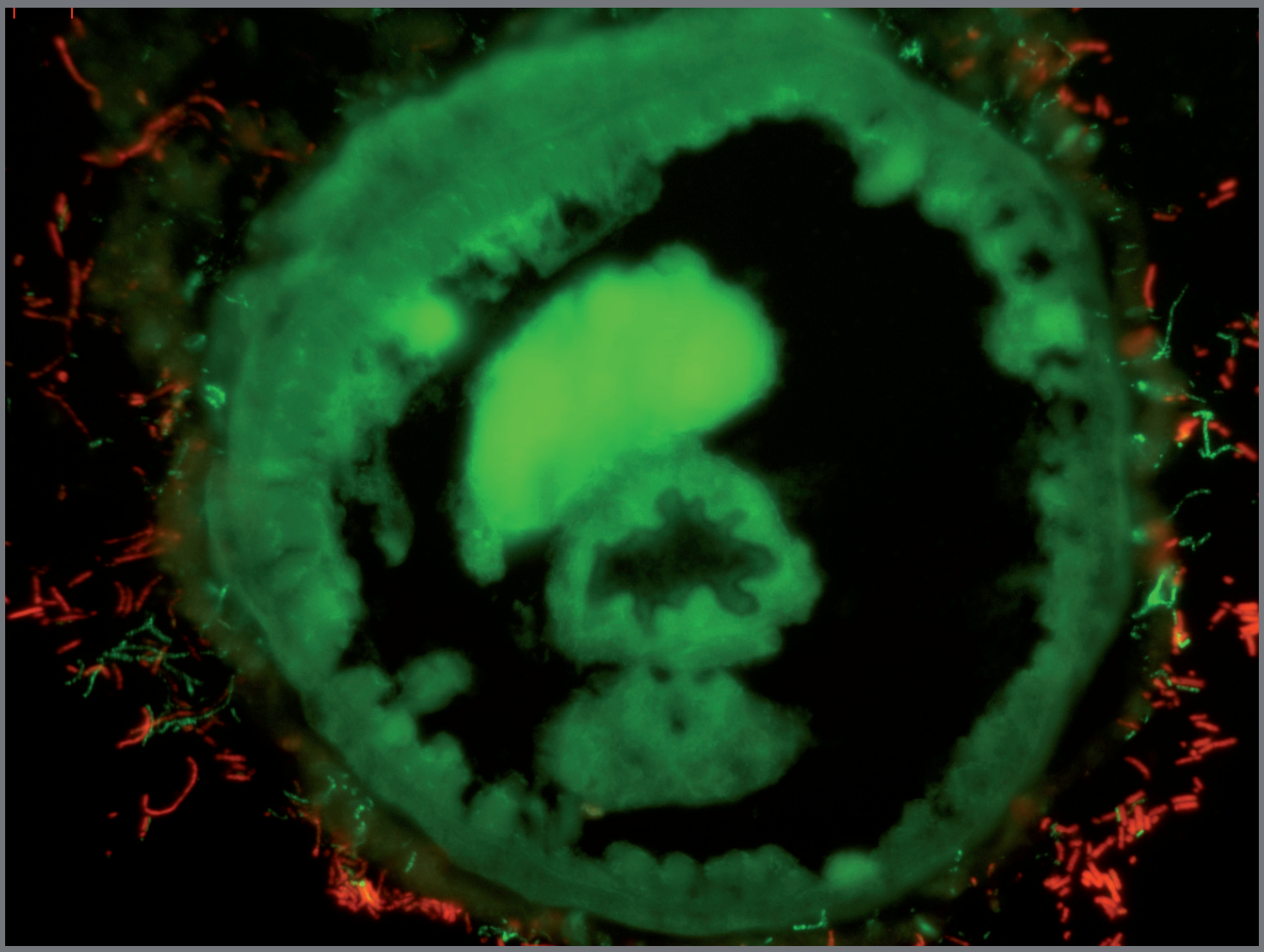


Characterization of bacterial endo- and ectosymbionts of oligochaete worms from marine sediments: Phylogeny and metabolic potential

Caroline Rühland

Characterization of bacterial endo- and ectosymbionts of oligochaete worms from marine sediments: Phylogeny and metabolic potential

Dissertation

zur

Erlangung des Grades eines

Doktors der Naturwissenschaften

- Dr. rer. nat. -

dem Bereich Biologie/Chemie der

Universität Bremen vorgelegt von

Caroline Rühland

Bremen 2010

Die vorliegende Doktorarbeit wurde am Max-Planck-Institut für Marine Mikrobiologie in Bremen angefertigt.

1. Gutachter: Prof. Dr. Rudolf Amann
2. Gutachter: Dr. Nicole Dubilier

Meiner Mutter

Table of Contents

Summary

Chemosynthetic marine environments have led to unique animal-bacteria associations. The discovery of chemosynthetic associations at hydrothermal vents and seeps in the deep-sea in the 1970s led to a turning point in symbiosis research. Only thereafter were chemosynthetic symbioses recognized to occur also in coastal sediments. In this thesis two obligate bacterial endosymbioses (1, 2) and a facultative ectosymbiosis (3) of gutless and gut-bearing marine oligochaetes from coastal sediments were characterized.

(1) Marine gutless oligochaetes lack a mouth, gut and excretory sytem. Instead they harbor specific bacterial consortia, in a layer between their cuticle and epidermis. The worms travel the redoxcline providing their primary sulfur-oxidizing symbionts with sulfide and oxygen. These symbionts use the energy gained by sulfide oxidation to fix CO_2 for producing organic compounds, from which the hosts also profits. Thus, the presence of the key electron donor sulfide is essential to the symbionts and their hosts that depend on them. The gutless oligochaete *Olavius algarvensis,* however, was found in coastal sediments of the mediterranean island Elba with extremely low sulfide concentrations. Molecular 16S rRNA analysis and fluorescence in situ hybridization (FISH) showed the presence of a Gammaproteobacterium belonging to the clade of known sulfur-oxidizing symbionts from gutless oligochaetes, and unexpectedly a Deltaproteobacterium for which the potential for sulfate reduction potential was suggested by successful amplification of the *dsrAB* gene. Sulfide production in the worms was confirmed by autoradiography and sulfate reduction rate measurements. Thus, the deltaproteobacterial symbionts were identified as sulfate reducers that provide the sulfide-oxidizing symbionts with an internal source of sulfide, explaining how the worms could live in a sulfide-poor environment.

(2) In the second part of this thesis, the symbiotic communities of the co-occurring hosts, *Olavius ilvae* and *O. algarvensis* were compared using molecular techniques. Extended 16S rRNA analysis, FISH, and catalyzed reporter deposition FISH (CARD-FISH) revealed that the symbiont community of *O. ilvae* was similar to that of *O. algarvensis* except for an additional spirochete in *O. algarvensis*. Both hosts harbored two gamma- and two deltaproteobacterial symbionts. Thioautotrophic and sulfate-reducing potential were demonstrated by comparative sequence analysis of genes indicative for sulfur metabolism (*aprA* and *dsrAB*) and autotrophic carbon fixation (*cbbL*) suggesting that in both host species not only the composition but also the function of the symbiotic community were similar with the potential for syntrophic sulfur cycling. The phylogenetic and metabolic similarity of symbionts in the two co-occurring but not closely related hosts suggested that sulfur syntrophy provided a selective advantage that enabled the worms to colonize sulfide-limited sediments.

(3) *Tubificoides benedii* is a marine oligochaete that has a functioning gut and gains its nutrition by ingesting sediment particles. It lives in eutrophic intertidal mudflats of the North Sea and is adapted to the extreme fluctuations of oxygen and sulfide in this habitat. Its posterior end is facultatively colonized by a dense cover of filamentous bacteria. In some of

the filaments the basal cells have penetrated the cuticle and are anchored within it, suggesting a specific association. Correlation of high bacterial abundance with sulfidic conditions and filament morphology resembling that of free-living sulfur oxidizers like *Thiothrix* led to the assumption that these ectofilamentous bacteria were sulfur oxidizers. Recovery of genes for autotrophic carbon fixation (*cbbL* and *cbbM*) and sulfur metabolism (*aprA*) in the third part of this thesis indicated chemosynthetic potential of the epibiotic community of *T. benedii*. Phylogentic analysis of the 16S rRNA genes and fluorescence in situ hybridization revealed that the filamentous community was dominated by two morphologically distinct phylotypes: A thicker Gammaproteobacterium was attached to the exterior of the cuticle while a thinner Epsilonproteobacterium penetrated it. Phylogenetic analyses revealed that both ectosymbionts belonged to clades that consisted nearly exclusively of bacteria associated with deep-sea invertebrates from hydrothermal vents such as the shrimp *Rimicaris exoculata*, the crab *Shinkaia crosnieri* and the barnacle *Vulcanolepas oshehai*. Such close relationships between symbionts from shallow-water and deep-sea marine hosts that are not closely related to each other are unusual, and indicate that these associations were apparently not influenced by biogeography or host affiliation but by environmental conditions, i.e. environments characterized by high fluctuations of oxidants and reductants.

This thesis contributed to symbiosis research with two unexpexted findings. Because of the toxicity of sulfide, bacterial sulfate reducers were considered unlikely candidates for invertebrate endosymbiosis. However, in this thesis sulfate-reducing Deltaproteobacteria were not only identified in two gutless oligochaetes but shown to fulfill an essential function in these symbioses engaging in an endosymbiotic sulfur cycle with the co-occurring sulfide-oxidizing symbionts. A second investigation revealed the similarity of a filamentous ectosymbiosis of a shallow-water worm to that of deep-sea invertebrates not described before.

Zusammenfassung

Der Fülle an energiereichen anorganischen Substraten hat fernab von Licht und Photosynthese in marinen chemosynthetischen Lebensräumen die Entwicklung außergewöhnlicher Bakterien-Tier-Assoziationen ermöglicht. Mit der Entdeckung chemosynthetischer Assoziationen an Hydrothermalquellen in der Tiefsee in den 1970er Jahren gelangte die Symbioseforschung zu einem bedeutenden Wendepunkt. Erst aufgrund dieser Entdeckung wurde im Zuge weiterer Forschungen wahrgenommen, dass chemosynthetische Symbiosen auch in Küstensedimenten vorkommen. In der vorliegenden Dissertationsarbeit wurden zwei obligate bakterielle Endo- (1, 2) und eine fakultative Ektosymbiose (3) mariner darmloser und darmtragender Oligochaeten aus Küstensedimenten charakterisiert.

(1) Darmlose Oligochaeten haben ein reduziertes Verdauungssystem, ihnen fehlen Mund, Darm und Ausscheidungsorgane. Stattdessen beherbergen sie verschiedene Bakterien zwischen Kutikula und Epidermis. Die Oligochaeten wandern im Sediment zwischen sulfidischen und oxidierten Schichten und ermöglichen damit ihren obligaten sulfidoxidierenden Primärsymbionten die Chemosynthese. Diese Symbionten nutzen die durch Chemosynthese gewonnene Energie zur CO_2-Fixierung und der Produktion von organischen Kohlenstoffen, von denen letztendlich auch der Wirt profitiert. So ist es essentiell für die Symbionten und damit auch ihren Wirt, dass ihr Habitat genügend Sulfid bietet. Der darmlose Oligochaet *Olavius algarvensis* jedoch lebt in mediterranen Küstensedimenten der Insel Elba, in denen die Sulfidkonzentration sehr gering ist. Molekulare 16S rRNA Analyse und FISH-Untersuchungen zeigten, dass diese Würmer ein Gammaproteobakterium beherbergten, das mit anderen thioautotrophen Primärsymbionten dieser Wirtsfamilie verwandt ist. Als Novum in marinen Evertebratensymbiosen besaß *O. algarvensis* jedoch zusätzlich ein Deltaproteobakterium, verwandt mit freilebenden sulfatreduzierenden Bakterien. Amplifizierung des *dsrAB* Gens bestärkten die Annahme, dass die Symbionten Sulfat reduzieren können. Mittels Autoradiographie und Sulfatreduktionsratenmessungen konnte eine interne Sulfidproduktion im Wurm nachgewiesen werden. So wurden die deltaproteobakteriellen Symbionten als Sulfatreduzierer, die den sulfidoxidierenden Primärsymbionten intern mit Sulfid versorgen, identifiziert. Ein interner syntropher Schwefelkreislauf zwischen Gamma- und Deltasymbionten kann demnach eine Erklärung dafür sein, wie der Wurm in diesem sulfidarmen Habitat leben kann.

(2) Im zweiten Teil dieser Arbeit wurde die Symbiontengemeinschaft einer zweiten Art aus Elbasedimenten, *O. ilvae*, mit der von *O. algarvensis* vergleichend charakterisiert. Eine ausführliche 16S rRNA Analyse sowie Fluoreszenz in situ Hybridisierung (FISH) und katalysierte FISH (CARD-FISH) ergaben, dass die beiden Wirte ähnliche Bakteriengemeinschaften beherbergten bis auf einen zusätzlichen Spirochaeten in *O. algarvensis*. Beide Wirtsarten besaßen zwei gamma- und zwei deltaproteobakterielle Symbionten. Das

Potenzial für Thioautotrophie beziehungsweise Sulfatatmung wurde durch die vergleichende Sequenzanalyse von Genen indikativ für Autotrophie (*cbbL*) und Schwefelmetabolismus (*aprA* and *dsrAB*) aufgezeigt. Die phylogenetische und physiologische Ähnlichkeit der beiden bakteriellen Symbiosegemeinschaften in den beiden nur entfernt verwandten aber gemeinsam vorkommenden Wirtsarten deuten darauf hin, dass Schwefelsyntrophie den Tieren einen ökologischen Vorteil zur Besiedlung sulfidarmer Sedimente erbrachte.

(3) *Tubificoides benedii* ist ein mariner Oligochaet mit Darm, der sich durch die Aufnahme von Sedimentpartikeln ernährt. *T. benedii* lebt in eutrophierten Wattenmeersedimenten und ist an starke Sauerstoff- und Sulfidfluktuationen angepasst. Sein Hinterende wird fakultativ durch einen dichten Bewuchs filamentöser Bakterien kolonisiert. Die Korrelation von Bakterienabundanzen mit Sulfidgehalt und die morphologische Ähnlichkeit der Filamente mit freilebenden sulfidoxidierenden Bakterien wie z.B. *Thiothrix* spp. führte in vorhergehenden Untersuchungen zu der Vermutung, dass auch die Ektobakterien Sulfidoxidierer sind. Die im dritten Teil dieser Studie erfolgreiche Amplifikation von Genen indikativ für Autotrophie (*cbbL*) und Schwefelmetabolismus (*aprA*) aus *T. benedii*-Hinterenden zeigten das Potenzial für Thioautotrophie der *T. benedii* besiedelnden bakteriellen Gemeinschaft. Mit Hilfe von phylogenetischen Analysen und FISH wurden die Filamente als ein dickeres Gammaproteobakterium, das auf der Kutikula aufsitzt und ein dünneres Epsilonproteobakterium, das die Kutikula durchdringt, identifiziert. Phylogenetische Analysen zeigten, dass beide Ektosymbionten phylogenetischen Gruppen angehörten, die nahezu nur aus Bakterien bestanden die in Gemeinschaft mit Tiefsee-Evertebraten, wie der Tiefseekrabbe *Rimicaris exoculata*, dem Krebs *Shinkaia crosnieri* und der Seepocke *Vulcanolepas oshehai*, leben. So eine nahe Verwandtschaft zwischen Ektosymbionten verschiedener Wirtstiere aus dem Flachwasser und der Tiefsee ist ungewöhnlich und deutet darauf hin, dass Biogeographie und Wirtsverwandtschaft in der Entstehung und Entwicklung dieser Assoziationen keine große Rolle spielten, sondern vielmehr die Umweltbedingungen, d.h. hohe Fluktuationen anorganischer Elektronendonatoren und -akzeptoren.

Diese Dissertationsstudie konnte mit zwei unerwarteten Entdeckungen zur Symbioseforschung beitragen. Aufgrund der toxischen Wirkung von Sulfid wurde es für unwahrscheinlich erachtet, dass Sulfatreduzierer als Endosymbionten von Evertebraten vorkommen. In dieser Studie wurden jedoch sulfatreduzierende Deltaproteobakterien nicht nur als Endosymbionten nachgewiesen, sondern auch gezeigt, dass sie eine essentielle Funktion haben können, indem sie einen endosymbiontischen Schwefelzyklus mit den sulfidoxidierenden Symbionten unterhalten. In einer weiteren Studie dieser Arbeit konnte eine große Ähnlichkeit zwischen der filamentösen Ektosymbiose eines Wattenmeerwurms und der Ektosymbiosen von Tiefseeinvertebraten gezeigt werden. Diese Ähnlichkeit ließ darauf schließen, dass sich diese Assoziationen unabhängig von der geographischen Lage und der Wirtsverwandtschaft in Habitaten, die sich durch starke Fluktuationen der Oxidantien und Reduktoren auszeichnen, entwickelt haben.

List of abbreviations

AprA	adenosine-5’-phosphosulfate reductase, alpha subunit
APS	adenosine-5'-phosphosulfate
ASW	artificial seawater
CARD	catalyzed reporter deposition
CBB	Calvin-Benson-Bassham
CbbL	rubisCO form I, large subunit
CbbM	rubisCO form II, large subunit
CoA	co-enzyme A
DNA	deoxyribonucleic acid
Dsr	dissimilatory sulfate reductase
Fcc	flavocytochrome *c* sulfide dehydrogenase
FISH	fluorescence in situ hybridization
kDA	kilo Dalton
LSM	laser scanning microscopy
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PHA	polyhydroxyalkanoate
PHB	poly-β-hydroxybutyric acid
RNA	ribonucleic acid
rRNA	ribosomal RNA
RubisCO	ribulose-1, 5-bisphosphate carboxylase/ oxygenase
SEM	scanning electron microscopy
SIMS	secondary ion mass spectrometry
SOR	sulfur-oxygenase-reducatse
SOX	sulfur-oxidizing bacteria
Sox	sulfur oxidation system of SOX
S_{ox}	oxidized sulfur compounds
sp.	species
spp.	several species
Sqr	sulfide:quinine oxidoreductase
SRB	sulfate-reducing bacteria
S_{red}	reduced sulfur compounds
TCA	tricarboxylic acid
TEM	transmission electron microscopy
TMAO	trimethylamine N-oxide

I Introduction

Microorganisms are extremely versatile and adaptable. With doubling times ranging from several hundred or an estimated 120 thousand years (Parkes and Wellsbury, 2004) to less than half an hour they have adapted to about any environment on earth from extremely barren to the lavish, eutrophic and anthrophogenically contaminated habitats, making up a major part of the Earth's biomass. Contact with other life forms led to the invasion of new habitats: microorganisms and later eukaryotes became hosts in symbiotic relationships. Symbiosis has strongly influenced evolution. In fact eukaryotes evolved through a series of symbiotic relationships. Chloroplasts were already in 1905 suggested to be derived from bacteria (Mereschowsky, 1905; Martin and Kowallik, 1999). Furthermore, mitochondria were first described by Altmann in 1890 as bacteria in a host cell (Altmann, 1890). Their nature as intracellular bacteria adjusted to life in a host cell was further propagated by Wallin in the 1920s (Wallin, 1923; Wallin, 1925). In 1970 this theory was revived as a hypothesis put forward that energy compartments such as mitochondria stem from bacterial endosymbioses (Margulis, 1970; Nakagawa and Takai, 2008).

1 Defining symbiosis

Symbiosis was first defined as the close living together of two unrelated species or organisms (de Bary, 1879). The novel non-parasitic nature of this alga-fungus association had been discussed previously by Schwendener (Schwendener, 1867). At about the same time the term 'symbiotism' was used to describe spatial proximity of different organisms in mycorrhizae (Frank, 1877). The term is derived from Greek *Sym* meaning 'with' and *biosis* 'living'. Such a long-term or permanent association can be 1) beneficial to one and harmful to the other organism in parasitism, 2) beneficial to one partner but indifferent to the other or indifferent to both (e.g. when they share the same food-source without affecting each other) in commensalism, and 3) beneficial to both in mutualism. Originally the term symbiosis was applied in a non-judgemental context; however, today "symbiosis" is often used in the sense of mutualism. These different concepts of symbiosis often originate from metabolically and phylogentically similar relationships. Transitions between parasitism, commensalism and mutualism can be continuous. Symbiosis should thus be referred to - as it will be in this thesis - in its original broad definition of different species living together in a

close physical and/or metabolic association. In general, the smaller partner in such an association is called the symbiont, while the larger is called the host.

1.1 Characterizing symbiosis

As bacteria can thrive virtually everywhere, contacts and interaction between microbes and eukaryotes are inevitable. Many forms of symbioses between prokaryotes and eukaryotes have evolved to a myriad of pathogenic, opportunistic, commensal and mutualistic bacterial associations. Fascinating adaptations to symbiotic life-style have arisen in co-evolution. Many parasites such as the malaria causing agent *Plasmodium* have complex life-cycles sometimes involving not only a final but also an intermediate or reservoir host. They also have several morphological and developmental stages including asexual and sexual proliferation (Mehlhorn and Piekarski, 1995). Sometimes even the host's behavior is influenced to guarantee the continuation of the symbiont's life-cycle. This has been shown mostly in parasitic species and is thought to occur as well in commensal symbionts (Jog and Watve, 2005). Certain changes in host behavior increase bacterial infection within a host population such as reduced dispersal behavior of female hosts of the spider *Erigone atra* (Goodacre et al., 2009) or intraspecific coprophagy of termites, i.e. eating faeces of mates for the acquisition of mutualistic gut flora (Kikuchi and Fukatsu, 2008), which can lead to complex social behavior (Nalepa et al., 2001; Minkley et al., 2006).

In a race of arms between the microbial invader and the host commonly arises as a result of contact and infection. The mechanisms of invasion and in response evasion or defense are continually improved. While in mammals antibodies counteract invading bacteria and B-cells "memorize" the antigen for a fast host-response, bacteria and viruses overcome this defense by continuously altering their surface structure. Intracellular bacterial symbioses challenge the immune system and medical efforts when pathogens such as *Rickettsia* 'hide' in host cells.

The symbiont benefits from a symbiotic lifestyle in several ways. The host offers access to substrates, including waste products as well as a safe habitat for the much smaller partner. In a mutualistic relationship the hosts in turn acquire new physiological traits through their symbionts. Sometimes these are defensive in nature, protecting the host against pathogens or predators, for example by production of antibiotics or toxins. In most cases the symbioses are nutritional, in which the symbiont supplies the host with carbon compounds and other vital products such as

amino acids and vitamins. The host can exploit new energy and nutritional sources through the metabolic potential of the prokaryotes such as the breakdown of complex carbon compounds (e.g. cellulytic bacteria in rumen and termite gut), photosynthesis or the coupling of oxidation of reduced inorganic substances and carbon fixation (chemosynthesis). The “acquired” foreign physiological capabilities often allow the host to colonize new habitats.

Symbioses are facultative or obligate (see also 1.3.5). In facultative symbioses the partners can live, grow and reproduce independently. The partners engage in associations when they come in contact and environmental conditions allow it. During the course of evolution a physiological characteristic of the symbiont can become vital to the host making the symbiosis obligate. An example for this is *Buchnera aphidicola*, a bacterial symbiont of aphids. This symbiont provides essential amino acids and vitamins to the aphids, which they can neither synthesize nor retrieve from their nutrition, phloem sap. The genome of this symbiont is extremely reduced, making a survival outside the host impossible (Moran, 2003). The symbiont has genes for the biosynthesis of amino acids essential for the host in its genome, while genes encoding enzymes for the synthesis of other non-essential amino acids are completely missing (Shigenobu et al., 2000). Apparently the aphid and *Buchnera* share amino acid biosynthesis (Eisen and International Aphid Genomics Consortium, 2010). Host and symbiont are interdependent and none can reproduce without the other (Baumann et al., 1995).

As mentioned above animal-bacteria associations can shift between parasitism, commensalism and mutualism. Human disease can be the result of such a shift. When bacterial fauna of the human gut is disturbed opportunistic bacteria which are normally kept at a low tolerable abundance by the healthy gut flora can proliferate and replace the latter. Furthermore, otherwise beneficial bacteria can acquire pathogenic traits through genetic transfer. In a healthy state *E. coli* and other heterotrophic bacteria are responsible for effectively breaking down food compounds and supplying essential amino acids and vitamins to their host. Yet, certain strains of *E. coli* have aquired ‘pathogenicity islands’ often through horizontal gene transfer, allowing them to cause infections (Oelschlaeger et al., 2002) or diarrhea (Mellies et al., 2001).

1.2 Marine symbioses

Highly diverse symbiotic lifestyles have been discovered in marine environments from beaches to remote areas of the deep-sea. In the euphotic zones besides photosynthetic symbioses such as corals hosting zooxanthellae, heterotrophic symbioses are common. In coastal areas organic matter is readily available, which is degraded by bacteria inhabiting guts of worms and sea urchins (heterotrophic symbioses). The bacteria help in breaking down complex substrates and provide essential nutrients. Sea urchins, for example, rely on their gut bacteria to digest coarse sea-weed. Furthermore the bacteria make up for the low nitrogen content of this diet by fixing nitrogen (N_2), (Barnes et al., 2001).

More extraordinary heterotrophic symbioses occur when large pieces of wood or animal carcasses sink to the seafloor. Wood-boring mussels of the family Teredinidae are commonly found on sunken wood. They host various heterotrophic intracellular bacteria in their gills that facilitate the degradation of the complex organic material such as cellulose (Luyten et al., 2006). Even in the vast organically depleted deep-sea, sunken whale bones are quickly colonized by bacteria. Some invertebrates have specialized on these rare, ephemeric habitats that form oases in otherwise substrate-limited marine areas such as *Osedax* annelid worms whose symbionts can degrade complex carbon compounds including whale oils and collagen (Goffredi et al., 2007).

1.3 Chemosynthetic symbioses

With its lack of light and thus photosynthetic primary production, low temperature and high pressure the deep-sea poses many challenges to higher organisms. For a long time the deep-sea was presumed to be scarcely populated by few well adapted organisms. This still holds true for the majority of the deep-sea. However, at hydrothermal vents and seeps where reduced chemicals are discharged, chemosynthetic primary production made the conquest of the deep-sea by diverse eukaryotic organisms possible. In the late 1970s, scientists on board the deep submersible vehicle Alvin discovered oases with a rich fauna closely associated with deep-sea hydrothermal vents on the Galapagos Ridge (Corliss and Ballard, 1977; Corliss et al., 1979). The discovery of animals such as the tube worm *Riftia pachyptila* living in symbioses with chemosynthetic bacteria that formed highly productive ecosystems revolutionized the understanding of symbiosis (Cavanaugh et al., 1981;

Felbeck, 1981). Until then only photosynthetic symbioses such as lichens or corals and heterotrophic symbioses such as rumen and gut bacteria were known. Furthermore, the deep-sea oases at hydrothermal vents and seeps are the only ecosystems - besides coral reefs where corals host zooxanthellae (photosynthetic dinoflagellates) – that are dominated by symbiotic production (Dubilier et al., 2008).

To date representatives of many animal and protists groups are known to engage in associations with chemosynthetic bacteria (see also Table 1, p17 and Table 2, pp 21-22): Chromalveolata (ciliates), Porifera (sponges), Mollusca (e.g. bivalves and gastropods), Annelida (e.g. polychaetes and oligochaetes), Nematoda (nematodes) and Arthropoda (e.g. crustaceans, amphipods), (Stewart et al., 2005; Cavanaugh et al., 2006; Dubilier et al., 2008; Zielinski, 2008). The advantage of harboring bacterial primary producers in chemically reduced environments has led to a myriad of animal-bacteria associations and cooperations. Due to the fact that at methane- and/ or sulfide-rich habitats these symbioses often dominate the ecosystem in abundance and turnover their biogeochemical importance can be considerable (Van Dover, 2000).

1.3.1 Defining chemosynthetic symbioses

In environments where highly reduced chemicals and oxidants are present, chemosynthesis can take place. Eukaryotic hosts often enhance bacterial chemosynthesis by physically, spatially or temporally bridging the substrates for their mutualistic bacteria. The chemosynthetic symbionts serve as the nutritional basis for the host, where either dissolved organic carbon compounds and other essential substances are passed to the host or the bacteria are taken up and lysed.

Electron donors and carbon substrates of the symbiotic bacteria can differ resulting in distinct terms to describe these processes. In chemoorganoheterotrophy organic compounds serve both as a carbon source and energy source to gain ATP. In contrast, autotrophic prokaryotes fix carbon dioxide (CO_2) and are thus independent of an external organic carbon source. Energy is derived from the oxidation of reduced inorganic compounds and is used to synthesize organic compounds in chemolithotrophy. Potential inorganic electron donors are reduced chemicals such as hydrogen (H_2), reduced sulfur compounds such as hydrogen sulfide (H_2S), iron (Fe^{2+}), and manganese (Mn^{2+}). In symbioses however, reduced sulfur compounds appear as the most common electron donor, followed by methane. Symbioses based

on hydrogen are rather exceptional, but have been reported (Takai et al., 2006; Zielinski, 2008), while the role of iron has been discussed but largely dismissed (Schmidt et al., 2009). As the energy used is based on chemicals instead of light it is often referred to as 'dark energy'. However, chemosynthesis is in not entirely independent of photosynthesis. Oxygen, the most common reducing agent, is originally a product of photosynthetic activity. While some bacteria can use alternative electron acceptors such as nitrate (see below 1.3.7), the majority of chemosynthetic symbionts use oxygen. Oxygen is often preferred as it maximizes energy gain through a high redox potential. Most importantly, animal hosts can only survive in aerobic environments. They need oxygen for respiration. Some can endure longer periods of oxygen limitation, but eventually they have to access oxygen. This is because anaerobic metabolism is much less energy efficient and leads to the accumulation of toxic waste products.

Methane, a simple carbon compound, is an important potential electron donor for bacteria. The Earth crust contains large amounts of this gas. It can be of abiogenic origin and is either mantle derived or is formed through low temperature water–rock interactions (Sherwood Lollar et al., 2006) or of biogenic origin from thermal decomposition of organic matter in deep oceanic sediments. Alternatively, methane is microbially synthesized by reduction of CO_2 in marine sediments and the deep subsurface (Sherwood Lollar et al., 2006). Methane is an organic molecule, therefore methanotrophic bacteria are not defined as chemoautotrophic but rather chemoorganoheterotrophic. The term chemosynthesis encompasses both life-styles. Some invertebrate hosts can engage in dual symbioses with sulfur oxidizers and methane oxidizers such as the snail *Ifremeria nautilei* (Galchenko et al., 1992), as well as some bathymodioline mussels, e.g. *Bathymodiolus azoricus*, and *B. puteoserpentis* (Distel et al., 1995; Duperron et al., 2006) and the *Bathymodiolus*-related mussel *Idas sp*. (Duperron et al., 2008).

Today even certain marine heterotrophic symbioses are mentioned in the context of chemosynthetic symbioses. Whale falls and sunken wood, as pointed out above, supply a habitat for various sulfide oxidizing symbioses through locally anaerobic decomposition of organic materials and the release of sulfide. As they also are habitat to heterotrophic symbioses such as that of siboglinid worms *Osedax* spp. and wood-boring mussels the latter are often included in discussions about chemosynthetic life.

1.3.2 Chemosynthetic habitats at vents and seeps

The chemosynthetic habitats of the deep-sea, hydrothermal vents and seeps, and shallow water vents and seeps will be discussed briefly. Special emphasis will be put on coastal sediments in a separate section as the objects of this study, marine oligochates, live in this habitat.

Hydrothermal vents

Along the mid ocean ridges new seafloor emerges from the spreading apart of tectonic plates and deep-sea hydrothermal vents can form. Cold seawater penetrates through cracks of the earth crust, is heated up and charged with minerals from the hot rock. Reaching temperatures of up to 407 °C (Haase et al., 2007) or even 464 °C (measured once at the South Mid-Atlantic Ridge (SMAR), (Koschinsky et al., 2008)), it pushes back up rising in a plume. Along the ridge also areas of diffuse flow exist, where the temperature is more moderate (about 5 °- 30 °C) due to the mixing of cold bottom sea-water with vent fluids. Many invertebrates such as tubeworms, limpets, clams and mussels settle at these sites. Highly reduced inorganic compounds from the vent fluids serve as electron donors that can be coupled with electron acceptors from the surrounding oxygenated sea-water for exergonic reactions with a large difference in redox potentials.

There are many differences in the geological and chemical settings of vents and these can influence the composition of the symbiotic communities. For example, ultramafic-hosted settings, e.g. the MAR sites Logatchev and Rainbow occur at slow-spreading centers and the vent fluids contain a high amount of dissolved hydrogen and methane. In contrast, basalt hosted vent systems at fast spreading ridges such as the East Pacific Rise contain lower amounts of these gases. Instead, their fluid chemistry is dominated by sulfide.

Cold Seeps

Cold seeps occur at passive and active continental margins at depths of 400 - 8000 m. Passive continental margins are non-seismic, while at active margins, an oceanic crust is pressed against another crust, oceanic or continental crust, and one plate crust is forced into subduction. High concentrations of methane and sulfide which originate from biological or thermogenic transformation of organic matter mix

with cold seawater and allow methane- and sulfur-oxidizing bacteria to accumulate. As at hydrothermal vents, many of these chemosynthetic bacteria are associated with animal hosts.

Animal communities of cold seeps are similar to those of hot vents and have a relatively low diversity compared to coastal environments. Still between the two environments, diversity indices showed significantly higher diversity at seeps than at hot vents (Turnipseed et al., 2003). While at the species level most animals are endemic to seeps or vents, this is less so at the genus level (Sibuet and Olu, 1998; Sibuet and Olu-Le Roy, 2002). Furthermore, the diversity of species decreases with depth (Sibuet and Olu-Le Roy, 2002).

Shallow-water vents and seeps

Hydothermal vent communities above 200 m are referred to as 'shallow-water' (Tarasov et al., 2005);(Dubilier et al., 2008). Shallow water vents and seeps occur worldwide at sites of volcanic activity, e.g. Iceland, Mediterranean or Japan. According to Tarasov et al. (Tarasov et al., 2005) there is a shift at 200 m depth in environmental parameters reflected in community structure and composition. Shallow-water hydrothermal vent communities in contrast to deep-sea vents have few if any vent obligate taxa. Only exceptionally do chemosynthetic symbioses dominate the biomass of shallow water habitats such as *Lamellibrachia satsuma* off the coast of Japan (Hashimoto et al., 1993; Kharlamenko et al., 1995; Tarasov et al., 2005). Shallow water vents are often inhabited by mussels (see below).

1.3.3 Chemosynthetic life in coastal sands and muds

After the discovery of deep-sea vent communities other reduced environments were reinvestigated and searched for symbiotic species. Indeed many eukaryotic species, often previously described to have anatomical abnormalities such as a reduced gut and/ or a bacterial layer, proved to host chemosynthetic bacteria. Coastal muds and sands are highly diverse ecosystems. In many coastal areas reducing sediments are habitat to invertebrates that have only in the last decades been recocgnized as chemosynthetic hosts.

Sulfate reducing bacteria (SRB) play an important role in the productivity of coastal sediments. With high organic input, oxygen is quickly depleted by aerobic heterotrophs. Sulfate is readily available in the marine environment and SRB oxidize

organic substrates while respiring sulfate. Thus reduced sulfur compounds, sulfide are released to the sediment. In the redoxcline they serve as electron donors for thioautotrophic bacteria. Availability of reduced sulfur and penetration of O_2 depend on the porosity of the sediment and the influence of tides or wave action. Dense and muddy sediments often only have a thin oxidized layer especially at low tide which is only a few millimeters thick. In sediments of larger grain size and strong wave action, oxygen can penetrate a few centimeters deep into the sediments. Here, overall sulfide concentrations might be lower than at most vents and seeps, while sulfate reduction can be high due to high organic input in coastal areas. It was suggested that the continuous supply of sulfide through sulfate reduction might be more important to symbiotic associations than the absolute concentrations (Dubilier et al., 2008).

Thus, coastal sediments provide a habitat for non-photosynthetic primary production as well, giving rise to associations between metazoans with chemosynthetic bacteria. Most hosts belong to the smaller macro- and meiofauna (small animals defined as passing through a 500 µm sieve but being retained on meshes of 40 - 64 µm size (Higgins and Thiel, 1988) and are in most cases much smaller than deep-sea invertebrate hosts. While the deep-sea annelid *R. pachyptila* reach up to two meters in length symbiotic worms from coastal sediments are easily overlooked. Symbiotic oligochaetes only measure up to a few centimeters and marine nematodes including those with obligate endo- or ectosymbionts are only seldom larger than 10 mm. This size difference can also be observed for other invertebrates. Coastal symbiotic mussels are generally smaller than their deep-sea relatives.

Diversity in coastal sandy sediments can be as high or even higher than at deep-sea sites (Dubilier et al., 2008). Several species of nematodes with endo- or ectosymbionts occur in this habitat (Ott et al., 2004), as well as gutless oligochaete worms with multiple endosymbionts (Dubilier et al., 2006), turbellarians and ciliates (Fenchel and Finlay, 1995; Dubilier et al., 2008). It is likely that many more animals of this habitat are candidate hosts such as copepods (personal observation). Some copepods from Elba sediment appeared transparent and with white inclusions underneath their thorax. Several mostly unidentified bacterial epibionts of diverse morphology have been observed on copepods and various other crustaceans, such as isopods, amphipods and decapods (Carman and Dobbs, 1997).

Adaptations of symbiotic hosts in sulfidic coastal sediments

To make both electron donors and electron acceptors accessible for their symbionts the host animals have - like their deep-sea counterparts (see below section 1.3.4 endosymbionts) - adapted with anatomical, physical, physiological and/or behavioral traits to bridge the oxic and anoxic, sulfide-rich sediment layers. Motile sediment dwellers such as nematodes and oligochaetes can travel above and below the redox-cline to alternately supply their symbionts with oxidants and reductants. This life style would cater to the ability of many sulfur-oxidizing bacteria to transiently store sulfur under anoxic conditions, which is then completely oxidized in oxic environments.

Some ciliates create a water current that reduces the boundary layer thickness and enhances contact with sulfide and oxygen for their ectosymbionts (Vopel et al., 2001; Røy et al., 2009). Chemosymbiotic clams that occur in inter- and subtidal sediment such as the lucinid *Anodontia edentula* living in sulfidic mangrove muds or thyrasid clams inhabiting reducing coastal North Sea sediments have developed alternative strategies. *A. edentula* buries 28-50 cm deep in the mud thus gaining direct contact to sulfide. Oxygen is transported towards the clam via oxygenated water from the surface through ventilation burrows (Lebata, 2001). Thyasirid clams such as *Thyasira (parathyasira) equalis, T. flexuosa, and T. sarsi* stay in the upper layers of the sediment and can extend their foot up to 30 times their body size to reach reduced sulfur in lower reduced layers for their symbionts (Dufour and Felbeck, 2003).

1.3.4 Symbiont location: ecto- and endosymbionts

Symbioses between animals and bacteria are morphologically and physically diverse. Bacterial endosymbionts live within the host organism. They can be located in specialized structures or organs in the host. Ectosymbionts, in contrast, attach to exterior soft or hard host surfaces.

Endosymbionts

Endosymbionts (Table 1) can occur intracellularly or extracellularly (Stewart et al., 2005). Various siboglinid tubeworms such as *Riftia, Escarpia* and *Lamellibrachia*, frenulates (*Siboglinum*, *Oligobrachia*) and Monilifera (*Sclerolinum*), host intracellular sulfur-oxidizing or methane-oxidizing symbionts (Dubilier et al., 2008). Early studies

Table 1. List of marine invertebrate hosts of (putative) chemosynthetic endosymbionts.

Phylum/ major group	Host	Common name	Symbiont-supporting tissue	Location	Habitat	Symbiont type
Ciliphora Polyhemonophora *Heterotrichida*	*Folliculinopsis*	Blue-mat ciliate	cytoplasm (also ectobionts)	Intracellular	hydrothermal vents	Unknown
Karyorelictea Kentrophoridae	*Kentrophorus*	Free-living ciliate	cytoplasm (also ectobionts)	Intracellular	Shallow water	Unknown
Porifera						
Class Demospongiae Family Cladorhizidae	*Cladorhiza*	Sponge	Skeletal matrix	Extracellular, intracellular	Cold seeps	Methanotroph
Plathyhelminthes Class Catenulida Family Retronectidae	*Paracatenula*	Mouthless flat worm	Trophosome	Intracellular	Shallow water	Chemoautotroph
Nematoda Monohysterida Siphonolaimidae	*Astomonema*	Mouthless nematode	Gut lumen	Extracellular	Shallow water	Chemoautotroph
Mollusca						
Class Aplacophora Simrothiellidae	*Helicoradomenia*	Worm mollusc	Epidermis (also ectobionts)	Intracellular	Hydrothermal vents	Unknown
Class Bivalvia						
Subclass Protobranchia, Family Solemyidae	*Solemya* *Archarax*	Clam	Gills	Intracellular	Reducing sediments, hydrothermal vents, cold seeps	Chemoautotroph
Sublass Heterodonta, Family Lucinidae	*Lucina* *Codakia*	Clam	Gills	Intracellular	Reducing sediments, cold seeps	Chemoautotroph
Family Thyasiridae	*Thyasira* *Maorhithyas*	Clam	Gills	Intracellular	Reducing sediments, cold seeps	Chemoautotroph
Family Vesicomyidae	*Calyptogena* *Vesicomya*	Clam	Gills	Intracellular	Hydrothermal vents, cold seeps	Chemoautotroph
Subclass Pteriomorphia Family Mytilidae	*Bathymodiolus* *Idas*	Mussel	Gills	Extracellular, intracellular	Hydrothermal vents, cold seeps	Chemoautotroph and/ or methano-troph
Class Gastropoda Family Provannidae Peltospiridae	*Ifremeria* *Chrysomallon*	Snail Snail	Gills Oesophageal gland	Intracellular	Hydrothermal vents	Chemoautotroph unknown
Annelida Class Polychaeta Family Siboglinidae[2] Vestimentifera[1]	*Riftia* *Escarpia* *Lamellibrachia*	Tube worm	Trophosome	Intracellular	Hydrothermal vents, cold seeps	Chemoautotroph
Monilifera[2] Frenultata[2]	*Sclerolinum* *Siboglinum* *Oligobrachia*	Tube worm Beard worm	Trophosome Trophosome	Intracellular Intracellular	Hydrothermal vents, seeps, whale falls, wood falls, fjords Hydrothermal vents, seeps, whale falls shallow water, fjords	Chemoautotroph/ Chemosynthetic[3]
incertae sedis[2] (*Osedax* spp.)		Bone-eating worm	root (ovisac)	Intracellular	Whale falls	Heterotroph[4]
Class Clitellata Family Naididae		Gutless oligochaete	Subcuticular	Extracellular	Reducing sediments	Chemoautotroph

Table based on (Stewart et al., 2005) and (Dubilier et al., 2008)
1 The non-taxonomic terms 'major and subgroups' used here as orders and families of chemosynthetic hosts still debated
2 Sytematics have been frequently revised for this group; most recently in Pleijel et al. these tube and beard worms were all placed in a single family, the Siboglinidae (Pleijel et al., 2009)
3 Contradictory evidence for presence of methanotrophic symbionts in siboglinid worms (Petersen and Dubilier, 2009a)
4 *Osedax* included because of close phylogenetic relationship to tubeworms and their chemosynthetic whale fall habitat

on the tubeworm *R. pachytila* revealed that the worm's physiology is completely modified to accommodate the bacteria and supply them with substrates (Cavanaugh, 1985). The bacteria are housed in a specialized organ, the trophosome, not found in symbiont-free relatives. Within the trophosome the symbionts are located in cytoplasmic vacuoles of specialized host cells, the bacteriocytes (Hand, 1987). Oxygen and sulfide are taken up through branched plumes and transported through the host vascular system by modified haemoglobin to the trophosome (Arp et al., 1987; Goffredi et al., 1997; Zal et al., 1998). Surprisingly the trophosomes of deep-sea worms might be ontogenetically of different origin. For vestimentiferan (today siboglinid) tubeworms mesodermal origin has been shown (Nussbaumer et al., 2006), while for frenulates and moniliferans development from endodermal gut tissue has been suggested. Consequently, this form of explicit specialization through host-symbiont coevolution has occured multiple times independently in convergent evolution (Dubilier et al., 2008).

In bivalves the bacteria live intra- or extracellularly in the gills. While the symbionts of the Solemyidae, Lucinidae and Vesicomyidae are intracellular, those of members of the Thyasiridae and Mytilidae are sometimes extracellular. Extracellular symbionts occur in the pandemic gutless oligochaetes. Here the bacteria live in a bacterial layer between cuticle and epidermal cells (Dubilier et al., 2006). Related symbionts also occur as extracellular endosymbionts in the gut lumen of nematodes (Musat et al., 2007).

Ectosymbionts

Sulfur-oxidizing ectosymbionts can appear as long white filaments (epi- or ectofilaments) attached to host surfaces such as the appendages and carapace of crustaceans (for overview see Table 2, pp. 21-22). Smaller rods or cocci are less readily detected, but can also occur on animal surfaces. Deep-sea galatheid crabs with epifliaments are the recently discovered Yeti Crab, *Kiwa hirsuta* (Goffredi et al., 2008), and *Shinkaia crosnieri* (Watsuji et al., 2008). On specialized appendages and within the gill chamber of the deep-sea vent shrimp *Rimicaris exoculata*, filamentous bacteria are regularly found as well (Schmidt et al., 2008; Petersen et al., 2009). The related caridean shrimp *Alvinocaris longirostris* harbors rods directly on the surface of its gill filaments, a unique epibiont position in deep-sea shrimp (Tokuda et al., 2008). Various ciliates such as *Zoothamnium* spp. have been shown to host specific

symbionts. Two *Zoothamnium* host species harbor each only one phylotype of ectosymbiont with the two ectosymbionts highly related to each other despite geographic distance of their hosts (Rinke et al., 2006; Rinke et al., 2009) . Nematodes can also host specific ectosymbionts (Stilbonematinae, (Bayer et al., 2009)). The nematode ectosymbionts are arranged in host specific patterns with bacterial morphotypes ranging from cocci to filaments (Polz et al., 1992). While in many cases it is unclear whether the hosts benefit or suffer from their ectosymbionts, nutritional function has been shown for the nematode ectosymbionts (Ott et al., 2004) and has been suggested for *R. exoculata* (Rieley et al., 1999).

On the other hand, in fish aquaculture and on crustaceans heavy colonization of the host with filamentous epibionts has in many cases been seen as 'infestations' compromising host health. In earlier publications these filaments were repeatedly identified as *Leucothrix* or *Thiothrix* (Johnson et al., 1971; Carman and Dobbs, 1997), mostly based on morphology which can be misleading (following section). *Leucothrix* and *Thiothrix* have formerly been classified as members of the order Thiotrichaceae which use sulfur for lithoheterotrophic, chemolithoautotrophic or mixotrophic growth (Garrity et al., 2005). However, the family name Thiotrichaceae is invalid, because it contains genera such as *Beggiatoa* belonging to other families (Euzéby, 2009). According to their phylogeny these free-living aquatic bacteria should simply be referred to as members of the *Leucothrix*-*Thiothrix* clade.

In the case of the amphipod *Urothea poseidonis* molecular identification verified the *Thiothrix* affiliation of the epifilaments (Gillan and Dubilier, 2004) as in the case of larval stages of the rock-lobster *Panulirus ornatus* (Payne et al., 2007). Here, epifilaments were identified (with fluorescence *in situ* hybridization (FISH)) as *Thiothrix* sp. and *Leucothrix* sp. bacteria and were detected on live and dead animals. Heavy infestation was assumed to contribute to larval mortality by affecting the immune response allowing opportunistic pathogenic *Vibrio* spp. to cause disease. Interestingly in that study other epifilamentous bacteria on these animals could not be identified suggesting a more diverse bacterial community.

Identification of bacterial ecto- and endosymbionts

It is important to note that phenotypic characteristics alone cannot be relied upon for the taxonomic identification of bacteria. Phylogentic studies have shown

that bacterial morphology and phylogeny are not neccessarily congruent. Misidentification by morphological traits can be corrected by molecular studies. The previous morphological identification of *Thiothrix* spp. filaments in the intestinal cecum of *Echinocardium chordatum* (Brigmon and De Ridder, 1998) was thus later corrected as *Desulfonema* spp. filaments (Thorsen et al., 2003). In other cases a single symbiotic phylotype was identified, where later extended studies with improved molecular techniques revealed multiple symbiont phylotypes. This was the case for studies of *R. exoculata* and gutless oligochaetes, both discussed below. With the help of FISH the abundance and specific locations of multiple symbionts can be identified. For example, two phylogenetically distinct subgroups of ectosymbionts of *Alvinella pompejana* were horizontally separated on individual dorsal expansions suggesting niche specialization (Campbell et al., 2006).

In general, specific FISH probing could also help to identify different persistent ectosymbiotic populations on host individuals and distinguish these 'true ectobiotic' sequences from those of 'contaminants' or loosely attached bacteria (e.g. in particles of detritus sticking to the host). This could clarify scattered phylogenetic patterns of invertebrate associated sequences (see section symbiont phylogeny below). To distinguish FISH identified sequences from pure bacterial clone sequences amplified from invertebrates, in this thesis I will refer to the latter as 'invertebrate associated' bacteria (sequences or clones).

1.3.5 Obligate versus facultative association - Degrees of dependency

Associations where the symbiont has taken over essential functions in its host physiology are obligate at least for the host: In chemoautotrophic symbioses the bacterial symbiont is often responsible for the nutrition of the host. Invertebrates that have completely adapted to a symbiotic lifestyle, eliminating their ability to take up food from the environment and digest it, depend on the bacteria for provision of organic molecules. Many chemosynthetic hosts such as vestimentiferans, gutless oligochaetes, mouthless nematodes and a hydrothermal vent gastropod have specialized symbiont-harboring organs or reduced digestive systems and their symbioses are considered obligate (Goffredi et al., 2004; Dubilier et al., 2006; Musat et al., 2007).

Table 2. List of ectosymbionts of invertebrate hosts in chemosynthetic environments

Phylum/ major group/subgroup[1]	Host	Common name	Location	Habitat	Symbiont type	16S rRNA phylogentic affiliation	Idenification of ectosymbiont
Ciliophora							
Oligohymenophora Peritrichida	*Zoothamnium*	Colonial ciliate	Cell surface	Shallow water	Chemoautotroph	γ-proteobacteria	group and specific FISH
Polyhemonophora Heterotrichida	*Folliculinopsis*	Blue-mat ciliate	Cell surface (also endosymbionts)	hydrothermal vents	Unknown	Unknown	-
Karyorelictea Kentrophoridae	*Kentrophorus*	Free-living ciliate	Cell surface (also endosymbionts)	Shallow water	Chemoautotroph[2]	Unknown	-
Nematoda Desmodorida Stilbonematinae	*Stilbonema* *Laxus* *Robbea*	Nematode	Cuticle	Shallow water	Chemoautotroph	γ-proteobacteria (Bayer et al., 2009)	group and specific FISH
Mollusca							
Aplacophora Simrothiellidae	*Helicoradomenia*	Worm mollusc	Sclerites and mantle cavity (also endosymbionts)	Hydrothermal vents	Unknown	α,γ-proteobacteria (Katz et al., 2006)	group FISH
Gastropoda Peltospiridae	*Chrysomallon*	Scaly foot snail	Sclerites	Hydrothermal vents	Unknown	ε,γ,δ, CFB and few other	-
Lepetodrilinae	*Lepetodrilus*	Limpet	Gill	Hydrothermal vents	Chemoautotroph	mostly γ-, few ε-proteo-bacteria (Bates, 2006)	specific FISH
Annelida Polychaeta							
Terebellida	*Alvinella*	Pompeii worm	Integument	Hydrothermal vents	Chemoautotroph	γ-, ε--proteobacteria	2 ε-specific FISH
Clitellata	*Tubificoides*	Sludge worm	Ecto- and endocuticular, posterior end	Shallow water	Unknown[3]	see results and discussion	see results and discussion
Arthropoda Decapoda Bresilidae*	*Alvinocaris*	Hydrothermal vent shrimp	gill surface	Hydrothermal vents	Unknown[4]	γ-, ε-proteobacteria	1ε-specific FISH
Alvinocarididae	*Rimicaris*	Hydrothermal vent	appendages in gill	Hydrothermal vents	Unknown[5]	γ-, ε-proteobacteria	specific FISH

Table 2 continued

Phylum/ major group/subgroup[1]	Host	Common name	Location	Habitat	Symbiont type	16S rRNA phylogentic affiliation	Idenification of ectosymbiont
Thalassinidea	*Pestarella*	vent shrimp Mudshrimp	chamber Setae	Shallow water (mud)	Unknown[6]	α-proteobacteria, CFB, Actinobacteria	-
Galatheoidea Kiwaidae	*Kiwa*	Yeti crab	Setae	Hydrothermal vents	Unknown	γ-,ε-,δ-proteobacteria, CFB and few other	ε-group FISH
	Shinkaia	Vent squat lobster	Setae	Hydrothermal vents	Chemosynthetic (both sulfur- and methane oxidizers[7])	α-,γ-,ε--proteobacteria	unpublished
Amphipoda							
Niphargidae	*Niphargus*	Cave crab	Exoskeleton	Sulfidic freshwater cave	Chemoautotroph[8]	γ-, ε-proteobacteria	γ-specific FISH
Urothoidae	*Urothoe*	Bulldozer lobster (bulldozerkreeftje)	Walking appendices	Shallow water	Unknown[9]	focus only γ-proteobacteria	γ-group and specific FISH
Cirripedia Eolepadidae	*Vulcanolepas*	Hydrothermal vent barnacle	Setae	Hydrothermal vents	Unknown[10]	α-,γ-,ε--proteobacteria, few other	group, 1ε-specific FISH

Table based on (Stewart et al., 2005) and (Dubilier et al., 2008), new references below.

* All endemic vent shrimps of the family Bresilidae are now referred to the family Alvinocaridae according to Martin (Martin and Haney, 2005).

1 The non-taxonomic terms 'major group and subgroup' used here as orders and families of chemosynthetic hosts are still debated.

2 (Fenchel and Finlay, 1989)

3 Bright and Giere et al. (Bright and Giere, 2005) cite unpublished and incomplete data for chemoautotrophic metabolism.

4 (Tokuda et al., 2008)

5 (Petersen et al., 2009)

6 (Demiri et al., In press)

7 (Watsuji and Takai, 2009)

8 (Dattagupta et al., 2009)

9 (Gillan and Dubilier, 2004)

10 (Suzuki et al., 2009)

Among chemosynthetic mussels the degree to which they depend on their symbionts for food may vary. Some have a reduced gut, e.g. *Bathymodilous mauritanicus* lacks an intestine (von Cosel, 1985) and for many a dependence on a significant amount of nutrition from their symbionts has been shown (van Dover, 1996). Nevertheless, filter feeding can also play a role for example in *B. thermophilus* (Page et al., 1991; Duperron et al., 2009). It has been suggested that carbon compounds are sufficiently supplied by the methanotrophic symbionts of *B. childressi* mussels (Childress et al., 1986), however filter feeding might serve for supplementary nutrition of larvae and adults (Tyler et al., 2007). There is physiological evidence that for optimal growth *B. childressi* acquire additional nitrogen by selective bacterioplankton uptake (Pile and Young, 1999).

Furthermore, many of the mussel endosymbioses display a certain plasticity with the presence or absence of methanotrophic bacteria termed ‘opportunistic’ (Duperron et al., 2009) allowing the host to respond to methane availability. Symbionts and their relative abundances may vary according to the environment. Other examples of less stringent associations are certain sponge-associated bacteria (Muscholl-Silberhorn et al., 2008) and some secondary symbionts of gutless oligochaetes (Dubilier et al., 2006). The later vary phylogenetically between host species, but some also vary within subpopulations of the same species as shown for *O. crassitunicatus* (Blazejak et al., 2005). In these facultative associations, the establishment of the association is possible in a favorable environment, but not essential to the survival of host or symbiont.

In contrast to the majority of their hosts, chemosynthetic symbionts are rarely shown to depend on a purely symbiotic lifestyle. Many bacteria are transmitted horizontally and recruited by their hosts from the environment, meaning they can also live freely. For these bacteria the association with the host is beneficial, but not crucial. Free-living stages of symbionts have been reported for symbioses such as *R. pachytila* (Harmer et al., 2008) and the clam *Codakia orbicularis* (Gros et al., 2003). For symbionts of bathymodioline mussels no proof for free-living stages exists yet, however a free-living form is thought to exist due to the environmental aquisition of their symbionts (Won et al., 2008; Duperron et al., 2009).

Nevertheless, the fact that chemosynthetic endosymbionts are hardly ever successfully cultivated indicates an intricate relationship between host and symbiont. As analyses independent of cultivation are often necessary to study chemosynthetic symbionts, most information stems from molecular methods. One way to assess the degree of dependency of the symbiont on the host is to sequence its genome. A reduced genome size, AT bias and loss of vital genes for survival in the environment like genetic repair systems are a sign for symbiont integration and co-dependency on the host. Often certain housekeeping genes are also lost while others are overexpressed. Most of these insights were gained from insect symbioses (see above, 1.1). Reductive genome evolution has recently also been reported for chemoautotrophic intracellular symbionts in two *Calyptogena* species with an AT bias and lack of repair genes (Kuwahara et al., 2008).

Elevated numbers of transposable elements and genetic rearrangement have been considered signs for the transition from free-living to symbiotic lifestyle, while long-established obligatory endosymbionts are mainly free of mobile elements (Moran, 2003; Moya et al., 2008). Chemoautotrophic symbionts that appear to be in transition from facultative to free-living lifestyle due to high numbers of transposase genes will be discussed for the *O. algarvensis* symbiosis (see Results and Discussion section 1.4). Moya et al. (2008) suggested that mobile elements initially increase as genes necessary for free-living stages become superfluous. Eventually, however they become detrimental and finally removed in the process of genome reduction. Obligate endosymbionts that have been transmitted vertically for long periods were considered to be the only bacteria without mobile genetic elements. This lack of mobile elements was attributed to the lack of exchange with other prokaryotes for example through horizontal gene transfer as well as to drastic genome reduction. Statistical analysis revealed that massive loads of mobile elements can appear and disappear in any group of bacteria and that this phenomenon is not related to any certain life-style apart from obligate endosymbiosis (Touchon and Rocha, 2007). However, an exception to this rule was found in an ancient insect symbiosis. The genomes of *Wolbachia*, obligate symbionts of aphids, have experienced multiple and distinct invasions by insertion sequences and intense transpositional activity of insertion sequences was found (Cordaux et al., 2008). Recently the genome of an obligate amoeba symbiont was fully sequenced and revealed a transposase content of 24%, while the genome appeared to be evolutionary stable (Schmitz-Esser et al.,

2010). Thus, transposases may be more common amongst obligate symbionts than previously thought.

Bacterial prerequisites for a symbiotic lifestyle

Bacterial symbionts need certain prerequisites such as the ability to attach and keep close contact to the host or pass host barriers into the body. This should involve a set of genes similar to those on pathogenicity islands, large genomic regions acquired by horizontal gene transfer, essential in invasion and virulence. Analogous genomic structures in mutualistic bacteria such as *Rhizobia* are called symbiotic islands (Uchiumi et al., 2004). Pathogenic and mutualsitic bacteria often use the same mechanisms for invasion and persistence (Dale and Moran, 2006). Bacterial attachment to cell surfaces is a key factor for symbiosis and pathogenicity. In Gram-negative bacteria bacterial pili extending out from the bacterial surface attach to host cell receptors, while adhesins attach firmly to the hosts cells. Virulence genes often include toxins that are less likely to occur in a commensal or mutualistic symbioses unless they provide protection to parasites or predators (‘vaccination behavior’, (Jog and Watve, 2005)).

On the other hand host susceptibility is another important factor in the establishment of host symbiont associations. Often host lectins are involved in bacterial recognition. One of the best described lectins in animals is the mannose- (or mannan-) binding lectin that can initiate a response cascade in the host (Sharon, 2008). As a defense mechanism the host can alter the surface proteins, selecting against or for certain bacteria. In turn the bacteria can again alter the adhesins in a ‘race of arms’ (Paracer and Ahmadjian, 2000). In mutualistic symbioses of marine nematodes a mannose/rhamnose-specific host lectin is thought to mediate recognition of the specific chemosynthetic symbionts (Nussbaumer et al., 2004). Similarly, a mannose-specific lectin may be involved in coral recognition of its symbiotic zooxanthellae (Vidal-Dupiol et al., 2009). In the bacterial symbiont *Vibrio fischeri* different strains are specific for squid or fish hosts. In a recent study a bacterial exopolysaccharide and mediator for biofilm formation was identified as the iniator for specific colonization. Here, a single regulatory gene was responsible for altering the bacterial host range (Mandel et al., 2009).

To understand how prokayotes interact with host cells genomic analysis can be very valuable as the complete genome of Candidatus *Amoebophilus asiaticus*, an obligate intracellular of amoeba symbiont recently showed (Schmitz-Esser et al., 2010). Here various gene families were identified hitherto only known from eukaryotes. The gene products such as ubiquitin specific proteases could allow the symbiont to interact with the host cell. Such adaptations to a symbiotic lifestyle were hypothesized in presenting a stepping stone in bacteria-eukaryote symbioses leading to the ability in infecting higher eukaryotes, e.g. becoming human pathogens (Schmitz-Esser et al., 2010).

Ectosymbioses: stepping stone for obligate endosymbioses?

In facultative ectosymbiotic associations the modes of attachment are probably less selective, less specific and more primitive (ancestral) than intricate recognition systems of host and symbiont that have developed over a long time scale in coevolution. Overall facultative associations likely represent an earlier step in the evolution of symbioses. The repeatedly observed attachments of bacteria such as *Leucothrix mucor* and *Thiothrix spp.* to crustaceans and macroalgae are probably less specific and might represent an ancestral mechanism. They occur on various hosts and also on non-living surfaces. However, as mentioned above for most of these associations specificity cannot be assessed as the identification was in most cases based on morphology only (Johnson et al., 1971; Oeschger and Schmaljohann, 1988; Carman and Dobbs, 1997).

It has long been assumed that ectosymbioses in general represent a less evolved stage than endosymbionts (Smith, 1979). However, there is substantial evidence that ectosymbioses per se are not necessarily more primitive and the hypothesis proposing all obligate chemoautotrophic endosymbioses to have originated from facultative ectosymbioses does not hold true. Phylogenetic clusters can be purely ecto- or endosymbiotic, but also mixed. Some ectosymbioses like those of the stilbonematid nematodes (see 1.3.4 ectosymbionts) can be highly specific and obligate. At the same time these ectosymbionts are closely related to endosymbionts of oligochaetes, hosts phylogenetically distant to the nematode hosts (Nussbaumer et al., 2004; Ott et al., 2004). Furthermore, recent studies on bathymodioline symbioses indicate that ectosymbioses are not more primitive than endosymbioses in this host

group. Thus it was concluded that there is more flexibility and plasticity in these symbioses than anticipated (Dubilier et al., 2008; Duperron et al., 2009).

1.3.6 Symbiont phylogeny

Many lineages of chemosynthetic symbionts are known. Their diversity was long underestimated until advanced molecular characterization of diverse symbioses resulted in the discovery of entirely new phylotypes. One example for novel phylogentically isolated symbionts are the recently described Gamma 4 symbionts of the oligochaete *Inanidrilus exumae* which falls distant from established symbiont clusters and free-living sulfide-oxidizing bacteria in the phylogenetic tree (Fig. 1), (Bergin et al., submitted).

Most chemoautotrophic and methanotrophic symbionts belong to the Gammaproteobacteria, but some sulfur-oxidizing symbionts to the Epsilonproteobacteria. More than 100 distinct 16S rRNA sequences from symbionts are available to date. Only for some of these symbionts sulfur-oxidizing capabilitiy has been shown directly and has been inferred for most others from indirect evidence (Dubilier et al., 2008). While most known gammaproteobacterial symbionts occur as endosymbionts most of the epsilonbacterial symbionts are ectosymbionts. Other bacterial lineages that can co-occur with these chemosynthetic bacteria belong to the Alpha-, Gamma- and Deltaproteobacteria, as well as the spirochetes or the *Cytophaga-Flavobacterium-Bacteroides* group (CFB). In the deep-sea mussel *Idas* sp.,for example, five gammaproteobacterial and one Bacteroidetes phylotypes were found (Duperron et al., 2008).

Gammaproteobacterial symbionts

Within the Gammaproteobacteria at least nine distinct clades of chemosynthetic symbionts have been described (Fig. 1, (Dubilier et al., 2008)). Unlike previously assumed, many symbiont clades are interspersed by free-living bacteria and not exclusively symbiotic. This is also the case for the so far molecularly identified methanotrophic symbionts, which belong to a single lineage, related to type I methanotrophs (Dubilier et al., 2008; Petersen and Dubilier, 2009a). There is, however, a phylogenetic distance between gammaproteobacterial sequence clades from coastal sediment symbioses such as that of the gutless oligochaetes and nematodes and those of deep-sea hosts. The oligochaete-nematode clade is moreover most closely

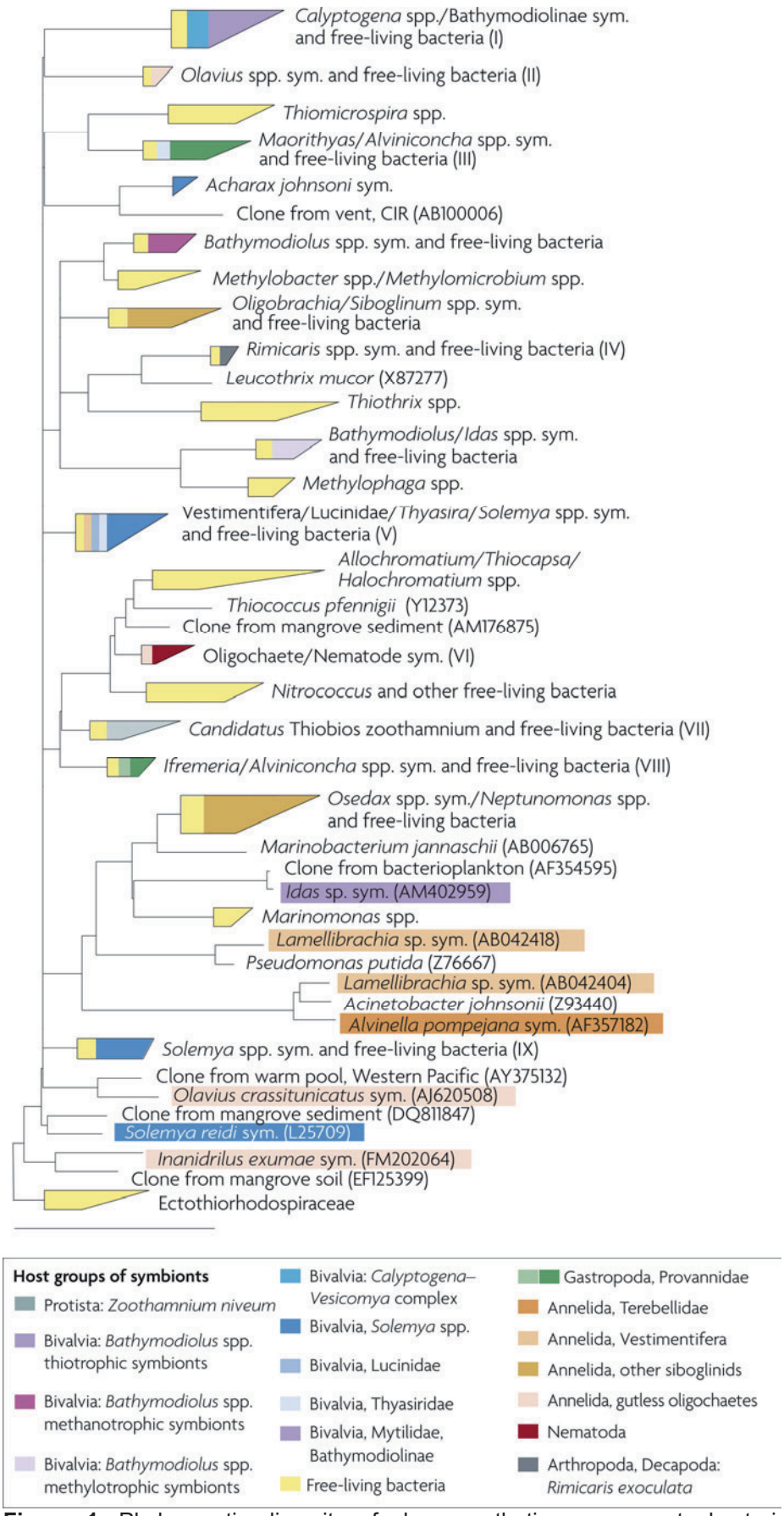


Figure 1. Phylogenetic diversity of chemosynthetic, gammaproteobacterial symbionts based on maximum likelihood analyses of their 16S rRNA sequences. Scale bar indicates 10% estimated sequence divergence. From (Dubilier et al., 2008).

related to free-living sulfur-oxidizing bacteria, the Chromatiaceae. These are anoxygenic phototrophic bacteria and typically occur in shallow water habitats like the worms, leading to the assumption that they share a common ancestor from these habitats (Dubilier et al., 2008).

Epsilonproteobacterial symbionts

Epsilonproteobacterial sequences have been retrieved from various deep-sea hydrothermal vent animals. Animal associated Epsilonproteobacteria belong to the marine Group I and II of the recently defined family Thiovulgaceae fam. nov. (Campbell et al., 2006). Free-living bacteria in these groups often dominate deep-sea hydrothermal sites (Campbell et al., 2003; Campbell et al., 2006). Cultured members of marine Group I and II are *Sulfurovum lithotrophicum* and *Sulfurimonas denitrificans* (previously *Thiomicrospira denitrificans* reclassified by Takai (Takai et al., 2006)). In the cladistics of Epsilonproteobacteria proposed by Corre et al. (2001) these bacteria fall into the epsilonproteobacterial groups F and B respectively (Inagaki et al., 2004; Nakagawa et al., 2005). *Sulfurovum lithotrophicum* is a chemolithoautotroph that oxidizes elemental sulfur or thiosulfate with nitrate or oxygen (Inagaki et al., 2004). *Sulfurimonas denitrificans* is also a chemolithoautotroph with similar metabolism. The genus *Sulfurimonas* was originally described to grow chemolithoautotrophically with CO_2 as carbon source and sulfide, elemental sulfur and thiosulfate as electron donors and O_2 as electron acceptor (Inagaki et al., 2003). With the inclusion of *S. denitrificans* and *S. paralvinellae*, the first deep-sea Epsilonproteobacterium capable of growth by both hydrogen and sulfur oxidation, the emended description also included hydrogen as a possible electron donor and nitrate and nitrite as possible electron acceptors (Takai et al., 2006). Genome analysis has shown potential for hydrogen and formate oxidation in *S. denitrificans* (Sievert et al., 2008b).

Members of the Nautiliaceae and Campylobacteraceae were isolated from the hydrothermal vent polychaete *Alvinella pompejana* tubes. Belonging to the former, *Nautilia lithotrophica* (Miroshnichenko et al., 2002), *Nautilia* sp. str. AM-H (Campbell et al., 2001; Campbell et al., 2006) and *Caminibacter hydrogenophilus* (Alain et al., 2002) are mixotrophs. They grow chemolithoautotrophically with H_2 or facultatively heterotrophic oxidizing formate with elemental sulfur. *C. hydrogenophilus* could also grow on complex organic compounds and use nitrate as electron acceptor. *Sulfuro-*

spirillum sp. str. Am-N belonging to the Campylobacteraceae is a heterotroph which uses formate and fumarate as electron donors and elemental sulfur as electron acceptor (Campbell et al., 2001).

In phylogenetic analyses 16S rRNA sequences derived from deep-sea invertebrates are either scattered throughout clades of free-living and animal associated sequences as in the recently published galatheid hydrothermal vent lobster *Shinkaia crosnieri* and the hydrothermal vent snails *Alviniconcha* spp. or form distinct monophyletic clades. Again here in situ identification would help in identifying which bacteria are the dominant ectosymbionts. In the deep sea hydrothermal vent shrimp *Rimicaris exoculata* the phylogenetic branching pattern of its Epsilon 1-5 ectosymbionts correlated with geographic distance, suggesting a radiation pattern (Petersen et al., 2009).

1.3.7 Symbiont energy metabolism

Chemosynthetic symbionts use various electron donors. In most cases reduced inorganic sulfur species (referred to as sulfur below) or methane, in some cases hydrogen (Zielinski et al., 2005) or possibly reduced metals such as iron (Schmidt et al., 2008) have been suggested. The electron acceptor is in most cases oxygen. The role of nitrate under anaerobic conditions has been discussed and might play a role in some gammaproteobacterial and epsilonproteobacterial symbionts as in their free-living relatives (Hentschel et al., 1996; Arndt et al., 2001; Hentschel and Felbeck, 1993; Pospesel et al., 1999; Minic and Hervé, 2004; Campbell et al., 2006). Nitrate is rarely the obligate electron acceptor as in *Lucinoma aequizonata* (Hentschel and Felbeck, 1995). Sulfur is often an intermediate product and can serve as an electron donor (Nelson and Hagen, 1995; Nakagawa and Takai, 2008) or in some symbionts under longer periods of anoxia as an electron acceptor (Duplessis et al., 2004).

Sulfur metabolism

Reduced sulfur species (H_2S, S^0 and $S_2O_3^{2-}$) are the most common electron donors in chemosynthetic symbioses (Stewart et al., 2005) and are oxidized in different or partly alternating pathways (Nelson and Hagen, 1995; Meyer et al., 2007; Nakagawa and Takai, 2008). A universal pathway does not exist (Kelly, 1988). Horizon-

tal gene transfer is assumed to have occurred between phylogenetically diverse bacterial lineages that have similar pathways (Meyer et al., 2007).

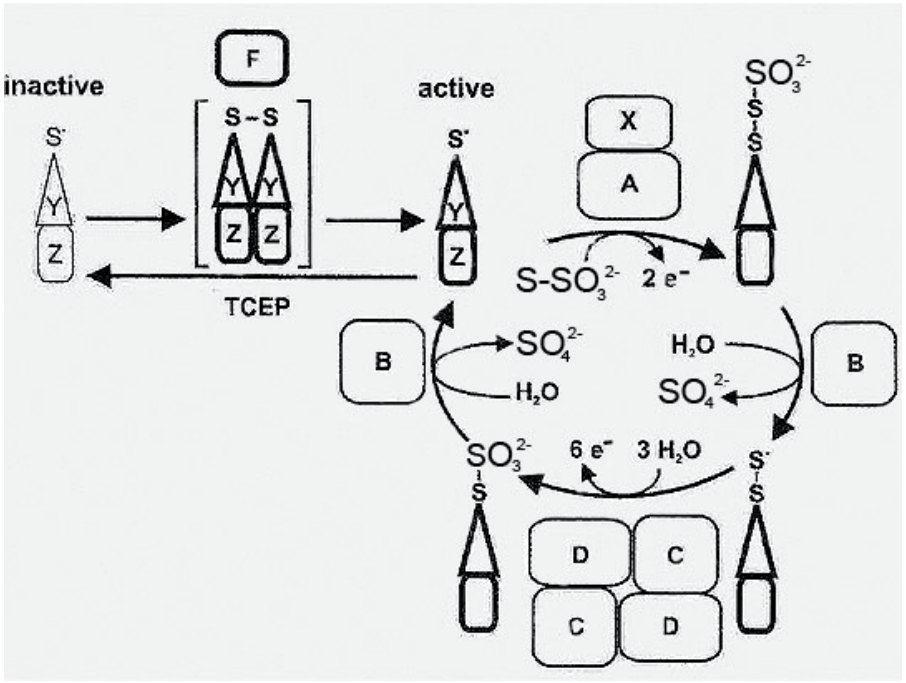


Figure 2. Model of the reaction cycle of thiosulfate oxidation by the Sox enzyme system of *Paracoccus pantotrophus* and reactivation of SoxYZ by the flavoprotein SoxF. The capital letters indicate the respective Sox proteins, the central proteins SoxYZ in its active form is indicated in boldface, and the inactive form is indicates in fine type. The SoxY-Y interprotein disulfide of the heterotetrameric SoxY-Y(Z)$_2$ represents a hypothetical intermediate in the transition of the inactive to active form of SoxYZ catalyzed by soxF. TCEP tris(2-carboxy-ethyl)phosphine. Model and legend from (Friedrich et al., 2008).

While most free-living sulfur oxidizers such as the gammaproteobacterial *Thiomicrospira crunogena* and deep-sea Epsilonproteobacteria have a full set of genes of the sulfur oxidation multienzyme complex (Sox system, Fig. 2) for the complete oxidation of H_2S, S^0, SO_3^{2-} and $S_2O_3^{2-}$ to SO_4^{2-} (Nakagawa and Takai, 2008), most gammaproteobacterial symbionts lack Sox(CD)$_2$, the sulfur dehydrogenase (Nakagawa and Takai, 2008). Instead the dissimilatory sulfite reductase (Dsr) pathway, originally discovered in sulfate reducing bacteria, is used in reverse direction to oxidize the stored sulfur (Fig. 3), (Meyer et al., 2007; Dahl et al., 2008; Nakagawa and Takai, 2008). The terminal oxidation of SO_3^{2-} to SO_4^{2-} is mediated by the adenosine phosphate reductase (APS reductase) which can also function in two directions (Meyer and Kuever, 2007). In the oxidative Apr pathway APS-reductase catalyzes the AMP-dependant oxidation of sulfite to adenosine-5'-phosphosulfate (APS). Pyrophosphate is then consumed with the release of sulfate and ATP by substrate level phosphorylation catalyzed by ATP sulfurylase (Meyer and Kuever, 2007). In addition to these pathways other mechanisms can be involved in the oxidation of sulfide to sulfur such as the flavocytochrome *c* sulfide dehydrogenase (FccAB) of *Candidatus* Endorfitia persephone, the symbiont of the hydrothermal vent tubeworm *Riftia pachyptila* (Robidart et al., 2008), that can also be found in (several) green and pur-

ple sulfur bacteria and Chromatiaceae (Kostanjevecki et al., 2000) and the sulfide:quinone oxidoreductase (Sqr) of *Candidatus* Ruthia magnifica and *Candidatus* Vesicomyosocius okutanii, symbionts of the hydrothermal vent clams *Calyptogena magnifica* and *Calyptogena okutanii* respectively (Harada et al., 2009).

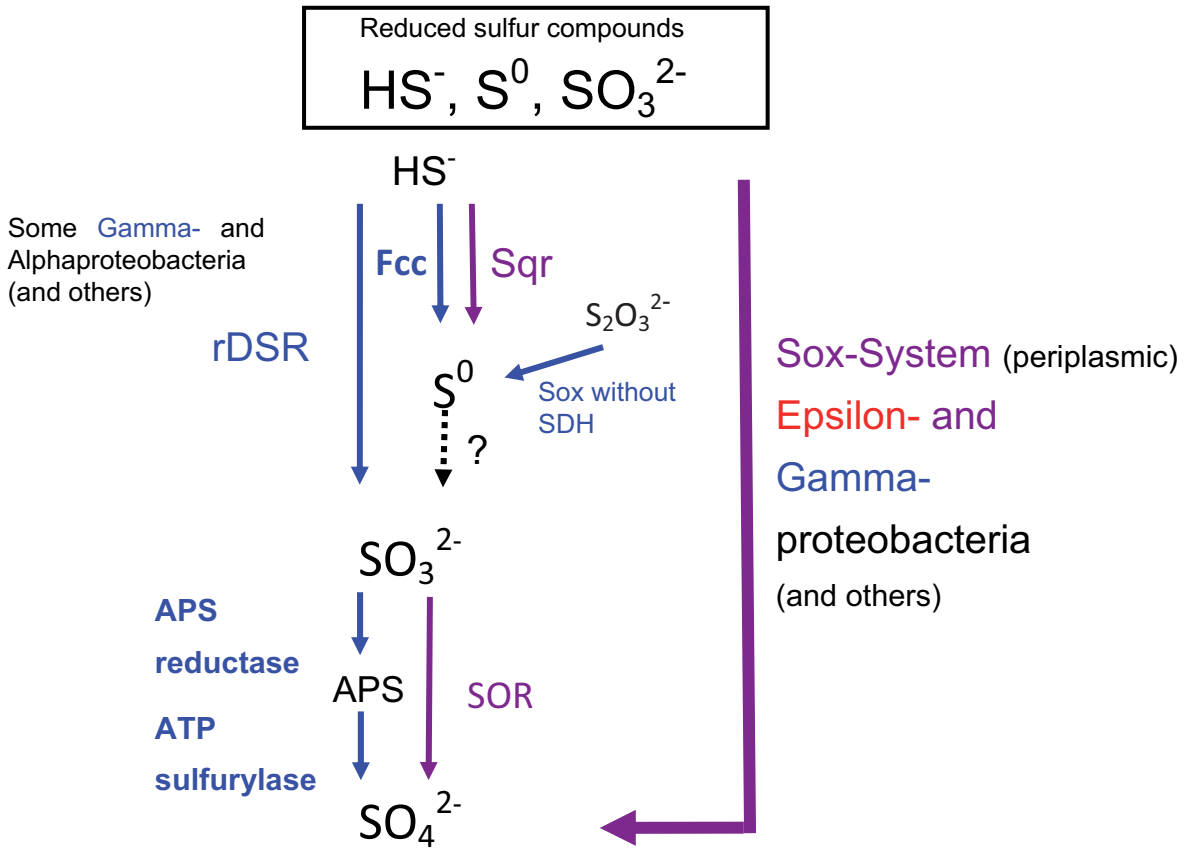


Figure 3. Sulfur oxidation pathways found in Gammaproteobacteria (blue) and Epsilonproteobacteria (in both, purple). The Sox system completely oxidizes reduced sulfur compounds to sulfate. Often symbiotic Gammaproteobacteria lack *soxCD* coding for the sulfur dehydrogenase (SDH) and thus only two electrons can be transfered from sulfide to sulfur or thiosulfate to sulfide. Instead many Gammaproteobacteria have a Dsr and Apr system (see text) with which sulfide can also be completely oxidized to sulfate. APS: sulfite to adenosine-5'-phosphosulfate; Fcc: flavocytochrome *c* sulfide dehydrogenase; rDSR: reverse dissimilatory sulfite reductase; Sqr: sulfide:quinone oxidoreductase; SOR: sulfur-oxygenase-reducatse. Modified from Nakagawa and Takai, 2008.

The presence of the genes *dsrAB*, coding for the dissimilatory sulfite reductase and *aprA* or *aprBA,* coding for the adenosine phosphate reductase, are widely used as indicators for dissimilatory sulfur metabolism. The encoded proteins of those genes from sulfate-reducing and sulfur-oxidizing bacteria are clearly separated in distinct clades. Another diagnostic gene, *soxB*, encodes the SoxB unit of the periplasmic Sox enzyme complex (SoxXABYZ(CD)$_2$). It is universal to all studied photo- and chemoautotrophic sulfur-oxidizing bacteria that form sulfur globules during thiosulfate oxidation (incomplete Sox enzyme system SoxXABYZ) and occurs also in bacteria that completely oxidize reduced sulfur species without sulfur globule inter-

mediates (complete Sox system SoxXABYZ$(CD)_2$). In general *soxB* phylogeny showed good correlation with 16S rRNA phylogeny in separating lineages despite some occasions of lateral gene transfer (Petri et al., 2001; Meyer et al., 2007). Unlike APS reductase and DSR, it also occurs in Epsilonproteobacteria. Some Epsilonproteobacteria also showed sulfur-oxygenase-reductase (SOR) activity for the oxidation of S^0, indicating that they either do not use the Sox system or use a modified version of it (Sievert et al., 2008a).

Alternative electron donors: methane, hydrogen, iron

Methane can be used as an electron donor by some chemosynthetic symbionts. Gammaproteobacterial methane oxidizers have been primarily found in bathymodioline mussels (Kochevar et al., 1992) and siboglinid worms (Schmaljohann and Flugel, 1987; Schmaljohann et al., 1990; Pimenov et al., 1999), sometimes in coexistence with gammaproteobacterial sulfur oxidizers (Fiala-Medioni et al., 2002, Duperron et al., 2006; Naganuma et al., 2005). Two pathways lead to the complete oxidation of methane to CO_2 via the intermediates methanol, formaldehyde, and formate. All molecularly investigated methane-oxidizing symbionts belong to one gammaproteobacterial clade related to type I methanotrophs (Petersen and Dubilier, 2009b). In general the C1-compounds are also used for the synthesis of higher carbon compounds (see 1.3.1). A widely used diagnostic gene is *pmoA* encoding the active site subunit of the particulate methane monooxygenase (Petersen and Dubilier, 2009b).

Hydrogen is an electron donor in autotrophic Deltaproteobacteria and is often transferred in microbial consortia. Recently, the gammaproteobacterial sulfur-oxidizing symbiont of the hydrothermal vent mussel *Bathymodiolus puteoserpentis* was shown to use hydrogen as an energy source (Zielinski, 2008). A possible diagnostic gene is *hynL* encoding the large subunit of a membrane-bound [NiFe]-hydrogenase, involved in respiratory hydrogen uptake. Finally, reduced iron has been suggested as an alternative electron donor for ectosymbionts of *R. exoculata* at the iron-rich Rainbow hydrothermal vent field on the Mid-Atlantic Ridge (Gloter et al., 2004; Zbinden et al., 2004), but a recent study suggests that symbiotic iron oxidation cannot outcompete abiogenic iron oxidation (Schmidt et al., 2009).

Carbon fixation pathways

Calvin Benson Bassham cycle

Gammaproteobacteria primarily use this pathway for CO_2 fixation. ATP and NADH, produced as energy sources in the oxidation of reduced chemicals, are used to convert carbon dioxide into carbohydrates. The key reaction is catalyzed by ribulose bisphosphate carboxylase (RubisCO). A CO_2 molecule is condensed with the 5-carbon molecule ribulose1,5-bisphosphate into a six-carbon intermediate that rapidly hydrolyzes into two molecules of 3-phosphoglycerate. This reaction is part of the Calvin cycle where ribulose1,5-bisphosphate is regenerated. RubisCo is commonly found in the cytoplasm and in some bacteria in organelles, the carboxysomes (Shively et al., 2003). Two forms of RubisCO are known: I and II. Chemoautotrophic gammaproteobacterial symbionts generally have Form IAq and Form IAc, some use Form IC and Form II enzymes or even a combination (Badger and Bek, 2008). Form II enzymes are less efficient in partitioning CO_2 and O_2 and are primarily used to enable the CBB pathway to balance the cell's redox potential according to growth conditions (Tabita et al., 2007). Indicator genes used in symbiont studies are *cbbL* for Form I and *cbbM* for Form II.

Reverse tricarboxylic acid cycle (rTCA or reverse Krebs cycle)

Many chemolithoautotrophic Epsilonproteobacteria (Campbell et al., 2003), some Gammaproteobacteria (Robidart et al., 2008; Markert et al., 2007), and some chemolithoautotrophic sulfate reducers (e.g. *Desulfobacter hydrogenophilus,* (Schauder et al., 1987)) as well as some archaea use this less energy consuming pathway. Because it involves oxygen-sensitive enzymes it is found in anaerobic and microaerophilic microbes only. In this cycle two molecules of CO_2 are used to synthesize one molecule of acetyl-CoA which can then be converted to pyruvate and phosphoenolpyruvate (PEP). PEP either regenerates the intermediates of the cycle or is used for gluconeogenesis (Campbell et al., 2006).

Reversibility is common to the rTCA cycle. In the presence of small organic molecules it is used in the oxidizing direction for energy generation (Campbell et al., 2006). Key enzymes are: the ATP citrate lyase (the gene *aclBA* is often used as an indicator for CO_2 fixation via the rTCA cycle), pyruvate synthase (pyruvate:ferredoxin oxidoreductase), ketoglutarate synthase (2-oxo-glutarate:ferredoxin oxidoreductase) and fumarate reductase.

Reductive acetyl-CoA pathway (Wood Ljungdahl Pathway)

Anaerobes such as methanogenic archaea, some acetogenic and some sulfate-reducing bacteria use this non-cyclic pathway. According to Ragsdale (1991) there are two parts to this pathway: (1) reduction of CO_2 to methyltetrahydrofolate and (2) formation of acetyl-CoA from methyltetrahydrofolate, a carboxyl donor and coenzyme A. Alternatively, carbon monoxide can be used to form acetyl-CoA. A two-carbon compound is formed from two one-carbon precursors. Key enzymes involved are the carbon monoxide dehydrogenase, formate dehydrogenase, 5,10 methenyl-terahydrofolate cyclohydrolase and 5,10-methylene-tetrahydrofolate reductase and a methyl-transferase (Ragsdale, 1991).

3-Hydroxypropionate pathway

Photolithoautotrophic bacteria of the genus *Chloroflexus* and some chemolithoautotrophic archaea (in a modified version) carboxylate acetyl-CoA to 3-hydroxypropionate, then reduce this to propionyl-CoA which is further converted via succinyl-CoA and CoA transferred to malyl-CoA. The latter is cleaved to glyoxylate, the fixation product, and acetyl-CoA, the primary CO_2 acceptor, is thus regenerated (Strauss and Fuchs, 1993). Key enzymes are acetyl-CoA carboxylase, 3-hydroxypropionate-CoA ligase and malyl-CoA lyase.

3-Hydroxypropionate/4-hydroxybutyrate pathway

A novel pathway which uses some of the same intermediates as the 3-Hydroxypropionate Pathway (enzymes are phylogenetically distinct) was described recently for anaerobic and microaerophilic archaea (Berg et al., 2007). It involves 4-hydroxybutyryl-CoA dehydratase, a radical, O_2-sensitive enzyme.

Bacterial autotrophy

There are several pathways with which microorganisms can fix carbon dioxide to build higher organic molecules (see inset, previous page). The most common carbon fixation pathways, also for chemosynthetic symbionts are the Calvin Benson Bassham Cycle (CBB) in Gammaproteobacteria and the reverse tricarboxylic acid cycle (rTCA) in Epsilonproteobacteria.

All CO_2 fixation mechanisms are energy consuming, especially the Calvin Benson Bassham Cycle (CBB cycle). Most bacteria with the respective metabolic potential refrain from carbon fixation when organic compounds are readily available from the environment. Genomic analyses have revealed heterotrophic potential in chemosynthetic symbionts such as *Candidatus* Endoriftia persephone (Robidart et al., 2008). Mixotrophy is also known from deep-sea Epsilonproteobacteria (Campbell et al., 2006).

2 Symbioses of coastal marine oligochaetes

Among the marine invertebrates that can engage in bacterial symbioses are gutless oligochaetes. Annelid worms in general are ubiquitous in terrestric and acquatic environments. Often they dominate extreme habitats with little oxygen or high sulfide concentrations that are generally toxic to higher eukaryotes. As inhabitants of reduced sediments, oligochaetes are potential hosts for chemoautotrophic bacteria. Indeed a large host group of gutless oligochaetes obligately hosts endosymbionts. Ectosymbioses with filamentous, possibly thioautotrophic bacteria from reduced environments have been reported only from one oligochaete: *Tubificoides benedii* (see 2.2).

2.1 Endosymbioses of gutless oligochaetes

More than 80 species of monophyletic origin are grouped in only two genera *Olavius* and *Inanidrilus.* They belong to the family Phallodrilinae (class Naididae, previously Tubficidae (Erséus, 2008)). Their worldwide distribution and the fact that they mostly occur in accessible shallow water make them easy and cost-effective to collect and thereby ideal candidates for symbiosis and evolutionary studies.

2.1.1 Oligochaete host distribution, habitat, phylogeny

Gutless oligochaetes are pandemic (collection sites Fig. 4), living in the pore waters of mostly shallow water sediments. The highest species diversity has been observed in calcareous coral reef sediments, but they also occur in silicate sediments and have been found in depths of up to several hundred meters off the coast of Peru (Blazejak et al., 2005). It is likely that due to their small size more species from other habitats remain undiscovered since specialists who can correctly identify them are rare. Their collection sites are generally considered sulfidic (Dubilier et al., 2006). The worms are most abundant in 5-15 cm sediment depth in suboxic or anoxic zones in concentrations of sulfide not exceeding 500 µM (Bright and Giere, 2005; Dubilier et al., 2006).

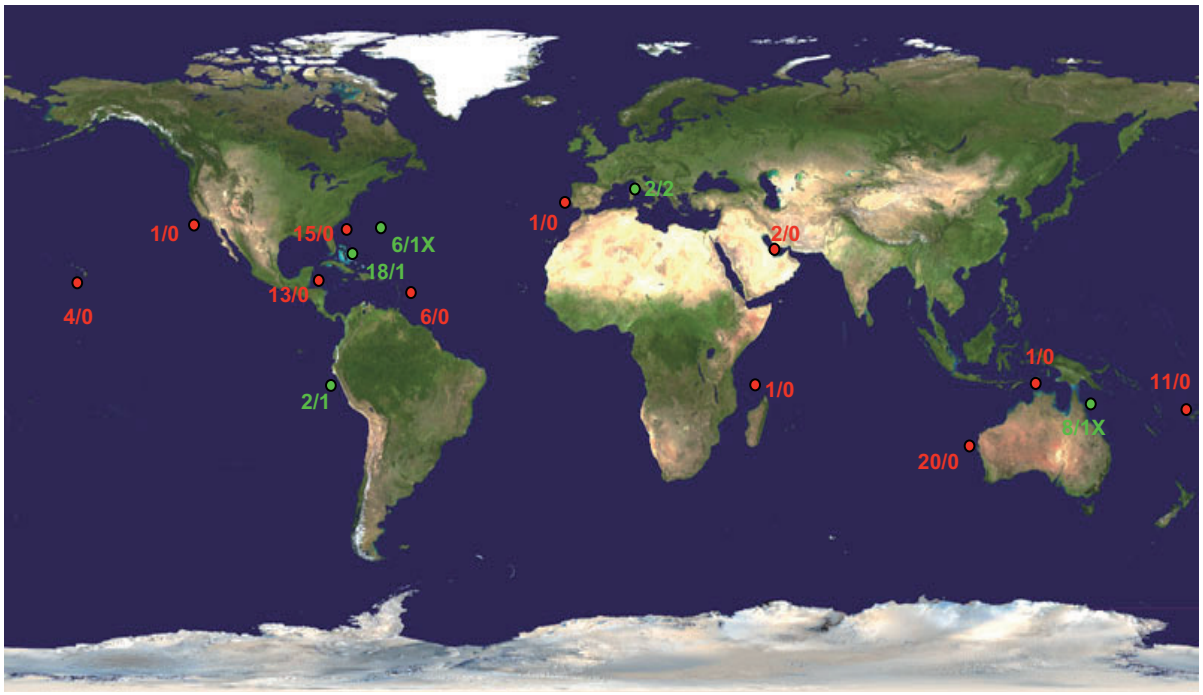


Figure 4. Distribution of described gutless oligochaetes worldwide. In green sites where at least one was analysed molecularly, in red sites where none of the symbiotic communites of the found oligochaetes were characterized molecularly yet with number of described oligochaetes versus molecularly characterized bacterial symbionts thereof to date. Symbionts identified at the beginning of this study indicated by asterisks (*). Picture modified from http://www.sage.wisc.edu/riverdata/.

Phylogentic studies show that all gutless oligochaetes stem from one common ancestor. Species range from primitive to highly developed forms. The genus *Inanidrilus* is monophyletic according to morphological (Erséus, 1984) and molecular studies (Nylander et al., 1999; Sjölin et al., 2005) while there is evidence that the genus *Olavius* is paraphyletic (Erséus and Bergfeldt, 2007).

2.1.2 Host morphology and endosymbiont location

Gutless oligochaetes are small worms of 0.1-0.3 mm diameter and 2-50 mm length (Fig. 5E). All described gutless oligochaetes lack mouth, gut and anus. Moreover, nephridia are completely absent. This is unique among free-living animals and extraordinary as these organs are responsible for the excretion of nitrogenous waste and essential in the marine environment for osmoregulation. Because of their extremely reduced digestive system, gutless oligochaetes were first assumed to absorb organic compounds from the environment through their body wall, since this had been previously suggested in other gutless worms, the long and thin frenulates (Southward et al., 1979). Later studies showed that indeed molecules of up to 70 kDa can pass the gutless oligochates' cuticle (Dubilier et al., 2006). Only after the discovery of the chemoautotrophic symbioses at hydrothermal vents, was the role of bacteria found in a dense multicellular layer between cuticle and epidermis (Fig. 5A-B) reassessed. It was then realized that the bacteria might provide nutrition (Giere, 1981).

It is due to the bacterial sulfur and polyhydroxybutyric acid (PHB) filled storage vesicles and their refraction of light that the worms appear white. This white appearance makes them easy to distinguish from non-symbiotic oligochaetes and resulted in the names of the first described species *Inanidrilus albidus* (albus: latin for white) and *I. leukodermatus* (leuko: Greek for white and derma: Greek for skin) in the 1970s before the discovery of their symbionts (Jamieson, 1977; Giere, 1979).

2.1.3 Symbiont morphology

All gutless oligochaetes investigated so far harbor large and small bacteria in the symbiont-containing layer between the worm's cuticle and epidermis (Fig. 5C, D). They are extracellular and embedded between epidermal extensions in the apical part. Using transmission electron microscopy (TEM) the bacteria have been estimated to reach at least 10^6 cells in an average adult worm comprising 25% of the hosts volume (Giere et al., 1995); The large bacterial morphotype is typically round or oval and 3-5 µm in diameter with large cellular inclusions. In addition a second smaller bacterial rod- or coccoid shaped morphotype about 0.5-1.5 µm in diameter devoid of inclusions has regularly been found. In some species, a third long, thin morphotype of about 1.8 up to 10 µm length and 0.3-0.4 µm in diameter occurs (Giere and Erséus, 2002; Bright and Giere, 2005; Giere et al., 1995; Giere and

Krieger, 2001). While first studies found only one 16S rRNA phylotype (Dubilier et al., 1995) later improved molecular and phylogenetic analyses revealed that the distinct morphotypes represent diverse 16S rRNA phylotypes (Dubilier et al., 1997b; Blazejak et al., 2006).

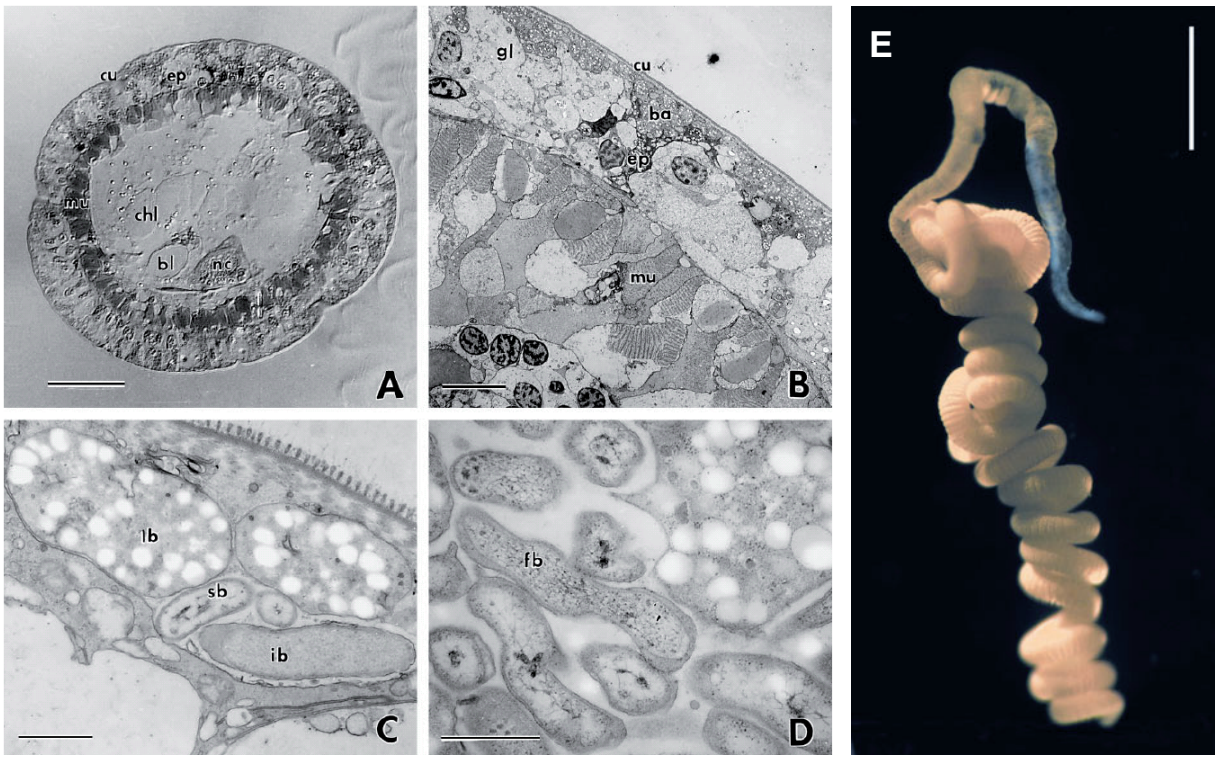


Figure 5. Exemplary pictures of a gutless oligochaete (*Olavius algarvensis*) in light microscopy (A, E) and TEM (B - D). Complete cross section through postgenital segment of *O. algarvensis* (A) showing cuticle (cu), epidermal layer (ep), muscle layer (mu), chloragog cells (chl), blood vessel (bl) and nerve cord (nc) and details of body wall (B-D). Bacterial symbionts are embedded in a layer between the cuticle and extensions of the epidermis (B). Large (lb) bacteria with inclusions, small, crescent-shaped bacteria (sb) and intermediate sized bacteria co-occur in the bacterial layer (C) with contorted filiform bacteria appearing in some sections. Scale bars in (A) 50 µm, (B) 10 µm, (C) 1.0 µm, (D) 0.5 µm and (E) 1 mm. (A-D) from Giere and Erséus, (2002), (E) picture C. Lott in Ruehland et al. (2006).

Bacterial fission was regularly observed in the large symbiotic morphotype (Giere and Krieger, 2001). In the basal region, lysis of bacteria has been observed (Giere et al., 1995; Giere and Krieger, 2001). This might be part of bacterial growth regulation and also part of the nutritional strategy of the host besides uptake of bacterial exudates (Dubilier et al., 2006; Woyke et al., 2006).

2.1.4 Symbiont transmission

Symbiont transmission can occur either horizontally between members of a population or vertically from parent to offspring: In gutless oligochaetes it is assumed to occur vertically, i.e. from the adult worm directly to its offspring, at least for the large symbiont (Krieger, 2000). Reaching full maturation the adults develop ‘genital

pads'. These paired sack-like pockets on the ventral side are filled with bacteria that are covered by a thin cuticle of the worm (Giere and Langheld, 1987). When eggs are released through the closely positioned oviparous, bacteria from rupturing genital pads are assumed to be released, thus colonizing the egg. This has been indicated in ultrastructural studies where eggs inside of the worm were free of bacteria while freshly laid eggs already contained bacteria between the inner and outer egg integument. Invasion of the egg cytoplasm was observed a few hours after deposition when bacteria accumulated at one pole and from there entered through the egg membrane (Krieger, 2000; Dubilier et al., 2006).

Whether the additional symbionts are also transmitted in this way is not clear. Repeated infections with phylogenetically diverse bacteria is likely to have occurred from the environment instead of an ancient common ancestor harboring all phylotypes which were then lost in speciation. While the Gamma 1 symbionts are closely related and monophyletic indicating a common ancestor the additional symbionts are highly diverse comprising several proteobacterial lineages (Fig. 6).

2.1.5 Primary symbionts

As mentioned above, initially the gammaproteobacterial symbiont Gamma 1 was thought to be the only symbiont of the gutless oligochaete *I. leukodermatus* with its different morphotypes belonging to one phylotype (Dubilier et al., 1995). With enhanced molecular techniques the large morphotype was linked to the Gamma 1 phylotype and additional phylotypes were identified (Dubilier et al., 1997b).

Gamma 1 phylogeny

All gutless oligochaetes described to date host a large bacterial morphotype. Phylogenetic analyses of this morphotype in 16 host species revealed that the symbionts are host specific but closely related. As these gammaproteobacterial symbionts occur in all gutless oligochaetes which rely on them for nutrition, they were called the primary Gamma 1 symbionts. Only recently a novel, distantly related Gammaproteobacterium, Gamma 4, of similar morphology as the Gamma 1 symbiont, has been found to replace the primary Gamma 1 symbiont in *Inanidrilus exumae* from coral reef sediments of the Bahamas (Fig. 6), (Bergin et al., submitted). The primary Gamma 1 symbionts of gutless oligochaetes fall into a clade with

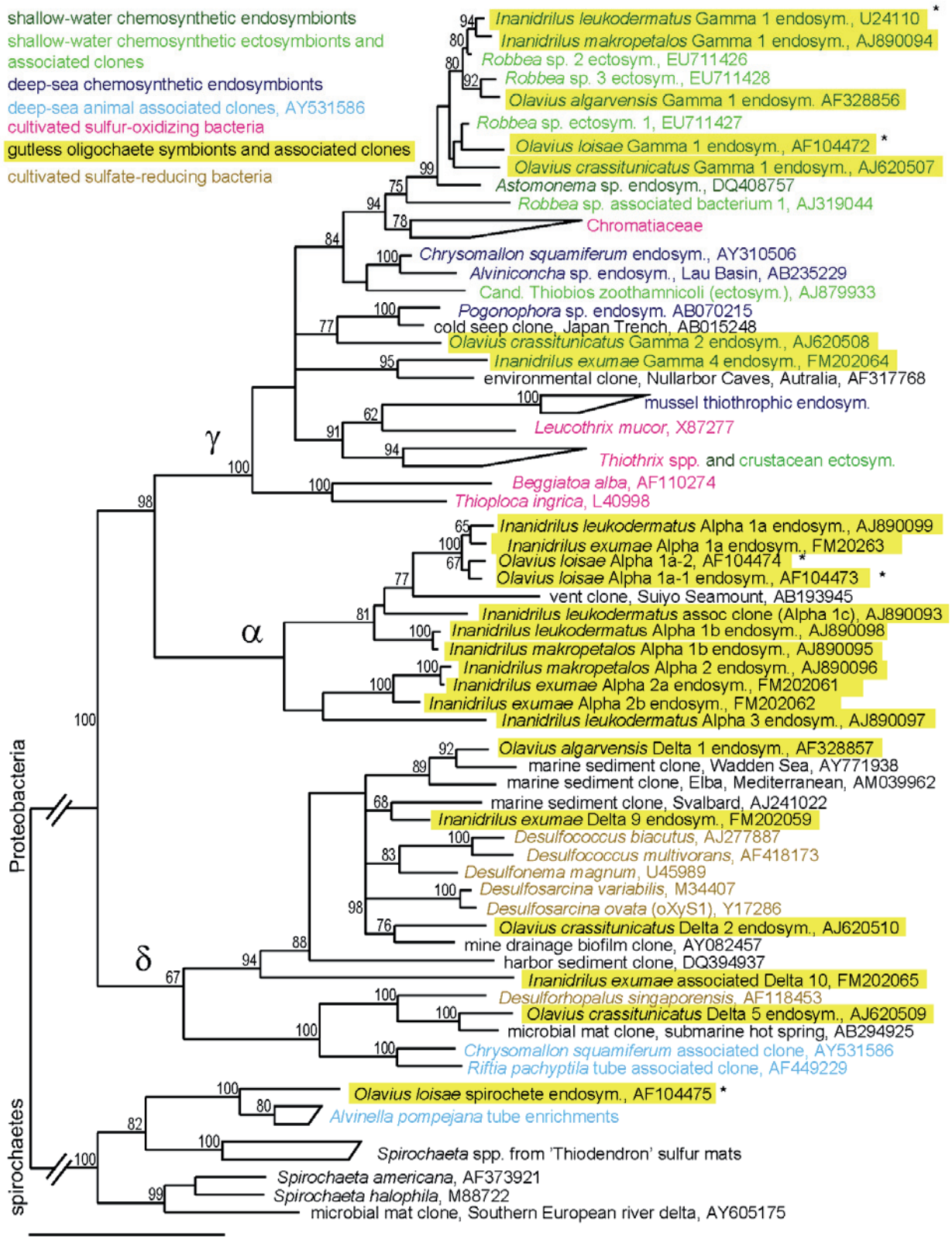


Figure 6. Phylogenetic diversity of endosymbionts of gutless oligochaetes known except those essential to the results of this study (shown in Results and Discussion).Tree based on maximum likelihood analyses of 16S rRNA sequences. Scale bar indicates 10% estimated sequence divergence. Sequences that were published at the beginning of this study are marked with asterisks (*).

endo- and ectosymbionts of marine nematodes that also occur in shallow water sediments. The closest free-living relatives are the Chromatiaceae (Fig. 6), purple sulfur bacteria that build sulfur globules as intermediates in the oxidation of sulfide. Some Chromatiaceae can also use thiosulfate or even hydrogen and in some cases organic molecules as electron donors (Imhoff et al., 1998).

Gamma 1 physiology

The Gamma 1 symbionts are considered obligatory for the host providing it with nutrition by using the energy derived from sulfur oxidation to fix carbon (Dubilier et al., 2006). They store sulfur in globules like their free-living relatives (Chromatiaceae) as demonstrated by ultrastructural studies (Krieger et al., 2000; Giere and Krieger, 2001). Autotrophy was demonstrated in uptake experiments showing the incorporation of inorganic carbon (Felbeck et al., 1983; Giere et al., 1988b) and immunohistochemical labelling of RubisCO (Krieger et al., 2000; Giere and Krieger, 2001). Additional evidence for thioautotrophy is presented in Chapters 1 and 2 (see also Results and Discussion section 1.1.1).

2.1.6 Secondary symbionts

Subsequent studies using the 16S rRNA approach showed the presence of not only a second bacterial symbiont but multiple and varying symbionts in gutless oligochaetes. The smaller morphotype can be of diverse phylogenetic origin (Fig. 6), either belonging to the Alpha- (Dubilier et al., 1997b; Blazejak et al., 2006), Gamma- (Blazejak et al., 2005), or Deltaproteobacteria (Blazejak et al., 2005), while the elongated morphotype found in some species belongs to the Spirochaeta (Blazejak et al., 2005; Dubilier et al., 2006). The majority of the alphaproteobacterial phylotypes fall within the Rhodospirillales and some are related to *Rhizobia*.

Alphaproteobacterial symbionts have been found to primarily co-occur with the Gamma 1 symbionts in hosts from biogenic calcareous sediments of the Bahamas, Bermudas, and the Great Barrier Reef (Blazejak et al., 2006). With the recent analysis of *I. exumae*, Deltaproteobacteria have been found to co-occur with Alphaproteobacteria (Bergin et al., submitted). Deltaproteobacterial symbionts were first found in gutless oligochaetes in my Diploma thesis (Mülders, 1999). Further

analyses were a main focus of this thesis, thus they will be discussed shortly below and more detailed in Results and Discussion (section 1.1.3 and 1.3).

Potential functions of secondary symbionts in gutless oligochaetes

For the alphaproteobacterial and spirochetal symbionts of gutless oligochaetes no detailed information on genetic potential is available to date. Possible functions suggested for the Alphaproteobacteria are the recycling of anaerobic waste products (Blazejak et al., 2006) or the use of organic sulfur compounds such as dimethylsulfoniopropionate (DSMP) and related compounds that occur in coral reef mucus and sediments as electron and carbon sources (Bergin et al., submitted). One of the alphaproteobacterial symbionts of *I. leukodermatus* was related to a nitrogen-fixing *Sinorizobium*, a symbiont of leguminose plants. However, potential for N_2-fixation by amplification of the indicator gene *nifH* could not be corroborated. As this symbiont occured only in *I. leukodermatus* and not in any other molecularly investigated oligochaete hosts it was also assumed not to play an essential role in nitrogen uptake (Blazejak et al., 2006; Dubilier et al., 2006). The role of additional gammaproteobacterial symbionts was elucidated by molecular and metagenomic analysis and will be discussed in Results and Discussion (section 1.1.2 and 1.3).

Spirochetal symbionts of gutless oligochaetes belong to a monophyletic clade most closely related to sequenced tube enrichment cultures from *Alvinella pompejana*, a deep-sea polychaete living at hydrothermal vents (M.A: Cambon-Bonavita, unpublished Data, e.g. GenBank AJ431238; (Dubilier et al., 2006). The closest free-living relative is *Spirochaeta isovalerica*, isolated from sulfidic muddy sediments and spirochetes from a Thiodendron sulfur mat. These spirochetes are obligate anaerobes or facultative microaerobes that ferment carbohydrates to acetate, ethanol, CO_2, and H_2 and are assumed to play a role in sulfur cycling by consuming sulfide to remove oxygen (Canale-Parola, 1992; Dubinina et al., 2004; Stephens et al., 2008). The spirochete symbionts of gutless oligochaetes could have a similar metabolism. However studies in termite symbioses revealed that the symbiotic bacteria have an alternative metabolism to their free-living relatives. Instead of a heterotrophic lifestyle they have the capacity for chemoautotrophy producing acetate from H_2 and CO_2 (Leadbetter et al., 1999) and some also have the ability to fix nitrogen (Lilburn et al., 2001). Clearly, hosts can benefit from such versatile symbionts. Further studies are

needed to identify the metabolic potential of spirochete symbionts in gutless oligochaetes.

2.1.7 Gutless oligochaetes in Mediterranean coastal sediments

O. algarvensis was originally discovered in sediments of the Algarvan coast of Portugal (Giere et al., 1998), and later found to occur in a patchy distribution adjacent to sea grass beds in coastal sediments of the Mediterranean island Elba (Mülders, 1999; Giere and Erséus, 2002). During this PhD thesis, *O. algarvensis* and four bacterial morphotypes in its bacterial layer were described (Fig. 5, (Giere and Erséus, 2002)). A large oval morphotype (length 2.6 µm, width 1.4 µm) resembled that of other gutless oligochaetes, while a rod- or crescent shaped bacterium was much smaller (length 1.1 µm, width 0.4 µm). A third morphotype was also rod-shaped but more stout and larger (length 1.5, width 0.6 µm) than the second morphotype. A fourth morphotype appeared filiform with a locally contorted cell wall (length 1.2 -2.4 µm, width 0.4 µm). While the large and third morphotype were found consistently in all sections the presence and abundance of the other two morphotypes varied.

The molecular analysis of this symbiosis in my Diploma thesis showed that next to the large Gamma 1 symbiont which belonged to the monophyletic group of primary sulfur-oxidizing Gamma 1 symbionts of gutless oligochaete these worms harbored deltaproteobacterial symbionts (later called Delta 1 symbiont), (Mülders, 1999). The large Gamma 1 symbiont belonged to the monophyletic group of primary sulfur-oxidizing Gamma 1 symbionts of gutless oligochaetes. The Delta 1 was closely related to sulfate-reducing marine bacteria. It was the first deltaproteobacterial endosymbiont discovered in marine invertebrates. Deltaproteobacteria were until then only known as gut bacteria and a novelty as invertebrate endosymbionts. Low external sulfide concentrations and amplification of bacterial dissimilatory sulfite reductase (DSR), an indicator gene for sulfate reduction, from *O. algarvensis* led to the assumption that these two symbionts interact in sulfur syntrophy in an endosymbiotic sulfur cycle (Fig. 7) (Mülders, 1999), discussed in detail in Results and Discussion (section 1.3).

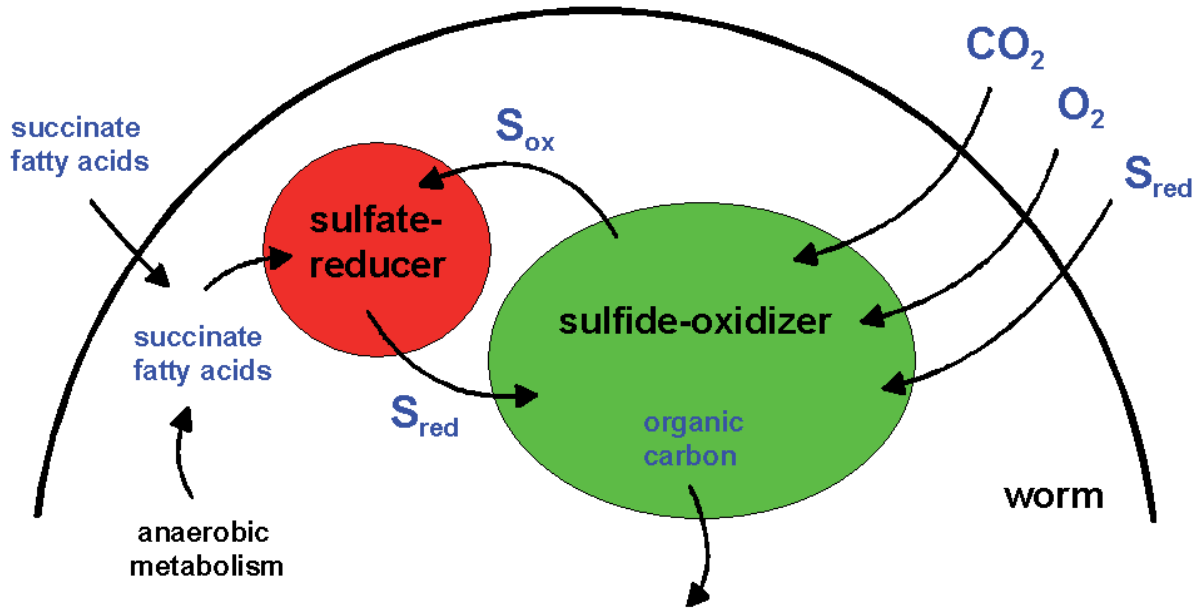


Figure 7. Schematic model of the hypothetical S-cycle in *O. algarvensis* as depicted in Mülders, 1999. The large sulfide oxidizer oxidizes reduced sulfur from the environment and sulfate reducers to gain energy for fixing carbon dioxide into higher organic compounds. The host benefits either by lysing the bacteria or taking up exuded carbon compounds. Under limited oxygen conditions the worm switches to anaerobic metabolism and released waste products are taken up by the sulfate reducer which oxidizes them with oxidized S compounds such as sulfate, readily available from the marine environment or the sulfide-oxidizer. For a net gain oxygen and energy rich compounds, such as fatty acids or reduced sulfur have to be taken up from the environment.

The discovery of a second gutless oligochaete host, *O. ilvae*, previously identified as *Inanidrilus bonomii* (Mülders, 1999), co-existing with *O. algarvensis*, gave an ideal opportunity to validate or reassess the hypothesis drawn for the ecology of the *O. algarvensis* symbiosis. This novel species, *O. ilvae* and its bacterial morphotypes were described by Giere and Erséus (2002). In *O. ilvae* a large morphotype (length 2.3 µm, width 1.3 µm) was similar to that of *O. algarvensis* but slightly smaller. A small morphotype was described as rod-shaped (length 1.3 -1.9 µm, width 0.4 µm). A third bacterial morphotype was of intermediate size and shape (length 1.6 µm, width 0.6 µm). In first molecular analyses of one *O. ilvae* individual a novel Gammaproteobacterium sequence falling into the group of Gamma 1 symbionts as well as the Delta 1 sequence and an additional unique deltaproteobacterial sequence were retrieved, suggesting similar but not identical symbiont composition in the two co-occuring host species (Mülders, 1999).

As presented in aims, this thesis further analysed symbiont composition, phylogeny, genetic, and physiological characteristics of these Mediterranean symbioses which are unique in occuring in an environment with little free sulfide.

2.2 Ectosymbioses of the gut-bearing Wadden Sea oligochaete *Tubificoides benedii*

While gutless oligochaete symbioses have been studied extensively culminating in the metagenomic study of *O. algarvensis* (Woyke et al., 2006) little is known of other chemosynthetic coastal oligochaete symbioses. *Tubificoides benedii* (d'Udekem, 1855) is so far the only coastal gut-bearing oligochaete that has been reported to associate with bacterial filaments as epibionts (see Chapter 3).

2.2.1 Distribution and habitat of *T. benedii*, Tubificinae (Naididae, Oligochaeta)

Tubificoides benedii is a small oligochaete ubiquitous to limnic and marine coastal, intertidal sediments that are subject to highly variable environmental factors. *Tubificoides* species occur worldwide with populations described from the Northeast and Northwest Atlantic, the Mediterranean (e.g. *Tubificoides vestibulatus)*, the Northeast Pacific, Heron Island, as well as the Gulf of Mexico, the Carribean and subtropical Asia (Baker, 1984; Milligan, 1991). *Tubificoides* populations also occur in seasonally variable abundances in sediments of the Northwestern Black Sea Shelf (Shurova, 2006).

Figure 8. Collection site (arrow) of *T. benedii* individuals at the Lister Haken, Sylt, North Sea, scale 500 m (Google maps).

T. benedii is a pioneer species and often dominates polluted coastal sediments. In previous experiments it had a high tolerance to hypoxic and sulfidic conditions (Giere et al., 1999). In the muddy sediments at the collection site of the worms studied here, at the Lister Haken of the island of Sylt (Fig. 8), the upper few millimetres were oxidized while the sediment layers below, where most worms occured in up to 5 cm depth were anoxic. Sulfide concentrations increasing with depth ranged between 5 and 150 µM (Thiermann et al., 1996), however, often exceeding 500 µmol (Giere et al., 1988a) and even increasing to 1 mM in late summer when algal mats extended over large parts of the intertidal mud flat (Thiermann et al., 1996; Dubilier et al., 1997a).

The negative effect of less energy generating anaerobic metabolism to which the worm had to switch in these oxygen depleted and sulfidic sediments was outweighed by a safe environment with high nutritional value, i.e. few predators and a rich supply of organic matter and bacteria (Giere et al., 1999). Like most other oligochaetes it ingested sediment rich in organic debris and bacteria for nutrition.

Its sulfide tolerating lifestyle and its habitat with a rich supply of reduced sulfur and temporal surges of oxygen made it a candidate for sulfur-based symbioses. Morphologically distinct filamentous bacteria resembling *Thiothrix* have been found to regularly attach to the oligochaetes' posterior end (see 2.2.3).

2.2.2 *T. benedii* physiology

In the sediment, the worms were positioned head down with the tail waving in the overlying water (Fig. 9A), (Dubilier, 1986) as most other tubificids (Guérin and Giani, 1996). Tail movement was considered a means to increase the oxygen levels in the immediate surrounding through circulation for their intestinal breathing that is also known from other tubificids (Dahl, 1960; Dubilier, 1986; Guérin and Giani, 1996). Their body had a red coloring due to the haemoglobin in their blood (Fig. 9B). In highly eutrophic sediments they often appeared black due to iron-sulfide precipitates in the mucus. While precipitates in the mucus between the papillae included sulfur, iron, silicon, and aluminium the cuticular matrix remained free of these (Giere et al., 1988a).

As both ends of the worm were free of mucus, H_2S could readily diffuse into the worm. It has been suggested that the mucus and regular shedding of the cuticle/mucus complex could only be of minor importance as a sulfide trap and detoxification mechanism (Dubilier, 1993). Furthermore, oxidation of sulfide to thiosulfate would not countervail against sulfide diffusion. Precipitation of sulfide by iron to iron sulfides also had no significant effect on hindering sulfide diffusion (Dubilier, 1993) leading to the question of how these worms coped with the fluctuating conditions with periodical hypoxic and sulfidic conditions.

Physiological experiments showed that these worms were effective oxyregulators with signs of severe hypoxia shown only at air saturation below 10% and minimal respiration still occurring at only 2% air saturation (Giere et al., 1999). Compared to other sediment worms it had a very low critical PO_2-value (equivalent to between 7.6% and 3.8% air saturation) where it switched from aerobic to anaerobic

metabolism, measured by mitochondrial succinate production (Giere et al., 1999). Under sulfidic and microaerobic conditions, anaerobic metabolism began at low sulfide conditions of about 30 µM, while under normoxic conditions respiration continued at concentrations up 300 µM sulfide (Giere et al., 1999). *T. benedii* appeared to cope with extreme conditions (low oxygen concentrations and temporarily high sulfide concentrations) by an effective circulation of blood of high oxygen affinity and a switch to energetically less favorable anaerobic metabolism under hypoxic conditions (Giere et al., 1999). In laboratory experiments succinate production (as an indicator for mitochondrial anaerobism) increased significantly after 12 hours. However, there was no significant change or damage in mitochondrial structure, even after 14 days of sulfidic and hypoxic conditions. This appeared quite unique as invertebrate mitochondria usually show signs of damage with severe hypoxia. Thus *T. benedii* mitochondria seemed to have an extraordinary high tolerance to these conditions (Dubilier, 1993). As mentioned above sulfide concentrations and oxygen limitation could reach extremes in tidal mud flats, especially in summer. However, these were temporal and *T. benedii* could revert easily to aerobic metabolism even during short and small increases of environmental oxygen (Giere et al., 1999).

It is unclear whether *T. benedii* can gain energy from reducing sulfur compounds without the aid of symbiotic bacteria by mitochondrial oxidation of sulfide to thiosulfate like its North Sea sediment cohabitating relative *Arenicola marina* (Völkel and Grieshaber, 1997) for which the involved enzymes were recently identified (Hildebrandt and Grieshaber, 2008). *A. marina* had furthermore a sulfide-insensitive mitochondrial cytochrome c oxidase (Völkel and Grieshaber, 1997).

2.2.3 Morphological characteristics of the association

As one of the smallest members of the macrofauna, *T. benedii* can grow to about 5.5 cm with a diameter of about 0.5 cm and have 75 to 100 body segments. Most internal structures are comparable to those of other tubificids. However, as a unique morphological peculiarity among tubificids these worms have mucus covered leaf-shaped cutaneous papillae that are regularly shed (Giere et al., 1988a). Ultrastructural studies revealed high abundances of diverse bacterial morphotypes populating the mucus membrane between the papillae, while none were found in the mucus of the clitellar region (Fig. 9C and D),(Giere et al., 1988a).

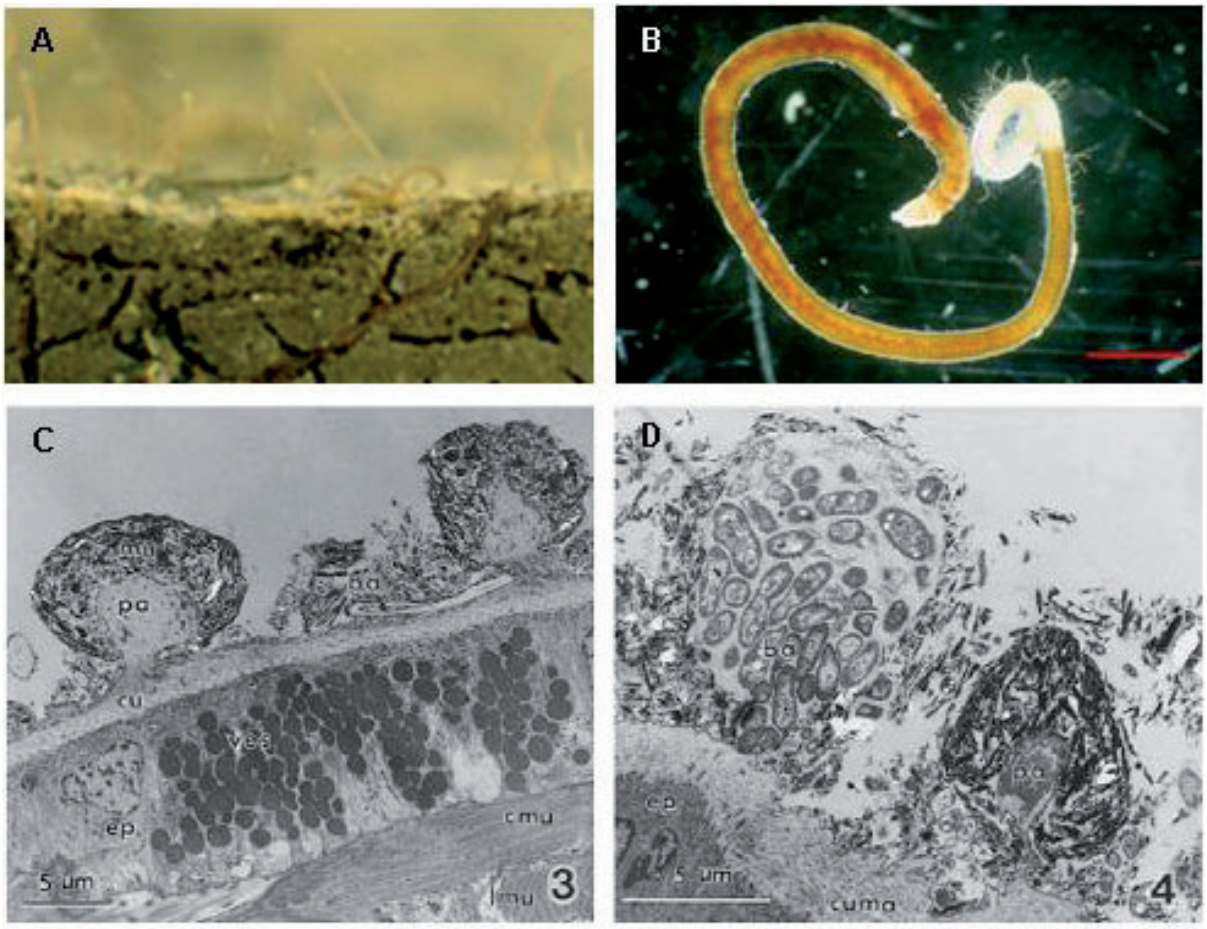


Figure 9. (A) *T. benedii* in laboratory sediment with tail ends waving in the overlying water for intestinal respiration. (B) Light microscopic image of an intact worm with filamentous bacteria colonizing the posterior end, scale bar 1 mm. (C) Cross section of body wall with cuticular papillae (pa) and detail thereof (D). Mucus (mu) is populated by various bacteria (*ba*). Below cuticle (*cu*) dark mucus vesicles (*ves*) in epidermis (*ep*) for regular release of mucus; *cmu* circular musculature, *lmu* longitudinal musculature, *cuma* cuticular matrix. (A, B) courtesy of N. Dubilier, (C, D) modified from Giere et al., 1988a.

Long filamentous bacteria were found to colonize the posterior end (Fig. 9B). Abundances and distribution of bacterial filaments in the host population were especially high in late summer and autumn when sulfide concentrations were at their peak. Bacterial filaments ranged in size between 10 to 80 µm length and 0.4 to 0.8 µm in diameter. They consisted of individual cells with an electron-light nuclear region in the center and granular cytoplasm along the multilayered cell wall (Fig. 10), (Dubilier, 1986). Globular electron-dense inclusions of 60 to 100 nm diameter occurred regularly in their cytoplasm. Filament morphology resembled that of sulfur-oxidizing bacteria such as *Thiothrix* and *Leucothrix* (Fig. 10). Due to the correlation of bacterial abundance with the presence of sulfide in the sediments and morphological resemblance to sulfur oxidizers, the filaments were proposed to be sulfur-metabolizing bacteria as well. However, no further studies on the identification of the bacteria and their metabolism had been done.

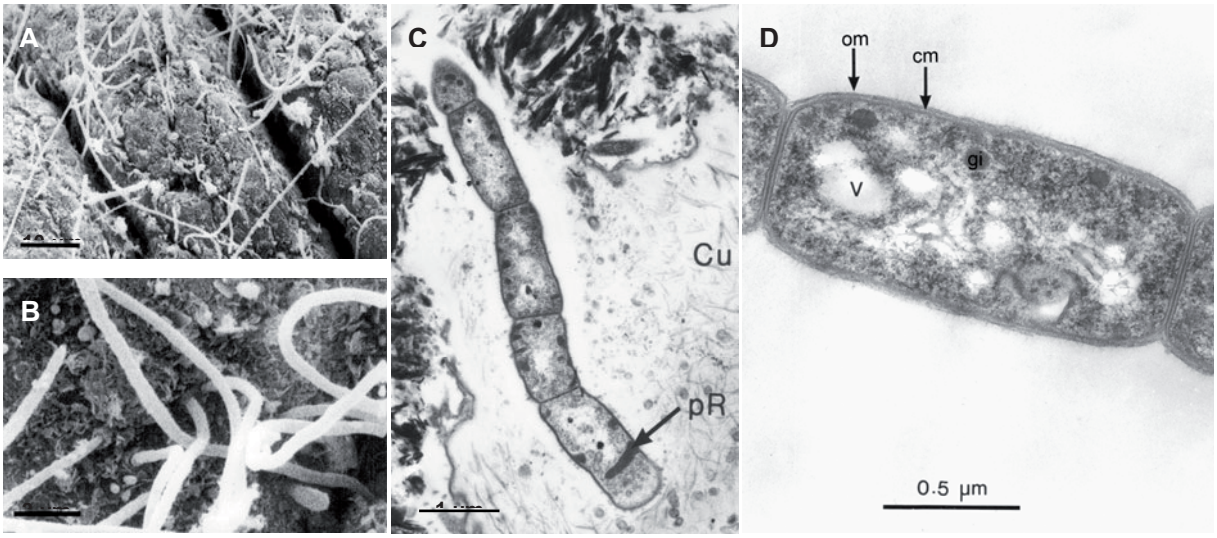


Figure 10. Bacterial filaments on *T. benedii* tail ends in SEM (A, B) and details therof in TEM (C, D). C: Filament embedded in cuticle (Cu). In the basal cell of this bacterial filament the periplasmic space (pR) extends into the inner cell. Note tiny globules around basal end. D: Bacterial cells contain vesicles (V) and globular elctron-dense inclusions (gi) and have an outer membrane (om) and cytoplamic membrane (cm). Modified pictures A,B from SEM by Renate Walter, University of Hamburg and C, D modified from (Dubilier, 1985).

2.2.4 Ecological implications of the associations

While very small globules surrounding the basal cells of the filaments suggest a reaction of the worm, physiological experiments have shown no difference between filament-colonized and filament-free worms (Dubilier, 1985, p 61). Pathogenic effects were also not observed and unlike *Tubifex tubifex* (Fischer and Horváth, 1977) no filaments were detected at cuticular lesions. The association was presumed to be commensal where the bacteria benefit from host excretions and its positioning between oxic and anoxic conditions, while the host stays unaffected (Dubilier, 1986). Identification of the bacteria and their metabolism was one aim of this thesis to elucidate the nature of this association.

3 References

Alain, K., Querellou, J., Lesongeur, F., Pignet, P., Crassous, P., Raguenes, G. et al. (2002) *Caminibacter hydrogeniphilus* gen. nov., sp. nov., a novel thermophilic, hydrogen-oxidizing bacterium isolated from an East Pacific Rise hydrothermal vent. *Int J Syst Evol Microbiol* **52**: 1317-1323.

Altmann, R. (1890) *Die Elementarorganismen und ihre Beziehungen zu den Zellen*. Leipzig: Veit.

Arndt, C., Gaill, F., and Felbeck, H. (2001) Anaerobic sulfur metabolism in thiotrophic symbioses. *J Exp Biol* **204**: 741-750.

Arp, A.J., Childress, J.J., and Vetter, R.D. (1987) The sulfide-binding protein in the blood of the vestimentiferan tube worm, *Riftia pachyptila*, is the extracellular hemoglobin. *J Exp Biol* **128**: 139-158.

Badger, M.R., and Bek, E.J. (2008) Multiple Rubisco forms in proteobacteria: their functional significance in relation to CO_2 acquisition by the CBB cycle. *J Exp Bot* **59**: 1525-1541.

Baker, H.R. (1984) Diversity and zoogeography of marine Tubificidae (Annelida, Oligochaeta) with notes on variation in widespread species. *Hydrobiologia* **115**: 191-196.

Barnes, R.S.K., Calow, P., Olive, P.J.W., Golding, D.W., and Spicer, J.I. (2001) Part 3: Invertebrate functional biology: Feeding. In *The invertebrates: A synthesis*. Malden, U.S.A.: Blackwell Publishers, pp. 209-237.

Bates, A.E. (2006) Population and feeding characteristics of hydrothermal vent gastropods along environmental gradients with a focus on bacterial symbiosis hosted by *Lepetodrilus fucensis* (Vetigastropoda). In *Dept. of Biology*. Victoria: University of Victoria.

Baumann, P., Lai, C.-Y., Baumann, L., Rouhbakhsh, D., Moran Nancy, A., and Clark Marta, A. (1995) Mutualistic associations of aphids and prokaryotes: Biology of the Genus *Buchnera*. *Appl Environ Microbiol* **61**: 1-7.

Bayer, C., Heindl, N.R., Rinke, C., Lücker, S., Ott, J.A., and Bulgheresi, S. (2009) Molecular characterization of the symbionts associated with marine nematodes of the genus *Robbea*. *Environ Microbiol Rep* **1**: 136-144.

Berg, I.A., Kockelkorn, D., Buckel, W., and Fuchs, G. (2007) A 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway in Archaea. *Science* **318**: 1782-1786.

Bergin, C., Brewig, N., Blazejak, A., Giere, O., Erséus, C., and Dubilier, N. (submitted) Replacement of the primary symbiont in the gutless marine worm *Inanidrilus exumae* by a sulfur oxidizer from a novel symbiont lineage.

Blazejak, A., Erséus, C., Amann, R., and Dubilier, N. (2005) Coexistence of bacterial sulfide oxidizers, sulfate reducers, and spirochetes in a gutless worm (Oligochaeta) from the Peru margin. *Appl Environ Microbiol* **71**: 1553-1561.

Blazejak, A., Kuever, J., Erséus, C., Amann, R., and Dubilier, N. (2006) Phylogeny of 16S rRNA, ribulose 1,5-bisphosphate carboxylase/oxygenase, and adenosine 5 '-phosphosulfate reductase genes from gamma- and alphaproteobacterial

symbionts in gutless marine worms (Oligochaeta) from Bermuda and the Bahamas. *Appl Environ Microbiol* **72**: 5527-5536.

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

Brigmon, R.L., and De Ridder, C. (1998) Symbiotic relationship of *Thiothrix* spp. with an echinoderm. *Appl Environ Microbiol* **64**: 3491-3495.

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

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

Campbell, B.J., Jeanthon, C., Kostka, J.E., Luther, G.W., III, and Cary, S.C. (2001) Growth and phylogenetic properties of novel bacteria belonging to the epsilon subdivision of the Proteobacteria enriched from *Alvinella pompejana* and deep-sea hydrothermal vents. *Appl Environ Microbiol* **67**: 4566-4572.

Canale-Parola, E. (1992) Free-living saccharolytic spirochetes: The genus *Spirochaeta*. In *The Prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer-Verlag, pp. 3524-3536.

Carman, K.R., and Dobbs, F.C. (1997) Epibiotic microorganisms on copepods and other marine crustaceans. *Micro Res Tech* **37**: 116-135.

Cavanaugh, C.M. (1985) Symbioses of chemoautotrophic bacteria and marine invertebrates from hydrothermal vents and reducing sediments. *Bull Biol Soc Wash*: 373-388.

Cavanaugh, C.M., McKiness, Z.P., Newton, I.L.G., and Stewart, F.J. (2006) Marine chemosynthetic symbioses. In *The Prokaryotes: An evolving electronic resource for the microbial community*. Dworkin, M., Falkow, S.I., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds). New York: Springer, pp. 475-507.

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

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

Cordaux, R., Pichon, S., Ling, A., Perez, P., Delaunay, C., Vavre, F. et al. (2008) Intense transpositional activity of insertion sequences in an ancient obligate endosymbiont. *Mol Biol Evol* **25**: 1889-1896.

Corliss, J.B., and Ballard, R.D. (1977) Oases of life in the cold abyss. *National Geographic Magazine* **152**: 440-453.

Corliss, J.B., Dymond, J., Gordon, L.I., Edmond, J.M., Herzen, R.P.V., Ballard, R.D. et al. (1979) Submarine thermal springs in the Galapagos Rift. *Science* **203**: 1073-1083.

Corre, E., Reysenbach, A.-L., and Prieur, D. (2001) ε-Proteobacterial diversity from a deep-sea hydrothermal vent on the Mid-Atlantic Ridge. *FEMS Microbiol Lett* **205**: 329-335.

d'Udekem, J. (1855) Nouvelle classification des Annélides sétigères abranches. *Bull Acad R Belg Cl Sci* **22**: 533-555.

Dahl, C., Schulte, A., Stockdreher, Y., Hong, C., Grimm, F., Sander, J. et al. (2008) Structural and molecular genetic insight into a widespread sulfur oxidation pathway. *J Mol Biol* **384**: 1287-1300.

Dahl, I.O. (1960) The oligochaete fauna of 3 Danish brackish water areas. *Meddr Danm Fisk Havunders* **26**: 1-20.

Dale, C., and Moran, N.A. (2006) Molecular interactions between bacterial symbionts and their hosts. *Cell* **126**: 453-465.

Dattagupta, S., Schaperdoth, I., Montanari, A., Mariani, S., Kita, N., Valley, J.W., and Macalady, J.L. (2009) A novel symbiosis between chemoautotrophic bacteria and a freshwater cave amphipod. *ISME J* **3**: 935-943.

de Bary, A. (1879) Die Erscheinung der Symbiose. In *Vortrag auf der Versammlung der Naturforscher und Ärzte zu Cassel*. Strassburg: Karl J.Trubner, pp. 1-30.

Demiri, A., Meziti, A., Papaspyrou, S., Thelassou-Legaki, M., and Kormas, K. (In press) Abdominal setae and midgut bacteria of the mudshrimp *Pestarella tyrrhena*. *Cent Eur J Biol*.

Distel, D.L., Lee, H.K.W., and Cavanaugh, C.M. (1995) Intracellular coexistence of methanotrophic and thioautotrophic bacteria in a hydrothermal vent mussel. *PNAS* **92**: 9598-9602.

Dubilier, N. (1985) Zur Ökophysiologie von *Tubificoides benedii* (Oligochaeta) aus sulfidischen Lebensräumen mit ergänzenden ultrastrukturellen Beobachtungen an endocuticulären Bakterien (Diplomarbeit). In *Fachbereich Biologie*. Hamburg: University of Hamburg.

Dubilier, N. (1986) Association of filamentous epibacteria with *Tubificoides benedii* (Oligochaeta, Annelida). *Mar Biol* **92**: 285-288.

Dubilier, N. (1993) Ökophysiologische und morphologische Anpassungen des marinen Oligochaeten *Tubificoides benedii* (d'Udekem) an Sauerstoffmangel und Sulfid. (PhD thesis). In *Fachbereich Biologie*. Hamburg: University of Hamburg.

Dubilier, N., Blazejak, A., and Ruehland, C. (2006) Symbioses between bacteria and gutless marine oligochaetes. In *Molecular Basis of Symbiosis*. Overmann, J. (ed). New York: Springer Verlag, pp. 251-275.

Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Micro* **6**: 725-740.

Dubilier, N., Giere, O., Distel, D.L., and Cavanaugh, C.M. (1995) Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (Oligochaeta, Annelida) by phylogenetic 16S rRNA sequence analysis and in situ hybridization. *Appl Env Microbiol* **61**: 2346-2350.

Dubilier, N., Windoffer, R., Grieshaber, M.K., and Giere, O. (1997a) Ultrasructure and anaerobic metabolism of mitochondria in the marine oligochaete *Tubificoides benedii*: Effects of hypoxia and sulfide. *Mar Biol* **127**: 637-645.

Dubilier, N., Amann, R., Erséus, C., Giere, O., Muyzer, G., Park, S., and Cavanaugh, C.M. (1997b) Phylogenetic diversity of bacterial endosymbionts in a gutless marine worm (Oligochaeta, Annelida). *Amer Zool* **37**: 196A.

Dubinina, G.A., Grabovich, M.Y., and Chernyshova, Y.Y. (2004) The role of oxygen in the regulation of the metabolism of aerotolerant spirochetes, a major component of "Thiodendron" bacterial sulfur mats. *Microbiology* **73**: 621-628.

Dufour, S.C., and Felbeck, H. (2003) Sulphide mining by the superextensile foot of symbiotic thyasirid bivalves. *Nature* **426**: 65-67.

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

Duperron, S., Lorion, J., Samadi, S., Gros, O., and Gaill, F. (2009) Symbioses between deep-sea mussels (Mytilidae: Bathymodiolinae) and chemosynthetic bacteria: diversity, function and evolution. *C R Acad Sci Biol* **322**: 298-310.

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

Duplessis, M.R., Ziebis, W., Gros, O., Caro, A., Robidart, J., and Felbeck, H. (2004) Respiration strategies utilized by the gill endosymbiont from the host lucinid *Codakia orbicularis* (Bivalvia: Lucinidae). *Appl Environ Microbiol* **70**: 4144-4150.

Eisen, J. and International Aphid Genomics Consortium, (2010) Genome sequence of the pea aphid *Acyrthosiphon pisum*. *PloS Biol* **8**: doi:10.1371/journal.pbio. 1000313.

Erséus, C. (1984) Taxonomy and phylogeny of the gutless Phallodrilinae (Oligochaeta, Tubificidae), with descriptions of one new genus and twenty-two new species. *Zool Scr* **13**: 239-272.

Erséus, C., and Bergfeldt, U. (2007) Six new species of the gutless genus *Olavius* (Annelida: Clitellata: Tubificidae) from New Caledonia. *Zootaxa* **1400**: 45-58.

Euzéby, J.P. (2009). List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. URL http://www.bacterio.cict.fr/t/thiotrichaceae.html

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

Felbeck, H., Liebezeit, G., Dawson, R., and Giere, O. (1983) CO_2 fixation in tissues of marine oligochaetes (*Phallodrilus leukodermatus* and *P. planus*) containing symbiotic, chemoautotrophic bacteria. *Mar Biol* **75**: 187-191.

Fenchel, T., and Finlay, B.J. (1989) Kentrophoros - a mouthless ciliate with a symbiotic kitchen garden. *Ophelia* **30**: 75-93.

Fenchel, T., and Finlay, B.J. (1995) *Ecology and evolution in the anoxic world*. Oxford: Oxford University Press.

Fiala-Medioni, A., McKiness, Z.P., Dando, P., Boulegue, J., Mariotti, A., Alayse-Danet, A.M. et al. (2002) Ultrastructural, biochemical, and immunological characterization of two populations of the mytilid mussel *Bathymodiolus azoricus* from the Mid-Atlantic Ridge: evidence for a dual symbiosis. *Mar Biol* **141**: 1035-1043.

Fischer, E., and Horváth, I. (1977) Cytochemical studies on the cuticle and epidermis of *Tubifex tubifex* Müll. with special regard to the localization of polysaccharides, heavy metals, and the DAB-reactivity. *Histochemistry* **54**: 259-271.

Frank, A.B. (1877) Ueber die biologischen Verhältnisse des Thallus einiger Krustenflechten. *(Cohn's) Beiträge zur Biologie der Pflanzen* **2**: 123-200.

Friedrich, C.G., Quentmeier, A., Bardischewsky, F., Rother, D., Orawski, G., Hellwig, P., and Fischer, J. (2008) Redox control of chemosynthetic sulfur oxidation of *Paracoccus pantotrophus*. In *Microbial Sulfur Metabolism*. Dahl, C., and Friedrich, C.G. (eds). Heidelberg: Springer Verlag, pp. 139-150.

Galchenko, V.F., Pimenov, N.V., Lein, A.Y., Galkin, S.V., Miller, Y.M., and Ivanov, M.V. (1992) Mixotrophic type of feeding of *Olgaconcha tufari* BECK (Gastropoda: Prosobranchia) from the active hydrothermal field of the Manus Basin (Bismarck Sea). *Doklady Akademii Nauk* **323**: 776-781.

Garrity, G.M., Bell, J.A., and Lilburn, T. (2005) Family I. Thiotrichaceae fam. nov. In *Bergey's Manual of Systematic Bacteriology*. Brenner, D.J., Krieg, N.R., and Staley, J.T. (eds). New York: Springer, p. p.131.

Giere, O. (1979) Studies on marine Oligochaeta from Bermuda, with emphasis on new *Phallodrilus* species (Tubificidae). *Cah Biol Mar* **20**: 301-314.

Giere, O. (1981) The gutless marine oligochaete *Phallodrilus leukodermatus* - structural studies on an aberrant tubificid associated with bacteria. *Mar Ecol Prog Ser* **5**: 353-357.

Giere, O., and Langheld, C. (1987) Structural organization, transfer and biological fate of endosymbiotic bacteria in gutless oligochaetes. *Mar Biol* **93**: 641-650.

Giere, O., and Krieger, J. (2001) A triple bacterial endosymbiosis in a gutless oligochaete (Annelida): ultrastructural and immunocytochemical evidence. *Invert Biol* **120**: 41-49.

Giere, O., and Erséus, C. (2002) Taxonomy and new bacterial symbioses of gutless marine Tubificidae (Annelida, Oligochaeta) from the Island of Elba (Italy). *Org Div Evol* **2**: 289-297.

Giere, O., Rhode, B., and Dubilier, N. (1988a) Structural peculiarities of the body wall of *Tubificoides benedii* (Oligochaeta) and possible relations of its life in sulphidic sediments. *Zoomorphology* **108**: 29-39.

Giere, O., Erséus, C., and Stuhlmacher, F. (1998) A new species of *Olavius* (Tubificidae, Phallodrilinae) from the Algarve Coast in Portugal, the first East Atlantic gutless oligochaete with symbiotic bacteria. *Zool Anz* **237**: 209-214.

Giere, O., Preusse, J.H., and Dubilier, N. (1999) *Tubificoides benedii* (Tubificidae, Oligochaeta) - a pioneer in hypoxic and sulfidic environments. An overview of adaptive pathways. *Hydrobiologia* **406**: 235-241.

Giere, O., Wirsen, C.O., Schmidt, C., and Jannasch, H.W. (1988b) Contrasting effects of sulfide and thiosulfate on symbiotic CO_2-assimilation of *Phallodrilus leukodermatus* (Annelida). *Mar Biol* **97**: 413-419.

Giere, O., Nieser, C., Windoffer, R., and Erséus, C. (1995) A comparative structural study on bacterial symbioses of Caribbean gutless Tubificidae (Annelida, Oligochaeta). *Acta Zoologica* **76**: 281-290.

Gillan, D.C., and Dubilier, N. (2004) Novel epibiotic *Thiothrix* bacterium on a marine amphipod. *Appl Environ Microbiol* **70**: 3772-3775.

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

Goffredi, S.K., Johnson, S.B., and Vrijenhoek, R.C. (2007) Genetic diversity and potential function of microbial symbionts associated with newly discovered species of *Osedax* polychaete worms. *Appl Environ Microbiol* **73**: 2314-2323.

Goffredi, S.K., Childress, J.J., Desaulniers, N.T., and Lallier, F.H. (1997) Sulfide acquisition by the vent worm *Riftia pachyptila* appears to be via uptake of HS^-, rather than H_2S. *J Exp Biol* **200**: 2609-2616.

Goffredi, S.K., Waren, A., Orphan, V.J., Van Dover, C.L., and Vrijenhoek, R.C. (2004) Novel forms of structural integration between microbes and a hydro-

thermal vent gastropod from the Indian Ocean. *Appl Environ Microbiol* **70**: 3082-3090.

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

Goodacre, S.L., Martin, O.Y., Dries, B., Hutchings, L., Woolley, C., Ibrahim, K. et al. (2009) Microbial modification of host long-distance dispersal capacity. *BMC Biology* **7**: doi:10.1186/1741-7007-1187-1132.

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

Guérin, C., and Giani, N. (1996) Analytical study of the locomotor and respiratory movements of tubificid worms by means of video recording. *Hydrobiologia* **333**: 63-69.

Haase, K.M., Petersen, S., Koschinsky, A., Seifert, R., Devey, C.W., Keir, R. et al. (2007) Young volcanism and related hydrothermal activity at 5°S on the slow-spreading southern Mid-Atlantic Ridge. *Geochem Geophys Geosyst* **8**.

Hand, S.C. (1987) Trophosome ultrastructure and the charcterization of isolated bacteriocytes from invertebrate-sulfur bacteria symbioses. *Biol Bull* **173**: 260-276.

Harada, M., Yoshida, T., Kuwahara, H., Shimamura, S., Takaki, Y., Kato, C. et al. (2009) Expression of genes for sulfur oxidation in the intracellular chemoautotrophic symbiont of the deep-sea bivalve *Calyptogena okutanii*. *Extremophiles* **13**: 895-903.

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

Hashimoto, J., Miura, T., Fujikura, K., and Ossaka, J. (1993) Discovery of vestimentiferan tube worms in the euphotic zone. *Zool Sci* **10**: 1063-1067.

Hentschel, U., and Felbeck, H. (1993) Nitrate respiration in the hydrothermal vent tubeworm *Riftia pachyptila*. *Nature* **366**: 338-340.

Hentschel, U., and Felbeck, H. (1995) Nitrate respiration in chemoautotrophic symbionts of the bivalve *Lucinoma aequizonata* is not regulated by oxygen. *Appl Environ Microbiol* **61**: 1630-1633.

Hentschel, U., Hand, S.U., and Felbeck, H. (1996) The contribution of nitrate respiration to the energy budget of the symbiont-containing clam *Lucinoma aequizonata*: A calorimetric study. *J Exp Biol* **199**: 427-433.

Higgins, P.R., and Thiel, H. (1988) *Introduction to the study of meiofauna*. Washington, D.C.: Smithsonian Inst. Press.

Hildebrandt, T.M., and Grieshaber, M.K. (2008) Three enzymatic activities catalyze the oxidation of sulfide to thiosulfate in mammalian and invertebrate mitochondria. *FEBS Journal* **275**: 3352-3361.

Imhoff, J.F., Suling, J., and Petri, R. (1998) Phylogenetic relationships among the Chromatiaceae, their taxonomic reclassification and description of the new genera *Allochromatium, Halochromatium, Isochromatium, Marichromatium, Thiococcus, Thiohalocapsa* and *Thermochromatium*. *Int J Syst Bacteriol* **48**: 1129-1143.

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

Inagaki, F., Takai, K., Kobayashi, H., Nealson, K.H., and Horikoshi, K. (2003) *Sulfurimonas autotrophica* gen. nov., sp. nov., a novel sulfur-oxidizing ε-proteobacterium isolated from hydrothermal sediments in the Mid-Okinawa Trough. *Int J Syst Evol Microbiol* **53**: 1801-1805.

Jamieson, B.G.M. (1977) Marine meiobenthic Oligochaeta from Heron and Wistari Reefs (Great Barrier Reef) of the genera *Clitellio*, *Limnodriloides* and *Phallodrilus* (Tubificidae) and *Grania* (Enchytraeidae). *Zool J Linn Soc* **61**: 329-350.

Jog, M., and Watve, M. (2005) Role of parasites and commensals in shaping host behaviour. *Curr Sci* **89**: 1184-1191.

Johnson, P.W., Sieburth, J.M., Sastry, A., Arnold, C.R., and Doty, M.S. (1971) *Leucothrix mucor* infestation of benthic Crustacea, fish eggs, and tropical algae. *Limnol Oceanogr* **16**: 962-969.

Katz, S., Cavanaugh, C.M., and Bright, M. (2006) Symbiosis of epi- and endocuticular bacteria with *Helicoradomenia* spp. (Mollusca, Aplacophora, Solenogastres) from deep-sea hydrothermal vents. *Mar Ecol Prog Ser* **320**: 89-99.

Kelly, D. (1988) Oxidation of sulphur compounds. In *The nitrogen and sulphur cycles*. Cole, J.A., and Ferguson, S.J. (eds). Cambridge: Cambridge University Press, pp. 65-98.

Kharlamenko, V.I., Zhukova, N.V., Khotimchenko, S.V., Svetashev, V.I., and Kamenev, G.M. (1995) Fatty acids as markers of food sources in a shallow-water hydrothermal ecosystem (Kraternaya Bight, Yankich Island, Kurile Islands). *Mar Ecol Prog Ser* **120**: 231-241.

Kikuchi, Y., and Fukatsu, T. (2008) Insect-bacterium mutualism without vertical transmission. In *Insect Symbiosis:* Bourtzis, K., and Miller, T.A. (eds). Boca Raton: CRC Press. Taylor and Francis Group, pp. 143-162.

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

Koschinsky, A., Garbe-Schoenberg, D., Sander, S., Schmidt, K., Gennerich, H.-H., and Strauss, H. (2008) Hydrothermal venting at pressure-temperature conditions above the critical point of seawater, 5°S on the Mid-Atlantic Ridge. *Geology* **36**: 615-618.

Kostanjevecki, V., Brigé, A., Meyer, T.E., Cusanovich, M.A., Guisez, Y., and Van Beeumen, J. (2000) A membrane-bound flavocytochrome c-sulfide dehydrogenase from the purple phototrophic sulfur bacterium *Ectothiorhodospira vacuolata*. *J Bacteriol* **182**: 3097-3103.

Krieger, J. (2000) Funktion und Übertragung endosymbiontischer Bakterien bei bakteriensymbiontischen, darmlosen marinen Oligochaeten - Function and transmission of the endosymbiotic bacteria in gultess marine oligochaetes. (PhD thesis). In *Fachbereich Biologie*. Hamburg: University of Hamburg, Germany.

Krieger, J., Giere, O., and Dubilier, N. (2000) Localization of RubisCO and sulfur in endosymbiotic bacteria of the gutless marine oligochaete *Inanidrilus leukodermatus* (Annelida). *Mar Biol* **137**: 239-244.

Kuwahara, H., Takaki, Y., Yoshida, T., Shimamura, S., Takishita, K., Reimer, J.D. et al. (2008) Reductive genome evolution in chemoautotrophic intracellular symbionts of deep-sea *Calyptogena* clams. *Extremophiles* **12**: 365-374.

Leadbetter, J.R., Schmidt, T.M., Graber, J.R., and Breznak, J.A. (1999) Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. *Science* **283**: 686-689.

Lebata, M.J.H.L. (2001) Oxygen, sulphide and nutrient uptake of the mangrove mud clam *Anodontia edentula* (family: Lucinidae). *Mar Pollut Bull* **42**: 1133-1138.

Lilburn, T.C., Kim, K.S., Ostrom, N.E., Byzek, K.R., Leadbetter, J.R., and Breznak, J.A. (2001) Nitrogen fixation by symbiotic and free-living spirochetes. *Science* **292**: 2495-2498.

Luyten, Y.A., Thompson, J.R., Morrill, W., Polz, M.F., and Distel, D.L. (2006) Extensive variation in intracellular symbiont community composition among members of a single population of the wood-boring bivalve *Lyrodus pedicellatus* (Bivalvia: Teredinidae). *Appl Environ Microbiol* **72**: 412-417.

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

Margulis, L. (1970) *The origin of eukaryotic cells*. New Haven: Yale University Press.

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

Martin, J.W., and Haney, T.A. (2005) Decapod crustaceans from hydrothermal vents and cold seeps: a review through 2005. *Zool J* **145**: 445-522.

Martin, W., and Kowallik, K.V. (1999) Annotated English translation of Mereschkowsky's 1905 paper ‘Ueber Natur und Ursprung der Chromatophoren im Pflanzenreiche’. *Eur J Phycol* **34**: 287-295.

Mehlhorn, H., and Piekarski, G. (1995) Haemosporidien. In *Grundriß der Parasitenkunde*. Stuttgart: Gustav Fischer Verlag, pp. 93-103.

Mellies, J.L., Navarro-Garcia, F., Okeke, I., Frederickson, J., Nataro, J.P., and Kaper, J.B. (2001) espC pathogenicity island of enteropathogenic *Escherichia coli* encodes an enterotoxin. *Am Soc Microbiol* **69**: 315-324.

Mereschowsky, C. (1905) Über die Natur und den Ursprung der Chromatophoren im Pflanzenreiche. *Biologisches Zentralblatt 25*: 593-604.

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

Meyer, B., Imhoff, J.F., and Kuever, J. (2007) Molecular analysis of the distribution and phylogeny of the *soxB* gene among sulfur-oxidizing bacteria - evolution of the Sox sulfur oxidation enzyme system. *Environ Microbiol* **9**: 2957-2977.

Milligan, M.R. (1991) Two new species of *Tubificoides*(Oligochaeta, Tubificidae) and new records of *T. brownae* and *T. imajimai* from the Gulf of Mexico and Caribbean, with a redescription of *T. bakeri*. *Zool Scripta* **20**: 339-345.

Minic, Z., and Hervé, G. (2004) Biochemical and enzymological aspects of the symbiosis between the deep-sea tubeworm *Riftia pachyptila* and its bacterial endosymbiont. *Eur J Biochem* **271**: 3093-3102.

Minkley, N., Fujita, A., Brune, A., and Kirchner, W. (2006) Nest specificity of the bacterial community in termite guts (*Hodotermes mossambicus*). *Insectes Sociaux* **53**: 339-344.

Miroshnichenko, M.L., Kostrikina, N.A., L'Haridon, S., Jeanthon, C., Hippe, H., Stackebrandt, E., and Bonch-Osmolovskaya, E.A. (2002) *Nautilia lithotrophica* gen. nov., sp. nov., a thermophilic sulfur-reducing epsilon-proteobacterium isolated from a deep-sea hydrothermal vent. *Int J Syst Evol Microbiol* **52**: 1299-1304.

Moran, N.A. (2003) Tracing the evolution of gene loss in obligate bacterial symbionts. *Curr Opin Microbiol* **6**: 512-518.

Moya, A., Pereto, J., Gil, R., and Latorre, A. (2008) Learning how to live together: genomic insights into prokaryote-animal symbioses. *Nature Rev Genet* **9**: 218-229.

Mülders, C. (1999) Molekularbiologische Charakterisierung von Bakterien assoziiert mit marinen Oligochaeten (Annelida). (Diplomarbeit). In *Fachbereich Biologie*. Hamburg: University of Hamburg.

Musat, N., Giere, O., Gieseke, A., Thiermann, F., Amann, R., and Dubilier, N. (2007) Molecular and morphological characterization of the association between bacterial endosymbionts and the marine nematode *Astomonema* sp. from the Bahamas. *Environ Microbiol* **9**: 1345-1353.

Muscholl-Silberhorn, A., Thiel, V., and Imhoff, J.F. (2008) Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. *Microb Ecol* **55**: 94-106.

Naganuma, T., Elsaied, H., Hoshii, D., and Kimura, H. (2005) Bacterial endosymbioses of gutless tube-dwelling worms in nonhydrothermal vent habitats. *Mar Biotech* **7**: 416-428.

Nakagawa, S., and Takai, K. (2008) Deep-sea vent chemoautotrophs: diversity, biochemistry and ecological significance. *FEMS Microbiol Ecol* **65**: 1-14.

Nakagawa, S., Takai, K., Inagaki, F., Hirayama, H., Nunoura, T., Horikoshi, K., and Sako, Y. (2005) Distribution, phylogenetic diversity and physiological characteristics of epsilon-Proteobacteria in a deep-sea hydrothermal field. *Environ Microbiol* **7**: 1619-1632.

Nalepa, C.A., Bignell, D.E., and Bandi, C. (2001) Detritivory, coprophagy, and the evolution of digestive mutualisms in Dictyoptera. *Insectes Sociaux* **48**: 194-201.

Nelson, D.C., and Hagen, K.D. (1995) Physiology and biochemistry of symbiotic and free-living chemoautotrophic sulfur bacteria. *Amer Zool* **35**: 91-101.

Nussbaumer, A.D., Fisher, C.R., and Bright, M. (2006) Horizontal endosymbiont transmission in hydrothermal vent tubeworms. *Nature* **441**: 345-348.

Nussbaumer, A.D., Bright, M., Baranyi, C., Beisser, C.J., and Ott, J.A. (2004) Attachment mechanism in a highly specific association between ectosymbiotic bacteria and marine nematodes. *Aquat Microb Ecol* **34**: 239-246.

Nylander, J.A.A., Erséus, C., and Kallersjo, M. (1999) A test of monophyly of the gutless Phallodrilinae (Oligochaeta, Tubificidae) and the use of a 573-bp region of the mitochondrial cytochrome oxidase I gene in analysis of annelid phylogeny. *Zool Scr* **28**: 305-313.

Oelschlaeger, T.A., Dobrindt, U., and Hacker, J. (2002) Pathogenicity islands of uropathogenic *E. coli* and the evolution of virulence. *Int J Antimicrobiol Ag* **19**: 517-521.

Oeschger, R., and Schmaljohann, R. (1988) Association of various types of epibacteria with *Halicryptus spinulosus* (Priapulida). *Mar Ecol Prog Ser* **48**: 285-293.

Ott, J., Bright, M., and Bulgheresi, S. (2004) Symbioses between marine nematodes and sulfur-oxidizing chemoautotrophic bacteria. *Symbiosis* **36**: 103-126.

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

Paracer, S., and Ahmadjian, V. (2000) In *Symbiosis: An Introduction to Biological Associations*. Oxford: Oxford University Press.

Parkes, J.R., and Wellsbury, P. (2004) Deep biospheres. In *Microbial diversity and bioprospecting* Bull, A.T. (ed). Washington, D.C.: ASM Press, pp. 120-129.

Payne, M.S., Hall, M.R., Sly, L., and Bourne, D.G. (2007) Microbial diversity within early-stage cultured *Panulirus ornatus* phyllosomas. *Appl Environ Microbiol* **73**: 1940-1951.

Petersen, J., and Dubilier, N. (2009a) Methanotrophic symbioses in marine invertebrates. *Environ Microbiol* **1**: 319-335.

Petersen, J., Ramette, A., Lott, C., Cambon-Bonavita, M.-A., Zbinden, M., and Dubilier, N. (2009) Dual symbiosis of the vent shrimp *Rimicaris exoculata* with filamentous gamma- and epsilonproteobacteria at four Mid-Atlantic Ridge hydrothermal vent fields. *Environ Microbiol*: doi:10.1111/j.1462-2920.2009.02129.x

Petersen, J.M., and Dubilier, N. (2009b) Symbiotic methane oxidizers. In *Handbook of Hydrocarbon and Lipid Microbiology*. Timmis, K.N. (ed): Springer Berlin Heidelberg, pp. 1977-1996.

Petri, R., Podgorsek, L., and Imhoff, J.F. (2001) Phylogeny and distribution of the *sox*B gene among thiosulfate-oxidizing bacteria. *FEMS Microbiol Lett* **197**: 171-178.

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

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

Pleijel, F., Dahlgren, T.G., and Rouse, G.W. (2009) Progress in systematics: from Siboglinidae to Pogonophora and Vestimentifera and back to Siboglinidae. *C R Biol* **332**: 140-148.

Polz, M.F., Felbeck, H., Novak, R., Nebelsick, M., and Ott, J.A. (1992) Chemoautotrophic, sulfur-oxidizing symbiotic bacteria on marine nematodes: morphological and biochemical characterization. *Microb Ecol* **24**: 313-329.

Pospesel, M.A., Hentschel, U., and Felbeck, H. (1999) Determination of nitrate in the blood of the hydrothermal vent tubeworm *Riftia pachyptila* using a bacterial nitrate reduction assay. *Deep-Sea Res I-Oceanogr Res Pap* **45**: 2189-2200.

Ragsdale, S.W. (1991) Enzymology of the acetyl-CoA pathway of CO2 fixation. *CRC Crit Rev Biochem Mol Biol* **26**: 261-300.

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

Rinke, C., Schmitz-Esser, S., Loy, A., Horn, M., Wagner, M., and Bright, M. (2009) High genetic similarity between two geographically distinct strains of the sulfur-oxidizing symbiont *Candidatus* Thiobios zoothamnicoli. *FEMS Microbiol Ecol* **67**: 229-241.

Rinke, C., Schmitz-Esser, S., Stoecker, K., Nussbaumer, A.D., Molnar, D.A., Vanura, K. et al. (2006) *Candidatus* Thiobios zoothamnicoli, an ectosymbiotic bacterium covering the giant marine ciliate *Zoothamnium niveum*. *Appl Environ Microbiol* **72**: 2014-2021.

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

Røy, H., Vopel, K., Huettel, M., and Jørgensen, B. (2009) Sulfide assimilation by ectosymbionts of the sessile ciliate *Zoothamnium niveum*. *Mar Biol* **156**: 669-677.

Ruehland, C., Bergin, C., Lott, C., and Dubilier, N. (2006) Darmlose marine Würmer: Symbiosen mit mikrobiellen Konsortien. *Biospektrum* **06**: 600-602.

Schauder, R., Widdel, F., and Fuchs, G. (1987) Carbon assimilation pathways in sulfate-reducing bacteria II. Enzymes of a reductive citric acid cycle in the autotrophic *Desulfobacter hydrogenophilus*. *Arch Microbiol* **148**: 218-225.

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

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

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

Schmidt, C., Corbari, L., Gaill, F., and Bris, N.L. (2009) Biotic and abiotic controls on iron oxyhydroxide formation in the gill chamber of the hydrothermal vent shrimp *Rimicaris exoculata*. *Geobiology* **7**: 454-464.

Schmitz-Esser, S., Tischler, P., Arnold, R., Montanaro, J., Wagner, M., Rattei, T., and Horn, M. (2010) The genome of the amoeba symbiont "*Candidatus* Amoebophilus asiaticus" reveals common mechanisms for host cell interaction among amoeba-associated bacteria. *J Bacteriol* **192**: 1045-1057.

Schwendener, S. (1867) Über die wahre Natur der Flechten. *Verhandlungen der Schweizerischen Naturforschenden Gesellschaft in Rheinfelden* **51**: 88-90.

Sharon, N. (2008) Lectins: past, present and future. *Biochem SocTrans* **36**: 1457-1460.

Sherwood Lollar, B., Lacrampe-Couloume, G., Slater, G.F., Ward, J., Moser, D.P., Gihring, T.M. et al. (2006) Unravelling abiogenic and biogenic sources of methane in the Earth's deep subsurface. *Chemical Geology* **226**: 328-339.

Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., and Ishikawa, H. (2000) Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* **407**: 81-86.

Shively, J.M., van Keulen, G., and Meijer, W.G. (2003) Something from almost nothing: carbon dioxide fixation in chemoautotrophs. *Annu Rev Microbiol* **52**: 191-230.

Shurova, N.M. (2006) The appearance in the Black Sea of Atlantic Oligochaeta *Tubificoides benedii* (Annelida, Oligochaeta) and peculiarities of its distribution on the Northwestern Black Sea shelf. *Vestnik Zoologii* **40**: 453-455.

Sibuet, M., and Olu, K. (1998) Biogeography, biodiversity and fluid dependence of deep-sea cold-seep communities at active and passive margins. *Deep-Sea Res II* **45**: 517-567.

Sibuet, M., and Olu-Le Roy, K. (2002) Cold seep communities on continental margins: Structure and quantitative distribution relative to geological and fluid venting patterns. In *Ocean Margin Systems*. Wefer, G., Billett, D., Hebbeln, D., Jørgensen, B.B., and Schlüter, M. (eds). Berlin, Heidelberg: Springer-Verlag, pp. 235-251.

Sievert, S.M., Hügler, M., Taylor, C.D., and Wirsen, C.O. (2008a) Sulfur oxidation at deep-sea hydorthermal vents. In *Microbial Sulfur Metabolism*. Dahl, C., and Friedrich, C.G. (eds). Heidelberg: Springer Verlag, pp. 238-258.

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

Sjölin, E., Erséus, C., and Kallersjo, M. (2005) Phylogeny of Tubificidae (Annelida, Clitellata) based on mitochondrial and nuclear sequence data. *Mol Phyl Evol* **35**: 431-441.

Smith, D.C. (1979) From extracellular to intracellular: the establishment of a symbiosis. *Proc R Soc Lond B* **204**: 115-130.

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

Stephens, E.A., Braissant, O., and Visscher, P.T. (2008) Spirochetes and salt marsh microbial mat geochemistry: Implications for the fossil record. In *Carnets de Géologie / Notebooks on Geology*.

Stewart, F.J., Newton, I.L.G., and Cavanaugh, C.M. (2005) Chemosynthetic endosymbioses: adaptations to oxic-anoxic interfaces. *Trends Microbiol* **13**: 439-448.

Strauss, G., and Fuchs, G. (1993) Enzymes of a novel autotrophic CO_2 fixation pathway in the phototrophic bacterium *Chloroflexus aurantiacus*, the 3-hydroxypropionate cycle. *Eur J Biochem* **215**: 633-643.

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

Tabita, F.R., Hanson, T.E., Li, H., Satagopan, S., Singh, J., and Chan, S. (2007) Function, structure, and evolution of the RubisCO-like proteins and their RubisCO homologs. *Microbiol Mol Biol Rev* **71**: 576-599.

Takai, K., Suzuki, M., Nakagawa, S., Miyazaki, M., Suzuki, Y., Inagaki, F., and Horikoshi, K. (2006) *Sulfurimonas paralvinellae* sp. nov., a novel mesophilic, hydrogen- and sulfur-oxidizing chemolithoautotroph within the Epsilonproteobacteria isolated from a deep-sea hydrothermal vent polychaete nest, reclassification of *Thiomicrospira denitrificans* as *Sulfurimonas denitrificans* comb. nov. and emended description of the genus *Sulfurimonas*. *Int J Syst Evol Microbiol* **56**: 1725-1733.

Tarasov, V.G., Gebruk, A.V., Mironov, A.N., and Moskalev, L.I. (2005) Deep-sea and shallow-water hydrothermal vent communities: Two different phenomena? *Chem Geol* **224**: 5-39.

Thiermann, F., Niemeyer, A.S., and Giere, O. (1996) Variations in the sulfide regime and the distribution of macrofauna in an intertidal flat in the North Sea. *Helgol Meeresunt* **50**: 87-104.

Thorsen, M.S., Wieland, A., Ploug, H., Kragelund, C., and H.P., N. (2003) Distribution, identity and activity of symbiotic bacteria in anoxic aggregates from the hindgut of the sea urchin *Echinocardium cordatum*. *Ophelia* **57**: 1-12.

Tokuda, G., Yamada, A., Nakano, K., Arita, N.O., and Yamasaki, H. (2008) Colonization of *Sulfurovum* sp. on the gill surfaces of *Alvinocaris longirostris*, a deep-sea hydrothermal vent shrimp. *Mar Ecol* **29**: 106-114.

Touchon, M., and Rocha, E.P.C. (2007) Causes of insertion sequences abundance in prokaryotic genomes. *Mol Biol Evol* **24**: 969-981.

Turnipseed, M., Knick, K.E., Lipcius, R.N., Dreyer, J., and Dover, C.L.V. (2003) Diversity in mussel beds at deep-sea hydrothermal vents and cold seeps. *Ecol Lett* **6**: 518-523.

Tyler, P., Young, C., Dolan, E., Arellano, S., Brooke, S., and Baker, M. (2007) Gametogenic periodicity in the chemosynthetic cold-seep mussel "*Bathymodiolus*" *childressi*. *Mar Biol* **150**: 829-840.

Uchiumi, T., Ohwada, T., Itakura, M., Mitsui, H., Nukui, N., Dawadi, P. et al. (2004) Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. *J Bacteriol* **186**: 2439-2448.

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

Van Dover, C.L., Desbruyères, D., Segonzac, M., Comtet, T., Saldanha, L., Fiala-Médioni, A., and Langmuir, C. (1996) Biology of the Lucky Strike hydrothermal field. *Deep-Sea Res Part I-Oceanogr Res Pap* **43**: 1509-1529.

Vidal-Dupiol, J., Adjeroud, M., Roger, E., Foure, L., Duval, D., Mone, Y. et al. (2009) Coral bleaching under thermal stress: putative involvement of host/symbiont recognition mechanisms. *BMC Physiology* **9**: 14.

Völkel, S., and Grieshaber, M.K. (1997) Sulphide oxidation and oxidative phosphorylation in the mitochondria of the lugworm *Arenicola marina*. *J Exp Biol* **200**: 83-92.

von Cosel, R. (1985) A new species of bathymodioline mussel (Mollusca, Bivalvia, Mytilidae) from Mauritania (West Africa), with comments on the genus *Bathymodiolus* Kenk & Wilson, 1985. *Zoosystema* **24**: 259-271.

Vopel, K., Pöhn, M., Sorgo, A., and Ott, J. (2001) Ciliate-generated advective seawater transport supplies chemoautotrophic ectosymbionts. *Mar Ecol Prog Ser* **210**: 93–99.

Wallin, I.E. (1923) The Mitochondria Problem. *Am Nat* **57**: 255-261.

Wallin, I.E. (1925) On the nature of mitochondria. IX. Demonstration of the bacterial nature of mitochondria. *Am J Anat* **36**: 131-149.

Watsuji, T., and Takai, K. (2009) Compositional and functional transition in epibiotic symbionts of *Shinkaia crosnieri*: morphology, structure, energy and carbon metabolisms during rearing. (Poster presentation). In *Goldschmidt Conference 2009 - Challenges to Our Volatile Planet*. Davos, Switzerland.

Watsuji, T., Nakagawa, S., Tsuchida, S., and Takai, K. (2008) Changes in epibiotic community structure and diversity of hydrothermal vent field animal *Shinkaia crosnieri* during long-term rearing. (Poster presentation). In *The 12th International Symposium on Microbial Ecology, ISME 12*. Cairns, Australia.

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

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

Zal, F., Leize, E., Lallier, F.H., Toulmond, A., Van Dorsselaer, A., and Childress, J.J. (1998) S-sulfohemoglobin and disulfide exchange: The mechanisms of sulfide binding by *Riftia pachyptila* hemoglobins. *PNAS* **95**: 8997-9002.

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

Zielinski, F. (2008) Geobiological coupling of hydrothermal vent fluids with endosymbiotic primary producers of Bathymodious mussels from hydrothermal vents on the Mid-Atlantic Ridge. In *Fachbereich Biologie/Chemie*. Bremen: University of Bremen, p. 160.

Zielinski, F., Pape, T., Wenzhöfer, F., Seifert, R., and Dubilier, N. (2005) Hydrogen may be an energy source for endosymbiotic bacteria of the vent mussel *Bathymodiolus puteoserpentis.* (Abstract). In *American Geophysical Union, Fall Meeting 2005*.

II Aims of this thesis

In this thesis, bacterial associations with oligochaetes from different coastal sediments were studied: 1) *Olavius algarvensis* and *O. ilvae*, gutless oligochaetes from coarse silicate sediments from the coast of Elba and 2) *Tubificoides benedii*, an oligochaete from the muddy tidal sediments of the Wadden Sea Coast of Sylt, Germany. The aim of this thesis was the identification of the bacterial symbionts, and to assess their abundance and reveal functional aspects of the symbioses.

1 Bacterial endosymbioses of gutless oligochaetes from Elba

In the first part of this study, the gutless oligochaete *O. algarvensis* from marine silicate sediments of the Mediterranean island Elba, Italy was investigated (Chapter 1). Previous molecular characterization of this marine worm had demonstrated that it not only hosts a Gamma 1 sulfur-oxidizing symbiont common to gutless oligochaetes, but also a deltaproteobacterial symbiont affiliated with free-living sulfate reducers (Mülders, 1999). This was the first time such a bacterium was found in a marine worm and surprising, because sulfide, the product of sulfate respiration, is potentially toxic to eukaryotic cells. Thus one aim was to verify the sulfate-reducing potential of the novel deltaproteobacterial symbiont by analysis of a gene diagnostic for sulfate reduction (*dsrAB*). In addition, sulfide production in the worm was investigated applying radiolabeled sulfate for subsequent autoradiography and measurement of sulfate reduction rates.

With the availability of enhanced molecular techniques the symbiosis of *O. algarvensis* was reinvestigated analyzing symbiont community and physiological potential of the symbionts (Chapter 2). A novel second gutless oligochaete, *O. ilvae*, was discovered co-occurring with *O. algarvensis* raising the question whether the two hosts share the same symbiont community. In order to assess similarities and differences in symbiont community and physiology, I used comparative molecular and phylogenetic analysis of 16S rRNA, and diagnostic genes for autotrophy (*cbbL* and *cbbM*) and sulfur metabolism (*aprA* and *dsrAB*). To verify that the retrieved 16S rRNA sequences originated from the symbionts of *O. algarvensis* and *O. ilvae* respectively, FISH and CARD-FISH was applied with group and specifically designed probes (16S rRNA approach, see Appendix). This was also applied for localization of the symbionts and assessment of relative abundance and thus significance of the individual symbionts (Chapter 2).

The above studies were based on culture-independent techniques. In addition, cultivation attempts were made to isolate the deltaproteobacterial symbionts and identify their substrate range. For this, anaerobic cultivation techniques established for the isolation of sulfate-reducing bacteria were applied. The results of these experiments did not lead to a publication and are presented in Results and Discussion.

2 Filamentous ectosymbionts of *Tubificoides benedii* from sulfidic muddy Wadden Sea sediment

The aim of the third part of this study was to identify the filamentous ectosymbionts of the Wadden Sea oligochaete *T. benedii* and to characterize the symbiont community of the mucus layer (Chapter 3). Previous studies suggested that the morphologically homogeneous filamentous bacteria attached to the posterior end belonged to the same genus (Dubilier, 1986). This assumption was checked using the 16S rRNA approach (see Appendix), identifying retrieved bacterial sequences by designing and applying specific probes in addition to common group probes. Phylogenetic analysis was used to elucidate whether the ectosymbiotic bacterial filaments are related to known symbionts of other oligochaetes and nematode ectosymbionts or if they are related to free-living bacteria known to inhabit Wadden Sea sediments.

A sulfur oxidation metabolism was suggested for the *T. benedii* ectosymbionts based on the correlation of highest bacterial densities with increased sulfide in the sediment, and the filaments' morphology resembling that of free-living sulfur oxidizers (Dubilier, 1986). Chemosynthetic potential of the bacterial community on *T. benedii* tail ends was assessed using diagnostic genes for sulfur metabolism and autotrophy. In addition, preliminary experiments for the immunohistochemical detection of RubisCO, a common CO_2-fixing enzyme in chemosynthetic Gamma-proteobacteria, were conducted to assign function to the bacterial ectosymbionts in situ.

Dubilier, N. (1986) Association of filamentous epibacteria with *Tubificoides benedii* (Oligochaeta, Annelida). *Mar Biol* **92**: 285-288.

Mülders, C. (1999) Molekularbiologische Charakterisierung von Bakterien assoziiert mit marinen Oligochaeten (Annelida). (Diplomarbeit). In *Fachbereich Biologie*. Hamburg: University of Hamburg.

III Chapter 1

Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm

In other long-distance migrants, arrival on the breeding grounds is also relatively insensitive to the temperature on arrival[19], although in some species the temperature on journey is a good correlate of arrival date[20,21]. Climatic factors may be important in fine-tuning the onset and speed of migration, but climate change differs between temperate and tropical latitudes[1], and therefore a response to environmental cues such as temperature for the onset and speed of migration may not lead to an adequate arrival date on the breeding grounds. Short-distance migrants may be more flexible in their response, because the circumstances on the wintering grounds will be a better predictor for the optimal arrival time on the breeding grounds and genetic variation has been shown for some of their migratory traits[22,23].

Large-scale climate change may thus form a serious threat to at least some of the numerous species that migrate from tropical wintering grounds to temperate breeding areas[24], because they arrive at an inappropriate time to exploit the habitat optimally, and face higher competition with resident species that may have increased in numbers through enhanced winter survival[25]. This may, in fact, be partly responsible for the decline of these species in western Europe[26]. □

Methods

Data collection

Data were collected from a nest-box breeding population of pied flycatchers in the Hoge Veluwe area, central Netherlands, between 1965 and 2000 (ref. 9). We analysed data from 1980 to 2000, because temperature increased most markedly after 1980. We used only nests that were considered to be first nesting attempts of females during that year (n = 1,892). Parents and chicks were ringed with uniquely numbered aluminium rings. Arrival data were obtained from a local amateur bird group, working within 10 km from the study area[27]. Members of this bird group recorded each year the first singing pied flycatcher, and the median first arrival date was used as approximation of arrival in the study area. The lack of an advancement in arrival date was confirmed by analysing the arrival date of the first male recorded in the study area from 1992 to 2000 ($F_{1,8}$ = 0.26, P = 0.63), the first ten males that arrived in an area nearby from 1980 to 1990 ($F_{1,10}$ = 0.02, P = 0.88), and the mean start of nest building of the first ten pied flycatcher nests each year in 1980–2000 ($F_{1,19}$ = 0.92, P = 0.35; start of egg-laying was estimated from the state of the nest during weekly checks).

Analyses

All analyses were performed with linear regression using two-tailed P values. In most cases we used annual means. In the analysis of the response of individual females to temperature, we used females that bred in at least 2 years (n = 273). In this analysis, female age was a factor for first known breeding or later breeding in the area (real age was not determined), because first year breeders normally breed later[14]. Individual is used here as a factor in an ANCOVA. Temperature used is the average of the mean daily temperatures from 16 April to 15 May recorded by the Royal Dutch Meteorological Institute (KNMI) at De Bilt (The Netherlands). The standardized selection differential is the mean laying date weighted for the number of recruits (offspring that return as breeding birds in the study area) each nest produced minus the mean laying date, divided by the standard deviation of laying date[17]. Selection differentials are given until 1998, because the number of recruits for later years is not yet known.

Received 1 November 2000; accepted 28 February 2001.

1. Houghton, J. T. *et al.* (eds) *Climate Change 1995* (Cambridge Univ. Press, Cambridge, 1996).
2. Beebee, T. J. C. Amphibian breeding and climate. *Nature* **374,** 219–220 (1995).
3. Crick, H. Q. P., Dudley, C., Glue, D. E. & Thomson, D. L. UK birds are laying eggs earlier. *Nature* **388,** 526 (1997).
4. Crick, H. Q. P. & Sparks, T. H. Climate change related to egg-laying trends. *Nature* **399,** 423–424 (1999).
5. Forchhammer, M. C., Post, E. & Stenseth, N. C. Breeding phenology and climate. *Nature* **391,** 29–30 (1998).
6. McCleery, R. H. & Perrins, C. M. …temperature and egg-laying trends. *Nature* **391,** 30–31 (1998).
7. Brown, J. L., Li, S. H. & Bhagabati, N. Long-term trend toward earlier breeding in an American bird: a response to global warming? *Proc. Natl Acad. Sci. USA* **96,** 5565–5569 (1999).
8. Gwinner, E. Circannual clocks in avian reproduction and migration. *Ibis* **138,** 47–63 (1996).
9. Visser, M. E., van Noordwijk, A. J., Tinbergen, J. M. & Lessells, C. M. Warmer springs lead to mistimed reproduction in great tits (*Parus major*). *Proc. R. Soc. Lond. B* **265,** 1867–1870 (1998).
10. Harrington, R., Woiwod, I. & Sparks, T. H. Climate change and trophic interactions. *Trends Ecol. Evol.* **14,** 146–150 (1999).
11. Visser, M. E. & Holleman, L. J. M. Warmer spring disrupt the synchrony of Oak and Winter Moth phenology. *Proc. R. Soc. Lond. B* **268,** 289–294 (2001)
12. Przybylo, R., Sheldon, B. C. & Merila, J. Climatic effect on breeding and morphology: evidence for phenotypic plasticity. *J. Anim. Ecol.* **69,** 395–403 (2000).
13. Stevenson, I. R. & Bryant, D. M. Climate change and constraints on breeding. *Nature* **406,** 366–367 (2000).
14. Lundberg. A. & Alatalo. R. V. *The Pied Flycatcher* (T. & A. D. Poyser. London. 1992).
15. Alatalo, R. V., Lundberg, A. & Glynn, C. Female Pied Flycatchers choose territory quality and not male characteristics. *Nature* **323,** 152–153 (1986).
16. Both, C. Density dependence of avian clutch size in resident and migrant species: is there a constraint on the predictability of competitor density? *J. Avian Biol.* **31,** 412–417 (2000).
17. Falconer, D. S. & Mackay, F. T. C. *An Introduction to Quantative Genetics* 4th edn (Longman Higher Education, Essex, 1996).
18. Buse, A., Dury, S. J., Woodburn, R. J. W., Perrins, C. M. & Good, J. E. G. Effects of elevated temperature on multi-species interactions: the case of Pedunculate Oak, Winter Moth and Tits. *Funct. Ecol.* **13** (suppl. 1), 74–82 (1999).
19. Mason, C. F. Long term trends in the arrival dates of spring migrants. *Bird Study* **42,** 182–189 (1995).
20. Huin, N. & Sparks, T. H. Spring arrival patterns of the Cuckoo *Cuculus canorus*, Nightingale *Luscinia megarhynchos* and Spotted Flycatcher *Musciapa striata* in Britain. *Bird Study* **47,** 22–31 (2000).
21. Huin, N. & Sparks, T. H. Arrival and progression of the Swallow *Hirundo rustica* through Britain. *Bird Study* **45,** 361–370 (1998).
22. Berthold, P., Helbig, A. J., Mohr, G. & Querner, U. Rapid microevolution of migratory behaviour in a wild bird species. *Nature* **360,** 668–670 (1992).
23. Pulido, F., Berthold, P. & van Noordwijk, A. J. Frequency of migrants and migratory activity are genetically correlated in a bird population: evolutionary implications. *Proc. Natl Acad. Sci. USA* **93,** 14642–14647 (1996).
24. Berthold, P., Fiedler, W., Schlenker, R. & Querner, U. 25-year study of the population development of Central European songbirds: A general decline, most evident in long-distance migrants. *Naturwissenschaften* **85,** 350–353 (1998).
25. Berthold, P. Patterns of avian migration in light of current global 'greenhouse' effects: a central European perspective. *Acta XX Congressus Internationalis Ornithologici* 780–786 (1990).
26. Myers, J. P. & Lester, R. T. *Global Warming and Biodiversity* (eds Peters, R. L. & Lovejoy, T. E.) 193–200 (Yale Univ. Press, New Haven, 1992).
27. Pilzecker, J. Een voorjaar maakt nog geen fenologie; Bewerking van de fenologiegegevens van Vogelwerkgroep Arnhem e. o. over de periode 1980–1997. Report No. 98-2 (Vogelwerkgroep Arnhem e. o., Arnhem, 1998).

Acknowledgements

J. H. van Balen and J. Visser managed the long-term study for many years. The board of National Park 'De Hoge Veluwe' kindly allowed us to work on their property. J. Pilzecker and R. Bijlsma allowed us to use some of their unpublished observations of flycatcher arrival. J. Harvey, C. M. Lessells and A. J. van Noordwijk commented on an earlier draft.

Correspondence and requests for materials should be addressed to M.E.V (e-mail: m.visser@cto.nioo.knaw.nl).

Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm

Nicole Dubilier*, Caroline Mülders*, Tim Ferdelman*, Dirk de Beer*, Annelie Pernthaler*, Michael Klein†, Michael Wagner†, Christer Erséus‡, Frank Thiermann§, Jens Krieger§, Olav Giere§ & Rudolf Amann*

* *Max Planck Institute of Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany*
† *Department of Microbiology, Technical University of Munich, D-85350 Freising, Germany*
‡ *Department of Invertebrate Zoology, Swedish Museum of Natural History, S-10405 Stockholm, Sweden*
§ *Zoological Institute and Zoological Museum, University of Hamburg, D-20146 Hamburg, Germany*

Stable associations of more than one species of symbiont within a single host cell or tissue are assumed to be rare in metazoans because competition for space and resources between symbionts can be detrimental to the host[1]. In animals with multiple endosymbionts, such as mussels from deep-sea hydrothermal vents[2] and reef-building corals[3], the costs of competition between the symbionts are outweighed by the ecological and physiological flexibility gained by the hosts. A further option for the coexistence of multiple symbionts within a host is if these benefit directly from one another, but such symbioses have not been previously described. Here we show that in the gutless marine oligochaete

***Olavius algarvensis*, endosymbiotic sulphate-reducing bacteria produce sulphide that can serve as an energy source for sulphide-oxidizing symbionts of the host. Thus, these symbionts do not compete for resources but rather share a mutualistic relationship with each other in an endosymbiotic sulphur cycle, in addition to their symbiotic relationship with the oligochaete host.**

Olavius algarvensis[4] is a small tubificid worm (0.2 mm × 20–30 mm) that is found in the Mediterranean at sediment depths of 5–15 cm in coarse-grained sands surrounding beds of sea grass. As in other gutless oligochaetes[5,6], two bacterial morphotypes occur in immediate proximity to one another just below the cuticle between extensions of the epidermal cells (Fig. 1). The larger morphotype (2.5 μm × 1.5 μm) contains numerous intracellular globules, whereas the smaller (1.1 μm × 0.7 μm) has no conspicuous inclusions.

We determined the phylogenetic identity of the *O. algarvensis* symbionts by using comparative 16S ribosomal RNA sequencing. We identified two dominant clone groups in the hosts, with minimal variations in the 16S rRNA sequences within each clone group (0.1–1.2%). Phylogenetic analyses revealed that the 16S rRNA sequences from these two groups are derived from the γ- and δ-subclasses of the Proteobacteria (Fig. 2a, b). The γ-proteobacterial sequence isolated from *O. algarvensis* consistently falls in a cluster with endosymbionts from other gutless oligochaetes such as *Olavius loisae*[7] and *Inanidrilus leukodermatus*[8] (96–97% sequence identity) in all treeing methods used. The δ-proteobacterial sequence is always placed within a subgroup of free-living sulphate-reducing bacteria (*Desulfococcus/Desulfonema/Desulfosarcina*) by all inference methods, with *Desulfosarcina variabilis* consistently identified as its closest relative (93% sequence identity).

Fluorescence *in situ* hybridization (FISH) confirmed that the γ- and δ-proteobacterial 16S rRNA sequences originated from the symbiotic bacteria in *O. algarvensis* (Fig. 3). The FISH signal from the probe specific to the γ-subclass of the Proteobacteria (GAM42a) and a species-specific probe based on the *O. algarvensis* γ-sequence (OalgGAM445) clearly originated from the larger bacterial symbiont, whereas the general *Desulfosarcina/Desulfococcus* probe (DSS658) and a probe targeting the *O. algarvensis* δ-sequence (OalgDEL136) consistently labelled the smaller bacterial symbiont.

The thioautotrophic nature (that is, sulphur-oxidizing, CO_2-fixing metabolism) of the γ-symbionts in *O. algarvensis* is suggested by their close evolutionary relationship to symbionts already characterized as thioautotrophic[8,9]. This assumption is corroborated by our results from immunocytochemical labelling with an antiserum directed against form I of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), the key CO_2-fixing enzyme. The antiserum consistently labelled the larger γ-symbionts but not the smaller δ-symbionts (see Supplementary Information). Further evidence for a thioautotrophic metabolism of the γ-symbionts is

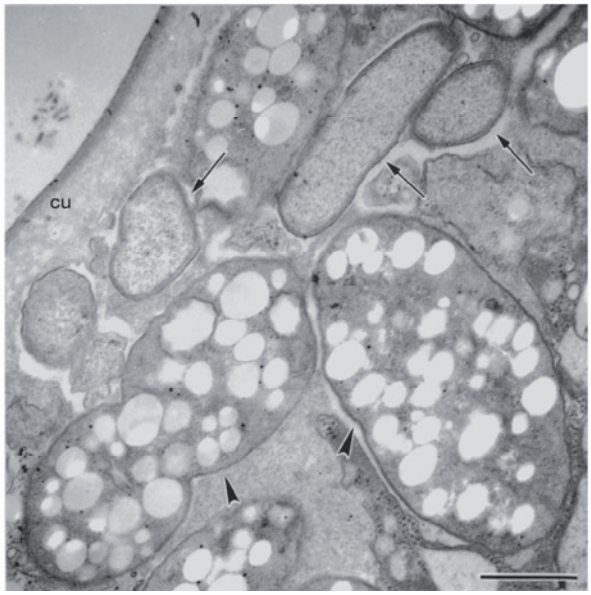


Figure 1 Transmission electron micrograph of bacterial endosymbionts in *O. algarvensis*. The symbionts occur just below the cuticle (cu) between extensions of the epidermal cells. The larger bacteria (arrowheads) contain numerous globules whereas the smaller bacteria (arrows) do not show any cytoplasmic inclusions. Scale bar, 1 μm.

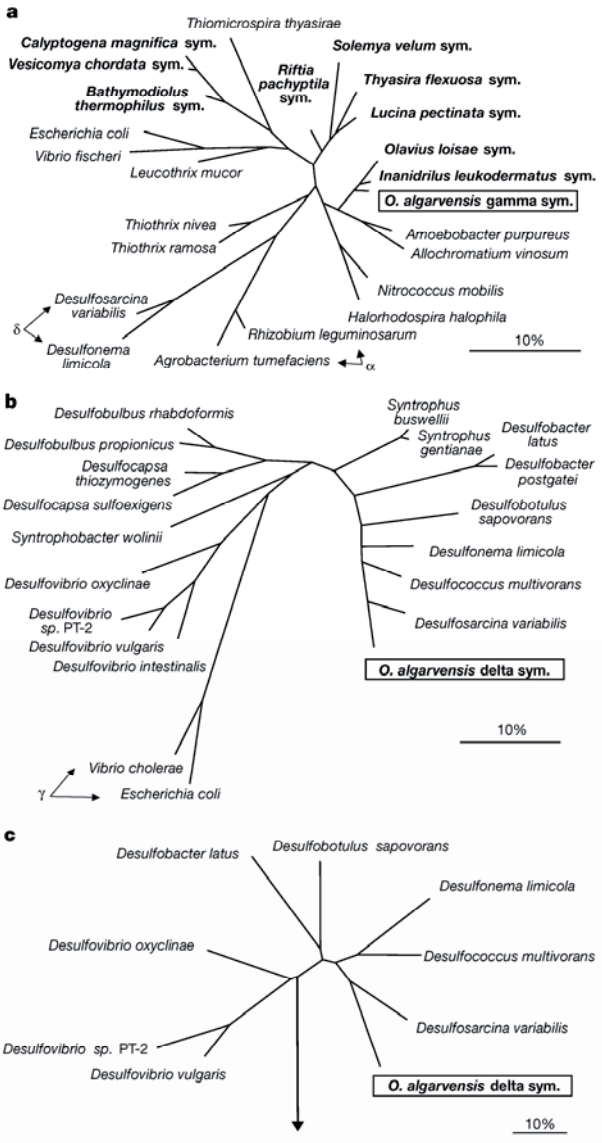


Figure 2 Phylogenetic relationships of the *O. algarvensis* symbionts based on maximum likelihood analyses. **a**, 16S rRNA sequences from the γ-subclass of Proteobacteria (α- and δ-Proteobacteria marked with arrows; chemoautotrophic symbionts (sym.) in bold type). **b**, 16S rRNA sequences from the δ-subclass of Proteobacteria (γ-Proteobacteria marked with arrows). **c**, DSR sequences based on a concatenated amino-acid alignment encompassing the DSR α- and β-subunit data sets. Arrow indicates published[16] and unpublished DSR sequences (M.W. *et al.*) not shown in tree. Scale bars indicate 0.10 expected substitutions per site.

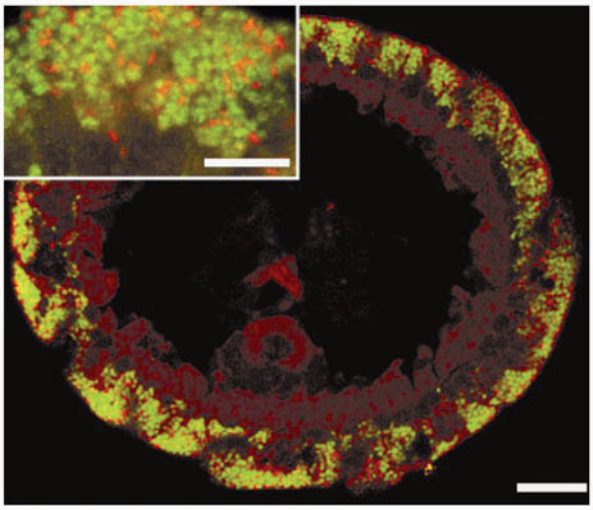


Figure 3 Fluorescence *in situ* hybridization of endosymbionts in *O. algarvensis* with oligonucleotide probes labelled with fluorochromes. γ-symbionts are green, δ-symbionts are red. Cross-section through entire worm. Hybridization with probes for the γ-subclass of the Proteobacteria and the *Desulfosarcina/Desulfococcus* group. Scale bar, 20 μm. Inset: body wall of the worms with symbiont-containing region. Hybridization with specific probes for the γ- and δ-symbionts. Scale bar, 10 μm.

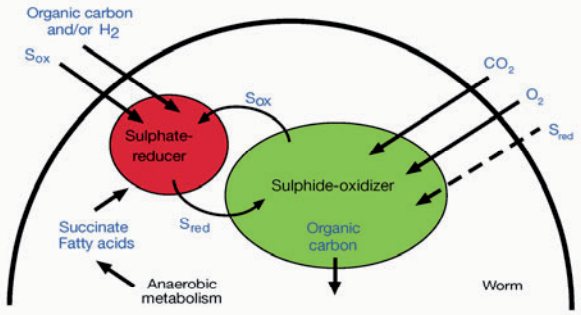


Figure 4 Model of the endosymbiotic sulphur cycle in *O. algarvensis* showing syntrophic cycling of oxidized and reduced sulphur compounds between the sulphate-reducing and sulphide-oxidizing symbionts. Under typical conditions of low sulphide flux from the sediment, sulphide produced internally by the sulphate reducers is used by the sulphide oxidizers as an electron donor for the autotrophic fixation of CO_2. Electron donors such as succinate and fatty acids can be supplied to the sulphate reducer internally by the worm during anaerobic metabolism. For net growth of the symbiotic association, external electron donors (organic carbon or H_2) are taken up from the sediment.

the high concentration of elemental sulphur in *O. algarvensis* (3.2 ± 1.7% dry weight; $n = 5$). Such large amounts of S^0 are characteristic for hosts with sulphide-oxidizing symbionts[10]. This corresponds well with electron microscopic spectroscopy studies that show the presence of sulphur in globules of the γ-symbionts (J.K., unpublished results).

The close evolutionary relationship of the δ-symbionts of *O. algarvensis* to free-living sulphate-reducing bacteria (SRB) suggests that these are also sulphate reducers. SRB have been described from termite guts[11] and the intestines of some mammals[12] and there is indirect evidence that they may occur as epibionts on some marine ciliates[13] and invertebrates[14,15]. However, SRB as endosymbionts have not been previously found in marine invertebrates and it has been suggested that such symbioses are unlikely because sulphide, their metabolic endproduct, is toxic to most aerobic organisms. We therefore used several methods to show that the δ-symbionts of *O. algarvensis* are indeed SRB and can actively respire sulphate in the worms.

The enzyme dissimilatory sulphite reductase (DSR) catalyses the reduction of (bi)sulphite to sulphide and is a good indicator for dissimilatory sulphate respiration, as it is only known to occur in sulphate-reducing prokaryotes[16]. Using specific primers, we successfully amplified the gene encoding DSR from *O. algarvensis*; no amplification products were obtained from negative controls with another gutless oligochaete host (*I. leukodermatus*) that does not harbour δ-proteobacterial symbionts. Comparative phylogenetic analyses (Fig. 2c) consistently showed that the DSR sequence from *O. algarvensis* is most closely related to *D. variabilis* (79% DNA sequence identity, 82% amino-acid identity), the free-living SRB most closely related to the δ-symbiont of *O. algarvensis* on the basis of 16S rRNA analyses (Fig. 2b). Previous studies have shown that 16S rRNA phylogenies of SRB agree well with their DSR phylogenies[16], indicating that the DSR sequence isolated from *O. algarvensis* originated from the δ-symbiont of this host and thus that this symbiont is a sulphate reducer.

To show that sulphate is actively reduced in *O. algarvensis*, we inserted silver needles through individual worms and incubated these in radiolabelled $^{35}SO_4^{2-}$ under microaerobic and aerobic conditions. After exposure of the needles to an autoradiographic film, blots from the needles inserted in live worms under microaerobic conditions showed a positive signal from ^{35}S-labelled sulphide that had precipitated on the needles, whereas under the same conditions a needle inserted in a formalin-fixed worm remained unlabelled (data not shown). This indicates that sulphate is reduced to sulphide during dissimilatory sulphate respiration by the δ-symbionts of *O. algarvensis* under microaerobic conditions. Sulphate respiration appears to be inhibited at high O_2 concentrations, on the basis of the absence of a sulphide precipitate on needles inserted in worms incubated under aerobic conditions.

We determined the sulphate reduction rates (SRRs) of the symbionts by incubating *O. algarvensis* in $^{35}SO_4^{2-}$ under microaerobic conditions (Table 1). In live worms, we measured SRRs of 53–

Table 1 Sulphate reduction rates in *O. algarvensis* specimens

Substrate	Worms	No. rep*	O_2 (μM)†	Sulphate reduction rate (pmol per worm per day)‡	Sulphate reduction rate (nmol cm^{-3} per day)§
Agar	Live	4	2–4	115 ± 76 (53/72/113/223)	1,474 ± 957 (690/935/1,440/2,830)
Agar	Live	1	200	<7.2	<80
Agar	Dead	1	2–4	<7.2	<80
Sand	Live	3	Micro-aerobic	638 ± 779 (115/266/1,534)	8,213 ± 9,917 (1,470/3,570/19,600)
Sand	Live	1	Aerobic	<7.2	<80
Sand	Dead	1	Micro-aerobic	<7.2	<80

Specimens were incubated in agar or sterilized sand.
*Number of replicate experiments; values in parentheses are rates measured in each replicate experiment.
† Measured at oligochaete surface in agar experiments (oxygen conditions in sand experiments are estimated from the agar experiments, as an identical experimental setup was used).
‡ Detection limit, 7.2 pmol per worm per day.
§ Detection limit: 80 nmol cm^{-3} per day.

1,534 pmol per worm per day, whereas SRRs in heat-killed worms under the same conditions were below detection limits. Sulphate was reduced to sulphide despite the absence of an external electron donor in the incubation medium. Endogenous electron donors that could have been used by the sulphate-reducing symbionts are fermentation products from the host such as succinate, propionate and acetate. These substrates accumulate during anaerobic metabolism under low oxygen concentrations in other marine tubificids[17] and many other aquatic invertebrates[18]. Under fully aerobic conditions, SRRs in live worms were below detection limits, indicating, as in the silver needle experiments, that high oxygen concentrations inhibit sulphate reduction. This corresponds well with observations on SRB in pure cultures, where most species are temporarily oxygen tolerant but not able to respire sulphate in the presence of high oxygen concentrations[19]. On the basis of the numbers of δ-symbionts in *O. algarvensis* as estimated by FISH, SRRs in these hosts (0.07–0.36 fmol per cell per day) are lower than those of SRB in pure cultures with saturating substrate concentrations (0.2-50 fmol per cell per day)[20] but in the same range as those estimated for free-living SRB in marine sediments (0.01–0.09 fmol per cell per day)[21]. SRRs in the worms on a volumetric basis are extremely high (690–19,600 nmol cm^{-3} per day) and comparable with rates measured in microbial mats (2,880–43,200 nmol cm^{-3} per day)[22].

To estimate the importance to the sulphide-oxidizing symbionts of internally produced sulphide compared with the import of external sulphide from the sediment, we compared the fluxes from these two sulphide sources. Dissolved sulphide concentrations in pore waters of *O. algarvensis* collection sites were extremely low: <14–76 nM (26 ± 21, $n = 9$) at 5–15 cm sediment depth, with no trend with sediment depth or location. Correspondingly, sulphide flux from the environment into the worm was <50–270 pmol per worm per day (93 ± 75, $n = 9$). Internal sulphide production from the sulphate-reducing symbionts on the basis of SRRs was 120–1,530 pmol per worm per day (640 ± 780, $n = 3$). Thus, internal sulphide production is typically considerably higher than sulphide flux from the sediment, indicating that under prevalent conditions this symbiosis appears to be independent of an external source of sulphide.

The coexistence of sulphate-reducing and sulphide-oxidizing bacteria as endosymbionts in *O. algarvensis* indicates that these are engaged in a syntrophic sulphur cycle in which oxidized and reduced sulphur compounds are recycled between the two symbionts (Fig. 4). For net growth of the symbiotic association, uptake of organic or inorganic sources of carbon and electron donors from the environment is required. As sulphide flux calculations indicate that the electron donor for the sulphide oxidizers is typically supplied internally, external reductants must be imported through the sulphate reducers. Given the metabolic diversity of SRB, in particular within the *Desulfosarcina* group, where both chemo-organotrophic and chemoautotrophic metabolism occurs, dissolved organic carbon and hydrogen are possible sources of reducing power. Migration of the worms between oxidized and reduced sediments, as described for other gutless oligochaetes[23], would provide the host and its sulphide-oxidizing symbionts with oxygen and the sulphate reducers with reductants. The benefits of this endosymbiotic sulphur cycle to its partners are clear. Cycling of oxidized and reduced sulphur compounds between the two symbionts would result in increased protein yields, as shown for continuous cultures with free-living SRB and sulphide-oxidizing bacteria[24]. Furthermore, fermentation products of the host that accumulate during anaerobic metabolism would provide the sulphate reducers with an ideal energy source, aid the hosts in the removal of these undesirable endproducts and recycle metabolites that would otherwise be lost to the symbiosis. A further advantage for the host and its thioautotrophic symbiont is that they are not limited by the external presence of reduced sulphur compounds, given the endogenous production of sulphide by the sulphate-reducing symbiont. Thus the uptake of a sulphate reducer may have enabled these hosts to colonize new habitats and extend their geographic distribution. □

Methods

For more details see Supplementary Information.

Specimens

O. algarvensis was collected in 1998–2000 from sediments at 6–8 m water depth in a bay off Capo di San Andrea (Elba, Italy) by SCUBA divers. *I. leukodermatus* specimens used as negative controls for the DSR amplifications were collected in Bermuda in 1997.

Pore water sulphide

Pore water was collected at 5, 10 and 15 cm depth at the *O. algarvensis* collection sites by SCUBA divers with immediate fixation of the samples in zinc acetate. In June 1999, October 1999 and January 2000, 1–2 ml of pore water per sample was collected and total sulphide concentrations were below the detection limit of 0.4 μM in all samples. In June 2000 the detection limit was lowered to 14 nM by collecting greater amounts of pore water (40–60 ml per sampling site) using samplers connected to evacuated serum vials containing zinc chloride. Concentrations of total sulphide were determined colorimetrically[25].

Transmission electron microscopy and immunocytochemistry

O. algarvensis individuals were fixed and prepared for electron microscopy as described[4]. For Rubisco immunocytochemistry, specimens were treated as described in ref. 9. In each worm ($n = 5$) 50–100 symbionts were examined for labelling response.

DNA analyses

Three *O. algarvensis* individuals (and two *I. leukodermatus* specimens for DSR controls) were prepared singly for polymerase chain reaction (PCR) as described in ref. 7. DNA was isolated from *D. variabilis* DSM 2060 as described[16]. Amplifications were performed with primers specific for the bacterial 16S rRNA genes (8F and 1507R) or the DSR genes of SRB (DSR1F and DSR4R)[16]. PCR products were cloned and grouped using amplified ribosomal DNA restriction analysis (ARDRA). Two or three clones per individual from dominant ARDRA groups were partially sequenced and at least one clone per individual from each ARDRA group was sequenced fully in both directions. Alignments, treeing and phylogenetic analyses (distance, parsimony and maximum likelihood) were performed with the ARB program (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/).

FISH

Five worms were fixed and prepared for FISH as described[7]. Sections were hybridized as described[7] with Cy3 and Cy5 labelled group-specific probes (GAM42a and DSS658) as well as two specific probes designed for this study (OalgGAM445: 5′-CTCGAGATCTTTCTTCCC-3′; OalgDEL136: 5′-GTTATCCCCGACTCGGGG-3′). Specificity of the probes was tested with reference strains as described[7].

$^{35}SO_4^{2-}$ incubations

For silver needle experiments worms were incubated in $Na^{35}SO_4^{2-}$ and 0.2-μm pore-size filtered seawater from the collection site. The medium was solidified with agar and the worms paralysed with lidocaine (2 mg ml^{-1}) to prevent excessive movements during insertion with silver needles (99.999% pure 50 μM Ag wire, tapered to a <1 μm tip). Incubations were run for 2–3 h under microaerobic (2–4 μM O_2) and aerobic (200 μM O_2) conditions with monitoring of oxygen concentrations with microsensors (two replicate experiments per O_2 concentration with one worm per incubation). In a control experiment at 2–4 μM O_2 with a dead worm, the specimen was fixed in 4% formalin in seawater and subsequently washed in filtered seawater. After removal, the needles were washed in 50 mM Na_2SO_4 solution and exposed to autoradiography film. Results were similar in replicate experiments.

For determination of SRRs we incubated five worms per experiment for 2–3 h in seawater with $Na^{35}SO_4^{2-}$ using agar or sand as substrates. Sand incubations were prepared and run in the same manner as the agar experiments (see above), but worms were not paralysed and moved freely in sand from the collection site that had been washed and combusted at 480 °C. Oxygen concentrations were not monitored during the sand incubations. For control experiments, specimens were heat killed in water at 70 °C for 10 min. SRRs were determined using the one-step acidic Cr-II method to separate reduced ^{35}S (ref. 26).

Elemental sulphur analyses

S^0 was extracted individually from five worms with methanol and quantified by high-performance liquid chromatography as described[27].

Flux calculations

Sulphide flux (Q) from the environment was calculated using the following equation[28]: $Q = 2\pi l D_{eff} C_p / \ln(1+2\delta/d)$, where the length of the worm (l) is 1 cm, the effective diffusion coefficient of total sulphide in sediment (D_{eff}) is 1.39×10^{-9} m^2 s^{-1}, C_p the concentration of total sulphide in the pore water, the mass boundary layer (δ) is 100 μm and the diameter

(d) of the worm is 200 μm (see Supplementary Information). All assumptions are conservative and result in an overestimation of sulphide flux from the sediment (see Supplementary Information). Internal sulphide production from the symbionts is based on SRRs measured in worms incubated in sand (Table 1), assuming that all sulphide produced is consumed by the sulphide-oxidizing symbionts. SRRs in the worms are assumed to be underestimated, given that no external electron donor was used and experimental conditions are suboptimal in comparison to the natural environment.

Received 25 September 2000; accepted 26 February 2001.

1. Maynard Smith, J. & Szathmáry, E. *The Major Transitions in Evolution* (Oxford Univ. Press, Oxford 1995).
2. Distel, D. L., Lee, H. K.-W. & Cavanaugh, C. M. Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel. *Proc. Natl Acad. Sci. USA* **92,** 9598–9602 (1995).
3. Rowan, R., Knowlton, N., Baker, A. & Jara, J. Landscape ecology of algal symbionts creates variation in episodes of coral bleaching. *Nature* **388,** 265–266 (1997).
4. Giere, O., Erséus, C. & Stuhlmacher, F. A new species of *Olavius* (Tubificidae, Phallodrilinae) from the Algarve Coast in Portugal, the first East Atlantic gutless oligochaete with symbiotic bacteria. *Zool. Anzeiger* **237,** 209–214 (1998).
5. Giere, O. & Langheld, C. Structural organisation, transfer and biological fate of endosymbiotic bacteria in gutless oligochaetes. *Mar. Biol.* **93,** 641–650 (1987).
6. Giere, O., Nieser, C., Windoffer, R. & Erséus, C. A comparative structural study on bacterial symbioses of Caribbean gutless Tubificidae (Annelida, Oligochaeta). *Acta Zool.* **76,** 281–290 (1995).
7. Dubilier, N. *et al.* Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochaete *Olavius loisae* (Annelida). *Mar. Ecol. Prog. Ser.* **178,** 271–280 (1999).
8. Dubilier, N., Giere, O., Distel, D. L. & Cavanaugh, C. M. Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (Oligochaeta, Annelida) by phylogenetic 16S rRNA sequence analysis and *in situ* hybridization. *Appl. Environ. Microbiol.* **61,** 2346–2350 (1995).
9. Krieger, J., Giere, O. & Dubilier, N. Localization of RubisCO and sulfur in endosymbiotic bacteria of the gutless marine oligochaete *Inanidrilus leukodermatus* (Annelida). *Mar. Biol.* **137,** 239–244 (2000).
10. Vetter, R. D. & Fry, B. Sulfur contents and sulfur-isotope compositions of thiotrophic symbioses in bivalve molluscs and vestimentiferan worms. *Mar. Biol.* **132,** 453–460 (1998).
11. Kuhnigk, T., Branke, J., Krekeler, D., Cypionka, H. & König, H. A feasible role of sulfate-reducing bacteria in the termite gut. *System. Appl. Microbiol.* **19,** 139–149 (1996).
12. Morvan, B., Bonnemoy, F., Fonty, G. & Gouet, P. Quantitative determination of H_2-utilizing acetogenic and sulfate-reducing bacteria and methanogenic archaea from digestive tracts of different mammals. *Curr. Microbiol.* **32,** 129–133 (1996).
13. Fenchel, T. & Ramsing, N. B. Identification of sulphate-reducing ectosymbiotic bacteria from anaerobic ciliates using 16S rRNA binding oligonucleotide probes. *Arch. Microbiol.* **158,** 394–397 (1992).
14. Bussmann, I. & Reichardt, W. Sulfate-reducing bacteria in temporarily toxic sediments with bivalves. *Mar. Ecol. Prog. Ser.* **78,** 97–102 (1991).
15. Cottrell, M. T. & Cary, C. S. Diversity of dissimilatory bisulfite reductase genes of bacteria associated with the deep-sea hydrothermal vent polychaete *Alvinella pompejana*. *Appl. Environ. Microbiol.* **65,** 1127–1132 (1999).
16. Wagner, M., Roger, A. J., Flax, J. L., Brusseau, G. A. & Stahl, D. A. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *J. Bacteriol.* **180,** 2975–2982 (1998).
17. Dubilier, N., Giere, O. & Grieshaber, M. K. Morphological and ecophysiological adaptations of the marine oligochaete *Tubificoides benedii* to sulfidic sediments. *Am. Zool.* **35,** 163–173 (1995).
18. Grieshaber, M. K., Hardewig, I., Kreutzer, U. & Pörtner, H.-O. Physiological and metabolic responses to hypoxia in invertebrates. *Rev. Physiol. Biochem. Pharmacol.* **125,** 43–147 (1994).
19. Barton, L. L. *Sulfate-Reducing Bacteria* (Plenum, New York, 1995).
20. Jørgensen, B. B. A comparison of methods for the quantification of bacterial sulfate reduction in coastal marine sediments. III. Estimation from chemical and bacteriological field data. *Geomicrobiol. J.* **1,** 49–64 (1978).
21. Sahm, K., MacGregor, B. J., Jørgensen, B. B. & Stahl, D. A. Sulphate reduction and vertical distribution of sulphate-reducing bacteria quantified by rRNA slot-blot hybridization in a coastal marine sediment. *Environ. Microbiol.* **1,** 65–74 (1999).
22. Canfield, D. E. & Des Marais, D. J. Biogeochemical cycles of carbon, sulfur, and free oxygen in a microbial mat. *Geochim. Cosmochim. Acta* **57,** 3971–3984 (1993).
23. Giere, O., Conway, N. M., Gastrock, G. & Schmidt, C. 'Regulation' of gutless annelid ecology by endosymbiotic bacteria. *Mar. Ecol. Prog. Ser.* **68,** 287–299 (1991).
24. van den Ende, F. P., Meier, J. & van Gemerden, H. Syntrophic growth of sulfate-reducing bacteria and colorless sulfur bacteria during oxygen limitation. *FEMS Microbiol. Ecol.* **23,** 65–80 (1997).
25. Cline, J. D. Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.* **14,** 454–458 (1969).
26. Fossing, H. & Jørgensen, B. B. Measurements of bacterial sulfate reduction in sediments: Evaluation of a single-step chromium reduction method. *Biogeochemistry* **8,** 205–222 (1989).
27. Ferdelman, T. G. *et al.* Sulfate reduction and methanogenesis in a *Thioploca*-dominated sediment off the coast of Chile. *Geochim. Cosmochim. Acta* **61,** 3065–3079 (1997).
28. Crank, J. *The Mathematics of Diffusion* (Oxford Univ. Press, New York, 1975).

Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank F. Widdel and B. Barker Jørgensen for reviewing this manuscript; E. Llobet-Brossa and A. Boetius for discussions; the Hydra Institute for Marine Sciences on Elba for logistical support during collection of the worms; and J. Wulf, D. Lange, G. Eickert and A. Eggers for technical support. The Max-Planck Society and the German Research Foundation (DFG) provided financial support.

Correspondence and requests for materials should be addressed to N.D. (e-mail: ndubilic@mpi-bremen.de). GenBank accession numbers: 16S rRNA: γ-Proteobacteria symbiont AF328856, δ-Proteobacteria symbiont AF328857; DSR: δ-Proteobacteria symbiont AF244995, *D. variabilis* AF191907.

Reproductive isolation caused by colour pattern mimicry

Chris D. Jiggins*†, Russell E. Naisbit*, Rebecca L. Coe‡ & James Mallet*†

* *The Galton Laboratory, University College London, 4 Stephenson Way, London NW1 2HE, UK*
† *Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Panama*
‡ *Downing College, The University of Cambridge, Cambridge CB2 1DQ, UK*

Speciation is facilitated if ecological adaptation directly causes assortative mating[1], but few natural examples are known. Here we show that a shift in colour pattern mimicry was crucial in the origin of two butterfly species. The sister species *Heliconius melpomene* and *Heliconius cydno* recently diverged to mimic different model taxa, and our experiments show that their mimetic coloration is also important in choosing mates. Assortative mating between the sister species means that hybridization is rare in nature, and the few hybrids that are produced are non-mimetic, poorly adapted intermediates. Thus, the mimetic shift has caused both pre-mating and post-mating isolation. In addition, individuals from a population of *H. melpomene* allopatric to *H. cydno* court and mate with *H. cydno* more readily than those from a sympatric population. This suggests that assortative mating has been enhanced in sympatry.

Mimicry is viewed mainly as a clear, visual demonstration of natural selection within species. But this was not always so: mimicry among Amazonian butterflies was originally presented as a striking example of speciation due to natural selection[2]. More recently, it has been argued that divergence in mimetic pattern can result in intermediates having low fitness because they are non-mimetic and, if colour pattern is also used in mate recognition, assortative mating. Therefore, both pre-mating and post-mating reproductive isolation might result from the evolution of mimicry[3–5]. Here we study mate choice in *Heliconius* butterflies, a group well known for Müllerian mimicry (mimicry between distasteful species)[2,4,5]. Closely related *Heliconius* species generally differ in mimetic colour pattern, as though adaptive radiation has occurred[6,7]. The sister species *H. melpomene* and *H. cydno* are sympatric throughout Central America and the Andean foothills, where they differ in mimicry (Fig. 1) and habitat use[8]. They occasionally hybridize and backcross in nature: hybrid females are sterile, but males are fertile and can be used in the laboratory to introgress genes between the species[8–10]. In most areas, *H. melpomene* mimics the black, red and yellow pattern of *H. erato*, whilst *H. cydno* mimics the black and white pattern of *H. sapho*. *Heliconius cydno* and *H. melpomene* separated in the last 10^6 years, much more recently than the non-sister species *H. sapho* and *H. erato* (Fig. 1)[11]. This and other evidence implies that *H. cydno* and *H. melpomene* have diverged to mimic *H. sapho* and *H. erato*, rather than vice versa[12].

Sympatric Panamanian *H. melpomene* and *H. cydno* did not mate with one another in choice experiments (Tables 1 and 2), although they will do so in no-choice tests[8–10]. Males from sympatric populations spent over 25 times longer courting virgin females of their own race than heterospecifics (Fig. 2). *Heliconius* females mate soon after eclosion, when they are unable to reject males, so that courtship and assortative mating is largely due to male choice[7]. To test whether males use mimetic colour pattern as a cue in choosing mates, we investigated the response of males to moving models made with either natural wings or coloured paper. Panama *H. melpomene* males approached *H. cydno* colour patterns about half as frequently as those of their own type, and were much less likely (2–4%) to court them (Fig. 3). Similarly, *H. cydno* males were a third as likely to court a *H. melpomene* pattern as their own type, although

Supplementary Information

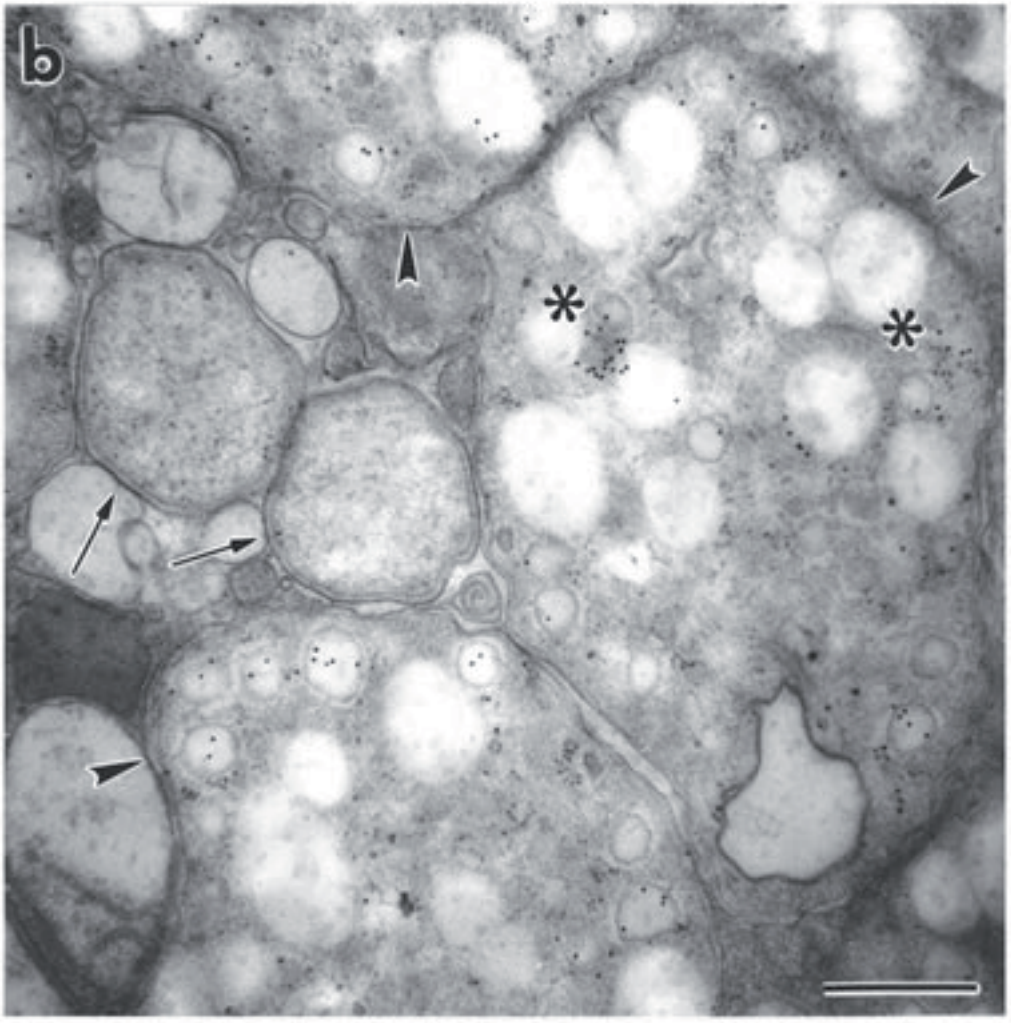


Figure 1 Immunocytochemical localization of form I RubisCO in bacterial endosymbionts of *Olavius algarvensis*. Only the larger symbionts (arrowheads) are labelled by the RubisCO antiserum (asterisks) while the smaller bacteria (arrows) and host tissue do not show immunoprecipitates. Scale bar = 0.4 µm.

Methods

Specimen collection. *O. algarvensis* specimens were collected by SCUBA diving in 1998-2000 from sediments surrounding sea grass beds at 6-8 m water depth in a bay off Capo di San Andrea (Elba, Italy). Worms were identified under a dissection microscope and fixed for electron microscopy, DNA analysis, and FISH, or transported live to the home laboratory in sediment from the collection site for experiments with radiotracers. The *Inanidrilus leukodermatus* specimens used as negative controls for the DSR amplifications were collected from shallow water sediments of Bermuda in 1997 and fixed in 96% ethanol for DNA analysis.

Pore water sulphide concentrations. Pore water from the *O. algarvensis* collection site was collected using samplers inserted at 5, 10, and 15 cm depth via SCUBA diving with immediate fixation of the samples in zinc acetate. In 6/99, 10/99, and 1/2000 1-2 ml of pore water per sample was collected and sulphide concentrations in all samples were always below the detection limit of 0.4 µM. In 6/2000 a higher detection limit was achieved by collecting

greater amounts of pore water (40-60 ml per sampling site) using samplers connected to evacuated serum vials containing 2 ml of 10% zinc chloride. In order to insure that only pore water from the desired sampling depth was drawn, no more than 2-5 ml pore water was sucked into a vial at one time, after which the sampler was reinserted to the same depth in 5-10 cm distance from the previous site. This procedure was repeated multiple times until a serum vial was filled with 40-60 ml of pore water. The contents of the vials were filtered through 0.2 μm Nucleopore filters, the filters placed in 2 ml of distilled water, and 0.16 ml of diamine reagent added for colorimetric detection[1]. This technique of precipitating and filtering zinc sulphide has been used to analyze nM concentrations of sulphide in open ocean waters[2].

Transmission electron microscopy and immunocytochemistry. *O. algarvensis* individuals were fixed in Trump's fixative and prepared for electron microscopical examination as described previously[3]. For immunocytochemistry, ultra-thin sections of worms were prepared as described in ref.[4] by hybridization with an antiserum directed against the large sub-unit of form I spinach RubisCO, followed by labelling with gold conjugated anti-rabbit IgG as a secondary antiserum. In each worm (n=5) between 50-100 symbionts were examined for labelling response. Specificity of the antiserum was tested as described previously[4] on sections with preimmune serum, and on immunoblots with preimmune serum and *Rhodospirillum rubrum*, which expresses form II RubisCO.

DNA preparation, PCR amplification, and phylogeny. Three *O. algarvensis* individuals (as well as two *I. leukodermatus* specimens for DSR negative controls) were prepared individually for PCR as described in ref. [5]. DNA was isolated from *Desulfosarcina variabilis* DSM 2060 as described previously[6]. Amplifications were performed with primers specific for the bacterial 16S rRNA genes (8F and 1507R) or the DSR genes of sulphate-reducing bacteria (DSR1F and DSR4R)[6]. PCR products were cloned and grouped using ARDRA with the restriction enzymes *Hae* III, *Sau* 3A I, and *Rsa* I. 2-3 clones per individual from dominant ARDRA groups were partially sequenced and at least 1 clone per individual from each ARDRA group was sequenced fully in both directions. For the 16S rRNA data set, sequences were aligned automatically using the ARB program (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/) and results corrected manually. For the DSR data set, nucleotides and deduced amino acid sequences were aligned and analysed as described previously[6]. Treeing and phylogenetic analyses were performed with the ARB program using distance matrix (neighbor joining with gaps treated as missing data), maximum parsiomony (DNAPARS with gaps treated as a fifth nucleotide state), and maximum likelihood (fastDNAml for DNA and ProtML for amino acids with gaps treated as unknown nucleotides or amino acids). For the 16S rRNA trees shown in Fig. 2a and 2b, 1515 sites (-lnLi=12974.43) and 1497 sites (-lnLi=12087.79) respectively were analysed. For the DSR tree shown in Fig. 2c, 646 sites were analysed with -lnLi=8016.48 in a tree without *Desulfonema limicola* and *Desulfovibrio oxyclinae*. The relatively short DSR sequences of these 2 species were added to the existing ML tree without changing its overall topology using the Parsimony Interactive Tool of the ARB package. *O. algarvensis* symbiont phylogenies were independent of the 3 treeing algorithms used and for DSR, whether DNA or amino acids were analysed.

FlSH. Five worms were fixed and stored as described previously[5]. After embedding in paraffin and sectioning, slides were pretreated and hybridized using methods described in ref. [5] with Cy3 and Cy5 labelled group-specific probes (GAM42a and DSS658) as well as 2

specific probes designed for this study (OalgGAM445: 5'-CTCGAGATCTTTCTTCCC-3'; OalgDEL136: 5'-GTTATCCCCGACTCGGGG–3'). Specificity of the probes was tested with reference strains (OalgGAM445: *Inanidrilus leukodermatus* gamma symbiont, 4 mismatches; OalgDEL136: *Desulfonema magnum* and *Rhodothermus marinus*, both 2 mismatches) and the formamide concentrations of the hybridizations adjusted accordingly as described previously[5].

$^{35}SO_4^{2-}$ incubations. For silver needle experiments worms were incubated in 0.2 µm filtered seawater from the collection site to which 7 Mbeq of carrier-free $Na^{35}SO_4^{2-}$ tracer was added to a specific activity of 218 Mbeq $mmol^{-1}$. The medium was solidified with 2% (w/v) low melting point agar and the worms paralysed with lidocaine (2 mg ml^{-1}) to prevent excessive movements of the worms during insertion with silver needles. The silver needles were made from 99.999% pure 50 µm Ag wire, tapered to a <1 µm tip, and inserted into the worms through a 10 µm glass capillary. Slow movements of the worms throughout the incubation procedure indicated that these were alive and viable. Incubations were run for 2-3 h under microaerobic (2-4 µM O_2) and aerobic (200 µM O_2) conditions with oxygen concentrations monitored during the incubations with microsensors (2 replicate experiments per O_2 concentration with 1 worm per incubation). In a control experiment at 2-4 µM O_2 with a dead worm, the specimen was fixed in 4% formalin in seawater and subsequently washed in filtered seawater to remove the formalin. After removal of the needles, these were washed carefully in 50 mM Na_2SO_4 solution, and exposed to autoradiography film for 2-3 weeks. Results were similar in replicate experiments.

For determination of sulphate reduction rates 5 worms per experiment were incubated for 2-3 hours in 0.2 µm filtered seawater from the collection site using agar or sand as a substrate. 7 Mbeq of carrier-free $Na^{35}SO_4^{2-}$ tracer was added to the same specific activity as above. Agar incubations were conducted as described with paralysed worms and monitoring of oxygen concentrations. In experiments with sand, incubation vials and medium were prepared for microaerobic and aerobic incubations in the same manner as the agar experiments, but worms were not paralysed and allowed to move freely in sand from the collection site that had been washed and combusted at 480°C for sterilization and to remove any potential organic substrates. Oxygen concentrations were not monitored during the sand incubations. For control experiments with dead worms, specimens were heat killed in water at 70°C for 10 min. Sulphate reduction rates were determined using the one step acidic Cr-II method to separate reduced ^{35}S[7].

Elemental sulphur analyses. Elemental sulphur was extracted individually from 5 worms with methanol and determined by HPLC as described previously[8].

Flux calculations. Sulphide diffusion flux (Q) from the pore water to the worms was calculated using the equation in ref. [9]. We assumed a cylindrical geometry of the worms with an outer, surrounding cylindrical mass boundary layer, so that diffusion flux was calculated through a hollow cylinder with an inner radius of a (radius of the worm) and an outer radius of b (radius of the worm plus the thickness of the boundary layer). Thus

$$Q_t = \frac{2\, l\, \pi\, D\, t\, (C_2 - C_1)}{\ln\,(b/a)}$$

where t is time, l the length of the worm, D the diffusion coefficient of total sulphide, C_2 the concentration of total sulphide at b, and C_1 at a. Assuming t=1 (mass flux per second), C_1=0 (all sulphide diffusing in from the environment is consumed, so that the concentration inside

the symbiont layer reaches 0), C_2 = the pore water sulphide concentration (C_p), the worm radius (r) is half the diameter (d) of the worm, and δ is the thickness of the mass boundary layer, Q in mol worm^{-1} sec^{-1} is:

$$Q = \frac{2\,l\,\pi\,D\,C_p}{\ln((d/2+\delta)/(d/2))} \text{ or } Q = \frac{2\,l\,\pi\,D\,C_p}{\ln(1+2\delta/d)} \text{ or } Q = \frac{2\,l\,\pi\,D\,C_p}{\ln(1+\delta/r)}$$

In essence, the mass boundary layer is a film of water surrounding the worm in which diffusion is the only mass transfer mechanism. The thickness of the boundary layer is dependent on the flow velocity, i.e. the lower the flow the thicker the boundary layer. We assumed that flow velocity is essentially negligible at sediment depths between 5-15 cm. Under stagnant conditions, the thickness of the boundary layer around a cylinder is equal to the radius of the cylinder, i.e. the radius of the worm (100 µm). Thus δ=r, so that

$$Q = \frac{2\,l\,\pi\,D\,C_p}{\ln(2)}$$

The length of the worms is approximately 1 cm. Since part of the worm surface is not exposed but covered by sand grains, we assumed that the effective exchange surface and thus the mass flux was further reduced to 60% (f = 0.6). The molecular diffusion coefficient of total sulphide in water is proportional to the molecular diffusion coefficient of O_2 in water by a factor of 0.64: $D(H_2S)=0.64\,D(O_2)$[10]. Therefore, at 40‰ salinity and 20°C (conditions at collection site of the worm) $D=1.26\,10^{-9}$ m^2 s^{-1}. The actual effective diffusion coefficient of total sulphide D_{eff} in the worm's environment is lower than in water because of the porosity and tortuosity of sediments. We conservatively estimate $D_{eff}=0.6\,D(H_2S)$:

$$Q = \frac{2\,l\,\pi\,D_{eff}\,C_p}{\ln(2)} \times f$$

Pore water sulphide concentrations in the worm's environment ranged between <14-76 nM. Thus, Q or sulphide flux from the environment into the worms ranged between <50-270 pmol worm^{-1} d^{-1}. These calculations overestimate the importance of sulphide flux from the environment: 1) we assumed that the sulphide concentration inside the symbiont layer is 0, but this is unlikely. No reliable data on biological sulphide oxidation kinetics exist, so we assumed 0-order kinetics. Sulphide concentrations in the worms are presumably higher than 0, so that flux from the environment should be lower than calculated, in proportion to the difference between sulphide concentrations inside and outside of the worm, 2) the mass transfer resistance through the cuticle of the worm is ignored; 3) the mass boundary layer was assumed to be equal to the diameter of the worm (200 µm), while the boundary layer thickness measured with microsensors was 250 µm; 4) the worm is not a perfect cylinder but curled, reducing effective exchange surface.

Internal sulphide flux from the symbionts is based on sulphate reduction rates measured in worms incubated in sand (Table 1), assuming that all sulphide produced is consumed by the sulphide-oxidizing symbionts. Sulphate reduction rates in the worms are assumed to be underestimated, given that no external electron donor was used and experimental conditions are suboptimal in comparison to the natural environment.

1. Cline, J.D. Spectrophotometric determination of hydrogen sulfide in natural waters. *Limnol. Oceanogr.* **14**, 454-458 (1969).

2. Cutter, G.A. & Oatts, T.J. Determination of dissolved sulfide and sedimentary sulfur speciation using gas chromatography-photoionization detection. *Anal. Chem.* **59**, 717-721 (1987).

3. Giere, O., Erséus, C. & Stuhlmacher, F. A new species of *Olavius* (Tubificidae, Phallodrilinae) from the Algarve Coast in Portugal, the first East Atlantic gutless oligochaete with symbiotic bacteria. *Zoologischer Anzeiger* **237**, 209-214 (1998).

4. Krieger, J., Giere, O. & Dubilier, N. Localization of RubisCO and sulfur in endosymbiotic bacteria of the gutless marine oligochaete *Inanidrilus leukodermatus* (Annelida). *Mar. Biol.* **137**, 239-244 (2000).

5. Dubilier, N.*, et al.* Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochaete *Olavius loisae* (Annelida). *Mar. Ecol. Prog. Ser.* **178**, 271-280 (1999).

6. Wagner, M., Roger, A.J., Flax, J.L., Brusseau, G.A. & Stahl, D.A. Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration. *J. Bacteriol.* **180**, 2975-2982 (1998).

7. Fossing, H. & Jørgensen, B.B. Measurements of bacterial sulfate reduction in sediments: Evaluation of a single-step chromium reduction method. *Biogeochem.* **8**, 205-222 (1989).

8. Ferdelman, T.G.*, et al.* Sulfate reduction and methanogenesis in a *Thioploca*-dominated sediment off the coast of Chile. *Geochim. Cosmochim. Acta* **61**, 3065-3079 (1997).

9. Crank, J. *The mathematics of diffusion* (Oxford University Press, New York, 1975).

10. Jørgensen, B.B., Revsbech, N.P., Blackburn, T.H. & Cohen, Y. Diurnal cycle of oxygen and sulfide microgradients and microbial photosynthesis in a cyanobacterial mat sediment. *Appl. Environ. Microbiol.* **38**, 46-58 (1979).

IV Chapter 2

Multiple bacterial symbionts in two species of co-occurring gutless oligochaete worms from Mediterranean sea grass sediments

Environmental Microbiology (2008) **10**(12), 3404–3416 doi:10.1111/j.1462-2920.2008.01728.x

Brief report

Multiple bacterial symbionts in two species of co-occurring gutless oligochaete worms from Mediterranean sea grass sediments

Caroline Ruehland,[1†] Anna Blazejak,[1†] Christian Lott,[1,2] Alexander Loy,[3] Christer Erséus[4] and Nicole Dubilier[1*]

[1]*Symbiosis Group, Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany.*

[2]*HYDRA Institute for Marine Sciences, Elba Field Station, Via del Forno 80, I-57034 Campo nell'Elba (LI), Italy.*

[3]*Department of Microbial Ecology, Faculty of Life Sciences, University of Vienna, Vienna, Austria.*

[4]*Department of Zoology, University of Gothenburg, Box 463, SE-40530, Göteborg, Sweden.*

Summary

Gutless oligochaete worms are found worldwide in the pore waters of marine sediments and live in symbiosis with chemoautotrophic sulfur-oxidizing bacteria. In the Mediterranean, two species of gutless oligochaete worms, *Olavius algarvensis* and *O. ilvae*, co-occur in sediments around sea grass beds. These sediments have extremely low sulfide concentrations (< 1 μM), raising the question if *O. ilvae*, as shown previously for *O. algarvensis*, also harbours sulfate-reducing symbionts that provide its sulfur-oxidizing symbionts with reduced sulfur compounds. In this study, we used fluorescence *in situ* hybridization (FISH) and comparative sequence analysis of genes for 16S rRNA, sulfur metabolism (*aprA* and *dsrAB*), and autotrophic carbon fixation (*cbbL*) to examine the microbial community of *O. ilvae* and re-examine the *O. algarvensis* symbiosis. In addition to the four previously described symbionts of *O. algarvensis*, in this study a fifth symbiont belonging to the *Spirochaetes* was found in these hosts. The symbiotic community of *O. ilvae* was similar to that of *O. algarvensis* and also included two gammaproteobacterial sulfur oxidizers and two deltaproteobacterial sulfate reducers, but not a spirochete. The phylogenetic and metabolic similarity of the symbiotic communities in these two co-occurring host species that are not closely related to each other indicates that syntrophic sulfur cycling provides a strong selective advantage to these worms in their sulfide-poor environment.

Received 6 May, 2008; accepted 2 July, 2008. *For correspondence. E-mail ndubilie@mpi-bremen.de; Tel. (+49) 4212028 932; Fax (+49) 4212028 580. †Authors contributed in equal parts to this publication.

Introduction

Gutless oligochaetes are small worms of less than 0.1–0.3 mm diameter and 2–50 mm length that are found worldwide in marine sediments (Bright and Giere, 2005; Dubilier *et al.*, 2006). With no mouth or gut, these worms are dependent on their symbiotic bacteria for nutrition. The endosymbionts are extracellular and occur in a thick layer between the cuticle and the epidermal cells of the worm. Enzyme assays, immunohistochemistry, uptake experiments with inorganic carbon and the presence of sulfur globules indicate that at least some of the bacterial symbionts are thiotrophic, using reduced sulfur compounds as electron donors, and fix CO_2 autotrophically to organic carbon compounds (Dubilier *et al.*, 2006). It is unclear if the transfer of these organic compounds to the host is the main mode of energy transfer or if digestion of the bacteria supplies the host with nutrients.

All gutless oligochaete species examined to date harbour thiotrophic *Gammaproteobacteria* called Gamma 1 symbionts that are related to free-living sulfur oxidizers of the family *Chromatiaceae* (Dubilier *et al.*, 1995; 1999; 2001; Blazejak *et al.*, 2005; 2006). Recently, the gutless oligochaete *Olavius algarvensis* (Giere *et al.*, 1998) from the Mediterranean island of Elba was discovered to harbour a second thiotrophic *Gammaproteobacterium*, called the Gamma 3 symbiont, as well as two deltaproteobacterial sulfate reducers, called Delta 1 and Delta 4 symbionts (Dubilier *et al.*, 2001; Woyke *et al.*, 2006). These symbionts are engaged in a syntrophic sulfur cycle

in which the sulfate reducers produce reduced sulfur compounds that are used by the sulfur oxidizers as an energy source. Sulfide concentrations are extremely low and rarely exceed 80 nM in the habitat of *O. algarvensis* (Dubilier *et al.*, 2001). This means that internal sulfide production from the sulfate-reducing symbionts greatly exceeds the flux of sulfide from the environment into the worms, showing the importance of the sulfate reducers for the thiotrophic symbiosis (Dubilier *et al.*, 2001).

The discovery of a second gutless oligochaete species, *Olavius ilvae* (Giere and Erséus, 2002), that co-occurs with *O. algarvensis* in the Elba sediments provides an ideal opportunity to examine if sulfate-reducing symbionts are common to chemosynthetic hosts living in these sulfide-poor environments. Using the full cycle rRNA approach that includes comparative 16S rRNA sequence analysis and fluorescence *in situ* hybridization (FISH), we examined the distribution, diversity and phylogeny of bacterial symbionts in *O. ilvae*. In addition, we re-examined the *O. algarvensis* symbiosis because only four symbiont phylotypes were identified in the metagenomic library of this host (Gamma 1 and 3, and Delta 1 and 4) while PCR analyses of the 16S rRNA gene indicated the presence of an additional spirochete phylotype (Woyke *et al.*, 2006). To better understand the metabolism of the symbionts in these hosts, we examined genes coding for enzymes diagnostic of sulfur metabolism and chemoautotrophy. For autotrophic CO_2 fixation via the Calvin–Benson–Bassham (CBB) cycle, *cbbL* and the *cbbM* genes, coding for the form I and II of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), were used as diagnostic markers (Elsaied and Naganuma, 2001; Blazejak *et al.*, 2006). The *aprA* gene, encoding the alpha subunit of adenosine-5′-phosphosulfate (APS) reductase, was used as an indicator for reductive and oxidative sulfur metabolism, as this gene is characteristic for both sulfate-reducing and many sulfur-oxidizing bacteria (Hipp *et al.*, 1997; Friedrich, 2002; Blazejak *et al.*, 2006). Finally, we examined the *dsrAB* genes, coding for the alpha and beta subunits of the dissimilatory (bi)sulfite reductase, as characteristic markers for dissimilatory sulfate/sulfite reduction (Wagner *et al.*, 2005; Zverlov *et al.*, 2005).

Results and discussion

The 16S rRNA gene clone libraries from eight *O. ilvae* and five *O. algarvensis* individuals showed a dominance of gamma- and deltaproteobacterial sequences. In addition, spirochete sequences were found in the *O. algarvensis* clone library (see Table S1 for clone library data and Table S2 for sequence identities to closest relatives).

By combining comparative 16S rRNA sequence analysis with FISH, we were able to clearly assign the dominant 16S rRNA sequences in the *O. ilvae* and *O. algarvensis* clone libraries to bacterial symbionts in these worms. The assignment of the metabolic indicator genes, *cbbL*, *aprA* and *dsrAB*, to a given symbiont was clear when sequences were identical or highly similar ($\geq$ 93.3% amino acid identity) to genes whose origin had been determined through metagenomic binning analyses (Woyke *et al.*, 2006). Otherwise, the phylogenetic position of a sequence was used to predict its origin. This approach is justified by the observation that in relatively closely related species (which is the case for oligochaete symbionts) the phylogeny of their *dsrAB* and *aprA* genes generally corresponds well with their 16S rRNA phylogeny (Zverlov *et al.*, 2005; Meyer and Kuever, 2007a,b). However, several studies have shown that phylogenetically diverse copies of a metabolic indicator gene can be present in a single species (e.g. for the *aprA* gene see Meyer and Kuever, 2007b; for *cbbL* see Scott *et al.*, 2006). Furthermore, the 16S rRNA phylogeny of a species is not always reflected in the phylogeny of its other genes, because of lateral gene transfer (Boucher *et al.*, 2003). We therefore distinguished between sequences whose assignment to a corresponding symbiont was unambiguous (based on their high similarity or identity to sequences whose origin was determined through metagenomic binning analyses) and sequences whose origin was inferred through their phylogeny, by placing the symbiont names of the latter in parentheses in the corresponding trees in Figs 3, 4 and 6.

Gammaproteobacterial endosymbionts

The dominant 16S rRNA sequences in both the *O. ilvae* and *O. algarvensis* clone libraries originated from their Gamma 1 symbionts (see FISH results below). The Gamma 1 sequences from both *O. algarvensis* and *O. ilvae* consistently fell in a monophyletic group of 16S rRNA sequences from the sulfide-oxidizing Gamma 1 symbionts of other gutless oligochaete species such as *Inanidrilus leukodermatus* and *Olavius crassitunicatus*, as well as symbionts of the marine nematodes *Laxus* sp. (Polz *et al.*, 1994) and *Astomonema* sp. (Musat *et al.*, 2007) (Fig. 1).

The Gamma 1 symbionts of *O. ilvae* and *O. algarvensis* were not most closely related to each other despite the fact that their hosts co-occur in the same sediments of the Mediterranean. Instead, their Gamma 1 symbionts were most closely related to symbionts from geographically distant oligochaete species: the *O. ilvae* Gamma 1 symbiont was most closely related to the Gamma 1 symbionts of *Olavius loisae* from the Australian Great Barrier Reef and *O. crassitunicatus* from the Peru margin, while the *O. algarvensis* Gamma 1 symbiont was most closely related to the Gamma 1 symbionts of *I. leukodermatus* from Bermuda and *Inanidrilus makropetalos* from the

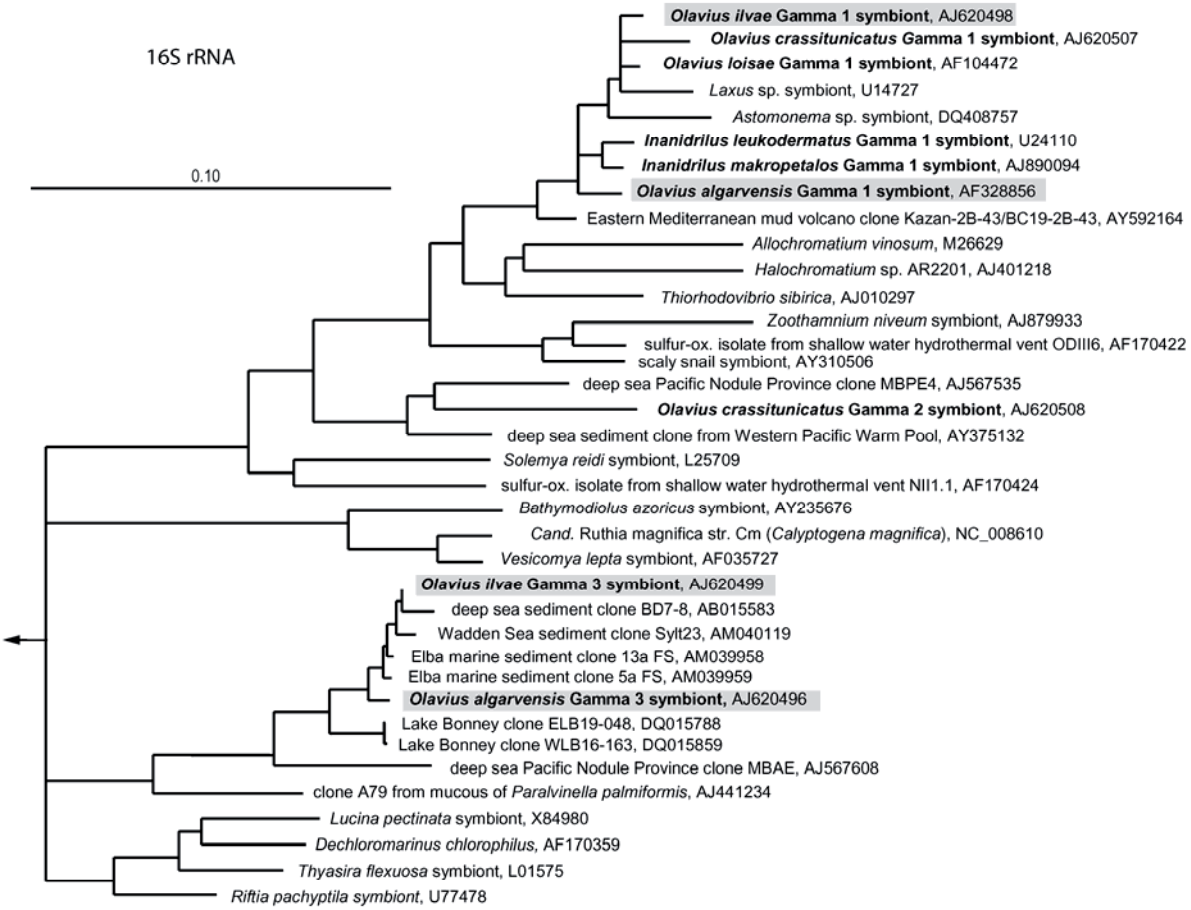


Fig. 1. Phylogenetic placement of the gammaproteobacterial symbionts from *O. algarvensis* and *O. ilvae* based on maximum likelihood (ML) analyses of 16S rRNA sequences (sequences from this study highlighted in grey; symbionts from gutless oligochaetes in bold). Nodes that differed between parsimony, neighbour-joining and ML analyses were collapsed to a consensus branch. Five deltaproteobacterial sequences were used as an out-group (arrow, AJ620497, AJ620509, M34407, AF418180, AJ620511). Scale bar = 0.10 estimated substitutions per site. The names of the oligochaete symbionts are derived from the bacterial group to which they belong (e.g. *Gamma-* or *Deltaproteobacteria*) with the number after their name denominating their position within each bacterial group. Symbionts with at least 95% sequence identity between their rRNA genes that were monophyletic in all three treeing analyses were given the same number. Alignments and phylogenetic analyses were performed with the ARB program (Ludwig *et al.*, 2004).

Bahamas (Fig. 1). This indicates that geography does not play an important role in determining the phylogenetic relationships of these symbionts. Interestingly, phylogenetic analyses based on host-specific mitochondrial cytochrome oxidase subunit I (COI) genes show that this is also true for their hosts (C. Erséus, A. Blazejak and N. Dubilier, unpubl. data). *Olavius ilvae* and *O. algarvensis* are more closely related to gutless oligochaetes from the Atlantic and Pacific Oceans than to each other. We are currently examining if co-speciation caused the phylogenetic patterns observed in oligochaete hosts and their Gamma 1 symbionts.

A second group of gammaproteobacterial sequences found in both host species originated from their Gamma 3 symbionts (Table S1). The Gamma 3 sequences of *O. ilvae* and *O. algarvensis* formed a clade separate from the Gamma 1 symbionts, and grouped with environmental clone sequences including those from sediments at the *O. ilvae* and *O. algarvensis* collection site (Fig. 1).

Fluorescence *in situ* hybridization with probes specific to the Gamma 1 and 3 16S rRNA sequences from *O. ilvae* and *O. algarvensis* (Table 1) confirmed their origin from symbionts in the layer between the cuticle and epidermis. Hybridization signals for probes specific to the Gamma 1 *O. algarvensis* and *O. ilvae* phylotypes were observed in all examined specimens and were consistent with the size, shape and distribution of the large bacterial morphotype (approximately 2.3–3 μm length and 1.4 μm width) observed in these hosts with transmission electron microscopy (Giere and Erséus, 2002) (Fig. 2B and F).

Table 1. Oligonucleotide probes used in this study.

Probe	Specificity	Probe sequence (5′–3′)	Position[a]	FA [%][b]	Reference organism	Literature reference
NON338	Antisense	ACT CCT ACG GGA GGC AGC	338–355	10 30 (HRP)	See Literature reference	Wallner *et al.* (1993)
Gam42a	*Gammaproteobacteria*	GCC TTC CCA CAT CGT TT	1027–1043[c]	30–35	See Literature reference	Manz *et al.* (1992)
DSS658	*O. algarvensis*/*O. ilvae* Delta 1, Delta 3 symbiont, *O. algarvensis* Delta 4 symbiont, *Desulfosarcina* spp. *Desulfofaba* sp., *Desulfococcus* spp., *Desulfofrigus* spp.	TCC ACT TCC CTC TCC CAT	658–685	30–60	See Literature reference	Manz *et al.* (1998)
OalgGAM1	*O. algarvensis* Gamma 1 symbiont	CTC GAG ATC TTT CTT CCC	445–462	10	*Inanidrilus leukodermatus* Gamma 1 symbiont, U24110 *O. ilvae* Gamma 1 symbiont, AJ620498	Dubilier *et al.* (2001)
Oilv/OcraGAM1	*O. ilvae*/*O. crassitunicatus* Gamma 1 symbiont	CAT ACT CTA GCC GAA CAG	643–660	10	*Inanidrilus triangulatus* Gamma 1 symbiont *O. algarvensis* Gamma 1 symbiont, AF328856	This study
Oalg/OilvGAM3	*O. algarvensis*/*O. ilvae* Gamma 3 symbionts, environmental clones such as: AB077346, AB015583, AB239038, AF424056, AF424077, AY133404, AJ567608, AM039958 (9), AM040119, AJ966594, AJ633950, AY580816, AY533996, DQ228658, DQ351743	CCG GAA TTC CAC TTG CCT	665–682	30	*Ifremeria nautilei* Gamma symbiont, AB189713	This study
OalgDEL1	*O. algarvensis* Delta 1 symbiont, environmental clone DQ395018	GTT ATC CCC GAC TCG GGG	136–153	10	*O. ilvae* Delta 1 symbiont, AJ620500 *Desulfonema magnum* (DSM 2077), U45989, *Rhodothermus marinus* (DSM 4252), X80994	Dubilier *et al.* (2001)
OilvDEL1	*O. ilvae* Delta 1 symbiont, environmental clone AY907763, AY710878	GTT ATC CCC GAT TCG GGG	136–153	30	*O. algarvensis* Delta 1 symbiont, AF328857, *D. magnum* (DSM 2077), U45989	This study
Oalg/OilvDEL3	*O. algarvensis*/*O. ilvae* Delta 3 symbiont	GTG CCT GCC TCC TGA AAG	1449–1465	30	*Desulfuromonas thiophila* (DSM 8987), Y11560	This study
OalgDEL4	*O. algarvensis* Delta 4 symbiont, environmental clones: DQ394892, DQ395063, EF061975, AB121109, AY499745, AY500008, AY822307, AY822331, DQ395004	GCC CAA CAA CTT CCG GTA	1427–1444	30	*Desulfobacterium indolicum* (DSM 3383), AJ237607	This study
SPIRO	*O. algarvensis*/*O. crassitunicatus*/ *O. loisae* spirochete symbionts, clones from termite gut: AB192148, AB192203, AB192256	GCT ATC CCC AAC CAA AAG	136–153	30 (HRP)	*Spirochaeta stenostrepta*, DSM 2028, M88724	This study

a. Position in the 16S rRNA of *E. coli*.
b. Formamide concentrations used in the FISH and catalysed reporter deposition (CARD) FISH (SPIRO, NON338 30%) hybridization buffer in percentage (v/v).
c. Position in the 23S rRNA of *E. coli*.
Further properties of the probes are available at probeBase (Loy *et al.*, 2007). Nine *O. algarvensis* and three *O. ilvae* specimens were prepared for FISH with monolabelled probes and CARD FISH with horseradish peroxidase (HRP)-labelled probes and tyramide signal amplification as described previously (Blazejak *et al.*, 2006). Specific and group oligonucleotide probes targeting the dominant 16S rRNA sequences found in *O. algarvensis* and *O. ilvae* were created with the ARB program (Ludwig *et al.*, 2004) and evaluated *in silico* by using BLAST (Altschul *et al.*, 1990) and the probe match tool of RDPII (Cole *et al.*, 2007). The specificity of the symbiont probes was tested against reference bacteria with 16S rRNA sequences containing one or more mismatches. General probes for the *Bacteria*, *Gammaproteobacteria* and a subgroup of the *Deltaproteobacteria* served as positive controls and the nonsense probe NON338 as a negative control, and hybridizations were performed at the formamide concentration ensuring specificity.

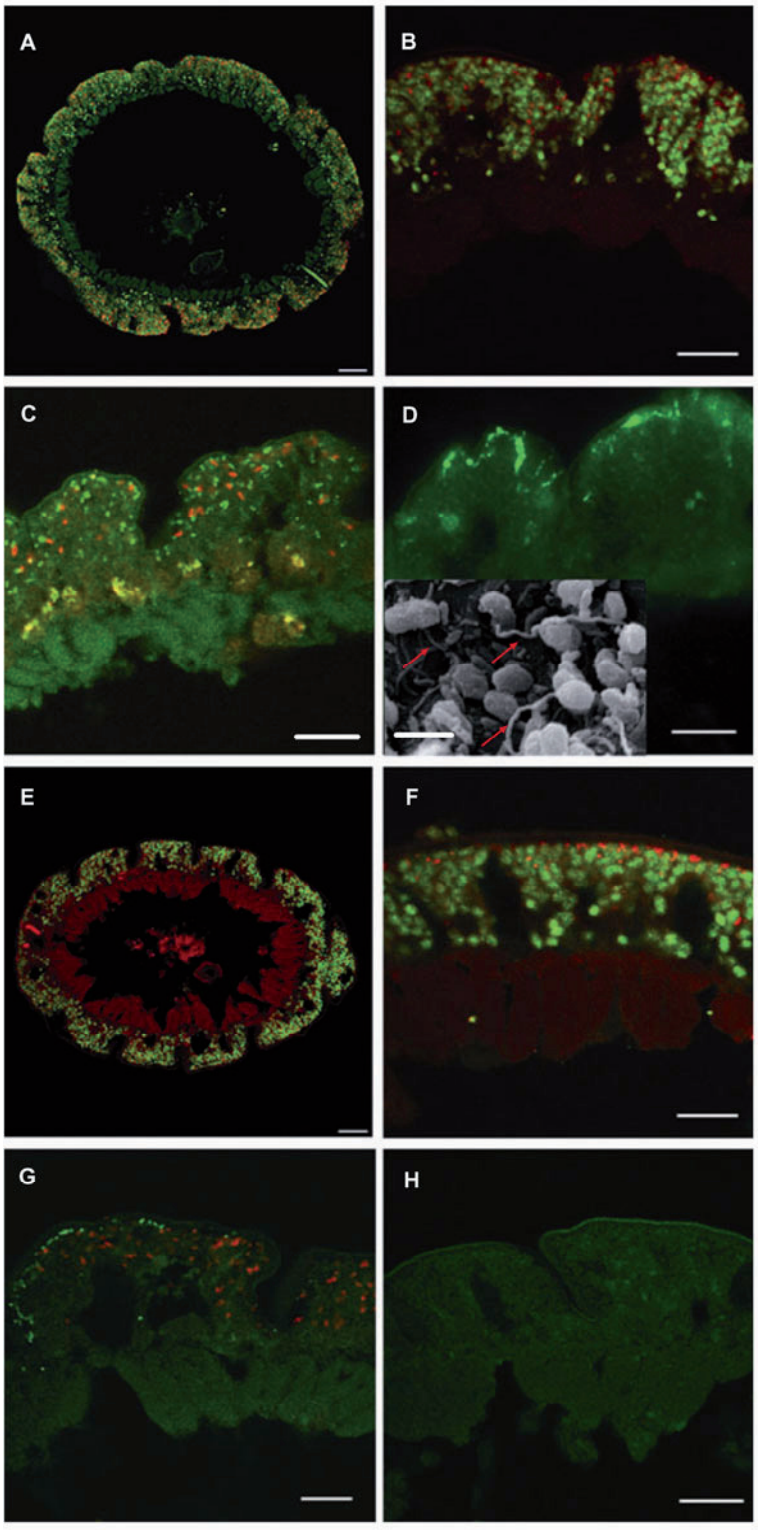


Fig. 2. Fluorescence *in situ* hybridization (FISH) identification of bacterial symbionts in *O. algarvensis* (A–D) and *O. ilvae* (E–H).
A and E. Cross-section through entire worm showing all gammaproteobacterial (green, Gam42a) and deltaproteobacterial (red, DSS658) symbionts.
B and F. The gammaproteobacterial symbionts. (B) *Olavius algarvensis* Gamma 1 (green, OalgGAM1) and Gamma 3 (red, Oalg/OilvGAM3) symbionts. (F) *Olavius ilvae* Gamma 1 (green, Oilv/OcraGAM1) and Gamma 3 (red, Oalg/OilvGAM3) symbionts.
C and G. The deltaproteobacterial symbionts. (C) *Olavius algarvensis* Delta 1 (red, OalgDEL1) and Delta 4 (green, OalgDEL4) symbionts. (G) *Olavius ilvae* Delta 1 (red, OilvDEL1) and Delta 3 (green, OilvDEL3) symbionts.
D and H. Spirochetes were found in *O. algarvensis* (shown in green using the probe SPIRO) but not in *O. ilvae* (H). Inset in (D) shows scanning electron microscopy image with red arrows showing spirochetes.
Scale bars: 20 μm in (A) and (E), 2 μm in inset (D), 10 μm in all other images. Fixation for FISH was carried out as described previously (Blazejak *et al.*, 2005). For scanning electron microscopy specimens of *O. algarvensis* were fixed in 4% glutaraldehyde buffered in 0.1 M Na-cacodylate, pH 7.3 and 3% sucrose, stored in fixative and post-fixed in buffered 1% OsO_4 solution.

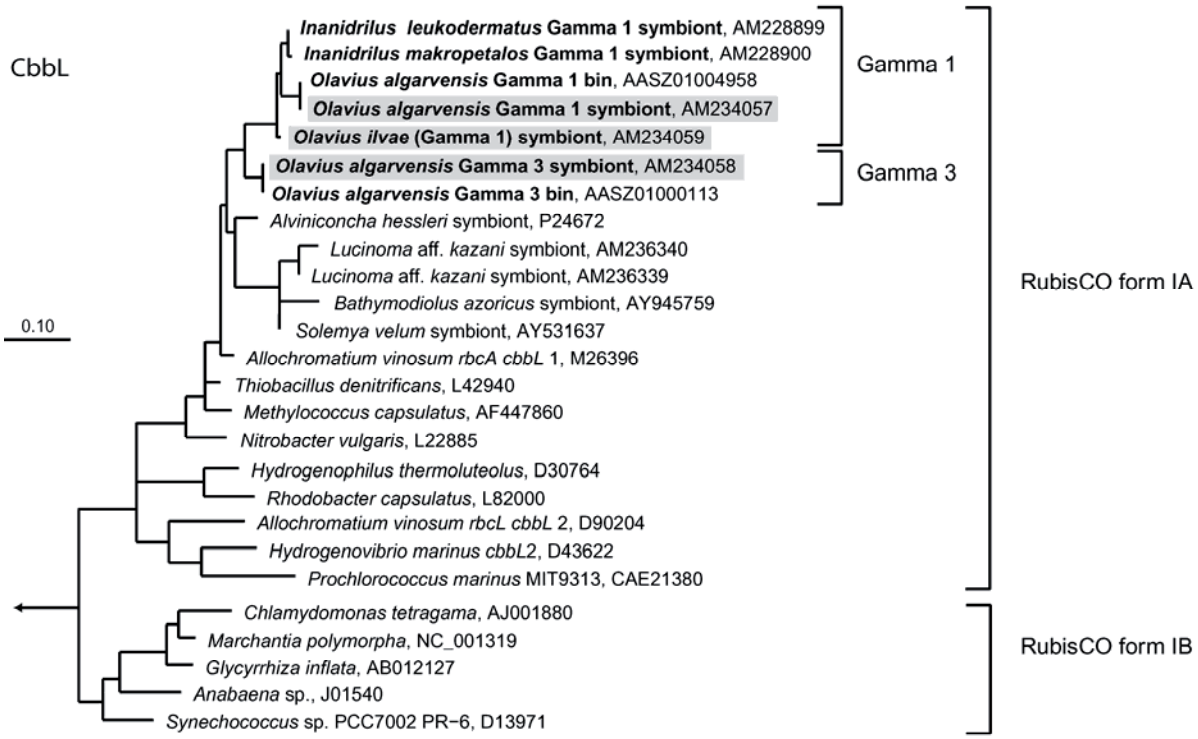


Fig. 3. CbbL consensus tree based on deduced amino acid sequences of the *cbbL* gene coding for the large-subunit of RubisCO (sequences from this study highlighted in grey; symbionts from gutless oligochaetes in bold; symbiont name of sequence whose origin was inferred through phylogeny is in parentheses). The *O. ilvae* (Gamma 1) symbiont sequence is assumed to have originated from the Gamma 1 symbiont of this host, because the sequence falls in a clade containing only sequences from Gamma 1 symbionts. CbbL sequences from form IC, ID and form II were used as out-groups (arrow, AJ001880, NC_001319, AB012127, J01540, D13971, M17744, M64624, M59080, X61918, X55372, X14171, AF047688, AF442518, D28135, X00286). Scale bar = 0.10 estimated substitutions per site. The phylogenetic tree was generated from sequences of 230 amino acids using the ML algorithm and a JTT model with a 25% positional conservation filter. Polytomic nodes were set for *sequences* for which positions varied with different filters.

Fluorescence *in situ* hybridization analyses with probes specific to the Gamma 3 phylotypes of *O. ilvae* and *O. algarvensis* showed that these symbionts were much smaller than the Gamma 1 symbionts (approximately 1 μm diameter; Fig. 2B and F). Their distribution and abundance varied in the two host species (Table 2). In *O. algarvensis*, the Gamma 3 symbionts were observed in seven of the nine specimens examined, occurred in roughly equal amounts as the Gamma 1 symbionts and were distributed evenly throughout the symbiotic region (Fig. 2B). In *O. ilvae*, all three individuals examined contained the Gamma 3 symbionts, but these were much less abundant than the Gamma 1 symbionts and occurred mainly just below the cuticle of the worms (Fig. 2F).

Genes characteristic for chemoautotrophic sulfur oxidation were found in both host species (Table S3), namely *cbbL* coding for RubisCO form I (Fig. 3) and *aprA* coding for APS reductase (Fig. 4), indicating that the Gamma 1 and 3 symbionts are thiotrophs. While the presence of two thiotrophic symbionts has been shown for *O. algarvensis* (Woyke *et al.*, 2006), this was not previously known for *O. ilvae*. Unexpectedly, we were not able to find a *cbbL* gene that we could clearly assign to the Gamma 3 symbiont of *O. ilvae* (Fig. 3), despite the analysis of more than 200 clones from three individuals (Table S3). However, the close phylogenetic relationship between the 16S rRNA and *aprA* genes of the *O. ilvae* Gamma 3 symbiont with those of the thiotrophic Gamma 3 symbiont of *O. algarvensis* supports the identification of the *O. ilvae* Gamma 3 symbiont as a sulfur oxidizer.

It is intriguing that both *O. algarvensis* and *O. ilvae*, as well as *O. crassitunicatus* from the continental margin of Peru, harbour two thiotrophic symbionts (Blazejak *et al.*, 2005). These three host species occur in abiogenic, silicate sediments. In contrast, the other host species we have examined to date, *O. loisae*, *I. leukodermatus* and *I. makropetalos*, live in biogenic, calcareous sediments and only have a single thiotrophic symbiont, the Gamma 1 symbiont found in all gutless oligochaetes (Dubilier *et al.*, 1999; Blazejak *et al.*, 2006). Analyses of additional host species from silicate and calcareous sediments

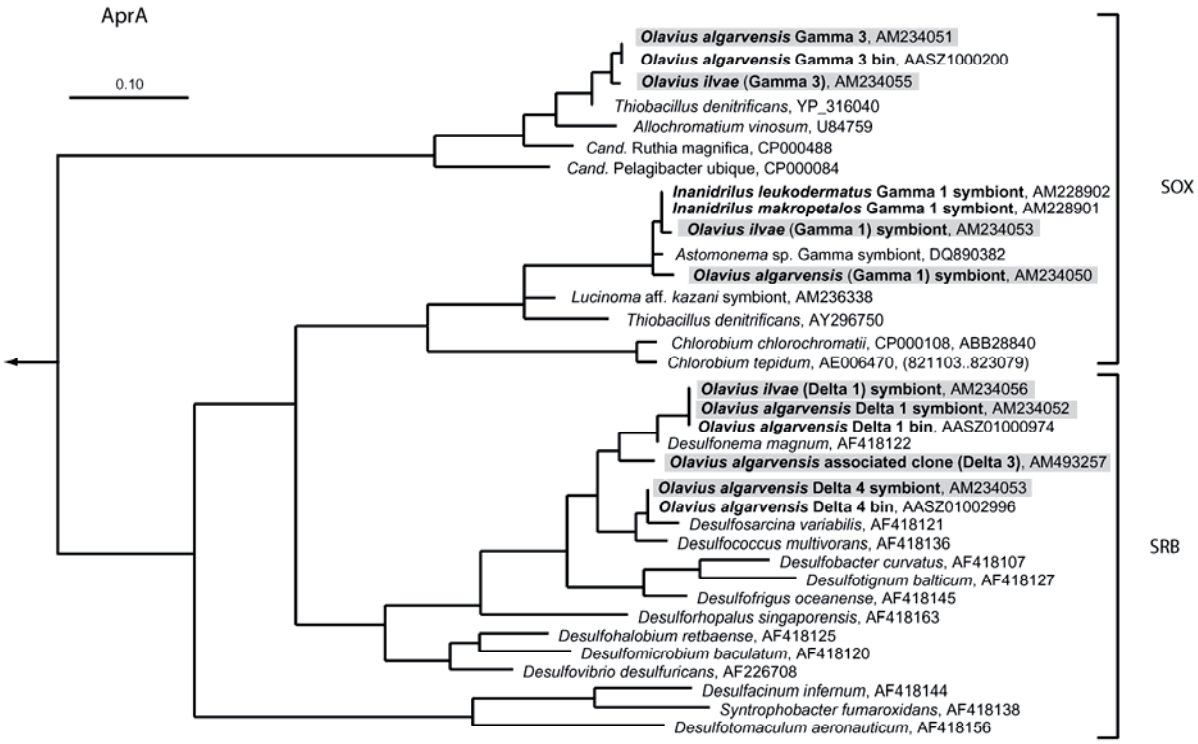


Fig. 4. AprA phylogeny based on deduced amino acid sequences of the *aprA* gene coding for the alpha subunit of APS reductase. The tree was generated from sequences of 131 amino acids using the ML algorithm and a JTT model with a 25% positional conservation filter. Sequences from this study are highlighted in grey, with those originating from symbionts in bold; symbiont names of sequences whose origin was inferred through phylogeny are in parentheses. Three AprA sequences from *Archaeoglobus* spp. served as an out-group (arrow, AE000782, AF418132, AF418134). The polytomic nodes were set for sequences whose positions varied with different filters. Scale bar = 0.10 estimated substitutions per site. SOX, sulfide-oxidizing bacteria; SRB, sulfate-reducing bacteria.

will reveal if the presence or absence of a second thiotrophic symbiont is related to their abiogenic or biogenic habitat.

Deltaproteobacterial endosymbionts

The dominant deltaproteobacterial sequences of *O. ilvae* and *O. algarvensis* belong to three distinct lineages called Delta 1, Delta 3 and Delta 4 (Fig. 5 and Table S1). The Delta 1 16S rRNA sequence from *O. ilvae* was most closely related to the *O. algarvensis* Delta 1 sequence (99.6% sequence identity). These two sequences formed a clade with the Delta 1 symbiont from *O. crassitunicatus* and a clone sequence from Arabian Sea picoplankton (Fig. 5). As for the Delta 1 sequences from *O. ilvae* and *O. algarvensis*, the Delta 3 16S rRNA sequences found in

Table 2. Morphology, abundance and distribution of the symbionts in *O. algarvensis* and *O. ilvae* observed with FISH.

		No. of individuals in which detected by FISH		% estimated abundance[b]		Distribution in symbiont region	
Symbiont	Cell dimension[a] (μm)	*O. algarvensis* ($n = 9$)	*O. ilvae* ($n = 3$)	*O. algarvensis*	*O. ilvae*	*O. algarvensis*	*O. ilvae*
Gamma 1	2.3–3 × 1.4	9	3	25–45%	70%	Even	Even
Gamma 3	1	7	3	25–30%	5–10%	Even	Mostly below cuticle
Delta 1	1–1.8	9	3	10–45%	20%	Even	Even
Delta 3	0.9–1.2	n.d.	3	n.d.	≤ 5%	n.d.	Mostly below cuticle
Delta 4	0.8–1.2	2	n.d.	25–30%	n.d.	Even	n.d.
Spirochete	0.2–0.3 × 5–12	9	n.d.	10%	n.d.	Patchy	n.d.

a. Cell dimensions are influenced by fixation and dehydration.
b. These numbers are based on non-quantitative observations.
n.d., not detected.

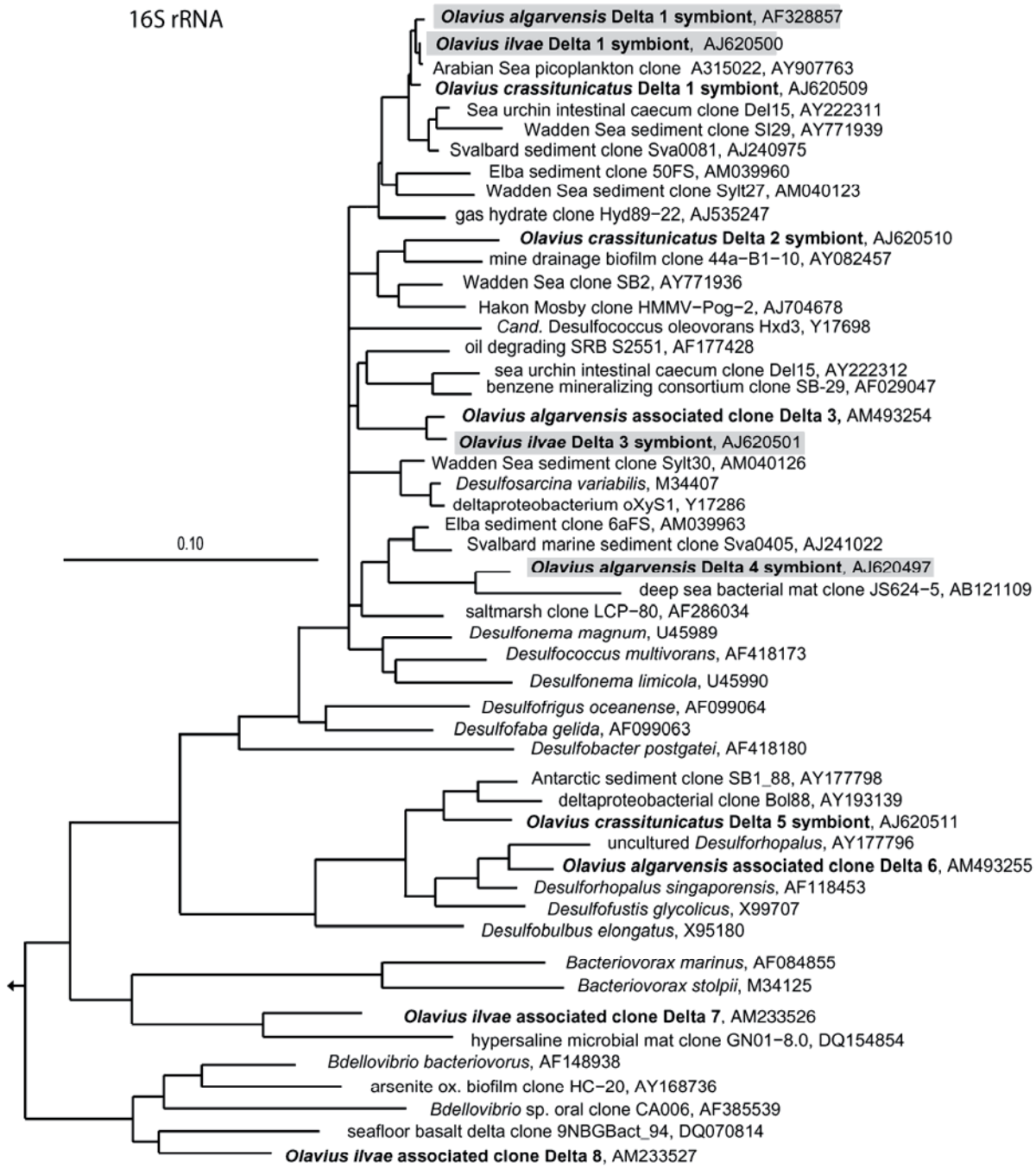


Fig. 5. Phylogenetic placement of the deltaproteobacterial 16S rRNA gene sequences from *O. algarvensis* and *O. ilvae*. Sequences from this study are highlighted in grey, with those confirmed to originate from symbionts using FISH highlighted in bold. Sequences not confirmed to originate from symbionts using FISH are called *O. algarvensis*- or *O. ilvae*-associated clones. Consensus tree based on maximum likelihood (ML) analyses of 16S rRNA sequences. Nodes that differed between parsimony, neighbour-joining and ML analyses were collapsed to a consensus branch. The relationships of the Delta 1, 3 and 4 sequences from *O. ilvae* and *O. algarvensis* to each other and to the closest cultivated *Desulfobacteraceae* were not stable and are therefore shown as a consensus branch. The *O. crassitunicatus* Delta 5 symbiont was formerly called the *O. crassitunicatus* Delta 3 symbiont in Blazejak and colleagues (2005). Five *Sphingomonas* spp. 16S rRNA sequences were used as an out-group (arrow, AJ416411, AB024289, U37341, AF281032, X97776). Scale bar = 0.10 estimated substitutions per site.

these two hosts were also closely related to each other (98.2% sequence identity). The Delta 3 sequences were most closely related to a group of environmental clone sequences that included 16S rRNA genes from bacteria in hydrocarbon-degrading consortia (Phelps *et al.*, 1998) and the gut nodules of the sea urchin *Echinocardium cordatum* (Da Silva *et al.*, 2006). The Delta 4 16S rRNA sequence from *O. algarvensis* was not closely related to other sequences from oligochaete hosts, and belonged instead to a separate lineage that included environmental clone sequences, some of which were from the collection site of the worms.

Fluorescence *in situ* hybridization with specific probes for the Delta 1 sequences (Table 1) showed that the Delta 1 symbionts were present in all individuals of both host species (Fig. 2C and G; Table 2). The probe specific to the Delta 3 sequence showed a signal in all *O. ilvae* individuals, but only a very small percentage ($\leq$ 5%) of the symbiotic microbial community belonged to the Delta 3 symbionts in this host (Fig. 2G; Table 2). Despite the presence of a Delta 3 sequence in *O. algarvensis* clone libraries (although only a single clone from one individual, Specimen 7 in Table S1), no signals for a Delta 3 symbiont were observed in *O. algarvensis*. Instead, two out of nine *O. algarvensis* individuals examined with FISH harboured the Delta 4 symbiont (Fig. 2C). This corresponds with our previous metagenomic analysis of *O. algarvensis*, in which we only found 16S rRNA genes for the Delta 1 and 4 symbionts (Woyke *et al.*, 2006).

Delta 1 symbionts have now been found in the two Mediterranean host species examined in this study as well as in the Peruvian species *O. crassitunicatus* (Blazejak *et al.*, 2005). The close phylogenetic relationship between the Delta 1 symbionts of these three host species (Fig. 5) despite their large geographic distances indicates that these associations are highly specific and not just casual relationships between the worms and *Deltaproteobacteria* from their habitat.

In contrast to the Delta 1 symbionts which occur in all members of both host species, the distribution of the Delta 3 and 4 symbionts from *O. ilvae* and *O. algarvensis* was variable between individuals (Table 2). Similarly, the additional deltaproteobacterial symbionts of *O. crassitunicatus* (Delta 2 and 5 in Fig. 5) also varied intraspecifically. This suggests that these additional deltaproteobacterial symbionts play a less important role in the association than the Delta 1 symbionts.

The close phylogenetic relationship of the deltaproteobacterial symbionts from *O. ilvae* and *O. algarvensis* to free-living sulfate-reducing bacteria (Fig. 5) suggests that the symbionts are also sulfate reducers. While this was previously shown for the Delta 1 and Delta 4 symbionts of *O. algarvensis* (Dubilier *et al.*, 2001; Woyke *et al.*, 2006), this study indicates that *O. ilvae* also harbours two sulfate-reducing symbionts, Delta 1 and Delta 3. Corresponding to the close relationship of the 16S rRNA sequences of the Delta 1 symbionts of *O. ilvae* and *O. algarvensis*, both AprA and DsrAB sequences of *O. ilvae* were closely related to the AprA and DsrAB sequences from the *O. algarvensis* Delta 1 symbiont, suggesting that these originated from the *O. ilvae* Delta 1 symbiont (Figs 4 and 6). For the Delta 3 symbiont of *O. ilvae*, a corresponding *aprA* was not found (Table S3), but the presence of *dsrAB* assumed to have originated from its Delta 3 symbiont (Fig. 6) suggests that the *O. ilvae* Delta 3 symbiont is also a sulfate reducer.

In addition to the *aprA* genes from the Delta 1 and Delta 4 symbionts of *O. algarvensis*, we found a third *aprA* sequence in this host (called *O. algarvensis* associated clone Delta 3 in Fig. 4, and *aprA* $D3_{alg}$ in Table S3). This gene was only found in Specimen 7, and correspondingly, we only found a 16S rRNA Delta 3 sequence in Specimen 7 (Table S1; Fig. 5). As described above, we could not find the Delta 3 16S rRNA sequence in *O. algarvensis* using FISH, but it is possible that a Delta 3 symbiont is present in *O. algarvensis* that only occurs in very low numbers or in a small percentage of the host population.

Three additional deltaproteobacterial 16S rRNA sequences were found in very low numbers in the clone libraries of *O. algarvensis* and *O. ilvae*, namely *O. algarvensis*-associated clone Delta 6, and *O. ilvae*-associated clones Delta 7 and 8 (Fig. 5 and Table S1). We did not develop FISH probes specific to these sequences because the amount of bacteria observed with the general eubacterial probe in the worms did not differ markedly from the sum of bacteria hybridized with specific probes for the symbionts described above. This indicates that these additional deltaproteobacterial sequences are rare or from non-symbiotic bacteria. The *O. algarvensis* Delta 6 sequence fell in a clade of sequences from sulfate-reducing bacteria of the family *Desulfobulbaceae* that also includes the *O. crassitunicatus* Delta 5 symbiont (formerly called *O. crassitunicatus* Delta 3 symbiont in Blazejak *et al.*, 2005). The *O. ilvae*-associated clones Delta 7 and 8 fall in clusters that include bacteria belonging to the *Bacteriovoracaceae* and the genus *Bdellovibrio*. Bacteria from these groups are parasites of free-living and symbiotic Gram-negative bacteria (Davidov and Jurkevitch, 2004). It is therefore possible that the *O. ilvae* Delta 7 and 8 sequences originated from parasites of the gamma- or deltaproteobacterial symbionts. While bacteria with a typical *Bdellovibrio*-like morphology (Shemesh and Jurkevitch, 2004) have not yet been observed in oligochaete symbionts, these have not been specifically searched for in ultrastructural analyses.

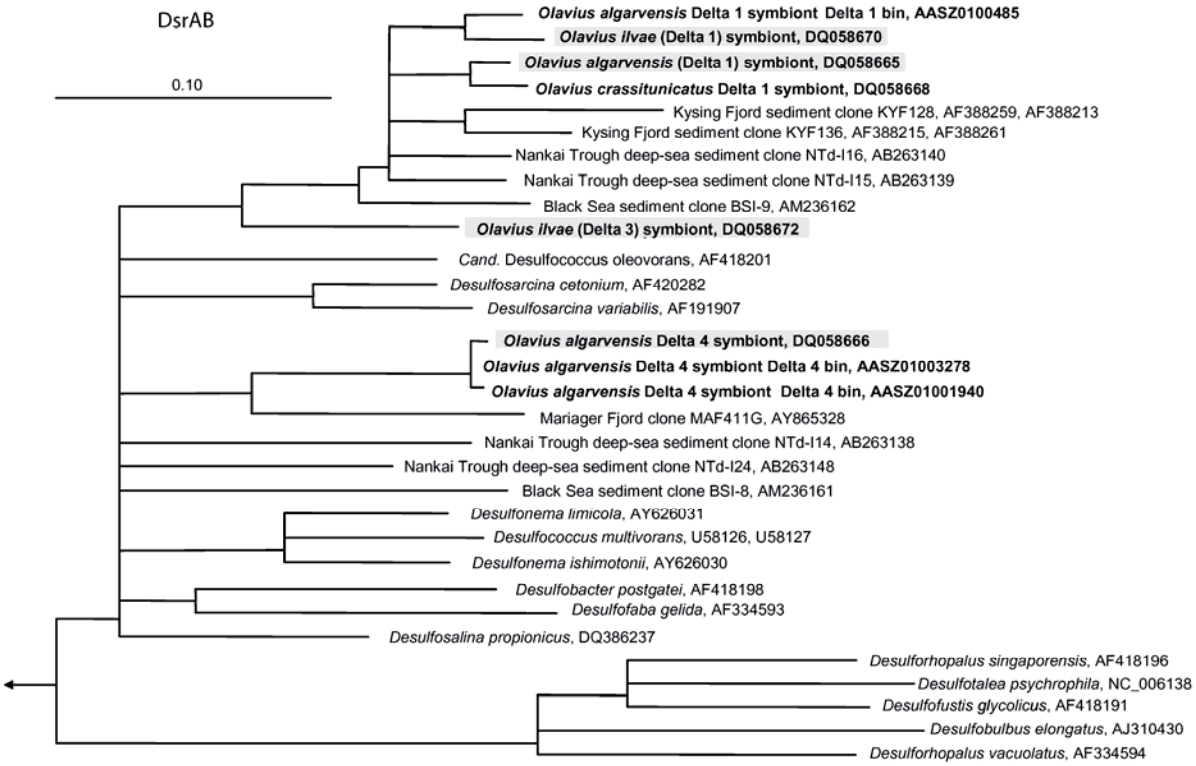


Fig. 6. DsrAB phylogeny based on deduced amino acid sequences of the *dsrAB* gene coding for the alpha and beta subunits of the dissimilatory (bi)sulfite reductase (sequences from this study highlighted in grey; symbionts from gutless oligochaetes in bold; symbiont names of sequences whose origin was inferred through phylogeny are in parentheses). Polytomic nodes in the DsrAB consensus tree connect branches for which a relative order could not be determined unambiguously by applying distance matrix, maximum parsimony and maximum likelihood treeing methods. The DsrAB sequence of *Desulfovibrio vulgaris* (U16723) was used as an out-group. Scale bar = 0.10 estimated substitutions per site as inferred from the distance matrix method.

Spirochete endosymbionts

The *O. algarvensis* spirochete 16S rRNA sequence from this study grouped with a monophyletic clade of spirochete symbiont sequences from the gutless oligochaetes *O. crassitunicatus* and *O. loisae* (Fig. 7). Their closest relatives were sequences from enrichment cultures of tubes from the hydrothermal vent polychaete *Alvinella pompejana*. Using a specific probe for all known oligochaete spirochete symbionts (Table 1), these bacteria were only observed in *O. algarvensis*, where they occurred regularly in all individuals examined (Fig. 2D, Table 2). Scanning electron microscopy analyses showed the elongated, spirochete-like shape of these bacteria (Fig. 2D, inset).

As discussed above for the Delta 1 symbionts, the close phylogenetic relationship of the spirochete symbionts from hosts found in geographically distant locations such as the Australian Great Barrier Reef (*O. loisae*), the Peru margin (*O. crassitunicatus*) and the Mediterranean suggests that these are widespread and specific to gutless oligochaetes and not just casual associates. However, the lack of spirochetes in *O. ilvae* that co-occurs in the same habitat with *O. algarvensis* as well as their absence in other gutless oligochaete species (Blazejak *et al.*, 2006) shows that these symbionts are not necessarily essential to the symbiotic association. Whether the presence of spirochetes provides a selective advantage to the host remains to be shown and cannot be clarified until their physiological role is known. As yet, there is no genomic analysis of oligochaete spirochetes.

Conclusions

Differences in the abundance and distribution of the co-occurring oligochaete symbionts indicate a dynamic evolutionary process in which some bacteria have become well established as symbionts while others are less stable members of the association. Co-occurring Gamma 1 and Delta 1 symbionts have now been found in the two Mediterranean species examined here as well as in *O. crassitunicatus* from the Peru margin (Blazejak *et al.*, 2005). The regular and persistent occurrence of these

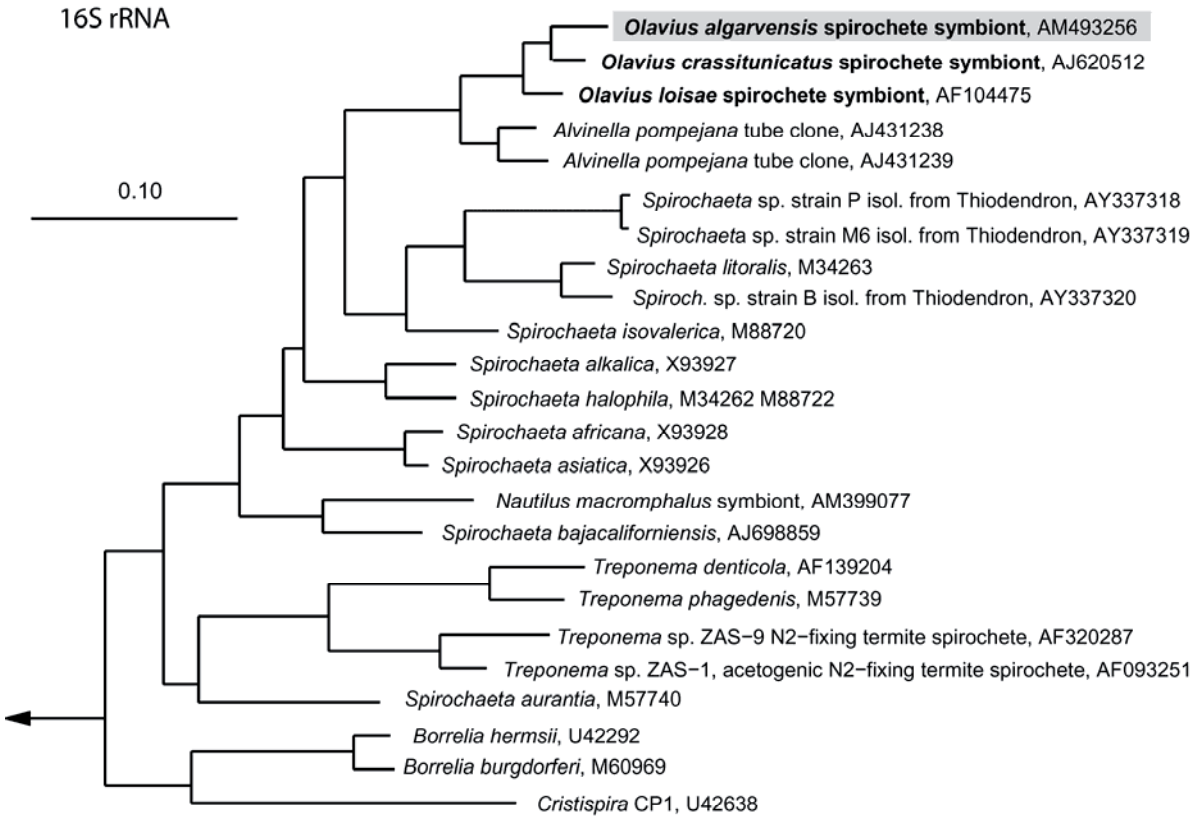


Fig. 7. Phylogenetic placement of the spirochete symbiont from *O. algarvensis* based on maximum likelihood (ML) analyses of 16S rRNA sequences (sequence from this study highlighted in grey; symbionts from gutless oligochaetes in bold face). *Escherichia coli* and *Vibrio fischeri* sequences were used as an out-group (arrow, AE016770, X56578). Scale bar = 0.10 estimated substitutions per site.

symbionts in these three host species suggests that they are essential for these hosts and that these associations are stable and well established. The additional gammaproteobacterial symbionts of these hosts (Gamma 2 and 3 in Fig. 1) occur fairly regularly within each host population, although some variability was observed in *O. algarvensis* (Table 2). The highest variability was observed in the abundance and distribution of the additional deltaproteobacterial symbionts of these three host species (Delta 3, 4 and 5 in Fig. 5), suggesting that these symbionts are less important, and that these associations are less stable. The phylogenetic diversity of these additional deltaproteobacterial symbionts (Fig. 5) indicates that these associations were established multiple times and independently of each other in convergent evolution.

As in other invertebrate hosts with multiple symbionts, little is currently understood about the selective advantage of harbouring different types of co-occurring bacteria. In gutless oligochaetes with sulfur-oxidizing and sulfate-reducing symbionts such as *O. algarvensis* and *O. ilvae*, it is clear that syntrophic sulfur cycling provides both the symbionts and their hosts with benefits (Dubilier *et al.*, 2001; Woyke *et al.*, 2006). Multiple symbionts with similar metabolic capabilities guarantee functional redundancy and increase the fitness of the host to respond to changes in the environment that might favour one symbiont species over the other. Particularly the provision of sulfide through sulfate-reducing symbionts is assumed to play a crucial role in these hosts, as sulfide concentrations in the Elba sediments are extremely low and rarely exceed a few micromolars (Dubilier *et al.*, 2001). It is intriguing that *O. algarvensis* and *O. ilvae* that are only distantly related to each other harbour a similar association of gamma- and deltaproteobacterial symbionts. This suggests that there was a strong selective pressure on these hosts to establish associations with sulfate-reducing bacteria to better supply their gammaproteobacterial symbionts with a continuous source of reduced sulfur compounds.

Acknowledgements

We thank Silke Wetzel, Jörg Wulf, Christian Baranyi, Daniela Gruber and Gerhard Spitzer for excellent technical assistance and the Microbial Genomics Group of the MPI Bremen,

especially Hanno Teeling, for bionformatic support and helpful discussions. We are grateful to Monika Bright and Waltraud Klepal, University of Vienna, for providing access to scanning electron microscopy facilities and to Michael Wagner, University of Vienna, for his helpful and insightful comments. This work was supported by the Max Planck Society, Munich, Germany. A.L. was supported by the European Community (Marie Curie Intra-European Fellowship within the 6th Framework Programme) and the FWF – Fonds zur Förderung der wissenschaftlichen Forschung (P18836-B17). C.E. was supported by a grant from the Swedish Research Council.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Blazejak, A., Erséus, C., Amann, R., and Dubilier, N. (2005) Coexistence of bacterial sulfide oxidizers, sulfate reducers, and spirochetes in a gutless worm (*Oligochaeta*) from the Peru margin. *Appl Environ Microbiol* **71:** 1553–1561.

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

Boucher, Y., Douady, C.J., Papke, R.T., Walsh, D.A., Boudreau, M.E.R., Nesbø, C.L., *et al.* (2003) Lateral gene transfer and the origins of prokaryotic groups. *Annu Rev Genet* **37:** 283–328.

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

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., *et al.* (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res* **35:** D169–D172.

Da Silva, S.G., Gillan, D.C., Dubilier, N., and De Ridder, C. (2006) Characterization by 16S rRNA gene analysis and *in situ* hybridization of bacteria living in the hindgut of a deposit-feeding echinoid (*Echinodermata*). *J Mar Biol Assoc UK* **86:** 1209–1213.

Davidov, Y., and Jurkevitch, E. (2004) Diversity and evolution of *Bdellovibrio*-and-like organisms (BALOs), reclassification of *Bacteriovorax starrii* as *Peredibacter starrii* gen. nov., comb. nov., and description of the *Bacteriovorax–Peredibacter* clade as *Bacteriovoracaceae* fam. nov. *Int J Syst Evol Microbiol* **54:** 1439–1452.

Dubilier, N., Giere, O., Distel, D.L., and Cavanaugh, C.M. (1995) Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (*Oligochaeta*, *Annelida*) by phylogenetic 16S rRNA sequence analysis and *in situ* hybridization. *Appl Environ Microbiol* **61:** 2346–2350.

Dubilier, N., Amann, R., Erséus, C., Muyzer, G., Park, S.Y., Giere, O., and Cavanaugh, C.M. (1999) Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochete *Olavius loisae* (*Annelida*). *Mar Ecol Prog Ser* **178:** 271–280.

Dubilier, N., Mulders, C., Ferdelman, T., de Beer, D., Pernthaler, A., Klein, M., *et al.* (2001) Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. *Nature* **411:** 298–302.

Dubilier, N., Blazejak, A., and Ruehland, C. (2006) Symbioses between bacteria and gutless marine oligochaetes. In *Molecular Basis of Symbiosis*. Overmann, J. (ed.). New York, NY, USA: Springer Verlag, pp. 251–275.

Elsaied, H., and Naganuma, T. (2001) Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* **67:** 1751–1765.

Friedrich, M.W. (2002) Phylogenetic analysis reveals multiple lateral transfers of adenosine-5′-phosphosulfate reductase genes among sulfate-reducing microorganisms. *J Bacteriol* **184:** 278–289.

Giere, O., and Erséus, C. (2002) Taxonomy and new bacterial symbioses of gutless marine *Tubificidae* (*Annelida, Oligochaeta*) from the Island of Elba (Italy). *Org Divers Evol* **2:** 289–297.

Giere, O., Erséus, C., and Stuhlmacher, F. (1998) A new species of *Olavius* (*Tubificidae*, *Phallodrilinae*) from the Algarve Coast in Portugal, the first East Atlantic gutless oligochaete with symbiotic bacteria. *Zool Anz* **237:** 209–214.

Hipp, W.M., Pott, A.S., Thum-Schmitz, N., Faath, I., Dahl, C., and Truper, H.G. (1997) Towards the phylogeny of APS reductases and sirohaem sulfite reductases in sulfate-reducing and sulfur-oxidizing prokaryotes. *Microbiology-UK* **143:** 2891–2902.

Johnson, J.M., Mason, K., Moallemi, C., Xi, H., Somaroo, S., and Huang, E.S. (2003) Protein family annotation in a multiple alignment viewer. *Bioinformatics* **19:** 544–545.

Loy, A., Kusel, K., Lehner, A., Drake, H.L., and Wagner, M. (2004) Microarray and functional gene analyses of sulfate-reducing prokaryotes in low-sulfate, acidic fens reveal cooccurrence of recognized genera and novel lineages. *Appl Environ Microbiol* **70:** 6998–7009.

Loy, A., Maixner, F., Wagner, M., and Horn, M. (2007) probeBase – an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Res* **35:** D800–D804.

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

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. *Syst Appl Microbiol* **15:** 593–600.

Manz, W., Eisenbrecher, M., Neu, T.R., and Szewzyk, U. (1998) Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by *in situ* probing with specific 16S rRNA targeted oligonucleotides. *FEMS Microbiol Ecol* **25:** 43–61.

Meyer. B., and Kuever, J. (2007a) Phylogeny of the alpha and beta subunits of the dissimilatory adenosine-5′-phosphosulfate (APS) reductase from sulfate-reducing prokaryotes – origin and evolution of the dissimilatory

sulfate-reduction pathway. *Microbiology* **153:** 2026–2044.

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

Musat, N., Giere, O., Gieseke, A., Thiermann, F., Amann, R., and Dubilier, N. (2007) Molecular and morphological characterization of the association between bacterial endosymbionts and the marine nematode *Astomonema* sp. from the Bahamas. *Environ Microbiol* **9:** 1345–1353.

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

Phelps, C., Kerkhof, L.J., and Young, L.Y. (1998) Molecular characterization of a sulfate reducing consortium which mineralizes benzene. *FEMS Microbiol Ecol* **27:** 269–279.

Polz, M.F., Distel, D.L., Zarda, B., Amann, R., Felbeck, H., Ott, J.A., and Cavanaugh, C.M. (1994) Phylogenetic analysis of a highly specific association between ectosymbiotic, sulfur-oxidizing bacteria and a marine nematode. *Appl Environ Microbiol* **60:** 4461–4467.

Schizas, N.V., Street, G.T., Coull, B.C., Chandler, G.T., and Quattro, J.M. (1997) An efficient DNA extraction method for small metazoans. *Mol Mar Biol Biotechnol* **6:** 381–383.

Scott, K.M., Sievert, S.M., Abril, F.N., Ball, L.A., Barrett, C.J., Blake, R.A., *et al.* (2006) The genome of deep-sea vent chemolithoautotroph *Thiomicrospira crunogena* XCL-2. *PLoS Biol* **4:** e383. doi:10.1371/journal.pbio.0040383.

Shemesh, Y., and Jurkevitch, E. (2004) Plastic phenotypic resistance to predation by *Bdellovibrio* and like organisms in bacterial prey. *Environ Microbiol* **6:** 12–18.

Wagner, M., Loy, A., Klein, M., Lee, N., Ramsing, N.B., Stahl, D.A., and Friedrich, M.W. (2005) Functional marker genes for identification of sulfate-reducing prokaryotes. *Methods Enzymol* **397:** 469–489.

Wallner, G., Amann, R., and Beisker, W. (1993) Optimizing fluorescent *in situ*-hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14:** 136–143.

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

Zverlov, V., Klein, M., Lucker, S., Friedrich, M.W., Kellermann, J., Stahl, D.A., *et al.* (2005) Lateral gene transfer of dissimilatory (bi)sulfite reductase revisited. *J Bacteriol* **187:** 2203–2208.

Supporting information

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

Table S1. 16S rRNA gene libraries from *O. ilvae* and *O. algarvensis* individuals. Worms were prepared individually for DNA extraction as described previously (Dubilier *et al.*, 1999) using the isolation protocol of (Schizas *et al.*, 1997). Amplification of the 16S rRNA genes was performed with the general bacterial primers 8F and 1492R (Muyzer *et al.*, 1995). At least one representative clone from each clone group was fully sequenced in both directions. Clones were grouped based on a sequence identity threshold of 99% for full sequences.

Table S2. Sequence identities of genes from *O. algarvensis* and *O. ilvae*. The 16S rRNA, *dsrAB*, *cbbL* and *aprA* sequences isolated from *O. algarvensis* and *O. ilvae* were submitted either to EBI or to GenBank. Nucleotide sequence accession numbers are presented. Amino acid identity values for protein-coding genes were calculated in PFAAT (Johnson *et al.*, 2003).

Table S3. Protein-coding gene libraries from *O. ilvae* and *O. algarvensis* individuals. Amplification, cloning and sequencing of the *dsrAB*, *cbbL*, *cbbM* and *aprA* genes were carried out as described previously (Loy *et al.*, 2004; Wagner *et al.*, 2005; Blazejak *et al.*, 2006). Full sequences within each clone group shared at least 98.4% sequence identity (% identical amino acids).

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

TABLE S1 16S rRNA gene libraries from *O. ilvae* and *O. algarvensis* individuals. Worms were prepared individually for DNA extraction as described previously (Dubilier et al., 1999) using the isolation protocol of Schizas et al. 1997. Amplification of the 16SrRNA genes was performed with the general bacterial primers 8F and 1492R (Muyzer et al., 1995). At least one representative clone from each clone group was fully sequenced in both directions. Clones were grouped based on a sequence identity threshold of 99% for full sequences.

	Number of clones													
Specimen	Gammaproteobacteria				Deltaproteobacteria								*Spirochaeta*	other*
	Gamma 1_{ilv}	Gamma $1_{a\,g}$	Gamma 3_{ilv}	Gamma 3_{alg}	Delta 1_{ilv}	Delta 1_{alg}	Delta 3_{ilv}	Delta $3_{a\,g}$	Delta 4_{alg}	Delta 6_{alg}	Delta $7_{\,v}$	Delta 8_{ilv}		
O. algarvensis 1	0	20	0	1	0	1	0	0	0	0	0	0	1	0
O. algarvensis 2	0	35	0	0	0	0	0	0	20	0	0	0	0	0
O. algarvensis 3	0	10	0	0	0	0	0	0	3	0	0	0	0	0
O. algarvensis 6	0	13	0	18	0	22	0	0	0	0	0	0	13	3
O. algarvensis 7	0	33	0	2	0	5	0	1	38	1	0	0	0	0
O. ilvae 1	15	0	0	0	2	0	3	0	0	0	0	0	0	0
O. ilvae 2	10	0	1	0	9	0	0	0	0	0	0	0	0	0
O. ilvae 3	0	0	0	0	55	0	0	0	0	0	0	0	0	0
O. ilvae 4	37	0	0	0	0	0	0	0	0	0	0	0	0	0
O. ilvae 5	35	0	0	0	0	0	18	0	0	0	0	0	0	0
O. ilvae 6	64	0	1	0	0	0	1	0	0	0	0	2	0	0
O. ilvae 7	35	0	0	0	8	0	6	0	0	0	0	0	0	0
O. ilvae 8	51	0	4	0	5	0	1	0	0	0	3	0	0	0

*other = Bacillales

TABLE S2 Sequence identities of genes from *O. algarvensis* and *O. ilvae*. The 16S rRNA, *dsrAB*, *cbbL*, and *aprA* sequences isolated from *O. algarvensis* and *O. ilvae* were submitted either to EBI or to Genbank. Nucleotide sequence accession numbers are presented below. Amino acid identity values for protein-coding genes were calculated in PFAAT (Johnson et al., 2003).

Gene	Host	Symbiont sequence	Accession number	Sequence with highest identity	Identity (%) *	Closest cultured organism	Identity (%) *
	O. algarvensis	Gamma 1	AF328856	*Inanidrilus leukodermatus* Gamma 1, U241´0	97.2	*Thiorhodovibrio winogradsky*, AB016986	94 3
		Gamma 3	AJ620496	Elba marine sediment clone 13a FS, AM039958	98.6		
		Delta 1	AF328857	*O. ilvae* Delta 1, AJ620500	99.6	*Desulfonema magnum*, U45989	93 ´
		Delta 4	AJ620497	Deep sea bacterial mat clone JS624-5, AB121109	92.8	*Desulfosarcina variabilis*, M34407	92 4
		associated clone Delta 3	AM493254	*O. ilvae* Delta 3, AJ620501	98.2	*Desulfosarcina variabilis*, M34407	92 7
		associated clone Delta 6	AM493255	*Desulforhopalus singaporensis*, AF118453	95.6		
16S rRNA		Sp rochete	AM493256	*O. crassitunicatus* spirochete symbiont, AJ620512	95.7	*Spirochaeta isovalerica*, M88720	86 ´
	O. ilvae	Gamma 1	AJ620498	*Laxus* sp. ectosymbiont, U14727	98.9	*Thiorhodovibrio winogradsky*, AB016986	94 ´
		Gamma 3	AJ620499	Wadden Sea sediment clone Sylt23, AM040119	98.8		
				O. algarvensis Gamma 3, AJ620496	98.1		
		Delta 1	AJ620500	Arabian Sea picoplankton clone A315022, AY771938	99.7	*Desulfonema magnum*, U45989	92 9
		Delta 3	AJ620501	*O. algarvensis* associated c one Delta 3, AM493257	98.2	*Desulfosarcina variabilis*, M34407	92 7
						Strain oxyS1, Y17286	92 7
		associated clone Delta 7	AM233526	Hypersaline microb al mat clone GN01-8.0, DQ154854	88.6	*Desulforhopalus singaporensis*, AF118453	83 4
		associated clone Delta 8	AM233527	Seafloor basalt clone, DQ070614	90.3	*Bdellovibrio bacteriovorus*, AF084850	88 6
	O. algarvensis	Gamma 1	AM234057	*Inanidrilus leukodermatus* Gamma 1, AM228899	96.8	*Allochromatium vinosum*, M26396	88 9
				O. ilvae Gamma 1 AM234059	96.3		
				O. algarvensis Gamma 1 bin, AASZ01004958 (115 AA)	100		
cbbL		Gamma 3	AM234058	*O. ilvae* Gamma 1 AM234059	92.6	*Allochromatium vinosum*, M26396	92 6
				O. algarvensis Gamma 3 bin, AASZ01000113 (217 AA)	100		
	O. ilvae	Gamma 1	AM234059	*Inanidrilus leukodermatus* Gamma 1, AM228899	98.2	*Allochromatium vinosum*, M26396	91 7
				Inanidrilus makropetalus Gamma 1, AM228900	97.1		
	O. algarvensis	Gamma 1	AM234050	*Inanidrilus leukodermatus* Gamma 1, AM228902	96.6	*Thiobacillus denitrificans*, AY296750	84 9
				Inanidrilus makropetalus Gamma 1, AM228901	96.6		
		Gamma 3	AM234051	*O. ilvae* Gamma 3 AM234055	98.3	*Thiobacillus denitrificans*, YP_316040	96 6
				O. algarvensis Gamma 3 bin, AASZ1000200 (117 AA)	100		
		Delta 1	AM234052	*O.ilvae* Delta 1, AM234056	100	*Desulfonema magnum*, AF418122	97 5
				O. algarvensis Delta 1 bin, AASZ01000974 (119 AA)	100		
aprA		Delta 4	AM234053	*O. algarvensis* Delta 4 bin, AASZ01002996 (24 AA)	100	*Desulfosarcina variabilis*, AF418121	97 5
						Desulfococcus multivorans, AF418136	96 6
		associated clone Delta 3	AM493257	*O. algarvensis* Delta 1, AM234052	91.6	*Desulfonema magnum*, AF418122	93 3
				O.ilvae Delta 1, AM234056	91.6		
	O. ilvae	Gamma 1	AM234054	*Inanidrilus leukodermatus* Gamma 1, AM228902	99.2	*Thiobacillus denitrificans*, AY296750	84 9
				Inanidrilus makropetalus Gamma 1, AM228901	98.3		

TABLE S2 continued

Gene	Host	Symbiont sequence	Accession number	Sequence with highest identity	Identity (%) *	Closest cultured organism	Identity (%) *
		Delta 1	AM234056	*O. algarvensis* Delta 1, AM234052	100	*Desulfonema magnum*, AF418122	97.5
				O. algarvensis Delta 1 bin, AASZ01000974	100		
	O. algarvensis	Delta 1	DQ08665	O. crassitunicatus Delta 1, DQ058668	95.5	*Desulfobacterium cetonicum*, AF420282	83.9
				O. algarvensis Delta 1 bin, AASZ0100485 (628 AA)	93.3		
dsrAB		Delta 4	DQ058666	*O. algarvensis* Delta 4 bin, AASZ01001940 (160 AA)	98.9	*Desulfobacterium cetonicum*, AF420282	83.9
				O. algarvensis Delta 4 bin, AASZ01003278 (460 AA)	98.5 - 99.3		
	O. ilvae	Delta 1	DQ058670	*O. algarvensis* Delta 1 bin, AASZ0100485 (628 AA)	95.5	*Desulfobacterium cetonicum*, AF420282	82.9
				O. algarvensis Delta 1, DQ08665	92.8		
		Delta 3	DQ058672	*O. algarvensis* Delta 1, DQ58665	84.9	*Desulfosarcina variabilis*, AF191907	83.2

* % identity for 16S rRNA gene based on nucleotide sequence and for *cbbL*, *aprA* and *dsrAB* based on amino acid sequence

TABLE S3 Protein-coding genes libraries from *O. ilvae* and *O. algarvensis* individuals. Amplification, cloning and sequencing of the *dsrAB*, *cbbL*, *cbbM* and *aprA* genes was carried out as described previously (Loy et al., 2004; Wagner et al., 2005; Blazejak et al., 2006). Full sequences within each clone group shared at least 98.4% sequence identity (% identical amino acids).

	Number of clones																	
	cbbL			*dsrAB*						*aprA*								
Specimen	*cbbL* G1$_{IV}$	*cbbL* G1$_{alg}$	*cbbL* G3$_{alg}$	*dsrAB* D1$_{IV}$	*dsrAB* D1$_{alg}$	*dsrAB* D3$_{IV}$	*dsrAB* D4$_{alg}$	*aprA* G1$_{IV}$	*aprA* G1$_{alg}$	*aprA* G3$_{IV}$	*aprA* G3$_{alg}$	*aprA* D1$_{IV}$	*aprA* D1$_{alg}$	*aprA* D3$_{alg}$	*aprA* D4$_{alg}$
O. algarvensis 1	-	-	-	0	0	0	4	-	-	-	-	-	-	-	-
O. algarvensis 2	-	-	-	0	0	0	5	-	-	-	-	-	-	-	-
O. algarvensis 3	-	-	-	0	0	0	6	-	-	-	-	-	-	-	-
O. algarvensis 4	-	-	-	0	1	0	3	-	-	-	-	-	-	-	-
O. algarvensis 5	0	31	6	-	-	-	-	0	2	0	23	0	3	0	14
O. algarvensis 6	0	53	7	-	-	-	-	0	14	0	50	0	9	0	2
O. algarvensis 7	0	77	0	-	-	-	-	0	42	0	4	0	1	1	42
O. algarvensis 8	-	-	-	0	1	0	0	-	-	-	-	-	-	-	-
O. ilvae 2	-	-	-	8	0	0	0	-	-	-	-	-	-	-	-
O. ilvae 3	-	-	-	7	0	0	0	-	-	-	-	-	-	-	-
O. ilvae 4	-	-	-	0	0	0	0	-	-	-	-	-	-	-	-
O. ilvae 5	-	-	-	0	0	9	0	-	-	-	-	-	-	-	-
O. ilvae 9	89	0	0	-	-	-	-	51	3	13	0	10	0	0	0
O. ilvae 10	71	0	0	-	-	-	-	84	3	6	0	0	0	0	0
O. ilvae 11	62	0	0	-	-	-	-	82	3	0	0	2	0	0	0

- not determined

V Chapter 3

Gamma- and epsilonproteobacterial ectosymbionts of a shallow-water marine worm are related to deep-sea hydrothermal vent ectosymbionts

please note: at the time of composing this thesis only the page proofs were available and are presented in this chapter.

emi_2256

Environmental Microbiology (2010) doi:10.1111/j.1462-2920.2010.02256.x

Gamma- and epsilonproteobacterial ectosymbionts of a shallow-water marine worm are related to deep-sea hydrothermal vent ectosymbionts

Caroline Ruehland and Nicole Dubilier*
Symbiosis Group, Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany.

Summary

The marine oligochaete worm *Tubificoides benedii* is often found in high numbers in eutrophic coastal sediments with low oxygen and high sulfide concentrations. A dense biofilm of filamentous bacteria on the worm's tail end were morphologically described over 20 years ago, but no further studies of these epibiotic associations were done. In this study, we used fluorescence *in situ* hybridization and comparative sequence analysis of 16S rRNA and protein-coding genes to characterize the microbial community of the worm's tail ends. The presence of genes involved in chemoautotrophy (*cbbL* and *cbbM*) and sulfur metabolism (*aprA*) indicated the potential of the *T. benedii* microbial community for chemosynthesis. Two filamentous ectosymbionts were specific to the worm's tail ends: one belonged to the *Leucothrix mucor* clade within the Gammaproteobacteria and the other to the Thiovulgaceae within the Epsilonproteobacteria. Both *T. benedii* ectosymbionts belonged to clades that consisted almost exclusively of bacteria associated with invertebrates from deep-sea hydrothermal vents. Such close relationships between symbionts from shallow-water and deep-sea hosts that are not closely related to each other are unusual, and indicate that biogeography and host affiliation did not play a role in these associations. Instead, similarities between the dynamic environments of vents and organic-rich mudflats with their strong fluctuations in reductants and oxidants may have been the driving force behind the establishment and evolution of these symbioses.

Received 17 November, 2009; accepted 8 April, 2010. *For correspondence. E-mail ndubilie@mpi-bremen.de; Tel. (+49) 421 2028 932; Fax ••.

Introduction

Until recently, the epibiotic microflora of animals was often thought to be just a consortium of casual associates, but it is now becoming increasingly clear that it consists of a highly specialized community of microbes that form specific and stable symbioses with their hosts (McFall-Ngai, 2008). In chemosynthetic environments such as hydrothermal vents or sulfide-rich sediments, some marine animals are regularly colonized by ectosymbiotic bacteria (Dubilier *et al.*, 2008; Goffredi, 2010). In coastal sediments these include nematode worms, amphipods and colonial ciliates all of which generally host only a single sulfur-oxidizing gammaproteobacterial symbiont (Gillan and Dubilier, 2004; Bayer *et al.*, 2009; Rinke *et al.*, 2009). Hosts from deep-sea hydrothermal vents such as shrimps, polychaete worms, crabs and barnacles often have a more diverse epibiotic community that is dominated by Gamma- and Epsilonproteobacteria (Goffredi *et al.*, 2008; Suzuki *et al.*, 2009; Watsuji and Takai, 2009; Petersen *et al.*, 2010). The role of these epibiotic bacteria is generally not well understood. They have been hypothesized to contribute to their hosts' nutrition (Rieley *et al.*, 1999; Rinke *et al.*, 2006; Suzuki *et al.*, 2009), detoxify sulfide and heavy metals (Alayse-Danet *et al.*, 1987; Prieur *et al.*, 1990), or provide defense against pathogenic microbes and predators (Gil-Turnes and Fenical, 1992; Goffredi *et al.*, 2004a).

The tubificid oligochaete *Tubificoides benedii* (d'Udekem, 1855) is a marine worm that is commonly found in coastal mudflats of the North Atlantic, especially those with a high input of organic matter (Timm and Erséus, 2009). As other tubificids (Guérin and Giani, 1996), *T. benedii* feeds head down in the sediment and uses its tail end for respiration by holding it above the sediment in the oxygenated seawater and moving it in a swaying motion (Dubilier *et al.*, 1995a). The worms can tolerate extended periods of low oxygen concentrations or anoxia by switching to an anaerobic metabolism, a strategy that is also used when sulfide concentrations become too high (Dubilier *et al.*, 1994).

Tubificoides benedii from mudflats in the Wadden Sea off the coast of Germany are covered with a morphologically diverse assemblage of ectobacteria in

emi_2256

the mucus layer covering their body wall (Giere and Rhode, 1987). Their tail ends are regularly colonized by a dense film of filamentous bacteria. Some of these penetrate the body wall and the basal cells are anchored in the cuticle just above the worm's epidermis, suggesting a highly specific association (Dubilier, 1986). Seasonal 'blooms' of the filamentous bacteria have been observed, with the densest abundance occurring in the summer and fall months when sulfide concentrations are high in the worm's habitat (Dubilier, 1986). This observation together with the morphological similarity of the filamentous bacteria to sulfur-oxidizing bacteria such as *Thiothrix* lead to the hypothesis that these ectofilaments might be chemoautotrophic sulfur oxidizers (Dubilier, 1986), but no further studies on this association followed. The purpose of this study was therefore to characterize the phylogeny and metabolic potential of the filamentous ectobionts of *T. benedii* using fluorescence *in situ* hybridization (FISH) and comparative sequence analysis of 16S rRNA genes as well as genes involved in chemoautotrophy (*cbbL, cbbM* and *aclB*) and sulfur metabolism (*aprA* and *soxB*).

Results and discussion

General bacterial diversity

Comparative 16S rRNA gene analyses and FISH of *T. benedii* tail ends revealed a diverse bacterial community. The filamentous ectosymbionts were identified as Gamma- and Epsilonproteobacteria (see below). As this study focuses on these ectosymbionts, all other bacteria associated with this worm are only briefly described here.

Gammaproteobacterial sequences dominated the 16S rRNA clone libraries (52.4%), Deltaprotobacteria (20.5%) and Cytophaga/Flavobacterium/Bacteroides (CFB; 19.2%) were also abundant, and 4% of the sequences belonged to the Epsilonproteobacteria (Table 1 and Figs S1 and S2). FISH analyses with group specific probes for Deltaproteobacteria and CFB (Table 2) confirmed that bacteria from these phyla colonized the mucus layer covering *T. benedii* tail ends (Fig. S3). Sequences found in only low abundance in single individuals made up only 2.7% of all 16S rRNA clone library sequences and belonged to the Acidobacteria, Verrucomicrobia, Planctomycetes and candidate Division OD1 (Table 1).

Phylogenetic analyses revealed that many of the gamma- and epsilonproteobacterial sequences from the *T. benedii* 16S rRNA clone libraries were most closely related to symbiotic and free-living bacteria from chemosynthetic environments (Fig. 1). Specific FISH probes were developed for all dominant gamma- and epsilonproteobacterial phylotypes (Tables 2 and S1). Aside from the filamentous ectosymbionts described below, the only other gamma- or epsilonproteobacterial phylotype from the 16S rRNA clone libraries that could be found with FISH was a rod- to cocci-shaped bacterium named the Gamma 2 ectobiont (Fig. S3). This phylotype was highly abundant in the 16S rRNA clone libraries (23% of all clones, Table 1), but with FISH, the Gamma 2 ectobiont was only observed occasionally in the mucus membrane of the worm's tail end. Phylogenetic analyses placed this ectobiont in a clade that included the Gamma 3 endosymbionts of the gutless oligochaete worms *Olavius ilvae* and *Olavius algarvensis* from Mediterranean seagrass sediments (Fig. 1) (Ruehland *et al.*, 2008). Such a close phylogenetic relationship between ecto- and endosymbionts of hosts that are separated by large geographic distances, live in different habitats, and are not closely related to each other have rarely been described but are not unprecedented. They have, for example, also been observed between the ectosymbionts of nematodes and the Gamma 1 endosymbionts of gutless oligochaetes (Bayer *et al.*, 2009).

Morphology and phylogeny of the filamentous ectosymbionts

Transmission electron microscopy (TEM) showed that two filamentous morphotypes co-occurred on *T. benedii* tail ends (Fig. 2). Thinner filaments of 0.4–0.65 μm penetrated the worm's cuticle, with the basal cells embedded within the cuticle just above the host's epidermis (Fig. 2B–D). Vesicle-like structures were regularly observed between the basal cell of these filaments and the worm's epidermal cells (Fig. 2D), indicating interactions between the filaments and host tissues (Dubilier, 1986). Thicker filaments of 0.9–1.1 μm were attached to the cuticle but were never observed penetrating it (Fig. 2B). FISH with probes specific to the Gamma 1 and Epsilon 1 sequences shown in Fig. 1 revealed that these originated from two filamentous morphotypes on the worm's tail (Fig. 2B–D). The diameters of these two filament types in FISH micrographs corresponded to those measured with TEM in the thinner and thicker morphotypes (0.4–0.7 μm for the Epsilon 1 and 0.7–1.3 μm for the Gamma 1).This indicates that the thinner filaments observed with TEM in the worm's cuticle belonged to the Epsilon 1 ectosymbionts. This conclusion is supported by FISH analyses that show the Epsilon 1 ectosymbionts embedded within the cuticle (Fig. 2H). The distribution and abundance of the two filamentous ectosymbionts varied considerably within and between individual worms. In some cross-sections only one filament type was observed, in others, especially towards the posterior end of the tail, both the Gamma 1 and Epsilon 1 ectosymbionts co-occurred in thick patches (Fig. 2E). Overall, the abundance of both ectosymbionts was equal based on

emi_2256

Table 1. Clone library sequences from *T. benedii* tail ends.

Gene/ protein	Clone group	Accession number	% in clone library	Closest relative	Identity BLAST (%)	Closest cultured relative	Identity BLAST (%)
16S rRNA	Gamma 1 ectosymbiont	GU197434	12.8	*Shinkaia crosnieri* setae associated clone, AB440175	95	*Leucothrix mucor*, X87277	90
	Gamma 2	GU197441	22.6	Sulfide-microbial incubator clone, DQ228657	97	*Dechloromarinus chlorophilus*, AF170359	90
n = 298	Gamma 3	GU197425	1.7	*Urothoe poseidonis* ectosymbiont, AY426613	95	*Thiothrix eikelboomii*, AB042541	91
	Gamma 4	GU197445	1.3	*Solemya velum* endosymbiont, M90415	94	*Thiobacillus prosperus*, EU653291	91
	Gamma 5	GU197422	12.0	Environmental clones, e.g. hydrothermal vent, AB252427	97	n.a.	
	Other Gamma		3.4				
	Epsilon 1 ectosymbiont	GU197436	1.3	*Pimicaris exoculata* Epsilon 3 ectosymbionts, e.g. FM203395	97	*Sulfurovum lithotrophicum*, AB091292	94
	Epsilon 2	GU197448	1.7	Environmental clones, e.g. methane seep, FJ264676	99	*Sulfurovum lithotrophicum*, AB091292	94
	Other Epsilon		1.0				
	Delta		20.5				
	CFB		19.2				
	Other		7.1				
CbbL	CbbL 1a	GU197482	54.0	*Solemya velum* endosymbiont, AAT01429	98	*Mariprofundus ferrooxydans*, EAU53852	89
	CbbL 1b	GU197481	10.3	*Solemya velum* endosymbiont, AAT01429 (prot)	96	*Mariprofundus ferrooxydans*, EAU53852	90
n = 165	CbbL 1c	GU197476	24.8	*Solemya velum* endosymbiont, AAT01429	93	*Mariprofundus ferrooxydans*, EAU53852	89
	CbbL 2	GU197479	4.8	>		*Mariprofundus ferrooxydans*, EAU53852	94
	CbbL 3	GU197478	3.6	>		*Ralstonia metallidurans*, ABF08384	92
	CbbL 4	GU197480	2.4	>		*Ralstonia metallidurans*, ABF08384	97
	CbbL 5	GU197477	0.6	>		*Thiobacillus denitrificans*, AAB70697	96
CbbM	CbbM 1	GU197394	2.3	*Lamellibrachia* sp. endosymbiont 2, BAA94433	93	*Thiobacillus denitrificans*, AAA99178	85
	CbbM 2	GU197398	4.0	*Lamellibrachia* sp. endosymbiont, CAQ63475	95	*Halothiobacillus neapolitanus*, AAD02442	91
n = 175	CbbM 3a	GU197396	90.3	*Lamellibrachia* sp. tube associated clone, CAQ63479	94	*Thiomicrospira crunogena*, DQ272535	86
	CbbM 3b	GU197397	2.3	*Lamellibrachia* sp. tube associated clone, CAQ63479	90	*Thiomicrospira halophila*, ABD52283	87
	cbbM 4	GU197395	1.1	>		*Rhodopseudomonas palustris*, ABD88443	86
AprA	AprA I1	GU197400	0.4	*O. ilvae* Gamma 3 symbiont, CAJ81245	96	*Thiobacillus thioparus*, ABV80092	94
	AprA II1	GU197399	0.4	*Ifremeria nautilei* symbiont, ABV80045	95	*Thiothrix nivea*, ABV80025	92
n = 242	AprA II2	GU197401	32.5	*Lamellibrachia* sp. endosymbiont, CAQ63492	91	*Thiothrix nivea*, ABV80025	90
	AprA II3	GU197406	0.8	Sea urchin gut clone, CAT03603	96	*Thiocapsa penderis*, ABV800015	89
	AprA II4	GU197404	60.1	*Oligobrachia haakonmosbiensis* endosymbiont, CAP03143	90	*Thiobacillus thioparus*, ABV80027	87
	AprA II5	GU197403	1.2	*Lucinoma* aff. *kazani* symbiont, CAJ85653	94	*Thiobacillus plumbophilus*, ABV80021	91
	AprA II6	GU197402	0.4	*Lucinoma* aff. *kazani* symbiont, CAJ85653	90	*Thiobacillus denitrificans*, ABV80031	88
	AprA SRB1	GU197405	4.1	>		*Desulfotomaculum thermoacetoxidans*, ABR92588	78

Four worms were prepared and analysed individually for the 16S rRNA, three for the *cbbL*, *cbbM* and *aprA* clone libraries (numbers in the first column show the total number of clones sequenced for each gene). Sequences were grouped together if they had at least 99.0% nucleotide identity (16S rRNA gene) or 95% amino acid identity (*cbbL*, *cbbM* and *aprA* genes). Only 16S rRNA gene sequences found in at least four clones and two individuals are shown (sequences at lower abundances are grouped under 'other').

n.a., •.

emi_2256

Table 2. Oligonucleotide probes used in this study (probes specific to the Gamma 1 and Epsilon 1 ectosymbionts in bold). Additional probe details are available at probeBase (Loy *et al.*, 2007).

Probe	Specificity	Probe sequence (5′-3′)	Position[a]	FA [%][b]	Reference organism
EUBI-III	Eubacteria	GCW GCC WCC CGT AGG WGT	338–355	40	(Daims *et al.*, 1999)
NON338	Antisense	ACT CCT ACG GGA GGC AGC	338–355	40	(Wallner *et al.*, 1993)
GAM42a	Gammaproteobacteria	GCC TTC CCA CAT CGT TT	1027–1043[c]	30–40	(Manz *et al.*, 1992)
EP404	*T. benedii* Epsilon 1 ectosymbiont and Epsilon 2 Epsilonproteobacteria (most Campylobacterales), various unc. bacteria including associated bacteria of *C. squamiferum*, *R. exoculata ectosymbionts*, *R. pachytila*	AAA KGY GTC ATC CTC CA	404–420	40	(Macalady *et al.*, 2006) [16]
TbGAM1-138	*T. benedii* Gamma 1 ectosymbiont associated bacteria of *V. osheai*, AB239761, *S. crosnieri*, e.g. AB440174, *K. hirsuta*, EU265799, vent clone AB464819	GGC TTG TCC CCC ACT ACT	138–155	40	*Ectothiorhodospira mongolicum*, other *T. benedii* epibionts
TbGAM1-444	*T. benedii* Gamma 1ectosymbiont associated bacteria of *S. crosnieri*, e.g. AB440175, *K. hirsuta*, EU265799, some environment clones e.g. AM778459, FJ169979	CTT AAC CCC TTC CTC ACA	444–461	40	nd
TbGAM2-447	*T. benedii* Gamma 2, invertebrate burrow clones, FJ753075 and FJ753097	AAG CTT AGG CTT TTC GTC	447–464	40	nd
TbEP1-744	*T. benedii* Epsilon 1 Epsilonproteobacteria, most *Sulfurovum*, including invertebrate associated clade, *C. squamiferum*, *V. osheai*, *A. pompejana* and *R. exoculata* Epsilon 1–5 ectosymbionts, few uncultivated, e.g. AF407203	TCT CAG CGT CAG TAC TGT	744–761	35	nd
TbEP1-94	*T. benedii* Epsilon 1 ectosymbiont associated bacteria of *S. crosnieri*, e.g. AB440164, *Rimicaris* sp., FM203397-99, environ. clones most deep sea, e.g. FJ905659	CCG TTC GCC ACT CGA CAG	95–99	40	*T. benedii* Gamma 1
CF319a	Most Flavobacteria, some Bacteroidetes, some Sphingobacteria	TGG TCC GTG TCT CAG TAC	319–336	40	(Manz *et al.*, 1996)
DELTA495a competitor	Most Deltaproteobacteria and most Gemmatimonadetes	AGT TAG CCG GTG CTT CCT AGT TAG CCG GTG CTT CTT	495–512	40	(Loy *et al.*, 2002)

a. Position in the 16S rRNA of *Escherichia coli*.
b. Formamide concentrations used in the FISH and CARD FISH hybridization buffer in % (v/v).
c. Position in the 23S rRNA of *E. coli*.
nd, not determined.

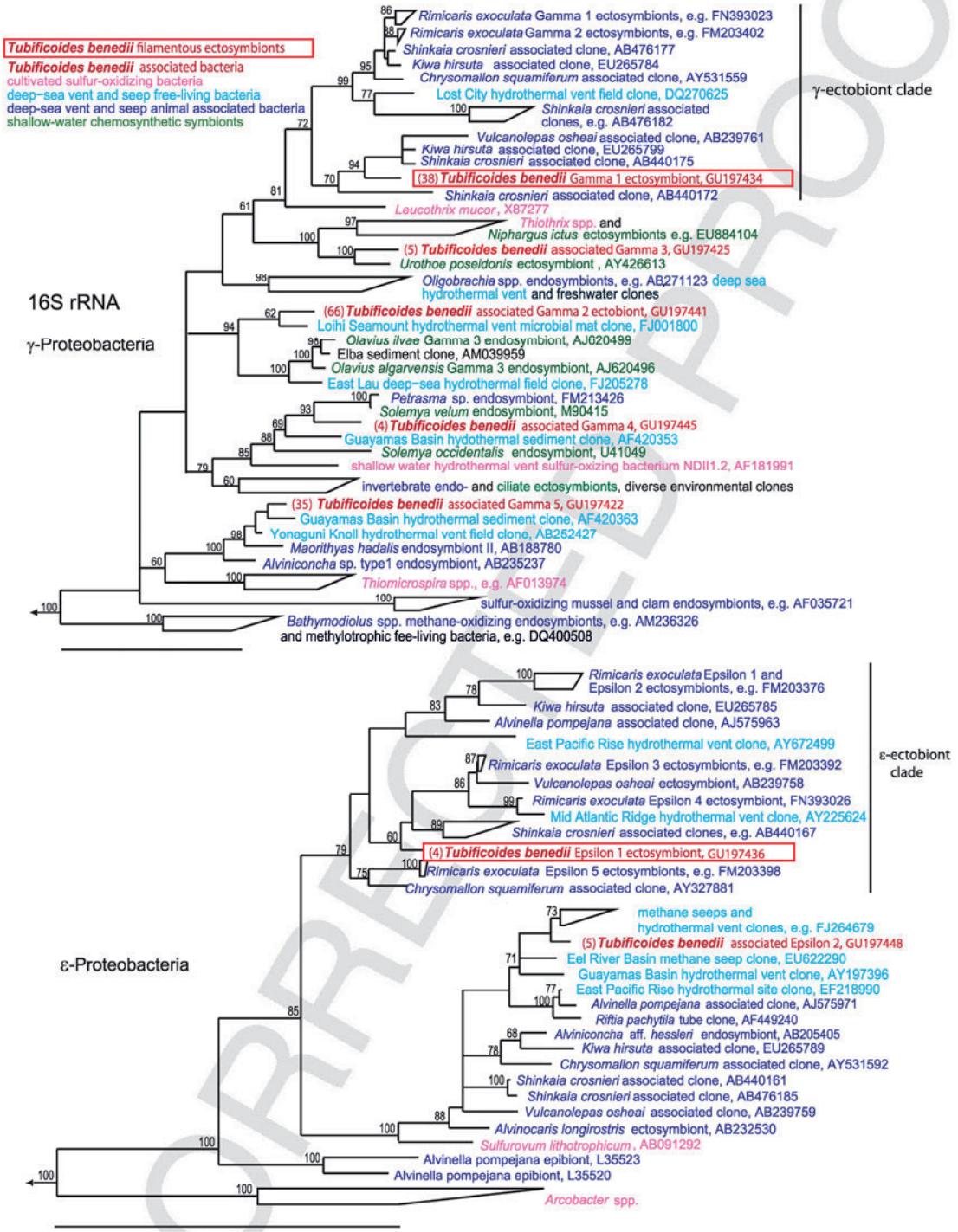


Fig. 1. Phylogenetic placement of Gamma- and Epsilonproteobacteria associated with *T. benedii* tail ends. Consensus tree based on maximum likelihood (ML) analyses of 16S rRNA sequences with nodes that differed in other treeing analyses collapsed to a consensus branch. Sequences from this study in red, in parentheses the number of sequences with > 99.0% to the given sequence. Bacterial sequences from the clone libraries that were not found with FISH on the worms were named '*T. benedii* associated'. Five deltaproteobacterial sequences were used as an outgroup (arrow). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes correspond to bootstrap values based on 1000 ML replicates (only values above 60% are shown).

emi_2256

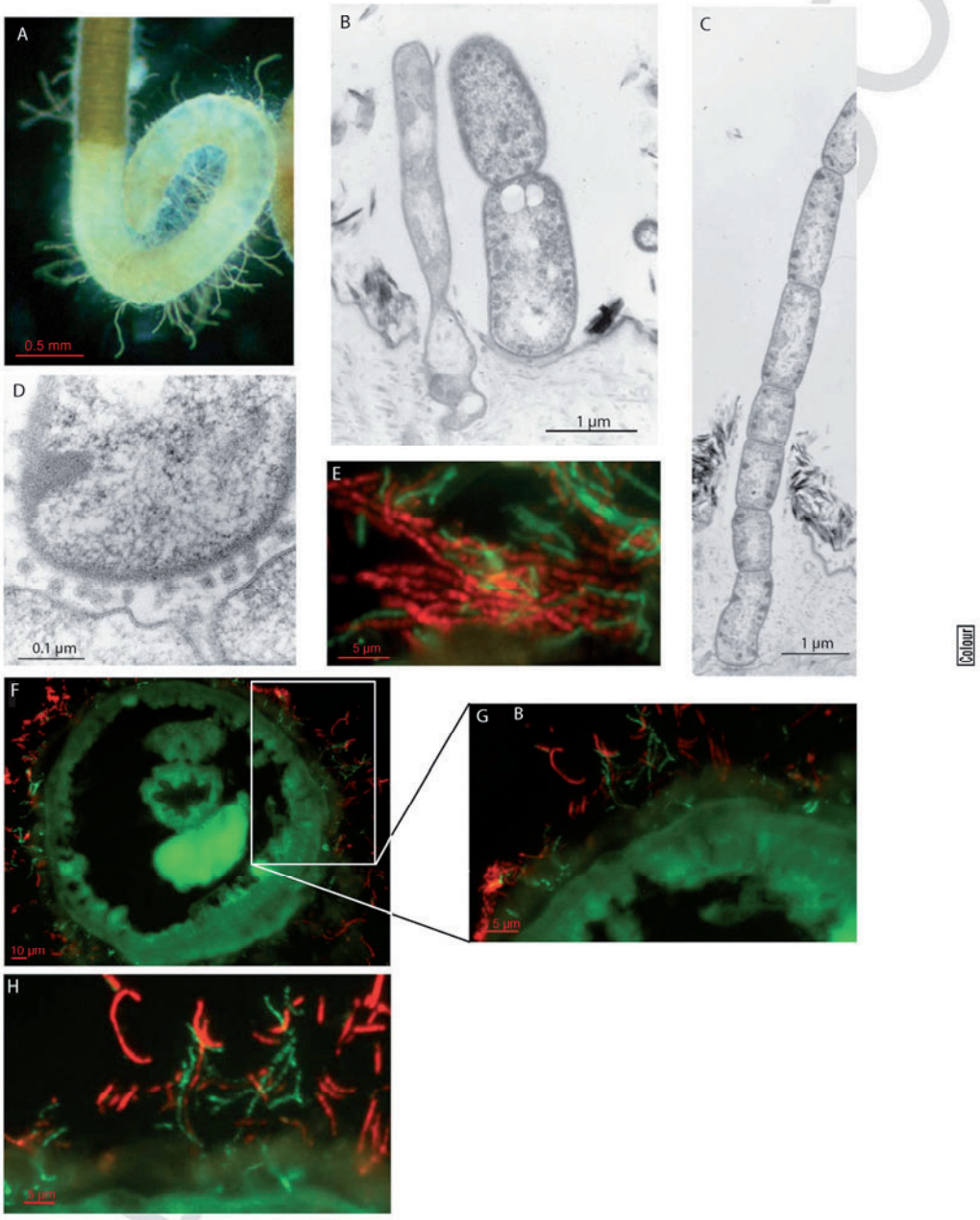


Fig. 2. Microbial community of *T. benedii* tail ends.
A. Light microscopy showing the numerous filaments on the worm's tail.
B–D. Transmission electron microscopy. Only the thinner filaments penetrate the cuticle (B,C), while the thicker filaments (B) are attached to the surface of the worm. D. Vesicle-like structure between the worm's epidermis and the basal cell of a thin filament.
E–H. Epifluorescence microscopy using FISH (E) and CARD-FISH (F–H). The thinner Epsilon 1 ectosymbionts are shown in green with the general epsilonproteobacterial probe EP404 (E) and the specific Epsilon 1 symbiont probe TbEP1-94 (F–H). The thicker Gamma 1 ectosymbionts are shown in red with the specific Gamma 1 symbiont probe TbGAM1-138 (E–H).

FISH analyses. The much lower abundance of Epsilon 1 sequences in the 16S rRNA clone libraries could have been caused by differences in the extraction efficiency of their DNA, primer mismatch and/or polymerase chain reaction (PCR) bias, as described from many other studies of mixed microbial communities (Gonzalez and Moran, 1997; Kanagawa, 2003; Sipos *et al.*, 2007; Hong *et al.*, 2009).

emi_2256

Phylogenetic analyses revealed that the Epsilon 1 ectosymbiont belonged to the newly established family Thiovulgaceae within the Epsilonproteobacteria (Campbell *et al.*, 2006) and the Gamma 1 ectosymbiont to the *Leucotrix/Thiothrix* group within the Gammaproteobacteria (Fig. 1). Both *T. benedii* ectosymbionts belonged to clades consisting nearly exclusively of bacteria associated with invertebrate hosts from deep-sea hydrothermal vents (γ- and ε-ectobiont clades in Fig. 1). These hosts included the galatheid crab *Shinkaia crosnieri* (Watsuji and Takai, 2009), the Yeti crab *Kiwa hirsuta* (Goffredi *et al.*, 2008), the scaly foot snail *Chrysomallon squamiferum* (Goffredi *et al.*, 2004b), the stalked barnacle *Vulcanolepas osheai* (Suzuki *et al.*, 2009), and the shrimp *Rimicaris exoculata* (Petersen *et al.*, 2010). Filamentous ectobionts have been described from all of these hosts but a clear assignment of sequences from the γ- and ε-ectobiont clades to bacteria with a filamentous morphology has only been shown for *R. exoculata* (Petersen *et al.*, 2010) and *V. osheai* (Suzuki *et al.*, 2009). Free-living filamentous bacteria of the genus *Thiothrix* fall in a more distantly related but neighbouring clade to the *T. benedii* Gamma 1 ectosymbionts (Fig. 1), and within this clade filamentous ectosymbionts have been identified from two other animal hosts, the marine amphipod *Urothoe poseidonis* (Gillan and Dubilier, 2004) and the freshwater cave amphipod *Niphargus ictus* (Dattagupta *et al.*, 2009).

The closest cultured relative of the *T. benedii* Gamma 1 ectosymbiont is the filamentous bacterium *Leucothrix mucor*, while the Epsilon 1 ectosymbiont is most closely related to the coccoid- to oval-shaped *Sulfurovum lithotrophicum* (Table 1, Fig. 1). Both *L. mucor* and *S. lithotrophicum* are sulfur oxidizers (Grabovich *et al.*, 1999; Inagaki *et al.*, 2004) and there is evidence for autotrophy and the use of reduced sulfur compounds as energy sources by the filamentous ectobionts of *R. exoculata* (Polz *et al.*, 1998) and *N. ictus* (Dattagupta *et al.*, 2009). The close relationship of the *T. benedii* ectosymbionts to symbiotic and free-living chemosynthetic bacteria and the sulfidic environment of the worms therefore led us to examine the potential of the *T. benedii* association to gain its energy through chemoautotrophic sulfur oxidation.

Metabolic potential for chemoautotrophy and sulfur oxidation

The metabolic potential of the *T. benedii* bacterial community was assessed by analysing protein-coding genes for sulfur metabolism (*aprA* and *soxB*) and chemoautotrophy (*cbbl* and *aclB*) (Table 1). The *aprA* gene, coding for the alpha-subunit of adenosine-5′-phosphosulfate (APS) reductase, is widespread in sulfur-oxidizing microorganisms including many Gammaproteobacteria, but has not yet been found in Epsilonproteobacteria (Meyer and Kuever, 2007). The majority of AprA sequences from the *T. benedii* microbial community (95% or 231 clones) belonged to the AprA lineage II of sulfur-oxidizig bacteria that includes sequences from both symbiotic and free-living Gammaproteobacteria (Meyer and Kuever, 2007). Two sequences were highly abundant within this lineage (*T. benedii* AprA II2 and II4) and both were closely related to AprA sequences from the sulfur-oxidizing gammaproteobacterial endosymbionts of tubeworms from cold seeps (Table 1, Fig. 3). Given the absence of the *aprA* gene from Epsilonproteobacteria, it is most likely that the AprA lineage II sequences from *T. benedii* originated from their gammaproteobacterial ectobionts, thus indicating their potential for the use of reduced sulfur compounds as an energy source. However, because of the incongruence of AprA phylogeny with 16S rRNA phylogeny, it is not clear which AprA sequence(s) might have originated from the *T. benedii* Gamma 1 ectosymbiont.

A small number of *T. benedii* AprA sequences (4%) belonged to the lineage of AprA sequences from sulfate-reducing bacteria (Fig. 3). In sulfate reducers, the enzyme functions in the opposite direction as in sulfur oxidizers (Meyer and Kuever, 2007). The presence of AprA sequences in *T. benedii* from the sulfate reducer lineage corresponds well with our 16S rRNA results showing that deltaproteobacterial sequences related to sulfate-reducing bacteria were abundant in the clone libraries (20%, see Table 1 and Fig. S2). Sulfate-reducing bacteria are well known as endosymbionts in gutless marine oligochaetes where they co-occur with sulfur-oxidizing endosymbionts and engage in syntrophic cycling of reduced and oxidized sulfur compounds (Dubilier *et al.*, 2001; Woyke *et al.*, 2006). Evidence for co-occurring sulfur-oxidizing and sulfate-reducing bacteria has also been described for the ectosymbiotic community of the Yeti crab *K. hirsuta* (Goffredi *et al.*, 2008).

To determine the potential of the Epsilon 1 ectosymbiont for sulfur oxidation, we attempted to amplify the *soxB* gene, coding for the SoxB component of the Sox enzyme complex. This gene is widespread among sulfur-oxidizing bacteria and has been found in all epsilonproteobacterial and some gammaproteobacterial sulfur oxidizers (Meyer *et al.*, 2007; Ghosh *et al.*, 2009). We were not able to amplify this gene from DNA extracted from *T. benedii* tail ends, despite the use of degenerate primers that work well for a phylogenetically wide range of bacteria (Petri *et al.*, 2001; Meyer *et al.*, 2007). It is possible that the concentrations of Epsilon 1 ectosymbiont DNA were too low for successful amplification. This symbiont was underrepresented in the 16S rRNA clone libraries (1.3%, Table 1), despite our FISH studies showing its high abundance (Fig. 2E,H).

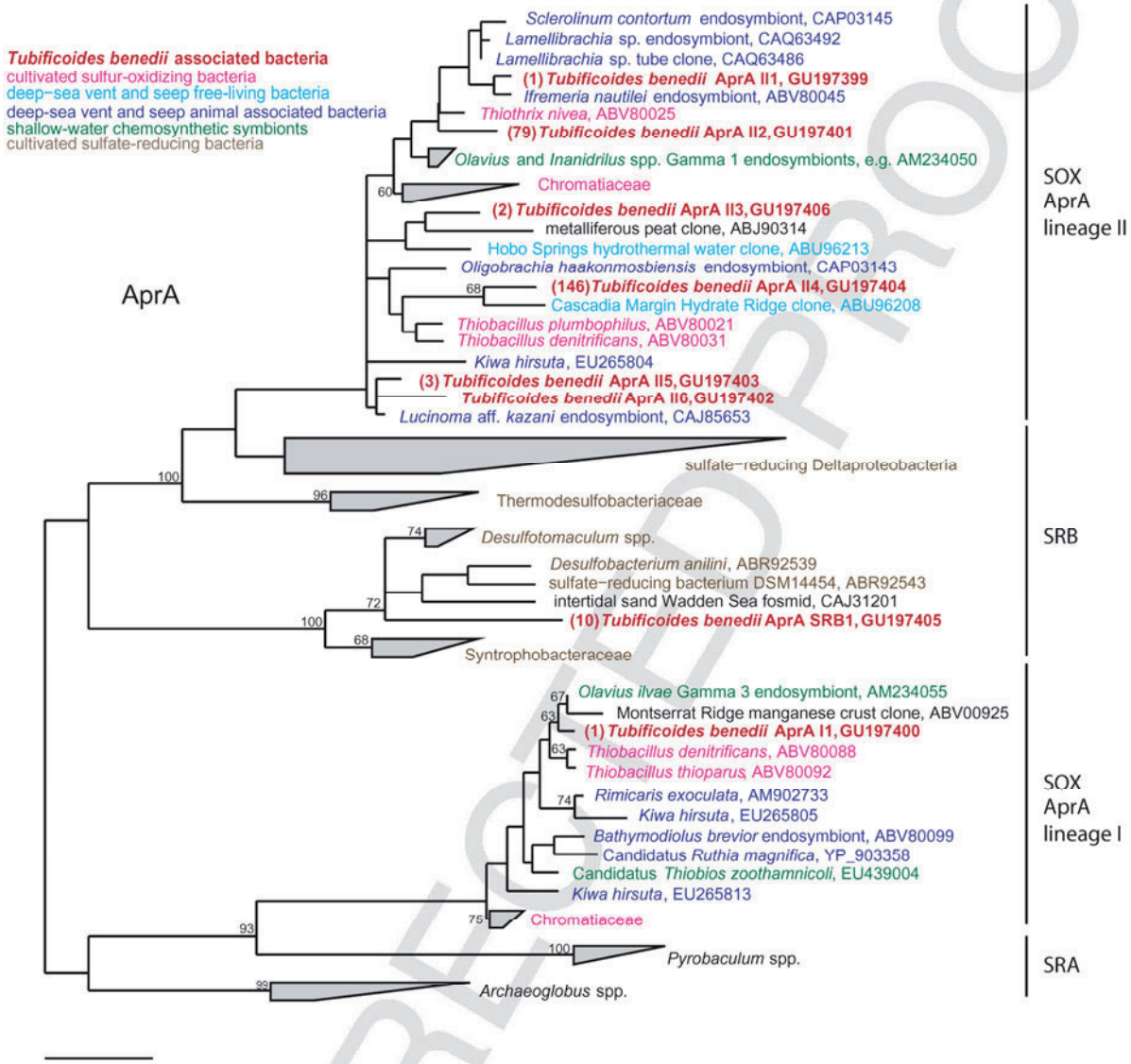


Fig. 3. AprA phylogeny based on ML analyses of deduced amino acid sequences of the *aprA* gene. Sequences from this study in red, in parentheses the number of sequences with > 95.0% to the given sequence. Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes show bootstrap values based on 100 ML replicates (only values above 60% are shown). SOX, sulfur-oxidizing bacteria; SRB, sulfate-reducing bacteria; SRA, sulfate-reducing archaea.

We assessed the potential of the *T. benedii* microbial community for autotrophic carbon fixation by analysing key genes of the Calvin Benson Bassham (CBB) cycle (*cbbL* and *cbbM*) and the reductive tricarboxylic acid (rTCA) cycle (*aclB*). The *cbbL* and *cbbM* genes, coding for the large subunits of the form I and II ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), respectively, are widespread among autotrophic organisms including many Gammaproteobacteria, but have not been found in Epsilonproteobacteria. Both genes could be amplified in DNA extracted from *T. benedii* tail ends, indicating the potential of its microbial community for autotrophy. The dominant sequences in the *cbbL* clone libraries were most closely related to sequences from the chemoautotrophic sulfur-oxidizing endosymbiont of the clam *Solemya velum*, while for *cbbM*, the dominant sequences grouped with sequences from bacteria associated with the seep tubeworm *Lamellibrachia* sp. (Fig. 4, Table 1). As with the *aprA* gene, given the many inconsistencies between phylogenetic trees based on *cbbL* and

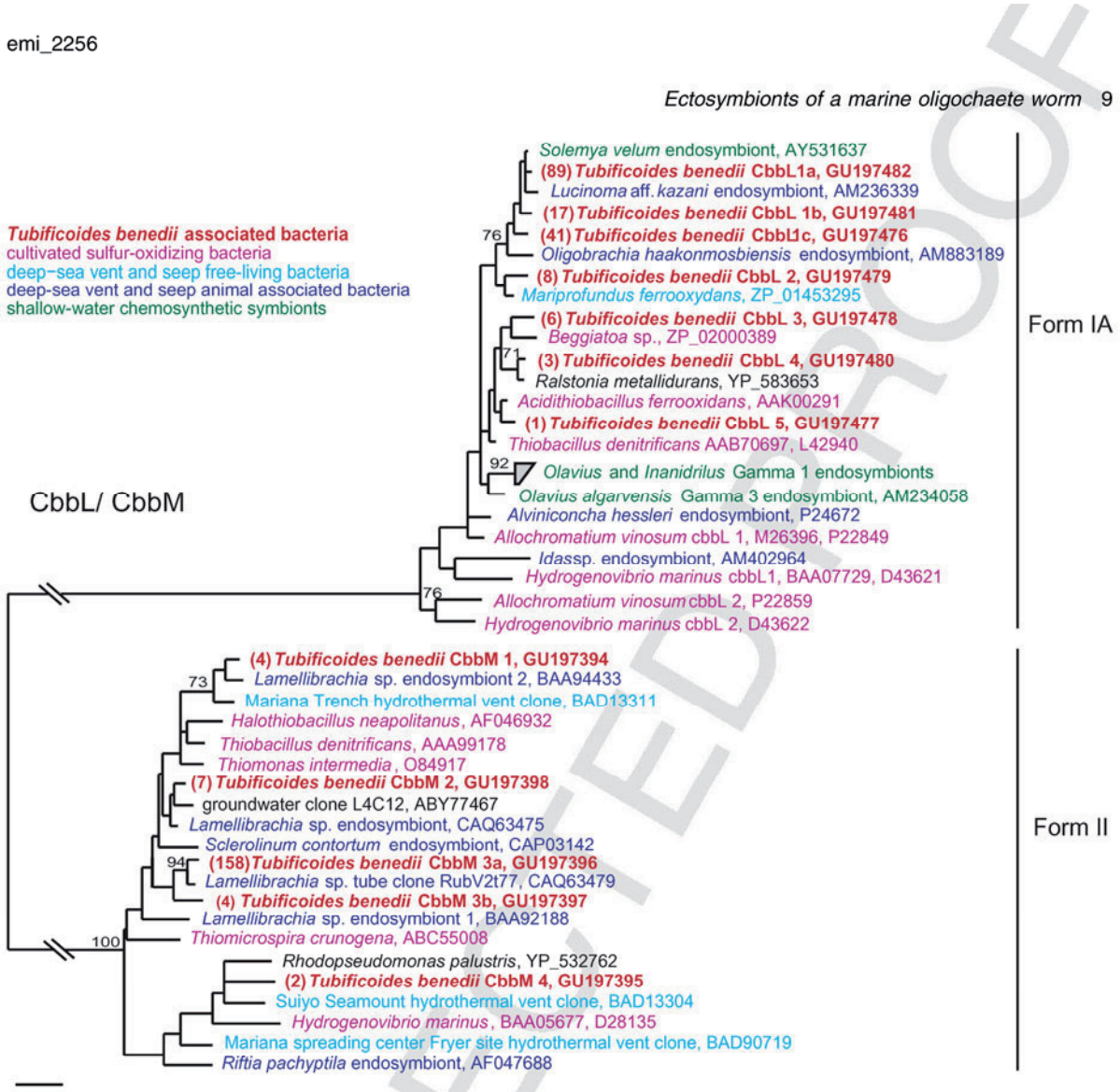


Fig. 4. RubisCO consensus tree based on ML analyses of deduced amino acid sequences of *cbbL* and *cbbM* genes (sequences from this study in red, in parentheses the number of sequences with > 95.0% to the given sequence). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes show to bootstrap values based on 100 ML replicates (only values above 60% are shown).

cbbM genes versus the 16S rRNA gene, it is not possible to identify which of these genes might have originated from the Gamma 1 ectosymbiont.

To examine the potential of the Epsilon 1 ectosymbiont for autotrophy, we examined the *aclB* gene, coding for the beta subunit of ATP citrate lyase. This gene is widespread in Epsilonproteobacteria including those commonly found at deep-sea hydrothermal vents but is not known from Gammaproteobacteria (Campbell *et al.*, 2006; Nakagawa and Takai, 2008). We were not able to amplify this gene from DNA extracts of *T. benedii* tail ends, paralleling the lack of amplification products for the indicator gene *soxB* for sulfur oxidation in Epsilonproteobacteria. The metabolism of *T. benedii's* epsilonproteobacterial ectobionts therefore remains unclear. Epsilonproteobacteria are highly versatile and can live autotrophically, as well as mixo- or heterotrophically (Campbell *et al.*, 2006). In the organic and sulfide-rich sediments in which *T. benedii* lives, any of these metabolisms are conceivable.

Nature of the T. benedii *ectosymbiont association*

The ectosymbiotic community associated with *T. benedii* has the potential for chemoautotrophic sulfur oxidation and it is possible that the symbionts fix inorganic carbon to organic carbon compounds that they pass on to their host. This has been suggested (but rarely proven) for other hosts with chemoautotrophic ectosymbionts such as stilbonematinid nematodes (Polz *et al.*, 1992; Ott *et al.*, 2004), the vent shrimp *R. exoculata* (Rieley *et al.*, 1999;

emi_2256

Zbinden and Cambon-Bonavita, 2003), the vent barnacle *V. osheai* (Suzuki *et al.*, 2009), the vent crab *S. crosnieri* (Watsuji and Takai, 2009) and the cave amphipod *N. ictus* (Dattagupta *et al.*, 2009). As mixo- or heterotrophs, the *T. benedii* ectosymbionts could use the waste products the host excretes during anaerobic metabolism such as succinate, acetate and propionate (Dubilier *et al.*, 1994) and recycle these back to the host as suggested for gutless oligochaetes (Dubilier *et al.*, 2001; Woyke *et al.*, 2006). They could also take up organic compounds directly from the environment and pass these or essential amino acids and vitamins to the host. However, *T. benedi* has a fully functional digestive system and like other aquatic oligochaetes ingests sediment and the organic material and microorganisms therein to gain nutrition. It is therefore hard to imagine that its ectobiotic community can play an important role in its nutrition in comparison to the organic matter available in its surroundings. Furthermore, only the basal cells of the filamentous ectosymbionts are in direct contact with the worm, and thus provide very little surface area for the exchange of organic compounds.

Another function hypothesized to play a role in symbioses with sulfur-oxidizing bacteria is that these detoxify hydrogen sulfide, a potent inhibitor of aerobic respiration, for their hosts (Somero *et al.*, 1989; Dattagupta *et al.*, 2009). However, our calculations show that diffusion rates of sulfide through the body wall of the worms are so fast that bacterial sulfide oxidation would not be able to outcompete it (Dubilier *et al.*, 1995a). Finally, it is also unlikely that the ectosymbionts are involved in pathogenic interactions, as we saw no differences in the vitality of worms lightly and heavily covered with the filaments in this study and an earlier one (Dubilier, 1986).

While the benefit of the ectosymbionts for *T. benedii* remains unclear, the advantages for these in associating with the worm are obvious. The sulfur-oxidizing ectosymbionts have access to an ideal environment on the worm's tail with access to sulfide rising up from the sediments and oxygen available from the water above the sediment surface. There is a strong selective advantage for sulfur-oxidizing bacteria in associating with animals that can provide them with both sulfide and oxygen. For example, Røy and colleagues (2009) showed that the sulfur-oxidizing symbionts of the ciliate *Zoothamnium niveum* take up 100 times more sulfide than bacteria on flat inert surfaces. This selective advantage is so strong that these types of associations have evolved multiple times in numerous lineages of sulfur-oxidizing bacteria and in a wide array of host groups (Dubilier *et al.*, 2008). For the mixo- and heterotrophic members of the *T. benedii* microbial community, the association with an animal that excretes large amounts of carbon and nitrogen waste compounds provides a rich source of nutrition, and this advantage is hypothesized to play a role in many associations between bacteria and marine invertebrates (Carman and Dobbs, 1997; Robidart *et al.*, 2008).

Specificity of the association

The *T. benedii* Gamma 1 and Epsilon 1 ectosymbionts belong to clades that consist almost exclusively of bacteria associated with hydrothermal vent invertebrates. This suggests that bacteria from these clades have developed an adaptive trait that enables them to easily colonize marine invertebrates. In the symbiotic associations between bioluminescent *Vibrio fisheri* and their marine hosts, Mandel and colleagues (2009) hypothesize that only a single regulatory gene was needed to confer free-living *V. fisheri* with the ability to colonize their hosts, possibly by 'switching "on" pre-existing capabilities for interacting with an animal'. Intriguingly, the closest cultured relative to the *T. benedii* Gamma 1 ectosymbiont, the filamentous sulfur oxidizer *L. mucor*, forms a basal node to the γ-ectobiont clade (Fig. 1). *Leucothrix mucor* has been described from surfaces as diverse as marine algae, fish eggs, and dead and live aquatic invertebrates (Johnson *et al.*, 1971; Sieburth, 1975; Carman and Dobbs, 1997; Payne *et al.*, 2007), suggesting a less specific interaction between these bacteria and the surfaces they colonize. This could indicate that there is a progression from these possibly ancestral *L. mucor* associations to the highly stable and specific associations within the γ-ectobiont clade. However, given that most studies used only morphological characteristics to identify the filamentous epibionts as *L. mucor*, unambiguous identification through 16S rRNA sequencing and FISH is needed to test this hypothesis.

Conclusions

Symbiotic associations with Epsilonproteobacteria have only been described from deep-sea invertebrates, making *T. benedii*, to our knowledge, the first invertebrate from shallow environments with symbiotic Epsilonproteobacteria. *Tubificoides benedii* is an opportunistic species that is well adapted to estuaries and mudflats with organic-rich sediments and rapid environmental fluctuations, including low oxygen and high sulfide concentrations (Giere, 2006). Similarly, Epsilonproteobacteria have been described as uniquely suited to thrive in extreme environments such as deep-sea hydrothermal vents, where they can rapidly colonize dynamic environments with suboxic to anoxic conditions (Campbell *et al.*, 2006). Thus, *T. benedii* and its epsilonproteobacterial ectosymbionts make good partners in sharing similar ecological niches.

Symbiotic associations with Gammaproteobacteria are widespread in both shallow-water and deep-sea chemosynthetic environments (Dubilier *et al.*, 2008). However,

emi_2256

the *T. benedii* Gamma 1 ectosymbiont belongs to a clade that consists exclusively of symbiotic and free-living bacteria from deep-sea hydrothermal vents, while the chemosynthetic symbionts of other hosts from shallow waters belong to clades distant from the γ-ectobiont clade (Fig. 1). Thus, *T. benedii* is, to date, unique in its symbiotic association with gamma- and epsilonproteboacterial symbionts related to those from deep-sea hydrothermal vents.

The discovery of the *T. benedii* ectosymbiosis shows that factors other than biogeography and host affiliation must have been the driving force behind the associations within the γ- and ε-ectobiont clades. Instead, the environment appears to have been crucial for the establishment and evolution of these ectosymbiotic associations, namely highly dynamic environments with strong fluctuations of oxidants and reductants. We hypothesize that symbioses with bacteria from these clades may be more widespread in shallow-water environments such as sulfide-rich intertidal mud flats than currently recognized. Morphological and molecular analyses of ectobionts from coastal marine sediments combined with analyses of their metabolic potential will be useful in providing a better understanding of the factors defining distribution patterns and function of associations between marine hosts and their symbiotic bacteria.

Experimental procedures

Specimen collection

Tubificoides benedii were collected in 1998 from Wadden Sea sediments at the Lister Haken in the Königshafen Bay on the Island of Sylt (55.03 N 8.10 E). The collection site is characterized by eutrophication and in the warmer months, massive green algal mats cover the sediment and lead to high sulfate reduction rates and sulfide concentrations (Kristensen *et al.*, 2000; Reise and Kohlus, 2008).

To ensure as little contamination as possible, worms were allowed to defecate their gut contents before fixation, and only worms with visibly clear guts were used. Individual specimens were rinsed three times in 0.2 µm filtered seawater and fixed as described below.

Electron microscopy

For TEM specimens were fixed and prepared as described previously (Dubilier, 1986).

Cloning and sequencing of 16S rRNA, aprA, soxB, cbbL, cbbM *and* aclB *genes*

The tail ends of four *T. benedii* individuals were prepared individually for PCR as described previously (Dubilier *et al.*, 1999) using the isolation protocol of Schizas and colleagues (1997). Briefly worms were digested with Proteinase K and DNA was extracted with Gene Releaser (BioVentures, Murfreesboro, TN, USA). Amplification, cloning and sequencing of the 16S rRNA, *cbbL*, *cbbM* and *aprA* genes was carried out as described previously (Blazejak *et al.*, 2006) with the following modifications: only 28–30 cycles and an extra reamplification procedure of five cycles. The annealing temperature for *cbbL* was set to 56°C instead of 48°C, for *aprA* to 58°C instead of 54°C. For the amplification of *cbbM*, the primers cbbMF_Els (Elsaied and Naganuma, 2001) and a modified cbbM1R (Blazejak *et al.*, 2006) with the sequence 5′ MGA GGT SAC SGC RCC RTG RCC RGC MCG RTG 3′ were used with an annealing temperature of 62°C. PCR of the *soxB* gene was carried out with soxB1446b and soxB432f with an annealing temperature of 47°C as described (Petri *et al.*, 2001). For *aclB* the primer combinations used were aclB892F or aclB275F with aclB1204R with an annealing temperature of 42°C (Campbell *et al.*, 2003; Takai *et al.*, 2005).

For all genes, PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and cloned using the pGEM-T/pGEM-T Easy Kit (Promega, Madison, WI, USA) or the TOPO Kit (Invitrogen, Paisley, UK) according to the manufacturers' protocols. Plasmid DNA was purified from overnight cultures using the QIAprep plasmid kit (QIAGEN, Hilden, Germany). Clones with correct insert size were partially sequenced (approximately 450–900 bp) and grouped according to phylogentic positioning and similarity values from a distance matrix in ARB (Ludwig *et al.*, 2004).

Sequencing reactions were run on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For each host individual, at least one representative clone from each dominant clone group was fully sequenced in both directions. Full sequences within each clone group shared at least 99.0% sequence similarity (% identical nucleotides) for the 16S rRNA gene and at least 95% sequence similarity (% identical amino acids) for the protein coding genes. Closely related sequences of representative sequences were identified with BLAST (Altschul *et al.*, 1990) queries and through phylogenetic analyses.

Phylogenetic analyses

16S rRNA chimeras were identified using CHIMERA_CHECK from the Ribosomal Database Project (Cole *et al.*, 2007) and by eye in sequence alignments, and trimmed or excluded from further analyses. Alignments were based on the 16S rRNA secondary structure without partitioning into stem and loops (Pruesse *et al.*, 2007). Phylogenetic analyses were performed with the ARB program (Ludwig *et al.*, 2004) and the online version of RaxML (Stamatakis *et al.*, 2008). For the 16S rRNA gene, phylogenetic trees were calculated with sequences no shorter than 1300 bp using neighbour-joining, parsimony and maximum likelihood (ML, HKY substitution model) as well as RaxML. Group filters for ML calculations (25%-, 30%-, 40%- and 50%-filters) were constructed from published gamma- and epsilonproteobacterial sequences in ARB. Bootstrap values for the gamma- and epsilonproteobacterial 16S rRNA trees were based on 1000 ML bootstraps calculated with ARB. Short sequences in the CFB and deltaproteobacterial 16S rRNA trees (Figs S1 and S2) were added to the ML-tree using parsimony with a positional variability filter, bootstraps resulted from whole tree ML calculations.

Phylogenetic trees of protein-coding genes were generated from translated gene sequences of 131 (*aprA*), 133 (*cbbM*) and 230 (*cbbL*) amino acids using the ML algorithm and a JTT model with a 25% positional conservation filter. Trees were reconstructed using the standard operating procedure for phylogenetic inference SOPPI (Peplies *et al.*, 2008), by visually comparing different methods, parameters and filters to identify the most stable tree topologies. All trees shown in this study were constructed based on ML analyses with nodes that were not stable (i.e. differed in more than two methods) collapsed to a consensus branch.

Accession numbers

The sequences from this study are available through GenBank under the accession numbers GU197394-GU197482.

Fluorescence in situ *hybridization (FISH)*

Worm tail ends were fixed, embedded and sectioned as described previously for gutless oligochaetes (Dubilier *et al.*, 1995b) with the omission of the redundant postfixation step with 4% paraformaldehyde during the rehydration process. Sections of *T. benedii* posterior ends were prepared for FISH with monolabeled cy3 and cy5 probes and catalyzed reporter deposition (CARD) FISH with horseradish peroxidase labeled probes and tyramide signal amplification (with the fluorescent dyes Alexa 488 and 633) as described previously (Blazejak *et al.*, 2006) with the following modifications: To decrease the loss of FISH signal in the mucous layer of the worm, additional digestion procedures were added. After 12 min of 0.3 M HCl, instead of a 5 min digestion with Proteinase K, the slides were immersed for 30 min to 1 h in 0.1% lysozyme in 0.1 M Tris/HCl / 0.05 M EDTA, 30 min to 1 h in 0.005% amylase in 1 × PBS (60 U ml^{-1}) and 5 min in 0.0005% Proteinase K in 20 mM Tris/HCl (all enzyme incubations at 37°C). Washing for CARD-FISH in 1 × SSC buffer was increased to 1 h. Probe concentrations in the hybridization buffer were 3.3 ng μl^{-1} in FISH and 0.05 ng μl^{-1} in CARD-FISH)

Specific and group oligonucleotide probes targeting the dominant gamma- and epsilonproteobacterial 16S rRNA sequences found in *T. benedii* clone libraries (Table 1) were created with the ARB program and checked against sequences in GenBank with BLAST and in the RDP database with Probe Match (Cole *et al.*, 2007) (Table 2). The specificity of the ectosymbiont probes was tested against reference bacteria with 16S rRNA sequences containing one or more mismatches unless otherwise noted. General probes for the Bacteria, Gammaproteobacteria, Deltaproteobacteria and the CFB served as positive controls and the nonsense probe NON338 as a negative control. Hybridizations were performed at formamide concentrations ensuring specificity for the targeted groups (Table 2). Images were recorded with the Axiovision camera (Zeiss) and optimized with the accompanying program AxioVision LE 4.5.

Acknowledgements

We thank Sabine Lenk for her help with *soxB* amplifications, and Silke Wetzel and Lisa Drews for excellent technical assistance. We are grateful to Claudia Bergin, Jan Küver and Sabine Lenk for fruitful discussions. This work was supported by the Max Planck Society, Munich, Germany.

References

Alayse-Danet, A.M., Desbruyères, D., and Gaill, F. (1987) The possible nutritional or detoxification role of the epibiotic bacteria of alvinellid polychaetes: review of current data. *Symbiosis* **4:** 51–62.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Bayer, C., Heindl, N.R., Rinke, C., Lücker, S., Ott, J.A., and Bulgheresi, S. (2009) Molecular characterization of the symbionts associated with marine nematodes of the genus *Robbea*. *Environ Microbiol Rep* **1:** 136–144.

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

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

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

Carman, K.R., and Dobbs, F.C. (1997) Epibiotic microorganisms on copepods and other marine crustaceans. *Microsc Res Tech* **37:** 116–135.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., *et al.* (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. *Nucleic Acids Res* **35:** D169–D172.

d'Udekem, J. (1855) Nouvelle classification des Annélides sétigères abranches. *Bull Acad R Belg Cl Sci* **22:** 533–555.

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

Dattagupta, S., Schaperdoth, I., Montanari, A., Mariani, S., Kita, N., Valley, J.W., and Macalady, J.L. (2009) A novel symbiosis between chemoautotrophic bacteria and a freshwater cave amphipod. *ISME J* **3:** 935–943.

Dubilier, N. (1986) Association of filamentous epibacteria with *Tubificoides benedii* (Oligochaeta, Annelida). *Mar Biol* **92:** 285–288.

Dubilier, N., Giere, O., and Grieshaber, M.K. (1994) Concomitant effects of sulfide and hypoxia on the aerobic metabolism of the marine oligochaete *Tubificoides benedii*. *J Exp Zool* **269:** 287–297.

Dubilier, N., Giere, O., and Grieshaber, M.K. (1995a) Morphological and ecophysiological adaptations of the marine oligochaete *Tubificoides benedii* to sulfidic sediments. *Am Zool* **35:** 163–173.

Dubilier, N., Giere, O., Distel, D.L., and Cavanaugh, C.M. (1995b) Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (Oligochaeta, Annelida) by phylogenetic 16S rRNA sequence analysis and *in situ* hybridization. *Appl Environ Microbiol* **61:** 2346–2350.

Dubilier, N., Amann, R., Erséus, C., Muyzer, G., Park, S.Y., Giere, O., and Cavanaugh, C.M. (1999) Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochete *Olavius loisae* (Annelida). *Mar Ecol Prog Ser* **178:** 271–280.

Dubilier, N., Mulders, C., Ferdelman, T., de Beer, D., Pernthaler, A., Klein, M., *et al.* (2001) Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. *Nature* **411:** 298–302.

Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6:** 725–740.

Elsaied, H., and Naganuma, T. (2001) Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* **67:** 1751–1765.

Ghosh, W., Mallick, S., and DasGupta, S.K. (2009) Origin of the Sox multienzyme complex system in ancient thermophilic bacteria and coevolution of its constituent proteins. *Res Microbiol* **160:** 409–420.

Giere, O. (2006) Ecology and biology of marine oligochaeta – an inventory rather than another review. *Hydrobiologia* **564:** 103–116.

Giere, O., and Rhode, B. (1987) Anatomy and ultrastructure of the marine oligochaete *Tubificoides benedii* (Tubificidae), with emphasis on its epidermis-cuticle-complex. *Hydrobiologia* **155:** 159–159.

Gillan, D.C., and Dubilier, N. (2004) Novel epibiotic *Thiothrix* bacterium on a marine amphipod. *Appl Environ Microbiol* **70:** 3772–3775.

Gil-Turnes, M.S., and Fenical, W. (1992) Embryos of *Homarus americanus* are protected by epibiotic bacteria. *Biol Bull* **182:** 105–108.

Goffredi, S. (2010) Indigenous ectosymbiotic bacteria associated with diverse hydrothermal vent invertebrates. *Environ Microbiol* (in press). doi:10.1111/j.1758-2229.2010.00136.x.

Goffredi, S.K., Barry, J.P., and Buck, K.R. (2004a) Vesicomyid symbioses from Monterey Bay (Central California) cold seeps. *Symbiosis* **36:** 1–27.

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

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

Gonzalez, J.M., and Moran, M.A. (1997) Numerical dominance of a group of marine bacteria in the alpha-subclass of the class Proteobacteria in coastal seawater. *Appl Environ Microbiol* **63:** 4237–4242.

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

Guérin, C., and Giani, N. (1996) Analytical study of the locomotor and respiratory movements of tubificid worms by means of video recording. *Hydrobiologia* **333:** 63–69.

Hong, S.H., Bunge, J., Leslin, C., Jeon, S., and Epstein, S.S. (2009) Polymerase chain reaction primers miss half of rRNA microbial diversity. *ISME J* **3:** 1365–1373.

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

Johnson, P.W., Sieburth, J.M., Sastry, A., Arnold, C.R., and Doty, M.S. (1971) *Leucothrix mucor* infestation of benthic Crustacea, fish eggs, and tropical algae. *Limnol Oceanogr* **16:** 962–969.

Kanagawa, T. (2003) Bias and artifacts in multitemplate polymerase chain reactions (PCR). *J Biosci Bioeng* **96:** 317–323.

Kristensen, E., Bodenbenderb, J., Jensenc, M.H., Rennenbergb, H., and Jensena, K.M. (2000) Sulfur cycling of intertidal Wadden Sea sediments (Konigshafen, Island of Sylt, Germany): sulfate reduction and sulfur gas emission. *J Sea Res* **43:** 93–104.

Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., *et al.* (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl Environ Microbiol* **68:** 5064–5081.

Loy, A., Maixner, F., Wagner, M., and Horn, M. (2007) probeBase – an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Res* **35:** D800–D804.

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

Macalady, J.L., Lyon, E.H., Koffman, B., Albertson, L.K., Meyer, K., Galdenzi, S., and Mariani, S. (2006) Dominant microbial populations in limestone-corroding stream biofilms, Frasassi Cave System, Italy. *Appl Environ Microbiol* **72:** 5596–5609.

McFall-Ngai, M. (2008) Are biologists in 'future shock'? Symbiosis integrates biology across domains. *Nat Rev Microbiol* **6:** 789–792.

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

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. *Syst Appl Microbiol* **15:** 593–600.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., and Schleifer, K.-H. (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacter-Bacteroides* in the natural environment. *Microbiology* **142:** 1097–1106.

Meyer, B., and Kuever, J. (2007) Molecular analysis of the distribution and phylogeny of dissimilatory adenosine-5′-phosphosulfate reductase-encoding genes (*aprBA*) among sulfur-oxidizing prokaryotes. *Microbiology* **153:** 3478–3498.

Meyer, B., Imhoff, J.F., and Kuever, J. (2007) Molecular analysis of the distribution and phylogeny of the *soxB* gene among sulfur-oxidizing bacteria – evolution of the Sox sulfur oxidation enzyme system. *Environ Microbiol* **9:** 2957–2977.

Nakagawa, S., and Takai, K. (2008) Deep-sea vent chemoautotrophs: diversity, biochemistry and ecological significance. *FEMS Microbiol Ecol* **65:** 1–14.

Ott, J., Bright, M., and Bulgheresi, S. (2004) Symbioses between marine nematodes and sulfur-oxidizing chemoautotrophic bacteria. *Symbiosis* **36:** 103–126.

Peplies, J., Kottmann, R., Ludwig, W., and Glöckner, F.O. (2008) A standard operating procedure for phylogenetic inference (SOPPI) using (rRNA) marker genes. *Syst Appl Microbiol* **31:** 251–257.

Petersen, J., Ramette, A., Lott, C., Cambon, Bonavita, M.-A., Zbinden, M., and Dubilier, N. (2010) Dual symbiosis of the vent shrimp *Rimicaris exoculata* with filamentous Gamma- and Epsilonproteobacteria at four Mid-Atlantic Ridge hydrotherma vent fields. *Environ Microbiol* (in press).

Petri, R., Podgorsek, L., and Imhoff, J.F. (2001) Phylogeny and distribution of the *sox*B gene among thiosulfate-oxidizing bacteria. *FEMS Microbiol Lett* **197:** 171–178.

Polz, M.F., Felbeck, H., Novak, R., Nebelsick, M., and Ott, J.A. (1992) Chemoautotrophic, sulfur-oxidizing symbiotic bacteria on marine nematodes: morphological and biochemical characterization. *Microb Ecol* **24:** 313–329.

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

Prieur, D., Chamroux, S., Durand, P., Erauso, G., Fera, P., Jeanthon, C., *et al.* (1990) Metabolic diversity in epibiotic microflora associated with the Pompeii worms *Alvinella pompejana* and *A. caudata* (Polychaetae: Annelida) from deep-sea hydrothermal vents. *Mar Biol* **106:** 361–367.

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

Reise, K., and Kohlus, J. (2008) Seagrass recovery in the Northern Wadden Sea? *Helgol Mar Res* **62:** 77–84.

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

Rinke, C., Schmitz-Esser, S., Stoecker, K., Nussbaumer, A.D., Molnar, D.A., Vanura, K., *et al.* (2006) *Candidatus* Thiobios zoothamnicoli, an ectosymbiotic bacterium covering the giant marine ciliate *Zoothamnium niveum*. *Appl Environ Microbiol* **72:** 2014–2021.

Rinke, C., Schmitz-Esser, S., Loy, A., Horn, M., Wagner, M., and Bright, M. (2009) High genetic similarity between two geographically distinct strains of the sulfur-oxidizing symbiont *Candidatus* Thiobios zoothamnicoli. *FEMS Microbiol Ecol* **67:** 229–241.

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

Røy, H., Vopel, K., Huettel, M., and Jørgensen, B. (2009) Sulfide assimilation by ectosymbionts of the sessile ciliate *Zoothamnium niveum*. *Mar Biol* **156:** 669–677.

Ruehland, C., Blazejak, A., Lott, C., Loy, A., Erséus, C., and Dubilier, N. (2008) Multiple bacterial symbionts in two species of co-occurring gutless oligochaete worms from Mediterranean sea grass sediments. *Environ Microbiol* **10:** 3404–3416.

Schizas, N.V., Street, G.T., Coull, B.C., Chandler, G.T., and Quattro, J.M. (1997) An efficient DNA extraction method for small metazoans. *Mol Mar Biol Biotechnol* **6:** 381–383.

Sieburth, J.M. (1975) *Microbial Seascapes*. Baltimore, MD, USA: University Park Press.

Sipos, R., Szekely, A.J., Palatinszky, M., Revesz, S., Marialigeti, K., and Nikolausz, M. (2007) Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis. *FEMS Microbiol Ecol* **60:** 341–350.

Somero, G.N., Childress, J.J., and Anderson, A.E. (1989) Transport, metabolism and detoxification of hydrogen sulfide in animals from sulfide-rich marine environments. *Rev Aquat Sci* **1:** 591–614.

Stamatakis, A., Hoover, P., and Rougemont, J. (2008) A rapid bootstrap algorithm for the RAxML web servers. *Syst Biol* **57:** 758–771.

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

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

Timm, T., and Erséus, C. (2009) *Tubificoides benedii* (Udekem, 1855). *Accessed through the World Register of Marine Species* [WWW document]. URL http://www.marinespecies.org/aphia.php?p=taxdetails&id=137571 (accessed on 13 August 2009).

Wallner, G., Amann, R., and Beisker, W. (1993) Optimizing fluorescent *in situ*-hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14:** 136–143.

Watsuji, T., and Takai, K. (2009) Compositional and functional transition in epibiotic symbionts of *Shinkaia crosnieri*: morphology, structure, energy and carbon metabolisms during rearing. *Goldschmidt Conference 2009 – Challenges to Our Volatile Planet. Poster presentation*. Davos, Switzerland.

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

emi_2256

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

Supporting information

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

Fig. S1. Phylogenetic placement of Cytophaga/Flavobacterium/Bacteroides16S rRNA bacterial sequences from *T. benedii* tail ends based on ML analyses (sequences from this study in red, in parentheses the number of sequences with > 99.0% to the given sequence). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes correspond to bootstrap values based on 100 ML replicates (only values above 60% are shown).

Fig. S2. Phylogenetic placement of deltaproteobacterial 16S rRNA sequences from *T. benedii* tail ends based on ML analyses (sequences from this study in red, in parentheses the number of sequences with > 99.0% to the given sequence). Five sphingo-bacterial sequences were used as an outgroup (arrow). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes correspond to bootstrap values based on 100 ML replicates (only values above 60% are shown).

Fig. S3. CARD-FISH images of epibacteria on *T. benedii* tail ends.

A. Cross section through the tail end showing the thick layer of bacteria covering the surface of the worm (eubacterial probe EUBI-III).

B. The general gammaproteobacterial probe (GAM42a, red) hybridized with rods and cocci in the mucus membrane, while the Gamma 1 ectosymbionts hybridized with specific probes (shown in green: TbGAM1-138) but not with the GAM42a probe.

C. The *T. benedii* Gamma 2 ectobionts (hybridized with the specific probe TbGAM2-447, green) were cocci-shaped and found occasionally in the mucous membrane. D.-E. Deltaproteobacteria (probe DELTA495a, red) and Bacteroidetes (probe CF319a, green) populated the worm's mucous layer. Deltaproteobacteria occurred singly as rods in groups of coccoid or oval shaped cells. Bacteroidetes mostly occurred in patches within the mucus and were often rod-shaped, and sometimes elongated or filamentous.

Table S1. Additional oligonucleotide probes used in this study that did not result in reproducible clear signals.

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

Supplementary Information

Table S1 Additional oligonucleotide probes used in this study that did not result in reproducible clear signals.

Probe	Targets	Probe sequence (5´-3´)	Position[a]
TbGAM3-826	*T. benedii Thiothrix* spp. Gamma 3	GTT GGA ACC AAC GGC TAG	826-843
TbGAM4-645	*T. benedii* Gamma 4	ATC AGA CTC GAG TCA AGC	645-662
TbGAM4-733	*T. benedii* Gamma 4, microbial mat clones, e.g. AM421142	GTC TTG ATC CAG GTA GCC	733-750
TbGAM5-645	*T. benedii* Gamma 5, environmental clones, e.g. AY592349	ACC ATA CTC TAG TCC GAC	645-662
TbGAM89IIF9-137	*T. benedii* single clone 89IIF9 environmental clones, e.g. AM292413	AGT TGT CCC CCT CTA CCA	137-154
TbGAM89IIF9-645	*T. benedii* single clone 89IIF9	GGC ATG CTA GAG TTT GGT	645-662
TbGAM86_06_1_3-841	*T. benedii* 2 clones, e.g. 86_06_1#3 invertebrate associated clones, e.g. *Shinkaia crosnieri*, AB440176 and *Capitella* sp., EU418468	CGT CAC TAA TCC CTC AAG	841-858
TbEP1+-67	*T. benedii* Epsilon 1 invertebrate associated clones, e.g. *Shinkaia crosnieri*, hydrothermal vent and volcanoe clones, e.g. AY075124	CAA GCA CTG CTG TTT CCG	67-86
TbEP2-184	*T. benedii* Epsilon 2 4*Riftia pachyptila* tube clones, e.g. AF449251, hydrothermal vent clones, e.g. AF420359, ammonia biofilter clone, AF090545	CCA ACT ACC ATT AAG GCA	184-194

[a] Position in the 16S rRNA of *E.coli*

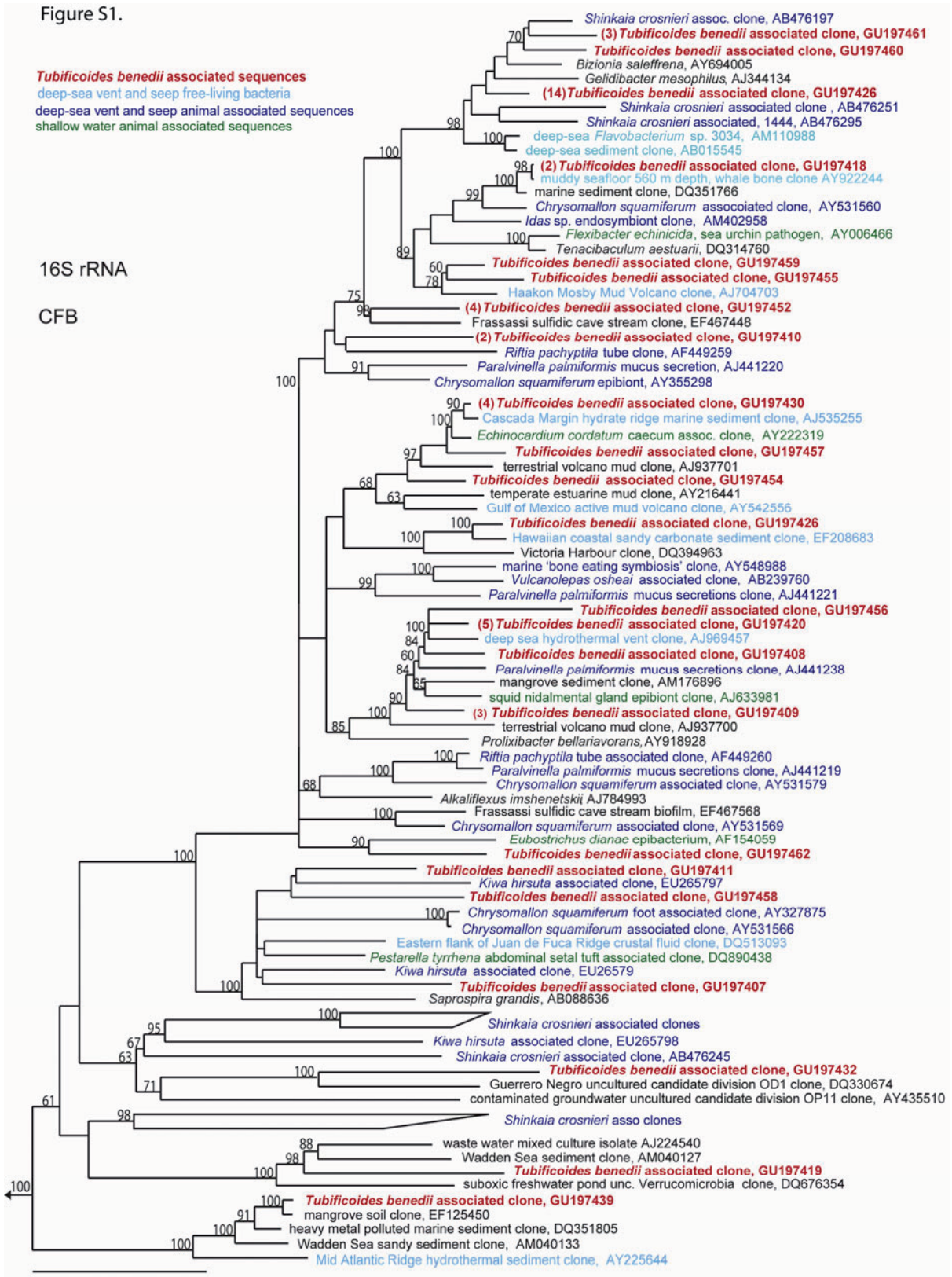


Fig. S1. Phylogenetic placement of CFB 16S rRNA bacterial sequences from *T. benedii* tail ends based on ML analyses (sequences from this study in red, in parentheses the number of sequences with >99.0% to the given sequence). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes correspond to bootsrap values based on 100 ML replicates (only values above 60% are shown).

Fig. S2. Phylogenetic placement of deltaproteobacterial 16S rRNA sequences from *T. benedii* tail ends based on ML analyses (sequences from this study in red, in parentheses the number of sequences with >99.0% to the given sequence). Five sphingobacterial sequences were used as an outgroup (arrow). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes correspond to bootstrap values based on 100 ML replicates (only values above 60% are shown).

Figure S3.

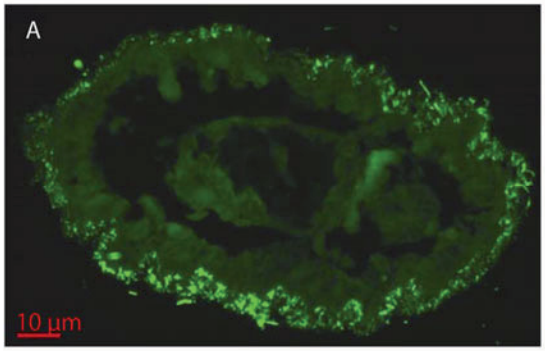
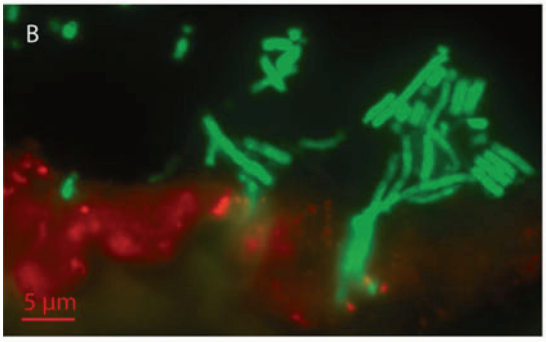
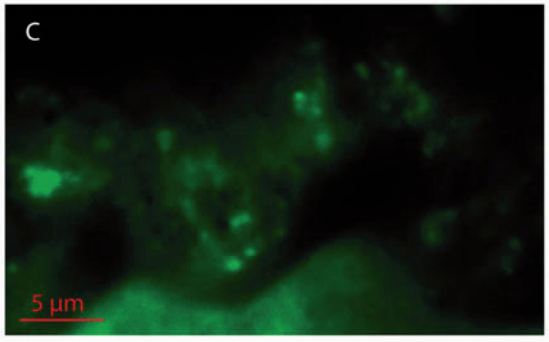
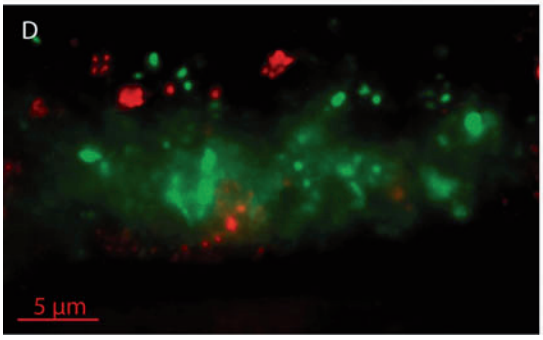
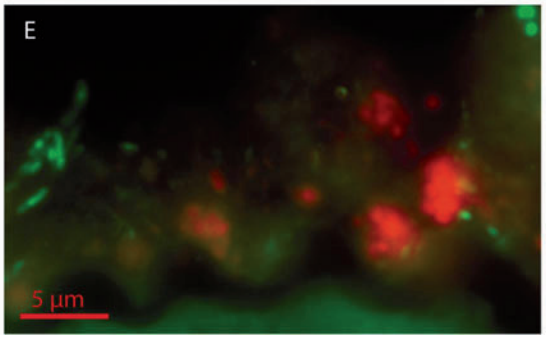


Fig. S3. CARD-FISH images of epibacteria on *T. benedii* tail ends. A. Cross section through the tail end showing the thick layer of bacteria covering the surface of the worm (eubacterial probe EUBI-III). B. The general gammaproteobacterial probe (GAM42a, red) hybridized with rods and cocci in the mucus membrane, while the Gamma 1 ectosymbionts hybridized with specific probes (shown in green: TbGAM1 138) but not with the GAM42a probe. C. The T. benedii Gamma 2 ectobionts (hybridized with the specific probe TbGAM2-447, green) were cocci-shaped and found occasionally in the mucous membrane. D.-E. Deltaproteobacteria (probe DELTA495a, red) and Bacteroidetes (probe CF319a, green) populated the worm's mucous layer. Deltaproteobacteria occurred singly as rods in groups of coccoid or oval shaped cells. Bacteroidetes mostly occurred in patches within the mucus and were often rod-shaped, and sometimes elongated or filamentous.









VI Results and Discussion

In this section results presented in the previous chapters are briefly summarized. Unpublished experiments and analyses of this thesis are shown. First, the symbioses of the two co-occurring Mediterranean gutless oligochaetes *O. algarvensis* and *O. ilvae* from sediments low in sulfide are discussed. Finally, the molecular ecology of ectosymbionts of the Wadden Sea oligochaete *T. benedii* is addressed.

1 Similar endosymbiotic communities in the co-occurring gutless oligochaetes *O. algarvensis* and the novel species, *O. ilvae*

Extensive molecular analyses and improved FISH techniques were used to study the symbiont community of *O. ilvae* and to re-examine the symbiont community of *O. algarvensis*. Phylogenetic diversity of the symbionts in both species (and other gutless oligochaetes) (Fig. 1) was higher than previously assumed. In addition to the two symbionts of *O. algarvensis*, Gamma 1 and Delta 1, described in my Diploma thesis (Mülders 1999) and Chapter 1 (Dubilier et al., 2001), additional symbionts were found to occur in *O. algarvensis.* Multiple symbionts were also identified in *O. ilvae* (Chapter 2, (Ruehland et al., 2008)). *O. algarvensis* and *O. ilvae* both harbored Gamma 1 and Delta 1 symbionts. Some *O. algarvensis* individuals also host Gamma 3 and Delta 4 symbionts as well as a spirochete. All examined specimens of the gutless oligochaete *O. ilvae* harbored four symbionts, i.e. Gamma 1, Delta 1, and also Gamma 3 and Delta 3 symbionts, but no spirochetes. Other sequences rarely occurred in the clone libraries suggesting a minor role of the corresponding organisms. This was supported by results from FISH where probes specific for the symbionts covered the symbiotic community as visualized with the general eubacterial probe EUB338 ((Chapter 2), (Ruehland et al., 2008)).

1.1 Physiology of *O. algarvensis* symbionts

The symbionts of *O. algarvensis* were extensively studied by comparative gene analysis, metagenomics (Woyke et al., 2006) and metaproteomics (Kleiner, 2008), which gave insight into the metabolic potential of the various gamma- and deltaproteobacterial symbionts. Molecular analysis suggested that the symbiont community of *O. ilvae* and their physiological potential is similar to that of the co-occurring *O. algarvensis* (see section 1.2). Therefore, the endosymbiotic system in

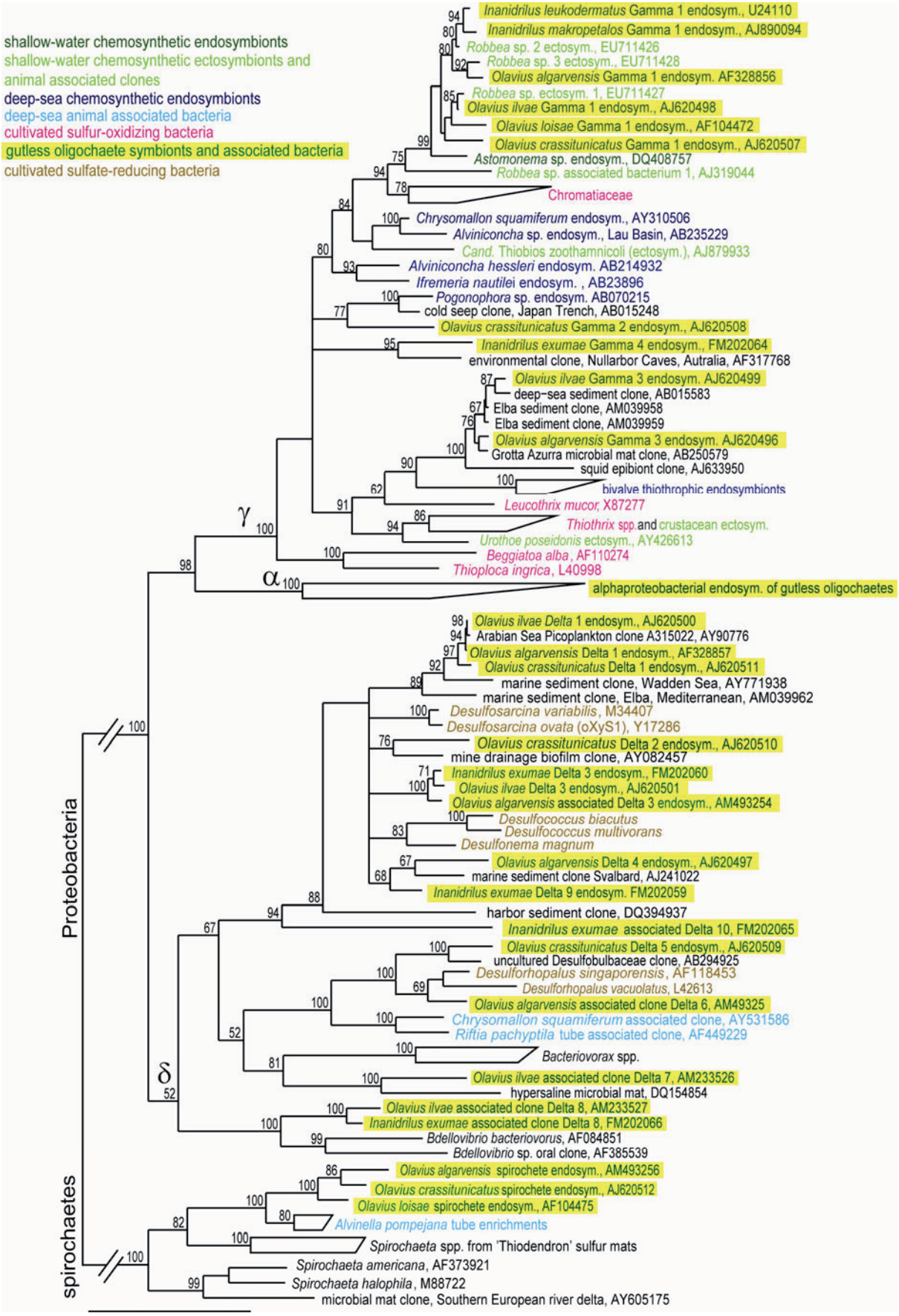


Figure 1. Phylogenetic diversity of oligochaete endosymbionts including sequences from this study. The 16S rRNA tree is based on ML analyses with 100 bootstraps. Scale bar indicates 10% estimated sequence divergence.

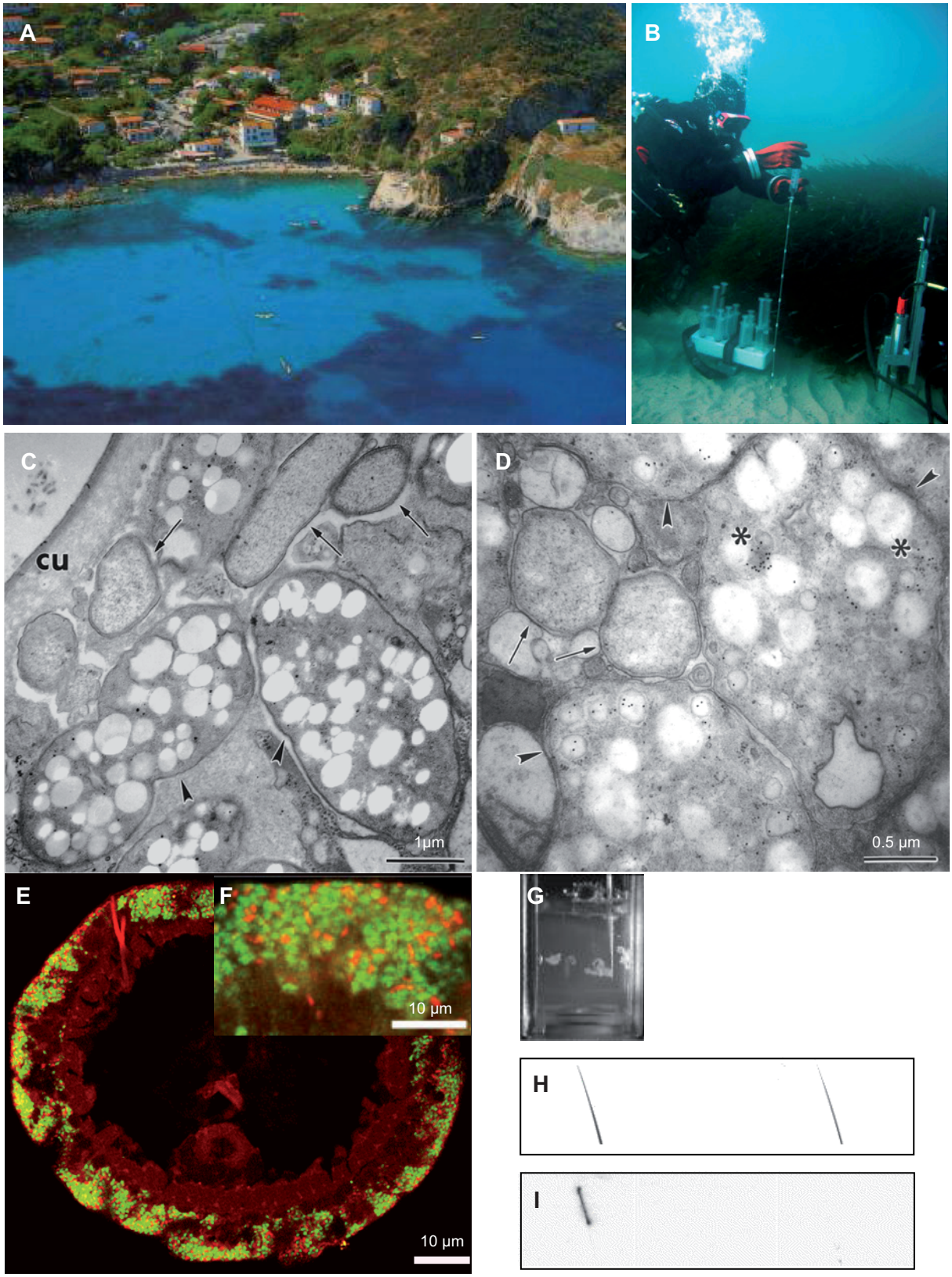
O. algarvensis may serve as a more general model for Mediterranean gutless oligochaete symbiosis.

1.1.1 Gamma 1 symbiont physiology

As in other gutless oligochaetes, the Gamma 1 symbiont of *O. algarvensis* corresponded to the large morphotype. Consistently, it affiliated with Gamma 1 symbionts of previously analysed gutless oligochaetes (Fig. 1 and Chapter 1 (Dubilier et al., 2001)). The oligochaete Gamma 1 symbionts form a sister clade to the Chromatiaceae, free-living phototrophic sulfur oxidizers. This phylogenetic affiliation suggested a thioautotrophic metabolism for the Gamma 1 symbionts. Thioautotrophic potential of the *O. algarvensis* Gamma 1 symbionts was first confirmed by immunohistochemistry and molecular evidence and more recently corroborated by metagenomic and proteomic analyses:

In the Gamma 1 symbiont the ribulose-1,5-bisphosphate carboxlase/ oxygenase (RubisCO) form I, an enzyme involved in CO_2 fixation, was localized (Figure 2 2D and Chapter 1 (Dubilier et al., 2001) Supplementary Information). The presence of genes involved in dissimilatory sulfur metabolism (the gene for the alpha subunit of the adenosin-5'-phosphosulfate reductase, *aprA*) and autotrophy (genes for the large subunit of RubisCO form I, *cbbL*) was confirmed in this study (see below 'Confirmation of sulfur cycling'). These genes and additional genes indicative of autotrophy and sulfur metabolism including dissimilatory sulfite reductase (*dsr*), flavocytochrome c sulfide dehydrogenase (*fcc*) and sulfur oxidation (*sox*) could be assigned to the Gamma 1 symbionts based on metagenomic binning analyses (Woyke et al., 2006). A sulfur storing potential similar to the sister clade Chromatiaceae was supported by the detection of both the gene and the protein of *sgpB* encoding a sulfur globule protein in the Gamma 1 symbiont (Woyke et al., 2006; Kleiner, 2008). This protein is involved in the formation of intracellular sulfur globules under limited oxidant supply.

The Gamma 1 symbiont seems to be metabolically versatile and is not limited to the use of O_2 and sulfur compounds, but can also use alternative electron acceptors and donors when redox conditions change. In the upper, oxygenized layers it can oxidize sulfur compounds with O_2. Migrating downwards in the sediment fumarate may serve as an electron acceptor with succinate and S_0 as products of incomplete oxidation. When moving upwards to the oxidized sediment layers the stored sulfur is further oxidized to sulfate (Woyke et al., 2006; Kleiner, 2008).


A
B
C
cu
1µm
D
0.5 µm
E
F
10 µm
10 µm
G
H
I

Finally, the Gamma 1 symbiont also has heterotrophic potential and could use host waste products as a carbon source. For example, in metaproteomic studies evidence for a modified version of the 3-hydroxypropionate pathway was found that indicates the uptake of acetate and propionate (M. Kleiner, personal communication). Furthermore, proteomic analyses supported previous evidence for granular polyhydroxyalkanoate (PHA) storage in the Gamma 1 symbiont by detection of several involved proteins (phasin (PhaP) and a PHA-synthase protein), (Kleiner, 2008). Thus, the Gamma 1 symbiont is adapted to fluctuating availabilities of electron donors and acceptors such as oxygen and sulfide when the worm moves through the sediment.

1.1.2 Gamma 3 symbiont physiology

While the phylogenetic affiliation with known sulfur-oxidizing bacteria already indicated sulfur metabolism for the Gamma 1 symbiont, the Gamma 3 symbiont of *O. algarvensis* was not related to known sulfur oxidizers based on 16S rRNA phylogeny (Fig. 1 and Chapter 2 (Ruehland et al., 2008)). However, data from molecular and metagenomic analyses revealed that Gamma 3 in *O. algarvensis*, like the Gamma 1, is a thioautotroph as well. All genes necessary for the oxidation of reduced sulfur (*dsr*), (*apr*), the gene for sulfate adenosyltransferase (*sat*), *fcc* and *sox*) and carbon fixation (*cbbL*) were assigned to the Gamma 3 symbiont (Woyke et al., 2006). In contrast to the Gamma 1 symbiont, the Gamma 3 symbiont does not appear to store sulfur, as no sulfur storage genes were found in the nearly complete genome, supporting previous ultrastructural studies where sulfur was only observed in the Gamma 1 symbiont (Woyke et al., 2006).

Figure 2 (opposite page). Sant' Andrea bay on the northern coast of Elba where the worms were collected (A). In situ measurements and collection of pore water samples for biogeochemical analysis (B), pictures C. Lott/ M. Weber from (Ruehland et al., 2006). TEM of bacterial endosmbionts in *O. algarvensis* (C) with larger bacteria (arrowheads) containing numerous globules, smaller (arrows) none; (cu) cuticle (from (Dubilier et al., 2001). Immunocytochemical localization of form I RubisCO (D). The larger symbionts (arrowheads) are labelled with an RubisCO antiserum (asterisks) (supplementary material for Dubilier et al (2001).FISH of *O. algarvensis* gamma- and deltaproteobacterial symbionts (green and red respectively) with group probes (E) for the Gammaproteobacteria (Gam42a) and *Desulfosarcina*/*Desulfococcus* group (DSS658), and with specific probes (inset, F) for *O. algarvensis* Gamma 1 and Delta 1 symbionts. To prove that sulfate reduction occurs actively in the worm we inserted silver needles into individual Elba worms (G) in incubations with radiolabelled $^{35}SO_4^{2-}$ under aerobic and microaerobic conditions. Precipitation occurred on needles from microaerobic experiments showing a blot on the autoradiographic film (H) while the formalin fixed control showed no positive signal (I), discussed but not shown in Dubilier et al. (2001).

While no genes for the use of oxygen as a terminal electron acceptor could be found (N. Dubilier and M. Kleiner, personal communication), the Gamma 3 symbionts uses nitrate as an electron acceptor, when oxygen becomes depleted in the deeper sediment. Nitrate respiration was indicated by genes involved in dissimilatory nitrate reduction in the Gamma 3 symbiont bin such as the genes for the periplasmic nitrate reductase (*nap*) and nitrite reductase (*nir*) (Woyke et al., 2006). The periplasmic nitrate reductase was also highly abundant in the metaproteome (M. Kleiner, personal communication) In nitrate depleted layers, fumarate or worm osmolytes such as trimethylamine *N*-oxide could also serve as electron acceptors as well as carbon and nitrogen sources. In this way the Gamma 3 symbiont is theoretically capable of sulfur oxidation in all layers inhabited by the worm making it a valuable symbiotic partner (Woyke et al., 2006).

1.1.3 Delta 1 and 4 symbiont physiology

The Delta 1 and Delta 4 symbionts of *O.algarvensis* are related to free-living, sulfate-reducing Deltaproteobacteria. The presence of such bacteria in an invertebrate symbiosis was unprecedented and unexpected. Until then these bacteria had only been found in the digestive systems of invertebrates. These organisms release sulfide which is a potent inhibitor of respiration and thus toxic to aerobic organisms. Therefore, active sulfate reducers were unlikely candidates for symbiosis with invertebrates. However, an internal supply of reduced sulfur compounds could be beneficial to thioautotrophic symbionts in a sulfide limited environment and could explain their presence in this symbiosis.

By autoradiography and detection of sulfate reduction we could show that hydrogen sulfide is produced in *O. algarvensis* under low oxygen conditions (Chapter1 (Dubilier et al., 2001)) and Fig. 2G, I, J). Furthermore, subsequent molecular and genomic studies revealed characteristic genes involved in sulfate reduction (such as *dsr,* periplasmic cytochrome c (*qmo*), and *apr*) in both the Delta 1 and 4 symbionts ((Woyke et al., 2006); Chapter 2 (Ruehland et al., 2008)). Delta 4 furthermore appears to have a complete pathway for tetrathionate reduction suggesting a possible exchange of sulfur intermediates with the sulfur oxidizers. This cycling of intermediates is energetically more favorable than the cycling of sulfate and sulfide (Woyke et al., 2006), because the activation of sulfate is very energy-consuming (with one ATP necessary for the conversion of sulfate to adenosine phosphosulfate).

Heterotrophy is the most common metabolism of the highly versatile free-living sulfate reducers. While few can live chemoautotrophically, most consume a wide range of organic substrates (Thauer et al., 2007). Such a substrate versatility was reflected in the presence of genes for transport and utilization of diverse carbohydrate substrates assigned to the deltaproteobacterial symbionts in the metagenomic analysis (Woyke et al., 2006). In this way the Delta symbionts could recycle the worm's waste products such as organic acids e.g. succinate formed during anaerobic metabolism, oxidizing these substrates in sulfate reduction. Nevertheless, for a net gain of the symbiosis and growth of the symbiotic partners external electron donors have to be taken up. However, so far, metabolomic analyses show no significant sources of easily available organic substrates in the pore waters of the worm's habitat (N. Dubilier, personal communication).

Furthermore, metagenomic analyses suggested that the deltaproteobacterial symbionts might have the potential to fix CO_2 via the reductive acetyl-CoA pathway (Woyke et al., 2006). While it has been discussed whether the involved enzymes might rather be used in heterotrophic metabolism (M. Kleiner, C. Wentrup, personal communication) the recent discovery of the expression of a carbon monoxide dehydrogenase (CO dehydrogenase) and hydrogenases in proteomic analyses corroborated the hypothesis for autotrophic potential of the Delta symbionts (M. Kleiner and N. Dubilier, personal communication).

1.2 Physiology of *O. ilvae* symbionts

The previous section discussed what is known about the different symbionts found in *O. algarvensis*. Molecular analysis revealed a similar symbiont composition in *O. ilvae*. To show the potential for sulfate respiration and thioautotrophy in both host species I comparatively analysed diagnostic genes. Indeed, genes involved in sulfur metabolism (*aprA, dsrAB*) and autotrophy (*cbbL*) in *O. algarvensis* and *O. ilvae* indicated sulfur syntrophy in both co-occuring host species. As described in Chapter 2 (Ruehland et al., 2008), phylogenetic analyses and metagenomic binning furthermore allowed the assignment of most genes and thus metabolic functions to the respective symbionts (Chapter 2, (Ruehland et al., 2008)). The close phylogentic position of the genes of gammaproteobacterial *O. ilvae* symbionts, Gamma 1 and 3, to the genes of Gamma 1 and 3 of *O. algarvensis*, suggested that they also possess thioautotrophic potential. Likewise, sulfur metabolism was also indicated by assign-

ment of *aprA* genes falling into the lineage of sulfate-reducing bacteria for *O. algarvensis* Delta 1 and 4 and *O. ilvae* Delta 1 symbionts. Additionally phylogenetic analysis of *dsr* genes suggested that this gene, indicative for sulfate reduction, was present in all deltaproteobacterial symbionts of *O. algarvensis* and *O. ilvae*. Therefore, the symbiotic communities found in the two co-occurring Mediterranean gutless oligochaetes from sediments low in sulfide have the potential to engage in sulfur syntrophy described below.

1.3 Syntrophy in the Mediterranean gutless oligochaete symbioses

The low sulfide concentrations in the habitat of *O. algarvensis* and *O. ilvae* (Chapter 1) might explain the importance of the sulfate reducers to this symbiosis (see also Introduction Chapter 1 (Dubilier et al., 2001), (Woyke et al., 2006), and Chapter 2 (Ruehland et al., 2008)). In short, the sulfate reducers provide reduced sulfur compounds by the respiration of sulfate or other oxidized sulfur compounds to oxidize organic substrates. The reduced sulfur compounds such as sulfide are oxidized by the Gamma symbionts to generate energy for carbon fixation. In this manner sulfur compounds could be constantly recycled.

In the absence of oxygen, sulfate is likely already reduced by the deltaproteobacterial symbionts under microaerobic conditions in the upper oxidized sediment layers. Here, the active sulfur oxidizers quickly respire the influxing oxygen for sulfur oxidation. Oxygen scavenging is one effect that allows the generally oxygen-sensitive sulfate reducers to thrive in free-living consortia in the presence of oxygen (Teske et al., 1996). Syntrophic cycling of oxidants and reductants yields higher growth yields for all involved partners, especially when intermediates are involved and the energy costly activation of sulfate for sulfate reduction is avoided (see above Delta 4 metabolism). This has been shown for various consortia of (anoxygenic photo- or aerobic chemoautotrophic) sulfur oxidizers and sulfate reducers (Biebl and Pfennig, 1978; van den Ende et al., 1997; Overmann and Gemerden, 2000). Furthermore, synergic effects of consortia on other heterotrophic bacteria have been shown in mixed cultures (Loka Bharati, 2004).

As an additional syntrophic effect, the Gamma 3 symbiont might produce hydrogen, which the Delta 1 might scavenge and use as energy source for autotrophic carbon fixation. Additional substrate cycling (e.g. fumarate/succinate between Gamma- and Delta symbionts) reduces the loss of energy–rich waste products by

channeling them back into the symbiosis. Waste products from the host such as volatile fatty acids from anaerobiosis or urea can serve as alternative substrates for the four symbionts and alleviate the host from the harmful accumulation of waste products (Woyke et al., 2006). Thus, substrate cycling poses advantages to the symbionts as well as the host.

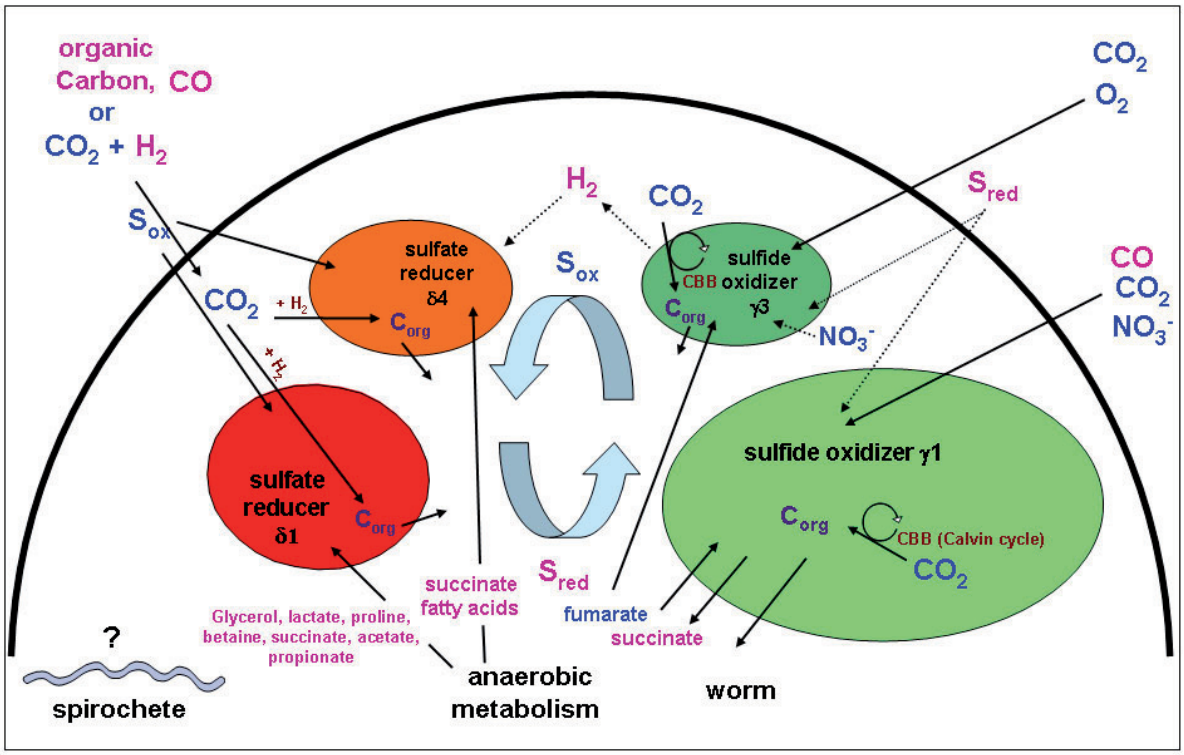


Figure 3. Simplified diagram of potential interactions of the symbiotic partners in the *O. algarvensis* symbiosis from sediments low in sulfide. The two sulfate-reducing symbionts (red) oxidize organic compounds (from worm waste products or the environment) or possibly hydrogen via sulfate reduction, producing sulfide, which is taken up by the sulfur oxidizers. The two sulfide oxidizers (green) use reduced sulfur compounds as an energy source for autotrophic fixation of CO_2 into organic compounds. Thus, sulfur compounds are constantly recycled. The function of the spirochete is unclear. Under anaerobic conditions the Gamma 1 and 3 sulfide-oxidizing symbionts have the ability to respire fumarate to oxidize H_2S to elemental sulfur. The produced succinate can serve as an electron donator for the Delta 1 and 4 sulfate-reducing symbionts, thereby recycling it to fumarate. Recent results from metabolomic analysis indicate that both sulfate reducers and the Gamma 3 symbiont might use carbon monoxide (CO) as an additional electron donator and carbon source. Furthermore, Gamma 3 most likely uses nitrate not oxygen as a terminal electron acceptor. For a more complete diagram and descriptions refer to Woyke, 2006. Diagram based on Dubilier (2001) and Woyke (2006). Inorganic electron donors in purple, inorganic electron acceptors in blue.

The complex syntrophic interactions and cycling of substrates gives the impression of a closed and self-sustaining system, however, this is thermodynamically impossible and the symbiosis is not a perpetuum mobile. For net growth additional energy equivalents such as hydrogen, reduced sulfur compounds or dissolved organic compounds need to be imported from the environment (Chapter 1 (Dubilier et al., 2001)). Uptake of substrates is possible as the cuticle is passable for molecules up to 70 kDa (Dubilier et al., 2006) and the surrounding sediments are full of organic

debris from the adjacent *Posidonia* sea grass beds. However, as mentioned above analyses of the sediment indicated that there are no easily available organic substrates like sugars, amino acids or organic acids in the Elba pore waters. Thus hydrogen and carbon monoxide (CO) may be the main electron donors.

1.4 Multiple symbionts: evolutionary dynamics in balancing of microniches and competition

For a long time symbioses with multiple symbionts had been considered to occur mainly in digestive systems with complex substrates. In chemoautotrophic symbioses the symbionts appeared to be specialized, host-specific and their population genetically homogenic. Competition for space and nutrients as well as strict vertical transmission or host selection seemed to outrule the establishment of multiple associations. However, in the last decade the advance of molecular methods revealed multiple symbionts occurring in a variety of chemosynthetic symbioses such as the endosymbioses of gutless oligochaetes.

In the *O. algarvensis* and *O. ilvae* symbioses the benefit of syntrophy is likely to outweigh the competition for space between gamma- and deltaproteobacterial symbionts. It is intriguing that at a first glance the additional Gamma- and Deltaproteobacteria seem to have a redundant function implying competition for resources while space already has to be shared between symbionts. This leads to two possible hypotheses: 1) The metabolism of the symbionts may slightly differ at a closer look. Within the symbiotic shuttling system as the worm travels through the different oxidized and reduced layers of the sediment, the symbionts could occupy physiological microniches. They may use different substrates or have different substrate affinities resulting in alternating production peaks depending on the environmental conditions. 2) However, when the ecophysiological strategies are too similar (or other reasons such as varying growth rates, antagonism of symbionts or host), competition could lead to the displacement of one symbiont during the course of evolution.

1) In favour of the first hypothesis are the results of the metagenomic study on *O. algarvensis* symbionts. As the worm moves through oxidized and reduced sediment layers, the gamma- and deltaproteobacterial symbionts express their metabolic versatility in the respective physiological niches resulting in a continued cycling of substrates. Various electron acceptors and donators are taken up during the migration and metabolites are released which are partitioned among symbionts. In the deeper, oxygen-free layers, the Gamma 1 symbiont replenishes the transient sulfur

storage by using alternative electron acceptors such as fumarate for sulfide oxidation. In the presence of oxygen the stored sulfur can be further oxidized. The Gamma 3 in turn apparently does not use oxygen as a terminal electron acceptor and does not store sulfur (M. Kleiner and N. Dubiler, personal communication). Instead it can use a wide palette of alternative electron acceptors such as nitrate and fumarate, but also worm osmolytes when oxygen is limited (see above Gamma 3 physiology).

The strategy to perform different energy-yielding processes in oxic and anoxic sediment layers is similar to the ecophysiology of filamentous sulfur oxidizing Gammaproteobacteria such as *Thioploca* and *Beggiatoa*. Both free-living sulfur oxidizers perform sulfide oxidation to sulfur with (stored) nitrate in the sulfidic, anoxic sediment layers, then move to the sulfide-free, oxic surface and further oxidize sulfur to sulfate using oxygen. Interestingly some *Thioploca* can engage in sulfur syntrophy with the sulfate reducer *Desulfonema* attached to their sheaths (Karavaiko et al., 2006).

The deltaproteobacterial symbionts also showed metabolic versatility. The Delta 4 symbiont uses intermediates of the sulfur cycle and the Delta 1 symbiont apparently takes up osmolytes and polyamines as well as mono-and dicarboxylates as suggested by metagenomic analysis (Woyke et al., 2006). Thus, all symbionts would contribute to the system by temporal and physiological niche partitioning. In addition to divergent substrate spectra the symbionts might exhibit gradual differences in affinities to the same substrate (e.g. sulfide) as shown for ammonia-oxidizing bacteria and archaea (Martens-Habbena et al., 2009).

Given a stable environment at evolutionary time scales, the symbioses might have reached equilibrium, where each symbiont serves a definite function. The symbiosis might function like a microcosm where competition is avoided by adaptation to microniches with phenotypic and ecological differentiation. An extreme case of such fine-scale evolution has recently been reported for co-existing free-living strains (*Salinibacter ruber*) identical in 16S rRNA and intergenic regions, but with genomic differences resulting in new ecotypes (Pena et al., 2010).

2) The symbiont composition varies through the recurrent uptake of bacteria from the environment, of which some are able to establish themselves as facultative or even obligate (at least for the host such as the Gamma 4 of *Inanidrilus exumae* replacing the Gamma 1 (Bergin et al., submitted), see below) symbionts. In this case we can consider the symbiosis as we see it now as representing just a moment in

time which is under constant change. If novel and established symbionts have redundant function competition could lead to displacement. Over the course of evolution novel symbionts could replace the primary symbionts or vice versa, the additional symbionts might be superseded by the primary symbionts. In the *O. algarvensis* specimen that harbored both, the Gamma 1 and Delta 1 as well as Gamma 3 and Delta 4 symbionts, the secondary symbionts appeared at least as abundant as the primary symbionts (Chapter 2 (Ruehland et al., 2008)). If the secondary symbionts are more efficient in uptake and substrate metabolism and exchange, they might outcompete the primary Gamma 1 and Delta 1 symbionts in the long run. A change in the environmental conditions might push the balance off to either side, favoring the primary Gamma 1 and Delta 1 or the secondary Gamma 3 and Delta 4 symbionts. Thus, the association with Gamma 3 and Delta 4 symbionts could have been established recently and might steadily spread in the population. Follow-up studies over a long term period to monitor the relative abundance of these symbionts in a given host population (same sampling site) are needed to address the stability of these multiple symbioses. Worm cultivation studies might also help in elucidating the fitness and versatility of the symbionts, monitoring their abundance and activity under different conditions.

Evolutionary aspects of the Mediterranean symbioses

The monophyly of the Gamma 1 symbionts indicates their common ancestry and a long evolutionary history is indicated through their co-speciation with oligochaete hosts. The Gamma 1 symbionts might have an advantage over other co-occurring symbionts as their association with the worms was the first one in this endosymbiosis. The adaptation to fluctuating substrate concentrations during the worm's vertical sediment migration might explain why the Gamma 1 symbiont has successfully established and maintained a stable symbiotic relationship with gutless oligochaetes over long evolutionary periods. They have co-evolved with the host which relies on them. Co-evolution in a long-term symbiotic relationship can lead in many ways to an optimization in synergy, symbiont transmission, recognition, and host-symbiont communication.

An indication for the transition from a facultative to an obligate symbiosis with vertical transmission could be an increased number of transposons in the symbiont genome (Plague et al., 2008). The Gamma 1 symbiont showed an unusually high

proportion and the Gamma 3 symbiont a smaller proportion of transposases indicating such a transition stage (Woyke et al., 2006). Other signs for an obligatory lifestyle are genome reduction, AT bias or loss of certain metabolic pathways. However, this did not seem to be the case for any of the oligochaete symbionts (Woyke et al., 2006). None of the symbiont genome was reduced in size as an indication for their obligatory state. Additionally, flagellar proteins were found in Delta 1, Delta 4 and Gamma 3 symbionts indicating a free living stage. However, flagellar proteins can also be responsible for communication functions (Shimoyama et al., 2009), essential in a symbiotic state. In previous studies sediment samples from the Elba site bore sequences highly similar to the secondary symbionts, Gamma 3 and Delta 1, and bacterial cells hybridized with the Gamma 1 and Gamma 3 probes indicating that these bacteria might occur as free-living species (Perner, 2003). Thus a recent acquisition at least of the secondary symbionts is conceivable. However, since eukaryotic cells were also detected in the samples the symbiont signals could have originated from ruptured hosts. At this point, it remains unclear whether some of the oligochaete symbionts have advanced states of dependency that might involve superior communication with the host and provide them with an advantage over other symbionts.

Even the Gamma 1 symbiont, although it is ubiquitous and considered obligate for gutless oligochaetes, can be replaced. Only recently it was revealed that a gutless oligochaete from calcareous coral reef sediments hosts an entirely novel gammaproteobacterial symbiont instead of the Gamma 1 symbiont. It was postulated that the Gamma 4 invaded the Gamma 1 symbiont hosting *I. exumae* ancestor, and that the two symbionts might have coexisted for a certain time (Bergin et al., submitted). Unlike for the Gamma 1 symbiont and Gamma 3 symbionts in *Olavius* spp., however, the morphology as well as the metabolism of the Gamma 1 and 4 sulfur-oxidizing symbionts is highly similar. Both are large and thus competition for space was presumably much more prevalent than between the large Gamma 1 and smaller Gamma 3 symbionts of the Mediterranean hosts. Unlike the Gamma 3 symbiont, both, the Gamma 1 and the Gamma 4 symbionts store sulfur globules, indicating redundant function in the symbioses (Bergin et al., submitted). Here, it appears as if the novel symbiont took over the functions of the Gamma 1 symbiont and eventually replaced it.

Environmental influences and symbiont composition

While under the current conditions in the Elba sediments niche partitioning appears to function well, shifts between activity and abundance of symbionts might change over short temporal intervals or seasonally in individual worms. External factors such as storms or human interference might pose sudden and drastic changes. In this case the cooperation of the multiple symbionts might be disturbed and lead to a shift in symbiont activity and eventually composition. Lack of activity has been observed previously, when the worms were kept in the laboratory over longer periods (more than two months). While deltaproteobacterial sequences could still be retrieved, no signal was obtained using specific probes for the Delta symbionts and group specific probes (DSS658), indicating that their activity was significantly reduced (Musat, 2006). It would be interesting to see whether the originally described *O. algarvensis* population from Portugal harbors similar symbionts or whether here environmental differences influenced symbiont composition.

Another possible factor determining fitness of the different symbionts and thus the symbiont community is susceptibility to predators such as viruses or *Bdellovibrio* and *Bacteriovax*. Substantial amounts of fosmid end reads had similarities to proteins of viral origin (Woyke et al., 2006) and sequences closely related to bacterial predators were present in the clone libraries (Chapter 2 (Ruehland et al., 2008; Kleiner et al., In press). These sequences were found in only in two specimens and invasions of symbionts could not be observed in ultrastructural studies, although these were not explicitly looked for. It is conceivable that rare events of predation or viral infection might lead to the elimination of susceptible bacterial symbionts.

1.5 Cultivation and enrichment of sulfate-reducing bacteria from Mediterranean gutless oligochaetes

Cultivation of bacteria allows the definition of physiological characteristics such as substrate spectra, temperature, oxygen and salinity optimum. Cultivation of the deltaproteobacterial symbionts would help in elucidating their role and their source of carbon in the symbiosis. Potential carbon sources could be substrates common to free-living SRB acquired through diffusion from the exterior. It had been demonstrated that molecules of up to 70 kDa could freely pass the cuticle, however, metabolomic analysis contradict this assumption (see above). Alternatively, the deltaproteobacterial symbionts could rely on carbon transfer from the gammaproteobac-

terial symbionts or on host metabolites. During anaerobiosis, oligochaetes produce organic acids such as succinate, proprionate and acetate. Alternatively, the Delta symbionts could thrive autotrophically by using hydrogen and fixing CO (see above section 1.1.3).

In this thesis I attempted to cultivate the deltaproteobacterial symbionts of gutless oligochaetes collected from Elba with microbiological techniques. Worms were washed four times in sterile filtered Elba sea-water, homogenized in 1 ml medium and used as the inoculum. Anaerobic agar dilution series (10^{-1} to 10^{-8}) were prepared as described (Widdel and Bak, 1992b). Alternatively, filtered seawater from the site as well as a defined bicarbonate-buffered and sulfide-reduced artificial sea-water media (Widdel and Bak, 1992a) were flushed with N_2/CO_2 (90:10) in culture flasks. Media were then inoculated with N_2/CO_2 (90:10) flushed pieces of single worms with and without various electron donors using three dilution steps. A variety of substrates such as volatile fatty acids and H_2 were added to the media (Table 1). To enhance growth of bacteria that need attachment, sterilized sand from the site was added. Incubations were kept in the dark at room temperature (~23°C). Colder incubation temperatures of 16°C yielded barely any growth.

Table 1. Substrates used in cultivation and enrichment of bacterial symbionts in liquid media and agar shakes

Substrate	agar shake	liquid media, no sediment	liquid media, sediment	liquid media, sediment, Dithionite
1mM acetate + H2/CO2				
8mM acetate				
5mM propionate				
5mM succinate				
2 mM succinate + 2mM propionate 3mM acetate				
2 mM succinate + 2mM propionate + 3mM acetate + 0,3g/L yeast extract				
control				

Growth of colonies was detected in agar shakes with acetate (10^{-1} and 10^{-5} dilution), propionate (10^{-1}) and H_2/CO_2 (10^{-1}). These colonies were transferred to liquid media

using the same substrates as their origin. Growth was also detected in synthetic media with the volatile fatty acid mixture. This culture had a strong smell of sulfide. Testing with $CuSO_4$ resulted in brown coloring as proof for the presence of sulfide, indicating growth of SRB. Under the microscope coccoid cells with one or two small circular structures at one site were visible. These cells resembled *Desulfovibrio* sp. in stationary phase (M. Mußmann, personal communication).

To verify whether these bacteria were the symbionts previously identified in *O. algarvensis* or *O. ilvae*, FISH was conducted with specific probes from this study. Other vials and transferrals from agar to liquid media which showed growth, but had no sign of sulfide production were also tested. Only the sulfide positive vial resulted in a positive signal with the *O. algarvensis* Delta 1 probe. No signal occurred with the 1 mismatch *O. ilvae* Delta 1 probe.

The colonies positive with the Delta 1 probe were further analysed based on 16S rRNA sequences. BLAST (Altschul et al., 1990) of the resulting sequence showed 99% similarity to *Desulfovibrio acrylicus*. As the morphology had already indicated *Desulfovibrio* and a contamination with this species in cultivation of free-living Deltaproteobacteria was common (M. Mußmann, personal communication) the experiment was not continued.

The Delta 1 symbiont might need the Gamma 1 symbiont for optimal growth. The cultivation of syntrophic cultures would require more sophisticated methods. As noted earlier, Gamma 1 and Delta 1 symbionts always co-occurred in the examined *O. algarvensis* individuals indicating a mutual dependency. Many syntrophic bacteria rely on their mutual partner to optimize energy yield and to grow faster e.g. by scavenging inhibitory waste products. Subsequent efforts to cultivate deltaproteobacterial symbionts by two colleagues (N. Musat, MPI Bremen, and A. Gaetjen, University of Oldenburg) also did not result in significant enrichment of the deltaproteobacterial symbionts. Obviously, the interactions between gamma- and deltaproteobacterial symbionts and maybe even the host are complex, making it difficult to mimic these natural conditions. However, free-living relatives from marine sediments are equally difficult to enrich. A continuous culture with a constant inflow of media and monitoring of concentrations of substrate and chemicals might be more successful in maintaining a co-culture.

2 Characterization of ectosymbionts on a gut-bearing oligochaete from muddy Wadden Sea sediments

At the beginning of this study gammaproteobacterial epibionts were known to occur on some marine nematodes from shallow coastal sediments, while long filaments from the deep-sea crab *R. exoculata* were identified as Epsilonproteobacteria (see ntroduction). Other reports of ectosymbionts on crustaceans often lacked phylogenetic identification. Previous studies on *T. benedii* epifilaments emphasized morphological resemblance to the gammaproteobacterial *Thiothrix* and *Leucothrix*, and it was assumed that all filaments belonged to the same genus (Dubilier, 1986). In this thesis the filamentous epibionts of *T. benedii* were identified as gamma- and epsilonproteobacterial ectosymbionts (Chapter 3 (Ruehland and Dubilier, 2010)).

2.1 *T. benedii* ectosymbionts are closely related to deep-sea invertebrate associated bacteria

Comparative 16S rRNA sequence analysis and FISH identified the dominant ectosymbionts as a filamentous Gammaproteobacterium (named the Gamma 1 ectosymbiont) and a filamentous Epsilonproteobacterium (named the Epsilon 1 ectosymbiont). Unexpectedly, both ectosymbionts were not closely related to known symbiotic or free-living shallow water bacteria but instead fell into clades dominated by bacteria associated with deep-sea invertebrates (Fig. 4 and 5). The *T. benedii* Gamma 1 ectosymbiont is the first described non-vent shallow water ectosymbiont whose 16S rRNA sequence falls into a clade with sequences from deep-sea invetrebrates such as the crab *Shinkaia crosnieri*, the crab *Kiwa hirsuta*, the shrimp *Rimicaris exoculata* and the barnacle *Vulcanolepas oshehai.* Furthermore, this is the first epsilonproteobacterial ectosymbiont found in shallow water sediments.

The *T. benedii* Epsilon 1 ectosymbiont affilated with a clade very similar to that of the Gamma 1 ectosymbiont. The two clades included bacterial sequences from the same deep-sea hydrothermal vent invertebrate hosts such as the species mentioned above. The hosts within the Gamma 1 and Epsilon 1 clades are not closely related to each other. Thus, these associations must have been established independently multiple times. Bacteria from these clades have likely specialized in establishing associations with invertebrates irrespective of their habitat and geography. Thus, neither host affiliation nor biogeography seem to have influenced these symbioses. Instead, environmental factors, i.e. highly fluctuating concentrations of oxi-

dants and reductants may have been a driving force in the establishment and evolution of these associations.

2.2 *Thiothrix/Leucothrix* and Thiovulgaceae - bacterial clades forming specific associations with invertebrates

The closest cultured relative of the Gamma 1 ectosymbiont is *Leucothrix mucor*. *Leucothrix* species attach to all kinds of surfaces (see Introduction 1.3.4). The next closest free-living relatives are *Thiothrix* spp. which have been shown to associate with invertebrate as well (Gillan and Dubilier, 2004; Dattagupta et al., 2009). Thus, colonization of surfaces seems to be a common trait to *Leucothrix*-*Thiothrix* bacteria. This seems to be even more pronounced within the invertebrate-associated clade the Gamma 1 ectosymbiont affiliates with which is dominated by invertebrate associated bacteria. Likewise the clade that the Epsilon 1 ectosymbiont belongs to within the Thiovulgaceae appears to be indigenous to invertebrate surfaces. Furthermore, members of these two ectosymbiont dominated clades co-occur on many invertebrates indicating that they form bacterial communities that characteristically inhabit invertebrate hosts. It has recently been suggested that bacteria of the Thiovulgaceae (Epsilonproteobacteria) and the *Leucothrix*-*Thiothrix* (Gammaproteobacteria) clades preferentially form associations with invertebrates (Goffredi, 2010).

For a long time marine ectosymbioses appeared to primarily consist of only single phylotypes (Polz et al., 1994; Polz and Cavanaugh, 1995; Polz et al., 2000; Rinke et al., 2006). With recent extensive molecular analyses of deep-sea invertebrates such as the crabs *Kiwa hirsuta* and *Shinkaia crosnieri* and the shrimp *Rimicaris exoculata* it was revealed that the associated bacterial layers of these animals often harbor a more diverse bacterial community (Goffredi et al., 2008; Watsuji et al., 2008; Petersen et al., 2009). This bacterial community can be limited to a few specific phylotypes as shown for *R. exoculata* where FISH with specific probes was implemented (Petersen et al., 2009)

These bacterial associations with deep-sea invertebrates are not random. While specific bacterial phylotypes attach to hosts of different phyla, only certain members of each phylum appear as suitable hosts. It has been reported that *K. hirsuta* specimen were densely covered with filaments, whereas other co-occurring crustaceans lacked such colonization. Thus, these associations might involve specific recognition interactions between bacteria and host similar to those reported for

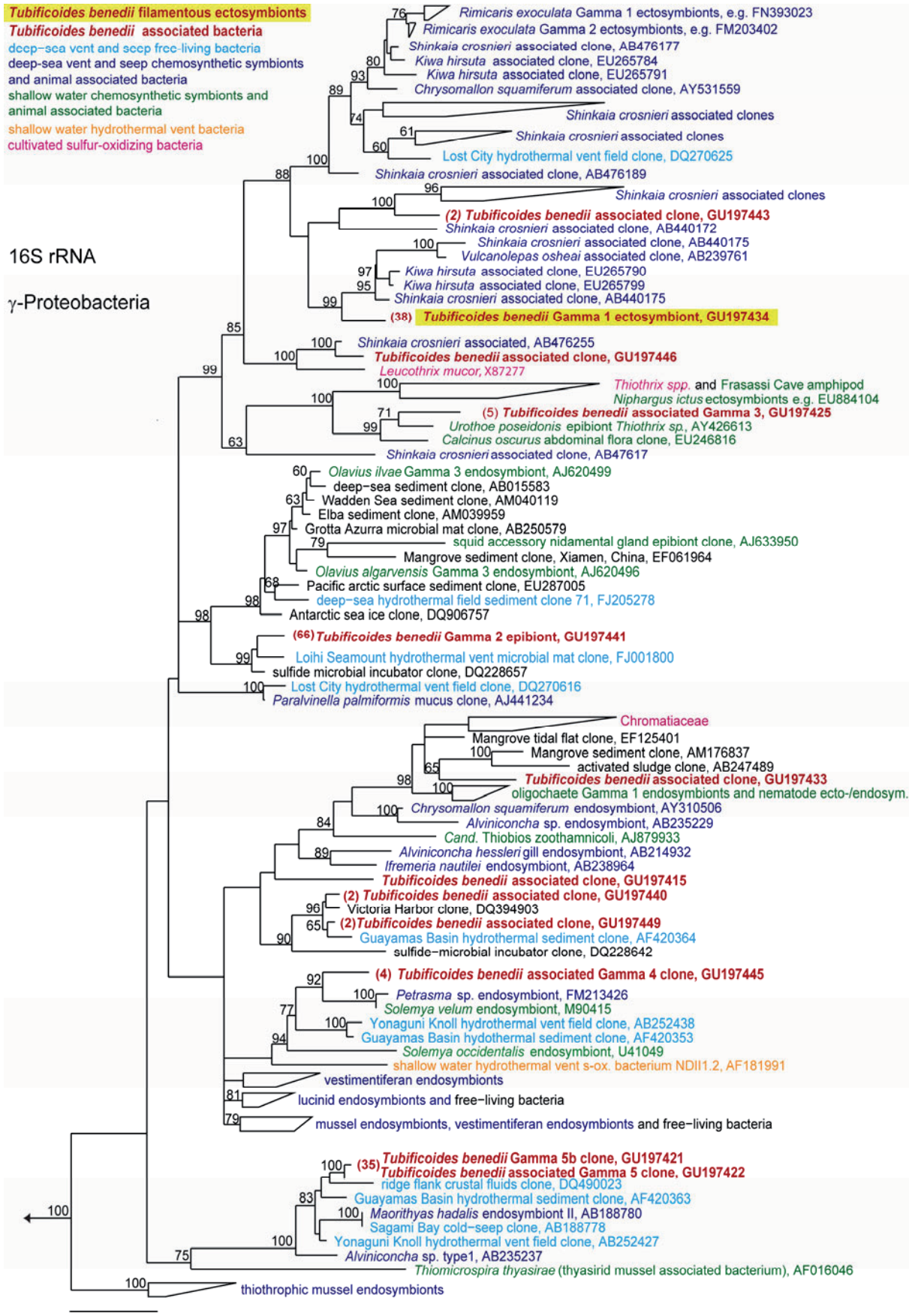


Figure 4. Phylogenetic placement of gammaproteobacterial 16S rRNA sequences from *T. benedii* tail ends based on a ML-tree (sequences from this study in red in parentheses the number of sequences with >99.0% to the given sequence). Bootstrap values based on 100 replicates. Scale bar = 0.10 estimated substitutions per site.

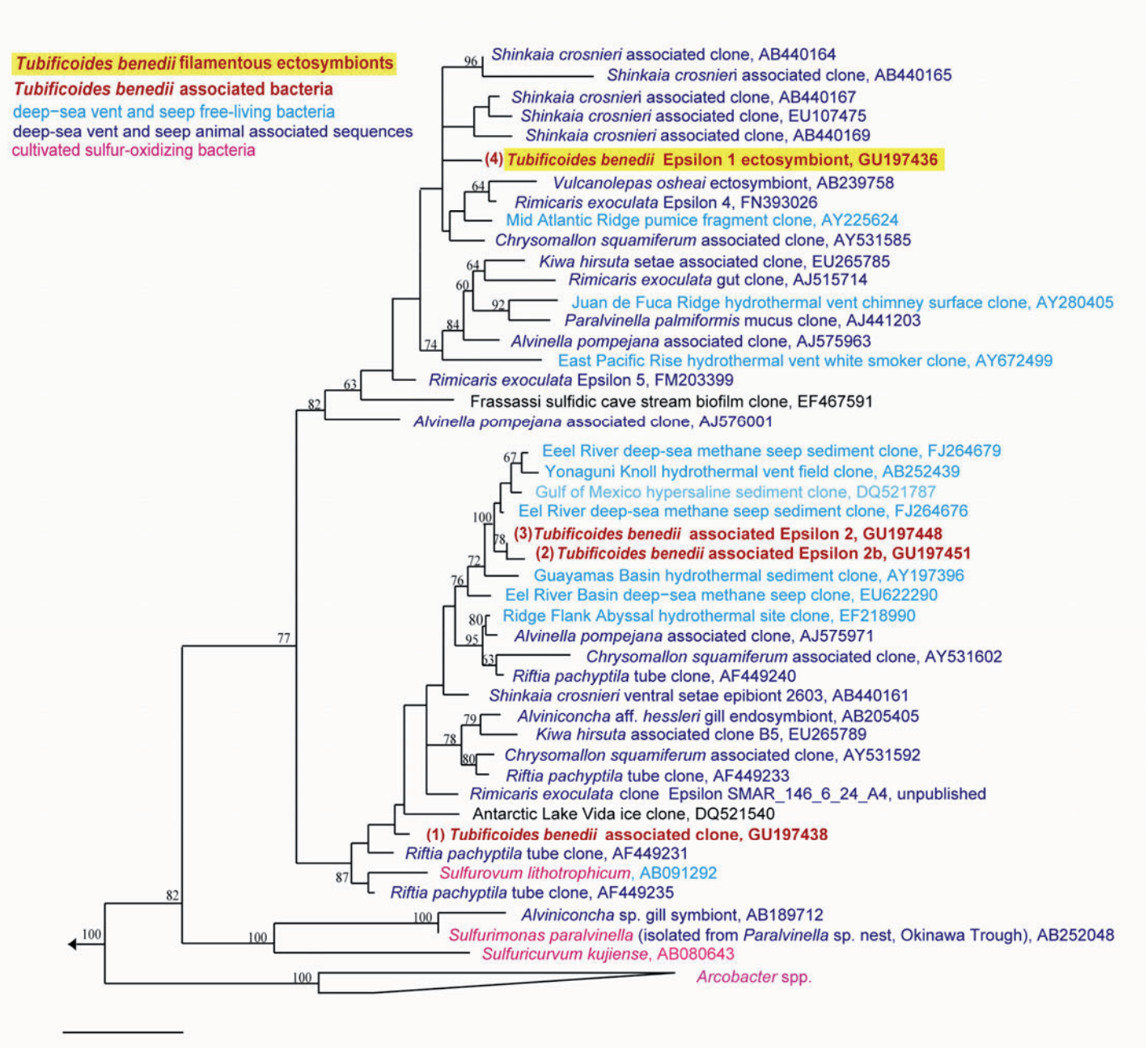


Figure 5. Phylogenetic placement of epsilonproteobacterial 16S rRNA sequences from *T. benedii* tail ends based on a ML-tree (sequences from this study in red in parentheses the number of sequences with >99.0% to the given sequence). Bootstrap values based on 100 replicates. Scale bar = 0.10 estimated substitutions per site.

the highly specific associations of some marine nematodes (Nussbaumer et al., 2004) and coral-dinoflagellate symbiosis involving lectin/glycan recognition systems. They might also involve regulatory mechanisms that are essential for successful for colonization as in *Vibrio fischeri* where one regulatory gene could alter its squid host range (Mandel et al., 2009). More studies (with specific FISH probes) in diverse chemosynthetic environments and the respective hosts are necessary to further investigate the presence of specific phylotypes. Additionally, regulatory genes and molecules involved in mechanisms mediating recognition and binding (such as

lectin/glycan systems) could be searched for molecularly, immunohistochemically or in cultivation experiments.

2.3 Host modification and possible interactions between *T. benedii* and its ectosymbionts

Some of the deep-sea invertebrates such as the shrimp *R. exoculata* have morphologically adapted to hosting ectofilaments. Appendages covered by bacteria were enlarged, suggesting that the host adapted to provide more space for bacterial ectosymbionts, possibly for eventually harvesting them (Petersen et al., 2009). While such an anatomical modification is not reported for *T. benedii*, the Epsilon 1 filament's penetration of the cuticle suggests a specific relationship. Unlike the *T. benedii* Gamma 1 filaments and other epibiotic bacteria, the Epsilon 1 ectosymbiont has gained access to the subcuticular space. This indicates a specialization for host invasion. The presence of globules around the bacterial base may further indicate communication or transfer of products between host and symbiont (Dubilier, 1986) .

Nevertheless, it remains unclear whether *T. benedii* benefits from this association by exchange of metabolites. Clearly, there is no dependency as the symbiosis is only facultative. The host like other sediment dwellers ingests bacterially enriched sediment and detritus for nutrition. If environmental conditions were to shift to constant high sulfide concentrations and the association were thus sustained over longer periods, it might prove beneficial to bacteria and worm to interact more tightly. They could cooperate in exchanging metabolites in mutualism, especially if the bacteria have autotrophic potential like many beneficial symbionts in chemosynthetic environments. Such mutualistic ectosymbioses have evolved in shallow water sediments in nematodes and ciliates with sulfur-oxidzing symbionts of different gammaproteobacterial lineages (see Introduction1.3.4 and 1.3.5).

2.4 Metabolic potential of the *T. benedii* ectosymbionts

As discussed in Chapter 3 (Ruehland and Dubilier, 2010) the bacterial community of *T. benedii* has the potential for autotrophy and sulfur metabolism. Both *cbbL* and *cbbM* genes encoding subunits of the CO_2-fixing enzyme RubisCO form I and II respectively, were successfully amplified from *T. benedii* tail ends. However, metabolic potential could not be resolved in detail for the *T. benedii* ectosymbionts, because the retrieved gene sequences could not be unambiguously assigned to sin-

gle 16S rRNA phylotypes. Immunohistochemical methods in combination with FISH could show which filaments actively fix carbon. In this study first attempts to assign autotrophic potential to specific *T. benedii* associated bacteria using RuBisCO form I and II antisera were made. In these preliminary experiments single filaments could be labelled indicating autotrophic metabolism (see next section). However, more controls (e.g. preimmune serum etc.) are necessary to rely on these results. Another possibility to identify autotrophic bacteria on the worm's posterior end would be cultivation experiments with labelled $^{14}C0_2$ followed by microautoradiography combined with FISH.

Preliminary immunohistochemical detection of autotrophic potential in situ: RubisCO form I and II antiserum-labelling of T. benedii *ectosymbionts*

HRP-conjugated antibodies against anti-RubisCO form I and II antisera were applied to sections of *T. benedii* tail ends. The RubisCO form I antiserum (Fig. 6A-C) labeled filamentous and small coccoid cells in the mucus membrane. Additional specific FISH failed. Whether the smaller cocci are the Gamma 2 epibionts which have a similar morphology (Gamma 2 epibionts described below) or the filaments of Gamma 1 origin can thus only be clarified in future studies optimizing additional hybridization with specific 16S rRNA targeting probes. However, the morphology of the filament in the RubisCO I immunohistochemic labeling is different from the typical Gamma 1 ectosymbiont. The filament is much thinner and thus might result form a different rare bacterial epibiont.

There was scattered labelling of filaments with the RubisCO form II antiserum on filaments resembling the thicker Gamma 1 ectosymbionts (see Fig. 2E-H in Chapter 3 (Ruehland and Dubilier, 2010), this section Fig. 6D). FISH with the Gamma 1 probe (targeting the 16S rRNA) after antiserum labelling was not successful. Differences in signal intensity and distribution (RubisCO II detection in the filaments was scattered and showed low signal intensities) could be due to the either cytosolic or organelle bound state of RubisCO (in carboxysomes, see Introduction1.3.7). So far only the morphological resemblance of the RubisCO form II labeled filaments (thick and long, see Fig. 2E-H in Chapter 3 (Ruehland and Dubilier, 2010) and this section Fig. 6D) with the Gamma 1 ectosymbionts suggests that the Gamma 1 ectosymbionts of *T. benedii* might have autotrophic potential. It is also possible that like many thiotrophic bacteria they use both forms of RubisCO (form I and II) and that the reac-

tion with the antibody for RubisCO form I failed because of methodological problems. However, this is highly speculative at this point.

There was also a strong rod shaped signal in the mucus membrane which might belong to the Gamma 2 epibiont again based on morphological resemblance (chapter 3 (Ruehland and Dubilier, 2010), Fig S3 and this study Fig. 6E). As true for all other bacteria that showed a signal in this immunohistochemic labeling only future studies with additional FISH will help to conclusively identify this bacterium.

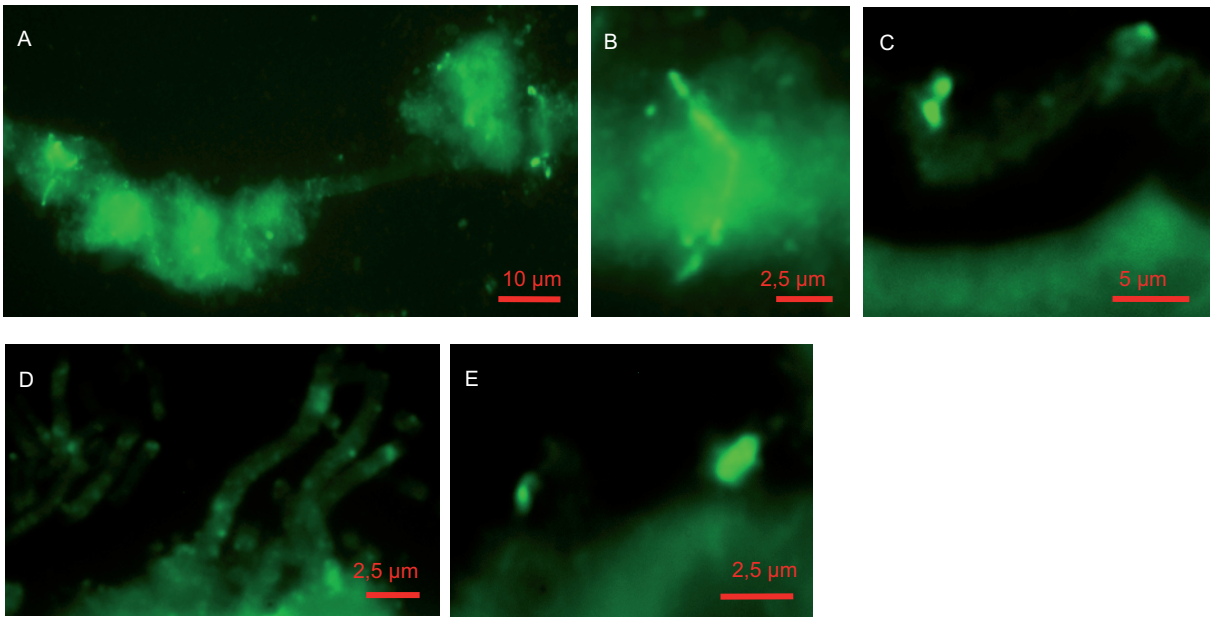


Figure 6. Flourescence microscopy images of immunocytochemical reaction of bacteria on *T. benedii* tail ends with RubisCO form I (A-C) and form II antisera (D, E). The RubisCO rabbit-antibody was labelled with HRP-conjugated anti-rabbit goat antibody. Signal intensity was amplified by HRP signal amplification with Alexa 488. These are only preliminary results. Further refinement and controls are necessary to improve and validate signals.

2.5 Epibiotic community in the mucus layer of *T. benedii*

In addition to the Gamma 1 and Epsilon 1 ectosymbionts, several other gamma- and epsilonproteobacterial were found in *T. benedii* 16S rRNA clone libraries (Fig. 4 and 5). I designed specific probes for all dominant and symbiont-related gamma- and epsilonproteobacterial sequences. An additional coccoid or small rod shaped gammaproteobacterial epibiont in the mucus membrane was identified and named *T. benedii* Gamma 2. All other specifically designed probes did not result in reproducible signals.

This lack of FISH signal from specific probes for gamma- and epsilonproteobacterial sequences could be due to several factors: 1) Contamination in the library as the worm mucus is sticky and sediment or detritus might not have been washed off completely. 2) De- and rehydration as well as additional enzymatic treatment and the

washing procedures for FISH might have decreased bacterial abundance within the mucus cover (washing off) or reduced signals by decomposition of cell membranes. 3) Activity of the additional bacteria was low and thus the bacteria had a low ribosome content (bacterial activity and ribosome content generally correlate (Srivastava and Schlessinger, 1990; Amann et al., 1995; Felske et al., 1996)). 4) Overall low abundances (either on some individuals only or only in some regions). 5) Signal diffusion in the mucus area might have increased any problems with low signal intensity. Despite various elongated enzymatic treatments signal diffusion could not be completely eliminated. Excessive digestion of mucus had to be prevented as it also damages the bacterial cells resulting in signal reduction again.

Other phylotypes from the *T. benedii* tail end 16S rRNA gene library belonged to the Bacteroidetes (Suppl. Fig. S1 in Chapter 3 (Ruehland and Dubilier, 2010)) or the Deltaproteobacteria (Suppl. Fig. S2 in Chapter 3 (Ruehland and Dubilier, 2010)). Hybridization with probes specific for these two groups resulted in regular signals in the mucus membrane (Suppl. Fig. S3 in Chapter 3 (Ruehland and Dubilier, 2010)). The phylogenetic positioning of sequences of Bacteroidetes and Deltaproteobacteria was very diverse in both groups the *T. benedii* sequences spread throughout the 16S rRNA trees. However, some CFB bacteria fell into clades with sequences from the same deep-sea hosts as those from the Gamma 1 and Epsilon 1 clades, namely, *Shinkaia crosnieri*, *Kiwa hirsuta*, and *Chrysomallon squamiferum*. In the deltaproteobacterial tree some sequences were related to sequences from gutless oligochaetes (Delta 3, Delta 5, Delta 6). This could be pure coincidence as members of these phyla are common inhabitants of marine sediments. To test whether certain phylotypes actually appear as epibionts on the surfaces or in mucus membranes while others are more or less ‘contaminants’, specific probes could be designed and applied (to all possible hosts available) in a comparative study.

However, associations of heterotrophic bacteria with animals in general are likely to be common, as the animal waste products and mucus zones offer plenty of substrates. The mucus is rich in host waste products and detritus loaded with organic matter easily sticks to it. Syntrophic relations between bacteria might further enhance the attractiveness of this habitat. As these areas are exterior host defenses are also probably less eminent.

3 Synthesis

A variety of bacteria-invertebrate associations exist ranging from loose, facultative ectobiosis to obligate endosymbiosis. In this study two marine oligochaete symbioses representing two ‘extremes’ were studied, an obligate endosymbiosis and a facultative ectosymbiosis. The advantage for the gutless oligochaetes seems to be obvious for the primary and some of the secondary symbionts (see below). The benefit for the hosts in the associations with additional symbionts such as Alphaproteobacteria and spirochetes in gutless oligochaetes, as well as ectosymbionts of gut-bearing oligochaetes remains to be resolved. For the bacteria however, associations with animals have several advantages and in general they are the initiators of associations when they first attach and in some occasions successfully invade the host.

In general, the short generation times of bacteria, their ability for gene exchange via horizontal gene transfer, gene rearrangements and thus increasing metabolic versatility enable them to rapidly adapt to various habitats. Often heat shock proteins, chaperones and DNA repair systems allow them to respond quickly to short term environmental stress such as higher temperature or radiation. Many free-living bacteria have resting stages. Some such as Clostridia, and members of the Bacteriales can form highly resistant spores, while others including some Gammaproteobacteria (various Pseudomonadales) have developed other structures such as cysts to endure long-term anabiosis (Suzina et al., 2006). This allows them to endure more intense or longer periods of stress such as famine. These resistant stages are also responsible for the passive dispersal via air, water currents and animals where at new destinations they await better conditions for revival (e.g., only recently, DNA of extremophiles was found in arctic sediments (Hubert et al., 2009)). To those bacteria that have the ability to pass immunological defences, animals present a safe space to settle and gain access to substrates.

Chemosynthetic symbionts, once engaged with an invertebrate host have an advantage over free-living bacteria. These specialized symbiotic bacteria are often hard to encounter in the environment, making up only a small proportion of the free-living bacterial community – if they possess free-living stages at all. However, they can reach high densities in or on host tissues which are often specialized to provide their symbionts with substrates. Often the symbionts are transmitted to the next host generation, giving them another advantage over the free-living bacteria. Less spe-

cialized heterotrophic bacteria can also benefit from associations with animals through a constant supply of host (or their symbionts') exudates (see above, 2.5). These associations are often commensal unless the host can benefit from detoxification effects of accumulating waste products, such as anaerobic metabolites and ammonia.

In chemosynthetic symbioses, the mutualistic nature of the relationship clearly presents an advantage to the host. While associations with one specialized bacterium might already provide an advantage to both partners, it has become clear in the last decade that most invertebrate associations are not limited to one bacterial symbiont. Instead, several phylotypes are present in many invertebrate associations. Their discovery was at first surprising and the function of some symbionts remains to be clarified, however, a myriad of new cultivation independent methods have greatly contributed to the understanding of symbioses with multiple symbionts. Bacteria taken up from the environment can aggregate with existing symbionts to build bacterial consortia that increase their own net gain through syntrophy (like their free-living analogues). By incorporation into the symbiosis metabolism they further increase host versatility. In this way the host is enabled to optimally exploit the substrates available in the environment which by itself it could not use.

Symbiotic systems are evolutionary processes that in the beginning might often rely on chance. For example, introduction of a new bacterium as a matter of chance might under certain environmental conditions not be advantageous, but very well so under different conditions, then helping the host to respond to changes such as fluctuating substrate availability. Symbioses are not static systems, but like any other biological organism, subject to dynamic environmental conditions, which they have to respond to. While some bacteria are lost others persist in a constant 'survival of the most cooperative' such as shown recently in a laboratory experiment with unrelated bacteria (Harcombe, 2010).

4 Concluding Remarks

In this PhD study associations of different oligochaetes with bacteria from Mediterranean and North Sea sediments were studied. Molecular investigation of the Mediterranean gutless oligochaete *Olavius algarvensis* revealed that it harbored multiple bacterial symbionts, at the time considered a rare phenomenon in marine chemosynthetic symbioses. Furthermore, deltaproteobacterial sulfate reducers were discovered, at the time surprising because sulfide is toxic to eukaryotes. However, this study demonstrated that sulfide produced in the worm by the deltaproteobacterial symbionts provided the sulfide-oxidizing symbionts with an energy source. Thus the deltaproteobacterial symbionts serve an essential function by enabling the internal cycling of reduced and oxidized sulfur compounds between themselves and the sulfur-oxidizing symbionts. This syntrophic sulfur cycle between the symbionts explained how the worm symbionts could successfully colonize sediments low in sulfide.

This study's comparative examination of the co-occurring *O. algarvensis* and *O. ilvae* showed that both hosts coped with sulfide limitation in a similar manner. Both harbor two distinct gamma- and deltaproteobacterial symbionts with the metabolic potential for sulfur syntrophy. As the hosts are not closely related and different deltaproteobacterial symbionts are involved, these associations must have been established independently of each other in convergent evolution. Thus, the *O. algarvensis* symbiosis - strange as it first appeared - is not the result of an exotic single event in evolution. Instead, environmental conditions have favored the establishment of endosymbioses with sulfate reducers in this habitat.

The identity of the filamentous *T. benedii* symbionts was unclear at the beginning of this study. The bacteria were speculated to be sulfur oxidizers based on their morphological resemblance to free-living sulfur oxidizers and sulfidic conditions in the worms' habitat. Using molecular methods and FISH the filamentous ectosymbionts were shown to belong to two phylotypes, a Gammaproteobacterium attached to the cuticle and an Epsilonproteobacterium penetrating it. Phylogenetic analyses revealed that both phylotypes were related to deep-sea ectosymbionts. Furthermore, Epsilonproteobacteria of this affiliation were only known from deep-sea environments and not previously described from Wadden Sea sediments. Thus, this study showed that these bacteria and their epibiotic associations with invertebrates are not limited

to the deep-sea. Instead, they are probably more wide spread than previously assumed. We concluded that these associations are influenced neither by host affiliation nor biogeography, but instead by the environmental conditions, such as strongly fluctuating oxidants and reductants in their habitat.

In conclusion, the results of this thesis indicated that in the different oligochaete symbioses studied, environmental factors had a strong impact on the composition of the symbiont community. Furthermore, both of these oligochaete populations from coastal marine habitats are ideal models to study symbioses with multiple symbionts as they are cheap and easy to collect. Gutless oligochaetes in general are ideal model systems to study the function of different bacteria in symbioses with multiple endosymbionts as the bacterial diversity is relatively low. *T benedii* in turn could serve as a model for ectosymbioses with Gamma- and Epsilonproteobacteria of the Gamma 1 and Epsilon 1 clades that appear characteristic also to deep-sea invertebrates.

5 Outlook

In this thesis, the symbiotic communities of *O. algarvensis* and *O. ilvae* and their metabolic potential were characterized. Building on these results, the next level would be to investigate if the identified metabolic genes are expressed and if so under which conditions, by looking at mRNA (transcriptomics) or protein expression patterns (proteomics). A first step in this direction was achieved in a metaproteomic study of the bacterial symbionts of *O. algarvensis* (Kleiner, 2008).

For localization of gene expression in situ, e.g. of RubisCO or Sox in the gammaproteobacterial symbionts, a combination of mRNA and 16S rRNA FISH could be applied (Pernthaler and Amann, 2004). Another possible method to validate metabolic function of particular symbionts is immunohistochemistry where tissue sections are treated with antisera against enzymes. This was already applied successfully to demonstrate the presence of the CO_2-fixing enzyme RubisCO in *O. algarvensis* Gamma 1 symbionts with gold-labeled antibodies and TEM-FISH (see Results and Discussion) and could be extended to other enzymes in question such as nitrate reductase. Identifying enzymes of the rTCA cycle or the reductive Acetyl CoA pathway could confirm the autotrophic potential of the deltaproteobacterial symbionts.

To investigate if substrates are metabolized by the symbionts as indicated by metagenomic and molecular results, for example urea by the gammaproteobacterial symbionts or volatile fatty acids by the deltaproteobacterial symbionts, the worms could be incubated with radiolabeled substrates. Subsequent microautoradiography with FISH could trace the uptake by the bacterial symbionts. In longer incubations, the substrates then might be further traced to the host thus demonstrating a mutualistic function of the symbionts. Fixed carbon in the symbioses has been tracked using radiolabeling and Nano-SIMS showing high CO_2 fixation activity of the primary symbionts (Bergin, 2009). Recent improvements in image analysis showed the transfer of freshly fixed carbon from the primary symbionts to the host (C. Bergin and L. Polerecky, personal communication). Future studies will focus on potential carbon and nitrogen substrates such as CO, acetate, propionate and urea to trace the fixation and uptake by the different symbionts and subsequent transfer to the host.

There was no information from the metagenomic studies on the spirochaetes' metabolism in *O. algarvensis*. However, these symbionts occur in various oligo-

chaetes worldwide and thus might play an important role for their hosts. To selectively investigate these symbionts, they could be isolated by micromanipulation (Fröhlich and König, 1999; Ishøy et al., 2006), a method already tested once for *I. leukodermatus* symbionts (Bergin, 2009), immunomagnetic cell capture (Pernthaler et al., 2008) or cell sorting using flow cytometry (Brehm-Stecher and Johnson, 2004). Another method is microfluidics (Chao and Ros, 2008) which is currenlty implemented by the Symbiosis Group in a cooperation with Paul Blainey to separate *O. algarvensis* symbionts. Single cells can also be separated from tissue by laser capture microdissection (LCM) (Espina et al., 2006). A combination of LCM with genomic techniques was proposed as an ideal way to study symbiont communities (Thornhill et al., 2008). Recently a complete symbiont genome was successfully assembled by single cell genomics (Woyke et al., 2010).

The same methods can be applied to the study of the filamentous ectosymbionts of *T. benedii* where first immunohistochemical localization of RubsisCO has been done in this thesis. This method needs to be optimized for RubisCO and used in junction with 16S rRNA FISH (results and discussion 2.3) and can then be extended to other enzymes. Another issue which has not been addressed experimentally, but is especially important in this facultative ectosymbiosis is recognition between symbiont and host and the factors leading to colonization. Does the host select and control the symbiotic population? Here, comparative genomics of symbiotic oligochaetes and related non-symbiotic oligochaetes might generate new insights. While differences in the symbiotic and nonsymbiotic host genetic components can already be a good indication, genes known to be involved in animal-host interaction could be specifically searched for in host and symbiont genomes, e.g. lectin-glycan systems that occur in a variety of animal-bacteria symbioses (Visick and McFall-Ngai, 2000; Bulgheresi et al., 2006; Wood-Charlson et al., 2006; Chaston and Goodrich-Blair, 2009). Other indicators for host-bacteria interaction are the type III secretion systems found in symbiont and pathogen genomes.

Experiments with cultured symbiotic and aposymbiotic hosts with different glycans could reveal whether they loosen or prevent attachment of bacteria by competing with the host receptor molecules. This has been successfully implemented to study the attachment mechanism of nematode ectosymbionts (Nussbaumer et al., 2004).

6 References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403-410.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143-169.

Bergin, C. (2009) Phylogenetic diversity and metabolic versatility of the bacterial endosymbionts in marine gutless oligochaete worms (PhD thesis). In *Fachbereich Biologie/ Chemie*. Bremen: University of Bremen.

Bergin, C., Brewig, N., Blazejak, A., Giere, O., Erséus, C., and Dubilier, N. (submitted) Replacement of the primary symbiont in the gutless marine worm *Inanidrilus exumae* by a sulfur oxidizer from a novel symbiont lineage.

Biebl, H., and Pfennig, N. (1978) Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. *Arch Microbiol* **117**: 9-16.

Brehm-Stecher, B.F., and Johnson, E.A. (2004) Single-cell microbiology: Tools, technologies, and applications. *Microbiol Mol Biol Rev* **68**: 538-559.

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

Chao, T.-C., and Ros, A. (2008) Microfluidic single-cell analysis of intracellular compounds. *J R Soc Interface* **5**.

Chaston, J., and Goodrich-Blair, H. (2009) Common trends in mutualism revealed by model associations between invertebrates and bacteria. *FEMS Microbiol Rev* **34**: 41-58.

Dattagupta, S., Schaperdoth, I., Montanari, A., Mariani, S., Kita, N., Valley, J.W., and Macalady, J.L. (2009) A novel symbiosis between chemoautotrophic bacteria and a freshwater cave amphipod. *ISME J* **3**: 935-943.

Dubilier, N. (1986) Association of filamentous epibacteria with *Tubificoides benedii* (Oligochaeta, Annelida). *Mar Biol* **92**: 285-288.

Dubilier, N., Blazejak, A., and Ruehland, C. (2006) Symbioses between bacteria and gutless marine oligochaetes. In *Molecular Basis of Symbiosis*. Overmann, J. (ed). New York: Springer Verlag, pp. 251-275.

Dubilier, N., Mulders, C., Ferdelman, T., de Beer, D., Pernthaler, A., Klein, M. et al. (2001) Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. *Nature* **411**: 298-302.

Espina, V., Wulfkuhle, J.D., Calvert, V.S., Van Meter, A., Zhou, W., Coukos, G. et al. (2006) Laser-capture microdissection. *Nat Protocols* **1**: 586-603.

Felske, A., Engelen, B., Nubel, U., and Backhaus, H. (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. *Appl Environ Microbiol* **62**: 4162-4167.

Fröhlich, J., and König, H. (1999) Rapid isolation of single microbial cells from mixed natural laboratory populations with the aid of a micromanipulator. *System Appl Microbiol* **22**: 249-257.

Gillan, D.C., and Dubilier, N. (2004) Novel epibiotic *Thiothrix* bacterium on a marine amphipod. *Appl Environ Microbiol* **70**: 3772-3775.

Goffredi, S. (2010) Indigenous ectosymbiotic bacteria associated with diverse hydrothermal vent invertebrates. *Environ Microbiol* doi:10.1111/j.1758-2229.2010.00136.x.

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

Harcombe, W. (2010) Novel cooperation experimentally evolved between species. *Evolution*: doi:10.1111/j.1558-5646.2010.00959.x.

Hubert, C., Loy, A., Nickel, M., Arnosti, C., Baranyi, C., Bruchert, V. et al. (2009) A Constant Flux of Diverse Thermophilic Bacteria into the Cold Arctic Seabed. *Science* **325**: 1541-1544.

Ishøy, T., Kvist, T., Westermann, P., and Ahring, B. (2006) An improved method for single cell isolation of prokaryotes from meso-, thermo- and hyperthermophilic environments using micromanipulation. *Appl Microbiol Biotechnol* **69**: 510-514.

Karavaiko, G., Dubinina, G., and Kondrat'eva, T. (2006) Lithotrophic microorganisms of the oxidative cycles of sulfur and iron. *Microbiology* **75**: 512-545.

Kleiner, M. (2008) Metaproteomics of the bacterial symbionts of the marine oligochaete worm *Olavius algarvensis*. In *Institute for Microbiology and Molecular Biology*. Greifswald: University of Greifswald, Germany.

Kleiner, M., Woyke, T., Ruehland, C., and Dubilier, N. (In press) The *Olavius algarvensis* metagenome revisited: lessons learned from the analysis of the low diversity microbial consortium of a gutless marine worm. In *Handbook of Molecular Microbial Ecology II: Metagenomics in Different Habitats.* de Bruijn, F.J. (ed). New Jersey: Wiley/Blackwell.

Loka Bharati, P.A. (2004) Synergy in the sulfur cycle: The biogeochemical significance of sulfate reducing bacteria in syntrophic associations. In *Marine Microbiology: Facets and opportunities*. Ramaiah, N. (ed): National Institute of Oceanography, Goa, pp. 27-35.

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

Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., and Stahl, D.A. (2009) Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* **461**: 976-979.

Musat, N. (2006) Molecular characterization of symbiotic associations between chemoautotrophic sulfur-oxidizing microorganisms and nematodes in shallow marine sediments. (PhD thesis). In *Fachbereich Biologie/Chemie*. Bremen: University of Bremen.

Nussbaumer, A.D., Bright, M., Baranyi, C., Beisser, C.J., and Ott, J.A. (2004) Attachment mechanism in a highly specific association between ectosymbiotic bacteria and marine nematodes. *Aquat Microb Ecol* **34**: 239-246.

Overmann, J., and Gemerden, H. (2000) Microbial interactions involving sulfur bacteria: implications for the ecology and evolution of bacterial communities. *FEMS Microbiol Rev* **24**: 591-599.

Pena, A., Teeling, H., Huerta-Cepas, J., Santos, F., Yarza, P., Brito-Echeverria, J. et al. (2010) Fine-scale evolution: genomic, phenotypic and ecological differentiation in two coexisting *Salinibacter ruber* strains. *ISME J*.

Perner, M. (2003) Biogeochemische und mikrobiologische Charakterisierung mariner Sedimente vor Elba - ein Beitrag zur ökosystemaren Analyse bakteriensymbiontischer Oligochaeten (Diplomarbeit). Biogeochemical and microbiological characterisation of marine sediments in Elba - a contribution to an ecosystematic analysis of oligochaetes with bacterial symbionts (Diploma thesis). In *Fachbereich Biologie*. Hamburg, Germany: University of Hamburg.

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

Pernthaler, A., Dekas, A.E., Brown, C.T., Goffredi, S.K., Embaye, T., and Orphan, V.J. (2008) Diverse syntrophic partnerships from deep-sea methane vents revealed by direct cell capture and metagenomics. *PNAS* **105**: 7052-7057.

Petersen, J., Ramette, A., Lott, C., Cambon-Bonavita, M.-A., Zbinden, M., and Dubilier, N. (2009) Dual symbiosis of the vent shrimp *Rimicaris exoculata* with filamentous gamma- and epsilonproteobacteria at four Mid-Atlantic Ridge hydrothermal vent fields. *Environ Microbiol*: doi:10.1111/j.1462-2920.2009.02129.x

Plague, G.R., Dunbar, H.E., Tran, P.L., and Moran, N.A. (2008) Extensive proliferation of transposable elements in heritable bacterial symbionts. *J Bacteriol* **190**: 777-779.

Polz, M.F., and Cavanaugh, C.M. (1995) Dominance of one bacterial phylotype at a mid-atlantic ridge hydrothermal vent site. *Proceedings of the National Academies and Sciences* **92**: 7232-7236.

Polz, M.F., Ott, J.A., Bright, M., and Cavanaugh, C.M. (2000) When bacteria hitch a ride. *Asm News* **66**: 531-539.

Polz, M.F., Distel, D.L., Zarda, B., Amann, R., Felbeck, H., Ott, J.A., and Cavanaugh, C.M. (1994) Phylogenetic analysis of a highly specific association between ectosymbiotic, sulfur-oxidizing bacteria and a marine nematode. *Appl. Environ. Microbiol.* **60**: 4461-4467.

Rinke, C., Schmitz-Esser, S., Stoecker, K., Nussbaumer, A.D., Molnar, D.A., Vanura, K. et al. (2006) *Candidatus* Thiobios zoothamnicoli, an ectosymbiotic bacterium covering the giant marine ciliate *Zoothamnium niveum*. *Appl Environ Microbiol* **72**: 2014-2021.

Ruehland, C., and Dubilier, N. (2010) Gamma- and epsilonproteobacterial ectosymbionts of a shallow-water marine worm are related to deep-sea hydrothermal vent ectosymbionts. *Environ Microbiol Rep* **in press**.

Ruehland, C., Bergin, C., Lott, C., and Dubilier, N. (2006) Darmlose marine Würmer: Symbiosen mit mikrobiellen Konsortien. *Biospektrum* **06**: 600-602.

Ruehland, C., Blazejak, A., Lott, C., Loy, A., Erséus, C., and Dubilier, N. (2008) Multiple bacterial symbionts in two species of co-occurring gutless oligochaete worms from Mediterranean sea grass sediments. *Environ Microbiol* **10**: 3404-3416.

Shimoyama, T., Kato, S., Ishii, S.i., and Watanabe, K. (2009) Flagellum Mediates Symbiosis. *Science* **323**: 1574.

Srivastava, A.K., and Schlessinger, D. (1990) Mechanism and regulation of bacterial ribosomal RNA processing. *Annu Rev Microbiol* **44**: 105-129.

Suzina, N.E., Mulyukin, A.L., Dmitriev, V.V., Nikolaev, Y.A., Shorokhova, A.P., Bobkova, Y.S. et al. (2006) The structural bases of long-term anabiosis in non-spore-forming bacteria. *Advances in Space Research* **38**: 1209-1219.

Teske, A., Sigalevich, P., Cohen, Y., and Muyzer, G. (1996) Molecular identification of bacteria from a coculture by denaturing gradient gel electrophoresis of 16S ribosomal DNA fragments as a tool for isolation in pure cultures. *Appl Environ Microbiol* **62**: 4210-4215.

Thauer, R.K., Stackebrandt, E., and Hamilton, W.A. (2007) Energy metabolism and phylogenetic diversity of sulphate-reducing bacteria. In *Sulphate-Reducing Bacteria - Environmental and Engineered Systems*. Barton, L.L., and Hamilton, W.A. (eds). Cambridge: Cambridge University Press, pp. 1-38.

Thornhill, D.J., Fielman, K.T., Santos, S.R., and Halanych, K.M. (2008) Siboglinidbacteria endosymbiosis: A model system for studying symbiotic mechanisms. *Commun Integr Biol* **1**: 163-166.

van den Ende, F.P., Meier, J., and van Gemerden, H. (1997) Syntrophic growth of sulfate-reducing bacteria and colorless sulfur bacteria during oxygen limitation. *FEMS Microbiol Ecol* **23**: 65-80.

Visick, K.L., and McFall-Ngai, M.J. (2000) An exclusive contract: specificity in the *Vibrio fischeri-Euprymna scolopes* partnership. *J Bacteriol* **182**: 1779-1787.

Watsuji, T., Nakagawa, S., Tsuchida, S., and Takai, K. (2008) Changes in epibiotic community structure and diversity of hydrothermal vent field animal *Shinkaia crosnieri* during long-term rearing. (Poster presentation). In *The 12th International Symposium on Microbial Ecology, ISME 12*. Cairns, Australia.

Widdel, F., and Bak, F. (1992a) Gram-negative mesophilic sulfate-reducing bacteria (1). In *The Prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer-Verlag, pp. 3352-3378.

Widdel, F., and Bak, F. (1992b) Gram-negative mesophilic sulfate-reducing bacteria. In *The prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York, Berlin, Heidelberg: Springer-Verlag New York Inc., pp. 3352-3389.

Wood-Charlson, E.M., Hollingsworth, L.L., Krupp, D.A., and Weis, V.M. (2006) Lectin/glycan interactions play a role in recognition in a coral/dinoflagellate symbiosis. *Cell Microbiol* **8**: 1985-1993.

Woyke, T., Tighe, D., Mavromatis, K., Clum, A., Copeland, A., Schackwitz, W. et al. (2010) One bacterial cell, one complete genome. *PLoS ONE* **5**: e10314.

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

VII Publications for this thesis

1. **Dubilier, N., Mülders, C., Ferdelman, T., de Beer, D., Hentschke, A., Klein M., Wagner, M., Erséus, C., Thiermann, F., Krieger, J., Giere, O., and Amann, R.** 2001. Endosymbiotic sulfate-reducing and sulfide-oxidizing bacteria in a gutless marine worm (Oligochaeta, Anneldia). 2001. Nature 411: 298-302.

   ND: experimental design and analysis, study supervision, writing of manuscript and revision; CR: experimental design and analysis, specimen collection and fixation, DNA extraction,16S rRNA gene library and phylogeny, probe design, FISH, sulfate reduction rates, autoradiography (silver needle experiment), manuscript revision; TF and DB: experimental design and analysis, sulfate reduction rates, microelectrode measurements, AH: confocal laser scanning microscopy, KM and MW: *dsrAB* gene library and phylogeny, manuscript revision; CE: specimen collection and species identification, manuscript revision; FT: specimen collection; JK: electron microscopy; OG: specimen collection, electron microscopy, manuscript revision; RA: manuscript revision

2. **Ruehland, C., Blazejak, A., Lott, C., Loy, A., Erséus, C., Dubilier, N.** 2008. Multiple bacterial symbionts in two species of co-occurring gutless marine worms from Mediterranean sea grass sediments. Environmental Microbiology. Published online 1 Sep 2008

   CR: experimental design and analysis, specimen collection, DNA extraction,16S rRNA, *cbbL*, and *aprA* gene libraries and phylogeny, probe design, FISH, writing of manuscript and revision; AB: specimen collection, DNA extraction, 16S rRNA, *cbbL* and *aprA* gene libraries, probe design, manuscript revision; CL: specimen collection, scanning electron microscopy analyses, manuscript revision; AL: *dsrAB* gene libraries and phylogeny, manuscript revision; CE: specimen collection and species identification, manuscript revision; ND: experimental design and analysis, study supervision, writing of manuscript and revision.

3. **Ruehland, C**., Dubilier, N. Epifilamentous bacteria of a Wadden Sea oligochaete are related to gamma- and epsilonproteobacterial deep-sea hydrothermal vent epibionts. In press.

   CR: experimental design and analysis, specimen collection and fixation, DNA extraction,16S rRNA, *cbbL*, and *aprA* gene libraries and phylogeny, probe design, (CARD-)FISH, writing of manuscript and revision; ND: experimental design and analysis, specimen collection and fixation, electron microscopy, study supervision, writing of manuscript and revision

VIII Additional publications

4. **Dubilier, N., Mülders, C., and Blazejak, A.** 2002. Eine ungewöhnliche Symbiose in einem darmlosen Meereswurm. BIOforum 3: 127-129

   CR: revision of manuscript, contribution of figures

5. **Behrens, S., Ruehland, C., Inacio, J., Huber, H., Fonseca, A., Spencer-Martins, I., Fuchs, B. M., and R. Amann.** 2003. In situ Accessibility of small-subunit rRNA of members of the domains Bacteria, Archaea, and Eucarya to Cy3-labeled oligonucleotide probes. Applied Environmental Microbiology 69:1748-1758.

   CR: probe design, hybridization, fluorescence assisted cell sorting

6. **Dubilier, N., Blazejak,A., Ruehland, C.** 2006. Symbioses between bacteria and gutless marine oligochaetes. In: J. Overmann (ed) Molecular Basis of Symbiosis. Springer Verlag, New York. 251-275.

   CR: data generation, revision of manuscript

7. **Woyke, T., Teeling, H., Ivanova, N.N., Richter, M., Hunteman, M., Gloeckner, F.O., Boffelli, D., Barry, K.W., Shapiro, H.J., Mussmann, M., Bergin, C., Ruehland, C., Amann, R., Anderson, I.J., Szeto, E., Kyrpides, N.C., Markowitz, V.M., Rubin, E.M., Dubilier, N.** 2006. Symbiosis insights through metagenomic analysis of a microbial consortium. Nature 443: 950-955.

   CR: member of manual annotation team

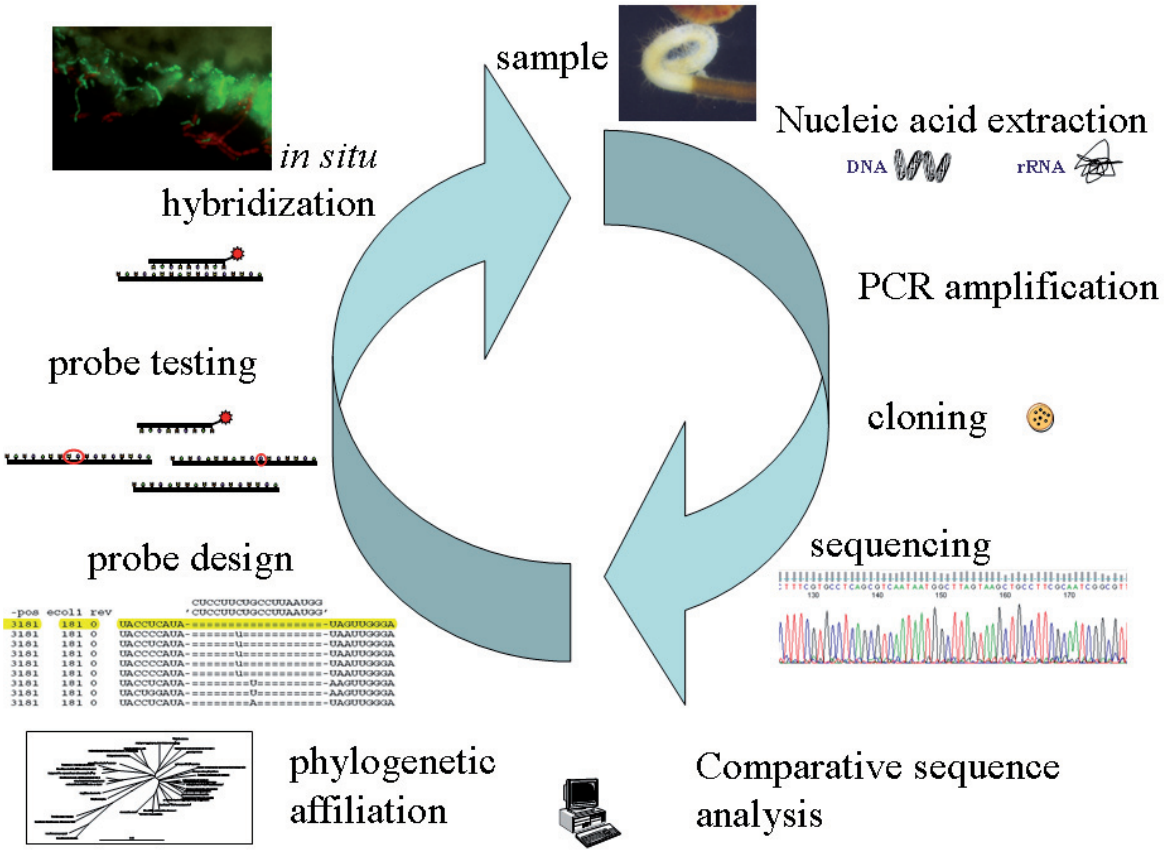
8. **Ruehland, C., Bergin, C., Lott, C., Dubilier, N.** 2006 Darmlose marine Würmer: Symbiosen mit mikrobiellen Konsortien. BIOspektrum 06:600-602

   CR: writing of manuscript, contribution of figures

9. **Kleiner, M., Woyke, T., Rühland, C., Dubilier, N.** 2010. The *Olavius algarvensis* metagenome revisited: lessons learned from the analysis of the low diversity microbial consortium of a gutless marine worm. In: Handbook of Molecular Microbial Ecology II; Metagenomics in Different Habitats. Ed: de Bruijn, F.J. Wiley/Blackwell.

   CR: phylogenetic analysis, contribution of figure and table, revision of manuscript

IX Appendix



The 16S rRNA approach (based on Amann et al. (1995))**:**

In the 16S rRNA approach bacterial DNA is extracted from environmental samples such as sediment or host tissue and phylogenetic and functional marker genes such as for the 16S rRNA are amplified using polymerase chain reaction. In inserting the different 16S rDNA genes into bacterial vectors such as plasmids, the genes are separated as each vector only takes up one copy. Cloning these vectors into chemically or electrically competent bacteria, allows the multiplication and storage of the genes which then can be extracted again and sequenced. Computer software such as the ARB program can then be used to phylogenetically analyze the genes (Ludwig et al., 2004). This program can also design specific probes for the bacterial 16S rRNA. New sequences are compared to those already in the public databases such as GenBank and ideally a region of about 18 oligomers with full match to the target- and at least one mismatch to nontarget-16S rRNA sequences is calculated. By labelling these oligomers with a fluorescent dye, the origin of the 16S rRNA can

be identified in situ (fluorescence in situ hybridization – FISH). By labeling the probes with DIG which is then detected by a horseradish peroxidase labeled antibody, the signal can subsequently be amplified by catalyzed reporter deposition with fluorochrome-labeled tyramides. This step increases the signal substantially and can be used when the monolabeled signal is too low. Thus, the 16S rRNA approach allows the assessment of the abundance of uncultivable and rare microorganisms from different environments in situ. This is important for ecological studies aiming to identify the key players in ecosystems because most organisms are to date unculturable.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 59: 143-169.

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

Danksagung

Ein großes Dankeschön an alle die zur Entstehung dieser Arbeit beigetragen haben! Für die freundliche Aufnahme in seine Arbeitsgruppe am MPI und die stete Unterstützung und das Vertrauen in meine Arbeit möchte ich mich ganz besonders bei Prof. Dr. Rudi Amann bedanken. Besonderer Dank gilt auch meiner Doktormutter, Dr. Nicole Dubilier, die mein Interesse, das vorher vor allem den Parasiten galt, für Symbiosen geweckt hat und mir die Arbeit am MPI und später in ihrer Arbeitsgruppe Symbiose ermöglicht hat. Im wissenschaftlichen Austausch konnte ich von ihrem analytischen Verstand und teils revolutionären Ideen profitieren. Symbiosen mariner darmloser Oligochaeten sind das Spezialgebiet von Prof. Dr. Giere und Prof. Dr. Christer Erséus, die meine Arbeit ebenfalls ein gutes Stück begleitet und unterstützt haben, wofür ich ihnen danken möchte.

Danke auch an die Mitglieder der Prüfungskommission Prof. Dr. Ulrich Fischer, Dr. Christian Borowski, Manuel Kleiner und Julia Zimmermann.

Prof. Dr. Friedrich Widdel und Dr. Ralf Rabus danke ich herzlich für die Einführung in die Welt der Sulfatreduzierer und deren anaerober Kultivierung. Bei Dr. Dirk de Beer und Dr. Tim Ferdelman bedanke ich mich für gemeinschaftlichen Laborarbeiten und anregende Diskussionen. Einen besonderen Dank für die Vermittlung eines mikrobiologischen Auslandsaufenthaltes und sein stets freundliches Interesse an und Enthusiasmus für meine Arbeit möchte ich Prof. Dr. Karlheinz Altendorf aussprechen.

Herzlicher Dank an Dr. Werner Armonies für die Benutzung seines Labors auf Sylt. Danke auch an das Team von HYDRA, Elba.

Sehr am Herzen liegt mir, mich für die große Einsatzbereitschaft und hochwertige Laborunterstützung vor allem durch Silke Wetzel zu bedanken. Auch Jörg Wulf, Daniela Lange und Lisa Kemp und viele andere fleißige Laborkräfte waren eine große Hilfe, die auf die kleinen und großen Fragen zwischendurch stets schnell antworteten oder gemeinsam eine Lösung suchten. Während meiner Schwangerschaften haben mich Dagmar Wöbken, Martha Schattenhöfer, Claudia Bergin und die schon erwähnten TAs super im Labor unterstützt. Ein großes Dankeschön hierfür.

Wie könnte ich die Kollegen der molekularen Ökologie, die ein paar Jahre lang meine Tage ausfüllten vergessen? Besonderer Dank für wissenschaftliche Unterstützung aber auch den einen oder anderen ‚comic relief' in der Teeküche möchte ich unseren Mollie-Dinosauriern aussprechen: Bernhard Fuchs und Frank-Oliver Glöckner. Desweiteren allergrößter Dank an meine Kollegin Claudia Bergin für Diskussionen und Korrekturlesen sowie einen eiskalten Spaziergang mit Hund an einer wunderschönen eisschschollen-bedeckten Elbe, den ich mir zwar vorgenommen, aber sonst nicht gemacht hätte. Viele weitere (Symbio-) Mollies haben mit Diskussionen, einigem Korrekturlesen zwischendurch - mit oder ohne Kaffee - oder metagenomic und computational problems geholfen. Danke dafür an Hanno Teeling, Katrin Knittel, Marc „Scully" Mussmann, Regina Schauer, Christian Lott, Jill Petersen, Anna Blazejak, Sebastian Behrens, Niculina Musat, Manuel Kleiner, Sabine Lenk, Renzo Kottmann, Michael Richter, Jost Waldmann, Quique Llobet-Brossa, Andreas Ellrott, Marga Bauer, Tanja Woyke und Thorben Stührmann.

Ohne die große Unterstützung, das Verständnis und die Geduld meiner Familie hätte ich diese Arbeit nicht geschafft! Liebevoller Dank gilt insbesondere Philipp, Paula und Pina und meiner Mutter Stephanie. Dem Rest meiner großen (Heuschrecken-) Familie ein Dankeschön für positives feedback und wunderschöne, regenerierende Urlaubspausen!