.* 1991, Ewing 1991), however this is the first time that a classical cold-seep fauna community was found coexisting with natural asphalt. The biology and microbiology of the northern GoM seeps is well studied (e.g. Fisher 1993, Cavanaugh *et al.*, 1987, Cavanaugh 1993, Carney *et al*. 2006, Cordes *et al*. 2005, 2007, Duperron *et al.* 2007), in contrast with the little studied southern GoM (Fig. 1). The presence of symbiotic bacteria in *Bathymodiolus* mussels and in escarpid tubeworms is one of the intensively studied topics in the nGoM. Three *Bathymodiolus* species in the northern Gulf of Mexico have been described*; B. childressi*, with methanotrophic bacteria, *B. heckerae* and *B. brooksi*, with a dual symbiosis of methanotrophic and thiotrophic bacteria. Also three different tubeworms genera are recognized and all of them bear thiotrophic symbionts: *Escarpia* sp., *Lamellibrachia* sp. and *Sepiophila* sp. The distribution of the different species varies along the GoM (Fig 1), in the Louisiana Slope *B. childressi*, *L. luymesi*, and *S. jonesi* are common and abundant; Atwater Canyon retain only *B. brooksi* and Missisipi Canyon only *B. childressi*. In the Florida Escarpment *B. heckerae*, *B. brooksi* and *E. laminata* are found. The Alaminos Canyon holds *B. brooksi*, *B. childressi*, and *E. laminata*. These species seem to be endemic of the GoM except *B. heckerae* that is found also in the Blake Ridge diapir (Salerno *et al*. 2005). All these species of both tubeworms and mussels get nutrients and benefits from their

chemosynthetic association using only sulfide, methane, or both as energy sources, no further sources are currently known.

Lipid analysis of mussels in the northern Gulf of Mexico, that only contain methanotrophic symbionts have been performed in former studies. It has been observed that depending on whether methane has a biogenic or a thermogenic source, the isotopic values of the tissue vary between -79 to -80 ‰ and -45 to -40 ‰, respectively (Jahnke *et al*. 1995, MacAvoy *et al*. 2002). Methane values for the thermogenic methane in the northern GoM are between -44 and -46 ‰ and for biogenic methane -64 to -65 ‰ (Sassen *et al*. 1999). At the Chapopote Knoll, the stable carbon isotope composition of methane is between -40 ‰ (in the asphalts) to -60‰ (in the sediments), representing a mixture of thermogenic and biogenic methane (Schubotz *et al*. *subm.*). In this study, we do genetic (phylogenetic and metabolic) and lipid analyses focusing on the question of whether the asphalt in Chapopote site might be shaping the community, in particular the bacterial symbiotic community of invertebrates, adding an extra carbon source. This habitat is a novel setting because of this heavy oil called asphalt. Asphalt has high amounts of asphaltenes that make it heavier than water. Thus, the oil stays in the seafloor, while in other settings the oil leaks upward to the water surface. It creates then an interface where hydrocarbons can be aerobically oxidized. Also, this is a system with new substrate for the megafauna to settle, as there are not just carbonates but also solid asphaltic formations.

Material and methods

Specimen collection

Mussels and vestimentiferan tubeworms were collected at the base of Chapopote Knoll with the ROV *Quest* aboard the RV *Meteor* during the M67/2 cruise (April 2005). *Bathymodiolus* mussels were collected from a mussel bed (21°53.98´N; 93°26.12`W) at a water depth of 2923 m. Four mussels were recovered during dive 83; two were identified as species 1 and the other two as species 2. The mussel gills were dissected immediately after recovery and segments were frozen and stored at -20°C for DNA or lipid extraction. Other fragments were fixed for FISH with 2% PFA and stored at 4°C in 0.5X PBS / 50% ethanol.
Tubeworms were collected in two different dives: dive 82 at 21°53.95´N; 93°26.23`W, 2918 m water depth, and dive 83 at 21°53.94´N; 93°26.25`W and water depth 2915 m. Three tubeworms from dive 82 (Tbw 1, Tbw 2 and Tbw 3) and one from dive 83 (Tbw 4) were extracted from their tube and different pieces were frozen for DNA extraction or fixed for FISH.

DNA extraction and PCR amplification

DNA was extracted from frozen tissue according to Zhou *et al*. (1996) with the following modifications. Briefly, 2 ml of extraction buffer and 20 µl proteinase K (20 mg/ml) were added to approximately 100 mg of sample, and incubated for 1.5 hours at 37°C. Then 200 µL of 20% SDS were added and incubated for 2 hr at 56°C. The liquid phase was recovered after centrifugation at 14000 g for 20 min and cleaned once with 1 V phenol/chloroform/isoamylalcohol (25:24:1) and a second time with chloroform/isoamylalcohol (24:1). DNA was precipitated with 0.6 V isopropanol and dissolved in Tris-EDTA buffer. The extracted DNA was used for both host and bacterial symbiont analysis.
Host COI genes were amplified using 36 PCR cycles. For the mussels the primers LCO-1560 and HCO-2148 (Jones *et al*. 2006) were used and for the tubeworms LCO1490 and

HCO2198 (Folmer *et al*. 1994). Bacterial 16S rRNA genes were amplified using 20 PCR cycles with universal primers GM3 (8F) and GM4 (1492R) (Muyzer *et al*. 1995). Metabolic marker genes were amplified using 23 PCR cycles. Primers aps1F and aps4R were used to amplify *aprA* gene (Blazejak *et al*. 2006) and the *pmoA* gene was amplified with A189F and MB661R primers (Costello & Lidstrom 1999). We tested all the ring-hydroxylating monooxygenases primers used by Baldwin *et al.* (2003), and only the RMO-F and RMO-R pair gave an amplification product of the expected size (see Table S2 for primer sequences and annealing temperatures). The expected 500 bp product was cloned and sequenced. We obtained sequences of 300 bp length which resulted to be a fragment of a methane/toluene/phenol hydroxylase.

Tubeworm genetic analysis

COI PCR products of the four tubeworms were directly sequenced from the PCR product, in both directions. Symbiont 16S rRNA and *aprA* genes of tubeworms 1 and 3 were cloned using the TOPO-TA system (Invitrogen) and analyzed. Genes *pmoA* and MTPH could not be amplified in the tubeworms.

Mussel genetic analysis

COI PCR products of the four mussels were directly sequenced in both directions. Symbiont genes of each mussel (16S rRNA, *aprA, pmoA* and MTPH were cloned using the TOPO-TA (Invitrogen) or pGEM-T Easy (Promega) cloning vectors. 16S rRNA clones were partially sequenced with primer 907RC (Muyzer & Smalla 1998) and representative clones from each host individual were chosen for full sequencing in both directions. Metabolic marker genes were sequenced with the respective primers (both strands). *aprA* and *pmoA* genes were analyzed in one individual of each species. MTPH was amplified from both individuals of *B. heckerae* but the product of only one individual could be successfully cloned and sequenced. Sequencing was performed using the BigDye terminator v3.1 Cycle Sequencing Kit along with the Genetic Analyzer Abiprism 3130 (Applied Biosystems).

Phylogenetic analysis

Sequences were analyzed with Sequencher (Genes Codes Corporation) and ARB (Ludwig *et al*. 2004) sofwares. 16S rRNA Sequences were aligned within the Ref_96 SILVA database (Pruesse *et al*. 2007). Databases for each metabolic marker gene were constructed with publicly available sequences for each gene, and the alignment was produced with CLUSTALW implemented in ARB (Ludwig *et al*. 2004). Phylogenetic trees were calculated with the ARB software. All sequence comparisons are given as percentage sequence identity (% similar nucleotides) after calculations of distance matrix. For tree reconstruction, only long sequences (~1400 bp for 16S rRNA, ~650 bp for COI, ~1200 bp for *aprA*, ~450 bp for *pmoA*) were used, except for the MTPH gene that we used the 300 bp sequence. Phylogenetic trees of 16S rRNA gene sequences were calculated by neighbor joining, maximum parsimony and maximum likelihood methods. As the differences in the resulting tree topographies were not significant we only present the maximum likelihood results. Sequences with more than 99.7% identity (% identical nucleotides) were grouped and are shown as one single sequence. To assess nodes robustness in the trees, 1000ML bootstrap replicates were run.

Probe design and testing

All probes used in this study were checked for mismatches against our sequences of interest and the Silva 96 SSU Ref database (Pruesse *et al.* 2007). The *Cycloclasticus* probe (Cypu-829) was originally used without formamide (Maruyama *et al.* 2003). We performed a formamide series between 10% and 70%, and the probe hybridized between 10% and 40% formamide. The probe NON338 (Amann *et al*. 1990) was used as a negative control and EUB338 (Wallner *et al.* 1993) as a positive control. Two new probes were designed (using the ARB Probe Design tool) to match the *Escarpia* symbiont, TbwT-643 and TbwT-139, both of which were tested for FISH at 20% formamide. Since these probes gave low intensity signals, we tested an HRP-labeled TbwT-643 probe. A 20% to 60% formamide series was

performed and signals were observed at all formamide concentrations (see probes and formamide concentrations for all probes in Table 1S).

FISH and CARD-FISH

A piece of tubeworms and gills were dehydrated in an ethanol series and embedded in low-melting temperature polyester wax (Steedman 1957). Wax cubes were cut into 5 or 6 µm sections with an RM2165 microtome (Leica, Germany) and mounted on Superfrost-Plus slides (Menzel-Gläser). Polyester wax was removed by washing three times in absolute ethanol (5 min each), and sections were rehydrated in a 96%-80%-70% ethanol series. Sections were permeabilized in Tris-HCl (20 mM, pH 8), proteinase K (0.05 mg ml-1 in Tris-EDTA, pH 8, at 37°C), and washed in MilliQ water (5 min each). For *in situ* hybridizations with fluorochrome- (FISH) or horseradish peroxidase (HRP)-labeled probes (CARD-FISH) and subsequent staining with DAPI, sections were processed as described previously (Duperron *et al*. 2007, Lösekann *et al*. 2008, Pernthaler *et al*. 2002).

Lipid biomarker/isotopic analysis

Lipids of freeze-dried and homogenized mussel gill and tubeworm tissue were extracted four times with a modified Bligh and Dyer method described in Sturt *et al.* (2004). Briefly, in the first two steps, dichloromethanol (DCM), methanol and a phosphate buffer (2:1:0.8) were added to the soft tissue and cell lysis was initiated during microwave extraction (15 min at 70°C; Brand), for the last two steps the phosphate buffer was exchanged with 0.5 M trichloroacetic acid. Total lipid extracts were collected after liquid-liquid extraction with DCM and deionized Milli-Q water. Aliquots of the total lipid extracts were saponified into free fatty acids and neutral lipids with aqueous 0.5M KOH in methanol (3 h at 80°C) following the protocol of Elvert *et al*. (2003). Fatty acids and neutral lipids were derivatized with Bis(trimethylsilyl)trifluoroacetamide (BSTFA), yielding trimethylsilyl (TMS) - derivatives, before analysis by gas chromatography. Structural identification of compounds was achieved using a GC-MSplus-DSQ system (Finnigan Trace). An injection standard

(squalane) was added for quantification purposes prior to analysis on a Thermo-Finnigan Trace GC coupled to a FID. Determination of compound specific stable carbon isotopic compositions was performed on a gas chromatograph coupled to an isotopic ratio mass spectrometer (GC-IRMS). Intact polar lipids (IPLs) were also analyzed with a HPLC-ESI-MS^n system as described previously (Sturt *et al*. 2004). All isotopic values are reported in the delta notation ($d^{13}C$) and are relative to the Vienna PeeDee Belemnite Standard. The isotopic compositions of the TMS-derivatives were corrected for the isotopic values of the methyl groups attached during derivatisation (-47.2‰). The standard deviation of replicates and an injection standard (hexatriacontan) was <1 ‰.

Statistics

The statistical analyses were performed with the SigmaStat software (version 3.5; Jandell Scientific, San Rafael, CA). To determine differences in isotopic fatty acid values ANOVA was performed. As the data were not normally distributed, comparisons were analyzed using the nonparametric Kruskal-Wallis ANOVA on ranks with Dunn´s method as the post hoc test.

Results

Host COI gene phylogeny

Cytochrome oxidase I (COI) gene sequences from the four tubeworms were >99.7% identical, and shared >99.1% identity with the described species *Escarpia laminata* from the northern Gulf of Mexico, *E. spicata* from the Guaymas and Santa Catalina Basins and with *E. southwardae* from the Zaire Margin (McMullin *et al*. 2003, Black *et al*. 1997, Feldman *et al*. 1998, Andersen *et al.* 2004). Four almost identical haplotypes were found within our sequences each with one synonymous substitution in three polymorphic sites. They fell within a well supported clade of *Escarpia* sequences (98% bootstrap support) and this clade grouped together with other seep vestimentiferans with 99% bootstrap support (Fig 2).
COI gene sequences of the mussels identified morphologically as *Bathymodiolus* sp. 1 were 99% identical and clustered with sequences from *B. heckerae* from the northern Gulf of Mexico (>98.4% identity). Sequences of the two mussels identified as *Bathymodiolus* sp. 2 were 99.4% identical and clustered with *B. brooksi* sequences from the northern Gulf of Mexico (>98.8% identity). From here on, we call *B. heckerae* to *Bathymodiolus* sp.1 and *B. brooksi* to *Bathymodiolus* sp.2.

Tubeworm symbiont 16S rRNA phylogeny and in situ localization

The 9 full sequences obtained from the 16S rRNA clone libraries of both analyzed tubeworms were identical between each other (a total of 209 partial sequences were analyzed and also identical) and with the endosymbionts of *Escarpia laminata* and *Lamellibrachia* sp. from the Northern GoM (Nelson & Fisher 2000, McMullin *et al*. 2003), and *E. spicata* and *Lamellibrachia barhami* from the Guaymas Basin (Vrijenhoek *et al.* 2007). These sequences together with the almost identical symbiont sequence of *L. columna* (99.9%) from Lau Basin, and *L. barhami* from Middle Valley (Nelson & Fisher 2000, McMullin *et al*. 2003) formed a well supported clade with a 100% value (Fig. 3b). The bacterial symbionts were clearly observed in sections of the tubeworm trophosome tissue with the specific probe TbwT-643.

They are present in highly abundant groups and patchily distributed in the trophosomal tissue (Fig. 5a).

Bathymodiolus heckerae symbionts and in situ localization

The *B. heckerae* 16S rRNA clone library was dominated by sequences close related to thiotrophic *B. heckerae* symbionts. Two different thiotrophic-related sequences were found with a 96.3% identity between them and one of them was identical to a sequence from the nGoM, and the second one had a 98.4% identity with the closest relative from the nGoM as well. We also obtained one methanotroph-related phylotype, which matched exactly with methanotrophic-related sequence from the nGoM (Fig. 3b). In addition to the above-described bacteria a *Cycloclasticus*-related species was found in the *B. heckerae* data-set which had a 97.9% identity with *Cycloclasticus spirillensus* and other cultivated *Cycloclasticus* sp. (see Fig. 3b). All different bacteria were localized with fluorescence *in situ* hybridization (FISH) in both individuals *B. heckerae* 1 and 2, including the *Cycloclasticus*-related species (Fig. 4 and Fig. S2). This last one showed very low fluorescence intensity with FISH therefore a CARD-FISH probe was used to have a more evident observation. The different endosymbionts were observed with specific probes for thiotrophic- and methanotrophic- *Bathymodiolus* symbiont (Table S1). The methanotrophic- and thiotrophic-related endosymbionts were co-localizing in the bacteriocytes showing a higher abundance of thiotrophic ones (4e-f, i-j). It is not clear in our observation if the biovolume of the thiotrophs is also larger than the methanotrophs due to the small size of the thiotrophs and the big size of the methanotrophs (Fig 4e). *Cycloclasticus*-related bacteria were co-localizing in *B. heckerae* with the other symbionts and with a triple hybridization it was estimated that the *Cycloclasticus*-related bacteria were making 6% of the total endosymbionts. These bacteria were not detected in other *Bathymodiolus* tissues, not *B. heckerae* from nGoM, nor *B. childressi* from the nGoM, neither *B. brooksi* from Chapopote.

Bathymodiolus brooksi symbionts and in situ localization

The *B. brooksi* 16S rRNA clone library was dominated by sequences close related to thiotrophic symbionts of *B. brooksi* from the nGoM. A single thiotrophic- and one methanotrophic-related phylotypes were obtained in this library (100% and 99.8% identities to respective sequences from the nGoM). In addition a *Psychromonas*-related phylotype was found and was most closely related to *Psychromonas profunda* (96.5% identity) and to a whale-fall clone sequence (96.7% identity). All the different endosymbionts were localized *in situ* with specific probes in both *B. brooksi* individuals. Thiotrophic- and methanotrophic-related symbionts were present both inside bacteriocytes. *Psychromonas*-related bacteria were present scarcely also within the mussel tissue; However, they do not seem to be in the same focus plain as the endosymbionts (Fig. S1).

Metabolic marker genes

The *aprA* gene coding for the alpha subunit of APS reductase was amplified in *B. heckerae*, *B. brooksi* and *Escarpia* sp. We analyzed 48 clones from *B. heckerae* 2 and as all the sequences were identical we decided to analyze only 2 to 8 clones from each host species. The sequences were identical between individuals for each species. The comparative sequence analysis (Fig. 5a) grouped *Bathymodiolus* spp. *aprA* sequences with other bathymodiolin spp. (72.5-93.9% identity). *Escarpia* sp. *aprA* grouped with other annelids (gutless oligochaetes) and *Astomonema* nematode symbionts (82.5-87.8%). The *pmoA* gene was only found in *Bathymodiolus* sp. No nucleotide differences were found within individuals of each *Bathymodiolus* species. Comparative sequence analysis (Fig. 5b) showed that *B. heckerae* and *B. brooksi pmoA* sequences are closely related to each other and to the other *Bathymodiolus* spp. (90–100% identity). Methane-oxidazing free-living bacteria sequences form a clade that is close-related (70-90%) to the *Bathymodiolus* group. The alpha subunit of the MTPH gene could be amplified only from both *B. heckerae* individuals (Fig. 5c-d), suggesting that *Cycloclasticus* bacteria are only present in this species.

Lipid analysis

The main fatty acids in all four investigated *Escarpia* species were 18:1ω7, 20:5, and 16:1ω7. Other major fatty acids were 16:0, 18:2, 20:4, 20:2, 20:1, 22:2 and 18:0. 18:1ω7 and 16:1w7 are known biomarkers for sulfur oxidizing bacteria (Conway & Capuzzo 1991). Within all samples 18:1ω7 and 16:1ω7 fatty acids are always most enriched and the longer and unsaturated the fatty acids the more depleted they are (Fig. S4). This observation is consistent with findings by Pond *et al.* (2002), who described a synthesis of elongated and desaturated fatty acids from the bacterial starting product. The values of this study for the $\delta^{13}C$ fatty acids of the *Escarpia* tubeworms range from -27‰ for the short chain fatty acids up to -43‰ for the longer or complex lipids, having no variation between individual compounds. Lower values might be due to the fractionation during the chain elongation that takes place as the two carbon acetyl groups are added to the carbon chain (Deniro & Epstein 1977, Monson & Hayes 1982) or when the desaturating enzymes create monounsaturated acids from saturated fatty acids (Monson & Hayes 1982, Abrajano *et al*. 1994). The stable carbon isotope composition of the weighted average mean of all fatty acids is -31.2 ± 1.5 ‰. When plotting mean values of any tubeworm fatty acid stable carbon isotopic composition against any other tubeworm it can be observed that all values plot on the 1:1 slope (see example in Fig. 6c). Three sterols dominated the neutral fraction, cholesterol, ergosta-dien-ol, and cholesta-dien-ol (For a complete overview of compounds see table S3). Sterols have the most depleted values, between -37.7 for cholesterol to -39.2‰ for ergosta-dien-ol.. The intact polar lipid composition of the *Escarpia* species is very complex, among the most abundant head groups are phosphatidylcholines (PC) and of phosphatidyl-ethanolamines (PE) as diacylglycerols and plasmalogens. Also present are phosphatidyl-serines (PS) and glycosidic ceramides (sphingolipids) between many other unknown.

The dominating fatty acids in both mussels were 16:1ω7 and 16:0. They also contained large amounts of 18:0, 16:1ω7, 20:1ω7 and polyunsaturated fatty acids 18:3, 18:2, 20:3, 20:2 and 22:2. 16:1ω7, 16:1ω7 and 20:1ω7, biomarkers that have been used as indicators for thiotrophy were detected in both mussel species. On the contrary, the methanotrophic lipid 16:1ω8 was only detected in *B. brooksi* , while diplopterol, which is also a marker for aerobic

methanotrophy (e.g. Hinrichs *et al*. 2003), was only observed in *B. heckerae*. Both mussels contain also 4-methyl sterols, which to date have only been found in methanotrophic bacteria (Jahnke *et al*. 1995, Schouten *et al*. 2000) and indeed they were always most depleted in ^{13}C (-54.9 to -50.9‰). *B. brooksi* additionally contained moderate amounts of lanosterol, and 4,4,-dimethylcholesta-dienol. The weighted average stable carbon isotope composition of all fatty acids for both *B. heckerae* species was -41.9‰ and -44.4‰ for the two *B. brooksi* species (see Table S3 for complete list of fatty acid isotopic values). The bulk gill tissue of both *B. brooksi* species is also approximately 2‰ lighter compared to both *B. heckerae* species, consisting of -42.9 and -40.8‰ in average, respectively. And as general observation the carbon isotopic values of the fatty acids compared to the bulk tissue have an average offset of ca. 2.5 ‰ for all compounds (Fig. 6a). Compound specific stable carbon isotopes of fatty acids cluster closely together but a general trend can be observed towards the more unsaturated the fatty acids the more depleted values they have (e.g 18:3 and 20:3 being approx. 3 ‰ lighter than the 18:1 and 20:1). When plotting the mean *B. heckerae* fatty acid stable carbon isotopic composition against the mean *B. brooksi* fatty acids it can be observed that all values plot below the 1:1 slope, indicating an average depletion of *B. brooksi* fatty acids in comparison to *B. heckerae* (Fig. 6b). This is supported by ANOVA values with $p = <0.001$ ($H=19,099$ with 3 degrees of freedom) and the Dunn´s method, showing significant differences between species but not between individuals of the same species. The analysis of intact polar lipids (IPLs) revealed the fatty acid combinations and head groups of the intact membrane lipids. Main polar headgroups of both *B. heckerae* and *B. brooksi* were composed PE, PC, phosphatidylphosphonoethanolamines (Phos-phono), and minor amounts of PS and phosphatidylinositols (PI). Phosphatidyl-glycerols (PG) were only detected in *B. heckerae*. In *B. brooksi* also minor amounts of the Sphingolipid ceramide-PE (PE-Cer) were detected (Table S3).

Discussion

Identity of tubeworms and mussels from the Chapopote asphalt seep

The chemosynthetic fauna present at Chapopote in the sGOM is reminiscent of cold seeps in the nGOM, dominated by tubeworms and mussels. In the first description of the Chapopote site by MacDonald *et al*. (2004), the tubeworms were identified morphologically as *Lamellibrachia* sp., and the mussels as *Bathymodiolus* sp.. Our comparative pylogenetic analysis of the COI genes of four tubeworms identified these as belonging to the genus *Escarpia*. Analysis of the four mussels sampled identified these as belonging to two separate species, one of which is closely related to *B. heckerae* from the nGOM, the other to *B. brooski* from the nGOM. The phylogenetic resolution of the COI gene worked well for bathymodiolin mussels, integrating the new species into a defined group, we could name the two species *B. heckerae* and *B. brooksi*. However, as observed before (for rev. see McMullin *et al. 2003*), the resolution of this gene is not sufficient for determining tubeworm species, especially within the escarpids that have a very similar COI sequence. However it gives a good definition of the tubeworm genera, in this case *Escarpia*. The phylogeny analysis of the tubeworms including other molecular markers (as the ND4 mitochondrial gene) would be needed to differentiate between *Escarpia* species. However, we can not let drop that vestimentiferan tubeworms have a remarkable plasticity (Black *et al.* 1997) and therefore *E. laminata*, *E. southwardae* and *E. spicata* could be the same species. To resolve this, a population genetic study would be required.

Phylogeny and in situ *distribution of previously described symbionts*

The endosymbiotic bacteria of the *Bathymodiolus* mussels and *Escarpia* tubeworms investigated in this study are, like the hosts themselves, closely related or even identical to their counterparts in the nGOM. Although a clone library might not be representative of the true abundance because of well-characterized method-biases (Reysenbach *et al*. 1992, Suzuki & Giovannoni 1996, Acinas *et al*. 2005) our clone library analysis showed a dual symbiosis

in both mussel species, with a dominant abundance of sulfur-oxidizers. This was corroborated with FISH observations (Fig. 4). The high abundance of thiotrophs contrasts with previous studies of *Bathymodiolus* mussels from the nGOM. In all nGOM mussels investigated to date, methanotrophic symbionts had a dominating abundance based on FISH and RNA slot blot hybridizations (Cavanaugh 1993, Fisher *et al*. 1993, Duperron *et al*. 2007). Methane concentrations were generally high at Chapopote, indicating that this was not the limiting factor for the presence of methanotrophic symbionts (MacDonald *et al*. 2004). Unfortunately, there are no geochemical data available for the site at which the mussels were sampled. Methane and sulfide availability in the mussel habitat has been shown to directly influence the relative abundance of thiotrophs and methanotrophs in the mussel gill tissue (Trask and Van Dover 1999, Fiala-Medioni *et al*. 2002, Salerno *et al*. 2005, Duperron *et al.* 2007, Riou *et al*. 2008). If methane is only delivered to the surface in short bursts during asphalt eruptions, and shorter hydrocarbons are the first to diffuse out of the asphalt, then there may not have been much methane available in the mussel habitat at the time of sampling. In these areas, sulfide may be more abundant than methane, which would explain why thiotrophs are relatively more abundant in the mussels at this site compared to the nGOM. At Chapopote, it might be important that the hydrocarbons are trapped below the bituminous formations because anaerobic hydrocarbon degradation (with sulfate) would support higher production of sulfide (Boetius 2005). It will be important in future studies to carry out punctual measurements of sulfide and methane concentrations in the different habitats to be able to link them to the symbiotic microbial community.

Metabolic capabilities of previously described symbioses

Based on the phylogenetic relationship of the *Escarpia* tubeworm symbionts to the sulfur-oxidizing symbionts of other tubeworms, we hypothesized that they are also sulfur-oxidizing chemolithoautotrophs. To investigate this further, we analyzed stable carbon isotopes and lipid profiles, and the presence of a key gene for sulfur oxidation, *aprA,* in the tubeworm tissues. Based on our geochemical analyses on the *Escarpia* tubeworms and the lipid profile showing 18:1w7 and 16:1w7 fatty acids presence in all four investigated individuals, we can

conclude that they contain thiotrophic symbionts. If the tubeworms rely entirely on their symbionts for their nutrition, we would expect the tubeworm tissue to have a carbon stable isotopic composition of around -29‰, based on the fractionation of CO_2 during chemoautotrophy (Fang *et al*. 1993). The $\delta^{13}C$ values of this study for the fatty acids of the *Escarpia* tubeworms are indeed around this value, except for the lower values for the longer fatty acids, which could be explained by host fractionation while synthesizing the longer fatty acids. The compound-specific stable carbon isotope values of *Escarpia* sp. show no significant differences between individuals (Fig. 6), indicating a similar nutritional strategy for all tubeworm individuals investigated. We were able to amplify and sequence an *aprA* gene from tubeworm trophosome tissue (Fig. 5a). Our *aprA* sequences clustered with those of free-living sulfur-oxidizing bacteria and the other thiotrophic symbionts, suggesting that this bacterial phylotype is capable of oxidizing sulfide. Before this study, there was only a single published *aprA* sequence available from lamellibrachid or escarpid tubeworms, from a tubeworm found at mud volcanoes in the Mediterranean (Duperron *et al*. 2009). The *aprA* sequences from other thiotrophic symbionts from deep-sea chemosynthetic ecosystems such as the clam and mussel symbionts fall into lineage I, as defined by Meyer & Kuever (2007). In contrast, our sequence from the Chapopote *Escarpia* tubeworm symbiont falls into lineage II, and clusters with sequences from symbionts of oligochaete worms found in shallow marine sediments (Fig. 5).

According to our metabolic marker and lipid analyses, both mussel species contain *pmoA*, *aprA* genes and lipids characteristic of thiotrophic (16:1w7 and 18:1w7) and methanotrophic bacteria (16:1w8, 16:1w5, and diplopterol). The *pmoA* gene separates well *B. heckerae* from *B. brooksi* bacterial phylotypes. Thus, the presence of the *pmoA* gene and the lipid profiles suggest that symbionts in the mussels have the potential to oxidize methane and use it as a source of energy and carbon, as was shown in previous studies for methanotrophy in bathymodiolin mussels (Fisher *et al.* 1987, 1993, Nelson *et al.* 1995, Cavanaugh 1993). The main isotopic values of both species are more enriched than typically observed for dual symbiont bearing mussels (i.e. methanotrophic and thiotrophic symbionts). Nevertheless, this is in direct correlation to the isotopic value of the carbon source in the Chapopote site. The heavier isotopic values for the mussel's lipids are explained by the combination of the

fractionation by methanotrophic bacteria that deplete by 10 - 20‰ (Barker & Fritz 1981), and thiotrophic bacteria that provoke a depletion by 24.4‰ (Scott *et al*. 2004). The $\delta^{13}C$ of the methane at Chapopote is between -41‰ and -70‰, and water column DIC has a $\delta^{13}C$ of approximately 0‰ (Gruber *et al*. 1999). We could say then, that the isotopic values that we got in this study could be the result of the uptake of the different carbon sources (CO_2, CH_4 and heavier hydrocarbons) in this complex symbiosis.

Novel symbionts in Bathymodiolus mussels, an adaptation?

Mussels in this novel environmental setting seem to have adapted well to this rare bituminous settling surface after their normal association to carbonates. And it could be that establishment of new symbioses helps this adaptation to happen. Independent of the host needs, the presence of potential new bacterial symbionts seem to be related to a high organic matter content in the environment, which in this case it does exist in Chapopote site and would explain the presence of *Psychromonas* bacteria in *B. brooksi* tissue, and of *Cycloclasticus*-related bacteria in *B. heckerae*. *Psychromonas* bacteria are heterotrophic organisms frequently found in cold-water sediments. Only once, another *Psychromonas*-related phylotype has been observed associated with an animal tissue, in the bones of a whale fall (Goffredi *et al*. 2004). *Cycloclasticus* is a common organism that blooms in oil spills, and it is commonly found and detected in water analysis (Kasai *et al.* 2002, Maruyama *et al*. 2003) . Here we suggest that the hydrocarbon load in the environment makes the presence of this *Cycloclasticus* bacterium possible, as they have been found also in oily sediments from shallow and deep waters. However, this is the first time to our knowledge that *Cycloclasticus* sp. is observed as an intracellular bacterium as it has been shown by our FISH analysis. We have showed here the presence of the gene for the MTPH enzyme in *B. heckerae*. We suggest that the presence of this gene is due to the *Cycloclasticus*-related bacteria. The association with the mussel host might provide this bacterium the advantage of being in an aerobic habitat with available nutrients (hydrocarbons flowing through the gills) and for the mussel this would provide a new nutrition source coming from the degradation of aromatic compounds, including a detoxification of them. We are not aware of any specific lipid

markers that have been found in the so far cultured *Cycloclasticus* spp. When analyzing the lipid data we can observe that all lipids are generally heavier in *B. heckerae* than in *B. brooksi* (Fig. 6). In fact, bulk tissue and compound specific stable carbon isotopes showed a mean average enrichment in $\delta^{13}C$ for *B. heckerae* in comparison to *B. brooksi*. As we discussed before it is clear that both mussels host a dual symbioses. Since the mussels co-occur, it is highly likely that the symbionts of both utilize the same carbon sources. In addition, the relative abundance of thiotrophs and methanotrophs is comparable for both species, indicating that the relative contribution of thiotrophy and methanotrophy to the nutrition of each mussel host is comparable. Based on these observations, we would expect them to have similar lipid isotopic values. However, *B. brooksi* is consistently lighter than *B. heckerae,* in bulk and compound-specific isotope analysis, and this is most likely explained by the contribution of the additional hydrocarbon degrading symbiont to host nutrition.

Concluding remarks

The sGOM Chapopote Knoll fauna is similar to that at the West Florida Escarpment, nGoM, having the presence of *B. heckerae*, *B. brooksi* and *E. laminata.* This gives an interesting comparison between hosts and symbionts of both places. This study shows that the input of hydrocarbons, derived probably from the asphalt, has directly influenced the diversity of symbionts found in the local chemosynthetic mussels. The use of improved molecular techniques and the discovery and investigation of novel environmental settings may reveal that this phenomenon is more common than previously assumed. The unique environmental conditions at Chapopote define not only the free-living microbial communities but also the symbiotic ones.

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Tables and figures

Fig. 1 Gulf of Mexico Basin. The most studied sites are shown with their respective *Bathymodiolus* and tube worm fauna.

Fig. 2a Phylogenetic affiliation of *Escarpia* tubeworms based on COI gene sequences. Maximum-likelihood tree showing vestimentiferan tubeworm species from vent and seep environments including the 5 individuals of this study (sequences highlighted in gray). Only bootstrap values greater than 70 % are shown.

Fig. 2b Phylogenetic reconstruction of bacterial symbionts of vestimentiferan tubeworms based on 16S rRNA gene sequences. Maximum-likelihood tree shows within the □-proteobacteria phylum, thiotrophic symbionts of seep and vent vestimentiferans. Only one phylotype was present in the two investigated tubeworms (in bold), and fell in group 1 (McMullin at al 2003) within *Escarpia spicata, Escarpia laminata* and *Lamellibrachia* spp.

Fig. 3a Phylogenetic affiliation of *Bathymodiolus* mussels based on COI gene sequences. Maximum likelihood tree showing *Bathymodiolus* spp. from vent and seep environments including the 4 individuals of this study (highlighted in gray). Only bootstrap values greater than 70 % are shown.

Fig. 3b Phylogenetic reconstruction of bacterial symbionts of *Bathymodiolus* mussels based on 16S rRNA gene sequences. Maximum-likelihood tree shows within the γ-proteobacteria phylum thiotrophic, *Cycloclasticus*-related, *Psychromonas*-related and methanotrophic bacteria. The sequences from this study are shown in bold, Note that *B. heckerae* individuals have two different thiotrophic phylotypes, one *Cycloclasticus*- and one methanotrophic-related phylotypes. In our *B. brooksi* datasets we have found one thiotrophic-, one *Psychromonas*, and one methanotrophic-related phylotypes.

Fig 4. *Bathymodiolus* mussels and *Escarpia* tubeworms from this study, and FISH images of bacteriocytes in the mussel gill filaments and in the tubeworm trophosome. (a) *Bathymodiolus brooksi* and *B. heckerae* mussels together with escarpid tubeworms settle in the asphaltic sediment at Chapopote cold seep in the southern Gulf of Mexico. Each metazoan species harbors its own specific bacterial phylotypes. (b) *Escarpia* tubeworms from this study bear chemoautotrophic tubeworm symbionts. (c) Localization of the symbionts (arrows) with a FISH specific probe through a tubeworm cross-section. (d-g) *B. heckerae* mussel and respective FISH images: *B. heckerae* shell has an elongated shape (d); Its filamentous gills house methanotrophic (pink) and thiotrophic (green) bacteria (e). Two different chemoautotrophic bacterial phylotypes have been recognized. The host nuclei are in blue, thiotrophs I in red, and thiotrophs II in yellow (f). A new hydrocarbon-degrader symbiont in green, is co-existing with the methanotrophic bacteria in blue and the thiotrophs in pink (g). (h-k) *B. brooksi* mussel and respective FISH images: the shape of the *B. brooksi* (h) shell is more round and smaller than *B. heckerae.*. When analyzed with FISH *B. brooksi* gill filaments (autofluorescence of the tissue is purple) house a methanotrophic bacterial phylotyope in red, and a thiotrophic one in green (i). A detail of (i) shows host nuclei in blue, methanotrophs in red and thiotrophs in green (j). A *Psychromonas*-related bacteria was found to be associated with *B. brooksi* gill tissue (k). Scale bars: (c, i) = 50µm; (d, h) = 5 cm; (e, f, g, k) = 5 µm; (j) = 10 µm.

Fig 5. Phylogenetic reconstruction of bacterial symbionts based on metabolic marker genes. Sequences highlighted in gray. (a) Maximum-likelihood tree based on the alpha subunit of the APS reductase gene (*aprA*) sequences. (b) Maximum-likelihood tree based on the alpha subunit of the particulate MMO gene (*pmoA*) sequences. We found this gene present only in *Bathymodiolus* spp. The sequences of this study grouped with former *Bathymodiolus* sequences. (c and d) Maximum-likelihood tree based on the phenol hydroxylase gene. We could amplify the gene in *B. heckerae* and not in *B.brooksi* (see d). The sequence fall within sequences related to high content hydrocarbon environments.

Fig 6. Stable carbon isotope measurements of lipids extracted from *B. heckerae*, *B. brooksi,* and *Escarpia* sp. tubeworms tissue. (a) Carbon isotope values of lipids (circles and bars) and bulk tissue (diamonds) from *Bathymodiolus* spp. and *Escarpia* sp. tissues. (b) *B. heckerae* carbon isotope values plotted against *B. brooksi* values. (c) *Escarpia* tubeworms B and 4 carbon isotope values plotted against A1 and A2.

Table S1. Primers and probes. All the oligonucleotides used in this study are listed and their anneling temperature or formamide concentration that were applied to them.

Table S2. Clone description of this study. In the case of the 16S rRNA gene the number of partial sequences are shown and complete ones are in parenthesis. No data means weather we did not obtained any sequence or we did not amplify from that individual. The dash meaning we run the PCR but no product was obtained.

Table S3. Isotopic composition and quantity (mg of lipid per g of tissue) of fatty acids from *Escarpia* tube worms

Table S4. Isotopic composition and quantity (mg of lipid per g of gill tissue) of fatty acids from *Bathymodiolus* mussels.

Fig. S1 Cross-section of gill filament of *B. brooksi.* (a) Methanotrophic-related bacteria (blue) and *Psychromonas*-related bacteria (orange). In gray the auto fluorescence of the tissue. *Psychromonas*-related bacteria are not abundant and seem to be filamentous-like bacteria. (Thiotrophic-related bacteria are not shown but they are similarly present as in *B. heckerae* tissue). (b) Psychromonas-related bacteria in pink and DAPI in blue.

Fig. S2 Cross-section through gill filaments of *B. heckerae*. (a) Thiotrophic-related bacteria T1 (in red), and T2 (in yellow). Scale bar in 40 μm. (b) A detail from a, scale bar 10 μm.

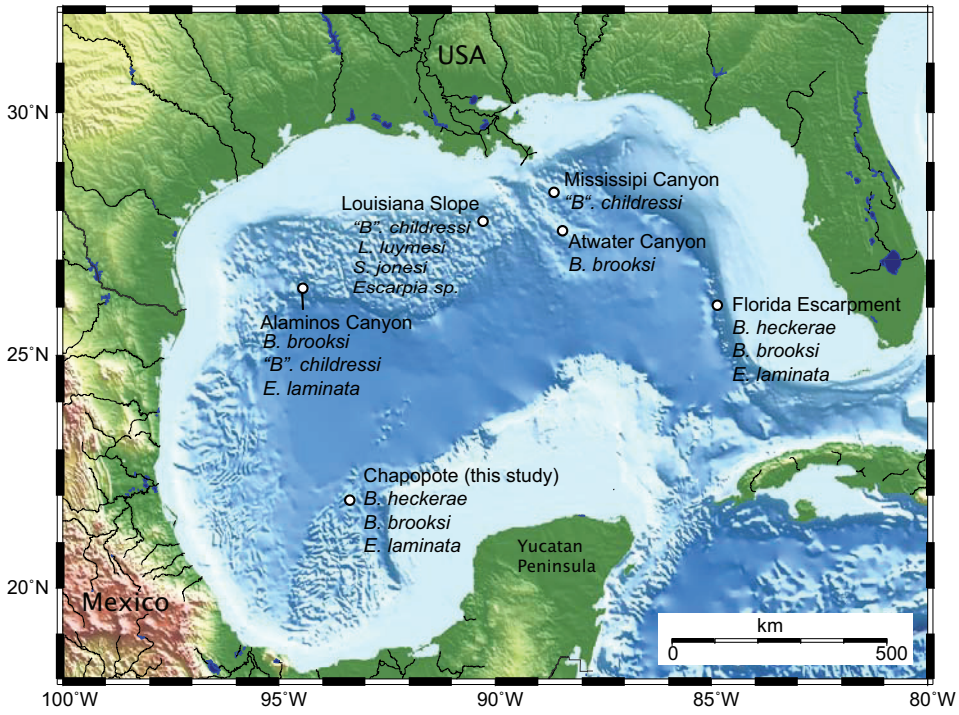


Fig. 1. Gulf of Mexico Basin. The most studied sites are shown with their respective *Bathymodiolus* mussels and tubeworm fauna.

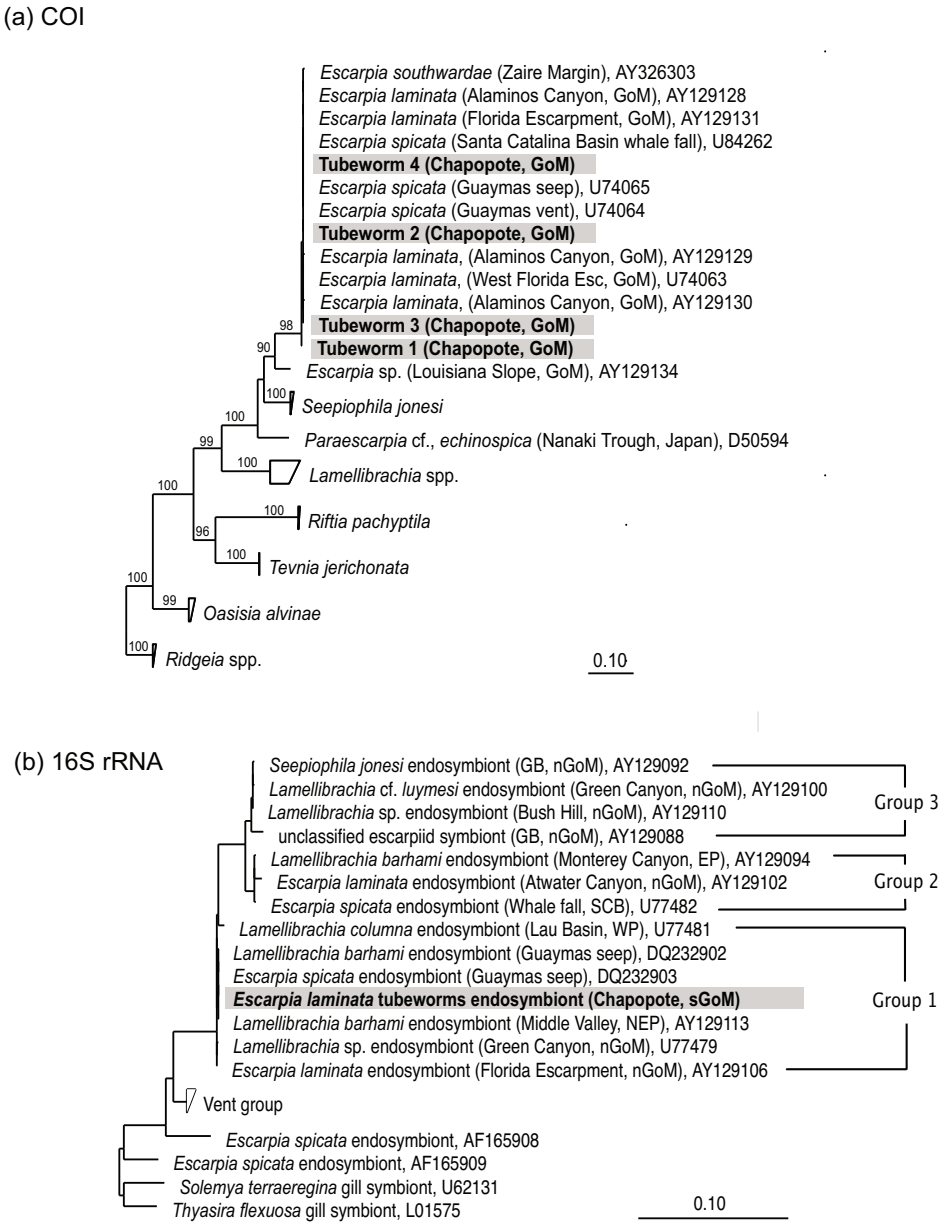


FIG 2. Phylogenetic affiliation of Escarpia tubeworms and their bacterial symbionts. (a) Tree based on COI gene sequences. Maximum-likelihood tree showing vestimentiferan tubeworm species from vent and seep environments including the 4 individuals of this study (sequences highlighted in gray). Only bootstrap values greater than 70 % are shown. (b) Tree based on 16S rRNA gene sequences. Maximum-likelihood tree shows within the gamma-proteobacteria phylum, thiotrophic symbionts of seep and vent vestimentiferans. Only one phylotype was present in the two investigated tubeworms (in bold), and fell in group 1 (McMullin at al 2003) within Escarpia spicata, Escarpia laminata and Lamellibrachia spp.

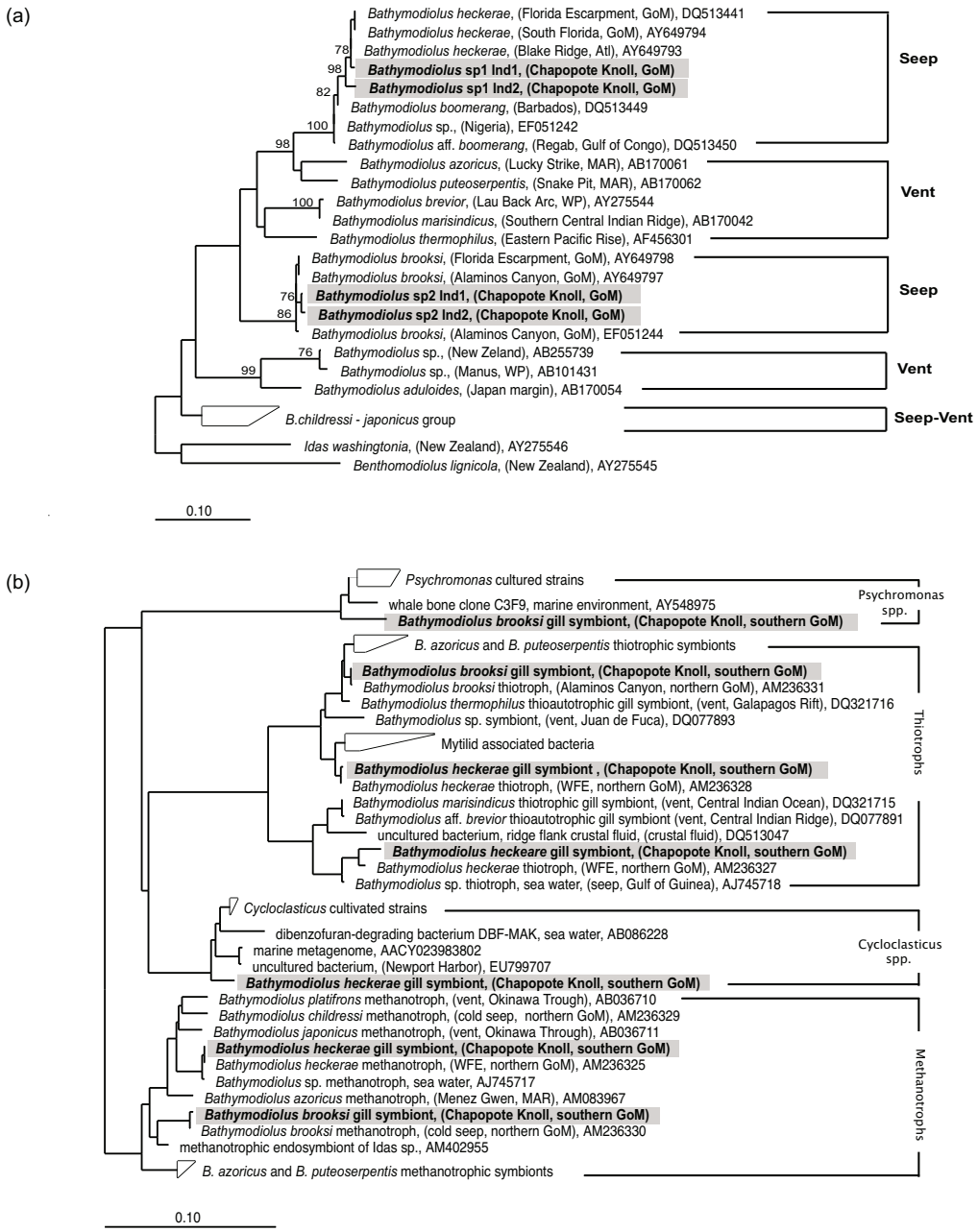


FIG 3. (a) Phylogenetic affiliation of *Bathymodiolus* mussels and their bacterial symbionts. (a) Tree based on COI gene sequences. Maximum likelihood tree showing *Bathymodiolus* spp. from vent and seep environments including the 4 individuals of this study (highlighted in gray). Only bootstrap values greater than 70 % are shown. (b) Phylogenetic reconstruction of bacterial symbionts of *Bathymodiolus* mussels based on 16S rRNA gene sequences. Maximum-likelihood tree shows within the Gammaproteobacteria phylum thiotrophic, *Cycloclasticus*-related, *Psychromonas*-related and methanotrophic bacteria. The phylotypes investigated in this study are shown in bold. Note that *B. heckerae* individuals have two different thiotrophic phylotypes, one *Cycloclasticus*-related and one methanotrophic phylotypes, and *B. brooksi* present only one thiotrophic, one *Psychromonas*-related, and one methanotrophic phylotypes.

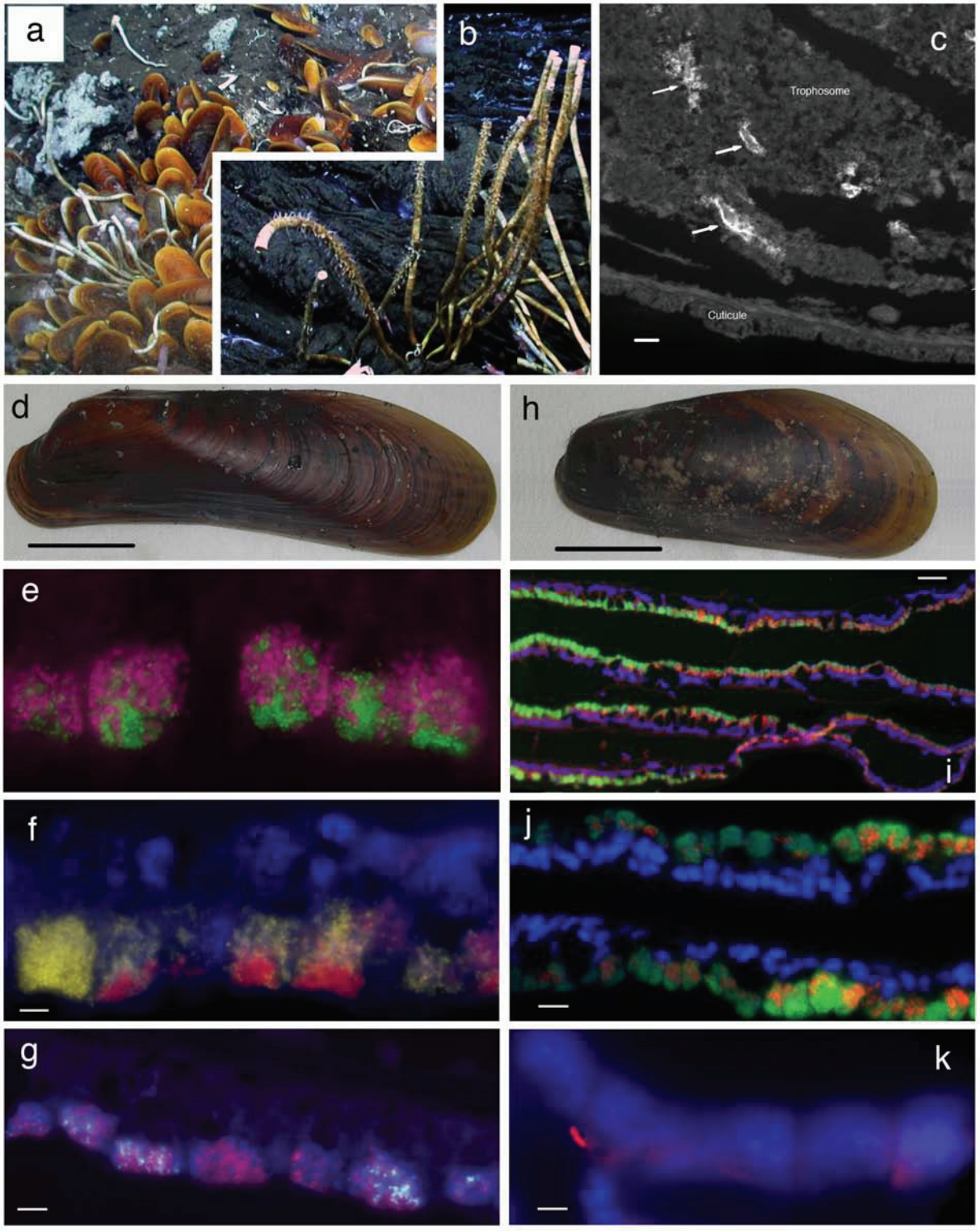


Fig. 4 Legend on the next page

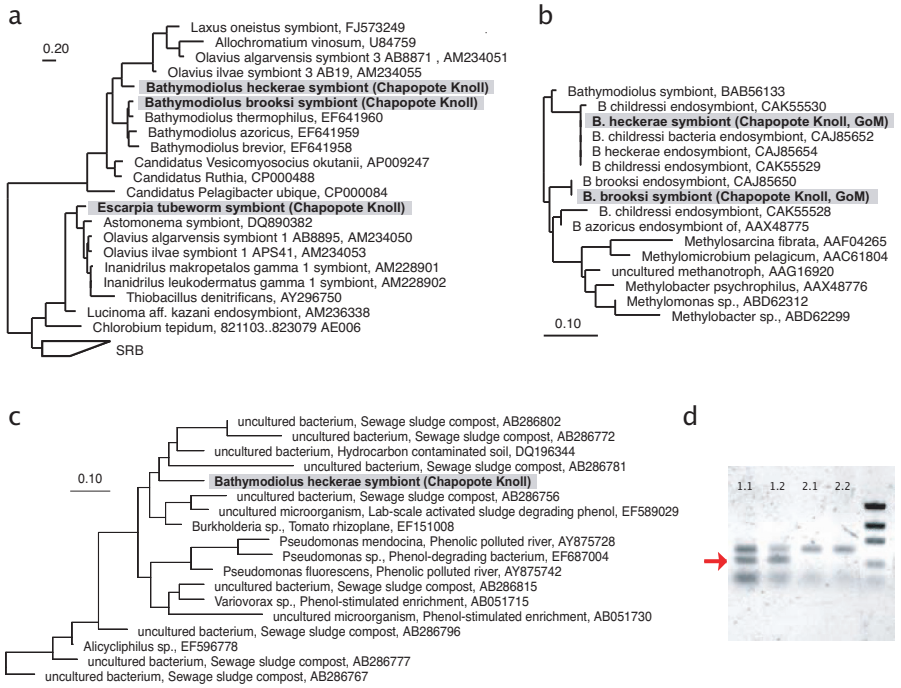
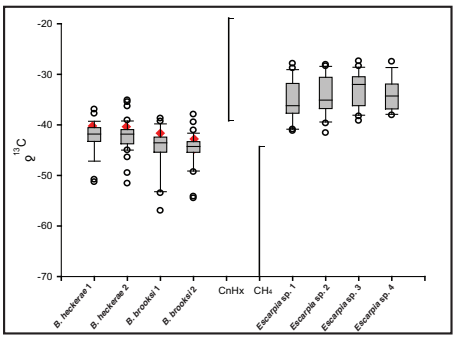
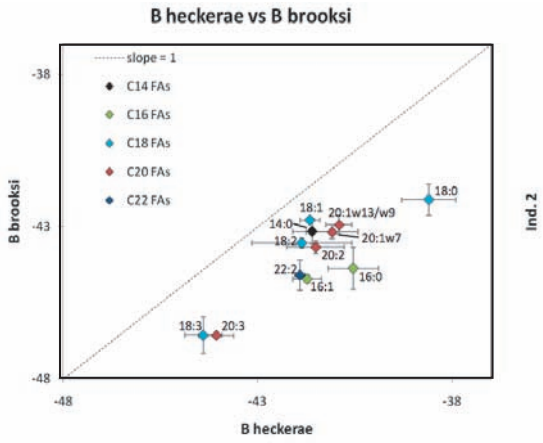


Fig. 5 Phylogenetic reconstruction of bacterial symbionts based on metabolic marker genes. The three sequences of this study are highlighted in gray. (a) Maximum-likelihood tree based on the alpha subunit of the APS reductase gene (*aprA*) sequences. (b) Maximum-likelihood tree based on the alpha subunit of the particulate MMO gene (*pmoA*) sequences. We found this gene present only in *Bathymodiolus* spp. The sequences of this study (in bold) grouped with former *Bathymodiolus* sequences. (c and d) Maximum-likelihood tree based on the phenol hydroxylase gene. We could amplify the gene in *B. heckerae* and not in *B.brooksi* (see d). The sequence of this study fall within sequences related to high content hydrocarbon environments.

Fig 4. Bathymodiolus mussels and Escarpia tubeworms from this study, and FISH images of bacteriocytes in the mussel gill filaments and in the tubeworm trophosome. (a) Bathymodiolus brooksi and B. heckerae mussels together with escarpid tubeworms settle in the asphaltic sediment at Chapopote cold seep in the southern Gulf of Mexico. Each metazoan species harbors its own specific bacterial phylotypes. (b) Escarpia tubeworms from this study bear chemoautotrophic tubeworm symbionts. (c) Localization of the symbionts (arrows) with a FISH specific probe through a tubeworm cross-section. (d-g) B. heckerae mussel and respective FISH images: B. heckerae shell has an elongated shape (d); Its filamentous gills house methanotrophic (pink) and thiotrophic (green) bacteria (e). Two different chemoautotrophic bacterial phylotypes have been recognized. The host nuclei are in blue, thiotrophs I in red, and thiotrophs II in yellow (f). A new hydrocarbon-degrader symbiont in green, is co-existing with the methanotrophic bacteria in blue and the thiotrophs in pink (g). (h-k) B. brooksi mussel and respective FISH images: the shape of the B. brooksi (h) shell is more round and smaller than B. heckerae.. When analyzed with FISH B. brooksi gill filaments (autofluorescence of the tissue is purple) house a methanotrophic bacterial phylotyope in red, and a thiotrophic one in green (i). A detail of (i) shows host nuclei in blue, methanotrophs in red and thiotrophs in green (j). A Psychromonas-related bacteria was found to be associated with B. brooksi gill tissue (k). Scale bars: (c, i) = 50µm; (d, h) = 5 cm; (e, f, g, k) = 5 µm; (j) = 10 µm.





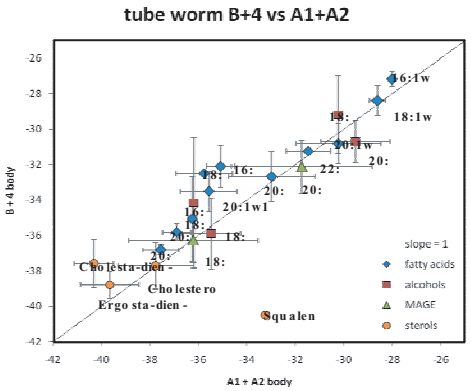
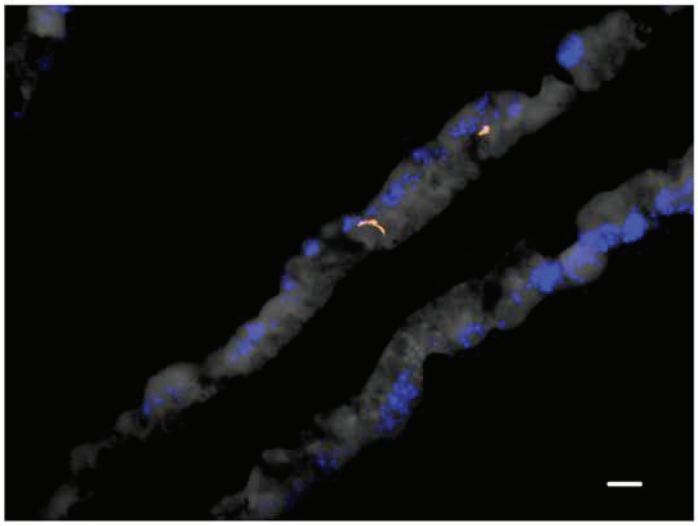


Fig. 6 Stable carbon isotope composition of lipids extracted from *B. heckerae, B. brooksi*, and *Escarpia* sp. tubeworms tissue. (a) Carbon isotope values of lipids (circles and bars) and bulk tissue (diamonds) from *Bathymodiolus* spp. and *Escarpia* sp. tissues. (b) *B. heckerae* carbon isotope values plotted against *B. brooksi* values. (c) Escarpia carbon isotope values tubeworms B and 4 plotted against A1 and A2.

Fig. 1S Cross-section through gill filament of B. brooksi. (a) Methanotrophic-related bacteria (blue) and Psychromonas-related bacteria (orange). In gray the auto fluorescence of the tissue. Psychromonas-related bacteria are not abundant and seem to be filamentous-like bacteria. (Thiotrophic-related bacteria are not shown but they are similarly present as in B. heckerae tissue). (b) Psychromonas-related bacteria in pink and DAPI in blue. Bar scales are 10 μm.



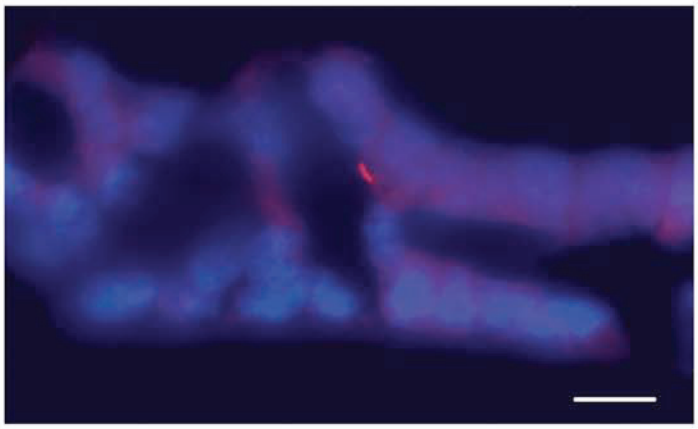
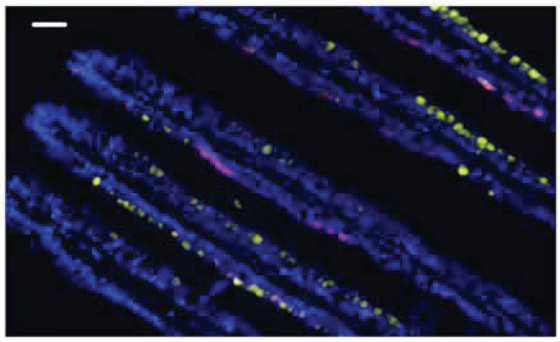
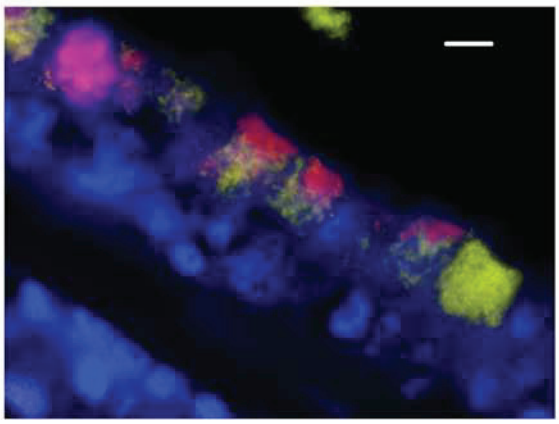


Fig. 2S Cross-section through gill filament of *B. heckerae*. (a) Thiotrophic-related bacteria T1 (in red), and T2 (in yellow). Scale bar in 40 μm. (b) A detail from a, scale bar 10 μm.





Gene	Primers	Primer sequence (5´-3´)	Anneling temperature	Ref.
16S rRNA	GM3 (8F)	AGAGTTTGATCATGGC	44	Muyzer et al., 1995
	GM4 (1492R)	TACCTTGTTACGACTT		Muyzer et al., 1998
COI	907RC	CCGTCAATTCCTTTGAGTTT	55	Jones et al., 2006
	LCO-1560	ATRCTDATTCGWATTGA		
	HCO-2148	CCYCTAGGRTCATAAAAAGA		
	LCO-1490	GGTCAACAAATCATAAAGATATTG	55	Folmer et al., 1994
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		
aprA	AprA-1-FW	TGGCAGATCATGATYMAYGG	54	Meyer and Kuever 2007
	AprA-5-RV	GCGCCAACYGGRCCRTA		
pmoA	A189F	GGNGACTGGGACTTCTGG	55	Holmes et al., 1995
	MB661R	CCGGMGCAACGTCYTTACC		
hydroxylase	RMO-F	TCTCVAGCATYCAGACVGACG	53	Baldwin et al., 2003
	RMO-R	TTKTCGATGATBACRTCCCA		
Species	**Probes**	**Probe sequence (5´-3´)**	**Formamide % (46°C)**	
B. heckerae TI and *B. brooksi* T	Bthio-193	CGAAGATCCTCCACTTTA	20	Duperron et al., 2007
B. heckerae TII	Bheck-193	AAGAGGGCTCCTTTT	20	Duperron et al., 2007
Bathymodiolus spp. methanotrophs	BangM-138	ACCAGGTTGTCCCCCACTAA	20	Duperron et al., 2005
Cycloclasticus spp.	Cypu-829	GGA AAC CCG CCC AAC AGT	20-50	Maruyama et al., 2003
Psychromonas spp.	Psychr-1453	GGTCATCGCCATCCCCGA	20	Eilers et al., 2000
Escarpia sp. thitrophs	Tbw-643	TCTACCACACTCTAGTCAGGCAG	20-60	This study
Escarpia sp. thitrophs	Tbw-139	TCCGAGTTGTCCCCCACTAC	20	This study

Table 1S. Primers and probes. All the oligonucleotides used in this study are listed and their anneling temperature or formamide concentration that were applied to them.

Species	Specimen	T1	T2	M	C	P	aprA	pmoA	hydrox
B. heckerae	1	119 (5)	56 (5)	5 (2)	3 (3)		6	42	3
	2	180 (3)	3 (1)				47	94	
B. brooksi	1	156 (3)		21 (2)		3 (3)	4	94	x
	2	111 (3)		63 (3)			4	89	x
Escarpia sp.	A1	140 (5)					2	x	-
	B1	69 (4)					2	x	-

Table 2S. Clone description of this study. In the case of the 16S rRNA gene the number of partial sequences are shown and complete ones are in parenthesis. No data means we did not obtained any sequence, dash that we did not try to amplify from that individual. The x means we run the PCR but no product was obtained.

	B.heck Ind.1			*B.heck Ind.2*			*B.brook Ind.1*			*B.brook Ind.2*		
Compound	mg lipid/g gill	MEAN	DEV	mg lipid/g gill	MEAN	DEV	mg lipid/g gill	MEAN	DEV	mg lipid/g gill	MEAN	DEV
?	0,12						0,23			0,05		
14:1	0,10			0,07	-44,2	0,6	0,17	-42,5		0,04	-43,6	
14:0	0,14	-39,3		0,06	-41,2	1,0	0,21	-43,8		0,04	-42,2	
15:0	0,14			0,03			0,16	-38,7		0,04	-38,0	
16:1ω8	0,00						10,69	-40,6	2,9	2,52	-49,3	4,4
16:1ω7	6,21	-41,2	1,0	2,18	-41,4	0,2	23,63	-45,0	0,1	5,07	-45,1	0,8
16:1ω5	0,69			0,08	-42,5	0,2	2,05			0,48	-45,0	0,7
16:2	0,32	-41,9	0,3	0,19	-41,6	0,7	0,08					
16:2	0,54									0,02		
16:0	1,97	-39,5	1,7	0,60	-41,6	0,4	4,87	-43,9	1,2	1,07	-44,3	0,6
17:1 ?	0,03						0,08			0,01		
17:0 ?	0,06						0,05			0,01		
17:0	0,16						0,27					
17:0	0,04									0,06	-39,5	0,1
18:3	2,58	-42,1	2,3	0,79	-44,7	0,8	1,09	-45,1	0,8	0,26	-46,3	0,2
18:3							0,29			0,09	-44,2	0,0
18:2	0,21	-45,2	0,8	0,07	-45,0	1,4	0,24	-42,2	0,9	0,08	-43,9	0,4
18:2	0,19	-40,0	0,5	0,09	-39,4	0,9	0,33	-42,4	1,0	0,11	-44,2	0,2
18:1ω11	0,14	-40,0	1,6	0,08	-41,3	0,8	0,36	-39,3	1,7	0,10	-43,8	0,6
18:1ω9	0,12	-41,8	3,1	0,11			1,08	-39,8	1,9	0,24	-41,1	0,2
18:1ω7	0,76	-40,5	1,0	0,32	-41,8	0,3	2,06	-41,9		0,44	-44,7	0,3
18:1ω5	0,14			0,02			0,35	-42,9		0,11	-54,5	0,1
18:0	0,23	-36,9	1,5	0,15	-39,1	0,7	0,85	-42,5	0,8	0,20	-42,7	0,1
?	0,06						0,06	-42,5				
?	0,24	-42,7	1,1				0,06					
20:3				0,10	-42,5	0,6						
20:3	1,32	-43,8	1,1	0,46	-43,8	0,3	0,05	-46,3	2,1	0,11	-46,7	0,6
20:2	0,51	-42,0	3,1	0,11	-44,0	1,3	0,78	-45,7	0,5	0,20	-43,1	2,0
20:2	1,75	-41,3	0,4	0,58	-42,0	0,5	1,70	-44,1	0,4	0,35	-44,4	0,4
20:2							0,32	-43,5	0,0	0,05	-45,4	0,3
20:1ω13	0,50	-41,1	0,5	0,16	-40,9	0,3	0,59	-43,2	0,3	0,13	-44,4	1,1
20:1ω9	0,36	-40,6	1,7	0,12	-44,1	2,6	0,80	-43,3	1,0	0,18	-42,7	0,2
20:1ω7	0,95	-40,7	0,9	0,36	-41,0	0,5	3,11	-43,2	0,6	0,63	-43,4	0,0
22:2				0,19	-42,0	0,7						
16:1 MAGE	0,18	-47,2	1,0	0,08	-46,4	1,1	0,11			0,03	-43,9	0,0
C16:1 MAGE				0,02	-43,9							
16:0 MAGE	0,11	-37,8		0,03	-49,5		0,12			0,03	-45,2	1,2
?	0,70	-41,8	0,7				1,00	-44,9	0,4	0,19	-44,5	0,4
C17 MAGE				0,00								
18:1 MAGE ?	0,04			0,01			0,01			0,00		
18:1 MAGE ?	0,05			0,01			0,05			0,01		
18:0 MAGE	0,05			0,02			0,14			0,05	-42,9	0,0
?	0,09	-43,8					0,14			0,02		
C20:2 MAGE	0,13	-42,0		0,02			0,00	-53,5	0,5	0,16	-48,9	1,4
C20:1 MAGE	0,14	-40,9		0,04	-41,7	0,6	0,51	-43,8	1,7	0,17	-43,9	1,1
Cholesterol	0,08	-42,9		0,01	-35,1	0,5	0,05			0,03		
4-Methyl-cholesta-N-en-3b-ol	0,13	-51,3		0,02			0,11	-57,0	0,6	0,12		
4-Methyl-cholesta-N,N-dien-3b-ol	3,76	-50,8	0,3	0,83	-51,6	0,5	3,79	-53,2	1,7	3,37	-54,2	1,5
4-Methyl-cholesta-N,N,N-trien-3b-ol	0,12			0,03			0,13			0,12		
?	0,06						0,06			0,16		
Lanosterol ?	0,06						0,16	-52,8	0,4	0,45	-46,4	0,5
Stigmasterol?	0,03						0,14	-48,8	1,2	0,40	-43,8	0,3
Diplopterol				0,01								
?				0,12	-42,4	0,1	0,03			0,10	-45,6	0,0
?				0,25	-43,0	0,3						
?	0,19	-41,1	0,1		-41,8	0,5	0,01					
?	0,06	-43,7	1,0	0,03	-43,7	0,7	0,004			0,02		
16:1 FAME				0,11	-41,3	0,4						
16:1 FAME				0,81	-45,0	0,0						
16:0 FAME				0,33	-40,9	0,8						
C18:3 FAME				0,16	-40,9	0,3						
C18:2 FAME				0,02								
C18:2 FAME				0,04								
C18:1 FAME				0,06	-35,5	0,2						
C18:1 FAME				0,13	-42,6	0,1						
C18:0 FAME				0,05	-40,3	0,0						
C20:3 FAME				0,08	-36,3	0,4						
C20:3 FAME				0,01								
C20:2 FAME				0,17	-43,3	0,3						
C20:2 FAME				0,23	-43,0	0,6						
C20:1 FAME				0,05	-39,9	0,5						
C20:1 FAME				0,16	-40,8	0,7						
C20:1 FAME				0,17	-41,7	0,7						

Table 4S. Isotopic composition and quantity (mg of lipid per g of gill tissue) of fatty acids from *Bathymodiolus* mussels

	Tube worm A1			Tube worm A2				Tube worm B				Tube worm 4			
	head		body	head		body		head		body1		head		body	
Compound	$\delta^{13}C$ (‰)	mg/g	$\delta^{13}C$ (‰)	mg/g	$\delta^{13}C$ (‰)	mg/g	$\delta^{13}C$ (‰)	mg/g	$\delta^{13}C$ (‰)	mg/g	$\delta^{13}C$ (‰)	mg/g	$\delta^{13}C$ (‰)	mg/g	$\delta^{13}C$ (‰)
14:0 FAs							-38,0								
16:1ω FAs	-28,8	4,93	-27,9	2,20	-29,1	2,19	-28,1	0,48	-28,2		-27,4	1,39	-26,83		-27,5
16:1ω FAs		0,13		0,10		0,08		0,06			-31,6	0,10			
16:0 FAs	-36,5	3,39	-34,7	2,03	-35,7	1,74	-35,5	0,42	-34,0		-32,0	1,52	-31,85		-33,3
18:2 FAs	-37,0	3,21	-36,1	1,60	-35,8	1,33	-36,4	0,28	-35,2		-33,2	1,30	-31,88		-36,0
18:2 FAs	-37,0	1,03		0,51		0,65		0,04				0,24			
18:1ω FAs	-28,8	14,55	-28,8	6,00	-28,7	5,82	-28,4	1,17	-28,8		-28,4	4,83	-27,11		-28,8
18:1ω FAs		0,35		0,32		0,21		0,05				0,13			
18:0 FAs	-33,3	1,31	-36,6	0,76	-34,6	1,05	-34,9	0,08	-35,1		-30,7	0,25	-34,23		
20:4 FAs	-40,0	2,98	-37,3	1,72	-35,8	1,82	-36,5	0,43	-36,1		-34,9	2,36	-36,46		-36,8
20:5 FAs	-38,1	4,77	-38,1	2,86	-36,2	2,20	-37,0	0,87	-36,6		-36,1	2,55	-37,33		-37,4
20:2 FAs	-33,5	2,14	-34,2	1,03	-32,9	1,16	-31,7	0,26	-33,0		-31,8	0,73	-31,34		-34,1
20:1ω FAs	-39,1	2,71	-36,4	1,51	-34,4	1,86	-34,7	0,19	-34,8		-33,2	0,71	-32,31		-34,5
20:1ω FAs	-32,1	2,75	-31,5	1,48	-30,1	2,62	-29,0	0,11	-30,7		-30,5	0,32	-30,77		-32,1
22:2 FAs		1,33	-32,1	0,82	-30,6	0,95	-30,8	0,26	-33,6		-31,6	0,71			
16:1 alcohol										0,17	-29,1				
16:0 alcohol		0,19	-36,2	0,03		0,04	-36,2	0,01	-32,6	0,46	-34,7	0,04	-27,70	0,16	-33,1
18:1 alcohol		0,43	-30,2	0,04		0,11	-30,3	0,02	-29,3	0,91	-29,1	0,06	-33,01	0,32	-29,0
18:0 alcohol	-42,0	0,45	-36,3	0,16	-36,4	0,19	-34,6	0,02	-35,2	0,18	-36,6	0,09	-32,26	0,28	-34,9
20:1 alcohol		0,51	-30,5	0,29	-30,8	0,21	-28,5	0,03	-32,6	0,14	-30,4	0,14	-29,42	0,21	-31,7
20:0 alcohol								0,01				0,10	-28,45		
18:1 MAGE								0,01	-36,8						
18:0 MAGE		0,18	-37,1	0,15	-35,6	0,14	-35,3	0,02	-36,1	0,09	-36,5	0,11	-31,80	0,07	-37,0
20:2 MAGE		0,05	-41,2			0,08	-41,6	0,01	-32,7						
20:1 MAGE	-24,7	0,22	-32,8	0,20	-32,3	0,16	-30,7	0,03	-32,4	0,08	-32,0	0,09	-37,24	0,09	-32,0
Cholesterol	-41,8	5,05	-38,9	5,29	-36,6	2,75	-36,7	0,87	-38,7	0,51	-37,6	3,72	-35,48	0,81	-37,9
Cholesta-dien-ol	-43,2	4,16	-40,9	3,81	-40,2	2,94	-39,7	0,53	-38,9	0,37	-38,3	1,93	-38,43	0,48	-36,1
Ergosta-dien-ol	-42,6	2,24	-40,7	2,03	-38,7	1,52	-38,8	0,48	-40,2	0,33	-39,2	2,25		0,47	-38,1

Table 3S. Isotopic composition and quantity (mg of lipid per g of tissue) of fatty acids from *Escarpia* tube worms

Manuscript II:
An intranuclear bacterial parasite in shallow water bivalves

Luciana Raggi, Dennis Fink and Nicole Dubilier

In Preparation

An intranuclear bacterial parasite in shallow water bivalves

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Keywords: Intranuclear bacteria, NIX, symbiosis, parasitic association, invertebrates

Running head: Intranuclear bacteria of bivalves.

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Intended as a Brief report in *Environmental Microbiology*

Abstract

Marine intranuclear bacteria have a potentially lethal effect on bivalve populations, thus we set out to look for the presence of intranuclear bacteria in economically important and commercially available bivalve species. These included oysters (*Crassostrea gigas*), razor clams (*Siliqua patula* and *Ensis directus*), blue mussels (*Mytilus edulis*), manila clams (*Venerupis philippinarum*), and common cockles (C*erastoderma edule*). 16S rRNA analysis and fluorescence *in situ* hybridization (FISH) revealed the presence of intranuclear bacteria in all investigated bivalves except oysters and blue mussels. A FISH probe targeting all currently known intranuclear *Gammaproteobacteria* was designed for future high-throughput analyses of marine invertebrates. Furthermore, primers were designed to quantify the abundance of intranuclear-related bacteria with real time PCR. Preliminary tests showed massive amounts of intranuclear bacteria in some bivalve species, raising the question as to whether these significantly affect not only the health of the bivalves, but possibly also of the humans that eat them.

Introduction

Bacteria that invade eukaryotic nuclei are commonly found in protists, but have rarely been observed in multicellular eukaryotes (Grandi et al. 1997; Arneodo et al. 2008). Recently, we described intranuclear bacteria in deep-sea hydrothermal vent and cold seep mussels of the genus *Bathymodiolus* (Zielinski et al. 2009). This bacterial parasite appears to infect a nucleus as a single cell, replicate in massive numbers and eventually cause the nucleus to burst, liberating thousands of bacteria with the possibility to infect contiguous nuclei. Phylogenetic analyses showed that these bacteria belong to a monophyletic clade of *Gammaproteobacteria* associated with marine animals as diverse as sponges, corals, bivalves, gastropods, echinoderms, ascidians, and fish. However, except for bathymodiolin mussels and a shallow water bivalve, the Pacific razor clam *Siliqua patula* (Kerk et al. 1992), none of these metazoa-associated bacteria have been shown to occur inside nuclei. When this intranuclear bacterium was first described, it was named "Nuclear Inclusion X" (NIX) (Elston, 1986), from here on we referrer to the monophyletic group described in Zielinski et al. (2009) as the NIX clade. Intranuclear non-Rickettsia-like bacteria have been observed by TEM in marine metazoans, such as sponges (Vacelet 1970, Friedrich et al. 1999*), Ruditapes (Tapes, Venerupis) decussatus* clams (Azevedo 1989*), Siliqua patula* razor clam (Elston 1986, Kerk et al. 1992) and

Bathymodiolus spp. mussels (Zielinski et al. 2009). With the exception of Kerk et al. and Zielinski et al., the observations were not associated with molecular data. It has been suggested that these intranuclear bacteria may cause massive mortality in bivalves, with potential impact on hatchery economics (Elston 1986, Ayres et al. 2004). Within this bacterial group there are cultivated bacteria, the *Endozoicomonas* sp. (Kurahashi and Yokota 2007) and surfactant-resistant bacteria (Plante et al. 2008), all of which were isolated from marine invertebrates. In the current study, we investigated the presence of these intranuclear bacteria in coastal consumable bivalves with both culture-dependent and -independent methods. In contrast with deep-sea bivalves, shallow-water bivalves have a diverse and abundant microflora. Molecular studies of this diversity are limited (e.g. Cavallo et al 2009), in part due to the fact that research on bacterial-bivalve interactions is largely focused on known human pathogenic bacteria (e.g *Vibrio* spp.). These studies have never reported the presence of bacteria related to the intranuclear group. We report here that NIX bacteria belonging to the monophyletic group are commonly present in bivalves and have a widespread distribution.

Material and Methods

Sampling site and processing

Bivalves were collected in April 2008 from intertidal zones in Sylt, Germany. *Ensis sp*. were collected in Königs Bay (Königshafen) and *Mytilus edulis, Cerastoderma sp*. and *Crassostrea gigas* at Oddewatt. Collection of *Ensis* sp. clams is traditionally known to be difficult due to their burying behavior. However, upon arrival to the beach, a small community of around 200 clams was observed on the surface of the intertidal sediment. *Siliqua patula* clams were collected from Kalaloch Beach on the Pacific coast of Washington State, USA. *Crassostrea gigas* (France and Mexico), and *Tapes semidecussatus* (Italy) were obtained in local markets in Bremen and Mexico City. The organisms were kept alive in seawater or on ice until further processing. The gills were dissected and segments were place in 96% ethanol and stored at -10°C for DNA analysis. Other fragments were fixed for FISH analysis with 1% PFA and stored at 4°C in 50% ethanol/PBS, and a third fragment was stored in 6% glycerol for cultivation attempts.

Cultivation

Three different media were used in solid (with 1.5 % Agar) and liquid presentations: Marine Medium 2216 (Difco), Minimum Medium with and without CTAB (0.1% yeast, 0.01% peptone, 1.5% agar, 100µm CTAB, dissolved in sea water), and WL Nutrient

Medium (Difco) in 3.5% NaCl. No NIX were recovered, but many other bacteria were (results of the cultivated bacteria will be presented in a separate report).

DNA extraction and PCR amplification

DNA was extracted from frozen or ethanol fixed tissue following the Zhou et al. (1996) protocol with small modifications. Briefly, 2 ml of extraction buffer and 20 µl proteinase K (20 mg/ml) were added to approximately 100 mg of tissue, and incubated 1.5 hours. 200 µL of 20% SDS were added and incubated 2 hr at 56°C. The liquid phase was recovered after centrifugation at 14000 g for 20 min and cleaned once with 1 V phenol/chloroform/isoamilalcohol (25:24:1) and a second time with chloroform/isoamylalcohol (24:1), precipitated with 0.6 V isopropanol and dissolved in TE buffer. DNA was extracted from a variety of animals, but we report here only those hosts for which a positive 16S rRNA sequence analysis confirmed the presence of NIX. The extracted DNA was used for both host and bacterial symbiont analysis. Hosts COI genes were amplified using 36 PCR cycles using the LCO1490 (5'-GGTCAACAAATCATAAAGATATTG-3´) and HCO2198 (5´-TAAACTTCAGGGTGACCAAAAAATCA-3´) primers (Folmer *et al*., 1994). Bacterial 16S rRNA genes were amplified using 20 PCR cycles with universal primers GM3 (8F) and GM4 (1492R) (Muyzer *et al*., 1995).

Quantification of bacteria: real time PCR

Primers for the amplification of a 100 bp fragment of the 16S rRNA were designed using the probe design tool of ARB (Ludwig et al., 2004). The primers matched all sequences in the NIX-clade, with no match to other sequences from bivalve-associated bacteria: Nix-721F (5'-AGTGGCGAAGGCGACACT-3') and Nix-805R (5'-GACATCGTTTACGGCGTGG-3'). Oligonucleotides were checked for their potential to form secondary structures using the analysis tool NetPrimer (http://www.premierbiosoft.com). For PCR conditions, the Eurogentech (Germany) protocol was followed. The annealing temperature was 60ºC and 50 cycles were used.

Phylogenetic analysis

COI products of the host invertebrates were directly sequenced (both strands). Bacterial 16S rRNA of each organism was cloned using the TOPO-TA system (Invitrogen) and clones were partially sequenced with primer 907RC (Muyzer et al., 1998). Clone sequences belonging to the NIX-clade from each host individual were chosen for full

sequencing in both directions. Sequence data were analyzed with Sequencher (Genes Codes Corporation), 16S rRNA sequences were aligned with the ARB/SILVA aligner (Pruesse et al. 2007), and phylogenetic trees were calculated with the ARB software (Ludwig et al., 2004). All sequence comparisons are given as percentage sequence identity (% similar nucleotides) after calculations of a Neighbor-Joining distance matrix. For tree reconstruction, only long sequences (~1400 bp) were used. Maximum likelihood phylogenetic tree was calculated using RAxML (GTRGAMMA distribution model, 100 bootstrap replicates).

FISH and CARD-FISH

A gill piece from each specimen was dehydrated in an ethanol series and embedded in low-melting polyester wax (Steedman, 1957). Wax cubes were sectioned with a Leica microtome (5-6 μm) and mounted on Plus Frosted Slides (Menzel-Gläser). Polyester wax was removed in three rinses in absolute ethanol (5 min each), and sections were rehydrated in a short ethanol series (3 concentrations). For better penetration of probes, sections were subsequently incubated in Tris-HCl (20 mM, pH 8), proteinase K (0.05 mg ml-1 in Tris-EDTA, pH 8, at 37°C), and washed in MilliQ water (5 min each). For *in situ* hybridizations with mono- or horseradish peroxidase (HRP)-labeled probes (CARD-FISH) and subsequent staining with DAPI, sections were processed as described previously (Zielinski et al 2009, Duperron et al 2007). The probes used in this study were designed based on the primer sequences. EUB338 probe was used as a positive control. Probe NON338 was used as a negative control for background autofluorescence. All hybridizations were performed using 20% formamide.

Results and Discussion

16S rRNA sequence analysis

In our 16S rRNA sequence analysis (Fig. 1), we obtained roughly 30 sequences from each reported host (Table 1), with the exception of *M. edulis, C. edule* and *T.* semidecussatus, with 4, 61 and 51 clone sequences, respectively. We found sequences belonging to the monophyletic group described in Zielinski et al. (2009), which we call the NIX-clade, in five of the bivalve species analyzed: *Ensis directus*, *Cerastoderma eduli*, *Crassostrea gigas*, *Siliqua patula*, *Tapes semidecussatus*. The amount and quality of DNA was limited, due to inefficient DNA extractions for some organisms. Two different NIX phylotypes were found in some of the bivalves, however the phylotypes of each host were nearly always different. Among the phylotypes found in this study (Figure 1), there are three cultivated strains isolated from a sponge (Nishijima et al. *in prep*), a sea slug (Kurahashi and Yokota 2007) and an echinoid (Becker et al 2007). These cultivated bacteria were not yet checked for *in situ* localization (personal communication from researchers), which, should they be confirmed as intranuclear, could pave the way towards physiological studies of intranuclear bacteria. All sequences in the NIX-clade except two (from marine water) belong to animal-associated bacteria (Figure 1). This suggests these bacteria are specialized at living within animal tissue and may get from them nutrients, protection and surface for attachment, parameters potentially essential for their life cycle. This study confirms previous findings that the NIX clade is a monophyletic group (Zielinski, et al. 2009).

In situ *localization and abundance*

We could localize intranuclear and not intranuclear bacteria belonging to the NIX-clade with specific probes (Table 2) in *S. patula*, *E. directus*, *C. edule*, and *T. semidecussatus*, which corresponded to only one of the phylotypes of each host (Fig. 2). We were not able to localize the second phylotype that occurred in *C. edule* and *E. directus*, as the specific probes (Cnix-643, Cnix-64, Cnix-1249 for *C. edule*, and Cnix-1249 for *E. directus* phylotype) did not show an intranuclear signal, and any positive signal other than nuclear could not be discern from background fluorescence. In the two different oysters, no positive signals with any bacterial probe (i.e. Eub-338, Gamma, and Tnix-64) were observed. In fact, the oyster tissue looked very clean (no bacteria or particles), which is likely due to the normal and extensive depuration process that oysters are put through for commercial use. In contrast, *C. edule* had a massive amount of particles and high

background, making it difficult to distinguish a positive signal outside nuclei. The second phylotype may be present, but could not be observed under these conditions. *S. patula* and *T. semidecussatus* frequently had massive infections, as did *C. edule*, though to a lesser degree (see Fig. 2-4). *T. semidecussatus* was unique in that NIX bacteria were found normally surrounding the nucleus and rarely inside (Fig. 2). These FISH observations suggest that NIX-bacteria are not exclusively intranuclear. However, as observed in *T. semidecussatus*, they may need to live associated with nuclei, potentially providing a mechanism for nucleus entry, easier than first thought.

The natural aggregation of NIX bacteria makes their quantification by FISH impossible. As such, we sought to develop a qPCR (real time PCR) based assay for high-throughput NIX-specific quantification, as described in the methods section. Preliminary results of qPCR are consistent with FISH observations (Table 1). *S. patula* had the highest quantity of NIXclade bacteria, as observed in both qPCR and *in situ* analyses (Fig 2X). According to the NIX life cycle proposed by Zielinski et al. (2009), the bacterial infections observed here are in the last developmental stage (Fig. 4). With the oligonucleotide probes designed in this study, it would now be possible to corroborate the presence of intranuclear-related bacteria in any of the organisms for which there is a published sequence, such as in the fish *Pomacanthus sextriatus*, the sponges *Petrosia ficiformis*, *Chondrilla nucula*, *Muricea elongata,* and the corals *Halichondria okadia, Erythropodium caribaeorum.*

Mass mortalities

What we observed in Sylt, a group of ~200 clams lying on the intertidal sediment is a low-scale mass mortalty. Some clams were dead and some did not perform their usual burying when feeling contact. The cause of the dying *Ensis* clams in Sylt coast is unclear. However, it is highly possible that the mortality was due to the presence of NIX-realted bacterial parasite. *Siliqua patula* razor clams have been observed to experience mass mortality related to a nuclear bacterial parasite (Elston 1986, Ayres et al. 2004) and it would not be surprising that a species as closely related as *E. directus* can become diseased by NIX bacteria as well. Typically, bacteria and parasites are persistent in certain species and it is a delicate equilibrium, likely dependent on environmental factors that keeps these organisms under control. An imbalance may cause certain parasites to bloom and opportunistically damage their host. As such, a NIX disease ecology or epidemiological study, requires a thorough assessment of environmental parameters, which, with the use of the qPCR primers designed and tested here, could be correlated with the emergence of the mass mortalities due to intracellular bacterial infections.

Conclusions

It is clear that the distribution of these NIX-related bacteria is widespread, not only biogeographically, as was suggested by Zielinski et al (2009), but also among phylogenetically diverse host invertebrates. The persistent occurrence of these bacteria suggests that they have both a parasitic stage *and* a non-parasitic host stage, some hosts appear to support high numbers of bacteria without presenting any physical disease. Is the symbiotic state of this bacteria-bivalve interaction in a host-commensal relationship rather than host-parasitic? Perhaps the observed association is a transition between these symbiotic concepts. The intranuclear presence of bacteria is an evolutionary process not well understood, requiring further ecological and physiological studies. Furthermore, the issue is of great importance due to the economical repercussions that mass mortality could have for aquaculture, as well as the consequences the infections of these bivalves have on human health.

Acknowledgements

We are grateful to R. Elston for providing the *Siliqua patula* samples and helpful discussions. To M. Duhaime for her constructive revision. This work was supported by the Max Planck Society, Germany. L.R. was supported by a CONACyT-DAAD scholarship.

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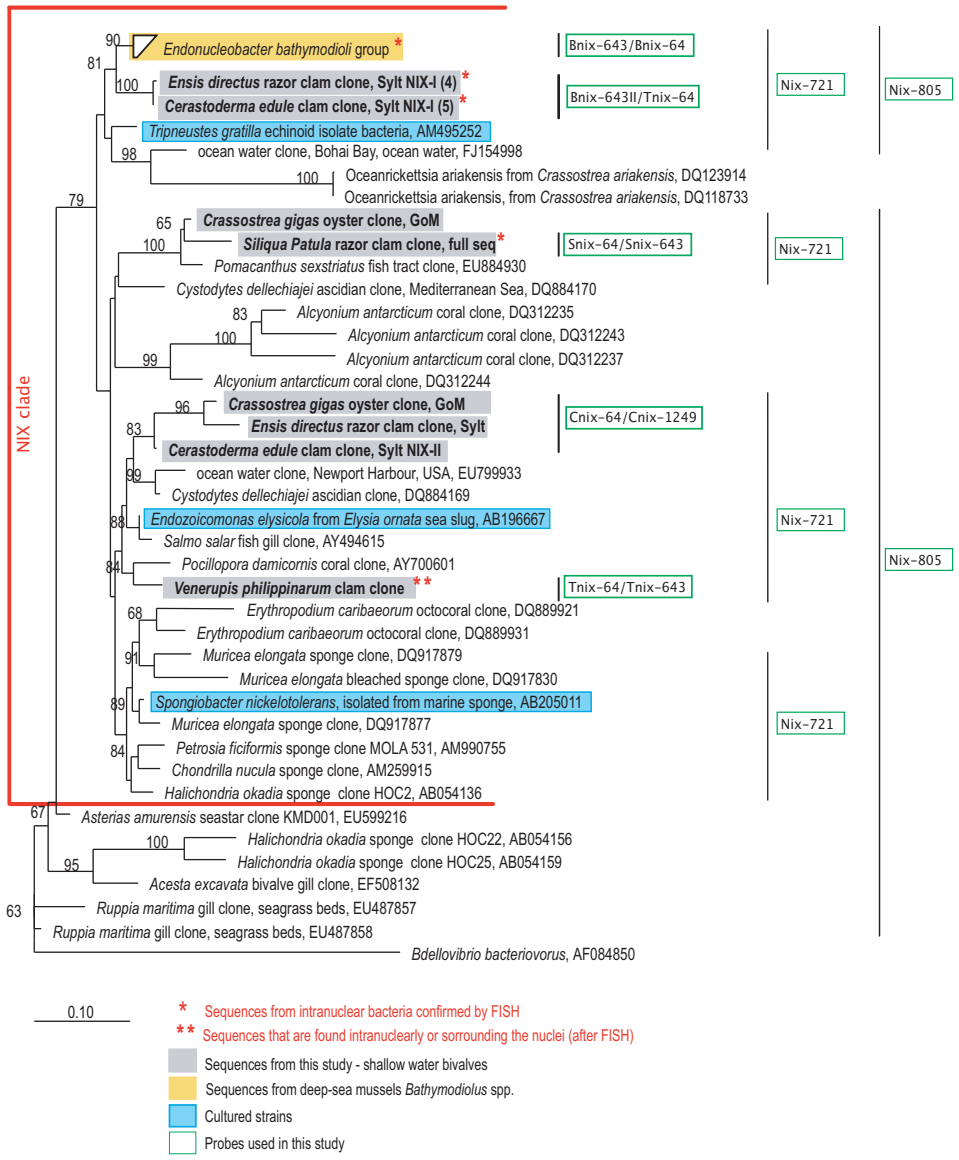
Tables and Figures

Figure 1.
16S rRNA phylogenetic tree based on maximum likelihood (RAxML) analysis. NIX-clade belongs to the Gammaproteobacteria. Sequences from this study (highlighted in grey) and all closely related sequences found in the literature, including the three cultivated strains (in blue) and the *Candidatus* Endonuclear bathymodioli (in yellow) are shown. Probes (in green square) designed to target each specific host were designed for use in FISH analysis and Nix-721 and Nix-805 for real time PCR. Sequences with a star (*) indicate nuclear phylotypes confirmed by FISH. The *T. semidecussatus* sequence (with two stars **) was found typically surrounding the nuclei and very rarely inside.

Figure 2-4
Fluorescence in situ hybridization (FISH) images of NIX bacteria in the different bivalves. We used two probes for detecting the precense of NIX bacteria. Only images with one probe are shown because they gave similar results. 2) Specific probes (Tnix-1249 and Tnix-64) for NIX in *T. semidecussatus*. Nuclei stained with DAPI (blue). FISH signals (with Tnix-1249) are in green. Scale bars: 10 µm. 3) FISH with specific probes (Tnix-64 and Bnix-643-II) for NIX in *C. edule*. DNA is stained with DAPI (host nuclei in blue). FISH signals (with Tnix-64) are in red. Bar scales in a and b: 20 µm, for c: 10 µm. 4) Specific FISH for razor clam sequences (signals in red and host nuclei in blue). (a-c) specific probes (Tnix-64 and Bnix-643_II) for *E. directu,* signals with Tnix-64 are shown. (d-e) and specific probes (Snix-64, Snix-643) for *S. patula*. FISH with Snix-64 is shown in images. Scale bars in a: 30 µm, in c: 10 µm, in d: 20 µm.

Table 1.
16S rRNA analysis. Number of clone sequences obtained from each host. NixI or NixII are different phylotypes in each host. Two different phylotypes could be found in *E.directus*, *C.edule* and *C. gigas*. The last line is the number of copies obtained in the qPCR assay.

Table 2
Oligonucleotide probes used in this study. Different probes matching each bivalve (green) and sometimes having a mistmatch (x). Matching squares are labelled with ‘nucl’, ‘not nucl’, or ‘both’ after FISH observation showed nuclear or not nuclear. In the case of *Ensis* sp. bacteria were localized both inside and outside nuclei with the same probe.



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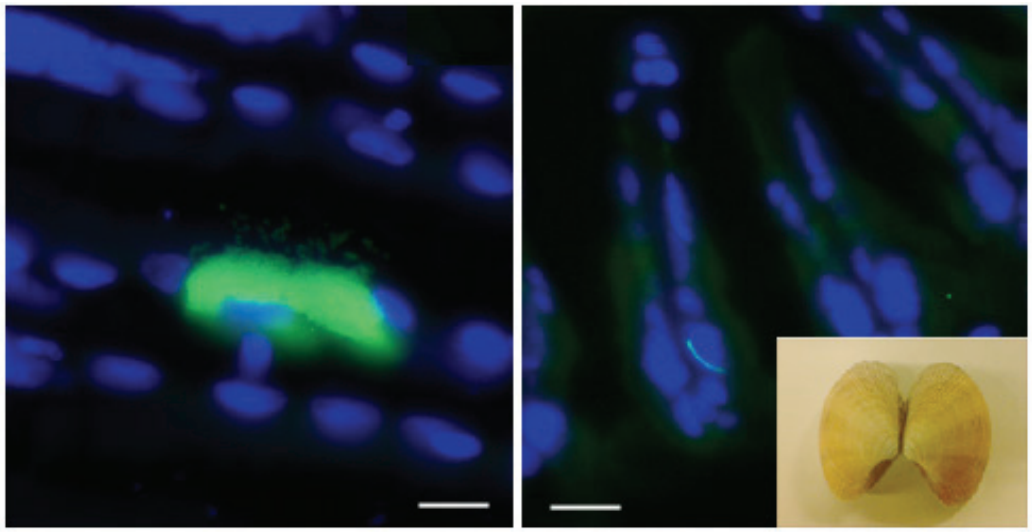

Fig. 2 FISH with specific probes (Tnix-1249 and Tnix-64) in *T. semidecussatus* gill tissue. In both images nuclei are stained with DAPI (blue). FISH signals (with Tnix-1249) are in green. Scale bar: 10 μm.

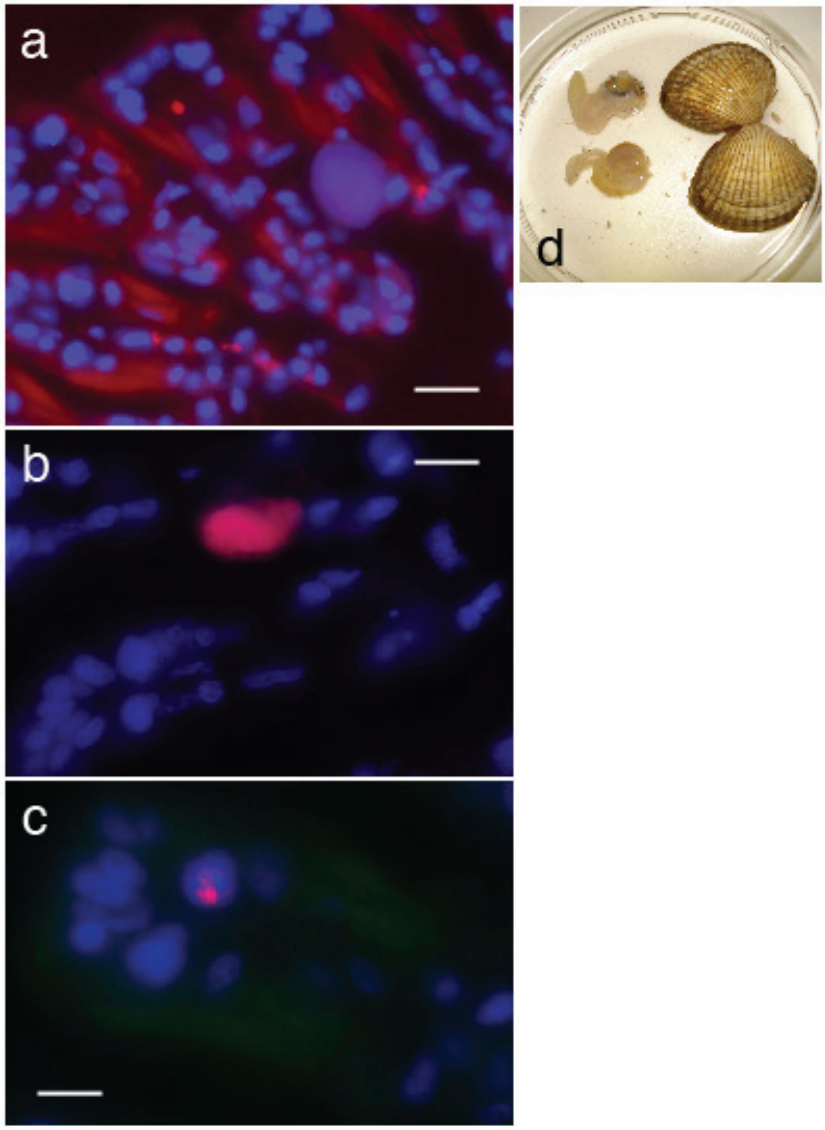

Fig. 3 FISH with specific probes (Tnix-64 and Bnix-643-II) in *C. edule* gill tissue. In a-c DNA is stained with DAPI (in blue), and FISH signals (with Tnix-64) are in red. Bar scales for a and b: 20 μm, for c: 10 μm. (a) image is without probe and a huge bacterial infection can be observed labeled with DAPI. Background fluorescence of host tissue is observed in red.

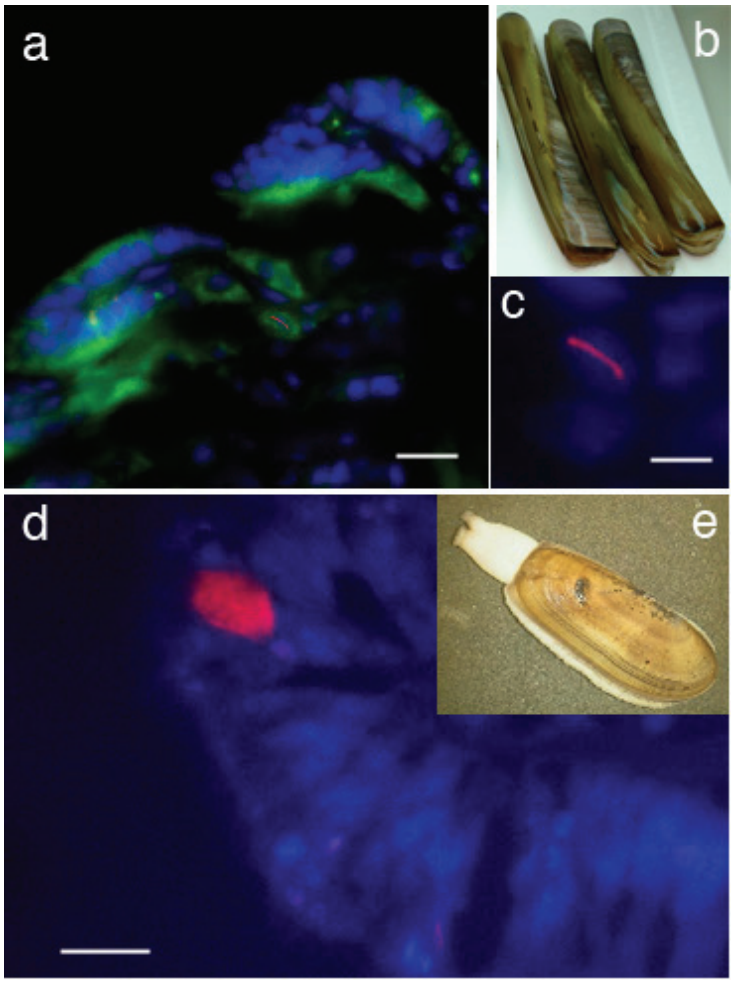

Fig. 4 Specific FISH for NIX-related sequences in razor clams (i.e. *E.directus* and *S. patula* gill tissue. Signals are in red and host nuclei labeled with DAPI in blue. (a-c) specific probes (Tnix-64 and Bnix-643-II) for *E. directus*, signals with Tnix-64 are shown. Background from tissue autofluorescence is seen in green. (d-e) specific probes (Snix-64, Snix-643) for *S. patula*. FISH with Snix-64 is shown in images (red). Autofluorescence of tissue is blue. Scale bars in a: 30 μm, in c: 10 μm, in d: 20 μm.

Host species	Nix	NixII	qPCR (copies/µl)
C. edule	49 (4)	10 (5)	8.31E+03
S. patula	20 (4)	0	5.65E+05
M. edulis	0	0	-
C. gigas (S)	4	1	1.03E+03
E. directus	8 (4)	1	2.42E+03
T. semidecussatus	29 (4)	0	1.56E+04
C. gigas (GoM)	1	0	-

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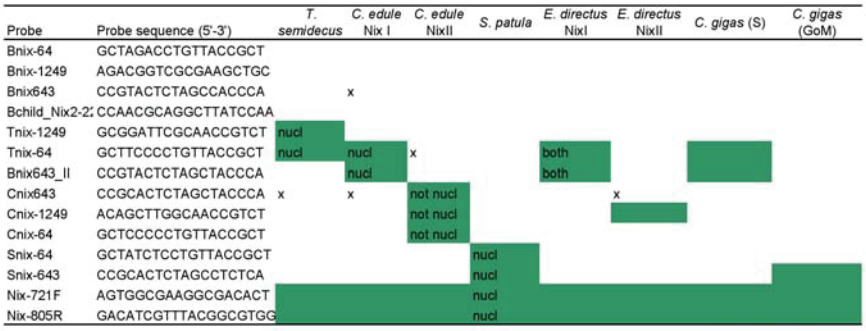

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Manuscript III:
Minireview: Bacterial diversity of shallow-water bivalves

Luciana Raggi-Hoyos, Nicole Dubilier

In Preparation

Minireview: Bacterial diversity in shallow-water bivalves

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Keywords: bacteria-bivalve interaction, symbiosis, invertebrates

Running head: Bacterial diversity in bivalves

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Abstract

Bacterial diversity in bivalves is a multidisciplinary topic, from biological and basic research to economical interests, including aquaculture and medicine. In this paper we review how these different disciplines have influenced the few bivalve microbiota studies available. We present new data from 16S rRNA analysis, including sequences from clone libraries and isolates, and fluorescence *in situ* hybridization (FISH) from shallow-water bivalves: *Ensis directus*, *Cerastoderma edule*, *Crassostrea gigas*, *Siliqua patula*, *Tapes semidecussatus, Mytilus edulis,* and *Mya arenaria,* localizing specific groups of bacteria associated with them. We also performed cultivations from *C. edule*, and *T. semidecussatus.* The isolated bacteria belong to bacteria phyla that are frequently found in organic rich environments: oil spills, bone-falls, feces or invertebrate tissue. Most of them belong to the Gammaproteobacteria group, and others to the Alphaproteobacteria, Bacteroidetes, Actinobacteria and Spirochetes. These associations might be occurring not only because of the surface that bivalves provide to bacteria, but also because the association could provide nutritional or protection benefits for both partners.

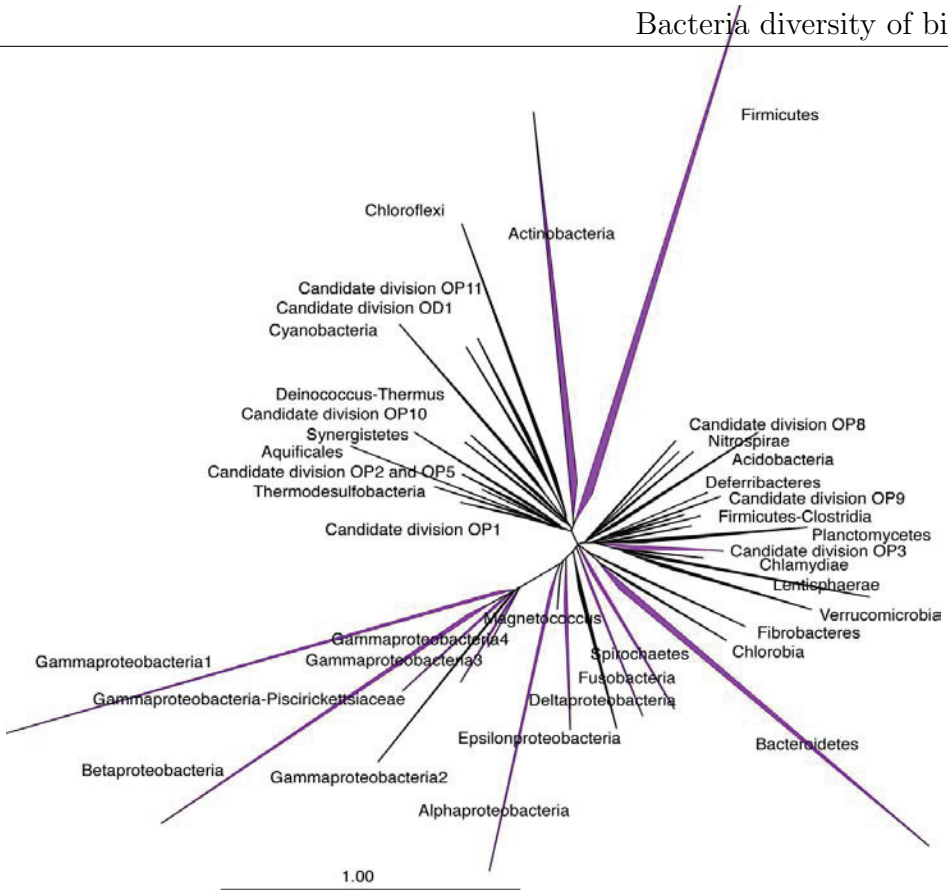

Fig. 1 Phylogentic tree of the main bacterial phyla based on 16S rRNA gene. Almost all existing bacteria phyla are visualized here. Branches in purple are the phyla for which bacterial species have been found associated to bivalves.

Bacteria-bivalve associations

Bacteria-bivalve association studies have been enriched by the discovery of intracellular bacterial symbionts of mussels and clams in the deep-sea. However, most studies have been focused on species with intracellular symbionts and there are scarce studies in the other associated bacteria. Through its history, two topics have inspired the study of bacteria-bivalve associations in shallow waters, the first one is the human pathogens that edible bivalves can concentrate (Kueh and Chan 1984, Prieur et al. 1990, Cabello et al 2005), and the second the economical importance that

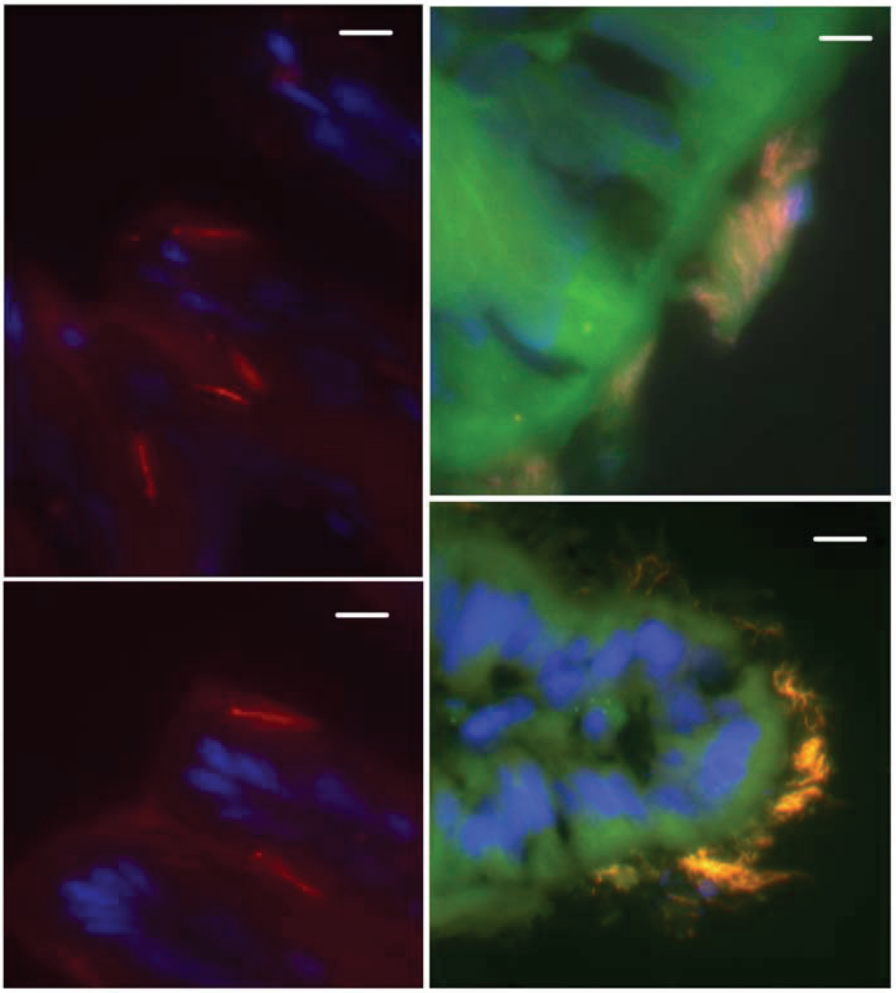

Fig. 2 *Mya arenaria* transversal sections of gill tissue in **left panels**. FISH with specific probes for spirochetes (Spiro-1400 – 5'-CTCGGGTGGTGTGACGGGCG–3'). In **right panels** *B. childressi*, with spirochete specific probe (Spiro-1400) in top image, and in image below with specific probe for epsilonproteobacteria (Epsy-682 – 5' CGGATTTTACCCCTACAC-3'). Scale bars: 10 µm in left panels and bottom right image; 5 µm in top right image.

healthy hatcheries have in aquaculture (Prieur 1987, Romero and Espejo 2001). In fact, because of these two reasons, already more than one century ago the depuration processes (cleaning based on maximizing the natural filtering activity of shellfish holding it in tanks of clean seawater) in particular for oysters were implemented. The study of these associations to use them as models for studying physiological interactions between eukarya and bacteria, and the possibility to study evolutionary pathways of symbiosis has seldom been the focus. With this last intention, we synthesize all the available information about bacteria and their association to shallow-water bivalves, and with molecular studies we contribute to the understanding of bacterial diversity in bivalves. the bacterial diversity of these living habitats. The first surprising thing that it is noted when reviewing described bacteria associated with bivalves is that their diversity is very broad and they cover almost all main branches of the Bacteria kingdom (see Fig. 1).

Microflora diversity

Pathogenic, commensal and beneficial bacteria have all been described as members of bivalve microflora. Most of the diversity studies performed with bivalves are culture-dependent (Lovelace et al. 1968, Murchelano and Brown 1968, Brisou 1962, Prieur 1981), meaning that the knowledge that we have about bivalve microflora diversity is limited to the estimated 1% bacterial population that is cultivable (Kjelleberg et al. 1993) or even less than 0.001% as described for oysters (Romero and Espejo 2001). Occurrence of bacteria has been studied intensively in the bivalve digestive tract (Prieur 1981, Minet et al. 1987) and in the crystalline style (Bernard 1970, Paster et al. 1996, Prieur et al. 1990) Various bacterial genera have been observed to be commonly

Table 1 Isolates from *T. semidecussatus*, *C. edule* and *E. directus.* Gill pieces stored in glycerol were homogenized and cultivated in three different media.

Isolate	Host	Medium[1]	Identity[2]	Closest relative	Acc.[3]
TI-1	*T. semidecussatus*	HNM	514/519 (99%)	*Kocuria sp. MOLA 658*	AM990771
TI-2	*T. semidecussatus*	HNM	360/367 (98%)	*Dermacoccus sp. Ellin183*	AF409025
TI-3	*T. semidecussatus*	HNM	650/650 (100%)	*Sulfitobacter sp. MOLA 8*	AM990784
TI-4	*T. semidecussatus*	HNM	912/964 (94%)	*Uncultured bacterium clone PL-10B7*	AY570580
TI-5	*T. semidecussatus*	HNM	788/800 (98%)	*Uncultured Vibrionaceae*	AY627367
TI-6	*T. semidecussatus*	HNM	926/1036 (89%)	*Alcanivorax sp. PA23*	EU647559
TI-7	*T. semidecussatus*	HNM	775/776 (99%)	*Pseudoalteromonas sp. BSs20060*	EU433327
CI-1	*C. edule*	HNM	1133/1169 (96%)	*Shewanella sp. P117*	EU195929
Vm1-1	*T. semidecussatus*	MM+CTAB	1084/1121 (96%)	*Vibrio sp. Sx2w6*	FJ025776
Vm1-2	*T. semidecussatus*	MM	916/921 (99%)	*Pseudoalteromonas sp. ArcN81*	GQ149233
Os1	*C. gigas*	MA	957/966 (99%)	*Krokinobacter diaphorus*	AB198089
Vm1-3	*T. semidecussatus*	MA	850/855 (99%)	*Vibrio lentus strain Sat201*	AY292936
Vm1-4	*T. semidecussatus*	MA	1124/1131 (99%)	*Vibrio sp. V201*	DQ146980
Hs1-1	*C. edule*	MA	887/890 (99%)	*Krokinobacter diaphorus*	AB198089
Hs1-2	*C. edule*	MA	747/759 (98%)	*Uncultured Silicibacter sp. clone Sc46*	EU375181
E1-1	*E.directus*	MA	640/665 (96%)	*Bacillus sp. FR-W11a2 clone*	FN395284
E1-2	*E.directus*	MA	883/897 (98%)	*Bacillus weihenstephanensis KBAB4,*	CP000903
Vm1-5	*T. semidecussatus*	MA+CTAB	1161/1166 (99%)	*Vibrio sp. S4639 16S*	FJ457601
Vm1-6	*T. semidecussatus*	MA+CTAB	1246/1268 (98%)	*Vibrio sp. S4639 16S*	FJ457601

[1]Media were used in solid (with 1.5 % Agar) and liquid presentations: MA - Marine Medium 2216 (Difco), MM - Minimum Medium with and without CTAB (0.1% yeast, 0.01% peptone, 1.5% agar, 100µm CTAB, dissolved in sea water), and HNM - WL Nutrient Medium (Difco) in 3.5% NaCl.

[2]This identity is with respect to closest relative of next column, it is shown first the number of base pairs ration of the isolate sequence over the closest relative.

[3]Accesión numbers correspond to the published closest relatives.

associated to shallow-water bivalves: *Vibrio, Pseudomonas, Spirochaetes, Achromobacter, Flavobacteria, Micrococcus, Bacillus* and also anaerobic bacteria *Bacteroidetes and Chlostridium.*

Many studies have focused on *Vibrio* spp. since they are facultative pathogens of humans and bivalves. *Vibrio* spp. are a regular component

of the microflora and they might have an "important ecological niche" (Prieur et al. 1990). Some have been related with the few described bivalve diseases like bacillary necrosis or brown ring disease (Grishkowsky and Liston 1974, Tubiash et al. 1970, Elston et al. 2008). Recently, a review on the diversity and pathogenesis of *Vibrio* spp. was made by Beaz-Hidalgo et al. (2010). With respect to bivalve lethality the only two associated species are *V. aestuarianus* and *V. splendidus*. Other species that seem to be commonly associated to bivalves are *V. alginolyticus* and *V. harveyi,* and it is been observed that environmental parameters such as salinity and temperature influence their diversity (Pujalte et al 1999, Arias et al 1999, Beaz-Hidalgo et al 2010). Using different media (High Nutrient Media, Minimum Media and Marine Agar, with or without CTAB [Cetyl trimethylammonium bromide]), we have been able to cultivate 18 different bacterial strains belonging to a variety of bacterial phyla (Table 1). As expected, we obtained *Vibrio* strains from *Tapes semidecussatus*, including in the media with CTAB, that was used before by Plante and coworkers (2008) to isolate surfactant-resistant bacteria with the aim of obtaining bacteria useful for environmental remediation. As already observed in previous studies (Rajagopalan & Sivalingan 1978, Sugita et al 1981) we could cultivate bacteria from the Actinobacteria phylum (*Kocuria* sp. and *Dermacoccus* sp.), and *Bacillus* sp. of the Firmicutes phylum. *Krokinobacter* sp. and *Alcanivorax* sp. isolates are of special interest because the first ones are bacteria that seem to be specialized in the degradation of organic matter (Khan et al. 2006) and the second are bacteria that use hydrocarbons as a sole carbon and energy source (Head et al. 2006). Many times coastal bivalves have been taken as a biological marker to assess pollution (e.g. Nishihama et al 1998, Bresler et al 1999, Verlecar et al 2006). For example, the presence of *Alcanivorax* bacteria in clam tissue indicates a strong oil contamination in North Sea beaches (Brakstad and Lodeng 2005). Many bacterial phylotypes were found in our 16S rRNA sequence analyses,

some are recurrent bacteria that have shown up in previous bivalve studies (Table 2). Most of these sequences are from gammaproteobacteria, as it has been seen in previous molecular studies of bivalve-associated bacterial communities (Schulze et al. 2006, Cavallo et al. 2009). We could detect groups of bacteria that seem to associate with high-content organic matter: oil spills, bone-falls, feces or invertebrate tissue. In addition to gammaproteobacteria there were also Alphaproteobacteria, Bacteroidetes, Actinobacteria and Spirochetes. We hypothesize that these bacteria are specialized in high-organic matter habitats and that further analysis of more individuals and more species will show a characteristic community perhaps host species-specific. Associated spirochaetes stand out because they seem to be a stable bacterial community in bivalves (Bernard et al. 1970, Paster et al. 1996, Prieur 1990, Margulis and Fester 1991). Our FISH observations (Fig 2) show them well established in bivalve gill tissue. It might be interesting to study the variability of these spirochetes species within the different bivalves. It is not clear so far if spirochetes have an ecological role or importance in the association with bivalves, but they seem to be ubiquitous microflora within the mollusk group (Prieur 1990) and perhaps the whole invertebrate group, as other spirochetes have been found in oligochaete worms (Ruehland et al. 2008). Margulis et al. (1991) named as symbionts the studied spirochetes in oysters.

Do intracellular symbionts reduce microflora diversity?

Six families of bivalves have been found to be associated with intracellular symbiotic bacteria: Vesicomyidae (*Calyptogena* , *Vesicomya)*, Mytilidae *(Bathymodiolus, Idas),* Solemyidae (*Solemya*, *Acharax)*, Lucinidae (*Lucina*, *Codakia)*, Thyasiridae (*Thyasira*), and Teredinidae (*Lyrodus*). All are deep-sea water organisms except for *Solemya, Thyasira* and lucinids*.* It is remarkable that these organisms associate with such a restricted

diversity of bacteria, perhaps due to the presence of the endosymbionts. However it is still possible that diversity is in fact not reduced and we have not been able to see it in cultures or in clone library analyses due to the high abundance of the bacterial symbionts. In our 16S rRNA analysis (sequencing and FISH) of *B. childressi*, we observed the presence of different bacteria associated with the tissue apart from the well characterized methanotrophic symbiont: an epsilon proteobacterium, a *Candidatus* Endonuclear bathymodiolin and a spirochete phylotype. This could suggest that the *B. childressi* recognition system is not as specific as in the other *Bathymodiolus* spp. In fact *B. childressi* is one of the lowest positioned bathymodiolin mussels in the COI phylogenetic analysis except for the latest described *B. sp*. from Juan de Fuca (Duperron et al. 2009) and could have an immunological system more similar to the shallow water bivalves. However, in-depth studies of other species might reveal that the diversity of bathymodiolin mussels is much higher than previously assumed.

Nutrition and protection

Bivalve gills provide an ideal habitat for bacteria, with protection from grazers and constant flow of nutrients. Bacteria might be complementing their host nutrition or contributing to metabolite production. Degradation of bacteria by bivalve enzymes has been observed and it seems that this degradation provide dissolved compounds (Birckbeck & McHenery 1982, Amouroux & Amouroux 1988) and improves bivalve nutrition (Samain et al. 1987). Bacteria could provide 5% to 10% of the carbon, and 20% of the nitrogen from the bivalve requirements (Prieur et al 1990). A stable microbiota could be providing protection to bivalves thanks to competition against other, potentially pathogenic bacteria. Also, microbiota could be secreting antimicrobial substances that have been observed to be commun in bacteria isolated from bivalves (Zheng et al. 2005).

Species	Vibrio	Pseudomonas	Achromobacter-Acinetobacter	Shewanella spp.	Enterobacteria	other Gamma	Micrococcus	Spirochetes	Bacteroidetes	Hydrocarbon-degraders	other Alpha	Epsilon	Chlamydia	Photobacterium	Proteolytic bact.	Fermentative bact.	Others	References
Oysters																		
Ostrea edulis							M											Noguchi 1921
Crassostrea gigas	CSCISCS	C		IS		S		ISS		ISS	ISS		C		C			Prieur 1981, Sugita et al 1981, Schultze et al. 2006, This study
C. virginica	C 25%	C31%					MC	C26%						C 87%				Noguchi 1921, Murchelano & Brown 1968
C. cuculata	C	C	C		C	C	C										*Neisseria, Corynobacterium*	Rajagopalan &Sivalingan 1978
Mussels																		
Mytilus edulis	CSCS					S		M					C		C			Noguchi 1921, Prieur 1981, this study
Mercenaria mercenaria																		
Mytilus viridis	C	C	C		C	C	C										*Neisseria, Corynobacterium*	Rajagopalan &Sivalingan 1978
Mytilus coruscus	C	C			C		C										*Moraxella*	Sugita et al 1981
Clams																		
Cerastoderma eduli						CS		S										this study
Tapes (Venerupis) spp.	CISSC	ISCCS		IS		CS	CS	M	IS	ECS	CS	IS		C		C	*Sulfitobacter*	Noguchi 1921, Prieur 1981, Sugita et al 1981, Schultze et al. 2006, This study
Siliqua patula																		
Ensis spp.	CS					S		M	S		S	S						Noguchi 1921
Mya arenaria	CCS	C	C			S		M	C			S					*Corynobacterium, Arthrobacter*	Noguchi 1921, Cundell & Young 1975
Mactra veneriformis	C	C			C		C	M										Noguchi 1921, Sugita et al 1981
Phacosoma japonicum	C	C			C													Sugita et al 1981
Scapharca broughtonii	C	C			C													Sugita et al 1981
Panopea abrupta	IS	IS		IS		IS												Schultze et al. 2006

Table 2 Bacteria found in the different bivalves. Studies where bacteria have been observed microscopically (M), cultured (C), isolated (I), or their 16S rRNA sequenced (S). Some letters are repeated because more than one study have characterized the species. In red the sequences and cultures from this study.

Summary and conclusions

Bacterial diversity in bivalves is a multidisciplinary topic, with from researches in biological, medical, aquaculture and basic research. Ecological studies with molecular techniques are scarce and they could help to disentangle the interaction patterns between the endemic microflora and the invasive ones, as well as the benefits that symbiotic or the harm that pathogenic bacteria bring along. It is important to understand the distribution of pathogenic bacteria in the marine environments to predict potential health concerns transmitted by seafood. Ecological parameters such as nutrient availability, temperature, and salinity influence the presence and persistence of bacteria. However we suggest that bacteria present in bivalves are not only randomly associated with their hosts. It is in part the result of the surrounding water community but also the result of a common evolution between host and bacteria that normally associate with invertebrates or occur in habitats with high organic matter content. We would need more studies to be able to say that these symbiotic bacteria, consistently associated with many diverse bivalves, are beneficial or harmful to their hosts.

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Manuscript IV: Widespread occurrence of an intranuclear parasite in bathymodiolin mussels

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Widespread occurrence of an intranuclear bacterial parasite in vent and seep bathymodiolin mussels

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Summary

Many parasitic bacteria live in the cytoplasm of multicellular animals, but only a few are known to regularly invade their nuclei. In this study, we describe the novel bacterial parasite "*Candidatus* Endonucleobacter bathymodioli" that invades the nuclei of deep-sea bathymodiolin mussels from hydrothermal vents and cold seeps. Bathymodiolin mussels are well known for their symbiotic associations with sulfur- and methane-oxidizing bacteria. In contrast, the parasitic bacteria of vent and seep animals have received little attention despite their potential importance for deep-sea ecosystems. We first discovered the intranuclear parasite "*Ca.* E. bathymodioli" in *Bathymodiolus puteoserpentis* from the Logatchev hydrothermal vent field on the Mid-Atlantic Ridge. Using primers and probes specific to "*Ca.* E. bathymodioli" we found this intranuclear parasite in at least six other bathymodiolin species from vents and seeps around the world. Fluorescence *in situ* hybridization and transmission electron microscopy analyses of the developmental cycle of "*Ca.* E. bathymodioli" showed that the infection of a nucleus begins with a single rod-shaped bacterium which grows to an unseptated filament of up to 20 μm length and then divides repeatedly until the nucleus is filled with up to 80 000 bacteria. The greatly swollen nucleus destroys its host cell and the bacteria are released after the nuclear membrane bursts. Intriguingly, the only nuclei that were never infected by "*Ca.* E. bathymodioli" were those of the gill bacteriocytes. These cells contain the symbiotic sulfur- and methane-oxidizing bacteria, suggesting that the mussel symbionts can protect their host nuclei against the parasite. Phylogenetic analyses showed that the "*Ca.* E. bathymodioli" belongs to a monophyletic clade of *Gammaproteobacteria* associated with marine metazoans as diverse as sponges, corals, bivalves, gastropods, echinoderms, ascidians and fish. We hypothesize that many of the sequences from this clade originated from intranuclear bacteria, and that these are widespread in marine invertebrates.

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Introduction

Bacteria inhabit every imaginable place on earth. With the evolution of the eukaryotes, numerous bacteria have established a symbiotic or parasitic relationship inside eukaryotic cells. Most of these bacteria live in the cytoplasm, but some have found their way into eukaryotic cell compartments, including mitochondria (Chang and Musgrave, 1970; Epis *et al*., 2008), chloroplasts (Wilcox, 1986; Schmid, 2003a,b) and nuclei (Maillet and Folliot, 1967; Grandi *et al*., 1997; Arneodo *et al*., 2008). Although eukaryotic cell compartments have been investigated for decades with light and electron microscopy, little is known about intracompartimental bacteria, particularly those that live in the nuclei of eukaryotes. Most commonly described from protists (Fokin, 2004; Görtz, 2006), almost nothing is known about intranuclear bacteria of metazoans. Rickettsial *Alphaproteobacteria* occasionally invade nuclei of their arthropod or mammalian hosts but occur mainly in the cytoplasm (Burgdorfer *et al*., 1968; Urakami *et al*., 1982; Pongponratn *et al*., 1998; Ogata *et al*., 2006). Apart from these facultative intranuclear *Rickettsia*, reports of intranuclear bacteria that are morphologically or phylogenetically distinct from the Rickettsiales are rare in

metazoans. Only a single 16S rRNA sequence is currently known from an intranuclear bacterium that does not belong to the Rickettsiales (Kerk *et al*., 1992). This *Gammaproteobacterium*, called 'Nuclear Inclusion X' (NIX), causes mass mortalities in the Pacific razor clam *Siliqua patula* (Elston, 1986; Ayres *et al*., 2004). A few morphological observations of non-Rickettsiales-like intranuclear bacteria have been described from the venerid clam *Ruditapes decussatus* (Azevedo, 1989), and two marine *Aplysina* sponges (formerly *Verongia*) (Vacelet, 1970; Friedrich *et al*., 1999).

Deep-sea hydrothermal vents and cold seeps have been studied extensively since their discovery 25–30 years ago. Given the high diversity of metazoans currently described from vents and seeps (Sibuet and Olu-Le Roy, 2002; Wolff, 2005; Desbruyères *et al*., 2006a), remarkably little is known about their parasites (de Buron and Morand, 2004). To date about a dozen metazoan macroparasites have been described (de Buron and Segonzac, 2006a,b). A few studies, using 18S rRNA-based molecular techniques, have suggested that there may be a high abundance of parasitic protist at vents (Atkins *et al*., 2000; Edgcomb *et al*., 2002; López-García *et al*., 2007), and the morphology of fungal, protist, bacterial and viral parasites has been described in more detail in some vent and seep mussels, clams and limpets (Powell *et al*., 1999; Terlizzi *et al*., 2004; Ward *et al*., 2004; Mills *et al*., 2005; Van Dover *et al*., 2007). These studies suggest the potential importance of parasites for vent and seep ecosystems, yet comprehensive investigations of the taxonomy, phylogeny, and life cycle of vent and seep parasites are still lacking.

Mussels of the genus *Bathymodiolus* (Bivalvia: Mytilidae) occur worldwide at deep-sea hydrothermal vents and cold seeps (Tarasov *et al*., 2005; DeChaine and Cavanaugh, 2006; Génio *et al*., 2008). In the absence of light and thus, photosynthetic carbon fixation, these mussels depend on chemosynthetic bacterial symbionts for their nutrition (Stewart *et al*., 2005; Cavanaugh *et al*., 2006). These endosymbionts occur in the mussel's gill tissue, in the cytoplasm of bacteriocytes that regularly alternate with symbiont-free intercalary cells (Fiala-Médioni and Le Pennec, 1987; Distel *et al*., 1995). Bathymodiolin mussels can harbour two types of chemosynthetic bacteria: a chemoautotrophic sulfur oxidizer, capable of fixing CO_2 in the presence of sulfide or thiosulfate as energy sources, and a methane oxidizer that uses methane as both a carbon and an energy source (Fisher *et al*., 1987; Nelson *et al*., 1995; Pimenov *et al*., 2002). Some mussel species harbour only thiotrophic or only methanotrophic symbionts, while other mussel species harbour both types and thus live in a dual symbiosis (DeChaine and Cavanaugh, 2006). The energy sources for the bacterial symbionts, reduced sulfur compounds and methane, are provided by the hydrothermal fluids at vents and through hydrocarbon seepage at cold seeps. Both the mussels and their chemosynthetic bacteria mutually benefit from their symbiosis: the mussel facilitates access to the reductants and oxidants that are necessary for energy production (such as sulfide, methane and oxygen) by supplying its symbionts with a constant fluid flow. In exchange, the bacterial symbionts provide carbon compounds that support the growth and maintenance of host biomass (Stewart *et al*., 2005; Cavanaugh *et al*., 2006). In addition to their symbiotic bacteria, some vent and seep mussels have recently been described that are colonized by parasites such as viruses, *Rickettsia*- and *Chlamydia*-like bacteria, ciliates, fungi and trematodes, but beyond their morphological description, almost nothing is known about these parasites (Powell *et al*., 1999; Ward *et al*., 2004; Van Dover *et al*., 2007).

This study describes a novel bacterial parasite of bathymodiolin mussels. Using comparative 16S rRNA sequence analysis, fluorescence *in situ* hybridization (FISH) and transmission electron microscopy (TEM), we show that this parasite lives in the nuclei of mussels from vents and seeps around the world. We describe the life cycle of this intranuclear parasite through reconstruction of its developmental cycle from a solitary cell to the proliferation of up to 80 000 bacteria within a single greatly enlarged nucleus. We propose the name "*Candidatus* Endonucleobacter bathymodioli" for this bacterium. The genus name '*Endonucleobacter*' translates freely into 'bacterium living inside the nucleus' and the species name '*bathymodioli*' refers to the host genus of vent and seep mussels, *Bathymodiolus*, in which we discovered this parasite.

Results

Discovery of intranuclear bacteria in Bathymodiolus puteoserpentis

Three gammaproteobacterial 16S rRNA phylotypes were found in gill tissues of *B. puteoserpentis* from the Logatchev hydrothermal vent field on the Mid-Atlantic Ridge (Fig. 1A). In addition to the sequences already known from the sulfur- and methane-oxidizing symbionts (Duperron *et al*., 2006), a novel 16S rRNA sequence was discovered ("*Candidatus* Endonucleobacter bathymodioli" in Fig. 1A). This novel sequence fell in a clade consisting of 16S rRNA sequences from bacteria associated with marine animals including sponges, corals, a sea slug, an ascidian, a sea urchin and a fish (93–97% identity, Table S1). This clade also included the sequence from the 'Nuclear Inclusion X' parasite of the Pacific razor clam *S. patula* (Kerk *et al*., 1992). The monophyly of this clade

was supported in both maximum likelihood and Bayesian analyses (support values: 60 and 1.00). The cultivated species within this clade were all heterotrophic: *Endozoicomonas elysicola*, a strictly aerobic and mesophilic bacterium from the gastrointestinal tract of the sea slug *Elysia ornata* (Kurahashi and Yokota, 2007), and three species from marine sponges, one rod-shaped (H425) and one spirillum-like (H262) bacterium (Sfanos *et al*., 2005), and *Spongiobacter nickelotolerans* (unpublished information from GenBank). The closest relatives to the clade containing "*Ca.* E. bathymodioli" were *Zooshikella gangwhensis*, a chemoorganotrophic, aerobic and halophilic isolate from sediments of a Korean tidal flat (Yi *et al*., 2003) (91% identity to the "*Ca.* E. bathymodioli" sequence from *B. puteoserpentis*) and two sequences from *Rickettsia*-like bacteria causing mass mortality in the oyster *Crassostrea ariakensis* (unpublished information in GenBank under accession numbers DQ118733 und DQ123914).

Fluorescence *in situ* hybridization analyses with probes specific to the "*Ca.* E. bathymodioli" phylotype showed that it originated from bacteria that occurred exclusively in the nuclei of *B. puteoserpentis* cells (Fig. 2). Gill tissues were most heavily colonized (Fig. 2A–D and F), but the bacteria were also observed in nuclei of the gut (Fig. 2E), digestive gland, labial palp, mantle and foot (data not shown). In the gill tissues, only nuclei of the symbiont-free intercalary cells were infected, whereas intranuclear bacteria were never observed in the bacteriocytes containing the thiotrophic and methanotrophic symbionts (Fig. 2B–D and F). Transmission electron microscopy analysis of gill filaments confirmed that the bacteria were inside the host nuclei (Fig. 3).

*Widespread occurrence of "*Ca. *E. bathymodioli" in deep-sea mussels*

Using PCR primers and FISH probes specific to the "*Ca.* E. bathymodioli" phylotype found in *B. puteoserpentis*, we searched for these bacteria in other bathymodiolin hosts from hydrothermal vents and cold seeps in the Atlantic Ocean, the Gulf of Mexico, and the Pacific Ocean (Fig. 4, Table 1, Table S2). We found "*Ca.* E. bathymodioli" in all host species except *B.* aff. *boomerang* (Southeast Atlantic) and *B. brevior* (West Pacific). All "*Ca.* E. bathymodioli" sequences were very closely related to each other with 98.8% identity between sequences on average and 98.1% identity between the two most distant phylotypes (Fig. 1B). In most host species, a single 16S rRNA phylotype dominated the clone libraries. Less dominant phylotypes that differed by at most 2 bp from the dominant phylotypes were also found in several host species. These phylotypes often co-occurred in the same host individuals and were shared between individuals (Table S2). In *B.* sp. from Wideawake (Mid-Atlantic Ridge), a single 16S rRNA clone was found with a sequence that differed by 1.9% (26 bp) from all other "*Ca.* E. bathymodioli" phylotypes found in this species (called 'minor phylotype III' in Table S2). This phylotype was most closely related to the sequence from *B.* aff. *thermophilus* (Pacific-Antarctic Ridge) (Fig. 1B). With the exception of this minor phylotype III, all other "*Ca.* E. bathymodioli" sequences fell into three clusters reflecting their geographic origins from the Mid-Atlantic Ridge, the Pacific-Antarctic Ridge, and the Gulf of Mexico (Fig. 1B). The correlation between genetic distances of the "*Ca.* E. bathymodioli" 16S rRNA phylotypes and geographical distances of sample locations was statistically significant for all phylotypes ($P = 0.04$ with minor phylotype III and $P = 0.01$ without minor phylotype III).

*Developmental cycle of "*Ca. *E. bathymodioli"*

Detailed FISH and TEM analyses of "*Ca.* E. bathymodioli" in *B. puteoserpentis* gill tissues revealed six distinct developmental stages (Figs 2, 3 and 5). In Stage 1, a single rod-shaped bacterium (1.8×0.4 μm) is present inside the nucleus (Fig. 2B and G). In Stage 2, the bacterium has grown to an unseptated filament of up to 18–20 μm length (Fig. 2H–J). In Stage 3, a loosely wrapped filamentous coil is visible inside the host nucleus (Fig. 2M). We could not clearly discern if this coil consists of several separate filaments or one long unseptated filament. However, on rare occasions we observed two filaments of equal length (Fig. 2L) and filaments that appeared to be in the process of longitudinal division in some nuclei (Fig. 2K), suggesting that the Stage 3 coil consists of several filaments. The host nuclei in Stage 3 have become more irregular in shape and chromatin is reduced to a thin layer along the nuclear membrane (Fig. 3A and D). Transverse binary fission of the filamentous coils leads to Stage 4 in which stacks of shorter filaments of up to 8–10 μm length fill the host nucleus (Fig. 2E and N). At this stage, host nuclei are at least two to three times the volume of uninfected nuclei. Repeated transverse binary fissions lead to the formation of numerous rods in Stage 5 with the nuclei further enlarged to about five times the volume of uninfected nuclei. Chromatin is nearly completely reduced and barely visible along some parts of the nuclear membrane (Fig. 3E). In Stage 6, the bacteria have reproduced massively forming a round to oval aggregate of up to 30 μm in diameter that is still surrounded by a membrane (Fig. 2P). At this stage, the infected nuclei are no longer visible within structurally intact host cells, but occur extracellularly within the gill epithelium. At average bacterial sizes of 1.8×0.4 μm, Stage 6 nuclei can contain between 10 000 and 80 000 bacteria. These aggregates are eventually disrupted and the bacteria released into the fluids surrounding the

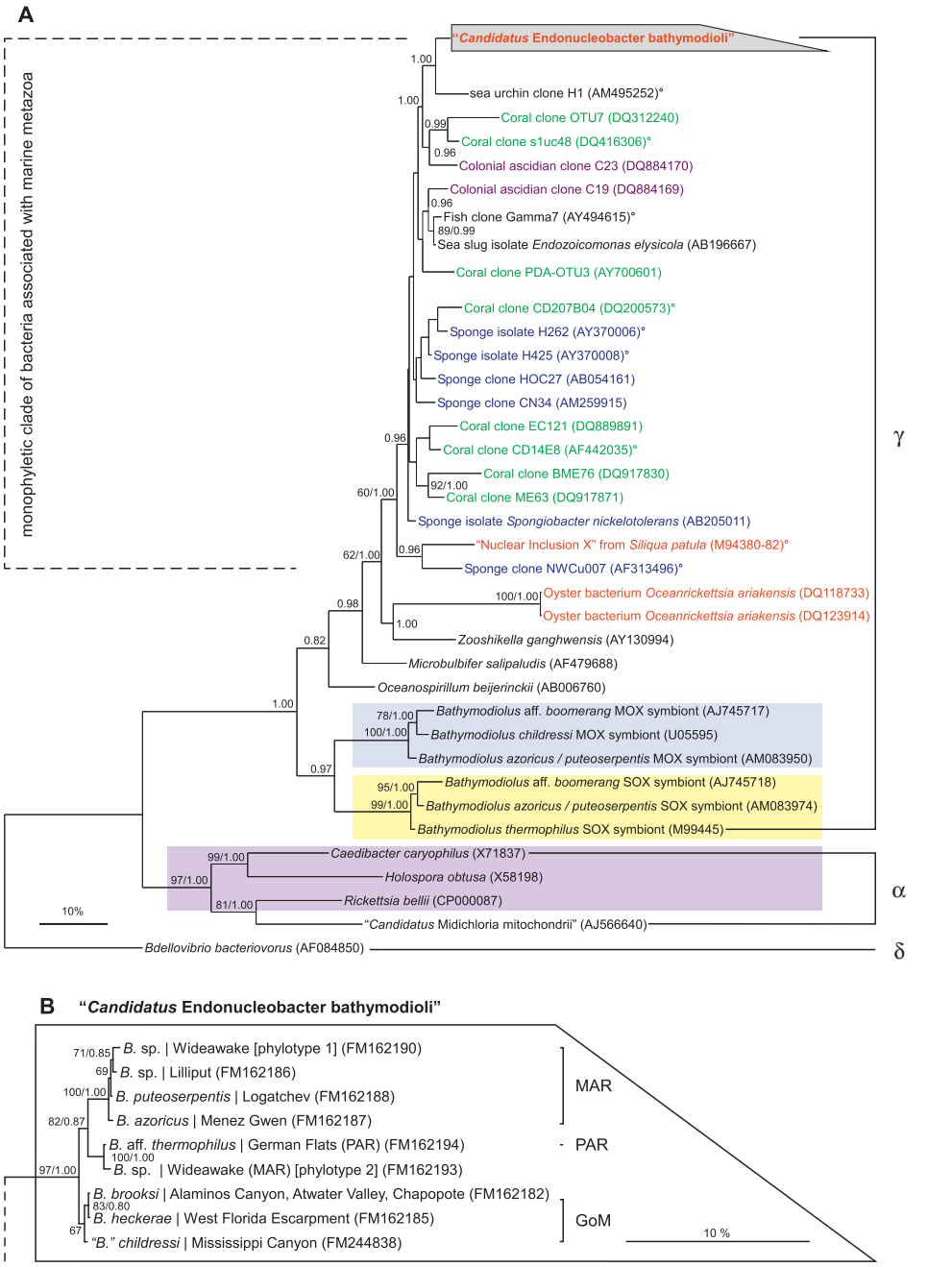

Fig. 1. 16S rRNA phylogenetic tree inferred from maximum likelihood and Bayesian analysis.
A. The tree shows the "*Candidatus* Endonucleobacter bathymodioli" clade (grey trapezoid) together with its closest relatives. Colours of sequences from marine invertebrates were assigned according to host phylogeny, with bacteria from bivalves shown in red, from sponges in blue, and from ascidians in purple (sea slug, sea urchin and fish bacteria are shown in black). Also included in the tree are (i) the closest free living relatives within the Oceanospirillales and Alteromonadales; (ii) the methane-oxidizing endosymbionts of bathymodiolin mussels (blue box); (iii) the sulfur-oxidizing endosymbionts of bathymodiolin mussels (yellow box); and (iv) intranuclear bacteria belonging to the Rickettsiales from ciliates and ticks (purple box). *Bdellovibrio bacteriovorus* was used as an outgroup. The phylogenetic reconstruction is based primarily on nearly full sequences. Partial sequences (508–983 bp) are marked (°). Methane- and sulfur-oxidizing symbionts of bathymodiolin mussels are labelled MOX and SOX respectively. Values at nodes represent maximum likelihood bootstrap values in percentage (first value) and posterior probabilities (second value). Scale bar represents 10% estimated base substitution. Only bootstrap values $\geq$ 60 and posterior probabilities $>$ 0.80 are shown. MAR, Mid-Atlantic Ridge; PAR, Pacific-Antarctic Ridge; GoM, Gulf of Mexico.
B. Tree of "*Ca.* E. bathymodioli" sequences from vent and seep mussels (collection sites of mussels are shown). The "*Ca.* E. bathymodioli" sequences form a monophyletic clade.

mussel gills. Three-dimensional examples of the developmental Stages 2, 3, 4 and 6 can be viewed as supporting animations (Videoes S1–S4). In addition to the detailed FISH and TEM analyses of the "*Ca.* E. bathymodioli" developmental stages in *B. puteoserpentis*, FISH analyses of *B. azoricus, B.* sp. (Wideawake), *B.* aff. *thermophilus*, *B. brooksi*, *B. heckerae,* and *"B." childressi* gill tissues showed the presence of similar developmental stages in these host species as well.

Discussion

Developmental cycle

The colonization of a nucleus requires several steps: the infection of the host cell, passage through the cytoplasm, and penetration of the nuclear membrane. None of these stages were observed in this study, presumably because these events occur on very short time scales and are therefore only rarely visible. The first discernible infection stage in our study was the presence of a single rod-shaped bacterium in the mussel nuclei that grows to an unseptated filament of about 20 μm and possibly longer (Stages 1 and 2 in Fig. 2G–J). This may be a characteristic feature of intranuclear bacteria. In the marine sponge *Aplysina*, intranuclear bacteria can form filaments up to 350 μm in length (Vacelet, 1970; Friedrich *et al*., 1999), and infectious forms of *Holospora obtusa* can reach 20 μm in length in the macronucleus of the ciliate *Paramecium caudatum* (Görtz, 2006). Unseptated filamentous growth has also been observed in *Bdellovibrio bacteriovorus*, a deltaproteobacterial predator of Gram-negative bacteria (Angert, 2005; Dworkin, 2006). The transition from Stage 2 to Stage 3 appears to take place through longitudinal fission (Fig. 2K). Longitudinal division is rare among bacteria and has only been described in the sulfur-oxidizing symbionts of three marine host genera, the nematode *Laxus* sp. (Polz *et al*., 1992; 1994), the gutless oligochaete *Olavius* (Giere and Krieger, 2001; Bright and Giere, 2005), and the sand-dwelling ciliates of the genus *Kentrophoros* (Fauré-Fremiet, 1951; Raikov, 1971).

The shift from filamentous growth to massive reproduction through transverse fission may be triggered by nutrient limitation. The chromatin of Stage 3 nuclei is greatly reduced to a thin layer along the nuclear membrane (Fig. 3A and D). Given that this reduction does not appear to be physically induced because the bacteria do not completely fill out the nucleus at this stage, it is likely that the bacteria have used the chromatin for nutrition (see below). In *B. bacteriovorus*, transition to the multiple fission phase occurs when the cytoplasm is consumed and the host's resources are exhausted (Horowitz *et al*., 1974; Angert, 2005; Lambert *et al*., 2006).

Multiple rounds of transverse binary fission between Stages 3 and 6 lead to massive swelling of the host nuclei. Greatly enlarged nuclei are typical for protists infected with intranuclear bacteria (Table S3) and have also been described in the Pacific razor clam *S. patula* infected with the intranuclear pathogen 'NIX' (Elston, 1986). It is intriguing that in two of the host species shown to be infected with "*Ca.* E. bathymodioli" in this study (*B. heckerae* and *B. puteoserpentis*), previous light microscopical analyses indicated the presence of hypertrophied nuclei in some tissues (Ward *et al*., 2004). Transmission electron microscopy and FISH analyses are needed to clarify if these nuclear distortions were caused by viruses, as suggested by Ward and colleagues (2004), or by intranuclear bacteria. Similarly, bacteria described as '*Rickettsia*-like' and '*Chlamydia*-like' based on light microscopical analyses of gill tissues of vent and seep mollusks (Powell *et al*., 1999; Terlizzi *et al*., 2004; Ward *et al*., 2004; Mills *et al*., 2005) might not be bacteria belonging to the *Rickettsia* and *Chlamydia* but rather "*Ca.* E. bathymodioli" parasites.

The completion of the "*Ca.* E. bathymodioli" cell cycle requires the release of the infected nuclei from the host cell. This most likely occurs through the destruction of the host cell and rupture of the host cytoplasmic membrane. In 'NIX'-infected *S. patula*, the host cells are ruptured by the greatly enlarged nuclei, indicating a greater resiliency of the nuclear over the cytoplasmic membrane (Elston, 1986). When the "*Ca.* E. bathymodioli"-infected nuclei become extracellular, they are still surrounded by a mem-

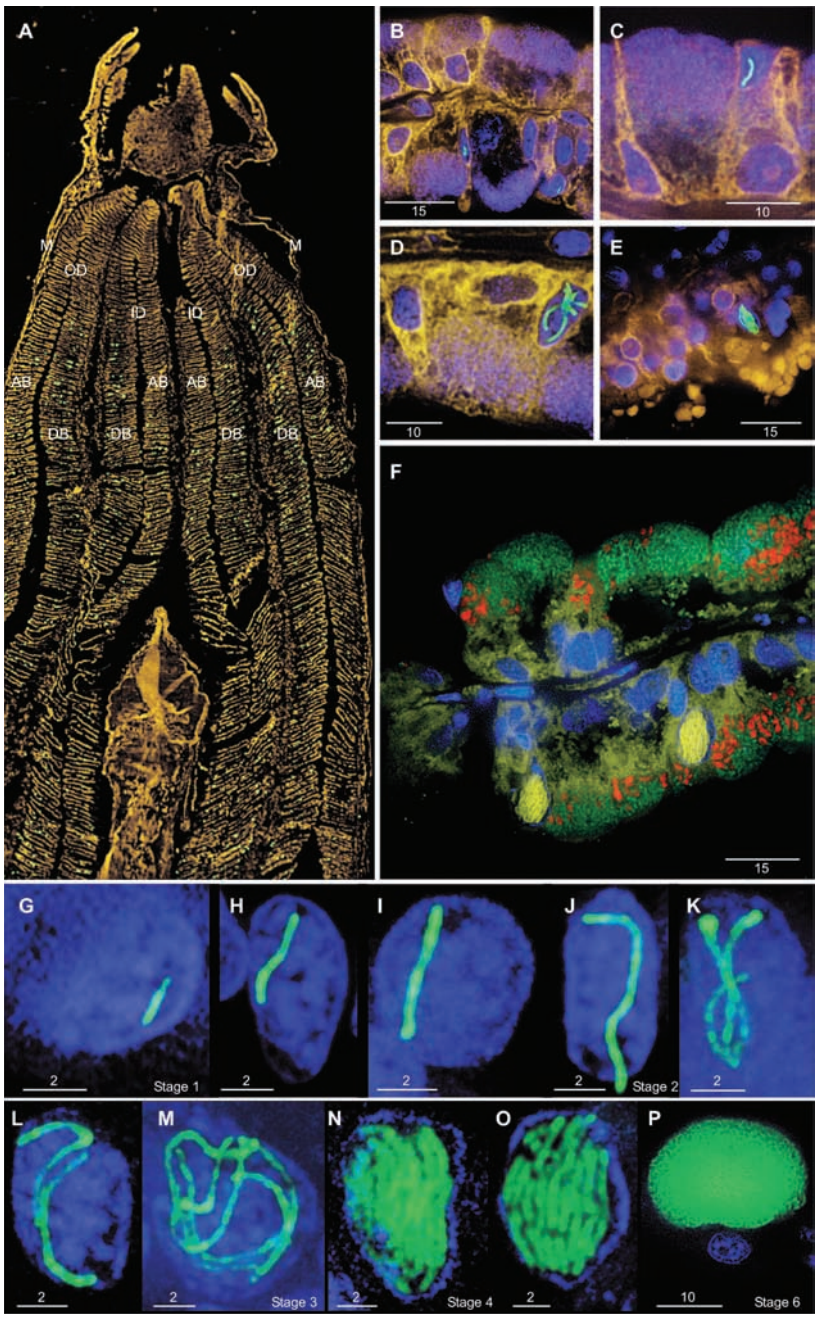

Fig. 2. "*Ca.* E. bathymodioli" in various mussel tissues and developmental stages. (This figure was prepared as an RGB image and converted to CMYK mode for print. The original RGB image is provided as Figure S1 in Supporting Information).
A. Cross-section through a juvenile mussel showing the distribution of the intranuclear bacterium throughout the gill tissue. Intranuclear bacteria are shown in green and mussel tissue appears in orange. The morphology results from staining nuclei and bacterial endosymbiotic DNA with DAPI which was assigned an orange colour. AB, ascending gill branch; DB, descending gill branch; ID, inner demibranch; M, mantle; OD, outer demibranch.
B–D. Non-ciliated gill tissue with intranuclear bacterium in intercalary cells which alternate with bacteriocytes.
E. Gut tissue. In images B–E intranuclear bacteria are shown in green and eukaryotic tissue is represented in yellow. Nuclei and bacterial endosymbiotic DNA in bacteriocytes appear in blue.
F. Non-ciliated gill tissue with intranuclear bacteria; intranuclear bacteria appear in bright yellow, whereas eukaryotic tissue is represented by a yellowish to brownish colour. Chemoautotrophic and methanotrophic bacterial endosymbionts in bacteriocytes are shown in green and red respectively. Nuclei were stained with DAPI and appear in blue.
G–P. Developmental stages of "*Ca.* E. bathymodioli" in *B. puteoserpentis* gill tissues. The intranuclear bacterium appears in green, the nucleus in blue. Images H–M result from projection of a stack of several two-dimensional layers onto one single layer reflecting the overall three-dimensional structure on a two-dimensional plane.
G–J. Series showing growth from a single short rod to a single filament in Stages 1 and 2.
K. Two overlapping filaments or filament in the process of longitudinal binary fission in transition from Stage 2 to Stage 3.
L. Two separate filaments (Stages 2–3).
M. Filamentous assembly consisting of either one single long coiled filament or several filaments (Stage 3).
N. Stacks of shorter filaments (Stage 4) resulting from transverse fissions of coiled filaments.
O. Long rods resulting from division of Stage 4 filaments.

brane. This membrane is most likely of nuclear origin, because it stained positively with 4,6-diamidino-2-phenylindole (DAPI), suggesting remnants of nuclear DNA along the inside of the membrane. Finally, the bacteria must escape from the membrane-surrounded Stage 6 nuclei, but how they do this remains unclear. Not unexpectedly, we only very rarely saw single extracellular "*Ca.* E. bathymodioli", as the washing procedure for the fixation of gill tissues would have removed most loosely attached cells. Little is known about how other intranuclear bacteria escape their hosts. In the ciliate *Paramecium caudatum*, infectious forms of the intranuclear bacterium *Holospora obtusa* make use of the division apparatus of the host nucleus to escape from the nucleus (Fokin, 2004; Görtz, 2006). In the bacterial predator *B. bacteriovorus*, 15 lipases have been identified that dissolve the outer membrane of its hosts and enable its release (Rendulic *et al*., 2004). We do not currently know if "*Ca.* E. bathymodioli" contribute actively to their release from the membrane-bound aggregate, for example, through the excretion of lipases, or if this process is passive, for example, through mechanical disruption of the greatly swollen aggregrate membrane.

Chromatin as a nutritional source

The disappearance of chromatin during the development of "*Ca.* E. bathymodioli" suggests that the host DNA with its surrounding chromosomal proteins provides the nutrition for the growing and reproducing parasites. Chromatin reduction has also been observed in protists infected with intranuclear bacteria (Table S3). Clearly, DNA provides a rich source of sugar, nitrogen and phosphorus. ATP for biomass synthesis could be acquired by breaking down host DNA or host nucleotides could be used for DNA synthesis. Recent studies have shown the importance of extracellular DNA as a nutritional source for free-living bacteria (Lennon, 2007; Pinchuk *et al*., 2008), but nothing is known about the metabolism of intranuclear bacteria and their genomes have not yet been sequenced. Although not an intranuclear bacterium but rather a bacterial parasite, *B. bacteriovorus* efficiently consumes its prey's cellular contents including nucleic material. It has been studied extensively (reviewed in Jurkevitch, 2006) and its genome contains 20 different deoxyribonuclease genes for DNA hydrolysis (Rendulic *et al*., 2004). A further source of nutrition for "*Ca.* E. bathymodioli" may also be cytosolic substrates, particularly during later developmental stages in which the extreme enlargement of the nuclear membrane might enable the leakage of substrates from the cytosol to the nucleus.

A few bacteria that fall within the monophyletic clade to which "*Ca.* E. bathymodioli" belongs have been cultivated: *E. elysicola* (isolated from a sea slug – Kurahashi and Yokota, 2007) and three bacteria isolated from marine sponges, *Spongiobacter nickelotolerans* (unpublished information from GenBank) and two unnamed species called H262 and H425 (Sfanos *et al*., 2005). Cultivation information is only available for *E. elysicola* which is an aerobic, mesophilic heterotroph but details for growth substrates of *E. elysicola* were not described and it is not clear if this species can grow on DNA alone (Kurahashi and Yokota, 2007).

Host–parasite–symbiont interactions

Both our FISH and TEM analyses showed that only symbiont-free intercalary gill cells were infected by "*Ca.* E. bathymodioli" whereas the nuclei of bacteriocytes con-

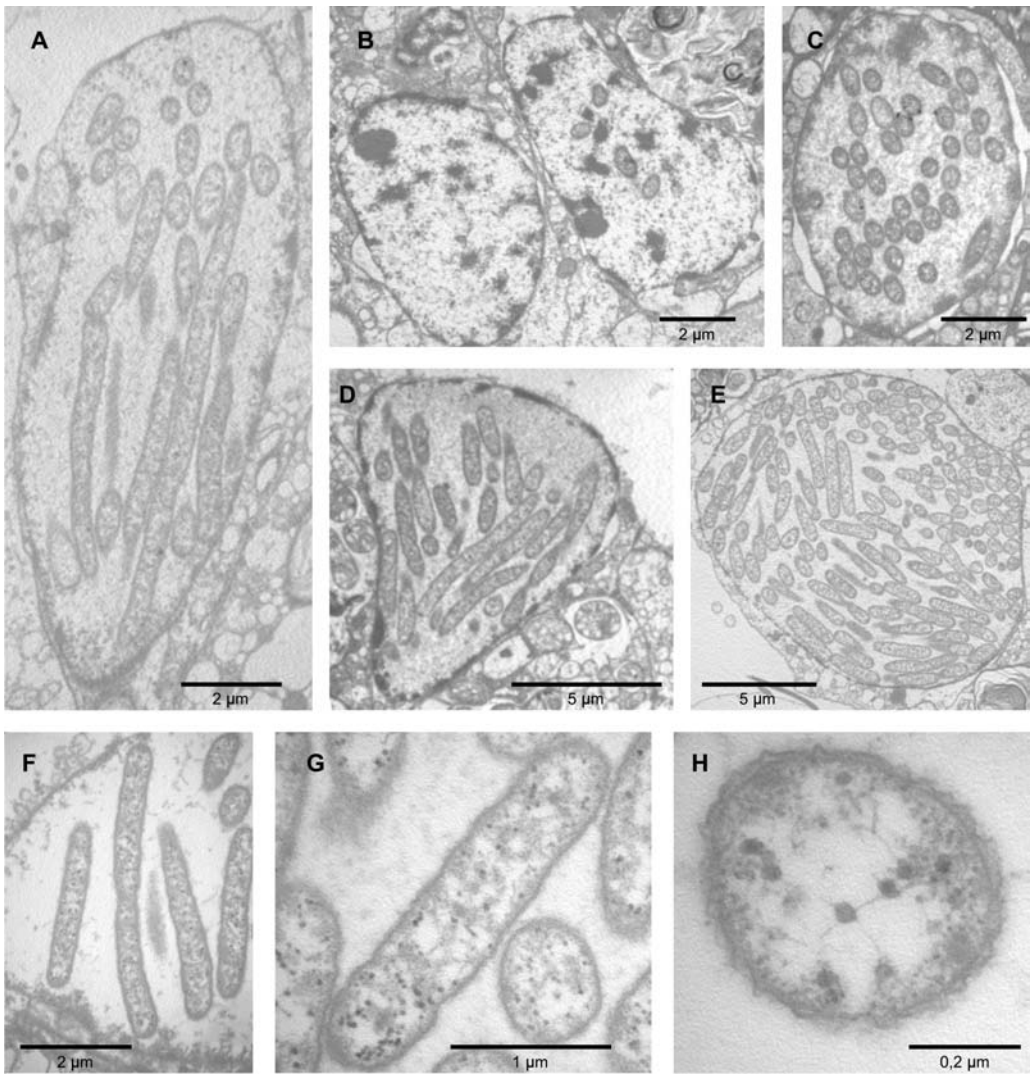

Fig. 3. Transmission electron microscopy images showing different stages in the development of "*Ca.* E. bathymodioli".
A. Oval elongated nucleus with intranuclear bacteria in longitudinal, transversal and cross-section most likely representing the filamentous assembly of Stage 3. Note the filamentous appearance of bacteria in this stage and that the filaments are unseptated.
B. Uninfected nucleus (left) and infected nucleus (right). The infected nucleus represents a cross-section of either a twisted Stage 2 filament or an early Stage 3 filamentous coil.
C. Stage 4 nucleus showing a stack of shorter filaments in cross-section.
D. Pear-shaped nucleus with intranuclear bacteria in longitudinal, transversal and cross-section most likely representing the multifilamentous coil of Stage 3. The nucleus, normally at the basal end of the cell when uninfected, is now at the apical end.
E. Swollen Stage 5 nucleus with bacteria in longitudinal and cross-section. Note the reduced length of each single bacterium as compared with the elongated form in Stage 3. Also notice the absence of chromatin except for narrow remnants along the nuclear membrane.
F. Dividing filament (middle) showing transverse binary fission.
G. Single bacterium showing electron dense particles distributed throughout the cell.
H. One single bacterium in cross-section. Note the inner and outer membrane characteristic of Gram-negative bacteria.

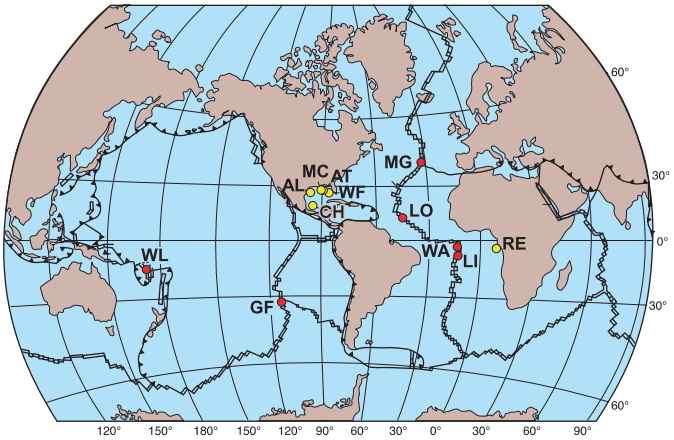

Fig. 4. Sampling sites of bathymodiolin mussels investigated in this study. Circles show hydrothermal vents (red) and cold seeps (yellow). MG, Menez Gwen; LO, Logatchev; WA, Wideawake; LI, Lilliput (Mid-Atlantic Ridge); AL, Alaminos Canyon; AT, Atwater Valley; CH, Chapopote; MC, Mississippi Canyon; WF, West Florida Escarpment (Gulf of Mexico); RE, Regab (Gabon Continental Margin); GF, German Flats (Pacific-Antarctic Ridge); WL, White Lady (North Fiji Back-Arc Basin).

taining the thiotrophic and methanotrophic endosymbionts were never infected. This suggests that these cells are protected against infection, either because the bacteriocytes differ from other host cells in a manner that prevents parasite infection or because the symbionts provide protection. How this protection might be afforded is not clear, but the consequences are substantial. As the bacteriocyte nuclei are not infected, it is likely that chemosynthetic energy production and carbon fixation by the endosymbiotic primary producers remains fully functional, thus assuring the nutritional supply of the host. While the infection may be deleterious to the intercalary cells, the overall health of the host does not appear to be significantly affected because its power plants, the bacteriocytes, are not infected. This assumption is supported by the fact that we have not observed mass mortality of the *B. puteoserpentis* population at the Logatchev hydrothermal vent field during the four research cruises we have had to this field between 2004 and 2007, despite the regular presence of "*Ca.* E. bathymodioli" within the population throughout this period. This is in contrast to the mass mortality caused by the intranuclear bacteria of the clam *S. patula* and the *Rickettsia*-like bacteria of the oyster *Crassostrea ariakensis* that do not have symbiotic bacteria in their gills (Elston, 1986; Ayres *et al.*, 2004; unpublished information in GenBank under accession numbers DQ118733 und DQ123914).

What is not currently known is if chemosynthetic symbionts protect their hosts against other parasites besides "*Ca.* E. bathymodioli". Mass mortality of symbiont-containing mussels and clams was observed at the Blake Ridge seep off the southeastern coast of the USA and morphological analyses of the clams and mussels showed that both were infested with eukaryotic and prokaryotic parasites (Ward *et al.*, 2004; Mills *et al.*, 2005). However, mortality could not be clearly linked to parasitism as shifts in seepage might also have caused mussel and clam deaths (Ward *et al.*, 2004; Mills *et al.*, 2005).

Global occurrence

Our studies show that "*Ca.* E. bathymodioli" occurs in seep and vent mussels from around the world. We could not find the intranuclear parasite in only two species, *B.* aff. *boomerang* from the Gabon continental margin and *B. brevior* from the West Pacific. It is possible that some bathymodiolin species are not infected by "*Ca.* E. bathymodioli". However, there are no shared characteristics between the two mussel species lacking "*Ca.* E. bathymodioli" that would explain why these hosts would not be infected with this global intranuclear parasite: Neither are they closely related to each other, nor do they occur in the same geographic region. An alternative explanation is that these species contained intranuclear bacteria at levels too low to be detected with PCR and FISH. Although we did not quantify infestation levels, we did observe differences in the abundances of "*Ca.* E. bathymodioli" both between hosts from different fields and between individuals of the same species (Table S2).

Biogeographical clusters

In the Gulf of Mexico, *B. brooksi* and *B. heckerae* share the same "*Ca.* E. bathymodioli" 16S rRNA phylotype despite the fact that these hosts are not closely related to each other (Jones *et al.*, 2006; Cordes *et al.*, 2007). Together with the fact that the "*Ca.* E. bathymodioli" phylotypes fall into three regional clusters that reflect the origin of their hosts, this suggests that host geography

Table 1. Bathymodiolin mussels investigated in this study and sampling sites.

Bathymodiolus species	Location	Cruise name	Recovery[a]	Month, year	Chief scientist	Reference	Sample ID	Depth	Latitude	Longitude
B. azoricus[b]	Menez Gwen	ATOS	Victor	Jun/Jul 2001	P. M. Sarradin	Sarradin *et al.* (2001)	MG3, 5, 10	850 m	37°51′N	31°31′W
B. puteoserpentis[b]	Logatchev/Irina 2	Hydromar I	Quest	Jan/Feb 2004	T. Kuhn	Kuhn *et al.* (2004)	35GTV-3	3016 m	14°45.1629′N	44°58.7478′W
							38ROV/6-5	3035 m		
							56ROV/6-j6	3035 m		
							66ROV/16-3	3053 m		
B. sp.[b]	Wideawake	Marsued II	Quest	Apr 2005	K. Haase	Haase *et al.* (2007)	109GTV/A-3	2998 m	4°48.64′S	12°22.36′W
							125ROV/1B-2	2987 m	4°48.6293′S	12°22.3524′W
							125ROV/7-4	2986 m	4°48.6275′S	12°22.3503′W
							125ROV/12-6	2987 m	4°48.6231′S	12°22.3515′W
B. sp.[b]	Lilliput/Main Lilliput	Marsued II	Quest	Apr 2005	K. Haase	Haase *et al.* (2005)	200ROV/9-3	1496 m	9°33.1783′S	13°11.8462′W
B. brevior[b]	White Lady/LHOS	Hyfiflux II	TV-grab	Aug/Sep 1998	P. Halbach	Halbach *et al.* (1999)	39GTV-6	1976 m	16°59.348′S	173°55.029′E
							66GTV-12	2009 m	16°59.449′S	173°54.940′E
							99GTV-9	2000 m	16°59.486′S	173°54.911′E
B. aff. *thermophilus*[b]	German Flats (38°S)[c]	Foundation 3	TV-grab	Jun/Jul 2001	P. Stoffers	Stecher *et al.* (2002); Stoffers *et al.* (2002)	30GTV-1-4	2212 m	37°47.4673′S	110°54.8675′W
B. aff. *boomerang*[d]	Gabon Continental Margin/Regab	Biozaire 2	Victor	Nov 2001	M. Sibuet	Duperron *et al.* (2005); Ondreas *et al.* (2005)	Ang M5	3150 m	5°52.8134′S	9°37.9419′E
B. heckerae[d]	West Florida Escarpment	Deep Seeps 11/I	Alvin	Oct 2003	C. R. Fisher and B. Carney	–	GoM M6	3284 m	26°02.00′N	84°55.03′W
B. heckerae[d]	Campeche Knolls/Chapopote	Meteor M67/2	Quest	Apr 2006	G. Bohrmann	Spiess and Bohrmann (2006)	Sp1.1, Sp1.2	2915 m	21°53.98′N	93°26.12′W
							Sp2.1, Sp2.2	2915 m		
B. brooksi[d]	Louisiana Continental Slope/Atwater Valley	Deep Seeps 11/I	Alvin	Oct 2003	C. R. Fisher and B. Carney	–	GoM M24	1893 m	27°34.10′N	88°29.8′W
B. brooksi[d]	Louisiana Continental Slope/Alaminos Canyon						GoM M29	2226 m	26°21.32′N	94°30.12′W
"B." childressi[d]							GoM M34			
"B." childressi[d]	Louisiana Continental Slope/Mississipi Canyon	Atlantis 15/3	Alvin	May 2006	C. R. Fisher	–	4178/mc853-2	1050 m	28°07.40′N	89°08.20′W

a. Samples were recovered using the remotely-operated vehicles Quest (Marum, University of Bremen, Germany) and Victor (Ifremer, France), the manned submersible Alvin (Woods Hole Oceanographic Institution, USA), and a TV-controlled grab (Oktopus GmbH Kiel, Germany).
b. Species from hydrothermal vents.
c. Vent field name according to Desbruyères and colleagues (2006b).
d. Species from cold seeps.

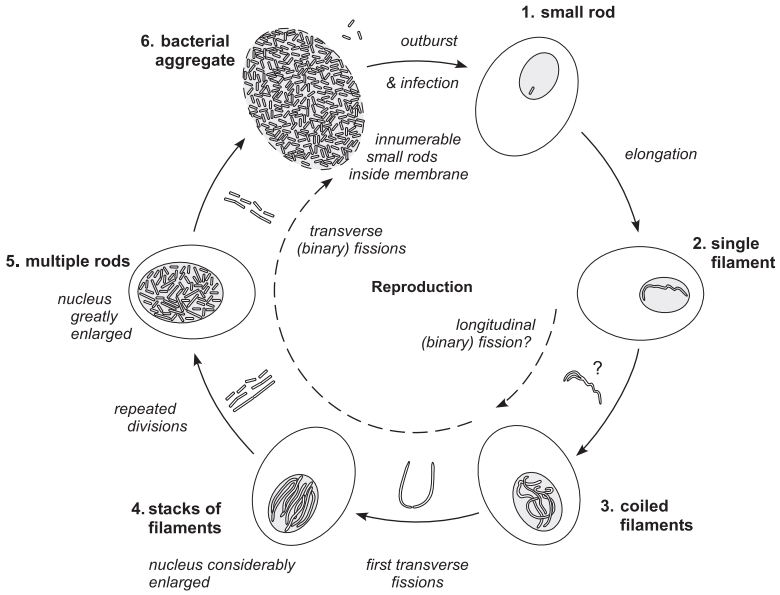

Fig. 5. Proposed developmental cycle of "*Candidatus* Endonucleobacter bathymodioli". The infection begins with a single rod-shaped bacterium inside the nucleus (Stage 1) that grows into an unseptated filament of up to 18–20 μm in length (Stage 2). Multiplication, possibly by longitudinal fission, yields several such filaments that are loosely wrapped in a filamentous coil (Stage 3). These subsequently form stacks of shorter filaments of up to 8–10 μm in length (Stage 4). Repeated transverse binary fissions lead to numerous rod-shaped bacteria (Stage 5). Further multiplication results in a voluminous membrane-surrounded bacterial aggregate containing up to 80 000 rod-shaped bacteria and the destruction of the host cell (Stage 6). These aggregates are eventually disrupted and the bacteria released.

and not cospeciation played a role in the establishment of these associations. This hypothesis is supported by our statistical analyses that showed a significant correlation between genetic and geographic distances. However, the investigation of more host species from different geographic regions, particularly the West Pacific, is needed before this hypothesis can be substantiated.

One mussel species, *B.* sp. from the Wideawake vent field on the southern Mid-Atlantic Ridge harboured two "*Ca.* E. bathymodioli" phylotypes that differed from each other by 1.9%. One falls into the Mid-Atlantic Ridge cluster whereas the other one falls in the Pacific-Antarctic Ridge cluster (Fig. 1B). This indicates that although most "*Ca.* E. bathymodioli" bacteria cluster according to their geography, there may have been transoceanic crossing in the past, in this case from vent sites on the Pacific-Antarctic Ridge to the southern Mid-Atlantic Ridge. Historical dispersal across ocean basins has been suggested for some bathymodiolin host species (Jones *et al.*, 2006; Olu-Le Roy *et al.*, 2007), and there is no reason to assume that it would not have occurred in their intranuclear bacteria. Extensive comparative analyses of the phylogeny of bathymodiolin hosts, their symbionts and their intranuclear bacteria will provide an ideal data set for a better understanding of historical dispersal patterns in these deep–sea associations.

Conclusions

This study shows that intranuclear bacteria are widespread in bathymodiolin mussels from hydrothermal vents and cold seeps. The 16S rRNA sequences of "*Ca.* E. bathymodioli" belong to a monophyletic clade that consists of sequences from bacteria associated with marine metazoans as diverse as sponges, corals, sea slugs, clams, ascidians, sea urchins and fish. Only one of the sequences within this clade is known to originate from an intranuclear bacterium, the previously described 'NIX' parasite from the razor clam *S. patula* (Elston, 1986; Kerk *et al.*, 1992; Ayres *et al.*, 2004) but morphological descriptions of intranuclear bacteria exist for other animal hosts within this clade, for example, from two species of *Aplysina* sponges (Vacelet, 1970; Friedrich *et al.*, 1999) and the venerid clam *Ruditapes decussatus* (Azevedo, 1989) (Table 2). We postulate that many of the sequences within this clade originate from intranuclear bacteria, and that these parasites are widespread in marine animals, including clams and other shellfish consumed by humans.

Table 2. Intranuclear bacteria described to date in Metazoa (excluding facultative intranuclear Rickettsiae).

Metazoa taxon *Host species*	Higher-ranking taxon Designation of bacteria inside nuclei	Shape	Size (µm)	Phylogeny	Reference
Demospongia *Aplysina aerophoba; A. cavernicola*	Porifera Intranuclear bacteria	Filamentous	150–350; at least 4.5	Gamma[a]	Vacelet (1970); Friedrich *et al.* (1999)
Bivalvia *Siliqua patula*	Mollusca 'Nuclear Inclusion X' (NIX)	Multilayered, membrane rich, complexly folded, partially cleaved	25 × 16	Gamma	Elston (1986); Kerk *et al.* (1992)
Rudtapes decussatus	Endonucleobiotic bacteria	Spherical to ellipsoidal	ø 1.3	nd	Azevedo (1989)
Bathymodiolus spp.	"*Ca.* Endonucleobacter bathymodioli"	Rod-shaped to filamentous	1.8 × 0.4; up to 20	Gamma	This study

a. Phylogeny based on FISH with general gammaproteobacterial probe, no. 16S rRNA sequence available.
nd, not determined.

Experimental procedures

Sampling sites and processing

Nine *Bathymodiolus* species from six deep-sea hydrothermal vent fields and five cold seep sites located in the Atlantic and Pacific Ocean were investigated (Fig. 4, Table 1). One to four individuals of each species were examined (Table 1). Upon recovery mussels were immediately transferred into chilled bottom sea water and processed as previously described (Duperron *et al.*, 2005; 2006; 2007). Briefly, gill tissue was frozen and stored at –20°C for DNA extraction as well as fixed for FISH and TEM investigations.

*Cloning and sequencing "*Ca. *E. bathymodioli" from* B. puteoserpentis *(Logatchev)*

Genomic DNA was extracted from gill tissue according to Zhou and colleagues (1996). PCR was performed using the universal bacterial primers GM3F and GM4R (Muyzer *et al.*, 1995) and a high-quality Taq DNA polymerase (error rate 2.7×10^{-5}; Eppendorf, Hamburg, Germany). Amplification products were purified and ligated into pGEM-T Easy vectors (Promega). One Shot TOP10 competent *E. coli* cells (Invitrogen) were subsequently transformed. A total of 1108 positive transformants from four individuals (277 clones per individual) were picked by blue/white screening and grown overnight in V96 MicroWell Plates (Nunc, Wiesbaden, Germany) containing 200 µl Luria–Bertani/ampicillin broth per well. A total of 1009 clones were screened for the right sized insert (252 clones per individual) by PCR using the M13F/M13R primer pair (Yanisch-Perron *et al.*, 1985) and 854 clones had an insert with the expected size of approximately 1500 bp. 384 clones with the right sized insert were partially sequenced (96 clones per individual). PCR products were purified in MultiScreen-HV plates (Millipore) using Sephadex G50 Superfine resin (Amersham Biosciences) and sequenced using the BigDye Terminator v2.0 Cycle Sequencing Kit along with the Genetic Analyzer Abiprism 3100 (Applied Biosystems). The GM3F oligonucleotide (Muyzer *et al.*, 1995) was used as sequencing primer. The resulting partial sequences were analysed with BioEdit Sequence Alignment Editor version 7.0 (Hall, 1999) using the ClustalW implementation (Thompson *et al.*, 1994). Uniquely occurring sequences were ignored. Repetitive sequences were grouped. Three clones per group and individual were fully sequenced by sequencing both the coding and non-coding DNA strands with the sequencing primers M13F, M13R (Yanisch-Perron *et al.*, 1985), 534R (Muyzer *et al.*, 1993), 518F, 1099F and 1193R (Buchholz-Cleven *et al.*, 1997). Cycle sequencing accessories, equipment and conditions were as described for partial sequencing. Sequences were assembled using Sequencher (Gene Codes Corporation, http://www.genecodes.com). The vectorial remnants and the primer sites were discarded.

*Cloning and sequencing "*Ca. *E. bathymodioli" from other bathymodiolin mussels*

Primers specifically targeting the 16S rRNA gene of "*Ca.* E. bathymodioli" (Logatchev) were designed using the reverse

and complementary sequence of probe Bnix64 as a forward primer (bnix-64F: AGCGGTAACAGGTCTAGC) and the sequence of probe Bnix1249 as a reverse primer (bnix-1267R: GCAGCTTCGCGACCGTCT) resulting in a 1203 bp amplification product (for probe design see below). A combination of the bnix-64F primer together with the universal bacterial reverse primer GM4R was eventually chosen to cover 1409 bp of the 16S rRNA gene. Steps for cloning, sequencing and analysing intranuclear bacteria belonging to "*Ca.* E. bathymodioli*"* were as described above for *B. puteoserpentis* with the exception that only 16 transformants per individual were picked and screened for the right sized insert, and the positive clones fully sequenced. The annealing temperatures for the bnix-64F/bnix-1267R and bnix-64F/GM4R primer pairs were 56°C and 45°C respectively. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit along with the Genetic Analyzer Abiprism 3130 (Applied Biosystems).

Accession numbers

The 16S rRNA gene sequences belonging to "*Candidatus* Endonucleobacter bathymodioli" have been registered at the EMBL database (Kulikova *et al.*, 2007) under accession numbers FM162182 to FM162195, and FM244838 (Table S2).

Phylogenetic reconstruction

Sequences were analysed using ARB (Ludwig *et al.*, 2004) and compared with the NCBI nucleotide database using nucleotide BLAST (McGinnis and Madden, 2004), the RDP-X database using the Sequence Match tool (Cole *et al.*, 2007), and the Silva rRNA database using the SINA webaligner (Pruesse *et al.*, 2007). Highly similar sequences were included in the analysis and aligned using ClustalX. Positions displaying more than 25% gaps as well as positions ambiguously aligned were removed from the analysis. The final alignment comprised 1416 positions. Phylogenetic analyses were performed using Bayesian as well as maximum likelihood analysis. The former was run using MrBayes 3 (v3.1.2) (Ronquist and Huelsenbeck, 2003) under a General Time Reversible model along with Gamma-distributed rates of evolution and a proportion of invariant sites. Analyses were performed for 2 000 000 generations using four parallel Monte Carlo Markov chains. Sample trees were taken every 1000 generations. Posterior probabilities calculated over 5000 best trees were used as support values for nodes in the tree. The maximum likelihood analysis was performed using PHYLIP based on 100 jumble replicates. To assess the robustness of nodes, 1000 ML bootstrap replicates were run. The alignment used for the phylogenetic analysis can be obtained from the EMBL database (accession number: Align_001264).

Statistical analyses

To test the hypothesis that genetic distances between "*Ca.* E. bathymodioli" 16S rRNA sequences were correlated with their geographical distances, a Mantel test was performed using the program R-package (Casgrain and Legendre, 2008). Geographical distances were estimated using Google Earth v.4 based on minimum oceanic distances between sample locations.

*Design of probes targeting "*Ca. *E. bathymodioli"*

Based on the 16S rRNA full sequence (1468 bp) of "*Ca.* E. bathymodioli" from *B. puteoserpentis* (Logatchev) three probes were designed using the probe design tool of ARB (Ludwig *et al.*, 2004). To verify their specificity, probes Bnix64 (GCTAGACCTGTTACCGCT), Bnix643 (CCGTACTCTAGC CACCCA) and Bnix1249 (GCAGCTTCGCGACCGTCT) were checked against the 16S dataset of the Ribosomal Database Project X using the online Probe Match tool (Cole *et al.*, 2007). The most recent probe match against the RDP-X dataset 10.4 (October 2008) revealed that probe Bnix64 targeted also 12 gammaproteobacterial sequences belonging to the Oceanospirillales and Alteromonadales. Probe Bnix643 targeted also 13 cyanobacterial sequences, five unclassified gammaproteobacterial sequences and one deltaproteobacterial sequence. However, probe Bnix1249 was confirmed to specifically target "*Ca.* E. bathymodioli" (Logatchev). With the amplification of more "*Ca.* E. bathymodioli" phylotypes from other bathymodiolin species it became evident that the probes Bnix1249 and Bnix643 had one mismatch each to some of the other intranuclear phylotypes (Table S2). All three probes are deposited in the oligonucleotide probe database 'probeBase' (Loy *et al.*, 2007) under accession numbers pB-01516 to pB-01518.

All probes were fluorescently labelled (biomers.net, Ulm, Germany). Specific hybridization conditions for all three probes were determined by varying the formamide concentration in the hybridization buffer (Pernthaler *et al.*, 2002). All probes hybridized equally well with the target organism at 35% formamide concentration. Probe EUB338 (Amann *et al.*, 1990) covering most bacteria was used as a positive control and the NON338 probe (Wallner *et al.*, 1993) as a control for background autofluorescence.

Fluorescence in situ *hybridization*

Subsamples of *B. puteoserpentis* tissues such as gill, gut, digestive gland, labial palps, mantle and foot were fixed in 1× phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) containing 2% paraformaldehyde at 4°C for 9–18 h. Samples were washed three times by placing them in fresh 1× PBS for 10 min each time and subsequently transferred into cold PBS/ethanol solution containing 1× PBS and pure ethanol in equal parts. Samples were kept at 4°C on board the research vessel, air-freighted back to the laboratory at 4°C and finally stored at −20°C. Fixed specimens were embedded in Steedman's Wax (Steedman, 1957) and sectioned with a microtome into 10 μm thick sections. The sections were placed onto Super-Frost slides (Fisher Scientific), dewaxed in three successive baths of absolute ethanol for 5 min each and air dried. Sections were then covered with 200 μl of hybridization buffer (Pernthaler *et al.*, 2002) containing fluorescently labelled oligonucleotide probes (5 ng μl^{-1} final concentration), covered with a glass coverslip and hybridized at 46°C for 10 min in a

histological microwave oven (Microwave Research and Applications, Laurel, MD, USA) with the power output set to 20%. The slides were rinsed in 1× PBS, MQwater, and absolute ethanol for 1 min each. Single hybridizations targeting specifically "*Ca.* E. bathymodioli" (Logatchev) were performed using probe Bnix1249 labelled with Cy3. For visualization of all bacteria in *B. puteoserpentis*, Cy3-labelled EUB338 was used (Amann *et al.*, 1990). Double hybridizations targeting eukaryotic 18S rRNA were performed using Cy5-labelled EUK516 (Amann *et al.*, 1990). Triple hybridizations targeting the chemoautotrophic endosymbiont, the methanotrophic endosymbiont and the eukaryotic 18S rRNA were performed using the probes BMARt-193 (Cy3), BMARm-845 (Cy5) (Duperron *et al.*, 2006) and EUK516 (Fluos). For quadruple hybridizations targeting additionally "*Ca.* E. bathymodioli" (Logatchev) Fluos-labelled Bnix1249 was used.

Deconvolution (restoration) microscopy

The air dried slides were embedded in a DAPI-amended mountant and evaluated on a DeltaVision RT Restoration Microscopy System (Applied Precision, Issaquah, WA, USA) using an Olympus IX71 (Olympus, Center Valley, PA, USA) equipped with appropriate filter sets for Cy3, Cy5, DAPI and fluorescein. For image capture and deconvolution the SoftWorx image analysis software was used (Applied Precision, Issaquah, WA, USA). Images were further processed and analysed using the Imaris software package (http://www.bitplane.com) that was also used to assign colours to the different wavelengths.

Transmission electron microscopy

Gill filaments of *B. puteoserpentis* from Logatchev were fixed in a modified Trump's fixative (McDowell and Trump, 1976) (0.05 M sodium cacodylate solution containing 2% glutaraldehyde and 2% paraformaldehyde, pH 7.3) and stored therein for several months. The tissues were dehydrated in an ethanol series and embedded in the acrylic resin LR White (Sigma). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Zeiss EM 109-S2. Transmission electron microscopy was performed on five adult specimens. Fifty-seven gill filaments were investigated in total.

*Estimation of "*Ca. *E. bathymodioli" numbers in Stage 6 aggregates*

The total number of "*Ca.* E. bathymodioli" cells in a Stage 6 aggregate was estimated by dividing the volume of an aggregate by the volume of a single rod-shaped bacterial cell. The volume of aggregates was calculated by assuming that these were spheres $\left(V = \frac{1}{6}\pi d^3\right)$. The diameter d of Stage 6 bacterial aggregates was determined from several FISH and four TEM images which ranged between 16 and 32 μm. The volume of single rod-shaped bacterial cells was calculated by assuming that these were cylinders capped at both ends by hemispheres $\left[\text{spherocylinder}, V = \frac{1}{2}\pi d^2\left(\frac{1}{2}h + \frac{1}{3}d\right)\right]$. The length h and diameter (width) d was determined for several intranuclear cells using TEM images (average size 1.8×0.4 μm, it follows that $h = 1.4$ μm, $d = 0.4$ μm).

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Supporting information

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

Fig. S1. Figure 2 of main document in original RGB mode (for figure caption, see Fig. 2 in main document).
Table S1. Closest relatives of "*Candidatus* Endonucleobacter bathymodioli" (Logatchev).
Table S2. Nucleotide differences of dominant and minor "*Ca.* E. bathymodioli" 16S rRNA sequences.
Table S3. Intranuclear bacteria described from protists.
Videos. Animations show stacks of 2D images created by moving the focal plane through the z-axis of 10 μm thick sections at 0.2 μm intervals. **S1:** developmental stage 2; **S2:** developmental stage 3; **S3:** developmental stage 4; S4: developmental stage 6.

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Part IV

Concluding remarks

Chapter 8

General Summary, Conclusions and Outlook

8.1 Symbiont diversity in Chapopote

My marine microbiology studies started after the discovery of the Chapopote Seep in the Gulf of Mexico. I finished my Bachelor studies analyzing the microbial diversity of sediment samples coming from this unusual site. It was quite interesting to see that microbial communities were different in two different but nearby spots within the site. It seemed that the presence/absence of oil in the samples was determining the microbial diversity profile. I was very excited when I was allowed to analyze the *Bathymodiolus* spp. coming from this same site as part of my PhD thesis work. These mussels had been studied all around the world and in particular in the northern Gulf of Mexico (GOM). Then, it was very interesting to get to characterize this *Bathymodiolus*-bacteria symbiosis in this new habitat. If the free-living bacterial community of two very close spots was already very different, I thought with more reasons the symbionts from two very distant sites on each side of the Gulf of Mexico would differ greatly. My knowledge about symbiosis was very limited at that time and I never took into account the very fine system that these associations are. Symbiosis is such a specific systems that when I analyzed the mussels from the southern GoM, mussels and their bacteria were exactly the same (based on standard COI and 16S rRNA analysis) as the ones from the north. 1000 miles of separation was meaning nothing for the symbiosis profile. However, two bacterial phylotypes, different to the classical symbionts, seemed to be present after the 16S rRNA sequence analysis.

They were indeed present after localization by FISH. The most surprising one was the phylotype related to cultivated *Cycloclasticus* sp. bacteria, because this bacterium seemed to cheat the host-symbiont recognition system and could co-habit with the chemosynthetic symbionts. Whether this new symbiosis described with detail in the body of this thesis is a stable association, we do not know. I have analyzed the two *B. heckerae* organisms where *Cycloclasticus* phylotype was found, but we would need to have a better sample collection to be able to do statistical analysis about the distribution of this new association. The re-visit of Chapopote site is essential to do a further characterization of this symbiosis. Experiments *in situ* could be then performed, as the injection of labeled hydrocarbons to site and the collection of the mussels to analyze C incorporation. Alternatively, incubation of fresh gills on board with the labeled hydrocarbons could be done. However, if this is not performed in a pressurized chamber and with all the abiotic factors controlled, the metabolic mechanisms might be very far from the original ones. It might also be interesting to try cultivation of bacteria from homogenized gills as not being an obligate symbiont might make easier to growth it on culture. Actually, the current knowledge of the *Bathymodiolin* mussels about transmission tells us that transmission in this system is horizontal (or environmental) and bacterial symbionts should have a free-living stage. With the right conditions the symbiotic bacteria should be cultivable. However, cultivation is not an easy task and therefore cultivation-independent methods are an alternative. Isolation or enrichment of symbionts with physical mechanisms are in development and this is opening the doors to have complete genomes to analyze genomic evolution and biochemical pathways important in the description of symbiotic interactions. But for performing any of these experiments that I mentioned here above, more *Bathymodiolus* mussels from Chapopote would be needed.

8.2 The S and P concept

The S concept is applicable to many of the bacteria associated with the bivalves studied in this thesis, while the thiotrophic and methanotrophic en-

dosymbionts of *Bathymodiolus* mussels are clearly P-symbionts. S-symbionts from this study could be *Cycloclasticus*-related phylotype, the NIX phylotypes and possibly all bacteria described in shallow-water mussels. Of course more physiological characterization to determine whether these bacteria are mutualists, commensalists, or parasites is still needed. Nonetheless, this study is one of the first molecular characterizations about bacterial diversity in bivalves. It is clear that bacteria like NIX-bacteria are S-symbionts as they are not always present in the organism, the host does not need them obligatorily and the bacteria have a parasitic behavior. For *Cycloclasticus*-bacterium we have a different set up as this bacterium might be becoming a P-symbiont. However, population ecology and physiology studies to analyse the specific recurrence and activity of this phylotype would be needed to describe this species as a P-symbiont. The shallow-water bivalves of this study seem to be associated only to S-symbionts, however, it is still an early hypothesis because not many studies are at hand. After this study and the previous ones there are some non-pathogenic bacteria that seem to be present regularly: *Vibrio* spp., *Shewanella* spp. and spirochetes. *Vibrio* species present in bivalves are most of the time non-pathogenic. I suggest they could be protecting pathogenic ones of infecting the host. Having then a mutualistic role as in the symbiosis of squid-*Vibrio*, where *Vibrio* has a function of protection. In this case, the *Vibrio* function would be through competition and not through luminescence. *Shewanella* are marine bacteria that have the ability to chelate metals, they might then be protecting bivalves from the accumulation of them. Spirochetes seem to be also well adapted to bivalves tissue. They are frequently present in bivalves and with a particular distribution. However the role of any of the non-chemosynthetic bacteria in bivalves has not been studied in a process-oriented way and there is the lack of information not just in the whole physiological direction but also in the pure phylogenetic characterization and biological distribution. I think the cultivation of bacteria associated to bivalves should be further established to then be able to do experiments with sterile bivalves (as treated with antibiotics) and not just observe the effect on the bivalves but study the active proteins and sugars to unveil the interaction processes.

8.3 Conclusions

This PhD thesis contributes to the understanding of the diversity of chemosynthetic and non-chemosynthetic bivalve symbioses. Bivalves are a worthy model to study symbiosis: 1) they have a simpler immunological system than vertebrates, 2) many of the species are large organisms which gives more area and biological material to work with, 3) they are vectors for transmission of bacterial and virus diseases to humans. And finally, 4) in the phylogenetic evolution of bivalves we can observe the different physiological behaviours with every different association: from extracellular heterotrophic to the intracellular chemosynthetic ones. Thus it is always important to analyze both bivalve and symbiotic bacteria phylogenies to be able to characterize the evolution of bivalve symbioses. Bivalve communication system might be similar within the whole group, then non-chemosynthetic bivalves could give us answers about the communication between bacteria and their bivalve hosts in a chemosynthetic symbiosis.

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Glossary

Bacteriocyte specialized cell of an eukaryotic organism that bears symbiotic bacteria.
Ectosymbiont inhabiting outside the organism.
Endosymbiont inhabiting inside the host cell (endocellularly).
Endogenous that live inside the organism, but not necessarily endocellularly.
P-symbiont the primary and most abundant symbiont in a host organism. Generally they are vertically transmitted endosymbionts.
S-symbiont the secondary and less abundant symbiont in a host organism. Generally they are horizontally transmitted and their presence is facultative.
Vertical transmission symbionts are transmitted from the parents to the offspring.
Horizontal transmission symbionts are taken from the environment whether direct or indirect contact with host related organism.
Facultative symbionts their presence is not obligatory for host survival.
Chemosynthesis - Chemosynthetic organisms convert one or more carbon molecules (usually carbon dioxide or methane) and nutrients into organic matter using methane (methanotrophs) or inorganic molecules such as hydrogen sulphide (thiotrophs) as a source of energy, rather than sunlight, as in photosynthesis.
Thiotrophy Thiotrophic organisms or sulphur oxidizers (also called chemoautotrophic) use reduced sulphur compounds (e.g. hydrogen sulphide) as electron donors and fix CO_2 to generate their organic matter.
Methanotrophy - is a special case of methylotrophy, using single-carbon compounds that are more reduced than carbon dioxide, as a carbon and energy source.
Autotroph - organism able to synthesize organic compounds from CO_2.
Heterotroph organism with a nutrition that is not based in CO_2 but in the uptake of organic compounds.

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Erklärung

Gem §6 (5) Nr. 1 - 3 Promotionsordnung erkläre ich hiermit, dass ich die Arbeit mit dem Titel:

Bacterial-invertebrate symbioses: from an asphalt cold seep to shallow waters

1. ohne unerlaubte fremde Hilfe angefertigt habe,

2. keine anderen, als die von mir angegebenen Quellen und Hilfsmittel benutzt habe,

3. die den benutzten Werken wrötlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Ebenfalls erkläre ich hiermit, dass es sich bei den von mir abgegebenen Arbeiten um drei identische Exemplare handelt.

Luciana Raggi Hoyos

Bremen,