Phylogenetic and functional characterization of symbiotic bacteria in gutless marine worms (Annelida, Oligochaeta)

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

dem Fachbereich Biologie/Chemie der
Universität Bremen
vorgelegt von

Anna Blazejak

Oktober 2005

Die vorliegende Arbeit wurde in der Zeit vom März 2002 bis Oktober 2005 am Max-Planck-Institut für Marine Mikrobiologie in Bremen angefertigt.

1. Gutachter: Prof. Dr. Rudolf Amann
2. Gutachter: Prof. Dr. Ulrich Fischer

Tag des Promotionskolloquiums: 22. November 2005

Contents

Summary

Symbioses between chemoautotrophic bacteria and eukaryotic hosts are widespread in marine environments. In most chemosynthetic endosymbioses, only a single, or at most two bacterial phylotypes co-occur within a host species.

In this study the phylogenetic and metabolic diversity of bacterial endosymbionts in gutless marine worms (Annelida, Oligochaeta) from different environments was investigated. Almost all host species harbor a gammaproteobacterial sulfur oxidizer indicating the importance of these Gamma 1 symbionts for the nutrition of the gutless oligochaetes. A second gammaproteobacterial symbiont and deltaproteobacterial symbionts were detected in hosts from coastal silicate sediments, while in hosts from calcareous sands alphaproteobacterial symbionts were identified. Spirochetes were found in hosts from both types of sediments.

The phylogenetic diversity of the bacterial symbionts mirrors their different metabolic capabilities. The Deltaproteobacteria have been identified as sulfate reducers and the secondary gammaproteobacterial symbionts are hypothesized to be sulfur oxidizers. Key genes involved in oxidative and reductive sulfur metabolism, CO_2 fixation via the Calvin-Benson-Bassham (CBB) cycle, and nitrogen metabolism were successfully detected. Based on phylogenetic analyses it was possible to make potential assignments of genes to a respective symbiont.

The use of comparative metagenomics gave first insights into the genome of a gutless oligochaete symbiont. A contiguous sequence of 51 kb from a bacterial artificial chromosome insert contained genes involved in significant metabolic pathways for these symbioses such as sulfur oxidation and CO_2 fixation via the CBB cycle indicating that this sequence originated from a thioautotrophic symbiont.

This study showed that the symbiotic community in marine gutless oligochaetes with at least three and as many as six different symbiotic phylotypes is much more complex than previously assumed. Despite the high phylogenetic diversity, these associations are clearly specific and stable for most phylotypes within a given host species.

Zusammenfassung

Symbiosen zwischen chemoautotrophen Bakterien und eukaryontischen Wirten sind weit verbreitet in marinen Lebensräumen. In den meisten chemosynthetischen Endosymbiosen ist innerhalb einer Wirtspezies nur ein einziger bakterieller Phylotyp zu finden oder es coexistieren höchstens zwei Phylotypen.

In dieser Studie zur phylogenetischen und metabolischen Diversität der bakteriellen Endosymbionten in darmlosen, marinen Würmern (Annelida, Oligochaeta) wurden Spezies aus unterschiedlichen Habitaten untersucht. Nahezu alle Wirtspezies beherbergen gammaproteobakterielle Schwefeloxidierer, ein Hinweis auf die Bedeutung dieser Gamma 1 Symbionten für die Ernährung der darmlosen Wirte. Ein zweiter gammaproteobakterieller Symbiont sowie deltaproteobakterielle Symbionten wurden in Wirten aus silikathaltigen Küstensedimenten detektiert, während in Wirten aus kalkhaltigen Sedimenten alphaproteobakterielle Symbionten identifiziert wurden. Spirocheten wurden in Wirten aus beiden Sedimenttypen gefunden.

Die phylogenetische Diversität der bakteriellen Symbionten spiegelt ihre metabolischen Fähigkeiten wider. Die Deltaproteobakterien sind Sulfatreduzierer, als Funktion der sekundären gammaproteobakteriellen Symbonten wird die Schwefeloxidation vermutet. Verschiedene Schlüsselenzyme des oxidativen und reduktiven Schwefelmetabolismus, der CO_2 Fixierung mittels Calvin-Benson-Bassham (CBB) Zyklus, und des Stickstoffmetabolismus wurden in diesen Symbionten erfolgreich detektiert. Phylogenetische Analysen der gefundenen Schlüsselenzyme ermöglichten eine potentielle Zuordnung der Gene zu den entsprechenden Symbionten.

Die Analyse einer Metagenomgenbank gewährte erste Einblicke in das Genom eines Symbionten. So trug z.B. ein Klon ein 51 kb großes Insert mit Genclustern, die maßgeblich für die Stoffwechselwege dieser Symbiosen, wie z.B. Schwefeloxidation und CO_2 Fixierung via CBB Zyklus, sind und auf einen Ursprung aus einem thiotrophen Symbionten hinweisen.

Diese Studie hat gezeigt, dass die symbiontische Gemeinschaft in den marinen, darmlosen Oligochaeten mit mindestens drei und höchstens sechs unterschiedlichen Phylotypen komplexer ist als früher angenommen. Trotz der hohen phylogenetischen Diversität sind diese Assoziationen eindeutig spezifisch und für die meisten Phylotypen innerhalb eines Wirtes stabil.

Part I:

Combined Presentation of Results

A Introduction

1 Definition and characteristics of symbiosis

The term “symbiosis” originates from the Greek word *sumbiosis* for “living together”. The first usage of this word in a biological context is attributed to the German lichenologist Albert B. Frank who carried out detailed studies of lichens, mutualistic associations of microscopic green algae or cyanobacteria and filamentous fungi (Frank, 1877). Only two years later, in 1879, the German scientist Anton de Bary defined symbiosis as the living together of differently named organisms and is now cited in almost all text books as having defined the term symbiosis (De Bary, 1879). De Bary included all cases of intimate associations between species in the term symbiosis including (i) mutualistic associations, in which all partners benefit from the relationship, (ii) parasitic associations, in which one of the organisms benefits at the expense of the other, and (iii) commensalistic associations, in which one partner benefits and the other is neither harmed nor benefited. These interactions were defined broadly by de Bary such as epiphytes growing on trees, insects pollinating flowers, harmful infections by bacteria, and beneficial consortia between plants and algae. Such a comprehensive definition caused confusion in the following years and some biologists argued for a more narrow definition in which symbiosis was limited to mutualistic associations and pathogenic associations should not be included.

Symbiosis and pathogenesis developed at very different rates during the 20th century. The immense consequences of pathogenic infections for human health promoted research on powerful therapeutic and preventive strategies. In contrast, the study of symbiotic interactions lagged behind, despite the fact that symbiosis was recognized as an important driving force in evolution, as exemplified by the endosymbiotic origin of mitochondria and chloroplasts (Margulis and Bermudes, 1985). One reason for the slower development of

research on mutualistic associations was surely their benign outcome without risk to humans and therefore no ensuing danger to the general public. A more general problem for both symbiotic and pathogenic bacteria was that they are often uncultivable and thus difficult to examine.

The recent development of molecular biological techniques, many of which are culture independent, provided important information about parasitic and symbiotic systems and profoundly modified the view of bacteria-host interactions (Gross et al., 2003; Ochman and Moran, 2001; Ruby et al., 2004). Particularly the sequencing of genomes of pathogens and symbionts has revealed similarities between genes used for pathogenic and symbiotic lifestyles. A good example is the recently sequenced genome of the symbiotic bacterium *Vibrio fischeri* that shows many parallels to that of its pathogenic congener *V. cholera* (Ruby et al., 2005).

What then does it take to become a pathogen or symbiont? The basic requirements involve similar mechanisms that facilitate the successful colonization of a host in both pathogenic and symbiotic bacteria. Common features include contacting and entering the host's body, growth and replication using nutrients from host, avoidance of host defense, persistence and replication, and finally exiting the old host and infecting a new one. Bacteria that can overcome these steps are successful, regardless of whether the ultimate outcome of the interaction is harmful, benign, or beneficial to the host. Only from the host's perspective are these distinctions critical (Ochman and Moran, 2001).

Today, several common strategies are considered to be involved in the establishment of symbiotic and pathogenic lifestyles. The most notable are a type III secretion system (Freiberg et al., 1997; Hueck, 1998), the release of tracheal cytotoxin (TCT) (Ruby et al., 2005), and the perception of sensor proteins required for bacterial colonization of the host or intraspecific cell density (quorum sensing) (Fidopiastis et al., 2002; Piper et al., 1993; Pirhonen et al., 1993). These but also other, here not further described criteria, have diminished significantly the once distinct differences between symbiotic and pathogenic

associations. Therefore, more and more scientists have reverted back to the original broad definition: symbiosis is the living together of different organisms.

The terms parasitism, mutualism, and commensalism describe symbiosis by distinguishing between the outcome of the association, which can range from harmful to beneficial. Another way to classify symbioses was developed by Starr (Starr, 1975), modified versions of which are still widely accepted. Important characteristics for this classification system are size and location of the partners as well as persistence, dependence, and specificity of the symbiosis. Furthermore, criteria such as the modes of interactions of the partners, the transmission of the symbionts, and the integration in the symbiosis are included in the scheme. For a general overview, these characteristics are summarized in Table 1.

2 Chemoautotrophic symbioses

Research on symbioses between chemoautotrophic bacteria and eukaryotic hosts has a very recent history compared to the long period of research on other symbioses such as those between photosynthetic organisms and marine invertebrates, or heterotrophic bacteria and insects. Since the first documentation of chemoautotrophic associations in 1981 (Cavanaugh et al., 1981; Felbeck, 1981a), their discovery has been fast paced, and they are now known to occur not only among diverse host groups but also different environments (Bright and Giere, 2005; Fisher, 1990; Giere, 1996; Ott, 1996; Van Dover, 2000). The characteristic feature of chemoautotrophic associations is that the bacterial symbionts use energy from the oxidation of reduced inorganic compounds to transform inorganic carbon into organic matter that in many cases serves the nutritional needs of the host. These chemolithoautotrophic (in short chemoautotrophic) bacteria are chemotrophic, they obtain energy from chemical sources; lithotrophic, their chemical energy source is inorganic; and autotrophic, their source for cellular carbon is inorganic. In most cases, the electron donors

Table 1. Characteristics used in the description of symbiosis (modified from Starr, 1975).

Characteristics	Involved features
Size of the partners	The partners in most symbioses are of unequal size; the larger is the host, and the smaller the symbiont.
Location of the symbionts	Symbionts are either external to the host (ectosymbiotic) or within the host (endosymbiotic). If the symbionts are endosymbiotic they can occur within the host cells (intracellular) or outside of them (extracellular).
Dependence on the symbiosis	Symbionts are obligate if they cannot survive without their partner, and facultative if they can exist in a free-living stage. Obligate symbionts depend on the symbiosis mostly for nutrients. However, organisms may also obtain physical protection from a symbiotic association or receive some other benefit, e.g., the light produced by symbiotic bacteria in marine fishes that is thought to be involved in the attraction of prey or even as camouflage.
Persistence of the symbiosis	Symbioses are persistent if the partners remain together for a long time, that is, through all stages of their life cycle or their contacts are frequent.
Specificity of symbiosis	Specificity refers to the degree of taxonomic difference between acceptable partners, and may vary from very low (where an organism can associate with members of more than one class or even phylum) to high or very high, where only a single species or 'strain' is acceptable. Presumably, the more highly evolved a symbiosis is, the longer symbionts have had to adapt to each other, and thus the more specific the association is.
Modes of interaction between the partners	The partners may interact in a range of ways including genetic, metabolic, and behavioral whereas the nutritional interactions are the most common.
Perpetuation of symbiosis	A host can acquire its symbiont either from the surrounding environment including co-occurring hosts (horizontal transmission) or directly from one generation to the next (vertical transmission). In that case, sexually reproducing hosts may evolve mechanisms by which symbionts are transferred to fertilized eggs or the cytoplasm of oocytes prior to fertilization.
Integration in the symbiosis	Formation of new structures or chemical compounds is displayed only in symbiosis but not in isolation. For example, legume roots and the red pigment leghemoglobin are only present in the symbiotic association of *Rhizobium* bacteria and legumes.

for these bacteria are reduced sulfur compounds such as hydrogen sulfide, elemental sulfur, or thiosulfate, and oxygen or nitrate as an electron acceptor. The sufficient supply of these substrates to the bacterial symbionts results in a successful association that enables the hosts to colonize sulfide rich environments in high densities, which are toxic to most other animals.

The benefit of harboring chemoautotrophic symbionts is most obvious for hosts which either completely lack a digestive system or in which it is greatly reduced. These animals are highly dependent on their symbionts for nutrition either from bacterial exudates or by digesting them. In contrast, the profit for eukaryotic hosts of harboring ectosymbionts on their body is not always clear, and while these have been suggested to provide nutrition in some hosts, free-living bacteria and other nutritional sources may be equally or more important (Cavanaugh, 1994; Polz et al., 1992).

The symbionts benefit from associating with eukaryotic hosts by the increased availability of substrates necessary for chemosynthesis, notably sulfide and oxygen. Free-living, sulfur-oxidizing chemoautotrophs are limited to those narrow zones in which both sulfide and oxygen coexist. By associating with eukaryotes, chemoautotrophs gain access to continuous supply of required substrates because the hosts are either big or active enough to span the discontinuity of sulfide or oxygen (e.g. in sediments) or they can pump water from both oxic and anoxic sources (e.g. turbulent flow environments) (Cavanaugh, 1994). In addition, the hosts provide the chemoautotrophic symbionts with shelter from predation by other organisms providing a safe habitat.

2.1 Habitats of chemoautotrophic symbioses

The first chemoautotrophic symbiosis was discovered at deep-sea hydrothermal vents in the vestimentiferan tubeworm *Riftia pachyptila* (Cavanaugh et al., 1981; Felbeck, 1981a; Felbeck et al., 1981b). Lacking a digestive system, these worms depend on obligate symbiosis with chemoautotrophic, sulfur-oxidizing bacteria that supply the host with the bulk of its nutritional needs (reviewed in Minic and

Hervé 2004). In the following years, extended research has resulted in the description of chemoautotrophic symbioses in many vent species including other vestimentiferan tubeworms, bivalve molluscs, provannid gastropods, and bresiliid shrimps (reviewed in Fisher 1990, Karl 1995, and Van Dover 2000).

Following the discovery of these chemoautotrophic symbioses that use reduced sulfur compounds, the question arose if other reduced substrates could support chemoautotrophic growth such as CH_4, H_2, NH_4, or Fe^{2+}. So far, methane oxidizers are the only group of bacteria that have been found in chemosynthetic associations with animals. A methanotrophic symbiosis was first reported in a mussel (Childress et al., 1986) and is now known from many bivalve mollusks and vestimentiferan tubeworms (Van Dover et al., 2002).

Chemoautotrophic symbioses are not only restricted to exotic sites such as hydrothermal vents. Rather, they are widespread in marine environments where sulfide and/or methane occur. Cold seeps are areas of the deep-ocean floor where methane and other hydrocarbon-rich fluid seepage occurs and where microbial oxidation of methane can lead to high sulfide concentrations (Sibuet and Olu, 1998). In this habitat, symbiotic invertebrates are associated with either sulfide- or methane-oxidizing bacteria, or both. The dominant seep hosts are large bivalves belonging to the families Vesicomyidae or Mytilidae and siboglinid tubeworms. Other symbiont-containing species also occur such as bivalves belonging to the Solemyidae, Thyasiridae, and Lucinidae families, and shrimps (Sibuet and Olu, 1998).

Other habitats in which chemoautotrophic symbioses occur are continental slope sediments, including sheltered sediments in inter- and subtidal zones (Ott, 1996; Reid and Brand, 1986), and coral reefs (Giere, 1996). In these sediments, hydrogen sulfide is produced by anaerobic degradation of organic matter coupled with sulfate reduction. In many of these habitats, oxygen and sulfide do not co-occur and the symbionts are dependent on their hosts for providing them with these substrates. The hosts bridge the gap between the upper oxic and the lower sulfidic sediments by various behavioral strategies. There are, e.g., worms that

migrate actively between these two zones (see also Chapter 3.3) or bivalves that construct ventilated burrows.

2.2 Diversity of hosts harboring chemoautotrophic bacteria

Chemoautotrophic ecto- or endosymbioses have been described in over 100 species from the following eukaryotic hosts: ciliates, molluscs, nematodes, arthropods, and annelids. In the Protista, only some ciliates, such as the marine peritrich *Zoothamnium* and the halotrich *Kentrophoros*, are known to harbor ectosymbiotic, chemoautotrophic bacteria (Bauer-Nebelsick et al., 1996; Fenchel and Finlay, 1989; Ott et al., 1998; Vopel et al., 2002).

In molluscs, all members of the bivalve families Lucinidae, Solemyidae, Vesicomyidae, some of the families Mytilidae and Thyasiridae, and some species of the class Gastropoda host chemoautotrophic and/or methanotrophic bacterial symbionts (reviewed in Fisher 1990, Childress and Fisher 1992, Karl 1995, Kelley et al. 2002). Despite different habitats, such as hydrothermal vents or shallow water sediments, all hosts have in common that their symbionts are located in the gills, either intracellulary in special bacteriocytes or among the microvilli of the bacteriocytes (Dufour, 2005; Fisher, 1990).

Among nematodes, chemoautotrophic symbioses are widespread ranging from ectosymbiotic associations within the subfamily Stilbonematinae to endosymbiotic associations within the genera *Astomonema, Parastomonema,* and *Rhaptothyreus* (Cavanaugh, 1994; Giere et al., 1995b; Kito, 1989; Ott et al., 1982; Ott, 1996; Riemann, 1993). In Stilbonematinae, the bacterial ectosymbionts are arranged as a multi-layered coat or as a single cell layer on the cuticle of the worms (Ott and Novak, 1989; Polz et al., 1992). The endosymbioses in nematodes are characterized by bacteria that completely fill the lumen of the modified gut (Giere et al., 1995b).

Within the Arthropoda, the shrimp *Rimicaris* is the best-studied host harboring chemoautotrophic ectosymbionts (Polz et al., 2000; Polz et al., 1999; Rieley et al., 1999; Van Dover et al., 1988). The symbionts grow in dense arrays

of filamentous bacteria that are present in highly specialized regions on setae attached to the hypertrophied mouthparts (Van Dover et al., 1988). The shrimp is hypothesized to rely on their epibionts by eating their own cultured microbial mats as a food source (Polz et al., 1999). Other epibionts occur in the gill chamber of the shrimp and may use iron as an electron donor for autotrophic CO_2 fixation (Zbinden et al., 2004).

In the phylum Annelida, worms of the classes Polychaeta and Clitellata are associated with chemoautotrophic bacteria. Most members of the polychete family Siboglinidae including the taxons Obturata (or Vestimentifera) and Frenulata (or Pognophora), and some members of the family Alvinellidae are described as symbiotic hosts (reviewed in Bright and Giere 2005). In Siboglinidae, the chemoautotrophic bacteria are found intracellulary in the symbiont-bearing organ, called the trophosome, whereas in Alvinnellidae they are present as ectosymbionts growing on the worm in dense filamentous arrays.

Within the Clitellata two genera of marine oligochaetes, *Olavius* and *Inanidrilus* are characterized by an obligate endosymbiosis with extracellular chemoautotrophs. These symbioses are the focus of this thesis and will be described in detail in Chapter 3.

2.2.1 Phylogenetic diversity of chemoautotrophic symbionts

Based on comparative 16S ribosomal RNA (rRNA) sequence analysis, chemoautotrophic symbionts belong mainly to the Gammaproteobacteria but are also present within the Epsilonproteobacteria. An overview of the phylogeny of these symbionts is shown in Figure 1.

Within the Gammaproteobacteria three major clusters are present, the first one exclusively consists of 16S rRNA sequences from mytilid and vesicomyid bivalve symbionts, the second from gutless oligochaetes and nematodes, and the third from vestimentiferan and pogonophoran tubeworms, bivalves, and the gastropod *Ifremeria nautilei*. Beside these three clusters, several other symbiotic sequences are distributed among the Gammaproteobacteria that were found in

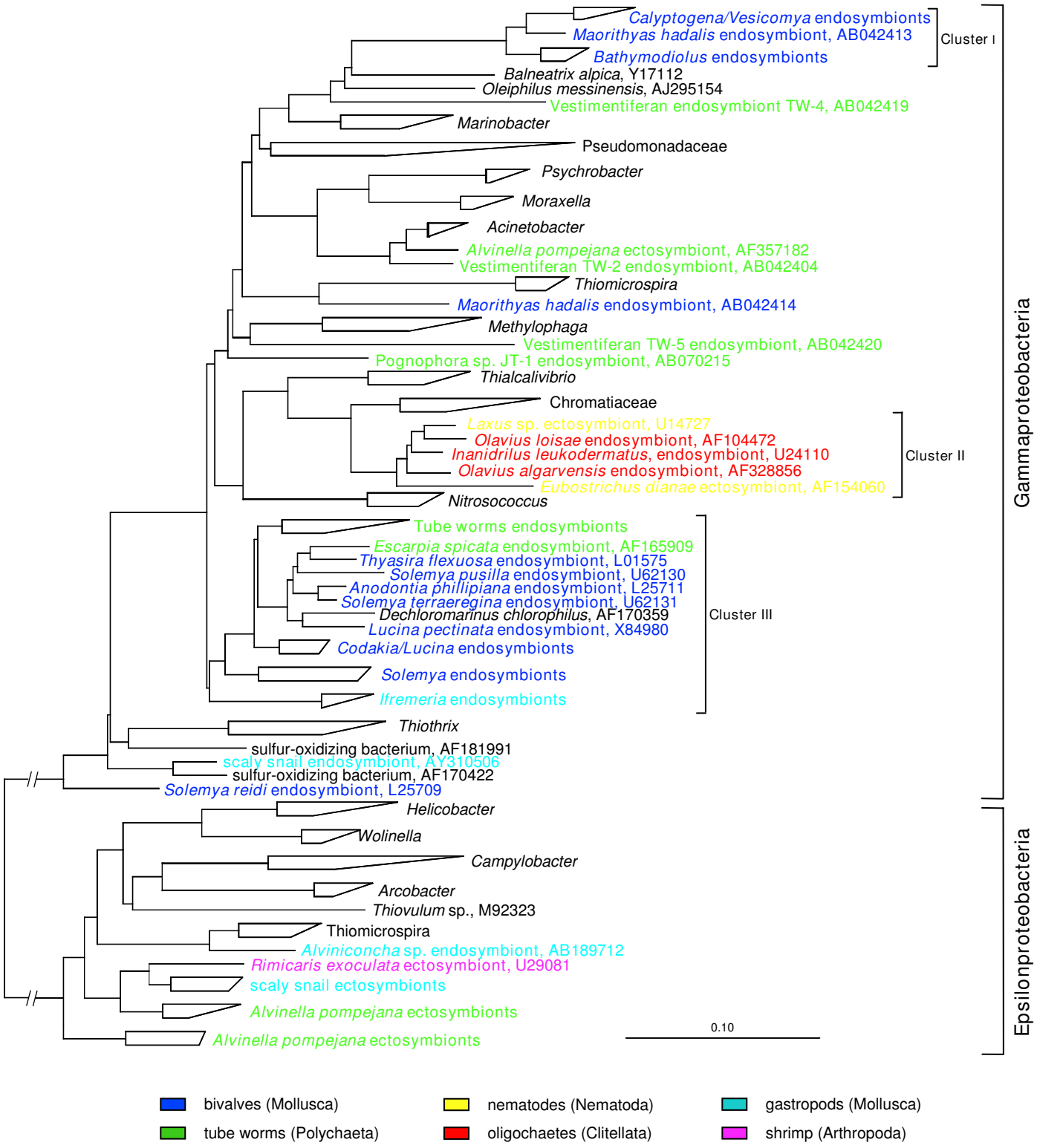


Figure 1. Phylogenetic tree showing relationships of chemoautotrophic symbionts to free-living bacteria based on parsimony analysis of their 16S rRNA sequences. For some of these sequences the symbiotic origin has not been proven by in situ hybridization studies. Hosts of the same phylum are shown in the same color (see legend). The scale bar represents 10% estimated sequence divergence. The distance between the Gamma- and Epsilonproteobacteria is an estimated 19% sequence divergence.

vestimentiferan and pognophoran tubeworms, the bivalves *Maorithyas hadalis* and *Solemya reidi*, the gastropod scaly snail, and the polychaete *Alvinella pompejana*.

Within the Epsilonproteobacteria 16S rRNA sequences of chemoautotrophic epibionts from the polychete worm *Alvinella pompejana* and the shrimp *Rimicaris exoculata* and a single endosymbiont from the gastropod *Alviniconcha* sp. belong to phylogenetic lineages that are distinct from each other. The high phylogenetic diversity of 16S rRNA sequences from chemoautotropic symbionts within the Gamma- and Epsilonproteobacteria with large groups of free-living bacteria interspersed between symbiotic sequences indicates that chemoautotrophic symbioses have evolved multiple times from different lineages of free-living, sulfur-oxidizing bacteria.

3 Symbiotic associations in gutless oligochaetes

Symbiotic associations between bacteria and gutless oligochaetes were first described by Olav Giere in 1981 (Giere, 1981), almost simultaneously with the discovery of the chemoautototrophic symbionts from the vent tubeworm, *Riftia pachyptila* (Cavanaugh et al., 1981; Felbeck, 1981a). Since then, extensive research has been in progress, most notably by the researchers Olav Giere (University of Hamburg, Germany), Christer Erséus (University of Göterborg, Sweden), and Nicole Dubilier (Max-Planck Institute for Marine Microbiology, Germany). Whereas Christer Erséus is an expert in taxonomy and phylogeny of these species, Olav Giere has focussed on morphological and physiological aspects, and Nicole Dubilier has concentrated on the molecular characterization of the bacterial symbionts.

3.1 Biogeography and phylogeny of the hosts

Gutless oligochaetes occur worldwide in pore waters of continental shelves. They have been found in high abundance and great diversity in shallow marine waters of tropical and subtropical coral reefs in the Atlantic, Caribbean, and Pacific

oceans (Erséus, 1984; Erséus, 1990; Erséus, 1992; Erséus, 2003). In particular two “hot spots”, the Caribbean and the Great Barrier Reef, possess a high number of gutless oligochaete species. Recently, these marine worms were also found in coastal areas of the Algarve and the Italian island Elba (Giere and Erséus, 2002; Giere et al., 1998). In contrast to the high diversity of species in most tropical waters, only a few are present in deeper (>100 m), colder waters off the Pacific and Atlantic coasts of North and South America, and in the eastern part of the Gulf of Mexico (Erséus, 1984; Erséus, 1991; Finogenova, 1986).

In spite of their broad geographical distribution, gutless oligochaetes are monophyletic, meaning that all species have descended from a common ancestor. The monophyly is demonstrated by morphological criteria (Erséus, 1984; Erséus, 1992) and by molecular analyses of host genes such as the 18S rRNA gene and the mitochondrial cytochrome oxidase subunit I (COI) and 16S rRNA genes (Erséus et al., 2002; Erséus et al., 2000; Nylander et al., 1999). All gutless oligochaetes (>80 species described) belong to either genus *Inanidrilus* or *Olavius*. The former is assumed to be monophyletic while the genus *Olavius* may be polyphyletic (C. Erséus, unpublished data). The widespread distribution and high diversity of gutless oligochaetes as well as their monophyly forms a solid base for studying the phylogeny and evolution of the associations with their symbionts.

3.2 The environment

The most common habitat of gutless oligochaetes are sulfidic calcareous sediments in warm water oceans. The best studied environments are coastal sediments off the island of Bermuda, which are populated by the species *I. leukodermatus* and *O. planus* (Giere, 1996; Giere et al., 1991; Giere and Langheld, 1987; Giere et al., 1982). The sand consists of heterogeneous medium grain size and is rich in degradable detritus, thus supplying the ambient habitat with dissolved organic matter (Giere et al., 1982). Due to the active decomposition of organic matter, the development of a reduced zone underneath

an oxic surface layer is supported. In these sediments, profiles of oxygen in the upper sediment layers and reduced sulfur compounds in the deeper sediment layers do not overlap. The worms require oxygen as an electron acceptor for their own respiration. In addition, they also have to ensure the respiration of their sulfur-oxidizing symbionts that need both oxygen or other oxidized compounds as an electron acceptor and reduced sulfur compounds as an electron donor. To bridge the gap between the oxic and anoxic environment the worms actively migrate between upper oxic and the lower sulfidic sediment layers (Giere et al., 1991). This model implies that sulfide is taken up in the anoxic deeper sediments layers, oxidized to sulfur by either nitrate from the environment or oxygen from the worm's hemoglobin, and the sulfur stored in the bacteria until the worms migrate to upper sediments layers where the sulfur could be fully oxidized to sulfate.

Interestingly, at almost all sites investigated, gutless oligochaetes are most dominant in the deeper sulfidic sediment layers at 5-15 cm below the sediment surface indicating their high adaptation to this anoxic habitat (Dubilier et al., 1994; Giere et al., 1991; Grieshaber and Völkel, 1998).

Recent studies showed that gutless oligochaetes are not only restricted to calcareous sediments but also occur in silicate sands, indicating that the sediment type is not crucial for colonization (Giere and Erséus, 2002; Giere et al., 1998; Giere and Krieger, 2001). For example, *O. crassitunicatus* occurs in silicate sediments of the oxygen minimum zone (OMZ) off the coast of Peru. This habitat is characterized by extremely low concentrations of oxygen in the bottom water (<1 µM) and sulfide-rich sediments (Giere and Krieger, 2001; Levin et al., 2002; Levin et al., 2003).

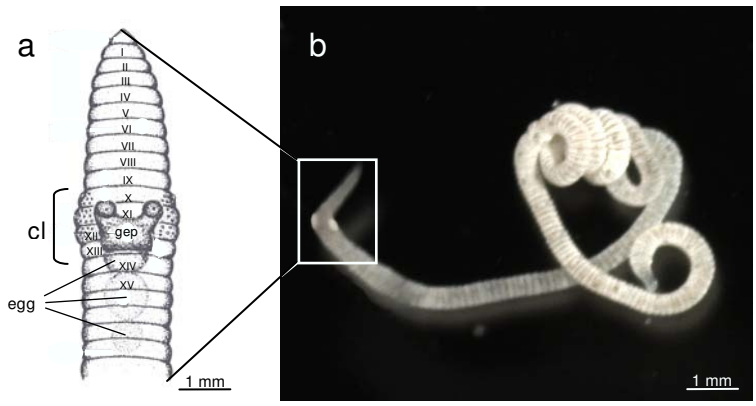
Another environment inhabited by gutless oligochaetes was found in silicate sands around the island of Elba in the Mediterranean (Giere and Erséus, 2002; Giere et al., 1998). In contrast to all other habitats in which sulfur-oxidizing symbioses are found, the Elba sediments revealed only traces of measurable hydrogen sulfide (<14-76 nM), raising the question if this provides an adequate supply of the required electron donor (Dubilier et al., 2001). One possible

explanation for the colonization of this unusual environment by the species *O. algarvensis* and *O. ilvae* is that the worms harbor sulfate-reducing symbionts which supply sulfide to the sulfur-oxidizing symbionts via internal production (Dubilier et al., 2001) (see Chapter 3.5).

3.3 Structure of the symbiosis

Gutless oligochaetes are small, segmented worms with a diameter of approximately 0.5 mm and varying lengths between 20-50 mm. Their morphological characterization and taxonomic classification relies mainly on differences of sexual organ structure within the clitellar region of these worms (segments1/2 X to XII) (Figure 2) and requires highly trained personnel (Erséus, 1984; Erséus, 1990; Erséus, 1991; Erséus, 1998; Erséus, 2003; Erséus and Baker, 1982; Erséus and Giere, 1995). The most remarkable feature of gutless oligochaetes, as the name implies, is their completely reduced digestive system without a mouth, gut, or anus (Giere, 1981). In addition, these worms are unique among all free-living gutless worms in lacking nephridia, excretory organs used to remove nitrogenous waste compounds and for osmoregulation (Giere, 1981). The nutritional needs of gutless oligochaetes are ensured by their symbionts either from their exudates or by their lysis that is visible in the basal region of the symbiont-containing region (Giere, 1985; Giere and Langheld, 1987). The association is certainly obligate for the host, as all gutless oligochaetes harbor symbionts. Whether a free-living stage of the symbionts exists is unclear.

The morphological structure of the symbiosis is similar in all gutless oligochaetes species examined to date. The bacterial symbionts occur extracellularly directly below the cuticle of the host between extensions of the epidermal cells (Giere, 1981). Their distribution along the body of the host is variable. While the symbiotic bacteria are less abundant in the very first anterior segments of the worm, they occur numerously along the vast majority of the rest of the worm filling the whole subcuticular space (Giere and Langheld, 1987).



Taxonomic classification

Phylum:	Annelida
Class:	Oligochaeta
Family:	Tubificidae
Subfamily:	Phallodrilinae
Genus:	*Inanidrilus*
Species:	*leukodermatus*

Figure 2. The gutless oligochaete *Inanidrilus leukodermatus*. (a) Schematic drawing of the anterior part of the worm. cl: clitellar region; gep: genital pad (modified from Giere 1987) (b) Mature live adult. Note the white color of the worm due to the cytoplasmic inclusions of the bacterial symbionts.

Up to three different bacterial morphotypes can coexist in a single host species (Dubilier et al., 1999; Giere and Krieger, 2001; Giere et al., 1995a) (Figure 3). Two of them, the larger and the smaller cell types, occur in all species studied, whereas the thin and long morphotype could only be detected in some species (Dubilier, 2003; Giere et al., 1995a). The larger morphotypes are oval bacteria with a diameter of 3 to 5 µm that are dominant in the symbiotic, subcuticular space. The cytoplasm of these cells is rich in intracellular inclusions including both non-membrane bound globules with polyhydroxybutyrate (PHB) and membrane-bound vesicles with sulfur (Giere and Krieger, 2001). These bacterial sulfur and PHB reserves give the hosts a white appearance that easily facilitates their identification within the diverse fauna found in the same habitat (Figure 2). The smaller bacterial morphotype is clearly distinct from the large one with a length of 0.5 to 1 µm and a width of approximately 0.25 µm (Giere et al., 1995a). These rod-shape bacteria have no cellular inclusions and occur either throughout the symbiont-containing region or are concentrated at the periphery directly below the cuticle. The thin and long morphotype is easily recognized by its long and thin cell shape (length, 10 µm; diameter, 0.3 µm) (Dubilier et al., 1999; Giere and Krieger, 2001; Giere et al., 1995a). As in the smaller morphotype, no cytoplasmic inclusions were observed in these bacteria.

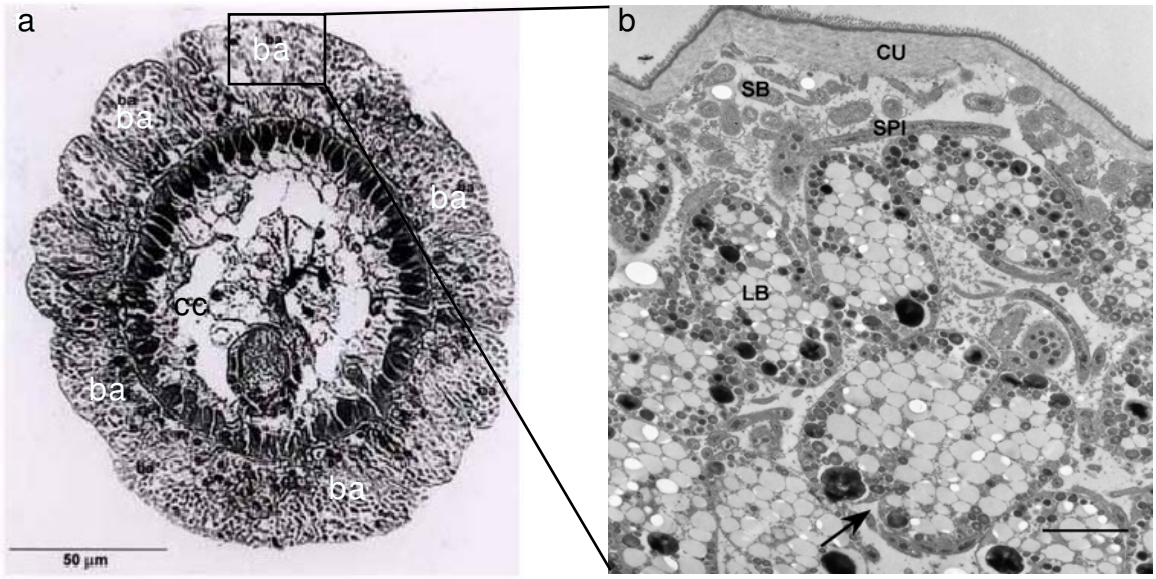


Figure 3. (a) Cross section through a gutless oligochaete. ba: subcuticular bacteria; cc: coelomic cavity containing ventral nerve cord, blood vessels, and chloragocytes. (b) Transmission electron micrograph showing cuticle (CU) and subcuticular space filled with three different bacterial morphotypes (LB = large, oval bacteria; SB = small, rod-shaped bacteria; SPI = long and thin bacteria). Note longitudinal fission in lower right large bacterium (arrow) (scale bar, 2 µm). Modified from Giere et al., 1989 and 2001.

3.4 Transmission of the symbionts

The transmission of at least some of the bacterial symbionts to the next host generation most probably takes place vertically, that is directly from the mature adult to their offspring (Giere and Langheld, 1987; Krieger, 2001). To date this transfer is likely for the large morphotype but it is unclear if this is also true for the other two morphotypes (Krieger, 2001). The mature worms deposit their eggs singly in a sticky mucus sheath into the sand. Between this mucus and the egg, bacteria are visible surrounding the egg. Several hours after egg deposition, the bacteria concentrate at one pole of the egg and penetrate the egg membrane (Krieger, 2001). In addition to the vertical transmission of symbionts, the deposition of the eggs into the sediment would offer free-living bacteria from the environment an opportunity to invade the egg. Thus, it is conceivable that some of the symbionts are inherited vertically from the parents, and some horizontally from the environment. Even if all phylotypes are inherited vertically in recent

oligochaetes, the first step in the development of these multiple symbioses may have originally evolved through horizontal infection of the egg, followed by a successful establishment as a symbiont and transfer to a vertical mode of infection (Dubilier et al., in press).

3.5 Molecular characterization of the symbionts

Although the morphology of the bacterial symbionts is well described these studies are not useful for investigating the phylogeny of the symbionts, as the morphology of bacteria cannot be used as a taxonomic criterium. The so-called full rRNA cycle (Figure 4) has emerged as a powerful technique for the molecular characterization of yet uncultivated bacteria such as the oligochaete symbionts (Amann, 1995). By combining comparative 16S rRNA sequence analysis and fluorescence in situ hybridization (FISH) with oligonucleotide probes targeting the 16S rRNA, the phylogenetic relationship as well as the identification and distribution of the symbionts can be revealed. At the beginning of this thesis, bacterial symbionts from three oligochaete species, *Inanidrilus leukodermatus* from Bermuda, *Olavius loisae* from the Great Barrier Reef, and *O. algarvensis* from Elba had been characterized using this approach (Dubilier et al., 1999; Dubilier et al., 1995; Dubilier et al., 2001). In these worms, the large morphotypes were identified as Gammaproteobacteria with a close phylogenetic relationship to each other. The closest relatives to this cluster of oligochaete symbionts are a chemoautotrophic ectosymbiont from a nematode and a clade of free-living, sulfur-oxidizing bacteria belonging to the family Chromatiaceae.

In contrast to the clear identity of the large morphotype, the smaller ones belong to different phylogenetic lineages. In *O. loisae,* the small bacterial symbionts were identified as Alphaproteobacteria related to free-living bacteria from the genus *Rhodovibrio* (Dubilier et al., 1999). In *O. algarvensis,* however, the smaller symbiont morphotype was identified as a sulfate-reducing bacterium of the Deltaproteobacteria (Dubilier et al., 2001).

The phylogeny of the thin, long bacterial morphotype was unknown at the beginning of this thesis. Although several 16S rRNA sequences belonging to the phylum Spirochaeta that is typically characterized by bacteria with a thin and long shape were isolated from *O. loisae*, the in situ identification with specific 16S rRNA oligonucleotide probes was not successful (Dubilier et al., 1999).

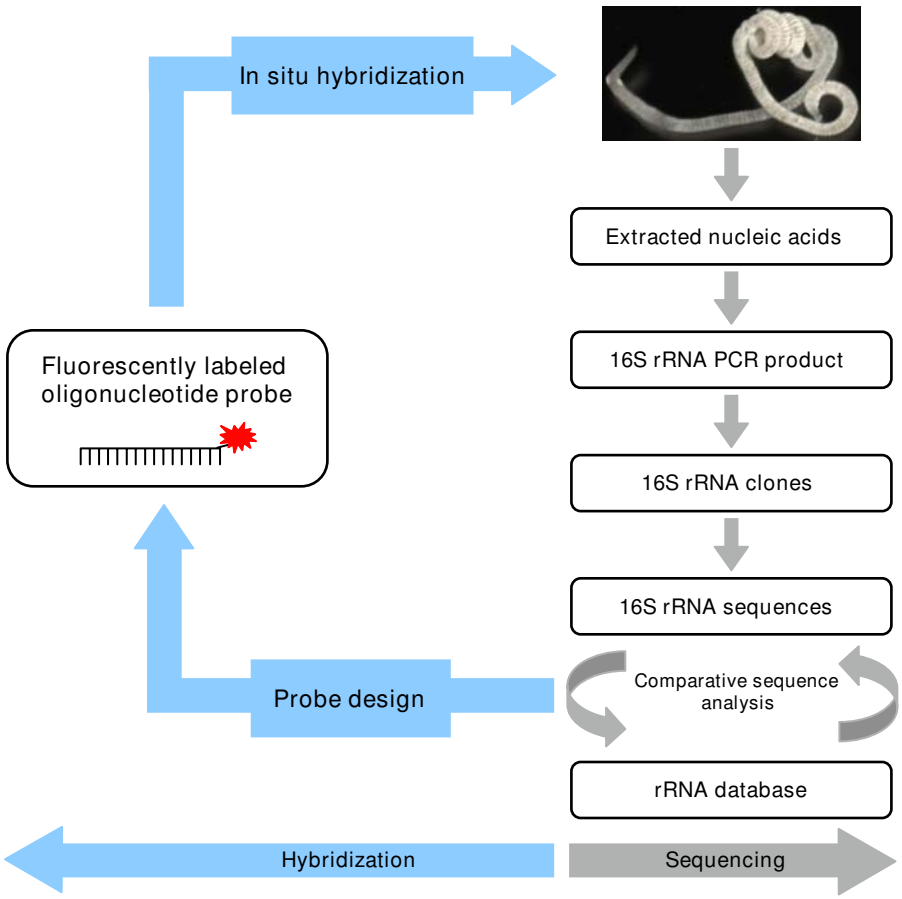


Figure 4. Flow chart showing the application of the full rRNA cycle on the uncultivable endosymbionts of gutless oligochaetes. Modified from Amann et al., 1995.

3.6 Function of the symbionts in gutless oligochaetes

Shortly after the discovery of the symbiotic bacteria in gutless oligochaetes it became apparent that this association is driven by bacterial chemosynthesis (Felbeck et al., 1983). It was found that the chemoautotrophic activity was carried out by the large oval bacteria belonging to the Gammaproteobacteria (Dubilier et

al., 2001; Giere and Krieger, 2001; Krieger et al., 2000). The thiotrophic nature of these symbionts is supported by the presence of sulfur in cytoplasmic globules and the immunocytochemical detection of form I ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the key CO_2-fixing enzyme (Dubilier et al., 2001; Giere and Krieger, 2001; Krieger et al., 2000). The role of the smaller rod-shaped bacterial symbionts belonging to the Deltaproteobacteria was also clarified (Dubilier et al., 2001). Their close phylogenetic relationship to free-living, sulfate-reducing bacteria, the presence of dissimilatory sulfite reductase (DSR), the key gene for dissimilatory sulfate reduction, and in vivo measurements of sulfate reduction in the symbiont-containing region all supported the conclusion that these symbionts are sulfate reducers. In contrast, the function of the small, rod-shaped symbionts belonging to the Alphaproteobacteria and the thin and long bacteria remains as yet unknown.

The simultaneous presence of sulfur-oxidizing and sulfate-reducing bacterial symbionts in the gutless oligochaete *O. algarvensis* attracted special attention (Dubilier et al., 2001). The coexistence of these two symbionts indicates an

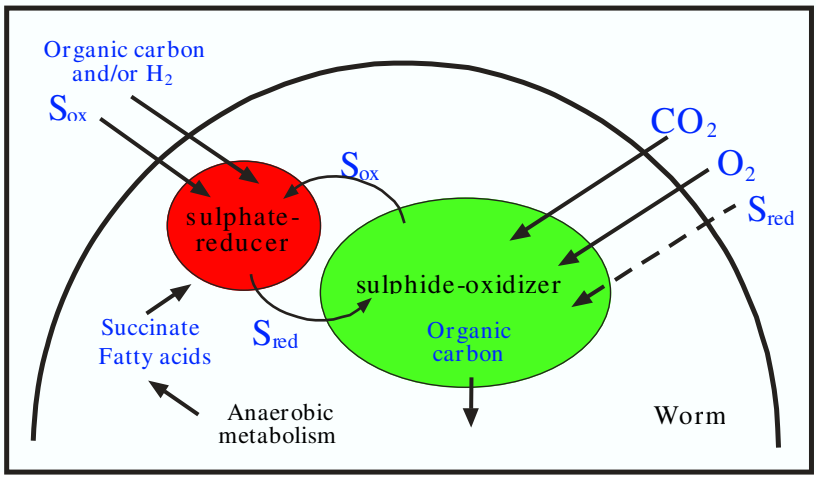


Figure 5. Model of the endosymbiotic sulfur cycle in *O. algarvensis* showing syntrophic cycling of oxidized and reduced sulfur compounds between the sulfate-reducing and sulfide-oxidizing symbionts. Under typical conditions of low sulfide flux from the sediment, sulfide produced internally by the sulfate reducers is used by the sulfide oxidizers as an electron donor for the autotrophic fixation of CO_2. Electron donors such as succinate and fatty acids can be supplied to the sulfate reducer internally by the worm during anaerobic metabolism. For net growth of the symbiotic association, external electron donors (organic carbon or H_2) are taken up from the sediment (Dubilier et al., 2001).

internal syntrophic sulfur cycle that had not been described previously in symbiotic associations. The sulfate reducers produce sulfide as a metabolic end product that can be used by the sulfur oxidizers for the autotrophic fixation of CO_2 (Figure 5). The internal production of sulfide is beneficial to the worm and its symbionts due to low concentrations of reduced sulfur compounds in the habitat of the host. Furthermore, the cycling of reduced and oxidized sulfur compounds between the two symbionts would result in increased protein yields, as shown for continuous cultures with free-living, sulfur-oxidizing and sulfate-reducing bacteria (van den Ende et al., 1997). Another profitable aspect for this association could be the recycling of fermentation products of the host that are produced during anaerobic metabolism and normally excreted in non-symbiotic worms, since these metabolites can provide the sulfate-reducing symbionts with an ideal energy source (Dubilier et al., 2001).

4 Goals of this thesis

At the beginning of this thesis, the symbiont phylogeny from only three gutless oligochaete species was known (Dubilier et al., 1999; Dubilier et al., 1995; Dubilier et al., 2001). One of the major goals of this Ph.D. thesis was to increase the knowledge of symbiotic diversity in gutless oligochaetes. To do this, the bacterial symbionts in species from different environments such as deeper and shallow waters of the continental shelf were examined using comparative 16S rRNA analysis and fluorescence in situ hybridization (FISH).

In the first part of this study symbiotic diversity in the gutless oligochaete *Olavius crassitunicatus* will be described. This species occurs in high abundance in silicate, oxygen deficient sediments in the East Pacific Ocean. Two *O. crassitunicatus* populations were studied that are geographically separated by 385 km on the Peru margin (water depth ~300 m).

In the second part of this thesis, bacterial symbionts from two gutless oligochaetes species, *Inanidrilus leukodermatus* and *I. makropetalos* that colonize calcareous sediments in shallow waters in the Atlantic Ocean were investigated. The species *I. leukodermatus* from Bermuda was reexamined because not all symbionts were identified in the first study (Dubilier et al., 1995) while the species *I. makropetalos* from Bahamas is newly studied. In addition to the diversity and phylogeny of the symbionts in these two hosts, PCR-based identification and phylogenetic relationship of symbiotic functional genes involved in autotrophic carbon dioxide fixation and sulfur metabolism were analyzed.

In the third part of this thesis a comparative metagenomic approach was used to gain insight into genome information from symbionts of two co-occurring gutless oligochaetes species, *O. algarvensis* and *O. ilvae*, from the Mediterranean. The major goal was to gain a better understanding of genes involved in significant pathways for these symbioses such as autotrophic carbon dioxide fixation and sulfur metabolism. For this purpose, a BAC library was constructed and analyzed.

B

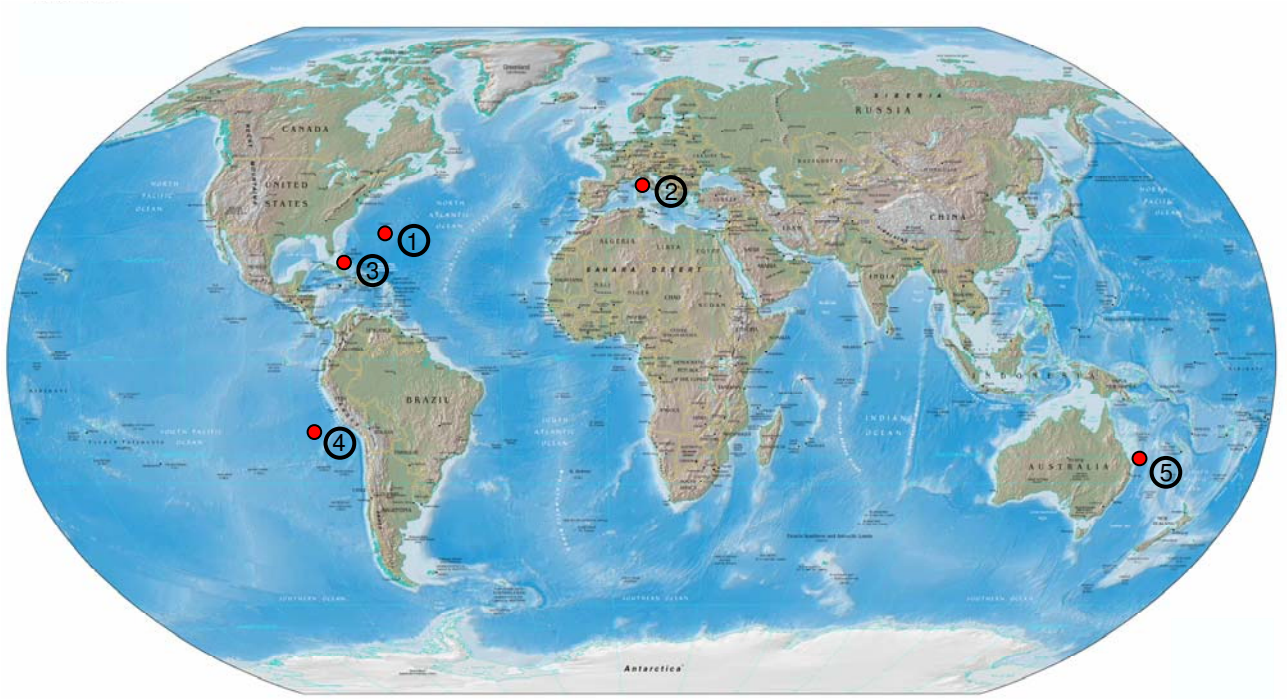
Results and Discussion

B Results and Discussion

1 Symbiotic community structure in gutless oligochaetes from different environments

1.1 Host species

The microbial diversity of bacterial symbionts was investigated in six oligochaetes species from the genera *Olavius* and *Inanidrilus* that colonize different habitats (Figure 6). Within the genus *Olavius,* the species *O. crassitunicatus* and *O. ilvae* were studied for the first time, while *O. algarvensis* was reexamined because of higher expected symbiotic diversity than assumed in a previous study (Dubilier et al., 2001). Although these three species are present in geographically distant habitats they all colonize silicate sediments. *O. crassitunicatus* is abundant in oxygen-deficient sediments (water depth ~300m) off the coast of Peru in the Southeast Pacific (Giere and Krieger, 2001), while *O. algarvensis* and *O. ilvae* co-occur in coastal sediments off the island of Elba in the Mediterranean (Giere and Erséus, 2002; Giere et al., 1998). Within the genus *Inanidrilus,* bacterial symbionts of the species *I. leukodermatus*, *I. makropetalos*, and *I. exumae* were characterized. *I. leukodermatus* was reexamined since not all bacterial symbionts could be identified in an earlier study (Dubilier et al., 1995), while the two other species were investigated for the first time. All three species occur in calcareous shallow water sediments, *I. makropetalos*, and *I. exumae* around Lee Stocking Island of the Bahamas (Northwest Atlantic) (Erséus, 2003), *and I. leukodermatus* off Bermuda in the North Atlantic (Giere, 1979; Giere, 1981).



1 *I. leukodermatus* (Bermuda)
2 *O. algarvensis* and *O. ilvae* (Elba)
3 *I. makropetalos* and *I. exumae* (Bahamas)
4 *O. crassitunicatus* (Peru)
5 *O. loisae* (Great Barrier Reef)

Figure 6: Habitats of gutless oligochaetes species whose symbiotic diversity was characterized in this and previous studies.

1.2 Molecular identification and phylogeny of the bacterial symbionts

For the molecular characterization of the bacterial symbionts at least six individuals from each species were investigated. The full rRNA cycle including comparative 16S rRNA gene analysis and fluorescence in situ hybridization (FISH) was applied for all examined species except for *I. exumae*. To characterize the symbionts of this host 16S rRNA gene libraries were constructed and FISH with general but not specific oligonucleotide probes was carried out.

1.2.1 Gammaproteobacterial symbionts

In all gutless oligochaetes species examined in this and earlier studies, the large, oval morphotype was identified as the Gamma 1 symbiont that occurs throughout the symbiont-containing region (Figure 7 a-b, e, and g). Based on comparative 16S rRNA gene analysis these symbionts belong to the Gammaproteobacteria

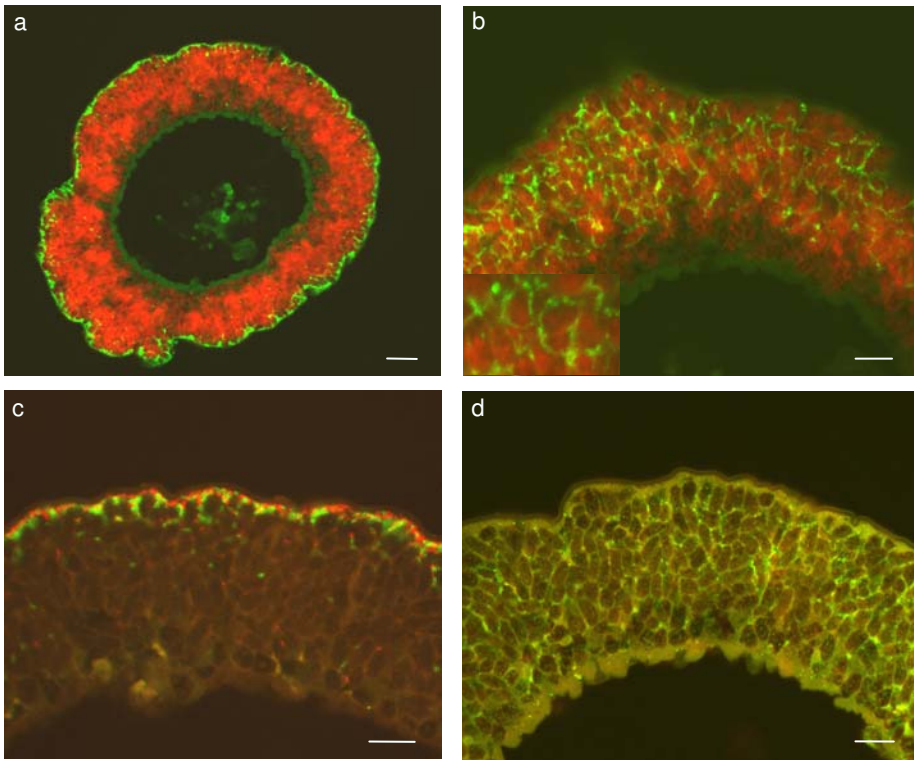
and are closely related to each other and to the ectosymbiont of the marine nematode *Laxus* sp. (Figure 8). This cluster of symbiotic sequences is related to a clone sequence isolated from the Kazan mud volcano in the Eastern Mediterranean and a clade of free-living bacteria belonging to the family Chromaciaceae.

To gain a better understanding of the phylogenetic relationships in the clade of Gamma 1 symbionts, two additional *Inanidrilus* and eight *Olavius* species were studied. Comparative 16S rRNA gene analysis showed that the Gamma 1 symbionts from *Inanidrilus* species form a monophyletic group (Figure 9). This corresponds well with molecular analyses of the host phylogeny that indicate that species of the genus *Inanidrilus* are monophyletic. However, to ensure this assumption investigation of more Gamma 1 symbionts from *Inanidrilus* hosts, in particular from different environments such as Mediterranean, Peru or the Great Barrier Reef, is needed.

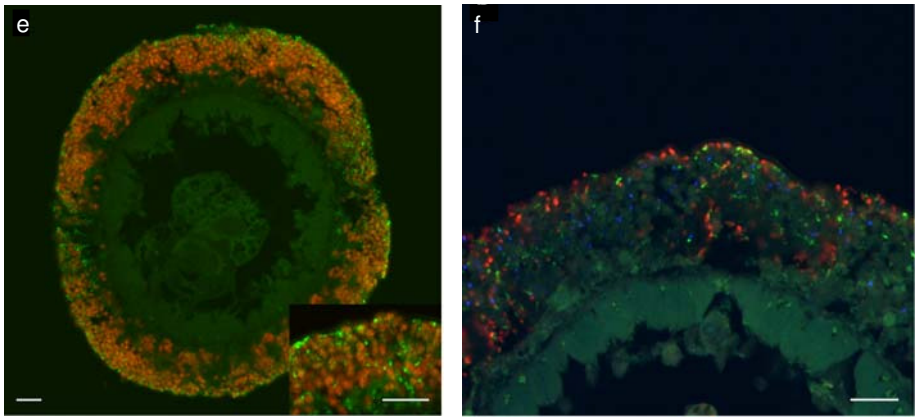
The Gamma 1 symbionts from *Olavius* species appear to be paraphyletic since they are clearly divided into two clusters, A and B (Figure 9). This phylogeny can not be explained solely by geographical location, as symbionts from species from the same site, such as *O. algarvensis* and *O. ilvae* from the Mediterranean island of Elba, are more closely related to symbionts from Bahamas than to each other. The phylogeny of the hosts may however play a role, as just suggested for *Inanidrilus* and indeed, the molecular phylogeny of

Figure 7. In situ identification of bacterial symbionts in *O. crassitunicatus* (a-d), *I. leukodermatus* (e-f), and *I. makropetalos* (g-h). The epifluorescence images show cross sections through the entire worm (a) (scale bar = 20 µm), and (e and g) (scale bar = 10 µm), and the symbiont containing region (b-d, f, and h) (scale bar = 10 µm). (a) Dual hybridization with GAM42a and DSS658/DSR651 probes, showing gammaproteobacterial symbionts in red and deltaproteobacterial symbionts in green. (b) Dual hybridization with OcraGAM1 and OcraGAM2 probes, showing the Gamma 1 symbionts in red and Gamma 2 symbionts in green. (c) Dual hybridization with OcraDEL1 and OcraDEL2 probes, showing the Delta 1 symbionts in green and Delta 2 symbionts in red. (d) Mono hybridization with the OcraSPI probe, showing spirochete symbionts in green. (e) Dual hybridization with InaGAM and IleuAlpha1a/IleuAlpha1b/IleuAlpha2 probes, showing gammaproteobacterial symbionts in red and alphaproteobacterial symbionts in green. Inset shows symbionts at higher magnification (scale bar = 10 µm). (f) Triple hybridization with Alpha1a, Alpha1b, and Alpha2 probes, showing the Alpha 1a symbionts in green, Alpha 1b symbionts in green, and Alpha 2 symbionts in blue. (g) Dual hybridization with InaGAM and ImakAlpha1a/ImakAlpha2 probes, showing gammaproteobacterial symbionts in red and alphaproteobacterial symbionts in green. Inset shows symbionts at higher magnification (scale bar = 10 µm). (h) Dual hybridization with ImakAlpha1a and ImakAlpha2 probes, showing the Alpha 1a symbionts in green and Alpha 2 symbionts in red.

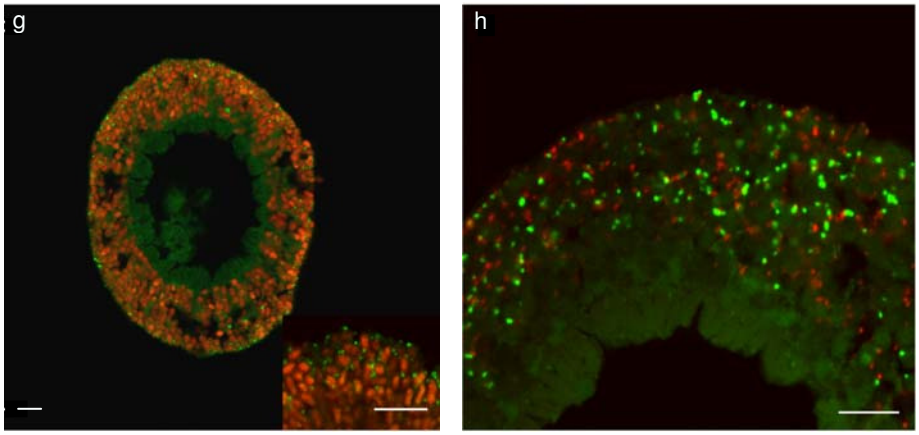
Olavius crassitunicatus



Inanidrilus leukodermatus



Inanidrilus makropetalos



Olavius species indicates that this genus is paraphyletic. A more detailed phylogenetic comparison of the Gamma 1 symbionts and their hosts is currently in progress and will reveal if cospeciation played a role in the establishment of these symbioses.

Inanidrilus exumae is the only host species in which a Gamma 1 symbiont has not been found (Figure 8). Clone libraries from this species were dominated by a different gammaproteobacterial phylotype (Table 2), affiliated to clone sequences isolated from aquatic microbial biofilms present in Australian caves, microbial communities of sponges, and hydrothermal vent sediments. The next relatives to

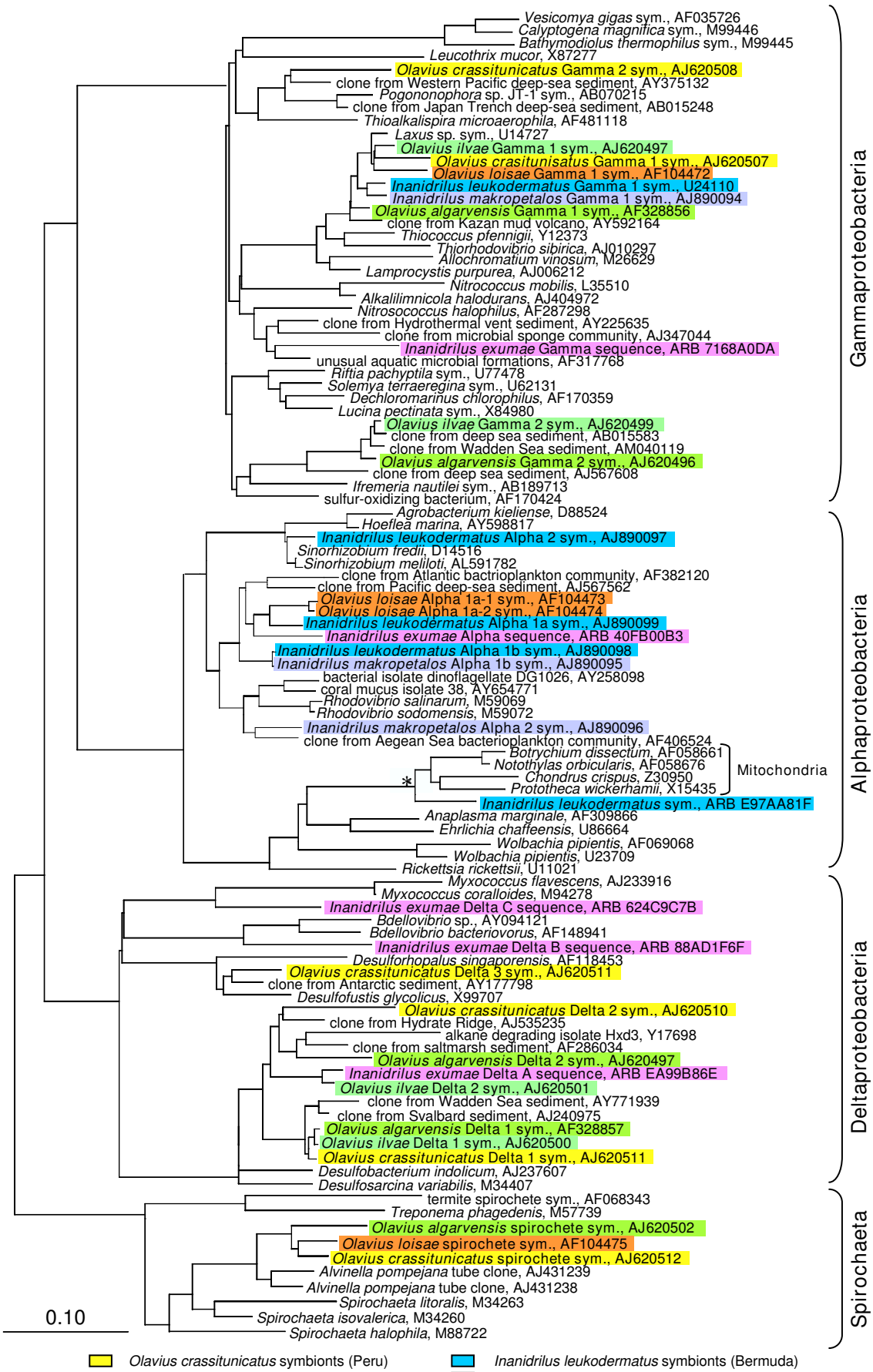
TABLE 2. 16S rRNA clone libraries from three *I. exumae* individuals

Source	No. of clones (% of total)					
	Total	Gamma	Delta A	Delta B	Delta C	Alpha
I. exumea 1	102	46 (45)	46 (45)	5 (5)	0	5 (5)
I. exumea 2	95	42 (45)	50 (51)	0	3 (3)	0
I. exumea 3	17	16 (94)	1 (6)	0	0	0

these sequences are marine, ammonia-oxidizing bacteria of the genus *Nitrosococcus* found primarily in brackish water environments (see also Chapter 1.4.2). In situ identification of the gammaproteobacterial symbionts was done with the general probe for Gammaproteobacteria, GAM 42a, and showed a hybridization pattern characteristic for gutless oligochaetes with Gamma 1 symbionts (Figure 10a). However, hybridizations with specific oligonucleotide probes for this sequence are needed to fully confirm if the *I. exumae* gammaproteobacterial symbiont belongs to a novel lineage of oligochaete symbionts.

In three host species, *O. algarvensis* and *O. ilvae* from the Mediterranean and *O. crassitunicatus* from the Southeast Pacific, a second gammaproteobacterial

Figure 8. Phylogenetic placement of the symbiotic bacteria in gutless oligochaetes based on parsimony analyses of 16S rRNA sequences. Symbionts from a given host species are shown in the same color (see legend). As many as six bacterial phylotypes can co-occur in a single host (e.g. *O. crassitunicatus*). Bar = 10% estimated sequence divergence. Below the asterisk the estimated sequence divergence is 48%.


Vesicomya gigas sym., AF035726
Calyptogena magnifica sym., M99446
Bathymodiolus thermophilus sym., M99445
Leucothrix mucor, X87277
Olavius crassitunicatus Gamma 2 sym., AJ620508
clone from Western Pacific deep-sea sediment, AY375132
Pogononophora sp. JT-1 sym., AB070215
clone from Japan Trench deep-sea sediment, AB015248
Thioalkalispira microaerophila, AF481118
Laxus sp. sym., U14727
Olavius ilvae Gamma 1 sym., AJ620497
Olavius crasitunisatus Gamma 1 sym., AJ620507
Olavius loisae Gamma 1 sym., AF104472
Inanidrilus leukodermatus Gamma 1 sym., U24110
Inanidrilus makropetalos Gamma 1 sym., AJ890094
Olavius algarvensis Gamma 1 sym., AF328856
clone from Kazan mud volcano, AY592164
Thiococcus pfennigii, Y12373
Thiorhodovibrio sibirica, AJ010297
Allochromatium vinosum, M26629
Lamprocystis purpurea, AJ006212
Nitrococcus mobilis, L35510
Alkalilimnicola halodurans, AJ404972
Nitrosococcus halophilus, AF287298
clone from Hydrothermal vent sediment, AY225635
clone from microbial sponge community, AJ347044
Inanidrilus exumae Gamma sequence, ARB 7168A0DA
unusual aquatic microbial formations, AF317768
Riftia pachyptila sym., U77478
Solemya terraeregina sym., U62131
Dechloromarinus chlorophilus, AF170359
Lucina pectinata sym., X84980
Olavius ilvae Gamma 2 sym., AJ620499
clone from deep sea sediment, AB015583
clone from Wadden Sea sediment, AM040119
Olavius algarvensis Gamma 2 sym., AJ620496
clone from deep sea sediment, AJ567608
Ifremeria nautilei sym., AB189713
sulfur-oxidizing bacterium, AF170424
Gammaproteobacteria
Agrobacterium kieliense, D88524
Hoeflea marina, AY598817
Inanidrilus leukodermatus Alpha 2 sym., AJ890097
Sinorhizobium fredii, D14516
Sinorhizobium meliloti, AL591782
clone from Atlantic bactrioplankton community, AF382120
clone from Pacific deep-sea sediment, AJ567562
Olavius loisae Alpha 1a-1 sym., AF104473
Olavius loisae Alpha 1a-2 sym., AF104474
Inanidrilus leukodermatus Alpha 1a sym., AJ890099
Inanidrilus exumae Alpha sequence, ARB 40FB00B3
Inanidrilus leukodermatus Alpha 1b sym., AJ890098
Inanidrilus makropetalos Alpha 1b sym., AJ890095
bacterial isolate dinoflagellate DG1026, AY258098
coral mucus isolate 38, AY654771
Rhodovibrio salinarum, M59069
Rhodovibrio sodomensis, M59072
Inanidrilus makropetalos Alpha 2 sym., AJ890096
clone from Aegean Sea bacterioplankton community, AF406524
Botrychium dissectum, AF058661
Nototylas orbicularis, AF058676
Chondrus crispus, Z30950
Prototheca wickerhamii, X15435
Mitochondria
*
Inanidrilus leukodermatus sym., ARB E97AA81F
Anaplasma marginale, AF309866
Ehrlichia chaffeensis, U86664
Wolbachia pipientis, AF069068
Wolbachia pipientis, U23709
Rickettsia rickettsii, U11021
Alphaproteobacteria
Myxococcus flavescens, AJ233916
Myxococcus coralloides, M94278
Inanidrilus exumae Delta C sequence, ARB 624C9C7B
Bdellovibrio sp., AY094121
Bdellovibrio bacteriovorus, AF148941
Inanidrilus exumae Delta B sequence, ARB 88AD1F6F
Desulforhopalus singaporensis, AF118453
Olavius crassitunicatus Delta 3 sym., AJ620511
clone from Antarctic sediment, AY177798
Desulfofustis glycolicus, X99707
Olavius crassitunicatus Delta 2 sym., AJ620510
clone from Hydrate Ridge, AJ535235
alkane degrading isolate Hxd3, Y17698
clone from saltmarsh sediment, AF286034
Olavius algarvensis Delta 2 sym., AJ620497
Inanidrilus exumae Delta A sequence, ARB EA99B86E
Olavius ilvae Delta 2 sym., AJ620501
clone from Wadden Sea sediment, AY771939
clone from Svalbard sediment, AJ240975
Olavius algarvensis Delta 1 sym., AF328857
Olavius ilvae Delta 1 sym., AJ620500
Olavius crassitunicatus Delta 1 sym., AJ620511
Desulfobacterium indolicum, AJ237607
Desulfosarcina variabilis, M34407
Deltaproteobacteria
termite spirochete sym., AF068343
Treponema phagedenis, M57739
Olavius algarvensis spirochete sym., AJ620502
Olavius loisae spirochete sym., AF104475
Olavius crassitunicatus spirochete sym., AJ620512
Alvinella pompejana tube clone, AJ431239
Alvinella pompejana tube clone, AJ431238
Spirochaeta litoralis, M34263
Spirochaeta isovalerica, M34260
Spirochaeta halophila, M88722
Spirochaeta
0.10
Olavius crassitunicatus symbionts (Peru)
Olavius ilvae symbionts (Mediterranean)
Olavius algarvensis symbionts (Mediterranean)
Olavius loisae symbionts (Australia)
Inanidrilus leukodermatus symbionts (Bermuda)
Inanidrilus makropetalos symbionts (Bahamas)
From Inanidrilus exumae isolated 16S rRNA sequences (Bahamas)

symbiont, Gamma 2, was detected. The Gamma 2 symbionts are much smaller (diameter approximately 1 µm) than the Gamma 1 symbionts (diameter 3-8 µm) and are located between these throughout the symbiont-containing region (Figure 7b). In contrast to the Gamma 1 symbionts, the 16S rRNA gene sequences of these symbiotic bacteria are not all clustered (Figure 8). The Gamma 2 symbionts of the two Mediterranean hosts form a clade with clone sequences from North Sea and Japan Trench sediments. The Gamma 2 symbiont from *O. crassitunicatus* is phylogenetically distant from the two other Gamma 2 symbionts, and is related to clone sequences from deep-sea sediments and a pogonophoran tubeworm in the Western Pacific.

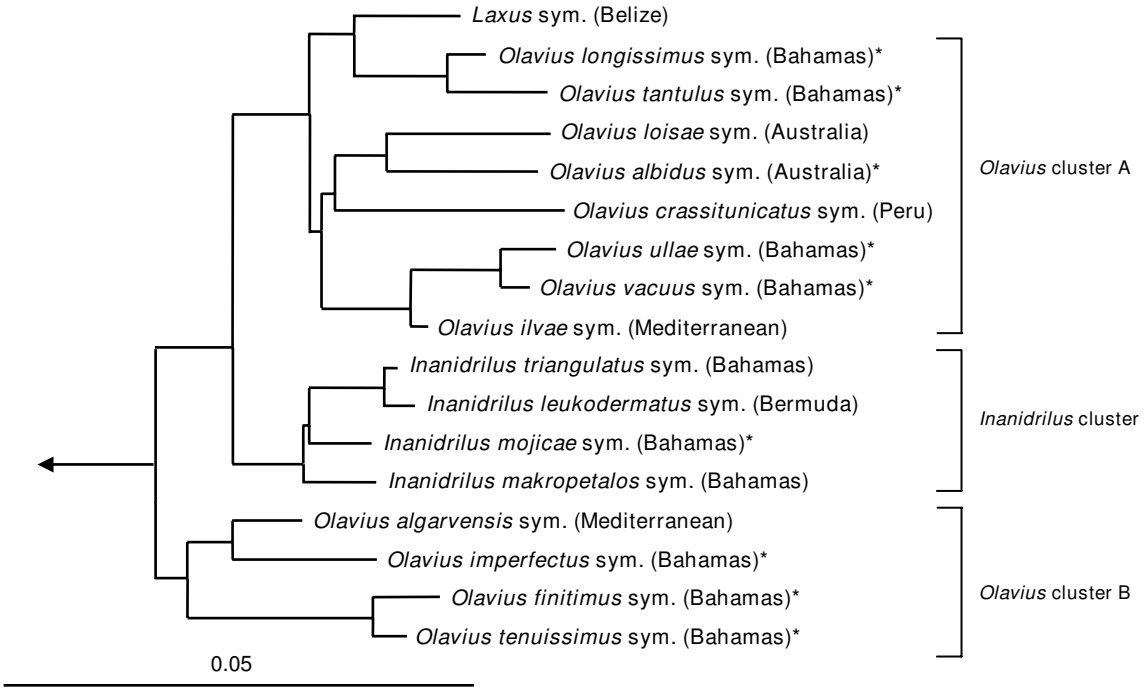


Figure 9. Phylogenetic tree of the chemoautotrophic, sulfur-oxidizing Gamma 1 symbionts of gutless oligochaetes, based on maximum-likelihood analyses of the 16S rRNA gene. Symbionts from *Inanidrilus* species form a monophyletic group, while those from *Olavius* species fall into 2 groups, cluster A and B, that are phylogenetically separate. Sequences marked with asterisk have not been confirmed with FISH. Bar = 5% estimated sequence divergence.

1.2.2 Deltaproteobacterial symbionts

The first deltaproteobacterial symbiont, Delta 1, was recently discovered in *O. algarvensis* (Dubilier et al., 2001). The present study demonstrates a higher diversity than previously assumed of these small, rod-shaped symbionts

(Figure 7c) with new lineages of deltaproteobacterial symbionts identified in the two co-occurring species from the Mediterranean, *O. algarvensis* and *O. ilvae*, and *O. crassitunicatus* from the Southeast Pacific. Although the phylogeny of these symbionts, Delta 1-3, differs they are all related to sulfate-reducing bacteria (Figure 8). The Delta 1 and 2 symbionts occur in all three host species, while the Delta 3 symbiont was only found in *O. crassitunicatus*. The Delta 1 symbionts are closely related to each other and form a monophyletic cluster. The next relatives to this clade of symbiotic sequences are clone sequences from the Wadden Sea and Svalbard sediments. The Delta 2 symbionts are not as closely related to each other as are the Delta 1 symbionts, however, they fall in the same cluster with two environmental clone sequences from marine sediments and an alkane degrading isolate. The two clusters with Delta 1 and 2 symbionts are related to the free-living, sulfate-reducing bacteria *Desulfosarcina variabilis* and *Desulfobacterium indolicum*. The Delta 3 symbiont of *O. crassitunicatus* is phylogenetically distinct from the Delta 1 and 2 symbionts, and is most closely related to clone sequences isolated from Antarctic sediments and the free living sulfate-reducing bacterium *Desulfofustis glycolicus*.

In *I. exumae* from the Bahamas three different 16S rRNA phylotypes, Delta A-C, belonging to the Deltaproteobacteria are present (Table 2). The Delta A sequence is dominant in the libraries of all three *I. exumae* individuals and is closely related to the Delta 2 symbiont from *O. ilvae,* suggesting that it originated from a symbiotic bacterium (Figure 8). The two other sequences, Delta B and C, were found in only a single *I. exumae* individual each. The Delta B sequence is related to the predacious bacteria of the genus *Bdellovibrio,* while the Delta C sequence is affiliated to free living, gliding bacteria of the genus *Myxococcus* (Figure 8). The unusual phylogeny of these sequences not previously described in gutless oligochaetes and their rare and irregular occurrence in the 16S rRNA gene libraries indicates that they may originate from contaminants instead of symbionts. In situ identification of deltaproteobacterial symbionts was done with the probe DSS658 that is generally used for Deltaproteobacteria belonging to the genera *Desulfosarcina*, *Desulfococcus, Desulfofaba*, and *Desulfofrigus*

(Figure 10b). The hybridization signal was limited to small bacteria within the symbiont-containing region indicating that the gutless oligochaete *I. exumae* harbors deltaproteobacterial symbionts. These bacteria occur primarily underneath the cuticle but were also sporadically present in the interior layers.

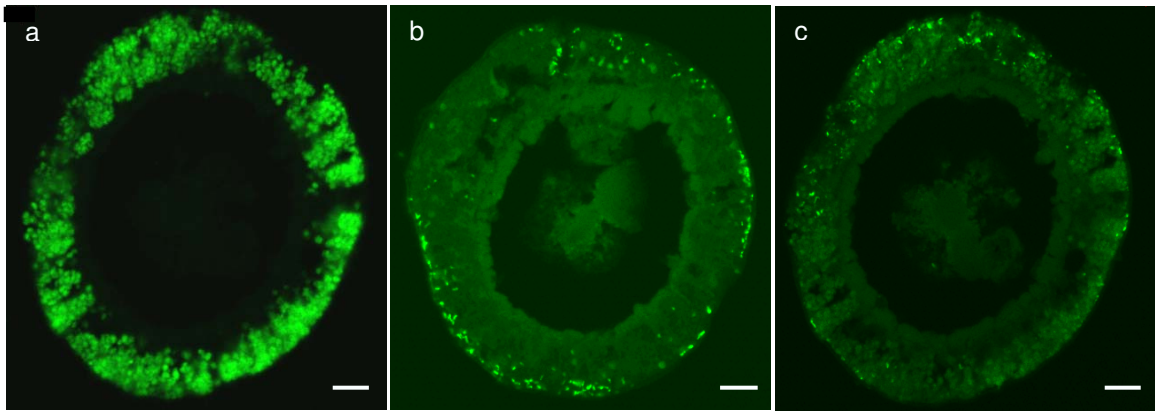


Figure 10. In situ identification of bacterial symbionts in *I. exumae*. The epifluorescence images show cross sections through the entire worm (scale bar = 10 µm). Hybridization with the GAM42a probe showing gammaproteobacterial symbionts (a), the DSS658 probe showing deltaproteobacterial symbionts (b), and the ALF968 probe showing alphaproteobacterial symbionts (c).

The final proof if the isolated deltaproteobacterial sequences originate from symbionts of *I. exumae* or they are contaminants will be clarified by FISH analysis with specific oligonucleotide probes.

1.2.3 Alphaproteobacterial symbionts

At the beginning of this study alphaproteobacterial symbionts had only been found in a single gutless oligochaetes species, *O. loisae* from the Australian Great Barrier Reef (Dubilier et al., 1999). The occurrence of these symbionts in *I. leukodermatus* from Bermuda and *I. makropetalos* from Bahamas argues for a more common nature of this symbiosis. All three host species harbor alphaproteobacterial symbionts belonging to a clade of relatively closely related bacteria called Alpha 1 symbionts (≥ 92.6% sequence similarity) (Figure 8). Within this clade, different phylotypes can co-occur in the same species such as Alpha 1a and 1b symbionts in *O. loisae* and *I. leukodermatus*. The next relatives to these symbiotic sequences are clone sequences isolated from Atlantic and

Arctic bacterioplancton communities and from Pacific deep-sea sediments. In addition to the Alpha 1 lineage of symbionts, *I. leukodermatus* and *I. makropetalos* harbor alphaproteobacterial bacteria called Alpha 2 that are phylogenetically distinct from each other (Figure 8). The Alpha 2 symbiont from *I. makropetalos* is related to the free-living, halophilic bacteria *Rhodovibrio salinarum* and *R. sodemensis* and bacteria isolated from dinoflagellate and coral mucus. The closest relatives of the Alpha 2 symbiont from *I. leukodermatus* are the plant-associated, nitrogen-fixing bacteria *Sinorhizobium meliloti* and *S. fredii*. The distribution and hybridization pattern of the alphaproteobacterial symbionts in gutless oligochaetes is similar. All of them are small rod-shaped bacteria that occur throughout the symbiont-containing region (Figure 7 e-h).

In *I. exumae* from the Bahamas, alphaproteobacterial sequences were found in one of the three individuals examined (Table 2). This sequence is closely related to the 16S rRNA gene sequences from the Alpha 1 symbionts of *I. leukodermatus*, *I. makropetalos*, and *O. loisae* indicating its origin from symbiotic bacteria (Figure 8). In situ identification of alphaproteobacterial symbionts in *I. exumae* was tested with the general probe for the Alphaproteobacteria, ALF968. This probe hybridized to small bacteria throughout the symbiont-containing region with a hybridization pattern similar to that from other hosts harboring alphaproteobacterial symbionts (Figure 10c). Although this indicates the presence of alphaproteobacterial symbionts in *I. exumae* species, hybridizations with oligonucleotide probes specific for the isolated alphaproteobacterial sequence are needed to fully confirm this result. *I. exumae* would be the first species of gutless oligochaetes examined to date that harbors both alphaproteobacterial and deltaproteobacterial symbionts.

1.2.3.1 Bacterial symbionts related to mitochondrial 16S rRNA gene sequences

In three out of six *I. leukodermatus* specimens, a highly unusual alphaproteobacterial sequence was found that is remarkably closely related to mitochondria (Figure 8). No other proteobacterial 16S RNA gene sequences are

as closely related to the mitochondrial 16S rRNA gene sequences as the bacterial sequence isolated from *I. leukodermatus*. To identify the source of this sequence seven specific probes were designed (Table 3) of which only the probe Ileumito140 showed a signal in some but not all host individuals

Table 3. Oligonucleotide probes used for the in situ identification of the mitochondria related alphaproteobacterial sequence from *I. leukodermatus*

Probe	Sequence (5'-3')	Position[a]
Ileu_mito_140	GGG TAC TTA TGC CTT GCC	140-156
Ileu_mito_329	CCG TAG AAA TCT GGG CCT	329-346
Ileu_mito_403	AAT GCC TTC ATC ACC CAC	403-420
Ileu_mito_564	ACC CAC CCT CTA CGC CTA	564-581
Ileu_mito_573	AAA ACA CCT ACC CAC CCT	573-590
Ileu_mito_1178	TGT CAT CGT CCC CTC CTT	1178-1195
Ileu_mito_1242	TGC GTC TCG CTG TAG TTG	1242-1259

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

In contrast to most of the other oligochaete symbionts that are regularly distributed throughout the symbiont-containing region, the Ileumito140 probe detected cells occurred in small clusters of 5-19 bacteria in the symbiont-containing region or as single cells in the coleomic cavity of the worm (Figure 11). The size and morphology of these rod-shaped bacteria at both locations is similar (approximately 1.5 to 2.8 µm x 0.8 µm). Bacterial cells have not been previously observed in the coleomic cavity of gutless oligochaetes. To fully confirm if these bacteria are the source of the mitochondria related 16S rRNA sequence, hybridizations with additional specific probes are needed, as it is highly unusual that only one out of seven probes showed a signal.

The phylogenetic placement of the unusual alphaproteobacterial sequence from *I. leukodermatus* changes the relationship between mitochondria and the order Rickettsiales. So far, members of the Rickettsiales were the closest relatives to mitochondria. Now, the closest relative is the alphaproteobacterial sequence isolated from *I. leukodermatus* indicating that the real phylogenetic relationship between the alphaproteobacterial and mitochondrial 16S rRNA

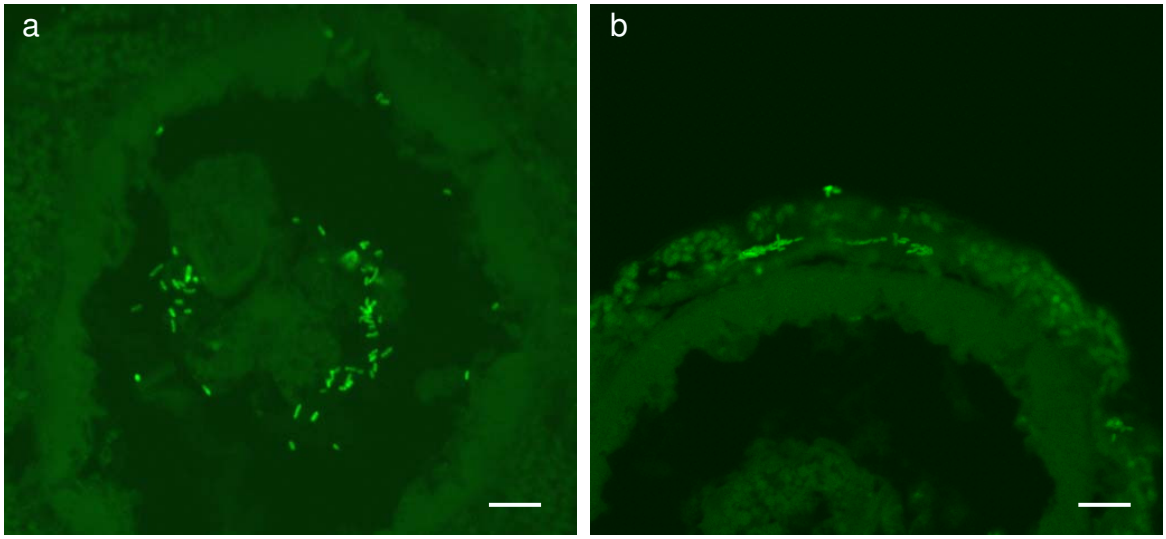


Figure 11. In situ identification of bacterial symbionts with the probe Ileumito140 in *I. leukodermatus* that are related to mitochondria. These bacteria were detected in the coleomic cavity of the host (a) and the symbiont-containing region between cuticle and epidermal layer (b) (scale bars = 10 µm).

sequences is not yet resolved. This is supported by data from the *Wolbachia pipientis* genome that indicates that mitochondria did not arise from the Rickettsiales, but rather might have arisen from a deeper branch in the Alphaproteobacteria (Cerdeño-Tárraga et al., 2004).

1.2.4 Spirochete symbionts

The first indication that spirochetes occur as symbionts in gutless oligochaetes was from a study of the species *O. loisae* (Dubilier et al., 1999). However, the authors were not able to confirm that their isolated spirochete 16S rRNA gene sequence originated from symbiotic bacteria. In this thesis FISH evidence for the presence of spirochetes in the symbiotic region of *O. crassitunicatus* and *O. algarvensis* is provided for the first time (Figure 7d). The hybridization signal from the spirochete probe was consistent with shape and distribution pattern of the long and thin bacterial morphotype described in ultrastructural studies of these host species (Giere and Erséus, 2002; Giere and Krieger, 2001). The symbiotic spirochete 16S rRNA gene sequences are closely related to each other and form a monophyletic group within the phylum Spirochaeta (Figure 8). Peripherally associated with these sequences are clone sequences obtained from tubes of

the hydrothermal vent polychaete *Alvinella pompejana*. The next relatives to this cluster of symbiotic sequences are the free living marine spirochetes *Spirochaeta isovalerica* and *S. litoralis*.

1.3 Conclusions

1.3.1 Phylogenetic diversity

The phylogenetic diversity of bacterial symbionts in gutless oligochaetes is much higher than previously assumed. In earlier studies, either a single phylotype (Dubilier et al., 1995) or two or three co-occurring bacterial phylotypes were found in each host species (Dubilier et al., 1999; Dubilier et al., 2001). This study shows that at least three and as many as six different symbiotic phylotypes can co-occur in the same species. One explanation for the previously unrecognized diversity is the ability to sequence much higher numbers of clones (300 versus 60 to 70 clones or direct sequencing in earlier studies) from the 16S rRNA gene libraries. Also the use of lower PCR cycle numbers decreased PCR bias and improved the chances of amplifying 16S rRNA gene sequences that are not dominant in the libraries. In addition, improved FISH techniques, such as catalyzed reporter deposition (CARD) FISH, enabled the identification of the otherwise not detectable symbiotic bacteria in the symbiont-containing region, such as small bacteria or ones with low rRNA content.

So far, except for sponges (Porifera) that harbor vast amounts of phylogenetically distinct ecto- and endosymbionts (reviewed in Lee et al. 2001), it is assumed that endosymbioses with multiple bacteria are rare among marine invertebrates (Maynard Smith and Szathmáry, 1995). Beside the dual symbioses of marine bivalves the coexistence of multiple endosymbionts has only been described in the vestimentiferan tubeworm *Lamellibrachia* sp. (Kimura et al., 2003a; Kimura et al., 2003b) and the wood-boring bivalve *Xylophaga atlantica* (Distel et al., 2002). Most recently, a bivalve from the Mediterranean seep with six coexisting symbionts in its gills was discovered (S. Dupperon, personal communication). These studies together with our studies on gutless oligochaetes

indicate that the symbiotic diversity in marine invertebrates has been underestimated and can be more complex than previously assumed.

1.3.2 Functional aspects of the bacterial symbionts

Despite the high phylogenetic diversity of symbionts in gutless oligochaetes, these associations are clearly specific and stable for most phylotypes within a given host species. The regular occurrence of the sulfur-oxidizing chemoautotrophic Gamma 1 symbionts in almost all gutless oligochaete species indicates their importance for the symbiotic association and strengthens the hypothesis that these bacteria are primary responsible for the nutrition of the host.

In contrast to the Gamma 1 symbionts all other detected symbiotic phylotypes occur in some but not all species. The Gamma 2 symbionts were only detected in hosts colonizing silicate sands suggesting that the mineral type of the sediment might be of importance for these symbioses. Their physiological nature is not currently known. However, the phylogenetic relationship of the Gamma 2 symbionts to bacterial clone sequences isolated from chemoautotrophic environments suggests their similar nature. Furthermore, molecular identification of genes involved in CO_2 fixation and oxidative sulfur metabolism suggests that these symbionts might be sulfur-oxidizing, chemoautotrophic bacteria (see 3.1.1.1 and 3.1.2.1). To harbor a second chemoautotrophic symbiont would be beneficial for these hosts because they could supply additional nutrition.

With the exception of *I. exumae*, all gutless oligochaetes harbor either Delta or Alpha symbionts as further symbionts. In these associations the sediment type could also play a role since Alphaproteobacteria were only found in hosts from calcareous sands, while Deltaproteobacteria occurred mainly in hosts from silicate sands. It would be interesting to find out if the different symbiotic phylotypes mirror the differences in microbial community structure in silicate and calcareous habitats of gutless oligochaetes.

The close evolutionary relationship of the deltaproteobacterial symbionts to free-living and symbiotic sulfate-reducing bacteria and the predominance of this

type of metabolism within the Deltaproteobacteria suggests that these symbionts also use sulfate as an electron acceptor. The first chemoautotrophic host known to harbor a deltaproteobacterial, sulfate-reducing symbiont was the gutless oligochaete *O. algarvensis* from sediments in the Mediterranean (Dubilier et al., 2001). In the Mediterranean sediments in which *O. algarvensis* and *O. ilvae* occur, sulfide concentrations are very low (< 1 µM) (Dubilier et al., 2001). This led to the suggestion that the sulfate-reducing symbionts in these hosts could provide the coexisting thioautotrophic symbionts with an internal source of sulfide. However, in the Peruvian sediments populated by *O. crassitunicatus,* sulfide was detectable by smell, suggesting concentrations in the mM range (Levin et al., 2002). The presence of sulfide in the habitat of *O. crassitunicatus* indicates that the role of the sulfate-reducing symbionts in these worms is not restricted to supplying sulfide for the thioautotrophic symbionts. One distinct difference between the sulfate-reducing symbionts of *O. algarvensis* and *O. ilvae*, and *O. crassitunicatus* is their distribution (Figure 12). In *O. algarvensis* and *O. ilvae*, the sulfate-reducing bacteria occur throughout the entire symbiont-containing region and are in close contact to the thioautotrophic symbionts. In

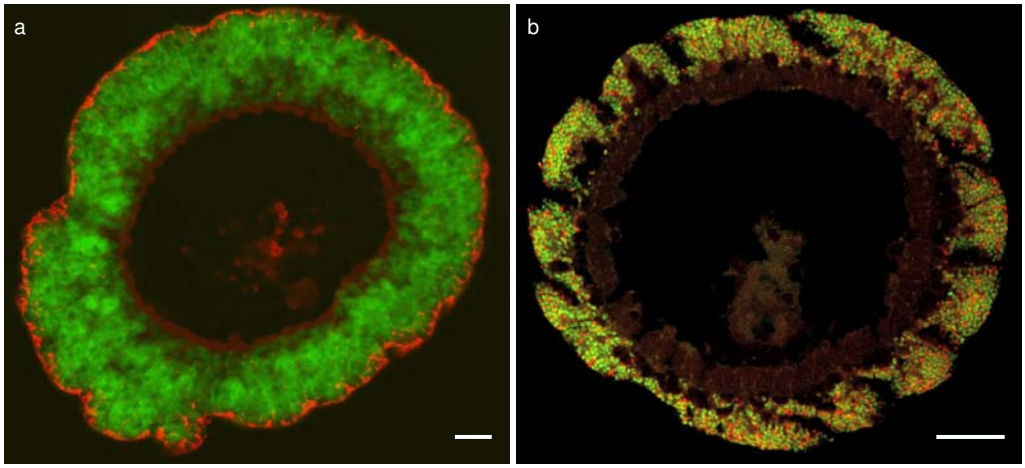


Figure 12. Fluorescence in situ hybridization (FISH) of bacterial symbionts in *O. crassitunicatus* (a) and *O. algarvensis* (b). Dual hybridization with Gam42a and DSS658/DSR651 probes, showing gammaproteobacterial symbionts in green (dark green in *O. crassitunicatus* and light green in *O. algarvensis*), and deltaproteobacterial symbionts in red. Note the different distribution of the symbionts in the two host species, with the deltaproteobacterial symbionts of *O. crassitunicatus* directly below the cuticle (this study), and those of *O. algarvensis* more evenly distributed throughout the symbiotic region (Dubilier et al., 2001). Bars: (a) = 10 µm, (b) = 20 µm.

contrast, in *O. crassitunicatus* the sulfate reducers occur almost exclusively in the outer region of the symbiont-containing region, just below the cuticle of the worm, and have little contact to the thioautotrophic symbionts. This suggests that the uptake of substrates from the environment, such as organic carbon, or hydrogen if the sulfate reducers are autotrophic, may play an important role in the *O. crassitunicatus* symbiosis.

Another role of the multiple sulfate-reducing bacteria in gutless oligochaetes could be the uptake of fermentative products from the host such as succinate, propionate, and acetate as suggested for the Delta 1 symbiont in *O. algarvensis* (Dubilier et al., 2001). The usage of these substrates would provide the sulfate reducers with a ideal energy source, aid the hosts in the removal of these undesirable endproducts and recycle metabolites that would otherwise be lost to the symbiosis.

The coexistence of two to three species of sulfate reducers in the same host could be explained by the absorption of different substrates by the particular symbionts. The usage of variable substrates is common among sulfate-reducing bacteria that are known to metabolize a wide variety of electron donators and carbon sources with different preferences (Widdel and Bak, 1992; Widdel and Pfennig, 1992).

In contrast to the well defined nature of the Delta symbionts, the metabolism of the Alpha symbionts occurring in hosts from calcareous sediments is uncertain. Most of the Alpha symbionts are related to the free living halophilic bacteria *Rhodovibrio salinarum* and *R. sodomensis* that are known to use fermentative products like lactate, acetate, malate, or pyruvate as a carbon source and/or energy source (Mack et al., 1993; Nissen and Dundas, 1984). These metabolites are produced by the worm when oxygen becomes limiting suggesting that they could be taken up by the alphaproteobacterial symbionts as a carbon source and/or energy source. As mentioned before, in gutless oligochaetes either Alpha or Delta symbionts coexist with the Gamma 1 symbionts and it is tempting to speculate that the Alpha symbionts might also

play a role in the recycling of anaerobic waste products of the worm as suggested for the Delta symbionts.

The spirochete symbionts were identified in some but not all gutless oligochaetes species present in silicate and calcareous sands indicating that the mineral type of the sediment is of minor importance for these symbioses. The function of spirochete symbionts is not currently known. The free-living, marine spirochetes *S. isovalerica* and *S. litoralis* consistently form a neighboring clade of the oligochaete spirochetes. These bacteria were isolated from sulfidic muddy sediments and are obligate anaerobes that ferment carbohydrates mainly to acetate, ethanol, CO_2, and H_2 (Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1973). While fermentation is one possible metabolic pathway of the oligochaete spirochetes, they could also have a completely different metabolism, just as the spirochete symbionts in termites that do not possess properties common to their closest free-living relatives within the genera *Treponema* (Breznak, 2002). Instead, termite spirochetes were recently discovered to be chemoautotrophic, using H_2 and CO_2 to produce acetate (Leadbetter et al., 1999). This type of metabolism would clearly be beneficial to the oligochaete hosts, providing them with an additional source of carbon and energy.

The phylogenetic diversity of bacterial symbionts in *I. exumae* differs from the symbiotic communities of all other gutless oligochaete species. This host, from calcareous sands of Bahamas, harbors in addition to the expected Gamma and Alpha symbionts, also symbiotic bacteria belonging to the Deltaproteobacteria. So far, these bacteria have only been detected in host species colonizing silicate sediments that in turn do not harbor symbiotic bacteria belonging to the Alphaproteobacteria. Also, the phylogeny of the *I. exumae* Gamma symbiont is unusual because it does not fall in the clade of Gamma 1 symbionts found in all other gutless oligochaetes. Instead, this symbiont is related to ammonia-oxidizing bacteria indicating that ammonia and not sulfur compounds might be used as electron donor. Further studies such as identification of key genes involved in ammonium oxidation and simultaneous

absence or presence of key genes involved in sulfur oxidation, could be helpful to provide an insight into the metabolic nature of the *I. exumae* Gamma symbionts.

2 Metabolic diversity of bacterial symbionts

Two different approaches were used to study the metabolic capabilities of the bacterial symbionts. The PCR based approach was used to identify functional genes of importance in the gutless oligochaetes symbioses and compare their phylogeny to homologous genes of free-living and symbiotic bacteria. The comparative metagenomic approach was used to gain insight into the genome of the symbionts to reveal the organization of genes involved in significant metabolic pathways of the oligochaete symbionts.

2.1 PCR based approaches

The main objective in this study was the characterization of key enzymes involved in sulfur and nitrogen energy metabolism, and CO_2 fixation. For a selective amplification of these genes specific primers were developed or published primers were used. Phylogenetic relationships of the isolated sequences were calculated based on their deduced amino acid sequences.

2.1.1 CO_2 fixation

2.1.1.1 Calvin-Benson-Bassham cycle

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) is one of the key enzymes involved in carbon dioxide fixation via the Calvin-Benson-Bassham (CBB) cycle and incorporates carbon dioxide into organic molecules. In chemoautotrophic symbionts two forms of RubisCO are present: form I encoded by the gene *cbbL* and form II by the gene *cbbM* (Cavanaugh and Robinson, 1996; Krieger et al., 2000; Robinson and Cavanaugh, 1995; Schwedock et al., 2004; Stein et al., 1990). These two forms differ in their molecular structure and their specificity for CO_2 (Tabita, 1988; Tabita, 1995; Watson and Tabita, 1997). In nature, the dominant form I RubisCO is subdivided into four classes A-D based

on inferred homology. Forms IA and IC are primarily present in Proteobacteria whereas forms IB and ID are more prevalent in cyanobacteria and chloroplasts.

In this study specific primers for genes encoding the RubisCO form I and II large-subunit were developed and used for amplification of these genes from four species of gutless oligochaetes: *O. algarvensis*, *O. ilvae*, *I. leukodermatus* and *I. makropetalos*. The *cbbL*-encoded RubisCO form I (~650 bp) was found in all four species, while the *cbbM*-encoded RubisCO form II was never detected indicating that form II RubisCO may not be present in these oligochaete symbionts.

The phylogenetic analysis of the oligochaete RubisCO form I sequences revealed that these belong to the form IA clade of RubisCO sequences (Figure 13). The next relatives of the oligochaete RubisCO sequences are those from chemoautotrophic, sulfur-oxidizing bacteria, such as symbionts of *Solemya velum* and *Alvinoconcha hessleri,* and from the free-living bacterium *Allochromatium vinosum*. Although RubisCO phylogeny often does not correspond with the 16S rRNA-based phylogeny due to lateral gene transfer (Shively et al., 1998), the close clustering of RubisCO IA sequences from chemoautotrophic, sulfur-oxidizing bacteria suggests that the oligochaete sequences originated from their chemoautotrophic symbionts.

Two phylogenetically distinct types of form IA RubisCO sequences were found *in O. algarvensis*, RubisCO 1 and 2, while only RubisCO 1 was found in *O. ilvae, I. leukodermatus* and *I. makropetalos*. The RubisCO 1 sequences from the four hosts form a cluster that is related to the RubisCO 2 sequence from *O. algarvensis*. The two *Inanidrilus* species harbor only a single gammaproteobacterial symbiont, Gamma 1, that is a chemoautotrophic sulfur oxidizer, and only a single RubisCO phylotype belonging to the RubisCO 1 group was found in these hosts. The close relationship of the RubisCO 1 sequences from *Inanidrilus* Gamma 1 symbionts with RubisCO 1 sequences from *O. algarvensis* and *O. ilvae* suggests that the *Olavius* RubisCO 1 sequences also originated from the Gamma 1 symbionts of these hosts. Correspondingly, the RubisCO 2 sequence in *O. algarvensis* may have originated from their Gamma 2

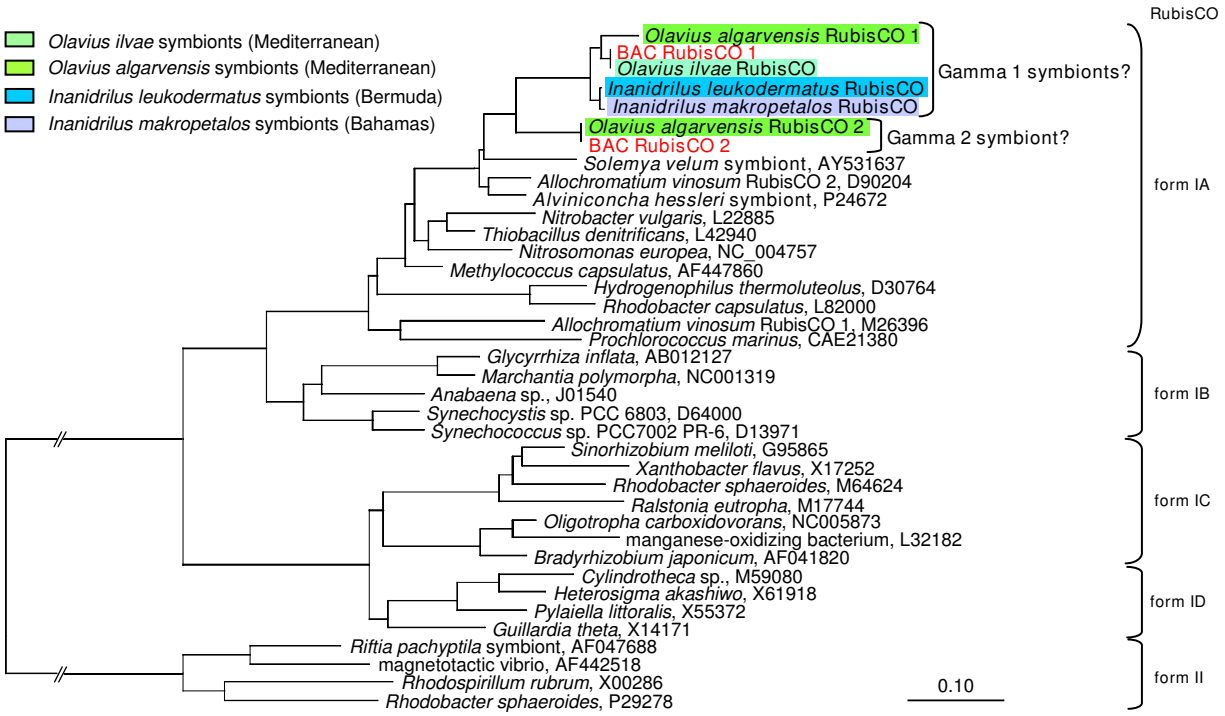


Figure 13. Phylogenetic placement of symbiotic RubisCO large-subunit sequences based on maximum likelihood analysis. Symbiotic sequences are listed in bold, with sequences from gutless oligochaetes colored according to host species (see legend). RubisCO sequences from the *Olavius* spp. BAC library are in red. The bar represents 10% estimated sequence divergence. The distance between RubisCO form I and II sequences is an estimated 126% sequence divergence.

symbionts. The lack of a RubisCO 2 sequence in *O. ilvae*, that also has Gamma 2 symbionts, could be explained by the fact that only 50 clones from a single individual were sequenced, and we are currently investigating more clones and individuals.

Alternatively, the RubisCO 1 and 2 sequences could have originated from the same symbiont. Two copies of form I RubisCO were reported in several species such as *Nitrobacter hamburgensis*, *Hydrogenvibrio marinus*, *Acidithiobacillus ferrooxidans*, and *Allochromatium vinosum* (Harris et al., 1988; Hayashi et al., 1998; Heinhorst et al., 2002; Kusian and Bowien, 1997; Shively et al., 1998; Viale et al., 1989).

2.1.1.2 Reverse tricarboxylic acid cycle

The reverse tricarboxylic acid (rTCA) cycle is an alternative CO_2 fixation pathway to the CBB cycle found in *Chlorobium* species, some members of the Delta- and

Epsilonproteobacteria, and some members of the thermophilic *Aquificales* order and archaeal *Thermoproteaceae.* Since gutless oligochaetes harbor in addition to the chemoautotrophic symbionts that fix CO_2 via the CBB cycle, also symbiotic bacteria of the Delta-, and Alphaproteobacteria and the phylum Spirochaeta, it was tested if the rTCA cycle could play a role in these symbioses. For this purpose specific primers for two key genes of the rTCA cycle, *aclB* encoding the ATP citrate lyase beta subunit and *oorA* encoding the 2-oxoglutarate ferredoxin oxidoreductase alpha subunit, were applied for the selective amplification of these genes (Campbell and Cary, 2004; Campbell et al., 2003). In the four examined oligochaetes species, *O. algarvensis*, *O. ilvae*, *I. leukodermatus*, and *I. makropetalos* no detectable amplification of these two key genes for the rTCA cycle was observed despite multiple PCR efforts with varying conditions. Consequently, CO_2 fixation via rTCA cycle might not play a role in symbionts of gutless oligochaetes, if it can be assumed that the specific primers match the template genes.

2.1.2 Energy metabolism

2.1.2.1 Sulfur energy metabolism

Sulfur energy metabolism plays an important role in symbiotic associations between bacteria and gutless oligochaetes because these hosts regularly harbor chemoautotrophic, sulfur-oxidizing symbionts, and some species also have sulfate-reducing bacteria. One of the key enzymes involved in the oxidative and reductive pathways of the sulfur metabolism is adenosine-5'-phosphosulphate (APS) reductase (Friedrich, 2002; Hipp et al., 1997). Depending on the mode of sulfur metabolism the enzymatic reaction can occur in two different directions. In sulfate reducers, APS reductase catalyzes the two-electron reduction of APS to sulfite and AMP. The reverse reaction, generating APS as a product, has been shown in the sulfur-oxidizing bacterium *A. vinosum* and other sulfur oxidizers (Sánchez et al., 2001). APS reductase consists of an alpha and beta subunit encoded by the genes *aprA* and *aprB*, respectively, that appears to form a 1:1 $\alpha\beta$

heterodimer. The *aprA* gene has been proposed as a useful phylogenetic marker for sulfate-reducing bacteria (Hipp et al., 1997). However, lateral gene transfer of *aprA* was observed among some sulfate-reducing bacteria, such as gram-positive and thermophilic, sulfate-reducing bacteria, that can blur the true phylogenetic relationship of *aprA* sequences despite their highly conserved nature (Friedrich, 2002). Little is known about the phylogeny of *aprA* from sulfur-oxidizing bacteria because only a few sequences from these bacteria are available in the database. These sequences form two phylogenetically distinct groups, one containing *aprA* sequences from *Thiobacillus denitrificans and Chlorobium tepidum*, the other the *aprA* sequence from *A. vinosum*.

For the specific amplification of the *aprA*-encoded APS reductase alpha subunit, primers developed by Jan Küver were used (Küver, unpublished data). In all examined oligochaetes species, *O. algarvensis*, *O. ilvae*, *I. leukodermatus*, and *I. makropetalos*, APS reductase sequences were isolated. Phylogenetic analysis placed these sequences into three distinct clusters with close relationships either to sulfur-oxidizing or sulfate-reducing bacteria (Figure 14).

The first cluster of APS reductase sequences, called APS 1 reductase, contains sequences from all four oligochaete species. The closest relatives to this clade of symbiotic sequences are those from the free-living, sulfur-oxidizing bacteria *T. denitrificans* and *C. tepidum,* indicating that the oligochaete sequences originated from their sulfur-oxidizing symbionts. The symbiotic APS reductase 1 clade contains sequences from hosts with only a single sulfur-oxidizing symbiont, Gamma 1 (*I. leukodermatus and I. makropetalos*) indicating that these sequences originated from their Gamma 1 symbionts. In hosts such as *O. algarvensis* and *O. ilvae* that also harbor Gamma 2 symbionts, two types of sequences with close relationship to sulfur-oxidizing bacteria were found, APS 1 and APS 2 reductase sequences. The close relationship of the *Olavius* APS 1 reductase sequences to those from the *Inanidrilus* Gamma 1 symbionts suggests that the *Olavius* APS 1 reductase sequences also originated from their Gamma 1 symbionts. Correspondingly, the *Olavius* APS 2 reductase sequences are assumed to have originated from the Gamma 2 symbionts of these hosts.

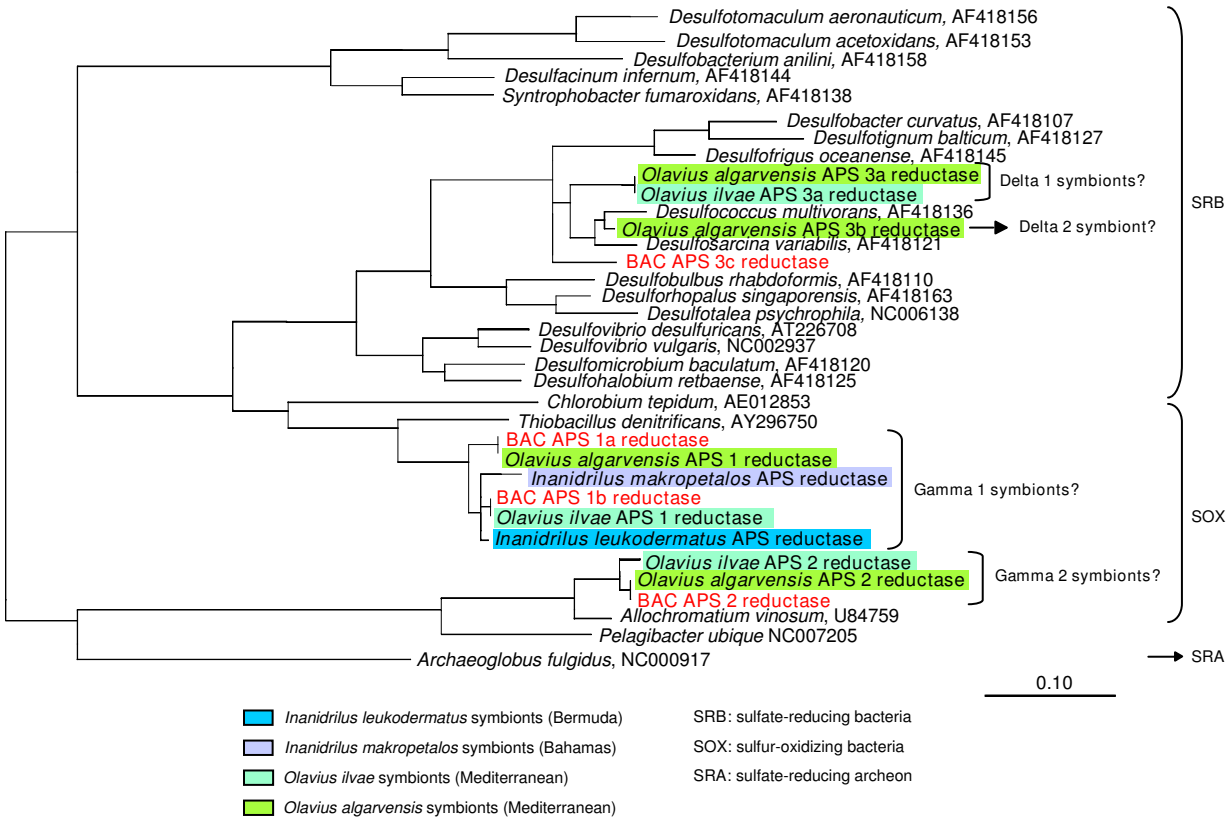


Figure 14. Phylogenetic placement of symbiotic APS reductase alpha-subunit sequences based on maximum likelihood analysis. Sequences from individual worms are shown in the corresponding color for the host species (see legend); sequences from the *Olavius* spp. BAC library are in red. The bar represents 10% estimated sequence divergence.

Alternatively, the APS 1 and 2 reductase sequences could have originated from the same symbiont. However, to date only a single APS reductase phylotype has been found in all bacteria with this gene (Friedrich, 2002).

The third cluster contains APS 3 reductase sequences that were only found in hosts with sulfate-reducing symbionts such as *O. algarvensis* and *O. ilvae.* The close phylogeny of these sequences to APS reductase sequences from the sulfate-reducing bacteria *Desulfosarcina variabilis* and *Desulfococcus multivorans* suggests their origin from the sulfate-reducing symbionts. The APS 3a reductase sequences from *O. algarvensis* and *O. ilvae* are identical at the amino acid level, differ however clearly in their nucleic acid sequences (96.2% sequence similarity). The close relationship of these two APS reductase sequences is congruent with the high similarity of the 16S rRNA sequences from the sulfate-reducing Delta 1 symbionts of *O. algarvensis* and *O. ilvae* (95.5%

sequence similarity) (Figure 14), indicating that the APS 3a reductase sequences originated from the Delta 1 symbionts of the *Olavius* hosts. Correspondingly, the APS 3b reductase sequence from *O. algarvensis* may have originated from the sulfate-reducing Delta 2 symbiont of this host. The lack of an APS 3b reductase sequence from the Delta 2 symbiont of *O. ilvae* could be explained by the small number of clones (50) and individuals (1) examined, and further sequencing is currently in progress. In summary, this study shows that the *aprA*-encoded APS reductase is a useful phylogenetic marker for identifying symbionts involved in oxidative and reductive sulfur metabolism.

2.1.2.1 Nitrogen energy metabolism

The ability to respire nitrate is widely spread among microorganisms. Nitrate is reduced to nitrite and then further to either gaseous compounds (denitrification) or to ammonia (ammonification) (Figure 15). The gaseous end products NO, N_2O, and N_2 of denitrification are released to the atmosphere leading to a loss of

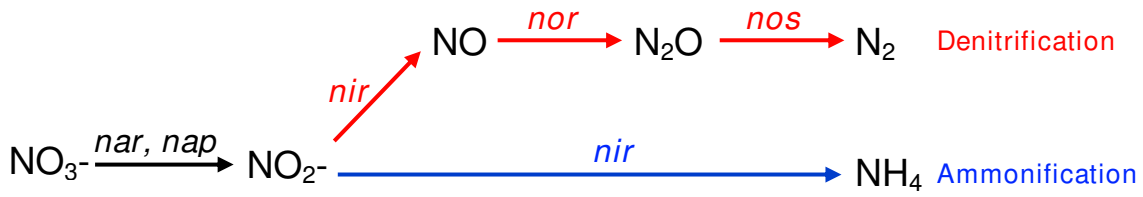


Figure 15. Scheme of dissimilatory nitrate reduction via two different pathways: in red denitrification and in blue ammonification. After the common initial step, the reduction of nitrate to nitrite, the following reduction steps are different. In denitrification, nitrite is reduced to gaseous end products, while in ammonification the end product is ammonium. Genes involved in nitrate respiration: *nar*: membrane bound nitrate reductase, *nap*: periplasmic nitrate reductase, *nir*: nitrite reductase either involved in denitrifcation or ammonification, *nor*: NO reductase, *nos*: N_2O reductase.

fixed nitrogen in the environment. The distribution of denitrification does not follow a distinct pattern among prokaryotes and is found in diverse subclasses of Proteobacteria and Archaea (Zumft, 1997). Defined as a physiological group, these facultative anaerobic microorganisms can switch from oxygen to nitrogen oxides as terminal electron acceptors when kept under anoxic conditions. One characteristic key enzyme for denitrification is the nitrite reductase. The reduction

of nitrite to NO can be catalyzed by the products of two different nitrite reductase genes, either *nirS* or *nirK*, that seem to be mutually exclusive in a given strain (Coyne et al., 1989).

Using specific primers for the *nirS* and *nirK* genes (Braker et al., 1998) it was investigated if denitrification could play a role in symbioses between gutless oligochaetes and their bacterial symbionts. These hosts harbor sulfur-oxidizing symbionts which require a terminal electron acceptor for the oxidation of reduced sulfur compounds. In free-living sulfur oxidizers, both oxygen and nitrate are common electron acceptors. Some species such as marine *Thioploca* reduce nitrate to ammonia (Otte et al., 1999), while others such *T. denitrificans* reduce nitrate to N_2 (Dalsgaard and Bak, 1992).

Two different gutless oligochaete species, *O. crassitunicatus* and *O. algarvensis,* were investigated with respect to the identification of the *nirS* and *nirK* genes that are crucial for denitrification. In all six studied *O. crassitunicatus* individuals identical *nirS* gene sequences (~890 bp) were obtained. In contrast, amplification of neither the *nirS* nor *nirK* gene was recorded in the four examined *O. algarvensis* specimens despite various PCR conditions tested. The *nirS*-encoded nitrite reductase from *O. crassitunicatus* shows highest identities to a homolog in the sulfur-oxidizing bacterium *T. denitrificans* (82% amino acid sequence identity) based on BLAST analysis. The presence of the *nirS* gene in *O. crassitunicatus* indicates that its sulfur-oxidizing symbionts might use nitrate as a terminal electron acceptor. This ability would be beneficial for the host and their symbionts since these worms colonize oxygen deficient sediments within the oxygen minimum zone (OMZ) off the coast of Peru and have to deal constantly with low oxygen concentration (<1 µM botton-water oxygen (Levin et al., 2002; Levin et al., 2003)). In the habitat of *O. crassitunicatus,* other sulfur oxidizing bacteria such as *Thioploca* spp. are present (Levin et al., 2002). These bacteria seem to use nitrate as a terminal electron acceptor (Otte et al., 1999) suggesting that nitrate respiration is an adaptive process in this oxygen deficient environment.

The absence of the *nirS*- and *nirK*-encoded nitrite reductase in *O. algarvensis* indicates that denitrifcation may not play a role in this symbiosis. *O. algarvensis* populates more oxygenated sediments around the Italian island Elba (Perner, 2003) and might not depend on nitrate as an alternative electron acceptor. However, nitrate respiration of the bacterial symbionts in this host can not be excluded because the primers used in this study may not have matched the target sequences of the nitrite reductase gene in this species. Furthermore, genes for key enzymes involved in the dissimilatory ammonification metabolism were not investigated.

2.2 Comparative metagenomics

The goal of the comparative metagenomic approach used in this study was to gain insight into the genome of the yet uncultivated symbionts from the two co-occurring gutless oligochaetes species *O. algarvensis* and *O. ilvae*. Particularly, operon organization of genes involved in metabolic pathways that are significant for these symbioses such as sulfur metabolism and CO_2 fixation of the bacterial symbionts was of high interest. The discovery of new, so far not described pathways, was a further goal.

2.2.1 Bacterial artificial chromosome library construction and analysis

The metagenomic approach was based on the analysis of a bacterial artificial chromosome (BAC) library that was constructed with DNA isolated from approximately 500 *O. algarvensis* and *O. ilvae* specimens harboring multiple symbionts. The pool of extracted genomes contained at least two eukaryotic (each estimated at 200 Mb) and at least nine bacterial symbiotic genomes, 5 of *O. algarvensis* and 4 of *O. ilvae* (each estimated at 5 Mb). The relative abundance of the two co-occurring hosts and thus of their various genomes was not known. However, previous studies showed that *O. algarvensis* species seem to be more abundant than *O. ilvae* species at the collection site off the island

Elba (Giere and Erséus, 2002) predicting an increased occurrence of genomes from *O. algarvensis* symbionts in the BAC library.

Rough estimates of the percentage of symbiotic and host genomes based on the number of bacterial (10^6) and eukaryotic cells (10^7) per worm suggested that more eukaryotic than prokaryotic sequences should be present in the BAC library. However, despite the fact that no steps were used to separate bacterial from eukaryotic DNA, the analysis of the BAC clones showed that the inserted sequences originated mainly from bacteria. This is based on BLAST results of end sequences from randomly chosen BAC clones indicating the apparent phylogenetic affinity of the inserted sequences. Approximately 37% of the analyzed end sequences were excluded from the phylogenetic analysis because of no similarities to the available sequences in the database. The source of these “no hit” sequences could be either bacterial or eukaryotic. As the database contains many more prokaryotic than eukaryotic sequences it is likely that the “no hit” sequences originated from the eukaryotes because of the lack of homologs in the database.

One possible reason for the smaller portion of eukaryotic sequences in the BAC library could be the preferential insertion of bacterial over eukaryotic DNA in BAC vectors. Circumstantial evidence indicates that with increasing insert size from small 3 kb shot-gun libraries to fosmids and BACs, the proportion of prokaryotic versus eukaryotic DNA increases (T. Woyke, personal communication).

The high proportion of bacterial sequences in the BAC library indicates an increased likelihood of identifying genes from the bacterial symbionts, and indeed, numerous symbiotic sequences *from O. algarvensis* and *O. ilvae* were found. Out of 1152 screened BAC clones, 16 contained symbiotic sequences: deltaproteobacterial 16S rRNA genes (3 clones), *aprA* genes coding for APS reductase involved in oxidative and reductive sulfur metabolism (5 clones), and *cbbL* genes coding for RubisCO involved in CO_2 fixation (8 clones) (Table 4). Comparative 16S rRNA analysis revealed that the deltaproteobacterial sequences originated from the Delta 2 symbionts of *O. algarvensis* and *O. ilvae*.

Table 4. Number of clones found in *O. algarvensis* and *O. ilvae* individuals as well as in the *Olavius* spp. BAC library for the 16S rRNA, RubisCO, and APS reductase genes. A total of 50 clones in a single individual each of *O. algarvensis* and *O. ilvae* and 1152 clones in the BAC library were screened. (-: investigated by Rühland et al., in prep)

Sequence phylotype	*O. algarvensis* (50 clones)	*O. ilvae* (50 clones)	*Olavius* spp. BAC library (1152 clones)
16S rRNA			
O. algarvensis Delta 2 phylotype	-	-	1
O. ilvae Delta 2 phylotype	-	-	2
RubisCO			
RubisCO 1			
O. algarvensis phylotype	43	0	0
O. ilvae phylotype	0	50	2
RubisCO 2	0	0	0
O. algarvensis phylotype	7	0	6
O. ilvae phylotype	0	0	0
APS reductase			
APS 1			
O. algarvensis phylotype	25	0	2
O. ilvae phylotype	0	31	1
APS 2			
O. algarvensis phylotype	14	0	0
O. ilvae phylotype	0	6	1
APS 3			
APS 3a			
O. algarvensis phylotype	3	0	0
O. ilvae phylotype	0	6	0
APS 3b			
O. algarvensis phylotype	2	0	0
O. ilvae phylotype	0	0	0
APS 3c			
O. algarvensis phylotype	0	0	0
O. ilvae phylotype	0	0	0
Olavius spp. BAC library	0	0	1

Three out of the four APS reductase sequences found in the BAC library were identical to APS reductase sequences isolated from single *O. algarvensis* and *O. ilvae* worms indicating their symbiotic origin (Figure 14). Only the origin of the APS 3c sequence could not be resolved because of no match with symbiotic APS sequences from single oligochaete worms. However, it is likely that this

APS sequence originated from a sulfate-reducing symbiont because of its close relationship to APS reductase sequences from sulfate-reducing bacteria (Figure 13). As only one APS reductase sequence was found for *O. ilvae*, despite the coexistence of two sulfate-reducing symbionts in this host, the BAC APS reductase 3c sequence may have originated from the second deltaproteobacterial symbiont of *O. ilvae*.

Two different types of RubisCO genes were identified in the BAC library, RubisCO 1 and 2 (Table 4). Both sequences are identical to RubisCO sequences isolated from either *O. algarvensis* or *O. ilvae* symbionts (Figure 13). The RubisCO 1 sequence most probably originated from the Gamma 1 symbiont of *O. ilvae,* while the source of the RubisCO 2 sequence is most likely the Gamma 2 symbiont of *O. algarvensis.* The BAC clone containing the *cbbl*-encoded RubisCO 2 sequence was fully sequenced. The genome information of this contiguous sequence is discussed in Chapter 2.2.2 below.

It can be summarized that within the BAC library 10 BAC clones were identified to contain partial genomes from *O. algarvensis* symbionts while only 5 BAC clones were shown to carry genomes from *O. ilvae* symbionts. The percentage of the symbiotic genomes from the two hosts corresponds well with the observation that *O. algarvensis* is more abundant than *O. ilvae* at the collection site.

2.2.2 Thioautotrophic metabolism of the *O. algarvensis* symbiont

The BAC clone Bac5g12 containing the symbiotic form IA RubisCO 2 sequence was fully sequenced. The source of this partial genome sequence was postulated on the basis of phylogenetic analysis of the included RubisCO sequence to have originated from the Gamma 2 symbiont of *O. algarvensis* (Figure 13). The function of the Gamma 2 symbionts in gutless oligochaetes is not clear. They are hypothesized to be chemosynthetic based on their 16S rRNA phylogeny that reveals a close relationship to bacterial clones isolated from chemosynthetic environments (Chapter 1.2.1). However, this highly speculative hypothesis is

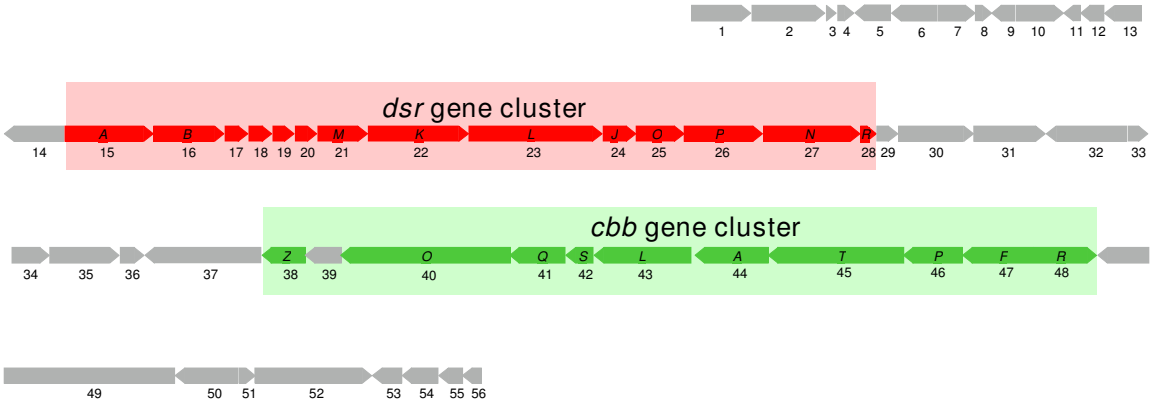


Figure 16. Gene organization in the contiguous sequence from the *O. algarvensis* symbiont (Bac5g12 insert). The sequence of 51 kb contains two clustered metabolic operons: a *dsr* gene cluster involved in intracellular sulfur oxidation (in red) and a *cbb* gene cluster taking part in CO_2 fixation (in green). Numbers below arrows represent annotated open reading frames (for detailed information see Table 2 on page 134).

based on phylogeny, which is not a good indicator for metabolism. The presence of two phylogenetically distinct APS reductase and RubisCO genes in *O. algarvensis* are further indicators that the Gamma 2 symbiont is chemoautotrophic, based on the assumption that one copy of each gene originated from the Gamma 1 and the other from the Gamma 2 symbiont of *O. algarvensis*. However, as discussed previously (p. 45), it is possible that these multiple gene copies originated from a single symbiont, namely the Gamma 1. Therefore, the Bac5g12 contig can not be unambiguously assigned either to the Gamma 1 or Gamma 2 symbiont, but we interpret the evidence as supporting an origin of this contig from the Gamma 2 symbiont of *O. algarvensis*. The possession of an additional chemoautotrophic symbiont is surely beneficial for the host since this could contribute supplementary nutrition.

The contiguous Bac5g12 sequence gave first insights into the genome of a symbiotic bacterium from a gutless oligochaete (Figure 16). Within this sequence two conspicuously gene clusters were detected: a *dsr* gene cluster involved in intracellular sulfur oxidation and a *cbb* gene cluster taking part in CO_2 fixation.

2.2.2.1 *Dsr* gene cluster

The Bac5g12 *dsr* gene cluster organized of 14 genes, *dsrABEFHCMKLJOPNR*, is nearly the same as in *Allochromatium vinosum* and *Thiobacillus denitrificans*, the only sulfur-oxidizing Proteobacteria whose *dsr* gene clusters were available in the database (March 2005). The sequence similarity between the *dsr* genes of these three bacteria as well as their conserved gene order indicates that this metabolic gene cluster has a significant function in sulfur oxidation. In the near future, it remains to be shown if this *dsr* gene cluster is more widely distributed in sulfur-oxidizing Proteobacteria, as genome sequencing from other species of this physiological group are currently in progress (e.g. *Thiomicrospira crunogena* and *Halorhodospira halophila* at DOE Joint Genome Institute (USA)).

2.2.2.2 *Cbb* gene cluster

The Bac5g12 *cbb* gene cluster consists of 10 adjacent genes, *cbbRFTPALSQOZ*, and is therefore the most contiguous *cbb* gene cluster currently known of form IA RubisCO (Kusian and Bowien, 1997; Schwedock et al., 2004; Shively et al., 1998). Unusual in the *cbb* gene cluster of the *O. algarvensis* symbiont are the additional *cbbFPTA* genes and thus the existence of both key enzymes for CO_2-fixation via CBB cycle, the *cbbLS*-encoded RubisCO and *cbbP*-encoded phosphoribulokinase, not present in other form IA RubisCO gene clusters. Another conspicuous observation was made by phylogenetic correlation of the symbiotic *cbb* genes. The *cbbRFPTA* genes from the first part of the cluster showed similar gene order and close relationship to homologs from *cbb* gene clusters of form IC RubisCO primarily present in Alpha- and some Betaproteobacteria. In contrast, the *cbbLSQOZ* genes of the second part were highly similar to homologs from *cbb* gene clusters of form IA RubisCO most abundant among Gamma- and Betaproteobacteria. Such an arrangement and relationship of genes within the same cluster indicates a hybrid cluster composed of two parts from different origins. Since the *cbb* gene cluster originated from a gammaproteobacterial symbiont and the *cbbRFPTA* genes are

related to homologs in Alphaproteobacteria, it is possible that these genes were acquired from such bacteria, either by the symbionts or their ancestors. Comparative sequence analysis of this *cbb* gene cluster from symbionts of other gutless oligochaetes and their free-living relatives would show how such a hybrid cluster evolved.

2.2.3 Novel genes

The discovery of novel genes was the purpose of end-sequencing of randomly chosen BAC clones. BAC inserts containing genes of interest were further sequenced by primer walking to obtain a longer sequence for an improved annotation. Within the BAC library several genes were found that indicated metabolic activities not previously known from the symbioses between the bacterial symbionts and gutless oligochaetes. Here, only the most interesting genes are described, which were assigned either to nitrogen or carbon metabolism.

Among genes involved in nitrogen metabolism, identification of the *ureFG*-encoded urease attracted our particular attention. Urease hydrolyzes urea to ammonia and carbon dioxide and is widely distributed among a number of bacteria, plants, fungi, and algae (Mobley and Hausinger, 1989). The possession of a microbial urease in bacterial symbionts of gutless oligochaetes could play an important role, if urea is a nitrogenous waste product of these worms that lack an excretory system. The symbiotic recycling of urea would bring advantages for both partners, the detoxification for the host and supply of the symbionts with a nitrogen source.

Among the genes involved in carbon metabolism in the BAC library the *ooxA*- and *ooxB*-encoded opine oxidase was of interest. In marine invertebrates this protein catalyzes the degradation of opines that are common products of anaerobic muscular metabolism (Pörtner, 2002). Since gutless oligochaetes are often exposed to anaerobic conditions, the formation of opine is possible. The possession of an opine oxidase by the symbionts would enable the bacteria to

utilize the waste product of the worm as a carbon source contributing to their nutrition. Thus no valuable molecules would be wasted.

Although the symbiotic source of the detected novel functional genes is putative, the discovery of these genes provides a basis for more detailed studies. To determine the origin of these genes in the symbioses their selective amplification with specific primers from *O. algarvensis* and *O. ilvae* individuals and their phylogenetic analysis would be useful for an assignment to individual symbionts.

3 Outlook

The analysis of the metagenomic BAC library from the two coexisting gutless oligochaete species, *O. algarvensis* and *O. ilvae,* provided insight into the genomes of their bacterial symbionts. Sequencing of many more BAC clones and their assembly may finally result in a contig carrying the gene of interest and a distinct phylogenetic marker such as the 16S rRNA gene. This would be the final prove for a correct assignment of the functional genes or gene clusters to a certain symbiont based on their phylogeny. This expensive approach with a high scale of sequencing throughput is currently in progress at the DOE Joint Genome Institute (USA) with fosmid libraries.

To confirm these assignments on the experimental level and examine if these genes are expressed, in situ analyses, such as messenger (m)RNA FISH or immunohistochemical staining with antibodies is important. For example, simultaneous hybridizations with a mRNA targeted probe specific for a functional gene and another probe specific for the 16S rRNA of a given symbiont would show clearly if a gene is expressed and if so, by which symbiont.

Another important goal is to gain a better understanding of the function of the alphaproteobacterial and the spirochaete symbionts in the gutless oligochaete associations. The often observed regular occurrence of alphaproteobacterial symbionts in species from both host genera (*Olavius* and *Inanidrilus*) and sites around the world suggests that they play an important role

in these associations. A comparative metagenomic approach of the symbiotic community of *I. leukodermatus*, a species with multiple alphaproteobacterial symbionts, would be an ideal candidate for finding genes that could provide clues of the metabolic capabilities of these symbionts.

C

References

Amann R. I. (1995) Fluorescently labelled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. *Molecular Ecology* 4, 543-554.

Bauer-Nebelsick M., Bardele C. F., and Ott J. A. (1996) Redescription of *Zoothamnium niveum* (Hemprich & Ehrenberg, 1831) Ehrenberg, 1838 (Oligohymenophora, Peritrichida), a ciliate with ectosymbiotic, chemoautotrophic bacteria. *European Journal of Protistology* 32, 18-30.

Braker G., Fesefeldt A., and Witzel K.-P. (1998) Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Applied and Environmental Microbiology* 64, 3769-3775.

Breznak J. A. (2002) Phylogenetic diversity and physiology of termite gut spirochetes. *Annual Meeting of the Society for Intergrative and Comparative Biology* 42, 313-318.

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

Campbell B. J. and Cary S. C. (2004) Abundance of reverse tricarboxylic acid cycle genes in free-living microorganisms at deep-sea hydrothermal vents. *Applied and Environmental Microbiology* 70, 6282-6289.

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

Cavanaugh C., M. (1994) Microbial symbiosis: Patterns of diversity in the marine environment. *American Zoologist* 34, 79-89.

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

Cavanaugh C. M. and Robinson J. J. (1996) CO_2 fixation in chemoautotrophic-invertebrate symbioses: expression of form I and form II RubisCO. In *Microbiol growth on C_1 compounds: Proceedings of the 8th International*

Symposium on Microbial Growth on C_1 Compounds (ed. M. E. Tabita and F. R. Lidstrom). Kluwer Academic Publishers.

Cerdeño-Tárraga A., Crossman L., and Parkhill J. (2004) New environments, versatile genomes. *Nature Reviews Microbiology* 2, 446-447.

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

Childress J. J. and Fisher C. R. (1992) The biology of hydrothermal vent animals: physiology, biochemistry, and autotrophic symbioses. *Oceanography and Marine Biology Annual Review* 30, 337-441.

Coyne M. S., Arunakumari A., Averill B. A., and Tiedje J. M. (1989) Immunological identification and distribution of dissimilatory heme cd1 and nonheme copper nitrite reductases in denitrifying bacteria. *Applied and Environmental Microbiology* 55, 2924-1931.

Dalsgaard T. and Bak F. (1992) Effect of acetylene on nitrous oxide reduction and sulfide oxidation in batch and gradient cultures of *Thiobacillus denitnificans*. *Applied and Environmental Microbiology* 58, 1601-1608.

De Bary A. (1879) Die Erscheinung der Symbiose. Verlag Karl J. Trubner, Strassburg, pp. 1-30.

Distel D. L., Beaudoin D. J., and Morrill W. (2002) Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve Lyrodus pedicellatus (Bivalvia: Teredinidae). *Applied and Environmental Microbiology* 68, 6292-6299.

Dubilier N. (2003) Multiple bacterial endosymbionts in gutless marine worms: competitors or partners? *Nova Acta Leopoldina* 333, 107-112.

Dubilier N., Amann R., Erséus C., Muyzer G., Park S. Y., Giere O., and Cavanaugh C. M. (1999) Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochaete *Olavius loisae* (Annelida). *Marine Ecology Progress Series* 178, 271-280.

Dubilier N., Giere O., Distel D. L., and Cavanaugh C. M. (1995) Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm

(Oligochaeta, Annelida) by phylogenetic 16S rRNA sequence analysis and in situ hybridization. *Applied and Environmental Microbiology* 61, 2346-2350.

Dubilier N., Giere O., and Grieshaber M. K. (1994) Concomitant effects of sulfide and hypoxia on the aerobic metabolism of the marine oligochaete *Tubificoides benedii*. *Journal of Experimental Zoology* 269, 287-297.

Dubilier N., Mülders C., Ferdelman T., de Beer D., Pernthaler A., Klein M., Wagner M., Erséus C., Thiermann F., Krieger J., Giere O., and Amann R. (2001) Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. *Nature* 411, 298-302.

Dubilier N., Blazejak A., and Rühland C. (in press) Symbioses between bacteria and gutless marine oligochaetes. In *In Molecular Basis of Symbiosis* (ed. J. Overmann). Springer-Verlag.

Dufour S. C. (2005) Gill anatomy and the evolution of symbiosis in the bivalve family Thyasiridae. *Biological Bulletin* 208, 200-212.

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

Erséus C. (1990) The marine Tubificidae (Oliogchaeta) of the barrier reef ecosystems at Carrie Bow Cay, Belize, and other parts of the Caribbean Sea, with descriptions of twenty-seven new species and revision of *Heterodrilus*,*Thalassodrilides* and *Smithsonidrilus*. *Zoologica Scripta* 19, 243-303.

Erséus C. (1991) Two new deep-water species of the gutless genus *Olavius* (Oligochaeta: Tubificidae) from both sides of North America. *Proceedings of the Biological Society of Washington* 104, 627-630.

Erséus C. (1992) A generic revision of the Phallodrilinae (Oligochaeta, Tubificidae). *Zoologica Scripta* 21, 5-48.

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

Erséus C. (2003) The gutless Tubificidae (Annelida, Oligochaeta) of the Bahamas. *Meiofauna Marina* 12, 59-84.

Erséus C. and Baker H. R. (1982) New species of the gutless marine genus *Inanidrilus* (Oligochaeta, Tubificidae) from the Gulf of Mexico and Barbados. *Canadian Journal of Zoology* 60, 3063-3067.

Erséus C. and Giere O. (1995) *Olavius nicolae*, a new gutless marine tubificid species (Oligochaeta) from Belize. *Proceedings of the Biological Society of Washington* 108, 491-495.

Erséus C., Källersjö M., Ekman M., and Hovmöller R. (2002) 18S rDNA phylogeny of Tubificidae and its constituent taxa (Clitellata): dismissal of the Naididae. *Molecular Phylogenetics and Evolution* 22, 414-422.

Erséus C., Prestegaard T., and Källersjö M. (2000) Phylogenetic analysis of Tubificidae (Annelida, Clitellata) based on 18S rRNA sequences. *Molecular Phylogenetics and Evolution* 15, 381-389.

Felbeck H. (1981a) Chemoautotrophic potential of the hydrothermal vent tube worm, *Riftia pachyptila* Jones (Vestimentifera). *Science* 213(4505), 336-338.

Felbeck H., Childress J. J., and Somero G. N. (1981b) Calvin-Benson cycle and sulfide oxidation enzymes in animals from sulfide-rich habitats. *Nature* 293, 291-293.

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

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

Fidopiastis P. M., Miyamoto C. M., Jobling M. G., Meighen E. A., and Ruby E. G. (2002) LitR, a new transcriptional activator in *Vibrio fischeri*, regulates luminescence and symbiotic light organ colonization. *Molecular Microbiology* 45, 131-143.

Finogenova N. (1986) Six new species of marine Tubificidae (Oligochaeta) from the continental shelf off Peru. *Zoologica Scripta* 15, 45-51.

Fisher C. R. (1990) Chemoautotrophic and methanotrophic symbioses in marine invertebrates. *Reviews in Aquatic Sciences* 2, 399-436.

Frank A. B. (1877) Über die biologischen Verhältnisse des Thallus einiger Krustenflechten. *Beitraege zur Biologie der Pflanzen* 2, 123-200.

Freiberg C., Fellay R., Bairoch A., Broughton W. J., Rosenthal A., and Perret X. (1997) Molecular basis of symbiosis between *Rhizobium* and legumes. *Nature* 387, 394-401.

Friedrich M. W. (2002) Phylogenetic analysis reveals multiple lateral transfers of adenosine-5'-phosphosulfate reductase genes among sulfate-reducing microorganisms. *Journal of Bacteriology* 184, 278-289.

Giere O. (1979) Studies on marine Oligochaeta from Bermuda, with emphasis on new *Phallodrilus* species (Tubificidae). *Cahiers de Biologie Marine* 20, 301-314.

Giere O. (1981) The gutless marine oligochaete *Phallodrilus leukodermatus*. Structural studies on an aberrant tubificid associated with bacteria. *Marine Ecology Progress Series* 5, 353-357.

Giere O. (1985) The gutless marine tubificid *Phallodrilus planus*, a flattened oligochaete with symbiotic bacteria. Results from morphologcial and ecological studies. *Zoologica Scripta* 14, 279-286.

Giere O. (1996) Bacterial endosymbioses in littoral worms. In *Biosystematics and Ecology Series,* (ed. F. Uiblein, J. Ott, and M. Stachowitsch), pp. 353-367.

Giere O., Conway N. M., Gastrock G., and Schmidt C. (1991) 'Regulation' of gutless annelid ecology by endosymbiotic bacteria. *Marine Ecology Progress Series* 68, 287-299.

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

Giere O., Erséus C., and Stuhlmacher F. (1998) A new species of *Olavius* (Tubificidae, Phallodrilinae) from the Algarve Coast in Portugal, the first

East Atlantic gutless oligochaete with symbiotic bacteria. *Zoologischer Anzeiger* 237, 209-214.

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

Giere O. and Langheld C. (1987) Structural organisation, transfer and biological fate of endosymbiotic bacteria in gutless oligochaetes. *Marine Biology* 93, 641-650.

Giere O., Liebezeit G., and Dawson R. (1982) Habitat conditions and distribution pattern of the gutless oligochaete *Phallodrilus leukodermatus*. *Marine Ecology Progress Series* 8, 291-299.

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

Giere O., Windoffer R., and Southward E. C. (1995b) The bacterial endosymbiosis of the gutless nematode, *Astomonema southwardorum*: utrastructural aspects. *Journal of the Marine Biological Association of the United Kingdom* 75, 153-164.

Grieshaber M. K. and Völkel S. (1998) Animal adaptations for tolerance and exploitation of poisonous sulfide. *Annual Review of Physiology* 60, 33-53.

Gross R., Hacker J., and Goebel W. (2003) The Leopoldina international symposium on parasitism, commensalism and symbiosis - common themes, different outcome. *Molecular Microbiology* 47, 1749-1758.

Harris S., Ebert A., Schutze E., Diercks M., Bock E., and Shively J. M. (1988) Two different genes and gene products for the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RubisCOase) in *Nitrobacter hamburgensis*. *Federation of European Microbiological Societies Microbiological Letters* 49, 267-271.

Harwood C. S. and Canale-Parola E. (1983) *Spirochaeta isovalerica* sp. nov., a marine anaerobe that forms branched-chain fatty acids as fermentation products. *International Journal of Systematic Bacteriology* 33, 573-579.

Hayashi N. R., Oguni A., Yaguchi T., Chung S.-Y., Nishihara H., Kodama T., and Igarashi Y. (1998) Different properties of gene products of three sets ribulose 1,5-bisphosphate carboxylase/oxygenase from a marine obligately autotrophic hydrogen-oxidizing bacterium, *Hydrogenovibrio marinus* strain MH-110. *Journal of Fermentation and Bioengineering* 85, 150-155.

Heinhorst S., Baker S. H., Johnson D. R., Davies P. S., Cannon G. C., and Shively J. M. (2002) Two copies of form I RuBisCO genes in *Acidithiobacillus ferrooxidans* ATCC 23270. *Current Microbiology* 45, 115-117.

Hespell R. B. and Canale-Parola E. (1973) Glucose and pyruvate metabolism of *Spirochaeta litoralis*, an anaerobic marine spirochete. *Journal of Bacteriology* 116, 931-937.

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

Hueck C. J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology and Molecular Biology Reviews* 62, 379-433.

Karl D. M. (1995) The Microbiology of Deep-Sea Hydrothermal Vents. CRC Press.

Kelley D. S., Baross J. A., and Delaney J. R. (2002) Volcanoes, fluids, and life at Mid-Ocean ridge spreading centers. *Annual Review of Earth and Planetary Sciences* 30, 385-491.

Kimura H., Higashide Y., and Naganuma T. (2003a) Endosymbiotic microflora of the vestimentiferan tubeworm (*Lamellibrachia* sp.) from a bathyal cold seep. *Marine Biotechnology*. 5, 593-603

Kimura H., Sato M., Sasayama Y., and Naganuma T. (2003b) Molecular characterization and in situ localization of endosymbiotic 16S ribosomal

RNA and RuBisCO genes in the pogonophoran tissue. *Marine Biotechnology* 5, 261-269.

Kito K. (1989) A new mouthless marine nematode from Fiji. *Journal of Natural History* 23, 635-642.

Krieger J. (2001) Funktion und Übertragung endosymbiontischer Bakterien bei darmlosen marinen Oligochaeten. *PhD Dissertation Universität Hamburg, Shaker-Verlag, Aachen*.

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

Kusian B. and Bowien B. (1997) Organization and regulation of cbb CO_2 assimilation genes in autotrophic bacteria. *Federation of European Microbiological Societies Microbiological Reviews* 21, 135-155.

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

Lee Y. K., Lee J.-H., and Lee H. K. (2001) Microbial symbiosis in marine sponges. *Journal of Microbiology* 39, 254-264.

Levin L., Gutièrrez D., Rathburn A., Neira C., Sellanes J., Muñoz P., Gallardo V., and Salamanca M. (2002) Benthic processes on the Peru margin: a transect across the oxygen minimum zone during the 1997-98 El Niño. *Progress in Oceanography* 53, 1-27.

Levin L. A., Rathburn A. E., Gutiérrez D., Muñoz P., and Shankle A. (2003) Bioturbation by symbiont-bearing annelids in near-anoxic sediments: Implications for biofacies models and paleo-oxygen assessments. *Palaeogeography, Palaeoclimatology, Palaeoecology* 199, 129-140.

Mack E. E., Mandelco L., Woese C. R., and Madigan M. T. (1993) *Rhodospirillum sodomense*, sp. nov., a Dead Sea *Rhodospirillum* species. *Archives of Microbiology* 160, 363-371.

Margulis L. and Bermudes D. (1985) Symbiosis as a mechanism of evolution: status of cell symbiosis theory. *Symbiosis* 1, 101-124.

Maynard Smith J. and Szathmáry E. (1995) The Major Transitions in Evolution. W.H. Freeman.

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

Mobley H. L. T. and Hausinger R. P. (1989) Microbial ureases: significance, regulation, and molecular characterization. *Microbiological Reviews* 53, 85-108.

Nissen H. and Dundas I. D. (1984) *Rhodospirillum salinarum* sp. nov., a halophilic photosynthetic bacterium isolated from Portuguese saltern. *Archives of Microbiology* 138, 251-256.

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

Ochman H. and Moran N. A. (2001) Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. *Science* 292, 1096-1098.

Ott J. and Novak R. (1989) Living at an interface: Meiofauna at the oxygen/sulfide boundary of marine sediments. In *Reproduction, genetics and distribution of marine organisms* (ed. J. S. Ryland and P. A. Tyler), pp. 415-422. Olsen and Olsen.

Ott J., Rieger G., Rieger R., and Enderes F. (1982) New mouthless interstitial worms from the sulfide system: symbiosis with prokaryotes. *Marine Ecology* 3, 313-333.

Ott J. A. (1996) Sulphide symbioses in shallow sands. In *Biology and ecolgy of shallow coastal waters* (ed. A. A. Eleftheriou, and Ch. J. Smith), pp. 143-147. Olsen and Olsen.

Ott J. A., Bright M., and Schiemer F. (1998) The ecology of a novel symbiosis between a marine peritrich ciliate and chemoautotrophic bacteria. *Marine Ecology* 19, 229-243.

Otte S., Kuenen J. G., Nielsen L. P., Paerl H. W., Zopfi J., Schulz H. N., Teske A., Strotmann B., Gallardo V. A., and Jorgensen B. B. (1999) Nitrogen, carbon, and sulfur metabolism in natural *Thioploca* samples. *Applied and Environmental Microbiology* 65, 3148-3157.

Perner M. (2003) Biogeochemische und mikrobiologische Charakterisierung mariner Sedimente vor Elba - ein Beitrag zur ökosystemaren Analyse bakteriensymbiontischer Oligochaeten. (Biogeochemical and microbiological characterisation of marine sediments from Elba - a contribution to an ecosystematic analysis of oligochaetes with bacterial symbionts). Master's Thesis. University of Hamburg, Germany.

Piper K. R., von Bodman S. B., and Farrand S. K. (1993) Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* 362, 448-450.

Pirhonen M., Flego D., Heikinheimo R., and Palva E. T. (1993) A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *The European Molecular Biology Organization Journal* 12, 2467-2476.

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

Polz M. F., Ott J. A., Bright M., and Cavanaugh C. M. (2000) When bacteria hitch a ride. *American Society for Microbiology News* 66, 531-539.

Polz M. F., Robinson J. J., Cavanaugh C. M., and Van Dover C. L. (1999) Trophic ecology of massive shrimp aggregations at a Mid-Atlantic hydrothermal vent site. *Limnology and Oceanography* 43, 1631-1638.

Pörtner H. O. (2002) Environmental and functional limits to muscular exercise and body size in marine invertebrate athletes. *Comparative Biochemical Physiology* 133, 303-321.

Reid R. G. B. and Brand D. G. (1986) Sulfide-oxidizing symbiosis in Lucinaceans: Implications for bivalve evolution. Veliger 29, 3-24.

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

Riemann F. (1993) The mouthless deep-sea nematode *Rhaptothyreus minor* sp. n. (Rhaptothyreidae, mermithoid nematodes of uncertain systematic position). *Zoologica Scripta* 22, 341-346.

Robinson J. J. and Cavanaugh C. M. (1995) Expression of form I and form II RubisCO in chemoautotrophic symbioses: implications for the interpretation of stable carbon isotope values. *Limnology and Oceanography* 40, 1496-1502.

Ruby E. G., Henderson B., and McFall-Ngai M. (2004) We get by with a little help from our (little) friends. *Science* 303, 1305-1307.

Ruby E. G., Urbanowski M., Campbell J., Dunn A., Faini M., Gunsalus R., Lostroh P., Lupp C., McCann J., Millikan D., Schaefer A., Stabb E., Stevens A., Visick K., Whistler C., and Greenberg E. P. (2005) Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. *Proceedings of the National Academy of Sciences* 102, 3004-3009.

Rühland C., Blazejak A., Loy A., Wagner M., Erséus C., Amann R., and Dubilier N. (in prep) Multiple symbioses with sulfide-oxidizers, sulfate-reducers, and spirochetes in two co-occurring species of gutless worm (Oligochaeta) from the Mediterranean.

Schwedock J., Harmer T. L., Scott K. M., Hektor H. J., Seitz A. P., Fontana M. C., Distel D. L., and Cavanaugh C. M. (2004) Characterization and expression of genes from the RubisCO gene cluster of the chemoautotrophic symbiont of *Solemya velum*: *cbbLSQO*. *Archives of Microbiology* 182, 18-29.

Shively J. M., van Keulen G., and Meijer W. G. (1998) Something from almost nothing: Carbon dioxide fixation in chemoautotrophs. *Annual Review of Microbiology* 52, 191-230.

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

Starr M. P. (1975) A generalized scheme for classifying organismic associations. In *Symbiosis* (ed. D. H. Jennings and D. L. Lee), pp. 1-20.

Stein J. L., Haygood M., and Felbeck H. (1990) Nucleotide sequence and expression of a deep-sea ribulose-1,5-bisphosphate carboxylase gene cloned from a chemoautotrophic bacterial endosymbiont. *Proceedings of the National Academy of Sciences* 87, 8850-8854.

Sánchez O., Ferrera I., Dahl C., and J. M. (2001) *In vivo* role of APS reductase in the purple sulfur bacterium *Allochromatium vinosum*. *Archives of Microbiology* 176, 301-305.

Tabita F. R. (1988) Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. *Microbiological Reviews* 52, 155-189.

Tabita F. R. (1995) The biochemistry and metabolic regulation of carbon metabolism and CO_2 fixation in purple bacteria. In *Anoxygenic photosynthetic bacteria* (ed. M. M. Tabita, R. E. Blankenship, and C. E. Bauer). Kluwer.

van den Ende F. P., Meier J., and van Gemerden H. (1997) Syntrophic growth of sulfate-reducing bacteria and colorless sulfur bacteria during oxygen limitation. *Federation of European Microbiological Societies Microbiology Ecology* 23, 65-80.

Van Dover C. L. (2000) The ecology of deep-sea hydrothermal vents. Princeton University Press.

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

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

Viale A. M., Kobayashi H., and Akazawa T. (1989) Expressed genes for plant-type ribulose 1,5-bisphosphate carboxylase/oxygenase in the photosynthetic bacterium *Chromatium vinosum*, which possesses two complete sets of the genes. *Journal of Bacteriology* 171, 2391-2400.

Vopel K., Reick C., Arlt G., Poehn M., and Ott J. (2002) Flow microenvironment of two marine peritrich ciliates with ectobiotic chemoautotrophic bacteria. *Aquatic Microbial Ecology* 29, 19-28.

Watson G. M. F. and Tabita F. R. (1997) Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a molecule for phylogenetic and enzymological investigation. *Federation of European Microbiological Societies Microbiology Letters* 146, 13-22.

Widdel F. and Bak F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. In *The Prokaryotes*, Vol. I (ed. A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer), pp. 3352-3378. Springer-Verlag.

Widdel F. and Pfennig N. (1992) The genus Desulfuromonas and other Gram-negative sulfur-reducing eubacteria. In *The Prokaryotes*, Vol. IV (ed. A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer), pp. 3379-3389. Springer-Verlag.

Zbinden M., Le Bris N., Gaill F., and Compère P. (2004) Distribution of bacteria and associated minerals in the gill chamber of the vent shrimp *Rimicaris exoculata* and related biogeochemical processes. *Marine Ecology Progress Series* 284, 237-251.

Zumft W. G. (1997) Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* 61, 533-616.

Part II:

Publications

A Contributions to the manuscript

(1) Anna Blazejak, Christer Erséus, Rudolf Amann, and Nicole Dubilier. 2005. Coexistence of bacterial sulfide oxidizers, sulfate reducers, and spirochetes in a gutless worm (Oligochaeta) from the Peru margin. Applied and Environmental Microbiology. 71: 1553-1561

Concept by A. B. and N. D., practical work and writing by A. B., and editorial help by C. E., R. A., and N. D.

(2) Anna Blazejak, Jan Kuever, Christer Erséus, Rudolf Amann, and Nicole Dubilier. Phylogeny of 16S rRNA, RubisCO, and APS reductase genes from gamma- and alphaproteobacterial symbionts in gutless marine worms (Oligochaeta) from Bermuda and Bahamas. *Manuscript submitted to Applied and Environmental Microbiology*.

Concept by A. B., practical work and writing by A. B., providing of APS reductase primers by J. K., and editorial help by J. K., C. E., R. A., and N. D.

(3) Anna Blazejak, Michael Richter, Zhanyou Xu, Amelia Rotaru, Michael Kube, Hong-Bin Zhang, Rudolf Amann, Frank Oliver Glöckner, and Nicole Dubilier. Metagenomic analysis of co-occurring symbionts in marine worms from the Mediterranean. *Manuscript in preparation*

Concept by A. B. and N. D., practical work by A. B., M. R., Z. X., A. R., M. K., writing by A. B., and editorial help by H-B. Z., R. A., F. O. G., and N. D.

(4) Caroline Rühland, Anna Blazejak, Alexander Loy, Michael Wagner, Christer Erséus, Rudolf Amann, Nicole Dubilier. Multiple symbioses with sulfur-oxidizers, sulfate-reducers and spirochetes in two gutless marine worms (Oligochaeta) Mediterranean sediments low in sulfide. *Manuscript in preparation.*

Concept by C. R. and N. D., practical working C. R., A. B. and A. L., writing by C. R., and editorial help by M. W., C. E., R. A., and N. D.

B Publications

1

Coexistence of bacterial sulfide oxidizers, sulfate reducers, and spirochetes in a gutless worm (Oligochaeta) from the Peru margin.

Anna Blazejak, Christer Erséus, Rudolf Amann, and Nicole Dubilier

Applied and Environmental Microbiology. 71: 1553-1561 (2005)

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2005, p. 1553–1561 Vol. 71, No. 3
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.3.1553–1561.2005

Coexistence of Bacterial Sulfide Oxidizers, Sulfate Reducers, and Spirochetes in a Gutless Worm (Oligochaeta) from the Peru Margin

Anna Blazejak,[1] Christer Erséus,[2]† Rudolf Amann,[1] and Nicole Dubilier[1]*

Max Planck Institute of Marine Microbiology, Bremen, Germany,[1] and Department of Invertebrate Zoology, Swedish Museum of Natural History, Stockholm, Sweden[2]

Received 22 March 2004/Accepted 1 October 2004

Olavius crassitunicatus **is a small symbiont-bearing worm that occurs at high abundance in oxygen-deficient sediments in the East Pacific Ocean. Using comparative 16S rRNA sequence analysis and fluorescence in situ hybridization, we examined the diversity and phylogeny of bacterial symbionts in two geographically distant *O. crassitunicatus* populations (separated by 385 km) on the Peru margin (water depth, ~300 m). Five distinct bacterial phylotypes co-occurred in all specimens from both sites: two members of the γ-*Proteobacteria* (Gamma 1 and 2 symbionts), two members of the δ-*Proteobacteria* (Delta 1 and 2 symbionts), and one spirochete. A sixth phylotype belonging to the δ-*Proteobacteria* (Delta 3 symbiont) was found in only one of the two host populations. Three of the *O. crassitunicatus* bacterial phylotypes are closely related to symbionts of other gutless oligochaete species; the Gamma 1 phylotype is closely related to sulfide-oxidizing symbionts of *Olavius algarvensis*, *Olavius loisae*, and *Inanidrilus leukodermatus*, the Delta 1 phylotype is closely related to sulfate-reducing symbionts of *O. algarvensis*, and the spirochete is closely related to spirochetal symbionts of *O. loisae*. In contrast, the Gamma 2 phylotype and the Delta 2 and 3 phylotypes belong to novel lineages that are not related to other bacterial symbionts. Such a phylogenetically diverse yet highly specific and stable association in which multiple bacterial phylotypes coexist within a single host has not been described previously for marine invertebrates.**

Gutless oligochaetes were first discovered 25 years ago in sediments of shallow coral reefs around Bermuda (20). As the name suggests, in these worms the digestive system is completely reduced, and the animals have no gut, anus, or nephridia (excretory system), raising the question of how they gain their nutrition. Shortly after the discovery of these worms, Giere (19) was able to show that they are associated with gram-negative bacteria that are hypothesized to provide a source of nutrition via chemosynthesis (16). To date, 80 gutless oligochaete species have been found in a wide array of habitats throughout the world and have been described. High numbers and great diversity have been found in shallow marine waters of tropical and subtropical coral reefs in the Atlantic, Caribbean, and Pacific oceans (10–13). Recently, gutless oligochaetes were also found in coastal areas of the Mediterranean Sea (21, 22). Only a few species occur in deeper (>100 m), colder waters off the Pacific and Atlantic coasts of North America, off the Pacific coast of South America, and in the eastern part of the Gulf of Mexico (13, 14, 17). Despite their ecological diversity, their worldwide distribution, and the high number of species, gutless oligochaetes are monophyletic (i.e., they descended from a single common ancestor) and belong to only two genera, *Inanidrilus* and *Olavius* (15, 36).

The morphologies of the symbiosis are very similar in all gutless oligochaetes examined to date. The bacterial symbionts occur just below the outer cuticle of the worm between extensions of the epidermal cells and are, in most cases, extracellular (19, 24). In all host species examined, two bacterial morphotypes have been described; in some host species, an additional third morphotype can also co-occur. One bacterial morphotype with a diameter of 3 to 5 μm has large intracellular inclusions; a second, smaller morphotype with a diameter of 0.5 to 1 μm has no conspicuous features; and a third morphotype, found in two host species, is very long and thin (length, 10 μm; diameter, 0.3 μm) (23, 24).

Comparative 16S rRNA analyses and fluorescence in situ hybridization (FISH) studies of the larger bacterial morphotype in three gutless oligochaete hosts, *Inanidrilus leukodermatus* (Bermuda), *Olavius algarvensis* (Mediterranean Sea), and *Olavius loisae* (Australia), showed that the symbionts belong to the gamma subclass of the *Proteobacteria* and are closely related to each other (7–9). The thioautotrophic nature of these symbionts is supported by the presence of sulfur in cytoplasmic globules of the bacteria and by immunocytochemical studies that showed the presence of form I ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the key CO_2-fixing enzyme (28). In contrast to the close evolutionary relationship of the large thiotrophic symbionts, the smaller, rod-shaped bacteria are phylogenetically diverse and can belong to the alpha or delta subclass of the *Proteobacteria* (7, 9). The phylogeny of the long, thin bacterial morphotype found in some hosts (23, 24) has not been studied yet.

Some of the more unusual habitats of gutless oligochaetes are organic matter-rich sediments in water that is 100 to 400 m deep in upwelling regions off the coast of Peru and Chile.

* Corresponding author. Mailing address: Max Planck Institute of Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany. Phone: 49 421 2028932. Fax: 49 421 2028580. E-mail: ndubilie@mpi-bremen.de.

† Present address: Department of Zoology, Göteborg University, Göteborg, Sweden.

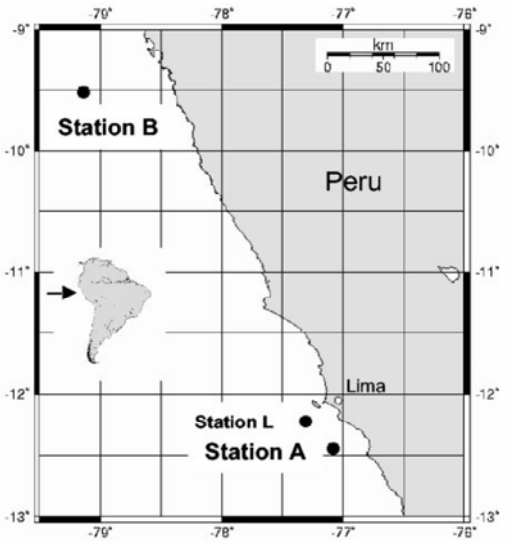


FIG. 1. Locations of *O. crassitunicatus* sampling sites on the Peru margin. The worms used in this study were collected at station A at a depth of 359 m and at station B at a depth of 270 m. The two stations are separated from each other by 385 km. Station L is a site sampled by Lisa Levin in 1998 at a depth of 305 m (12°22.7′S, 77°29.1′W); this station was called station A by Levin et al. in reference 30 and station 1 by Levin et al. in reference 31. Specimens from the Levin cruise were used by Giere and Krieger (23) for their ultrastructural studies of *O. crassitunicatus*.

Despite extremely low bottom-water oxygen concentrations (<1 μM), the gutless oligochaete *Olavius crassitunicatus* FINOGENOVA (17) is the dominant member of the infaunal community, occurring at densities as high as 13,500 individuals m^{-2} (30, 31). Morphological studies have shown that *O. crassitunicatus* harbors three structurally distinct types of extracellular bacterial symbionts (23). Large oval bacteria (3.5 by 7.3 μm) with intracellular inclusions fill the major part of the subcuticular space, whereas smaller rod-shaped bacteria (0.7 by 1.9 μm) are found in a peripheral position directly under the cuticle. A third long, thin bacterial morphotype (0.3 to 0.4 by 9.1 μm) occurs in the spaces between the large oval bacteria (23).

In this study, comparative 16S rRNA sequence analysis and FISH were used to examine the diversity and phylogeny of symbionts in two geographically separated (by 385 km) *O. crassitunicatus* populations from the Peru margin. We discovered a phylogenetically diverse symbiont population consisting of five or six co-occurring bacterial phylotypes that appears to be highly specific and stable in both host populations. The term symbiosis is used here in the nonrestrictive sense as defined by de Bary (5), namely, the "living together of differently named organisms."

MATERIALS AND METHODS

Specimen collection. *O. crassitunicatus* specimens were collected in June 2000 off the Peruvian coast in the Pacific Ocean. Samples were collected from two stations separated by ~385 km; at station A (12°43.93′S, 77°07.96′W) the water depth was 359 m, and at station B (9°51.52′S, 79° 12.74′W) the water depth was 270 m (Fig. 1). The worms were extracted from the sediment by decantation with seawater and were identified under a microscope. Only active and intact worms were used. Specimens were fixed in 70% ethanol for DNA analyses and for FISH as described previously (8) and were stored at 4°C.

DNA preparation and PCR amplification. Three *O. crassitunicatus* individuals each from stations A and B were prepared individually for PCR. The specimens were rinsed three times in MilliQ water, and DNA was isolated as described by Schizas et al. (40) by a protocol in which proteinase K was used for digestion and the reagent GeneReleaser (BioVentures, Murfreesboro, Tenn.) was used for DNA purification. Amplification was performed with primers specific for the bacterial 16S rRNA gene (primers 8F and 1492R [35]). Template DNA (1 to 2 μl) was added after the PCR mixture (total volume, 100 μl) was heated to 80°C to avoid nonspecific annealing of the primers to nontarget DNA. The following thermocycling conditions were used: one cycle at 80°C for 5 min; 30 cycles at 95°C for 1 min, 40°C for 1 min, and 72°C for 3 min; and one cycle at 72°C for 10 min.

Cloning and sequencing. PCR products from the six host individuals were cloned separately by using a TA cloning kit (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. For screening of 16S rRNA genes, 100 clones per individual were randomly picked and controlled for the correct insert size by PCR with the vector primers M13F and M13R. For template DNA, a small amount of cells from each clone colony was picked with a sterile toothpick and resuspended in 10 μl of sterile water. One to two microliters of this template DNA, after preheating to 95°C for 5 min, was amplified by PCR as described above by using a 30-μl (total volume) mixture. PCR products of the correct size (~1,500 bp) were screened by partial sequencing of 300 to 500 bp by using the vector primer M13F. Sequencing reactions were performed by using ABI BigDye and an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, Calif.). Sequences were aligned and compared by using the Bioedit program (www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were grouped together in a clone family if they exhibited ≥99% sequence identity (percentage of identical nucleotides). For each host individual, a representative clone from each clone family was prepared by using a QIAprep plasmid kit (QIAGEN, Hilden, Germany) and was nearly fully sequenced in both directions (1,498 bp).

Phylogenetic analysis. The 16S rRNA symbiont sequences from *O. crassitunicatus* were checked against sequences in the GenBank database by using BLAST (1) for similarity searches. The 16S rRNA sequence data were analyzed by using the ARB software package (www.arb-home.de). Phylogenetic trees were calculated by performing parsimony, distance, and maximum-likelihood analyses with different sets of filters. For tree reconstruction only sequences with more than 1,400 bp were used.

FISH. Three *O. crassitunicatus* specimens each from stations A and B were prepared for FISH analysis of bacterial endosymbionts as described previously (8), with the following modifications: in the prehybridization treatments, the tissue sections were incubated for 6 min instead of 10 min in xylene, ethanol, HCl, and proteinase K, and the postfixation step in 4% formaldehyde was omitted. FISH with monolabeled fluorescent oligonucleotide probes was used for the bacterial symbionts belonging to the δ-*Proteobacteria* by following the general protocol for FISH described by Pernthaler et al. (38). Due to weak signals of the symbionts belonging to the γ-*Proteobacteria* and *Spirochaeta*, an enhanced FISH detection method, catalyzed reporter deposition (CARD) FISH with horseradish peroxidase (HRP)-labeled probes and tyramide signal amplification, was used, as described by Schönhuber et al. (41). Briefly, after pretreatment as described above, tissue sections were prehybridized in hybridization buffer (41) for 20 min at 35°C, and this was followed by hybridization with the HRP-labeled probe for 3 h at 35°C. After the sections were washed for 15 min at 35°C in washing buffer (41), they were equilibrated for 15 min at room temperature in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate; pH 7). The moist tissue sections were incubated with the amplification solution (1× phosphate-buffered saline [pH 7.3], 0.0015% [vol/vol] H_2O_2, 1% Alexa Fluor 350, 488, or 546 dye [Molecular Probes, Leiden, The Netherlands]) for 20 min at 37°C in the dark and rinsed in 1× SSC for 15 min at room temperature. After air drying, tissue sections were embedded in the mounting fluid Vecta Shield (Vecta Laboratories, Burlingame, Calif.) and stored for microscopic evaluation at −20°C for <1 to 2 days. For dual and triple hybridizations, the CARD FISH protocol was repeated two or three times with the same sections by using different probes and Alexa dyes. To do this, after the last washing step the tissue sections were covered with 0.01 M HCI for 10 min at room temperature to inactivate the HRP. After the tissue sections were washed for 3 min in sterile water, they were hybridized with another probe as described above.

The oligonucleotide probes designed in this study target 16S rRNA sequences of bacterial symbionts of *O. crassitunicatus* (Table 1). The probes were checked against sequences in the GenBank database by using BLAST (1) and against

TABLE 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5′-3′)	Position[a]	Formamide concn (%, vol/vol)[c]		Reference
				FISH	CARD FISH	
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	338–355	35	55	2
GAM42a	γ-*Proteobacteria*	GCCTTCCCACATCGTTT	1027–1043[b]	35	55	33
NON338	Negative control	ACTCCTACGGGAGGCAGC	338–355	10	30	43
DSS658	*Desulfosarcina* spp., *Desulfofaba* spp., *Desulfococcus* spp., *Desulfofrigus* spp.	TCCACTTCCCTCTCCCAT	658–685	60	70	34
DSR651	*Desulforhopalus* spp.	CCCCCTCCAGTACTCAAG	651–668	35	70	34
OcraGAM1	Gamma 1 symbionts	GCATACAGACAGAGGCCC	200–215		40	This study
OcraGAM2	Gamma 2 symbionts	CTGCGCTCCCAAAGGCAC	1024–1041		55	This study
OcraDEL1	Delta 1 symbionts	CGTCAGCACCTGGTGATA	467–484	20		This study
OcraDEL2	Delta 2 symbionts	CATGCAGATTCTTCCCAC	443–471	20		This study
OcraDEL3	Delta 3 symbionts	TTTCATAGAGCTTCCCGG	999–1016	20		This study
OcraSPI	Spirochete symbionts	GCTATCCCCAACCAAAAG	136–153		40	39

[a] Position in the 16S rRNA of *Escherichia coli* unless indicated otherwise.
[b] Position in the 23S rRNA of *E. coli.*
[c] Formamide concentration in hybridization buffer.

small-subunit rRNA sequences in the Ribosomal Database Project by using CHECK-PROBE (37). The specificity of the probes designed for the γ-proteobacterial and δ-proteobacterial symbionts was tested with symbionts from another gutless oligochaete, *Olavius ilvae* (21), that have one or two mismatches in their 16S rRNA compared to the specific probes for *O. crassitunicatus* (39). The OcraGAM1, OcraDEL3, and OcraDEL2 probes failed to hybridize to the *O. ilvae* symbionts even in the absence of formamide, indicating the specificity of these probes at low stringencies. The signals from the OcraDEL1 and OcraGAM2 probes were not visible with 15 and 50% formamide, respectively, while the probe signals were still strong at the same formamide concentrations in hybridizations with *O. crassitunicatus* symbionts. The specificity of the OcraSPI probe was tested with the reference strain *Spirochaeta stenostrepta* DSM2028 (one mismatch in the probe target region), and the results showed that there was no signal in the absence of formamide (but there was a strong signal with probe EUB338), indicating the high level of specificity of the OcraSPI probe.

The general *Bacteria* probe EUB338, the general γ-*Proteobacteria* probe GAM42a, and the δ-*Proteobacteria* probes DSS658 and DSR651 were used as positive controls, and the nonsense probe NON338 was used as a negative control. All hybridizations were performed with formamide concentrations that ensured high specificity (Table 1).

Nucleotide sequence accession numbers. The symbiont 16S rRNA sequences from *O. crassitunicatus* have been deposited in the GenBank database under accession numbers AJ620507 (Gamma 1 symbiont), AJ620508 (Gamma 2 symbiont), AJ620509 (Delta 1 symbiont), AJ620510 (Delta 2 symbiont), AJ620511 (Delta 3 symbiont), and AJ620512 (spirochete symbiont).

RESULTS

Clone library analysis. Bacterial 16S rRNA sequences from three *O. crassitunicatus* specimens each from stations A and B were grouped into six distinct clone families; a total of 573 clones were analyzed (Table 2). Within each clone family, the level of sequence identity was never less than 99.7% (percentage of identical nucleotides). Phylogenetic analyses (see below) revealed that two clone families belong to the γ-*Proteobacteria* (Gamma 1 and 2), three clone families belong to the δ-*Proteobacteria* (Delta 1, 2, and 3), and one clone family belongs to the spirochetes.

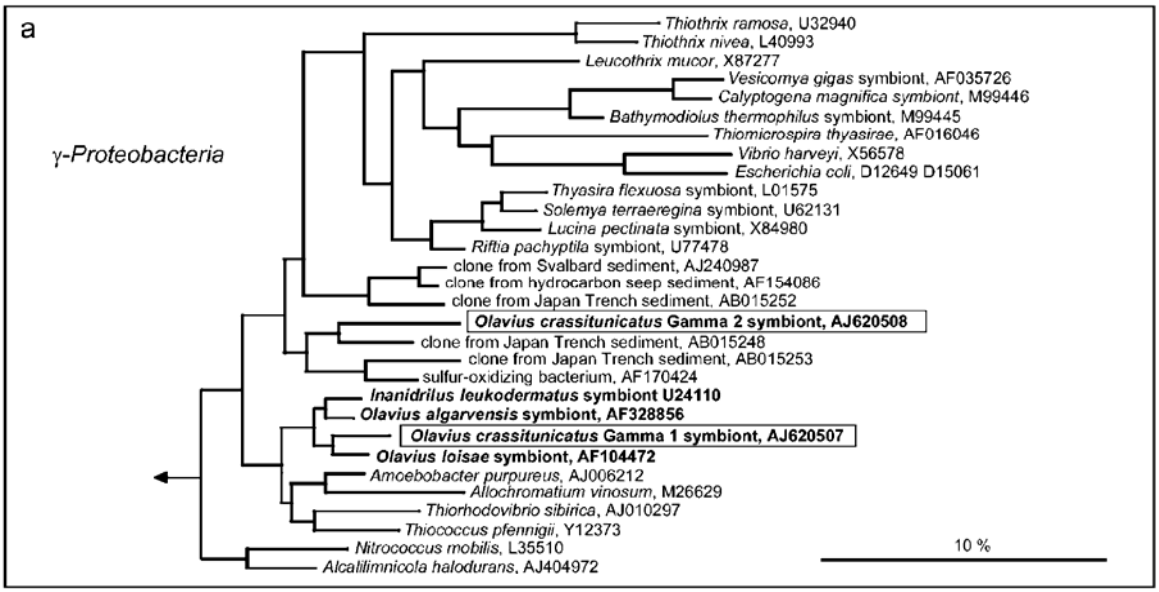
Sequences belonging to the Gamma 1 and 2 clone families were found in all hosts from stations A and B. For the Delta 1 and 2 clone families, as well as the spirochetes, at least two of three *O. crassitunicatus* individuals from both stations harbored sequences belonging to these groups. In contrast, sequences belonging to the Delta 3 clone family were found only in individuals from station A.

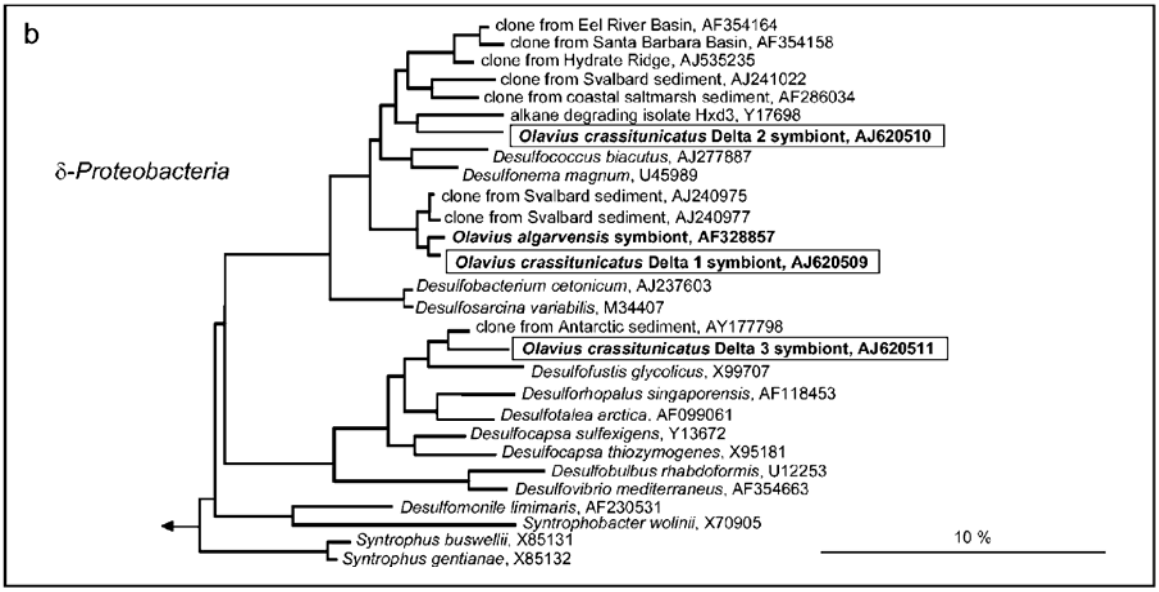
Phylogenetic analysis. Parsimony, distance, and maximum-likelihood analyses of the 16S rRNA sequences from the six *O. crassitunicatus* clone families confirmed that these sequences belonged to two phylogenetically distinct groups of the γ-*Proteobacteria*, three phylogenetically distinct groups of the δ-*Proteobacteria*, and one phylogenetically distinct group of the spirochetes. The six *O. crassitunicatus* sequences are unique to this host and differ from the sequences of symbionts from other host species or free-living bacteria.

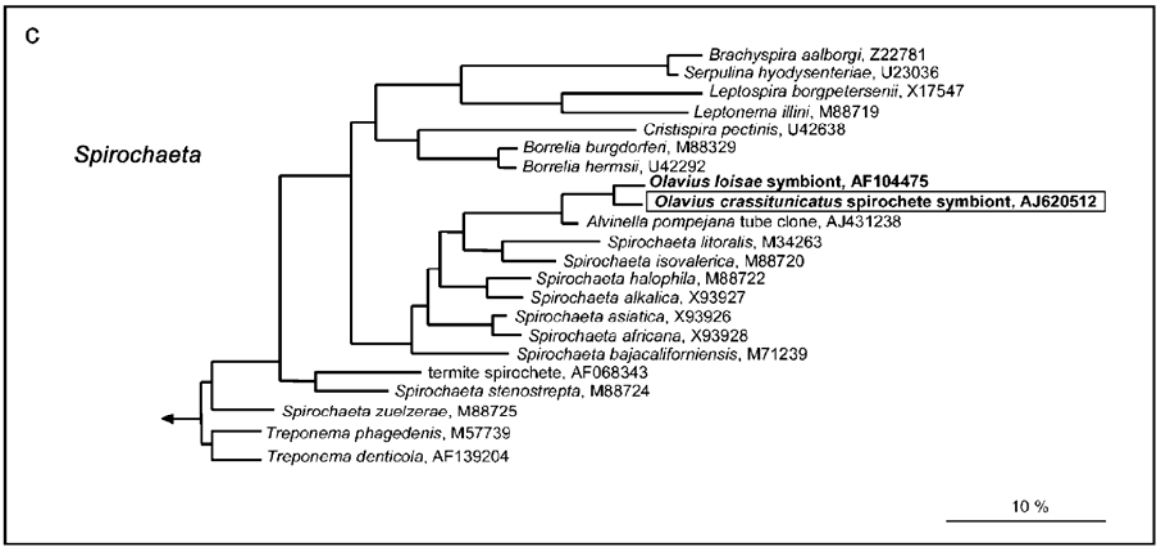
In all phylogenetic analyses, the *O. crassitunicatus* Gamma 1

TABLE 2. 16S rRNA clone libraries from six *O. crassitunicatus* individuals

Worm no.	No. of clones (% of total)						
	Total	Gamma 1	Gamma 2	Delta 1	Delta 2	Delta 3	*Spirochaeta*
Station A							
1	101	12 (12)	25 (25)	18 (18)	9 (9)	33 (33)	4 (4)
2	90	18 (20)	27 (30)	10 (11)	14 (16)	20 (22)	1 (1)
3	90	25 (28)	25 (28)	19 (21)	0 (0)	18 (20)	3 (3)
Station B							
1	109	3 (3)	70 (64)	36 (33)	0 (0)	0 (0)	0 (0)
2	77	9 (12)	38 (49)	21 (27)	7 (9)	0 (0)	2 (3)
3	106	13 (12)	20 (19)	32 (30)	37 (35)	0 (0)	4 (4)


a
γ-Proteobacteria
Thiothrix ramosa, U32940
Thiothrix nivea, L40993
Leucothrix mucor, X87277
Vesicomya gigas symbiont, AF035726
Calyptogena magnifica symbiont, M99446
Bathymodiolus thermophilus symbiont, M99445
Thiomicrospira thyasirae, AF016046
Vibrio harveyi, X56578
Escherichia coli, D12649 D15061
Thyasira flexuosa symbiont, L01575
Solemya terraeregina symbiont, U62131
Lucina pectinata symbiont, X84980
Riftia pachyptila symbiont, U77478
clone from Svalbard sediment, AJ240987
clone from hydrocarbon seep sediment, AF154086
clone from Japan Trench sediment, AB015252
Olavius crassitunicatus Gamma 2 symbiont, AJ620508
clone from Japan Trench sediment, AB015248
clone from Japan Trench sediment, AB015253
sulfur-oxidizing bacterium, AF170424
Inanidrilus leukodermatus symbiont U24110
Olavius algarvensis symbiont, AF328856
Olavius crassitunicatus Gamma 1 symbiont, AJ620507
Olavius loisae symbiont, AF104472
Amoebobacter purpureus, AJ006212
Allochromatium vinosum, M26629
Thiorhodovibrio sibirica, AJ010297
Thiococcus pfennigii, Y12373
Nitrococcus mobilis, L35510
Alcalilimnicola halodurans, AJ404972
10 %


b
δ-Proteobacteria
clone from Eel River Basin, AF354164
clone from Santa Barbara Basin, AF354158
clone from Hydrate Ridge, AJ535235
clone from Svalbard sediment, AJ241022
clone from coastal saltmarsh sediment, AF286034
alkane degrading isolate Hxd3, Y17698
Olavius crassitunicatus Delta 2 symbiont, AJ620510
Desulfococcus biacutus, AJ277887
Desulfonema magnum, U45989
clone from Svalbard sediment, AJ240975
clone from Svalbard sediment, AJ240977
Olavius algarvensis symbiont, AF328857
Olavius crassitunicatus Delta 1 symbiont, AJ620509
Desulfobacterium cetonicum, AJ237603
Desulfosarcina variabilis, M34407
clone from Antarctic sediment, AY177798
Olavius crassitunicatus Delta 3 symbiont, AJ620511
Desulfofustis glycolicus, X99707
Desulforhopalus singaporensis, AF118453
Desulfotalea arctica, AF099061
Desulfocapsa sulfexigens, Y13672
Desulfocapsa thiozymogenes, X95181
Desulfobulbus rhabdoformis, U12253
Desulfovibrio mediterraneus, AF354663
Desulfomonile limimaris, AF230531
Syntrophobacter wolinii, X70905
Syntrophus buswellii, X85131
Syntrophus gentianae, X85132
10 %


c
Spirochaeta
Brachyspira aalborgi, Z22781
Serpulina hyodysenteriae, U23036
Leptospira borgpetersenii, X17547
Leptonema illini, M88719
Cristispira pectinis, U42638
Borrelia burgdorferi, M88329
Borrelia hermsii, U42292
Olavius loisae symbiont, AF104475
Olavius crassitunicatus spirochete symbiont, AJ620512
Alvinella pompejana tube clone, AJ431238
Spirochaeta litoralis, M34263
Spirochaeta isovalerica, M88720
Spirochaeta halophila, M88722
Spirochaeta alkalica, X93927
Spirochaeta asiatica, X93926
Spirochaeta africana, X93928
Spirochaeta bajacaliforniensis, M71239
termite spirochete, AF068343
Spirochaeta stenostrepta, M88724
Spirochaeta zuelzerae, M88725
Treponema phagedenis, M57739
Treponema denticola, AF139204
10 %

sequence consistently fell in a cluster with the sequences of endosymbionts from other gutless oligochaetes, such as *Inanidrilus leukodermatus*, *O. loisae*, and *O. algarvensis* (≥95.6% sequence identity) (Fig. 2a). The closest relatives of this cluster of oligochaete symbionts are a clade of free-living bacteria belonging to the family *Chromatiaceae*. The second γ-proteobacterial sequence found in *O. crassitunicatus*, Gamma 2, is not closely related to those of other symbiotic γ-*Proteobacteria* (Fig. 2a). This sequence was consistently grouped with a clone sequence obtained from deep-sea sediments in the Japan Trench (90.3% sequence identity) by all inference methods. The relationship of this sequence to other 16S rRNA sequences varied depending on the treeing method used. Maximum-likelihood and distance analyses identified a clade containing a sulfur-oxidizing bacterium isolated from a shallow water hydrothermal vent in the Mediterranean Sea (89.8% sequence identity) and a clone sequence from deep-sea sediments in the Japan Trench (89.4% sequence identity) as the closest relatives. In parsimony analyses, the sister group of the Gamma 2 sequence is a cluster of clone sequences isolated from permanently cold sediments off the coast of Svalbard, Japan Trench sediments, and hydrocarbon seep sediments (≥86.2% sequence identity).

The three δ-proteobacterial sequences from *O. crassitunicatus*, Delta 1, 2, and 3, are phylogenetically distinct from each other (Fig. 2b). The Delta 1 sequence is most closely related to the sequence of the sulfate-reducing endosymbiont of the gutless oligochaete *O. algarvensis* as determined by all treeing methods (98.6% sequence identity). The neighboring clade of these two sequences includes clone sequences isolated from permanently cold sediments off the coast of Svalbard (97.8 to 97.6% sequence identity).

The *O. crassitunicatus* Delta 2 and 3 sequences are not closely related to other δ-proteobacterial symbiont sequences. The closest relative of the Delta 2 sequence as determined by all inference methods is the sequence of the alkane-degrading bacterial strain Hxd3 isolated from an oil tank (90.9% sequence identity). The neighboring branches of these two sequences were consistently identified as a cluster of clone sequences from sediments obtained above gas hydrates in Eel River, the Santa Barbara Basin, and Hydrate Ridge and a cluster of clone sequences from sediments obtained off the coast of Svalbard and coastal salt marsh sediments (91.2 to 89.9% sequence identity) as determined by all treeing methods. The Delta 3 sequence is most closely related to a clone sequence isolated from Antarctic sediments (94.7% sequence identity) and the sequence of the free-living sulfate-reducing bacterium *Desulfofustis glycolicus* (92.4% sequence identity).

The *O. crassitunicatus* spirochete sequence was consistently grouped with the sequence of the spirochete endosymbiont of the gutless oligochaete *O. loisae* from the Australian Great Barrier Reef in all three phylogenetic analyses (95.4% sequence identity) (Fig. 2c). Peripherally associated with these two sequences is a clone sequence obtained from tubes of the hydrothermal vent polychaete *Alvinella pompejana* (92.9% sequence identity).

In situ identification. FISH with oligonucleotide probes confirmed that the six 16S rRNA sequences isolated from *O. crassitunicatus* originated from bacteria in the symbiont-containing region between the cuticle and the epidermis of the worm (Fig. 3). FISH studies showed that the two γ-proteobacterial symbionts (Gamma 1 and 2), two of the δ-proteobacterial symbionts (Delta 1 and 2), and the spirochete co-occurred in all three specimens from stations A and B. The third δ-proteobacterial symbiont, Delta 3, was found only in the three specimens from station A.

The general probe for γ-*Proteobacteria*, GAM42a, as well as the specific probes OcraGAM1 and OcraGAM2 for the Gamma 1 and 2 symbionts, respectively, hybridized to bacteria throughout the symbiont-containing region (Fig. 3a and b). The hybridization patterns of the two specific probes were distinctly different. The signal from the OcraGAM1 probe was consistent in size, shape, and distribution with the bacteria described as the large morphotype in *O. crassitunicatus* by Giere and Krieger (23) (Fig. 3b). The OcraGAM2 probe hybridized to much smaller bacteria that were approximately 1 μm long and located in the spaces between the large morphotypes (Fig. 3b).

The hybridization signal from the δ-proteobacterial probes DSS658 and DSR651 was limited to small bacteria in the peripheral area of the symbiont-containing region directly beneath the cuticle (Fig. 3a and f). These general probes are used for identification of δ-*Proteobacteria* belonging to the genera *Desulfosarcina*, *Desulfofaba*, *Desulfococcus*, *Desulfofrigus*, and *Desulforhopalus* and also targeted the δ-proteobacterial sequences Delta 1 to 3 isolated from *O. crassitunicatus*. A similar hybridization pattern was observed with the specific probes for the three δ-proteobacterial sequences, OcraDEL1, OcraDEL2, and OcraDEL3, confirming that these sequences originated from δ-proteobacterial symbionts beneath the cuticle of the worm. Dual hybridizations with the OcraDEL probes showed that the Delta 1 and 2 symbionts occurred in approximately equal numbers in specimens from both station A and station B (Fig. 3c), while the Delta 3 symbionts were much rarer and were found only in specimens from station A (Fig. 3d).

The specific probe for the spirochete sequence isolated from *O. crassitunicatus*, OcraSPI, hybridized to long, thin bacteria located between the large Gamma 1 symbionts (Fig. 3e and f). The hybridization signal from this probe was consistent with the shape and distribution pattern of the long, filiform bacterial morphotype described by Giere and Krieger (23) in *O. crassitunicatus.*

DISCUSSION

This study revealed the coexistence of five to six distinct bacterial phylotypes in the body wall of the gutless marine oligochaete *O. crassitunicatus*. By using comparative 16S rRNA

FIG. 2. Phylogenetic placement of bacterial symbionts in *O. crassitunicatus* based on 16S rRNA sequences: maximum-likelihood trees of members of the γ-*Proteobacteria* (a), δ-*Proteobacteria* (b), and *Spirochaeta* (c). Symbionts of gutless oligochaetes are indicated by boldface type, and the *O. crassitunicatus* symbionts are enclosed in boxes. Bar = 10% estimated sequence divergence.

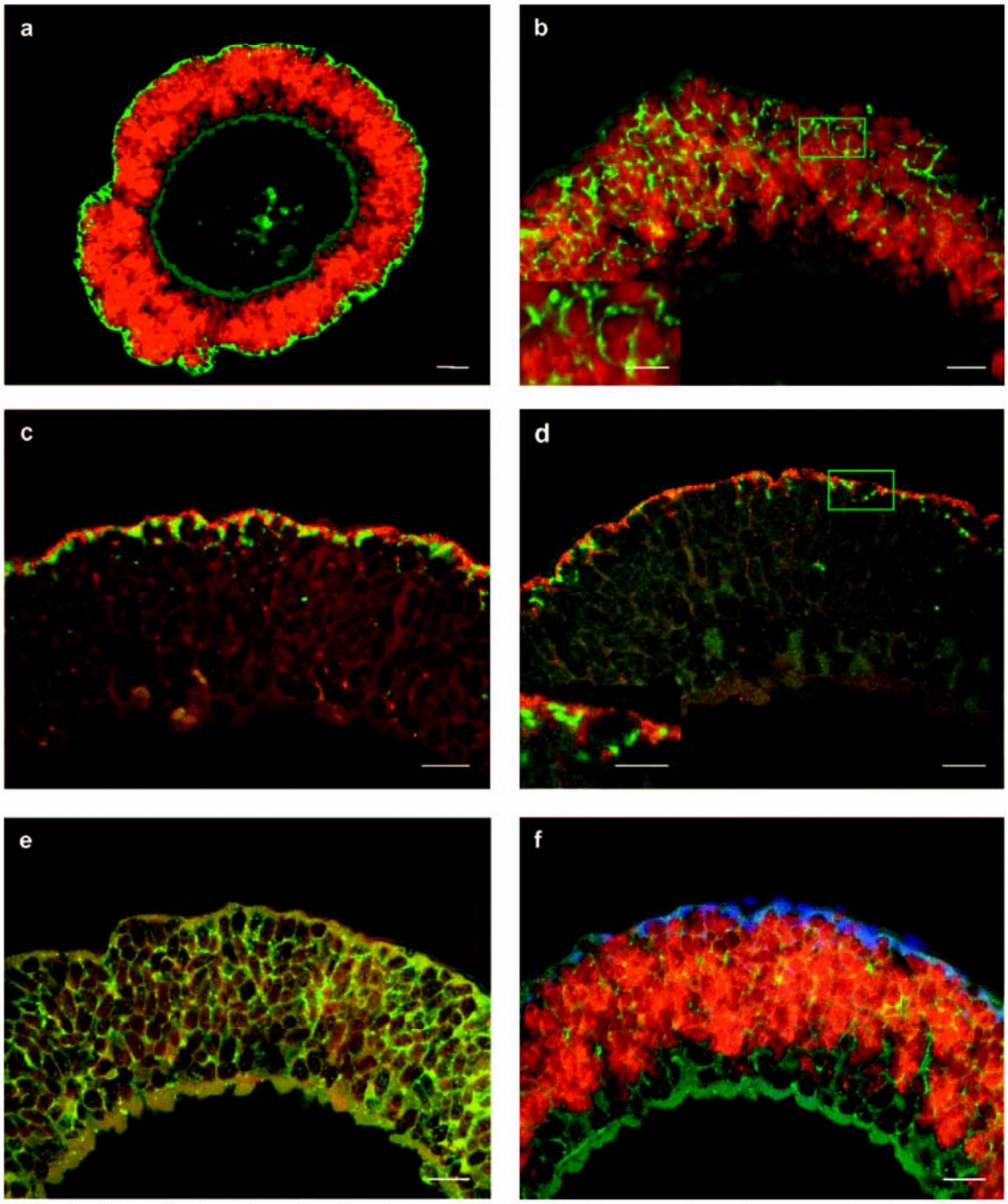


FIG. 3. In situ identification of bacterial symbionts in *O. crassitunicatus*. The epifluorescence images show cross sections through the entire worm (a) (scale bar, 20 μm) and the symbiont-containing region of the worm's body wall (b to f) (scale bars, 10 μm). All worms shown were from station A; the only exception is the worm shown in panel c, which was from station B. (a) Dual hybridization with the GAM42a and DSS658/DSR651 probes, showing γ-proteobacterial symbionts (red) and δ-proteobacterial symbionts (green). (b) Dual hybridization with the OcraGAM1 and OcraGAM2 probes, showing the Gamma 1 symbionts (red) and Gamma 2 symbionts (green). The inset shows an enlargement of the area enclosed by a box (scale bar, 5 μm). (c) Dual hybridization with the OcraDEL1 and OcraDEL2 probes, showing the Delta 1 symbionts (green) and Delta 2 symbionts (red). (d) Dual hybridization with the OcraDEL1/OcraDEL2 and OcraDEL3 probes, showing the Delta 1 and Delta 2 symbionts (red)

sequence analysis and fluorescence in situ hybridization, two symbionts belonging to the γ-*Proteobacteria*, three symbionts belonging to the δ-*Proteobacteria*, and one spirochete symbiont were identified in this host species. Despite this high diversity, the association between the bacterial endosymbionts and *O. crassitunicatus* is clearly specific within a given host population, as all individuals from stations A and B harbored the same five or six phylotypes. Furthermore, the association appears to be highly specific and stable in geographically distant host populations, given the co-occurrence of the same five bacterial phylotypes in worms separated from each other by 385 km.

Such great diversity of multiple endosymbiont species has not been observed previously in oligochaete symbioses. In previous studies, either a single phylotype (8) or two or three co-occurring bacterial phylotypes were described for each host species (7, 9). The ability to sequence a much larger number of clones in this study (573 clones versus 60 to 70 clones or direct sequencing in previous studies), in addition to improved FISH techniques, such as CARD FISH, is the most likely explanation for the discovery of this previously unrecognized diversity. Indeed, recent studies have suggested that there is similar diversity in other gutless oligochaete species (Blazejak and Dubilier, unpublished data), including species previously assumed to harbor only one to three bacterial phylotypes (7, 8, 9).

The FISH analyses in this study of the Gamma 1 bacteria in *O. crassitunicatus* indicate that this symbiont is the same as the large, oval bacterial morphotype found by Giere and Krieger (23) in another *O. crassitunicatus* population from Peru sediments (station L in Fig. 1). In both the specimens of Giere and Krieger (23) and the worms studied here, these bacteria were unusually large (diameter, 7 to 10 μm), in contrast to other gutless oligochaetes, in which this morphotype is at most only one-half as big (diameter, 3 to 5 μm) (24). The thioautotrophic (i.e., sulfide-oxidizing, CO_2-fixing) nature of this symbiont was confirmed by Giere and Krieger (23), who used immunocytochemistry to show the presence of the CO_2-fixing enzyme RubisCO and spectroscopy to identify sulfur in intracellular deposits. The close phylogenetic relationship of the *O. crassitunicatus* Gamma 1 symbiont to chemoautotrophic symbionts of other gutless oligochaetes and the monophyly of this group as determined by all treeing methods are further indications that the *O. crassitunicatus* Gamma 1 bacteria are autotrophic, sulfide-oxidizing symbionts.

The occurrence of a second γ-proteobacterial symbiont, like Gamma 2 in *O. crassitunicatus*, has not been described previously for other oligochaete species, which so far have been found to harbor only a single Gamma 1-like symbiont (7–9). Endosymbiotic associations with more than one γ-proteobacterial phylotype have been observed in the wood-boring mussel *Lyrodus pedicellatus* (6) and in the cold-seep thyasirid clam *Maorithyas hadalis* (18). The function of these multiple symbionts in these bivalves is unclear, just as the metabolism of the novel *O. crassitunicatus* Gamma 2 phylotype remains to be determined. The Gamma 2 symbiont belongs to a phylogenetic group that includes clone sequences from cold-seep communities in the Japan Trench (32), as well as a chemoautotrophic, sulfur-oxidizing isolate obtained from a shallow hydrothermal vent in the Mediterranean Sea (42), suggesting that the Gamma 2 symbiont might also participate in chemosynthetic pathways. Studies of functional genes involved in this symbiosis, such as genes coding for RubisCO forms I and II and enzymes involved in sulfur metabolism, such as adenosine-5′-phosphosulfate reductase (*aprA*), as well as in dissimilatory nitrate reduction (nitrite reductase; *nirK*), are in progress.

Three phylogenetically distinct δ-proteobacterial symbionts were found in *O. crassitunicatus*. The close evolutionary relationship of these symbionts to free-living and symbiotic sulfate-reducing bacteria and the predominance of this type of metabolism within the δ-*Proteobacteria* suggest that these symbionts also use sulfate as an electron acceptor. The first chemoautotrophic host known to harbor a δ-proteobacterial symbiont was the gutless oligochaete *O. algarvensis* obtained from sediments in the Mediterranean Sea. In this species, only a single δ-proteobacterial symbiont was found, and it was identified as a sulfate reducer based on molecular and physiological data (9). In *O. crassitunicatus*, the 16S rRNA sequence of the Delta 1 symbiont is very closely related to that of the sulfate-reducing symbiont of *O. algarvensis* (98.6% sequence identity) (9). In contrast, the Delta 2 and 3 symbionts belong to novel lineages not previously known to occur in symbiotic associations.

In the Mediterranean Sea sediments in which *O. algarvensis* occurs, sulfide is not detectable by smell, and measurements have shown that the sulfide concentrations are very low (<1 μM) (9). This led to the suggestion that the sulfate-reducing symbiont of *O. algarvensis* could provide the thioautotrophic symbiont of this host with an internal source of sulfide (9). In this study, sulfide concentrations were not measured at the two sampling sites where *O. crassitunicatus* was obtained, but at station A the sediments clearly smelled of sulfide. At another site off the coast of Peru where *O. crassitunicatus* was reported to occur in high numbers together with the free-living, sulfide-oxidizing bacteria *Thioploca* spp. (station L in Fig. 1), sulfide was also detected by smell (30). The presence of sulfide in the habitat of *O. crassitunicatus* suggests that the role of the sulfate-reducing symbionts in these worms is not restricted to supplying sulfide for the thioautotrophic symbionts. One distinct difference between the *O. algarvensis* symbiosis and the *O. crassitunicatus* symbiosis is the distribution of the sulfate reducers. In *O. algarvensis*, the sulfate-reducing bacteria occur throughout the entire symbiont-containing region and are in close contact with the thioautotrophic symbionts. In contrast, in *O. crassitunicatus* the sulfate reducers occur almost exclusively in the outer part of the symbiont-containing region, just below the cuticle of the worm, and have little contact with the thioautotrophic symbionts. This suggests that the uptake of substrates such as organic carbon, or hydrogen if the sulfate reducers are autotrophic, from the environment may play an important role in the *O. crassitunicatus* symbiosis. This could

and the Delta 3 symbionts (green). The inset shows an enlargement of the area enclosed by a box area (scale bar, 5 μm). (e) Monohybridization with the OcraSPI probe, showing spirochete symbionts (green). (f) Triple hybridization with the GAM42a, DSS658/DSR651, and OcraSPI probes, showing the two γ-proteobacterial symbionts (red), the three δ-proteobacterial symbionts (blue), and the spirochete symbionts (yellow). The muscle tissue of the worm at the bottom of the panel appears to be bluish green because of autofluorescence at mixed wavelengths.

also explain the presence of two or three δ-proteobacterial phylotypes in these hosts. Sulfate-reducing bacteria are known to metabolize a wide variety of electron donors and carbon sources, including low-molecular-weight compounds from the fermentative breakdown of biomolecules, various aromatic compounds (44), and even hydrocarbons (26). Multiple sulfate-reducing symbionts with different preferences for carbon compounds and electron donors could provide nutritional versatility that broadens the spectrum of nutritional sources available to the *O. crassitunicatus* association.

A symbiotic association between a spirochete and a gutless oligochaete was first suggested to occur in *O. loisae* from the Australian Great Barrier Reef (7). However, the authors were not able to unambiguously confirm that the spirochete 16S rRNA sequence isolated from *O. loisae* originated from symbiotic bacteria because the specific probes developed for the spirochete sequence did not hybridize to bacteria in the body wall of *O. loisae*. In this study a spirochete was successfully identified in the symbiont-containing region of a gutless oligochaete by using CARD FISH and the spirochete-specific probe OcraSPI. The hybridization signal from the spirochete probe was consistent with the shape and distribution pattern of the long, thin bacterial morphotype described in the ultrastructural study of *O. crassitunicatus* specimens from station L (Fig. 1) (23), indicating that spirochetes consistently occur as symbionts in these hosts.

The spirochete symbionts of *O. crassitunicatus* and *O. loisae* are closely related to each other (95.4% sequence identity) and form a monophyletic group as determined by all three treeing methods used. The close relationship of these two symbionts despite the great geographic distance between the two hosts and the differences in their habitats (deep-water slope sediments versus shallow coral reef sediments) indicates that the spirochete symbiosis is integral to the oligochaete hosts and independent of geographic or environmental factors. Indeed, studies of other gutless oligochaetes have shown that spirochetes regularly occur as symbionts in these hosts (39).

The oligochaete spirochetes fall on a neighboring branch with a clone sequence (accession no. AJ431238) isolated from the tubes of the hydrothermal vent polychaete *Alvinella pompejana* (M. A. Cambon-Bonavita, unpublished data). (Another spirochete sequence that also originated from *A. pompejana* tubes [accession no. AF180309] and was previously suggested to be most closely related to the *O. loisae* spirochete [4] does not fall within the oligochaete clade but rather is related to *Spirochaeta alkalica* based on BLAST and treeing analyses; this sequence was not included in the trees shown here due to its short length, 474 bp.) The free-living marine spirochetes *Spirochaeta isovalerica* and *Spirochaeta litoralis* consistently form a neighboring clade of the oligochaete spirochetes. These bacteria were isolated from sulfidic muddy sediments and are obligate anaerobes that ferment carbohydrates mainly to acetate, ethanol, CO_2, and H_2 (25, 27). While fermentation is one possible metabolic pathway of the oligochaete spirochetes, they could also have a completely different metabolism, just as the spirochete symbionts in termites do not have properties of their closest free-living relatives in the genus *Treponema* (3). Instead, termite spirochetes were recently found to be chemoautotrophic and to use H_2 and CO_2 to produce acetate (29). This type of metabolism should clearly be beneficial to the oligochaete hosts, providing them with an additional chemoautotrophic symbiont as a source of carbon and energy.

Of the six different bacterial phylotypes that coexist in *O. crassitunicatus*, three belong to clades in which only symbionts occur: (i) the Gamma 1 symbionts, (ii) the Delta 1 symbionts, and (iii) the spirochetes. Within these three clades, the host species are separated by large geographic distances (Bermuda, Mediterranean Sea, Australia, and Peru) and come from very different habitats (coral reef sediments, coarse-grained coastal sands, and deep-water silty muds). The symbionts in these three clades, however, are closely related to each other (>95 to 98% sequence identity), indicating that within each clade the symbionts descended from a common ancestor. Three bacterial phylotypes found in *O. crassitunicatus* belong to novel lineages that have not been found previously in symbiotic associations (Gamma 2, Delta 2, and Delta 3).

All symbiont phylotypes except Delta 3 appear to be highly specific and stable both within a given population and between host populations. However, variation can clearly occur between populations, as seen in the Delta 3 phylotype, which occurred only in hosts from station A. There are two explanations for the intraspecific variation of the Delta 3 symbiont: (i) this symbiont originally occurred in both populations and was lost by hosts at station B, or (ii) this symbiont never occurred in the population at station B, so that the association was established independently by the hosts at station A. Future studies of other *O. crassitunicatus* populations from the Peru and Chile margin, as well as a better understanding of the acquisition of symbionts, should help answer these and other questions concerning the establishment and evolution of symbioses in oligochaete hosts.

ACKNOWLEDGMENTS

We are grateful to Daniela Riechmann for collecting the worms and to Julia Herglotz for technical assistance.

This work was supported by the Max Planck Society, Munich, Germany.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targetcd oligonuclcotidc probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
3. **Breznak, J. A.** 2002. Phylogenetic diversity and physiology of termite gut spirochetes. Intergr. Comp. Biol. **42:**313–318.
4. **Campbell, B. J., and S. C. Cary.** 2001. Characterization of a novel spirochete associated with the hydrothermal vent polychaete annelid, *Alvinella pompejana*. Appl. Environ. Microbiol. **67:**110–117.
5. **de Bary, A.** 1879. Die Erscheinung der Symbiose. Verlag von Karl J. Trubner, Strassburg, Germany.
6. **Distel, D. L., D. J. Beaudoin, and W. Morrill.** 2002. Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve *Lyrodus pedicellatus* (Bivalvia: Teredinidae). Appl. Environ. Microbiol. **68:**6292–6299.
7. **Dubilier, N., R. Amann, C. Erséus, G. Muyzer, S. Y. Park, O. Giere, and C. M. Cavanaugh.** 1999. Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochaete *Olavius loisae* (Annelida). Mar. Ecol. Prog. Ser. **178:**271–280.
8. **Dubilier, N., O. Giere, D. L. Distel, and C. M. Cavanaugh.** 1995. Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (Oligochaeta, Annelida) by phylogenetic 16S rRNA sequence analysis and in situ hybridization. Appl. Environ. Microbiol. **61:**2346–2350.
9. **Dubilier, N., C. Mülders, T. Ferdelman, D. de Beer, A. Pernthaler, M. Klein, M. Wagner, C. Erséus, F. Thiermann, J. Krieger, O. Giere, and R. Amann.** 2001. Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. Nature **411:**298–302.

10. **Erséus, C.** 1992. A generic revision of the Phallodrilinae (Oligochaeta, Tubificidae). Zool. Scr. **21:**5–48.
11. **Erséus, C.** 2003. The gutless Tubificidae (Annelida, Oligochaeta) of the Bahamas. Meiofauna Mar. **12:**59–84.
12. **Erséus, C.** 1990. The marine Tubificidae (Oliogchaeta) of the barrier reef ecosystems at Carrie Bow Cay, Belize, and other parts of the Caribbean Sea, with descriptions of twenty-seven new species and revision of *Heterodrilus, Thalassodrilides* and *Smithsonidrilus.* Zool. Scr. **19:**243–303.
13. **Erséus, C.** 1984. Taxonomy and phylogeny of the gutless Phallodrilinae (Oligochaeta, Tubificidae), with descriptions of one new genus and twenty-two new species. Zool. Scr. **13:**239–272.
14. **Erséus, C.** 1991. Two new deep-water species of the gutless genus *Olavius* (Oligochaeta: Tubificidae) from both sides of North America. Proc. Biol. Soc. Wash. **104:**627–630.
15. **Erséus, C., M. Källersjö, M. Ekman, and R. Hovmöller.** 2002. 18S rDNA phylogeny of Tubificidae and its constituent taxa (Clitellata): dismissal of the Naididae. Mol. Phylogenet. Evol. **22:**414–422.
16. **Felbeck, H., G. Liebezeit, R. Dawson, and O. Giere.** 1983. CO_2 fixation in tissues of marine oligochaetes (*Phallodrilus leukodermatus* and *P. planus*) containing symbiotic, chemoautotrophic bacteria. Mar. Biol. **75:**187–191.
17. **Finogenova, N.** 1986. Six new species of marine Tubificidae (Oligochaeta) from the continental shelf off Peru. Zool. Scr. **15:**45–51.
18. **Fujiwara, Y., C. Kato, N. Masui, K. Fujikura, and S. Kojima.** 2001. Dual symbiosis in the cold-seep thyasirid clam *Maorithyas hadalis* from the hadal zone in the Japan Trench, western Pacific. Mar. Ecol. Prog. Ser. **214:**151–159.
19. **Giere, O.** 1981. The gutless marine oligochaete *Phallodrilus leukodermatus.* Structural studies on an aberrant tubificid associated with bacteria. Mar. Ecol. Prog. Ser. **5:**353–357.
20. **Giere, O.** 1979. Studies on marine Oligochaeta from Bermuda, with emphasis on new *Phallodrilus* species (Tubificidae). Cah. Biol. Mar. **20:**301–314.
21. **Giere, O., and C. Erséus.** 2002. Taxonomy and new bacterial symbioses of gutless marine Tubificidae (Annelida, Oligochaeta) from the Island of Elba (Italy). Org. Divers. Evol. **2:**289–297.
22. **Giere, O., C. Erséus, and F. Stuhlmacher.** 1998. A new species of *Olavius* (Tubificidae, Phallodrilinae) from the Algarve Coast in Portugal, the first East Atlantic gutless oligochaete with symbiotic bacteria. Zool. Anz. **237:**209–214.
23. **Giere, O., and J. Krieger.** 2001. A triple bacterial endosymbiosis in a gutless oligochaete (Annelida): ultrastructural and immunocytochemical evidence. Invertebr. Biol. **120:**41–49.
24. **Giere, O., C. Nieser, R. Windoffer, and C. Erséus.** 1995. A comparative structural study on bacterial symbioses of Caribbean gutless Tubificidae (Annelida, Oligochaeta). Acta Zool. **76:**281–290.
25. **Harwood, C. S., and E. Canale-Parola.** 1983. *Spirochaeta isovalerica* sp. nov., a marine anaerobe that forms branched-chain fatty acids as fermentation products. Int. J. Syst. Bacteriol. **33:**573–579.
26. **Heider, H., A. M. Spormann, H. R. Beller, and F. Widdel.** 1999. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol. Rev. **22:**459–473.
27. **Hespell, R. B., and E. Canale-Parola.** 1973. Glucose and pyruvate metabolism of *Spirochaeta litoralis*, an anaerobic marine spirochete. J. Bacteriol. **116:**931–937.
28. **Krieger, J., O. Giere, and N. Dubilier.** 2000. Localization of RubisCO and sulfur in endosymbiotic bacteria of the gutless marine oligochaete *Inanidrilus leukodermatus* (Annelida). Mar. Biol. **137:**239–244.
29. **Leadbetter, J. R., T. M. Schmidt, J. R. Graber, and J. A. Breznak.** 1999. Acetogenesis from H_2 plus CO_2 by spirochetes from termite guts. Science **283:**686–689.
30. **Levin, L., D. Gutiérrez, A. Rathburn, C. Neira, J. Sellanes, P. Muñoz, V. Gallardo, and M. Salamanca.** 2002. Benthic processes on the Peru margin: a transect across the oxygen minimum zone during the 1997–98 El Niño. Prog. Oceanogr. **53:**1–27.
31. **Levin, L. A., A. E. Rathburn, D. Gutiérrez, P. Muñoz, and A. Shankle.** 2003. Bioturbation by symbiont-bearing annelids in near-anoxic sediments: implications for biofacies models and paleo-oxygen assessments. Palaeogeogr. Palaeoclimatol. Palaeoecol. **199:**129–140.
32. **Li, L., C. Kato, and K. Horikoshi.** 1999. Microbial diversity in sediments collected from the deepest cold-seep area, the Japan trench. Mar. Biotechnol. **1:**391–400.
33. **Manz, W., R. Amann, W. Ludwig, M. Wagner, and K.-H. Schleifer.** 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. Syst. Appl. Microbiol. **15:**593–600.
34. **Manz, W., M. Eisenbrecher, T. R. Neu, and U. Szewzyk.** 1998. Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol. Ecol. **25:**43–61.
35. **Muyzer, G., A. Teske, C. O. Wirsen, and H. W. Jannasch.** 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. **164:**165–172.
36. **Nylander, J. A. A., C. Erséus, and M. Källersjö.** 1999. A test of monophyly of the gutless Phallodrilinae (Oligochaeta, Tubificidae) and the use of a 573 bp region of the mitochondrial cytochrome oxidase I gene in analysis of annelid phylogeny. Zoo. Scr. **28:**305–313.
37. **Olsen, G. J., R. Overbeck, N. Larson, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W. M. Kuan, T. J. Macke, Y. Xing, and C. R. Woese.** 1992. Ribosomal Database Project. Nucleic Acids Res. **20:**2199–2200.
38. **Pernthaler, J., F. O. Glöckner, W. Schönhuber, and R. Amann.** 2001. Fluorescence in situ hybridization with rRNA-targeted oligonucleotides probes. Methods Microbiol. **30:**207–226.
39. **Rühland, C., A. Blazejak, A. Loy, M. Wagner, C. Erséus, R. Amann, and N. Dubilier.** Unpublished data.
40. **Schizas, N. V., G. T. Street, B. C. Coull, G. T. Chandler, and J. M. Quattro.** 1997. An efficient DNA extraction method for small metazoans. Mol. Mar. Biol. Biotechnol. **6:**381–383.
41. **Schönhuber, W., B. Zarda, S. Eix, R. Rippka, M. Herdman, W. Ludwig, and R. Amann.** 1999. In situ identification of cyanobacteria with horseradish peroxidase-labeled, rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **65:**1259–1267.
42. **Sievert, S. M., G. Muyzer, and J. Küver.** 1999. Novel sulfur-oxidizing bacteria from a shallow submarine hydrothermal vent, most closely related to obligate symbionts of invertebrates. Ph.D. dissertation. University of Bremen, Bremen, Germany.
43. **Wallner, G., R. Amann, and W. Beisker.** 1993. Optimizing fluorescent *in situ*-hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry **14:**136–143.
44. **Widdel, F., and F. Bak.** 1992. Gram-negative mesophilic sulfate-reducing bacteria, p. 3352–3389. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes, vol. IV. Springer-Verlag New York, N.Y.

2

Phylogeny of 16S rRNA, RubisCO, and APS reductase genes from gamma- and alphaproteobacterial symbionts in gutless marine worms (Oligochaeta) from Bermuda and Bahamas

Anna Blazejak, Jan Kuever, Christer Erséus, Rudolf Amann,
and Nicole Dubilier

Applied and Environmental Microbiology. 72: 5527-5536 (2006)

Phylogeny of 16S rRNA, RubisCO, and APS reductase genes from gamma- and alphaproteobacterial symbionts in gutless marine worms (Oligochaeta) from Bermuda and Bahamas

Anna Blazejak[1], Jan Kuever[1,2], Christer Erséus[3], Rudolf Amann[1]and Nicole Dubilier[1*]

[1] Max Planck Institute of Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany

[2] Present Address: Bremen Institute for Materials Testing, Paul-Feller-Str. 1, D-28199 Bremen, Germany

[3] Göteborg University, Box 463, SE-405 30 Göteborg, Sweden

Key words: symbiosis, chemoautotrophy, fluorescence in situ hybridization (FISH), chemosynthesis, sulfur oxidizers

* Corresponding Author: Nicole Dubilier. Mailing Address: Max Planck Institute of Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany. Tel: +49-(0)421-2028932. E-mail: ndubilie@mpi-bremen.de

Abstract

Gutless oligochaetes are small marine worms that live in obligate associations with bacterial endosymbionts. While the symbionts from hosts belonging to the genus *Olavius* have been described in several species, little is known of the symbionts from the host genus *Inanidrilus*. In this study, the diversity of bacterial endosymbionts in *I. leukodermatus* from Bermuda and *I. makropetalos* from Bahamas was investigated using comparative sequence analysis of phylogenetic and function genes, and fluorescence in situ hybridization. As in all other gutless oligochaetes examined to date, *I. leukodermatus* and *I. makropetalos* harbor large, oval shaped bacteria identified as Gamma 1 symbionts. The presence of genes coding for ribulose-1,5-bisphosphate carboxylase/oxygenase form I (*cbbL*) and adenosine-5'-phosphosulphate reductase (*aprA*) supports earlier studies indicating that these symbionts are chemoautotrophic sulfur oxidizers. Alphaproteobacteria, previously only described in the gutless oligochaete *O. loisae* from the West Pacific, coexist with the Gamma 1 symbionts in both *I. leukodermatus* and *I. makropetalos* with the former harboring four and the latter two alphaproteobacterial phylotypes. The presence of these symbionts in hosts from such geographically distant oceans as the Atlantic and Pacific suggests that symbioses with alphaproteobacterial symbionts may be widespread in gutless oligochaetes. The high phylogenetic diversity of bacterial endosymbionts in 2 species of the genus *Inanidrilus*, previously only known from members of the genus *Olavius*, shows that the stable coexistence of multiple symbionts is a common feature in gutless oligochaetes.

Introduction

Gutless oligochaetes are small worms of about 0.5 mm diameter and 2 - 5 cm length that occur worldwide in marine sediments with the highest diversity found in tropical and subtropical coral reefs (9-13, 15, 18, 19). They belong to a phylogenetically closely related group, consisting of only two genera, *Inanidrilus* and *Olavius*, within the subfamily Phallodrilinae. All worm species described so far, 25 *Inanidrilus* and 56 *Olavius* species (10), live in an obligate association with

endosymbiotic bacteria. The lack of both a digestive and an excretory system led to the assumption that the symbiotic bacteria provide their hosts with a source of nutrition (17). Enzyme assays and uptake experiments with inorganic carbon indicated that at least some of the bacterial symbionts are thiotrophic, i.e. use reduced sulfur compounds to fix CO_2 into organic carbon compounds (14). It is assumed that the transfer of these organic compounds to the host is the main mode of energy transfer although digestion of the bacteria may also supply nutrients.

The bacterial symbionts occur in a thick layer just below the outer cuticle of the worm in an extracellular space above the epidermal cells of the worm (21). A phylogenetically diverse assemblage of bacteria co-occurs within the symbiotic layer, with up to six rRNA phylotypes identified in a single host species (2). All gutless oligochaetes harbor a large (3 x 8 µm) thiotrophic symbiont with numerous sulfur and polyhydroxybutyric acid inclusions (3). These symbionts are found in all host species, and form a closely related cluster of 16S rRNA sequences within the Gammaproteobacteria (6). Coexisting with these primary symbionts, are smaller bacteria (0.7 x 1.9 µm) without any conspicuous inclusions that can belong to the Gamma-, Alpha-, or Deltaproteobacteria (2, 5, 8). While the metabolism of these smaller bacteria is not clear for the gamma- and alphaproteobacterial symbionts, the deltaproteobacterial symbionts have been identified as sulfate reducers, assumed to be engaged in a syntrophic sulfur cycle with the thiotrophic symbiont (2, 8). In some host species, spirochetes have also been identified as members of the oligochaete symbiont community (2, 5, 20).

The phylogeny of symbionts from the genus *Olavius* has been described in three host species, *O. loisae* from Australia (5), *O. algarvensis* from Mediterranean (8), and *O. crassitunicatus* from Peru (2), but only in a single species from the genus *Inanidrilus*, *I. leukodermatus* (7). *I. leukodermatus* occurs in high abundance in calcareous sands of coral reefs around the island Bermuda in the northwest Atlantic and the morphology, physiology, and ecology of this species has been studied intensively (7, 14, 17, 18, 21, 24). The symbionts of

this species were the first to be characterized using molecular methods, and only a single thiotrophic gammaproteobacterial phylotype was identified despite the presence of multiple bacterial morphotypes (7). More recent studies on other gutless oligochaete species using improved methods of 16S rRNA analysis and FISH have demonstrated that their multiple bacterial morphotypes correspond to multiple bacterial phylotypes (6). We therefore re-examined the symbionts in *I. leukodermatus* and extended these analyses to include another *Inanidrilus* species from the northwest Atlantic, *I. makropetalos*, that occurs in calcareous sands of coral reefs around the island Bahamas (10).

In addition to the phylogenetic characterization of the *Inanidrilus* symbionts we also investigated functional genes coding for ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the key enzyme for autotrophic CO_2 fixation in the Calvin-Benson-Bassham (CBB) cycle, and adenosine-5'-phosphosulphate (APS) reductase, an enzyme involved in sulfur metabolism. Two distinct forms of RubisCO have been found in chemoautotrophic symbionts, form I for which the *cbbL* gene and form II for which the *cbbM* gene is used as a functional marker (4, 24, 29, 34, 35). Form I RubisCO differs from form II structurally and is better adapted to CO_2 fixation under aerobic conditions (36, 37).

APS reductase is used in both the reductive and oxidative mode of sulfur metabolism and is therefore found in both sulfate reducers and sulfur oxidizers (16, 23). In sulfate reducers, APS reductase catalyzes the two-electron reduction of APS to sulfite and AMP, and in sulfur oxidizers the reverse reaction (31). APS reductase consists of an alpha and beta subunit encoded by the genes *aprA* and *aprB*, respectively, that form a 1:1 $\alpha\beta$ heterodimer. The *aprA* gene has been proposed as a useful phylogenetic marker for bacteria involved in oxidative and reductive sulfur metabolism (23). The phylogeny of the *aprA* gene has been well studied in sulfate reducers (17), but nothing is known about the phylogeny of *aprA* from sulfur-oxidizing bacteria. Only a few sequences from free-living sulfur oxidizers are currently available in the database and previous to this study, none were known from symbiotic sulfur oxidizers. The recently sequenced genome of *Pelagibacter ubique*, an alphaproteobacterium belonging to the SAR11 clade of

marine bacterioplankton, revealed that this species also has the APS reductase genes *aprA* and *aprB*. Given the extreme streamlining of the *P. ubique* genome it is likely that the APS reductase plays an important role in this species. *P. ubique* is not known to oxidize reduced inorganic sulfur compounds but is assumed to use dimethylsulfoniopropionate (DMSP) as a carbon source (22). It is therefore likely that APS reductase is used by *P. ubique* and other DMSP degrading bacteria to oxidize sulfite, which is highly toxic for living cells.

Materials and Methods

Specimen collection. *Inanidrilus leukodermatus* specimens were collected in June 1998 in Harrington Sound, Bermuda (Fig. 1). *Inanidrilus makropetalos* specimens were collected in April 1999 off Lee Stocking Island in the Bahamas (Fig. 1). The worms were extracted from shallow water (< 3 m) sediments by decantation with seawater and identified under the microscope. Specimens were fixed in 95% EtOH for DNA analyses, and for FISH as described previously (7), and stored at 4°C.

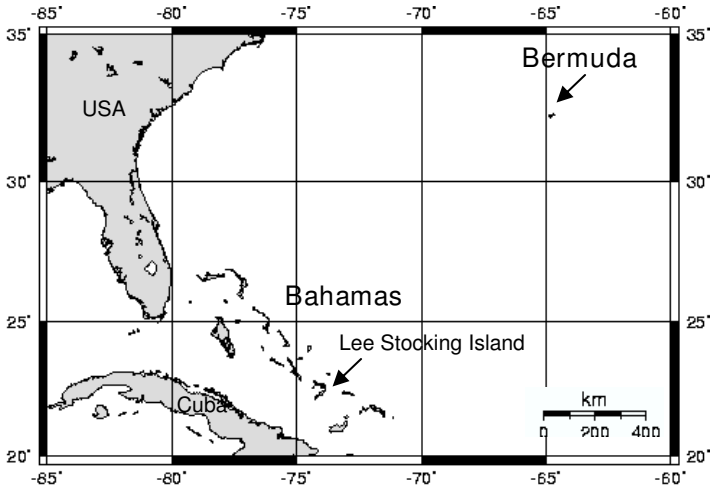


Figure 1. Map of sampling sites in the northwest Atlantic. *I. leukodermatus* worms were collected in Bermuda and *I. makropetalos* off Lee Stocking Island (Bahamas).

DNA preparation. Six *I. leukodermatus* and three *I. makropetalos* individuals were prepared individually for PCR. Specimens were rinsed 3 times in MilliQ, and DNA isolated as described by Schizas et al. (32) in a protocol using Proteinase K for digestion and the reagent GeneReleaser (BioVentures, Murfreesboro, USA) for DNA purification.

Polymerase chain reaction (PCR) amplification.

16S rRNA gene. Amplifications were performed with primers specific for the bacterial 16S rRNA gene (8F and 1492R; (26)). Template DNA (1-2 µl) was added after preheating the PCR mix (100 µl total volume) to 80°C to avoid nonspecific annealing of the primers to nontarget DNA. The following thermocycling conditions were used: 1 cycle at 80°C for 5 min; 27 cycles at 95°C for 1 min, 40°C for 1 min, and 72°C for 3 min; and 1 cycle at 72°C for 10 min.

Functional genes. The functional genes *cbbL* coding for RubisCO form I, *cbbM* for RubisCO form II, and *aprA* for APS reductase were examined. For the specific amplification of RubisCO form I and II genes the following primers were designed from available *cbbL* and *cbbM* sequences in the database: cbbLF (5'-CACCTGGACCACVGTBTGG-3') and cbbLR (5'-CGGTGYATGTGCAGCAGCAT5CCG-3') for the *cbbL* gene and cbbMF (5'-ATCATCAARCCSAARCTSGGYCTGCG-3') and cbbM1R (5'-GAGGTSACSGCRCCRTGRCCRGCMCGRTG-3') or cbbM2R (5'-GAGGTSACSGCRCCRTGRCC-3') for the *cbbM* gene. For the specific amplification of the *aprA* gene the primers aps1F and aps4R (Kuever, unpublished data) were used. Template DNA (0.5 µl) was added to the PCR mix (50 µl total volume). The following thermocycling conditions were applied: 1 cycle at 95°C for 3 min; 27 cycles at 95°C for 1 min, 48°C (for *cbbL* and *cbbM* genes) or 54°C (for *aprA* genes) for 1 min, and 72°C for 3 min; and 1 cycle at 72°C for 5 min. The PCR products from each individual were purified using the QIAquick

PCR Purification Kit (Qiagen, Hilden, Deutschland) and directly sequenced in both directions (~696 bp for *cbbL* genes or ~396 bp for *aprA* genes).

Cloning and sequencing. 16S rRNA PCR products from each individual were cloned separately using the TA Cloning Kit (Invitrogen, Breda, Netherlands) according to the manufacturer's protocol. For screening of 16S rRNA genes, clones were randomly picked and controlled for the correct insert size (~ 1500 bp) by PCR with the vector primers M13F and M13R. For template DNA, a small amount of cells from each clone colony was picked with a sterile toothpick and resuspended in 10 µl sterile water. After preheating of this suspension to 95°C for 5 min, template DNA was amplified by PCR as described above using a total volume of 30 µl. Clones with PCR products of the correct size were prepared using the Montage®Plasmid Miniprep$_{96}$ Kit (Millipore, Bedford, USA) and screened by partial sequencing of 300-500 bp using the 16S rRNA primer GM1F. Sequencing reactions were run using ABI BigDye on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA). Sequences were aligned and compared using the Bioedit Program (www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were grouped together in a clone family if they shared ≥ 99% sequence identity (% identical nucleotides). For each host individual, a representative clone from each clone family fully sequenced in both directions.

Phylogenetic analyses. The 16S rRNA, *cbbL,* and *aprA* sequences were checked against sequences in GenBank using BLAST (1) for similarity searches. The sequence data were analyzed using the ARB software package (www.arb-home.de).

Phylogenetic trees for 16S rRNA sequences were calculated using parsimony, distance, and maximum-likelihood analyses with different filter sets considering only sequences with a length of at least 1255 bp.

Phylogenetic trees for the RubisCO gene were generated from amino acid sequences using maximum-likelihood analyses with a 25% amino acid frequency

filter. The leading and tailing amino acids stretches of full length sequences were excluded from the analyses, eliminating those regions found in some but not all RubisCO sequences. The partial RubisCO form I sequences (*cbbL*, 211 amino acids) from this study were added to the maximum-likelihood tree using maximum parsimony. The phylogeny of the *aprA* gene was calculated from partial (131) amino acid sequences using maximum-likelihood analyses with a 25% amino acid frequency filter.

Fluorescence in situ hybridization (FISH). Six *I. leukodermatus* and three *I. makropetalos* specimens were prepared for FISH analyses of bacterial endosymbionts as described previously (2). The detection of the symbionts was performed by CARD (catalyzed reporter deposition) FISH with horseradish peroxidase (HRP) labelled probes and tyramide signal amplification as described by Schönhuber et al. (33). The tissue sections were hybridized with the HRP labelled probe for 3 h at 35°C. After washing for 15 minutes at 35°C in washing buffer (see (33)), the sections were equilibrated for 15 minutes at room temperature in PBS buffer (phosphate buffered saline, pH 7.3). The moist tissue sections were incubated with the amplification solution (1x phosphate buffered saline, pH 7.3; 0.0015% v/v H_2O_2; and 1% Alexa Fluor 488, 546 or 633 Dyes (Molecular Probes, Leiden, Netherlands)) for 10 minutes at 37°C in the dark, and rinsed in PBS buffer for 15 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 for < 1-2 days. For dual and triple hybridizations, the CARD FISH protocol was repeated 2-3 times on the same sections using different probes and Alexa dyes. For this purpose, after the last washing step the tissue sections were covered with 0.01 M HCl for 10 min at room temperature to inactivate the HRP. After washing for 3 min in sterile water, tissue sections were hybridized with another probe as just described.

The oligonucleotide probes designed in this study target 16S rRNA sequences isolated from *I. leukodermatus* and *I. makropetalos* (Table 1). The

probes were checked against sequences in GenBank using BLAST (1) and against small-subunit rRNA sequences in the Ribosomal Database Project using PROBE MATCH (28) and contained at least 1 mismatch to all entered sequences. The general Bacteria probe EUB338, the general Gammaproteobacteria probe GAM42a, and the general Alphaproteobacteria probe ALF968 were used as positive controls, and the antisense probe NON338 as a negative control. All hybridizations were performed at formamide concentrations ensuring high specificity (Table 1).

Table 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5`? 3`)	Position[a]	CARD-FISH [FA][c]	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	338-355	55	(2)
GAM42a	Gammaproteobacteria	GCCTTCCCACATCGTTT	1027-1043[b]	55	(27)
NON338	negative control	ACTCCTACGGGAGGCAGC	338-355	30	(42)
ALF968	Alphaproteobacteria, several members of the Deltaproteobacteria	GGTAAGGTTCTGCGCGTT	968-985	40	(29)
IleuGAM1	Gamma 1 sym. in *I. leukodermatus*	TCTGACTTATTCGGCCGCCTAC	581-602	40	(8)
InaGAM1	Gamma 1 sym. in *I. leukodermatus* and *I. makropetalos*	CGCTCCCGAAGGCACCTA	1020-1038	55	this study
IleuALF1a	Alpha 1a sym. in *I. leukodermatus*	CTCGGGTCCCCGCGACCG	999-1016	45	this study
IleuALF1b	Alpha 1b sym. in *I. leukodermatus*	CTCCCGGACTCGAGCACA	646-663	45	this study
IleuALF2	Alpha 2 sym. in *I. leukodermatus*	GAATGTCTCCACTCTCCG	1004-1021	45	this study
ImakALF1a	Alpha 1a sym. in *I. makropetalos*	TCCGGTCTCCGCGACCCC	998-1015	55	this study
ImakALF2	Alpha 2 sym. in *I. makropetalos*	ATCGCCACCCATTGTCAC	1244-1261	55	this study

[a] Position in the 16S rRNA of *Escherichia coli*
[b] Position in the 23S rRNA of *E. coli*
[c] Formamide concentrations in the hybridization buffer in % (v/v)

Nucleotide sequence accession numbers. The 16S rRNA sequences from *Inanidrilus leukodermatus* were submitted to GenBank under accession numbers AJ890100 (Gamma 1 symbiont), AJ890099 (Alpha 1a symbiont), AJ890098 (Alpha 1b symbiont), AJ890093 (Alpha 1c clone associated with *I. leukodermatus*), and AJ890097 (Alpha 2 symbiont), and from *Inanidrilus makropetalos* under accession numbers AJ890094 (Gamma 1 symbiont), AJ890095 (Alpha 1b symbiont), and AJ890096 (Alpha 2 symbiont). Functional sequences, isolated from *Inanidrilus leukodermatus,* were submitted to GenBank under accession numbers xxx (*aprA* gene) and xxx (*cbbL* gene) and from

Inanidrilus makropetalos under accession numbers xxx (*aprA* gene), and xxx (*cbbL* gene).

Results

Clone library analysis

Bacterial 16S rRNA sequences from six *I. leukodermatus* specimens were grouped into five distinct clone families with a total of 510 clones analyzed (Table 2). Within each clone family, sequence similarity was never less than 99.6%. Phylogenetic analyses (see below) revealed that in *I. leukodermatus* one clone family belongs to the Gammaproteobacteria (Gamma 1) and four to the Alphaproteobacteria (Alpha 1a, 1b, 1c, and 2). Sequences belonging to the Gamma 1 clone family were found in all individuals. For the Alpha 1a, Alpha 1b, and Alpha 2 clone families, at least three out of six *I. leukodermatus* individuals harbored sequences belonging to these groups. In contrast, sequences belonging to the Alpha 1c clone family were present in only a single individual.

Bacterial 16S rRNA sequences from the three *I. makropetalos* specimens (318 clones analyzed) were grouped into three distinct clone families, with at least 99.6% sequence similarity within each clone family (Table 2). Phylogenetic

Table 2. 16S rRNA clone libraries from six *I. leukodermatus* and three *I. makropetalos* individuals (No. of clones: number of clones analyzed, Gamma 1: Gammaproteobacteria, Alpha 1a, 1b, 1c, and Alpha 2: Alphaproteobacteria)

Worms	No. of clones	Gamma 1	Alpha 1a	Alpha 1b	Alpha 1c	Alpha 2
I. leu. 1	78	54 (69%)	16 (21%)	2 (3%)	6 (8%)	0 (0%)
I. leu. 2	99	57 (58%)	29 (29%)	0 (0%)	0 (0%)	13 (13%)
I. leu. 3	93	33 (36%)	3 (3%)	31 (33%)	0 (0%)	26 (28%)
I. leu. 4	93	78 (84%)	0 (0%)	5 (5%)	0 (0%)	10 (11%)
I. leu. 5	53	48 (91%)	0 (0%)	3 (6%)	0 (0%)	2 (4%)
I. leu. 6	94	79 (84%)	0 (0%)	15 (16%)	0 (0%)	0 (0%)
I. mak. 1	101	55 (56%)	2 (2%)	-	-	41 (42%)
I. mak. 2	114	95 (83%)	0 (0%)	-	-	19 (17%)
I. mak. 3	103	92 (89%)	3 (3%)	-	-	8 (8%)

analyses (see below) confirmed that in *I. makropetalos* one clone family belongs to the Gammaproteobacteria (Gamma) and two to the Alphaproteobacteria (Alpha 1a and 2). Sequences belonging to the Gamma 1 and Alpha 2 clone families were found in all three *I. makropetalos* individuals and from the Alpha 1a clone family in two out of the three specimens examined.

Phylogenetic analyses

16S rRNA gene. Parsimony, distance, and maximum-likelihood analyses of the 16S rRNA sequences from the five *I. leukodermatus* and three *I. makropetalos* clone families confirmed that these belong to one bacterial group of the Gammaproteobacteria and five phylogenetically distinct bacterial groups of the Alphaproteobacteria. The five *I. leukodermatus* and three *I. makropetalos* sequences are unique to these hosts and differ from those of symbionts from other host species or free-living bacteria.

Gammaproteobacterial symbionts. The 16S rRNA sequence of the *I. leukodermatus* Gamma 1 symbiont from this study differed from the *I. leukodermatus* Gamma sequence described in an earlier study (8) by 3 nucleotides (positions 82, 861, 862 based on *E. coli* numbering). All 3 nucleotides are in stem regions and led to miss-pairings in the earlier study. Furthermore, these sites are conserved in all Gamma I symbionts and the nucleotides in this study are in consensus with all other Gamma 1 symbionts, indicating that these 3 substitutions were caused by PCR or sequencing error in the earlier study (8).

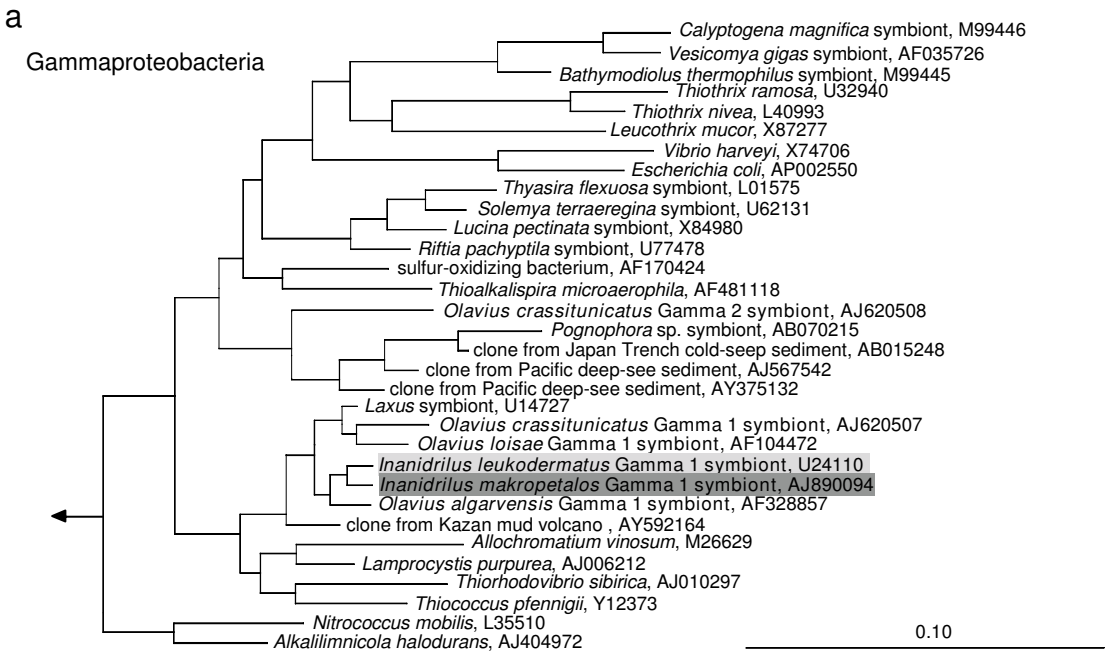
In all phylogenetic analyses, the *I. makropetalos* and the *I. leukodermatus* Gamma 1 sequences consistently fall together (98.5% sequence similarity) (Fig. 2a). The *Inanidrilus* Gamma 1 sequences fall in the same cluster as the Gamma 1 symbionts from *Olavius* hosts and the ectosymbiont of the marine nematode *Laxus* sp. (≥ 95.7% sequence similarity) (Fig. 2a). These symbiotic sequences, together with a clone sequence isolated from the Kazan mud volcano

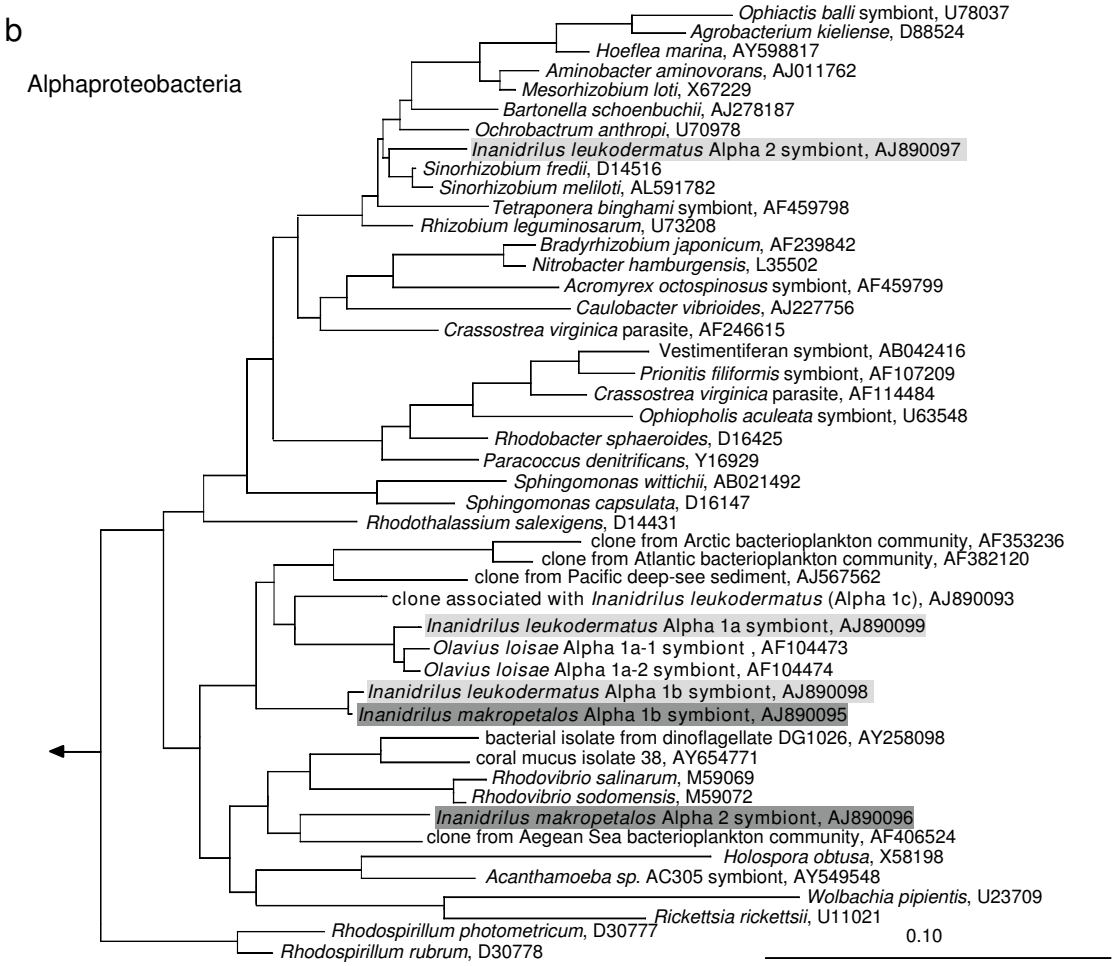
in the eastern Mediterranean, are most closely related to a clade of free-living, phototrophic, sulfur-oxidizing bacteria from the family Chromatiaceae.

Alphaproteobacterial symbionts.The alphaproteobacterial sequences from *I. leukodermatus* and *I. makropetalos* (Alpha 1a and 2), belong to five phylogenetically distinct bacterial groups, Alpha 1a, 1b, 1c, and two Alpha 2 lineages (Fig. 2b). The Alpha 1a-c sequences from both species are clustered (≥ 92% sequence similarity) whereas the Alpha 2 sequences are phylogenetically separate from the Alpha 1 sequences (≥ 88.3% sequence similarity) and from each other.

Within the Alpha 1 group, *I. leukodermatus* contained sequences belonging to all three subgroups Alpha 1a, 1b, and 1c, while in *I. makropetalos* only sequences belonging to the Alpha 1b group were found. The Alpha 1a sequence from *I. leukodermatus* is most closely related to the Alpha1a endosymbionts of the gutless oligochaete *Olavius loisae* from the Australian Great Barrier Reef in all treeing methods (≥ 97.78% sequence similarity). The closest relatives of these sequences are the Alpha 1c sequence from *I. leukodermatus* (≥ 92% sequence similarity) and a cluster of clone sequences isolated from Atlantic and Arctic bacterioplankton communities and from Pacific deep-see sediments (≥ 97.2% sequence similarity). The Alpha 1b *I. leukodermatus* and *I. makropetalos* sequences consistently grouped together in all 3 phylogenetic analyses (95.5% sequence similarity). These two sequences are distantly related to the Alpha 1a clade.

Fig. 2. Phylogenetic placement of bacterial symbionts in *I. leukodermatus* and *I. makropetalos* based on 16S rRNA sequences. Maximum likelihood trees of members of the (a) Gammaproteobacteria and (b) Alphaproteobacteria. Symbionts of gutless oligochaetes are listed in bold type, with the *I. leukodermatus* symbionts boxed in bright gray and *I. makropetalos* symbionts in dark gray. The bars represent 10% estimated sequence divergence.


a
Gammaproteobacteria
Calyptogena magnifica symbiont, M99446
Vesicomya gigas symbiont, AF035726
Bathymodiolus thermophilus symbiont, M99445
Thiothrix ramosa, U32940
Thiothrix nivea, L40993
Leucothrix mucor, X87277
Vibrio harveyi, X74706
Escherichia coli, AP002550
Thyasira flexuosa symbiont, L01575
Solemya terraeregina symbiont, U62131
Lucina pectinata symbiont, X84980
Riftia pachyptila symbiont, U77478
sulfur-oxidizing bacterium, AF170424
Thioalkalispira microaerophila, AF481118
Olavius crassitunicatus Gamma 2 symbiont, AJ620508
Pognophora sp. symbiont, AB070215
clone from Japan Trench cold-seep sediment, AB015248
clone from Pacific deep-see sediment, AJ567542
clone from Pacific deep-see sediment, AY375132
Laxus symbiont, U14727
Olavius crassitunicatus Gamma 1 symbiont, AJ620507
Olavius loisae Gamma 1 symbiont, AF104472
Inanidrilus leukodermatus Gamma 1 symbiont, U24110
Inanidrilus makropetalos Gamma 1 symbiont, AJ890094
Olavius algarvensis Gamma 1 symbiont, AF328857
clone from Kazan mud volcano , AY592164
Allochromatium vinosum, M26629
Lamprocystis purpurea, AJ006212
Thiorhodovibrio sibirica, AJ010297
Thiococcus pfennigii, Y12373
Nitrococcus mobilis, L35510
Alkalilimnicola halodurans, AJ404972
0.10


b
Alphaproteobacteria
Ophiactis balli symbiont, U78037
Agrobacterium kieliense, D88524
Hoeflea marina, AY598817
Aminobacter aminovorans, AJ011762
Mesorhizobium loti, X67229
Bartonella schoenbuchii, AJ278187
Ochrobactrum anthropi, U70978
Inanidrilus leukodermatus Alpha 2 symbiont, AJ890097
Sinorhizobium fredii, D14516
Sinorhizobium meliloti, AL591782
Tetraponera binghami symbiont, AF459798
Rhizobium leguminosarum, U73208
Bradyrhizobium japonicum, AF239842
Nitrobacter hamburgensis, L35502
Acromyrex octospinosus symbiont, AF459799
Caulobacter vibrioides, AJ227756
Crassostrea virginica parasite, AF246615
Vestimentiferan symbiont, AB042416
Prionitis filiformis symbiont, AF107209
Crassostrea virginica parasite, AF114484
Ophiopholis aculeata symbiont, U63548
Rhodobacter sphaeroides, D16425
Paracoccus denitrificans, Y16929
Sphingomonas wittichii, AB021492
Sphingomonas capsulata, D16147
Rhodothalassium salexigens, D14431
clone from Arctic bacterioplankton community, AF353236
clone from Atlantic bacterioplankton community, AF382120
clone from Pacific deep-see sediment, AJ567562
clone associated with Inanidrilus leukodermatus (Alpha 1c), AJ890093
Inanidrilus leukodermatus Alpha 1a symbiont, AJ890099
Olavius loisae Alpha 1a-1 symbiont , AF104473
Olavius loisae Alpha 1a-2 symbiont, AF104474
Inanidrilus leukodermatus Alpha 1b symbiont, AJ890098
Inanidrilus makropetalos Alpha 1b symbiont, AJ890095
bacterial isolate from dinoflagellate DG1026, AY258098
coral mucus isolate 38, AY654771
Rhodovibrio salinarum, M59069
Rhodovibrio sodomensis, M59072
Inanidrilus makropetalos Alpha 2 symbiont, AJ890096
clone from Aegean Sea bacterioplankton community, AF406524
Holospora obtusa, X58198
Acanthamoeba sp. AC305 symbiont, AY549548
Wolbachia pipientis, U23709
Rickettsia rickettsii, U11021
Rhodospirillum photometricum, D30777
Rhodospirillum rubrum, D30778
0.10

Both host species contained Alpha 2 sequences that were phylogenetically distinct from each other. In *I. leukodermatus* the relationship of the Alpha 2 sequence to other 16S rRNA sequences varied with the treeing method used. In maximum likelihood analyses the closest relatives were the nitrogen-fixing symbionts, *Sinorhizobium meliloti* and *S. fredii* (≥ 95.7% sequence similarity), while parsimony and distance analyses placed the Alpha 2 sequence within a clade of bacteria belonging to the genera *Mesorhizobium*, *Aminobacter*, *Hoeflea*, and *Agrobacterium*, and symbionts from the marine brittle star *Ophiactis balli* and the ant *Tetraponera binghami*.

The phylogeny of the Alpha 2 sequence from *I. makropetalos* was similar in maximum likelihood and parsimony analyses, with a clone sequence from an Aegean Sea bacterioplankton community as the closest relative (91.1% sequence similarity). These two sequences are most closely related to the free leaving, halophilic bacteria *Rhodovibrio salinarum* and *R. sodomensis* (≥ 89.31% sequence similarity) and bacteria isolated from a dinoflagellate and coral mucus (≥ 90.2% sequence similarity). In distance analyses the *I. makropetalos* Alpha 2 sequence does not cluster with the Aegean Sea clone sequence, but otherwise the relationships to the next relatives are similar to those shown in Fig. 2b.

Functional genes. The *cbbL* gene of RubisCO form I was amplified from all six *I. leukodermatus* and three *I. makropetalos* individuals. The sequences from each host species were identical between individuals. The *cbbM* gene of from II RubisCO was not detectable in either species despite multiple PCR assays under varying conditions. Comparative phylogenetic analyses showed that the *I. leukodermatus* and *I. makropetalos cbbL* sequences are closely related to each other (99.5% amino acid sequence identity) (Fig. 3a). The closest relatives to these sequences are RubisCO form IA sequences from gammaproteobacterial sulfur-oxidizing endosymbionts of the snail *Alvinoconcha hessleri*, the clam *Solemya velum*, and the free-living sulfur-oxidizing bacterium *Allochromatium vinosum* (*cbbL*-2) (≥89% amino acid sequence identity). *A. vinosum* has two copies of *cbbL* sequences of which only *cbbL*-2 is expressed (38).

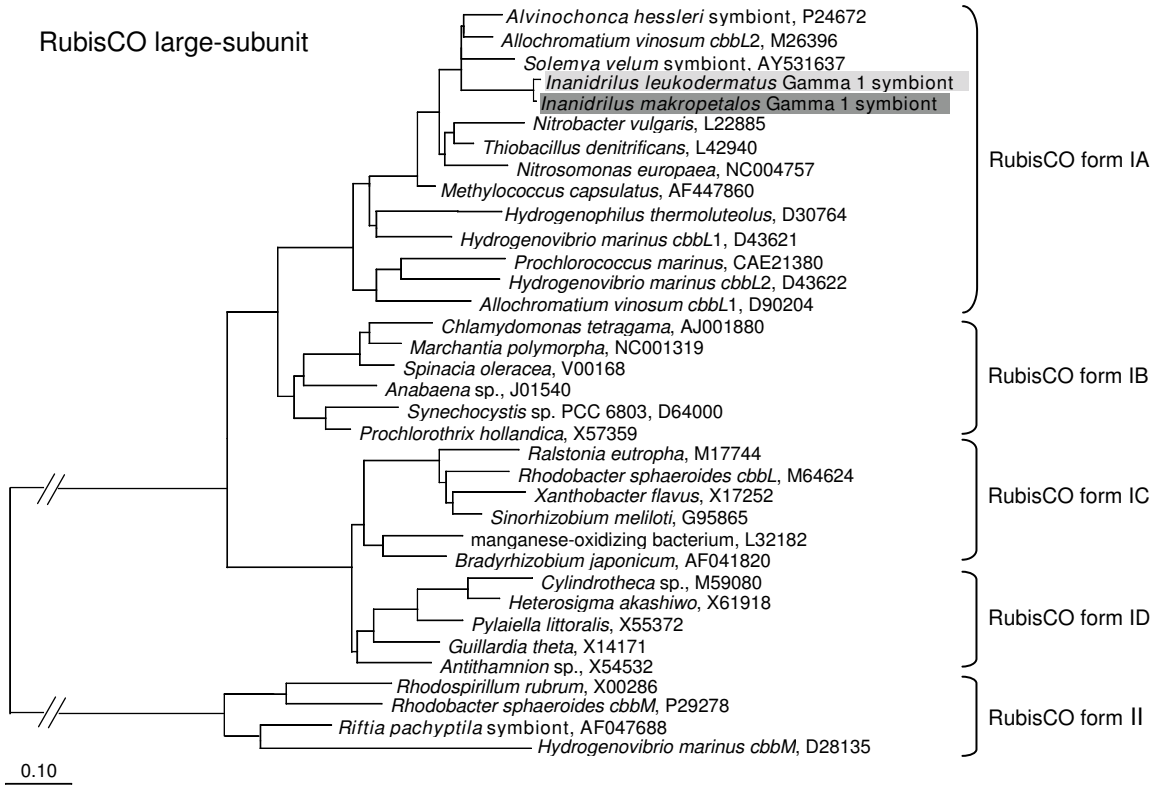
The *aprA* gene, coding for the alpha subunit of APS reductase, was found in all six *I. leukodermatus* and three *I. makropetalos* specimens and showed no sequence variation within each host species. The *I. leukodermatus* and *I. makropetalos aprA* sequences are closely related to each other (99.2% amino acid sequence identity) (Fig. 3b). In the absence of *aprA* sequences from other symbiotic sulfur-oxidizing bacteria, the closest relatives to the two *Inanidrilus* sequences are *aprA* sequences from free-living sulfur-oxidizing bacteria such as the betaproteobacterium *Thiobacillus denitrificans* (86.6% amino acid sequence identity) and *Chlorobium tepidum* of the green sulfur bacteria phylum (60% amino acid sequence identity).

In situ identification

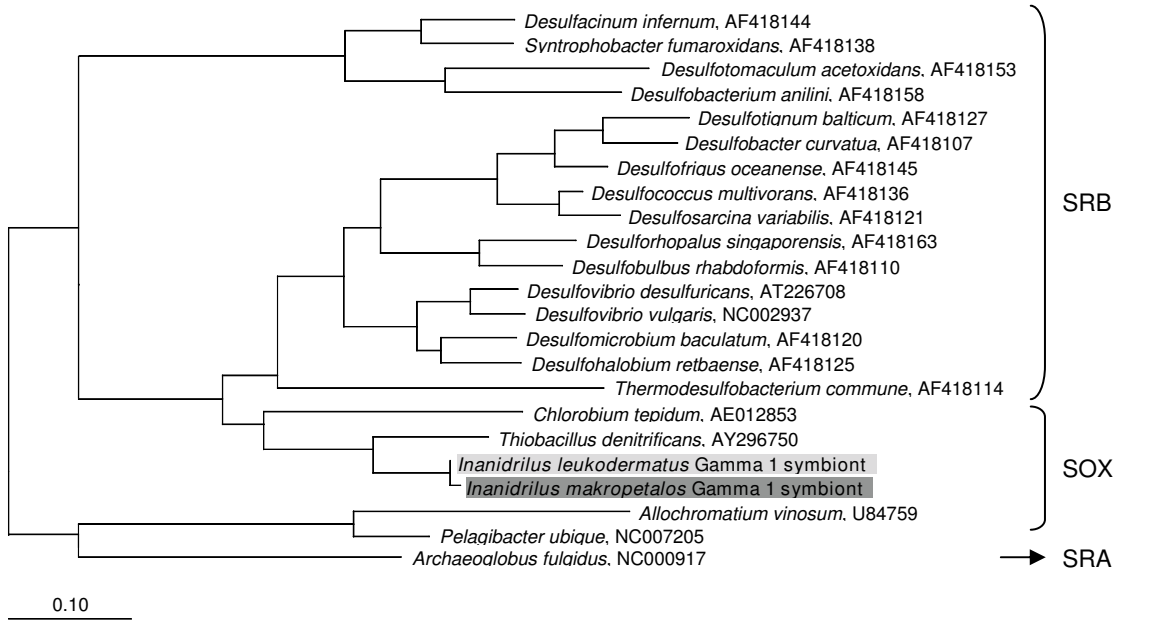
Fluorescence in situ hybridization (FISH) with oligonucleotide probes confirmed that four of the five 16S rRNA phylotypes isolated from *I. leukodermatus* and all three phylotypes isolated from *I. makropetalos* originated from bacteria in the symbiont-containing region between the cuticle and the epidermis of the worm (Fig. 4a, c). In *I. leukodermatus,* the gammaproteobacterial symbiont (Gamma 1) and three alphaproteobacterial symbionts (Alpha 1a, Alpha 1b, and Alpha 2) co-occurred in all six specimens. The Alpha 1c 16S rRNA sequence found in a single *I. leukodermatus* specimen, was not observed in any of the six examined worms despite the design of six specific probes and multiple in situ hybridizations under different conditions. This indicates that the Alpha 1c sequence originated from a contaminant or from a symbiont that is very rare. In *I. makropetalos*, all three bacterial phylotypes, the gammaproteobacterial symbiont (Gamma1) and the two alphaproteobacterial symbionts (Alpha 1a and Alpha 2) were observed to coexist in all examined host specimens.

The hybridization pattern of the Gamma 1 symbionts in the symbiont-containing region of *I. leukodermatus* and *I. makropetalos* was similar (Fig. 4a and c). In both species, the general probe for Gammaproteobacteria, GAM42a, and the specific probe InaGAM for the Gamma 1 sequences in these worms hybridized to large, oval bacteria throughout the symbiont-containing region (Fig. 4a and c), described as the large morphotype in *I. leukodermatus* (7, 21). The

a


RubisCO large-subunit
Alvinochonca hessleri symbiont, P24672
Allochromatium vinosum cbbL2, M26396
Solemya velum symbiont, AY531637
Inanidrilus leukodermatus Gamma 1 symbiont
Inanidrilus makropetalos Gamma 1 symbiont
Nitrobacter vulgaris, L22885
Thiobacillus denitrificans, L42940
Nitrosomonas europaea, NC004757
Methylococcus capsulatus, AF447860
Hydrogenophilus thermoluteolus, D30764
Hydrogenovibrio marinus cbbL1, D43621
Prochlorococcus marinus, CAE21380
Hydrogenovibrio marinus cbbL2, D43622
Allochromatium vinosum cbbL1, D90204
Chlamydomonas tetragama, AJ001880
Marchantia polymorpha, NC001319
Spinacia oleracea, V00168
Anabaena sp., J01540
Synechocystis sp. PCC 6803, D64000
Prochlorothrix hollandica, X57359
Ralstonia eutropha, M17744
Rhodobacter sphaeroides cbbL, M64624
Xanthobacter flavus, X17252
Sinorhizobium meliloti, G95865
manganese-oxidizing bacterium, L32182
Bradyrhizobium japonicum, AF041820
Cylindrotheca sp., M59080
Heterosigma akashiwo, X61918
Pylaiella littoralis, X55372
Guillardia theta, X14171
Antithamnion sp., X54532
Rhodospirillum rubrum, X00286
Rhodobacter sphaeroides cbbM, P29278
Riftia pachyptila symbiont, AF047688
Hydrogenovibrio marinus cbbM, D28135
RubisCO form IA
RubisCO form IB
RubisCO form IC
RubisCO form ID
RubisCO form II
0.10

b


APS alpha-subunit reductase
Desulfacinum infernum, AF418144
Syntrophobacter fumaroxidans, AF418138
Desulfotomaculum acetoxidans, AF418153
Desulfobacterium anilini, AF418158
Desulfotignum balticum, AF418127
Desulfobacter curvatus, AF418107
Desulfofrigus oceanense, AF418145
Desulfococcus multivorans, AF418136
Desulfosarcina variabilis, AF418121
Desulforhopalus singaporensis, AF418163
Desulfobulbus rhabdoformis, AF418110
Desulfovibrio desulfuricans, AT226708
Desulfovibrio vulgaris, NC002937
Desulfomicrobium baculatum, AF418120
Desulfohalobium retbaense, AF418125
Thermodesulfobacterium commune, AF418114
Chlorobium tepidum, AE012853
Thiobacillus denitrificans, AY296750
Inanidrilus leukodermatus Gamma 1 symbiont
Inanidrilus makropetalos Gamma 1 symbiont
Allochromatium vinosum, U84759
Pelagibacter ubique, NC007205
Archaeoglobus fulgidus, NC000917
SRB
SOX
SRA
0.10

specific probe IleuGAM for the Gamma 1 symbionts in *I. leukodermatus* (7, 21) detected these symbionts in *I. leukodermatus* but not in *I. makropetalos*.

The hybridization signal of the general probe for Alphaproteobacteria, ALF968, was limited to small bacterial cells distributed throughout the entire symbiont region of *I. leukodermatus* and *I. makropetalos*. The hybridiziation patterns of the probes specific to the Alpha symbionts of *I. leukodermatus*, Alpha1a, 1b, and 2 (Fig. 4b), and of *I. makropetalos*, Alpha1b and 2 (Fig. 4d), corresponded to those from the general alphaproteobacterial probe ALF968, indicating that all alphaproteobacterial symbionts in the worms were identified with the specific probes.

While the distribution of the Gamma 1 symbionts in the symbiont-containing region was similar in the two host species, the distribution of the Alpha symbionts was different. The two Alpha symbionts of *I. makropetalos,* Alpha 1b and 2, were observed in all cross sections of the three individuals. In contrast, the distribution of three Alpha symbionts of *I. leukodermatus*, Alpha1a, 1b, and 2, varied between individuals and within an individual, with some cross sections showing the coexistence of all 3 symbionts and some showing only 1 or 2 of the Alpha symbionts. These differences appeared to be random, with no obvious patterns of coexistence between the 3 Alpha symbionts observed.

Discussion

As in all other gutless oligochaetes examined to date, *I. leukodermatus* and *I. makropetalos* harbor large, oval shaped bacteria identified as Gamma 1 symbionts (Fig. 2a and 4). These symbionts are more closely related to each other than to the Gamma 1 symbionts from hosts belonging to the genus *Olavius*.

Figure 3. Phylogenetic placement of symbiotic RubisCO large-subunit (a) and APS alpha-subunit reductase (b) sequences from *I. leukodermatus* and *I. makropetalos* based on maximum likelihood analyses. The sequence divergence, showed between the RubisCO form I and II members, do not correspond to the calculated divergence. The bars represent 10% estimated sequence divergence, except for the estimated divergence between RubisCO form I and II sequences, which corresponds to 124%.

The close phylogenetic relationship of the *Inanidrilus* Gamma 1 symbionts could originate from the close relationship of their hosts, that is have been caused by cospeciation. Alternatively, their biogeography may have played a role as the two host species are geographically closest to each other (both Northwest Atlantic) than to the *Olavius* species from the Mediterranean and the Pacific Oceans. Additional studies on Gamma 1 symbionts from both *Inanidrilus* and *Olavius* host species are currently in progress to better understand the role of biogeography and cospeciation in these associations.

The Gamma 1 symbionts of gutless oligochaetes have been identified as chemoautotrophic sulfur oxidizers based on their close phylogenetic relationship to free-living sulfur oxidizers, the presence of sulfur in their cells, and immunohistochemical studies showing the presence of form I RubisCO in these symbionts (8, 24). This study provides further evidence that these symbionts are thiotrophic based on the presence of the *cbbL* gene of form I RubisCO and the *aprA* gene of APS reductase. The close relationship of the *I. leukodermatus* and *I. makropetalos cbbL* and *aprA* genes to those of free-living and symbiotic sulfur oxidizers indicates that the oligochaete sequences originated from their sulfur-oxidizing Gamma 1 symbionts. An alternative explanation, that some or all of the alphaproteobacterial symbionts of these hosts are sulfur-oxidizing chemoautotrophs and that they are the source of these functional genes is unlikely. The oligochaete Alpha symbionts are not related to known alphaproteobacterial sulfur oxidizers and in *I. leukodermatus* it has been shown that these small bacteria do not contain sulfur and are not labeled by a form I RubisCO antiserum (24).

Previous to this study, alphaproteobacterial symbionts had only been found in a single gutless oligochaete species, *O. loisae* from Australian Great Barrier Reef (5). The presence of these symbionts in two species from the host genus *Inanidrilus* that are geographically very distant from *O. loisae* indicates that symbioses with alphaproteobacterial symbionts may be more widespread in gutless oligochaetes than previously assumed. The Alpha 1 symbionts occur in all three host species with alphaproteobacterial symbionts and are relatively

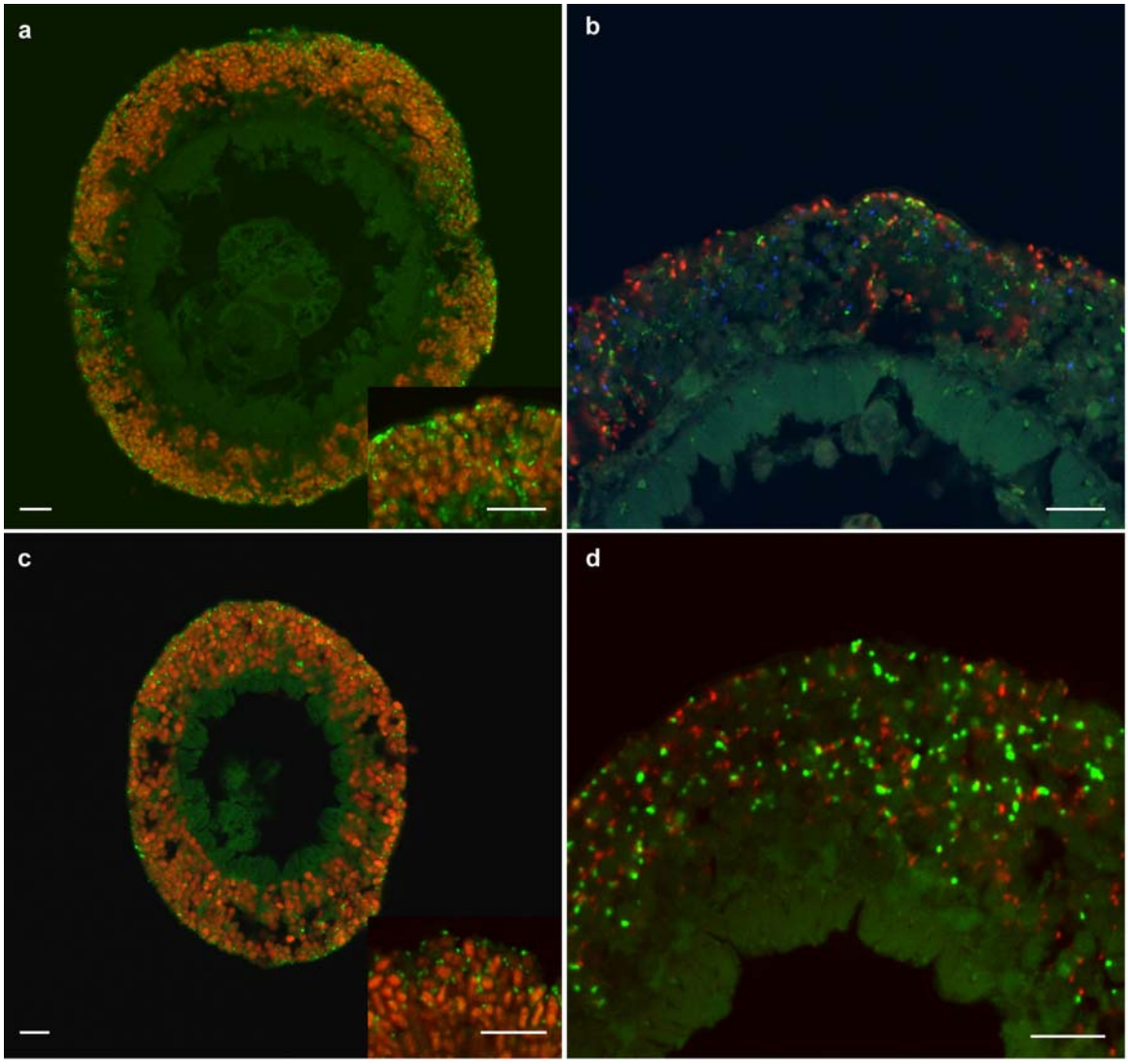


Figure 4. In situ identification of bacterial symbionts in *I. leukodermatus* (a and b) and *I. makropetalos* (c and d). Epifluorescence images show a cross-section through the entire worm and the symbiont-containing region of the worm's body wall (all scale bars, 10 µm). a) Dual hybridization with InaGAM and IleuAlpha1a/IleuAlpha1b/IleuAlpha2 probes, showing gammaproteobacterial symbionts in red and alphaproteobacterial symbionts in green. Inset shows symbionts at higher magnification. b) Triple hybridization with Alpha1a, Alpha1b, and Alpha2 probes, showing the Alpha 1a symbionts in green, Alpha 1b symbionts in green, and Alpha 2 symbionts in blue. c) Dual hybridization with InaGAM and ImakAlpha1a/ ImakAlpha2 probes, showing gammaproteobacterial symbionts in red and alphaproteobacterial symbionts in green. Inset shows symbionts at higher magnification. d) Dual hybridization with ImakAlpha1a and ImakAlpha2 probes, showing the Alpha 1a symbionts in green and Alpha 2 symbionts in red.

closely related to each other (≥ 92.6% sequence similarity). Within this clade, different phylotypes can co-occur in the same species such as Alpha 1a and 1b symbionts in *O. loisae* and *I. leukodermatus*. In addition to the Alpha 1 lineage of symbionts, *I. leukodermatus* and *I. makropetalos* harbor additional alphaproteobacterial symbionts, Alpha 2 symbionts, that are phylogenetically distinct from each other and the Alpha 1 symbionts.

The metabolism of the alphaproteobacterial symbionts is not currently known. The closest free-living relatives of the Alpha 1 symbionts of both host species and the Alpha 2 symbionts of *I. makropetalos* are the halophilic bacteria *Rhodovibrio salinarum* and *R. sodomensis* (≥ 89.2% sequence similarity). Both species are photoheterotroph under anoxic conditions, while only *R. salinarum* can also grow in the dark under aerobic conditions as a chemoheterotroph (25, 27). Tests for photoautotrophic growth with reduced sulfur compounds were negative for both species (25, 27). The common feature of these bacteria is their use of fermentative products such as lactate, acetate, succinate, malate or pyruvate as an electron and carbon source during anaerobic growth (25, 27). These metabolites are produced by marine invertebrates when oxygen concentrations become limiting and are excreted in animals without symbionts. In gutless oligochaetes with sulfate-reducing deltaproteobacterial symbionts, it was suggested that these might take up the anaerobic waste products of their hosts, thus recycling these valuable carbon compounds (8, 30). It is intriguing that in all gutless oligochaetes, either alpha- or deltaproteobacterial symbionts coexist with the Gamma 1 symbionts, and it is tempting to speculate that the alphaproteobacterial symbionts might play a similar role as the deltaproteobacterial symbionts by recycling the anaerobic waste products of the worms.

The Alpha 2 symbiont from *I. leukodermatus* is most closely related to nitrogen-fixing *Sinorhizobium* symbionts of leguminous plants (≥ 95.7% sequence similarity), suggesting that the Alpha 2 symbionts might also fix N_2. However, numerous attempts to amplify the *nifH* gene, involved in bactarial N_2-fixation, were unsuccessful (N. Dubilier and J. Zehr, unpublished data). Since this

symbiont occurs only in *I. leukodermatus*, it does not appear to be essential for nitrogen uptake, as other oligochaete hosts are clearly able to acquire nitrogen without this Alpha 2 symbiont.

The association between the multiple symbiotic bacteria and the gutless oligochaetes *I. leukodermatus* and *I. makropetalos* is highly specific and stable within each host species. FISH analyses showed that the symbionts occur regularly in all examined individuals. In addition, the 16S rRNA sequences of the Gamma 1 symbiont from *I. leukodermatus* worms collected in 1992 (7) and in 1998 (this study) are identical (with the exception of 3 nucleotide substitutions caused by sequencing error), indicating a high specificity and evolutionary stability of this symbiont over this period of time. A high phylogenetic diversity of symbiotic bacteria was first observed in the gutless oligochaete *O. crassitunicatus* that harbors up to six bacterial phylotypes (2). This study shows that the stable coexistence of multiple endosymbionts is not limited to a single species but rather appears to be a common feature in oligochaete symbioses.

Acknowledgments

We are grateful to Jens Krieger for collecting worms and to Christina Zaluski and Silke Wetzel for technical assistance. In Bermuda, Wolfgang Sterrer, the Director of the Bermuda Aquarium, Museum, and Zoo graciously provided logistical support for collecting worms, and the Bermuda Biological Station of Research provided laboratory space. In Bahamas, we thank the Caribbean Marine Research Center for support and lodging while collection worms. The Max Planck Society, Munich, Germany provided financial support for which we are grateful. This study is contribution No. xxx of the Bermuda Aquarium, Museum, and Zoo and No. xxx of the Caribbean Marine Research Center.

References

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

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

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

4. Cavanaugh, C. M., and J. J. Robinson. 1996. CO_2 fixation in chemoautotrophic-invertebrate symbioses: expression of form I and form II RubisCO. *In* M. E. a. T. Lidstrom, F. R. (ed.), Microbiol growth on C_1 compounds: Proceedings of the 8th International Symposium on Microbial Growth on C_1 Compounds. Kluwer Academic Publishers, Dordrecht.

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

6. Dubilier, N., A. Blazejak, and C. Rühland. In press. Symbioses between bacteria and gutless marine oligochaetes. *In* J. Overmann (ed.), In Molecular Basis of Symbiosis. Springer-Verlag, New York.

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

8. Dubilier, N., C. Mülders, T. Ferdelman, D. de Beer, A. Pernthaler, M. Klein, M. Wagner, C. Erséus, F. Thiermann, J. Krieger, O. Giere, and R. Amann. 2001. Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. Nature 411:298-302.

9. Erséus, C. 1992. A generic revision of the Phallodrilinae (Oligochaeta, Tubificidae). Zool. Scr. 21:5-48.

10. Erséus, C. 2003. The gutless Tubificidae (Annelida, Oligochaeta) of the Bahamas. Meiofauna Marina 12:59-84.

11. Erséus, C. 1990. The marine Tubificidae (Oliogchaeta) of the barrier reef ecosystems at Carrie Bow Cay, Belize, and other parts of the Caribbean Sea, with descriptions of twenty-seven new species and revision of *Heterodrilus*,*Thalassodrilides* and *Smithsonidrilus*. Zool. Scr. 19:243-303.

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

13. Erséus, C. 1991. Two new deep-water species of the gutless genus *Olavius* (Oligochaeta: Tubificidae) from both sides of North America. Proc. Biol. Soc. Wash. 104:627-630.

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

15. Finogenova, N. 1986. Six new species of marine Tubificidae (Oligochaeta) from the continental shelf off Peru. Zool. Scr. 15:45-51.

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

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

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

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

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

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

22. Giovannoni, S. J., H. J. Tripp, S. Givan, M. Podar, K. L. Vergin, D. Baptista, L. Bibbs, J. Eads, T. H. Richardson, M. Noordewier, M. S. Rappe, J. M. Short, J. C. Carrington, and E. J. Mathur. 2005. Genome streamlining in a cosmopolitan oceanic bacterium. Science 309:1242-1245.

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

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

25. Mack, E. E., L. Mandelco, C. R. Woese, and M. T. Madigan. 1993. *Rhodospirillum sodomense*, sp. nov., a Dead Sea *Rhodospirillum* species. Arch. Microbiol. 160:363-371.

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

27. Nissen, H., and I. D. Dundas. 1984. *Rhodospirillum salinarum* sp. nov., a halophilic photosynthetic bacterium isolated from Portuguese saltern. Arch. Microbiol. 138:251-256.

28. Olsen, G. J., R. Overbeck, N. Larson, T. L. Marsh, M. J. McCaughey, M. A. Maciukenas, W. M. Kuan, T. J. Macke, Y. Xing, and C. R. Woese. 1992. Ribosomal Database Project. Nucleic Acids Res. 20:2199-2200.

29. Robinson, J. J., and C. M. Cavanaugh. 1995. Expression of form I and form II RubisCO in chemoautotrophic symbioses: implications for the interpretation of stable carbon isotope values. Limnol. Oceanogr. 40:1496-1502.

30. Rühland, C., A. Blazejak, A. Loy, M. Wagner, C. Erséus, R. Amann, and N. Dubilier. In prep. Multiple symbioses with sulfide-oxidizers, sulfate-reducers, and spirochetes in two co-occurring species of gutless worms (Oligochaeta) from the Mediterranean. In preparation .

31. Sánchez, O., I. Ferrera, C. Dahl, and M. J. 2001. *In vivo* role of APS reductase in the purple sulfur bacterium *Allochromatium vinosum*. Arch. Microbiol. 176:301-305.

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

33. Schönhuber, W., B. Zarda, S. Eix, R. Rippka, M. Herdman, W. Ludwig, and R. Amann. 1999. In situ identification of cyanobacteria with horseradish peroxidase-labeled, rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. 65:1259-1267.

34. Schwedock, J., T. L. Harmer, K. M. Scott, H. J. Hektor, A. P. Seitz, M. C. Fontana, D. L. Distel, and C. M. Cavanaugh. 2004. Characterization and expression of genes from the RubisCO gene cluster of the chemoautotrophic symbiont of *Solemya velum*: *cbbLSQO*. Arch. Microbiol. 182:18-29.

35. Stein, J. L., M. Haygood, and H. Felbeck. 1990. Nucleotide sequence and expression of a deep-sea ribulose-1,5-bisphosphate carboxylase gene cloned from a chemoautotrophic bacterial endosymbiont. Proc. Natl. Acad. Sci. 87:8850-8854.

36. Tabita, F. R. 1995. The biochemistry and metabolic regulation of carbon metabolism and CO_2 fixation in purple bacteria. *In* M. M. T. Blankenship R. E., Bauer C. E. (ed.), Anoxygenic photosynthetic bacteria. Kluwer, Dordrecht.

37. Tabita, F. R. 1988. Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol. Rev. 52:155-189.

38. Viale, A. M., H. Kobayashi, and T. Akazawa. 1989. Expressed genes for plant-type ribulose 1,5-bisphosphate carboxylase/oxygenase in the photosynthetic bacterium *Chromatium vinosum*, which possesses two complete sets of the genes. J. Bacteriol. 171:2391-2400.

3

Metagenomic analysis of co-occurring symbionts in marine worms from the Mediterranean

Anna Blazejak, Michael Richter, Zhanyou Xu, Amelia E. Rotaru, Hong-Bin Zhang, Michael Kube, Rudolf Amann, Frank Oliver Glöckner, and Nicole Dubilier

Manuscript in preparation

Metagenomic analysis of co-occurring symbionts in marine worms from the Mediterranean

Anna Blazejak[1], Michael Richter[1], Zhanyou Xu[2], Amelia E. Rotaru[1], Hong-Bin Zhang[2], Michael Kube[3], Rudolf Amann[1], Frank Oliver Glöckner[1], and Nicole Dubilier[1*]

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

[2] Department of Soil and Crop Sciences and Crop Biotechnology Center, Texas A&M University, Texas, USA

[3] Max Planck Institute for Molecular Genetics, Ihnestraße 63/73, 14195 Berlin, Germany

Key words: symbiosis, *Olavius,* sulfide-oxidizing bacteria, Dsr, RubisCO, Oligochaeta

* Corresponding Author: Nicole Dubilier. Mailing Address: Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany. Phone: +49-(0)421-2028932. E-mail: ndubilie@mpi-bremen.de

Abstract

Gutless marine oligochaetes have completely reduced their digestive and excretory systems and live in obligate association with multiple bacterial symbionts. In comparison to the well characterized phylogenetic diversity of the symbiotic bacteria, the study of their metabolic diversity is limited by the fact that these bacteria are yet uncultivable. The use of comparative metagenomics gave insight into the metabolic diversity of endosymbionts in the two co-occurring gutless oligochaetes, *Olavius algarvensis* and *O. ilvae* from the Mediterranean. The constructed BAC library, prepared with genomic DNA from pooled worms, was dominated by sequences with high similarity to bacterial genes, indicating that these originated from the oligochaete symbionts. Known functional genes such as *aprA* involved in sulfur metabolism and *cbbL* in CO_2 fixation were identified by screening of the BAC library, and assigned to symbionts of either *O. algarvensis* or *O. ilvae* using single worm analysis. Complete sequencing of a BAC clone revealed gene clusters involved in intracellular sulfur oxidation and CO_2 fixation via the Calvin-Benson-Bassham (CBB) cycle. Genes involved in carbon and nitrogen pathways not yet known to play a role in the oligochaete symbionts were obtained by random end-sequencing of BAC inserts.

Introduction

Gutless oligochaetes are small marine worms of 0.2 mm diameter and 1-2 cm length that occur world wide in coastal sediments (9, 15, 18). They are unique in having established highly specific and stable associations with multiple endosymbiotic bacteria located extracellulary in a thick layer just below the cuticle between extensions of epidermal cells. This symbiosis is clearly obligate for the host given their complete reduction of a digestive and excretory system. The hosts depend on their symbiotic bacteria for their nutrition, either by taking up organic compounds provided by the symbionts or by directly digesting the symbionts.

The symbiotic community of gutless oligochaetes is well described based on culture independent methods such as comparative 16S rRNA and fluorescence

in situ hybridisation (FISH) analysis (6, 7, 17, 19, 20, 57). All worm species examined to date harbor chemoautotrophic sulfur-oxidizing symbionts belonging to the Gammaproteobacteria. Evidence for their thioautotrophic metabolism is provided by (i) the close relationship of these symbionts to free-living chemoautotrophic bacteria based on comparative 16S rRNA analysis (6, 16, 17, 19, 57), (ii) the occurrence of sulfur in the bacterial cytoplasm (20, 28), and (iii) the presence of key enzymes characteristic for CO_2 fixation and sulfur oxidation (22, 28). In addition to these thioautotrophic symbionts, up to five other bacterial phylotypes belonging to the Alpha-, Delta-, and Gammaproteobacteria, or the Spirochaeta, can coexist in the same host.

In gutless oligochaetes it has been shown that deltaproteobacterial symbionts are sulfate-reducing bacteria (6, 20, 57). The coexistence of both sulfate-reducing and sulfide-oxidizing symbionts in the same host suggests that these are engaged in a syntrophic sulfur cycle. The sulfate reducers produce reduced sulfur compounds as a metabolic end product that can be used by the sulfide oxidizers for the autotrophic fixation of CO_2. The internal production of sulfide is beneficial for the host and its thioautotrophic symbionts in environments with low concentrations of reduced sulfur compounds. Furthermore, waste compounds produced by the host during anaerobic metabolism, and otherwise excreted in non-symbiotic worms, can be recycled by the host (20, 57).

The syntrophic sulfur cycle was first described in the gutless oligochaete *O. algarvensis* (20). This species co-occurs with another gutless oligochaete, *O. ilvae*, in shallow water sediments off the island of Elba (Italy) in the Mediterranean (27). Molecular characterisation of the *O. algarvensis* and *O. ilvae* symbionts revealed that both hosts harbor two gammaproteobacterial symbionts, Gamma 1 and 2, and two deltaproteobacterial symbionts, Delta 1 and 2, with a spirochete symbiont only found in *O. algarvensis* (20, 57). The Gamma 1 symbionts are found in all gutless oligochaetes and are chemoautotrophic sulfur oxidizers, and the Delta 1 and 2 symbionts are sulfate reducers. The function of the Gamma 2 and spirochete symbionts is not yet known.

The study of metabolic capabilities of these bacterial symbionts is limited by the fact that the symbionts have resisted any cultivation attempts. In addition to PCR based studies of functional genes, metagenomic analyses can provide valuable information on the metabolism of yet uncultivated microbes. Metagenomic approaches have been used to study the diversity of genes (e.g. cellulases (33)), metabolic pathways (e.g. antibiotic synthesis (56)), free-living and symbiotic bacteria and archaea (43, 52, 62), microbial communities (e.g. acid mine drainage biofilm (66) and environmental samples (5, 65, 67)).

In the present study, a metagenomic approach was used to investigate the metabolic diversity of bacterial endosymbionts in *O. algarvensis* and *O. ilvae* analysing a BAC library created without physical separation of the symbiotic partners. Functional genes such as adenosine-5´-phosphosulfate (APS) reductase involved in sulfur metabolism and ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO) involved in CO_2 fixation, as well as symbiont specific 16S rRNA genes were used as phylogenetic anchors for the screening of the BAC library. Random end-sequencing of BAC clones was used to discover genes not previously identified in these symbioses using PCR based approaches. Two BAC clones of 18 and 51 kb containing functional genes involved in CO_2 fixation and sulfur oxidation were fully sequenced to gain a better understanding of thiotrophic metabolism in the oligochaete symbiosis.

Materials and Methods

Specimen collection. *O. algarvensis* and *O. ilvae* were collected in October 2001 in the bay of Capo di San Andrea (Elba, Italy). The worms were extracted from the sediment by decantation with seawater. After washing with sterile seawater specimens were frozen and stored at -80°C until DNA extraction. Large numbers of worms (~520 specimens) were needed to isolate sufficient DNA for BAC library construction. Time constraints made it impossible to identify such a large number of worms to the species level, so that the BAC library contained both *O. algarvensis* and *O. ilvae* DNA. However, *O. algarvensis* is more

abundant at the collection site (27). For PCR analyses of functional genes, single worm individual per species were collected and identified using the cytochrom oxidase I (COI) gene (50).

BAC library construction and analysis.

Library construction. High molecular weight (HMW) DNA was isolated from a pooled sample of 520 worm specimens according to Wu et al. (70). The frozen worms were homogenised in liquid nitrogen, transferred into ice-cold STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 10 mM EDTA, pH8.0) with 30 mM β-mercaptoethanol and kept on ice for 10 minutes with gentle stirring. The cell suspension was filtered through 1-2 layers of miracloth to remove large pieces of cell debris and centrifuged at 5,000 rpm, and 4°C for 10 minutes to pellet the cells. The cells were once washed with STE buffer without β-mercaptoethanol and collected by centrifugation as above. The cell pellet was resuspended in 1-2 ml STE buffer without β-mercaptoethanol per gram tissue wet weight, pre-warmed at 45°C for 5 minutes, and mixed with an equal volume of molten 1% low melting point (LMP) agarose in STE buffer at 45°C. The cell-agarose mixture was aliquoted into ice-cold 100 µl plug molds and kept on ice for 10 minutes to allow the plugs to solidify. The plugs were transferred into lysis buffer (0.5 M EDTA, pH 9.0, 1% lauryl sarcosine, and 0.5-1 mg/ml proteinase K) at approximately 1 ml lysis buffer per 100 µl plug, and incubated under mild shaking at 50°C for 24-48 hours. The agarose plugs were washed twice for 30 minutes each in 10-20 volumes of ice-cold TE buffer (10 mM Tris-HCL, 1.0 mM EDTA, pH 8.0), three times for one hour each in 10-20 volumes of ice-cold TE supplemented with 0.1 mM PMSF (phenylmethyl sulfonyl fluoride), and three times for one hour each in 10-20 volumes of TE on ice. The DNA in these agarose plugs was stored at 4°C for several months without significant degradation.

The BAC library was constructed as described previously (70, 71). In brief, DNA fragments of desired size (100-200 kb) were selected after pulse-field gel electrophoresis (PFGE) analysis. The DNA fragments were recovered from the gel by electroelution in dialysis tubing (12,000-14,000 daltons molecular weight

exclusion, Gibco BRL, USA) and ligated to the cloning vector pECBAC1 (Gibco BRL, USA). Ligated DNA was transformed into *Escherichia coli* strain ElectroMAX DH10B competent cells (Gibco BRL, USA) by electroporation. Recombinant transformants were randomly selected, and BAC DNA was isolated, digested with *Not*I and subjected to size analysis by PFGE.

Library analysis

Library screening. The BAC library was screened by PCR for *cbbL* and *cbbM* encoding form I and II RubisCO, for *aprA* encoding the alpha-subunit of the APS reductase, and for deltaproteobacterial 16S rRNA genes. The following primers were used: for the *cbbL* gene cbbL1bF and cbbL2cR (7), for the *cbbM* gene cbbM1aF and cbbM2R or cbbM2aR (7), for the *aprA* gene APS1F and APS4R (Küver, unpublished data), and for the deltaproteobacterial 16S rRNA symbiont genes 8F (48) and DSS658R (41). Attempts to screen for gammaproteobacterial and spirochete symbionts using 16S rRNA gene specific primers were not successful.

For PCR screening, four BAC clones were pooled, and 1 µl template of this cell mixture was added to the PCR mix (30 µl total volume). The following thermocycling conditions were applied: 1 cycle at 95°C for 4 min; 35 cycles at 95°C for 1 min, 48°C (for *cbbL* genes), 54°C (for *aprA* genes), or 55°C (for deltaproteobacterial 16S rRNA genes) for 1 min, and 72°C for 2 min; and 1 cycle at 72°C for 4 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and directly sequenced in both directions (~696 bp for *cbbL* genes, ~396 bp for *aprA* genes, and ~660 bp for 16S rRNA).

BAC sequencing. About 850 BAC clones were randomly chosen for insert end-sequencing. BAC DNA was prepared according to the manual by Hong-Bin Zhang (http://hbz7.tamu.edu/homelinks/tool/protocol.htm). For sequencing, 150 ng BAC DNA, 1.5 µl 5x buffer (400 mM Tris-HCL, pH 9, 10 mM $MgCl_2$), 1 µl 10 µM Sp6 or T7 vector primer, and 1.5 µl BIG Dye Terminator Sequencing Mix Version 3 (Applied Biosystems, USA) were filled up with PCR water to a final

volume of 15 µl. The following cycle sequencing conditions were applied: initial 5 min denaturation at 95°C, followed by 99 cycles of 30 sec denaturation at 95°C, 10 sec annealing at 56°C (T7 primer) or 50°C (Sp6 primer), and 4 min elongation at 60°C (ramp: 1°C/s).

Selected BAC inserts of interest were further sequenced by primer walking. Except for the annealing temperature, which was adjusted for each primer, the same cycle sequencing conditions as for end-sequencing were used.

Two BACs were chosen for full sequencing using a shotgun approach based on plasmid libraries with 1.5 and 3.5 kb inserts; sequence coverage was more than 10-fold. Resulting reads were assembled by Phrap44 and manually finished in GAP4 (www.sanger.ac.uk). The quality of the sequence data was completed to reach a maximum of one error in 10.000 bases.

Sequence analysis.

For the analysis of the end-sequences and BAC inserts the GenDB 2.0 annotation software was used (42). For function predictions Blastp was used (1) against the non-redundant database from NCBI and against the SWISS-PROT database (8). In additional Pfam (4) and InterPro (47) searches were performed. Transmembrane helices in proteins were determined by using TMHMM V2.0 (46) and signal peptides were predicted by using SignalP V2.0 (49).

The end-sequences were translated in six reading frames and imported into GenDB. All reading frames were analyzed with the tools described above and reading frames with significant tool results were considered as protein coding sequences. For all of these protein coding sequences the best Blastp against NCBI-nr hit has been taken for the apparent phylogenetic affiliation.

On the BAC-insert a ORF prediction was carried out by using Glimmer (13, 58). All predicted ORFs were analyzed by the described bioinformatic pipeline and followed by a manual annotation.

AprA and *cbbL* libraries from *O. algarvensis* and *O. ilvae.*

BAC inserts containing *aprA* or *cbbL* genes originated either from *O. algarvensis* or *O. ilvae* symbionts because both species were pooled for the construction of

the BAC library. To determine from which host species these sequences originate, *aprA* and *cbbL* libraries from single worms identified as either *O. algarvensis* or *O. ilvae* using the host COI gene were constructed. DNA was extracted from single worms as described previously (6). The *aprA* and *cbbL* genes were amplified with the same primers used for the screening of the BAC library (see above). 1 µl template DNA was added to the PCR mix (50 µl total volume). The following thermocycling conditions were used: 1 cycle at 95°C for 4 min; 25 cycles at 95°C for 1 min, 56°C (for the *cbbL* gene), or 54°C (for the *aprA* gene), 1 min, and 72°C for 2 min; and 1 cycle at 72°C for 4 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and cloned using the TOPO-TA cloning kit (Invitrogen, Breda, Netherlands) according to the manufacturer's protocol. For screening of the *aprA* and *cbbL* genes 50 clones per individual were randomly picked and controlled for the correct insert size by PCR using the vector primers M13F and M13R. Positive clones were fully sequenced in both directions.

Phylogenetic analysis.

The partial *cbbL* and *aprA* sequences were checked against the sequences in non-redundant protein database using the BLAST algorithm (2) for similarity searches. The sequence data were analyzed using the ARB software package (www.arb-home.de).

Phylogenetic trees of the RubisCO large-subunit, including RubisCO form I (*cbbL*) and RubisCO form II (*cbbM*) were generated from amino acid sequences using the maximum-likelihood algorithm with a 25% positional conservation filter The leading and tailing amino acid stretches of full length sequences were excluded from the analysis, eliminating those regions found in some but not all RubisCO sequences. The partial RubisCO form I sequences (*cbbL*, 211 amino acids) from this study were added to the maximum-likelihood tree using maximum parsimony. The APS alpha-subunit reductase tree was calculated based on partial amino acid sequences (*aprA,* 131 amino acids) using maximum-

likelihood algorithm. For tree reconstruction a 25% positional conservation filter was used.

Results

Library characterization.

The metagenomic BAC library constructed from DNA directly extracted from 520 pooled worms contained XX clones with an average insert size of about 50 kb. Of these, 1152 BAC clones were analyzed for this study.

Blastp analysis of 1662 end-sequences revealed an extraordinary predominance of genes of bacterial origin in the BAC library (Figure 1). The relative percentage of sequences with highest similarity to bacterial genes increased from 87% at e-values $\leq e^{-3}$ to 97% at e-values $\leq e^{-50}$. For 525 out of these 1662 end-sequences no similarities ($> e^{-3}$) to sequences deposited in the GenBank databases were found.

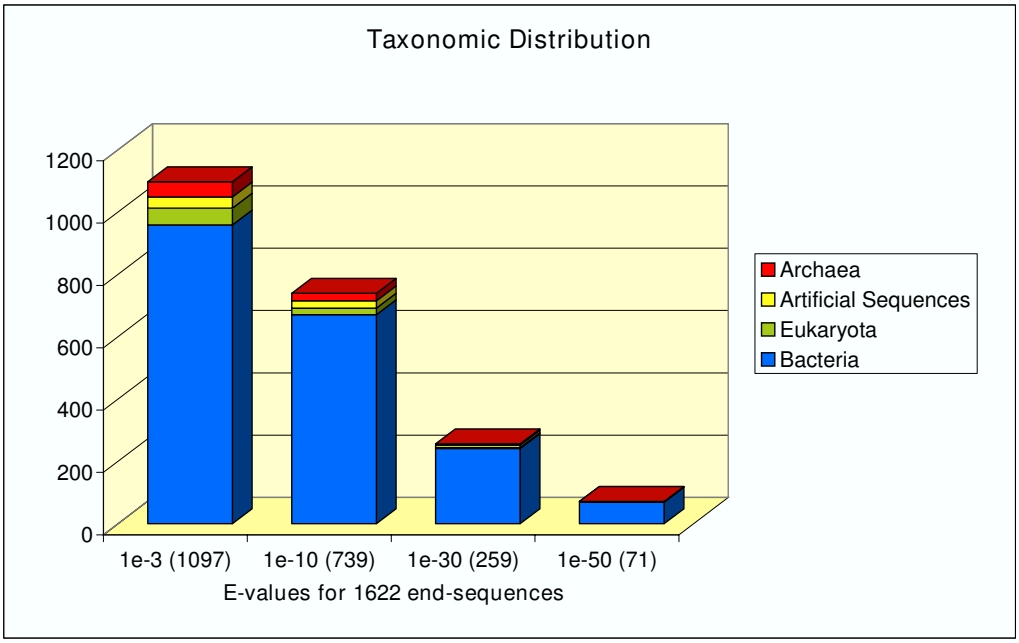


Figure 1. Taxonomic distribution of end-sequences from the *Olavius* spp. BAC library based on BLAST e-values.

Library screening.

16S rRNA gene. Three clones out of the 1152 BAC clones analyzed contained deltaproteobacterial 16S rRNA genes based on PCR products of ~650 bp length (Table 1). Two of these were 100% identical to the 16S rRNA gene sequence from the *O. ilvae* Delta 2 symbiont and one 100% identical to the *O. algarvensis* Delta 2 symbiont (57).

Form I RubisCO. Of the 1152 screened BAC clones, 8 were positive for the *cbbL* gene that is encoding form I RubisCO (Table 1). Two different RubisCO form I sequences, BAC RubisCO 1 and 2, with 94% amino acid (aa) sequence similarity were found with two clones containing the BAC RubisCO 1 and six containing the BAC RubisCO 2 sequence. BAC clones carrying the gene for form II RubisCO were not found.

APS reductase. Five clones out of the 1152 screened BAC clones contained the *aprA* gene coding for the alpha subunit of the APS reductase (Table 1). The sequences of two clones were identical (BAC APS 1A). The remaining three clones (BAC APS 1B, 2, and 3C) differed from each other as well as from the BAC APS 1A sequence.

Phylogeny of functional genes and assignment to *O. algarvensis* or *O. ilvae* symbionts.

To identify the origin of the form I RubisCO and APS reductase genes from the BAC library of pooled *O. algarvensis* and *O. ilvae* worms to the correct species, these genes were characterized in single individuals of both species and their phylogeny compared to sequences from the database.

Form I RubisCO. Form I RubisCO sequences obtained by screening of the *Olavius* spp. BAC library and the *O. algarvensis* and *O. ilvae* individuals fall into two distinct phylogenetic groups with RubisCO 1 and 2 type sequences (Figure 2). In *O. algarvensis,* both RubisCO 1 and 2 sequences were found, while in *O. ilvae* only RubisCO 1 sequences were revealed. The BAC library RubisCO 1 sequence is 100% identical to the RubisCO 1 sequence from

O. ilvae, while the BAC library RubisCO 2 sequence is 100% identical to the RubisCO 2 sequence isolated from *O. algarvensis* (both at the nucleotide level). This indicates that the BAC library RubisCO 1 sequence originated from *O. ilvae* symbionts, while the RubisCO 2 sequence originated from *O. algarvensis* symbionts.

Table 1. Number of clones found in *O. algarvensis* and *O. ilvae* individuals as well as in the *Olavius* spp. BAC library for the 16S rRNA, RubisCO, and APS reductase genes. A total of 50 clones in a single individual each of *O. algarvensis* and *O. ilvae* and 1152 clones in the BAC library were screened. (-: investigated by Rühland et al., in prep)

Sequence phylotype	*O. algarvensis* (50 clones)	*O. ilvae* (50 clones)	*Olavius* spp. BAC library (1152 clones)
16S rRNA			
O. algarvensis Delta 2 phylotype	-	-	1
O. ilvae Delta 2 phylotype	-	-	2
RubisCO			
RubisCO 1			
O. algarvensis phylotype	43	0	0
O. ilvae phylotype	0	50	2
RubisCO 2	0	0	0
O. algarvensis phylotype	7	0	6
O. ilvae phylotype	0	0	0
APS reductase			
APS 1			
O. algarvensis phylotype	25	0	2
O. ilvae phylotype	0	31	1
APS 2			
O. algarvensis phylotype	14	0	0
O. ilvae phylotype	0	6	1
APS 3			
APS 3a			
O. algarvensis phylotype	3	0	0
O. ilvae phylotype	0	6	0
APS 3b			
O. algarvensis phylotype	2	0	0
O. ilvae phylotype	0	0	0
APS 3c			
O. algarvensis phylotype	0	0	0
O. ilvae phylotype	0	0	0
Olavius spp. BAC library	0	0	1

Phylogenetic analyses of these RubisCO sequences shows that they belong to the form IA RubisCO sequences from Proteobacteria (Figure 2). The *O. algarvensis* and *O. ilvae* RubisCO 1 sequences are most closely related to each other (96.3% aa sequence similarity) and form a cluster with the RubisCO sequences from symbionts of two other gutless oligochaete species, *Inanidrilus leukodermatus* and *I. makropetalos* (≥96% aa sequence similarity). The closest relative to this clade is the RubisCO 2 sequence from *O. algarvensis* (92.6% aa sequence similarity). The cluster of form IA RubisCO 1 and 2 sequences from gutless oligochaetes is related to form IA RubisCO sequences from thiotrophic endosymbionts of *Solemya velum* and *Alviniconcha hessleri* and one of the two form IA RubisCO genes in the free-living thiotroph *Allochromatium vinosum* (≥87.6% aa sequence similarity). *A. vinosum* has two copies of form IA RubisCO

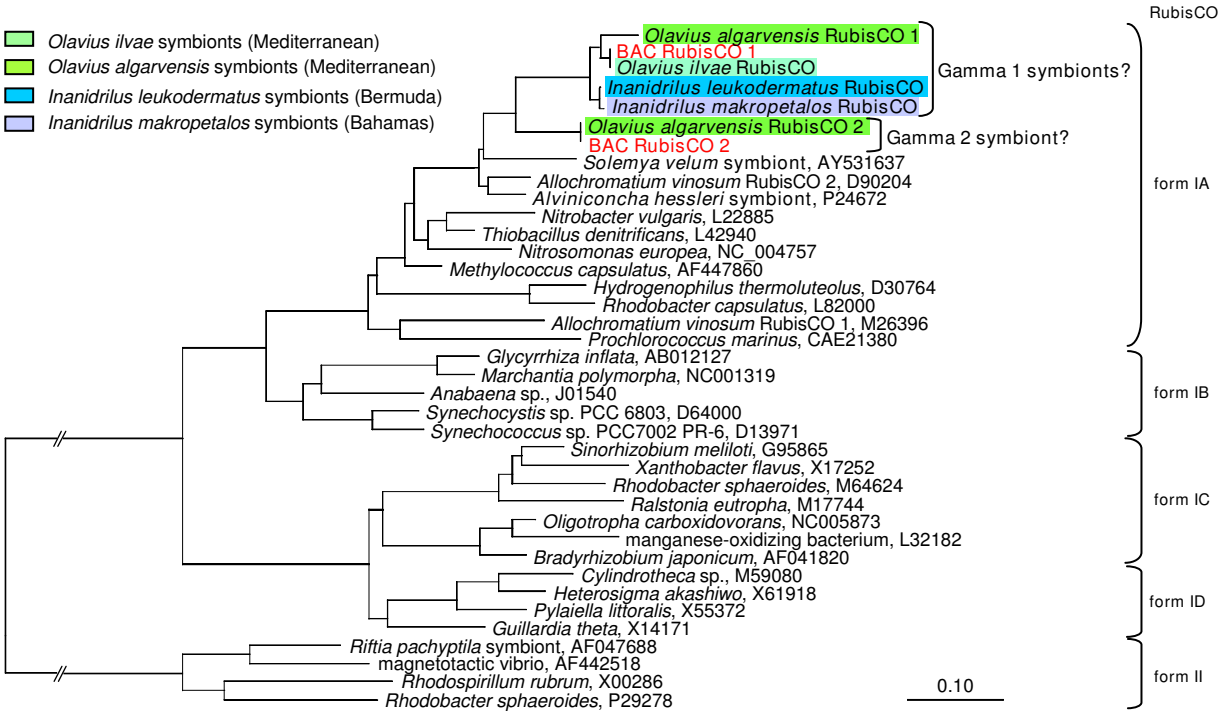


Figure 2. Phylogenetic placement of symbiotic RubisCO large-subunit sequences based on maximum likelihood analysis. Symbiotic sequences are listed in bold, with sequences from gutless oligochaetes colored according to host species (see legend). RubisCO sequences from the *Olavius* spp. BAC library are in red. The bar represents 10% estimated sequence divergence. The distance between RubisCO form I and II sequences is an estimated 126% sequence divergence.

of which only RubisCO 2 has been shown to be expressed (68).

APS reductase. APS reductase sequences from *O. algarvensis* and *O. ilvae* symbionts fall into three phylogenetically distinct groups of APS reductase sequences called APS 1-3. (Figure 3). Two of these are most closely related to thiotrophic bacteria, APS 1 and 2, while APS 3 oligochaete sequences fall within a clade of sequences from sulfate-reducing bacteria. In the APS 1 group, two BAC sequences are 100% identical to *O. algarvensis aprA* sequences, while one BAC sequence is 100% identical to *O. ilvae aprA* sequences (both at the nucleotide level), allowing a clear assignment of the BAC genes to the corresponding host species. The *O. algarvensis* and *O. ilvae* APS 1 sequences are most closely related to *aprA* sequences from two other gutless oligochaetes,

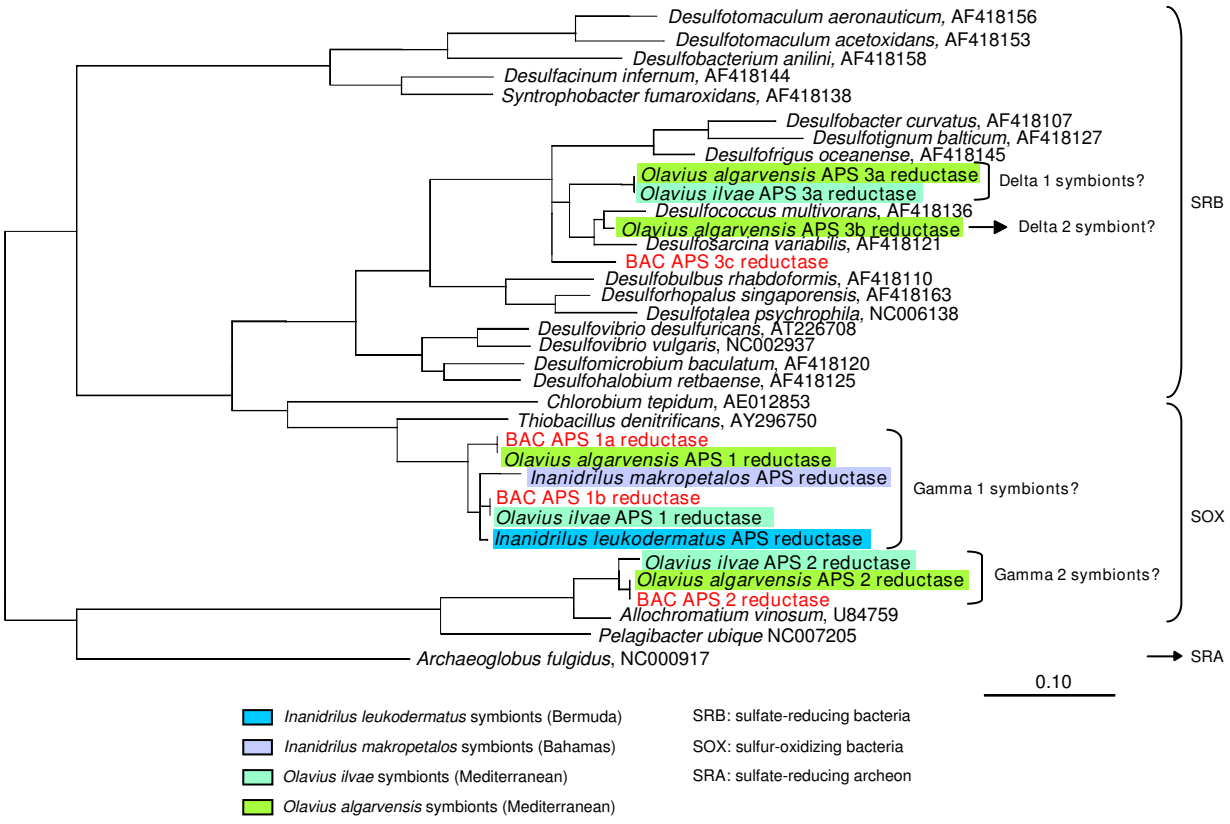


Figure 3. Phylogenetic placement of symbiotic APS reductase alpha-subunit sequences based on maximum likelihood analysis. Sequences from individual worms are shown in the corresponding color for the host species (see legend); sequences from the *Olavius* spp. BAC library are in red. The bar represents 10% estimated sequence divergence.

Inanidrilus leukodermatus and *I. makropetalos* (≥96% aa sequence similarity). This clade of oligochaete symbiont sequences is related to *aprA* sequences from the free-living sulfur-oxidizing bacteria *Thiobacillus denitrificans* and *Chlorobium tepidum* (≥71% aa sequence similarity).

The second clade of oligochaete symbiont *aprA* sequences, APS 2, is most closely related to the *aprA* sequence from the free-living sulfur oxidizer *A. vinosum* (≥93% aa sequence similarity). The BAC library APS 2 sequence is 100% identical at the nucleotide level to the APS 2 sequence from *O. algarvensis* indicating that this BAC sequence originated from *O. algarvensis* symbiont. The next relative to these sequences is the APS 2 sequence from *O. ilvae* (98% aa sequence similarity).

The APS 3 oligochaete symbiont sequences are most closely related to *aprA* sequences from sulfate-reducing bacteria. The APS 3a symbiont sequences are identical at the amino acid level, but differ by 3.8% at the nucleotide level in the two species. The APS 3 sequences are most closely related to *aprA* genes from the free-living sulfate-reducing bacteria *Desulfosarcina variabilis* and *Desulfococcus multivorans* (≥91.2% aa sequence similarity), and to an APS 3b sequence found only in an *O. algarvensis* individual (93.6% aa sequence similarity). A third *aprA* sequence from the APS 3 group, APS 3c, was only found in one clone from the BAC library and is most closely related to *aprA* sequences from the free-living, sulfate-reducing bacteria *Desulfotignum balticum*, *Desulfobacter curvatus*, and *Desulfofrigus oceanense* (≥79.3% aa sequence similarity).

Analysis of fully sequenced and end-sequenced BAC inserts.

Full sequencing. Two BAC clones were selected for full sequencing based on the presence of genes characteristic of thiotrophic bacteria. Bac5g12 contained a form I RubisCO encoding *cbbL* gene with 100% nucleotide identity to the RubisCO 2 sequence from *O. algarvensis* (Figure 2), indicating that this BAC insert originated from *O. algarvensis* symbiont. Bac4a12 contained a *dsrK* gene coding for a protein involved in sulfur metabolism. The gene was detected by

insert end-sequencing of randomly chosen BAC clones. Full sequencing of these two clones showed that the Bac4a12 insert of 18 kb was 100% identical to the Bac5g12 insert of 51.7 kb so that only the latter is described further.

The insert of Bac5g12 contained 56 ORFs. The deduced amino acid sequences of 35 ORFs revealed similarities to functional proteins in the databases whereas 10 ORFs showed only similarities to hypothetical proteins. Eleven ORFs had no similarity to current GenBank database entries. (Table 2). The predicted ORFs putatively coding for functional proteins are organized in three distinct gene clusters, the *dsr* gene cluster containing 14 genes coding for enzymes involved in sulfur energy metabolism, the *cbb* gene cluster containing 10 genes encoding enzymes involved in CO_2 fixation, and the *acr* gene cluster composed of 2 genes coding for a multidrug efflux transport system. The proteins encoded by the remaining 9 genes play a role in different metabolic pathways and are not clustered.

Carbon metabolism.

Cbb locus. The *cbb* locus of the Bac5g12 sequence consists of 10 genes, *cbbRFPTALSQOZ*, involved in CO_2 fixation via the Calvin-Benson-Bassham (CBB) cycle (Figure 3a). Two enzymes encoded by the *cbb* gene cluster are characteristic for the CBB cycle. The first is riboluse-1,5-bisphosphate carboxylase/oxygenase (RubisCO) which consists of the large and small subunit encoded by the adjacent *cbbL* and *cbbS* genes, and the second is the *cbbP*-encoded phosphoribulokinase (PRK). Downstream of the *cbbLS* BAC cluster *cbbQ* and *cbbO* are located whose protein products are implicated in the posttranslational enhancement of the RubisCO activity (31). Next to these genes, a hypothetical protein of unknown function and a *cbbZ* gene encoding phosphoglycolate phosphatase are located.

Upstream of the *cbbLS* cluster, *cbbA* and *cbbT* genes encoding fructose-bis-P-aldolase and transketolase, respectively, and the *cbbF* gene coding for the fructose-1-6-bis-phposphatase are present. In addition to the CBB cycle, these enzymes can also participate in the pentose-phosphate pathway and two of

them, CbbA and CbbF, are used in glycolysis, gluconeogenesis, and fructose and mannose metabolism. The *cbb* cluster is flanked by a *cbbR* gene encoding a transcriptional regulator that is transcribed in the opposite direction of all other *cbb* genes.

Similarity searches of Cbb protein sequences deduced from ORFs on Bac5g12 revealed that proteins encoded by the first part of the cluster, *cbbRFPTA*, show

TABLE 2. Annotated ORFs in the *Olavius algarvensis* symbiont Bac5g12[a]

Open Reading Frame	Nucleotide range (protein size, in no. of amino acids)	Dir[b]	Most similar homolog blastp vs nr[c]			Interpro/Pfam References[e]	Predicted gene	Predicted function
			Organism	E-value	Acc. no.[d]			
1	86-904 (272)	+	*Acinetobacter* sp. ADP1	2e-37	YP_047876	—	—	Conserved hypothetical protein
2	1112-2257 (381)	+	*Thiobacillus denitrificans* ATCC 25259	2e-83	ZP_00333469	PF04143	—	Integral membrane protein
3	2250-2390 (46)	+	—	—	—	—	—	Hypothetical protein
4	2404-2610 (68)	+	—	—	—	—	—	Hypothetical protein
5	3202-2618 (194)	-	*Thiobacillus denitrificans* ATCC 25259	2e-23	ZP_00334399	—	—	Conserved hypothetical protein
6	4013-3231 (260)	-	*Thiobacillus denitrificans* ATCC 25259	9e-35	ZP_00334577	—	—	Conserved hypothetical protein
7	4450-5034 (194)	+	*Idiomarina loihiensis* L2TR	3e-18	YP_155925	—	—	Conserved hypothetical protein
8	5126-5293 (55)	+	—	—	—	—	—	Hypothetical protein
9	5613-5290 (107)	-	—	—	—	—	—	Hypothetical protein
10	5635-6306 (223)	+	*Synechococcus elongatus* PCC 6301	3e-40	YP_172192	SSF47240	—	Protein belonging to 2OG-Fe(II) oxygenase superfamily
11	6516-6313 (67)	-	—	—	—	—	—	Hypothetical protein
12	6888-6547 (113)	-	*Thiobacillus denitrificans* ATCC 25259	7e-17	ZP_00334566	PF04358	—	DsrC protein homolog
13	7607-6924 (227)	-	*Thiobacillus denitrificans* ATCC 25259	1e-18	ZP_00334396	—	—	Conserved hypothetical protein
14	8570-7617 (317)	-	*Thiobacillus denitrificans* ATCC 25259	1e-47	ZP_00334400	—	—	Conserved hypothetical protein
15	8898-10220 (440)	+	*Allochromatium vinosum*	0.0	AAC35394	TIGR02064	*dsrA*	Dissimilatory siroheme sulfite reductase alpha subunit
16	10279-11349 (356)	+	*Allochromatium vinosum*	0.0	AAC35395	TIGR02066	*dsrB*	Dissimilatory siroheme sulfite reductase beta subunit
17	11362-11754 (130)	+	*Allochromatium vinosum*	1e-46	AAC35396	PF02635	*dsrE*	Intracellular sulfur oxidation protein DsrE
18	11758-12162 (134)	+	*Allochromatium vinosum*	2e-36	ACC35397	PF02635	*dsrF*	Intracellular sulfur oxidation protein DsrF
19	12173-12481 (102)	+	*Allochromatium vinosum*	6e-25	ACC35398	PF04077	*dsrH*	DsrH
20	12520-12852 (110)	+	*Allochromatium vinosum*	4e-42	ACC35399	PF04358	*dsrC*	DsrC
21	12924-13682 (252)	+	*Thiobacillus denitrificans* ATCC 25259	4e-64	ZP_00333742	PF02665	*dsrM*	DsrM
22	13675-15165 (496)	+	*Allochromatium vinosum*	0.0	AAC35401	SSF46548	*dsrK*	DsrK
23	15192-17144 (650)	+	*Allochromatium vinosum*	0.0	AAG13082	PR00419	*dsrL*	DsrL
24	17147-17659 (170)	+	*Thiobacillus denitrificans* ATCC 25259	1e-19	ZP_00333739	—	*dsrJ*	DsrJ
25	17656-18411 (251)	+	*Thiobacillus denitrificans* ATCC 25259	4e-69	ZP_00333738	PF00037	*dsrO*	DsrO
26	18414-19607 (397)	+	*Thiobacillus denitrificans* ATCC 25259	4e-97	ZP_00333737	PF03916	*dsrP*	DsrP
27	19611-21026 (471)	+	*Thiobacillus denitrificans* ATCC 25259	1e-122	ZP_00333736	—	*dsrN*	DsrN
28	21023-21340 (105)	+	*Allochromatium vinosum*	4e-16	AAG13087	—	*dsrR*	DsrR
29	21437-21832 (131)	+	—	—	—	—	—	Hypothetical protein
30	21864-23093 (409)	+	Dechloromonas aromatica RCB	2e-38	ZP_0014961	PF02518	—	Two-component system sensor kinase
31	23330-24496 (388)	+	*Thiobacillus denitrificans* ATCC 25259	5e-56	ZP_00335105	SSF54292	—	Protein containg 2Fe-2S iron-sulfur cluster binding domain
32	25928-24546 (460)	-	*Pseudomonas* sp. ADP	1e-136	AAK50323	PF01609	—	Putative transposase
33	26079-26414 (111)	+	—	—	—	—	—	Hypothetical protein
34	26411-27073 (220)	+	*Thiobacillus denitrificans* ATCC 25259	2e-43	ZP_00333727	SSF52172	—	Two-component response regulator
35	27323-28564 (413)	+	*Yersinia pseudotuberculosis* IP32953	1e-136	YP_069749	PF00270	—	Protein belonging to DEAD/DEAH box helicase family
36	28566-28832 (88)	+	—	—	—	—	—	Hypothetical protein
37	30640-28877 (587)	-	*Chlorobium tepidum* TLS	0.0	NP_661913	—	*soxB*	SoxB
38	31302-30640 (220)	-	*Thiobacillus denitrificans* ATCC 25259	6e-50	ZP_00333497	TIGR01449	*cbbZ*	Phosphoglycolate phosphatase
39	31838-31299 (179)	-	—	—	—	—	—	Hypothetical protein
40	34375-31853 (840)	-	*Solemya velum* gill symbiont	0.0	AAT01432	—	*cbbO*	CbbO
41	35231-34398 (277)	-	*Solemya velum* gill symbiont	1e-125	AAT01431	PF07728	*cbbQ*	CbbQ
42	35597-35241 (118)	-	*Allochromatium vinosum*	1e-50	P22850	PF00101	*cbbS*	Ribulose-1,5-bisphosphate carboxylase/oxygenase smal subunit
43	37034-35610 (474)	-	*Thiobacillus denitrificans* ATCC 25259	0.0	ZP_00334530	PF00016	*cbbL*	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit

44	38138-37056 (360)	-	*Rhodospirillum rubrum*	1e-153	ZP_00267929	TIGR01521	*cbbA*	Fructose-bisphosphate aldolase class-II
45	40165-38156 (669)	-	*Brucella melitensis* 16M	0.0	NP_539228	TIGR00232	*cbbT*	Transketolase
46	41055-40180 (291)	-	*Nitrobacter vulgaris*	1e-116	P37100	PF00485	*cbbP*	Phosphoribulokinase
47	42120-41065 (351)	-	*Bradyrhizobium japonicum* USDA 110	1e-76	NP_769221	PF00316	*cbbF*	Fructose-1-6-bisphosphatase
48	42182-43114 (310)	+	*Rhodopseudomonas palustris* CGA009	9e-59	CAE269	PF03466	*cbbR*	HTH-type transcriptional regulator cbbR
49	46348-43130 (1072)	-	*Magnetococcus* sp. MC-1	0.0	ZP_00289984	PF00873	—	AcrB/AcrD/AcrF family protein
50	47304-46345 (319)	-	*Magnetococcus* sp. MC-1	9e-55	ZP_00289111	TIGR01730	—	AcrA/AcrE family protein
51	47294-47419 (41)	+	—	—	—	—	—	Hypothetical protein
52	47511-49310 (599)	+	*Rubrobacter xylanophilus*	2e--63	ZP_00187680	PF03190	—	Protein containing DUF255
53	49971-49486 (161)	-	*Bradyrhizobium japonicum* USDA 110	8e-07	NP_772324	—	—	Conserved hypothetical protein
54	50609-49968 (213)	-	*Rubrivivax gelatinosus*	4e-50	ZP_00242217	—	—	Conserved hypothetical protein
55	51001-50606 (131)	-	*Bradyrhizobium japonicum* USDA 110	3e-15	NP_772326	—	—	Conserved hypothetical protein
56	51264-51067 (65)	-	—	—	—	—	—	Hypothetical protein, membrane

[a] Dsr, dissimilatory siroheme sulfite reductase, HDH, helix-turn-helix
[b] Dir, direction of transcription, upstream (+) or downstream (-).
[c] Hits were obtained from blastp comparison of predicted proteins from contig Bac5g12 to non-redundant GenBank protein database
[d] Accession numbers from NCBI database (http://www.ncbi.nlm.nih.gov/Entrez/index.html).
[e] References relate to INTERPRO (http://ebi.ac.uk/interpro/; (lit)) and PFAM databases (home-page; [Bateman, 2002 #6867]).

highest similarities to homologs in Alphaproteobacteria. In contrast, proteins encoded by the second part of the *cbb* gene cluster, *cbbLSQOZ*, are most similar to protein homologs in Gamma- and Betaproteobacteria.

Cbb locus structure. Comparison of the *cbb* locus on Bac5g12 with those in the databases shows conserved organization of the two form I RubisCO encoding genes in all bacteria, *cbbL* always followed by *cbbS*. In contrast, the adjacent *cbb* genes are present in some but not all bacteria and show variable locations. Of the numerous *cbb* loci in the database, a selection is shown in Figure 3a. The genes *cbbLSQO* appear to be clustered in most of the form IA RubisCO gene clusters. The *cbbQ* and *cbbO* genes do not occur in the *cbb* gene clusters of bacteria with form IC RubisCO. Instead *cbbX* gene is present, which encodes a protein assumed to be necessary for RubisCO expression (26). Despite of their different structures CbbX and CbbQO proteins seem to have a similar function in enhancing the expression of RubisCO. The downstream of *cbbL* located c*bbRFPTA* genes are clustered in *cbb* loci of form IC RubisCO from some Alphaproteobacteria such as the plant-associated *B. japonicum* and *S. meliloti* and the free-living *O. carboxidovorans*. In contrast, these genes are not clustered in *cbb* loci of form IA RubisCO, with the exception of the *cbb* locus on Bac5g12.

Sulfur metabolism.

Dsr locus. The *dsr* locus on Bac5g12 is composed of 14 genes, *dsrABEFHCMKLJOPNR* that have been shown to encode proteins participating in the oxidation of intracellular sulfur (11, 54) (Figure 3b). All protein sequences deduced from the *dsr* locus show highest similarities to homologs present in the free-living chemoautotrophic sulfur oxidizers *Allochromatium vinosum* of the Gammaproteobacteria and *Thiobacillus denitrificans* of the Betaproteobacteria. The *dsrAB* genes encode two subunits, alpha and beta, of the dissimilatory siroheme sulfite reductase. The adjacent *dsrEFHC* and *dsrR* genes encode small soluble cytoplasmic proteins of unknown function. The derived proteins of *dsrMKOPJ* genes resemble a membrane-spanning electron-transporting complex. All these proteins contain either heme groups or [Fe-S] clusters. The *dsrL* gene encodes a predicted NADPH:acceptor oxidoreductase and the *dsrN*-encoded protein resembles a cobyrinic acid a,c-diamide synthase that is assumed to be involved in the biosynthesis of siro(heme)amide, the prosthetic group of the sulfite reductase.

Dsr locus structure. Comparison of the BAC *dsr* locus to the three available *dsr* loci of sulfur-oxidizing bacteria in the database revealed that the BAC locus structure is highly similar to that of *A. vinosum* and *T. denitrificans* (Figure 3a). The only difference is the *dsrS* gene of *A. vinosum,* which occurs downstream of its *dsrR* gene (11). This gene encodes a small soluble cytoplasmic protein of unknown function and was not found on the BAC insert or the draft assembly of the *T. denitrificans* ATCC 25259 genome. In contrast to these three similar clusters, the *dsr* locus in the sulfur-oxidizing green sulfur bacterium *Chlorobium tepidum* is divided into two *dsr* gene clusters, in which genes and gene order differ from those of the BAC sequence, *A. vinosum*, and *T. denitrificans*.

DsrC paralog. In addition to the DsrC protein deduced from ORF 20 in the *dsr* gene cluster, a second sequence with high similarity to a DsrC protein was deduced from ORF 12, with 35% aa sequence identity to the ORF 20 protein. However, the strictly conserved cysteine in the C-terminus of DsrC

Figure 3. Comparison of *cbb* and *dsr* gene clusters from the *O. algarvensis* symbiont Bac5g12 to correspondent gene clusters in the database. (a) Bac5g12 *cbb* locus versus selected proteobacterial *cbb* loci of the form IA and IC RubisCO group. (b) Bac5g12 dsr locus versus the available *dsr* loci from sulfur-oxidizing bacteria in the database.

which is assumed to have a catalytic function in the metabolism of sulfur compounds (54) is missing in the ORF 12 protein indicating another protein function. DsrC proteins without the strictly conserved cysteine are found in organisms such as *Escherichia coli* and *Haemophilus influenzae,* which do not contain dissimilatory sulfite reductase but can synthesize assimilatory sirohaem sulfite and nitrite reductases (45). This suggests that by ORF12 encoded protein is a DsrC paralog.

SoxB. The deduced protein of ORF 37 showed highest similarity to sulfur oxidation (Sox) protein B in the sulfur-oxidizing bacterium *C. tepidum*. This protein is part of a multienzyme complex involved in thiosulfate-oxidation. In all other sulfur oxidizers, the *soxB* gene is clustered with other *sox* genes (23). In contrast, no further *sox* genes were observed upstream and downstream of the *soxB* gene in the BAC insert.

Multidrug efflux pump.

Acr locus. The adjacent ORFs 49 and 50 encode members of the AcrA/AcrE and AcrB/AcrD/AcrF families, respectively, showing highest similarities to corresponding sequences in the magnetotactic bacterium *Magnetococcus* sp. MC-1. Both proteins are involved in a proton motive force (PMF) dependent multidrug efflux system that can apparently handle a wide range of structurally dissimilar compounds (51).

Conserved hypothetical proteins.

Two conserved hypothetical proteins encoded in ORFs 54 and 55 are only known to occur in two other bacteria, both of which are nitrogen-fixing autotrophs: the plant-associated *Bradyrhizobium japonicum* and the free-living *Rubrivivax gelatinosus*.

End-sequencing. 9 BAC end-sequences were chosen for primer walking based on genes encoding proteins of potential importance to the symbiosis (Table 3).

Sulfur metabolism.

Dissimilatory sulfite reductase (Dsr). On Bac1h4, bp 106-918 were most similar to *dsrEF* genes encoding DsrE and DsrF proteins involved in intracellular sulfur oxidation (11, 54). These enzymes differed by 56-69% (aa identity) to the corresponding homologs on the *O. algarvensis* Bac5g12 sequence indicating that these *dsrEF* genes originated from another oligochaete symbiont in *O. algarvensis* or *O. ilvae*.

Nitrogen metabolism.

Urease. The deduced proteins of the *ureFG* genes on Bac8D1 (bp 7-930) show highest similarity to urease accessory proteins UreF and UreG in the halophilic bacterium *Vibrio parahaemolyticus*. Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide and the proteins. UreF and UreG proteins play a role in the incorporation of nickel into the urease apoenzyme.

Table 3. Annotated ORFs from end-sequencing of selected clones from the *Olavius* spp. BAC library.

BAC clone	Nucleotide range	Dir[b]	Most similar homolog blastp vs nr[c]			Interpro/Pfam References[e]	Predicted gene	Predicted function
			Organism	E-value	Acc. no.[d]			
			SULFUR METABOLISM					
Dsr								
Bac1H4	106-495	-	*Alochromatium vinosum*	3e-64	AAC35396	PF02635	*dsrE*	Intracellular sulfur oxidation protein DsrE
	511-918	-	*Alochromatium vinosum*	8e-49	AAC35397	PF02635	*dsrF*	Intracellular sulfur oxidation protein DsrF
			NITROGEN METABOLISM					
Urease								
Bac8D1	7-302	+	*Vibrio parahaemolyticus*	8e-18	BAB13790		*ureF*	Urease accessory protein UreF
	337-930	+	*Vibrio parahaemolyticus*	1e-89	BAB13791	TIGR00101	*ureG*	Urease accessory protein UreG
Cyanophycin synthetase								
Bac10H10	1-1302	-	*Francisella tularensis* SCHU S4	e-136	YP_170103		*cphA*	Cyanophycin synthetase
			CARBON METABOLISM					
Opine oxidase								
Bac8g3	2-679	+	*Polaromonas* sp. JS666	e-53	ZP_00362102		*ooxB*	Opine oxidase subunit B
	703-981	+	*Burkholderia fungorum* LB400	5e-17	ZP_00277219		-	-
	1018-1641	+	*Bordetella bronchiseptica* RB50	1e-41	NP_887540		*ooxA*	Opine oxidase subunit A

[a] Dsr, dissimilatory siroheme sulfite reductase
[b] Dir, direction of transcription, upstream (+) or downstream (-).
[c] Hits were obtained from blastp comparison of predicted proteins from contig Bac5g12 to non-redundant GenBank protein database
[d] Accession numbers from NCBI database (http://www.ncbi.nlm.nih.gov/Entrez/index.html).
[e] References relate to INTERPRO (http://ebi.ac.uk/interpro/; (lit)) and PFAM databases (home-page; [Bateman, 2002 #6867]).

Cyanophycin. In Bac10h10, a *cphA* gene encoding cyanophycin synthetase was annotated for bp 1-1302 with highest similarity to the corresponding homolog in the human pathogen *Francisella tulariensis*. This protein catalyzes the ATP-dependent polymerization of arginine and aspartate to multi-L-arginyl-poly-L-aspartic acid as a storage polymer for both nitrogen and carbon.

Carbon metabolism.

Opine oxidase.

The *ooxA* and *ooxB* genes on Bac8g3 (bp 2-1641) encode the opine oxidase subunits A and B. Both proteins show the highest similarity to homologs in Betaproteobacteria such as the cis-dichlorathane degrading *Polaromonas* sp. (*ooxA*) and the pathogen *Bordetella bronchiseptica* (*ooxB*). Opine oxidase catalyzes the degradation of opines that are products of the NAD(P)H-dependent reductive condensation between an α-keto acid and the α- or ω-NH_2 group of an amino acid. In marine invertebrates opines are a common product of anaerobic metabolism (29, 53).

Discussion

Construction and analysis of the BAC library

The analysis of the BAC library showed a high fraction of genes of bacterial origin, despite the fact that no separation steps to isolate the DNA from bacterial symbionts and eukaryotic hosts were used. This was unexpected because rough estimates of symbiont and host cell numbers suggested that eight times more eukaryotic than prokaryotic genomes occur in the worms. The reason for the high portion of bacterial sequences could be preferentially insertion of bacterial DNA into BAC vectors.

The BAC library contained numerous symbiotic sequences from *O. algarvensis* and *O. ilvae,* indicating a high yield of genomes from symbionts in the library. Out of 1152 analyzed BAC clones, 16 contained symbiotic sequences such as 16S rRNA genes, *aprA* genes coding for adenosine-5'-phosphosulfate

(APS) reductase, and *cbbL* genes coding for ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). Some genes were 100% identical at the nucleotide level in several different BAC clones, e.g. the *cbbL* gene from the *O. algarvensis* symbiont of which identical copies were found in six BAC clones. Such a high number of identical sequences is surprising given the pooled nature of the DNA sample, with roughly 10^6 bacteria per worm and 520 worms used for DNA isolation. Assuming that no cloning bias occurred, this indicates little to no heterogeneity of the symbiont population, at least for important functional genes such as *cbbL* gene.

Carbon dioxide fixation

RubisCO

Carbon dioxide fixation by the chemoautotrophic, sulfur-oxidizing bacteria of gutless oligochaetes is essential for the transfer of carbon from the symbionts to the host (20-22, 39). The enzyme RubisCO is characteristic for CO_2 fixation via the Calvin-Benson-Bassham (CBB) cycle and was used as a phylogenetic marker in this study. In chemoautotrophic symbionts, two forms of RubisCO have been found: form I encoded by the gene *cbbL* and form II by the gene *cbbM* (10, 39, 55, 59, 61). These two forms differ in their molecular structure and their specificity for CO_2 (63, 64, 69). The highly distributed form I RubisCO is subdivided into four classes based on inferred homology. Forms IA and IC are primarily present in Proteobacteria whereas forms IB and ID are more prevalent in cyanobacteria and chloroplasts.

Two phylogenetically distinct types of RubisCO form IA sequences were found *in O. algarvensis*, BAC RubisCO 1 and 2, while only RubisCO 1 was found in *O. ilvae*. The RubisCO 1 sequences are most closely related to form IA RubisCO sequences from gammaproteobacterial chemoautotrophic, sulfur-oxidizing symbionts of two other gutless oligochaetes, *I. leukodermatus* and *I. makropetalos* (7). These two *Inanidrilus* species harbor only a single gammaproteobacterial symbiont, Gamma 1, that is a chemoautotrophic sulfur oxidizer, and only a single RubisCO phylotype belonging to the RubisCO 1 group

was found in these hosts (7). The close relationship of the RubisCO 1 sequences from *Inanidrilus* Gamma 1 symbionts with RubisCO 1 from *O. algarvensis* and *O. ilvae* suggests that the *Olavius* RubisCO 1 sequences originated from the Gamma 1 symbionts of these hosts. Correspondingly, the RubisCO 2 sequence in *O. algarvensis* may have originated from their Gamma 2 symbionts. The lack of a RubisCO 2 sequence in *O. ilvae*, that also has Gamma 2 symbionts, could be explained by the fact that only 50 clones from a single individual were sequenced, and we are currently investigating more clones and individuals.

Alternatively, the RubisCO 1 and 2 sequences could originate from the same symbiont. Two copies of form I RubisCO were reported in several species such as *Nitrobacter hamburgensis*, *Hydrogenvibrio marinus*, *Acidithiobacillus ferrooxidans*, and *Allochromatium vinosum* (30, 32, 34, 40, 60, 68).

Cbb locus

Full sequencing of the BAC clone containing a RubisCO 2 sequence from one of the two gammaproteobacterial symbionts of *O. algarvensis* revealed an entire *cbb* locus. This symbiotic gene cluster consists of 10 adjacent genes and is the most contiguous *cbb* gene cluster currently known within the form IA RubisCO clusters (40, 59, 60). In contrast to other form IA RubisCO clusters that do not contain the *cbbP* gene, the *O. algarvensis cbb* gene cluster contains this gene coding for phosphoribulokinase (PRK), and thus carries both key enzymes for CO_2-fixation via the CBB cycle, PRK and the *cbbLS*-encoded RubisCO.

The arrangement and relationship of genes within the symbiont *cbb* locus indicates that it could be a hybrid composed of two parts from different origins. While the *cbbLSQOZ* genes showed similar gene order and close phylogenetic relationship to homologs from *cbb* locus of form IA RubisCO the *cbbRFPTA* genes from the first part of the locus correspond in gene order and phylogeny to homologs from *cbb* locus of form IC RubisCO.

Sulfur metabolism

APS reductase

Since *O. algarvensis* and *O. ilvae* harbor sulfur-oxidizing and sulfate-reducing symbionts, sulfur energy metabolism plays an important role in these symbiotic associations (20, 57). One of the key enzymes involved in the oxidative and reductive pathway of sulfur metabolism is adenosine-5'-phosphosulfate (APS) reductase (24, 35). Depending on the mode of sulfur metabolism two different directions of the enzymatic reaction occur. In sulfate reducers, APS reductase catalyzes the two-electron reduction of APS to sulfite and AMP. The same enzyme activity is used for the reverse reaction in a variety of sulfur oxidizers generating APS as the product. APS reductase is encoded by two subunits, alpha and beta (*aprAB*), that appear to form a 1:1 $\alpha\beta$ heterodimer. Since both subunits are highly conserved, phylogenetic analyses of this enzyme can be used to gain information on their evolution (25).

Two types of APS sequences occur in *O. algarvensis* and *O. ilvae* that are related to sulfur-oxidizing bacteria, APS 1 and 2. The APS 1 sequences are most closely related to *aprA* genes from symbionts in the gutless oligochaetes, *I. leukodermatus* and *I. makropetalos* (7). As described above the two *Inanidrilus* species contain only a single gammaproteobacterial thiotroph, the Gamma 1 symbiont, and only a single *cbbL* and *aprA* phylotype has been found in these worms suggesting that these originated from the Gamma 1 symbionts. The close phylogenetic relationship of the *O. algarvensis* and *O. ilvae* APS 1 sequences to the *aprA* genes from the *Inanidrilus* symbionts indicates that these also originated from the Gamma 1 symbionts. Correspondingly, the APS 2 sequences related to *A. vinosum* could originate from the Gamma 2 symbionts of *O. algarvensis* and *O. ilvae*. It is highly unlikely that they originated from the sulfate-reducing bacteria symbionts as the APS 2 sequences are unrelated to the *aprA* genes of sulfate-reducing bacteria. Another unlikely explanation is that the APS 1 and 2 sequences are multiple genes from a single symbiont, as two sets of these genes in a single organism have not been observed to date (24).

The close phylogenetic relationship of the APS 3 sequences from *O. algarvensis* and *O. ilvae* to the *aprA* genes of sulfate-reducing bacteria suggests that these originated from the sulfate-reducing symbionts in the worms. Two species of sulfate-reducing symbionts occur in each host, Delta 1 and Delta 2. The two APS 3 sequences from *O. algarvensis*, APS 3A and B, are therefore assumed to originate from the Delta 1 and 2 symbionts from this species, but a clear assignment of the two *aprA* genes to the two symbionts is not possible. In *O. ilvae*, only a single APS 3 sequence was found that originated from either Delta 1 or 2 symbiont. The assignment of the BAC APS 3 sequence is not possible, as this sequence differs from those found in individual worms. The close phylogenetic relationship of this sequence to those from free-living, sulfate-reducing bacteria indicates that it originates from a sulfate reducer that has not yet been identified in the APS reductase libraries from single worms. As a second *aprA* gene was not found in *O. ilvae*, it is possible that the BAC sequence originated from this "missing" *O. ilvae* sulfate-reducing symbiont.

Dsr locus

The entire *dsr* gene cluster involved in sulfur metabolism was revealed by sequencing of the Bac5g12 insert from the *O. algarvensis* symbiont. This locus consists of 14 genes, *dsrABEFHCMKLJOPNR* that play an essential role in the oxidation of intracellulary stored sulfur, an obligate intermediate during the oxidation of sulfide to thiosulfate (11, 54). All symbiont *dsr* genes are closely related to homologs from the sulfur-oxidizing bacteria *A. vinosum* and *T. denitrificans* which are the only ones available in the database. Gene order is also highly conserved, which is surprising since these bacteria differ in their phylogeny and life-style. *A. vinosum* and *T. denitrificans* are free-living bacteria of the Gamma and Betaproteobacteria, while the gammprotebacterial symbionts live in obligate symbiosis with *O. algarvensis*. The high similarity of these three *dsr* gene clusters indicates their importance in the oxidation of sulfur. As more genomes from sulfur-oxidizing bacteria become available, it will be intriguing to see if this gene organization is broadly distributed within the Proteobacteria.

A second *dsr* gene cluster, *dsrEF*, was identified by end-sequencing of the BAC clones. These *dsr* genes are similar but not identical (56-69% aa sequence identity) to those of the Bac5g12 *dsr* locus indicating their origin from another sulfur-oxidizing symbiont or the existence of a second copy of a *dsr* locus. The latter is less unlikely since multiple sets of *dsr* loci do not occur in *A. vinosum* and *T. denitrificans*.

Sulfur oxidation protein SoxB

The soxB-encoded protein is part of a multienzyme complex involved in the wellstudied and widely distributed pathway for the oxidation of thiosulfate to sulfate (3, 23, 36). Thiosulfate is a reduced sulfur compound that could be used by the thiotrophic symbionts as an electron donor. The *soxB* gene occurred between the *dsr* and *cbb* loci of the *O. algarvensis* Bac5g12. Surprisingly, no further *sox* genes were observed upstream and downstream of the *soxB* gene that are regularly present in sox operons of other sulfur-oxidizing bacteria (23). To demonstrate a functional pathway of thiosulfate oxidation in *O. algarvensis* symbiosis, identification of additional *sox* genes is needed.

Metabolic island

The contiguous sequence from *O. algarvensis* symbiont contains *dsr* gene cluster involved in intracellular sulfur oxidation and *cbb* gene cluster participating in the CO_2 fixation demonstrating the sulfur-oxidizing, chemoautotrophic nature of this bacterium. The adjacent location of these two metabolic clusters, separated to each other by only 9.3 kb, is conspicuous and it is intriguing to speculate for a putative genomic island encoding functions for a sulfur-oxidizing chemoautotrophic life-style. Among prokaryotic bacteria genomic islands are common and confer the owner with different advantages including for example iron uptake, antibiotic resistance, expression of toxins, nitrogen fixation, or degradation and metabolism of certain compounds (14).

Nitrogen metabolism

End sequencing revealed the genes *ureFG*, predicted to code for accessory proteins of urease. Urease hydrolyzes urea to ammonia and CO_2 and is a widely distributed enzyme among a number of bacteria, plants, fungi, and algae (44). It is not known how gutless oligochaetes excrete their nitrogenous waste compounds as they lack an excretory system. It is tempting to speculate that the oligochaetes excrete nitrogen in the form of urea, as shown in the vent tube *Riftia pachyptila* (12) that could be taken up and degraded by the symbionts. The symbiotic recycling of urea would be advantageous for both partners, by allowing the detoxification of nitrogenous waste for the host and by supplying the symbionts with a rich source of nitrogen.

Another enzyme involved in nitrogen metabolism found in the BAC library was *cphA*-encoded cyanophicin synthetase. This protein catalyses the polymerization of arginine and aspartate as a storage polymer for both nitrogen and carbon and is predominantly present in cyanobacteria but also in several non-cyanobacterial species (37, 38). The ability to accumulate two amino acids, arginine and aspartate, in bacterial symbionts would imply their active biosynthesis or import. In the case of active biosynthesis, the amino acids could be stored in the form of cyanophicin as a nitrogen and carbon source or exported to the host. In contrast, import would indicate supply of the symbionts with arginine and aspartate from the environment or by the host. Exchange of amino acids between symbiotic partners was reported in obligate associations between aphids and bacteria of the genus *Buchnera* that is also conceivable in the endosymbiotic coexistence of bacteria and gutless oligochaetes (Shigenobu et al., 2000).

Carbon metabolism

The enzyme opine oxidase was identified by the presence of the coding genes *ooxA* and *ooxB*. In marine invertebrates this protein catalyzes the degradation of opines, common end products of anaerobic muscular metabolism (53). Gutless oligochaetes are often exposed to anaerobic conditions and could produce opine

as an anaerobic metabolite. The opine oxidase of the symbionts would enable them to utilize the waste products of the worm that would otherwise be excreted, allowing the recycling of valuable carbon compounds.

Conclusion

Comparative metagenomics has provided a successful approach to investigate the metabolic diversity of uncultivable bacterial symbionts in the gutless oligochaetes species *O. algarvensis* and *O. ilvae*. In the BAC library, functional symbiotic genes involved in oxidative and reductive sulfur metabolism and in CO_2 fixation were identified and could be clearly assigned to symbionts from the two host species. Full sequencing of a 51 kb BAC from the thiotrophic symbiont of *O. algarvensis* revealed a large metabolic island containing significant metabolic pathways such as intracellular sulfur oxidation encoded by the *dsr* gene cluater and CO_2 fixation via the CBB cycle encoded by the *cbb* gene cluster. Random end-sequencing of BAC clones identified novel genes involved in nitrogen and carbon metabolism not described previously in these symbioses.

References

1. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.
2. Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.
3. Bamford, V. A., S. Bruno, T. Rasmussen, C. Appia-Ayme, M. R. Cheesman, B. C. Berks, and A. M. Hemmings. 2002. Structural basis for the oxidation of thiosulfate by a sulfur cycle enzyme. The EMBO J. 21:5599-5610.

4. Bateman, A., E. Birney, L. Cerruti, R. Durbin, L. Etwiller, and S. R. Eddy. 2002. The Pfam Protein Families Database. Nucleic Acids Res. 30:276-280.
5. Beja, O., L. Aravind, E. V. Koonin, M. T. Suzuki, A. Hadd, L. P. Nguyen, S. B. Jovanovich, C. M. Gates, R. A. Feldman, J. L. Spudich, E. N. Spudich, and E. F. DeLong. 2000. Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science 289:1902-1906.
6. Blazejak, A., C. Erséus, R. Amann, and N. Dubilier. 2005. Coexistence of bacterial sulfide-oxidizers, sulfate-reducers, and spirochetes in a gutless worm (Oligochaeta) from the Peru margin. Appl. Environ. Microbiol. 71:1553-1561.
7. Blazejak, A., J. Küver, R. Amann, and N. Dubilier. Phylogeny of 16S rRNA, RubisCO, and APS reductase genes from Gamma and Alpha proteobacterial symbionts in gutless marine worms (Oligochaeta) from Bermuda and Bahamas. In preparation .
8. Boeckmann, B., A. Bairoch, R. Apweiler, M. C. Blatter, A. Estreicher, E. Gasteiger, M. J. Martin, K. Michoud, O. D. C., I. Phan, S. Pilbout, and M. Schneider. 2003. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. Nucleic Acids Res. 31:365-370.
9. Bright, M., and O. Giere. 2005. Microbial symbiosis in Annelida. Symbiosis 38:1-45.
10. Cavanaugh, C. M., and J. J. Robinson. 1996. CO_2 fixation in chemoautotrophic-invertebrate symbioses: expression of form I and form II RubisCO. Lidstrom, M. E. and Tabita, F. R. (ed.), Microbiol growth on C_1 compounds: Proceedings of the 8th International Symposium on Microbial Growth on C_1 Compounds. Kluwer Academic Publishers, Dordrecht.
11. Dahl, C., S. Engels, A. S. Pott-Sperling, A. Schulte, J. Sander, Y. Lübbe, O. Deuster, and D. C. Brune. 2005. Novel genes of the *dsr* gene cluster and evidence for close interaction of DSR proteins during sulfur oxidation in the phototrophic sulfur bacterium *Allochromatium vinosum*. J. Bacteriol. 187:1392-1404.

12. De Cian, M.-C., M. Regnault, and F. H. Lallier. 2000. Nitrogen metabolites and related enzymatic activities in the body fluids and tissues of the hydrothermal vent tubeworm *Riftia pachyptila*. J. Exp. Biol. 203:2907-2920.
13. Delcher, A. L., D. Harmon, S. Kasif, O. White, and S. L. Salzberg. 1999. Improved microbial gene identification with GLIMMER. Nucleic Acids Rev. 27:4636-4641.
14. Dobrindt, U., B. Hochhut, U. Hentschel, and J. Hacker. 2004. Genomic islands in pathogenic and environmental microorganisims. Nature Rev. 2:414-424.
15. Dubilier, N. 2003. Multiple bacterial endosymbionts in gutless marine worms: competitors or partners? Nova Acta Leopol. 333:107-112.
16. Dubilier, N., R. Amann, C. Erséus, C. M. Cavanaugh, and O. Giere. 1999. Molecular evolution of the symbiosis between gutless marine oligochaetes and their bacterial symbionts. Zoology 102:6.
17. Dubilier, N., R. Amann, C. Erséus, G. Muyzer, S. Y. Park, O. Giere, and C. M. Cavanaugh. 1999. Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochaete *Olavius loisae* (Annelida). Mar. Ecol. Prog. Ser. 178:271-280.
18. Dubilier, N., A. Blazejak, and C. Rühland. In press. Symbioses between bacteria and gutless marine oligochaetes. *In* J. Overmann (ed.), In Molecular Basis of Symbiosis. Springer-Verlag, New York.
19. Dubilier, N., O. Giere, D. L. Distel, and C. M. Cavanaugh. 1995. Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm (Oligochaeta, Annelida) by phylogenetic 16S rRNA sequence analysis and in situ hybridization. Appl. Environ. Microbiol. 61:2346-2350.
20. Dubilier, N., C. Mülders, T. Ferdelman, D. de Beer, A. Pernthaler, M. Klein, M. Wagner, C. Erséus, F. Thiermann, J. Krieger, O. Giere, and R. Amann. 2001. Endosymbiotic sulphate-reducing and sulphide-oxidizing bacteria in an oligochaete worm. Nature 411:298-302.

21. Felbeck, H., J. J. Childress, and G. N. Somero. 1981b. Calvin-Benson cycle and sulfide oxidation enzymes in animals from sulfide-rich habitats. Nature 293:291-293.
22. Felbeck, H., G. Liebezeit, R. Dawson, and O. Giere. 1983. CO_2 fixation in tissues of marine oligochaetes (*Phallodrilus leukodermatus* and *P. planus*) containing symbiotic, chemoautotrophic bacteria. Mar. Biol. 75:187-191.
23. Friedrich, C. G., D. Rother, F. Bardischewsky, A. Quentmeier, and J. Fischer. 2001. Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism? Appl. Environ. Microbiol. 67:2873-2882.
24. Friedrich, M. W. 2002. Phylogenetic analysis reveals multiple lateral transfers of adenosine-5 '-phosphosulfate reductase genes among sulfate-reducing microorganisms. J. Bacteriol. 184:278-289.
25. Fritz, G., H. Buchert, K. Huber, K. O. Stetter, and P. M. H. Kroneck. 2000. Adenylylsulfate reductases from archaea and bacteria are 1:1 alpha beta heterodimeric iron-sulfur flavoenzymes-high similarity of molecular properties emphasizes their central role in sulfur metabolism. FEBS Lett. 473:63-66.
26. Gibson, J. L., and F. R. Tabita. 1997. Analysis of the *cbbXYZ* operon in *Rhodobacter sphaeroides*. J. Bacteriol. 179:663-669.
27. Giere, O., and C. Erséus. 2002. Taxonomy and new bacterial symbioses of gutless marine Tubificidae (Annelida, Oligochaeta) from the Island of Elba (Italy). Org. Divers. Evol. 2:289-297.
28. Giere, O., and J. Krieger. 2001. A triple bacterial endosymbiosis in a gutless oligochaete (Annelida): ultrastructural and immunocytochemical evidence. Invertebr. Biol. 120:41-49.
29. Grieshaber, M. K., I. Hardewig, U. Kreutzer, and H.-O. Pörtner. 1994. Physiological and metabolic responses to hypoxia in invertebrates. Rev. Physiol. Biochem. Pharmacol. 125:43-147.

30. Harris, S., A. Ebert, E. Schutze, M. Diercks, E. Bock, and J. M. Shively. 1988. Two different genes and gene products for the large subunit of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RubisCOase) in *Nitrobacter hamburgensis*. FEMS Microbiol. Letters 49:267-271.

31. Hayashi, N. R., H. Arai, T. Kodama, and Y. Igarashi. 1997. The novel genes, *cbbQ* and *cbbO*, located downstream from the RubisCO genes of *Pseudomonas hydrogenothermophila*, affect the conformational states and activity of RubisCO. Biochem. Biophys. Res. Commun. 265:177-183.

32. Hayashi, N. R., A. Oguni, T. Yaguchi, S.-Y. Chung, H. Nishihara, T. Kodama, and Y. Igarashi. 1998. Different properties of gene products of three sets ribulose 1,5-bisphosphate carboxylase/oxygenase from a marine obligately autotrophic hydrogen-oxidizing bacterium, *Hydrogenovibrio marinus* strain MH-110. J. Ferment. Bioeng. 85:150-155.

33. Healy, F. G., R. M. Ray, H. C. Aldrich, A. C. Wilkie, and L. O. Ingram. 1995. Direct isolation of functional genes encoding celulases from the microbial consortia in a thermophilic, anaerobic digester maintained on lignocellulose. Appl. Microbiol. Biotechnol. 43:667-674.

34. Heinhorst, S., S. H. Baker, D. R. Johnson, P. S. Davies, G. C. Cannon, and J. M. Shively. 2002. Two copies of form I RuBisCO genes in *Acidithiobacillus ferrooxidans* ATCC 23270. Curr. Micriobiol. 45:115-117.

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

36. Kelly, D. P., J. K. Shergill, W.-P. Lu, and A. P. Wood. 1997. Oxidative metabolism of inorganic sulfur compounds by bacteria. Antonie van Leeuwenhoek 71:95-107.

37. Krehenbrink, M., F.-B. Oppermann-Sanio, and A. Steinbüchel. 2002. Evaluation of non-cyanobacterial genome sequences for occurance of genes encoding proteins homologous to cyanophycin synthetase and

cloning of an active cyanophycin synthetase from *Acinetobacter* sp. strain DSM 587. Arch. Microbiol. 177:371-380.

38. Krehenbrink, M., and A. Steinbüchel. 2004. Partial purification and characterization of a non-cyanobacterial cyanophycin synthetase from *Acinetobacter calcoaceticus* strain ADP1 with regard to substrate specificity, substrate affinity and binding to cyanophycin. Microbiology 150:2599-2608.
39. Krieger, J., O. Giere, and N. Dubilier. 2000. Localization of RubisCO and sulfur in endosymbiotic bacteria of the gutless marine oligochaete *Inanidrilus leukodermatus* (Annelida). Mar. Biol. 137:239-244.
40. Kusian, B., and B. Bowien. 1997. Organization and regulation of cbb CO_2 assimilation genes in autotrophic bacteria. FEMS Microbiol. Rev. 21:135-155.
41. Manz, W., M. Eisenbrecher, T. R. Neu, and U. Szewzyk. 1998. Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol. Ecol. 25:43-61.
42. Meyer, F., A. Goesmann, A. C. McHardy, D. Bartels, T. Bekel, and J. Clausen. 2003. GenDB - an open source genome annotation system for prokaryote genomes. Nucleic Acids Res. 31:2187-2195.
43. Meyerdierks, A., M. Kube, T. Lombardot, K. Knittel, M. Bauer, F.-O. Glöckner, R. Reinhardt, and R. Amann. 2005. Insights into the genomes of archaea mediating the anaerobic oxidation of methane. Environ. Microbiol.
44. Mobley, H. L. T., and R. P. Hausinger. 1989. Microbial ureases: significance, regulation, and molecular characterization. Microbiol. Rev. 53:85-108.
45. Molitor, M., C. Dahl, I. Molitor, U. Schäfer, N. Speich, R. Huber, R. Deutzmann, and H. G. Trüper. 1998. A dissimilatory sirohaem-sulfite-reductase-type protein from the hyperthermophilic archaeon *Pyrobaculum islandicum*. Microbiology 144:529-541.

46. Moller, S., M. D. R. Croning, and R. Apweiler. 2001. Evaluation of methods for the prediction of membrane spanning regions. Bioinformatics 17:646-653.

47. Mulder, N. J., R. Apweiler, T. K. Attwood, B. A., A. Bateman, D. Binns, P. Bradley, P. Bork, P. Bucher, L. Cerutti, R. Copley, E. Courcelle, U. Das, R. Durbin, W. Fleischmann, J. Gough, D. Haft, N. Harte, N. Hulo, D. Kahn, A. Kanapin, M. Krestyaninova, D. Lonsdale, R. Lopez, I. Letunic, M. Madera, J. Maslen, J. McDowall, A. Mitchell, A. N. Nikolskaya, S. Orchard, M. Pagni, C. P. Ponting, E. Quevillon, J. Selengut, C. J. Sigrist, V. Silventoinen, D. J. Studholme, R. Vaughan, and C. H. Wu. 2005. InterPro, progress and status in 2005. Nucleic Acids Res. 33.

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

49. Nielsen, H., J. Engelbrecht, S. Brunak, and v. H. G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. 10:1-6.

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

51. Paulsen, I. T., M. H. Brown, and R. A. Skurray. 1996. Proton-dependent multidrug efflux systems. Microbiological reviews :575-608.

52. Piel, J. 2002. A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. Proc. Natl. Acad. Sci. 99:14002-14007.

53. Pörtner, H. O. 2002. Environmental and functional limits to muscular exercise and body size in marine invertebrate athletes. Com. Bioche. Physiol. A 133:303-321.

54. Pott, A. S., and C. Dahl. 1998. Sirohaem sulfite reductase and other proteins encoded by genes at the *dsr* locus of *Chromatium vinosum* are involved in the oxidation of intracellular sulfur. Microbiology 144:1881-1894.

55. Robinson, J. J., and C. M. Cavanaugh. 1995. Expression of form I and form II RubisCO in chemoautotrophic symbioses: implications for the interpretation of stable carbon isotope values. Limnol. Oceanogr. 40:1496-1502.

56. Rondon, M. R., P. R. August, A. D. Bettermann, S. F. Brady, T. H. Grossman, M. R. Liles, L. K.A., B. A. Lynch, I. A. MacNeil, C. Minor, C. L. Tiong, M. Gilman, M. S. Osburne, J. Clardy, J. Handelsman, and R. M. Goodman. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl. Environ. Microbiol. 66:2541-2547.

57. Rühland, C., A. Blazejak, A. Loy, M. Wagner, C. Erséus, R. Amann, and N. Dubilier. Multiple symbioses with sulfide oxidizers, sulfate reducers, and spirochetes in two co-occurring species of gutless worms (Oligochaeta) from the Mediterranean. In preparation .

58. Salzberg, S. L., A. L. Delcher, S. Kasif, and O. White. 1998. Microbial gene identification using interpolated Markov models. Nucleic Acids Res. 26:544-548.

59. Schwedock, J., T. L. Harmer, K. M. Scott, H. J. Hektor, A. P. Seitz, M. C. Fontana, D. L. Distel, and C. M. Cavanaugh. 2004. Characterization and expression of genes from the RubisCO gene cluster of the chemoautotrophic symbiont of *Solemya velum*: *cbbLSQO*. Arch. Microbiol. 182:18-29.

60. Shively, J. M., G. van Keulen, and W. G. Meijer. 1998. Something from almost nothing: Carbon dioxide fixation in chemoautotrophs. Annu. Rev. Microbiol. 52:191-230.

61. Stein, J. L., M. Haygood, and H. Felbeck. 1990. Nucleotide sequence and expression of a deep-sea ribulose-1,5-bisphosphate carboxylase gene cloned from a chemoautotrophic bacterial endosymbiont. Proc. Natl. Acad. Sci. 87:8850-8854.

62. Stein, J. L., T. L. Marsh, K. Y. Wu, H. Shizuya, and E. F. DeLong. 1996. Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine Archaeon. J. Bacteriol. 178:591-599.

63. Tabita, F. R. 1995. The biochemistry and metabolic regulation of carbon metabolism and CO_2 fixation in purple bacteria. *In* M. M. Tabita, Blankenship R. E., Bauer C. E. (ed.), Anoxygenic photosynthetic bacteria. Kluwer, Dordrecht.

64. Tabita, F. R. 1988. Molecular and cellular regulation of autotrophic carbon dioxide fixation in microorganisms. Microbiol. Rev. 52:155-189.

65. Tringe, S. G., C. von Mering, A. Kobayashi, A. A. Salamov, K. Chen, H. W. Chang, M. Podar, J. M. Short, E. J. Mathur, J. C. Detter, P. Bork, P. Hugenholtz, and E. M. Rubin. 2005. Comparative metagenomics of microbial communities. Science 308.

66. Tyson, G. W., J. Chapman, P. Hugenholtz, E. E. Allen, R. J. Ram, P. M. Richardson, V. V. Solovyev, E. M. Rubin, D. S. Rokhsar, and J. F. Banfield. 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428:37-43.

67. Venter, J. C., K. Remington, J. F. Heidelberg, A. L. Halpern, D. Rusch, J. A. Eisen, D. Wu, I. Paulsen, K. E. Nelson, W. Nelson, D. E. Fouts, S. Levy, A. H. Knap, M. W. Lomas, K. Nealson, O. White, J. Peterson, J. Hoffman, R. Parsons, H. Baden-Tillson, C. Pfannkoch, Y.-H. Rogers,

and H. O. Smith. 2004. Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66-74.

68. Viale, A. M., H. Kobayashi, and T. Akazawa. 1989. Expressed genes for plant-type ribulose 1,5-bisphosphate carboxylase/oxygenase in the photosynthetic bacterium *Chromatium vinosum*, which possesses two complete sets of the genes. J. Bacteriol. 171:2391-2400.
69. Watson, G. M. F., and F. R. Tabita. 1997. Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: a molecule for phylogenetic and enzymological investigation. FEMS Microbiol. Letters 146:13-22.
70. Wu, C., Z. Xu, and H.-B. Zhang. 2004. DNA Libraries, p. 385-425. *In* R. A. Meyers (ed.), Encyclopedia of Molecular Cell Biology and Molecular Medicine, vol. 3. Wiley-VCH, Weinheim.
71. Zhang, H.-B. 2000. Construction and manipulation of large-insert bacterial clone libraries. Manual, Texas A&M University, Texas, USA .

4

Multiple symbioses with sulfur oxidizers, sulfate reducers, and spirochetes in two gutless marine worms (Oligochaeta) from Mediterranean sediments low in sulfide

Caroline Rühland*, Anna Blazejak*, Alexander Loy, Michael Wagner, Christer Erséus, Rudolf Amann, and Nicole Dubilier

* Both authors contributed equally to the manuscript

Manuscript in preparation

Abstract

Olavius algarvensis, a gutless marine oligochaete from Mediterranean sediments low in sulfide, is known to host sulfur-oxidizing Gammaproteobacteria and sulfate-reducing Deltaproteobacteria, suggesting a syntrophic sulfur cycle in this worm (Dubilier et al. 2001). In this study we identified bacterial endosymbionts of a second host species, *Olavius ilvae*, from the same habitat. Two symbionts of this host, Gamma 1 and Delta 1, are closely related to the described symbionts of *Olavius algarvensis*. In addition second gamma- and a second deltaproteobacterial symbiont phylotype have been detected in *Olavius ilvae.* They are phylogenetically distinct to the Gamma 1 and Delta 1 group. Reinvestigation of *Olavius algarvensis* revealed a second gamma and a second delta symbiont in this host as well. While the novel Gamma 2 symbiont sequences of both hosts group together in a non-symbiotic clade with sequences from marine sediment, the new Delta 2 symbiont sequences were only distantly related to each other. As a fifth symbiotic bacterium a spirochete was found in *O. algarvensis*. So far this high diversity of bacterial symbiont phylotypes in gutless oligochaetes is unique among marine endosymbiosis. Considering previous studies and the data of the present study four symbiotic bacterial groups of gutless oligochaetes emerge: The Alpha-, Gamma- and Deltaproteobacteria as well as the spirochetes are regular symbionts of oligochaete species. Based on the current datasets Gamma 1, Alpha 1, Delta 1 and the spirochete symbiont sequences form symbiont specific clades, while the additional symbionts fall into non-symbiotic groups.

C List of Publications

Anna Blazejak, Christer Erséus, Rudolf Amann, and Nicole Dubilier. 2005. Coexistence of bacterial sulfide oxidizers, sulfate reducers, and spirochetes in a gutless worm (Oligochaeta) from the Peru margin. Applied and Environmental Microbiology. 71: 1553-1561

Anna Blazejak, Jan Kuever, Christer Erséus, Rudolf Amann, and Nicole Dubilier. Phylogeny of 16S rRNA, RubisCO, and APS reductase genes from gamma- and alphaproteobacterial symbionts in gutless marine worms (Oligochaeta) from Bermuda and Bahamas. Applied and Environmental Microbiology. 72: 5527-5536

Anna Blazejak, Michael Richter, Zhanyou Xu, Amelia Rotaru, Michael Kube, Hong-Bin Zhang, Rudolf Amann, Frank Oliver Glöckner, and Nicole Dubilier. Metagenomic analysis of co-occurring symbionts in marine worms from the Mediterranean. *Manuscript in preparation.*

Caroline Rühland*, Anna Blazejak*, Alexander Loy, Michael Wagner, Christer Erséus, Rudolf Amann, Nicole Dubilier. Multiple symbioses with sulfur-oxidizers, sulfate-reducers and spirochetes in two gutless marine worms (Oligochaeta) Mediterranean sediments low in sulfide. *Manuscript in preparation.*

* Both authors contributed equally to the manuscript

Nicole Dubilier, Anna Blazejak, and Caroline Rühland. Molecular basis of symbiosis: Symbioses between bacteria and gutless marine oligochaetes. Springer-Verlag, In: J. Overmann (ed) Molecular Basis of Symbiosis. Springer Verlag, New York. Pp 251-275

Christer Erséus, Anna Blazejak, Mari Källersjö, Bodil Cronholm and Nicole Dubilier. Coevolution in gutless marine oligochaetes (Annelida, Clitellata,

Tubificidae) and their chemoautotrophic symbionts, inferred by analysis of DNA data. *Manuscript in preparation.*

Ursula Werner, Anna Blazejak, Paul Bird, Gaby Eickert, Dirk de Beer. Photosynthesis in coral reef sediments (Heron Reef, Australia). *Manuscript in preparation.*

Duperron Sebastien, Claudia Bergin, Frank Zielinski, Anna Blazejak, Annelie Pernthaler, Zoe P. McKiness, Eric DeChaine, Colleen Cavanaugh and Nicole Dubilier. 2006 A dual symbiosis shared by two mussel species, *Bathymodiolus azoricus* and *B. puteoserpentis* (Bivalvia: Mytilidae), from hydrothermal vents along the northern Mid-Atlantic Ridge. Environmental Microbiology. 8: 1441-1447

Danksagung

Die vorliegende Arbeit wurde in der Abteilung Molekulare Ökologie am Max-Planck-Institut für Marine Mikrobiologie in Bremen unter der Leitung von Herrn Prof. Dr. Rudolf Amann von März 2002 bis Oktober 2005 angefertigt.

Ich möchte mich herzlich bei Herrn Prof. Dr. Rudolf Amann für die freundliche Aufnahme in seine Arbeitsgruppe und seine Unterstützung bedanken. Insbesondere bin ich dankbar, daß mir die Teilnahme an dem "Microbial diversity" Kurs in Woods Hole ermöglicht wurde, der mir neue Einsichten verschaffte und daher unvergeßlich bleiben wird.

Vielen Dank auch an Herrn Prof. Dr. Ulrich Fischer, der das Zweitgutachten dieser Arbeit übernimmt, sowie den weiteren Mitgliedern des Prüfungskomitees: Herrn Prof. Dr. Rudolf Amann, Frau Dr. Nicole Dubilier, Herrn Prof. Thomas Hoffmeister, Tina Lösekann, und Pamela Ohlf für Ihre Zeit und Mühe.

Mein ganz besonderer Dank gilt Frau Dr. Nicole Dubilier für die Betreuung der Arbeit, gerade in der letzten, intensiven Phase. Nicole ist es gelungen, mich für die wunderbare Symbioseforschung von kleinen, weißen Würmchen zu begeistern. Ihr Enthusiasmus, Ideenreichtum und ein ‘rund um die Uhr’ offenes Ohr für neue Konzepte haben diese Arbeit maßgeblich bereichert.

Vielen Dank an meine Bürokollegen, Katrin, Caroline, Christine und Torben für das kreative Arbeitsklima und die tatkräftige Hilfe, meine Weggenossen Tina, Caroline, Falk, Cecilia und Chris, sowie der gesamten Abteilung der molekularen Ökologie. An dieser Stelle ein besonderes Dankeschön sowohl an Katrin Knittel, Christine Beardsley, Tina Lösekann, Michael Richter, Anke Meyerdierks und Marc Mußmann für Ihre Hilfsbereitschaft als auch Silke Wetzel, Amelia Rotaru, Wiebke Krämer und Julia Herglotz für Ihre praktische Unterstützung. Durch Euch ist Vieles einfacher geworden!

Mojej kochanej rodzinie dziękuje z całego serca za to że cały czas wierzyli we mnie und meinem liebsten Ted für seine unendliche Geduld und Liebe.