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Acquisition of a novel sulfur-oxidizing symbiont in the gutless marine worm

*Inanidrilus exuma*

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Abstract

Gutless phallodrilines are marine annelid worms without a mouth or gut, which live in an obligate association with multiple bacterial endosymbionts that supply them with nutrition. In this study, we discovered an unusual symbiont community in the gutless phallodrine Inanidrilus exumae that differs markedly from the microbiome of all other 22 examined host species. Comparative 16S rRNA gene sequence analysis and fluorescence in situ hybridization revealed that I. exumae harboured co-occurring gamma-, alpha- and deltaproteobacterial symbionts, while all other known host species harbour gamma- and either alpha- or deltaproteobacterial symbionts.

Surprisingly, the primary chemoautotrophic, sulfur oxidizer, Ca. Thiosymbion, which occurs in all other gutless phallodrine hosts, does not appear to be present in I. exumae. Instead, I. exumae harboured a bacterial endosymbiont that resembled Ca. Thiosymbion morphologically and metabolically, but originated from a novel lineage within the Gammaproteobacteria. This endosymbiont, named Gamma 4 symbiont here, had a 16S rRNA gene sequence that differed by at least 7% from those of other free-living and symbiotic bacteria, and by 10% from Ca. Thiosymbion. Sulfur globules in the Gamma 4 symbiont cells, as well as the presence of genes characteristic for autotrophy (cbbL) and sulfur oxidation (aprA), indicate that this
symbiont is a chemoautotrophic sulfur oxidizer. Our results suggest that a novel
lineage of free-living bacteria was able to establish a stable and specific association
with I. exumae, and appears to have displaced the Ca. Thiosymbion symbionts
originally associated with these hosts.

Importance

All 22 gutless marine phallodrine species examined to date live in a highly
specific association with endosymbiotic, chemoautotrophic sulfur oxidizers called Ca.
Thiosymbion. These symbionts evolved from a single common ancestor and represent
the ancestral trait for this host group. They are transmitted vertically and assumed to
be in transition to becoming obligate endosymbionts. It is therefore surprising that
despite this ancient, evolutionary relationship between phallodrine hosts and Ca.
Thiosymbion, in I. exumae these symbionts are apparently no longer present. They
appear to have been displaced by a novel lineage of sulfur-oxidizing bacteria only
very distantly related to Ca. Thiosymbion. This study, thus, highlights the remarkable
plasticity of both animals and bacteria in establishing beneficial associations: the
phallodrine hosts were able to acquire and maintain symbionts from two very
different lineages of bacteria, while sulfur-oxidizing bacteria from two very distantly
related lineages were able to independently establish symbiotic relationships with
phallodrine hosts.

Introduction

Symbioses are essential for the ecology and evolution of eukaryotes, but the
processes involved in symbiosis initiation and maintenance are still only poorly
understood (1, 2). Stable, long-lasting and specific associations between symbionts and their hosts are common in vertically transmitted symbionts (inheritance of the symbiont from the parent). In such associations, if the symbionts are consistently and strictly transmitted to the host, co-diversification occurs and is reflected in congruent phylogenies of the symbionts and their hosts (3, 4). However, strict vertical transmission over long evolutionary time periods, while well-known from some insect symbioses, has rarely been observed in marine symbioses (4).

In the beneficial association between gutless, marine phallodrilines (oligochaetes, Annelida, Clitellata, Naididae *sensu* (5)) and their bacterial endosymbionts, the hosts lack a mouth, gut, and an excretory system, and are dependent on their symbionts for nutrition and waste recycling. The primary symbionts in all gutless phallodrine worms examined to date are large (2 – 7 µm) sulfur-storing Gammaproteobacteria, previously called Gamma 1 symbionts, and now named *Candidatus* Thiosymbion (6). All individuals of a given host species share a highly similar *Ca.* Thiosymbion phylotype, with > 99% 16S rRNA gene sequence similarity. Among host species, the 16S rRNA gene sequences of *Ca.* Thiosymbion are closely related to each other (> 94.7% identity) and have evolved from a single common ancestor (6). Evidence for the chemoautotrophic metabolism of *Ca.* Thiosymbion includes the presence of sulfur globules (7), uptake experiments showing the incorporation of inorganic carbon (8, 9), immunohistochemical labelling of one of the key enzymes for CO₂ fixation, ribulose-1,5-bisphosphate carboxylase/oxygenase (7, 10, 11), and more recently, metagenomic and proteomic analyses revealing the expression of pathways used for the fixation of inorganic carbon and the use of reduced sulfur compounds as an energy source (12, 13).
The primary Ca. Thiosymbion symbionts co-occur with secondary symbionts that are much smaller (0.7 – 1.5 µm). These secondary symbionts are rod- and cocci-shaped, and belong to the Gamma-, Delta- or Alphaproteobacteria, while secondary symbionts with an elongated, spiral-shaped morphotype belong to the spirochetes (14). The secondary gammaproteobacterial symbionts are sulfur oxidizers, while the deltaproteobacterial symbionts are sulfate reducers. The sulfate-reducing symbionts provide the sulfur-oxidizing symbionts with reduced sulfur compounds, thus allowing their hosts to live in sediments with little or no environmental sulfide (11, 12, 15). The metabolism of the alphaproteobacterial and spirochete symbionts remains unclear (15, 16).

The dominant mode of symbiont transmission in gutless phallodrilines is vertical. Morphological studies indicated that both the primary and secondary symbionts are passed vertically from the parent worm to the offspring in a smear infection during the deposition of the egg in the sediment environment (17, 18). However, a recent analysis of Ca. Thiosymbion from 22 phallodrine host species found only weak congruence between symbiont and host phylogenies, and little evidence for cospeciation (6). This indicates that repeated events of symbiont displacement through switching of Ca. Thiosymbion between host species have occurred in gutless phallodrilines (6).

In this study, we describe a gutless phallodrine in which Ca. Thiosymbion does not appear to be present, namely Inanidrilus exumae Erséus, 2003 from the Bahamas (19, 20). We hypothesize that Ca. Thiosymbion was displaced in I. exumae, but not through host switching. Instead, these hosts appear to have taken up sulfur-oxidizing bacteria from a novel lineage only very distantly related to the ancestral Ca. Thiosymbion of gutless phallodrilines.
Results and Discussion

Our morphological and molecular analyses revealed an unusual symbiotic community in *Inanidrilus exumae*, consisting of co-occurring gamma-, alpha- and deltaproteobacterial symbionts (Fig. 1, Fig. 2, Fig. S1). In the five host species whose symbiont communities have been examined so far, alpha- and deltaproteobacterial symbionts appeared to be mutually exclusive (15, 16, 21, 22). Furthermore, we found no evidence for the presence of the primary symbiont, *Ca. Thiosymbion* in *I. exumae*. This is surprising because in the 22 gutless phalldrine species examined to date, these always harboured *Ca. Thiosymbion* symbionts (6). In contrast, *I. exumae* harbours a sulfur oxidizer that resembles *Ca. Thiosymbion* in appearance and function, but belongs to a lineage of Gammaproteobacteria not previously known to be associated with gutless phalldrinines or other eukaryotic hosts. In the following, we will focus on the morphology, phylogeny and potential function of this novel gammaproteobacterial symbiont of *I. exumae*. A brief description of the phylogeny and possible function of the secondary alpha- and deltaproteobacterial symbionts of *I. exumae*, is provided in the Supplemental Material.

Morphology and phylogeny of the Gamma 4 symbiont

Only a single gammaproteobacterial 16S rRNA phylotype, which we named Gamma 4, was found in the clone libraries from *I. exumae* worms (Table 1). Out of a total of 734 sequenced clones from seven host individuals, we never found a sequence that belonged to the *Ca. Thiosymbion* clade. In previous studies of other gutless
The 16S rRNA primers we used in this study (8F and 1492R) (21, 22).

FISH provided further support for our assumption that the Gamma 4 symbiont is the only Gammaproteobacterium present in *I. exumae*. FISH with a probe specific to the 16S rRNA gene sequence of the Gamma 4 symbiont (IexuGAM4, Table 2) showed that this sequence originated from large oval-shaped bacteria (2 – 3 µm length and 1-2 µm width) that were highly abundant and dominated the symbiont-containing region in all host individuals examined (Fig. 1A and D). Dual FISH hybridization with the specific IexuGAM4 probe and the general probe for Gammaproteobacteria (GAM42a, Table 2) showed a complete overlay of the hybridization signals, with both probes hybridizing to the same large oval-shaped morphotype (Fig. 1D). These results indicate that the Gamma 4 symbionts are the only Gammaproteobacteria present in *I. exumae*, and that these hosts lack the *Ca. Thiosymbion* found in all other examined gutless phalodrine species.

Transmission electron microscopy showed that the ultrastructure of the Gamma 4 symbiont was remarkably similar to that of *Ca. Thiosymbion* symbionts (Fig. 1I and J). Like *Ca. Thiosymbion*, the Gamma 4 symbiont was the largest (2 – 3 µm) and most abundant morphotype of the symbiotic community, and its cells were also filled with large electron-dense globules (Fig. 1I).

Comparative phylogenetic analyses of 16S rRNA gene sequences revealed that the Gamma 4 symbiont belongs to a novel lineage of Gammaproteobacteria not previously shown to be associated with animal or plant hosts (Fig. 2). While the phylogenetic resolution of the gammaproteobacterial tree was not well defined at the basal nodes (Fig. 2), we never observed any clustering of the Gamma 4 symbiont sequence with the *Ca. Thiosymbion* clade in our analyses. Indeed, the 16S rRNA
gene sequence of the Gamma 4 symbiont differed from sequences belonging to the

Ca. Thiosymbion clade by more than 10% (Fig. 2). The closest uncultured relative

with a sequence divergence of 7% was a sediment clone from a beach in the Cies

Islands, off the coast of northern Spain (JF344692). The closest cultured relatives with

a sequence divergence of 9 - 10% were sulfur-storing Ectothiorhodospiraceae, and

bacteria from the genera Nitrosococcus and Methyllococcus.

Indications for autotrophic sulfur oxidation by the Gamma 4 symbiont

Despite their divergent phylogeny, Ca. Thiosymbion and the I. exumae

Gamma 4 symbionts not only share highly similar morphologies, but also appear to

have similar functional roles as chemoautotrophic sulfur oxidizers. As shown for

other chemoautotrophic symbioses, the cbbL gene, coding for one of the key proteins

of the Calvin-Benson-Bassham (CBB) cycle, the ribulose-1,5-bisphosphate

carboxylase/oxygenase (RubisCO) form I large subunit, was present in I. exumae

(Fig. 3A, Table 1) (23). The cbbL sequence obtained from I. exumae grouped with

sequences from other gammaproteobacterial chemoautotrophs such as free-living

Chromatiaceae and sulfur-oxidizing symbionts from other marine invertebrates. It is

therefore likely that the I. exumae cbbL sequence originated from the I. exumae

Gamma 4 symbiont.

Evidence for the potential of the Gamma 4 symbiont to oxidize reduced sulfur

compounds was provided by Raman spectroscopy analyses, which revealed sulfur in

the cells of these symbionts (Fig. 1K, S2.1, S2.2). Moreover, we amplified aprA genes

(encoding AprA, the alpha subunit of adenosine-5'-phosphosulfate (APS) reductase)

related to those of free-living and symbiotic sulfur-oxidizing bacteria from I. exumae
individuals (Fig. 3B, Table 1). Sequences belonging to two phylogenetically distinct
APS reductase lineages, AprA I and II, were found in *I. exumae* (Fig. 3B). We assume
that the sequences from both the AprA I and II originated from the Gamma 4
symbiont, as no other gammaproteobacterial sulfur oxidizers were found in *I. exumae*,
and the alphaproteobacterial symbionts of gutless phallodrilines do not appear to have
an APS reductase (22). The presence of two gene loci for AprA has been shown for
several free-living sulfur-oxidizing bacteria and is therefore not unusual (24). Meyer
and Kuever (2007) (24) hypothesized that the presence of two gene loci might provide
physiological versatility in habitats with oscillating oxygen and sulfide
concentrations. This may well be the case for *I. exumae* and other gutless
phallodrilines, which migrate between upper oxidized and lower sulfidic sediment
layers.

Symbiont replacement in *I. exumae*?

What are the evolutionary events that might explain the presence of a novel
sulfur-oxidizing symbiont and the absence of the ubiquitous *Ca. Thiosymbion* in
*I. exumae*? *Ca. Thiosymbion* is present in all 22 gutless phallodrilines species
examined to date from habitats around the world, including six host species from the
Bahamas, of which some co-occur with *I. exumae* (6, 20). All *Ca. Thiosymbion* 16S
rRNA gene sequences are closely related to each other and belong to a monophyletic
clade (6). The phallodrine hosts have also evolved from a single common ancestor,
based on morphological (25, 26) and molecular data (27, 28). Furthermore, *I. exumae*
is not an early-diverging or basal species within the gutless phallodrinines, but rather
closely related to other *Inanidrilus* species, which form a monophyletic group within
the gutless phallodrilines (Fig. 4). Since all gutless phallodrilines, including the four
Inanidrilus species closely related to I. exumae (Fig. 4), harbour Ca. Thiosymbion symbionts (6, 14), the most parsimonious conclusion is that the ancestor of I. exumae also harboured a Ca. Thiosymbion symbiont.

How could the Gamma 4 symbiont have displaced Ca. Thiosymbion in I. exumae? We envision the following three successive scenarios that could explain how the ancestral symbiont of I. exumae was displaced. In the first step, when Ca. Thiosymbion was still the primary symbiont, the ancestors of the Gamma 4 symbiont must have been able to enter and persist in I. exumae at some point in their evolutionary history. The early developmental stages of the worms were the most likely window of opportunity for infection by bacteria from the environment. Gutless phalodrilines lay single eggs into the surrounding sediment. These remain attached to the parent worm, are fertilized with sperm and coated with symbiotic bacteria from the parent worm in a smear infection, and then encased in a cocoon, which is eventually deposited in the sediment (17, 18). Free-living bacteria from the sediment could easily become encased within the cocoon during this process and colonize the developing embryo.

During a second, transition phase, the Gamma 4 bacteria and the Ca. Thiosymbion symbiont may have co-existed in I. exumae. In some gutless phalodrine species, Ca. Thiosymbion co-occurs with secondary sulfur-oxidizing Gammaproteobacteria, called Gamma 2 and 3 symbionts (15, 16). However, these secondary sulfur-oxidizing symbionts are much smaller than Ca. Thiosymbion and occur in the small interstitial spaces between the large Ca. Thiosymbion cells, so that competition for space does not appear to occur. Also, they have functional differences that may allow niche separation: The Gamma 3 symbiont of O. algarvensis, for example, uses nitrate as an electron acceptor while Ca. Thiosymbion uses oxygen (12,
Furthermore, the *O. algarvensis* Gamma 3 symbiont can use additional electron donors such as carbon monoxide, which cannot be used by its *Ca. Thiosymbion* symbiont, thereby reducing competition for energy sources (13, 29).

In the third and final step of displacement, the Gamma 4 symbiont appears to have outcompeted *Ca. Thiosymbion* in *I. exumae*, at least in the host population we examined (sixteen individuals from the same collection site were examined with molecular methods or FISH). While it is possible that our methods were not sensitive enough to detect residual, very low numbers of *Ca. Thiosymbion* cells in the *I. exumae* individuals we examined, these hosts were clearly dominated by Gamma 4 symbionts. Niche separation between *Ca. Thiosymbion* and Gamma 4 symbionts may not have been sufficient to allow their co-dominance in *I. exumae*. However, other factors could also explain the displacement of *Ca. Thiosymbion*, such as a massive viral infection event, a strong competitive advantage of the Gamma 4 symbiont over *Ca. Thiosymbion*, or harmful mutations in the ancestral *Ca. Thiosymbion* population.

Recent studies have shown that symbiont displacement is not as rare as previously assumed. Even in associations in which vertical transmission of symbionts occurs over long evolutionary times, acquisition of symbionts from novel lineages of environmental bacteria and symbiont displacement can occur occasionally in both aquatic and terrestrial symbioses (30–38). In the gutless phalodriline symbioses, Zimmermann et al. (2016) (6) revealed that displacement of *Ca. Thiosymbion* may have occurred numerous times. However, in the 22 phalodriline species analysed by Zimmermann et al. (2016) (6), displacement appears to have always occurred within the *Ca. Thiosymbion* clade, that is, the ancestral *Ca. Thiosymbion* of a given host species was displaced by a *Ca. Thiosymbion* from another host species. *I. exumae* is the only species in which we found indications for the displacement of *Ca.*
Thiosymbion by a novel, phylogenetically distinct lineage of bacteria not closely related to Ca. Thiosymbion. Genomic, transcriptomic and proteomic analyses of the Gamma 4 symbionts are needed to better understand the factors that allowed these bacteria to successfully colonize and persist in *I. exumae*.

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Material and Methods

Site description and specimen collection

*Inanidrilus exumae* specimens were collected from shallow water sediments off Lee Stocking Island, Bahamas, in April 1999. *I. exumae* co-occurred with several other gutless phallodriline species in a water depth of about 3 m in sediments that were largely composed of fine calcareous sands (20). The worms were extracted by decantation and identified under a microscope. In total sixteen specimens were divided for different analyses, eight were fixed for DNA extraction in 80% ethanol (7 for analysis of bacterial genes and 1 for analysis of host genes), another eight individuals were cut and either fixed for TEM or for FISH as described previously (16, 39). Samples were stored at 4°C.

DNA preparation and PCR amplification

For DNA extraction and subsequent PCR of bacterial genes, seven individual worms were prepared singly. Specimens were rinsed in MilliQ water, and DNA was isolated as described previously (6, 21) following the method of Schizas and colleagues (40). The bacterial 16S rRNA genes were amplified with primers specific for the bacterial 16S rRNA gene 8F and 1492R (41) using Taq DNA polymerase (Eppendorf, Hamburg, Germany). The bacterial 16S rRNA genes of individuals *I. exumae* 1 and 2 were amplified applying the reconditioning approach (42, 43) under the following conditions: initial denaturation at 96 °C for 5 min, 15+5 and 15+7 cycles for *I. exumae* 1 and *I. exumae* 2, respectively at 96 °C 1 min, 44 °C 2 min and 72 °C 3 min, followed by a final elongation of 10 min at 72 °C. PCR conditions for *I. exumae* 3, 4 and 5 were as described previously (22), PCR conditions for *I. exumae*...
6 and 7 were: initial denaturation at 94 °C for 5 min, 30 cycles at 94 °C 1 min, 42 °C 1.5 min and 72 °C 2 min, followed by a final elongation of 30 min at 72 °C. The PCR protocols differed due to protocol improvements in the course of our biodiversity studies during the last decade and sample availability.

Genes coding for Rubisco form I and APS reductase were PCR amplified with 30 and 33 cycles, respectively. The following primers were used: cbbLF (5'-
CACCTGGACCACVGTBTGG-3') and cbbLR (5'-
CGGTGYATGTGCAGCAGCATICCG-3') for cbbL (22) and aps1F (5'-
TGGCAGATCATGATYMAYGG-3') and aps4R (5'-GCGCCAACYGGRCCRTA-3')
for aprA, with the annealing temperature for aprA at 60 °C and 48 °C for cbbL (22).

Host genes were amplified and sequenced (Table 3) from DNA extracted from a single individual as previously described (6).

**Cloning and sequencing**

PCR products for all bacterial genes (16S rRNA, cbbL and aprA) were cloned separately for each individual using the pCR®4-TOPO® plasmids and TOP10 chemocompetent cells (Invitrogen, Carlsbad, CA) according to the manufacturers protocol. Clones were selected for the correct insert size, sequenced, and sequences grouped in clone groups as described in (44). PCR products for amplified host genes were sequenced directly.

**Phylogenetic analyses of symbiont sequences**

Sequences were checked with BLAST (45, 46) for similarity searches. Chimeras were identified using CHIMERA_CHECK from the Ribosomal Database Project.
Sequences were trimmed at the 5’ and 3’ end and only nearly full-length 16S rRNA gene sequences including outgroup sequences were considered for tree calculations (>1200 bp) using the ARB software package (48) and the SILVA SSU Ref, release_NR99_119 July 2014 (49). Sequence similarity of the nucleotide sequences was calculated by distance matrix analysis excluding the primer region. Phylogenetic trees for 16S rRNA gene sequences were calculated using Bayesian inference (MrBayes v. 3.2) (50) and maximum-likelihood-based methods (PHYML) provided within the ARB software package as described previously (6). We used the substitution model GTR for both analyses. Trees for alpha-, gamma- and deltaproteobacterial symbionts were calculated separately and consensus trees were constructed based on the information of the Bayesian inference and maximum likelihood analyses. Node stability was evaluated using posterior probabilities (pp, Bayesian inference).

The phylogenies of the aprA and cbbL genes were generated from partial sequences of deduced amino acid sequences with 134 and 101 compared amino acids positions, respectively. Sequences for each gene were aligned separately using MAFFT provided within the ARB software package and 5’ and 3’ end-trimmed. For phylogenetic tree reconstruction, we used maximum likelihood analyses (PHYML with LG and RAxML with JTT) and the bootstrapping algorithm in RAxML (51), as well as Bayesian inference (50). For the Bayesian inference analyses, the optimal model of amino acid evolution for AprA and CbbL was determined using prottest3 (https://github.com/ddarriba/prottest3) (LG + G for both proteins). The protein alignments were imported into MrBayes v. 3.2 and run in duplicate runs with four
chains each (one hot and three cold) until convergence (26 M generations for AprA and 50 M generations for CbbL). Trees were sampled every 1000 generations, and were then summarized in a majority-rule consensus using a burnin value of 20%. Clade posterior probabilities were plotted onto the ML trees shown in Figure 3.

**Phalodriline host phylogeny**

The mitochondrial (mt) 12S, mt16S, mtCOI as well as the nuclear 18S and 28S rRNA, and ITS genes of 22 gutless phalodrilines and five gut-bearing annelids submitted by Zimmermann et al. (6) as well as the genes from *I. exumae* (Table 3) were used for phylogenetic reconstruction. Sequences for each gene were aligned separately using MAFFT version 7 (52) with the Q-INS-I setting (53), alignments were manually adjusted, and 5' and 3' end-trimmed using BioEdit as described in Zimmermann et al. (6).

The optimal substitution model for each alignment was assessed and phylogenetic trees were reconstructed using Bayesian inference (MrBayes v. 3.2) (50) as described previously (6). Node stability was evaluated using posterior probabilities (pp, Bayesian inference) and bootstrap support (100 RaxML rapid bootstrap runs) with values above 0.80 considered significant.

**Data accessibility**

All sequences were submitted to GenBank and are available under the accession numbers given in the figures and tables.

**Fluorescence in situ hybridization**
Parts of eight *I. exumae* individuals were fixed and prepared for fluorescence *in situ* hybridization (FISH) as described previously (16) with the slight modification that we used xylol instead of Roti®-Histol (Carl Roth, Karlsruhe, Germany).

Symbionts were detected by CARD (catalyzed reporter deposition) FISH as described before (54) with slight modifications: Tissue sections were hybridized with the HRP-labelled probe for 2.5 h at 46 °C. After washing for 15 min at 48 °C in washing buffer, the sections were equilibrated for 20 min at room temperature in phosphate-buffered saline (PBS, pH 8.0). The moist tissue sections were incubated with amplification solution (1x PBS pH 8.0, 2M NaCl, 0.1% Blocking Reagent in 100 mM maleic acid buffer pH 7.5, 0.0015% [vol/vol] H$_2$O$_2$; and 1% Alexa Fluor 488, 546, or 633 dye [Molecular Probes, Leiden, The Netherlands]) for 30 min at 46°C in the dark and rinsed in 1x PBS buffer for at least 20 min at room temperature. For dual and triple hybridizations, the CARD FISH protocol was repeated two or three times on the same sections using different probes and Alexa dyes and the HRP was inactivated after each hybridization round with 0.01 M HCl for 10 min at room temperature after the last washing step (16).

Oligonucleotide probes and formamide concentrations used in this study are listed in Table 2. Probes designed with ARB were checked for *in silico* specificity against sequences in GenBank using BLAST, and against rRNA sequence databases using ProbeCheck (55). The specificity was also tested experimentally against mismatch 16S rRNA gene sequences of either reference strains or symbionts. General probes for Bacteria (EUB338 I-III), Gammaproteobacteria (GAM42a,) and a subgroup of the Deltaproteobacteria (DSS658) were used as positive controls, and the antisense probe NON338 was used as a negative control. All hybridizations were performed at formamide concentrations ensuring the highest possible specificity.
Transmission electron microscopy (TEM)

Parts of eight *I. exmuae* worms were TEM-fixed and washed in 0.05 M Na-cacodylate and post-fixed in osmium tetroxide. After dehydration in an acetone series, specimens were embedded in Spurr resin (56) and the worms’ middle part containing the symbiont region sectioned on an ultramicrotome. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 902A (39).

Raman spectroscopy

Raman spectroscopy was done on parts of two of the individuals used for FISH analyses as described in Eichinger et al. (57). More details on material and methods as well as results and discussion can be found in the supplemental material.
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Fig. 1. Bacterial symbionts in *Inanidrilus exumae*. A) – F): FISH images of the body wall of *I. exumae*. A) The Gamma 4 symbionts (red, probe IexuGam4), deltaproteobacterial symbionts (blue, probe DSS658) and alphaproteobacterial symbionts (light green, combined probes ImakALF1b, IexuALFb, and IecuALFd) co-occur in the body wall of the worm. The symbiont-free parts of the worm's body wall are visible in green due to their high autofluorescence. B) Delta 3 symbiont (green, probe Oalg/OilvDEL3). C) Delta 9 symbiont (green, probe OalgDEL4). D) Double hybridization with the Gamma 4 probe IexuGAM4 (red) and the general gammaproteobacterial probe GAM42a (green) shows a complete overlay of both probes (yellow), indicating that the Gamma 4 symbionts were the only Gammaproteobacteria present in *I. exumae*. E) The Alpha 1a (green, probe IexuALFd) and Alpha 2a (red, probe ImakALF1b) symbionts always co-occurred in the two examined individuals. F) The Alpha 2b symbiont (green, IexuALFb) was never observed to co-occur with the other alphaproteobacterial symbionts. Scale bars in A) – F): 5 µm. G) and H) Differential interference contrast images of *I. exumae*. G) Cross section through an entire worm. The white box shows the part of the body wall shown at higher magnification in the following panel to the right. H) The large Gamma 4 symbionts are visible in the body wall and fill the entire symbiont-containing region. I) TEM image of *I. exumae*: The Gamma 4 symbionts have large electron-dense globules, some of which contained sulfur, based on Raman analyses (see Fig. 1K and Supplement), and have a highly similar morphology to that of *Ca. Thiosymbion* (shown in (J)). J) TEM image of *Ca. Thiosymbion* in *O. ilvae*. Scale bars: 10 µm (G), 5 µm (H), 1 µm (I, J). K) Raman microspectroscopy: one clear sulfur peak is visible at 475 cm$^{-1}$ in the symbiont-containing region of *I. exumae*. Raman
spectra of host tissues without symbionts did not have a peak at 475 cm\(^{-1}\) or the two other peaks characteristic for S8 (and S6) sulfur (Fig S2) (57–60).

**Fig. 2.** Phylogenetic analyses of the Gamma 4 symbiont of *Inanidrilus exumae* based on 16S rRNA gene sequences.

Sequences obtained in this study are framed with a red box, sequences from gutless phallodriline symbionts are highlighted in yellow and sequences belonging to the *Ca.* Thiosymbion clade are highlighted in purple. The consensus tree shown is based on maximum likelihood analysis. Branching orders that were not supported in both calculation methods are shown as multifurcations. Scale bars represent 10% estimated phylogenetic divergence for non-multifurcation branches. Numbers within the polygons show the number of bacterial species concatenated in the node.

**Fig. 3.** Phylogenetic affiliation of the large subunit of the RubisCO form I \(cbbL\) (A) and the APS reductase \(aprA\) (B) genes from *Inanidrilus exumae* based on deduced amino acid sequences. Based on close phylogenetic relationships to free-living and symbiotic sulfur-oxidizing bacteria, we assume that the \(cbbL\) sequence and the \(aprA\) sequences from lineages AprA I and II originated from the Gamma 4 symbiont, while a fifth \(aprA\) sequence most likely originated from a deltaproteobacterial symbiont. Asterisks show bacteria that have \(aprA\) gene sequences from both AprA lineages I and II. Sequences obtained in this study are framed with a red box, sequences from *Ca.* Thiosymbion are highlighted in purple, and sequences from other gutless phallodriline symbionts are highlighted in yellow. Scale bars represent 10% estimated phylogenetic divergence for non-multifurcation branches. Numbers in the polygons show the number of bacterial species concatenated in the
node. Black or white filled circles indicate maximum likelihood bootstrap values, while percentages show posterior probabilities from Bayesian inference.

Figure 4. Phylogenetic tree of gutless phalodrilies using Bayesian inference analysis of six concatenated genetic markers for the host (mt12S, mt16S, 18S, 28S rRNA, mtCOI, ITS). Posterior probability (pp) values are indicated at nodes. Scale bar represents 10% estimated phylogenetic divergence for non-multifurcation branches. 18S rRNA: KP943792 - KP943817, 28S rRNA: KP943818 - KP943844, mtCOI: KP943845 - KP943866, ITS: KP943867 - KP943884, mt12S rRNA: KP943885 - KP943908, mt16S rRNA: KP943909 - KP943931
Table 1. Number of partial 16S rRNA, *aprA* and *cbbL* gene sequences from cloned PCR products from *Inanidrilus exumae*. Sequences that shared > 99% identity were grouped as a single phylotype. One or more clones of each phylotype and individual were sequenced in both directions for the almost full length 16S rRNA gene sequence, and for partial *aprA* and *cbbL* gene sequences. n.a. not analyzed.

| Inanidrilus exumae specimen | Clone family/phylogroup | #1 | #2 | #3 | #4 | #5 | #6 | #7 |
|-----------------------------|-------------------------|----|----|----|----|----|----|----|
| Gammaproteobacterial symbiont | Gamma 4                 | 72 | 156| 46 | 78 | 16 | 0  | 6  |
| Alphaproteobacterial symbionts | Alpha 1a                | 0  | 5  | 0  | 0  | 0  | 13 | 54 |
|                             | Alpha 2a                | 0  | 0  | 5  | 0  | 0  | 13 | 11 |
|                             | Alpha 2b                | 61 | 27 | 0  | 1  | 0  | 13 | 11 |
| Deltaproteobacterial symbionts | Delta 3                | 0  | 0  | 46 | 75 | 0  | 23 | 5  |
|                             | Delta 9                 | 5  | 1  | 0  | 0  | 0  | 0  | 0  |
| Associated bacteria         | Delta 8                 | 0  | 0  | 11 | 1  | 0  | 0  | 0  |
|                             | Delta 10                | 0  | 0  | 0  | 0  | 3  | 0  | 0  |
| *aprA*                      | *aprA* Ia               | 4  | 4  | n.a.| n.a.| n.a.| n.a.| n.a.|
|                             | *aprA* Ib               | 2  | 5  | n.a.| n.a.| n.a.| n.a.| n.a.|
|                             | *aprA* IIa              | 7  | 11 | n.a.| n.a.| n.a.| n.a.| n.a.|
|                             | *aprA* IIb              | 11 | 4  | n.a.| n.a.| n.a.| n.a.| n.a.|
| *aprA* SRB                  | 4  | 0  | n.a.| n.a.| n.a.| n.a.| n.a.|
| *cbbL*                      | *cbbL*                  | n.a.| n.a.| n.a.| n.a.| 29 | 18 |
### Table 2: Symbiont-specific and general oligonucleotide probes used in this study.

| Probe       | Target and specificity                                                                 | Probe sequence (5'→3') | Position\(^a\) | FA [\(^\%\)] | Literature reference |
|-------------|----------------------------------------------------------------------------------------|-------------------------|----------------|--------------|---------------------|
| NON338      | Antisense, background control                                                          | ACT CCT AGG GGC AGC     | 338-355        | 10-30        | (61)               |
| GAM42a      | Gammaproteobacteria                                                                     | GCC TTC CCA CAT CGT TT  | 1027-1043\(^b\) | 30 - 35      | (62)               |
| DSS658      | *E. exuere*-Delta 3, *O. algarvensis* and *O. ilvae* Delta 3 symbiont; Delta 3 symbiont, *O. algarvensis* Delta 4 symbiont, *Dehalococcoides* spp., *Dehalogenococcus* spp. | TCC ACT TCC TTC TCC CAT | 658-685 | 50 - 60 | (63) |
| IexuGAM4    | *E. exuere* Gamma 4                                                                     | ATT CCG CCT CCC TCT ACC GGA | 657-677 | 50          | this study          |
| IexuALFd    | *E. exuere* Alpha 1a, *O. loisae* Alpha 1a and Alpha 1a-2 symbiont, *I. leucodermate* Alpha 1a symbiont | GTA CCC GCC CAA ACC CGA | 1131-1147 | 30          | this study |
| IexuALFb    | *E. exuere* Alpha 2a, *I. makropetalos* Alpha 2 symbiont                                | TCC GGT CTC GCG GAC CCC | 999-1014 | 35          | (22)               |
| IexuALFb    | *E. exuere* Alpha 2b, DQ662742, EU133383, AJB10382, AY326683, DQ648967                 | TCT GGT CTC CGC GAC CGG | 999-1014 | 30          | this study          |
| Oalg/Dol DEL3 | *E. exuere* Delta 3, *O. algarvensis* Delta 4 symbiont                               | GTG CCT GCC TTC TGA AAG | 1449-1465 | 30          | (15)               |
| OalgDEL4    | *E. exuere* Delta 9, *O. algarvensis* Delta 4 symbiont, AB121099, EF91973, DQ350563, EU290060, EU290067, DQ395004, DQ394892, GCC CAA CCA CTT CGC GTA | 1427-1444 | 30 | (15) |

\(^a\) position in the 16S rRNA of *Escherichia coli*

\(^b\) Percent formamide (FA) concentration (\(^v/v\)) used in the CARD-FISH hybridization buffer

\(^c\) position in the 23S rRNA of *E. coli*
Table 3. Amplified host genes and accession numbers from one *Inanidrilus exumae* specimen.

| Sample ID | Species (taxonomic identification) | mt12S   | mt16S   | 18S    | 28S    | mtCOI   | ITS     |
|-----------|-----------------------------------|---------|---------|--------|--------|---------|---------|
| CE73      | *I. exumae*                       | MF991272| MF991273| MF991275| MF991276| MF991274| MF991277|
