Phylogenetic diversity and metabolic versatility of the bacterial endosymbionts in marine gutless oligochaete worms

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Claudia Bergin

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Front: *Olavius* sp., Elba (picture courtesy Christian Lott); nanoSIMS image showing the uptake of ^{13}C by the Gamma 1 symbionts of *Olavius* sp. Elba; geographic distribution of gutless oligochaetes
Back: Horseshoe Beach, Bermuda

Wollte ich Vollkommenheit anstreben,
würde mein Buch nie fertig.
Tai T'ung, 13. Jhdt.

Die Physik [respektive Biologie, die Autorin]
erklärt die Geheimnisse der Natur nicht,
sie führt sie auf tiefer liegende Geheimnisse zurück.
Carl Friedrich von Weizsäcker (1912 – 2007)

Die Untersuchungen zur vorliegenden Arbeit wurden am Max-Planck-Institut für Marine Mikrobiologie in Bremen durchgeführt.

1. Gutachter: Dr. Nicole Dubilier
2. Gutachter: Prof. Dr. Michael Friedrich

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Summary

Marin gutless oligochaete worms (Annelida, Phallodrilinae) live in an obligate association with bacterial endosymbionts. Each host, belonging to one of the two genera *Olavius* or *Inanidrilus*, harbours a specific, but morphologically, phylogenetically and metabolically diverse symbiont community. The primary symbionts of gutless oligochaetes, called Gamma 1, are large chemoautotrophic sulfur-storing bacteria that form a monophyletic clade within the Gammaproteobacteria and had been found in all host species studied so far. Secondary symbionts of gutless oligochaetes belong to the Alpha-, Gamma- and Deltaproteobacteria and to the Spirochaetes. In this PhD thesis the diversity and function of gutless oligochaete symbiont communities was investigated.

In a first part, the phylogenetic and metabolic diversity of *I. exumae* was studied. The symbiont community of this host differed markedly from that of other gutless oligochaetes. Sulfate-reducing deltaproteobacterial symbionts co-occurred with alpha-proteobacterial symbionts in this host, showing that these do not mutually exclude each other as previously assumed. Furthermore, a large novel gammaproteobacterial symbiont only distantly related to the Gamma 1 symbionts, but morphologically similar, dominated the symbiont community, while no indication was found for a Gamma 1 symbiont. The presence of sulfur and genes diagnostic for autotrophy and sulfur oxidation indicate that this new symbiont is a sulfur-storing chemoautotroph. Thus, the novel symbiont seems to share its morphology and its function with the Gamma 1 symbionts and may have replaced the Gamma 1 symbiont in *I. exumae*.

To learn more about the ecophysiology of gutless oligochaete symbioses, the autotrophic activity was investigated in a second project with tracer incubation experiments. Analyses of radiolabelled inorganic carbon uptake and sulfur content of individual *Olavius algarvensis* worms showed that in the presence of oxygen, internally stored sulfur was used as an energy source for the incorporation of inorganic carbon into biomass. In the absence of oxygen or sulfur, inorganic carbon was taken up at lower rates. The electron donors and electron acceptors used under anoxic conditions could not be unambiguously identified. However, increased carbon fixation occurred in the presence of nitrate, sulfide and thiosulfate in a few worms.

Identification of the autotrophic symbionts in the *O. algarvensis* symbiont community was achieved in a third project by applying in situ hybridization combined with microautoradiography (MARFISH) or high resolution mass spectrometry (nanoSIMS-HISH). The Gamma 1 symbionts immediately incorporated inorganic carbon into biomass under oxic conditions in the absence of external energy sources suggesting the usage of internally stored sulfur as electron donor. Uptake rates of individual cells varied, but were on average in the range of those found for free-living sulfur bacteria and chemoautotrophic symbionts. For the first time, the autotrophic symbiont could be directly identified and the inorganic carbon uptake analyzed for individual symbionts within the gutless oligochaete symbiosis.

Zusammenfassung

Marine darmlose oligochaete Würmer (Annelida, Phallodrilinae) leben in Symbiose mit bakteriellen Endosymbionten. Jeder Wirt, der zum Genus *Olavius* oder zum Genus *Inanidrilus* gehört, beherbergt seine spezifische, jedoch morphologisch, phylogenetisch und physiologisch diverse Symbiontengemeinschaft. Die Primärsymbionten der darmlosen Oligochaeten, sogenannte Gamma-1, sind große chemoautotrophe schwefelspeichernde Gammaproteobakterien, die eine monophyletische Gruppe bilden und bisher für alle untersuchten Wirtsarten beschrieben wurden. Sekundärsymbionten gehören zu den Alpha-, Gamma- und Deltaproteobakterien und zu den Spirocheten. Das Thema dieser Arbeit war die Diversität und Funktion der Symbiontengemeinschaften darmloser Oligochaeten.

In einem ersten Projekt wurde die Symbiontengemeinschaft von *I. exmuae* untersucht. Die Gemeinschaft dieses Wirts unterschied sich deutlich von allen bisher bekannten Symbiontengemeinschaften darmloser Oligochaeten. Anders als bisher angenommen, schließen sich alphaproteobakterielle und sulfatreduzierende deltaproteo-bakterielle Symbionten nicht aus und kommen in *I. exumae* gemeinsam vor. Darüberhinaus fand sich kein Hinweis auf das Vorkommen eines Gamma-1-Symbionten. Stattdessen dominierte ein neuer großer gammaproteobakterieller Symbiont, der nur entfernt mit den Gamma-1-Symbionten verwandt ist, diesen jedoch morphologisch ähnelt. Der Nachweis von Schwefel sowie von Genen, die diagnostisch sind für Autotrophie und Schwefeloxidation, impliziert, dass der neue Symbiont ein autotropher Schwefeloxidierer ist. Damit gleicht dieser neue Symbiont dem Gamma-1-Symbionten morphologisch und funktionell und scheint den Gamma-1-Symbionten in *I. exumae* ersetzt zu haben.

Die Bedeutung der Autotrophie für die Symbiose wurde in dieser Arbeit am Beispiel von *O. algarvensis* in Inkubationsexperimenten untersucht. Die Analyse des Schwefelgehalts einzelner Würmer sowie des Einbaus von radioaktiv-markiertem CO_2 in Biomasse zeigte, dass der gespeicherte Schwefel in Anwesenheit von Sauerstoff als Energiequelle für die autotrophe CO_2-Fixierung genutzt wurde. Ohne Sauerstoff oder Schwefel war die CO_2-Fixierung reduziert. Mögliche Elektronendonatoren und Elektronenakzeptoren unter anoxischen Bedingungen konnten nicht eindeutig identifiziert werden. Jedoch zeigten einzelne Würmer deutlich erhöhte CO_2-Aufnahmeraten mit Nitrat, Sulfid und Thiosulfat.

Die Identifizierung der autotrophen Symbionten von *O. algarvensis* und die Analyse der CO_2-Aufnahme auf Einzelzellebene gelang mit einer Kombination aus in situ-Hybridisierung und Mikroautoradiographie (MARFISH) sowie mit hoch auflösender Massenspektrometrie (nanoSIMS-HISH). Unter oxischen Bedingungen in Abwesenheit externer Energiequellen fixierten die Gamma-1-Symbionten von *O. algarvensis* CO_2. Fixierungsraten variierten stark für einzelne Gamma-1-Symbionten und lagen im Mittel in der Größenordnung von freilebenden Schwefelbakterien und chemoautotrophen Symbionten. Damit konnten in dieser Arbeit zum ersten Mal die autotrophen Symbionten in der Oligochaetensymbiose identifiziert und deren Aufnahmerate bestimmt werden.

List of abbreviations

APS	adenosine-5'-phosphosulfate
ASW	artificial seawater
CARD	catalyzed reporter deposition
CBB	Calvin-Benson-Bassham
CO	carbon monoxide
CODH	carbon monoxide dehydrogenase
DAPI	4',6-diamidino-2-phenylindol
DNA	deoxyribonucleic acid
FACS	fluorescence assisted cell sorting
FISH	fluorescence in situ hybridization
h	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
kb	kilo base pairs
kDA	kilo Dalton
LSC	liquid scintillation counting
MAR	microautoradiography
MDA	multiple displacement amplification
MIMS	multiple isotope mass spectrometry
min	minute
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pH	minus the decimal logarithm of the hydrogen ion activity in an aqueous solution
PHA	polyhydroxyalkanoate
PHB	poly-β-hydroxybutyric acid
RNA	ribonucleic acid
rRNA	ribosomal RNA
RubisCO	ribulose-1, 5-bisphosphate carboxylase/ oxygenase
SIMS	secondary ion mass spectrometry
sp.	species
spp.	several species
TCA	tricarboxylic acid
TMAO	trimethylamine N-oxide

Part A - Combined presentation of results

1 Introduction

1.1 Symbiosis

"Symbiosis has a major role in shaping the evolution and diversity of eukaryotic organisms (Margulis 1993). Remarkably, it is only in recent times that there has been an emerging recognition that most eukaryotic organisms are intimately associated with a complex community of beneficial microbes that are essential for their development, health and interactions with the environment (Ruby et al. 2004)."[1]

Symbioses are defined as close, long-term or permanent associations between two or more species. The botanists Albert Bernhard Frank (Frank 1877) and Anton de Bary (De Bary 1879) introduced this term to biology to describe the dual nature of lichens as associations between algae and fungi. At that time, lichens were considered plants and symbiosis was a rather revolutionary idea. The term symbiosis was originally defined broadly and included associations such as epiphytes growing on trees. Since then it has been redefined to describe only beneficial associations. Today, many scientists have returned to a slight modification of the original definition that includes three types of associations: i) mutualistic or beneficial, where all symbiotic partners benefit, ii) commensalistic or neutral, where one partner benefits and the other is neither harmed nor benefited and iii) parasitic or harmful, in which one of the organisms benefits at the expense of the other. In symbioses of different sized partners, the larger partner is defined as the host and the smaller partner as the symbiont. Symbiotic associations can be obligate or facultative and are manifested on the genetic, metabolic or behavioural level. In mutualistic symbioses, the benefits to the host and symbionts include nutrition (the most common interaction), defence, sexual fitness or protection from predators (Moya et al. 2008, Starr 1975).

Marine chemosynthetic symbioses have a very recent history in symbiosis research. Nevertheless, they have made an impressive entrance. The first chemosynthetic symbioses were discovered about three decades ago in the deep sea (Cavanaugh et al. 1981, Felbeck 1981, Rau 1981), a habitat, until then, believed to be a hostile, dark, food-limited desert. This point of view changed with the discovery of hydrothermal vents, where many of the remarkably diverse fauna live in symbiosis with bacteria (van Dover 2000). Today, chemosynthetic symbioses are known to exist in a wide range of marine habitats, with a tremendous degree of diversity in bacterial symbiont species, as well as host species, which represent numerous phyla and a variety of adaptations to their symbiotic life. These aspects will be introduced in the following chapters.

[1] Adapted from Woyke et al. 2006

1.2 Symbioses between marine invertebrates and chemosynthetic bacteria

The first marine deep-sea symbiotic host discovered was the gutless tubeworm *Riftia pachyptila*, probably the best-known example of a chemosynthetic symbiosis. This symbiosis was soon described as being chemoautotrophic. Chemoautotrophy is short for chemolithoautotrophy and describes the ability to derive energy for autotrophic carbon fixation from the oxidation of chemical compounds, as opposed to phototrophy, in which light is used as an energy source (Table 1.1). Chemosynthesis is, in the strict sense, the synthesis of organic material from inorganic carbon and water, with energy derived from the chemical oxidation of simple inorganic compounds such as reduced sulfur species. In the more commonly used and encompassing sense, methanotrophy is included, where the organic molecule methane serves as both the energy and carbon source. Strictly speaking, however, methanotrophic bacteria are chemoorgano-heterotrophs, because methane is commonly defined as an organic compound[2]. (although its origin can be either biotic or abiotic).

Table 1.1: Classification of physiological strategies using different sources of carbon and energy.

Physiological type	Carbon source	Energy source
Photoautotroph	inorganic C	sunlight
Photoheterotroph	organic C	sunlight
Chemolithoautotroph	inorganic C	inorganic
Chemosynthetic	inorganic C and/ or methane	inorganic compounds or methane
Facultative chemolithoautotroph	inorganic or organic C	inorganic or organic compounds
Chemolithoheterotroph	organic C	inorganic compounds
Chemoorganoheterotroph	organic C	organic C

Chemosynthetic symbionts known today are either sulfur-oxidizing or methane-oxidizing bacteria. Both are dependent on the supply of reduced compounds such as sulfide (in this work, the term sulfide refers to total dissolved sulfide: H_2S, HS^- and S^{2-}) or methane as their energy sources and oxygen as electron acceptor. Other energy sources such as hydrogen, ammonia and iron, or other electron acceptors such as nitrate have been considered, but not yet conclusively shown to be important or used in symbioses for biomass generation (Cavanaugh et al. 2006). In many symbioses the chemosynthetic role of the symbionts was only inferred from ultrastructural, biochemical or genetic analyses, and remains to be proven.

Both host and symbiont benefit from their respective associations. The host species are well adapted to provide their symbionts with electron donors and electron acceptors, which may be temporarily or spatially separated in the habitat. The symbionts are often also protected from predators. In return the host receives its nutrition from the symbiont

[2] The terms organic and inorganic are historic with organic compounds originally defined to be synthesized only by living organisms.

via transfer of organic compounds or lysis of symbiont cells. This allows the host to dwell in otherwise often hostile habitats where organic matter can be limiting.

1.2.1 Habitats of marine chemosynthetic symbioses

Habitats for chemosynthetic symbioses can be as extreme and different as shallow water sulphidic sediments, tidal mud flats and mangrove swamps, coral reef sediments, reducing sediments on continental shelves, mud volcanoes or deep sea hydrothermal vents and cold seeps (Figure 1.1). Deep-sea hydrothermal vents were the first habitat discovered to house chemosynthetic symbioses. This ecosystem is unique in that it relies on chemosynthesis rather than photosynthesis, which drives most ecosystems on earth. Hydrothermal fluids are discharged from the seafloor, either exiting as hot fluids through chimney-like structures known as black smokers, or as diffusive, low temperature flows (van Dover 2000). These fluids provide the chemosynthetic community with reduced compounds, such as sulfide and methane, while oxygen is available from the surrounding seawater. However, this chemosynthetically driven ecosystem is not independent of photosynthesis. Many microorganisms and all vent animals depend on oxygen, which is produced photosynthetically in the euphotic zones.

Cold seeps are an additional deep-sea habitat and often characterized by thick sediment layers. When the sediment is compressed, fluids and gases are expelled transporting reduced inorganic compounds to the sediment surface, where they support a chemosynthetic community. Sulfide and methane are mostly of biogenic origin due to microbial activity in the sediment, although thermogenic reactions do occur (Sibuet and Olu 1998). Some chemosynthetic host families occur at both vents and seeps (Dubilier et al. 2008) while at the species level most species are endemic to one or the other habitat type.

Whale and wood falls are short-term habitats. Whale and wood falls provide the organic-poor deep-sea with a sudden input of organic material when a dying whale, wood of terrestrial origin transported by rivers and currents, or even a wooden shipwreck sink to the ocean floor. When the lipid-rich whale bones and the wood degrade, sulfide builds up, supporting the development of a chemosynthetic community (Smith and Baco 2003). Whale and wood falls can attract a specialized animal community, among them the symbiont-bearing bone-eating worm *Osedax* (see below).

Vents, seeps, whale and wood falls also occur in shallow waters. However, the characteristic vent and seep symbioses are rare at these sites, while phototrophic and heterotrophic communities dominate (Tarasov et al. 2005). The more typical shallow-water habitats for chemosynthetic symbioses are sulfidic sediments. Their chemosynthetic communities were discovered only shortly after the deep-sea vents (Felbeck et al. 1981). Today shallow-water chemosynthetic symbioses are known from

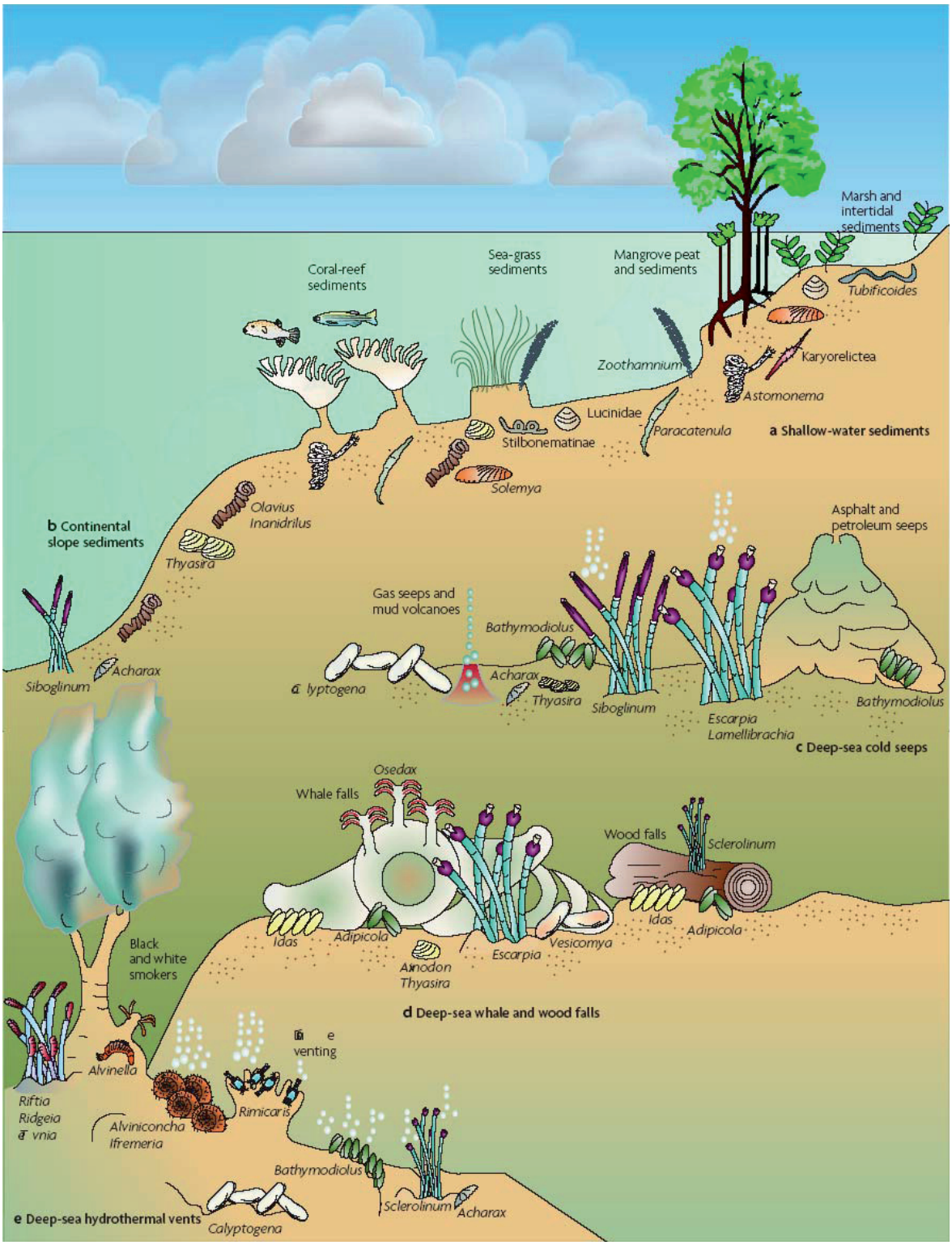


Figure 1.1: Habitats with chemosynthetic symbioses. (Fig. adapted from Dubilier, Bergin and Lott 2008)

tidal mud flats, coral reef sediments, sea grass beds and sewage outflows (Figure 1.1, reviewed in Cavanaugh et al. 2006, Dubilier et al. 2008). Shallow-water sediments are characterized by oxic overlaying water and often oxidized upper sediment layers, while

deeper sediment layers are anoxic and sulfidic due to microbial degradation of organic matter and sulfate-reducing activity. The overlapping zone where both oxidant and reductant are accessible ("chemocline") can be either very narrow or very broad. Symbiotic clams and small annelid and nematode worms frequently occur in these shallow-water habitats.

1.2.2 The host – adaptation and diversity

Host adaptation

The variety of habitats where chemosynthetic symbioses occur is reflected in the variety of host adaptations to supply their symbionts with electron donors and electron acceptors. When reductants and oxidants like oxygen and sulfide are temporarily or spatially separated, e.g. in shallow-water sulfidic sediments or at cold seeps, the host must bridge this gap. Smaller animals, such as nematodes or the gutless oligochaetes (see Chapter 1.3), move up and down between the oxic and sulfidic sediment layers, and many symbionts store sulfur and/ or carbon to withstand times of substrate limitation. An alternative strategy to overcome the gap is used by some bivalves including *Solemya* species, which build Y-shaped burrows. Sitting at the intersection, the bivalve pumps seawater through the upper part of the burrow for oxygen supply while sulfide is accessible via the lower part of the burrow (Scott and Cavanaugh 2007). Vesicomyid clams, on the other hand, bury their foot in the sediment where they take up sulfide via a specific sulfide-binding protein (Zal et al. 2000). Oxygen is accessible in the water column and enters via the clam's siphon. In a similar way, some siboglinid tubeworms bury their extended roots deep in the sulfidic sediment to scavenge for sulfide, while oxygen is locally present in the surrounding seawater and taken up via the plume, which is well supplied with blood (reviewed in Dubilier et al. 2008). At vent sites, oxygen, methane and sulfide are accessible from the surrounding water. Tubeworms at these sites, e.g. *R. pachyptila*, take up oxygen, nitrate and sulfide via their branchial plume. Bound to special high affinity haemoglobins, these compounds are transported to the trophosome where the symbionts are located (reviewed in Dubilier et al. 2008). Bathymodiolin mussels lack such specialized adaptations (Nelson and Fisher 1995). Accordingly, their symbionts are located in the apical part of the bacteriocytes in the gill tissue where they are in closer contact with the circulating water which transports the substrates required for symbiosis functioning (van Dover 2000).

Host diversity

Marine chemosynthetic symbioses are known from seven host phyla (Dubilier et al. 2008). The symbionts can be ecto- or endosymbionts. Ectosymbionts are associated with the surface of their host, while endosymbionts are either intracellular or extracellular within the host (van Dover 2000). The intracellular endosymbionts are

restricted to specialized cells referred to as bacteriocytes (Fiala-Médioni and Metivier 1986, Fiala-Médioni et al. 1986b). Some hosts have evolved specialized organs such as the trophosome in siboglinid tubeworms (reviewed in Bright and Giere 2005). Seldom endo- and ectosymbionts co-occur within a single host. An overview of chemosynthetic hosts and their symbionts is given below.

Protozoa associated with bacteria can be found in deep-sea and shallow-water habitats (Kouris et al. 2007, Ott et al. 2004a, Ott et al. 1998). Although a symbiotic association for several ciliates has been suggested (Ott et al. 2004a), only the symbiosis of the ciliate *Zoothamnium niveum* has been studied in detail, which inhabits mangrove peat. Its ectosymbiont *Candidatus* Thiobios zoothamnicoli was identified as a thioautotrophic sulfur-storing bacterium (Ott et al. 1998, Rinke et al. 2006, Rinke et al. 2007). Within the protozoa symbioses, clearly more remains to be discovered and investigated.

Descriptions of sponges associated with chemosynthetic bacteria are rare despite extensive studies of the microorganisms associated with sponges (reviewed in Taylor et al. 2007). A *Cladorhiza* species was found to harbour a methane-oxidizing symbiont and derives at least some of its nutrition via methanotrophy (Vacelet and Boury-Esnault 2002).

Little is known about Platyhelminthes with chemosynthetic symbionts. The mouthless *Paracatenula* is a shallow water flatworm. Its symbionts are located in a modified gut, where the presence of sulfur implies a thiotrophic metabolism for the symbionts (Gruber and Ott 2005, Ott et al. 1982).

Chemosynthetic host species of the Nematoda inhabit primarily shallow-water reducing sediments and are most abundant around the redox potential boundary layer (reviewed in Ott et al. 2004a, b). The nematode hosts can harbour either ecto- or endosymbionts. Ectosymbionts are associated with the Stilbonematinae where coccoid to rod-shaped bacteria form a dense host-species or host-genus specific pattern on the host cuticle (Polz et al. 1992). This symbiont coat can either be a mono-layer as on *Catanema*, *Laxus*, *Robbea* and some *Leptonemella* species, or multi-layered as on *Stilbonema* and other *Leptonemella* species (reviewed in Ott et al. 2004a, b). The monolayered symbiotic coat on worms of the genus *Eubostrichus* is special as the symbionts are filamentous bacteria (Polz et al. 1992). The Stilbonematinae symbionts are sulfur-oxidizing chemoautotrophs. They store sulfur and possibly polyhydroxyalkanoates (PHA; Hentschel et al. 1999, Polz et al. 1992). The hosts graze upon their symbiont for nutrition (Ott and Novak 1989, Ott et al. 1991, Polz et al. 1992). Grazing to meet their nutritional needs is not an option for nematode hosts that harbour endosymbionts. Endosymbionts occur within the genera of *Astomonema*, *Parastomonema* and *Rhaptothyreus*, the latter a deep-sea representative (reviewed in Ott et al. 2004a, b). The symbionts completely fill the lumen of the host's modified gut and have chemoautotrophic potential (Giere et al. 1995b, Musat et al. 2007).

Molluscan hosts of the Bivalvia and Gastropoda are widespread and occur in a variety of habitats, from shallow-water sulfidic sediments and whale falls to deep-sea hydrothermal vents. Within the Bivalvia, vesicomyid and bathymodiolin species are deep-sea seep and vent inhabitants. The bathymodiolin species *Idas* and *Adipicola* have been found at seeps, whale and wood falls (Dubilier et al. 2008, Duperron et al. 2008b). Representatives of the Lucinidae, Solemyidae and Thyasiridae occur at deep-sea and shallow-water sites. While all species of the Lucinidae, Solemyidae, Vesicomyidae and Bathymodiolinae harbour symbionts (reviewed in Cavanaugh et al. 2006), only some species of the Thyasiridae are symbiotic (Dufour 2005). Most bivalve hosts harbour endosymbionts within bacteriocytes in their enlarged gills. In contrast, most symbiotic Thyasiridae are associated with ectosymbionts. The hosts' dependence on their symbionts for nutrition is indicated by an often much reduced gut, although filter feeding can play a role in some species (Page et al. 1990, Page et al. 1991, von Cosel 2002). The majority of bivalve host species harbour a single sulfur-oxidizing symbiont (Dubilier et al. 2008). Some *Bathymodiolus* mussels are associated with a methane-oxidizing symbiont (Cary et al. 1988, Childress et al. 1986, Fisher et al. 1987). Interestingly, a few bathymodiolin mussels have established dual symbioses with a sulfur and methane oxidizer (Distel et al. 1995, Duperron et al. 2006, Fiala-Médioni et al. 2002, Fisher et al. 1993), or symbiont communities with up to six different symbionts of which at least one is a sulfur- and one a methane-oxidizing symbiont (Duperron et al. 2007, Duperron et al. 2008a).

The snails *Ifremeria nautilei* and *Alviniconcha hessleri* (Gastropoda, Mollusca) occur at hydrothermal vents. They are associated with chemoautotrophic endosymbionts located in their gills. *I. nautilei* may in addition harbour a methanotrophic symbiont (Dubilier et al. 2008). The symbionts of the vent gastropod *Lepetodrilus fucensis*, a limpet, are epibionts and embedded in the gill epidermis. This host is able to graze and feed on suspended matter and its symbionts seem not to be its major food source (Bates 2007). The recently discovered "scaly snail" is different in that the endosymbionts are not located within or next to the gill. Instead, they reside in an enlarged esophageal gland (Goffredi et al. 2004). In addition to these endosymbionts, a number of microbes are associated with the exterior of this snail, in particular with the iron scales on the snail's foot (Goffredi et al. 2004, Waren et al. 2003).

Within the Annelida, host species belonging to the Polychaeta occur at deep-sea habitats. The alvinellid polychaetes *Alvinella pompejana* and *A. caudata* inhabit and often dominate hydrothermal vents of the Eastern Pacific. The diverse filamentous episymbiotic bacteria of *A. pompejana* are located on the host's dorsal surface with their ends embedded in a mucus-like structure, although differently shaped bacteria can also occur (reviewed in Bright and Giere 2005). Genes for autotrophy, sulphur oxidation, denitrification and amino acid synthesis were found in the epibiotic community, and a

nutritional role has been suggested for the *A. pompejana* symbiosis (Grzymski et al. 2008).

In contrast to the alvinellid polychaetes, the symbionts of siboglinid polychaetes reside within host bacteriocytes. The siboglinids comprise four subgroups, of which the Vestimentifera, Monilifera and Frenulata inhabit vents, seeps, and whale and wood falls. They have evolved a trophosome harbouring the endosymbionts, whereas in *Osedax* species the symbionts are located in a modified root (Bright and Giere 2005, Goffredi et al. 2005). Almost all siboglinid endosymbionts are sulfur oxidizers, with the well-known *R. pachyptila* symbiont being the best studied. Only one tubeworm, *Siboglinum poseidoni* appears to be associated with a methane-oxidizing symbiont (Schmaljohann and Flugel 1987). The presence of a methane-oxidizing symbiont in *S. contortum* (Pimenov et al. 2000) could not yet be confirmed (Lösekann et al. 2008). In contrast to the chemosynthetic siboglinid symbionts, the *Osedax* symbionts are assumed to be heterotrophic (Goffredi et al. 2005). *Osedax* worms are found exclusively on whale bones (Goffredi et al. 2005, Rouse et al. 2004).

Shallow-water representatives within the Annelida are the oligochaetes. Currently the only described host with ectosymbionts, *Tubificoides benedii*, lives in sulphidic North Atlantic coastal tidal flat sediments (Dubilier 1986, Dubilier et al. 1995). Rod- to coccoid-shaped bacteria inhabit the mucus surrounding the body of this oligochaete worm. Filamentous bacteria are restricted to the very posterior end of the worm (Dubilier 1986, Giere et al. 1988a), where they intrude the host's epidermal layer based on ultrastructural analysis, indicating a very close interaction of these filamentous bacteria with their host (Dubilier 1986). A chemoautotrophic role for at least some of the symbionts was suggested (Dubilier et al. 1997).

Gutless oligochaetes are small worms with almost worldwide distribution. This host group is the only one known so far in which all species studied harbour at least three phylogenetically distinct bacterial endosymbionts. The gutless oligochaete symbiosis is the focus of this work and will be presented in detail in Chapter 1.3.

Within the Arthropoda, the few known chemosynthetic host species all carry ectosymbionts. The vent shrimp *Rimicaris exoculata* (Alvinocarididae, Decapoda) is abundant at many Mid-Atlantic Ridge vent sites. Its filamentous symbionts are located on the external carapace, the mouthparts, and the inner surfaces of its enlarged gill chamber (Zbinden et al. 2004). The role of the symbionts for the host's nutrition is unclear (reviewed in Schmidt et al. 2008). Another arthropod host has been recently discovered. This "hairy" deep-sea crab *Kiwa hirsuta* (Gallatheoidea, Decapoda) from the Pacific Antarctic Ridge is packed with morphologically and phylogenetically diverse bacteria (Goffredi et al. 2008). A chemosynthetic potential has been suggested for this association, although it could not be unambiguously linked to one of the bacteria present (Goffredi et al. 2008).

1.2.3 The symbionts – adaptation and diversity

Symbiont adaptation

Remarkably little is known about how the symbionts have adapted to their symbiotic lifestyle through metabolic or genomic modifications. While symbiont metabolism via methanotrophy or chemoautotrophy is at least in part understood, it is unclear how the symbionts provide their host with nutrition. Possible means of host nutrition are the lysis of symbiont cells and digestion by the host, the translocation of organic carbon, or a combination of both (Cavanaugh et al. 2006, Fisher 1990). Lysis of symbiont cells was suggested based on ultrastructural observations of lysosomal structures (degenerate stages of bacteria) in the basal part of bacteriocytes of bivalves, in the peripheral bacteriocytes in the trophosome of *R. pachyptila* (Barry et al. 2002, Bright and Sorgo 2003, Cavanaugh et al. 1992, Fiala-Médioni and Metivier 1986, Fiala-Médioni et al. 1986b, Frenkiel et al. 1996), and in the symbiotic region of oligochaetes (Giere and Langheld 1987, Giere and Milligan 1989). The detection of lysosomal and protein-degrading enzymes in bivalve gills and tubeworm tissue further supports that symbionts are digested by the host (Boetius and Felbeck 1995, Fiala-Médioni et al. 1994, Nelson et al. 1995, and references therein). Host nutrition via symbiont digestion was also linked to slow transfer and incorporation of symbiont derived compounds into host biomass (Fisher 1990, Fisher and Childress 1992). In contrast, quick turnover rates and incorporation of labelled carbon into host tissues have been associated with a transfer of symbiont metabolites to the host (Bright et al. 2000, Fisher and Childress 1986). The potential metabolites have not yet been identified, nor have the genes or enzymes which are involved in nutrient transfer. However, compounds such as malate, succinate and other organic acids were excreted by the *R. pachyptila* symbionts and might be taken up by the host (Felbeck and Turner 1995, Felbeck and Jarchow 1998).

Genomic modifications are seen in some obligate symbionts. Typical genomic changes in symbionts compared to free-living relatives are highly biased nucleotide base composition with elevated frequencies of adenine and thymine (AT bias) (Silva et al. 2007), loss of genes, and reduced genome size. The genomes of two deep-sea vesicomyid clam symbionts showed these modifications (Kuwahara et al. 2007, Newton et al. 2007). Other symbionts seem to lack such characteristic modifications: neither reduced genome size nor AT bias were found in tubeworm and oligochaete symbionts (Robidart et al. 2008, Woyke et al. 2006).

Genomic modifications seem to reflect the mode of symbiont transmission. Symbionts are transmitted either vertically, that is from parent to offspring, or transmitted horizontally, where symbionts are taken up from the environment each generation. Horizontally transmission was found for the chemoautotrophic sulfur-oxidizing symbionts of *R. pachyptila* (Nussbaumer et al. 2006), lucinid clams (Gros et al. 1996, Gros et al. 2003), and *Bathymodiolus* mussels (Won et al. 2003). Environmentally (or horizontally) transmitted symbionts do not show apparent genome

modifications like genome reduction or AT bias in contrast to vertically transmitted symbionts, such as the vesicomyid clam symbionts. However, genomic data on symbionts is still scarce and other correlations might be found.

Although there is strong evidence for symbiont-host specificity for both vertically and horizontally transmitted symbionts within chemosynthetic symbioses, the genetic or biochemical nature of symbiont recognition and acquisition are barely understood. For a few nematode symbioses it could be shown that lectins are involved in ectosymbiont acquisition (Bulgheresi et al. 2006, Nussbaumer et al. 2004).

Symbiont diversity

Symbiont-host specificity is partly reflected by the symbiont's phylogenetic affiliation. Marine chemosynthetic symbionts belong primarily to the Gammaproteobacteria, based on comparative 16S ribosomal RNA (rRNA) sequence analysis. Several clades comprising sulfur-oxidizing symbionts are known which, in most cases, form host-specific clades (Dubilier et al. 2008). The chemoautotrophic symbionts of *Solemya* species are one exception, as their 16S rRNA sequences are scattered throughout the Gammaproteobacteria (Figure 1.4, Dubilier et al. 2008, Krueger and Cavanaugh 1997). The methane-oxidizing symbionts of bathymodiolin mussels form their own distinct clade within the Gammaproteobacteria (Dubilier et al. 2008). Only a few chemosynthetic symbionts are known from the epsilon subdivision of the Proteobacteria. They are filamentous ectosymbionts of *A. pompejana* (Cary et al. 1997, Haddad et al. 1995), *R. exoculata* (Polz and Cavanaugh 1995, Zbinden et al. 2008), and *T. benedii* (Rühland et al., unpublished data), and the endosymbionts of *Alviniconcha* species (Suzuki et al. 2005, Urakawa et al. 2005). Most of the symbiont clusters are interspersed with free-living bacteria and are separated from another by clusters of free-living bacteria. This strongly indicates that the ability to form chemosynthetic symbioses with marine invertebrate hosts has evolved independently several times (Dubilier et al. 2008).

The host associated symbiont diversity has been determined via in situ identification methods only for a few associations, with endosymbiotic diversity much better understood than ectosymbiotic diversity. Most endosymbioses consist of a single chemosynthetic symbiont, most commonly a sulfur oxidizer, and less often a methane oxidizer (Dubilier et al. 2008). A number of mytilid mussels have evolved dual symbioses with gammaproteobacterial sulfur- and methane-oxidizing symbionts co-occurring within the same bacteriocyte e.g. *Bathymodiolus azoricus* and *B. puteoserpentis* (see above, Dubilier et al. 2008). Very few host species are associated with additional symbionts, which can belong to the Gamma-, Alpha- or Deltaproteobacteria, the Bacteroidetes, or the Spirochaetes, and are not necessarily chemosynthetic. Examples of host species harbouring symbiont communities are the "seep" mussel *Bathymodiolus heckerae*, with up to four symbionts (Duperron et al.

2007); the bathymodiolin mussel *Idas* sp., with six symbionts (Duperron et al. 2008a); and gutless oligochaetes, all species of which harbour at least three phylogenetically different symbionts (Blazejak et al. 2005, Blazejak et al. 2006, Dubilier et al. 2006, Ruehland et al. 2008).

1.3 The gutless oligochaete symbiosis

Gutless oligochaete worms were first described from shallow water sediments (Erséus 1979a, Erséus 1979b, Giere 1979, Giere 1981) at the same time that the deep-sea symbiosis of the gutless tubeworm *R. pachyptila* was discovered. The thick bacterial layer under the oligochaete host cuticle (Giere 1981, Giere 1985, Richards et al. 1982) and the chemoautotrophic potential of this symbiosis (Felbeck et al. 1983, Giere et al. 1988b) were recognized a few years later. Today, gutless oligochaetes are known primarily from shallow-water habitats around the world. Characteristic of these chemosynthetic endosymbioses is the remarkable diversity of the symbiotic community.

1.3.1 Phylogeny, biogeography and ecology of the host

Gutless oligochaetes are small, about 2 cm long, segmented marine worms that belong to the Annelida (Clitellata, Naididae, Phallodrilinae). More than 80 species have been analyzed taxonomically, structurally and genetically (Figure 1.2 and Table C.2), with many more not yet described. Gutless oligochaetes appear to be monophyletic, that is all species have descended from a common ancestor. This is supported by morphological observations (Erséus 1984, Erséus 1992) and comparative gene analyses of the host nuclear gene for 18S rRNA and mitochondrial genes for 16S rRNA and cytochrome oxidase I (Erséus et al. 2002, Nylander et al. 1999, Sjölin et al. 2005). All species described today belong to one of two genera, *Olavius* or *Inanidrilus*. While the genus *Inanidrilus* is monophyletic, the genus *Olavius* is likely paraphyletic (C. Erséus unpublished data, Nylander et al. 1999).

Gutless oligochaetes predominate in tropical and subtropical marine sediments, but they are also present in more temperate regions and colder waters (Figure 1.2 and Table C.2). Some species are widely distributed, whereas others are endemic, and at many sites several species co-occur (Erséus 1992). “Hot spots” of abundance and diversity are shallow-water coral reef-influenced sediments in the Caribbean and the Great Barrier Reef of Australia (Erséus 1984, Erséus 1990, Giere et al. 1995a).

The best-studied gutless oligochaete habitats are the shallow-water sediments around Bermuda. The sediment consists of fairly fine to medium-sized calcareous sand that is often covered by coarse shell particles (Giere et al. 1982, Giere et al. 1991). Organic matter settles easily and is degraded in the sediment, resulting in a sulfidic zone underneath the oxic surface layer. *I. leukodermatus* is very abundant in these sediments,

with estimated densities of up to 80,000 individuals per m^2 (Giere et al. 1982). The maximal density was found in the suboxic to anoxic sediment layers at about 5-10 cm depth with a sulfide concentration in the range of 2-32 µM (Dubilier et al. 2006, Giere et al. 1982). The worms spend most of the time just below the redox cline (Giere et al. 1991, Grieshaber and Volkel 1998) and presumably adapt to anoxic life by switching to an anaerobic metabolism (Dubilier et al. 2006, Giere et al. 1984). Although gutless worms can survive long periods of anoxia (C. Lott unpublished data, Dubilier et al. 2006, Giere et al. 1984), both they and their chemoautotrophic symbionts require occasional access to oxygen. By migrating up and down in the sediment, the worms are able to fulfil the host and symbiont needs for oxygen and give the sulfur-oxidizing symbionts access to reduced sulfur compounds (Giere et al. 1991). Gutless oligochaetes thus resemble those whales that move upwards to surface waters only to take a deep breath before "disappearing" again for some time, hunting for food.

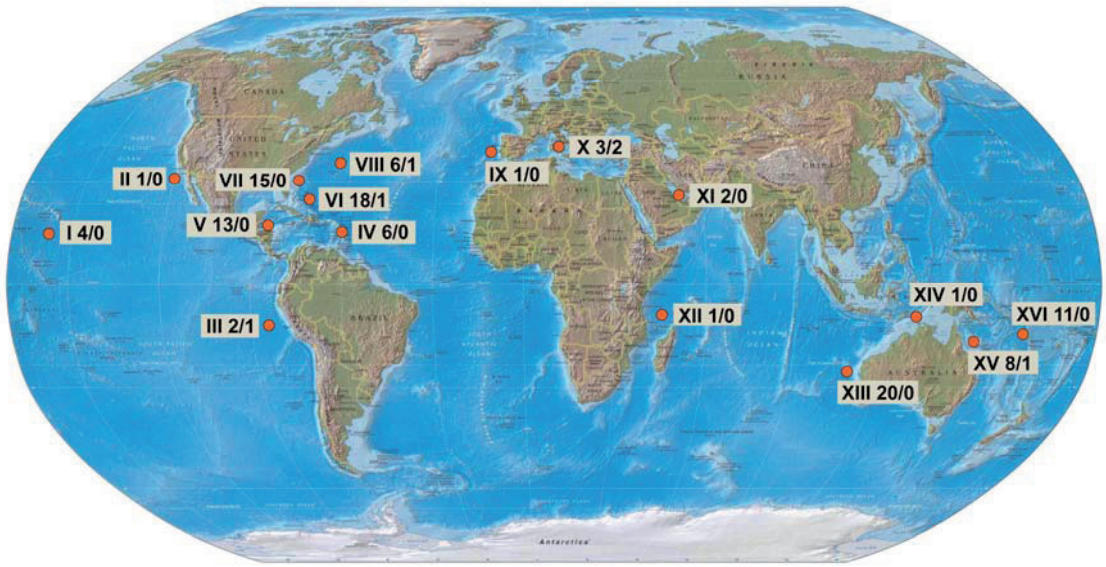


Figure 1.2: Geographic distribution of gutless oligochaetes and described symbiont communities. For each region, the labels on the map show number of host species described/ number of host species in which the symbiont community has been identified. Some sampling sites were combined in one geographical region for better visualization. Several of the more than 80 described species are found in more than one geographical region; and species from the same region do not necessarily co-occur (e.g. they inhabit different beaches or islands). A comprehensive list of described gutless oligochaete species and references is given in the supplemental materials C.1. For references for described symbiont communities see Table 1.2. N – North; S – South; NW – Northwest; NE – Northeast; W – West; E – East.

I	N Pacific, Hawaii
II	N Pacific, American W coast
III	S Pacific, American W coast
IV	Caribbean Sea, Barbados and Guadeloupe
V	Caribbean Sea, Belize reef
VI	Caribbean Sea, Bahamas
VII	NW Atlantic, American NE coast and Florida W coast
VIII	NW Atlantic, Bermuda
IX	NE Atlantic, Portugal
X	Mediterranean Sea, Italy, Elba and Sicily
XI	Indian Ocean, Arabian Sea, Persian Gulf
XII	W Indian Ocean, Comoro Island
XIII	E Indian Ocean, Australia W coast
XIV	Australia Northern Territories
XV	S Pacific, Australia, Great Barrier Reef
XVI	S Pacific, New Caledonia, Fiji and Solomon Islands

A similar distribution pattern of gutless oligochaetes was found in silicate sediments off the coast of the Italian island of Elba. This habitat, however, is different from the Bermuda sediments. The co-occurring worms at this site, *O. algarvensis* and *O. ilvae*, experience a relatively deep penetration of oxygen into the sediment and only trace amounts of free sulfide at sediment depths of 20 cm and deeper (C. Lott, unpublished data, Dubilier et al. 2001, Perner 2003). It has been suggested that the chemoautotrophic symbionts meet their requirements for reduced sulfur not from the environment but rather from co-occurring sulfate-reducing symbionts, thus creating a syntrophic sulfur cycle within this gutless oligochaete symbiosis (Chapter 1.3.4, Dubilier et al. 2001, Ruehland et al. 2008).

In contrast, *O. crassitunicatus* inhabits sediments off the coast of Peru that are characterized by extremely low oxygen concentrations in the bottom water paired with high sulfide concentrations in the top sediment layers (Giere and Krieger 2001, Levin et al. 2002, Levin et al. 2003). The worms are found in the upper 1-5 cm (Giere and Krieger 2001, Levin et al. 2002, Levin et al. 2003), indicating that in oxygen-poor sediments, the worms prefer the upper, more oxidized layers.

1.3.2 Morphology and anatomy of gutless oligochaetes

Gutless oligochaetes are well adapted to their life in the porewater of marine sediments. These worms are thin and long with a diameter of about 0.1 to 0.3 mm and a length of 10 to 30 mm, with the longest reaching up to 40 mm (*O. longissimus*, Giere 1979). Gutless oligochaetes appear almost entirely bright white, making them easily distinguishable from other meiofauna. This particular coloration was noted in the first species descriptions of *I. albidus* (Latin: albus white, Giere 1979) and *I. leukodermatus* (Greek: leukos – white, derma – skin, Jamieson 1977). The first segments are pale to transparent up to approximately the clitellar region (segment ½ X to XII, Figure 1.3), where the sexual organs are located. The structure of the "complex and elaborate" sexual organs in the hermaphrodite gutless oligochaetes is the most important feature for their morphological identification and taxonomic classification (Giere 2006). The digestive system of gutless oligochaetes is – as the name implies – completely reduced. Mouth, gut and anus are lacking (Giere 1981) and so, remarkably, nephridia are absent as well, making gutless oligochaetes unique among free-living animals (Giere 1981). Nephridia are excretory organs used by annelids to remove nitrogenous waste compounds and for osmoregulation. Symbionts of gutless oligochaetes might therefore not only be useful for host nutrition but may also play an important role in recycling host waste products (Chapter 1.3.4).

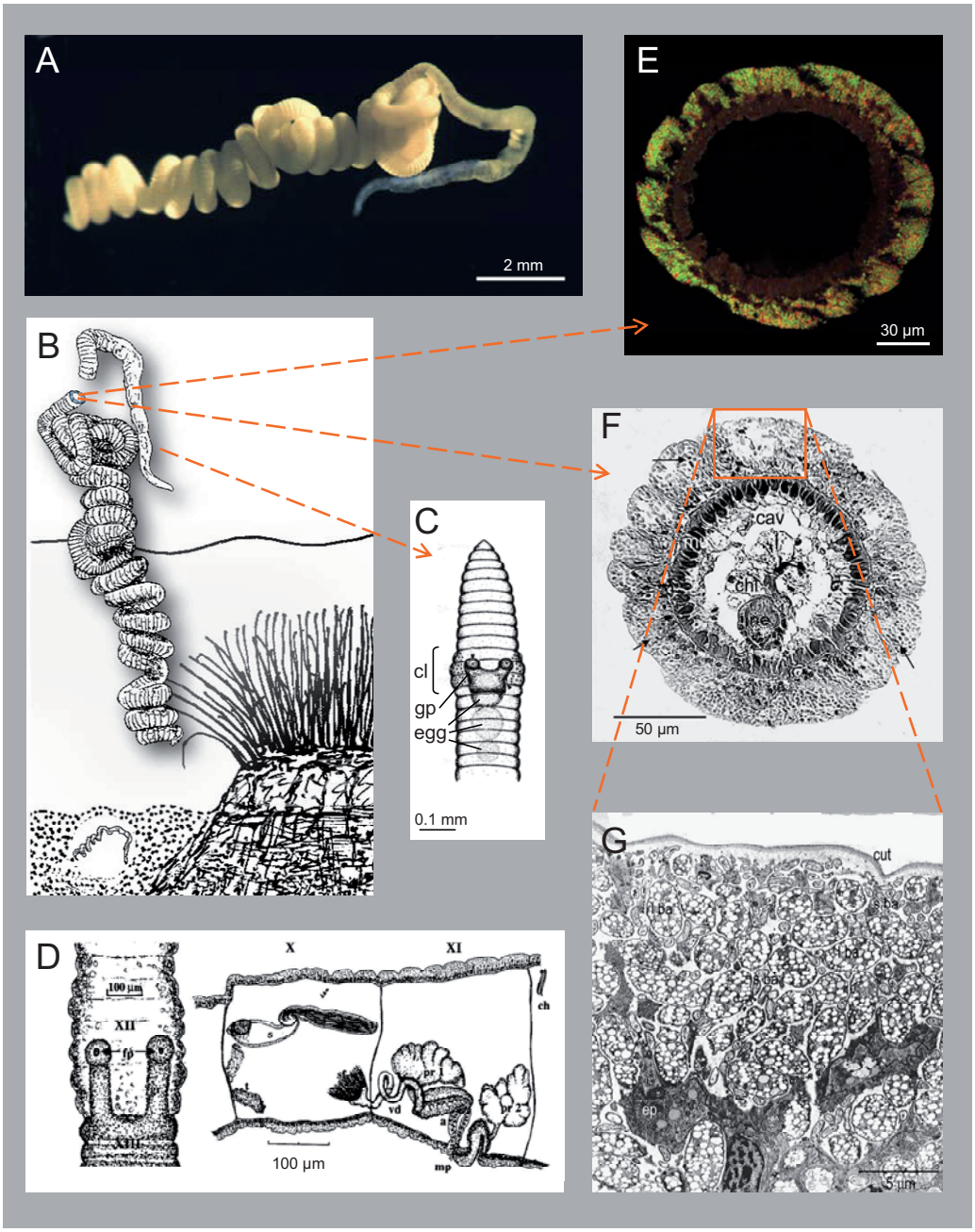


Figure 1.3: The gutless oligochaete symbiosis, habitus and morphology. Habitus of a gutless oligochaete, real life (A) and schematic (B). (C) Front part of a gutless oligochaete (cl – clitellar region, gp – genital pads). (D) Schematic drawings of segments X and XI of a gutless oligochaete (fp – female pores, a – atrium, ch – chaeta, mp – male pore, pr 1 – anterior prostate, pr 2 – posterior prostate, s – spermatheca, sf – sperm funnel, t – testis, vd – vas deferens). (E) Cross section through a gutless oligochaete: the bacterial symbionts, stained in green and red, sit between the epidermal cells and the cuticle. (F) Cross section through a gutless oligochaete, arrows pointing to the bacterial symbionts in the symbiotic region (arrows; cav – coelomic cavity). (G) Close-up of the symbiotic region, granules are visible as white areas within the larger bacterial symbionts (cut – cuticle). (Figures adapted from Dubilier et al. 2008 (B), Giere and Langheld 1987 (C), Giere and Erséus 2002 (D), Giere 2006 (F and G), figure (A) courtesy of C. Lott.)

1.3.3 Structure of the gutless oligochaete symbiosis

All gutless oligochaetes harbour bacterial symbionts in a consistent pattern. Their symbionts are located just beneath the cuticle between extensions of epidermal cells (Dubilier et al. 2006, Richards et al. 1982). This symbiotic region is packed with bacterial cells of different morphotypes, with small bacteria more or less distributed between large ones. The symbionts make up an estimated 25% of the total volume of the host, with at least 10^6 bacterial cells per individual (Giere et al. 1995a). While symbionts are rare in the very first segments of the host, they are densely packed and most abundant from the postgenital region onward (Giere and Langheld 1987). This symbiont distribution is recognizable by the white outer appearance of the host in the postgenital region, which is the result of reflected light from inclusion bodies (see below) in the large bacterial morphotypes (Giere 1985, Richards et al. 1982).

The large morphotypes are oval-shaped bacteria and common to all gutless oligochaetes. These symbionts are on average 3 x 1.5 µm in size (Giere 1985, Giere et al. 1995a, Giere and Erséus 2002), though some can be as large as 7.30 x 4.47 µm, as in *O. crassitunicatus* (Giere and Krieger 2001). Inclusion bodies within the cells are either membrane bound sulfur globules or non-membrane bound polyhydroxybutyric acid (PHB) storage bodies (Figure 1.3G, Giere and Langheld 1987, Giere and Krieger 2001, Krieger et al. 2000). At least two other morphotypes co-occur with the large morphotypes. These are smaller rod-shaped or coccoid bacteria and thin elongated morphotypes. Small morphotypes are found together with the large bacteria in all host species studied. Their size range is 1.1-1.8 x 0.32-0.6 µm (Giere et al. 1995a, Giere and Krieger 2001, Giere and Erséus 2002). Internal cellular structures are mostly absent from the small morphotypes, although electron-dense vesicles were present in small symbiont morphotypes of some host species (Giere 1985, Richards et al. 1982). The elongated morphotypes can be up to 9 µm long, 0.3 µm thin and have yet been described for a few host species only (Dubilier et al. 1999, Giere and Krieger 2001).

Molecular phylogeny and identification of the symbionts

Despite the vast number of host species described and identified, the symbiont community has been studied in detail in only a few species (Table 1.2). During the last decade, the bacterial symbionts of six host species were identified by the cultivation-independent full cycle ribosomal RNA (rRNA) approach (Amann et al. 1995). In this approach, comparative 16S rRNA gene sequence analysis and fluorescence in situ hybridization (FISH) with oligonucleotide probes targeting the 16S rRNA are combined to assess the phylogenetic relationship of the symbionts, their identity, and their distribution within the host tissue.

Table 1.2: Gutless oligochaete-host species and their symbiont communities, which have been identified based on morphology, 16S rRNA analysis and/ or identification by fluorescence in situ hybridization (FISH). Empty fields in the symbiont columns represent data which has not yet been investigated or published.

Host	Sampling site	Symbionts					Reference [a]
		MT	16S rRNA and FISH				
			γ	α	δ	Spiro	
O. crassitunicatus	Pacific, Peru	3	2	-	2-3	1	Blazejak et al. 2005, Finogenova 1986, Giere and Krieger 2001
O. loisae	Great Barrier Reef, Australia	3	1	1	-	1	Dubilier et al. 1999
O. albidus	Great Barrier Reef, Australia	2	x	x			Dubilier et al. 2006
O. algarvensis	Elba, Italy	3	2	-	2	1	Giere and Erséus 2002, Ruehland et al. 2008
O. ilvae	Elba, Italy	3	2	-	2	-	Giere and Erséus 2002, Ruehland et al. 2008
I. leukodermatus	Bermuda	2	1	3	-	-	Blazejak et al. 2006, Giere et al. 1982
I. planus	Bermuda	2					Bright and Giere 2005
I. makropetalus	Bahamas	2	1	2	-	-	Blazejak et al. 2006
I. exumae	Bahamas		x[b]	x	x	-	This study
O. triangulatus	Bahamas		x				Dubilier et al. 2006
O. vacuus	Bahamas	2	x				Dubilier et al. 2006
O. longissimus	Bahamas		x				Dubilier et al. 2006
O. tantulus	Bahamas		x				Dubilier et al. 2006
O. ullae	Bahamas		x				Dubilier et al. 2006
I. mojicae	Bahamas		x				Dubilier et al. 2006
O. imperfectus	Bahamas		x				Dubilier et al. 2006
O. finitimus	Bahamas		x				Dubilier et al. 2006
O. tenuissimus	Bahamas		x				Dubilier et al. 2006

MT: # of different morphotypes (MT) based on ultrastructure; γ: Gamma; α: Alpha; δ: Delta; Spiro: spirochete; [a] additional information taken from Bright and Giere 2005; [b] morphotype similar to Gamma 1 symbiont, but Gamma 1 related 16S rRNA sequence was not found (compare Chapter 2.1); x: symbiont 16S rRNA analyzed, but sequence not published (compare Dubilier et al. 2006).

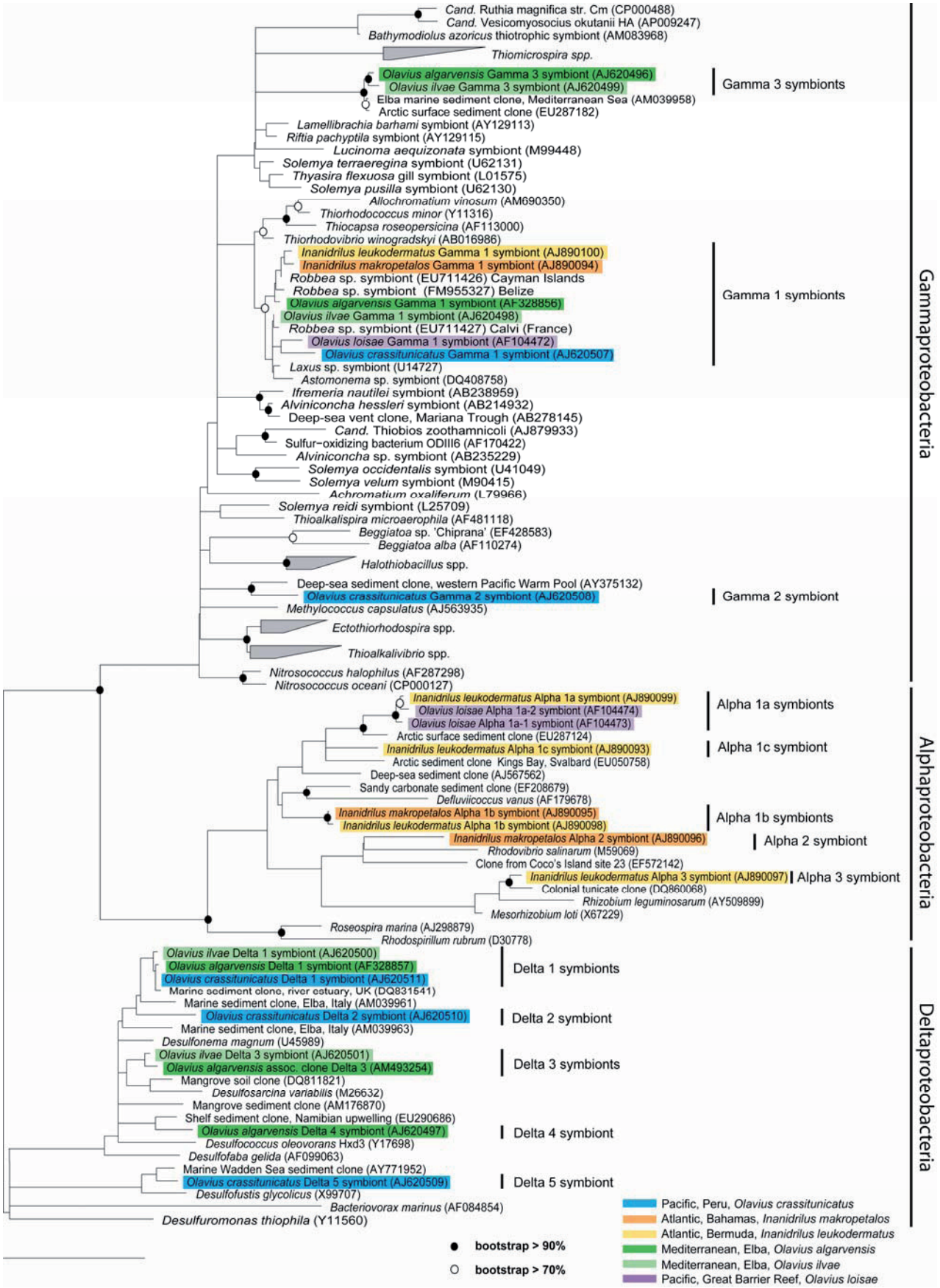
The large morphotypes in gutless oligochaetes have been consistently identified as symbionts belonging to a monophyletic clade within the Gammaproteobacteria, now called Gamma 1 symbionts. This clade is closely affiliated with the Chromatiaceae and also includes a few nematode symbionts (Musat et al. 2007, Polz et al. 1994). The smaller, rod-shaped or coccoid symbionts are phylogenetically diverse and belong to the Alpha-, Gamma- or Deltaproteobacteria. The long filiform symbionts were affiliated with the Spirochaetes. Most of these symbionts from different host species form symbiont-specific clades, some of which include free-living bacteria (Figure 1.4). Several symbiont clades within the Gamma-, Alpha-, Deltaproteobacteria, and one clade

within the Spirochaetes are known today. A few symbionts were identified that are not associated with a symbiont clade. Despite this diversity, the symbiont community of each host species is species-specific and can differ even between co-occurring host species (Blazejak et al. 2005, Blazejak et al. 2006, Ruehland et al. 2008).

Symbiont transmission

Symbiont transmission is a very important factor for sustaining a specific symbiosis, but is, as yet, barely understood. The transmission must guarantee the specific transfer or uptake of symbionts for each new generation, especially when gutless oligochaete species co-occur. Specificity is further hampered by the way gutless oligochaetes progenite. In contrast to other oligochaetes which build cocoons around their eggs, gutless worms deposit a single egg, protected by a mucus sheath, into the sediment (Giere and Langheld 1987) where bacteria from the surrounding sediment could infect the egg. Such a horizontal mode of transmission might have been common during the early evolutionary stages of the gutless oligochaete symbiosis. Some symbionts might still be transmitted horizontally as indicated by the presence of genes coding for flagellar proteins, the absence of an AT bias and the non-reduced genome size (Woyke et al. 2006).

There is strong indication though that at least some symbionts are transmitted vertically or are in a transition stage from environmental to vertical transmission. Giere and Langheld (1987) and Krieger (2001) showed that bacteria were present between the mucus layer surrounding the egg and the egg itself. The bacteria penetrate the egg membrane a few hours after egg deposition, and young worms already harbour all their symbionts (Giere and Langheld 1987). As bacteria have not yet been found anywhere inside the worm aside from the symbiotic region and the genital pads, it is unclear where and when the transfer of symbionts occurs. The genital pads filled with bacteria are disrupted during egg deposition, and thus may be a source of symbiont infection of the freshly laid eggs (Giere and Langheld 1987, Krieger 2001). Vertical transmission might be further indicated by a high number of mobile elements within the *O. algarvensis* Gamma 1 symbiont bin (Woyke et al. 2006).


Cand. Ruthia magnifica str. Cm (CP000488)
Cand. Vesicomyosocius okutanii HA (AP009247)
Bathymodiolus azoricus thiotrophic symbiont (AM083968)
Thiomicrospira spp.
Olavius algarvensis Gamma 3 symbiont (AJ620496)
Olavius ilvae Gamma 3 symbiont (AJ620499)
Elba marine sediment clone, Mediterranean Sea (AM039958)
Arctic surface sediment clone (EU287182)
Gamma 3 symbionts
Lamellibrachia barhami symbiont (AY129113)
Riftia pachyptila symbiont (AY129115)
Lucinoma aequizonata symbiont (M99448)
Solemya terraeregina symbiont (U62131)
Thyasira flexuosa gill symbiont (L01575)
Solemya pusilla symbiont (U62130)
Allochromatium vinosum (AM690350)
Thiorhodococcus minor (Y11316)
Thiocapsa roseopersicina (AF113000)
Thiorhodovibrio winogradskyi (AB016986)
Inanidrilus leukodermatus Gamma 1 symbiont (AJ890100)
Inanidrilus makropetalos Gamma 1 symbiont (AJ890094)
Robbea sp. symbiont (EU711426) Cayman Islands
Robbea sp. symbiont (FM955327) Belize
Olavius algarvensis Gamma 1 symbiont (AF328856)
Olavius ilvae Gamma 1 symbiont (AJ620498)
Robbea sp. symbiont (EU711427) Calvi (France)
Olavius loisae Gamma 1 symbiont (AF104472)
Olavius crassitunicatus Gamma 1 symbiont (AJ620507)
Gamma 1 symbionts
Laxus sp. symbiont (U14727)
Astomonema sp. symbiont (DQ408758)
Ifremeria nautilei symbiont (AB238959)
Alviniconcha hessleri symbiont (AB214932)
Deep-sea vent clone, Mariana Trough (AB278145)
Cand. Thiobios zoothamnicoli (AJ879933)
Sulfur-oxidizing bacterium ODIII6 (AF170422)
Alviniconcha sp. symbiont (AB235229)
Solemya occidentalis symbiont (U41049)
Solemya velum symbiont (M90415)
Achromatium oxaliferum (L79966)
Solemya reidi symbiont (L25709)
Thioalkalispira microaerophila (AF481118)
Beggiatoa sp. 'Chiprana' (EF428583)
Beggiatoa alba (AF110274)
Halothiobacillus spp.
Deep-sea sediment clone, western Pacific Warm Pool (AY375132)
Olavius crassitunicatus Gamma 2 symbiont (AJ620508)
Gamma 2 symbiont
Methylococcus capsulatus (AJ563935)
Ectothiorhodospira spp.
Thioalkalivibrio spp.
Nitrosococcus halophilus (AF287298)
Nitrosococcus oceani (CP000127)
Gammaproteobacteria
Inanidrilus leukodermatus Alpha 1a symbiont (AJ890099)
Olavius loisae Alpha 1a-2 symbiont (AF104474)
Olavius loisae Alpha 1a-1 symbiont (AF104473)
Alpha 1a symbionts
Arctic surface sediment clone (EU287124)
Inanidrilus leukodermatus Alpha 1c symbiont (AJ890093)
Alpha 1c symbiont
Arctic sediment clone Kings Bay, Svalbard (EU050758)
Deep-sea sediment clone (AJ567562)
Sandy carbonate sediment clone (EF208679)
Defluviicoccus vanus (AF179678)
Inanidrilus makropetalos Alpha 1b symbiont (AJ890095)
Inanidrilus leukodermatus Alpha 1b symbiont (AJ890098)
Alpha 1b symbionts
Inanidrilus makropetalos Alpha 2 symbiont (AJ890096)
Alpha 2 symbiont
Rhodovibrio salinarum (M59069)
Clone from Coco's Island site 23 (EF572142)
Inanidrilus leukodermatus Alpha 3 symbiont (AJ890097)
Alpha 3 symbiont
Colonial tunicate clone (DQ860068)
Rhizobium leguminosarum (AY509899)
Mesorhizobium loti (X67229)
Roseospira marina (AJ298879)
Rhodospirillum rubrum (D30778)
Alphaproteobacteria
Olavius ilvae Delta 1 symbiont (AJ620500)
Olavius algarvensis Delta 1 symbiont (AF328857)
Olavius crassitunicatus Delta 1 symbiont (AJ620511)
Delta 1 symbionts
Marine sediment clone, river estuary, UK (DQ831541)
Marine sediment clone, Elba, Italy (AM039961)
Olavius crassitunicatus Delta 2 symbiont (AJ620510)
Delta 2 symbiont
Marine sediment clone, Elba, Italy (AM039963)
Desulfonema magnum (U45989)
Olavius ilvae Delta 3 symbiont (AJ620501)
Olavius algarvensis assoc. clone Delta 3 (AM493254)
Delta 3 symbionts
Mangrove soil clone (DQ811821)
Desulfosarcina variabilis (M26632)
Mangrove sediment clone (AM176870)
Shelf sediment clone, Namibian upwelling (EU290686)
Olavius algarvensis Delta 4 symbiont (AJ620497)
Delta 4 symbiont
Desulfococcus oleovorans Hxd3 (Y17698)
Desulfofaba gelida (AF099063)
Marine Wadden Sea sediment clone (AY771952)
Olavius crassitunicatus Delta 5 symbiont (AJ620509)
Delta 5 symbiont
Desulfofustis glycolicus (X99707)
Bacteriovorax marinus (AF084854)
Desulfuromonas thiophila (Y11560)
Deltaproteobacteria
bootstrap > 90%
bootstrap > 70%
Pacific, Peru, Olavius crassitunicatus
Atlantic, Bahamas, Inanidrilus makropetalos
Atlantic, Bermuda, Inanidrilus leukodermatus
Mediterranean, Elba, Olavius algarvensis
Mediterranean, Elba, Olavius ilvae
Pacific, Great Barrier Reef, Olavius loisae

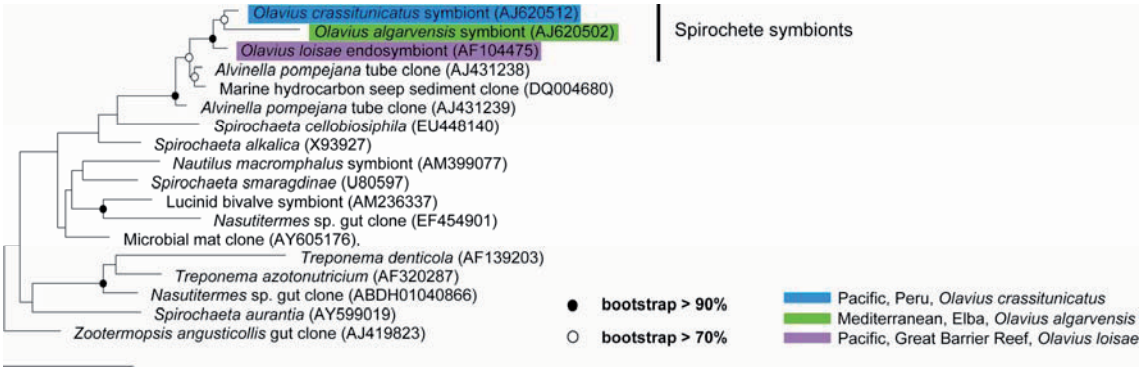


Figure 1.4: Phylogenetic affiliation of the gutless oligochaete proteobacterial (previous page) and spirochete (above) symbionts based on 16S rRNA sequences. Symbiont 16S rRNA sequences from different gutless oligochaete host species are pointed out by a colour code. Phylogenetic reconstruction was based on maximum likelihood. Branching orders that were not supported in most calculation methods are shown as multifurcations. Scale bars represent 10% estimated phylogenetic divergence for non-multifurcation branches.

1.3.4 Function of the gutless oligochaete symbiosis

After three decades of oligochaete research, the precise functioning of this symbiosis and each symbiont's role is still not completely understood. The chemoautotrophic nature of the gutless oligochaete symbioses was soon recognized in *I. leukodermatus*. Assays for enzymes involved in autotrophy and sulfur metabolism (Felbeck et al. 1983), uptake experiments with radiolabelled bicarbonate (Felbeck et al. 1983, Giere et al. 1988b), sulfide and thiosulfate usage, sulfate production and the presence of stored sulfur (Giere et al. 1988b) were strong indications of the presence of a sulfur-oxidizing chemoautotrophic symbiont. Furthermore, a positive immuno-cytochemical labelling for the enzyme ribulose-1,5-bisphosphate carboxylase/ oxygenase (RubisCO) form I, essential for autotrophy, and stored sulfur have been found thus far exclusively within the large morphotype of *I. leukodermatus* and other host species (Dubilier et al. 2001, Giere and Krieger 2001, Krieger et al. 2000). Thus, the chemoautotrophic sulfur-oxidizing activity was assigned to the large morphotype, identified as Gamma 1 symbionts.

The potential metabolism of the alphaproteobacterial and the spirochete symbionts is still unknown, whereas the role of the deltaproteobacterial symbionts as sulfate reducers has been elucidated (Dubilier et al. 2001). A sulfur syntrophy was suggested for the symbionts of *O. algarvensis* and *O. ilvae*, which inhabit the sulfide-poor sediments off the coast of Elba (Dubilier et al. 2001, Ruehland et al. 2008). The sulfate-reducing deltaproteobacterial symbionts provide the co-occurring sulfur-oxidizing symbionts with sulfide or other reduced sulfur compounds. The sulfur-oxidizing symbionts complete this syntrophic sulfur cycle by oxidizing the reduced sulfur compounds to sulfur or sulfate. The latter could in turn be used by the sulfate reducer,

creating a full sulfur cycle within this minute system of the oligochaete host's body (Dubilier et al. 2001, Ruehland et al. 2008).

The recent metagenomic study of the *O. algarvensis* symbionts brought remarkable insight to the workings of this symbiosis. The syntrophic sulfur cycle hypothesis was supported by the presence of genes coding for enzymes involved in sulfate reduction and the oxidation of reduced sulfur compounds (Woyke et al. 2006). The metagenome also revealed the presence of a second sulfur-oxidizing gammaproteobacterial symbiont and a second sulfate-reducing deltaproteobacterial symbiont that may also be engaged in the sulfur cycling.

Besides sulfur cycling, exchange of other intermediates and novel metabolic pathways were predicted by the metagenomic study (Woyke et al. 2006 and Figure 1.5). When oxygen is not available, the worms excrete waste products of their anaerobic metabolism such as succinate. Succinate could theoretically be used as an electron donor by the sulfate-reducing symbionts and the corresponding product, fumarate, could serve as an electron acceptor for the sulfur-oxidizing symbionts under anoxic conditions. Another possible electron acceptor for the sulfur-oxidizing symbionts under anoxic conditions could be nitrate. Hydrogen could also be exchanged. After being released by the smaller sulfur-oxidizing symbionts during pyruvate oxidation hydrogen might serve as an energy source for the sulfate-reducing symbionts. It is known that free-living sulfate-reducing bacteria can indeed use the energy provided by hydrogen oxidation for autotrophic growth (Matias et al. 2005).

The metagenome further supported autotrophy in both sulfate-reducing and both sulfur-oxidizing symbionts (Woyke et al. 2006). Genes coding for enzymes required for different autotrophic carbon fixation pathways, the Calvin-Benson-Bassham (CBB) cycle in the sulfur-oxidizing symbionts, and the reductive acetyl-coenzyme A and reductive tricarboxylic acid cycle in the sulfate-reducing symbionts, were found. This autotrophic capacity would make the *O. algarvensis* symbiosis essentially independent of external organic carbon sources, though uptake of organic material through the host's cuticle is possible (Giere et al. 1982, Liebezeit et al. 1983).

The nutrition of the host might function via excretion of organic compounds from the symbionts or via lysis of the symbiont cells. Symbionts in digestive and lytic stages were frequently observed in the inner area of the symbiotic region (Giere and Langheld 1987, Giere and Milligan 1989). In addition to providing their host with nutrition, an important role of the symbionts could be the recycling of host waste products, such as urea (Giere et al. 1984, Richards et al. 1982), compounds used for osmoregulation such as glycine betaine or trimethylamine N-oxide (TMAO), and products of its anaerobic metabolism such as succinate or propionate. Indeed, a wide range of transporters were abundant in the metagenome (Woyke et al. 2006).

The interactions suggested and possible internal recycling of reductants, oxidants and carbon sources within the *O. algarvensis* symbiosis are remarkable, and are

currently not known to exist in other chemosynthetic symbioses. The seemingly redundant set of symbiotic metabolisms (such as sulfur oxidation, sulfate reduction, autotrophy) may be advantageous, as the symbionts may be active under different environmental conditions. While the worm travels up and down in the sediment exploiting all the advantages of its diverse symbionts (Woyke et al. 2006).

The metagenome has spurred many hypotheses concerning symbiont metabolism. It remains to be shown though, which pathways are really used or are important in the gutless oligochaete symbiosis. Methods to study function, identity and activity of symbionts, as well as recent technological developments will be the focus of the next section.

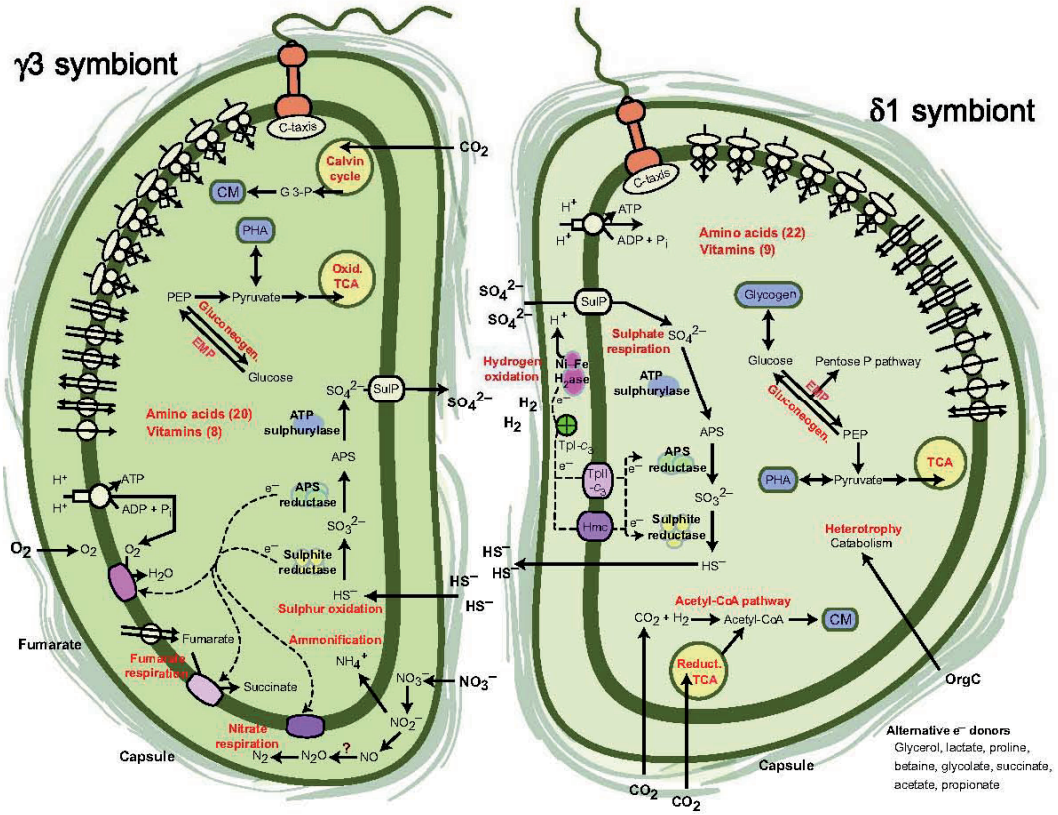


Figure 1.5: Metabolic reconstruction for two symbionts of *O. algarvensis* based on metagenomic analyses. (Fig. adapted from Woyke et al. 2006). APS: adenosine 5'-phosphosulfate; CM: cell material; CoA: coenzyme A; C-taxis: chemotaxis; EMP: Embden-Meyerhoff pathway; G 3-P: glyceraldehyde 3-phosphate; H_2ase: hydrogenase; Hmc: high-molecular-weight cytochrome c; orgC: organic compounds; PEP: phosphoenolpyruvate; PHA: polyhydroxyalkanoates; TCA: tricyclic acid; TpI/II-c_3: type I/II tetrahaem cytochrome c_3; TRAP: tripartite ATP-independent periplasmic. SulP: sulfate permease. A question mark (?) indicates the lack of nitric oxide reductase in the γ3 genome bin.

1.4 Studying marine chemosynthetic symbioses

Studying marine chemosynthetic symbiosis was and still is hampered by the lack of culturability of the bacterial symbionts. It can be further complicated by the presence of more than one symbiont. A suite of methods (e.g. ultrastructural, biochemical, molecular biological, bioinformatic) has been used over the last decades to investigate the identities of the symbionts and their metabolic capacities and activities. Methods which were important and useful in the past and still are will briefly be introduced. In addition novel techniques and improvements that are promising tools already used in microbial ecology but have not yet been applied to marine chemosynthetic symbioses will be presented.

1.4.1 Morphological and biochemical analyses

Microscopy enabled the discovery of the first symbiotic associations. By careful investigation the Swiss botanist Simon Schwendener found that lichens consist of a very close association of a fungus and an alga rather than a single organism (Schwendener 1969). Accordingly the first chemoautotrophic symbioses were identified by (ultrastructural) microscopy. The discovery of symbiotic bacteria within the gutless tubeworm *R. pachyptila* explained the observed chemoautotrophic activity that could then be assigned to the symbionts rather than to the host (Cavanaugh et al. 1981, Felbeck 1981). Stable isotope signatures showed that non-photosynthetic carbon was used by *R. pachyptila* as a carbon source "implying a role for chemoautotrophy in tubeworm autotrophy" (Rau 1981). Activities of enzymes involved in sulfur oxidation and carbon fixation were detected in the trophosome tissue of the tubeworm (Felbeck 1981). All these indications combined were evidence for the first marine chemoautotrophic symbiosis.

Ultrastructural analyses can – aside from symbiont localization - provide valuable information about symbiont function. Internal membranes typical for methane-oxidizing bacteria (Cavanaugh et al. 1987) or inclusion bodies e.g. of sulfur (Chapter 1.3.3) can indicate certain metabolic pathways. However, information about the physiological properties and identity of the symbiont is very limited. The physiological activity can be assessed with natural stable isotope analyses, enzyme assays, and incubation experiments with labelled and unlabelled substrates, while identity and metabolic potential can be assessed with molecular tools (see below).

Natural stable carbon isotope compositions have been used to elucidate nutritional pathways and food sources in host-symbiont associations as they can reflect the in situ carbon source of the host. The stable carbon isotope composition $\delta^{13}C$ [‰] is expressed as the ratio of the isotope composition of the sample to the isotope composition of a standard with known isotope composition. 'Heavier' samples are enriched in ^{13}C compared to the standard or another sample and have a more positive $\delta^{13}C$. The isotope

composition of primary producers, including chemosynthetic symbionts, is the result of a distinct discrimination of the different carbon-fixing enzymes against the heavier isotope which leads to a shift in isotope composition between reactant and product (fractionation). The consumer though generally keeps the $\delta^{13}C$ of its carbon food source (DeNiro and Epstein 1978). Therefore stable carbon isotope values were and are valuable to distinguish if host nutrition relies on carbon produced by photoautotrophs, chemoautotrophs or methanotrophs as these often have distinct $\delta^{13}C$. Marine phytoplankton has generally $\delta^{13}C$ of –18 to –28‰ (Cavanaugh et al. 2006), chemoautotrophic mussel symbioses often, though not always, have $\delta^{13}C$ values in the range of –27 to –35‰, and vestimentiferan tubeworm symbioses of –9 to –16‰ (Childress and Fisher 1992). Methane usually has a much lighter $\delta^{13}C$ than carbon dioxide. Thus, very light stable carbon isotope composition of –39‰ to –75‰ implies that the host feeds on carbon derived from a methane-oxidizing symbiont (MacAvoy et al. 2002, Paull et al. 1985). These distinct values could in many cases identify methanotrophic and chemoautotrophic symbioses. Often though, $\delta^{13}C$ are influenced by factors, such as substrate concentrations or carbon fixation rates or unusual $\delta^{13}C$ of the carbon sources, resulting in $\delta^{13}C$ of the symbiosis much heavier or much lighter than expected (Fisher 1990). Due to these difficulties stable carbon isotope values of chemosynthetic symbioses should be complemented with information on the possible carbon sources and their $\delta^{13}C$, carbon assimilation pathways, (theoretical) enzymatic fractionation as well as possible internal (re-)cycling of inorganic carbon.

Enzyme assays target the enzymatic capacities of symbioses and thus can complement stable isotope data. Enzymes involved in carbon fixation and sulfur metabolism were analyzed in fresh or frozen material, whole animals, symbiont-containing tissue and enriched symbiont fractions (reviewed in Cavanaugh et al. 2006, Nelson and Fisher 1995). The observed enzyme activities are evidence that a particular enzyme is potentially active and indicate that the corresponding pathway is functional in the symbiosis. Enzymes that have been studied include RubisCO and phosphoribulokinase, which are essential for autotrophy via the CBB cycle (Table 1.3). APS reductase, ATP sulfurylase and rhodanese are involved in sulfur metabolism, although not necessarily indicative of disimilatory sulfur-oxidation and energy generation. Methane monooxygenase (MMO) would be diagnostic for methanotrophy, but due to enzymatic instability methyldehydrogenase (MeDH) was frequently used instead (Cavanaugh et al. 1987, Cavanaugh et al. 1992). MeDH catabolizes the second step in methane oxidation and is characteristic of methylotrophy. Nitrate reductase activity was investigated in some symbioses (Felbeck et al. 1981, Felbeck et al. 1983), though assimilatory and dissimilatory nitrate reduction cannot be distinguished from enzyme assays alone. Enzyme activities can differ between sample preparations, and interpretation of the data is often hampered by the presence of host enzymes. A good

Table 1.3: Overview on enzymes and genes that have been analyzed in marine chemosynthetic symbiosis research as indicator for autotrophy, sulfur, methane, and nitrogen metabolism. n.a. not yet analyzed in chemosynthetic symbiosis research.

Metabolism	Enzyme	References	Gene	References
Autotrophy				
Part of CBB cycle, CO_2 incorporation	RubisCO	1, 2	*cbbL*, *cbbM*	3, 4, 5, 6, 7, and more
Part of CBB pathway, posphotransferase	Ru5P kinase	1	*cbbP*	n.a.
Part of rTCA cycle, citrate cleavage	ATP citrate lyase	n.a.	*aclBA*	8
Sulfur oxidation				
(Sulfite oxidation)	APS reductase	1, 2	*aprA*	3
Sulfate and ATP generation from APS	ATP sulfurylase	1, 2	*sopT*	4, 9
Sulfite oxidation	Sulfite oxidase	2	n.a.	
Thiosulfate cleavage	rhodanese	1, 2	n.a.	
Sulfur oxidation	rDSR	n.a.	*dsrAB*	10
Methane oxidation				
Methane oxidation	pMMO	n.a.	*pmoA*	4, 11, 12
Methanol oxidation	Methanol dehydrogenase	13, 14, 15	n.a.	
Nitrate reduction				
Nitrate reduction	Nitrate reductase	1, 2	n.a.	
Nitrite reduction	Nitrite reductase	n.a.	*nirS*, *nirK*	16

RubisCO: ribulose-1,5-bisphosphate carboxylase/ oxygenase; Ru5P kinase: Ribulose 5-phospate kinase (phosphoribulokinase); APS: adenosine-5'-phosphosulfate; ATP: adenosine-5'-triphosphate; rTCA: reductive tricarboxylic acid, rDSR: reverse operating sirohaem dissimilatory sulfite reductase; pMMO: particulate methane monooxygenase. In parantheses suggested pathways. [1]Felbeck et al. 1981, [2]Felbeck et al. 1983, [3]Blazejak et al. 2006, [4]Elsaied et al. 2006, [5]Robinson et al. 1998, [6]Schwedock et al. 2004, [7]Stein et al. 1990, [8]Campbell et al. 2003, [9]Laue and Nelson 1994, [10]Ruehland et al. 2008, [11]Duperron et al. 2007, [12]Pernthaler and Amann 2004, [13]Cavanaugh et al. 1987, [14]Cavanaugh et al. 1992, [15]Fisher et al. 1987, [16]Blazejak 2006

overview of the pitfalls of enzyme assays on marine chemosynthetic symbioses is given by Nelson and Fisher (1995).

Incubation experiments can address some of these inconsistencies in enzyme activity studies. Substrate uptake, turnover and fluxes in the symbiosis can be studied qualitatively and quantitatively using labelled or unlabelled compounds and can provide information on whether a substrate is needed and used for growth, assimilated without supporting growth, or serves as an energy source or reductant (e.g. Anderson et al. 1987, Girguis et al. 2002, Girguis and Childress 2006, Kochevar et al. 1992). Data from different studies are, however rarely easy to compare and results can vary by orders of magnitude for the same host species when different samples are incubated, such as whole animals, symbiont-containing tissue, enriched symbiont fractions or cell homogenates. Attention must be paid to potential interactions between the host and the symbiont (Nelson and Fisher 1995). For example, sulfide oxidation by host

mitochondria and symbionts both lead to sulfide usage, making the interpretation of bulk tissue experiments and the distinction between host and symbiont activity difficult.

Radiolabelled substrates are used in so-called tracer experiments. A radioactive compound is applied in trace amounts to track symbiont metabolic activities. This technique was used to investigate into which organic molecules the autotrophically fixed inorganic carbon was incorporated first (Felbeck 1983, Felbeck et al. 1983). In microautoradiography (MAR) tracer experiments are combined with microscopy to elucidate and visualize the site of carbon incorporation into biomass within a chemosynthetic symbiosis (Chapter 1.4.3). The samples are incubated with a radiolabelled substrate, such as ^{14}C-labelled inorganic carbon before the tissue is sectioned and exposed to an autoradiographic emulsion. During exposure, silver grains are formed next to those cells that incorporated the radioactive label. This method, combined with time series, illustrated that after addition of the labelled inorganic carbon the label appeared very quickly in the symbiont-containing tissue of *R. pachyptila* (Bright et al. 2000) and *Solemya reidi* (Fisher and Childress 1986). Within only a few hours, the label was found in host tissue, demonstrating that a nutrient transfer from the symbiont to the host occurred rapidly (Bright et al. 2000, Fisher and Childress 1986). In a similar approach, substrate incorporation was followed in the shipworm symbiosis, applying stable, non-radioactive isotopes as label (Lechene et al. 2007). Visualization of the incorporated substrate was achieved by highly sensitive secondary ion mass spectrometry (SIMS) with high mass and high spatial resolution (Chapter 1.4.3, Lechene et al. 2007).

The methods presented above revealed a variety of physiological characteristics of chemosynthetic symbionts. However, most of these methods lack the possibility to assign the observed activity to one symbiont when symbiont communities are considered. Isolation and cultivation would allow the physiological properties of symbionts to be studied in detail. Unfortunately, only very few symbionts have been cultivated so far including the nitrogen-fixing shipworm symbiont (Distel et al. 2002, Waterbury et al. 1983) and the squid symbiont *Vibrio fischeri* (Nyholm and McFall-Ngai 2004). The vast majority of symbionts, and all marine chemosynthetic symbionts, have defied cultivation. Even symbionts with a free-living stage, such as those of lucinid clams (Gros et al. 1996, Gros et al. 2003) and *R. pachyptila* (Cary et al. 1993, Harmer et al. 2008, Nussbaumer et al. 2006) have not yet been cultured. The few reported isolation and cultivation attempts only isolated contaminants from clams (Distel and Wood 1992, Wood and Kelly 1989a, Wood and Kelly 1989b) and gutless oligochaetes (C. Rühland, A. Gittel, unpublished data, Musat 2006). Thus, other methodological approaches were needed to identify symbionts in symbiont communities and to assign a specific function.

1.4.2 Nucleic acid-based molecular biology methods

Morphology- and cultivation-independent identification of symbionts (Table 1.4.) was made possible with the advent of RNA- and DNA-based methods. In particular, the full cycle rRNA approach (Capter 1.3.2, Amann et al. 1995) opened a new era in microbial ecology and in symbiosis research, revealing an as yet unknown and immense microbial diversity, the so-called 'uncultivated majority' (Rappe and Giovannoni 2003, Whitman et al. 1998). In this approach, the 16S small subunit rRNA (SSU rRNA) serves as a phylogenetic marker and identifier for both culturable and 'unculturable' microbes. The 16S rRNA gene sequences are retrieved by PCR amplification from mixed genomic DNA using universal primers, and are then cloned and sequenced. Oligonucleotide probes are designed specifically to match the 16S rRNA of a target organism. A fluorescently labelled probe is hybridized with the target molecule (FISH) in the environmental sample and the target organisms is then microscopically visualized and identified (Amann et al. 1995). This approach ensures that the 16S rRNA sequences obtained were indeed from a symbiont and not from a contaminant. For most symbioses, the presence of a single symbiont could be confirmed, even when different morphotypes were observed, as in *R. pachyptila* (Distel et al. 1988, Nussbaumer et al. 2006). Other symbioses appeared more complex, with two or three morphotypes identified as three to six phylogenetically different symbionts (Blazejak et al. 2005, Blazejak et al. 2006, Duperron et al. 2007, Duperron et al. 2008a, Ruehland et al. 2008). A FISH protocol with improved sensitivity, so-called catalyzed reporter deposition (CARD) of fluorescently labelled tyramides (CARD-FISH) (Pernthaler et al. 2002, Pernthaler et al. 2004) enabled the identification of symbionts that had previously defied FISH detection, such as the spirochetes in gutless oligochaetes (Blazejak et al. 2005, Ruehland et al. 2008).

In most cases, the symbiont SSU rRNA gene sequences form clades (Chapter 1.2.3), and thus their phylogenetic position can suggest their functional activities, with the caveat that even closely related species can have very different lifestyles. The physiological properties of symbionts lacking a related cultured isolate remain elusive. Functional activities of the symbionts can be inferred from protein-coding genes diagnostic for characteristic metabolic pathways. Genes of interest for marine chemosynthetic symbioses investigated in recent years were those involved in sulfur metabolism (*aprA*, *sopT*, *dsrAB*), autotrophy (*cbbL*, *cbbM*, *aclBA*) and methane oxidation (*pmoA;* Table 1.3). Gene sequences were retrieved from clone libraries of the PCR products which were amplified from genomic DNA. The presence of diagnostic genes and their phylogenetic affiliation can help identify the metabolic capacities of the symbiont. The protein-coding genes can, in most cases, be assigned to a symbiont, either because only a single symbiont is present or via phylogenetic affiliation of the gene. Closely related symbionts within one host, or lateral gene transfer - common in many gene families - can make a clear assignment difficult. In symbiont communities,

the PCR product often cannot be linked unambiguously to a specific symbiont (e.g. Goffredi et al. 2008, Ruehland et al. 2008). Furthermore, genes might be missed due to primer mismatch and limited datasets for primer development. In conclusion, the information retrieved from such small insert libraries is limited.

Large-insert clone libraries (40 to 100 kb or more) can provide contextual data, such as operon structure and gene arrangements. These libraries are generated via direct cloning of environmental DNA without PCR amplification and can be analyzed for novel genes and pathways. The directed sequencing approach screens for genes of interest and the respective clones are then fully sequenced. Often phylogenetic marker genes, such as the 16S rRNA gene or phylogenetic affiliation of genes on the insert, as determined by similarity searches (such as via the BLASTx algorithm), help classify the DNA fragment. This approach has not become a standard tool in marine chemosynthetic symbiosis research and only a few such studies exist (Blazejak 2006, Campbell et al. 2003, Rotaru 2005).

The time intensive method of screening large insert clone libraries has largely been replaced by so-called 'shotgun' sequencing that allows a much higher throughput of genomic and metagenomic sequencing (Hugenholtz and Tyson 2008). While genome sequencing comprises the genetic information of single organisms, the metagenomic approach encompasses the genomes of (almost) all (micro)organisms in a particular habitat. Thus, this approach is applied to target the genetic information of 'uncultured' microorganisms and mixed communities (Handelsman et al. 1998). In contrast to the rapidly increasing number of microbial genomes and metagenomes, just two genomes and three metagenomes of marine chemosynthetic symbioses have been sequenced so far: the endosymbionts of two deep-sea clams, and the metagenomes of a tubeworm symbiosis, an alvenellid ectosymbiosis and the gutless oligochaete endosymbiosis (Grzymski et al. 2008, Kuwahara et al. 2007, Newton et al. 2007, Robidart et al. 2008, Woyke et al. 2006).

Symbiont genomic and metagenomic approaches are hampered by the presence of host tissue and DNA. To reduce the amount of contaminating host DNA, separation of host and symbiont can be realized by methods such as density gradient centrifugation (Newton et al. 2007, Woyke et al. 2006). This approach is especially effective for the very dense sulfur-storing symbionts (Distel and Felbeck 1988a). Interestingly, the cloning of large inserts from host-symbiont community DNA seems to be another separation step as symbiont DNA was preferentially cloned over host DNA (T. Woyke, personal communication, Blazejak 2006). Bioinformatics tools are required to separate the DNA of individual genomes in a metagenomic sample. The *O. algarvensis* symbiont metagenome contained the genetic information of four symbionts plus some contaminating host and bacterial DNA. Bioinformatic binning approaches successfully classified the sequence reads into four symbiont-specific clusters and unbinned reads (Woyke et al. 2006). Such binning can be achieved by GC content or di- and

oligonucleotide patterns of the DNA, but can be hampered by similar DNA signatures and when horizontal gene transfer occurs. Another drawback of metagenomics is that shorter DNA fragments often cannot be classified reliably (Hugenholtz and Tyson 2008). Short DNA fragments can be a problem in diverse habitats where assembly of sequence reads is difficult. As such, closed genomes (fully assembled into one genome sized contig) cannot be obtained from metagenome studies (e.g. Sleator et al. 2008, Warnecke and Hugenholtz 2007). An overview of (bioinformatics) tools in metagenomics can be found in Mavromatis et al. (2007) and Kunin et al. (2008).

One way around some of the bioinformatic obstacles in symbiont communities is to further separate the symbionts. Such separation was achieved for some mixed bacterial communities via flow sorting, fluorescence assisted cell sorting (FACS; Brehm-Stecher and Johnson 2004), and by filtration (Baker et al. 2006), and could work for symbiont communities as well (e.g. Caro et al. 2007). The enriched samples can be further analyzed by PCR or genome sequencing. As such enriched samples typically do not provide sufficient DNA for genome sequencing, whole genome amplification (WGA) by multiple displacement amplification (MDA) can be used to increase the amount of DNA in small samples (reviewed in Binga et al. 2008, Lasken 2007). The potential of this approach was nicely demonstrated for termite symbionts. Hongoh and colleagues (Hongoh et al. 2008a, b) sampled the bacterial intracellular symbionts of a single protozoan termite symbiont by micromanipulation, amplified the DNA before genome sequencing and were able to close the genome. Another approach applied MDA to enriched samples using the 16S rRNA as an identifier and hook to capture a specific aggregate consisting of bacteria and archaea (Pernthaler et al. 2008). Drawbacks of MDA are amplification bias, chimera formation, primer-primer interactions and contaminating DNA, especially for small amounts of biomass or DNA such as in single cell approaches (Ishoey et al. 2008, Lasken and Stockwell 2007, Zhang et al. 2006).

1.4.3 Combining identity and function

DNA-based methods including single cell genomics and metagenomics provide insight into potential metabolic pathways. However, those approaches are limited, because they cannot tell which genes are actually expressed and which metabolic pathways are of importance. Expression studies of mRNA and protein analyses have been used to characterize microbial isolates and are now beginning to be applied to microbial communities as metatranscriptomics (reviewed in Warnecke and Hess 2009) and metaproteomics (Lo et al. 2007, Ram et al. 2005, Sowell et al. 2008, Wilmes et al. 2008).

The first proteomic study on chemosynthetic symbioses investigated the *R. pachyptila* symbiont and demonstrated that proteins of starved and well-fed symbionts were differentially expressed (Markert et al. 2007). The *R. pachyptila*

symbiont can be enriched rather easily from the trophosome as it is the only symbiont present, and proteins can be extracted in sufficient quantities. A very recent proteomics approach by Kleiner (2008) aimed at studying the *O. algarvensis* symbiont community. Symbiont separation was achieved with high-resolution density gradient centrifugation. Subsequent proteomic analysis of these fractions enriched in the different symbionts allowed assigning identified proteins to the symbionts based on the distinct fractions they were derived from (Kleiner 2008).

Methods beyond DNA-based "omics" target mRNA and proteins and are often combined with imaging techniques, which enable single-cell resolution (Table 1.4). Gene expression or protein presence can be assessed by polynucleotide probes targeting the mRNA (mRNA-FISH; Elsaied et al. 2002) or by antibodies against the enzyme of interest (immunolabelling; Cavanaugh et al. 1988). In a recent study, mRNA-FISH was applied to show the expression of the *pmoA* by methane-oxidizing mussel symbionts that were simultaneously identified via a specific 16S rRNA probe (Pernthaler and Amann 2004, Pernthaler and Pernthaler 2005).

Linking metabolic processes to particular symbionts at the single-cell level has been made possible with recent techniqual developments and the combination of methods. MAR, Raman spectroscopy and nanoSIMS analyses make use of labelled substrates, identification via in situ hybridization (ISH) and visualization via microscopy or nanoSIMS. While MAR is restricted to radioactive isotopes, radioactive as well as stable isotopes can be used for Raman spectroscopy and nanoSIMS. MAR visualizes radiation from a radioactive substrate incorporated into biomass in the form of silver grains after exposure to a radiation-sensitive photographic emulsion (Nielsen et al. 2003b). Raman spectroscopy is based on the analysis of scattered photons after a laser light hit the sample. The spectrum of the scattered laser light of a given sample shows characteristic peaks for different (biological) molecules in the cell. Incorporation of labelled substrates is visible as a shift in some pronounced peaks of this spectrum. NanoSIMS analyzes the masses of secondary ions emitted from the sample after bombardment with a primary ion beam (Kuypers and Jørgensen 2007, Lechene et al. 2006). A system of electrostatic collectors similar to microscopical lenses allows imaging of the mass distribution and abundance in the sample. Uptake of a labelled substrate is detectable as an increased isotope ratio of the sample compared to the natural or background isotope ratio.

Quantification of the metabolic processes in individual cells is theoretically possible for all three methods (Huang et al. 2004, Musat et al. 2008, Nielsen et al. 2003a, Sintes and Herndl 2006). In combination with in situ hybridization, Raman

Table 1.4: Overview on methods, which combine identity and function in microbial ecology with focus on marine chemosynthetic symbioses. Non-symbiotic examples and references are only given when no studies on marine chemosynthetic symbioses are known.

Method	Target		Example
	Identity	Function	
DNA-based			
Gene FISH	16S rRNA	gene	marine plankton[1]
Single cell PCR	16S rRNA	gene	termite hindgut microbiota[2], marine plankton[3]
Genomics (enriched)	database, 16S rRNA	genome	Clam symbionts[4, 5], termite gut protozoen[6, 7] symbiont
Genomics (single cell)	databases, 16S rRNA	genome	soil microbiota[8, 9]
Metagenomics	databases, 16S rRNA	metagenome	*O. algarvensis* symbiont community[10], *R. pachyptila* symbiont[11], *Alvinella pompejana* ectobiont community[12], enriched syntrophic bacterial archaeal association[13]
mRNA-based			
mRNA FISH	16S rRNA	mRNA	*Lamellibrachia* sp., *B. azoricus* and *B. puteoserpentis* symbioses [14, 15, 16]
Metatranscriptomics	databases, metagenome, phylogenetic classification	mRNA	soil, surface water picoplankton, coastal water mesocosm (reviewed in[17])
Protein-based			
Immunolabelling	cell features (TEM)	protein	Clam and oligochaete symbioses[18, 19, 20, 21]
Immuno FISH	16S rRNA	protein	no studies known from microbial ecology
Metaproteomics	16S rRNA, metagenome	protein	*O. algarvensis* symbiosis[22]
Metabolite-based			
MAR	in situ localization	metabolite	*Solemya reidi* and *R. pachyptila* symbioses[23, 24]
MARFISH	16S rRNA	metabolite	Marine and freshwater plankton, activated sludge, biofilm (reviewed in[25, 26]), *O. algarvensis* symbiosis (this study)
Raman FISH	16S rRNA	metabolite	groundwater biofilm[27]
NanoSIMS	in situ localization	metabolite	shipworm *Lyrodus pedicellatus* symbiont[28], filamentous cyanobacteria *Anabaena oscillarioides*[29]
NanoSIMS with ISH	16S rRNA	metabolite	bioreactor microbial community[30], coculture of *Anabaena* and *Rhizobium*[31], oral biofilm[31], plankton[32], *O. algarvensis* symbiosis (this study)

[1] Moraru et al. 2009, [2]Ottesen et al. 2006, [3]Stepanauskas and Sieracki 2007, [4]Kuwahara et al. 2007, [5]Newton et al. 2007, [6]Hongoh et al. 2008a, [7]Hongoh et al. 2008b, [8]Kvist et al. 2007, [9]Podar et al. 2007, [10]Woyke et al. 2006, [11]Robidart et al. 2008, [12]Grzymski et al. 2008, [13]Pernthaler et al. 2008, [14]Elsaied et al. 2002, [15]Pernthaler and Amann 2004, [16]Pernthaler and Pernthaler 2005, [17]Warnecke and Hess 2009, [18]Cavanaugh et al. 1988, [19]Giere and Krieger 2001, [20]Dubilier et al. 2001, [21]Krieger et al. 2000, [22]Kleiner 2008, [23]Fisher and Childress 1986, [24]Bright et al. 2000, [25]Wagner et al. 2006, [26] Okabe et al. 2004, [27]Huang et al. 2007, [28]Lechene et al. 2007, [29]Popa et al. 2007, [30]Behrens et al. 2008, [31]Li et al. 2008, [32]Musat et al. 2008.

spectroscopy (Huang et al. 2007), MAR[3] (reviewed in Okabe et al. 2004, Wagner et al. 2006) and nanoSIMS (Behrens et al. 2008, Li et al. 2008, Musat et al. 2008) can be applied to mixed communities and identify the active population. MAR and nanoSIMS combined with CARD-FISH were applied in the present thesis and will be explained in more detail in Chapter 2.3.

1.5 Aims

The present PhD thesis investigated aspects of the phylogenetic and metabolic diversity of the gutless oligochaete symbiosis. As gutless oligochaete symbionts cannot be cultured outside their host, cultivation-independent approaches were applied.

Inanidrilus exumae co-occurs with several other gutless oligochaete species off the coast of Lee Stocking Island in the Bahamas. While 18 host species from the Bahamas were morphologically identified, the symbiont community of only one host has been investigated in detail (Blazejak et al. 2006). The phylogenetic diversity of the symbiont community of *I. exumae* was studied applying the full cycle rRNA approach. In addition, the metabolic potential of the *I. exumae* symbionts was investigated. Protein-coding genes indicative of autotrophy and sulfur metabolism were amplified by PCR and their phylogenetic affiliation studied. The results of this part of the thesis led to a manuscript for publication, in which the details on methods and results can be found. A brief summary of the results, additional discussion on this topic followed by an outlook is provided in Chapter 2.1.

The metabolic diversity of the symbionts in the gutless oligochaete *Olavius algarvensis* was addressed in incubation tracer experiments. The experiments were designed based on the recent metagenomic study, which had revealed that the two sulfur-oxidizing and the two sulfate-reducing symbionts of *O. algarvensis* have autotrophic potential (Woyke et al. 2006). Incubations were set up to investigate which energy sources would support inorganic carbon fixation in the *O. algarvensis* symbiosis. Therefore, live worms were incubated under oxic and anoxic conditions in the presence and absence of different electron donors and electron acceptors. Radiolabelled bicarbonate was applied as a tracer to follow the uptake of inorganic carbon into biomass under different incubation conditions. Incorporated label was analyzed by liquid scintillation counting (LSC) for bulk analyses. In addition, elemental sulfur stored within the *O. algarvensis* symbiosis was analyzed by high performance liquid chromatography (HPLC) and its influence on inorganic carbon uptake was investigated.

[3] MARFISH (Microautoradiography fluorescence in situ hybridization) was described under different names: MAR-FISH (Ito et al. 2002, Lee et al. 1999), STAR-FISH (substrate tracking autoradiography FISH; Ouverney and Fuhrman 1999), and MICRO-FISH (Microautoradiography-FISH; Cottrell and Kirchman 2000).

As these incubation experiments did not result in a manuscript intended for publication, an extended introduction and discussion is presented in Chapter 2.2.

In addition to bulk analyses of inorganic carbon uptake in the *O. algarvensis* symbiosis, single-cell tools were applied to identify those symbionts that incorporated the inorganic carbon. The focus was on the chemoautotrophic symbionts of *O. algarvensis* and the question of whether both symbionts were able to fix inorganic carbon under oxic conditions without external energy sources. Two approaches were used, MAR and nanoSIMS, and both methods were combined with in situ hybridization to identify the symbionts within the oligochaete microbial community. Aside from qualitative identification of the active symbionts in *O. algarvensis*, uptake rates were calculated for individual symbiont cells. The results from the nanoSIMS analyses are presented in a publication. As both methods used in this thesis are rather new techniques to microbial ecology and have not yet been applied to marine chemosynthetic symbioses, background information for both approaches as well as some methodological details are given in Chapter 2.3.

Another approach studying symbiont metabolic diversity targeted the symbiont genomes of *Inanidrilus leukodermatus*. *I. leukodermatus* harbours a Gamma 1 symbiont and up to three phylogenetically distinct alphaproteobacterial symbionts. To gain insight into the metabolic capacities of this symbiosis a large insert (fosmid) library was generated. Fosmid clones were screened for genes involved in autotrophy and energy generation as well as for 16S rRNA coding genes. Selected fosmids were sequenced and gene arrangement was compared with genomic data from symbionts and free-living bacteria. The preliminary results, discussion and suggestion for future experiments are presented in Chapter 2.4.

2 Results and Discussion

The results and discussion part is divided into four chapters, each chapter with a brief introduction and, if necessary, some methodological information preceding the results and their discussion. Instead of a general outlook each chapter includes an outlook focussing on the respective topic of that chapter. The results and discussion part is summed up in a short general conclusion.

2.1 A peculiar symbiont community in *Inanidrilus exumae*

The Caribbean Sea is a hot spot of diversity and abundance of gutless oligochaetes. The Belize barrier reef (Erséus 1990, Erséus and Giere 1995) and the sediments around the Bahamas island Lee Stocking Island (Erséus 2003) have been studied most intensively. To date 18 oligochaete species are known from Lee Stocking Island (Erséus 2003), where they inhabit shallow water sandy sediments. At all sites several species co-occur, with some species endemic to one site, and others more widely distributed (Erséus 2003). The species richness and distribution makes this an excellent site to study the biogeography and evolution of these small marine symbiotic worms.

In contrast to the host diversity, the symbiont community has been investigated in detail for only one species, *Inanidrilus makropetalos* (Blazejak et al. 2006). However, several gutless oligochaete hosts from the Bahamas and other habitats were screened for Gamma 1 symbiont 16S rRNA sequences in a study aimed at elucidating the evolution of the host and the primary Gamma 1 symbionts (C. Erséus, unpublished data, Blazejak 2006). Gamma 1 sequences were derived from 16 *Olavius* and *Inanidrilus* species, with 9 sequences originating from Bahamas species. Both gutless oligochaetes and the primary Gamma 1 symbionts are monophyletic. A detailed analysis of potential co-speciation is in progress (C. Erséus, unpublished data). For the Bahamas gutless oligochaete *I. exumae* no Gamma 1 sequence could be obtained, but a different gammaproteobacterial sequence was found in 16S rRNA clone libraries (Blazejak 2006).

I. exumae was selected for this study as the second host from Bahamas beside *I. makropetalos* (Blazejak et al. 2006) because of its potentially unusual symbiont community. *I. exumae* inhabits subtidal fine sandy sediments together with a few other gutless oligochaete species, including *I. makropetalos*, all being rather small, less than 10 mm long (Erséus 2003). A “high degree of microhabitat partitioning” was suggested in view of the high number of co-occurring species (Erséus 2003).

2.1.1 Phylogeny and function of the *I. exumae* symbionts

Comparative 16S rRNA sequence analysis and fluorescence in situ hybridization of the *I. exumae* symbiosis revealed six symbionts belonging to the Gamma-, Alpha- and Deltaproteobacteria (Figure 2.1 and 2.2). The symbiont community differed between specimens, with no one host individual bearing all six symbionts. However, at least one symbiont of each proteobacterial subdivision was identified in each specimen studied (except one specimen, for which only 16 clones in total could be retrieved, all of which being gammaproteobacterial 16S rRNA sequences, manuscript 2). One of the two delta- and all three alphaproteobacterial symbionts were closely related to other oligochaete symbionts.

Deltaproteobacterial symbionts

The deltaproteobacterial symbionts Delta 3 and Delta 9 of *I. exumae* are most likely sulfate reducers, based on their close phylogenetic affiliation with the sulfate-reducing symbionts of *O. ilvae* and *O. algarvensis* and free-living sulfate-reducing bacteria within the Desulfobacteraceae (Figure 2.2). The presence of the APS reductase-encoding gene *apr*A is another strong hint for a sulfate-reducing capacity within the *I. exumae* symbiosis (Figure 2.3). The deduced protein sequence grouped with AprA sequences from sulfate-reducing free-living bacteria. Only one AprA sequence was found in the clone libraries of the two *I. exumae* specimens analyzed. For these two specimens 16S rRNA sequences for the Delta 9 symbiont and associated deltaproteobacterial clones but none for the Delta 3 symbiont were obtained (manuscript 2 and Brewig 2006). Thus the *apr*A sequence from *I. exumae* may derive from the Delta 9 symbiont. DNA from specimens yielding Delta 3 symbiont sequences was not available for analysis of protein-coding genes.

Alphaproteobacterial symbionts

Three alphaproteobacterial symbionts were identified in *I. exumae*, Alpha 1a, Alpha 2a and Alpha 2b. All three are closely related to gutless oligochaete alphaproteobacterial symbionts (Figure 2.2). The function of these symbionts remains elusive. It was suggested that they fulfil a similar role as the deltaproteobacterial symbionts by recycling host waste products (manuscript 2, Blazejak et al. 2006). In addition, the alphaproteobacterial symbionts might store carbon as polyhydroxy-alkanoates (PHA) or glycogen, as inferred from the presence of small granules in the alphaproteobacterial symbionts of *I. leukodermatus* (Giere and Langheld 1987). Such storage compounds are common in free-living bacteria including Alphaproteobacteria (Liebergesell et al. 1991, Meyer et al. 2006, Wong and Liu 2007).

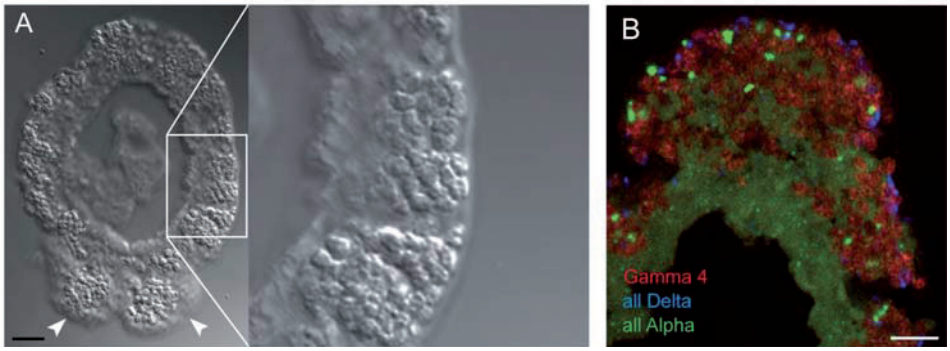


Figure 2.1: In situ identification of the *I. exumae* symbionts. (A) Differential interference contrast image of a cross section through an entire worm, arrowheads marking the genital pads, and magnification of the inset to the right. (B) Epifluorescence image of the symbiotic region showing a triple hybridizations with the probes IexuGAM4, DSS658 and combined ImakALF1b, IexuALFb and IexuALFd probes targeting the Gamma 4 symbiont (red), deltaproteobacterial symbionts (blue) and alphaproteobacterial symbionts (green), respectively. Scale bar: 10 µm (A) and 5 µm (B).

It is remarkable that almost all alphaproteobacterial symbionts of gutless oligochaetes fell within symbiont clades (Figure 2.2). The Alpha 2a and Alpha 2b symbionts of *I. exumae* form the Alpha 2 clade with the Alpha 2 symbiont of the co-occurring *I. makropetalos*. The two *I. exumae* Alpha 2 symbionts were never found within the same *I. exumae* specimen, suggesting that they might exclude each other's presence. However, material for FISH was very limited, and more individuals need to be analyzed for a thorough investigation of symbiont distribution patterns in *I. exumae*. The third alphaproteobacterial symbiont of *I. exumae*, Alpha 1a, was affiliated with a clade comprising symbionts of *I. leukodermatus* from Bermuda and *O. loisae* from the Great Barrier Reef. This Alpha 1a clade includes symbionts from habitats around the world, in contrast to the Alpha 2 clade, which consists of symbionts from the Bahamas only. The Alpha 1c and the Alpha 3 symbiont of *I. leukodermatus* were not affiliated with any of the other symbiont clades (Figure 2.2). Further studies of gutless oligochaete symbiont communities and symbiont diversity would clearly shed more light on the evolution of symbiont clades and their geographic distribution.

A novel gammaproteobacterial primary symbiont

A new gammaproteobacterial primary symbiont was identified in *I. exumae*. This Gamma 4 symbiont resembled the Gamma 1 symbiont in its form, size and content of storage globules. Comparative 16S rRNA sequence and CARD-FISH analyses strongly indicate that in *I. exumae* the Gamma 1 symbiont is absent (manuscript 2).

The Gamma 4 symbiont is only distantly related to the Gamma 1 symbiont clade and other thioautotrophic symbionts (>10% sequence divergence), and forms a new symbiont clade within the Gammaproteobacteria (Figure 2.2). The closest relatives are clones from diverse habitats, including caves and deep-sea, arctic and shallow water

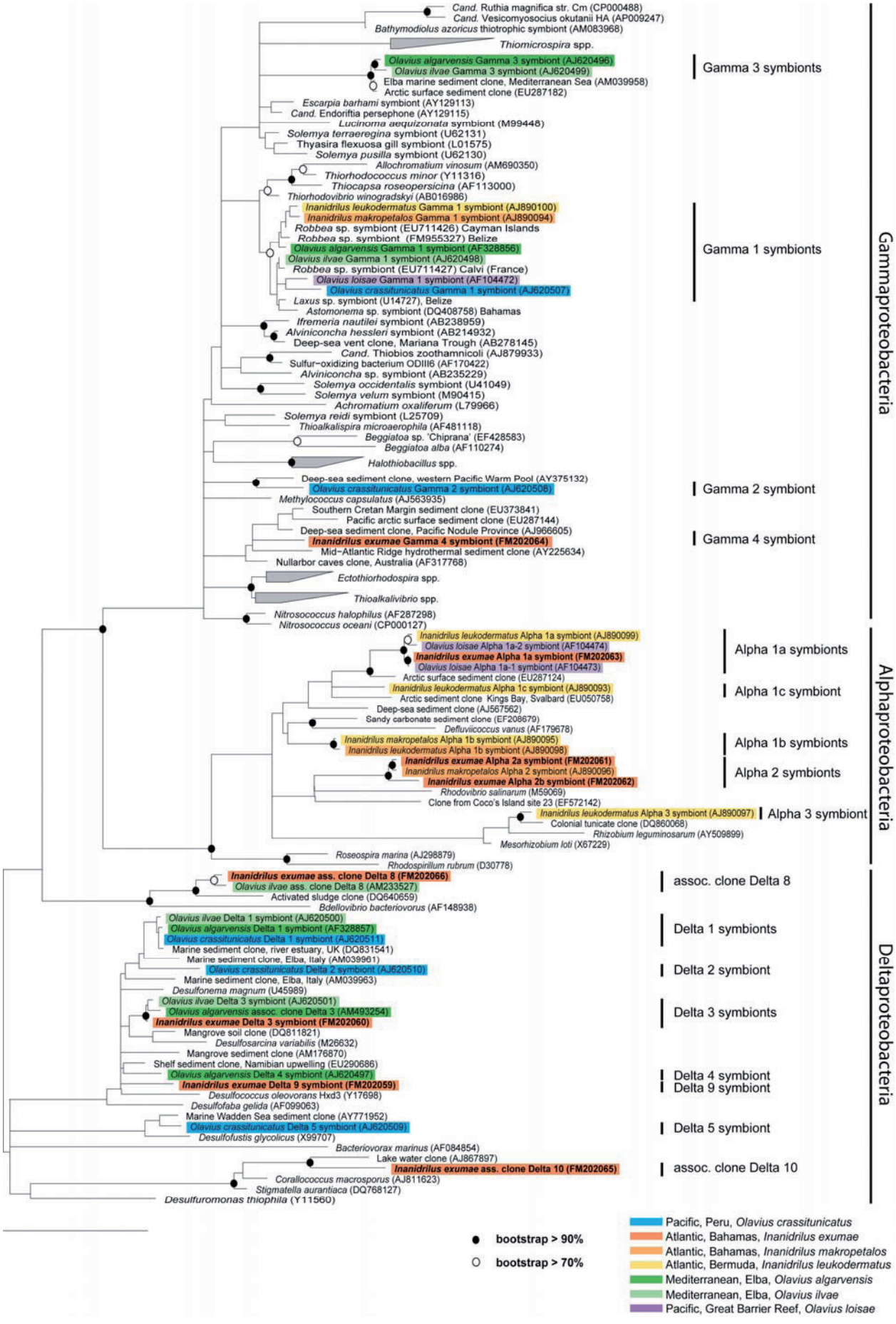

Cand. Ruthia magnifica str. Cm (CP000488)
Cand. Vesicomyosocius okutanii HA (AP009247)
Bathymodiolus azoricus thiotrophic symbiont (AM083968)
Thiomicrospira spp.
Olavius algarvensis Gamma 3 symbiont (AJ620496)
Olavius ilvae Gamma 3 symbiont (AJ620499)
Elba marine sediment clone, Mediterranean Sea (AM039958)
Arctic surface sediment clone (EU287182)
Escarpia barhami symbiont (AY129113)
Cand. Endoriftia persephone (AY129115)
Lucinoma aequizonata symbiont (M99448)
Solemya terraeregina symbiont (U62131)
Thyasira flexuosa gill symbiont (L01575)
Solemya pusilla symbiont (U62130)
Allochromatium vinosum (AM690350)
Thiorhodococcus minor (Y11316)
Thiocapsa roseopersicina (AF113000)
Thiorhodovibrio winogradskyi (AB016986)
Inanidrilus leukodermatus Gamma 1 symbiont (AJ890100)
Inanidrilus makropetalos Gamma 1 symbiont (AJ890094)
Robbea sp. symbiont (EU711426) Cayman Islands
Robbea sp. symbiont (FM955327) Belize
Olavius algarvensis Gamma 1 symbiont (AF328856)
Olavius ilvae Gamma 1 symbiont (AJ620498)
Robbea sp. symbiont (EU711427) Calvi (France)
Olavius loisae Gamma 1 symbiont (AF104472)
Olavius crassitunicatus Gamma 1 symbiont (AJ620507)
Laxus sp. symbiont (U14727), Belize
Astomonema sp. symbiont (DQ408758) Bahamas
Ifremeria nautilei symbiont (AB238959)
Alviniconcha hessleri symbiont (AB214932)
Deep-sea vent clone, Mariana Trough (AB278145)
Cand. Thiobios zoothamnicoli (AJ879933)
Sulfur-oxidizing bacterium ODIII6 (AF170422)
Alviniconcha sp. symbiont (AB235229)
Solemya occidentalis symbiont (U41049)
Solemya velum symbiont (M90415)
Achromatium oxaliferum (L79966)
Solemya reidi symbiont (L25709)
Thioalkalispira microaerophila (AF481118)
Beggiatoa sp. 'Chiprana' (EF428583)
Beggiatoa alba (AF110274)
Halothiobacillus spp.
Deep-sea sediment clone, western Pacific Warm Pool (AY375132)
Olavius crassitunicatus Gamma 2 symbiont (AJ620508)
Methylococcus capsulatus (AJ563935)
Southern Cretan Margin sediment clone (EU373841)
Pacific arctic surface sediment clone (EU287144)
Deep-sea sediment clone, Pacific Nodule Province (AJ966605)
Inanidrilus exumae Gamma 4 symbiont (FM202064)
Mid-Atlantic Ridge hydrothermal sediment clone (AY225634)
Nullarbor caves clone, Australia (AF317768)
Ectothiorhodospira spp.
Thioalkalivibrio spp.
Nitrococcus halophilus (AF287298)
Nitrococcus oceani (CP000127)
Inanidrilus leukodermatus Alpha 1a symbiont (AJ890099)
Olavius loisae Alpha 1a-2 symbiont (AF104474)
Inanidrilus exumae Alpha 1a symbiont (FM202063)
Olavius loisae Alpha 1a-1 symbiont (AF104473)
Arctic surface sediment clone (EU287124)
Inanidrilus leukodermatus Alpha 1c symbiont (AJ890093)
Arctic sediment clone Kings Bay, Svalbard (EU050758)
Deep-sea sediment clone (AJ567562)
Sandy carbonate sediment clone (EF208679)
Defluviicoccus vanus (AF179678)
Inanidrilus makropetalos Alpha 1b symbiont (AJ890095)
Inanidrilus leukodermatus Alpha 1b symbiont (AJ890098)
Inanidrilus exumae Alpha 2a symbiont (FM202061)
Inanidrilus makropetalos Alpha 2 symbiont (AJ890096)
Inanidrilus exumae Alpha 2b symbiont (FM202062)
Rhodovibrio salinarum (M59069)
Clone from Coco's Island site 23 (EF572142)
Inanidrilus leukodermatus Alpha 3 symbiont (AJ890097)
Colonial tunicate clone (DQ860068)
Rhizobium leguminosarum (AY509899)
Mesorhizobium loti (X67229)
Roseospira marina (AJ298879)
Rhodospirillum rubrum (D30778)
Inanidrilus exumae ass. clone Delta 8 (FM202066)
Olavius ilvae ass. clone Delta 8 (AM233527)
Activated sludge clone (DQ640659)
Bdellovibrio bacteriovorus (AF148938)
Olavius ilvae Delta 1 symbiont (AJ620500)
Olavius algarvensis Delta 1 symbiont (AF328857)
Olavius crassitunicatus Delta 1 symbiont (AJ620511)
Marine sediment clone, river estuary, UK (DQ831541)
Marine sediment clone, Elba, Italy (AM039961)
Olavius crassitunicatus Delta 2 symbiont (AJ620510)
Marine sediment clone, Elba, Italy (AM039963)
Desulfonema magnum (U45989)
Olavius ilvae Delta 3 symbiont (AJ620501)
Olavius algarvensis assoc. clone Delta 3 (AM493254)
Inanidrilus exumae Delta 3 symbiont (FM202060)
Mangrove soil clone (DQ811821)
Desulfosarcina variabilis (M26632)
Mangrove sediment clone (AM176870)
Shelf sediment clone, Namibian upwelling (EU290686)
Olavius algarvensis Delta 4 symbiont (AJ620497)
Inanidrilus exumae Delta 9 symbiont (FM202059)
Desulfococcus oleovorans Hxd3 (Y17698)
Desulfofaba gelida (AF099063)
Marine Wadden Sea sediment clone (AY771952)
Olavius crassitunicatus Delta 5 symbiont (AJ620509)
Desulfofustis glycolicus (X99707)
Bacteriovorax marinus (AF084854)
Lake water clone (AJ867897)
Inanidrilus exumae ass. clone Delta 10 (FM202065)
Corallococcus macrosporus (AJ811623)
Stigmatella aurantiaca (DQ768127)
Desulfuromonas thiophila (Y11560)
Gamma 3 symbionts
Gamma 1 symbionts
Gamma 2 symbiont
Gamma 4 symbiont
Alpha 1a symbionts
Alpha 1c symbiont
Alpha 1b symbionts
Alpha 2 symbionts
Alpha 3 symbiont
assoc. clone Delta 8
Delta 1 symbionts
Delta 2 symbiont
Delta 3 symbionts
Delta 4 symbiont
Delta 9 symbiont
Delta 5 symbiont
assoc. clone Delta 10
Gammaproteobacteria
Alphaproteobacteria
Deltaproteobacteria
bootstrap > 90%
bootstrap > 70%
Pacific, Peru, Olavius crassitunicatus
Atlantic, Bahamas, Inanidrilus exumae
Atlantic, Bahamas, Inanidrilus makropetalos
Atlantic, Bermuda, Inanidrilus leukodermatus
Mediterranean, Elba, Olavius algarvensis
Mediterranean, Elba, Olavius ilvae
Pacific, Great Barrier Reef, Olavius loisae

Figure 2.2 (previous page): Phylogenetic reconstruction based on 16S rRNA sequences showing the phylogenetic affiliations of the gamma,- alpha,- and deltaproteobacterial symbionts of *I. exumae*. Sequences from gutless oligochaete symbionts are pointed out by a colour code with sequences from this study shown in bold face. Designation of symbionts and clone sequences from *I. exumae* refer to their phylogenetic affiliation with previously described gutless oligochaete symbiont clades. Sequences without such affiliations were assigned new numbers in ascending order. The asterisk indicates a node that was well supported in the majority of treeing calculations. The consensus tree shown is based on a tree generated by maximum likelihood analysis. Branching orders that were not supported in most calculation methods are shown as multifurcations. Scale bar represents 10% estimated phylogenetic divergence for non-multifurcation branches.

sediments. The next cultivated relatives of the Gamma 4 symbiont, with more than 9% sequence divergence, are sulfur-storing bacteria including *Ectothiorhodospira* species, alkalophilic sulfur oxidizers such as *Thioalkalispira* species, and the ammonia-oxidizing *Nitrosococcus* species (Figure 2.2).

Function of the novel symbiont

The Gamma 4 symbiont appears to share a functional role with the Gamma 1 symbionts as a sulfur-oxidizing chemoautotroph. Although its phylogenetic affiliation does not clearly indicate a potential metabolism, the majority of the closest cultivated relatives are sulfur-storing sulfur-oxidizing bacteria. The presence of sulfur globules within the Gamma 4 cells (manuscript 2) characterizes this symbiont as a sulfur bacterium and indicates a sulfur-oxidizing capacity. Analysis of protein-coding genes further corroborated the chemoautotrophic potential of the Gamma 4 symbiont. Genes coding for the large subunit of the RubisCO (*cbbL*) and for the oxidative APS reductase (*aprA*), indicative of an autotrophic, sulfur-oxidizing metabolism, were amplified from *I. exumae*. Based on the phylogenetic affiliation, these genes most likely derive from the Gamma 4 symbiont, although horizontal gene transfer of these genes cannot be completely ruled out.

The ability to oxidize ammonia was investigated because ammonia-oxidizing *Nitrosococcus* species were among the close relatives of the Gamma 4 symbiont. The gene *amoA*, coding for one subunit of the ammonium monooxygenase, was chosen as a target gene. However, two sets of primers (Holmes et al. 1995, Purkhold et al. 2000) and several different amplification conditions did not result in the expected sequence (Brewig 2006). This suggests that the Gamma 4 symbiont uses reduced sulfur compounds rather than ammonia as electron donor.

A remarkable difference between the Gamma 4 symbiont and most other marine chemoautotrophic symbionts was the presence of several copies of one protein-coding gene, *aprA* (Figure 2.3). So far, only one other study reported a similar variety for a marine chemosynthetic symbiont. The mussel *Bathymodiolus childressi* harbours a single methanotroph-related symbiont. However, five genes coding for the *pmoA*, a gene characteristic for methanotrophic bacteria, were found (Duperron et al. 2007). Multiple copies of one gene or operon are not rare in free-living bacteria, however, and

are often suggested to be of ecophysiological advantage (Meyer and Kuever 2007a, Scott et al. 2006, Stolyar et al. 1999). The Gamma 4 symbiont not only has two different APS reductase loci (this work, Meyer and Kuever 2007a), but each *apr*A gene is also present in two slightly different sequence variations. These sequences might derive either from different Gamma 4 strains and/or from multiple APS reductase loci in a single strain. Such variability on the nucleotide level is surprising for an oligochaete symbiont and would be in contrast to the very low strain variability (frequency of polymorphic sites) found for the *O. algarvensis* symbionts (Woyke et al. 2006).

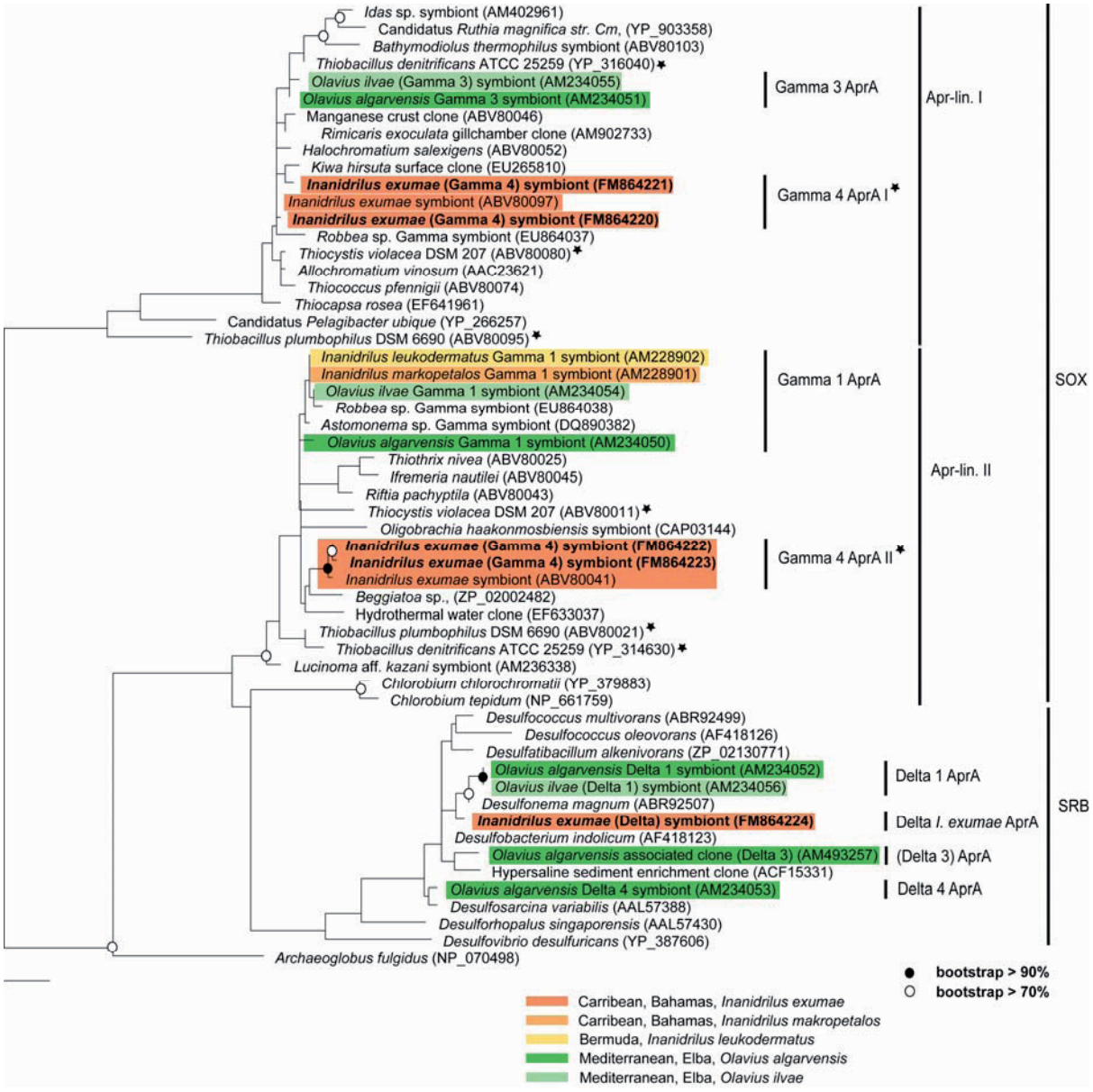


Figure 2.3: Phylogenetic affiliation of the alpha subunit of the APS reductase (AprA) from *I. exumae* based on deduced amino acid sequences. The symbiont name of sequences whose origin was inferred through phylogeny is in parentheses. Five distinct *aprA* sequences were found in *I. exumae*, resulting in five translated protein sequences. Asterisks indicate AprA sequences from organisms with two *aprA* loci. SOX, sulfur-oxidizing bacteria; SRB, sulfate-reducing bacteria. See legend of Figure 2.2 for additional information.

2.1.2 A novel primary symbiont in *I. exumae*

The novel primary symbiont of *I. exumae* seems to share both its morphology and its chemoautotrophic capacity with the Gamma 1 symbionts. It is the first example of a marine chemoautotrophic symbiosis in which a host group has established an association with two unrelated symbiont clades as its primary symbionts. Almost all (primary) chemoautotrophic symbionts known fall into host-genus specific clades (Dubilier et al. 2008). Even when lateral symbiont acquisition occurred, as in a symbiosis which symbionts are generally transmitted horizontally (Stewart et al. 2008), or when symbionts are acquired locally (Vrijenhoek et al. 2007, Won et al. 2003), these symbionts were still retrieved from the same 16S rRNA symbiont clade. One other host group in which the symbionts are not phylogenetically clustered within one clade is *Solemya* whose symbionts are scattered among the chemoautrotrophic symbiont clades (Figure 1.4, Dubilier et al. 2008).

Symbiont acquisition and replacement of the primary symbiont by a symbiont from another phylogenetic lineage have been found in non-chemoautotrophic symbioses, such as the association of anaerobic ciliates with methanogenic archaeal symbionts (van Hoek et al. 2000). The authors proposed that the symbionts were acquired by a common ancestor of the ciliates, prior to radiation, and that acquisition and replacement of symbionts occurred as a reaction to niche adaptation of the host (van Hoek et al. 2000). Such niche adaption might also have occurred in the *I. exumae* symbiosis.

The high prevalence of the Gamma 1 symbionts in gutless oligochaetes and the phylogenetic position of *I. exumae* which is not deep-branching (C. Erséus, unpublished data), indicate that the Gamma 1 symbiont may represent the ancestral state of the gutless oligochaete symbiosis and the Gamma 4 symbiont was taken up later. Over time the Gamma 1 symbiont might have been lost by competition or mere coincidence (manuscript 2). It is also possible that *I. exumae* never harboured a Gamma 1 symbiont and the Gamma 4 was the first and only primary symbiont. Yet another possibility might be that the Gamma 1 symbiont was lost before the Gamma 4 symbiont was taken up. The current data however point to symbiont replacement being a likely scenario for *I. exumae*, but further investigation is needed to answer this question.

2.1.3 Conclusion

It may be advantageous for host organisms to leave the chance open for symbiont acquisition and replacement. Very tight co-evolution might be a dead end for both the host and the symbiont, for whom genetic exchange becomes restricted due to limited interaction with free-living bacteria and genome size can be much reduced (Moran 2007, Perez-Brocal et al. 2006). Indeed, strict co-evolution of host and symbiont might be rather the exception than the rule in mutualistic host-microbe associations (Mueller and Rabeling 2008). The presence of a primary symbiont from a novel symbiont lineage

indicates a high plasticity in symbiont specificity of gutless oligochaetes allowing them to take up symbionts not closely related to symbionts of co-occurring species. This plasticity of the gutless oligochaetes' symbiont community is further reflected in the diversity of symbiont community compositions and their physiological features, and might be one factor that enables niche adaptation and thus the co-occurrence of several gutless oligochaete species.

2.1.4 Outlook

The remarkable symbiont community of *I. exumae* demonstrated that there is still a great deal to discover regarding symbiont diversity in gutless oligochaetes. So far, each new host investigated has revealed novel symbionts, and even novel symbiont clades. Nevertheless the symbiont community is host species-specific and differs even between co-occurring species, such as *I. exumae* and *I. makropetalos*.

To better understand the evolution and biogeography of this unique symbiosis, more data on the symbiont community of co-occurring hosts and hosts from different habitats is clearly needed. Will clades such as the Alpha 2 remain local as more symbionts are identified? Are there undiscovered lineages of free-living bacteria within clades that so far include only symbionts? It would be of particular interest to find out if more gutless oligochaetes harbour a Gamma 4 symbiont or if indeed this symbiont acquisition in *I. exumae* was a single event in the evolution of the gutless oligochaete symbiosis. It is also possible that other host groups beside gutless oligochaetes have established an association with symbionts closely related to the Gamma 4 symbiont as is the case for the Gamma 1 clade. The Gamma 1 clade comprises not only the gutless oligochaete symbionts but also ecto- and endosymbionts from nematodes (Figure 2.2).

A free-living symbiont stage has not yet been found for any gutless oligochaete symbiont, possibly due to the fact that the microbial community of only one habitat of gutless oligochaetes has been investigated so far. This study yielded several clones closely related to some of the *O. algarvensis* symbionts (Perner 2003). Studying the microbial community of Bahamas sediments would provide valuable information on the presence and abundance of free-living bacteria related to the *I. exumae* symbionts. The so-called pyro-tag approach targeting a certain region of the 16S rRNA could provide a fast and deep overview of the microbial diversity in a given habitat (e.g. Huse et al. 2008, Neufeld et al. 2008, Sogin et al. 2006). In combination with novel sequencing technologies that allow high through-put analysis with a reasonable sequence length of 450 base pairs this approach could detect sequences which occur in low abundance (Sogin et al. 2006), which might include those from free-living symbiont stages or very closely related bacteria.

Comparing symbiont genomes with those from a very closely related free-living bacterium might provide insight into symbiont adaptations on the genome level. Probes

designed for such sequences which originate from free-living symbionts or closely related free-living bacteria could be used to isolate those cells for (single) genome analyses by methods such as cell capture (Pernthaler et al. 2008), flow sorting (Podar et al. 2007), micromanipulation (Hongoh et al. 2008a) or microfluidics (Marcy et al. 2007). Genome comparison from an evolutionary perspective would be exciting also for the Gamma 4 symbiont and the Gamma 1 symbionts. Differences in genome size reduction, GC content or the load of mobile elements in the respective genomes may support the assumption that the Gamma 4 symbiont has been acquired more recently and that the Gamma 1 symbiont represents the ancestral state. Even more exciting would be to track ecophysiological differences between the Gamma 1 and Gamma 4 symbiont. Such differences might be seen at least partly in protein-coding genes present in the genomes, their gene neighbourhood and operon structure. This might reveal why the Gamma 4 and not the Gamma 1 symbiont dominates the symbiont community in *I. exumae*. The multiple APS reductase loci in the Gamma 4 symbiont, provided they derive from a single symbiont, are already one obvious difference, although their physiological role or advantage remains elusive.

Ecophysiological differences between the primary and other gutless oligochaete symbionts might be seen on the genome level, but probably more so in their metabolic activity. Physiological studies on the gutless oligochaete symbioses have been rare and hampered as gutless oligochaetes cannot be maintained for extended periods of time under laboratory conditions, proliferation has not yet been achieved and symbionts cannot be cultivated. In recent years, methods combining ecophysiological studies and identification of ‘unculturable’ microorganisms in situ have been developed. These methods are promising tools for studying the function of the gutless oligochaete symbiont community and were applied in this thesis (Chapter 2.3).

2.2 Inorganic carbon uptake by the *Olavius algarvensis* symbiosis

Autotrophy in marine chemoautotrophic symbioses

Marine chemoautotrophic symbioses depend on the carbon-fixing ability of their bacterial symbionts for nutrition. While some host species can also feed on organic compounds taken up from the surrounding seawater, the symbionts are an essential food source in most associations. The contribution of symbiont-derived carbon to host nutrition was estimated to be at least 50% for a shallow-water clam (Dando et al. 1986), and can be as high as 100% for the *Riftia pachyptila* and the *Solemya reidi* symbioses (Anderson et al. 1987, Fisher et al. 1989, Stewart et al. 2005).

Symbiotic autotrophic carbon fixation in marine chemoautotrophic symbioses relies on energy generated from the oxidation of reduced sulfur compounds such as sulfide and thiosulfate (reviewed in Cavanaugh et al. 2006, Nelson and Fisher 1995). In addition, most marine chemoautotrophic symbionts store elemental sulfur. It was suggested that this stored sulfur serves as an energy source when external reduced sulfur compounds become limiting. However, noticeable uptake of inorganic carbon in the absence of external reduced sulfur compounds has only been shown in a few symbioses (Dando et al. 1986, Giere et al. 1988b, Schiemer et al. 1990).

The most favourable electron acceptor supporting autotrophy and used by almost all chemoautotrophic symbionts is oxygen. Only the symbiont of the clam *Lucinoma aequizonata* has so far been shown to use nitrate rather than oxygen as electron acceptor (Hentschel et al. 1993, Nelson and Fisher 1995). Carbon fixation did not increase with nitrate in the absence of oxygen, though, so the role of nitrate respiration in this symbiosis remains unclear (Hentschel et al. 1993, Girguis et al. 2000, Nelson and Fisher 1995). Sulfur has also been suggested to serve as electron sink, indicated by sulfide excretion when oxygen becomes limiting (Duplessis et al. 2004). But as for nitrate, the importance of sulfur for inorganic carbon fixation in chemoautotrophic marine symbioses has not yet been shown.

Autotrophy in gutless oligochaetes

Due to the lack of mouth and gut gutless oligochaetes were first assumed to feed on organic compounds taken up from the surrounding porewater through their body wall (Dubilier et al. 2006, Felbeck et al. 1983). Indeed, compounds as large as 70 kDa can cross the gutless oligochaete cuticle by diffusion (J. Krieger and N. Dubilier unpublished data in Dubilier et al. 2006). Yet uptake of organic carbon was slow in comparison with the incorporation of inorganic carbon (Felbeck et al. 1983, Liebezeit et al. 1983). Even more so, inorganic carbon uptake rates in the presence of oxygen were in the range of those in other chemoautotrophic symbioses. This indicates an important role of autotrophy in the gutless oligochaete symbiosis (Felbeck et al. 1983, Giere et al. 1988b).

The uptake of inorganic carbon by the gutless oligochaete symbiosis is affected by the presence of one or both of the internal storage compounds, poly-β-hydroxybutyric acid (PHB) and sulfur. Reduced carbon fixation rates were linked with reducd PHB and sulfur content (Giere et al. 1988b). Such depleted storage compounds were recognizable by a pale appearance of the worms in contrast to white worms which had much higher sulfur and PHB contents (Giere et al. 1988b). The majority of worms freshly collected in the field appear white, although pale to transparent worms occur occasionally (personal observation, Giere et al. 1982).

Most of what is known about the ecology and physiology of gutless oligochaete symbioses was derived from studies on a single host species, *Inanidrilus leukodermatus* from Bermuda (Felbeck et al. 1983, Giere et al. 1988b, Giere et al. 1991, Liebezeit et al. 1983). Little is known about the physiology of oligochaete symbioses from other habitats and with different symbiont communities (Dubilier et al. 2001).

Evidence for autotrophy in the Olavius algarvensis *symbiosis*

The habitat and the symbiont community of *O. algarvensis* from the Mediterranean Sea differ from those of *I. leukodermatus*. While *I. leukodermatus* inhabits sulfidic, calcareous sediments that are not limited in organic matter (Giere et al. 1982), *O. algarvensis* lives in silicate sediments where sulfide and organic matter are present in very low concentration in the upper 20 cm (C. Lott, unpublished data, Dubilier et al. 2001, Perner 2003). The symbiont community of *I. leukodermatus* consists of the chemoautotrophic sulfur-oxidizing Gamma 1 symbiont, and co-occurring alphaproteobacterial symbionts of as yet unknown function (Blazejak et al. 2006). In contrast, *O. algarvensis* harbours four symbionts with autotrophic potential, two gammaproteobacterial sulfur-oxidizing and two deltaproteobacterial sulfate-reducing symbionts, based on PCR-based and metagenomic analyses (Ruehland et al. 2008, Woyke et al. 2006). Possible electron donors and acceptors for the *O. algarvensis* symbiont community differ in part from those known for marine chemoautotrophic symbioses (Woyke et al. 2006), and will be summarized briefly in the following paragraphs.

Electron donors used by the two sulfur-oxidizing symbionts of *O. algarvensis*, Gamma 1 and Gamma 3, are most likely reduced sulfur compounds such as sulfide and thiosulfate and possibly stored elemental sulfur (Woyke et al. 2006). Elemental sulfur is known to be present in Gamma 1 symbionts (Giere and Krieger 2001, Krieger et al. 2000) and a gene coding for a sulfur globule protein was found in the Gamma 1 symbiont bin (Woyke et al. 2006). It is unknown, however, whether the Gamma 3 symbiont can store elemental sulfur.

The energetically most favourable electron acceptor used by the sulfur-oxidizing symbionts is oxygen. However, gutless oligochaetes spend most of the time in the anoxic zone. Therefore, alternative electron acceptors might play a role when oxygen

becomes limiting. The chemoautotrophic symbionts would depend on the ability to use electron acceptors such as nitrate, fumarate or TMAO for anaerobic respiration (Woyke et al. 2006) to maintain an autotrophic metabolism. Aside from anaerobic respiration, the uptake of organic compounds as carbon and/ or energy sources might also be possible (Woyke et al. 2006).

The sulfate-reducing symbionts use sulfate as electron acceptor. Autotrophy might be supported by energy derived from the oxidation of hydrogen (Woyke et al. 2006). A heterotrophic metabolism would be an additional possible metabolic feature of the sulfate-reducing symbionts (Woyke et al. 2006).

Several of the suggested electron donors and electron acceptors might be provided within the *O. algarvensis* symbioses (Chapter 1.3.4, Dubilier et al. 2001, Woyke et al. 2006). Internally available substrates can include sulfide and other reduced sulfur compounds, fumarate, TMAO or hydrogen, as well as a variety of organic carbon compounds (Woyke et al. 2006).

Aim of the project

The aim of this project was to study the physiology of the *O. algarvensis* symbiosis, scrutinizing hypotheses of the metagenome study, and complementing field studies on in situ porewater analyses, species distribution and abundance (investigated by C. Lott). The focus was the inorganic carbon uptake as an important feature of the *O. algarvensis* symbiosis under different experimental conditions, investigating the influence of stored elemental sulfur on inorganic carbon uptake by analyzing sulfur content and carbon uptake of pale and white worms (Figure 2.4), and the effect of different electron donors and electron acceptors, which might support carbon fixation in the *O. algarvensis* symbiosis.

Figure 2.4: White (left panel) and pale (right panel) gutless oligochaetes. (Picture courtesy of C. Lott.)

Experimental set-up and analyses

Gutless oligochaetes[4] were sampled off the coast of the Italian island Elba and maintained in the laboratory for several weeks to months (see supplemental materials for details on collection and maintenance Chapter C.2). The worms were incubated under oxic and under anoxic conditions in sterile-filtered seawater from the sampling site or artificial seawater. Radiolabelled ^{14}C-bicarbonate was added as a tracer. Incubations were carried out in the absence and in the presence of different electron acceptors (nitrate, fumarate, and TMAO), electron donors (sulfide, thiosulfate, and hydrogen) and organic carbon compounds (acetate, lactate, and succinate). Anoxic incubations were pre-incubated for 1 h at room temperature before the ^{14}C-bicarbonate tracer was added. In this text the term anoxic refers to those incubations that were set-up with medium prepared anoxically. However, traces of oxygen might have been present in the incubation vials, so the term severely hypoxic might be more appropriate as anoxic refers to oxygen-free conditions.

After incubation for four or six hours for most experiments (or as otherwise stated), worms were washed in sterile seawater. The length and width of the worms were measured and the volume calculated. Elemental sulfur was extracted from whole worms with methanol and analyzed by high performance liquid chromatography (HPLC). The same worms were further processed for bulk ^{14}C-inorganic carbon uptake analyses by liquid scintillation counting (LSC). Some samples were in addition analyzed by beta-imager analysis. Uptake of inorganic carbon (nmol C) and inorganic carbon uptake rates (nmol C h^{-1}) were calculated based on the incorporated radioactivity and the specific activity in the sample medium. Values were normalized to worm size as mm^3. Further details on experimental set-up and substrates used can be found in the supplemental materials Chapter C.2.

Presentation and discussion of the results is divided into three parts: i) inorganic carbon uptake in the presence of oxygen without external energy sources and influence of stored elemental sulfur on inorganic carbon uptake, ii) inorganic carbon uptake in the presence of external electron acceptors, anoxic, and iii) inorganic carbon uptake in the presence of external electron donors, anoxic. This is followed by a general discussion and an outlook.

[4] Three gutless oligochaete species (*O. algarvensis*, *O. ilvae* (Giere and Erséus 2002), and a not yet described third species (C. Lott, unpublished data)) co-occur at this sampling site. Because *O. algarvensis* was the most abundant species during most samplings and *O. ilvae* harbours a very similar symbiont community (Ruehland et al. 2008), it was not distinguished between these two species for the incubation experiments. The third species was found in very low abundances only (C. Lott, unpublished data) and was thus not considered to be of major concern.

2.2.1 Inorganic carbon uptake with oxygen in the absence of external energy sources

Sulfur content of white versus pale worms

The elemental sulfur (S) content of white and pale *O. algarvensis* worms was compared to confirm that worms' appearance correlated with the presence or absence of this storage compound. Elemental sulfur concentrations in white worms ranged from about 10 nmol S mm^{-3} to more than 100 nmol S mm^{-3}. In contrast, sulfur could not be detected in pale worms, even when ten worms were pooled for sulfur analysis. Thus, the white (or pale) appearance of the worms was a good indicator for the presence (or absence) of stored sulfur - that is, for the presence (or absence) of a potential internal energy source.

Influence of stored elemental sulfur on inorganic carbon uptake

The presence of stored sulfur strongly affected the ability of the *O. algarvensis* symbiosis to take up inorganic carbon. White worms with stored elemental sulfur incorporated inorganic ^{14}C-labelled carbon into biomass when incubated under oxic conditions in the absence of external energy sources. These worms were already clearly labelled with ^{14}C after 30 min incubation (Figure 2.5). The carbon uptake increased from less than 1 nmol mm^{-3} h^{-1} after 30 min to up to 80 nmol mm^{-3} h^{-1} after 4 h. In contrast, there was little or no carbon uptake by pale worms without detectable sulfur in the absence of an external energy source (Figure 2.5 and Figure 2.6). Inorganic carbon uptake did not correlate linearly with the sulfur content apart from its presence or absence (Figure 2.6).

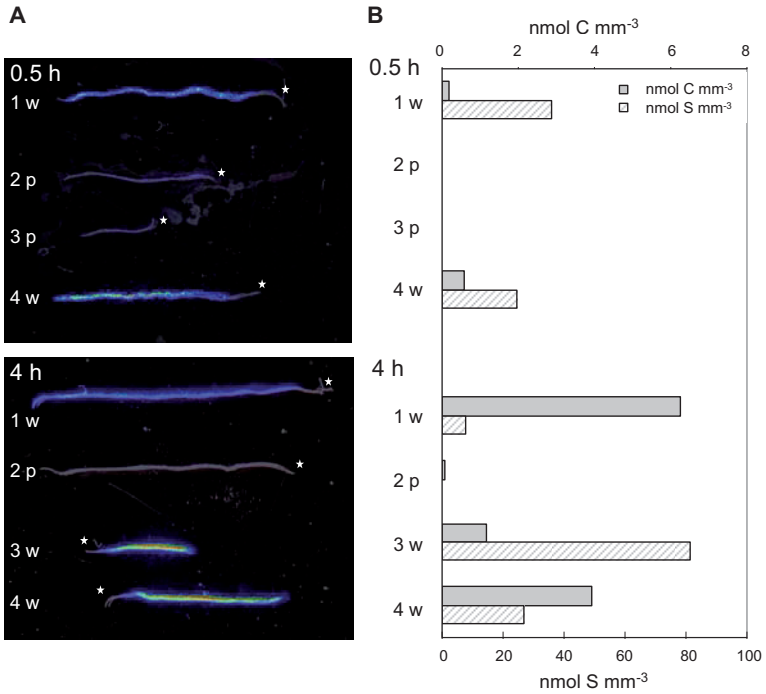


Figure 2.5: Uptake of ^{14}C-labelled inorganic carbon by white (w) and pale (p) *O. algarvensis* in the absence of external energy sources under oxic conditions. (A) Overlay of beta-imager and photographic images showing the ^{14}C-inorganic carbon uptake in colour code as decays per minute after 0.5 h and 4 h incubation. ^{14}C incorporation, indicated by blue (low) to red (high) colour, does not occur in the first segments of the worms where only very few symbionts are present (asterisk). (B) Carbon uptake and sulfur content for the same worms as in (A) show that carbon fixation occured in the presence of elemental sulfur.

Discussion

The *O. algarvensis* symbiosis fixed carbon under oxic conditions in the presence of internal stored sulfur (no external substrate added). In the absence of sulfur (pale worms) and external energy sources, almost no inorganic carbon was incorporated, indicating that the sulfur served as an energy source for inorganic carbon fixation. This carbon fixation activity was most likely carried out by the sulfur-oxidizing chemoautotrophic symbionts of *O. algarvensis*, which can oxidize the stored sulfur in the presence of oxygen. At this point it remains unclear, though, whether the large Gamma 1 symbiont, the smaller Gamma 3 symbiont, or both incorporated the inorganic carbon under these conditions (manuscript 3 and Chapter 2.3).

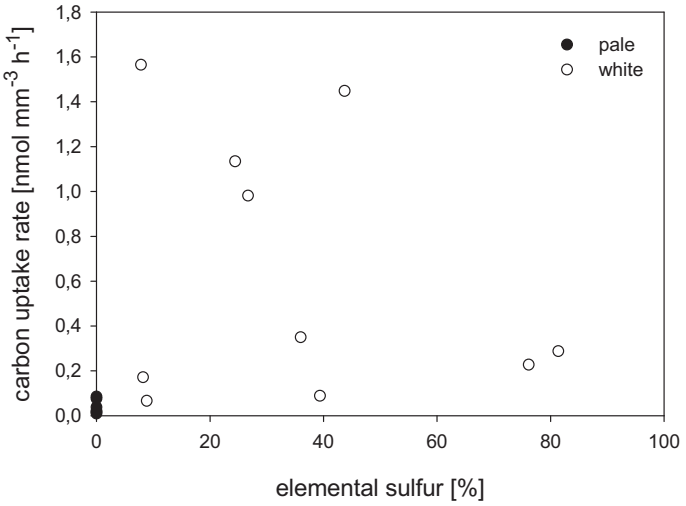


Figure 2.6: Influence of stored elemental sulfur on the uptake of inorganic ^{14}C-labelled carbon by white and pale *O. algarvensis* worms. The sulfur content and the amount of carbon taken up by individual worms show no linear correlation. Pale worms n = 6, white worms n = 10.

Carbon fixation rates of the *O. algarvensis* symbiosis were lower than those of other marine chemoautotrophic symbioses. Assuming a worm fresh weight of 150 µg (Felbeck et al. 1983), inorganic carbon uptake rates of the *O. algarvensis* symbiosis were about 0.6 µmol h^{-1} per g worm, and thus lower than those of the *I. leukodermatus* symbiosis of about 30 µmol g^{-1} h^{-1} (Giere et al. 1988b). Carbon fixation rates by bivalve and tubeworm symbionts were highest only in the presence of sulfide or thiosulfate with 1.3 to 70 µmol g^{-1} h^{-1} (e.g. Anderson et al. 1987, Cavanaugh 1983, Fisher et al. 1989, Girguis et al. 2002, Girguis and Childress 2006, Scott and Cavanaugh 2007, see also discussion in Chapter 2.2.4). However direct comparison of carbon fixation rates is difficult as uptake rates were calculated based on wet weight, dry weight or protein weight and assumptions must be made to convert these rates for comparison.

The dependence of the *O. algarvensis* symbiosis on internal energy sources, presumably stored sulfur, for inorganic carbon uptake and uptake rates that were only slightly lower than those of other marine chemoautotrophic symbioses suggests that the observed carbon fixation was a result of autotrophic and not heterotrophic carbon fixation. Heterotrophic or anapleurotic carboxylation reactions are a mean to replenish

metabolic intermediates (e.g. in the TCA cycle by the enzyme phosphoenolpyruvate carboxylase). Thus, heterotrophic carbon fixation occurs in virtually all organisms.

Inorganic carbon uptake in the absence of external energy sources occurred in several marine chemoautotrophic symbioses (Belkin et al. 1986, Distel and Felbeck 1988b, Fiala-Médioni et al. 1986a, Fisher et al. 1989, Girguis and Childress 2006, Schiemer et al. 1990). However, often uptake rates were low and the presence of internal energy sources was either not analyzed or merely estimated by trophosome or gill colour (e.g. Pflugfelder et al. 2005, Vetter 1985). Thus, the importance of internal sulfur as energy source for carbon fixation has so far been shown most clearly only for the gutless oligochaete symbioses of *I. leukodermatus* and *O. algarvensis*, and ectosymbiotic nematodes (this work, Giere et al. 1988b, Schiemer et al. 1990).

Inorganic carbon uptake rates varied for individual *O. algarvensis* worms even for those incubated in the same vial, and they did not correlate with the sulfur content other than by presence or absence. One reason might be that the initial sulfur content of individual worms was unknown as the elemental sulfur could only be analyzed at the end of the incubation and the amount of consumed sulfur during the incubation was not analyzed and cannot be estimated from sulfur analyses alone. Sulfur, though, was not limiting in these short term (4 to 6 h) incubation experiments. In general, it takes several days until formerly white gutless oligochaetes appear pale, and even then sulfur can still be detected (personal observation, C. Lott, unpublished data, Häusler 2008).

An alternative explanation for the lack of correlation between sulfur content and carbon fixation rates is given by incubation experiments that investigated sulfide and oxygen consumption and carbon dioxide flux of the *R. pachyptila* symbiosis. While sulfide oxidation and oxygen consumption were coupled, inorganic carbon fluxes or uptake did not correlate linearly with either of these (Girguis and Childress 2006, Fisher et al. 1989). It is known for free-living sulfur bacteria that carbon fixation and energy generation are not tightly coupled (Kelly 1989). This would explain why no correlation between carbon fixation rates and final sulfur concentration was found in this study. However, a good correlation of inorganic carbon uptake with sulfide concentration has been found for the *Alviniconcha sp.* symbiosis (Henry et al. 2008)

To conclude, under oxic conditions without an external energy source no or little inorganic carbon was incorporated by the *O. algarvensis* symbiosis when internal sulfur stores were depleted. Carbon fixation occurred in those worms with detectable amounts of elemental sulfur. Thus, internal sulfur stores served as energy source for autotrophic carbon fixation in *O. algarvensis*. This activity was most likely carried out by the chemoautotrophic sulfur-oxidizing symbionts.

2.2.2 Inorganic carbon uptake in the presence of alternative external electron acceptors

Results

To find out whether the *O. algarvensis* symbiosis is able to sustain autotrophic carbon fixation in the absence of oxygen as an electron acceptor for the energy yielding oxidation of sulfur, worms were incubated without oxygen and electron donor. Nitrate, fumarate and TMAO were supplied as alternative electron acceptors. The results shown below were derived from several independent anoxic incubation experiments and are therefore presented as % of the unsupplemented control analyzed in each experiment.

Inorganic carbon was taken up by the *O. algarvensis* symbiosis under anoxic conditions in the absence of an external substrate. Addition of alternative electron acceptors did not increase the average carbon fixation rates above those of the unamended control. Some individual worms, though, showed a slight increase in carbon fixation in the presence of 1 mmol/l fumarate or TMAO (Figure 2.7). Oxygen had the strongest effect on inorganic carbon uptake and carbon fixation rates were high in some worms, though not in all (Figure 2.7). The presence or absence of sulfur did not seem to influence the uptake of inorganic carbon under anoxic conditions. White and pale worms showed similar uptake rates with and without externally supplied electron acceptors, but data for pale worms was limited.

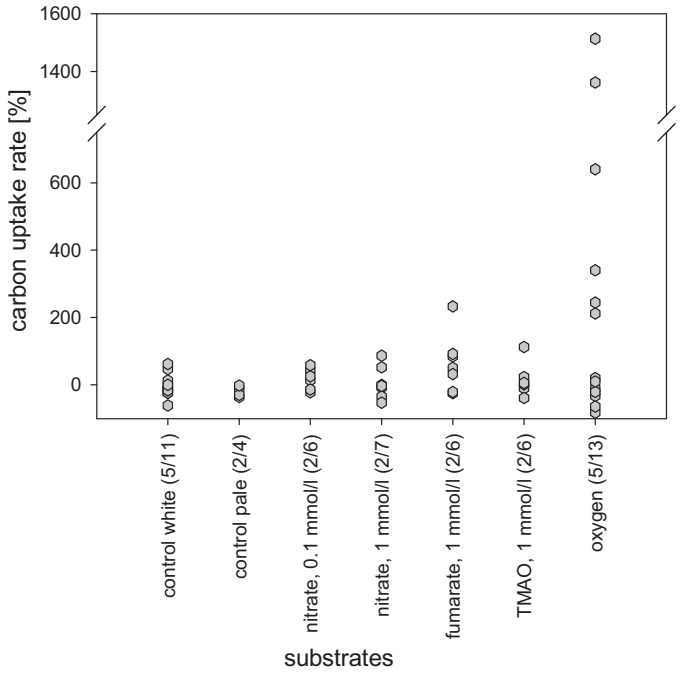


Figure 2.7: ^{14}C-inorganic carbon fixation rates by the *O. algarvensis* symbiosis under anoxic incubation conditions in the presence of different electron acceptors. Values were obtained from up to five independent incubations and are therefore given in % of the unsupplemented control in the different experiments for comparison (average of control was set as 100 = 0%, no uptake = -100%). Carbon fixation rates in *O. algarvensis* were – on average – not affected by the addition of different substrates compared to the unsupplemented controls of *O. algarvensis* except for oxygen. (number of incubations with the given concentration / total number of worms analyzed).

Discussion

It was surprising that none of the alternative electron acceptors added stimulated carbon uptake under anoxic conditions. This can have several causes, which will be explained in the following paragraphs. 1) Electron acceptors were provided or stored

internally and external substrates were not able to increase inorganic carbon fixation. 2) Substrate concentrations used in these experiments were too low to stimulate carbon fixation. 3) Nitrate, fumarate and TMAO were not used as electron acceptors by the chemoautotrophic sulfur-oxidizing symbionts of *O. algarvensis* to support autotrophy under the experimental conditions. These substrates might still be reduced or metabolized as nitrogen or carbon sources. 4) A suitable electron donor was lacking or limiting. 5) Last, the fitness and symbiont composition of the symbiosis might have influenced the symbiosis' performance during the incubations. These last two possible explanations are discussed in the general part (Chapter 2.2.4).

Electron acceptors provided internally. Electron acceptors might have been provided internally making the symbiosis independent from external substrates for a certain time and external stimulation difficult in short term incubations. Indeed, preliminary results suggest that nitrate is accumulated within some *O. algarvensis* worms to up to 150-fold above porewater concentration (about 75 µM nitrate in the porewater, C. Lott, unpublished data). This is an even higher nitrate accumulation than reported for *R. pachyptila* blood and the nitrate-reducing *Lucinoma aequizonata* symbiosis with 20 to 50-fold and 10-fold, respectively (Hentschel et al. 1996), although well below the several 1,000-fold accumulation achieved by free-living nitrate-storing sulfur bacteria (Fossing et al. 1995, McHatton et al. 1996, Schulz et al. 1999). It is unclear at this point where nitrate might be stored in the *O. algarvensis* symbiosis. Large vacuoles known from nitrate-storing sulfur bacteria (Fossing et al. 1995, McHatton et al. 1996, Schulz et al. 1999) have not yet been described for any of the gutless oligochaete symbionts in ultrastructural analyses.

Fumarate and TMAO might also be available within the *O. algarvensis* symbiosis. Fumarate could be produced internally by succinate oxidation. Succinate is an early metabolite in invertebrates that switch from aerobic to anaerobic metabolism (Dubilier et al. 1995, Grieshaber et al. 1994). When excreted by the host, succinate could be oxidized by the sulfate-reducing symbionts and the resulting fumarate might be used by the sulfur-oxidizing symbionts as electron acceptor. TMAO is found in marine invertebrates as an osmoregulatory compound (Yancey et al. 2002) and might serve as electron acceptor for the sulfur-oxidizing symbionts in *O. algarvensis* (Woyke et al. 2006). Whether fumarate and TMAO were indeed available for the sulfur-oxidizing symbionts in *O. algarvensis* is not known.

Substrate concentration. Limitation of electron acceptor could be another explanation for the lack of carbon fixation stimulation. Concentrations in the present work though were in the range of those used in marine chemosynthetic symbiosis research (e.g. Girguis and Childress 2006, Hentschel and Felbeck 1993, Hentschel et al. 1999). Although much lower than those usually applied for microbial cultures and isolates (e.g. Proctor and Gunsalus 2000), concentrations of 1 mmol/l were probably

well above environmentally relevant concentrations. Thus, externally provided electron acceptors should not have been limiting.

Substrates used as electron acceptor supporting autotrophy. There is good evidence for nitrate being metabolized by the gutless oligochaete symbiosis. Nitrate reductase activity (Felbeck et al. 1983) and nitrite accumulation (Hentschel et al. 1999) were found in gutless oligochaete species. Both enzyme activity and nitrite accumulation, though, were described for several marine chemosynthetic symbioses (Felbeck et al. 1981, Hentschel et al. 1993, Hentschel and Felbeck 1993, Hentschel et al. 1999), but correlation with or stimulation of autotrophic carbon fixation by nitrate was either not investigated or not affected (Hentschel et al. 1993, Scott and Cavanaugh 2007). Calculations of nitrate reduction and carbon fixation stoichiometry indicate that nitrate is reduced and assimilated in the *R. pachyptila* symbiosis to fulfil the nitrogen needs of symbiont and host, and that nitrate reduction does not provide energy for autotrophic carbon fixation (Girguis et al. 2000, Nelson and Fisher 1995).

A strong indication for dissimilatory nitrate reduction in the *O. algarvensis* symbiosis was the presence of a set of genes in the Gamma 3 metagenome bin that codes for proteins involved in dissimilatory nitrate reduction (Woyke et al. 2006). Several of these proteins were recently identified in a proteome study of the *O. algarvensis* symbiosis (M. Kleiner, C. Wentrup, N. Dubilier, unpublished data). While most marine chemoautotrophic symbionts might use nitrate predominantly as nitrogen source (see Nelson and Fisher 1995), the Gamma 3 symbiont of *O. algarvensis* could use nitrate as electron acceptor under oxygen limiting conditions and support autotrophy. Indeed, individual worms had increased inorganic carbon uptake rates in the presence of nitrate compared to the unsupplemental control in incubations slightly different from those presented above (Chapter 2.2.3 and 2.2.4).

Support for TMAO and fumarate reduction in the gutless oligochaete symbiosis is less strong than for nitrate. Genes potentially encoding TMAO and fumarate reductases were found in the *O. algarvensis* metagenome (Woyke et al. 2006), but their substrate specificity is unclear. Only one study on marine chemoautotrophic symbioses reported TMAO reductase activity. As only the enzyme activity was analyzed in this study, it remains unclear whether carbon fixation is enabled by TMAO reduction (Hentschel and Felbeck 1995).

Nitrate and TMAO reduction could have another function in marine symbioses. Using these alternative electron acceptors might be an oxygen-saving reaction by the symbionts thus avoiding competition for oxygen between host and symbiont. This has been suggested for a clam symbiosis (Hentschel et al. 1993). Another example is the squid-*Vibrio fischeri* symbiosis where oxygen is saved for the oxygen-demanding bioluminescent activity of the symbiont while symbiont metabolism uses nitrate as electron acceptor (Proctor and Gunsalus 2000).

Elemental sulfur is another possible internal substrate which might serve as an electron sink during temporary anoxia, as has been suggested for tubeworm and clam symbioses (Arndt et al. 2001, Duplessis et al. 2004), and for free-living sulfur-storing bacteria (Nelson and Castenholz 1981, Schmidt et al. 1987). This, however, is unlikely the case for gutless oligochaetes. Several observers have reported that white gutless oligochaetes turn pale when maintained under oxic conditions for a few days (personal observation, Giere et al. 1991), indicating that internal sulfur stores were consumed. Sulfur stores are replenished when reduced sulfur compounds are available, for example in reduced sediments, and the worms appear white again after several days to weeks (personal observation, Giere et al. 1991). In contrast, worms remain bright white when kept anoxic for extended periods of time (C. Wentrup, C. Lott, personal communication), indicating that sulfur stores are not used up in the absence of oxygen. Similar observations have been made for ectosymbiotic nematodes (Schiemer et al. 1990). Thus, sulfur – although internally available – is unlikely to serve as internal electron acceptor under anoxic conditions.

The addition of oxygen as electron acceptor had the strongest affect on inorganic carbon uptake by the *O. algarvensis* symbiosis resulting in the highest uptake rates of individual worms. Thus, with oxygen internal electron donors can be used to generate energy for carbon fixation, which could not be oxidized under anoxic conditions. However, most worms though showed no increase in uptake rates. This variability might be due to physiological differences among individual worms and their symbionts. As the worms migrate up and down between oxidized and reduced sediment layers, the actual need for oxygen, internal substrate concentrations and fluxes might change, depending whether they were on the way up or on their way down prior to the incubation experiment.

Electron donor lacking or limiting. Suitable electron donors might have been limiting under the conditions used as inorganic carbon uptake rates were low in most anoxic incubations. Incubations with externally provided electron donors under anoxic conditions and a mixture of donor and acceptor are presented below and indicate that indeed substrate limitation might be one possible explanation for the lack of carbon fixation stimulation (Chapter 2.2.3).

In conclusion, under the experimental conditions used externally supplied electron acceptors did not play a major role for inorganic carbon uptake of the *O. algarvensis* symbiosis in the absence of external electron donors except for oxygen. Although internal storage or production of electron acceptors might be possible, the most likely explanation is that a suitable electron donor was limiting. The electron acceptor or acceptors enabling inorganic carbon uptake under anoxic conditions remained unidentified from these experiments.

2.2.3 Inorganic carbon uptake in the presence of external inorganic electron donors

Results

To address the question whether electron acceptors were provided internally and electron donors were lacking in the above experiments, anoxic incubatons were carried out with different electron donors and combinations of electron donors and acceptors. Reduced sulfur compounds (sulfide and thiosulfate) were added as potential electron donors for the autotrophic sulfur-oxidizing symbionts, and hydrogen for the sulfate-reducing symbionts of *O. algarvensis*. Acetate was added to see whether (heterotrophic) carbon fixation would be stimulated by the addition of an organic carbon compound.

Neither sulfide nor thiosulfate, however, stimulated on average inorganic carbon uptake under anoxic conditions (Figure 2.8), although uptake rates of individual worms incubated with sulfide at 50 and 200 µmol/l or thiosulfate at 1 mmol/l were slightly increased compared to the control. Hydrogen had no or a slightly inhibitory effect on the overall uptake of inorganic carbon. Acetate did also not stimulate inorganic carbon uptake.

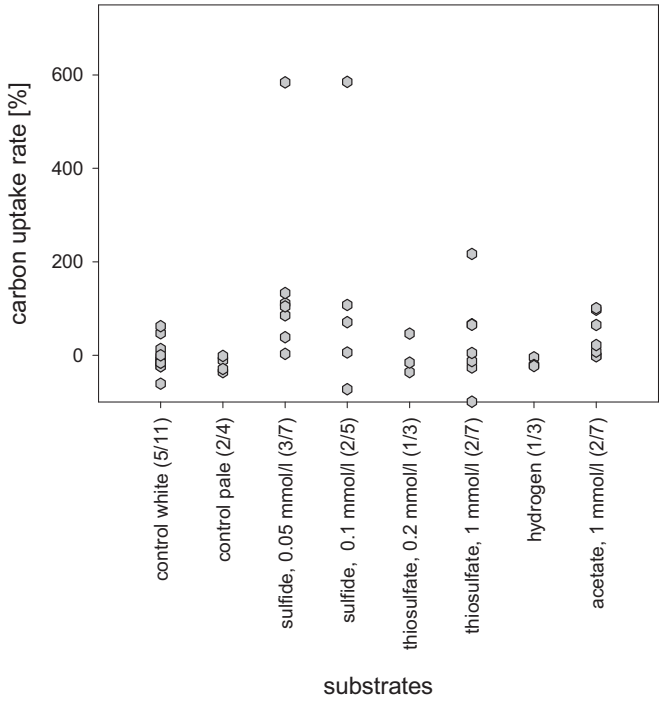


Figure 2.8: ^{14}C-inorganic carbon fixation rates by the *O. algarvensis* symbiosis under anoxic incubation conditions in the presence of different electron donors. See Figure 2.7 for further explanations.

When combining external electron donors and acceptors, sulfide and nitrate or thiosulfate and oxygen strongly increased inorganic carbon uptake in one of three and two of three individuals tested, respectively (Figure 2.9). Combinations with TMAO, fumarate and hydrogen (Table C.1 in the supplemental materials) did not influence inorganic carbon uptake (not shown).

Discussion

The addition of electron donors did not stimulate inorganic carbon uptake noticeably in the *O. algarvensis* symbiosis under anoxic conditions, although sulfide and thiosulfate could enhance carbon fixation in individual worms. These results are similar to those seen for the electron acceptor incubations where addition of substrates had little or no influence on carbon fixation rates, and basically the same assumptions

can be made, which are discussed below. Possible external substrate limitation has been discussed above and seems unlikely.

Electron donors provided internally. The assumption that some electron donors might be provided internally is supported by sulfate reduction rates and flux calculations (Dubilier et al. 2001). Internal sulfide production by the sulfate-reducing symbionts of *O. algarvensis* was estimated to exceed sulfide influx from the surrounding sediment, were sulfide concentrations are low (Dubilier et al. 2001). Thus, internal sulfide production might be – for a certain time – sufficient for the sulfur-oxidizing symbionts, making sulfide uptake from the environment almost unnecessary. This internal sulfur loop would be particularly advantageous in the sulfide-poor habitat of *O. algarvensis* and might have occurred in the incubation experiments presented. High sulfate concentrations in the anoxic medium used might have even stimulated high sulfate-reduction rates by the sulfate-reducing symbionts resulting in high sulfide production (or other reduced sulfur compounds) provided that an energy source for sulfate reduction was available. This sulfide (or other intermediates) could have been used as internal electron donor by the sulfur-oxidizing symbionts.

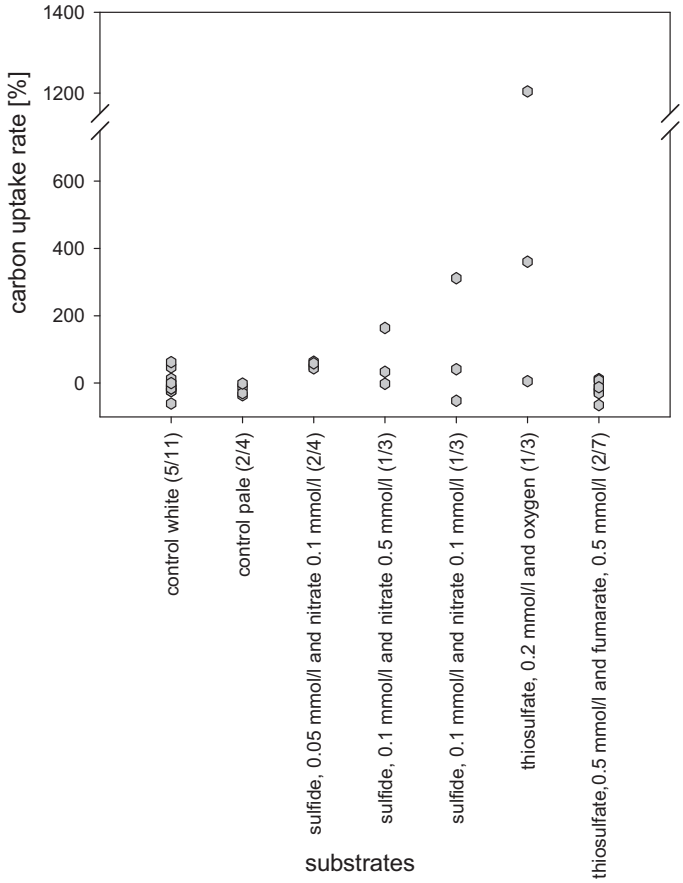


Figure 2.9: ^{14}C-inorganic carbon fixation rates by the *O. algarvensis* symbiosis under anoxic incubation conditions in the presence of combinations of electron donors and electron acceptors. See Figure 2.7 for further explanations.

Thiosulfate would be another possible internal electron donor. Many symbiotic and non-symbiotic marine invertebrates produce thio-sulfate as a means of sulfide detoxification (Grieshaber and Volkel 1998, O'Brien and Vetter 1990, Powell and Somero 1986). This was also proposed for a gut-bearing, ectosymbiotic marine oligo-chaete (Thiermann et al. 1996). Thiosulfate can be accumulated in symbiotic hosts after being exposed to sulfide or thiosulfate, and much beyond the usually low porewater concentrations (Anderson et al. 1987, Hentschel et al. 1999). It is unknown how gutless oligochaetes detoxify sulfide and if thiosulfate is accumulated or generated

at sufficient rates considering the low in situ sulfide concentrations (C. Lott, unpublished data, Dubilier et al. 2001, Perner 2003).

Stored sulfur is present in white oligochaetes and might serve as internal electron donor for the sulfur-oxidizing symbionts. However, field observations, incubation experiments with oxygen, and similar inorganic carbon uptake rates for pale and white worms under anoxic conditions suggest that sulfur is used as electron donor under oxic conditions. Under anoxic conditions, sulfur stores are rather recharged than consumed.

Substrates used as electron donor supporting autotrophy. Most worms incubated with sulfide or thiosulfate did not display an increase in inorganic carbon uptake rates compared to the unsupplemented control. It was suggested that further stimulation is not possible by external reduced sulfur compounds when internal sulfur stores were present in the symbiosis (Hentschel et al. 1999). Individual worms though had increased inorganic carbon uptake rates under anoxic conditions in the presence of sulfide and thiosulfate. This suggests that stimulation was possible and that the oxidation of these reduced sulfur compounds provided energy for inorganic carbon uptake, most likely carried out by the chemoautotrophic sulfur-oxidizing symbionts of *O. algarvensis*. Even higher stimulation of uptake rates were detected when sulfide and thiosulfate were combined with the electron acceptors nitrate and oxygen, indicating that both electron acceptors and electron donors might have been limiting in unsupplemented incubations and that stimulation of carbon fixation with external substrates was possible in at least some worms. Suggestions why these large differences between individual worm uptake rates might have occurred are presented in Chapter 2.2.4.

Hydrogen was investigated as potential electron donor that can support autotrophy in the sulfate-reducing symbionts. Even this substrate might have been provided by the symbiosis (Chapter 1.3.4, Woyke et al. 2006) and could explain why the addition of hydrogen did not affected carbon fixation in the *O. algarvensis* symbiosis. Another explanation might be that the sulfate-reducing symbionts of *O. algarvensis* do not live autotrophically. Especially under anoxic conditions they could benefit from the waste products the worm excretes when switching to an anaerobic metabolism. Metagenomic analysis and preliminary proteome data have identified a wide range of genes coding for enzymes involved in transporting carbon compounds across the membrane (M. Kleiner, C. Wentrup, N. Dubilier, unpublished data, Woyke et al. 2006). This suggests that a heterotrophic metabolism for the sulfate-reducing symbionts is not only energetically more advantageous, but also more likely. Only under starving conditions the sulfate-reducing symbionts might switch to autotrophy.

Finally, acetate was added as a heterotrophic carbon fixation control. However, inorganic carbon uptake did not increase in the presence of an organic carbon compound. Thus, acetate did not stimulate anapleurotic nor autotrophic carbon fixation under the conditions used.

Electron acceptor lacking or limiting. Lack of electron acceptor might explain the lack of carbon fixation stimulation. This is indicated by the results of the mixed incubations with sulfide and nitrate, and thiosulfate and oxygen, which showed a strong increase in inorganic carbon uptake rates in individual worms when electron donor and electron acceptor were added. However, only very few worms were analyzed under these conditions, thus more data is clearly needed to confirm this result.

Preliiminiary data from another experiment, which was meant as control experiment for oxygen consumption studies, supports the assumption that electron acceptors and donors were limiting in the anoxic incubations (Figure 2.10). Based on oxygen consumption rates of *O. algarvensis* (Häusler 2008) these incubations were depleted in oxygen already after a few hours. The almost constant carbon uptake rate of the control worms independent of their sulfur content indicates that oxygen as electron acceptor for the oxidation of stored sulfur was used up. Thus oxidation of sulfur stopped and with that, autotrophic carbon fixation (Figure 2.10). However, when external substrates were added, in particular thiosulfate and nitrate, carbon fixation rates increased above the rates of the control worms even when sulfur contents were low.

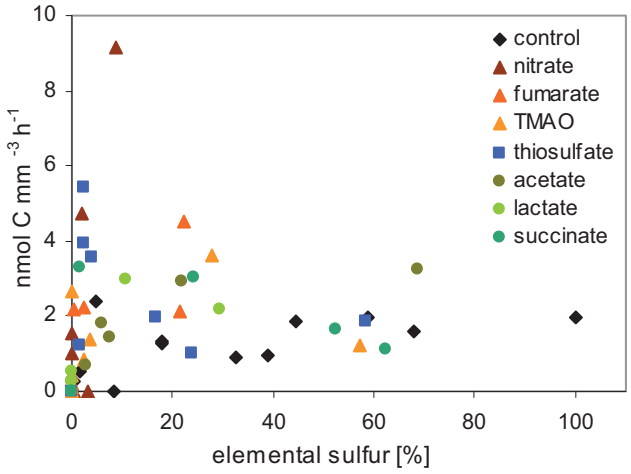


Figure 2.10: Elemental sulfur content and ^{14}C-inorganic carbon fixation rates of the *O. algarvensis* symbiosis in the presence of different electron donors, electron acceptors and organic carbon compounds under oxygen limiting conditions. Oxygen was used up within the first half of the 6 h incubation based on oxygen consumption rates (Häusler 2008). Worms in this experiment were collected in the field a few days prior to the incubation experiment and were not maintained in the laboratory as the worms used in most other experiments. All substrates were added to a final concentration of 1 mM.

To summarize, the addition of external electron acceptors had little to no effect on inorganic carbon uptake for most worms studied. Sulfide and thiosulfate though could increase uptake rates in individual worms. These substrates were most likely used by the sulfur-oxidizing symbionts. When both external electron donors and electron acceptors were added, sulfide, thiosulfate, nitrate and oxygen can stimulate carbon uptake, indicating that electron donor and electron acceptor were limiting in most anoxic incubations.

2.2.4 General discussion and conclusion

Two remarkable results were obtained from the anoxic incubations. On the one hand, the majority of worms analyzed did not show an increase in inorganic carbon uptake rates when external substrates were added. On the other hand, individual worms exhibited very high uptake rates under the same conditions. This high variability is unlikely to be caused by incubation conditions as it was found for worms incubated in the same vial. It rather suggests that the fitness of the symbiosis or the symbiont composition of the individual worms might have caused these differences and the lack of carbon fixation stimulation.

Indication for an impaired fitness of the symbiosis were low uptake rates and low incorporated radioactivity detected in most incubations (compare rates of this project with those in Figure 2.10 and manuscript 3, which were 2 to 40 times higher). This low activity might be attributed to the use of "old" worms, which were kept in the laboratory for several weeks to months prior to incubation. "Old" worms were on average smaller than those collected in the field, they were less white and seemed "weaker", which is manifested in a more fragile habitus making handling them more difficult and sediment sticking more easily to the worms. However, these worms showed "normal" behaviour as they were actively moving and curled up quickly when disturbed.

Another explanation could be that the total number of symbionts was reduced or symbiont populations were lost during maintenance. Indeed, when specimens of *I. leukodermatus* were incubated for five days under oxic, organic carbon-limited conditions, the symbiotic region, analysed with CARD-FISH, was considerably thinner compared to freshly collected worms (personal observation). Symbiont loss due to maintenance conditions has also been described for bivalve symbioses (Kádár et al. 2005, Lechaire et al. 2008). Thus, suboptimal maintenance of the worms could have influenced symbiont abundance and affected the incubation experiments.

The symbiont composition of the individual worms might have played a role in carbon fixation. Indeed, the Gamma 3 symbiont was not found in all *O. algarvensis* individuals investigated with FISH (Ruehland et al. 2008). Furthermore, it was not distinguished in this work between the two co-occurring host species *O. algarvensis* and *O. ilvae* as both species generally harbour two sulfur-oxidizing chemoautotrophic and two sulfate-reducing symbionts. The gammaproteobacterial symbionts Gamma 1 and Gamma 3 of both hosts are closely related, indicating similar metabolic capacities. The deltaproteobacterial symbionts though differ between host species, with Delta 1 and Delta 4 in *O. algarvensis* and Delta 1 and Delta 3 in *O. ilvae* (Ruehland et al. 2008). Clone libraries and a metagenomic study though strongly indicate, that a Delta 3 symbiont might be present in *O. algarvensis* as well (Ruehland et al. 2008, Woyke et al. 2006). A clear difference in the symbiont community of both hosts is the presence of a spirochete in *O. algarvensis* which has not been found in *O. ilvae* and which metabolic capacities are unknown (Ruehland et al. 2008). Thus, differences in carbon fixation

rates might have been caused by differences in the symbiont community composition of individual worms. Worms used in this work were not analyzed for their symbiont composition as this cannot be combined with bulk analyses when whole worms are considered. While the host species could be identified prior to an incubation experiment, analyzing only parts of the worm for inorganic carbon uptake or FISH would probably not reflect the true picture of carbon fixation and symbiont community composition as little is known about symbiont distribution in the host.

How could the daily life of a gutless oligochaete in the Mediterranean sediment look like when considerung what is currently known about the *O. algarvensi*s symbiosis? Under oxic conditions that is, when the worms are in the upper, oxidized sediment layers, presumably only for short time periods, carbon is fixed into cell material using energy generated from the oxidation of stored sulfur with oxygen. This activity is most likely due to the sulfur-oxidizing chemoautotrophic symbionts of *O. algarvensis*. The symbionts incorporating inorganic carbon with sulfur and oxygen for energy generation could be identified in this thesis (Chapter 2.3). Alternatively or in addition to sulfur reduced sulfur compounds could be oxidized and support carbon fixation as was shown for thiosulfate. Under anoxic conditions inorganic carbon might be incorporated with energy derived from sulfide or thiosulfate oxidation, but not sulfur, and with nitrate as electron acceptor. If both or only one of the two co-occurring chemoautotrophic symbionts fixes carbon under anoxic conditions remains to be shown. Internal substrates might have been provided, as carbon fixation occurred in the absence of electron donor, electron acceptor and even in the absence of both. These internal substrates were not identified, but internally produced reduced sulfur compounds and stored nitrate might be possible candidates.

Internal substrate storage or production is advantageous in the nutrient-poor habitat of *O. algarvensis* where only sporadic input of nitrate or organic matter occurs. This ability would allow the gutless oligochaete symbiosis to continue autotrophy for a certain time when external supply of energy source and electron acceptor was low. However, for a net gain, and thus for survival and growth of the symbiosis, once in a while, energy has to be taken up by the symbiosis, as a biological system is not a perpetuum mobile and energy is lost over time.

2.2.5 Outlook

Experimental set-up

In the following some suggestions are made that could help to improve the incubation experiments. First of all, worm maintenance conditions were not ideal as they led to less active worm symbioses compared to those collected freshly in the field. Freshly collected worms should be preferred over laboratory maintained worms.

Maintenance needs to be improved, if the use of freshly collected worms is not feasible. Ideally, worms would be kept in natural sediment in cores of at least 15 cm depth, with slightly sulfidic deeper layers and fully oxidized upper layers. This would force the worms to migrate up and down maintaining fitness of all their symbionts (see also supplemental materials C.2).

Pale worms are a good non-sulfur control, but care should be taken in choosing pale worms for incubation experiments. Most of the pale worms used in this work were kept in the laboratory for months. Slightly pale worms, which have been maintained under oxic conditions for a week to ten days only, might be a better choice than those worms which were used in the present work. Although slightly pale worms still had stored elemental sulfur left (C. Lott, unpublished data, Häusler 2008), differences in metabolism compared to fully white worms were already seen (Häusler 2008).

The high variability in carbon uptake rates might be reduced by "synchronizing" the worms prior to incubation. One hour pre-incubation as done in the experiments presented might not have been long enough. Pre-incubation of several hours improved the reproducibility of incubation experiments with symbiotic nematodes (Hentschel et al. 1999). A lower variability in carbon uptake was also seen when gutless oligochaetes were kept fully anoxic for 12 to 24 h before an oxic incubation experiment (manuscript 3). This might work for anoxic incubations as well.

Suggestions for future incubation experiments

A very important next step would be inhibition experiments for which fresh worms should be used (see above) to ensure high uptake rates, because often complete inhibition is not feasible, and reduction in carbon fixation might be difficult to detect at low carbon fixation rates. As a first step, autotrophic carbon fixation of the sulfur-oxidizing symbionts of *O. algarvensis* via the CBB cycle would be inhibited by glyceraldehyde or 6-phosphogluconate (Fisher et al. 1987, Robinson and Cavanaugh 1995). This experiment is required as an additional conformation that the observed carbon fixation was indeed autotrophic. In a second step, sulfate reduction would be targeted with e.g. molybdate, commonly used as inhibitor for sulfate reduction. This might show which role internal production of reduced sulfur compounds plays in the *O. algarvensis* symbiosis under anoxic conditions and whether it influences carbon fixation.

The amount of internally produced and stored substrates, such as nitrate, thiosulfate and sulfide, would provide valuable information for better understanding the functioning of the *O. algarvensis* symbioses and the results of this study. Nitrate analyses are being investigated (C. Lott, unpublished data). In addition, the role of the carbon storage compound PHB in marine chemosynthetic symbioses deserves more attention. PHB concentrations were reduced in pale gutless oligochaetes compared to white worms (Giere et al. 1988b). This change in concentration was also found in the

Gamma 1 symbionts of pale and white worms in staining experiments (C. Lott and C. Bergin, unpublished data). It was suggested that PHB functions as electron sink when oxygen or other nutrients are limiting (Steinbuchel and Schlegel 1991, Trainer and Charles 2006), but it is not known if the reduced carbon fixation rates in pale worms are linked with the PHB content or only with the presence or absence of stored sulfur.

The bulk analyses applied in this study are in general a good screening tool to find out which substrates are incorporated or might support inorganic carbon uptake. However, they provide only indirect evidence for the roles of particular symbionts in the gutless oligochaetes symbiosis. Other methods are needed to identify those symbionts that were responsible for the uptake of inorganic carbon. Two methods were applied in this thesis and the results are described in the next section (Chapter 2.3).

2.3 Single-cell studies on the *Olavius algarvensis* symbiosis

Inorganic carbon is taken up by the *Olavius algarvensis* symbiosis in the absence of external electron donors under oxic or anoxic conditions, as shown by tracer incubation experiments (Chapter 2.2). However, these bulk analyses did not allow autotrophic activity to be unambiguously assigned to a specific symbiont. Furthermore, such analyses cannot tell whether carbon is transferred from the autotrophic symbiont to the host or to other symbionts.

Two single-cell tools, microautoradiography (MAR) and nanoscale cesondary ion imaging (nanoSIMS), were used in this work to address these question for the *O. algarvensis* symbiosis under specified incubation conditions. Oxic conditions without externally supplied energy source were chosen, which should favour the autotrophic sulfur-oxidizing symbionts. Carbon fixation under these conditions has been demonstrated for the *O. algarvensis* (Chapter 2.2) and *I. leukodermatus* symbioses (Giere et al. 1988b). As mentioned above (Chapter 2.2), two chemoautotrophic symbionts co-occur in *O. algarvensis* (Ruehland et al. 2008), in contrast to *I. leukodermatus*, which harbours the sulfur-storing Gamma 1 symbiont as the probably only chemoautotrophic symbiont (Blazejak et al. 2006). While all Gamma 1 symbionts store sulfur, it is not known whether the *O. algarvensis* Gamma 3 symbionts do. Electron-dense vesicles of unknown composition were found by ultrastructural analyses within small symbiont cells of *O. algarvensis*, although these cells have not yet been identified as Gamma 3 symbionts (C. Lott, unpublished data). It is also possible that the Gamma 3 symbiont has access to internally produced energy sources.

MAR was performed on *O. algarvensis* incubated with radiolabelled inorganic carbon (^{14}C), and nanoSIMS analysis on samples incubated with ^{13}C-labelled inorganic carbon. Both methods were combined with in situ hybridization for specific

identification of the different symbiont populations. As the methods and experimental procedures differ, both tools and the respective results are described in separate chapters, first the MAR approach, second the nanoSIMS approach, followed by a general discussion and outlook.

2.3.1 Single-cell analysis with MARFISH

Background information

MAR combined with FISH or CARD-FISH (hereafter referred to as MARFISH) can link the phylogenetic identity and activities of microorganisms with single-cell resolution (Nielsen et al. 2003b). This technique has been applied to diverse habitats such as freshwater lakes, marine plankton, nitrifying biofilms and activated sludge to study the activity of 'unculturable' microorganisms (reviewed in Okabe et al. 2004, Wagner et al. 2006). For MARFISH analysis, the sample is incubated with a radiolabelled substrate, fixed and then hybridized with specific probes targeting the 16S rRNA of phylogenetic groups of interest. Samples are processed either on filters glued to slides or placed directly on cover slips. After exposure to a photographic emulsion, the MAR signal (in form of silver grains) and the FISH or CARD-FISH signal can be visualized and evaluated microscopically (Figure 2.11). No MARFISH study on symbioses has yet been reported. Autoradiography without cell identification though was applied to a few marine chemosynthetic symbioses (Chapter 1.4.1, Bright et al. 2000, Fisher and Childress 1986).

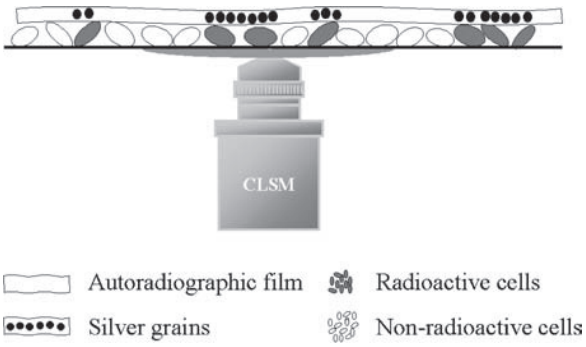


Figure 2.11: Schematic presentation of MARFISH. A radiolabelled substrate incorporated by microbial cells causes the formation of silver grains after exposure to a radiation-sensitive emulsion that covers the cells. The silver grains visible as black areas around the cells can be visualized microscopically. This method makes active cell populations directly visible when combined with FISH identification applying specific oligonucleotide probes. (Fig. adapted from Nielsen et al. 2003b).

Experimental procedure and analysis

O. algarvensis worms were incubated under oxic conditions in the absence of externally provided energy sources (for details on incubation set-up see supplemental materials Chapter C.2). Incubations were run for 6 h in the dark. After incubation, worms were rinsed in sterile seawater and homogenized prior to the MARFISH procedure. The homogenization step was necessary to separate cells for unambiguous assignment of the MAR signal to individual cells. The reason is that the silver grains are

deposited on top, at the edge of and around the labelled cells, and can be found up to 4 µm from the cell when ^{14}C is used (Nielsen et al. 2003a), thereby covering the neighbouring cells. Thus, densely packed communities such as biofilms or symbiont-containing tissue require dilution or homogenization (Nielsen et al. 2003b).

Worms were homogenized freshly in 50 µl sterile seawater and before fixation as fixation resulted in stiff worms that were difficult to homogenize completely. Worm homogenates were then fixed, filtered (see below), and stored at -20°C. If filtration directly after fixation was not possible, fixed samples were stored for not longer than four weeks at -80°C as prolonged storage can result in loss of radioactivity when frozen and thawed repeatedly (Nielsen et al. 2003a). All worm homogenates were analysed for bulk ^{14}C uptake prior to MARFISH. For this purpose one aliquot of 5 – 10 µl per homogenized sample was diluted in sterile phosphate-buffered saline (PBS) and filtered through a nitrocellulose filter, washed twice with ice-cold trichloroacetic acid and analyzed by LSC.

Samples for MARFISH were filtered through a polycarbonate filter (type GTTP) and washed twice with sterile PBS. Filters were dried in the dark and stored at -20°C until analysis. The MARFISH protocol followed Alonso and Pernthaler (2005). Briefly, filters were hybridized according to the CARD-FISH protocol (Pernthaler and Pernthaler 2007), counterstained with the DNA stain 4',6-diamidino-2-phenylindol (DAPI), dried and glued to glass slides. Filters were then covered with a mixture of agar and a radiation-sensitive emulsion and exposed at 4°C in the complete darkness for several days. The exposure time for the ^{14}C-incubated worm samples was adjusted to seven days for the best signal-to-noise ratio. The CARD-FISH signal was in most cases excellent after the MAR procedure. DNA staining with DAPI was modified by adding the detergent sodium dodecyl sulfate at a final concentration of 1% to the DAPI staining solution. This modification slightly enhanced the very weak DAPI signal of the Gamma 1 symbionts.

Results

MARFISH-treated samples showed a good cell separation and distribution on the filter. All symbiont types, including the spirochete symbionts, could be identified in all samples analyzed. Symbiont-specific clusters of several tens to more than hundred cells of Gamma 1 or deltaproteobacterial symbionts were often found. Leftover tissue fragments that were not completely homogenized were also present and these sometimes enclosed symbiont cells.

Incorporation of ^{14}C-labelled inorganic carbon into symbiont biomass was visible as dense areas of silver grains in the vicinity of cells after 6 h incubation (black areas in Figure 2.12). These silver-grain areas were associated with the large Gamma 1 symbiont cells, while none of the other symbiont cells were labelled (Figure 2.12). Thus, the Gamma 1 symbiont was the only symbiont in the *O. algarvensis* symbiont

community that detectably fixed inorganic carbon within 6 h under oxic conditions when no external energy source was available.

Uptake of inorganic carbon varied, not only between worms but also between individual Gamma 1 symbionts. Variability between worms was found by bulk analyses (Chapter 2.2) and indicated by MARFISH results. Semiquantitative investigation of the MARFISH samples showed that between 70-95% of the Gamma 1 symbionts in individual worms incorporated inorganic carbon. These worm to worm differences of symbiont activity might partly explain the variations seen in bulk carbon uptake for individual worms.

Variability between individual Gamma 1 symbionts was indicated by the number and size of and the area covered by silver grains associated with the symbionts. Silver grains were abundant and very dense around some Gamma 1 cells, while only a few grains were observed associated with other Gamma 1 cells within the same host individual (Figure 2.12). This variability suggests that the Gamma 1 symbionts within the same host specimen had distinct carbon uptake rates. These differences were further investigated and quantified by nanoSIMS (Chapter 2.3.2).

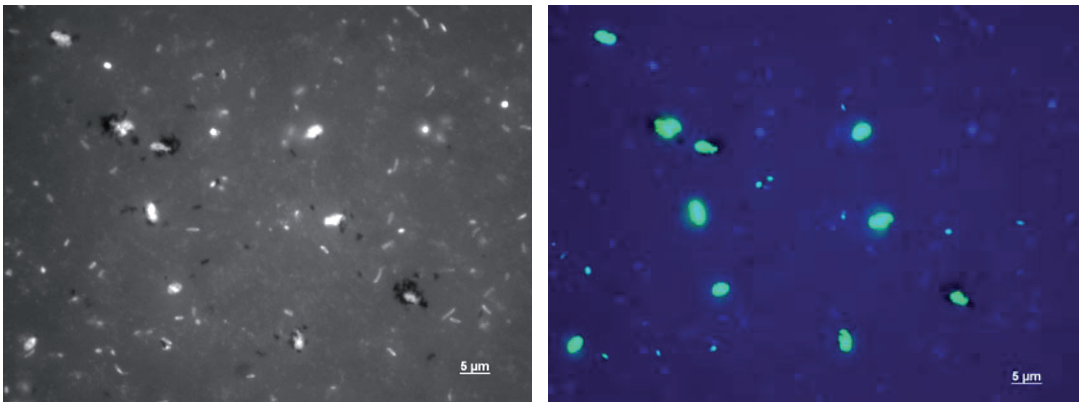


Figure 2.12: Microautoradiographic (MAR) image (left panel) of homogenized *O. algarvensis* which were incubated for 6 h with ^{14}C-labelled bicarbonate under oxic conditions in the absence of external electron donors. Combined MAR and CARD-FISH image (right panel), in green gammaproteobacterial symbionts, in blue DAPI-stained DNA, here the deltaproteobacterial symbionts. The larger gammaproteobacterial cells (bright green) are the Gamma 1 symbionts which were the only symbionts that showed detectable inorganic carbon uptake under the conditions used.

2.3.2 Single-cell analysis with nanoSIMS

Background information

NanoSIMS is a rather new technology that combines multiple isotope mass spectrometry (MIMS) with high spatial mass resolution (Lechene et al. 2006). With its high resolution imaging in the nanometer range, this technique has recently reached the microbial world as even small microbial cells can be visualized and analyzed (Kuypers and Jørgensen 2007). The principle of this technique is the bombardment of the sample

with a primary ion beam under high vacuum (Figure 2.13). Neutral and positively or negatively charged atoms and atomic clusters are ejected from the sample. Ionized particles are collected in a secondary ion beam, separated according to their masses and detected, thus yielding information about the elemental or isotopic composition of the sample. Up to seven masses from the same field can be detected simultaneously with the newest generation of nanoSIMS. By applying an electrostatic collection system to the secondary ion beam, analogous to a lens in a light microscope, the topological information about the ions' origin is retained from the sample to the detector and an image of the sample can be generated with quantitative information for each mass (Lechene et al. 2006). From this information, isotope ratios and uptake rates can be calculated, making this instrument a valuable tool for studying the ecophysiology of ('uncultured') microorganisms (Kuypers and Jørgensen 2007).

Basically all stable and radioactive isotopes can be analyzed with nanoSIMS. Carbon and nitrogen isotopes are routinely measured to identify the biomass in the sample. In addition, both elements are frequently used in incubation tracer experiments to track microbial activity and substrate uptake, applying the stable isotopes of these elements, ^{13}C and ^{15}N, or the radioactive carbon isotope, ^{14}C. Carbon is mostly detected as $^{12}C^-$ or $^{13}C^-$. Nitrogen itself does not ionize and is therefore analyzed as $^{12}C^{14}N^-$ or $^{12}C^{15}N^-$. The high mass resolution of the nanoSIMS can distinguish between the isobars $^{13}C^-$ from $^{12}C^1H^-$, and $^{13}C^{14}N^-$ from $^{12}C^{15}N^-$ (Lechene et al. 2006), which is essential for labelling experiments.

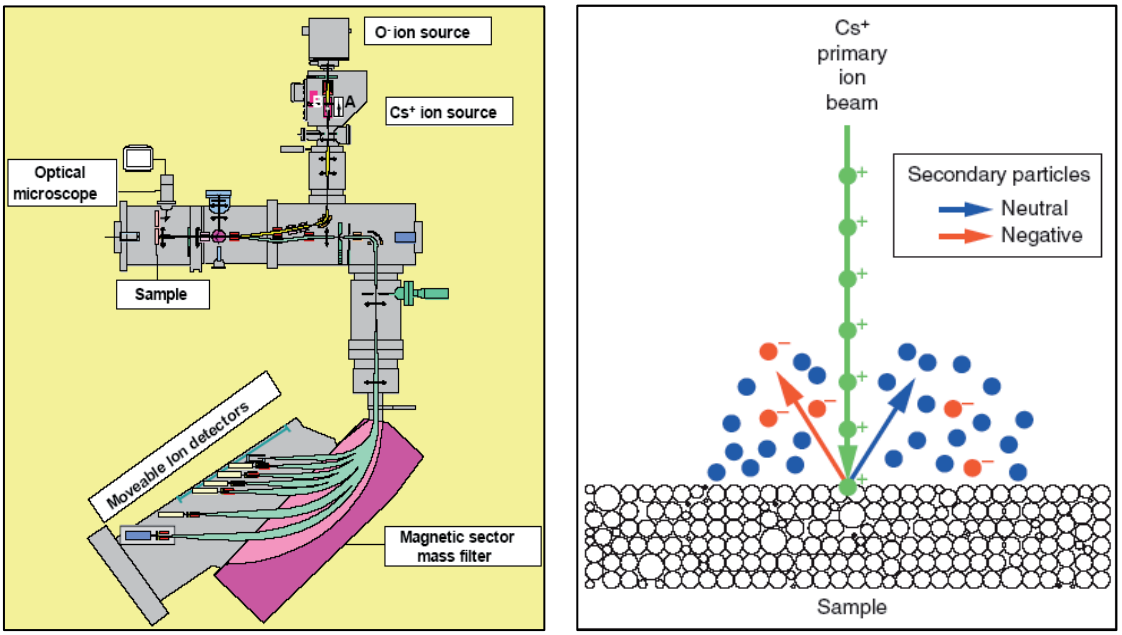


Figure 2.13: The principle of nanoSIMS: The sample is placed in an ultra-high vacuum chamber and bombarded with a primary ion beam. The ions of the beam collide with the sample surface resulting in ionization of surface atoms and molecules. Secondary ions are collected and separated into detectors specific to their masses. Up to seven masses can be analyzed simultaneously from the same field. (Figures adapted from CAMECA (left) and Lechene et al. 2006 (right)).

NanoSIMS has recently been combined with in situ hybridization for the phylogenetic identification of microorganisms in mixed communities (Behrens et al. 2008, Li et al. 2008, Musat et al. 2008). In situ hybridization for nanoSIMS cannot make use of a fluorescent or other dye moiety if it consists only of those elements that biological samples are made of, such as carbon, hydrogen and nitrogen, as the signal is not distinguishable from that retrieved from the biomass. Halogens or other elements that are easily ionized and usually rare in biological samples have been applied as in situ signal in nanoSIMS analyses (Behrens et al. 2008, Li et al. 2008, Musat et al. 2008). Halogens can be synthesized into the oligonucleotide probe (Li et al. 2008) or, when applying the CARD-FISH protocol for increased sensitivity, into the tyramide (Behrens et al. 2008, Musat et al. 2008). If the oligonucleotide probe or the tyramide carries in addition a fluorescent label, the samples can be quality checked for successful hybridization with an epifluorescence microscope prior to nanoSIMS analysis.

Aim of this project

The analysis of the *O. algarvensis* symbioses with nanoSIMS-HISH (nanoSIMS with halogen in situ hybridization) had two goals: i) quantification of the carbon uptake differences at the single cell level seen with MARFISH on worm homogenates and ii) quantification of symbiotic inorganic carbon uptake in the intact host tissue (not possible with MARFISH). The latter goal was also of interest for understanding and following the transfer of carbon compounds from the autotrophic symbionts to neighbouring symbionts and host tissue.

Experimental procedure

O. algarvensis specimens were incubated in artificial seawater (ASW) under oxic conditions between 10 min and up to 25 h (for details see manuscript 3). The ASW was prepared without any organic or inorganic carbon source to ensure that most of the inorganic carbon in the ASW was labelled. Prior to each experiment, ^{13}C-labelled bicarbonate was added to the medium to a final concentration of 2.5 mmol/l. No energy source or electron acceptor besides oxygen was present in the medium.

At the end of the incubations, worms were rinsed in carbon-free ASW, then fixed and embedded (see below) or homogenized and fixed as described for the MARFISH samples (Chapter 2.3.1). Whole worms were embedded in paraffin or Steedman's wax, and placed on either rinsed or untreated silicon chips, or on gold-palladium sputtered polycarbonate filters.

The different section carriers, silicon chips or filters, were compared for best adherence and best preservation of the section overall structure and shape during the CARD-FISH/HISH procedure. Silicon chips were manually cut from silicon wafers (doped with antimony) with a glass cutter into square-shaped chips of 7.2 x 7.2 mm^2 to fit into the sample holder used for the nanoSIMS instrument. The small pieces were

cleaned and washed with acetone, methanol, isopropanol, ethanol and water and dried under nitrogen gas (as described in http://www.nrims.hms.harvard.edu/protocols/Washing_and_Sterilizing_of_Silicon_Pieces.pdf, but omitting the sonication). Sections were placed on the rinsed silicon chips and warmed for one hour to remove excess water and enhance adherence of the sections to the silicon chip. During hybridization of 2.5 h, many sections were lost (that is, fell off the chips) with worse results after longer hybridization times of 5 h. Also, sections were often torn after the hybridization procedure. Much better results were achieved with silicon chips that were cut and only treated with nitrogen gas to remove silicon dust without any rinsing. The best results were obtained with Steedman's wax sectioned on sputtered filters. These polycarbonate filters with a pore size of 0.2 µm were the same that are used for standard FISH (Pernthaler and Pernthaler 2007). The filters were sputtered with a gold-palladium mix prior to use to provide a conducting surface that is needed to minimize charging of the sample during the primary ion beam bombardment. Sections on filters remained almost perfectly in shape, handling of filters during the hybridization procedure was easier, and the processing of several samples at once was faster than for sections on chips. Paraffin sections were not tested on filters, but would presumably also work well.

Worm sections on silicon chips and on filters were de-waxed prior to CARD-FISH as described previously (manuscript 2, Pernthaler and Pernthaler 2005). Two CARD-FISH hybridization buffers (Pernthaler et al. 2002, Pernthaler and Pernthaler 2007) and two different hybridization times (2.5 and 5 h) were tested for the worm sections as the first in situ signals retrieved during nanoSIMS analyses were very weak. The two hybridization buffers differed in their content of blocking reagents, however, no clear difference was observed in signal intensity using either of the two buffers. The hybridization buffer which contained additional blocking reagents (Pernthaler et al. 2002) was then chosen for further experiments, because it reduced background signal of samples on filters (N. Musat, personal communication). Increase of hybridization time did not improve the signal, confirming results of earlier optimization tests for regular CARD-FISH which showed best results after 2.5 h hybridization (personal observation). Washing steps with PBS and the amplification step were increased to 30 min compared to the standard protocol (Pernthaler et al. 2002).

Homogenate samples were filtered onto gold-palladium sputtered filters. Samples were further processed for CARD-FISH as the worm sections with 2.5 h hybridization. All hybridized samples were quality checked under an epifluorescence microscope for successful hybridization. Samples were then loaded into the nanoSIMS instrument for analysis.

NanoSIMS and data analysis

Analyses were run on a NanoSIMS 50 or NanoSIMS 50L instrument (CAMECA, Paris, France) between May 2007 and January 2009 in collaboration with the MPI

Mainz and the Nutrient Group, MPI Bremen. Secondary ion images were simultaneously generated for the following masses: $^{12}C^-$ for the natural occurring ^{12}C, $^{13}C^-$ for the ^{13}C isotope indicating inorganic carbon uptake, $^{12}C^{14}N^-$ for the biomass, and $^{19}F^-$ for the ^{19}F signal of the in situ hybridization. During analysis, the primary ion beam sputtered a field of choice several times, thereby generating a number of layers. These layers were accumulated by adding all counts per pixel per mass. The data and images were processed using the CAMECA Win-Image processing software (CAMECA), ImageJ (National Institutes of Health, USA) and Matlab (MathWorks Inc.).

Regions of interest were drawn manually around individual symbiont cells within tissue sections and homogenate samples of *O. algarvensis*, for host tissue and background control (filter). In tissue sections individual cells were often not clearly distinguishable. In this case, several regions were drawn within the symbiotic region, enclosing more than one putative symbiont cell. ^{13}C enrichment was calculated as $^{13}C/^{12}C$ sample ratio over $^{13}C/^{12}C$ background ratio for the regions of interest.

Results of worm homogenate analyses

O. algarvensis homogenate samples hybridized with the general bacterial probe EUB338 (Daims et al. 1999), the general gammaproteobacterial probe GAM42a (Manz et al. 1998) and the Gamma 1 symbiont-specific probe OalgOilvGAM1 (Ruehland et al. 2008) showed clear and specific $^{19}F^-$ signals in the nanoSIMS analysis. The hybridization signal of the Gamma 3 symbiont-specific probe OalgOilvGaAM3 (Ruehland et al. 2008) was too weak for nanoSIMS detection. Therefore, the Gamma 3 symbionts were identified based on the GAM42a ^{19}F signal and by size discrimination from the larger Gamma 1 symbionts.

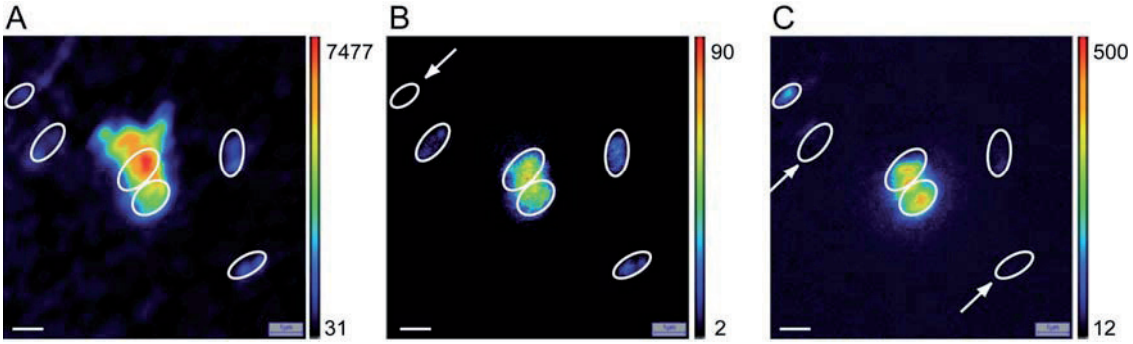


Figure 2.14: Uptake of ^{13}C-inorganic carbon by the *O. algarvensis* symbiosis analyzed on worm homogenates after incubation with ^{13}C-labelled bicarbonate as sole carbon source for 25 h. (A) $^{12}C^{14}N^-$ showing the biomass. (B) $^{19}F^-$-in situ signal of the gammaproteobacterial symbionts, the arrow marks an unlabelled deltaproteobacterial symbiont. (C) The $^{13}C/^{12}C$ ratio shows strong ^{13}C enrichment in the large Gamma 1 symbiont cells (center), and weak labelling in Gamma 3 symbionts. Note that the colour code in (C) shows the ^{13}C enrichment in pixels and not in %. Scale bar 2 µm.

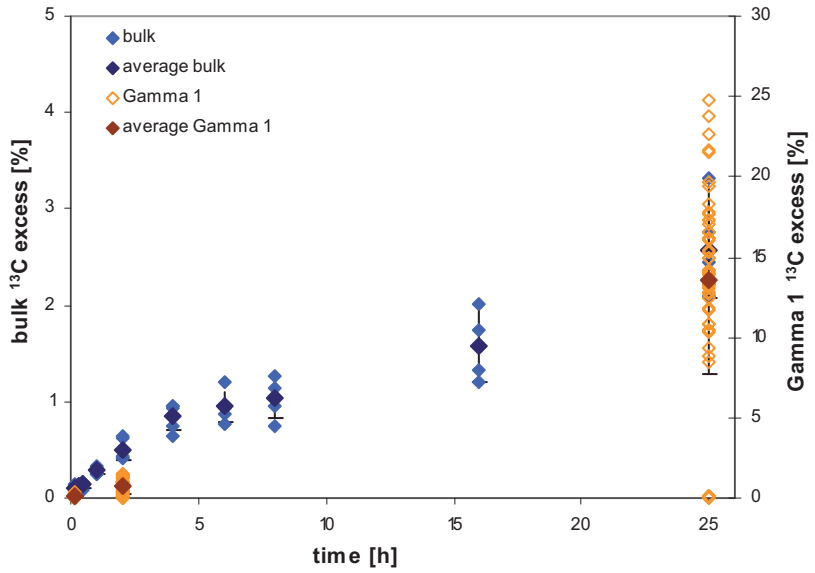


Figure 2.15: Uptake of ^{13}C-inorganic carbon by the *O. algarvensis* symbiosis (bulk, n = 5) and by individual Gamma 1 symbionts (n = 55, 41, 50 for 10 min, 2 h and 25 h, respectively) when incubated in oxic artificial seawater and inorganic carbon as sole carbon source with no external energy source. Data is presented as ^{13}C excess over background control.

NanoSIMS analyses showed a clear enrichment of ^{13}C over the natural ^{13}C abundance in the Gamma 1 symbionts of *O. algarvensis* when incubated with ^{13}C-labelled bicarbonate as the only carbon source (Figure 2.14 and 2.15). The ^{13}C enrichment increased over time from 0.14% ± 0.07 after 10 min to 12.55% ± 6.83 after 25 h incubation (Figure 2.15). The enrichment in ^{13}C varied for individual Gamma 1 symbionts. The majority of Gamma 1 cells incorporated ^{13}C-inorganic carbon while a very few cells remained unlabelled (manuscript 3, Figure 2.15). Preliminary data showed that after 25 h incubation other symbiont cells were slightly enriched in ^{13}C (Fiugure 2.14).

Results of worm section analyses

NanoSIMS analysis allowed the identification of the autotrophic symbionts within the host tissue and showed that the symbiotic region of *O. algarvensis* was strongly enriched in ^{13}C after 6 h incubation in ^{13}C-labelled bicarbonate as the only carbon source (Figure 2.16). The distribution pattern of the ^{13}C enrichment matched with the distribution of the in situ $^{19}F^{-}$ signal for the probe GAM42a targeting the chemoautotrophic symbionts of *O. algarvensis* (Figure 2.16). The ^{13}C-incorporation was restricted to the symbiotic region and clearly visible in the shape of large cells that corresponded well with the size and shape of the Gamma 1 symbionts (large, 2 - 3 µm, oval cells). Host tissue did not show detectable enrichment in ^{13}C even after 25 h incubation (not shown).

Enrichment in ^{13}C and uptake rates were calculated based on the regions of interest drawn within the symbiont-containing region and were very similar to results obtained with homogenized samples with 11.91 ± 8.76 after 25 h compared to 12.55% ± 6.8 for Gamma 1 symbionts in homogenates (Figure 2.15 and 2.17). As for homogenized

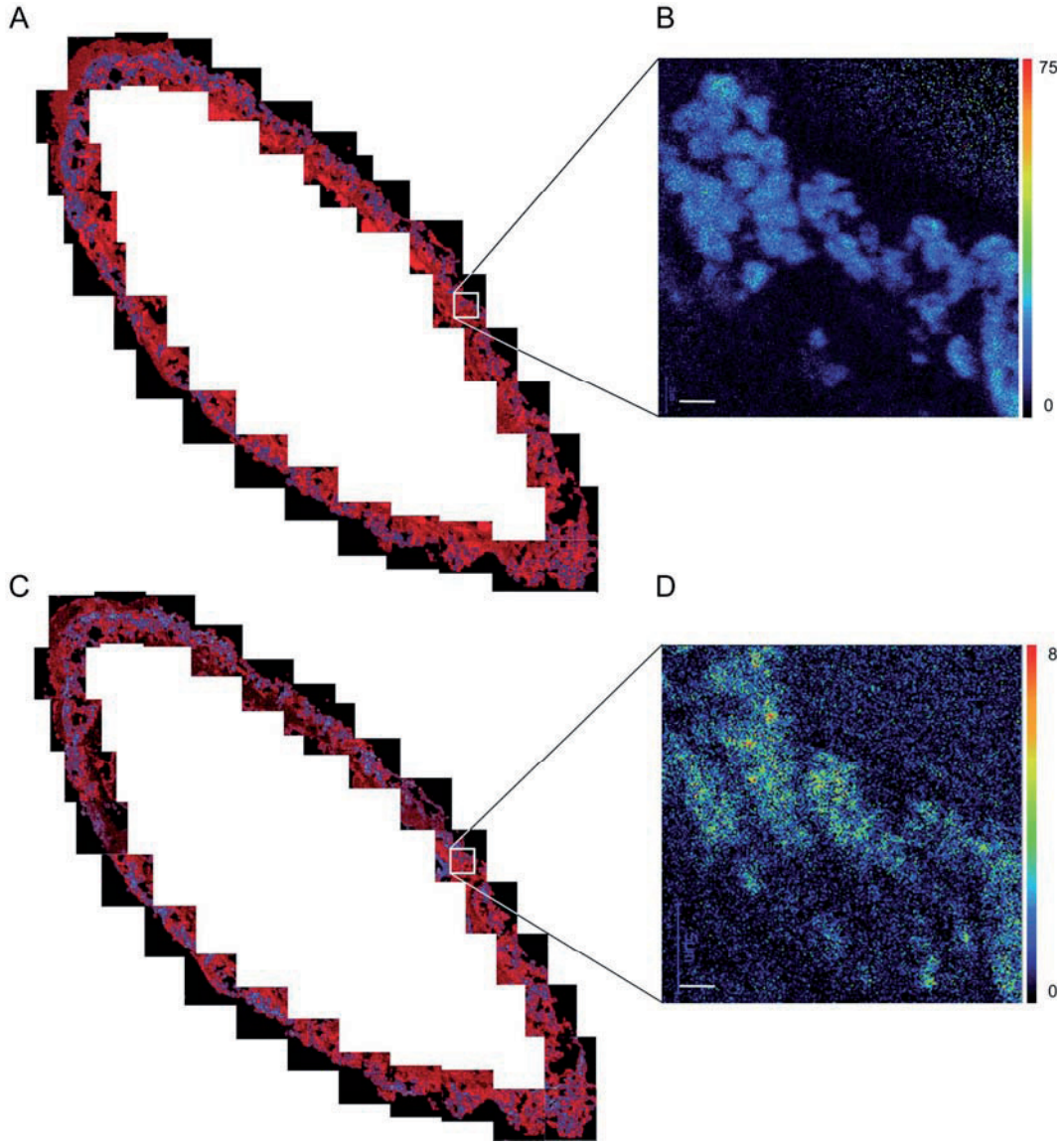


Figure 2.16: Composite nanoSIMS images of a cross section through an entire *O. algarvensis* worm (A, C) and close up of the symbiotic region (B, D). Worms were incubated for 6 h under oxic conditions in artificial seawater with ^{13}C-labelled bicarbonate as sole carbon source and without an externally provided energy source. The $^{13}C/^{12}C$ ratio (A, B) and the in situ $^{19}F^-$ signal of the general gammaproteobacterial probe GAM42a (C, D) are shown. Uptake of ^{13}C-inorganic carbon occurred exclusively in the symbiotic region (blue in A and B) and ^{13}C enrichment appeared in cell-like shapes (B). Note that the colour code in (B) shows the ^{13}C enrichment in pixels and not in %. Scale bar 2 µm (B, D).

samples, not all ^{19}F-labelled Gamma 1 symbionts in the symbiont region were enriched in ^{13}C (Figure 2.17).The weak hybridization signal of the Gamma 3 symbiont-specific probe did not allow the identification of these smaller symbionts within the tissue sections. Using the general gammaproteobacterial probe and cell sizes and shapes to distinguish between the larger Gamma 1 and the smaller Gamma 3 symbionts, as done in the homogenized samples, was not feasible for tissue sections. Here, the cells are densely packed and oriented 3-dimensionally, so partial views of large cells cannot always be distinguished from complete views of small cells and vice versa.

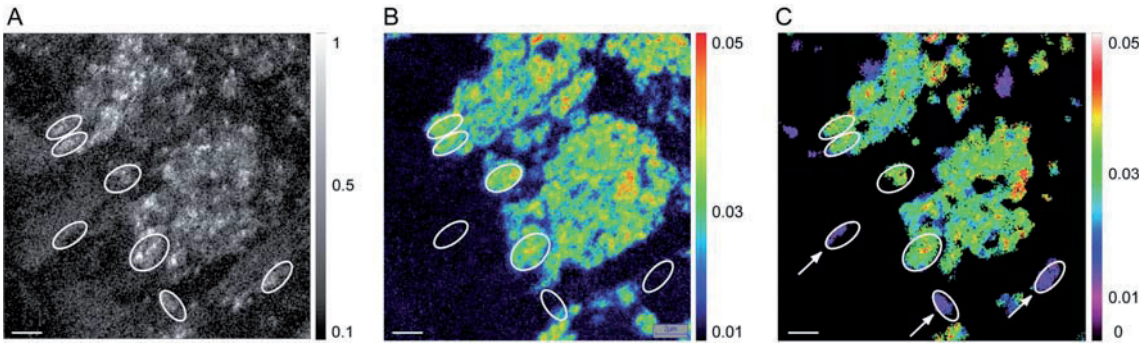


Figure 2.17: Uptake of ^{13}C-inorganic carbon by Gamma 1 symbionts in *O. algarvensis* after 2 h incubation under oxic conditions. (A) $^{19}F^-$ in situ hybridization signal; (B) $^{13}C/^{12}C$ ratio showing the ^{13}C enrichment; (C) $^{19}F^-$ in situ signal was used as a mask over the $^{13}C/^{12}C$ ratio. The majority of Gamma 1 symbionts incorporated ^{13}C-inorganic carbon, but some cells remained unlabelled, indicated by arrows (C). A $^{13}C/^{12}C$ ratio of 0.01 equates the natural abundance ratio. Scale bar 2 µm.

2.3.3 Discussion

The fast uptake of inorganic carbon by the Gamma 1 symbionts in *O. algarvensis* in the presence of oxygen and stored sulfur confirmed that this symbiont is a sulfur-oxidizing chemoautotrophic symbiont, as has been inferred previously from ultrastructural, biochemical and molecular data. In contrast to the Gamma 1 symbionts, the Gamma 3 symbionts showed only slight enrichment after 25 h incubation. One possible explanation for the low uptake of labelled carbon of this symbiont could be that the Gamma 3 symbionts do not store sulfur and would therefore lack an electron donor under the conditions used. Other internal energy sources which might be used by the Gamma 3 symbionts when living autotrophically, such as sulfide or thiosulfate, were not available externally and probably also not internally, as the sulfate-reducing symbionts of *O. algarvensis* were unlikely to reduce sulfate under oxic conditions. Therefore, the slight enrichment seen in the Gamma 3 symbionts after 25 h can be the result of heterotrophic carbon fixation, uptake of freshly synthesizid organic carbon from the Gamma 1 symbiont or a combination of both.

The high spatial resolution of the nanoSIMS enabled the analysis of substrate uptake within the intact symbiont-containing region, and analysis of the $^{13}C/^{12}C$ of host tissue was possible. However, there was no indication of carbon transfer to host tissue. This result is in contrast to some marine chemoautotrophic symbioses, where carbon transfer occurs within few hours (Bright et al. 2000, Fisher and Childress 1986). Such a quick transfer was explained with the translocation of soluble organic compounds ("milking", Chapter 1.2.3). A slow labelling of host tissue (within a day or longer) was attributed to lysis and digestion of the symbionts as the mode of host nutrition (Fiala-Médioni et al. 1986a, Fisher and Childress 1992, Streams et al. 1997). Additional indication for symbiont lysis and digestion were lysosomal activity, the presence of numerous lysozymes in symbiont-bearing host tissue and high protease activity (Boetius

and Felbeck 1995, Fiala-Médioni and Metivier 1986, Fiala-Médioni et al. 1986b, Fiala-Médioni et al. 1994, Nelson et al. 1995).

Indeed, in gutless oligochaetes, lysozomal structures are abundant in the basal, inner part of the symbiotic region (Giere 1985, Giere and Langheld 1987, Giere et al. 1995a, Giere and Erséus 2002) and estimations based on ultrastructural analyses found on average 8-10% with up to 24% of the symbionts undergoing digestion (Giere et al. 1995a). It is therefore likely that lysis and digestion rather than symbiont "milking" plays a role in *O. algarvensis* nutrition, but longer incubations with labelled carbon are needed to confirm this hypothesis.

2.3.4 Conclusion and outlook

This study has shown that the Gamma 1 symbiont of *O. algarvensis* is an autotrophic sulfur oxidizer that fixes inorganic carbon at a high rate presumably using its own stored elemental sulfur as energy source in the presence of oxygen. Furthermore, the hypothesis that the Gamma 1 and Gamma 3 symbionts of *O. algarvensis* do not share the same ecological niche (Woyke et al. 2006) was corroborated. And for the first time, the activity of a marine chemoautotrophic symbiont within a symbiont community was visualized and its uptake determined on the single cell level.

MARFISH and nanoSIMS-HISH were equally successful in symbiont activity detection and visualization with single-cell resolution. With both methods the Gamma 1 symbiont was identified as the dominant autotrophic symbiont in the *O. algarvensis* symbiont community. Both MARFISH and nanoSIMS have their advantages and disadvantages though.

MARFISH allows the simultaneous processing of several samples, and semi-automated counting of MAR-positive cells is possible (Alonso and Pernthaler 2005, Cottrell and Kirchman 2003, Pernthaler et al. 2003). This method however is limited by the use of radiolabelled compounds. Nitrogen cannot be studied, as a radioactive nitrogen isotope with a sufficient half life does not exist. Furthermore, special regulations apply for radioactive substrates, limiting their application in the field. In addition, quantification of substrate uptake in MAR samples is difficult. Although protocols for quantification of carbon uptake and rate measurements for single cells based on the silver grains have been developed and applied (Nielsen et al. 2003a, Sintes and Herndl 2006), the necessary standardization for each sample can be complicated and time consuming. The 3-dimensional distribution of the silver grains within the emulsion surrounding the active cell further hampers accurate quantification. Finally, the lateral resolution is too low when using strong beta-emitting isotopes such as ^{14}C. Dilution or homogenization is therefore needed to analyse the activity of single cells in

tightly packed microbial communities such as biofilms and symbiont-containing tissue (this work, Nielsen et al. 2003b).

NanoSIMS allows the usage and analysis of radioactive and stable isotopes as labels, even simultaneously. The advantage of using stable isotopes is that working and incubating samples in the field is possible without special permits. Furthermore, the lateral resolution of nanoSIMS is sufficient to analyse and quantify symbiotic activity within host tissues (this work, Lechene et al. 2007) with enough accuracy to calculate uptake rates (manuscript 3, Musat et al. 2008). However, in situ signal intensities can be weak, especially in mixed, dense communities such as symbioses or biofilms (this work, Behrens et al. 2008). Best results are currently obtained with the fluorochrome Oregon Green® 488-X (Molecular Probes, Inc.) that has been used in this work. Other dyes and ISH methods have been tested, but all showed even lower in situ signal intensities (Behrens et al. 2008, Li et al. 2008). Clearly, optimization is needed for future research on communities whose members exhibit weak HISH signals. One way to achieve this could be the analysis of the complete cell, not just a few nanometer-thick layers, to gather all in situ signals within the cell, This, however, is an instrument time consuming approach. Optimization can also target the specific probes by designing new probes for the Gamma 3 symbiont; the use of helper probes, which are unlabelled oligonucleotides that support the access to the target region on the rRNA by opening to secondary structures (Fuchs et al. 2000); or exchanging one or more nucleotides with peptide nucleic acids (PNA) or locked nucleic acids (LNA), which have a higher affinity to complementary bases (in Amann and Fuchs 2008). NanoSIMS is, at least for now, not a high-throughput tool, and searching for the cells of interest in a small field of view and the tuning of the instrument for best mass resolution can be very time-consuming. Ideally, both, MARFISH and nanoSIMS-HISH, are applied in parallel where possible, using MARFISH as a qualitative screening tool to identify the active populations and their abundance, and nanoSIMS for quantification of single cell uptake rates.

Future experiments regarding the physiology of the gutless oligochaete symbiosis, experimental improvements and control experiments were suggested in Chapter 2.2.5. Symbionts which are active, that is, which incorporate carbon or nitrogen substrates, under anoxic conditions, in the presence of different electron donors and electron acceptors can now be identified. Aside from autotrophy, organic substrates might be investigated to find out which compounds are used by the sulfate-reducing symbionts. It is now also possible to study the nitrogen cycling within the *O. algarvensis* such as urea excretion by the host and possible uptake by the symbionts (Woyke et al. 2006).

One additional very interesting question to answer is the source of host nutrition. As a first step, longer incubations should be performed to study the transfer of freshly synthesized carbon to the host and co-occurring symbionts. Pulse-chase experiments with ^{13}C-labelled bicarbonate under oxic conditions with up to five days chase have been done on *O. algarvensis* and *I. leukodermatus* and the samples await analysis.

2.4 Metagenomic analysis of the *Inanidrilus leukodermatus* symbiosis

The gutless oligochaete *Inanidrilus leukodermatus* was one of the first marine chemoautotrophic host species discovered from a shallow-water habitat. Its habitat conditions and ecophysiology have been studied (Giere et al. 1982, Giere et al. 1984, Giere et al. 1991), and a recent investigation of its symbiont community identified a Gamma 1 symbiont co-occurring with alphaproteobacterial symbionts (Blazejak et al. 2006). Genes diagnostic for autotrophy and sulfur oxidation were found indicating a chemoautotrophic sulfur-oxidizing potential of the Gamma 1 symbiont, but little else is known about symbiont function (Blazejak et al. 2006).

In this project a large-insert library generated from the *I. leukodermatus* symbiosis community DNA was analyzed for genes involved in autotrophy and energy metabolism (see below). The aim of this project was to gain insight into the arrangement and operon structure of these genes, and to compare the gene organisation to genomic and metagenomic data of the *O. algarvensis* symbiosis, other marine chemosynthetic symbionts and free-living bacteria.

Autotrophy via the Calvin-Benson-Bassham cycle

Autotrophy in marine chemoautotrophic symbioses can occur via several pathways, of which the Calvin-Benson-Bassham (CBB) cycle is widely distributed within the marine gammaproteobacterial sulfur-oxidizing symbionts. Ribulose-1,5-bisphosphate carboxylase/ oxygenase (RubisCO), one of the key enzymes of this pathway, assimilates carbon dioxide into organic molecules. Two different forms of RubisCO are known, form I and form II. Only form I, encoded by the genes *cbbL* and *cbbS* for the large and the small subunit, respectively (Elsaied and Naganuma 2001), has been found in gutless oligochaete symbionts (Blazejak et al. 2006, Ruehland et al. 2008, Woyke et al. 2006). In this study *cbbL* was used as a marker gene for autotrophy.

Energy metabolism

Three enzymes involved in the oxidation of reduced sulfur compounds were analyzed in this project, the reverse-operating adenosine-5'-phosphosulfate (APS) reductase (Meyer and Kuever 2007a); the reverse-operating sirohaem dissimilatory sulfite reductase (rDSR; Loy et al. 2009); and SoxB, which is involved in thiosulfate oxidation (Friedrich et al. 2001, Meyer et al. 2007). APS reductase (Apr) is a heterodimer consisting of the two subunits alpha and beta (Meyer and Kuever 2007a). This enzyme was suggested to oxidatively bind sulfite to AMP, generating APS, the reverse of the well-studied APS reduction reaction found in sulfate-reducing bacteria (Meyer and Kuever 2007b). rDSR is a heterotetramer with two alpha- and two beta-subunits encoded by the neighbouring genes *dsrA* and *dsrB*, which are part of a large rDSR gene cluster (Loy et al. 2009). Mutagenesis studies indicate that rDSR is necessary for sulfur oxidation in sulfur-storing bacteria (Pott and Dahl 1998). SoxB, a

monomer with sulfate thioesterase activity, is part of a multienzyme complex (Sox), the branched thiosulfate-oxidation pathway, and the polythionate degradation pathway (Meyer et al. 2007). Genes coding for Apr, rDSR and SoxB are present in sulfur-oxidizing free-living gamma- and alphaproteobacteria as well as in marine chemoautotrophic symbionts, including those of gutless oligochaetes (Kuwahara et al. 2007, Loy et al. 2009, Meyer et al. 2007, Meyer and Kuever 2007a, Newton et al. 2007, Woyke et al. 2006).

Carbon monoxide dehydrogenase (CODH) was analyzed because a gene coding for the large subunit of a CODH was found via endsequencing of the *I. leukodermatus* fosmid library (T. Woyke, unpublished data). CODH catalyzes the oxidation of carbon monoxide (CO) to carbon dioxide. Two forms exist, an aerobic CODH employing molybdenum as co-factor, and an anaerobic CODH containing nickel (King and Weber 2007). Aerobic CODHs can be further distinguished as form I (OMP-type), which has been verified to oxidize CO and is present in carboxydotrophic and other CO-oxidizing bacteria (King 2003a); and a putative form II (BMS-type), who's activity has not yet been demonstrated, but which is the only CODH found in CO-oxidizing bacteria and thus should be a functional CODH (King 2003a).

Phylogenetic marker

The 16S rRNA gene of the three alphaproteobacterial symbionts, Alpha 1a, Alpha 1b and Alpha 3, of *I. leukodermatus* was targeted as a marker gene for these symbionts. Neighbouring genes of the rRNA operon might provide information on the metabolic features of these symbionts.

2.4.1 Analysis of the *I. leukodermatus* fosmid library

I. leukodermatus specimens were collected off Bermuda, high molecular weight DNA was extracted from 200 worms, and a fosmid library generated (fosmid library generation by T. Woyke). In the present work, more than 8000 fosmid clones were combined into several hundred pooled fosmid clones via a robot pipeline. The pooling combined rows and columns of as well as complete 96-well microtitre plates in a way that allowed tracking back from positive PCR reactions of the pooled fosmids to single positive fosmid clones (roboter and pipetting pipeline set-up by A. Ellrott). The pooled fosmids were screened for the genes of interest. Single fosmids were identified based on the pooling scheme and analyzed by PCR and direct sequencing of the PCR-product to verify that they coded for the gene of interest. Primers were chosen from the literature or designed based on the symbiont 16S rRNA sequences (Table 2.1 and supplemental materials Chapter C.3). The specificity of the primers was tested on *I. leukodermatus* community DNA.

The screening of the pooled fosmid clones provided positive PCR products for the following genes: *aprA*, *cbbL*, *soxB*, *coxL* and all three 16S rRNAs (Table 2.1). Direct sequencing of several *cbbL* and *aprA* PCR products showed that these were 100% identical at the amino acid level to those amplified from individual *I. leukodermatus* specimens (Blazejak et al. 2006), and thus originated most likely from the Gamma 1 symbionts. The 16S rRNA sequences obtained were almost identical to those of the *I. leukodermatus* alphaproteobacterial symbionts (Table 2.1). Slight differences between sequences though were seen on the nucleotide level. Two *cbbL*-, one *aprA*-, one Alpha 1b- and one Alpha 3-containing fosmid were selected for full sequencing. Two additional fosmids from the endsequencing project (T. Woyke, unpublished data) were combined with the *cbbL* fosmid data.

Sequences amplified with the *soxB* primers showed highest identities to *soxB* (sulfate thioesterase/ sulfate thiohydrolase) from Chromatiaceae species, including *Thiorhodococcus minor* and *Allochromatium vinosum*. The *I. leukodermatus soxB*-like sequences therefore derive most likely from the Gamma 1 symbiont, as this symbiont is related to these Chromatiaceae species. However, horizontal gene transfer of the SoxB gene cannot be excluded (Meyer et al. 2007). Sequences affiliated with *soxB* sequences from free-living alphaproteobaceria were not found in the *I. leukodermatus* fosmid library. No single fosmid with a *soxB* gene was identified.

A *coxL*-related sequence was obtained by amplification with primers specific for the OMP-type but not the BMS-type CODH. The deduced protein sequence showed the highest identity to the large CODH subunit of *Roseobacter* species (Table 2.1). A single fosmid with a *coxL* sequence was identified and fully sequenced.

PCR amplification with primers targeting the large *dsrAB* and the small *dsrB* fragments was not successful despite several PCR attempts with varying conditions. This was surprising as an rDsrAB gene assigned to the Gamma 1 symbiont was successfully amplified from *O. algarvensis* community DNA and found in the metagenome of the *O. algarvensis* symbiosis (Loy et al. 2009, Woyke et al. 2006). Primer pairs though might not have matched with the *I. leukodermatus* Gamma 1 symbiont *dsrAB*.

Despite the high number of positive-screened pooled fosmid clones, in particular for *cbbL*, *aprA* and *soxB*, few positive single fosmids were found with only one fosmid for *coxL* and none for *soxB*. It is unclear at this point why the detection of genes by PCR amplification in single fosmids was less successful than for pooled fosmids.

Table 2.1: Summary of genes investigated in this study and their presence in the *I. leukodermatus* genomic DNA, pooled and single fosmids, determined by PCR amplification. Best protein BLAST hit and % identity are given.

Gene	Target	Genomic DNA	Pooled fosmids	Single fosmids	Best BLAST hit	%
16S rRNA Alpha 1a	Alpha 1a symbiont	x	x	0	*I. leukodermatus* Alpha 1a and Alpha 1b symbiont	99-100
16S rRNA Alpha 1b	Alpha 1b symbiont	x	x	x	*I. leukodermatus* Alpha 1b symbiont	100
16S rRNA Alpha 3	Alpha 3 symbiont	x	x	x	*I. leukodermatus* Alpha 3 symbiont[a]	100
cbbL	large subunit of RubisCO	x	x	x	*cbbL* of *I. leukodermatus* Gamma 1 symbiont	100
aprA	alpha subunit of oxidative APS reductase	x	x	x	*aprA* of *I. leukodermatus* Gamma 1symbiont	100
soxB	SoxB component	x	x	0	sulfate thioesterase/ sulfate thiohydrolase (*soxB*) of *Thiorhodococcus minor*	68
dsrAB	alpha and beta subunit of the rDSR	0	0	0	-	-
dsrB	beta subunit of the rDSR	0	0	0	-	-
coxL	large subunit of the CODH	x	x	x	*coxL* of *Roseobacter* sp.	79

[a] former Alpha 2 symbiont of *I. leukodermatus* (accession number AJ890097).
RubisCO: Ribulose-1,5-bisphosphate carboxylase/ oxygenase; APS: adenosine-5'-phosphosulfate; Sox: periplasmic thiosulfate-oxidizing Sox enzyme complex; rDSR: reverse operating sirohaem dissimilatory sulfite reductase; CODH: carbon monoxide dehydrogenase.

2.4.2 Analysis of fosmid sequences and genome comparison

The fosmid sequences were loaded into IMG/M-ER for automated gene prediction and annotation (Markowitz et al. 2008). Tools provided within IMG/M-ER were used for gene neighbourhood comparison. The sequence annotation revealed remarkable differences between the fosmids. Predicted open reading frames (ORFs) on the fosmids with *cbbL*, *aprA* and Alpha 3 16s rRNA were often short, separated by extended non-coding regions. Furthermore, these fosmids carried up to five transposable elements on a length of 22 to 40 kb. Assembly was difficult, in particular for the *cbbL* fosmid, which remained in few contigs dispite applying different assembly methods. In contrast, the *coxL* fosmid contained no transposable elements and the Alpha 1b 16S rRNA fosmid only a single one. On both fosmids there were only very short non-coding regions between ORFs.

Alphaproteobacterial fosmids

16S rRNA. The two alphaproteobacterial 16S rRNA fosmids encoded the rRNA operons of the Alpha 1b and the Alpha 3 symbiont with 16S, 23S and 5S rRNA as well as tRNA. ORFs upstream and downstream of the Alpha 1b rRNA operon included genes coding for aminotransferase, arginase and a C4-dicarboxylate transporter. The Alpha 3 fosmid encoded for an oligopeptide/ dipeptide transporter and recombinase A, a protein involved in homologous recombination and DNA repair.

CoxL. The *coxL* fosmid coded for the CODH locus *coxMSL*. The deduced amino acid sequence of the *coxM* showed the highest sequence identity to the medium CODH subunit of *Oligotropha carboxidovorans* (62%). The small CODH subunit found on the *coxL* fosmid had 75% amino acid identity to a (2Fe-2S)-binding protein of the alphaproteobacterium *Stappia aggregata* and ≤ 72% to small CODH subunits of *Roseobacter* species. The protein sequence coded by *coxL* shared up to 79% identical amino acids with large CODH subunits of *Roseobacter* species and 75% with the CoxL of *O. carboxidovorans*. The sequence of the active site of OMP-type CODH (King 2003a) was present in the aminoc acid sequence. Neighbouring ORFs to the *coxMSL* encode for accessory proteins of CO oxidation, such as the putative *coxC* upstream and *coxDEF* downstream to the CODH locus (Figure 2.18). In addition, two ABC-type transporter systems were present on the fosmid, one for nitrate/ sulfonate/ bicarbonate, and one for molybdenum, the co-factor of the aerobic CODH. The presence of the CODH locus, the high sequence identities of the CODH subunits with aerobic CODH from CO-oxidizing free-living bacteria, and the adjacent genes suggest a functional form I OMP-type CODH on the *I. leukodermatus coxL* fosmid.

A CODH operon and a *coxMSL* locus coding for an OMP-type CODH were also found in the *O. algarvensis* Gamma 3 symbiont bin (Figure 2.18, Woyke et al. 2006). It would be intriguing if gutless oligochaete symbionts use CO, as CO is toxic for multicellular organisms. CO has a higher affinity for haemoglobin than oxygen, thereby blocking the oxygenation of tissues and inhibiting the mitochondrial electron transport chain by binding cytochromes. Furthermore, in situ CO concentrations in marine sediments are usually low, although high local CO production can occur as a result of abiological degradation and other processes, providing sufficient CO for CO-oxidizing microorganisms. Usage of internally produced CO might be another possible CO source. Indeed, CO-oxidizing bacteria that use internal CO, exist as mammalian parasites (King 2003b).

Aerobic CO oxidation would provide the gutless oligochaete symbionts with another way of energy generation which has not yet been found in marine chemosynthetic symbioses. CO oxidation occurs in autotrophic and in heterotrophic or mixotrophic free-living bacteria and is coupled to oxygen reduction thereby generating energy. In heterotrophic bacteria, CO oxidation provides supplemental energy and

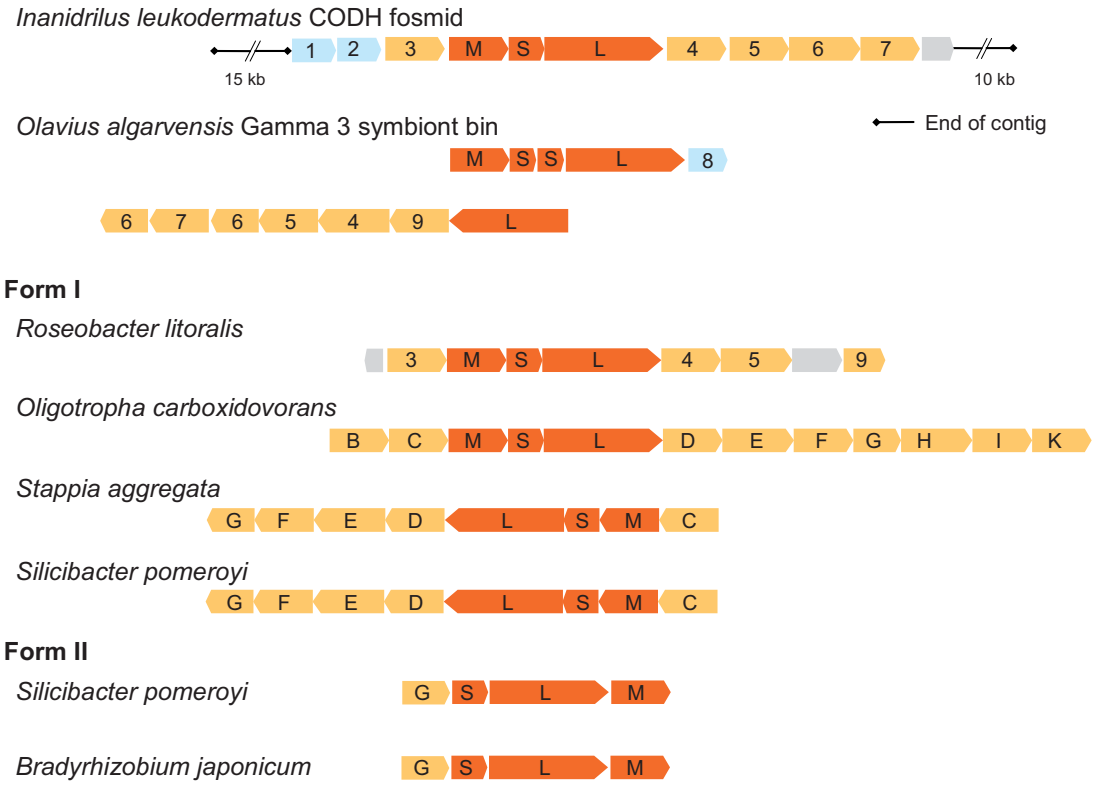


Figure 2.18: Genomic organization of the *coxMSL* locus in the *I. leukodermatus* fosmid, in the *O. algarvensis* Gamma 3 symbiont bin, and in CO-oxidizing free-living bacteria. CODH-coding genes in red, accessory genes in orange, genes coding for proteins with function prediction in blue, and hypothetical proteins in grey. ORF numbers: 1 and 2: acyl-CoA dehydrogenase; 3: integral membrane sensor protein/ putative *coxC*; 4: ATPase/ putatitve *coxD*; 5: putative *coxE*; 6: putative *xdhC*/*coxF*; 7: molybdopterin-binding protein; 8: ABC transporter ATPase component; 9: putative *coxG*. (Data from IMG/M-ER and King and Weber 2007).

allows organic carbon compounds to be saved for assimilation, which is of particular interest in oligotrophic environments and has been repeatedly suggested for species of the marine alphaproteobacterial *Roseobacter* clade (King and Weber 2007, Moran et al. 2004, Wagner-Dobler and Biebl 2006). In addition, CO oxidation can enhance heterotrophic CO_2 fixation by providing an additional carbon source as these bacteria often lack enzymes for autotrophic carbon fixation. The absence of a second RubisCO in *I. leukodermatus* besides the Gamma 1 RubisCO, and the absence of genes diagnostic for the rTCA cycle (Blazejak 2006) indicates that the alphaproteobacterial symbionts are heterotrophic. Thus, CO oxidation might generate supplemental energy in the gutless oligochaete symbiosis, provided that the *coxMSL* was obtained from one of the alphaproteobacterial symbionts of *I. leukodermatus*.

Only a single *coxL* sequence was found in the *I. leukodermatus* community fosmid library which might have originated from a free-living contaminant and not from one of the alphaproteobacterial symbionts. Evidence for a symbiont derived CODH could be provided by analysing several individual *I. leukodermatus* for the *coxL* gene, which

should give (almost) identical *coxL* sequences assuming low strain variability in gutless oligochaete symbiont communities. Another approach would be single-gene detection in combination with 16S rRNA FISH (Moraru et al. 2009) to localize the gene within a specific symbiont. Finally, detection of gene expression by mRNA-FISH (Pernthaler and Amann 2004) could confirm that the *coxMSL* locus indeed belongs to and is expressed by one of the *I. leukodermatus* symbionts.

Gamma 1 symbiont fosmids

CbbL. The largest contig of the *cbbL* fosmid contigs encoded the *cbbLS* locus. Although genes involved in CO_2 fixation are often clustered, no other Cbb genes were found on any of the *I. leukodermatus* fosmid contigs. In contrast, the *O. algarvensis* Gamma 3 symbiont RubisCO locus consists of 10 genes (*cbbRFPTALSQOZ*), including another essential gene for autotrophy via the CBB cycle - *cbbP*, encoding the phosphoribulokinase (Blazejak 2006, Woyke et al. 2006). Only spare information exists on the RubisCO locus of the *O. algarvensis* Gamma 1 symbiont. A Gamma 1 *cbbL* was located on a very short contig containing only the *cbbL* sequence, which could not be assembled with other fragments of the metagenome. Similarly, a *cbbS* assigned to the Gamma 1 symbiont bin was located at the end of a contig with no sequence information on the other side (Woyke et al. 2006). The fact that the Gamma 1 symbionts of gutless oligochaetes are closely related suggests that the *cbb* loci of the *O. algarvensis* and the *I. leukodermatus* Gamma 1 symbionts are similar, although verification is needed.

The difference between *cbb* loci of the two chemoautotrophic symbionts of gutless oligochaetes, Gamma 1 and Gamma 3 leads to speculate about their role within the symbiosis. Scott et al. (2006) found that in obligately autotrophic bacteria the RubisCO-encoding genes are not clustered together with genes coding for other CBB cycle enzymes, whereas in facultative autotrophs, *cbbP* forms an operon with the RubisCO genes (Scott et al. 2006). Based on this assumption, the primary symbionts of gutless oligochaetes might be obligate autotrophs, while the Gamma 3 symbionts might be facultative autotrophs.

The *cbbLS* locus of the Gamma 1 symbiont differed from that of known autotrophs in which *cbbLS* is generally followed by *cbbO* and/ or *cbbQ*, genes coding for proteins possibly involved in RubisCO assembly (Scott et al. 2006, and references therein). One or both genes were also present and adjacent to the RubisCO encoding genes in the clam symbiont genomes of *Candidatus* Ruthia magnifica (Newton et al. 2007) and *Candidatus* Vesicomya okenii (Kuwahara et al. 2007). The *I. leukodermatus* Gamma 1 symbiont *cbbLS* locus was instead flanked by transposable elements (see below).

AprA. The *aprBA* locus on the *aprA* fosmid was arranged similar to the *cbbLS* locus. Transposable elements were located upstream and downstream of the *aprBA* locus, which does not resemble known *aprBA* locus neighbourhoods. AprBA of sulfur-oxidizing bacteria fall in two groups, lineage I including the oligochaete Gamma 1

symbiont AprBA, and lineage II with Gamma 3 and clam symbiont AprBA (Figure 2.3, Meyer and Kuever 2007a). Lineage I *aprBA* are often directly preceeded by a gene coding for the adenylylsulfate reductase (*sat*) (Meyer and Kuever 2007a), while in bacteria and symbionts with a lineage II AprBA a membrane anchor protein *(aprM)* is located between *sat* and the *aprBA* locus (Meyer and Kuever 2007a). Although *sat* was present on the *I. leukodermatus aprA* fosmid, this gene was not close to the *aprBA* locus.

Mobile elements. It is remarkable that those genes considered coding for important enzymes in the chemoautotrophic primary symbiont of *I. leukodermatus* are surrounded by mobile elements. Mobile elements are generally rare in free-living bacteria and eliminated in obligate host-associated bacteria (Moran and Plague 2004). A high or increased load of transposable elements compared to a progenitor has been proposed to indicate a transition stage from a facultative to an obligate, often intracellular life style (Moran and Plague 2004, Plague et al. 2008). Such a transition might also be the case for the *O. algarvensis* Gamma 1 symbiont with 20.5% of its genome encoding for transposases (Woyke et al. 2006). The observation that genes thought to be essential for the Gamma 1 symbiont metabolism are flanked by mobile elements could hint to processes of genome reorganisation and optimization of gene arrangement which might eventually result in gene loss and genome size reduction in the Gamma 1 symbionts.

Similarly, the Alpha 3 symbiont could also be in such a transition stage. The Alpha 3 16S rRNA fosmid coded for as many transposable elements as the Gamma 1 symbiont fosmid contigs. However, genome information on the alphaproteobacterial symbionts is limited and further analyses are needed.

2.4.3 Conclusion

Screening of the *I. leukodermatus* fosmid library and analysis of selected fosmids provided some insight into gene arrangement and operon structure of the Gamma 1 and alphaproteobacterial symbionts. The function of the alphaproteobacterial symbionts though still remains elusive. Protein-coding genes on the alphaproteobacterial 16S rRNA fosmids did not indicate specific metabolic features except a heterotrophic metabolism. The operon coding for the aerobic CODH, if derived from one of the alphaproteobacterial symbionts of *I. leukodermatus*, is an exciting result as it would be a novel source of energy for the gutless oligochaete symbiosis. Future studies need to verify the origin of the CODH-coding gene locus and the usage of CO as energy source in the *I. leukodermatus* symbiosis.

This work confirmed the chemoautotrophic sulfur-oxidizing capacity of the Gamma 1 symbiont of *I. leukodermatus* and corroborated that the genomes of the primary symbionts of gutless oligochaetes are packed with mobile elements. It remains

to be investigated though, if the high load of mobile elements indeed indicates that the Gamma 1 symbiont only 'recently' became an obligate symbiont.

2.4.4 Outlook

DNA-based investigation of symbiont function

The alphaproteobacterial symbionts of gutless oligochaetes deserve further investigation to elucidate their functional role within this symbiosis. Large insert library screening is limited as primer design depends on a reasonable large database and still, genes of interest might be missed due to primer mismatches. Another possible direction to investigate metabolic capacities on the DNA level would be genomic analysis. In addition to library screening, an enrichment of the alphaproteobacterial symbionts was tried for *I. leukodermatus* by density gradient centrifugation prior to DNA extraction (modified after Woyke et al. 2006). The fractions retrieved were analysed with CARD-FISH to identify those dominated by alphaproteobacterial symbionts. Enriched fractions from several gradients were combined for genomic DNA extraction. A 3 kb library was generated from this DNA and checked for purity with a 16S rRNA library. Sequencing and phylogenetic analysis of about 300 16S rRNA clones showed that the library was contaminated with sequences related to Bacteroidetes, which accounted for 30% of the 16S rRNA sequences. Therefore the 3 kb library was not investigated further.

Enrichment by density gradient centrifugation is an efficient method to separate symbionts from the host and from each other. It works best, though, for symbionts which differ clearly in those features affecting the behaviour in a density gradient. The three alphaproteobacterial symbionts of *I. leukodermatus* would have been enriched in the same fractions as they are of about the same size and lack large inclusion bodies. Thus, the library generated would have been a mixed DNA sample of all alphaproteobacterial symbionts.

To avoid such a mixed symbiont DNA sample and to circumvent contamination with free-living bacteria, individual symbiont cells from *I. leukodermatus* were picked by micromanipulation for single cell genome amplification via MDA (Fröhlich and König 1999, Ishøy et al. 2006, Kvist et al. 2007). In collaboration with T. Woyke (Joint Genome Institute, USA) several large and a few small cells were picked, the latter being more difficult to recognize, because of the smaller size and the lack of large inclusion bodies which easily identified the Gamma 1 symbionts (Figure 2.19). After MDA of the single cell DNA, 16S and 18S rRNA was PCR-amplified as a first estimate for purity of the DNA. The 16S rRNA sequences of several Gamma 1 and one alphaproteobacterial symbiont cells were not clean, indicating contaminating DNA in the sample. In contrast, clean 16S rRNAs were often identified as *Delftia acidovorans*, a betaproteobacterium, which was repeatedly found in amplified DNA when small amounts of initial DNA

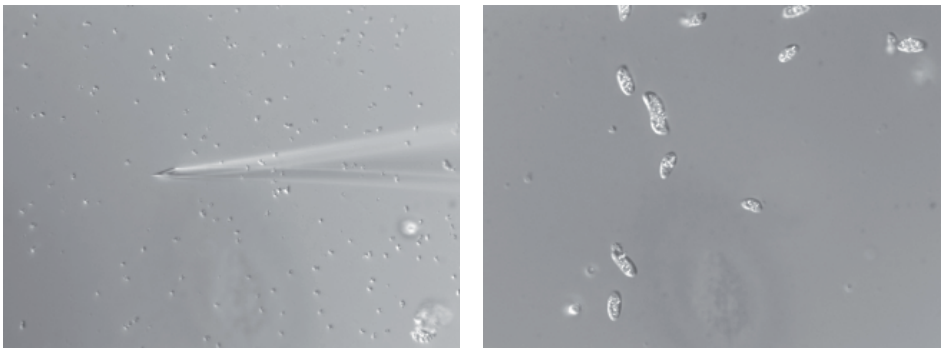


Figure 2.19: DIC images of Gamma 1 symbionts next to a glass capillary used for micromanipulation (left panel) and close-up of the Gamma 1 symbionts (right panel). Gamma 1 symbionts were easily recognizable by their cell size of 2-3 µm length and their inclusion bodies.

were used, and likely derives from the MDA reagents (T. Woyke, personal communication, Pernthaler et al. 2008, Stepanauskas and Sieracki 2007). The amplified contaminated symbiont DNA was not further investigated. For future experiments collecting larger quantities of DNA prior to MDA reaction can avoid the risk of amplifying exogenous contaminating DNA, and could be achieved by methods such as flow sorting, FACS or magnetoFISH (Chapter 1.4.2).

Non-DNA-based investigation of symbiont function

Studying the metabolic capacities of the *I. leukodermatus* (and of other gutless oligochaetes) symbionts beyond (meta-) genomics might include meta-transcriptomics and metaproteomics. One advantage of investigating the metatranscriptome is the possibility to amplify the mRNA (reviewed in Warnecke and Hess 2009), if needed, which cannot be done for proteome analyses. Although the risk of amplification bias has to be considered, a general idea about expressed genes can be gained and might be useful for habitats where several oligochaete species co-occur and collecting large numbers of a single species for proteome analysis is not feasible.

Incubation experiments with labelled or unlabelled substrates would provide information on the substrates used and assimilated by the symbionts (Chapter 2.2.5 and 2.3). For the alphaproteobacterial symbionts of gutless oligochaetes, incubation with DMS, DMSO or DMSP might be promising as the ability to use these substrates as electron acceptor, electron donor or sulfur and/or carbon source is widely distributed within marine Alphaproteobacteria (Bentley and Chasteen 2004, Hatton et al. 2005, Howard et al. 2008). MARFISH studies of ^{35}S-labelled DMSP uptake by marine plankton indicated an important role of Alphaproteobacteria in DMSP metabolism (Vila et al. 2004, Zubkov et al. 2002). Similarly, the usage of these organosulfur compounds or CO (Tolli et al. 2006) could be analyzed in incubation experiments combined with MARFISH (Chapter 2.3.1) and/ or nanoSIMS-HISH (Chapter 2.3.2).

2.5 General conclusions

In this PhD work the diversity and function of the symbiont communities in gutless oligochaetes was investigated and some pieces of information were added to the gutless oligochaete symbiosis puzzle. For the first time the primary Gamma 1 symbiont thought to be ubiquitous to gutless oligochaetes was not found in a host species. Instead, molecular investigation revealed that a novel gammaproteobacterial symbiont, called Gamma 4, dominated the symbiont community of *Inanidrilus exumae*. Though morphologically similar, but only distantly related to the Gamma 1 symbiont, the Gamma 4 symbiont probably fulfils a similar role as a chemoautotrophic sulfur-storing symbiont. In addition, a novel symbiont composition of co-occurring delta- and alphaproteobacterial symbionts not previously described was found in *I. exumae*. Most of the symbionts identified in *I. exumae* were affiliated with known oligochaete symbiont clades. However, new symbiont lineages, including the Gamma 4 symbiont, were identified. These results corroborate the high versatility of symbiont communities in gutless oligochaetes, which is unprecedented in marine chemosynthetic symbioses.

The importance of the Gamma 1 symbiont for the gutless oligochaete symbiosis is indicated by its presence in nearly all host species studied previously, and by the fact that the novel primary symbiont in *I. exumae* seems to fulfil the same "core function" as sulfur-storing chemoautotroph. Physiological studies in this work investigated these core functions, the autotrophic sulfur-oxidizing capacities of the *Olavius algarvensis* symbiosis by labelling experiments. Under oxic conditions and in the absence of an external electron donor, inorganic carbon was taken up at rates similar to other marine chemoautotrophic symbioses, indicating the importance of this metabolism for the symbiosis. Stored sulfur was most likely used as electron donor for carbon fixation in the presence of oxygen. Electron donors and electron acceptors that enabled carbon fixation under anoxic conditions could not be unambiguously identified. Possible explanations are that substrates were produced or stored within the symbiosis for a limited time, that electron donor, electron acceptor or both were limiting, or an impaired fitness of the symbiosis due to maintenance conditions.

Two chemoautotrophic sulfur-oxidizing symbionts, the Gamma 1 and the Gamma 3 symbiont co-occur in *O. algarvensis*, which hampered a clear assignment of the observed autotrophic activity to one or both symbionts in bulk measurements alone. Identification of the active symbiont population, and moreover the visualization and quantification of carbon uptake on the single-cell level was achieved by applying MARFISH and nanoSIMS-HISH. Under oxic conditions without external energy sources, the Gamma 1 symbiont immediately fixed inorganic carbon. Other symbionts, including the Gamma 3 symbiont, were only weakly labelled after 25 h incubation. Poor incorporation of labelled carbon is most likely the result of heterotrophic carbon

fixation or transfer of freshly synthesized carbon from the Gamma 1 symbionts to co-occurring symbionts rather than autotrophic carbon fixation. Host tissue remained unlabelled, even after 25 h. Thus, host nutrition based on symbiont-fixed carbon is probably slow, suggesting that the host retrieves its carbon by symbiont lysis and digestion rather than by transfer of carbon compounds.

Although transfer of carbon from symbiont to host has not yet been observed in gutless oligochaetes, autotrophy and sulfur oxidation seem to be important metabolic features of this symbiosis as shown by uptake experiments. Interestingly, genes coding for these core functions of the Gamma 1 symbiont showed an unusual gene arrangement, and were flanked by mobile elements. The role of these mobile elements in the symbiont's functioning is yet unknown, though it might indicate genome reorganisation and a recent transition from a facultative to an obligate symbiotic lifestyle.

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Part B - Publications and manuscripts

List of publications and manuscripts with author's contribution

(1) Dubilier, N., **Bergin C.**, Lott C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. Nature Reviews Microbiology **6**: 725-740.

Concept N.D., phylogenetic analyses and trees C.B., table C.B. and C.L., figures C.L., manuscript writing N.D., editorial revision C.B. and C.L.

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

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(3) **Bergin, C.**, Lott, C., Polerecky, L., Musat, N., Vagner, T., Kuypers, M.M.M., Dubilier, N. Single-cell analysis of autotrophic carbon fixation in the symbiotic community of the gutless marine worm *Olavius algarvensis* using nanoSIMS. (in preparation)

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(4) Woyke T., Teeling H., Ivanova N.N., Hunteman M., Richter M., Gloeckner F.-G., Boffelli D, Anderson I.J., Barry K.W., Shapiro H.J., Szeto E., Kyrpides N.C., Mussmann M., Amann R., **Bergin C.**, Ruehland C., Rubin E.R. and Dubilier N. (2006). Symbiosis insights through metagenomic analysis of a microbial consortium. Nature 443: 950-955.

C.B. was a member of the manual annotation consortium

(5) Rühland C., **Bergin C.**, Lott C., Dubilier N. (2006). Symbiosen mit mikrobiellen Konsortien. Biospektrum: 600-602.

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Symbiotic diversity in marine animals: the art of harnessing chemosynthesis

Nicole Dubilier, Claudia Bergin, Christian Lott

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FOCUS ON SYMBIOSIS

REVIEWS

Symbiotic diversity in marine animals: the art of harnessing chemosynthesis

Nicole Dubilier, Claudia Bergin* and Christian Lott*‡*

Abstract | Chemosynthetic symbioses between bacteria and marine invertebrates were discovered 30 years ago at hydrothermal vents on the Galapagos Rift. Remarkably, it took the discovery of these symbioses in the deep sea for scientists to realize that chemosynthetic symbioses occur worldwide in a wide range of habitats, including cold seeps, whale and wood falls, shallow-water coastal sediments and continental margins. The evolutionary success of these symbioses is evident from the wide range of animal groups that have established associations with chemosynthetic bacteria; at least seven animal phyla are known to host these symbionts. The diversity of the bacterial symbionts is equally high, and phylogenetic analyses have shown that these associations have evolved on multiple occasions by convergent evolution. This Review focuses on the diversity of chemosynthetic symbionts and their hosts, and examines the traits that have resulted in their evolutionary success.

Chemolithoautotrophic
Chemolithoautotrophic organisms use a chemical compound as an energy source, an inorganic compound, such as sulphide, as an electron donor and an inorganic carbon source (usually carbon dioxide) to synthesize organic carbon.

Phototrophic
Phototrophic organisms, such as plants, use light to gain energy.

Heterotrophic
Heterotrophic organisms, such as humans, use an organic source of carbon.

**Symbiosis Group, Max Planck Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany.*
‡HYDRA Institute for Marine Sciences, Elba Field Station, Fetovaia, Via del Forno 80, I-57034 Campo nell'Elba (LI), Italy.
Correspondence to N.D.
e-mail: ndubilie@mpi-bremen.de
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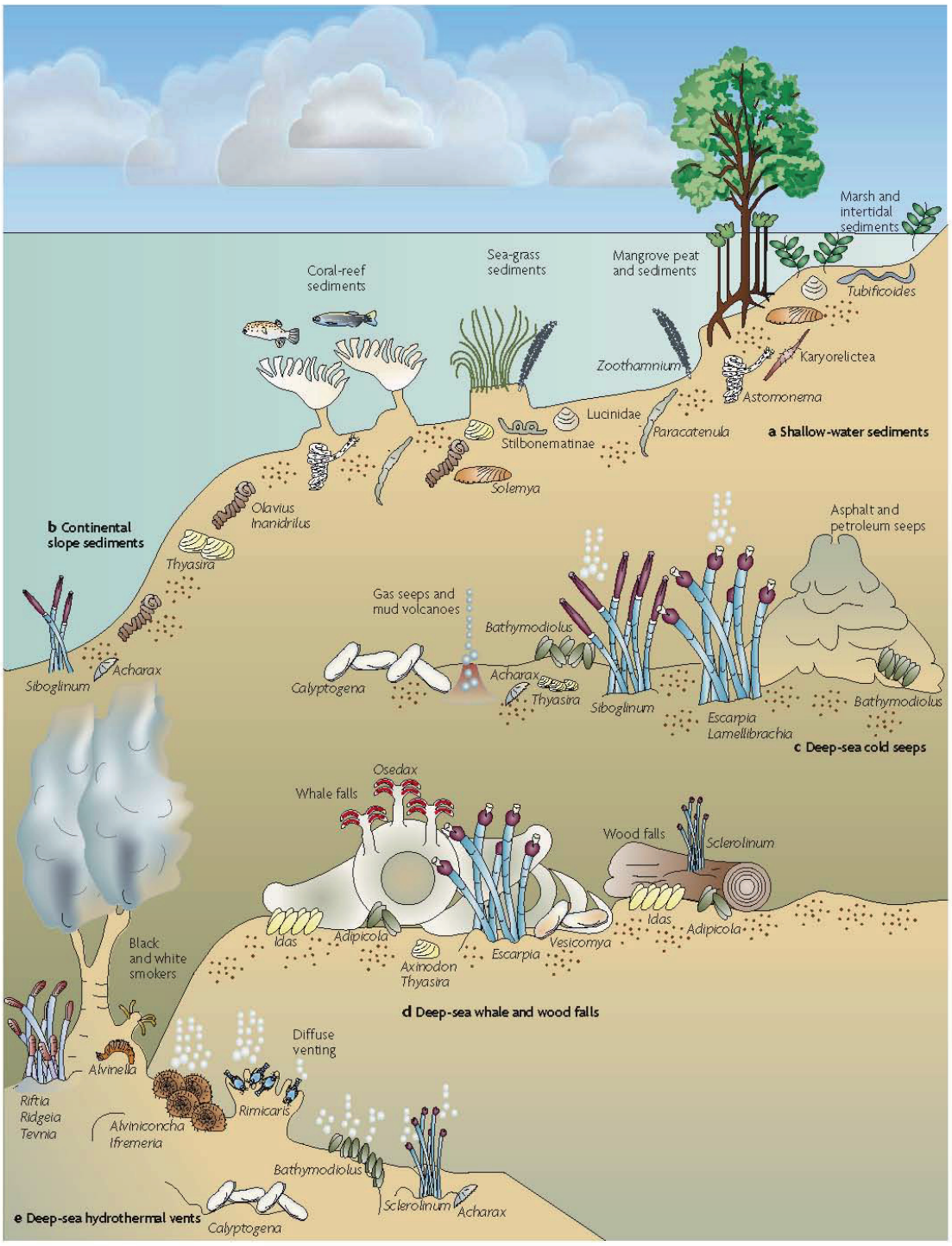
Long before the gutless pogonophore tube worm *Riftia pachyptila* was discovered at hydrothermal vents on the Galapagos Rift in the late 1970s, marine worms from the phylum Pogonophora that lack both a mouth and gut were known to scientists[1]. It was assumed that these gutless worms, which were first found in the deep-sea sediments of the Pacific, gained their nutrition by taking up dissolved organic compounds through their tentacles or body wall[2]. The *R. pachyptila* worms discovered at the Galapagos vents were quickly identified as members of the phylum Pogonophora. But it was not until they were examined more closely that it became apparent that they obtain their nutrition from endosymbiotic bacteria. Histological and enzymatic analyses revealed that the *R. pachyptila* endosymbionts are chemolithoautotrophic, sulphur-oxidizing bacteria, and it was proposed that they use reduced sulphur compounds from the vent fluids as electron donors and fix carbon dioxide autotrophically to synthesize organic compounds that are passed on to the host (reviewed in REFS 3,4). Before the discovery of *R. pachyptila*, only phototrophic symbioses (such as those in corals) and heterotrophic symbioses (such as those in rumen associations) had been characterized, and *R. pachyptila* was thus the first host in which a chemoautotrophic symbiosis was discovered.

It is surprising in retrospect that it took the discovery of these symbioses in the deep sea for scientists to realize that chemosynthetic symbioses occur worldwide in a wide range of habitats, including easily accessible habitats, such as shallow-water coastal sediments. As so often occurs in science, the incredible diversity of species in our own backyard was overlooked[5]. The discovery of the *R. pachyptila* symbiosis triggered a search for chemosynthetic associations in other environments, particularly in habitats with sulphide concentrations that are as high as those of vents such as sewage outfalls and organic-rich mud flats. In addition to looking for symbioses in other vent animals, scientists also searched the literature for descriptions of free-living animals that have a reduced digestive system and could therefore be potential hosts. Only a few years after the discovery of *R. pachyptila*, it became clear that chemosynthetic symbioses are ubiquitous, in environments that range from hydrothermal vents, whale and wood falls, cold seeps, mud volcanoes and continental margins, to shallow-water coastal sediments[4,6] (FIG. 1). Animals from at least seven different phyla are currently known to harbour chemosynthetic symbionts, with hundreds of host species now described and the discovery of many more expected. Although the diversity of chemosynthetic symbionts was long underestimated, molecular methods have recently revealed that many different lineages of bacteria can establish chemosynthetic symbioses, and most recently, genomic and proteomic analyses have revealed the remarkable range of different metabolic pathways that chemosynthetic symbionts use to gain energy from the environment and feed their hosts. This Review describes the diversity of chemosynthetic habitats, hosts and symbionts, and discusses various explanations for the evolutionary success of chemosynthetic symbioses.



Chemoautotrophic
The term chemoautotrophic is often used as a synonym for chemolithoautotrophic. However, some chemoautotrophs use organic compounds as electron donors; these organisms are called chemoorganoautotrophs.

Chemosynthetic
Describes two types of organisms: chemolitho-autotrophs (for example, sulphur oxidizers) and methane oxidizers. These organisms convert one or more carbon molecules (usually carbon dioxide or methane) into organic matter using the oxidation of inorganic compounds (for example, sulphide) or methane as a source of energy. Both symbiotic and free-living chemosynthetic microorganisms are primary producers; they form the basis of the food chain at vents and seeps.

Thiotrophic
An organism that uses reduced sulphur compounds, such as sulphide, as electron donors is called a thiotroph or sulphur oxidizer.

Meiofauna
Small free-living invertebrates that live in marine and fresh-water sediments. Meiofauna do not constitute a defined taxonomic rank but rather are a group of benthic animals that are defined by their size (in general, these organisms can pass through a 1 mm sieve, but are retained on a 0.45 μm sieve).

Habitat diversity

The most well known habitats for chemosynthetic symbioses are those in the deep sea (FIG. 1). Deep-sea hydrothermal vents were the first habitats in which chemosynthetic primary production was shown to fuel large animal communities that are considered to be among the most productive on the Earth[3]. Most of this biomass is in the form of animals that are associated with symbionts, and dominant species are vestimentiferan tube worms, bathymodiolin mussels, vesicomyid clams and shrimp (TABLE 1). Only a few years after the discovery of the vent fauna, similar communities were discovered at cold seeps (FIG. 1) in the Gulf of Mexico[4]. Both vents and seeps are characterized by high concentrations of reduced energy sources, such as sulphide and methane, in close proximity to oxidants, such as oxygen, nitrate and sulphate. In chemosynthetic habitats in which animals occur, oxygen must be present, because free-living animals can only tolerate anoxia for limited time periods. Furthermore, only oxygen has clearly been shown to function as an electron acceptor for chemosynthetic symbionts, although the role of nitrate in symbiont respiration is still debated[7,8]. Thus, deep-sea vent and seep communities are not completely independent of photosynthetic primary production, as the oxygen in the deep sea originates from photosynthesis in surface waters.

When organic matter falls to the deep-sea floor in the form of whale carcasses or sunken wood (called whale and wood falls) (FIG. 1), it supports chemosynthetic communities for limited periods of time[6]. At whale falls, both the sediment around the whale, as well as the whale bones, become highly sulphidic owing to microbial degradation of organic-rich whale remains. This attracts a highly specialized and diverse assemblage of animals, some of which contain bacterial symbionts and are restricted to whale falls, including the gutless siboglinid worm *Osedax*, as well as others that are also found at vents and seeps, such as mussels, clams and vestimentiferan tube worms. Wood falls can also be colonized by animals that have symbionts[9], although evidence for a thiotrophic metabolism has only recently been shown in some of these symbioses[10]. Perhaps the most unusual habitats in which chemosynthetic symbioses have been found are shipwrecks. In a 1,100-metre-deep shipwreck off the coast of Spain, rotting beans in the hold of the ship produced enough sulphide for the growth of vestimentiferan tube worms[11]. *Lamellibrachia* tube worms were also collected from a 2,800-metre-deep shipwreck in the Mediterranean in association with decomposing paper in the ship's mailroom[12].

All deep-sea habitats support chemosynthetic symbioses, but only at vents and seeps do these associations dominate the biomass and form large standing crops. At whale and wood falls, chemosynthetic symbioses form only a small part of the animal community[6]. In shallow waters (commonly defined as 0–200 metres deep), where primary production is almost always driven by phototrophy, animal communities can usually gain enough energy from heterotrophy, and chemosynthetic symbioses never dominate the community. The only known exception is the tube worm *Lamellibrachia satsuma*, which dominates vents that are approximately 100 metres deep off the coast of Japan[13]. All other shallow-water hydrothermal vents are largely devoid of a typical vent fauna, and chemosynthetic symbioses occur only occasionally[14]. Likewise, the faunal communities of shallow-water seeps differ from those in deeper waters and are not dominated by chemosynthetic symbioses, although some hosts (mainly bivalves) with chemosynthetic symbionts can occur occasionally[14]. Whale falls in shallow waters lack the chemosynthetic vesicomyid clams found at deep-sea whale falls, but the gutless worm *Osedax* has been found on whale falls in waters as shallow as 30 metres[15–17]. As suggested by Little *et al.*[18], a clear distinction between shallow-water and deep-water chemosynthetic communities might not accurately reflect their ecology; instead these communities form a continuum in which heterotrophic communities dominate in shallow waters and autotrophic communities dominate at deep-sea sites.

Although the search for, and discovery of, shallow-water vents and seep symbioses did not begin until the 1990s, shallow-water coastal sediments with high sulphide concentrations were some of the first habitats in which chemosynthetic symbioses were searched for after the discovery of deep-sea vent symbioses. Reid and Bernard[19], inspired by the description of the large gutless tube worms at vents, but unaware of their association with chemosynthetic bacteria, described the gutless condition of a *Solemya* clam, found in pulp-mill effluents. Shortly afterwards, chemoautotrophic, sulphur-oxidizing symbionts were discovered in *Solemya velum*, which lives in sulphide-rich eel-grass sediments, and *Solemya reidi*, which lives in sulphidic sewage-outfall sediments (reviewed in REF. 4). Other sulphide-rich habitats in which chemosynthetic symbioses have been found include mangrove muds[20–22] and sediments in upwelling regions[23].

What is not commonly known is that in some shallow-water environments with extremely low sulphide concentrations (<5 μM), the diversity of chemosynthetic symbioses can be as high as, or even higher than, at vents and seeps (Fig. 1). For example, in coarse-grained sediments that surround sea-grass beds off the island of Elba in the Mediterranean, chemosynthetic symbionts occur in or on ciliates, turbellarian *Paracatenula*-like worms, three species of gutless oligochaetes[24] and several nematode species (stilbonematinids and *Astomonema*) (J. Ott, C.L. and N.D., unpublished observations). These animals are small and are often only known to meiofauna specialists. Other low-sulphide habitats with a high

◀ Figure 1 | **Chemosynthetic symbioses in different marine habitats.** Chemosynthetic symbioses occur in a wide range of marine habitats, including shallow-water sediments (**a**), continental slope sediments (**b**), cold seeps (**c**), whale and wood falls (**d**), and hydrothermal vents (**e**). Some host groups are found in only one habitat (such as *Osedax* on whale bones), whereas others occur in several different environments (such as thyasirid clams, which are found in shallow-water sea-grass sediments and in the deep sea at cold seeps, whale falls and hydrothermal vents). The animals are not drawn to scale; for example, *Idas* and *Adipicola* mussels are much smaller than *Bathymodiolus* mussels.

Table 1 | Marine chemosynthetic symbioses

Phylum or major group	Subgroups*	Host‡	Common name	Symbiont location	Habitat§	Symbiont type‖	Refs#
Ciliophora	Oligohymenophora Peritrichida	*Zoothamnium*	Colonial ciliate	Epibiotic; cell surface	Shallow water	Sulphur-oxidizing symbiont	22
Ciliophora	Polyhymenophora Heterotrichida	*Folliculinopsis*	Blue-mat ciliate	Epibiotic and endobiotic; cell surface and cytoplasm	Vents	Unknown	41
Ciliophora	Karyorelictea Kentrophoridae	*Kentrophoros*	Free-living ciliate	Epibiotic and endobiotic; cell surface and cytoplasm	Shallow water	Unknown	42,126
Porifera	Demospongiae Cladorhizidae	*Cladorhiza*	Sponge	Intracellular and extracellular	Seeps	Methane-oxidizing symbiont	127
Platyhelminthes	Catenulida Retronectidae	*Paracatenula*	Mouthless flat worm	Intracellular; trophosome	Shallow water	Sulphur-oxidizing symbiont	128,129
Nematoda	Desmodorida Stilbonematinae	*Stilbonema Laxus*	Nematode	Epibiotic	Shallow water	Chemoautotrophic symbiont	26,130
Nematoda	Monhysterida Siphonolaimidae	*Astomonema*	Mouthless nematode	Endosymbiont; gut lumen	Shallow water	Sulphur-oxidizing symbiont	26, 27,130
Mollusca	Aplacophora Simrothiellidae	*Helicoradomenia*	Worm mollusc	Epibiotic and endocuticular; sclerites and mantle cavity	Vents	Unknown	131
Mollusca	Bivalvia Solemyidae	*Solemya Acharax*	Awning clam	Intracellular; gill	Vents, seeps, wood falls and shallow water	Sulphur-oxidizing symbiont	17,19, 28
Mollusca	Bivalvia Lucinidae	*Lucina Codakia*	Clam	Intracellular; gill	Vents, seeps and shallow water	Sulphur-oxidizing symbiont	20,29, 30,132
Mollusca	Bivalvia Thyasiridae	*Thyasira Maorithyas*	Clam	Extracellular; gill Intracellular; gill	Vents, seeps, whale falls and shallow water	Sulphur-oxidizing symbiont	36,39, 133
Mollusca	Bivalvia Vesicomyidae	*Calyptogena Vesicomya*	Clam	Intracellular; gill	Vents, seeps and whale falls	Sulphur-oxidizing symbiont	61,62, 120,121
Mollusca	Bivalvia Mytilidae	*Bathymodiolus Idas*	Mussel	Intracellular and extracellular; gill	Vents, seeps, whale falls and wood falls	Sulphur-oxidizing and methane-oxidizing symbionts	17,47, 48,51, 81
Mollusca	Gastropoda Provannidae	*Alviniconcha Ifremeria*	Snail	Intracellular; gill	Vents	Sulphur-oxidizing and methane-oxidizing symbionts	72,73, 75,82, 134
Mollusca	Gastropoda Lepetodrilinae	*Lepetodrilus*	Limpet	Epibiotic; gill	Vents	Chemoautrophic symbiont¶	135
Mollusca	Gastropoda Peltospiridae	Not named yet	Scaly foot snail	Intracellular; oesophageal gland	Vents	Unknown	136
Annelida	Polychaeta Terebellida	*Alvinella*	Pompeii worm	Epibiont; integument	Vents	Chemoautrophic symbiont¶	46,70, 71
Annelida	Polychaeta Vestimentifera	*Riftia Lamellibrachia Escarpia*	Tube worm	Intracellular; trophosome	Vents, seeps, whale falls and wood falls	Sulphur-oxidizing symbiont	46,53, 91,93, 137,138
Annelida	Polychaeta Monilifera	*Sclerolinum*	Tube worm	Intracellular; trophosome	Vents, seeps and wood falls	Sulphur-oxidizing symbiont	46,77, 78,137
Annelida	Polychaeta Frenulata	*Siboglinum Oligobrachia*	Beard worm	Intracellular; trophosome	Vents, seeps, wood falls and shallow water	Sulphur-oxidizing and methane-oxidizing symbionts	46,78, 137,139, 140
Annelida	Polychaeta *incertae sedis*	*Osedax***	Bone-eating worm	Intracellular; root (ovisac)	Whale falls	Heterotroph	15,54, 55
Annelida	Clitellata Phallodrilinae	*Inanidrilus Olavius*	Gutless oligochaete	Extracellular; subcuticular	Shallow water	Sulphur-oxidizing and sulphate-reducing symbionts	24,25, 40,90
Annelida	Clitellata Tubificinae	*Tubificoides*	Sludge worm	Epibiotic	Shallow water	Sulphur-oxidizing symbiont	46
Arthropoda	Decapoda Alvinocarididae	*Rimicaris*	Hydrothermal vent shrimp	Epibiotic; gill chamber	Vents	Chemoautrophic symbiont¶	44,45
Arthropoda	Decapoda Galatheoidea	*Kiwa*	Yeti crab	Epibiotic; setae	Vents	Unknown	141

*The orders and families to which chemosynthetic hosts belong are still under debate, and therefore the non-taxonomic term subgroup is used here. ‡An example of one or more genera is listed. §Shallow water includes all marine habitats less than 200 metres deep. ‖Based on enzymatic, molecular or stable-isotope data. ¶Function inferred from phylogenetic data. #If possible, recent literature was chosen. **_Osedax_ hosts are included here, even though they have heterotrophic symbionts, because they are closely related to tube worms with chemosynthetic symbionts. For additional literature, see Cavanaugh and colleagues[4].

diversity of chemosynthetic hosts include coral-reef sediments, in which gutless oligochaetes[25], nematodes[26,27] and many symbiotic bivalves, such as solemyid[28], lucinid and thyasirid clams[29,30], are regularly found. Both the Elba sediments and coral-reef sands have a high porosity, which results in high inputs of oxygen and organic carbon from the water column to the sediment. Thus, sulphate-reduction rates can be high in these sediments, but sulphide concentrations remain low in the upper sediment layers because of the unusually deep penetration of oxygen[31,32]. For the symbiotic associations, a constant supply of sulphide (sulphide flux) might be more important than the absolute concentration of sulphide.

Host diversity

A remarkable number of animals have established symbioses with chemosynthetic symbionts (TABLE 1). Given that only a small percentage of the deep sea has been explored, many more vents, seeps, and whale and wood falls remain to be discovered, and correspondingly, the number of chemosynthetic host species will increase with time[33]. In addition to discovery-based research, the use of whale[16,17] and cow bones[34], as well as large pieces of wood[35], for colonization experiments can allow the discovery of new chemosynthetic host species. In shallow-water habitats, the diversity of host species has been well described in some groups; for example, in the lucinids[30], thyasirids[36] and gutless oligochaetes[37]. However, in these host groups, the symbiotic associations of only a few species have been investigated in detail[38–40], whereas the symbionts of hundreds of chemosynthetic host species remain to be described. Another host group that has been largely overlooked is the Protozoa, for which only a few symbioses have been described[21,22,41,42]. Given their high abundance in many sulphide-rich habitats[43], these hosts are good candidates for chemosynthetic symbioses.

Morphological diversity. The morphological diversity of chemosynthetic associations is also high, which reflects the adaptive flexibility of both the animals and the microorganisms in these associations (FIGS 2–5; TABLE 1). Epibionts can be attached to a specific part of an animal, such as in the vent shrimp, in which they occur mainly on the mouth appendages and in the gill chamber[44,45], or can cover almost the entire surface of the animal, such as in nematodes[26]. Endobionts can be extracellular, such as in gutless oligochaetes, in which they occur just below the cuticle of the body wall[40], or intracellular, such as in many bivalves[4] and siboglinid tube worms[46]. Within many host groups, the episymbiotic or endosymbiotic location is consistent in all species, but within some host groups, associations can range from episymbiotic to endosymbiotic between species. For example, in thyasirid and bathymodiolin bivalves the symbionts are always associated with the gills in adult specimens, but their location can vary between species. In thyasirid clams, the symbionts are extracellular in all investigated host species, with the exception of *Maorithyas hadalis*, which has intracellular symbionts (reviewed in REF. 36). In bathymodiolin mussels, most if not all members of the genus *Bathymodiolus* have intracellular endosymbionts, whereas in some *Idas* and *Adipicola* species, the symbionts are epibiotic and are attached to the outside of the gill cells[47,48]. It has been suggested that episymbiosis represents a more primitive evolutionary stage than endosymbiosis, and indeed some studies indicate that at least *Idas* spp. form an ancestral group within the bathymodiolin mussels[9,49,50]. However, our unpublished analyses of bathymodiolin phylogeny, as well as those of Duperron *et al.*[51], show that *Idas* and *Adipicola* species are no more primitive than bathymodiolin hosts with endosymbionts. Furthermore, our analyses of 16S ribosomal RNA (rRNA) symbiont phylogeny show that bathymodiolin episymbionts are not ancestral to bathymodiolin endosymbionts, but instead fall randomly between endosymbiotic lineages (FIG. 4). This suggests that the morphological location of a symbiont is not always a conserved trait and that both the host and the symbiotic bacteria are more flexible in their ability to establish episymbiotic and endosymbiotic associations than previously assumed.

Another example of the morphological plasticity of chemosynthetic associations is siboglinid worms. In the three siboglinid tube worm groups Monilifera, Frenulata and Vestimentifera, the bacteria are housed in the interior of the worm in an organ called the trophosome[46,52]. In vestimentiferans (for example, *Riftia*, *Lamellibrachia* and *Escarpia*), the trophosome is massive, extends throughout the entire trunk region and is densely packed with bacteria (in *R. pachyptila*, the symbionts account for at least 25% of the trophosome volume[46]). By contrast, in moniliferans (*Sclerolinum*) and frenulates (for example, *Oligobrachia* and *Siboglinum*), the trophosome is small, only occupies the posterior region of the trunk and contains few bacteria (the symbionts make up less than 1% of the total worm volume[46]). Intriguingly, the origin of the trophosomal tissue might differ between tube worm groups: Nussbaumer *et al.*[53] showed that the vestimentiferan trophosome develops from mesodermal tissue, rather than from endodermal gut tissue as previously assumed. In frenulates and moniliferans, the simple two-layered structure of the trophosome suggests that it originated from endodermal gut tissue, although this has not been proven. If this is the case, the symbiont-housing trophosome might have evolved from two different tissues by convergent evolution. In the fourth group of siboglinid worms, the whale-bone inhabitants of the genus *Osedax*, yet another strategy for housing the symbionts has evolved. They are located in elaborate posterior roots that invade the whale bones[54], and remarkably, the morphological origin of these roots is the egg-containing tissues (ovisac) of *Osedax*[55].

Behavioural and physiological strategies. In addition to morphological diversity, the behavioural and physiological strategies used by animals to supply their symbionts with both reductants and oxidants vary even within closely related host groups. For the vent tube worms, both sulphide and oxygen are present in the fluids that surround their anterior ends, and the animals use their gill-like branchial plumes to obtain both reductants and oxidants. The plumes are packed with haemoglobin-containing blood vessels and thus are bright red. These

Epibiont
A symbiont that lives on the surface of its host.

Endobiont
A symbiont that lives inside its host.

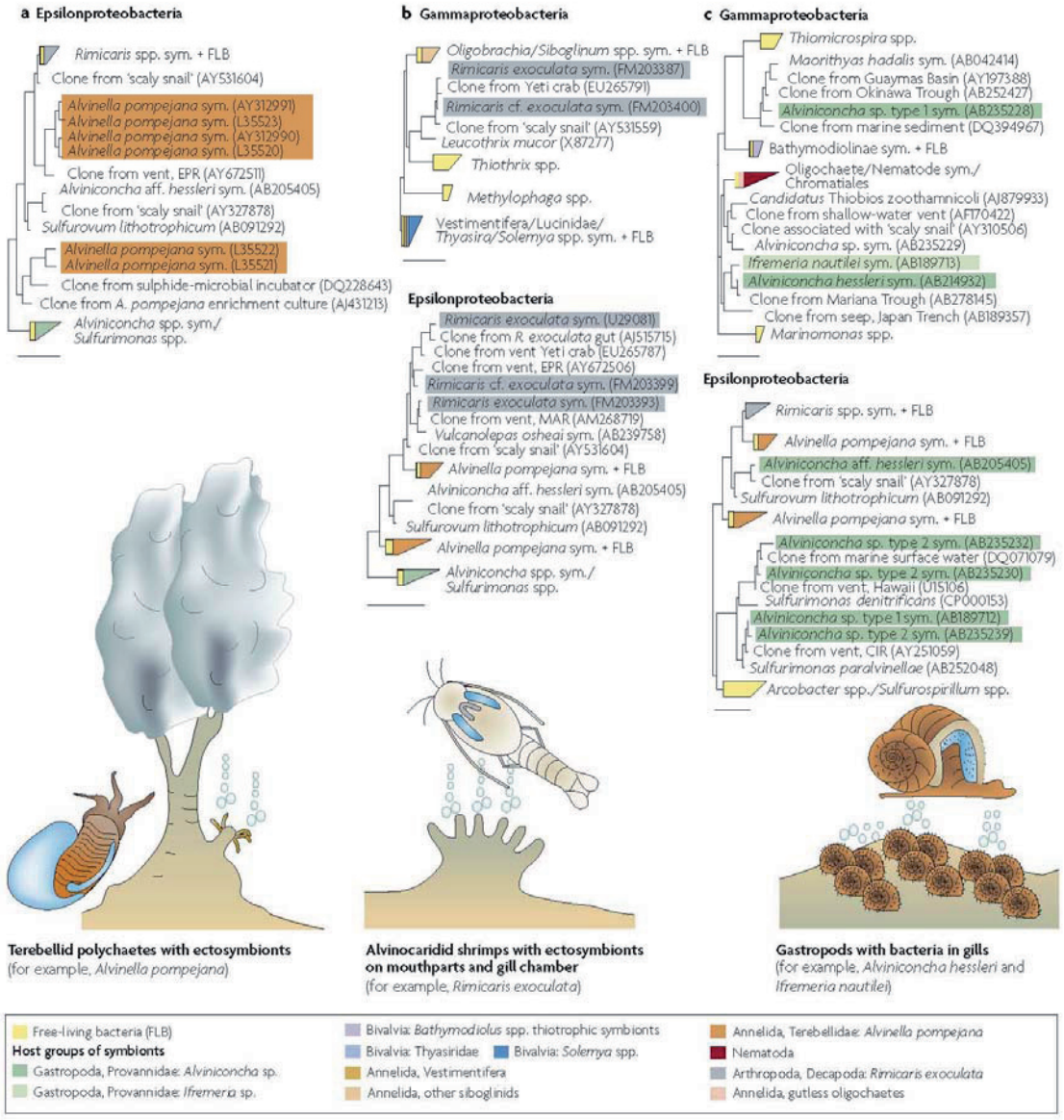


Figure 2 | **Symbioses found at deep-sea hydrothermal vents.** Symbiotic bacteria colonize different parts of the host body (coloured blue). Ectosymbionts are found on the dorsal surface of the polychaete worm *Alvinella* (**a**) and on the mouthparts and gill chamber of the vent shrimp *Rimicaris* (**b**). Endosymbionts occur intracellularly in the gill tissues of gastropod snails (**c**). Symbiont phylogeny is based on maximum likelihood analyses of 16S ribosomal RNA (rRNA) gene sequences. Symbionts from the same host group are shown in the same colour, and free-living bacteria (FLB) are shown in yellow. One representative sequence is shown if several sequences of the same host species are highly similar (for example, *I. nautilei* or *R. exoculata*). Sequences of almost full length were chosen from the ARB or SILVA database[124] (release 94; March 2008), aligned and analysed with the ARB software package. The scale bar indicates 10% estimated sequence divergence. Numbers in brackets are the accession numbers of the 16S rRNA sequences. For more details of the biology and biogeography of vent animals see REF. 125. CIR, Central Indian Ridge; EPR, East Pacific Rise; MAR, Mid-Atlantic Ridge, sym., symbionts.

Box 1 | **Methodological challenges of characterizing symbiotic bacteria**

Although many have tried, it has not yet been possible to culture a chemosynthetic symbiont (as unsuccessful experiments are rarely published, there is no definite information about cultivation attempts). It is astonishing that chemosynthetic symbionts, particularly those that are epibiotic or known to occur in a free-living stage, such as the symbionts of lucinid clams[100] or the hydrothermal-vent tube worm *Riftia pachyptila*[53,92] have not yet been isolated into pure culture. The identification of genes for heterotrophic metabolism in the *R. pachyptila* symbiont led Robidart and colleagues[91] to suggest the use of an organic carbon source instead of an inorganic one in the cultivation medium. Alternatively, enrichment cultures with minimal amounts of host tissue or co-occurring symbionts, or long-term incubation experiments, such as those used to characterize the metabolism of microbial consortia[112], would be valuable for characterizing chemosynthetic symbionts, but have not yet been described.

Revealing the true diversity of a microbial community is a challenge, even in low-diversity ecosystems, such as symbiotic associations[113]. Only in the last few years has the previously unrecognized diversity of symbionts been discovered in chemosynthetic hosts, largely because of methodological progress. Sequencing has become less costly and more clones and individuals can be examined. Furthermore, methods for decreasing PCR bias and artefacts have led to a better and more even representation of phylotypes in clone libraries[114,115], and enhanced fluorescence *in situ* hybridization (FISH) techniques, such as catalysed reported deposition (CARD)–FISH, have recently improved the ability to detect members of a community that are in low abundance[116]. Finally, novel sequencing strategies, such as massively parallel DNA pyrosequencing[117], now allow more comprehensive analyses of highly variable genetic markers from large numbers of individuals and a wide range of habitats and geographical locations. These are needed to distinguish between artefactual variability caused by patchy sampling and true diversity that is derived from host specificity, biogeography or environmental factors.

haemoglobins can bind and transport oxygen, sulphide and nitrate to the symbionts in the highly vascularized trophosome[56–58]. At cold seeps, oxidants and reductants are more spatially separated than at vents: oxygen is present in the surrounding sea-water at the anterior end of the worms, whereas sulphide remains mostly in the sediment, where the worm sits, and must be obtained through its posterior end. Seep vestimentiferans have extended roots with which they can not only gain sulphide from deep sediment layers, but can also release sulphate to enhance sulphide production around their roots[59]. Animals that can move easily, such as small oligochaete and nematode worms, bridge the physical gap between oxidants and reductants by migrating between the upper oxidized and lower reduced sediment layers[40,60]. In bivalves, some clams, such as the vesicomyids, use their foot, which is well supplied with blood, to dig for sulphide in the sediment or in vent cracks, while their siphon lies in the oxygenated sea-water[61]. Their blood stores and transports oxygen that is bound to haemoglobin, as well as sulphide that is bound to a specific protein in the serum, to the symbionts in the gill tissues[62]. Other clams, such as *Solemya* spp., build Y-shaped burrows to access sulphide from below and oxygen from above[4], and their gills contain intracellular haemoglobins that can bind oxygen and sulphide[63]. Bathymodiolin mussels lack specific proteins in their blood that can bind oxygen, sulphide or methane, and are therefore dependent on the diffusion of dissolved gasses from sea water into their gills to take up reductants and oxidants. Mussel beds, however, can disperse the hydrothermal fluids that diffuse from narrow fissures laterally for distances of several metres, resulting in a large increase in the areas in which both dissolved oxygen and hydrogen sulphide are available[64]. Thyasirid clams use their foot to form burrows, and interestingly, the length and number of burrows that are formed by different species is related to the concentration of hydrogen sulphide in the sediment[65]. The thyasirid foot can extend up to 30 times the length of the shell[65], an extreme example of a morphological adaptation of an animal to a symbiosis.

Symbiont diversity

Until recently, the diversity of chemosynthetic symbionts was considerably underestimated. Molecular tools for investigating microbial diversity (BOX 1) were still in their infancy in the 1980s when the first chemosynthetic symbioses were discovered. The first rRNA sequences from the sulphur-oxidizing endosymbionts of *R. pachyptila*, *Calyptogena magnifica*, *Bathymodiolus thermophilus* and lucinid clams were gained through laborious RNA extraction and reverse transcription sequencing of 5S[66] and 16S rRNA[67] in the mid-to-late 1980s. Only a single 16S rRNA symbiont phylotype was found in each host species, indicating a high degree of specificity between host and symbiont[67]. Modern DNA-sequencing technology has led to a dramatic increase in the number of 16S rRNA sequences from free-living and symbiotic microorganisms. More than 100 16S rRNA sequences from sulphur-oxidizing symbionts are now available in the databases, although the ability to oxidize reduced sulphur compounds has only been shown for a few of these symbionts and for most has been inferred from indirect evidence.

Most sulphur-oxidizing symbionts belong to the Gammaproteobacteria (FIG. 6). Previous phylogenetic analyses clustered gammaproteobacterial sulphur-oxidizing symbionts into only a few clades, in which few sequences from free-living bacteria were found[4,68]. Our most recent phylogenetic analyses of these symbionts revealed at least nine phylogenetically distinct clades, most of which were interspersed with sequences from free-living bacteria (FIG. 6). This indicates that sulphur-oxidizing symbioses have evolved on multiple occasions and did so independently of each other, from many different groups of bacteria. For example, nematode symbionts and the Gamma 1 symbionts of gutless oligochaetes evolved from a common ancestor that is shared with anoxygenic, phototrophic sulphur oxidizers, such as *Thiococcus pfennigii* and *Allochromatium vinosum*, whereas the symbionts of the vent snail *Alviniconcha* sp. and the seep mussel *Maorithyas hadalis* share a common ancestor with free-living sulphur oxidizers of the genus *Thiomicrospira* (FIG. 6). It is intriguing that the members of all three host groups with symbionts that are related to phototrophic sulphur oxidizers — oligochaetes, stilbonematinid nematodes and *Astomonema* species — occur in shallow waters in which these phototrophic bacteria are widespread.

It has recently been suggested that some chemosynthetic symbionts have descended from pathogenic ancestors[68]. Our analyses showed that the closest relatives of chemosynthetic symbionts were non-pathogenic bacteria or free-living bacteria from marine environments (FIGS 2–6). To our knowledge, all known bacterial

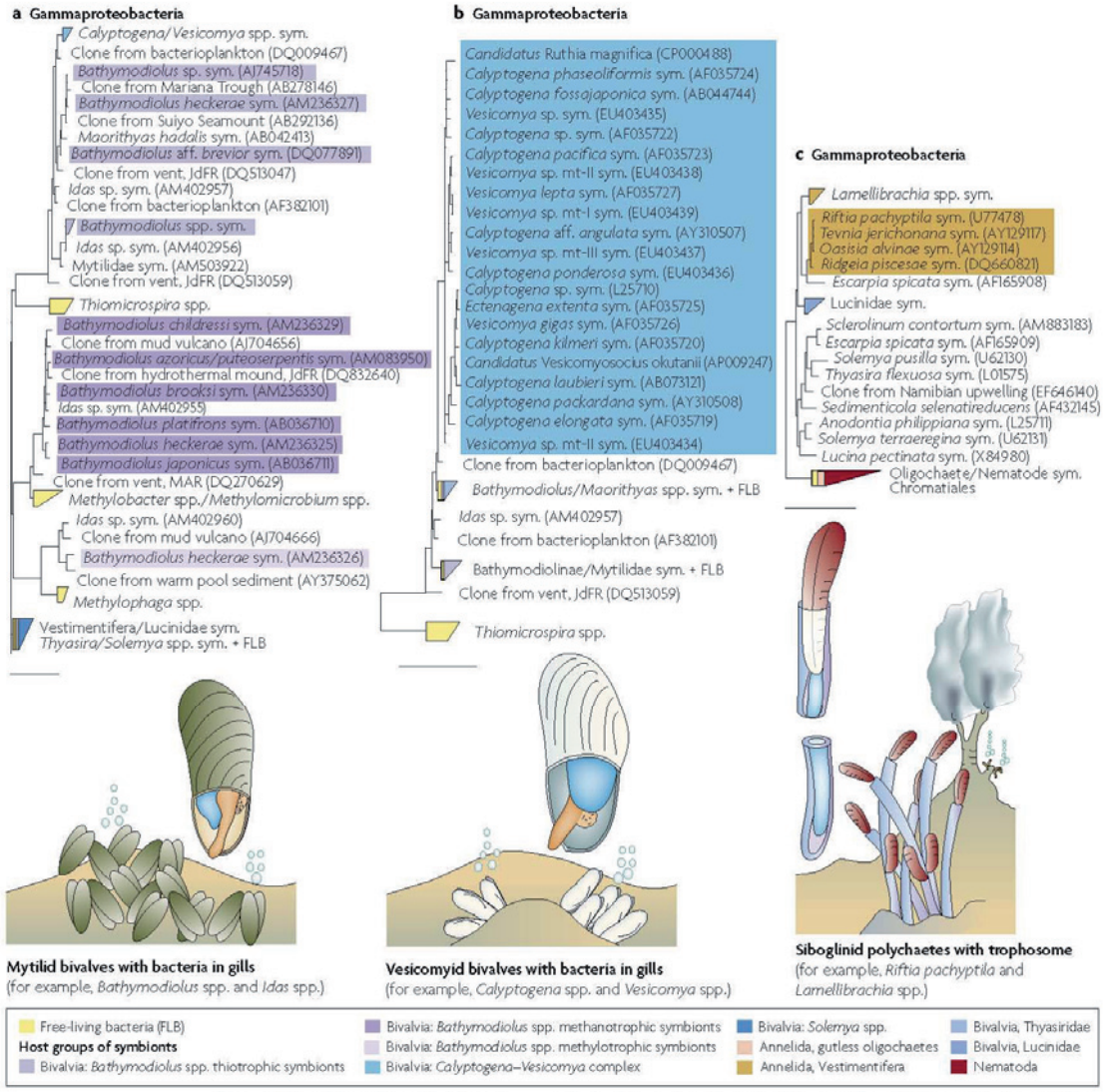


Figure 3 | **Symbioses found both at deep-sea hydrothermal vents and cold seeps.** The gill tissues of bathymodiolin mussels (**a**) and vesicomyid clams (**b**), as well as the trophosomes of siboglinid tube worms and beard worms (**c**), are colonized by endosymbionts (symbiont-containing tissues are coloured blue). Symbiont phylogeny is based on maximum likelihood analyses of 16S ribosomal RNA (rRNA) gene sequences. Symbionts from the same host group are shown in the same colour, and free-living bacteria (FLB) are shown in yellow. Sequences of almost full length were chosen from the ARB or SILVA database[124] (release 94; March 2008), aligned and analysed with the ARB software package. The scale bar indicates 10% estimated sequence divergence. Numbers in brackets are the accession numbers of the 16S rRNA sequences. For more details of the biology and biogeography of vent animals see REF. 125. JdFR, Juan de Fuca Ridge; MAR, Mid-Atlantic Ridge, sym., symbionts.

pathogens from animals are heterotrophs. The hypothesis of a pathogenic ancestor would therefore imply that the heterotrophic pathogen acquired the ability to gain energy from sulphide or methane after the establishment of the symbiosis. A more parsimonious explanation is that the ancestors of chemosynthetic symbionts gained energy from chemoautotrophy or methanotrophy before the establishment of the symbiosis. This hypothesis is supported by our analyses, which show that many of the closest relatives of chemosynthetic symbionts are

free-living sulphur or methane oxidizers, or are found in chemosynthetic habitats (FIGS 2–6).

Some sulphur-oxidizing symbionts, such as those from the vent shrimp *Rimicaris exoculata*[69], the Pompeii worm *Alvinella*[70,71] and some *Alviniconcha* species[72,73], belong to the Epsilonproteobacteria (FIG. 2). As for many gammaproteobacterial symbionts, there is little direct evidence that these epsilonproteobacteria use reduced sulphur compounds as an energy source.

A few years after the first molecular identification of thiotrophic symbionts, the first 16S rRNA sequence of a methanotrophic symbiont from the seep mussel *Bathymodiolus childressi* was published[74]. All currently published sequences from methanotrophic symbionts cluster in a single clade within the Gammaproteobacteria, with free-living methane oxidizers of the genera *Methylobacter* and *Methylomicrobium* as their sister group (FIGS 3,6). To date, all published methanotrophic symbiont sequences are from bathymodiolin mussels (FIG. 3). However, morphological and enzymatic data, as well as stable-isotope analyses, indicate that other hosts, such as the vent snail *Ifremeria nautilei*[75], and siboglinid tube worms, such as *Siboglinum poseidoni*[76] and *Sclerolinum contortum*[77], also have methanotrophic symbionts. Our 16S rRNA sequence analyses and fluorescence *in situ* hybridization (FISH) confirm that *I. nautilei* has a methanotrophic symbiont (discussed below). However, our molecular analyses of the symbionts in *S. contortum* indicate that this host only harbours sulphur-oxidizing symbionts[78].

Multiple co-occurring symbionts. The first host species in which more than one endosymbiont was found were *Bathymodiolus* mussels from cold seeps in the Gulf of Mexico and vents on the Mid-Atlantic Ridge: morphological and enzymatic data[4,79], as well as comparative 16S rRNA sequence analysis and FISH[80], showed that sulphur-oxidizing and methane-oxidizing symbionts co-occur within the same cells of the mussel's gills. Dual symbioses with thiotrophic and methanotrophic symbionts have now been described in five *Bathymodiolus* species from cold seeps in the Gulf of Mexico, as well as vents and seeps in the Atlantic[4,81]. The only other host in which thiotrophic and methanotrophic symbionts are known to coexist (based on morphological and stable-isotope analyses) is the provannid snail *I. nautilei* from vents in the West Pacific (reviewed in REF. 75). Only 16S rRNA sequences from thiotrophic symbionts of *Ifremeria* have been published[72,82] (FIG. 2), although our analyses show that thiotrophic and methanotrophic symbionts co-occur in *I. nautilei* from vents in the North-Fiji back arc basin (C. Borowski, H. Urakawa and N.D., unpublished observations).

Progress in the molecular techniques that have been used to detect microbial diversity (BOX 1) has led to the realization that more than two endosymbionts can co-occur in both deep-sea and shallow-water hosts. *Bathymodiolus heckerae*, a mussel from cold seeps in the Gulf of Mexico, was previously assumed to have only two symbionts, based on morphological and physiological studies[4]. Our studies show that this host has four co-occurring symbionts in its gills (FIG. 3): two phylogenetically distinct thiotrophs (only one of these is shown in FIG. 3), one methanotroph and a novel methylotroph-related phylotype[83]. The cold-seep mussel *Idas* sp. has six co-occurring bacterial symbionts[47]: four are from the same phylogenetic groups as the *B. heckerae* symbionts (FIG. 3), but two have not yet been found in chemosynthetic hosts (one phylotype is from the Bacteroidetes and the other is from a novel gammaproteobacterial lineage). The discovery of this unexpected symbiont diversity has implications for the study of biogeography and co-speciation patterns in bathymodiolin mussels, which can only be correctly interpreted if the full diversity of symbionts is known.

Gutless oligochaetes are also host to multiple co-occurring symbionts (FIG. 5). Two to three bacterial morphotypes were identified in ultrastructural analyses[46], but our studies show that these hosts can harbour as many as six co-occurring symbionts that belong to diverse bacterial groups, including the Gammaproteobacteria, Deltaproteobacteria and Alphaproteobacteria, as well as the *Spirochaeta*[23–25,40]. The high morphological diversity of the epibiotic bacterial symbionts of alvinellid worms[70] and *Rimicaris* shrimp[44,45] indicates that their phylogenetic diversity is probably much higher than the few phylotypes currently described from these hosts[69,71,84]. In fact, our analyses show that in addition to the described epsilonproteobacterial ectosymbiont of *R. exoculata*, these vent shrimp are densely colonized by a gammaproteobacterial ectosymbiont (J. M. Struck and N.D., unpublished observations) (FIG. 2).

Diversity at the strain level. Analyses of more variable genetic markers than 16S RNA, such as the internal transcribed spacer (ITS) region or DNA fingerprinting techniques can provide a higher degree of resolution and reveal diversity at the strain or substrain level. This is crucial for a better understanding of the roles of host specificity, the environment and geography in symbiont diversity. For example, 16S rRNA analyses show that the symbionts of some hosts, such as those of vestimentiferan tube worms[85,86], some lucinid clams[38] and *Bathymodiolus* mussels from vents on the northern Mid-Atlantic Ridge[81] are promiscuous and can be shared between related host species. An analysis of the ITS sequences of symbionts from eight vestimentiferan host species from different vents and seep sites showed that these were highly similar, but fingerprinting analyses indicated a high degree of variability, and host specificity was detected at the sub-strain level in most species[87]. By contrast, the strain variability (based on ITS analyses) of the sulphur-oxidizing symbionts of *Bathymodiolus* mussels from the northern Mid-Atlantic Ridge[88] does not seem to be related to host specificity, but rather to the geographical location of the symbionts[89].

Metabolic diversity. Just as molecular analyses have led to the discovery of unrecognized phylogenetic diversity, genomic and proteomic analyses are beginning to reveal

Methanotrophic
An organism that uses methane as an energy and carbon source is called a methanotroph or methane oxidizer.

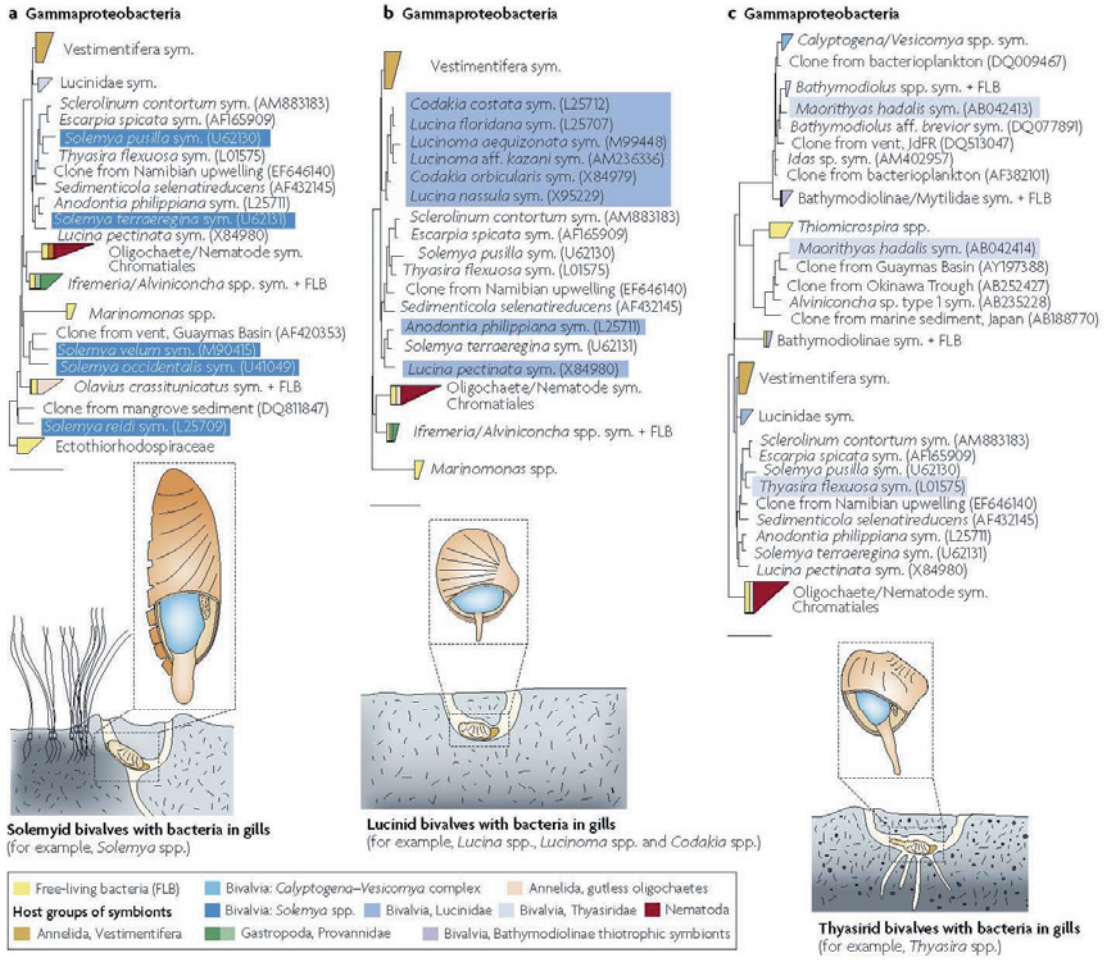


Figure 4 | **Symbioses in bivalves from shallow-water habitats.** The symbionts are intracellular in the gills of solemyid (**a**) and lucinid clams (**b**). All thyasirid clams (**c**) have extracellular symbionts that occur between microvilli of epithelial cells, except for *Maorithyas hadalis*, which has intracellular symbionts. The symbiotic bacteria of bivalves always occur on or in their gill tissues (coloured blue). Symbiont phylogeny is based on maximum likelihood analyses of 16S ribosomal RNA (rRNA) gene sequences. Symbionts from the same host group are shown in the same colour, and free-living bacteria (FLB) are shown in yellow. Sequences of almost full length were chosen from the ARB or SILVA database[124] (release 94; March 2008), aligned and analysed with the ARB software package. The scale bar indicates 10% estimated sequence divergence. Numbers in brackets are the accession numbers of the 16S rRNA sequences. JdFR, Juan de Fuca Ridge; sym., symbionts.

Syntrophy
Strictly defined, syntrophy describes a nutritional relationship between two organisms that combine their metabolic capabilities to use a substrate that neither could use alone. In this Review, we use syntrophy loosely to describe the beneficial exchange of products between two or more organisms.

the metabolic diversity of chemosynthetic symbionts. The first genomes to be sequenced from chemosynthetic symbionts were from a metagenomic analysis of the four co-occurring symbionts of the gutless oligochaete *Olavius algarvensis*[90]. These analyses showed that two of the symbionts are sulphur oxidizers and two are sulphate reducers, and that these symbionts are engaged in a mutually beneficial syntrophy that involves the exchange of reduced and oxidized sulphur compounds. The two sulphur-oxidizing symbionts have the potential to fix inorganic carbon autotrophically, and unexpectedly, the two sulphate-reducing symbionts also have genes for the autotrophic fixation of carbon dioxide. Thus, these four symbionts can provide their host with multiple sources of carbon. Results from our proteomic analyses of the *O. algarvensis* association confirm that the symbionts use many of the autotrophic pathways that we predicted based on the metagenome (M. Kleiner, N. C. Verberkmoes, H. Teeling, M. Hecker, T. Schweder and N.D., unpublished observations).

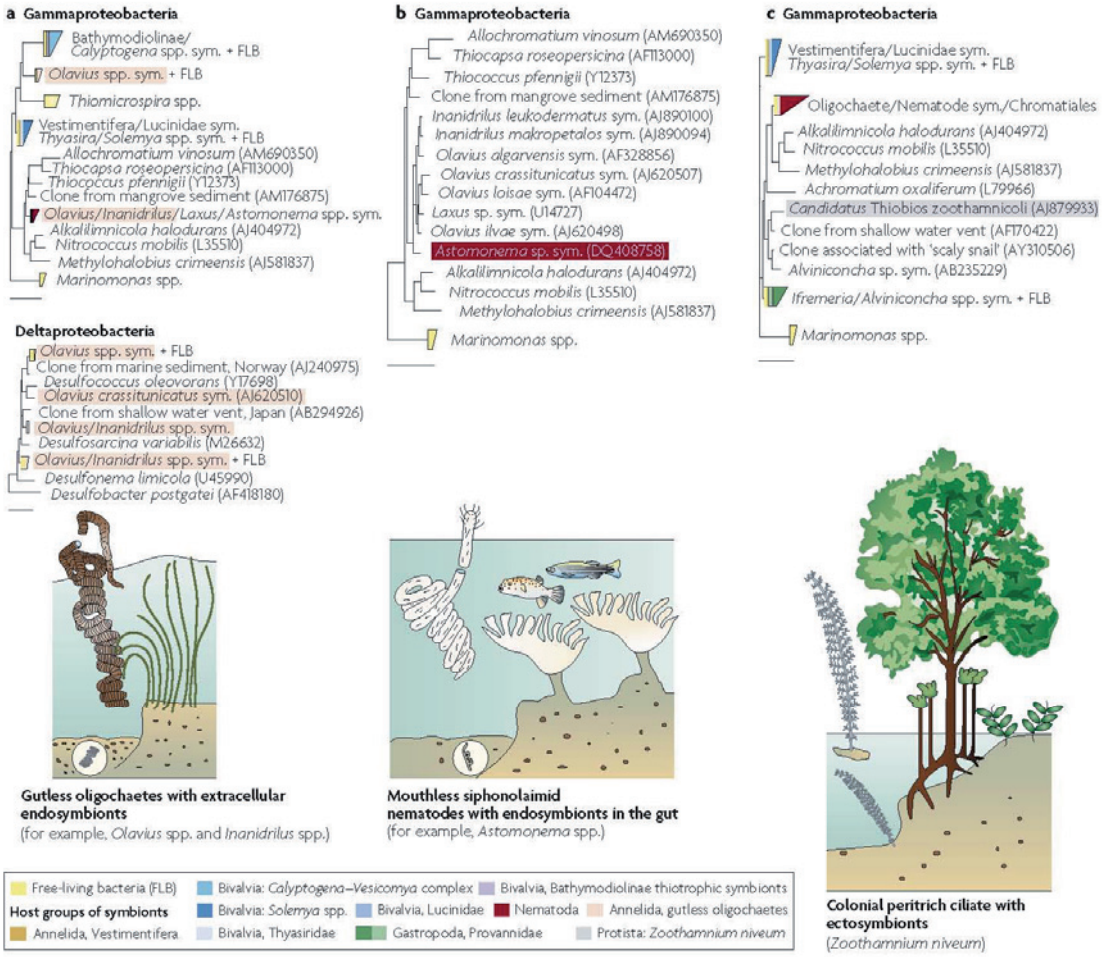


Figure 5 | **Symbioses in worms and protists from shallow-water habitats.** In the gutless oligochaetes *Olavius* and *Inanidrilus* (**a**), the bacteria are endosymbiotic (but not intracellular), and occur just below the cuticle in an extracellular space above the epidermal cells, whereas in the nematode *Astomonema* (**b**) they occupy the entire gut lumen. In the colony-forming ciliate *Zoothamnium niveum* (**c**), the ectosymbionts cover the entire surface of the colony. The symbiont-containing regions of each host group are coloured blue. Symbiont phylogeny is based on maximum likelihood analyses of 16S ribosomal RNA (rRNA) gene sequences. Symbionts from the same host group are shown in the same colour, and free-living bacteria (FLB) are shown in yellow. Sequences of almost full length were chosen from the ARB or SILVA database[124] (release 94; March 2008), aligned and analysed with the ARB software package. The scale bar indicates 10% estimated sequence divergence. Numbers in brackets are the accession numbers of the 16S rRNA sequences. Sym., symbionts.

Genomic analyses of the *R. pachyptila* symbiont revealed that as well as possessing the genes that are needed for chemoautotrophic sulphur oxidation, this symbiont can also live heterotrophically. Heterotrophy might be the preferred mode of nutrition during the free-living stage of the *R. pachyptila* symbiont[91] and would explain why it is found at off-axis sites where there is no apparent sulphide[92]. The proteome of the *R. pachyptila* symbiont is the first, and to date only, chemosynthetic proteome to be described[93]. In addition to the known pathway of inorganic carbon fixation through the Calvin cycle, Markert and colleagues[93] discovered that *R. pachyptila* also uses the reductive tricarboxylic acid cycle (rTCA) to fix inorganic carbon. The rTCA cycle requires less energy than the Calvin cycle, and correspondingly, symbionts with depleted energy sources owing to low sulphur contents expressed more of the proteins that are involved in the rTCA cycle than

Box 2 | Symbiont transmission

The term transmission is used to describe how hosts acquire their symbionts. Differences in transmission have a major effect on symbiont diversity, phylogeny and evolution. Two strategies for symbiont transmission are distinguished. In vertical transmission, the symbionts are passed from one generation to the next, through the direct transmission of symbionts from the parent to the egg or embryo. In horizontal transmission, symbiont transmission is independent of host reproduction, and the symbionts are either taken up from the environment or from co-occurring hosts. Obligate symbionts that have been transmitted vertically for long evolutionary time periods have reduced genomes compared with their free-living relatives[94,110]. During vertical transmission, only a limited number of symbionts are passed from one generation to the next. These population bottlenecks lead to an increase in genetic drift that results in increased rates of neutral and deleterious mutations[110]. The lack of effective natural selection for purging these mutations results in gene loss. Reductive genome evolution does not depend on an intracellular location of the symbiont and can also occur in symbionts that are extracellular[110].

Vertical transmission has been proposed for three groups of chemosynthetic hosts based on morphological observations: gutless oligochaetes (reviewed in REF. 40), and solemyid and vesicomyid clams (reviewed in REF. 4). The recently sequenced genomes of the intracellular symbionts of two vesicomyid clams are considerably reduced (1.0 – 1.2 Mb) and are the smallest known genomes from autotrophic bacteria[120,121]. Genes for autotrophy and sulphur metabolism are present, but genes for DNA recombination and repair are lacking, as in other vertically transmitted intracellular symbionts that have undergone genome reduction[122]. The genomes of the extracellular oligochaete symbionts are not reduced (~4.7–13.6 Mb), but exceptionally high numbers of transposable elements in the Gamma 1 and Delta 1 symbionts[90] suggest that these are vertically transmitted and in an early stage of genome reduction[123].

those with higher sulphur contents[93]. This study shows the power of proteomics for gaining insights into the metabolic pathways that are used by symbionts to adapt to different environmental conditions.

Explaining the diversity

Chemosynthetic symbioses are not the only associations in which previously unrecognized diversity is now being discovered; in many other hosts, including humans, molecular tools are revealing the hidden diversity of the microbial biome[94,95]. As discussed below, several factors influence the diversity of microbial symbioses.

Transmission. The way in which symbionts are transmitted from one host to the next (BOX 2) can have an important role in determining the diversity of symbioses. Symbionts that are transmitted vertically usually have low levels of strain variability, as they no longer exchange genetic material with free-living members of their community. In associations with strict vertical transmission, symbionts and their hosts show parallel or congruent phylogenies and co-speciate. Vesicomyid clams acquire their symbionts vertically, and parallel patterns of host and symbiont genetic variation and phylogeny are generally observed in this group[96–98]. However, strict vertical transmission can be disrupted when symbionts are exchanged between host species or when new symbionts are acquired from the environment. For example, lateral acquisition of symbionts has recently been shown for vesicomyid clams from vents in the North-Eastern Pacific[99]. Lateral acquisition leads to the introduction of new genetic material in divergent host lineages and can therefore increase symbiont heterogeneity.

Population bottleneck
An evolutionary event in which the size of a population is greatly reduced.

Although horizontally transmitted symbionts are usually genetically more diverse than those that are transmitted vertically, this is not always the case. The *R. pachyptila* symbiont is acquired horizontally from the environment[83], but ITS analyses of three host individuals collected at the same site indicated high levels of homogeneity between symbionts[92]. As the ITS variability of the free-living stage of the symbionts was not investigated, it is not clear if this homogeneity reflects a limited genetic variability of these symbionts in the environment or a highly selective colonization process. The latter seems likely given that these symbionts enter the juvenile worms through the skin and fewer than 20 bacteria infect each host individual[83]. Morphological changes to host tissues that occur immediately after infection of *R. pachyptila* juveniles are suggestive of apoptosis[83], and are reminiscent of colonization patterns that are observed in pathogenic infections, and indicate strong selection for a highly specific mode of partner recognition. Lucinid clams are another host group in which symbionts are transmitted horizontally[100]. Despite horizontal transmission, the symbiont populations of lucinid clams show little to no variability in their 16S rRNA sequences, even between different host species[38], although strain variability has not yet been examined.

Bathymodiolin mussels and gutless oligochaetes can harbour as many as six co-occurring symbiont phylotypes. In mussels, evidence for horizontal transmission of sulphur-oxidizing symbionts is based on morphological and genetic data[88,89], as well as experiments that showed the loss and reacquisition of symbionts in adult mussels[101]. In addition, phylogenetic analyses argue against vertical transmission, as there is no evidence of co-speciation between the hosts and their sulphur-oxidizing and methane-oxidizing symbionts[4,50]. This flexibility in the uptake of symbionts, which seems to be possible throughout the entire life cycle, could explain the high diversity of symbionts in these hosts. For gutless oligochaetes there is morphological and genetic evidence for vertical transmission of the dominant symbionts (BOX 2), but the less-dominant symbionts could be acquired horizontally from the environment during deposition of the eggs into the surrounding sediments[40,102]. These mixed-transmission modes could lead to homogeneity in the vertically transmitted symbionts and heterogeneity in the horizontally transmitted symbionts. The horizontally transmitted symbionts could regularly provide the vertically transmitted symbionts with a source of fresh genetic material through the transfer of genes and phages, and therefore cause the vertically transmitted symbionts to be more genetically variable than if they occurred alone[103].

Biogeography and ecology. Habitat type and geographical location also influence the diversification of chemosynthetic symbioses. In the vestimentiferan tube worm *Escarpia spicata*, hosts from vents, seeps and wood falls harbour different 16S rRNA symbiont phylotypes[86]. Won *et al.*[88] argue that this type of 'opportunistic environmental acquisition' provides ecological flexibility by allowing the host to take up bacteria that are optimally adapted to their local environment. Geographical location influences

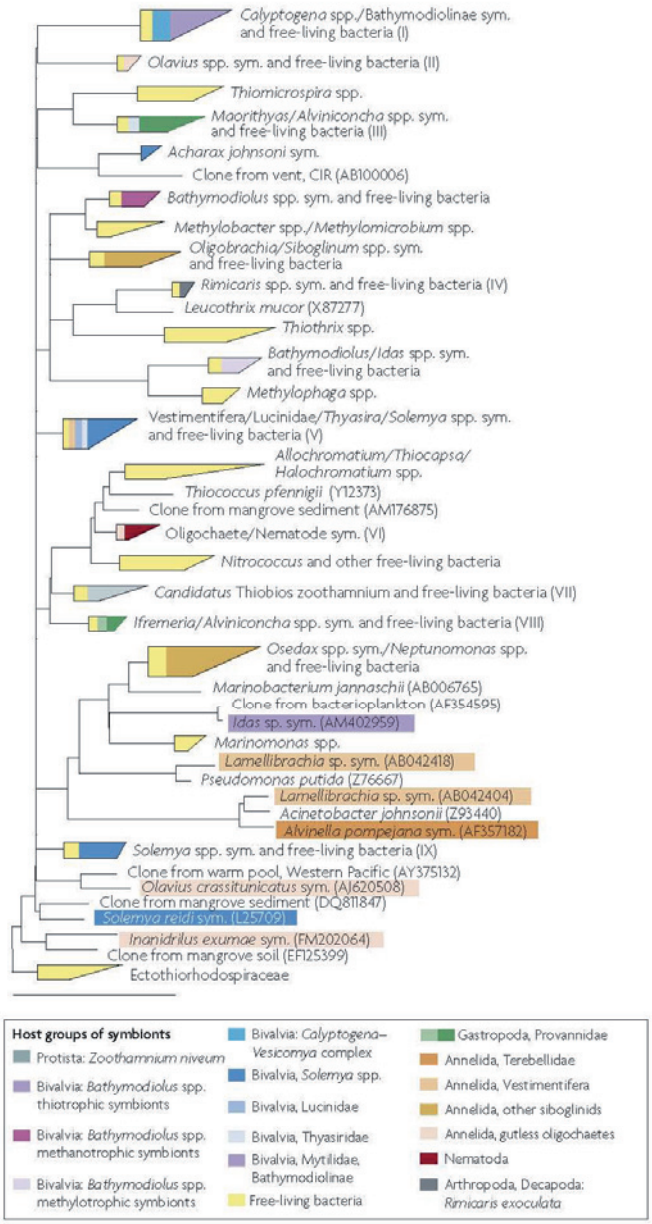


Figure 6 | **Phylogenetic diversity of gammaproteobacterial, chemosynthetic symbionts based on their 16S ribosomal RNA gene sequences.** Symbionts from the same host group are shown in the same colour, and free-living bacteria are shown in yellow. Symbiont phylogeny is based on maximum likelihood analyses of 16S ribosomal RNA (rRNA) gene sequences. Sequences of almost full length were chosen from the ARB or SILVA database[124] (release 94; March 2008), aligned and analysed with the ARB software package. The scale bar indicates 10% estimated sequence divergence. Numbers in brackets are the accession numbers of the 16S rRNA sequences. CIR, Central Indian Ridge; I–IX, clades with chemoautotrophic symbionts. Sym., symbionts.

both symbiont diversity (for example, in bathymodiolin mussels from the Mid-Atlantic Ridge[89]) and host diversity. Geographical barriers between vents at back-arc basins in the West Pacific might be the cause of the genetic variability of *Ifremeria* snail populations[104], and might also have influenced their symbiont populations[82]. At much longer time-scales, such as tens of millions of years, movement of tectonic plates and the closure of ancient oceans, such as the Tethys Sea, led to biological isolation with subsequent diversification of vent and seep fauna[3,33,105].

Evolutionary theory. Evolutionary theory can elucidate both the advantages and disadvantages of diversity in symbiotic associations (reviewed in REFS 106,107). Competition between the symbionts for space and resources are proposed to destabilize associations with various symbiont genotypes, as this causes reduced fitness of the host. Furthermore, selective pressure on the host to exclude less effective symbiont genotypes leads to symbiont uniformity. Another argument is that multiple symbionts lead to parasitism, as selection favours symbionts that make maximum use of nutrients and other host resources[108].

The following two theories provide explanations for the selective advantage of harbouring multiple symbionts. First, in unstable environments, a host might derive more benefit from one type of symbiont under one set of conditions than from another type of symbiont under a different set of conditions. Thus, access to genetically diverse symbionts allows hosts to adapt to changes in the environment. Second, in associations in which symbionts gain their nutrition from the environment, multiple symbionts do not compete for host-derived resources. Instead, multiple symbionts with different metabolic pathways can partition resources and cooperate in the use of resources. Support for these arguments comes from the dual symbioses of bathymodiolin mussels with sulphur-oxidizing and methane-oxidizing bacteria. The acquisition of symbionts that use two different energy sources allows the mussels to colonize unstable vent and seep habitats that contain variable concentrations of sulphide and methane. Correspondingly, mussels might be able to regulate the relative abundance of these two symbionts according to the relative amounts of these two energy sources in their habitat[109,110]. In addition, the dual symbionts do not compete with each other for their energy source, and might even be able to cooperate; for example, through the transfer of inorganic carbon from the methanotrophic to the thiotrophic symbiont[111]. The symbioses of gutless oligochaetes in which sulphur-oxidizing and sulphate-reducing symbionts co-occur also provide support for both of these theories. The acquisition of sulphate-reducing symbionts has enabled these hosts to colonize sediments in which sulphide concentrations can vary considerably over time and space, as the sulphate reducers can supply the co-occurring sulphur-oxidizing symbionts with an internal source of sulphide[31]. In addition, the sulphur-oxidizing and sulphate-reducing symbionts do not seem to compete for host-derived resources, but instead cooperate in the use of resources by exchanging oxidants and reductants, as well as participating in sulphur syntrophy[90].

Conclusions

As costs for sequencing decrease, unlimited opportunities for studying chemosynthetic symbioses lie ahead. To date the genomes of symbionts from only four hosts have been described. The sequencing of many more symbionts from a wide array of hosts and environments will allow comparative analyses of the genetic similarities and differences within this highly diverse group of chemosynthetic bacteria. Sequencing of host genomes is now also feasible, and could provide the exciting possibility of examining how both the host and the symbiont have adapted to each other and whether genetic exchange has occurred between the symbiotic partners. Other 'omic' analyses of chemosynthetic symbioses (for example, proteomics) are still in their infancy or have not yet begun (for example metabolomics), but will eventually reveal the range of metabolic pathways that are used by symbionts to gain energy from the environment and provide their hosts and co-occurring symbionts with metabolites and nutrition. Advances in single-cell methods that combine imaging techniques with metabolic analyses, such as NanoSIMS and Raman microscopy, will allow the uptake and distribution of substrates from the environment to be investigated as well as the rates at which these substrates are acquired, and will be particularly valuable for associations with multiple symbionts to tease apart the role of the different bacterial partners. Equally important is the development of improved *in situ* methods for analyses of environmental factors that are important to the symbioses; for example, the concentrations and flux of symbiotic energy sources, such as methane and sulphide, at deep-sea vents and seeps. Finally, despite the importance of these technological advances, we will always need the expertise of taxonomists to recognize, identify and characterize marine protists and invertebrates and reveal the remarkable diversity of chemosynthetic symbioses.

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FURTHER INFORMATION
Nicole Dubilier's homepage: http://www.mpi-bremen.de/en/Nicole_Dubilier.html
ALL LINKS ARE ACTIVE IN THE ONLINE PDF

Replacement of the primary symbiont in the gutless marine worm *Inanidrilus exumae* by a sulfur oxidizer from a novel symbiont lineage

Claudia Bergin, Nancy Brewig, Anna Blazejak,
Olav Giere, Christer Erséus, Nicole Dubilier

Manuscript in preparation

Replacement of the primary symbiont in the gutless marine worm *Inanidrilus exumae* by a sulfur oxidizer from a novel symbiont lineage

C. Bergin[1], N. Brewig[1†], A. Blazejak[1‡], O. Giere[2], C. Erséus[3], N. Dubilier[1*]

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

[2]Biozentrum Grindel
Zoologisches Institut und Zoologisches Museum
Universität Hamburg
Martin-Luther-King-Platz 3
D-20146 Hamburg, Germany

[3]Department of Zoology
University of Gothenburg
Box 463
SE-405 30 Göteborg, Sweden

[†]Present address: Department of Immunology
Bernhard Nocht Institute for Tropical Medicine
Bernhard-Nocht-Str. 74
20359 Hamburg, Germany

[‡]Present address: Arbeitsbereich Geomikrobiologie (B1.3)
Bundesanstalt für Geowissenschaften und Rohstoffe (BGR)
Stilleweg 2
30655 Hannover, Germany

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Running title: Symbiont replacement in a gutless worm

Summary

Gutless marine oligochaetes live in obligate associations with multiple bacterial endosymbionts. The primary symbionts of these hosts, called Gamma 1 symbionts, have been found in all 16 host species examined so far. These symbionts are chemoautotrophic sulfur oxidizers and form a monophyletic clade within the *Gammaproteobacteria*. Secondary symbionts that co-occur with the primary symbionts are smaller and belong to the *Gamma*-, *Delta*-, *Alphaproteobacteri*a or the *Spirochaete*s.

The gutless oligochaete *Inanidrilus exumae* from coral reef sediments of the Bahamas has an unusual symbiont community that differs markedly from all other host species. Comparative 16S rRNA sequence analysis and fluorescence in situ hybridization revealed that *I. exumae* harbors co-occurring alpha- and deltaproteobacterial symbionts, while all other host species harbor either alpha- or deltaproteobacterial but never both. This shows that *Deltaproteobacteria* can occur as symbionts in gutless oligochaetes living in calcareous sediments, in contrast to previous studies in which only *Alphaproteobacteria* were found in hosts from these habitats.

Unexpectedly, we were not able to detect the Gamma 1 symbiont in *I. exumae*. Instead, these hosts harbored a bacterial symbiont, called Gamma 4, which was morphologically highly similar to the Gamma 1 symbionts but phylogenetically distinct from these. The Gamma 4 symbiont differed by 10% from the Gamma 1 symbionts, and by at least 7% from all other 16S rRNA sequences, with *Nitrosococcus* and *Methylococcus*/*Methylocaldum* species, and sulfur-storing bacteria of the *Ectothiorhodospiraceae* as the closest cultured relatives. Despite its highly divergent phylogeny, the Gamma 4 symbiont apparently shares the same function in the symbiosis as the Gamma 1 symbiont: sulfur globules in the symbiont cells as well as characteristic genes for autotrophy (*cbbL*) and sulfur oxidation (*aprA*) indicate that the novel symbiont is a chemoautotrophic sulfur oxidizer. We hypothesize that the Gamma 4 symbiont has displaced the Gamma 1 symbiont in *I. exumae*, either by outcompeting it or by chance during population bottlenecks in vertical symbiont transmission.

Introduction

Gutless oligochaetes of the genera *Inanidrilus* and *Olavius* are a diverse and widespread group of marine invertebrates that live in symbiosis with multiple bacteria. These small worms occur worldwide in many marine sediments and are most commonly found in tropical and sub-tropical coral reef sediments (for reviews see (Bright and Giere, 2005; Dubilier et al., 2006). All gutless oligochaetes live in obligate symbiosis with endosymbiotic bacteria that occur in a thick layer in the body wall just below the cuticle. As implied by the word gutless, these hosts lack a digestive system and no longer have a mouth or gut, depending instead on their symbionts for nutrition (Giere, 1981).

The symbiotic community of gutless oligochaetes is phylogenetically diverse and consists of *Proteobacteria* from the gamma, delta, and alpha subdivisions as well as spirochetes. The oligochaete symbionts are host-specific: each host species harbors a distinct assemblage of 5 - 6 16S rRNA phylotypes that is specific to the species and does not occur in other host species. The primary symbiont in all gutless oligochaetes examined to date is a large (2 – 7 μm) sulfur-storing bacterial morphoptype, called the Gamma 1 symbiont. The Gamma 1 symbionts of gutless oligochaetes are closely related to each other (>95.7% 16S rRNA sequence identity) and form a monophyletic clade within the *Gammaproteobacteria*, with free-living sulfur oxidizers of the family *Chromatiaceae* as their closest relatives. The Gamma 1 symbionts have been identified as chemoautotrophic sulfur oxidizers based on the presence of sulfur globules (Giere and Krieger, 2001), uptake experiments showing the incorporation of inorganic carbon (Felbeck et al., 1983; Giere et al., 1988), immunohistochemical labeling of ribulose-1,5-bisphosphate carboxylase/oxygenase (Krieger et al., 2000; Giere and Krieger, 2001) (Dubilier et al., 2001), and recent metagenomic analyses (Woyke et al., 2006).

The Gamma 1 symbionts co-occur with so-called secondary symbionts that are much smaller (0.7 – 1.5 μm). These secondary symbionts are rod- and cocci-shaped and have been identified as gamma-, delta- or alphaproteobacterial symbionts, while elongated morphotypes belong to the spirochetes (Dubilier et al., 2006). The secondary gammaproteobacterial symbionts are sulfur oxidizers, while the deltaproteobacterial symbionts are sulfate reducers. The sulfate-reducing symbionts provide the primary (Gamma 1) and secondary gammaproteobacterial sulfur oxidizers with reduced sulfur compounds, thus allowing their hosts to live in sediments with little or no environmental sulfide (Dubilier et al., 2001; Woyke et al., 2006; Ruehland et al., 2008). The metabolism of the alphaproteobacterial and spirochete symbionts remains unclear (Blazejak et al., 2005; Ruehland et al., 2008).

As part of our biodiversity studies of gutless oligochaete symbioses, we examined the bacterial community of *Inanidrilus exumae* from the Bahamas, a hotspot of diversity for gutless oligochaetes (Giere et al., 1995; Erséus, 2003). *I. exumae* individuals were collected at Lee Stocking Island in the Bahamas, where they co-occurred with several other gutless oligochaete species in shallow-water coral reef sediments (Erséus, 2003). We used comparative 16S rRNA sequence analysis, fluorescence in situ hybridization (FISH) and ultrastructural analyses to examine the diversity and distribution of the symbionts in *I. exumae*. In addition, we investigated the potential of the symbionts for autotrophy and sulfur metabolism by examining two protein-coding genes. For autotrophy via the Calvin-Benson-Bassham (CBB) cycle, *cbbL* was used as a functional marker gene, which codes for the ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) form I large subunit (Elsaied and Naganuma, 2001; Blazejak et al., 2006). As a marker for reductive and oxidative sulfur metabolism the *aprA* gene, coding for the alpha-subunit of adenosine-5′-phosphosulfate reductase (APS reductase) was examined

(Hipp et al., 1997; Friedrich, 2002; Blazejak et al., 2006). APS reductase catalyzes the reduction of APS to sulfite in sulfate reducers and functions in the opposite direction in sulfur oxidizers. The phylogeny of the *aprA* gene is congruent with its function, with sequences from sulfate reducers forming a clade distinct from the sequences found in sulfur oxidizers (Meyer and Kuever, 2007).

Results and Discussion

Comparative 16S rRNA gene analysis and FISH revealed an unusual symbiotic community in *Inanidrilus exumae*. This is the first oligochaete host species in which we found co-occurring alpha- and deltaproteobacterial symbionts. *I. exumae* is also the first gutless oligochaete that does not have the Gamma 1 symbiont found in all other gutless oligochaetes. Instead, it harbors a novel gammaproteobacterial symbiont resembling the Gamma 1 symbiont in appearance and function.

Co-occurring alpha- and deltaproteobacterial symbionts in *Inanidrilus exumae*

All previously examined oligochaete hosts have either *Alpha*- or *Deltaproteobacteria* as secondary symbionts, while in *I. exumae*, symbionts from these two proteobacterial subgroups co-occur in the symbiont-containing region. Clone libraries of the 16S rRNA gene from seven *I. exumae* individuals (Table 1) contained sequences belonging to both the *Delta*- and the *Alphaproteobacteria* (the gammaproteobacterial symbionts are discussed below).

Deltaproteobacterial symbionts

Of the four deltaproteobacterial sequences found in the clone libraries, the Delta 3 and Delta 9 were identified as symbionts using FISH and specific probes (Table 2). These symbionts were small, rod-shaped bacteria that occurred mostly in the periphery of the symbiont containing region just below the worm's cuticle (Fig. 1B and C). In phylogenetic analyses, both deltaproteobacterial symbionts belonged to the *Desulfobacteraceae* (Fig. 2A). The Delta 3 symbiont of *I. exumae* was closely related to the Delta 3 symbiont of the gutless oligochaete *Olavius ilvae* and a 16S rRNA sequence found in clone libraries of the gutless oligochaete *O. algarvensis* called "*O. algarvensis* associated clone Delta 3" (Ruehland et al., 2008). (The latter was not confirmed to have originated from a symbiont with FISH. We therefore called this sequence and all other sequences that could not be identified as symbionts with FISH "associated clones"). The second deltaproteobacterial symbiont Delta 9 was most closely related to environmental clone sequences from marine sediments, but its exact position within the *Desulfobacteraceae* varied depending on the phylogenetic method used (Fig. 2A).

Table 1: Number of partial 16S rRNA, *aprA* and *cbbL* gene sequences obtained from cloned PCR products from *I. exumae*. Sequences that shared >99% identity were grouped in a clone family. One or more clones of each phylotype and individual were sequenced in both directions for the almost full length 16S rRNA sequence, and partial *aprA* and *cbbL* sequences. n.d. not determined due to limited material.

		***Inanidrilus exumae* specimen**						
	Clone family/ phylotype	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**
Gammaproteobacterial symbiont	Gamma 4	72	156	46	78	16	0	6
Alphaproteobacterial symbionts	Alpha 1a	0	5	0	0	0	13	54
	Alpha 2a	0	0	5	0	0	13	11
	Alpha 2b	61	27	0	1	0	0	0
Deltaproteobacterial symbionts	Delta 3	0	0	46	75	0	23	5
	Delta 9	5	1	0	0	0	0	0
Associated	Delta 8	0	0	11	1	0	0	0
	Delta 10	0	0	0	3	0	0	0
aprA	*aprA* Ia	4	4	n.d.	n.d.	n.d.	n.d.	n.d.
	aprA Ib	2	5	n.d.	n.d.	n.d.	n.d.	n.d.
	aprA IIa	7	11	n.d.	n.d.	n.d.	n.d.	n.d.
	aprA IIb	11	4	n.d.	n.d.	n.d.	n.d.	n.d.
	aprA SRB	4	0	n.d.	n.d.	n.d.	n.d.	n.d.
cbbL	*cbbL*	n.d.	n.d.	n.d.	n.d.	n.d.	29	18

Two further deltaproteobacterial 16S rRNA gene sequences were found in the *I. exumae* clone libraries (Table 1), but probes specific to these sequences did not show FISH signals. The Delta 8 associated clone from *I. exumae* belonged to the Bdellovibrio and was most closely related to the Delta 8 associated clone from *O. ilvae* (Fig. 2A). Rühland et al. (2008) speculated that the *O. ilvae* Delta 8 sequence originated from a Bdellovibrio-like parasite of the worm's bacterial symbionts. As in this study, however, no FISH evidence for these bacteria was found. The Delta 10 associated clone was most closely related to environmental clone sequences from a lake and a bioreactor (Fig. 2A) with members of the *Myxococcales* as the closest cultivated bacterial relatives.

The close phylogenetic relationship of the Delta 3 and 9 symbionts of *I. exumae* to sulfate-reducing symbionts of other gutless oligochaetes and free-living sulfate reducers suggest that the *I. exumae* deltaproteobacterial symbionts are also sulfate reducers. This conclusion is supported by the presence of an *aprA* gene in *I. exumae* that fell within the lineage of AprA sequences from sulfate-reducing prokaryotes (Fig. 3). Only a single AprA sequence belonging to the reductive AprA lineage was found in the *I. exumae* clone libraries despite the presence of two phylogenetically distinct deltaproteobacterial symbionts, Delta 3 and 9. This AprA sequence fell in a clade that contained the AprA sequences from Delta 1 symbionts of gutless oligochaetes, although this relationship

was not supported by bootstrap analyses (Fig. 3). The phylogeny of this AprA sequence therefore does not indicate if it originated from the Delta 3 or 9 symbiont. The presence of sulfate-reducing bacteria in *I. exumae* suggests that these provide the symbiosis with reduced sulfur compounds as shown for the gutless oligochaetes *O. algarvenis* and *O. ilvae* from the Mediterranean (Dubilier et al., 2001; Woyke et al., 2006; Ruehland et al., 2008). Sulfide concentrations are extremely low in the Mediterranean sediments in

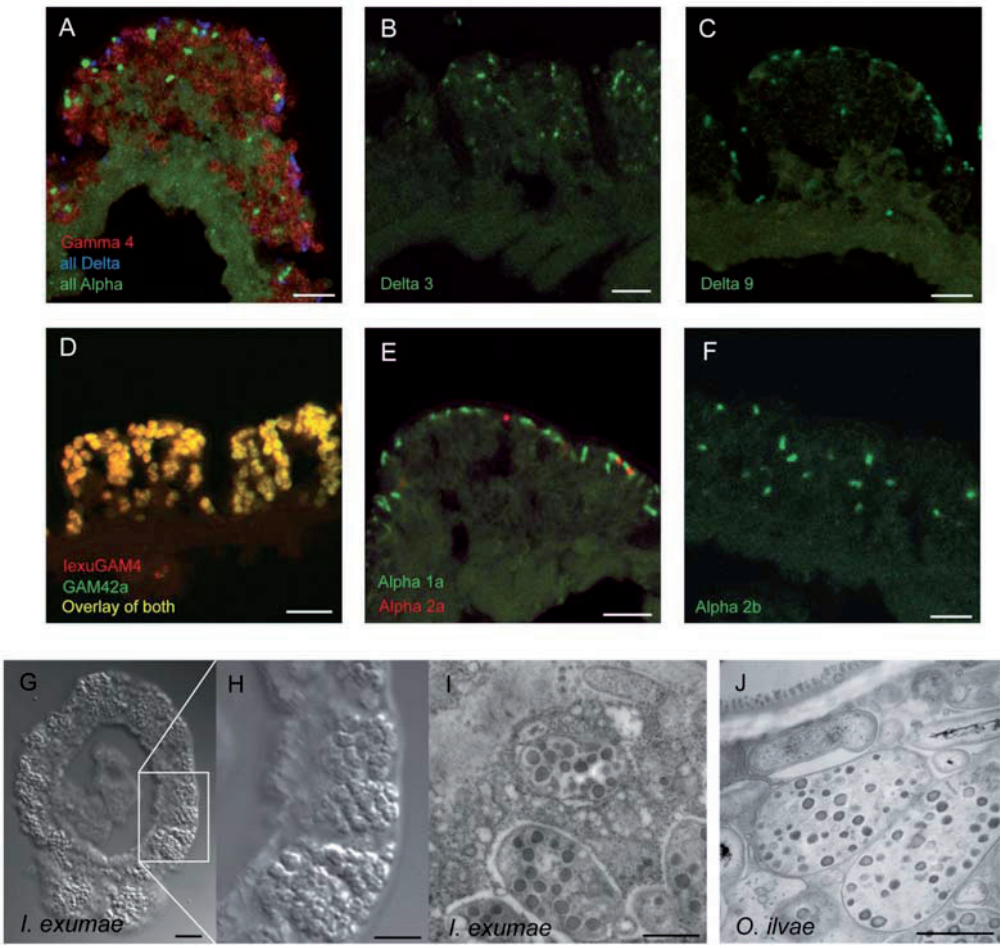

Fig. 1. Bacterial symbionts in *I. exumae*. FISH images are shown in A) – F), Light and electron microscopy images in G) – J). A) Epifluorescence image showing the body wall of *I. exumae* with the co-occurring Gamma 4 symbionts (red), deltaproteobacterial symbionts (blue) and alphaproteobacterial symbionts (green). B) Delta 3 symbiont (green, probe Oalg/OilvDEL3). C) Delta 9 symbiont (green, probe OalgDEL4). D) The Alpha 1a (green, probe IexuALFd) and Alpha 2a (red, probe ImakALF1b) symbionts co-occurred in all sections studied. E) The Alpha 2b symbiont (green, IexuALFb) did not co-occur with the other alphaproteobacterial symbionts. F) Double hybridization with the Gamma 4 probe IexuGAM4 (green) and the general gammaproteobacterial probe GAM42a (red) shows a complete overlay of both probes (yellow), indicating that the Gamma 4 symbionts are the only *Gammaproteobacteria* present in *I. exumae*. Scale bars in A) – F): 5 µm. G) and H) Differential interference contrast images. G) Cross section through an entire worm. The white box encloses the part of the body wall shown at high magnification in the following panel to the right. H) The large Gamma 4 symbiont cells are visible in the body wall and fill the entire symbiont-containing region. I) The ultrastructure of the Gamma 4 symbionts is highly similar to that of the Gamma 1 symbionts (shown in the following panel to the right), with large electron-dense globules, some of which contained sulfur. J) Gamma 1 symbionts in *O. ilvae*. Scale bars: 10 µm (G), 5 µm (H), 1 µm (I, J).

which *O. algarvensis* and *O. ilvae* occur, but nothing is known about the sulfide concentrations in the sediments at the *I. exumae* collection site.

Alphaproteobacterial symbionts

In addition to deltaproteobacterial symbionts we found three alphaproteobacterial 16S rRNA sequences in *I. exumae* clone libraries, Alpha 1a, Alpha 2a, and Alpha 2b (Table 1). FISH with probes specific to these sequences confirmed that all three originated from symbionts (Table 2, Fig. 1E and F). The Alpha 1a and Alpha 2a symbionts co-occurred and were abundant in the periphery of the symbiont-containing region (Fig. 1E). In contrast, the Alpha 2b symbiont was evenly distributed throughout the symbiont-containing regions (Fig. 1F) and was not observed to co-occur with the two other alphaproteobacterial symbionts. (In the 16S rRNA clone library (Table 1), however, sequences from the Alpha 2b and 1a symbionts co-occurred in *I. exumae* specimen 2, indicating that these symbionts are not always mutually exclusive.) All three alphaproteobacterial symbionts were most closely related to symbionts from other gutless oligochaete species (Fig. 2B). The closest cultured relatives were *Defluviicoccus vanus* and *Rhodovibrio* species within the *Rhodospirillales*.

All *I. exumae* individuals of this study harbored at least one alphaproteobacterial symbiont, indicating that these are important if not essential for the symbiosis. As with the alphaproteobacterial symbionts of other gutless oligochaetes, their role in the association is unknown (Dubilier et al., 1999; Blazejak et al., 2006). One possibility is that these recycle anaerobic waste products of their hosts such as acetate and propionate as suggested for the deltaproteobacterial symbionts of gutless oligochaetes (Dubilier et al., 2001; Woyke et al., 2006). The closest cultured relatives of the Alpha 1 and 2 symbionts of gutless oligochaetes, *D. vanus* and *Rhodovibrio* species (Fig. 2B), use a wide range of organic compounds as carbon sources and electron donors (Nissen and Dundas, 1984) including acetate (Burow et al., 2007) . In addition to recycling host waste products, the alphaproteobacterial symbionts could also benefit the association by metabolizing organic sulfur compounds such as dimethylsulfoniopropionate (DMSP), dimethylsulfide (DMS), and dimethysulfoxide (DMSO). High concentrations of DMSP and DMS are found in coral mucus and in coral reef sediments (Broadbent and Jones, 2004), where gutless oligochaetes with alphaproteobacterial symbionts occur most commonly (Dubilier et al., 2006). The use of these organic sulfur compounds as both electron donors and/or a carbon source is widespread in bacteria (Schäfer, 2007; Stefels et al., 2007; Howard et al., 2008), and common in members of the *Alphaproteobacteria* (Hatton et al., 2005; Howard et al., 2008). DMSO is abundant in the marine environment as a chemical and biological oxidation product of DMS (Bentley and Chasteen, 2004; Hatton et al., 2005) and would be an energetically favorable electron acceptor under conditions where neither oxygen nor nitrate are available in the sediments inhabited by the gutless oligochaetes.

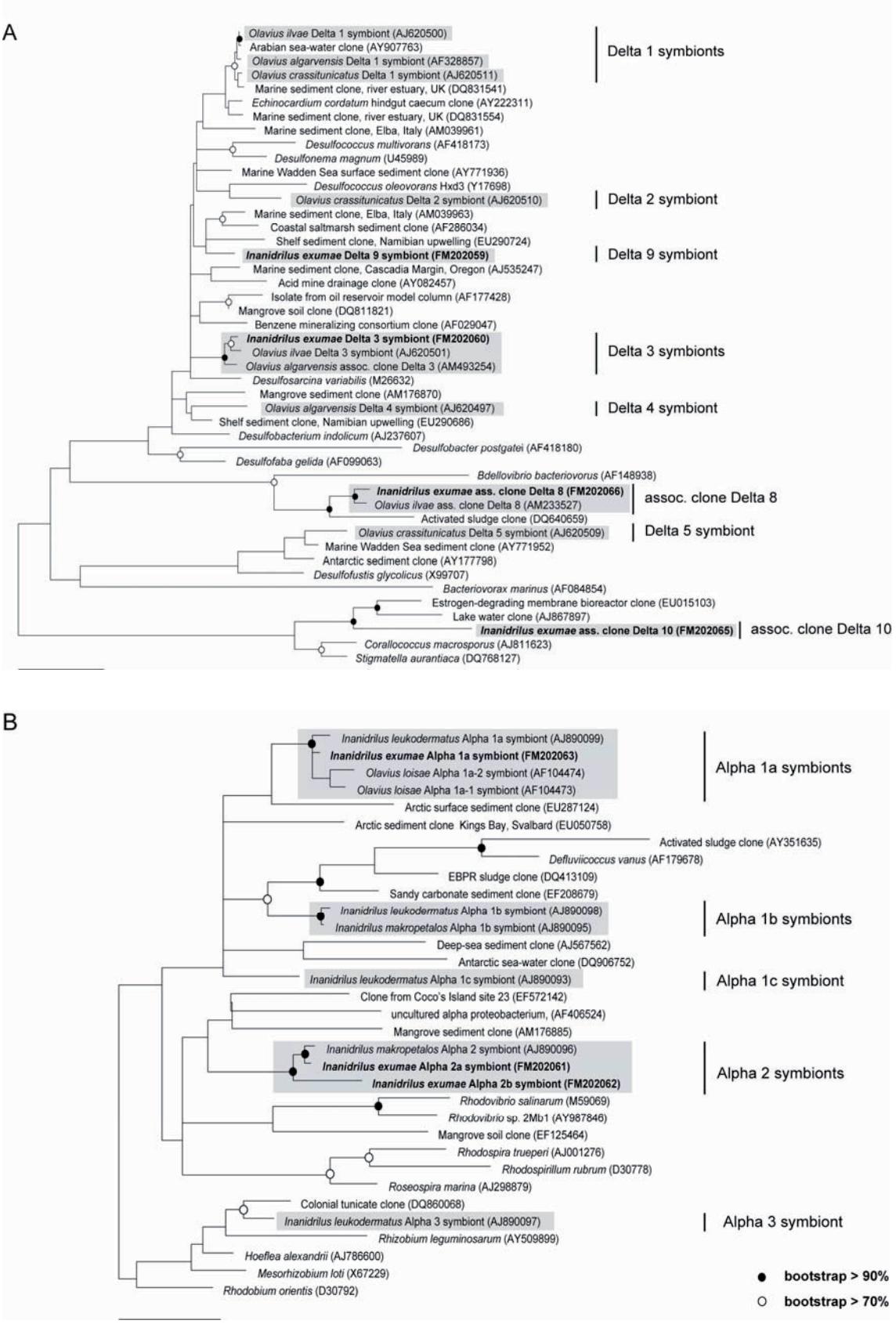
A
Olavius ilvae Delta 1 symbiont (AJ620500)
Arabian sea-water clone (AY907763)
Olavius algarvensis Delta 1 symbiont (AF328857)
Olavius crassitunicatus Delta 1 symbiont (AJ620511)
Marine sediment clone, river estuary, UK (DQ831541)
Echinocardium cordatum hindgut caecum clone (AY222311)
Marine sediment clone, river estuary, UK (DQ831554)
Marine sediment clone, Elba, Italy (AM039961)
Desulfococcus multivorans (AF418173)
Desulfonema magnum (U45989)
Marine Wadden Sea surface sediment clone (AY771936)
Desulfococcus oleovorans Hxd3 (Y17698)
Olavius crassitunicatus Delta 2 symbiont (AJ620510)
Marine sediment clone, Elba, Italy (AM039963)
Coastal saltmarsh sediment clone (AF286034)
Shelf sediment clone, Namibian upwelling (EU290724)
Inanidrilus exumae Delta 9 symbiont (FM202059)
Marine sediment clone, Cascadia Margin, Oregon (AJ535247)
Acid mine drainage clone (AY082457)
Isolate from oil reservoir model column (AF177428)
Mangrove soil clone (DQ811821)
Benzene mineralizing consortium clone (AF029047)
Inanidrilus exumae Delta 3 symbiont (FM202060)
Olavius ilvae Delta 3 symbiont (AJ620501)
Olavius algarvensis assoc. clone Delta 3 (AM493254)
Desulfosarcina variabilis (M26632)
Mangrove sediment clone (AM176870)
Olavius algarvensis Delta 4 symbiont (AJ620497)
Shelf sediment clone, Namibian upwelling (EU290686)
Desulfobacterium indolicum (AJ237607)
Desulfobacter postgatei (AF418180)
Desulfofaba gelida (AF099063)
Bdellovibrio bacteriovorus (AF148938)
Inanidrilus exumae ass. clone Delta 8 (FM202066)
Olavius ilvae ass. clone Delta 8 (AM233527)
Activated sludge clone (DQ640659)
Olavius crassitunicatus Delta 5 symbiont (AJ620509)
Marine Wadden Sea sediment clone (AY771952)
Antarctic sediment clone (AY177798)
Desulfofustis glycolicus (X99707)
Bacteriovorax marinus (AF084854)
Estrogen-degrading membrane bioreactor clone (EU015103)
Lake water clone (AJ867897)
Inanidrilus exumae ass. clone Delta 10 (FM202065)
Corallococcus macrosporus (AJ811623)
Stigmatella aurantiaca (DQ768127)
Delta 1 symbionts
Delta 2 symbiont
Delta 9 symbiont
Delta 3 symbionts
Delta 4 symbiont
assoc. clone Delta 8
Delta 5 symbiont
assoc. clone Delta 10
B
Inanidrilus leukodermatus Alpha 1a symbiont (AJ890099)
Inanidrilus exumae Alpha 1a symbiont (FM202063)
Olavius loisae Alpha 1a-2 symbiont (AF104474)
Olavius loisae Alpha 1a-1 symbiont (AF104473)
Arctic surface sediment clone (EU287124)
Arctic sediment clone Kings Bay, Svalbard (EU050758)
Activated sludge clone (AY351635)
Defluviicoccus vanus (AF179678)
EBPR sludge clone (DQ413109)
Sandy carbonate sediment clone (EF208679)
Inanidrilus leukodermatus Alpha 1b symbiont (AJ890098)
Inanidrilus makropetalos Alpha 1b symbiont (AJ890095)
Deep-sea sediment clone (AJ567562)
Antarctic sea-water clone (DQ906752)
Inanidrilus leukodermatus Alpha 1c symbiont (AJ890093)
Clone from Coco's Island site 23 (EF572142)
uncultured alpha proteobacterium, (AF406524)
Mangrove sediment clone (AM176885)
Inanidrilus makropetalos Alpha 2 symbiont (AJ890096)
Inanidrilus exumae Alpha 2a symbiont (FM202061)
Inanidrilus exumae Alpha 2b symbiont (FM202062)
Rhodovibrio salinarum (M59069)
Rhodovibrio sp. 2Mb1 (AY987846)
Mangrove soil clone (EF125464)
Rhodospira trueperi (AJ001276)
Rhodospirillum rubrum (D30778)
Roseospira marina (AJ298879)
Colonial tunicate clone (DQ860068)
Inanidrilus leukodermatus Alpha 3 symbiont (AJ890097)
Rhizobium leguminosarum (AY509899)
Hoeflea alexandrii (AJ786600)
Mesorhizobium loti (X67229)
Rhodobium orientis (D30792)
Alpha 1a symbionts
Alpha 1b symbionts
Alpha 1c symbiont
Alpha 2 symbionts
Alpha 3 symbiont
bootstrap > 90%
bootstrap > 70%

Fig. 2., opposite page: Phylogenetic analyses of the A) delta- and B) alphaproteobacterial symbionts of *I. exumae* based on their 16S rRNA gene sequences. Sequences obtained in this study are shown in bold face, sequences from gutless oligochaete symbionts are highlighted in grey. Designation of symbionts and clone sequences from *I. exumae* in this tree and all others shown in this study refer to their phylogenetic affiliation with previously described gutless oligochaete symbiont clades. Sequences without such affiliations were assigned new numbers in ascending order. The consensus trees shown are based on maximum likelihood analysis. Branching orders that were not supported in most calculation methods are shown as multifurcations. Scale bars represent 10% estimated phylogenetic divergence for non-multifurcation branches.

Co-occurrence of alpha- and deltaproteobacterial symbionts

In all oligochaete host species examined before this study (two *Inanidrilus* and four *Olavius* species), either alpha- or deltaproteobacterial symbionts were found together with the Gamma 1 symbionts in each host. We hypothesized that sediment type influences the distribution of these secondary symbiont, because we only found alphaproteobacterial symbionts in hosts from biogenic calcareous sediments such as the Bahamas, Bermuda, and the Great Barrier Reef (Dubilier et al., 1999; Blazejak et al., 2006) while deltaproteobacterial symbionts only occurred in hosts from non-biogenic silicate sediments such as Mediterranean seagrass beds (Dubilier et al., 2006; Ruehland et al., 2008). This study shows that there are exceptions to this habitat pattern: the *I. exumae* examined here live in calcareous sediments and harbor not only *Alphaproteobacteria* but also co-occurring *Deltaproteobacteria*. Although the distribution of the three alpha- and two deltaproteobacterial symbionts varied in the *I. exumae* population with all symbionts rarely co-occurring within the same individual, each host specimen harbored at least one alpha- and one deltaproteobacterial symbiont. This shows that alpha- and deltaproteobacterial symbionts do not mutually exclude each other and it is possible that they complement each other or interact beneficially with each other to the advantage of the symbiosis.

A novel gammaproteobacterial symbiont in *Inanidrilus exumae*

Morphology and phylogeny of the Gamma 4 symbiont

Only a single gammaproteobacterial sequence, called Gamma 4, was found in the 16S rRNA clone libraries of all seven *I. exumae* individuals (Table 1). FISH with a probe specific to the Gamma 4 sequence (IexuGAM4, Table 2) showed that it originated from a large oval-shaped bacterium that was highly abundant and dominated the symbiont-containing region in all examined sections and individuals (Fig. 1A and D). Dual FISH hybridizations with the specific IexuGAM4 probe and the general probe for Gammaproteobacteria GAM42a showed a complete overlay of the hybridization signals in all sections, with both probes hybridizing to the same large oval-shaped morphotype (Fig. 1D). This indicates that the Gamma 4 symbionts are the only Gammaproteobacteria present in *I. exumae* and that this species lacks the Gamma 1 symbiont found in all other gutless oligochaetes.

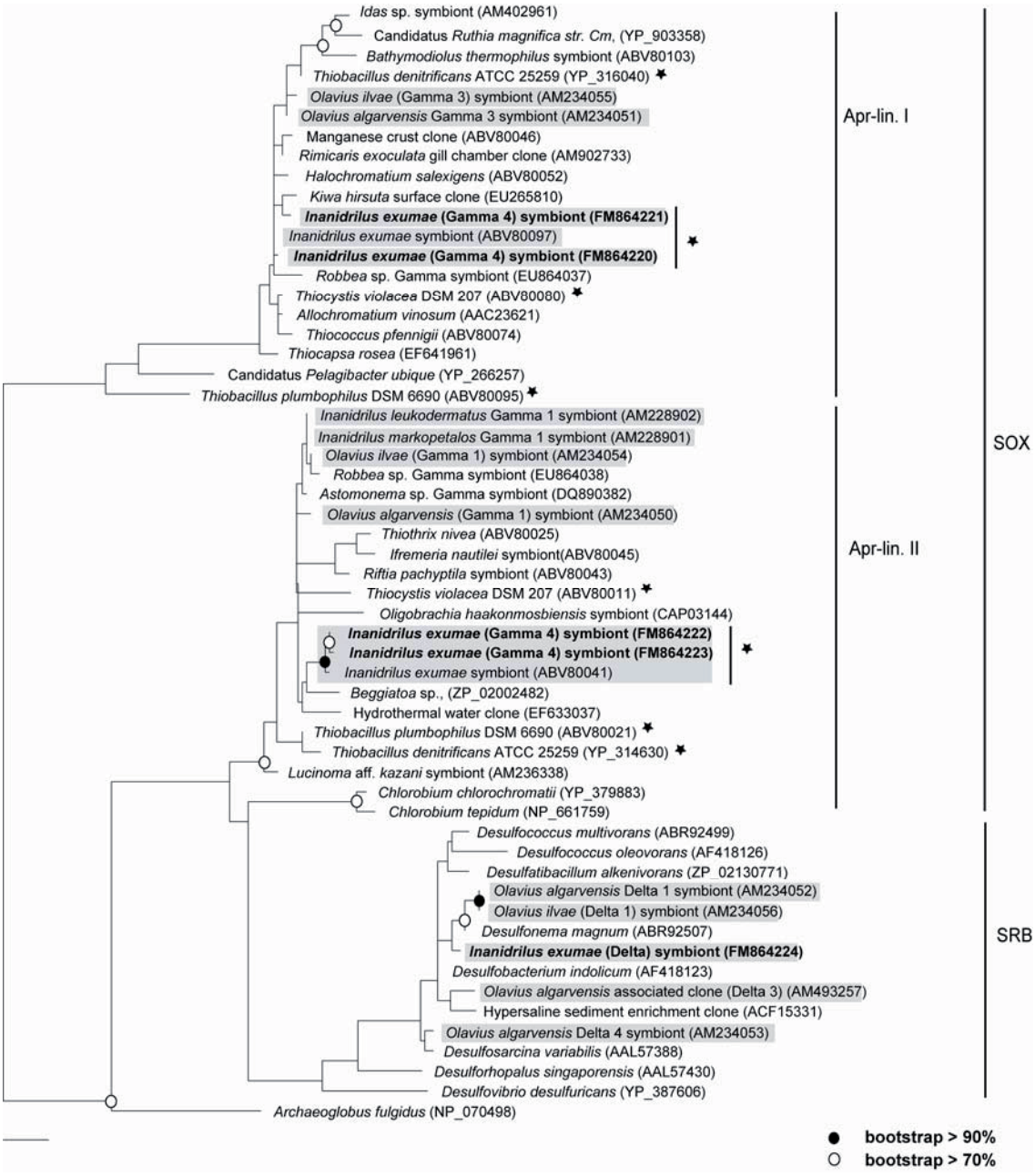

Fig. 3. Phylogenetic affiliation of the alpha subunit of the APS reductase *aprA* genes from *I. exumae* based on deduced amino acid sequences. Symbiont name of sequence whose origin was inferred through phylogeny is in parentheses. Five distinct *aprA* sequences were found in *I. exumae* resulting in five translated protein sequences. (The *I. exumae* sequences ABV80097 and ABV80041 are from Meyer and Kuever, 2007.) Two AprA sequences of *I. exumae* within the Apr I and two within the Apr II lineage were very closely related (>99%). Asteriks indicate AprA sequences from organisms with two *aprA* operons. SOX, sulfur-oxidizing bacteria; SRB, sulfate-reducing bacteria. Additional information see legend of Fig. 2.

Transmission electron microscopy (TEM) showed that the ultrastructure of the Gamma 4 is highly similar to that of the Gamma 1 symbiont (Fig. 1I and J). Like the Gamma 1 symbiont, the Gamma 4 symbiont is the largest morphotype within the symbiotic community (2 – 3 μm), and its cells are also filled with large electron-dense

globules (Fig. 1I). Both symbionts are highly abundant and dominate the symbiont-containing region.

Phylogenetic analyses revealed that the Gamma 4 symbiont belongs to a novel symbiont lineage (Fig. 4). The 16S rRNA sequence of the Gamma 4 symbiont did not fall within the group of sequences belonging to the Gamma 1 symbiont clade, and differed from these by more than 10% (Fig. 4). Instead, it grouped consistently with clone sequences from caves and marine sediments including hydrothermal vent sediments, and showed a divergence of at least 7% to all other 16S rRNA sequences (Fig. 4). The closest cultured relatives with a sequence divergence of about 9 - 10% were *Nitrosococcus* and *Methylococcus*/*Methylocaldum* species, and sulfur-storing bacteria of the *Ectothiorhodospiraceae*, but the phylogenetic relationship of these bacteria to the Gamma 4 symbiont was not consistent between treeing methods.

The Gamma 4 symbiont is a potential chemoautotrophic sulfur oxidizer

Despite their divergent phylogeny, the Gamma 1 and Gamma 4 symbionts not only share a similar morphology, but also appear to have the same functional role as chemoautotrophic sulfur oxidizers. The autotrophic potential of the Gamma 4 symbiont is indicated by the presence of the *cbbL* gene coding for the RubisCO form I large subunit in the worms (Fig. 5). The *I. exumae* CbbL sequence was not closely related to sequences from Gamma 1 symbionts which group together in a clade, nor did it fall with the CbbL sequences from the secondary gammaproteobacterial symbiont (Gamma 3) of the gutless oligochaete. Instead, the *I. exumae* CbbL sequence was most closely related to the CbbL sequence from the sulfur-oxidizing symbiont of the hydrothermal vent snail *Alviniconcha hessleri* (Fig. 5).

The potential of the Gamma 4 symbiont to use reduced sulfur compounds as an energy source is supported by the detection of sulfur in the Gamma 4 symbiont using electron spectroscopic imaging (Keyser and Giere, unpublished data) and the presence of *aprA* genes related to those of free-living and symbiotic sulfur-oxidizing bacteria (Fig. 3). As with CbbL, the *I. exumae* AprA sequences were not closely related to those from Gamma 1 symbionts. Two phylogenetically distinct groups of AprA sequences belonging to the AprA I and II lineages were found in *I. exumae* (Fig. 3). This corresponds well with a previous comprehensive study of APS reductase genes in sulfur-oxidizing bacteria in which these two phylogenetically distinct genes were found in *I. exumae* (Meyer and Kuever, 2007). We assume that both the AprA I and II sequences originated from the Gamma 4 symbiont as no other gammaproteobacterial sulfur oxidizers are present in *I. exumae* and the alphaproteobacterial symbionts of gutless oligochaetes do not appear to have an APS reductase (Blazejak et al., 2006). The presence of two gene loci for APS reductase has been shown for several free-living sulfur oxidizing bacteria and is therefore not unusual (Meyer and Kuever, 2007). Meyer and Kuever (2007) hypothesized that the presence of two gene loci might provide

physiological versatility in habitats with oscillating oxygen and sulfide concentrations, which may well be the case for *I. exumae* and other gutless oligochaetes that migrate between upper oxidized and lower sulfidic sediment layers.

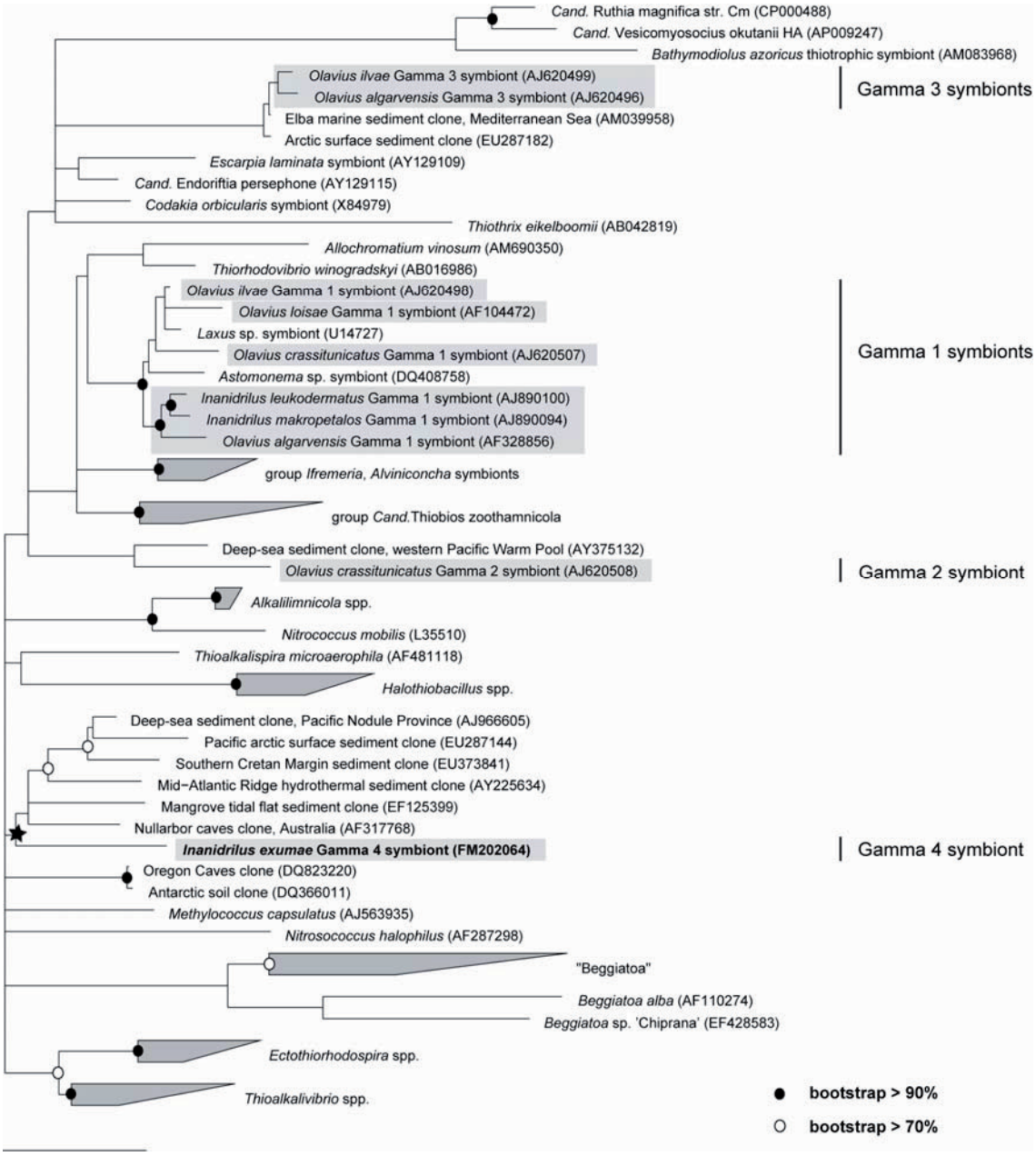

Fig. 4. Phylogenetic analysis of the gammaproteobacterial symbiont of *I. exumae* based on 16S rRNA gene sequences. The asterisk indicates a node that was well supported in the majority of the treeing calculations. Additional information see legend of Fig. 2.

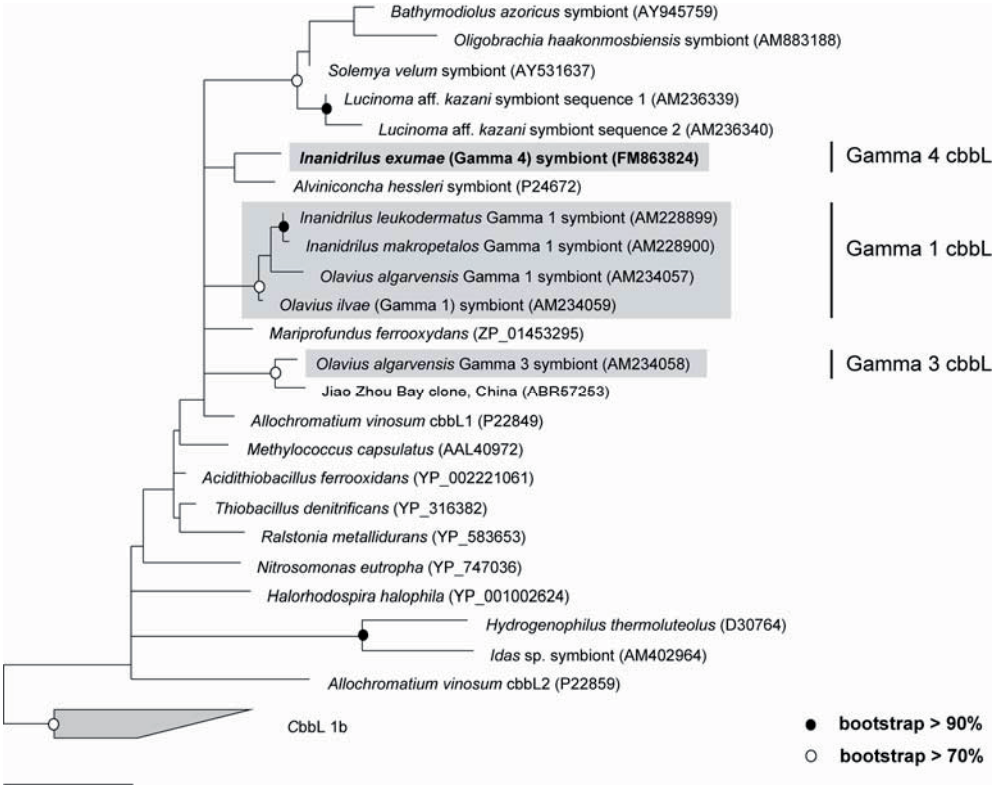

Fig. 5. Phylogenetic affiliation of the RubisCO form I *cbbL* gene from *I. exumae* based on deduced amino acid sequences. Additional information see legend of Fig. 2 and 3.

Symbiont replacement in I. exumae

What are the evolutionary models that can explain the presence of a novel sulfur-oxidizing symbiont and the absence of the ubiquitous Gamma 1 symbiont in *I. exumae*? The Gamma 1 symbionts are present in all 16 gutless oligochaete species examined to date from habitats around the world, including 9 host species from the Bahamas (Dubilier et al., 2006) of which some co-occur with *I. exumae* (Erséus, 2003). All Gamma 1 symbiont 16S rRNA sequences are closely related to each other and fall within a monophyletic clade in all phylogenetic analyses. The oligochaete hosts are also monophyletic, based on morphological (Erséus, 1984, 1992) and molecular data (Nylander et al., 1999; Sjölin et al., 2005). The phylogeny of the Gamma 1 symbionts is congruent with that of their hosts (Dubilier et al., 2006), indicating that these have cospeciated over an extended evolutionary time. Together these results support the conclusion that the Gamma 1 symbionts are ancestral to the gutless oligochaete associations, that is, the ancestral oligochaete host was associated with a Gamma 1 symbiont.

The phylogenetic position of *I. exumae* within the gutless oligochaetes is not deep-branching or basal (Erséus, unpublished data), indicating that this species is not primitive, or ancestral to all other gutless oligochaetes. It is therefore likely that the immediate ancestor of *I. exumae* also harbored a Gamma 1 symbiont, given the ubiquity

of this symbiont in all other host species and its presumably ancestral state. How then could the Gamma 4 symbiont have replaced the Gamma 1 symbiont in these hosts?

Two modes of symbiont transmission appear to co-exist in gutless oligochaetes. Morphological studies indicate that at least some of the symbionts are transmitted vertically from one generation to the next (Giere and Langheld, 1987; Krieger, 2000). Metagenomic analyses of the symbiont genomes of the gutless oligochaete provide support that the Gamma 1 symbiont is transmitted vertically based on an unusually high proportion of transposable elements in the Gamma 1 genome (Woyke et al., 2006). High numbers of transposons in symbionts indicates that these are in transition to an obligate endosymbiotic lifestyle with vertical transmission (Plague et al., 2008). The secondary symbionts of *O. algarvensis* do not have such high numbers of transposable elements and their genomes are not reduced, indicating that these may be taken up horizontally from the environment (Woyke et al., 2006). The egg-laying behavior of gutless oligochaetes in which these are deposited into the surrounding sediment would certainly provide ample opportunities for free-living bacteria to invade the egg (Giere and Langheld, 1987).

The coexistence of both vertical and horizontal symbiont transmission in gutless oligochaetes could explain how these gain novel symbionts. We postulate the following scenario: the ancestor of *I. exumae* originally harbored a Gamma 1 symbiont but was invaded by the free-living ancestor of the Gamma 4 symbiont at some point. It is possible that the Gamma 1 and 4 symbionts then co-existed in *I. exumae* for some time. In other gutless oligochaetes, Gamma 1 symbionts co-exist with secondary sulfur-oxidizing Gammaproteobacteria, called Gamma 2 and 3 symbionts (Blazejak et al., 2005; Ruehland et al., 2008). However, these secondary sulfur-oxidizing symbionts are much smaller than the Gamma 1 symbionts and therefore do not appear to compete for space, with the smaller Gamma 2 and 3 symbionts occurring in small pockets between the large Gamma 1 symbionts. Also, they appear to differ functionally: the Gamma 1 symbionts store sulfur in large vesicles in their cells while the Gamma 2 and 3 symbionts do not appear to have these sulfur vesicles (Giere and Erseus, 2002). In contrast, the Gamma 1 and the Gamma 4 symbionts are both large and both store sulfur. It is therefore possible that these competed for space and metabolic resources in *I. exumae*. The novel Gamma 4 symbiont may have been able to 'out-compete' the Gamma 1 symbiont, for example through a higher versatility of sulfur oxidation because of its two APS reductase enzymes. Alternatively, the replacement of the Gamma 1 by the Gamma 4 symbiont could be a mere coincidence caused by population bottlenecks during vertical transmission in which only a few or no Gamma 1 but many Gamma 4 symbionts were transmitted.

The exchange or replacement of symbionts is not uncommon in nature and was recently described in the chemosynthetic associations between vesicomyid deep-sea clams and their sulfur-oxidizing symbionts (Stewart et al., 2008). Vesicomyid clams

harbor only a single intracellular symbiont that is transmitted vertically and has a reduced genome (Kuwahara et al., 2007; Newton et al., 2007). Vesicomyid symbiont and host phylogeny is generally congruent, indicating cospeciation in these associations (Peek et al., 1998). However, even within this group of hosts with a long evolutionary history of vertical symbiont transmission, there is evidence for occasional instances of lateral symbiont transfer in some host species (Stewart et al., 2008). Symbiont replacement has also been described in insect symbioses where it has occurred occasionally in some insect lineages (Lefèvre et al., 2004) and regularly in others (Fukatsu and Ishikawa, 1996; Currie et al., 2003). As with *I. exumae*, the evolutionary processes that lead to these symbiont replacements are not well understood and are therefore often speculative. More insight in how this symbiont replacement came about could be gained from the sequencing of the Gamma 4 symbiont genome and its comparison with the Gamma 1 symbiont genome to reveal the similarities and differences between these phylogenetically distinct yet physiologically similar symbionts.

Acknowledgements

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Table 2. Symbiont-specific and general oligonucleotide probes used in this study.

Probe	Target and specificity[a]	Probe sequence (5'-3')	Position[b]	FA [%][c]	Reference
NON338	Antisense, background control	ACTCCTACGGGAGGCAGC	338-355	10-30	Wallner et al., 1993
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	1027-1043[d]	30 - 35	Manz et al., 1998
DSS658	***I. exumae* Delta 3, Delta 9**, *O. algarvensis* and *O. ilvae* Delta 1 symbiont, Delta 3 symbiont, *O. algarvensis* Delta 4 symbiont, *Desulfosarcina* spp., *Desulfofab*a sp., *Desulfococcus* spp., *Desulfofrigus* spp.	TCCACTTCCCTCTCCCAT	658-685	50 - 60	Manz et al., 1998
IexuGAM4	***I. exumae* Gamma 4**	ATTCCGCCTCCCTCTACCGTA	657-677	50	this study
IexuALFd	***I. exumae* Alpha 1a**, *O. loisae* Alpha 1a-1 and Alpha 1a-2 symbiont , *I. leukodermatus* Alpha 1a symbiont	GTACCCGGCCAAACCCGA	1131-1147	30	this study
ImakALF1b	***I. exumae* Alpha 2a**, *I. makropetalos* Alpha 2 symbiont	TCCGGTCTCCGCGACCCC	999-1014	35	Blazejak et al., 2006
IexuALFb	***I. exumae* Alpha 2b**, DQ062742, EU133383, AJ810382, AY326603, DQ648967	TCTGGTCTCCGCGACCGG	999-1014	30	this study
Oalg/OilvDEL3	***I. exumae* Delta 3**, *O. algarvensis* and *O. ilvae* Delta 3 symbiont	GTGCCTGCCTCCTGAAAG	1449-1465	30	Ruehland et al., 2008
OalgDEL4	***I. exumae* Delta 9**, *O. algarvensi*s Delta 4 symbiont, AB121109, EF061975, DQ395063, EU290686, EU290687, DQ395004, DQ394892,	GCCCAACAACTTCCGGTA	1427-1444	30	Ruehland et al., 2008

[a] symbionts and accession number for environmenta clones

[b] position in the 16S rRNA of *Escherichia coli*

[c] Percent formamide (FA) concentration (v/v) used in the CARD-FISH hybridization buffer

[d] position in the 23S rRNA of *E. coli*

Material and Methods

Site description and specimen collection

Inanidrilus exumae specimens were collected from shallow water sediments off Lee Stocking Island, Bahamas, in April 1999. *I. exumae* co-occurred with several other gutless oligochaete species in a water depth of about 3 m in sediments that were largely composed of fine calcareous sands (Erséus, 2003). The worms were extracted by decantation and identified under a microscope. Nine specimens were fixed for DNA extraction in 80% ethanol and for FISH and TEM as described previously (Dubilier et al., 1995; Blazejak et al., 2005). Samples were stored at 4°C.

DNA preparation and PCR amplification

For DNA extraction and subsequent PCR, individual worms were prepared singly. Specimens were rinsed in MilliQ water, and DNA was isolated as described by Schizas et al. (1997). In short, proteinase K was used for digestion and the reagent GeneReleaser (BioVentures, Murfreesboro, Tenn) for DNA purification.

The bacterial 16S rRNA genes were amplified with primers specific for the bacterial 16S rRNA gene 8F and 1492R (Muyzer et al., 1995) using Taq DNA polymerase (Eppendorf, Hamburg, Germany). The 16S rRNA genes of individuals *I. exumae* 1 and 2 were amplified applying the reconditioning approach (Thompson et al., 2002; Acinas et al., 2004) under the following conditions: initial denaturation at 96°C for 5 min, 15+5 and 15+7 cycles for *I. exumae* 1 and *I. exumae* 2, respectively at 96°C 1 min, 44°C 2 min and 72°C 3 min, followed by a final elongation of 10 min at 72°C. PCR conditions for *I. exumae* 3, 4 and 5 were as described previously (Blazejak et al., 2006), PCR conditions for *I. exumae* 6 and 7 were: initial denaturation at 94°C for 5 min, 30 cycles at 94°C 1 min, 42°C 1.5 min and 72°C 2 min, followed by a final elongation of 30 min at 72°C. PCR were carried out between 2003 and 2008, and varying PCR conditions are due to improvements made during this time.

Genes coding for the RubisCO form I and APS reductase were PCR amplified with 30 and 33 cycles, respectively. The following primers were used: cbbLF (5'-CACCTGGACCACVGTBTGG-3') and cbbLR (5'-CGGTGYATGTGCAGCAGCAT ICCG-3') for *cbbL* (Blazejak et al., 2006) and aps1F (5'-TGGCAGATCATGATYMA YGG-3') and aps4R (5'-GCGCCAACYGGRCCRTA-3') for *aprA*, with the annealing temperature for *aprA* at 60°C and 48°C for *cbbL* (Blazejak et al., 2006).

Cloning and sequencing

PCR products for all genes (16S rRNA, *cbbL* and *aprA*) were cloned separately for each individual using the pCR®4-TOPO® plasmids and TOP10 chemo competent cells (Invitrogen, Carlsbad, CA) according to the manufacturers protocol. Clones were randomly picked, resuspended in PCR grade water and selected for the correct insert size by PCR with vector primers. Sequencing reactions were run using ABI BigDye on

an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned and compared using the BioEdit program (www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were grouped together in a clone group if they shared more than 99% sequence identity (percent identical nucleotides). For each host individual, representatives of each clone group were fully sequenced in both directions.

Phylogenetic analyses

Sequences were checked with BLAST (Altschul et al., 1997; Tatusova and Madden, 1999) for similarity searches. Chimeras were identified using CHIMERA_CHECK from the Ribosomal Database Project (RDP) (Cole et al., 2003) and manually in sequence alignments, and were excluded from further analysis.

For final sequence alignment and phylogenetic tree reconstruction, the ARB software package (Ludwig et al., 2004) and the rRNA database SILVA SSU Ref, release_94 July 2008 (Pruesse et al., 2007) were used. Only nearly full-length 16S rRNA sequences including outgroup sequences were considered for tree calculation (>1200 bp). Sequence similarity of the nucleotide sequences was calculated by distance matrix analysis excluding the primer region.

Phylogenetic trees for 16S rRNA gene sequences were calculated with the neighbour-joining (with Jukes Cantor and Felsenstein correction) and maximum-likelihood (PHYML with HKY and GTR) tools provided within the ARB software package and combined with filters excluding highly variable regions. In addition, the bootstrapped maximum likelihood algorithm RAxML (Stamatakis et al., 2008) was used. Trees for alpha-, gamma- and deltaproteobacterial symbionts were calculated separately with 227, 306 and 244 sequences, respectively, including outgroup sequences of the *Bacteroidetes* group. Consensus trees were built based on an ML tree.

The phylogeny of the *aprA* and *cbbL* genes was generated from partial sequences of deduced amino acid sequences with 130 and 230 compared amino acids positions and 338 and 99 sequences, respectively, including outgroup sequences. Maximum-likelihood (ProML with JTT and PAM, RAxML with JTT) and neighbour-joining (Kimura) analyses were performed with a 25% amino acid frequency filter. Branching patterns that were not supported by most analyses are shown as multifurcations (see Fig. 2 - 5).

FISH

Two *I. exumae* individuals were fixed and prepared for FISH as described previously (Blazejak et al., 2005) with slight modification replacing xylol with Roti®-Histol (Carl Roth, Karlsruhe, Germany). Symbionts were detected by CARD (catalyzed reporter deposition) FISH with horseradish peroxidase (HRP)-labelled probes and tyramide signal amplification as described before with slight modifications (Pernthaler and

Pernthaler, 2007). Tissue sections were hybridized with the HRP-labelled probe for 2.5 h at 46°C. After washing for 15 min at 48°C in washing buffer, the sections were equilibrated for 20 min at room temperature in phosphate-buffered saline (PBS, pH 8.0). The moist tissue sections were incubated with amplification solution (1x PBS pH 8.0, 2 M NaCl, 0.1% Blocking Reagent in 100 mM maleic acid buffer pH 7.5, 0.0015% [vol/vol] H_2O_2; and 1% Alexa Fluor 488, 546, or 633 dye [Molecular Probes, Leiden, The Netherlands]) for 30 min at 46°C in the dark and rinsed in PBS buffer for at least 20 min at room temperature. After air drying, tissue sections were embedded in the mounting fluid Vecta Shield (Vecta Laboratories, Burlingame, CA) and stored for microscopic evaluation at -20°C until analysis. For dual and triple hybridizations, the CARD-FISH protocol was repeated two or three times on the same sections using different probes and Alexa dyes. To inactivate the HRP after each hybridization round, tissue sections were covered with 0.01 M HCI for 10 min at room temperature after the last washing step. Following another washing step for 3 min in sterilized water, tissue sections were hybridized as just described.

Oligonculeotide probes and formamide concentrations used in this study are listed in Table 2. Probes designed with ARB were checked for in silico specificity against sequences in GenBank using the BLAST search algorithm, and against rRNA sequence databases using ProbeCheck (Loy et al., 2008). The specificity was also tested experimentally against mismatch 16S rRNA sequences of either reference strains or symbionts. General probes for *Bacteria* (EUB338 I-III), *Gammaproteobacteria* (GAM42a,) and a subgroup of the *Deltaproteobacteria* (DSS658) were used as positive controls, and the antisense probe NON338 was used as a negative control. All hybridizations were performed at formamide concentrations ensuring the highest possible specificity.

TEM

The TEM-fixed *I. exmuae* worms were washed in a 0.05 M NA-cacodylatesolution and post-fixed in osmiumtetroxide. After dehydration in an acetone series, specimens were embedded in Spurr resin and sectioned on an ultramicrotome. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 902A (Dubilier et al., 1995).

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Single-cell analysis of autotrophic carbon fixation in the symbiotic community of the gutless marine worm *Olavius algarvensis* using nanoSIMS

Claudia Bergin, Christian Lott, Lubos Polerecky, Niculina Musat,
Tomas Vagner, Marcel M. M. Kuypers, Nicole Dubilier

Manuscript in preparation

Single-cell analysis of autotrophic carbon fixation in the symbiotic community of the gutless marine worm *Olavius algarvensis* using nanoSIMS

C. Bergin[1‡], C. Lott[1,2‡], L. Polerecky[3], N. Musat[4],
T. Vagner[4], M.M.M. Kuypers[4], N. Dubilier[1*]

[1]Symbiosis Group
Max Planck Institute for Marine Microbiology
Bremen, Germany

[2]HYDRA Institute for Marine Sciences
Elba Field Station
Campo nell'Elba (LI), Italy

[3]Microsensor Group
Max Planck Institute for Marine Microbiology
Bremen, Germany

[4]Nutrient Group
Max Planck Institute for Marine Microbiology
Bremen, Germany

‡ Authors contributed in equal parts to this publication

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Running title: single-cell view on symbiont autotrophy

Abstract

The marine gutless oligochaete worm *Olavius algarvensis* harbors a phylogenetically and metabolically diverse community of co-occurring bacterial endosymbionts. Based on biochemical and molecular analyses, two symbionts are chemoautotrophic sulfur oxidizers, the Gamma 1 symbiont, common to nearly all gutless oligochaetes, and a secondary symbiont called Gamma 3. Earlier studies showed that the Gamma 1 symbionts store sulfur, which was suggested to serve as energy source for autotrophic carbon fixation. These studies, however, could not resolve if the Gamma 3 symbiont can store sulfur. In this study we followed the autotrophic carbon fixation and carbon transfer in the *O. algarvensis* symbiosis at the single-cell level to identify the autotrophic symbiont population. Live worms were incubated with ^{13}C-labelled inorganic carbon under oxic conditions without an external electron donor for up to 25 h. By combining high resolution mass spectrometry (nanoSIMS) with in situ hybridization we were able to identify the symbionts that incorporated ^{13}C. Only the Gamma 1 symbionts fixed carbon under the conditions used, and we propose that internally stored sulfur served as energy source. Carbon fixation rates of the Gamma 1 symbionts showed a high variability, indicating physiological differences between cells. Co-occurring symbionts, including the Gamma 3, were slightly enriched only after 25 h. This suggests that the Gamma 3 symbionts are not able to store and use sulfur as energy source for autotrophic carbon fixation under the experimental conditions. Host tissue remained unlabelled even after 25 h. It is therefore unlikely that symbiont "milking", in which organic compounds are transferred to the host, plays a role in *O. algarvensis*, as this mode of nutritional transfer occurs within hours in other symbioses. Instead, a slow lysis and digestion of the symbionts may be the main mode of nutrient transfer.

Introduction

Marine gutless oligochaetes of the genera *Olavius* and *Inanidrilus* are a widespread group of invertebrates that live in obligate symbiosis with a phylogenetically and metabolically diverse bacterial endosymbiont community. The symbiosis is driven by chemoautotrophy indicated by the fast uptake and incorporation of radiolabelled inorganic carbon (Giere *et al.,* 1988), and enzyme activities diagnostic of carbon fixation and sulfur oxidation (Felbeck *et al.,* 1983). This chemoautotrophic activity was assigned to the primary symbiont, a large sulfur-storing morphotype common to nearly all gutless oligochaetes studied, based on morphological, molecular and biochemical analyses (Blazejak *et al.,* 2006, Dubilier *et al.,* 2001, Giere & Krieger 2001, Krieger *et al.,* 2000, Ruehland *et al.,* 2008, Woyke *et al.,* 2006). The primary symbionts of the different host species form a monophyletic clade within the *Gammaproteobacteria*, the so-called Gamma 1 clade and are closely related to free-living sulfur oxidizers of the family *Chromatiaceae* (Ruehland *et al.,* 2008).

Despite these indications for chemoautotrophy in gutless oligochaetes very little is known about the physiology of this symbiosis. Studies are rare and limited to a single host species, *I. leukodermatus* from Bermuda (Felbeck *et al.,* 1983, Giere *et al.,* 1988). Giere *et al.* (1988) could show that inorganic carbon was taken up by the *I. leukodermatus* symbiosis in the absence of externally provided electron donors indicating the use of stored sulfur as energy source. Within the *I. leukodermatus* symbiont community the primary Gamma 1 symbiont is presumably the only chemoautotrophic sulfur-oxider (Blazejak *et al.,* 2006), and the observed carbon uptake might be solely assigned to this symbiont.

Olavius algarvensis harbours a second gammaproteobacterial, chemoautotrophic sulfur oxidizer co-occurring with the Gamma 1 symbiont, based on recent PCR-based and metagenomic studies (Ruehland *et al.,* 2008, Woyke *et al.,* 2006). This secondary, so-called Gamma 3 symbiont is only distantly related to the Gamma 1 symbiont and much smaller in size, but can be as abundant as the Gamma 1 symbiont (Ruehland *et al.,* 2008). It is not clear, if the Gamma 3 symbiont stores sulfur. Vesicles of unknown composition were observed in ultrastructural analyses within small symbiont cells of *O. algarvensis* (C. Lott, unpublished observation).

In this study we analyzed the uptake and transfer of labelled inorganic carbon in the *O. algarvensis* symbiosis under oxic conditions without externally supplied energy sources to investigate, wether both the Gamma 1 and the Gamma 3 symbionts can use internal energy sources for autotrophic carbon fixation. The incorporation of labelled inorganic carbon was studied with bulk carbon isotope analyses. In addition we applied a novel approach that combines high resolution secondary ion mass spectrometry (nanoSIMS) with specific halogen in situ hybridization (HISH) (Musat *et al.,* 2008). This combination allowed us to distinguish between the Gamma 1 and Gamma 3 symbiont and to analyze inorganic carbon uptake for individual symbiont cells. We were thus able to not only deduce symbiotic carbon fixation from bulk analyses, but to show single-cell activity within a symbiont community.

Material and methods

O. algarvensis worms were collected off the coast of the island of Elba, Mediterranean Sea, and used for incubation experiments within 48 h. The worms were transferred to carbon-free oxygenated artificial seawater. The only carbon source was ^{13}C-labelled bicarbonate and was added to the medium immediately prior to the incubation. No electron donor was present in the medium. Incubations were run for 25 h with ten time points for bulk carbon isotope analyses (10, 20, 30 min, 1, 2, 4, 6, 8, 16 and 25 h) and three time points for single cell analyses (10 min, 2 h and 25 h). Worms for single cell analyses were either embedded and sectioned or homogenized. Sections and homogenates were placed on filters and symbionts were hybridized via catalyzed

reporter deposition fluorescence in situ hybridization (CARD-FISH) according to Pernthaler *et al.* (2002) using a halogenated reporter molecule (HISH) (Musat *et al.,* 2008).

Uptake of ^{13}C-inorganic carbon by individual cells was analyzed with nanoSIMS. Briefly, secondary ion images of naturally abundant $^{12}C^{14}N^-$ for the biomass, of $^{12}C^-$ and $^{13}C^-$ for calculation of the $^{13}C/^{12}C$ ratio, and of $^{19}F^-$ for the HISH signal were recorded simultaneously. Regions of interest were drawn manually around individual cells, and by image analysis based on the in situ ^{19}F signal that was used as a mask. For each region of interest the $^{13}C/^{12}C$ ratio was calculated and compared with the natural $^{13}C/^{12}C$ ratio. Carbon uptake rates were estimated for individual symbiont cells based on the $^{13}C/^{12}C$ ratios, an estimated carbon content of the cells, and the mean cell biovolume. Details can be found in the supplemental materials.

Results

Bulk carbon isotope analyses of whole *O. algarvensis* worms showed enrichment in ^{13}C by 0.11% ± 0.03 over the untreated control already after 10 min incubation (Table 1). The ^{13}C enrichment of the worms increased to 2.57% ± 0.49 after 25 h. The initial carbon uptake rate of about 41 nmol h^{-1} per mm^{-3} worm after 10 min decreased to 15 nmol h^{-1} per mm^{-3} worm after 4 h and remained at this rate (Table 1 and Figure 1).

In addition to bulk measurements, we analyzed the uptake of inorganic carbon in individual symbiont cells by nanoSIMS. Several symbionts in worm homogenates were already slightly enriched in ^{13}C after 10 min incubation (Figure 1 and 2). After 25 h, many symbiont cells were strongly labelled with ^{13}C, while other cells were unlabelled or only slightly enriched. Applying HISH using group- and symbiont-specific probes showed that only the Gamma 1 symbionts incorporated the labelled inorganic carbon already after 10 min and the majority of the Gamma 1 symbionts were highly enriched in ^{13}C after 25 h (Figure 2).

The ^{13}C enrichment of Gamma 1 symbionts increased from 0.14% ± 0.07 after 10 min incubation to up to 24% for individual cells after 25 h (average 12.55% ± 6.83, Table 1) with a high cell-to-cell variability. The initial uptake rates decreased slightly from 124 ± 61 amol h^{-1} per Gamma 1 cell after 10 min to 77 ± 42 amol h^{-1} per cell after 25 h (Table 1 and Figure 1). Very similar results were obtained when analysing symbiont ^{13}C-incorporation in tissue sections (not shown). By using the HISH signal as a mask over the $^{13}C/^{12}C$ ratio we could distinguish enriched Gamma 1 symbionts from unlabelled Gamma 1 cells, which is also evident from a pixel histogram showing two distinct peaks (Figure 3).

Table 1. ^{13}C enrichment and inorganic carbon uptake rates for *O. algarvensis* worms and individual Gamma 1 symbionts. Mean and standard deviation are given for three time points of the 25 h incubation experiment. In parentheses the number of replicates for bulk analyses and the number of individual cells analyzed (taken from two individually homogenized worms) is given.

	Whole worms		Gamma 1 symbionts	
	^{13}C excess [%]	Uptake rate [nmol C (mm worm)$^{-3}$ h^{-1}]	^{13}C excess [%]	Uptake rate [amol C cell^{-1} h^{-1}]
10 min	0.11 ± 0.03 (5)	41.1 ±13.7 (5)	0.14 ± 0.07 (55)	124 ± 61 (55)
2 h	0.50 ± 0.12 (5)	22.7 ± 6.8 (5)	0.77 ± 0.47 (41)	59 ± 35 (41)
25 h	2.57 ± 0.49 (5)	12.3 ± 2.5 (5)	12.55 ± 6.83 (50)	81 ± 35 (50)

The in situ hybridization signal of the Gamma 3 symbiont-specific probe was not strong enough for identification in nanoSIMS analyses. Nevertheless, we could distinguish the small Gamma 3 cells from co-occurring symbionts by applying the gammaproteobacterial probe GAM42a, and from the large Gamma 1 symbionts by cell size and shape in homogenized samples (Figure 2). Indeed, Gamma 3 symbionts were slightly labelled with ^{13}C after 25 h incubation (preliminary data). Identification of the Gamma 3 symbionts was not possible in tissue sections.

The symbiont-containing region in *O. algarvensis* was heavily enriched after 25 h incubation. However, host tissue close to and further away from the symbionts remained unlabelled during the length of the incubation.

Discussion

The *O. algarvensis* symbioses quickly incorporated inorganic carbon in the absence of external energy sources, and we were able to identify the Gamma 1 symbionts as the only symbionts that immediately fixed carbon at high rates under the conditions used. This indicates that the energy for inorganic fixation must have been provided internally and we suggest that the stored sulfur was being used as such. Sulfur as energy source for autotrophy has been shown for free-living sulfur-storing bacteria by growth in the absence of external reduced sulfur compounds (Otte *et al.*, 1999), and was suggested for marine chemoautotrophic symbioses as well. However, carbon uptake rates without external reduced sulfur compounds were usually low in bivalve and tubeworm symbioses, and sulfide or thiosulfate were needed to obtain substantial carbon fixation rates (Belkin *et al.*, 1986).

Bulk uptake rates of inorganic carbon with stored sulfur as energy source showed higher rates for *O. algarvensis* with 53 to 150 µmol h^{-1} per g worm wet weight, (assuming a fresh weight of 150 µg per worm, Felbeck *et al.*, 1983) than for *I. leukodermatus* with about 30 µmol h^{-1} per g worm wet weight (Giere *et al.,* 1988). This difference might have been caused by different incubation conditions. Uptake rates

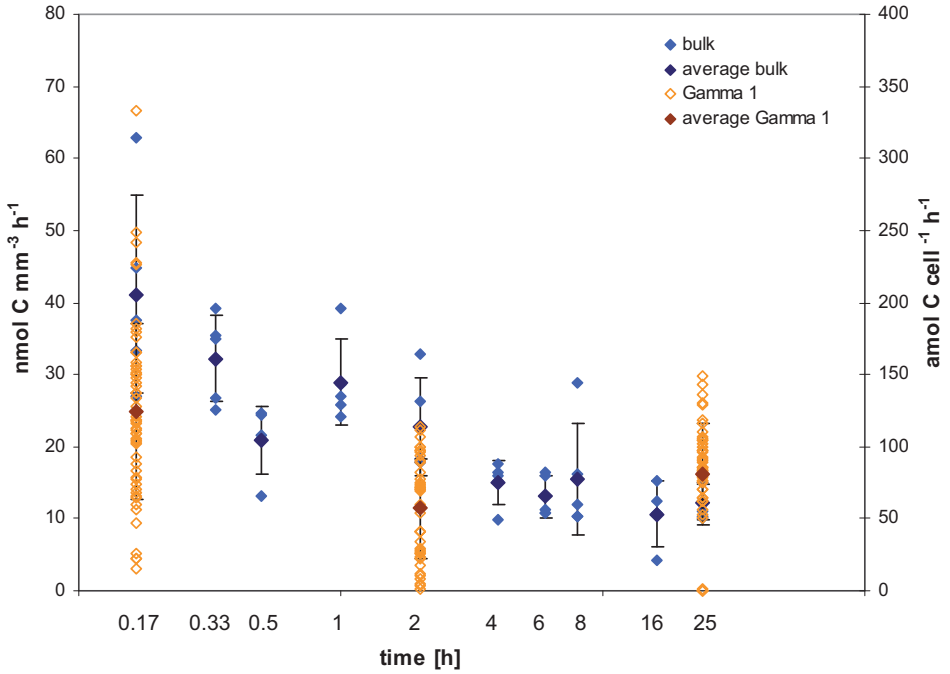

Figure 1. ^{13}C-inorganic carbon uptake rates of the *O. algarvensis* symbiosis under oxic conditions without an external energy source, shown for whole worms (bulk measurements), individual Gamma 1 symbiont cells, and bulk and single cell average uptake rates. X-axis in log scale.

were in the range of other marine chemoautotrophic symbioses which fixed carbon with up to 70 µmol C h^{-1} per g wet weight gill tissue in the presence of sulfide or thiosulfate (Scott & Cavanaugh 2007). Thus, gutless oligochaete symbioses achieve carbon uptake rates with internal energy sources, which are as high as those of chemoautotrophic symbioses supplemented with sulfide or thiosulfate. Unfortunately, direct comparison of uptake rates is difficult as carbon fixation rates depend on the incubation settings, on the sample investigated (symbiont enrichments, symbiont containing host tissue or whole animals), and on the normalization of the rates to mg protein, g wet weight, g dry weight or per animal.

When comparing carbon fixation rates per cell, the Gamma 1 symbionts had on average higher rates than other marine chemoautotrophic symbionts. Inorganic carbon uptake rates for the symbiont of the vent tubeworm *Riftia pachyptila* with sulfide as energy source were in the range of 2 – 25 amol h^{-1} per cell, based on rates per g tissue and estimated symbiont abundance (Cavanaugh *et al.,* 1981, Fisher *et al.,* 1989, Belkin *et al.*, 1986). Similarly, the *Solemya velum* symbiont fixed carbon at estimated 1.2 amol h^{-1} per cell (Cavanaugh, 1983), and the chemoautotrophic symbiont of the deep-sea mussel *Bathymodiolus thermophilus* with 8 – 18 amol h^{-1} per cell in the presence of thiosulfate (Belkin *et al.,* 1986). Uptake rates of the *O. algarvensis* Gamma 1 symbionts though might be slightly overestimated as the cell carbon content could only be assumed. Still, chemoautotrophic symbionts of different hosts showed comparable

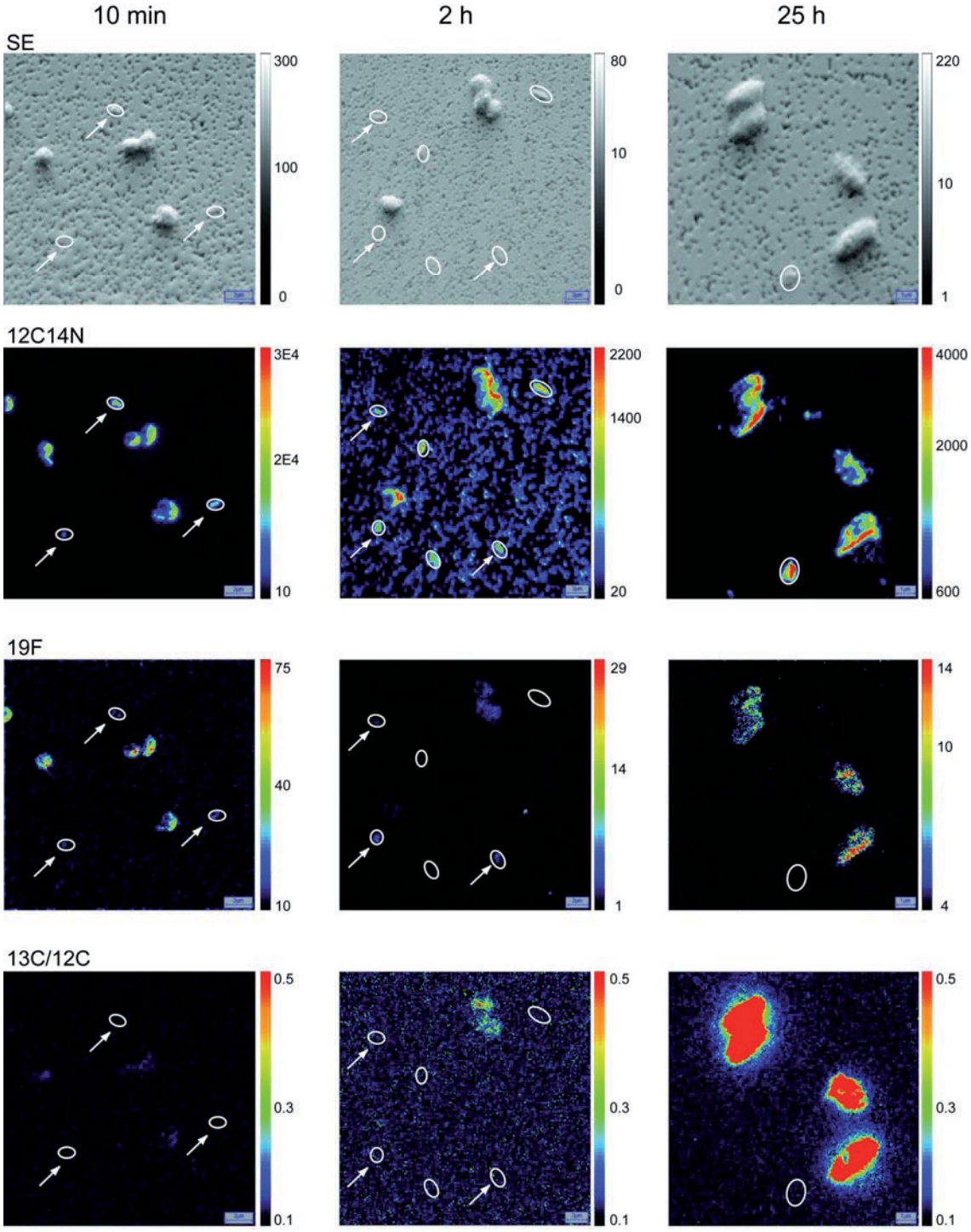

Figure 2. Uptake of ^{13}C-inorganic carbon by the *O. algarvensis* symbionts in the absence of external electron donors and organic carbon sources under oxic conditions. For each time point (from left to right: 10 min, 2 h and 25 h) parallel secondary electron and secondary ion images are shown: SE: secondary electron image; $^{12}C^{14}N$: biomass; ^{19}F: in situ hybridization signal; $^{13}C/^{12}C$: ^{13}C enrichment. The in situ hybridization targeted all symbionts (EUB338 probe, 10 min) and the gammaprotoebacterial symbionts (GAM42a probe, 2 h and 25 h). Large cells represent the Gamma 1 symbionts, circled cells mark Gamma 3 and deltaproteobacterial symbionts, and arrows indicate those cells that showed HISH signals. Scale bars: 0.2 µm (10 min and 2 h) and 1 µm (25 h). The color code gives the abundances of the masses analyzed ($^{12}C^{14}N$ and ^{19}F) and the ratio ($^{13}C/^{12}C$) with black = low and red = high.

carbon fixation rates. These rates though were lower than those of the free-living green sulfur bacteria *Lamprocystis purpurea* and *Chromatium okenii* which had uptake rates between 0.1 and 200 fmol $cell^{-1}$ h^{-1} and which have been analyzed recently with nanoSIMS (Musat *et al.,* 2008).

The high variability in ^{13}C uptake rates by individual Gamma 1 symbionts was intriguing. Single-cell variability in activity within a given bacterial population was observed for e.g. bacterial cultures (Strovas *et al.,* 2007 and references therein), free-living fresh-water bacteria (Musat *et al.,* 2008) and a marine chemoautotrophic symbiosis (Caro *et al.,* 2007). Such variability can be caused by genomic heterogeneity in phylogenetically identical populations (Jaspers & Overmann 2004). However, the Gamma 1 symbionts are obligate endosymbionts and thus are genetically more homogenous than free-living bacterial populations as indicated by a very low level of nucleotide polymorphisms in the metagenome of the *O. algarvensis* symbionts (Woyke *et al.,* 2006).

Non-genetic heterogeneity could be an alternative explanation for the observed variability (Tolker-Nielsen *et al*., 1998). Differences in their physiological state, including substrate availability such as oxygen access or sulfur content might have affected carbon uptake rates of individual Gamma 1 symbionts. Oxygen access though did most likely not differ for the individual symbionts. We assume that the oxygen penetrates the worm from the outside and enters the symbiont-containing region via the cuticle. Yet, we did not observe a gradient in carbon uptake for individual cells from the outer to the inner zone of the symbiont-containing region (Figure 3). The sulfur content for individual Gamma 1 symbionts could not yet be analyzed, but future studies will investigate single-cell sulfur content and its influence on inorganic carbon uptake rates. Other, unknown physiological differences, age or life history can also affect the performance of individual cells (Brookes *et al.,* 2000, Strovas *et al.,* 2007 and references therein).

The second chemoautotrophic sulfur-oxidizing symbiont of *O. algarvensis*, the Gamma 3 symbiont, showed a slight uptake of ^{13}C-labelled carbon after 25 h. The very slow uptake indicates that these symbionts were not autotrophic under the conditions used. The lack of a suitable electron donor might be one explanation. Thus, the Gamma 3 symbionts did not store sulfur and had no access to the sulfur stores of the Gamma 1 symbiont or intermediates transferred from the Gamma 1 symbiont. Other possible energy sources for autotrophy such as reduced sulfur compounds were not provided internally under these experimental conditions (Dubilier *et al.,* 2001, Woyke *et al.,* 2006). Thus, the incorporated carbon was most likely obtained via uptake of freshly synthesized organic carbon compounds transferred from the Gamma 1 symbionts, by heterotrophic carbon fixation, or both. This result corroborates the assumption that the two co-occurring chemoautotrophic sulfur-oxidizing symbionts of *O. algarvensis* are

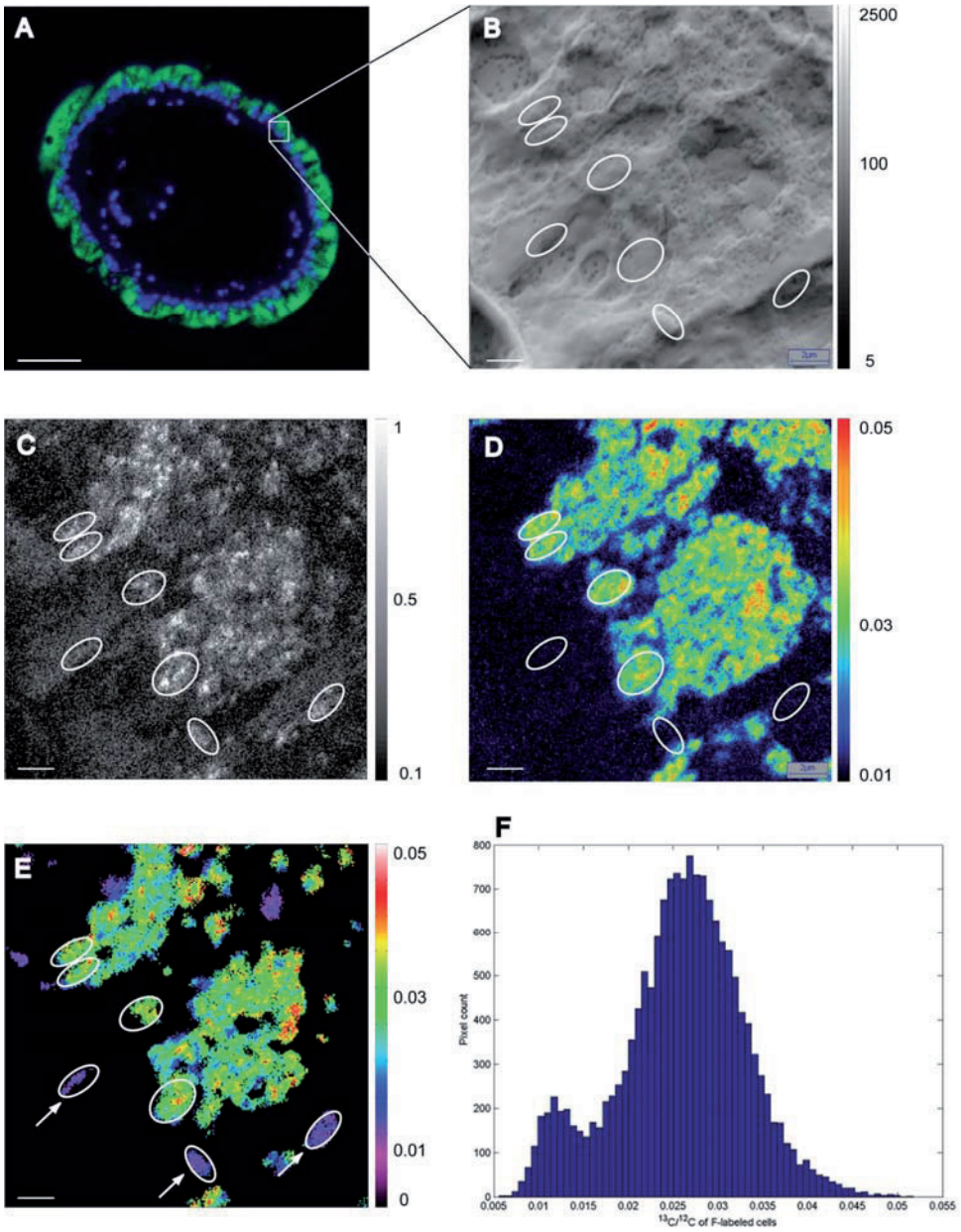

Figure 3. NanoSIMS analysis of the Gamma 1 symbionts embedded in the tissue of *O. algarvensis* after 2 h incubation with inorganic ^{13}C. (A) Fluorescenc in situ hybridization of a cross section through an entire worm showing the gammaproteobacterial symbionts (green) in the symbiotic region and host cell nuclei (blue). (B) Secondary electron image of the symbiont-containing region. (C) ^{19}F in situ signal of the gammaproteobacterial symbionts. (D) $^{13}C/^{12}C$ ratio shows clear ^{13}C enrichment. (E) The ^{19}F signal was used as a mask over $^{13}C/^{12}C$ showing that most symbionts identified by FISH incorporated ^{13}C, except for the cells marked by arrows (E). A clear discrimination between labelled and unlabelled cells is also seen in the histogram (F). Scale bars 50 µm (A), 2 µm in (B) – (E). Color code in D and E shows the ratio of ^{13}C over ^{12}C.

not autotrophic under the same environmental conditions and might fill different niches in this symbiosis (Woyke *et al.,* 2006).

It was surprising that there was no enrichment in ^{13}C of host tissue even after 25 h incubation. Assuming that the worm feeds on its symbionts, freshly fixed ^{13}C-labelled

carbon should be transferred from the Gamma 1 symbionts and incorporated into host tissue at some point of time, but this probably takes longer in the gutless worm symbiosis than the investigated 25 h. A slow transfer of carbon compounds between symbiont and host indicates that the feeding of the host occurs via symbiont lysis and digestion (Cavanaugh *et al.,* 2006, Fisher & Childress 1992) rather than by transfer of nutrients from the symbionts to the host, which is usually much faster and occurs within minutes to hours (Bright *et al.,* 2000, Fisher & Childress 1986, Popa *et al.,* 2007). Future studies will apply longer incubations to investigate the carbon transfer within the *O. algarvensis* symbiosis.

It is not clear why the inorganic carbon uptake rates of the *O. algarvensis* symbiosis decreased over time. It could not have been caused by a dilution effect of ^{13}C inorganic carbon with ^{12}C from atmospheric inorganic carbon, as this dilution effect was incorporated in our calculation. Also oxygen depletion could not be the cause as the worms were incubated under fully oxygenated conditions.

Alternative explanations for the decrease in inorganic carbon uptake rates are regulation of carbon fixation or decreased fitness of the symbiosis. Regulation of carbon fixation could occur, when internal sulfur levels reach a threshold. At this concentration inorganic carbon is then no longer fixed at maximal rates. However, sulfur content of individual worms remained constant over time, although the variability was high (C. Lott, unpublished). Fixation rates could also be down-regulated when enough carbon storage compounds are synthesized. Finally, a decreased fitness of the symbiosis could have caused the lower uptake rates. Gutless oligochaetes spend most of their time in anoxic sediments and are assumed to move into the upper oxidized sediment layers only for brief periods. Thus, the extended time period in an oxic environment and the artificial incubation conditions might have led to an increased stress to the symbiosis and thus cause depressed inorganic carbon uptake rates.

Conclusion and outlook

Under oxic conditions the Gamma 1 symbionts in *O. algarvensis* fixed inorganic carbon at high rates by using stored sulfur as energy source with high cell-to-cell variability indicating different physiological states of the cells. The second chemoautotrophic symbiont of *O. algarvensis* did not fix inorganic carbon under the conditions used possibly due to lack of energy sources under the conditions used. Carbon transfer to the host did not occur within 25 h, thus symbiont lysis and digestion by the host might be the way of nutrition.

For the first time in marine chemoautotrophic symbiosis research we could not only deduce symbiotic autotrophy from bulk analyses, but identify a chemoautotrophic symbiont and analyze its specific uptake of labelled inorganic carbon at the single-cell level. We were also able to calculate carbon uptake rates for individual cells without

cultivation or enrichment of the symbionts. We can now study the function of the different symbiont populations in gutless oligochaetes and address question such as which energy sources and electron acceptors are used and support autotrophy, and which carbon or nitrogen sources are taken up by which of the symbiont populations.

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Supplemental materials

Sampling

Individuals of *Olavius algarvensis* were sampled off the coast of the island of Elba, Italy, in a water depth of about 5 – 6 m by Scuba diving. Three species of gutless oligochaetes co-occur at this site, *Olavius algarvensis*, *Olavius ilvae* and a third species that has not been named yet. For our experiments we did not differentiate between those three species, since the third species was found only in low abundances, while the other two share similar symbionts and should therefore show comparable metabolic features. Only intact, white and actively moving worms were chosen for all experiments.

Incubation

Prior to the incubation, all worms used were kept fully anoxic over night. The worms were then washed carefully in carbon-free artificial seawater (ASW: 0.756 mmol/l KBr, 8.05 mmol/l KCl, 10 mmol/l $CaCl_2$, 27.89 mmol/l $MgCl_2$, 27.6 mmol/l $MgSO_4$, 451 mmol/l NaCl, 4.67 µmol/l NH_4Cl, 1.47 µmol/l KH_2PO_4,). Incubations were done in ASW with 2.5 mmol/l ^{13}C-labelled sodium bicarbonate as sole carbon source (98 atom % ^{13}C, Sigma Aldrich Inc.) for 25 h. Time series were run with the following ten time points: 10, 20, 30 min, 1, 2, 4, 6, 8, 16, and 25 h plus a control (t = 0). For each time point samples were collected for bulk carbon and nanoSIMS analyses. Worms were checked for integrity after incubation. The length and diameter of each worm was measured with ImageJ (National Institutes of Health, USA) except for those worms used for nanoSIMS analysis.

Samples for carbon bulk analyses were placed in silver cups with three worms combined as one replicate, and stored at -20°C. Samples for nanoSIMS analyses were treated as described below.

Carbon bulk analysis

The abundance of ^{13}C was analyzed applying standard techniques (Musat *et al.,* 2008). Five replicates per time point were analyzed, consisting of three worms each.

Correction for dilution

Subsamples were taken from the incubation medium after 10 min, 2 h and 25 h for isotope analysis of total dissolved inorganic carbon in the medium. All ^{13}C uptake values were corrected for the observed dilution of the ^{13}C label in the medium over time.

In situ hybridization of homogenates and tissue sections

Worms for nanoSIMS were washed in sterile seawater and either embedded as whole worms or homogenized immediately. Whole worms and homogenates were fixed in 4%

paraformaldehyde for 3 h, washed three times in sterile seawater and transferred into 50% ethanol (whole worms) for storage at 4°C, or stored in 70% ethanol at -20°C.
The whole worms were cut in three to four pieces and the pieces were embedded next to each other in Steedman's wax. Embedded samples were cut in 5 µm sections, placed on gold-palladium coated polycarbonate filters (Musat *et al.,* 2008) and de-waxed (Pernthaler & Pernthaler 2005). Aliquots of the homogenates were pipetted onto gold-palladium coated polycarbonate filters.
Catalyzed reporter deposition (CARD) fluorescence in situ hybridization (FISH) followed the protocol by Pernthaler *et al.* (2002) applying a tyramide that was synthesized from the fluorine-containing dye Oregon Green® 488-X (Molecular Probes, Inc.) (Musat *et al.,* 2008). The following oligonucleotide probes were used: EUB I-III (Daims *et al.,* 1999) as positive control, Gam42a (Manz *et al.,* 1998), NON338 (Wallner *et al.,* 1993) as negative control, and OalgGam1 and Oalg/OilvGam3 as symbiont specific probes (Ruehland *et al.,* 2008). All hybridized samples were quality checked for successful hybridization by their fluorescence signal under an epifluorescence microscope.

NanoSIMS analyses of worm homogenates and worm tissue sections
Single cell analyses were run on a nanoSIMS 50L (CAMECA, Paris, France) for hybridized worm homogenates and worm sections for three time points (10 min, 2 and 25 h). Samples were analyzed in January and May 2009. The primary ion beam had a size of approximately 150 nm and the sample was sputtered with a dwelling time of 1 ms per pixel. The primary current was 4.7 pA Cs^+ during acquisition for most images. Data generated with the nanoSIMS was processed with the CAMECA WinImage software (CAMECA), ImageJ, and Matlab (MathWorks, Inc.).

Calculation of biovolume and single cell carbon uptake rates
Symbiont cell biovolume was calculated based on ultrastructural analyses and mean symbiont sizes given in (Giere & Erséus 2002). Carbon uptake rates for the Gamma 1 symbionts were calculated using the $^{13}C/^{12}C$ ratios, the mean biovolume of the symbiont cells and an assumed carbon content of 4.5 fmol C/μm^3 (determined for *Lamprocystis purpurea* cultures, Musat *et al.,* 2008)).

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Part C - Supplemental Material

C.1 Comprehensive list of gutless oligochaete species

Today 62 *Olavius* and 26 *Inanidrilus* species are known to literature (May 2009). The following two tables list these species, their geographic distribution and respective references.

The genus *Olavius*

Species	Species description	Collection site	References
abrolhosensis	Erséus, 1997	E Indian Ocean, W Australia	Erséus, 1997a
albidoides	Erséus, 1997	E Indian Ocean, W Australia	Erséus, 1997a
albidus	(Jamieson, 1977)	S Pacific, Great Barrier Reef	Erséus, 1979b, 1984, Jamieson, 1977
algarvensis	Giere, Erséus & Stuhlmacher, 1998	NE Atlantic, Portugal	Giere *et al.*, 1998
		Mediterranean, Elba	Giere & Erséus, 2002
alius	Erséus, 1984	NW Atlantic, Bermuda	Erséus, 1984
amplectens	Erséus & Bergfeldt, 2007	S Pacific, New Caledonia	Erséus & Bergfeldt, 2007
avisceralis	(Erséus, 1981)	S Pacific, Great Barrier Reef	Erséus, 1981, 1984
		E Indian Ocean, W Australia	Erséus, 1997a
avitus	Erséus, 2003	Carribean Sea, Bahamas	Erséus, 2003
bullatus	Finogenova, 1986	S Pacific, off Peru	Finogenova, 1986
capillus	Erséus, 1997	E Indian Ocean, W Australia	Erséus, 1997a
caudatus	(Erséus, 1979)	NW Atlantic, Florida	Erséus, 1979b, 1984
clavatus	(Erséus, 1981)	S Pacific, Great Barrier Reef	Erséus, 1981, 1984
		Australia, Northern Territories	Erséus, 1997b and reference therein
		E Indian Ocean, W Australia	Erséus, 1993, 1997a and reference therein
comorensis	(Erséus, 1981)	W Indian Ocean, Comoro Island	Erséus, 1981, 1984
cornuatus	Davis, 1984	NW Atlantic, Massachusetts	Davis, 1984, Erséus, 1984
crassitunicatus	Finogenova, 1986	S Pacific, off Peru	Finogenova, 1986
curtus	Erséus, 2003	Carribean Sea, Bahamas	Erséus, 2003
fidelis	Erséus & Bergfeldt, 2007	S Pacific, New Caledonia	Erséus & Bergfeldt, 2007
filithecatus	(Erséus, 1981)	S Pacific, Great Barrier Reef	Erséus, 1981, 1984
finitimus	Erséus, 1990	Carribean Sea, Bahamas	Erséus, 2003
		Carribean Sea, Belize	Erséus, 1990a
fredi	Erséus, 1997	E Indian Ocean, W Australia	Erséus, 1997a
furinus	Erséus, 2003	Carribean Sea, Bahamas	Erséus, 2003
		NW Atlantic Florida, Atlantic coast	Erséus, 1979b, 2003
fusus	Erséus, 1993	E Indian Ocean, W Australia	Erséus, 1993

Table continued

Species	Species description	Collection site	References
geniculatus	(Erséus, 1981)	S Pacific, Great Barrier Reef	Erséus, 1981, 1984
		S Pacific, Fiji	Erséus, 1984
gierei	Erséus, 1997	E Indian Ocean, W Australia	Erséus, 1997a
hamulatus	Erseús, 1997	E Indian Ocean, W Australia	Erséus, 1997a
hanssoni	Erséus, 1984	S Pacific, Solomon Islands	Erséus, 1984
		E Indian Ocean, W Australia	Erséus, 1997a
ilvae	Giere & Erséus, 2002	Mediterranean, Elba	Giere & Erséus, 2002
imperfectus	Erséus, 1984	Carribean Sea, Bahamas	Erséus, 2003
		Carribean Sea, Belize	Erséus, 1984
		NW Atlantic, W coast Florida	Erséus, 1990a
isomerus	Erséus & Bergfeldt, 2007	SW Pacific, New Caledonia	Erséus & Bergfeldt, 2007
latus	Erséus, 1986	NW Atlantic, E coast Florida	Erséus, 1986a
lifouensis	Erséus & Bergfeldt, 2007	S Pacific, New Caledonia	Erséus & Bergfeldt, 2007
loisae	Erséus, 1984	S Pacific, Great Barrier Reef	Erséus, 1984
longissimus	(Giere, 1979)	Carribean Sea, Bahamas	Erséus, 2003
		Carribean Sea, Belize	Erséus, 1984, 1990a
		NW Atlantic, Bermuda	Erséus, 1984, Giere, 1979
macer	Erséus, 1984	NW Atlantic, Florida E coast GoM	Erséus, 1984
manifae	Erséus, 1986	Arabian Sea, Gulf Coast of Saudi Arabia	Erséus, 1985, 1986b
mokapuensis	Erséus & Davis, 1989	N Pacific, Hawai	Erséus & Davis, 1989
montebelloensis	Erséus, 1997	E Indian Ocean, W Australia	Erséus, 1997a
muris	Erséus, 2003	Carribean Sea, Bahamas	Erséus, 2003
nicolae	Erséus & Giere, 1995	Carribean Sea, Bahamas	Erséus, 2003
		Carribean Sea, Belize	Erséus & Giere, 1995
nivalis	Erséus & Bergfeldt 2007	S Pacific, New Caledonia	Erséus & Bergfeldt, 2007
paraloisae	Erséus & Bergfeldt 2007	S Pacific, New Caledonia	Erséus & Bergfeldt, 2007
parapellucidus	Erséus & Davis, 1989	N Pacific, Hawai	Erséus & Davis, 1989
patriciae	Erséus, 1993	E Indian Ocean, W Australia	Erséus, 1993, 1997a
pellucidus	Erséus, 1984	Carribean Sea, Barbados	Erséus, 1984
		Carribean Sea, Guadeloupe	Erséus, 1984
planus	(Erséus, 1979)	Carribean Sea, Bermuda	Erséus, 1979b, 1984
pravus	Erséus, 1990	Carribean Sea, Belize	Erséus, 1990a
prodigus	Erséus, 1993	E Indian Ocean, W Australia	Erséus, 1993
productus	Erséus, 1997	E Indian Ocean, W Australia	Erséus, 1997a
propinquus	Erséus, 1984	S Pacific, Fiji	Erséus, 1984, 2008
rallus	Erséus, 1991	NW Atlantic North Carolina	Erséus, 1991

Table continued			
Species	**Species description**	**Collection site**	**References**
rottnestensis	Erséus, 1993	E Indian Ocean, W Australia	Erséus, 1993
separatus	Erséus, 1993	E Indian Ocean, W Australia	Erséus, 1993
soror	Erséus, 2003	Carribean Sea, Bahamas	Erséus, 2003
sp.		Carribean Sea, Belize	Giere *et al.*, 1995
sp. B	Erséus, 1986	NW Atlantic, Florida	Erséus, 1986a
strigosus	Erséus & Davis, 1989	N Pacific, Hawai	Erséus & Davis, 1989
tannerensis	Erséus, 1991	NE Pacific California	Erséus, 1991
tantulus	Erséus, 1984	Carribean Sea, Bahamas	Erséus, 2003
		Carribean Sea, Belize	Erséus, 1984, 1990a
tenuissimus	(Erséus, 1979)	Carribean Sea, Bahamas	Erséus, 2003
		Carribean Sea, Belize	Erséus, 1984, 1990a
		NW Atlantic, Bermuda	Erséus, 1979b
		NW Atlantic: Florida, Atlantic and Golf coast, North Carolina, New Jersey, Massachusetts	Erséus, 1979b, 1984
ullae	Erséus, 2003	Carribean Sea, Bahamas	Erséus, 2003
ulrikae	Erséus, 2008	E Indian Ocean, W Australia	Erséus, 2008
vacuus	Erséus, 1990	Carribean Sea, Bahamas	Erséus, 2003
		Carribean Sea, Belize	Erséus, 1990a
		NW Atlantic E and W coast of Florida, Virginia	Erséus, 1986a, 1990a
valens	Erséus, 1997	E Indian Ocean, W Australia	Erséus, 1997a
verpa	Erséus, 1986	Arabian Sea, Gulf Coast of Saudi Arabia	Erséus, 1985, 1986b

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Species	**Species description**	**Collection site**	**References**
aduncosetis	Erséus, 1984	Carribean Sea, Belize	Erséus, 1990a
		NW Atlantic, Bermuda	Erséus, 1984
asagittatus	Erséus, 1997	E Indian Ocean, W Australia	Erséus, 1997a
belizensis	Erséus, 1984	Carribean Sea, Belize	Erséus, 1984, 1990a
bonomii	Erséus, 1984	Mediterranean Sea, Italy, Sicily	Erséus, 1984
bulbosus	Erséus, 1979	NW Atlantic Florida	Erséus, 1979a, b, 1984, 2003
carterensis	Erséus, 1984	S Pacific, Great Barrier Reef	Erséus, 1984
dutchae	Erséus & Davis, 1989	N Pacific, Hawai	Erséus & Davis, 1989
elaboratus	Erséus, 1990	E Indian Ocean, W Australia	Erséus, 1990b
ernesti	Erséus, 1984	NW Atlantic Florida Golf coast	Erséus, 1984, 1986a
extremus	(Erséus, 1979)	NW Atlantic Florida, E	Erséus, 1979b, 1984, 2003

Table continued			
Species	**Species description**	**Collection site**	**References**
exumae	Erséus, 2003	Carribean Sea, Bahamas	Erséus, 2003
falcifer	Erséus & Baker, 1982	Carribean Sea, Barbados	Erséus, 1984, Erseus & Baker, 1982
fijijensis	Erséus, 1984	S Pacific, Fiji and Solomon Islands	Erséus, 1984
gustavsoni	Erséus, 1984	Carribean Sea, Barbados	Erséus, 1984
leukodermatus	(Giere, 1979)	Carribean Sea, Belize	Erséus, 1984, 1990a
		NW Atlantic, Bermuda	Erséus, 1984, Giere, 1979
makropetalos	Erséus, 2003	Carribean Sea, Bahamas	Erséus, 2003
manae	Erséus, 1984	S Pacific, Fiji	Erséus, 1984
mexicanus	Erséus & Baker, 1982	NW Atlantic Florida, E and W coast	Erséus, 1984, Erseus & Baker, 1982
mojicae	Erséus, 2003	Carribean Sea, Bahamas	Erséus, 2003
reginae	Erséus, 1990	Carribean Sea, Belize	Erséus, 1990a
renaudae	Erséus, 1984	Carribean Sea, Guadeloupe	Erséus, 1984
scalprum	Erséus, 1984	Carribean Sea, Belize	Erséus, 1984, 1990a
speroi	Erséus, 1984	Carribean Sea, Bahamas	Erséus, 2003
		Carribean Sea, Barbados	Erséus, 1984
triangulatus	Erséus, 1984	Carribean Sea, Bahamas	Erséus, 2003
		NW Atlantic Florida	Erséus, 1984
vacivus	Erséus, 1984	NW Atlantic Florida Golf coast	Erséus, 1984, 1986a
wasseri	Erséus, 1984	S Pacific, Great Barrier Reef	Erséus, 1984

W – West; NE – Northeast; SW – Southwest; NW – Northwest; E – East; S – South; N – North.

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C.2 Incubation experiments

Collection and maintenance of the worms

Olavius sp. specimens (see foot note Chapter 2.2) were collected off the coast of the Italian island of Elba. The sampling sites with high worm densities were in the vicinity of sea grass beds. Worms were retrieved by scuba diving by co-workers of the HYDRA Institute for Marine Science in a water depth of about 5-6 m. Worms were maintained in plastic jars or buckets containing sediment and seawater from the sampling site at 18-23°C. Previous attempts to maintain worms in the lab were successful in keeping the worms alive for several months to over a year in plastic jars, when slightly sulfidic conditions were guaranteed by adding pieces of organic matter to the sediment, which was degraded microbially thereby generating black spots indicative of sulfide production (Musat, 2006). During maintenance the sulfate-reducing symbionts, though still present as indicated by the corresponding positive PCR product sequences, seem to have become almost completely inactive under these conditions as indicated by the absence of a FISH signal (Musat, 2006). Maintenance in rather oxic sediment (no generation of black spots) also kept worms alive. In these sediments the sulfate-reducing symbionts seem to be still active (positive FISH signal, personal observation). The worms, however, turned pale after several weeks with no detectable stored sulfur. These worms often seem "weak", easier to brake and more delicate to handle, though they were still actively moving (personal observation). In all experiments white worms were needed. For this purpose, pale worms were incubated for at least two weeks in test tubes with slightly sulfidic sediment. Fresh sediment from the sampling site was used and spiked with organic matter (dried *Ulva lactuca*) to create a sulfidic area before pale worms were added. The worms were observed frequently within this black area and became white within the two weeks incubation. This set-up was also used to concentrate worms prior to an incubation experiment making the collection of a number of worms prior to the incubation much faster. Still, the search for, and washing of the worms took up to two hours between starting to collect worms until the start of the incubation. Worms used for incubations were sorted into multi-well plates in about 1 ml oxic or anoxic sterile seawater without sediments or glass beads until transfer to the incubation vial.

Incubation experiments

Incubation experiments were carried out in 5 ml Hungate tubes that were closed with rubber stopper and lid. These tubes and size were chosen for better handling, so that the worms could be easily and carefully placed onto the bottom of the tube with either needle or pipette. Sterile baked glass beads (0.4-0.6 mm in diameter, 0.1 g) served as sediment replacement. Previous studies emphasized the importance of sediment in incubation experiments to avoid local anoxic conditions, which appear, when the worms

would clump together without sediment (Felbeck *et al.*, 1983, Giere *et al.*, 1988, Krieger, 2001) and for the worms' well-being. Indeed, after only a few hours in plain seawater without sediment or sediment surrogate, the worms became less active and usually did not survive 24 h (personal observation). For dead controls worms were either killed by fixation with formaldehyde or ZnAcetate prior to incubation experiments.

Oxic incubations

Incubations were carried out in 1 ml oxidized sterile filtered seawater from the sampling site with air as headspace. The incubation time was 30 min to 22 h in the dark after the tracer (sodium bicarbonate [^{14}C] (5.0 mCi 1.8 GBq/mmol), PerkinElmer, Waltham, MA, USA) was added. The experiment was stopped by opening the incubation tube, sampling 0.5 ml medium for specific activity determination, before emptying the contents into a Petri dish where the worms were checked for their integrity and washed three times in sterile filtered seawater before further analyses. The specific activity in the incubation as cpmB or Bq per nmol dissolved inorganic carbon (DIC) was determined by liquid scintillation counter (LSC, see below) for the 0.5 ml aliquot of medium after the incubation.

Anoxic incubations

Anoxic incubations were carried out in artificial seawater (ASW: 0.756 mmol/l KBr, 8.05 mmol/l KCl, 10 mmol/l $CaCl_2$, 27.89 mmol/l $MgCl_2$, 27.6 mmol/l $MgSO_4$, 451 mmol/l NaCl, 2.5 mmol/l NaHCO3, 4.67 µmol/l NH_4Cl, 1.47 µmol/l KH_2PO_4, trace elements (Widdel & Pfennig, 1981, modified) either buffered with HEPES (10 mmol/l, pH 7.6) or without buffer. The seawater was prepared anoxic. Dithionite and ascorbate were added as reductant in some experiments with resazurin as redox indicator. When no reductant was added special care was taken to ensure that only trace amounts of oxygen were present in the medium. Therefore the anoxic seawater was used only as long as no gas bubbles were seen in the storage flask (storage possible for approximately a month). Constant N_2 flushing to prevent oxygen from penetrating into the medium while adding the worms to the tube resulted in disturbance of the worms by spinning around due to the small volumes used for the incubations. This spinning was avoided by setting up the incubation in an anaerobic chamber (N_2:CO_2 90:10). Therefore, the worms were collected from their sediment jars, washed in ASW, checked for integrity under a binocular (dissecting scope) and placed into the sterile Hungate tube containing 0.1 g glass beads using a pipet (wide opening of tips) in a total volume of 50 or 100 µl anoxic ASW. Tubes containing the worms were transferred to the anaerobic chamber, where the substrates (Table C.1) and the rest of ASW to 1 ml were added. The tubes were closed with rubber stopper and lid. Incubation tubes were pre-incubated for one hour at room temperature in the dark before they were supplemented

with the ^{14}C-bicarbonate tracer. The pre-incubation should help the worms and symbionts to adapt to the incubation conditions and ensure that oxygen was almost completely used up. The total incubation volumes for anoxic incubations were 1 ml with a headspace of N_2:CO_2 (90:10) for most incubations (Table C.2). LSC and sulfur analyses are described below.

Post-incubation analyses of elemental sulfur and ^{14}C-carbon uptake

The stored elemental sulfur of individual worms was determined for most experiments. After incubation and washing of the worms, sulfur was extracted from individual worms with methanol (Roth, Karlsruhe, Germany). The worms were removed from the methanol and the methanol was analyzed for elemental sulfur on an HPLC (SYKAM, Fürstenfeldbruck, Germany), Zorbax ODS 5 µM vertex column (Knauer, Berlin, Germany) with methanol as eluent as described previously (Ferdelman *et al.*, 1997).

All ^{14}C-incubation experiments were analyzed in a LSC (2500 TR LSC, Packard, Ramsey, MN, USA). Worms from the methanol or from the incubation were placed into scintillation vials with 0.5 ml 6 N HCl to remove any unbound carbon. After three times of thorough mixing and 10 min evaporation, scintillation cocktail (Ultima Gold, PerkinElmer, Waltham, MA, USA) was added and the vials were thoroughly mixed before the amount of incorporated ^{14}C for individual worms was analyzed. A quench correction for the HCl was not needed as determined with a quench curve.

For a few incubations worms were also analyzed in a beta- imager that provides a picture of the incorporated label distribution in the worm. Worms for beta-imager analyses were washed, killed and placed into the beta-imager (Micro Imager V2, Biospace Mesures, Paris, France). Data were acquired with BV acquisition ™ Version 7.X (Biospace Mesures, Paris, France) and processed with β–vision ™ 4.2 (Biospace Mesures, Paris, France).

Table C.1: Substrates and concentrations used in oxic and anoxic incubations of the *O. algarveniss* symbiosis (see Chapter 2.2).

Incubation	Electron donor	Concentration [µmol/l]	electron acceptor*	Concentration [µmol/l]
oxic	none			
	thiosulfate	1000		
			nitrate	1000
			fumarate	1000
			TMAO	1000
	acetate	1000		
	lactate	1000		
	succinate	1000		
anoxic	none		none	
	sulfide	50, 100, 200		
	sulfide	50, 100	nitrate	100, 200, 500
			nitrate	100, 200, 500
	thiosulfate	200, 1000		
	acetate	200, 1000		
			fumarate	200, 1000
	thiosulfate	500	fumarate	500
			TMAO	1000
	H_2	500		
	H_2	500	fumarate	1000
	H_2	500	TMAO	1000

* Oxic medium was oxygen saturated with an oxygen concentration of approximately 200 µmol/l.

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C.3 Primers used for the fosmid library screening

Primer	Sequence[1] 5'-3'	Annealing T [°C]	References
16S rRNA			
Alpha 1a_768 fwd	ACGCCGTAAACGATGAGTGCTAGA	55°C	This study
Alpha 1a_1101 rev	AAACCCGATGGTAACTAAGGGCGA	55°C	This study
Alpha 1b_407 fwd	AGCTCTTTCACCCGTGACGATGAT	55°C	This study
Alpha 1b_1123 rev	TGTCACCGGCAGTTTCTTCAGAGT	55°C	This study
Alpha 2_ 762fwd	TTAGCCGTCGGTCAGTTGACTGTT	55°C	This study
Alpha 2_1256 rev	TGCAGAGTGCAATCCGAACTGAGA	55°C	This study
RubisCO (*cbbL*)			
cbbLF_1b fwd	CACCTGGACCACVGTBTGG	See ref.	Blazejak *et al.*, 2006
cbbLR_2c rev	CGGTGYATGTGCAGCAGCATICCG	See ref.	Blazejak *et al.*, 2006
APS reductase (*aprA*)			
aps1F	TGGCAGATCATGATYMAYGG	See ref.	Blazejak *et al.*, 2006
aps4R	GCGCCAACYGGRCCRTA	See ref.	Blazejak *et al.*, 2006
SoxB			
soxB432F fwd	GAYGGNGGNGAYACNTGG	See ref.	Petri *et al.*, 2001
soxB1446B rev	CATGTCNCCNCCRTGYTG	See ref.	Petri *et al.*, 2001
rDSR			
dsrA 240F	GGNTAYTGGAARGGYGG	See ref.	Lenk, 2006
dsrB 808R	CCDCCNACCCADATNGC	See ref.	Lenk, 2006
dsrB 403F	CAYACNCARGGNTGGY	See ref.	Lenk, 2006
dsrB 403R	ARCCANCCYTGNGTRTG	See ref.	Lenk, 2006
CODH (*coxL*)			
coxL_OMPf fwd	GGCGGCTT[C/T]GG[C/G]AA[C/G]AAGGT	See ref.	King, 2003
coxL_BMSf fwd	GGCGGCTT[C/T]GG[C/G]TC[C/G]AAGAT	See ref.	King, 2003
coxL_O/Br rev	[C/T]TCGA[T/C]GATCATCGG[A/G]TTGA	See ref.	King, 2003

F, fwd: forward; R, rev: reverse primer.

[1]Ambiguity base codes: R = A, G; Y = C, T; B = C, G, T; N = A, T, C, G; I = Inosin/Hypoxanthin

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