universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Molecular identification and life cycle of the giant ectosymbiont of *Eubostrichus dianaea*

verfasst von

Nika Pende

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 444
Studienrichtung lt. Studienblatt:	Diplomstudium Ökologie
Betreut von:	Univ.-Prof. i.R. Dr. Jörg Ott

Inhaltsverzeichnis

To dream is happiness;

to wake is life.

Victor Hugo

EINLEITUNG

Symbiose

Symbiose, aus dem Griechischen abgeleitet („*syn*– zusammen“ und „*bios*– leben“), bezeichnet in der Biologie jegliches Zusammenleben von Individuen unterschiedlicher Art. Dieser Begriff wurde von dem Arzt und Botaniker Anton de Bary 1878 auf der *51. Versammlung Deutscher Naturforscher und Ärzte* in Kassel eingeführt (Bary, 1879). Er gebrauchte Symbiose als Überbegriff für verschiede Kategorien, basierend auf den Auswirkungen für den jeweiligen Symbiosepartner. Wenn ein erheblicher Größenunterschied zwischen den Symbiosepartnern besteht, wird der größeren Partner als Wirt und der kleinere als Symbiont bezeichnet.

Wie schon erwähnt, gibt es verschiedene Formen von Symbiosen, die abhängig von der Art der Interaktion der Symbiosepartner kategorisiert werden (McFall-Ngai and Gordon, 2006). So beschreibt Mutualismus das Zusammenleben zweier Organismen, bei dem beide einen Nutzen haben. Einige Beispiele dafür sind Flechten (Symbiose zwischen Algen und Pilzen), Cnidaria mit intrazellulären Zooxanthellen (Protisten), die Interaktion von Ameisen und Blattläusen, Bienen und Blütenpflanzen, Putzerfischen und ihre „Klienten“, etc. Es lässt sich auch eine Unterscheidung nach Art des Nutzens treffen, so gibt es Mutualismen, die der Fortpflanzung, dem Schutz vor Fressfeinden, dem Entfernen von Parasiten dienen oder trophischer Natur sind. Jedoch handelt es sich selten um nur eine Art des Nutzens, oft ist es eine Kombination aus mehreren.

Beim Kommensalismus zieht nur ein Partner einen Vorteil aus dem Zusammenleben, während es für den zweiten Partner keine Auswirkungen hat, z.B. Coronulidae (Cirripedia), deren Larven sich auf den Körpern von Bartenwalen festsetzen und mitgetragen werden (beide ernähren sich von Plankton).

Bei Parasitismus hat ein Partner auf Kosten des Anderen einen Vorteil, wobei der eine Partner auch letal geschädigt werden kann. Es gibt viele Beispiele für parasitäre Interaktionen: Mikroorganismen die Krankheitserreger sind (Malariaerreger *Plasmodium*), Pilze, Plattwürmer (Plathelminthes) mit den Klassen Bandwürmer (Cestoda) und Saugwürmer (Trematoda), Fadenwürmern (Nematoda), Zecken, Schlupfwespen,

Wucherpflanzen, etc. Es lassen sich auch Übergangsstadien zwischen den einzelnen Kategorien von Symbiosen finden und abhängig von externen Faktoren kann sich die Beziehung der Partner zueinander verändern (aus Mutualismus wird Parasitismus und umgekehrt) (Ahmadjian and Paracer, 1986).

Die Übertragung von Symbiont auf den Wirt kann auf zwei Arten vonstatten gehen. Bei einer horizontalen Übertragung wird der Symbiont der jeweiligen Wirtsgeneration aus der Umwelt erneut aufgenommen. Wenn der Symbiont einer Wirtsgeneration an die nächste weitergegeben wird, z.B. über die Gameten des Wirtes, so spricht man von einer vertikale Übertragung.

Eine weitere Unterscheidung von Symbiose kann auf Grund der räumlichen Beziehung zwischen den beiden Partnern getroffen werden. So handelt es sich um eine Ektosymbiose, wenn sich der Symbiont außerhalb des Wirts befindet. Wichtig bei dieser Art von Symbiose ist, dass der Symbiont noch immer mit der Umwelt interagieren kann. Bei einer Endosymbiose lebt der Symbiont im Körper des Wirts. Hier kann zwischen einer interzellulären Symbiose, in der der Partner im Wirtsgewebe lebt oder einer intrazellulären, in der sich der Symbiont innerhalb der Wirtszellen befindet unterschieden werden (McFall-Ngai and Gordon, 2006). In einigen Fällen von Endosymbiosen leben symbiotische Bakterien in speziellen Organen bzw. Organteilen oder Wirtszellen. Im Falle der interzellulären Symbiosen zwischen Leguminosen, insbesondere Pflanzen der Familie der *Fabaceae* und Knöllchenbakterien (Rhizobien) aus der Familie der *Rhizobiaceae*, bilden die symbiotischen Bakterien spezielle Zellorganellen. Die Rhizobien vermehren sich und wandeln sich anschließend zu s.g. Bacterioiden um, die von den infizieren Pflanzenzellen in Membranen gehüllt werden und so das Symbiosom bilden.

Weitere Charakterisierungen können im Bezug auf den Grad der Abhängigkeit, entweder fakultative oder obligat und auf die Spezifität, also ob nur zwei oder noch mehr Partner in die Symbiose involviert sind, getroffen werden (McFall-Ngai and Gordon, 2006).

Chemoautotrophe Symbiosen

In Habitaten, die weder über photosynthetische Primärproduktion, noch über organischen Zufluss von Außen verfügen, müssen alternative Energiegewinnungsformen angewandt werden. In solchen Habitaten kommen vermehrt chemoautotrophe Organismen vor, die

Wasserstoff oder anorganische Schwefelverbindungen wie Sulfide oxidieren um Kohlenstoff zu fixieren.

Viele dieser Lebensräume sind marin und sind durch das Vorkommen von Sulfid charakterisiert. In der Tiefsee am mittelozeanischen Rücken finden sich sog. „hot vents“. In deren Nähe wird Wasser durch die Reaktion mit dem reduzierten Mantelgestein mit Metallen (Eisen, Mangan) und Sulfid angereichert. Dadurch wird den dort vorkommenden Organismen ermöglicht diese Verbindungen zu nutzen. In den „Cold seeps“ und den sog. „whale falls“ (Walkadaver und deren Überresten, die auf den Meeresgrund sinken) zersetzen Sulfat-reduzierende Bakterien organische Verbindungen, als dessen Produkt Sulfid entsteht.

In Flachwassersedimenten findet man eine Chemokline, die sog. Redox Potenzial Diskontinuität (RPD), welche die sauerstoffreiche obere Sedimentschicht (wenige Millimeter bis Zentimeter) von der darunterliegenden anoxischen Sulfidschicht trennt (Fenchel and Riedl, 1970). Die Tiefe und Dicke der RPD-Schicht kann durch Wasserbewegungen, die primären Produktion tagsüber oder der Respiration nachts schwanken. Die in diesem Habitat vorkommenden chemoautotrophen Bakterien benötigen sowohl Sauerstoff, als auch Sulfid und müssen daher die Distanzen im Milli- bis Zentimeter Bereich überwinden.

Viele der chemoautotrophen Bakterien leben in Symbiose mit Vielzellern als Wirt, der ihnen dabei hilft diese Distanzen zu überbrücken und somit eine Versorgung mit Sulfid und Elektronenakzeptoren gewährleistet. Bei den Symbionten handelt es sich meistens um Gamma- und Epsilon- Proteobakterien, die Wirte können z.B.: Ziliaten, Nematoden, Anneliden, Arthropoda oder auch Mollusken sein (Dubilier et al., 2008).

Die *Stilbonematid* Symbiose

Die kleine Unterfamilie der *Stilbonematinae* (*Desmodoridae*, Nematoda) ist insofern einzigartig, da sie ektosymbiotische Schwefel-oxidierende Bakterien auf ihrer Kutikula tragen (reviewed in (Ott et al., 2004a, 2004b). Sie kommen besonders häufig in tropischen Meeren vor, nur wenigen Zentimeter unterhalb der Sedimentoberfläche in geschützten, oft kalkhaltigem Sand (Ott and Novak, 1989; Ott et al., 1991). Die *Stilbonematinae* zeigen ein characteristisches Migrationsverhalten, in dem sie sich zwischen der sauerstoffreichen oberen Sandschicht und dem darunterliegenden, anoxischen, schwefelwasserstoffhaltigen Sediment bewegen. Dieses Verhalten ermöglicht den Bakterien den Zugang zu Sauerstoff als Elektronenakzeptor, sowie zu Sulfid (oder auch anderen Schwefelverbindungen wie z.B.

Thiosulfat) als Elektronendonor. Beide Bestandteile werden für den Prozess der Schwefeloxidation gebraucht und die daraus resultierende Energie wird für die Kohlenstofffixierung verwendet (Hentschel et al., 1999; Ott et al., 1991). Die Bakterien scheinen den Wirt nicht nur mit Nährstoffen zu versorgen (Ott et al., 1991), sondern können auch ein Schutzfunktion einnehmen, indem sie ihn vor Schwefelvergiftung bewahren (Hentschel et al., 1999). Soweit bis heute bekannt ist, gehören alle ektosymbiotischen Bakterien der *Stilbonematinae* (außer die von *Eubostrichus dianaea*, bis zu dieser Studie unbekannt, siehe unten) zu den *Gammaproteobacteria* (Bayer et al., 2009). Diese bilden gemeinsame mit den Schwefel-oxidierenden Endosymbionten, der darmlosen marinen Oligochaeten (Polz et al., 1994) und der ebenfalls mundlosen Nematoden Gattung *Astomonema sp.* (Musat et al., 2007) eine Gruppe, das sog. Marine Oligochaete and Nematode Thiotrophic Symbiont (MONTS) cluster (Heindl et al., 2011).

Eubostrichus dianaea[1] Symbiose

Der marine freilebende Nematode *Eubostrichus dianaea* (Hopper and Cefalu, 1973) gehört ebenfalls zu den *Stilbonematinae.* Charakteristisch für die Spezies *E. dianaea* (Genus etabliert von (Greeff, 1869) ist, dass die Bakterien ein einheitliches „Fell" um den Körper des Wurms formen (SEM; Fig. 1A) (Ott et al., 1991). Ultrastrukturanalysen haben gezeigt, dass diese >100 µm langen Filamente in der Kutikula fest verankert sind (J. A. Ott persönliche Kommunikation). Weiters kann man an der Basis stäbchenförmige Bakterien finden (SEM; Fig. 1B, C) (Polz et al., 1992).

Vorhergehende Analysen von *E. dianaea* zeigten, dass die epibiotische bakterielle Gemeinschaft ein hohes Maß an bakterieller Diversität und Heterogenität auf einzelnen, aber auch zwischen verschiedenen Individuen aufweist. Jedoch konnte keine der 16S rRNA-gen Sequenzen zu einem ungeteilten filamentösen Bakterium, welches die *E. dianaea*-assoziierte Mikrobiota zu dominieren scheint, zugeteilt werden (Polz et al., 1999). Von hier an werden wir den gigantischen filamentösen Ektosymbionten als *Eds* bezeichnen.

[1] *Eubostrichus dianaea* wird häufig in anderen Arbeiten fälschlicherweise *Eubostrichus dianae* genannt.

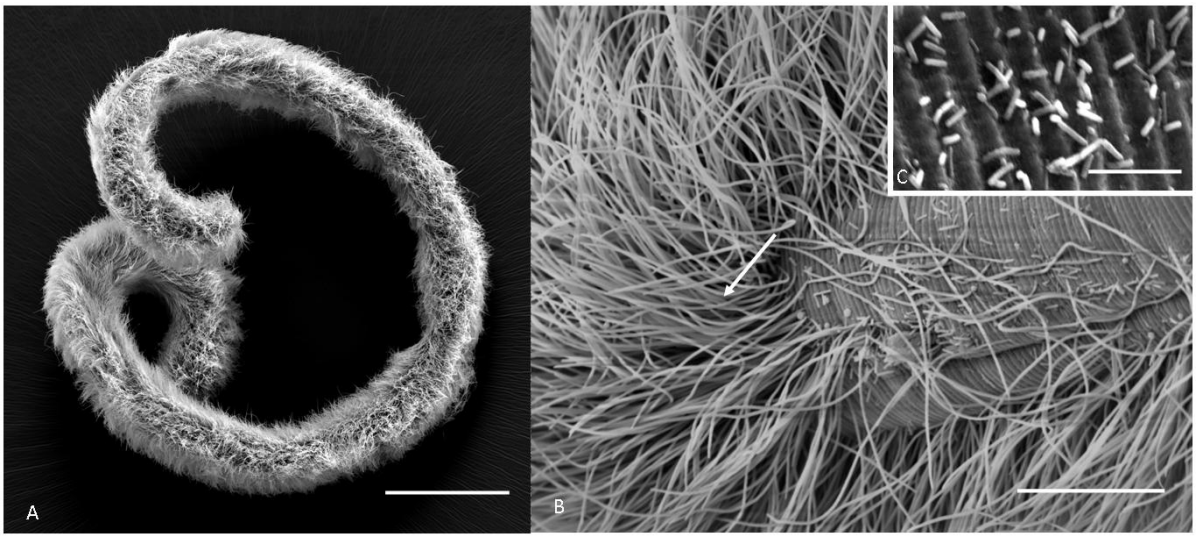

Figure 1 Scanning electron micrographs of *E. dianaea* (A) and of the bacteria attached to the cuticle, arrow points at the long filamentous ectosymbiont (B). In (C) higher magnification of the smaller interspersed rod-shaped bacteria. Scale bar is 200 µm in (A), 20 µm in (B) and 5 µm in (C).

Ultrastrukturelle Studien (TEM) von *E. dianaea* deuten darauf hin, dass Eds sphärische cytoplasmatische Einschlüsse besitzt (Polz et al., 1992). Mit Hilfe von Raman Mikrospektroskopie konnte gezeigt werden, dass diese Cluster von elementarem Schwefel (S_8) sind. Die Schwefeleinschlüsse sind in der bakteriellen Schicht des Nematoden lokalisiert (Himmel et al., 2009), während im Umgebungswasser kein elementarer Schwefel nachgewiesen werden konnte (Maurin et al., 2010). Wie schon oben erwähnt, sind alle bis heute beschriebenen *Stilbonematinae* obligat mit Schwefel-oxidierenden chemoautotrophen Ektosymbionten assoziiert. Schwefeleinlagerungen von chemoautotrophen Endosymbionten wurden schon früher in verschiedenen marinen Evertebraten beschrieben, wie z.B. in einigen Bivalven (Vetter, 1985), Oligochaeten (Giere et al., 1988) und im Plattwurmgenus *Paracatenula* (Gruber-Vodicka et al., 2011). Diese Ergebnisse deuten darauf hin, dass Eds vermutlich Schwefel speichern und auch oxidieren kann.

Nicht nur Eds ist ein bemerkenswertes Beispiel für überdimensional große Bakterien, sondern auch die symbiotischen Bakterien von *E.* cf. *parasitiferus* (ein weiterer Vertreter des Genus *Eubostirchus* aus der Unterfamilie der *Stilbonematinae*). Seine halbmondförmigen ektosymbiotischen Bakterien sind spiralig um den Körper des Wurms angeordnet und mit

beiden Enden an diesen angeheftet (Ott et al., 1991). DAPI-Färbungen dieser Bakterien zeigten das Vorhandensein von mehreren Nucleoiden (bis zu 16) in jeder einzelnen Zelle (Polz et al., 1992). Interessanterweise, konnte bis zur heutigen Studie weder in den überdimensionalen ektosymbiotischen Zellen von *E.* cf. *parasitiferus,* noch in denen von Eds ein Art von Zellteilung festgestellt werden. Deswegen wurde angenommen, dass die Bakterien beider Nematoden eine beachtliche Vergrößerung durch Längenwachstum erfahren, jedoch erfolgt dies ohne Zellteilung, möglicherweise ausgelöst durch Moleküle, die von den Nematoden selbst abgesonderte werden (Polz et al., 1992).

Weitere Beispiele für große symbiotische Bakterien

Bakterien, deren Wachstum sehr stark beeinflusst wird, können Endosymbionten von Pflanzen, Insekten oder auch Vertebraten sein.

In *Medicago* wandeln sich die *Rhizobien* in Stickstoff-Fixierende Bakterioide um, die dann von den infizierten Pflanzenzellen von einer Membran umhüllt werden und so die Knöllchen an den Pflanzenwurzeln bilden. Die symbiotischen Knöllchenzellen sind polyploid, da von der Pflanze produzierte Stoffe die bakterielle Zellteilung inhibieren und mehrere Zyklen von Endoreduplikation steuern (Mergaert et al., 2006; Van de Velde et al., 2010). Endoreduplikation ist ein Art modifizierter Zellzyklus, bei dem eine Replikation des Genomes stattfindet (S Phase), jedoch ohne dabei die Mitose und die Cytokinese (M Phase) durchzumachen. Daraus resultierend, wachsen die Zellen zu extremer Größe. Die pflanzlichen Stoffe sind Knöllchenspezifische Cystein-reiche (engl. nodule-specific cysteine-rich, NCR) Peptide, die eigens auf den Endosymbionten anzielen. Die NCR Peptide sind in der Lage in die bakterielle Membran und das Cytosol einzudringen, wo sie die Prädestination der Zelle manipulieren (Van de Velde et al., 2010).

Ein anderes Beispiel für die Kontrolle des Wirts über das Wachstum des Symbionten und dessen Reproduktion wurde in den Rüsselkäfergenus *Sitophilus* beobachtet. Der Rüsselkäfer sondert ein anti-mikrobielles Peptid, das sog. Coleoptericin-A (ColA) ab, welches selektiv die endosymbiotischen Bakterien in den Bakteriomen im Gewebe der mesenterischen Caeca und in den Ovarien des Käfers beeinflusst. ColA inhibiert die Zellteilung, wodurch die Bakterien zu langen Filamenten auswachsen (Login et al., 2011). Überdies wurde angenommen, dass dieses Peptid als Immunverteidigung gegen mikrobielle Eindringlinge agiert und gleichzeitig den Endosymbionten auf die Bakteriome beschränkt. ColA kommt

nicht nur gemeinsam mit den Endosymbionten im Bakteriom vor, sondern hat auch eine regulierende Funktion auf die Bakterienzahl und den Standort ihres Vorkommens (Login et al., 2011).

Im Darmtrakt der Doktorfische lässt sich das symbiotische Bakterium *Epulopiscium spp.* finden, welches ebenfalls zu extremer Größe wächst. Dieser Endosymbiont hat eine multiple intrazelluläre Nachwuchsproduktion, bei der intrazelluläre Tochterzellen im Cytoplasma der Mutterzelle heranwachsen. Es wird angenommen, dass sie in das umgebende Freiwasser entlassen werden, indem sie die Mutterzellenwand durchdringen und sie dabei zerstören (Angert, 2012; Mendell et al., 2008; Miller et al., 2012).

Einfluss von Wirt auf Symbiontenwachstum

Alle oben genannten Beispiele von ungewöhnlichem Größenwachstum von bakteriellen Symbionten haben die Gemeinsamkeit, dass die Bakterien sich nicht teilen und somit auch keine kanonische, binäre Zellteilung durchmachen. Im Falle des pflanzlichen Symbionten und des Endosymbionten des Rüsselkäfers wurde gezeigt, dass die Cytokinese durch den Wirt blockiert wird (Login et al., 2011; Van de Velde et al., 2010). Dies wirft die Frage auf, ob eine Art von Cytokinese-Blockade auch in Eds stattfindet, wie schon von Polz et al. 1992, 1999 angenommen wurde. Dies wäre das erste Beispiel für ein kontrolliertes bakterielles Wachstum in einer Ektosymbiose. In der Regel wird diese Form von Verbindung als weniger eng betrachtet, als die der Endosymbiose.

Tatsächlich weiß man von alle bekannten Ektosymbionten, alle *Stilbonematinae*-assoziierten mit eingeschlossen, dass sie sich teilen. In den meisten Bakterien wird die Zellteilung oder auch Cytokinese durch das Divisome, eine makromolekulare Maschinerie (Den Blaauwen et al., 1999) deren Formierung durch die Polymerisation des Tubulin-homologs FtsZ (Aarsman et al., 2005) induziert wird, durchgeführt. In dem stäbchenförmigen Modell-Gammaproteobakterium *Escherichia coli* formiert sich das FtsZ Protein selbst zu einer ringartigen Struktur (den Z-Ring), welcher mit der cytoplasmatischen Membran assoziiert ist. In *E. coli* Zellen befindet sich dieser Ring für gewöhnlich in der Mitte der Zelle, senkrecht zur Längsachse (Adams and Errington, 2009). Nach der Z- Ringformierung, beginnt sich dieser einzuschnüren, dabei treibt er die Teilung der Zelle voran, welche in zwei gleichen Tochterzellen resultiert. Nachdem FtsZ in allen uns bekannten *Gammaproteobacteria* essentiell für die binäre Zellteilung ist, haben wir es als Marker für diese ausgewählt. Ein

weiteres Protein, welches wir ausgewählt haben ist das bakterielle Actin-homolog MreB, welches einen der bedeutendsten Bestandteile des Cytoskeletts darstellt. Dieses Protein steuert die Synthese und den Einbau von Peptidoglykan in der Zellwand von stäbchenförmigen Bakterien und seine Lokalisierungsmuster könnten uns eine Einblick darüber gewähren, wie der Symbiont wächst (Erickson, 2001).

Die Absicht dieser Studie war es, (1) den gigantischen filamentösen Ektosymbionten von *E. dianaea* molekular zu identifizieren und (2) zu verstehen, ob er auf dem Wurm wächst und sich reproduziert, und wenn ja, wie dies von statten geht.

ABSTRACT

The marine free-living nematode *Eubostrichus dianaea* (*Stilbonematinae, Nematoda*) carries giant filamentous bacteria on its cuticle, which form an ordered “fur” around its body. Furthermore, at the base of the non-septated filaments, small rod-shaped bacteria could be detected. Using sequencing analysis and FISH we molecularly identified the giant and the small ectosymbiotic cells as sulfur-oxidizing *Gammaproteobacteria*. 16S rRNA-based phylogenetic analysis revealed that they belong to the Marine Oligochaete and Nematode Thiotrophic Symbionts (MONTS) cluster. In order to assess if (and how) the giant bacteria reproduce while attached to the host, we choose the well-studied tubulin homolog FtsZ as a marker for canonical cell division. Dividing rod-shaped *Gammaproteobacteria* usually place a constricting FtsZ ring at midcell, perpendicular to their long axis. Additionally, the actin homolog MreB directs peptidoglycan synthesis and may be used to visualize cell wall growth sites. We amplified the *ftsZ*-gene and showed that *E. dianaea* expresses both the FtsZ and MreB proteins by western blot analysis. Immunostaining revealed FtsZ polymerization into a ring at mid-cell, together with a dotted but homogenously distributed MreB localization pattern in all stages. DAPI stainings showed that each giant cell is polyploidy with up to 16 nucleoids. This is the first report of a over 100 µm-long Gammaproteobacterium which – after homogenous growth throughout its length and equal segregation of multiple nucleoids – places a FtsZ ring at mid-site (transverse binary fission).

INTRODUCTION

The marine free-living nematode *Eubostrichus dianaea* (Hopper and Cefalu, 1973) belongs to the small subfamily of *Stilbonematinae (Desmodoridae, Nematoda),* which are unique in carrying ectosymbiotic sulfur-oxidizing bacteria on their cuticle (reviewed in (Ott et al., 2004a, 2004b). They are especially abundant in sheltered tropical calcareous sands a few centimeters below the sediment surface (Ott and Novak, 1989; Ott et al., 1991). Characteristically, the *Stilbonematinae* migrate between oxygenated surface sand and the deeper, anoxic, sulfidic sediment. This behavior enables the bacteria to access both oxygen, as an electron acceptor, and sulfide (or other sulfur compounds e.g. thiosulfate) as electron donor. Both are needed for the process of sulfur-oxidation and the resulting energy is used for carbon fixation (Hentschel et al., 1999; Ott et al., 1991). The bacteria not only seem to provide their hosts with nutrients (Ott et al., 1991), but may also protect them against sulfide poisoning (Hentschel et al., 1999). Up to the present study all *Stilbonematinae*-associated bacteria (except for those previously reported for *Eubostrichus dianaea*, see below) belong to the *Gammaproteobacteria* (Bayer et al., 2009) and they cluster together with the sulfur-oxidizing endosymbionts of marine gutless oligochaetes (Polz et al., 1994) and of the as well gutless nematode genus *Astomonema sp.* (Musat et al., 2007), in the so called marine oligochaete and nematode thiotrophic symbiont (MONTS) cluster (Heindl et al., 2011).

In the case of *E. dianaea*, previous analysis of its epibiotic bacterial community revealed that it has a high level of bacterial diversity and heterogeneity on each and among different individuals. Surprisingly, none of the 16S rRNA-gene sequences could be attributed to the non-septated filamentous bacterium, which appears to dominate the *E. dianaea*-associated microbiota (Polz et al., 1999). This forms an ordered “fur” around the worm’s body (Fig. 1A) (Ott et al., 1991), which is characteristic for the species *E. dianaea* (genus established by (Greeff, 1869). Ultrastructural analysis showed that this >100µm long filamentous bacterium is attached to its cuticle by “holdfasts” (J. A. Ott personal communication). Further, at its base, rod-shaped bacteria could be detected (SEM; Fig. 1B, C) (Polz et al., 1992). From here on we will refer to the giant filamentous ectosymbiont as *Eds*. Ultrastructural studies (TEM) of *E. dianaea* indicate that Eds contain spherical cytoplasmic inclusions (Polz et al., 1992). Raman microspectrometry applied to *E. dianaea*

was able to detect clusters of elemental sulfur (S_8) and localize them in the bacterial coat of the nematode (Himmel et al., 2009; Maurin et al., 2010). As already mentioned above, all *Stilbonematinae* characterized so far are obligatory associated with sulfur-oxidizing chemoautotrophic ectosymbionts. Sulfur granules of obligatory chemoautotrophic endosymbionts have been described before in various marine invertebrates, for example in some bivalves (Vetter, 1985), oligochaetes (Giere et al., 1988) and in the flatworm genus *Paracatenula* (Gruber-Vodicka et al., 2011). These data suggest that Eds may store and oxidize sulfur.

Beside the Eds, another remarkable example of oversize bacteria within the marine nematodes is *E.* cf. *parasitiferus*. Its crescent-shaped ectosymbiotic bacteria are arranged spirally and attached with both ends around the worm's body (Ott et al., 1991). DAPI staining of these bacteria revealed the presence of several nucleoids (up to 16) in each cell (Polz et al., 1992). Interestingly, up to the present study, no division stages could be detected neither in the oversized ectosymbiotic cells of *E.* cf. *parasitiferus* nor in Eds. It was therefore assumed that the bacteria of both nematodes undergo considerable growth by elongation without cell division, possibly inhibited by nematode-secreted molecules (Polz et al., 1992).

Bacteria that grow to extraordinary sizes may also be *endo*symbionts of plants, insects or vertebrates. In *Medicago,* the *Rhizobium* cells are transformed into nitrogen-fixing bacteroids. The infected plant cells are covering them with a membrane and thereby they are forming nodules on the plant roots. The symbiotic nodule cells are polyploid due to plant factors that block bacterial cell division and trigger several cycles of endoreduplication (Mergaert et al., 2006; Van de Velde et al., 2010). Endoreduplication is a modified cell cycle, in which replication of the genome (S phase) takes place, but without undergoing mitosis and cytokinesis (M phase) and as a result the endosymbiotic cells grow to extreme size. The plant factors are nodule-specific cysteine-rich (NCR) peptides that are specially targeting the endosymbiont. The NCR peptides are able to enter the bacterial membrane and cytosol, where they manipulate the cell fate (Van de Velde et al., 2010). Another example of host control of symbiont growth and reproduction was observed in the weevil genus *Sitophilus*. The weevil secretes an antimicrobial peptide called coleoptericin-A (ColA) that selectively targets endosymbiotic bacteria within the bacteriomes in the tissues of mesenteric caeca and of ovaries of the weevil. ColA is inhibiting the cell division and consequently the bacteria

grow into giant filaments (Login et al., 2011). Furthermore, it was assumed that the weevil ColA peptide acts as the first defense against microbial intrusion in insects and that it may retain the endosymbiotic cells within the bacteriomes. ColA not only appears to colocalize with the endosymbiont in bacteriomes, but has also a regulatory function on bacteria number and location (Login et al., 2011). *Epulopiscium spp.* bacteria that can be found in the intestinal tract of the surgeonfish grow as well to extremely large sizes. These endosymbionts produce multiple intracellular offspring where active intracellular daughter cells grow within the mother cell cytoplasm. They are eventually released by perforating the mother cell envelope and thereby destroying it (Angert, 2012; Mendell et al., 2008; Miller et al., 2012).

All aforementioned examples of plant, insect and vertebrate *endo*symbionts grow to extraordinary sizes, because they do not divide by canonical binary fission. In the case of plant and weevil endosymbionts, cytokinesis is blocked by the host (Login et al., 2011; Van de Velde et al., 2010). We therefore wondered whether cytokinesis block also occurs in Eds as hypothesized by Polz et al. 1992, 1999. If this were the case, this would be the first example of bacterial growth control in an *ecto*symbiosis, a form of association commonly considered less intimate than endosymbiosis.

Indeed, all known ectosymbionts including all *Stilbonematinae*-associated ones*,* have so far been reported to divide. In order to answer this question, we searched for a binary fission ´molecular marker`. Cell division or cytokinisis in most bacteria is achieved by the divisome, a macromolecular machine (Den Blaauwen et al., 1999) whose assembly is initiated by the polymerization of the tubulin homolog FtsZ (Aarsman et al., 2005). In the model rod-shaped gammaproteobacterium *Escherichia coli* the FtsZ protein self-assembles into a ring-like structure (the Z-ring) associated to the cytoplasmic membrane. In *E. coli* cells the ring is usually positioned at midcell, perpendicular to the longitudinal axis (Adams and Errington, 2009). After the self-assembly the Z-ring starts to constrict, thereby driving the division of the cell, which results in two equal daughter cells. FtsZ is essential for binary fission in all known *Gammaproteobacteria* and we therefore chose it as a marker for binary fission. Another symbiont protein that we have chosen to visualize is the bacterial actin homologue MreB, which is a major component of the cytoskeleton. This protein directs the synthesis and insertion of peptidoglycan in the cell wall of rod-shaped bacteria and its localization pattern may inform about how the symbiont grows (Erickson, 2001).

The aims of this study were (1) to molecularly characterize the *E. dianaea* giant filamentous ectosymbiont and (2) to understand if it grows and reproduces on the worm and, in case it does, how.

METHODS

Nematode collection

Specimens of *E. dianaea* were collected in December 2011 and January 2012 in approximately 1 m depth from a sand bar off Twin Cays, Belize (16° 49' 00" N, 88° 06' 00" W). The worms were extracted from the sand by stirring the sand in seawater and pouring the supernatant through a 63-µm-pore-size mesh sieve. The content of the net was transferred into a Petri dish and single individuals were then picked by hand using fine tweezers under a dissecting microscope. For genomic DNA (gDNA) extraction and Western blotting, batches of freshly collected nematodes were flash frozen in liquid N_2. For Fluorescence In Situ Hybridization (FISH) and Immunostaining worms were fixed in methanol. All samples were deep-frozen for transportation and storage.

gDNA extraction

The genomic DNA of *E. dianaea* was extracted as described in (Mortazavi et al., 2010) with slight volume adaptations to maintain the ratio between nematodes and solutions used in this protocol. A batch of ca. 120 deep-frozen *E. dianaea* specimens was transferred to a 2ml tube containing 1ml of worm lysis-buffer. To this, 40 µl of 20 mg/ml Protease K were added and mixed by inversion. The solution was incubated at 62°C for 60 min to disintegrate the worms, while isopropanol was pre-chilled at -20°C. During the incubation the tubes were mixed 4-5 times by gentle inversion. Subsequently, 160 µl of 5M NaCl were added and mixed by thorough inversion and then 160 µl of CTAB/NaCl solution (10 % CTAB (Sigma M.7635) in 0.7M NaCl) were added, the solution was incubated 10 min at 37°C. Half of it was transferred into another 2 ml tube. The gDNA was extracted with one volume of chloroform and then phenol/chloroform, using gentle inversion, centrifugation and recovering aqueous phase. DNA was precipitated by adding one volume of pre-chilled isopropanol and was visible as a pellet upon 10 min of centrifugation at 13,000 rpm. After three washes with 70 % ethanol and 5 min centrifugation, supernatant was removed and the pellet air dried before resuspending overnight in 70 µl of TE buffer + 2 µl of RNAse A, at 4°C. On the next day the DNA solution was incubated 2 h at 37°C to drive RNAse activity to its conclusion. The solutions were pooled again and 8 µl of 20 % SDS, 4 µl of 0.5M ETDA pH 8.0 and 8 µl of Protease A was added. Subsequently the solutions were mixed by gentle inversion and

incubated at 62°C for 2 h. 7 µl of 5M NaCl was added and mixed by inversion, before extracting twice with phenol/chloroform and once chloroform. The gDNA was washed again three times in 70 % ethanol, air-dried and resuspended in 40 µl of TE buffer over night at 4°C.

16S rRNA- and *ftsZ* genes PCR amplification

1,499 nt-long fragments of the 16S rRNA-gene were amplified by PCR with bacterial primers 616V (5´-AGAGTTTGATYMTGGCTC-3´; (Juretschko et al., 1998) and 1492R (5´-GGYTACCTTGTTACGACTT-3´; (Kane et al., 1993). Further 1,106 nt-long fragments of the ftsZ gene were amplified using degenerate primers ftsz 1F (5'- GCVGTVATYAARGTBATCGG -3') and ftsz 2.1R (5'- GCYGGRATRTCSAGRTAATC -3'). As template 2 µl of gDNA were used in 50 µl PCR reactions, that contained 5 µl of 10X Dream Taq buffer, 5 µl of 2mM dNTPs, 1 µl of 1mM each forward and reverse primer, 0.5 µl of Taq polymerase and 35.5 µl of sterilized double distilled water. The cycling conditions of the 16S rRNA amplification were: 94°C initial denaturation for 4 min, 35 loops with each 45 sec denaturation at 94°C, 30 sec annealing at 49°C and 1 minute and 45 sec elongation time at 72°C, followed by a final elongation of 10 min at 72°C.

For the FtsZ rRNA a touchdown PCR with following conditions was performed: 94°C initial denaturation for 3 min, 8 loops with each 45 sec of denaturation at 94°C, 45 sec annealing from 58°C decreasing to 50°C with each loop and 1 minute and 15 sec elongation time at 72°C, followed by 27 loops with each 45 sec of denaturation at 94°C, 45 sec annealing at 50°C and 1 minute and 15 sec elongation time at 72°C, ending with a final elongation of 10 min at 72°C. Afterwards PCR products were checked on a 1% agarose gel and then photographed under UV light.

Cloning

PCR products with the expected fragment sizes of approximately 1.5 kb for the 16S rRNA and 1.1 kb for the *ftsZ*-gene were loaded on a 1 % agarose gel, cut out and then purified using the MinElute PCR Purification Kit (Qiagen, USA). Purified fragments were cloned into pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen Life Technologies, Germany). 5 clones containing the *ftsZ*-gene fragment were randomly picked and screened for the right insert. 4 were fully sequenced in both directions. One forward and one reverse sequence were

obtained by direct sequencing of the *ftsZ*-gene fragment. All the sequences were aligned and compared with CodonCode Aligner 1.6.3 software. 32 clones containing the 16S rRNA fragment were randomly picked and screened for the right insert. We found 17 sulfur reducing bacteria (13 *Delta*- and one *E. topiarius* associated sulfur reducing bacteria; clones were >99.9% identical), 7 sulfur oxidizing bacteria, 3 *Cytophaga spp.* and 6 Rhodospirillales bacteria clones (4 Rhodospirillales *Alphaprotebacteria*, one deep-sea Rhodospirillales A*lphaproteobacterium* and one Riegeria-like Rhodospirillales; clones were >99.9% identical).

Plasmid multiplication and purification

Cultures from each colony with the right insert were set overnight at 37°C and 200 rpm in culture tubes with 5 ml of sterile LB broth. The next day the cultures were pelleted by centrifugation at 4,500 rpm for 10 min at 4°C and the supernatant was discarded. Plasmids were isolated from the bacterial pellets using the E.Z.N.A. Plasmid Mini Kit II – Spin Protocol. Afterwards the concentration of purified plasmid was measured with the NanoDrop (Thermo Fisher Scientific, USA).

Sequencing

For the sequencing reaction 1 µl of plasmid with a concentration between 150 and 400ng/µl was used. The total reaction volume of 10 µl contained 1.5 µl of BigDye, 1µl of buffer, either 1 µl of 20µM M13F or M13R primer and 5.5 µl of sterile double distilled water. The cycling conditions were: 40 loops with 20 sec of denaturation at 96°C, 10 sec of annealing at 48°C and 4 min of elongation time at 60°C.

16S rRNA-gene based phylogenetic analysis

The 16S rRNA sequences of *E. dianaea* sulfur oxidizing ectosymbiont were compared with gammaproteobacterial sequences found in Genbank using BLAST (Altschul et al., 1990) with a minimal sequence similarity cutoff of 95% to the *E.dianaea* symbiont. This set of sequences included all full length MONTS cluster phylotypes (Heindl et al., 2011) and several closely related environmental sequences. Selected *Chromatiacae* were used as an out-group. The sequences were aligned with the online version of MAFFT version 7 (Katoh and Standley, 2013); http://mafft.cbrc.jp/alignment/server/index.html), with the Q-INS-i strategy (consid-

ers secondary structure of RNA) and the parameter for the scoring matrix for nucleotide sequences was 1PAM.

The sequence alignment file created by MAFFT was analysed at the phylogeny.fr portal http://www.phylogeny.fr/version2_cgi/phylogeny.cgi using the maximum likelihood based PhyML (Dereeper et al., 2008) for tree reconstruction and node support test. Chosen parameters: app. likelihood-Ratio-Test (aLRT) was SH-like, substitution model was GTR+Gamma+INV-model. Using the same MAFFT alignment file two additional phylogenetic reconstructions were generated using the software MEGA (Tamura et al., 2011) – one with the distance based neighbor joining (NJ) algorithm (500 boot straps) and one using the maximum parsimony algorithm (100 boot straps). A node support > than 0.8 was considered significant for all methods and the node support values of the three methods were indicated in the presented NJ tree if a node was supported by > 0.5 by in least one method.

Clone Fluorescence *In Situ* Hybridization (FISH) and FISH on whole or sonicated worms

By using the probe design tool of the ARB software package (Ludwig et al., 2004) and the SILVA ssu_jano4_corrected database (Pruesse et al., 2007), with the sequences added, FISH probes specially targeting the 16S rRNA-gene of *E. dianaea* were designed by N. R. Heindl. FISH was performed according to (Manz et al., 1992). A detailed overview of all probes and formamide concentrations used in the different experiments is given in Table 1.

Clone FISH was performed to determine the optimum hybridization conditions of the *E. dianaea* filamentous ectosymbiont-specific probe. Ligation, transformation, PCR screen, overnight cultures, plasmid purification, sequencing and sequence data analysis were performed as described above. 1 µl (ca. 500 ng/µl) of the plasmid carrying the *E. dianaea* 16S rRNA-gene was diluted 1:7.5 with sterile double distilled water. Afterwards 2 µl of the plasmid solution were electroporated into 100 µl of *E. coli* cells (in 10 % glycerol). Cells were regenerated in 250 µl SOC medium at 37°C and 550 rpm for 1h, subsequently plated on 0.1 mg/ml kanamycin LB agar plates and incubated overnight at 37°C. On the next day some colonies were picked and a colony screen was carried out with M13F and M13R primers. Clones with the expected insert size were sequenced and grown in 5 ml 0.1 mg/ml kanamycin LB broth overnight at 37°C and 200 rpm. On the next day 1 ml of the overnight cultures was transferred into an autoclaved Erlenmeyer flask with 100 ml 0.1 mg/ml kanamycin LB broth and grown in a water bath at 37°C to an OD_{600} of 0.4. Afterwards by

adding 100 µl of 1M IPTG the expression of the cloned 16S rRNA-gene was immediately induced. The cultures were grown another hour at 37°C before adding 100 µl of 170 mg/ml chloramphenicol and growing 4 h at 37°C. Subsequently the cultures were pelleted, washed twice in 1x PBS, fixed in 4 % PFA for 1 h at 4°C, washed again and stored in 1 vol 1x PBS plus 1 vol of 96 % ethanol at -20°C.

One drop of poly-L-lysin was placed in each well of a Teflon coated microscopy slides and air dried, to ensure the adhesion of the cells to the slide. Afterwards 2 µl of the clone suspension were applied onto the slides and air dried. 20 µl of 10 % hybridization buffer (0.9M NaCl, 20 mM TrisHCl (pH 8.0), 0.001 % SDS, 10 % formamide) were applied to each well with cells on it. 2 µl of 30 ng of Cy3 labelled *E. dianaea* giant ectosymbiont-specific Eds214 probe, and 2 µl of 50 ng of FLUOS labelled eubacteria-specific Eub338 probe were added on one slide. On another slide 2 µl of 30 ng of Cy3 labelled of a probe differing from Eds214 for a central A to T substitution and therefore containing a single nucleotide mismatch with respect to the target sequence (Eds214mis), were applied together with 2 µl of 50 ng of FLUOS labelled Eub338 probe. The slide were put into a 50 ml tube with a hybridization buffer-soaked paper inside and incubated at 46°C in a hybridization oven. After 3 h of incubation, the slide was transferred into the corresponding washing buffer (70mM NaCl, 20mM Tris·HCl (pH 8.0), 0.125M EDTA), incubated for 10 min at 48°C, rinsed in ice cold water and dried quickly under a weak air stream. Before putting a cover slip, cells were mounted in the anti-fading medium Vectashield (Vector Labs). The slides were examined with a Nikon epifluorescence microscope and the clone with the strongest Cy3 signal was chosen by eye. Afterwards, we performed a formamide series (0 %, 10 %, 20 %, 25 %, 30 %, 35 %, 40 %, 45 % and 55) for all used FISH probes to determine stringent hybridization conditions as described above. Evaluation of the signal intensity was done on a Leica TCS-NT confocal laser scanning microscope, where the gain, o-set and zoom values were adjusted to the brightest fluorescent sample. The formamide concentration resulting in the strongest Cy3 with the Eds214 probe and simultaneously weakest with the Eds214 mismatch probe was chosen for stainings of whole worms.

Several methanol-fixed *E. dianaea*, were put in different wells of a Teflon coated slide, air dried, coated in warm 0.1 % agarose and air dried again. Afterwards 20 µl of 40 % hybridization buffer was added to each well of the slide together with 2 µl of each fluorescent probe, 30 ng/µl of Eds214 (Cy3) or Eds214mis (Cy3), 30 ng/µl of Gam42a (Cy5)

and 50 ng/µl of Eub338 (FLUOS). The slides were then put into a 50 ml tube with a hybridization buffer soaked paper inside and incubated at 46°C in a hybridization oven over night. On the next day the slides were transferred into the corresponding washing buffer (70mM NaCl, 20mM Tris·HCl (pH 8.0), 0.125M EDTA), incubated for 10 min at 48°C, rinsed in ice cold water and dried quickly under a weak air stream. Before putting a cover slip, worms were mounted in the anti-fading medium Vectashield (Vector Labs).

To dissociate ectosymbiont cells from the worms, 15 methanol-fixed specimens of *E. dianaea* were sonicated for 40 sec in a 0.5 ml tube containing 30 µl of methanol, which led to a detachment of the filamentous bacteria. Afterwards 20 µl of methanol with ectosymbiont cells were put on poly-L-lysin covered slides and air dried. FISH, was performed as above.

Table 1. Probes used for FISH

Probe	Specificity	Sequence/5' modification	Target RNA	Position[1]	Formamide percentage/incubation time (h)/probe concentration (ng/µl)	Reference
EUB338	Most bacteria	5´-GCTGCCTCCCGTAGGAGT -3´ fluorescein	16S	338-355	40%/12/3.8	(Amann et al., 1990)
GAM42a	Gammaproteo-bacteria	5´-GCCTTCCCACATCGTTT-3´ Cy5	23S	1027-1043	40%/12/2.4	(Manz et al., 1992)
Eds214	*E. dianaea* ectosymbiont	5'-GCTCATCATCATAGCGGAA -3' Cy3	16S	214-235	40%/12/2.4	This study
Eds214mis	1 mismatch to the *E. dianaea* ectosymbiont	5'-GGCTCATCATCTTAGCGGAAG-3' Cy3	16S	214-235	40%/12/2.4	This study
EdsSRB193	*E. dianaea* associated SRB	5'-CTCCAAACAATCGCTTGCAAGC-3' Fam	16S	193-215	40%12/3.8	This study
EdsSRB64	*E. dianaea* associated SRB	5'-TGCAAGCAACCCCTTTCTCGTT-3'	16S	64-86	40%12/3.8	This study

[1]16S rRNA position, *E. coli* numbering (Brosius et al., 1978)

Antibodies and Western blot

We used a mouse monoclonal antibody, which recognizes a C-terminal epitope of *E. coli* FtsZ (the C-terminal 15 aa of *E. coli* are identical to those of the ectosymbiont FtsZ except for a single aa) (Voskuil et al., 1994)and a rabbit polyclonal antibody, which recognizes a *E. coli* MreB (Karczmarek et al., 2007), a kind gift from Tanneke den Blaauwen). To extract proteins, approximately 50 µl of packed, deep-frozen, symbiotic *E. dianaea* or *L. oneistus*, or *E. coli* cell pellets (from app. 1 ml overnight culture) were grinded in an equal volume of NuPAGE 4x LDS sample buffer to which 5 % 2-mercaptoethanol was previously added (Invitrogen, Germany). The solutions were heated for 5 min at 95°C, centrifuged for 10 min at 14,000g and the supernatant transferred into a now tube. Following the solutions were loaded on a NuPAGE 4-12% Bis-Tris precast gel (Invitrogen, Germany) and the proteins were separated by reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using MOPS as running buffer. By following the descriptions in the Invitrogen NuPAGE Techincal Guide, the proteins were transferred to a Hybond ECL nitrocellulose membrane (GE Healthcare, UK). Afterwards the membrane was cut into stripes and these were blocked in 1x PBS plus 5 % non-fat milk (PBS/milk) for 45 min at room temperature. Primary antibody (1:500) incubation was performed in PBS/milk overnight at 4°C, with a negative control stripe incubated with PBS/milk only in parallel. On the next day, the membrane stripes were washed in PBS/milk three times, 10 min each and subsequently incubated for 1 h at room temperature with a horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibody (both 1:1000; Amersham Biosciences) in PBS/milk. To remove unspecifically bound 2ry antibodies, the stripes were washed three times in PBS/milk and one time in PBS/0.1% $Tween_{20}$. ECL Plus detection reagents (GE Healthcare, UK) were used according to the manufacturers' protocol and the emitted light detected on photographic films (Amersham Biosciences).

Immunostaining

Immune fluorescence was performed according to (Bulgheresi et al., 2006).

Methanol-fixed *E. dianaea* were placed in the wells of a Teflon coated slide and washed three time 10 min in PBS plus 0.1 % $Tween_{20}$ (PBS-T). Afterwards bacterial peptidoglycan was permeabilized by incubating 30 min with 0.1 % lysozyme and washing 10 min in PBS-T. Worms were blocked for 1 h at room temperature with blocking solution (PBS-T/2% BSA).

The worms were incubated with the primary antibodies anti-FtsZ in blocking solution (1:500) and anti-MreB in blocking solution (1:500) overnight. On the next day they were washed three times in PBS-T and then blocked 1 h in blocking solution with anti-mouse and anti-rabbit secondary antibody (both 1:500). They were subsequently washed three times in 1x PBS and stained with DAPI (5 µg/µl in McIlvain's citric acid-phosphate buffer, pH 4.5) for 1 h. To remove unbound DAPI, a last wash in McIlvain's citric acid-phosphate buffer for 1 h was performed. Slides were mounted in Vectashield (Vector Labs) and examined on a Leica TCS-NT confocal laser scanning microscope.

To stain worm-dissociated ectosymbiont cells of 15 specimen of *E. dianaea* were put into a 0.5 ml tube containing 25 µl of PBS-T. After performing immunostaining and DAPI staining exactly as above but in the tube, instead of on the slide, worms in the tube were sonicated for 40 sec, which led to a detachment of the ectosymbiont. 3 µl of the bacterial solution were mixed with 1 µl the mounting medium Vectashield (Vector Labs) on a 0.1 % agarose covered slide and closed with a cover slip.

RESULTS

The giant E. dianaea ectosymbiont belongs to the marine oligochaete and nematode thiotrophic symbiont (MONTS) cluster

In order to identify the giant *E. dianaea* ectosymbiont *(Eds)*, we constructed a bacterial 16S rRNA-gene library. Sequencing of 32 clones resulted in the identification of fourteen sulfur-reducing bacteria (>99.9% identity among their relative 16SrRNA-gene sequences), seven sulfur-oxidizing bacteria (>99.9% identity among their relative sequences), tree *Cytophaga* and six *Rhodospirillales* bacteria. The 16S rRNA sequences attributable to the sulfur-oxidizing bacteria were compared and aligned with GenBank sequences that had > 95 % sequence similarity with them, including those of other stilbonematid and oligochaete symbionts, as well as those of bacteria belonging to the *Chromatiaceae* and other uncultured *Gammaproteobacteria*. The resulting 16S rRNA-gene-based phylogenetic tree shows the relationship of the *E. dianaea* ectosymbiont with the above-mentioned symbionts (Fig. 2). According to the phylogenetic analysis, the *E. dianaea* sulfur-oxidizing bacterium is most closely related to the *E. topiarius*-associated bacteria and belongs to the MONTS cluster.

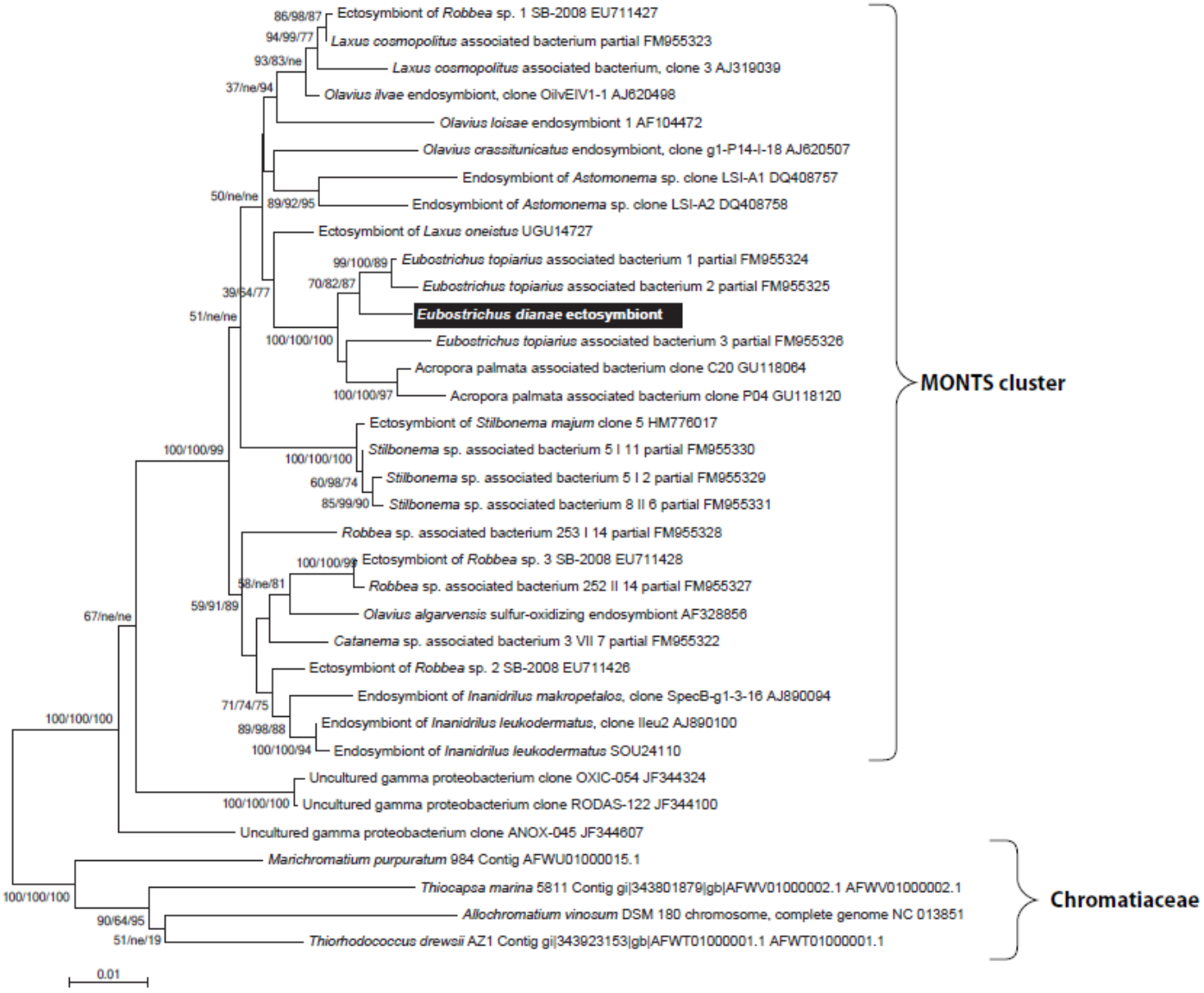

Figure 2 16S rRNA-gene-based phylogenetic consensus tree of maximum likelihood based PhyML, distance based neighbor joining (NJ) algorithm (500 boot straps) and maximum parsimony algorithm (100 boot straps) showing the relationship of the *E. dianaea* ectosymbiont with other stilbonematid and oligochaete symbionts, as well as other bacteria belonging to the *Chromatiaceae* and uncultured *Gammaproteobacteria* (with > 95% sequence similarity). A node support > than 0.8 was considered significant for all methods and the node support values of the three methods were indicated in the presented NJ tree if a node was supported by > 0.5 by in least one method. Scale bar represents 1% estimated sequence divergence.

To confirm that the MONTS 16S rRNA sequences obtained in our library originated from the Eds, we sought to stain it with a FISH specific probe targeting them (Eds214 probe). We first performed clone FISH in order to determine the optimum hybridization conditions for this

probe (Manz et al., 1992). 40 % formamide concentration resulted in the strongest Eds214 probe signal, and the weakest Eds214mis probe signal in engineered *E. coli* cells (Fig. 3I, K).

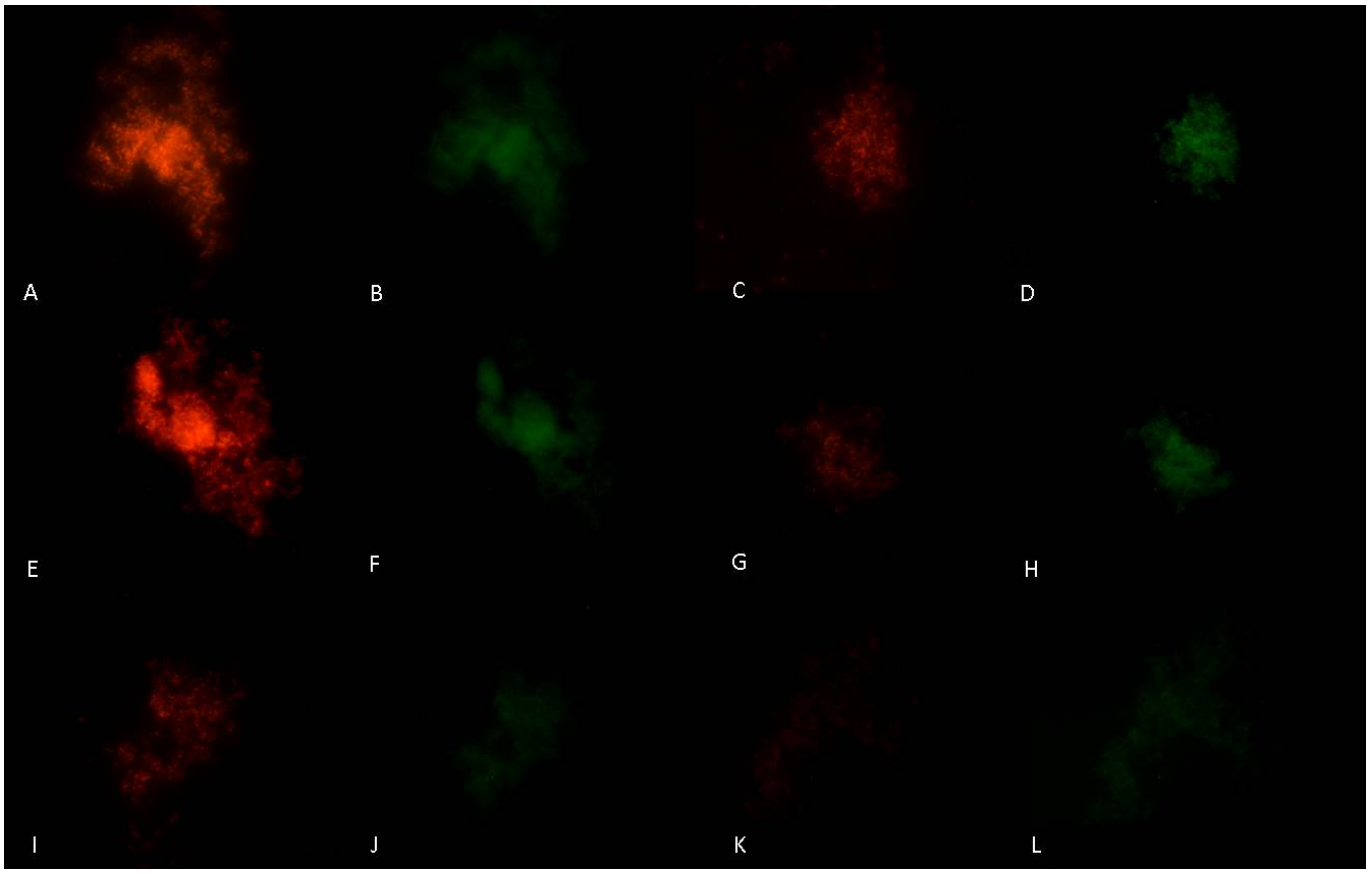

Figure 3 Clone Fluorescence *in situ* hybridization (FISH) epifluorescence micrographs. *E. coli* cells were hybridized in the presence of 20% (A-D) or 30% (E-H) or 40% formamide (I-L). (A, B, E, F, I and J) show staining with ectosymbiont-specific probe (red) and *Eubacteria*-specific probe (green). (C, D, G, H, K and L) are stained with a probe containing a single nucleotide mismatch with respect to the ectosymbiont-specific probe (red) and Eubacteria-specific probe (green).

Therefore we chose these conditions for FISH staining of *Eubostrichus*-associated bacteria.
By using whole mount FISH on *E. dianaea* individuals we showed that all filamentous bacteria attached to the worm were triple stained by the eubacterial probe EUB338, by the *Gammaproteobacteria*-specific probe GAM42a and by the respective *E. dianaea* ectosymbiont-specific probe (Fig. 4).

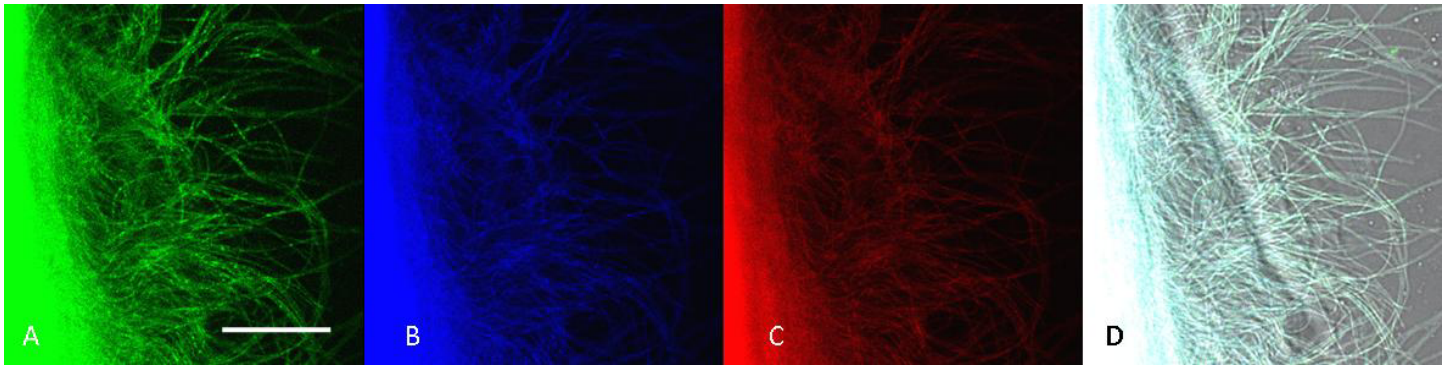

Figure 4 Fluorescence *in situ* hybridization (FISH) confocal micrographs of *E. dianaea* ectosymbionts attached to the worm surface. Each filamentous ectosymbiont is triple stained with a *Eubacteria*-specific probe (green) (A), a Gammaproteobacteria-specific probe (blue) (B) and an ectosymbiont-specific probe (red) (C). (D) is an overlay picture of (A)-(C) together with the corresponding Bright Field microscopy picture. Scale bar is 25 µm.

In contrast, the control with the ectosymbiont-specific mismatch probe Eds214mis showed a highly reduced fluorescence signal (Fig. 5). This clearly indicates that the MONTS 16S rRNA sequences obtained in our library can be attributed to Eds.

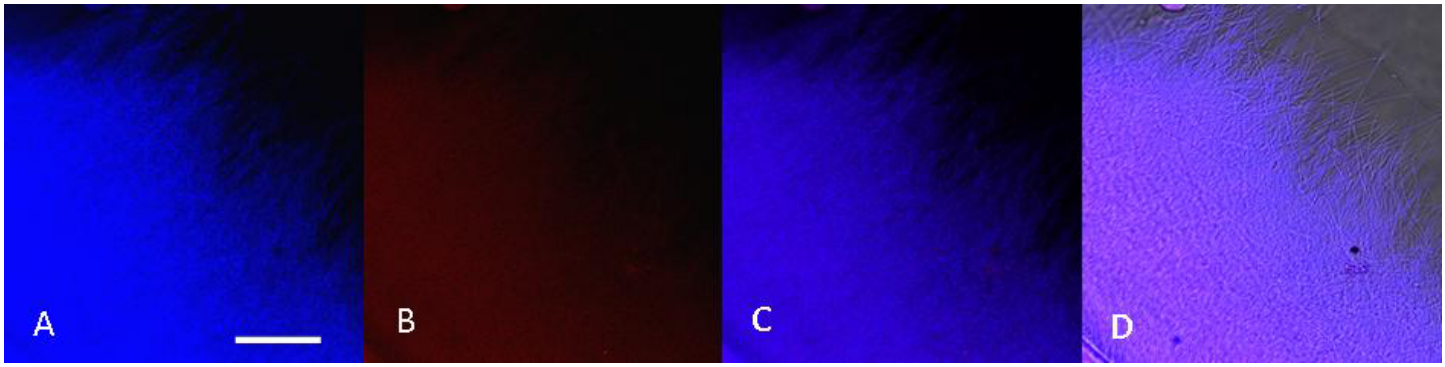

Figure 5 Fluorescence *in situ* hybridization (FISH) confocal micrographs of *E. dianaea* ectosymbiont cells attached to the worm surface. Each filament is stained with a *Gammaproteobacteria*-specific probe (blue) (A), but not with Eds214mis (red; see text) (B). (C) is an overlay picture of (A) and (B). In (D) the overlay is together with the corresponding Bright Field microscopy picture. Scale bar is 25 µm.

All ectosymbiotic bacteria appear to belong to the same phylotype as Eds irrespectively from their size

Given that non-Eds bacteria were undetectable via FISH on whole mount worms, we dissociated cuticle-associated bacteria via ultrasonication to assess if the small bacteria apparent in SEM are younger stages of Eds i.e. to assess if these sulfur-oxidizing bacteria may grow on the nematode host. FISH performed on bacteria detached from the worms

showed that all dissociated ectosymbiont cells from *E. dianaea*, irrespective of their size, were double stained with a *Gammaproteobacteria*-specific probe GAM42a and the *E. dianaea* sulfur-oxidizing bacteria targeting probe Eds214 (Fig 6).

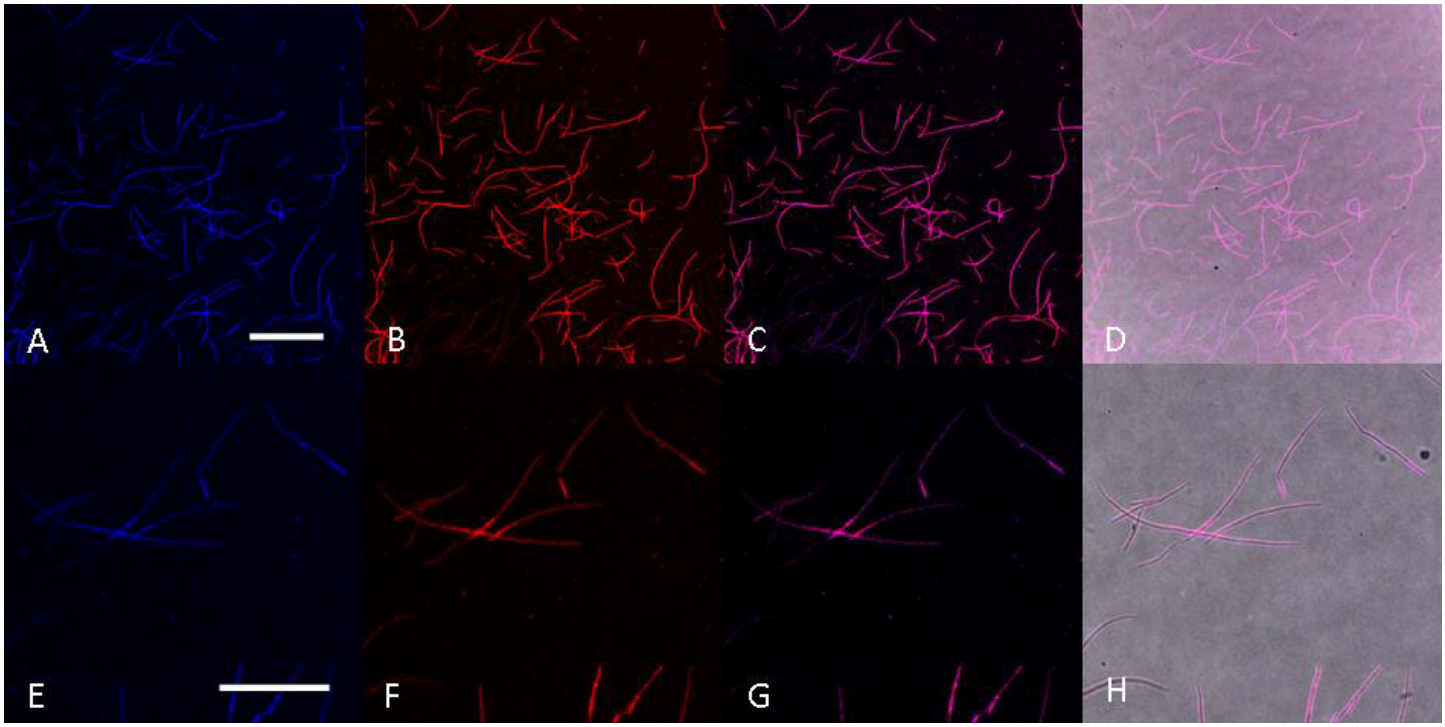

Figure 6 Fluorescence *in situ* hybridization (FISH) confocal micrographs of dissociated ectosymbiont cells of *E. dianaea*. All filaments, irrespective of their size, are stained with a *Gammaproteobacteria*-specific probe (blue) (A) and (E), as well as with an ectosymbiont-specific probe (red) (B) and (F). (C) and (G) are overlay pictures of both probes. (D) and (H) is the overlay together with the corresponding Bright Field microscopy picture. Scale bar is 50 µm (A)-(C) and 25 µm (D)-(F).

As a further control, we performed FISH with two different probes targeting sulfur-reducing (SRB) bacteria (EdsSRB193 and EdsSRB64), which were also present in the clone library, together with a eubacterial probe (EUB338) and a *Gammaproteobacteria*-specific probe (GAM42a). Only these latter two probes gave a signal, whereas the SRB-specific probes did not result in any detectable FISH signal (Fig. 7). These results show that all bacteria, whether long and filamentous or small and rod-shaped, belong to the same 16S rRNA-phylotype.

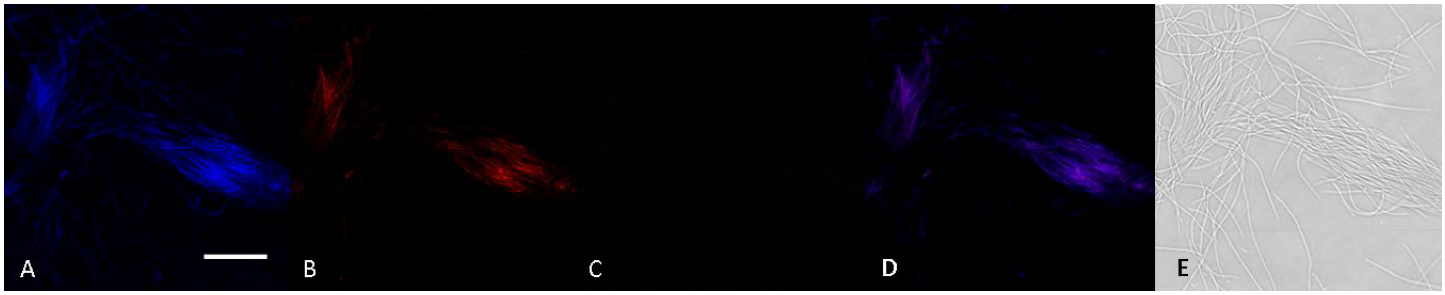

Figure 7 Fluorescence *in situ* hybridization (FISH) confocal micrographs of dissociated ectosymbiont cells of *E. dianaea*. All filaments, irrespective of their size, are stained with a *Gammaproteobacteria*-specific probe (blue) (A), as well as with an ectosymbiont-specific probe (red) (B), but no signal with SRB (green) (C). (D) is an overlay picture of (A)-(C) and (E) is the corresponding Bright Field microscopy picture. Scale bar is 25µm.

Amplification and alignment of the ftsZ gene from Eds

In the model gammaproteobacterium *E. coli* cell division is initiated by polymerization of the FtsZ protein into a ring and the constriction of this Z-ring (see Introduction). We wanted to find out if the gene encoding for this protein is also present in Eds. Therefore we homology cloned the *ftsZ*-gene from *E. dianaea* gDNA by PCR amplification with degenerate primers and the reaction resulted in a product with the expected size of approximately 1.158 nt (Fig. 8).

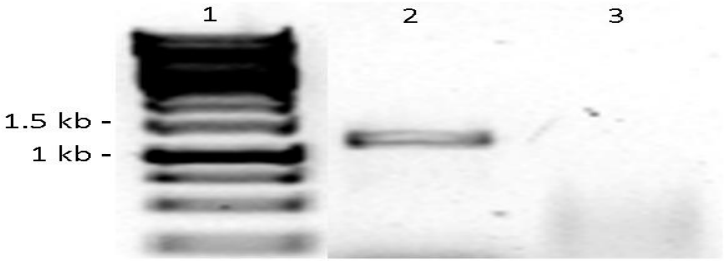

Figure 8 Homology cloning of the *E. dianaea ftsZ* gene. Approximately 1.1 kb-long PCR fragment obtained form genomic DNA of *E. dianae* with ftsZ 1F and ftsZ 2.1R degenerate primers (lane 2). Lane 1: 1 kb ladder, lane 3: negative control displaying no amplification with ftsZ 1F and ftsZ 2.1R degenerate primers on ultrapure water as template.

The obtained product was cloned and the inserts of four colonies were sequenced in both directions. All the sequences were identical, except for their length. The longest sequence

was translated (386 aa) and compared with *L. oneistus* symbiont and *E. coli* FtsZ sequences (Fig. 9). The Eds FtsZ protein had 59% sequence identity with the one of *E. coli* and 79% with the one of *L. oneistus* symbiont.

Figure 9 Protein sequence alignment of *E. dianaea*, *L. oneistus* and *E. coli* FtsZ. Asterisk (*) indicates positions which have a single, fully conserved residue, colon (:) indicates conservation between groups of strongly similar properties-scoring (> 0.5 in the Gonnet PAM 250 matrix) and period (.) indicates conservation between groups of weakly similar properties-scoring (=< 0.5 in the Gonnet PAM 250 matrix).

Eds expresses the FtsZ protein

To test if Eds expresses the FtsZ protein a Western blot of symbiotic nematode protein extracts with mouse monoclonal anti-*E. coli* FtsZ antibody was performed together with protein extracts of the symbiotic stilbonematid *L. oneistus* and of *E. coli*, as positive controls. The predicted molecular mass of FtsZ is 40 kDa. In *E. coli*, *L. oneistus* and *E. dianaea* protein extracts the anti-FtsZ antibody specifically recognized a band of approximately 40 kDa, which

corresponds to the predicted MW of *E.coli* FtsZ (Fig. 10, lanes 1 to 3). No bands appeared if the same Western blot was probed with only anti-mouse secondary antibodies (Fig. 10, lanes 4 to 6). This means that FtsZ is expressed in Eds as well as in *L. oneistus* symbionts (confirming what recently published in Leisch et al., 2012) and, as expected, in the positive control *E. coli*.

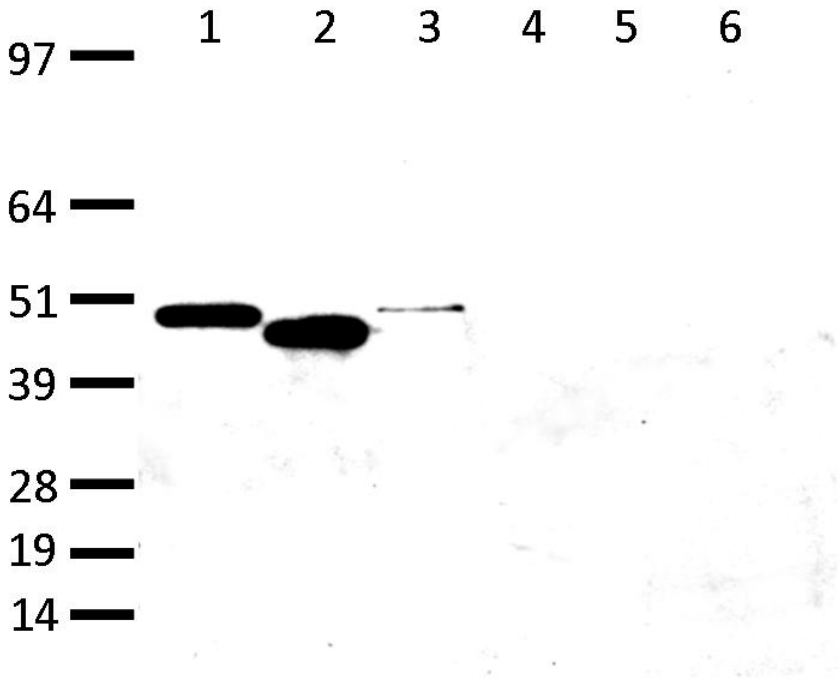

Figure 10 Western blots of symbiotic nematode and *E. coli* protein extracts. *L. oneistus* protein extracts (lanes 1 and 4), *E. coli* protein extract (lanes 2 and 5, control) and *E. dianaea* protein extract (lanes 3 and 6) probed with a mouse monoclonal anti *E. coli* FtsZ antibody (lane 1, 2 and 3, respectively) and secondary antibody alone (lane 4, 5 and 6, respectively). Numbers indicate apparent MW expressed in kDa.

Additionally the expression of the MreB protein was tested on a Western blot of the same protein extracts mentioned above with a rabbit polyclonal *E. coli* MreB antibody (Karczmarek et al., 2007). The predicted molecular mass of MreB is 37 kDa. As in the case of FtsZ, in all three protein extracts the anti-MreB antibody specifically recognized a band of approximately 39 kDa, which corresponds to the predicted MW of *E. coli* MreB (Fig. 11, lanes 1, 3 and 5). No bands appeared if the same Western blot was probed with only anti-rabbit secondary antibodies (Fig. 11, lanes 2, 4 and 6). This result shows that MreB is expressed in both stilbonematids.

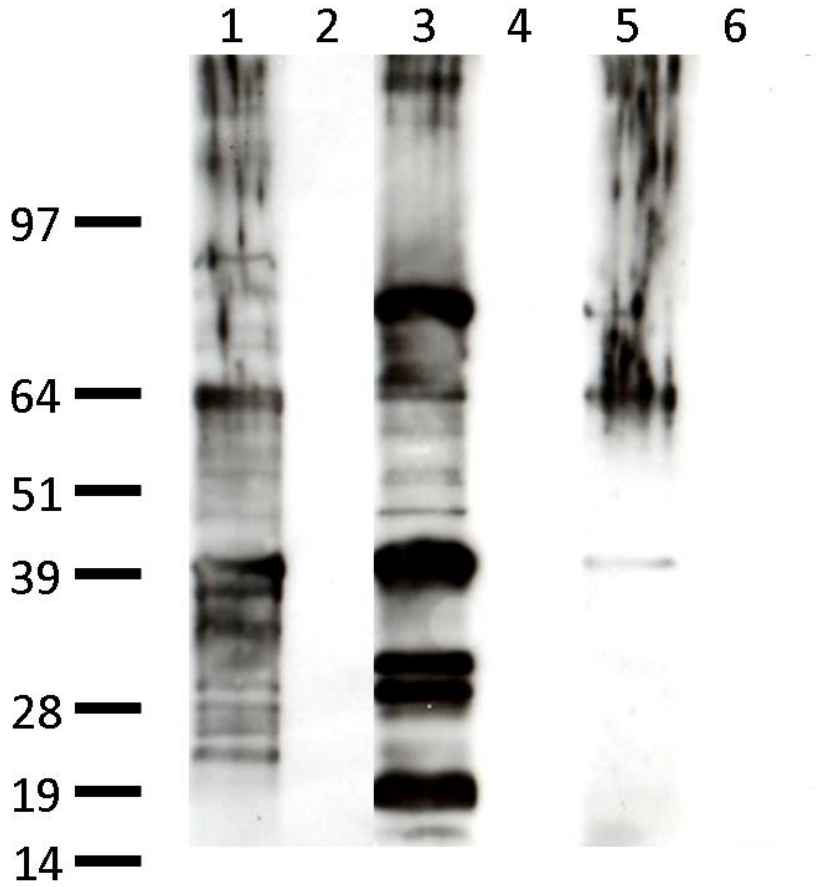

Figure 11 Western blots of symbiotic nematode and *E. coli* protein extracts. *L. oneistus* protein extracts (lanes 1 and 2), *E. coli* protein extract (lanes 3 and 4, control) and *E. dianaea* protein extract (lanes 5 and 6) probed with a rabbit polyclonal anti *E. coli* MreB antibody (lane 1, 3 and 5, respectively) and secondary antibody alone (lane 2, 4 and 6, respectively). Numbers indicate apparent MW expressed in kDa.

Eds is dividing by FtsZ-based binary fission

We immunostained *E. dianaea*-associated bacteria with FtsZ-antibody to visualize its localization pattern. The laser scanning confocal microscope images (Fig 12) showed four different kinds of FtsZ signal; (0) not detectable; (1) punctuate, diffuse; (2) punctuate, concentrated; (3) FtsZ polymerizes into a ring at Eds midcell. Immunostaining with MreB-antibody showed a diffuse and dotty distribution of the MreB protein throughout the cell membrane of filamentous bacteria (Fig. 12). This suggests that Eds cell wall grows uniformly along its length instead of polarly. In stages 1-3, prior to FtsZ ring assembly, the genetic material is compacted and segregated into two areas as revealed by DAPI staining.

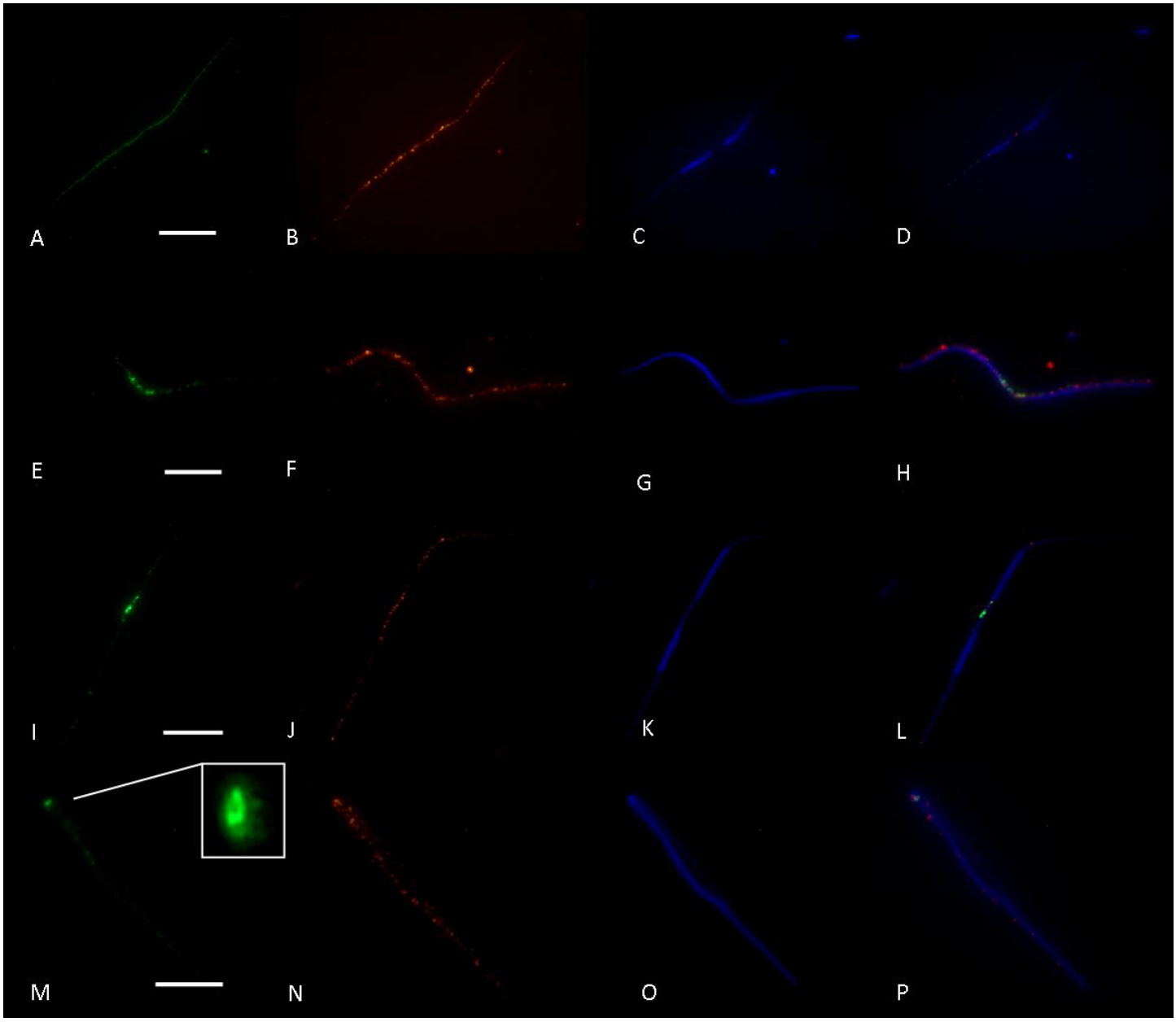

Figure 12 *E. dianaea* ectosymbiont FtsZ and MreB localization pattern. Epifluorescence micrographs of dissociated bacteria of *E. dianaea*, immunostained with an anti- *E. coli* FtsZ antibody and Alexa488-conjugated secondary anti-mouse antibody (green) (A, E, I and M), an anti- *E. coli* MreB antibody and Alexa555-conjugated secondary anti-rabbit antibody (red) (B, F, J and N) and DAPI (blue) (C, G, K and O). In (M) the scare shows an enlargement of the Z-ring. (D, H, L and P) are overlay pictures of both probes together with DAPI. Scale bars represent 10 µm.

In particular, at stage 0 up to 16 nucleoid-like structures may appear (Fig. 13). These can either be found in the middle of the filament or equally segregated into the two halves of the filament (i.e. eight in each half). Taken together these data suggest that Eds may divide by FtsZ-based symmetric, transverse fission.

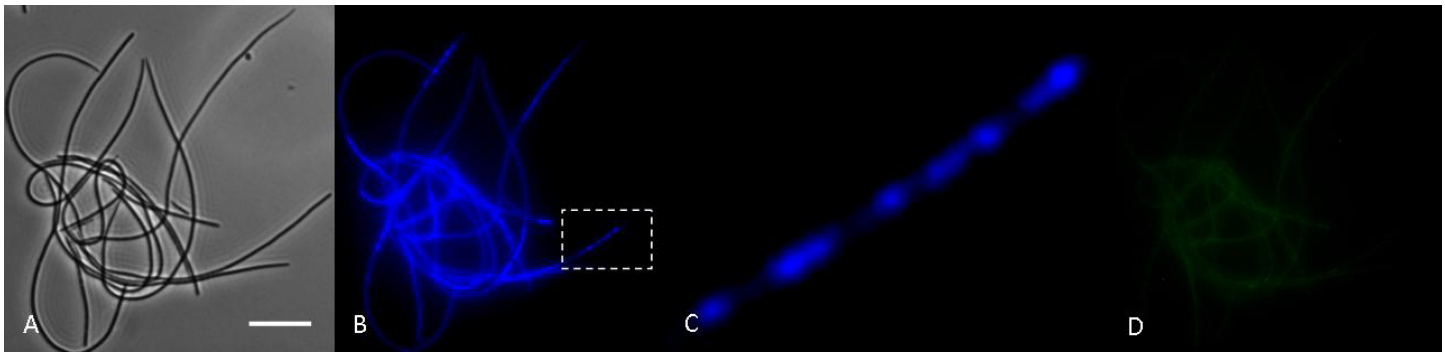

Figure 13 DNA localization in non-dividing Eds. Confocal micrographs of dissociated ectosymbiont cells of *E. dianaea*. (A) Bright Field picture of filamentous bacteria. (B) is filaments stained with DAPI (blue) and (C) is enlargement of filament region with several nuclei visible. (D) is filaments stained with anti- *E. coli* FtsZ antibody and Alexa488-conjugated secondary anti-mouse antibody (green). Scale bars represent 10µm.

DISCUSSION

In this study we molecularly characterized the giant epibiotic bacterium coating the marine nematode *E. dianaea* (Eds). Cloning, sequencing and phylogenetic analysis of the Eds 16S rRNA-gene showed that Eds clusters with other SOB. In particular, it falls into the MONTS cluster (Heindl et al., 2011), which comprises other *Eubostrichus*, stilbonematid- and oligochaete-associated SOB. MONTS are most closely related to free-living SOB from the *Chromatiaceae* family. FISH confirmed that the SOB 16S rRNA-gene sequences obtained in our library can be assigned to Eds. It also revealed that all the oversize filamentous bacteria detected on the nematode cuticle are Eds.

FISH of ectosymbiotic cells dissociated from the nematodes prior mounting revealed that, not only all the giant filamentous bacteria are Eds, but that all the bacteria we found associated to *E. dianaea*, irrespective of their size are Eds. In other words, all detectable *Eu-* or *Gammaproteobacteria* were belonging to the same phylotype, because they were recognized by the probe specifically targeting the SOB 16S rRNA obtained from the library. The fact that Eds appear to be the dominant, if not the only, ectosymbiont is in stark contrast with the former study by (Polz et al., 1999), which showed a high level of bacterial diversity associated to the worm. However, all bacterial sequences obtained in the former study originated from bacteria that can be commonly found in marine environments and FISH analysis could not assign them to any *E. dianaea*-associated bacterium. Consistently, we also obtained a heterogeneous 16S rRNA-gene library, containing among others, SRB sequences which we could not assign to any *E. dianaea*-associated bacteria by FISH (Fig. 7). The lack of SOB sequences in the library constructed in the previous study could be due to a PCR bias. The discrepancy between the heterogeneity of our *E. dianaae* 16S rRNA-gene library and the apparent phylogenetic homogeneity of the bacterial coat when applying FISH, could be explained as follows: (1) non-Eds bacteria are too rare to be detected by FISH; (2) non-Eds bacteria are not associated to the cuticle but to other body regions such as the gut. Taken together, although we cannot exclude that bacteria other than Eds may occasionally be found on the nematode surface, all *E. dianaea* epibionts visualized in our study belong to a single phylotype. Moreover, Eds appears to grow on the nematode as this also hosts non-giant, Eds probe-positive bacteria.

The presence of multiple nucleoids (polyploidy) was already observed in several organisms, e.g. in the *E.* cf. *parasitiferus* ectosymbiotic bacteria (Polz et al., 1992) or in the endosymbiont of the weevil genus *Sitophilus* (Login et al., 2011). Here, it was proved that polyploidy is due to a weevil-secreted, antimicrobial peptide (ColA) that inhibits cell division. It was shown that all bacteria are polyploid and that the number of chromosomes correlates with the size of the bacteria, e.g. in *Nardonella* 120 chromosomes in a 200 µm-long giant cell. Similarly, in symbiotic rhizobia endoreduplication is triggered by host-secreted, nodule-specific cysteine-rich (NCR) peptides (Van de Velde et al., 2010). Endoreduplication, like it is the case in the weevil endosymbiont or in the *Rhizobium* symbionts (Mergaert et al., 2006), could be necessary to support the large size of the filamentous Eds. The investigation on nematode-secreted peptides should be pursued, since it might be possible that some of them or other host-produced molecules mediate cytokinesis inhibition/endoreduplication.

Beside the assumption that Eds might grow on the worm into filaments, FtsZ immunostaining revealed that Eds may divide by binary fission. We observed different FtsZ signals,, from not-detectable to ring-like (the latter signal was only observed in about 10 % of the cells). In non-dividing Eds the nucleoids were found in the middle of the filament (Fig. 13), whereas in dividing filaments the DNA was compacted and segregated into two distinct areas (Fig. 12 G and K). This DNA high compaction into distinct nucleoids could facilitate DNA segregation prior FtsZ ring positioning and binary fission.

To get a closer insight into the cell division of *Eds* and the positioning of the Z-ring additional studies on the cell division proteins should be done. In the *Gammaproteobacteria* the positioning of the FtsZ-ring depends on the MinCDE-system, which prevents aberrant division at the cell poles (Adams and Errington, 2009). MinC prevents FtsZ polymerization via two distinct mechanisms. In *E. coli* cells the inhibitory activity of MinCD is restricted to the poles, which is driven by MinE. MinE stimulates periodic oscillations of MinC and MinD along the long axis and thereby only allows the positioning of the FtsZ-ring at midcell (Lutkenhaus, 2007). A modification or a host induced inhibition of the Min-system on one pole of the cell could maybe cause asymmetric Z-ring formation and cell division and thereby relay the attachment of both, mother and daughter cell on the host's cuticle.

Since its discovery *E. dianaea* unusual appearance inspired very accurate morphological analyses (Hopper and Cefalu, 1973). On the other hand, the molecular and cellular mechanisms underlying its association with its symbiotic bacteria are not known:

why Eds become so big and what is the resulting ecological advantage for both, host and symbiont? How is the FtsZ ring placed in the middle of a giant filament? How is the equal segregation of several nucleoids achieved? This study is a first step in better understanding the growth and reproduction mechanisms of Eds.

Conclusions

Eds was molecularly identified as a sulfur-oxidizing bacterium clustering with other stilbonematid symbionts into the MONTS-cluster. This result fits very well with the lifestyle of stilbonematid nematodes and the sulfur granules found in the ectosymbiotic filaments in previous studies. Additionally, we showed that all detectable bacteria belong to one single phylotype. The presence of the *ftsZ*-gene and of a FtsZ protein that can polymerize into a ring indicate that Eds – despite being oversize - may undergo binary fission. The fact that the giant bacteria are polyploid and may segregate equal numbers of nucleoids in their daughter cells is also remarkable. Taken together, this is the first report of a >100 µm polyploid *Gammaproteobacteria* which divides by symmetric transverse binary fission.

REFERENCES

Aarsman, M.E., Piette, A., Fraipont, C., Vinkenvleugel, T.M., Nguyen-Disteche, M., and den Blaauwen, T. (2005). Maturation of the Escherichia coli divisome occurs in two steps. Mol Microbiol *55*, 1631-1645.

Adams, D.W., and Errington, J. (2009). Bacterial cell division: assembly, maintenance and disassembly of the Z ring. Nat Rev Microbiol *7*, 642-653.

Ahmadjian, V., and Paracer, S. (1986). Symbiosis: An introduction to Biological Associations (Hanover, N. H., University Press of New England).

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

Amann, R.I., Krumholz, L., and Stahl, D.A. (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol *172*, 762-770.

Angert, E.R. (2012). DNA replication and genomic architecture of very large bacteria. Annu Rev Microbiol *66*, 197-212.

Bary, A. (1879). Die Erscheinung der Symbiose. Vortrag gehalten auf der Versammlung Deutscher Naturforscher und Aerzte zu Cassel (K. J. Trübner).

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

Brosius, J., Palmer, M.L., Kennedy, P.J., and Noller, H.F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli. Proc Natl Acad Sci U S A *75*, 4801-4805.

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

Den Blaauwen, T., Buddelmeijer, N., Aarsman, M.E., Hameete, C.M., and Nanninga, N. (1999). Timing of FtsZ assembly in Escherichia coli. J Bacteriol *181*, 5167-5175.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M*., et al.* (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res *36*, W465-469.

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

Erickson, H.P. (2001). Cytoskeleton. Evolution in bacteria. Nature *413*, 30.

Fenchel, T.M., and Riedl, R.J. (1970). The sulfide system: a new biotic community underneath the oxidized layer of marine sand bottoms. Marine Biology (Berlin) 7(3): 255-268.

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

Greeff, R. (1869). Untersuchungen liber einige Formen des Arthropoden- und Wurm-Typus. Arch Gesch Naturwiss *35*, 117- 118.

Gruber-Vodicka, H.R., Dirks, U., Leisch, N., Baranyi, C., Stoecker, K., Bulgheresi, S., Heindl, N.R., Horn, M., Lott, C., Loy, A*., et al.* (2011). Paracatenula, an ancient symbiosis between thiotrophic Alphaproteobacteria and catenulid flatworms. Proc Natl Acad Sci U S A *108*, 12078-12083.

Heindl, N.R., Gruber-Vodicka, H.R., Bayer, C., Lucker, S., Ott, J.A., and Bulgheresi, S. (2011). First detection of thiotrophic symbiont phylotypes in the pelagic marine environment. FEMS Microbiol Ecol *77*, 223-227.

Hentschel, U., Berger, E.C., Bright, M., Felbeck, H., and Ott, J. (1999). Metabolism of nitrogen and sulfur in ectosymbiotic bacteria of marine nematodes (Nematoda, Stilbonematinae). Marine Ecology Progress Series 183, 149-158.

Himmel, D., Maurin, L.C., Gros, O., and Mansot, J.L. (2009). Raman microspectrometry sulfur detection and characterization in the marine ectosymbiotic nematode Eubostrichus dianae (Desmodoridae, Stilbonematidae). Biol Cell *101*, 43-54.

Hopper, B.E., and Cefalu, R.C. (1973). Free-living nematodes from Biscayne Bay, Florida, V. stilbonematinae: contributions to the taxonomy and morphology of the genus Eubostrichus greeff and related genera. Trans Am Micros Soc 4, 578–591.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Roser, A., Koops, H.P., and Wagner, M. (1998). Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-like bacteria as dominant populations. Appl Environ Microbiol *64*, 3042-3051.

Kane, M.D., Poulsen, L.K., and Stahl, D.A. (1993). Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. Appl Environ Microbiol *59*, 682-686.

Karczmarek, A., Martinez-Arteaga, R., Alexeeva, S., Hansen, F.G., Vicente, M., Nanninga, N., and den Blaauwen, T. (2007). DNA and origin region segregation are not affected by the transition from rod to sphere after inhibition of Escherichia coli MreB by A22. Mol Microbiol *65*, 51-63.

Katoh, K., and Standley, D.M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol.

Login, F.H., Balmand, S., Vallier, A., Vincent-Monegat, C., Vigneron, A., Weiss-Gayet, M., Rochat, D., and Heddi, A. (2011). Antimicrobial peptides keep insect endosymbionts under control. Science *334*, 362-365.

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

Lutkenhaus, J. (2007). Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. Annu Rev Biochem *76*, 539-562.

Manz, W., Amann, R., Ludwig, W., Wagner, M., and Schleifer, K.-H. (1992). Phylogenetic Oligodeoxynucleotide Probes for the Major Subclasses of Proteobacteria: Problems and Solutions. Systematic and Applied Microbiology *15*, 593-600.

Maurin, L.C., Himmel, D., Mansot, J.L., and Gros, O. (2010). Raman microspectrometry as a powerful tool for a quick screening of thiotrophy: an application on mangrove swamp meiofauna of Guadeloupe (F.W.I.). Mar Environ Res *69*, 382-389.

McFall-Ngai, M.J., and Gordon, J.I. (2006). Experimental models of symbiotic host-microbial relationships: understanding the underpinnings of beneficience and the origins of pathogenesis. In Evolution of Microbial Pathogens, H. Steven Seifert, and Victor J. DiRita, eds. (Washington, D.C., ASM Press).

Mendell, J.E., Clements, K.D., Choat, J.H., and Angert, E.R. (2008). Extreme polyploidy in a large bacterium. Proceedings of the National Academy of Sciences *105*, 6730-6734.

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., Mausset, A.E., Barloy-Hubler, F., Galibert, F., Kondorosi, A.*, et al.* (2006). Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium-legume symbiosis. Proc Natl Acad Sci U S A *103*, 5230-5235.

Miller, D., Suen, G., Clements, K., and Angert, E. (2012). The genomic basis for the evolution of a novel form of cellular reproduction in the bacterium Epulopiscium. BMC Genomics *13*, 265.

Mortazavi, A., Schwarz, E.M., Williams, B., Schaeffer, L., Antoshechkin, I., Wold, B.J., and Sternberg, P.W. (2010). Scaffolding a Caenorhabditis nematode genome with RNA-seq. Genome Res *20*, 1740-1747.

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

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

Ott, J., Bright, M., and Bulgheresi, S. (2004b). Marine microbial thiotrophic ectosymbioses. Oceanography and Marine Biology: An Annual Review 42, 95-118.

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, J.S. Ryland, and P.A. Tyler, eds. (Fredensbourg, Olsen & Olsen).

Ott, J.A., Novak, R., Schiemer, F., Hentschel, U., Nebelsick, M., and Polz, M. (1991). Tackling the sulfide gradient: a novel strategy involving marine nematodes and chemoautotrophic ectosymbionts. Pubblicazioni della Stazione Zoologica di Napoli I Marine Ecology 12, 261–279.

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

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

Polz, M.F., Harbison, C., and Cavanaugh, C.M. (1999). Diversity and heterogeneity of epibiotic bacterial communities on the marine nematode Eubostrichus dianae. Appl Environ Microbiol *65*, 4271-4275.

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

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol *28*, 2731-2739.

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., Farkas, A., Mikulass, K., Nagy, A., Tiricz, H*., et al.* (2010). Plant peptides govern terminal differentiation of bacteria in symbiosis. Science *327*, 1122-1126.

Vetter, R.D. (1985). Elemental sulfur in the gills of three species of clams containing chemoautotrophic symbiotic bacteria: a possible inorganic energy storage compound. Mar. Biol. 88: 3342.

Voskuil, J.L., Westerbeek, C.A., Wu, C., Kolk, A.H., and Nanninga, N. (1994). Epitope mapping of Escherichia coli cell division protein FtsZ with monoclonal antibodies. J Bacteriol *176*, 1886-1893.

Zusammenfassung (see Abstract)

In dieser Arbeit wurden die riesigen epibiotischen Bakterien (Eds), die auf dem marinen Nematoden *E. dianaea* leben, molekular identifiziert. Nach Klonierung und Sequenzierung zeigte die phylogenetische Analyse der Eds 16S rRNA-Gene, dass Eds in eine Gruppe mit anderen Schwefel-oxidierenden Bakterien (SOB) fällt. Im Besonderen gehört Eds zum MONTS Cluster, welches auch andere *Eubostrichus*-, Stilbonematid- und Oligochaeten-assoziierte SOB beinhaltet. Mit Hilfe von Fluoreszenz *in situ* Hybridisierung (FISH) konnte gezeigt werden, dass die gefundenen SOB 16S rRNA-Gene aus der Gen-Bibliothek zu Eds gehören. Weiters zeigten FISH-Versuche von dissoziierten ektosymbiontischen Zellen, dass nicht nur alle überdimensionalen filamentösen Bakterien auf der Nematodenkutikula, sondern auch die kleineren Bakterien, unabhängig von ihrer Größe und Morphologie, auch Eds sind. Die heterogene 16S rRNA Gen-Bibliothek enthielt auch Sequenzen von Schwefel-reduzierenden Bakterien (SRB), die jedoch mit FISH zu keinem *E. dianaea* assoziierten Bakterium zugeteilt werden konnten. Die Diskrepanz zwischen der Heterogenität unserer 16S rRNA Gen-Bibliothek und der augenscheinlichen phylogenetischen Homogenität des bakteriellen Mantels kann (1) dadurch erklärt werden, dass nicht-Eds Bakterien zu selten sind um sie mit FISH nachzuweisen und (2), dass nicht-Eds nicht unbedingt mit der Kutikula assoziiert sind, sondern auch mit anderen Körperregionen wie z.B.: dem Darm. Zusätzlich scheint Eds auf dem Nematoden zu wachsen, da auch kleine Zellen mit der Eds-Sonde ein Fluoreszenzsignal zeigten. Ein weiterer Anhaltspunkt dafür ist die Präsenz von multiplen Zellkernen (Polyploidie). In anderen Organismen wie Rhizobien wurde gezeigt, dass die Polyploidie durch Endoreduplikation entsteht. Diese wird durch den Wirt sekretierte Proteine induziert, was auch hier der Fall sein könnte. Weitere Anstrengungen zur Erforschung von Nematod-sekretierten Peptiden und anderen Molekülen sollten angestellt werden, da diese möglicherweise die Cytokinese-Inhibierung/ Endoreduplikation vermitteln könnten.

Um auf die zweite Frage einzugehen, ob sich Eds teilen, wurde zuerst PCR mit FtsZ-spezifischen Primern mit genomische DNA von *E. dianaea* durchgeführt. Nachdem das Ftsz-Gen in der DNA nachgewiesen werden konnte, wurde die Expression dieses Gen mittels Western blot mit monoklonalen Maus Anti-Ftsz Antikörpern in Nematoden-extrakten getestet. Da das FtsZ-Gen exprimiert wird, haben wir Immunfluoreszenzuntersuchungen mit FtsZ-Antikörpern durchgeführt, um das Protein zu visualisieren und lokalisieren. Die

Immunfluoreszenzuntersuchungen zeigten, dass sich Eds durch binäre Teilung vermehrt. Es konnten vier verschiedene Stadien des FtsZ-Signals gezeigt werden: (0) nicht nachweisbares Signal; (1) gepunktet, diffus; (2) gepunktet, konzentriert und (3) ringförmige Struktur in etwa in der Zellmitte. Nur ein kleiner Teil der filamentösen Bakterien scheinen sich zu teilen, in etwa 10% der Eds-Population. In nicht-teilenden Eds befanden sich multiple Nucleoide in der Mitte der Zelle. In sich teilenden Filamenten war die DNA verdichtet und teilte sich in zwei bestimmte Zonen auf. Diese DNA-Verdichtung in getrennte Nucleoide könnte die DNA-Segregation vor der Positionierung des FtsZ-Rings und der binären Teilung erleichtern.

Immunfluoreszenzuntersuchungen mit MreB-Antikörpern zeigten eine diffuse und gepunktete Verteilung des MreB Proteins durchgehend in der ganzen Zellmembran der filamentösen Bakterien. Das weist daraufhin, dass die Zellwände von Eds gleichmäßig entlang ihrer Längsachse, und nicht polar, wachsen. Um einen näheren Einblick in die Zellteilung von Eds und die Positionierung des Z-Rings zu erhalten, sollten noch weiter Studien an anderen bei der Zellteilung beteiligten Proteinen durchgeführt werden.

Acknowledgements

First I want to thank my two supervisors Jörg Ott and Silvia Bulgheresi, who gave me the opportunity to work on this interesting and challenging topic. During my work for the diploma thesis I got an intense insight in the scientific work and research. Special thanks go to Silvia. She is not only a very warm and helpful supervisor, but also a great and creative scientist. In the moments when everything seemed to go wrong Silvia gave me the support and strength to continue. By sometimes leaving me on purpose alone with my work, she forced me to think about problems and solve them on my own and thereby she promoted my scientific skills. Jörg Ott was always the spirit and force, who held our Shallow Water Symbiosis Group together. Without his pioneering work on the marine nematodes this project wouldn't even exist.

At this point I would like to thank my dear (partly former) members of the Shallow Water Symbiosis Group: Harald Gruber-Vodicka, Niko Leisch, Ulrich Dirks, Amir Schmidt und Lisa Bauer. Their feedback after every presentation was helping me to refine my style, making me more confident and relaxed while presenting. They were also always very helpful in the lab and they gave me some constructive comments. I am also very grateful to Harald who helped me with my phylogenetic analysis and who took the time to explain me the tools and programs in order to be able to do it myself.

I would also like to thank very much the Department of Genetics in Ecology and Christa Schleper, who provided me with a perfectly equipped working space. But more important than the equipment were the people there. The dear colleagues and the nice atmosphere made it a real pleasure to work in this department. I can't think of any better working place. Many thanks go to my colleagues and friends Amir and Lisa. The inspiring conversations and their humour made me enjoy my stay in the lab.

The Department of Marine Biology under the supervision of Gerhard Herndl gave me the chance to get insight in different fields of Marine Biology and to profit of their knowledgability. My dream to become a marine Biologist finally turned into reality

I have to thank Silvia again for the opportunity not only to go on a field trip to Belize, but also to work in a bilateral project between Austria and Montenegro. The trip to the Smithsonian field station in Belize made me see where ´´my`` nematodes are coming from and gave me the chance to work in the field. Also the two trips to Kotor, Montenegro and

the work with the people there was a rewarding experience. My thanks also go to the Austrian Science Fond (FWF) for financing the project P22470, which made the work on my diploma thesis possible.

And last, but not least I would like to thank my family for their support, it's the best family in the world and without it I wouldn't have make it so far. My brother Marko was not only a mental support, but he was also always explaining me the genetics aspects in my work and helped me to solve some problems. I have to thank my dear friend Veronika, who pushed me during my studies and helped me to find my way in the bureaucracy jungle. Also I have to thank Danae, who corrected the German part of my diploma thesis although she had to deal with her own one. At the end a special thanks goes to my boyfriend Olivier, who went with me through my motivation ups and downs in writing the thesis. He was not only trying to understand what I am doing (since he comes from a completely different field), but also giving me inspiring comments on my manuscript and thereby making it better.

Curriculum vitae

Nika Pende

born, 1988 in Zagreb, Croatia.

2006-2013 Study of Ecology (with focus on Marine Ecology) and of Zoology (with focus on Anatomy and Morphology), at the University of Vienna, Austria.

2011-2013 Diploma student at the Department of Genetics in Ecology, Faculty of Life Sciences, University of Vienna; expected date of graduation: Mai 2013.

2012-2013 Erasmus student at the Université Pierre et Marie Curie, Paris, France.

Further education and Internships:

Dec. 2012-Feb.2013 Two months lab internship at CNRS - MNHN (Centre national de la recherche scientifique - Museum national d'Histoire naturelle), team Adaptation aux Milieux Extrêmes (AMEX) under the supervision of PhD Sébastien Duperron, Université Pierre et Marie Curie, Paris, France. Subject: Bivalves (*Mytilidae)* associated symbiotic bacteria and microbial biodiversity of deep sea submerged wood samples.

Oct. 2012 Bioinformatics course (held by Genoscreen), organized by PhD Sébastien Duperron at the Université Pierre et Marie Curie, Paris, France. Subject: Methods and tools to analyze big dataset of high throughput pyrosequencing (sequences obtained from microbial community of deep sea submerged wood samples).

Sep. 2011 Marine ecology internship: Tidal influenced community shifts of bacterioplankton and impact of a storm event in coastal North Sea waters, NIOZ, Texel, Netherlands

Oct. 2010 Practical **c**ourse: Molecular analysis of gene expression, at the Cancer Research Institute, Medical University of Vienna.

May 2010 PADI (Professional Association of Diving Instructors) Divemaster certification (allows guiding people under water and assisting diving instructors during courses).

Apr. 2010 Project internship: Practice in Fossil and Recent Coral Reefs in the Golf of Aqaba, Egypt. Subject: Differences in distribution of conchiferous molluscs in coral reefs.

Feb. 2010 Project internship: Ecology and Behaviour of Insects in Costa Rica, field station La Gamba, Costa Rica. Subject**:** Activity under poor light conditions in correlation to eye size in stingless bees

Sep. 2008 Marine Biology course at the Hydra Institute on Elba, Italy. Subject: General Marine Biology course, with examination of material, which was previously collected by scuba diving.

Research field trips:

Dec. 2011- Jan. 2012 3 weeks sampling at the Smithsonian field station in Carrie Bow Cay, Belize

2011 2 weeks sampling at the Marine Biology Institute, Kotor, Montenegro (Project Nr. ME 01/2011, OeAD)

2012 one week sampling at Marine Biology Institute, Kotor, Montenegro (Project Nr. ME 01/2011, OeAD)

Oral presentations:

Science Day of the Vienna Ecology Center, March 2012, Vienna, Austria: "Molecular characterization of an *Oceanospirillales* bacterium associated to the *Laxus oneistus* male reproductive system".

7th International Symbiosis Society meeting, 22 - 28 July, 2012, Krakow, Poland: "Can you hear me? How the marine nematode *Laxus oneistus* may perceive and respond to microbes" (speaker: S. Bulgheresi)

Publications:

Leisch,N., Verheul, J., Heindl, N.R., Gruber-Vodicka, H.R., **Pende, N.,** den Blaauwen, T., and Bulgheresi, S. (2012). Growth in width and FtsZ ring longitudinal positioning in a gammaproteobacterial symbiont. Curr Biol *22*, R831-832.

Pende N., Leisch N., Heindl N.R., Gruber-Vodicka H.R., Bulgheresi S. Molecular identification of two oversize, filamentous ectosymbonts, in preparation.

Pende, N., Gruber-Vodicka H.R., Leisch N. and Bulgheresi, S. Molecular identification of an *Oceanospirillales* bacterium associated to *Laxus oneistus* male reproductive organ, in preparation.