Symbiosis insights through metagenomic analysis of a microbial consortium

Tanja Woyke[1,2], Hanno Teeling[3], Natalia N. Ivanova[1], Marcel Hunteman[3], Michael Richter[3], Frank Oliver Gloeckner[3,4], Dario Boffelli[1,2], Iain J. Anderson[1], Kerrie W. Barry[1], Harris J. Shapiro[1], Ernest Szeto[1], Nikos C. Kyrpides[1], Marc Mussmann[3], Rudolf Amann[3], Claudia Bergin[3], Caroline Ruehland[3], Edward M. Rubin[1,2,†] & Nicole Dubilier[3,†]

[1]*DOE Joint Genome Institute, Walnut Creek, California 94598, USA.*

[2]*Lawrence Berkeley National Laboratory, Genomics Division, Berkeley, California 94720, USA.*

[3]*Max Planck Institute for Marine Microbiology, 28359 Bremen, Germany.*

[4]*International University Bremen, 28759 Bremen, Germany.*

† Correspondence should be addressed to: N. D. (ndubilie@mpi-bremen.de) and E. M. R. (EMRubin@lbl.gov).

Symbioses between bacteria and eukaryotes are ubiquitous, yet our understanding of the interactions driving these associations is hampered by our inability to cultivate most host-associated microbes. Here, we used a metagenomic approach to describe four co-occurring symbionts from the marine oligochaete *Olavius algarvensis*, a worm lacking a mouth, gut, and nephridia. Shotgun sequencing and metabolic pathway reconstruction revealed that the symbionts are sulfur-oxidizing and sulfate-reducing bacteria, all of which are capable of carbon fixation, providing the host with multiple sources of nutrition. Molecular evidence for the uptake and recycling of worm waste products by the symbionts suggests how the worm could eliminate its excretory system, an adaptation unique among annelid worms. We propose a model which describes how the versatile metabolism within this symbiotic consortium provides the host with an optimal energy supply as it shuttles between the upper oxic and lower anoxic coastal sediments which it inhabits.

Symbiosis plays a major role in shaping the evolution and diversity of eukaryotic organisms[1]. Remarkably, only recently has there been an emerging recognition that most eukaryotic organisms are intimately associated with a complex community of beneficial microbes that are essential for their development, health, and interactions with the environment[2]. This renaissance in symbiosis research stems from advances in molecular approaches that have enabled the study of natural microbial consortia using cultivation-independent methods[3-5]. Metagenomic analyses have provided a new dimension in the study of community organization and metabolism in natural microbial communities[6-10]. To date, however, genomic analyses of symbiotic microbes from eukaryotes have been confined to individual strains (for the only exception see Wu *et al.*[11]), limiting our ability to understand the intricate interactions involving communication, competition, and resource partitioning that shape symbiotic microbial communities.

Here, we used random shotgun sequencing and nucleotide-signature based binning to study the symbiotic community in *Olavius algarvensis*. This marine worm belongs to a group of oligochaetes (phylum Annelida) that lack a mouth, gut, and anus, and are unique among annelid worms in having reduced their nephridial excretory system[12]. They live in obligate and species-specific associations with multiple extracellular bacterial endosymbionts located just below the worm cuticle[12]. Since the symbionts have yet to be grown in culture, their phylogeny has only been accessible through 16S rRNA analysis and fluorescence in situ hybridization (FISH)[13,14]. *O. algarvensis* lives in coastal Mediterranean sediments and harbors a chemoautotrophic sulfur-oxidizing Gammaproteobacterium (γ1 symbiont) and a deltaproteobacterial sulfate reducer (δ1 symbiont), recently shown to be engaged in an endosymbiotic sulfur cycle[14]. An additional gamma- and deltaproteobacterial symbiont (γ3 and δ4 symbionts) of unknown function occur consistently in these hosts, and in some individuals a spirochete has been observed as a minor part of the symbiotic consortium (see Supplementary Figure S1a)[12].

Given that most chemosynthetic hosts harbor only one or two bacteria, the associations of gutless oligochaetes with multiple symbionts are remarkable, raising a series of questions about their interactions with each other, their host, and the environment. How does the symbiosis compensate for the loss of digestive and excretory systems in the host, what do the various partners gain from this relationship, and is it mutually obligate? What is the selective advantage for *O. algarvensis* in harboring multiple symbiotic partners? Our metagenomic analyses explain how the bacterial consortium meets the energy and waste management needs of its oligochaete host. We describe how resource partitioning between the phylogenetically diverse symbionts benefits both the symbionts and the worm in the heterogeneous environment. Finally, we propose a model showing that the selective advantage of harboring multiple symbionts lies in their ability to supply their host with energy from an abundant and diverse supply of reducing equivalents and electron acceptors as it shuttles between the oxidized and reduced sediment layers.

Metagenomic data analysis and binning

Pooled samples of 200 *O. algarvensis* specimens per library were shotgun sequenced (see Supplementary Information, Supplementary Fig. S1b) and the sequences assembled using the whole-genome shotgun assembler JAZZ[15] (Supplementary Methods, Supplementary Fig. S2). To assign the metagenomic scaffolds to their phylotype origin, we used a combinatory binning approach based on intrinsic DNA signatures (see Supplementary Methods). Binning of the *Olavius* spp. symbionts' metagenome resulted in the formation of four distinct clusters (Fig. 1). The presence of the corresponding rRNA operons, which represented the only rRNA operons found in these bins and the assembly, enabled us to identify them as the *O. algarvensis* symbionts δ1, δ4, γ1, and γ3 (Fig. 1, Supplementary

Table S1, Supplementary Table S2 providing a comparison to other symbiont genomes). This study illustrates the usefulness of our nucleotide frequency method for the assignment of metagenomic scaffolds from a natural microbial community to a phylotype when using shotgun sequencing. It would not have been possible to reconstruct genome assemblies of the four symbionts based purely on GC contents and scaffold read depth, and without any closely related, fully sequenced reference genome.

Symbiont bin assignments based on 16S rRNA genes were confirmed by phylogenetic analysis of predicted proteins within each cluster of scaffolds (Supplementary Fig. S3), and the clusters were furthermore supported by the distribution of 49 single-copy genes. Although the populations are not clonal, the frequencies of polymorphic sites found in the four symbiont bins, ranging from 0.01-0.1% (Supplementary Table S4), were rather low compared to environmental microbes such as those from the acid mine drainage[8]. The following discussion describing the metabolism of the symbionts is based on the genes found in each symbiont bin. With a focus on capturing the core metabolic pathways present in the community, it is of minor importance if the genes within each bin originated from a single strain or represent a pan-genome of several very closely related strains[16].

Carbon and energy metabolism

Gammaproteobacterial symbionts. Chemoautotrophic symbionts feed their hosts by providing them with organic carbon from autotrophic CO_2 fixation driven by oxidation of reduced inorganic compounds such as sulfide. In agreement with previous studies indicating the chemoautotrophic, sulfur-oxidizing nature of the *O. algarvensis* γ1 symbiont[12], our analysis of the γ1 bin revealed the presence of genes required for autotrophic CO_2 fixation via the Calvin-Benson-Bassham cycle using type I ribulose 1,5-

bisphosphate carboxylase-oxygenase (RubisCO) (*cbb*), the oxidation of reduced sulfur compounds (such as *dsr*, *fcc* and *sox*), and the storage of sulfur in globules (*sgpB* encoding one of three known sulfur globule proteins).

Unexpectedly, our metagenomic analyses revealed, that the γ3 symbiont of *O. algarvensis* is also a sulfur-oxidizing chemoautotroph. Several gutless oligochaete species are known to harbor γ3 symbionts but the metabolism of these bacteria was previously unknown and the benefit of harboring additional Gammaproteobacteria is unclear. The nearly complete genomic sequence for the *O. algarvensis* γ3 symbiont obtained in this study is notable, as this is the first sequenced genome from a chemoautotrophic symbiont. The γ3 bin carries all the genes required for a thiotrophic (sulfur-oxidizing) metabolism including those needed for the oxidation of reduced sulfur compounds (including *dsr*, *apr*, *sat*, *fcc*, and *sox*) as well as autotrophic CO_2 fixation by means of genes closely related to but phylogenetically distinct from the γ1 symbiont (Fig. 2). The absence of sulfur globule proteins in the near complete γ3 bin suggests that these symbionts do not store sulfur, supporting transmission electron microscopy analyses showing that only γ1 symbionts contain sulfur globules (Giere, pers. communication). In addition to using oxygen as an electron acceptor, the presence of *nap* and *nir* gene clusters suggests that the γ3 symbionts couple oxidation of reduced sulfur compounds to dissimilatory nitrate reduction under oxygen-limiting conditions (Fig. 2). In deeper sediment layers with neither oxygen nor nitrate both Gammaproteobacteria have the ability to use fumarate as an electron acceptor for the oxidation of reduced sulfur compounds (Fig. 2).

Deltaproteobacterial symbionts. The presence of genes characteristic of dissimilatory sulfate reduction (such as *dsr*, *qmo*, and *apr*) in both the δ1 and δ4 bins suggests that these symbionts are sulfate-reducing bacteria that use oxidized sulfur

compounds such as sulfate as an electron acceptor, thereby producing sulfide (Fig. 2). In addition to the syntrophic cycling of sulfate and sulfide between the gamma- and deltaproteobacterial *O. algarvensis* symbionts[14], intermediate sulfur compounds such as tetrathionate and thiosulfate may be cycled between the symbionts. The δ4 symbiont appears to be able to reduce sulfur compounds of intermediate oxidation states, as suggested by the presence of a multi-heme cytochrome most closely related to tetrathionate reductase of *Shewanella oneidensis*[17] and located in a chromosomal cluster with molybdopterin-dependent dehydrogenase related to thiosulfate reductase of *Wolinella succinogenes*. Cycling of intermediate sulfur compounds is energetically more favorable than the exchange of sulfide and sulfate, as shown previously in experiments with free-living sulfate reducers and sulfur oxidizers[18].

Heterotrophy is important in sulfate-reducing bacteria and correspondingly we found in the δ1 bin genes for the transport and utilization of a large variety of carbohydrate substrates, including uronic acids (glucuronate, galacturonate and fructuronate), xylose, fructose, dihydroxyacetone, and polyols (mannitol, sorbitol and glycerol). While all sulfate-reducing bacteria are heterotrophic, only some fix CO_2 and it is intriguing that both deltaproteobacterial symbionts are capable of autotrophic carbon fixation via the reductive acetyl-coenzymeA (CoA) pathway, as well as via the reductive tricarboxylic acid (TCA) cycle (Fig. 2). Thus, *O. algarvensis* has established an association with four symbionts that are all capable of providing it with organic carbon through three different autotrophic pathways.

One of the most common electron donors for autotrophic sulfate-reducing bacteria is hydrogen. We found gene clusters for periplasmic Ni-Fe hydrogenases, transmembrane high-molecular-weight cytochrome c (hmc) complex, and tetraheam type II tetrahaem cytochrome c_3 (TpII-*c_3*) in the bins of both sulfate-reducing symbionts, as well as TpI-*c3*

in the δ1 bin (Fig. 2). This is a compelling indication for the uptake and oxidation of molecular hydrogen using sulfate as an electron acceptor[19]. It is not clear if hydrogen is provided by the γ-symbionts. Within the γ3 bin, we found genes encoding a pyruvate ferredoxin oxidoreductase (POR), typically used in an alternative route for pyruvate oxidation[20] and indicative of hydrogen release from low-potential ferredoxins. Released hydrogen could subsequently be taken up by the sulfate-reducing symbionts leading to hydrogen syntrophy within the microbial consortium. Alternative electron donors to hydrogen include glycerol, lactate, proline and betaine, and potentially glycolate and other 2-hydroxy acids, as well as succinate, acetate and propionate.

Symbiont host interactions

"Feeding" of the host. In other chemosynthetic associations, the symbionts provide their hosts with nutrition using either reduced sulfur compounds or methane as their energy source[21]. In the *O. algarvensis* symbiosis, both reduced sulfur compounds and hydrogen can be used as energy sources, and all four symbionts have the potential to fix CO_2 into organic carbon via autotrophy. In addition, the sulfate-reducing symbionts can feed the oligochaete host through heterotrophy by taking up dissolved organic carbon compounds from the environment. Largely all amino acids and a variety of vitamins can be synthesized by the symbionts to provide their host with these required nutrients (Supplementary Table S5). Nutrient transfer to the host is likely to occur via intracellular uptake and digestion of the bacteria, as the number of genes encoding amino acid exporters was not elevated in the symbionts when compared to those of free-living bacteria and the only known family of sugar exporters[22] was not encoded in any of the symbiont bins. This conclusion is supported by morphological analyses showing symbiont lysis in the basal region of the worm's epidermis[23].

Host waste recycling. The reduction of nephridia in the oligochaete host, used for the excretion of nitrogenous waste compounds and osmoregulation, suggests that its symbionts have adapted to carry out these functions. Most aquatic organisms excrete ammonium but urea, which also functions as a common organic osmolyte, has also been found in marine worms with symbionts, such as the hydrothermal vent worm *Riftia pachyptila*[24]. The genome bins for the δ1, δ4 and γ3 symbionts encode a bidirectional uniporter (Amt family transporter[25]) for the counter ion-independent and energy-independent uptake of ammonium. A likely urea ABC transporter for urea uptake is present in the γ3 bin, adjacent to an urease operon encoding genes involved in urea hydrolysis. Ammonium and urea uptake by the symbionts would not only aid the host in the removal of these toxic waste products, but also lead to the conservation of valuable nitrogen by the symbionts (Fig. 3).

Marine invertebrates are typical osmoconformers, maintaining their cell volume largely with organic osmoregulatory compounds such as amino acids, taurine, glycine betaine, trimethylamine *N*-oxide (TMAO), and urea, as well as polyols and sugars[26]. The presence of gene clusters encoding proteins used for taurine and glycine betaine import and catabolism in the γ3 bin may indicate the use of these osmolytes as both, a carbon and nitrogen source[27] (Fig. 3). Furthermore, we found pathways for TMAO degradation in the *O. algarvensis* symbionts. Microbial TMAO degradation includes its conversion to trimethylamine by TMAO reductase and further demethylation of trimethylamine either by trimethylamine and dimethylamine dehydrogenases found in Bacteria[28] or by trimethylamine, dimethylamine and monomethylamine methyltransferases found mostly in Archaea[29]. Genes coding for the key enzymes in both pathways were present in the γ3 and δ1 symbionts, suggestive of their TMAO catabolic activity (Fig. 3). The availability of the osmolyte TMAO would furthermore be particularly advantageous for the sulfur-

oxidizing symbionts that could use this organic carbon compound as an alternate electron acceptor in the absence of oxygen or nitrate. As just described, the γ3 bin encodes enzymes likely to be involved in the reduction of TMAO, although their specificity for this substrate could not be clearly identified.

Polyamines are essential in all organisms for DNA stabilization, DNA replication, and cell proliferation[30] and represent additional products of host protein breakdown. We found abundant gene clusters encoding ABC transporters for the uptake of the polyamines putrescine and spermidine in the δ1 bin, and putrescine in the γ3 bin (Fig. 3).

Finally, evidence is provided for the recycling of host fermentation waste products such the dicarboxylate succinate, as well as the monocarboxylates acetate and propionate. Pathways for their utilization and a variety of potential dicarboxylate transporters, and likely monocarboxylate transporters were found in all four symbiont bins. The δ1 bin encodes 23 tripartite ATP-independent periplasmic (TRAP)-T family dicarboxylate transporters[31], some of which are likely involved in monocarboxylate and dicarboxylate transport (Fig. 3).

A mutually obligate relationship?

The lack of a digestive and excretory system in *O. algarvensis* means that its symbionts are crucial for its survival. But is this relationship mutually obligate or can the symbionts survive outside of the host in a free-living stage? Several pieces of evidence from the sequence data and the metabolic reconstruction analyses suggest a lack of evidence for obligate host-dependence of any of the symbionts. This includes the observation that the genomes of the extracellular bacteria do not show AT-bias or genome size reduction (Supplementary Table S2) and there was no obvious loss of essential metabolic pathways

in the δ1 or γ3 bins suggestive of host-dependence, as is the case for many obligate host-associated bacteria[32,33]. Finally, we found genes required for cell motility via a flagellum in the δ1, δ4, and γ3 bins. While this evidence suggests that the symbionts associated with the worm may have a free-living stage, the presence of a remarkable number of transposases in the γ3 and γ1 symbiont bins (7.5% and 20.5% respectively; Supplementary Tables S4, S6) suggests that these symbionts may be in transition to an obligate symbiotic lifestyle. Bacteria which have recently evolved into obligate symbionts show an increase in frequency of mobile elements, representing a source for chromosomal rearrangements and gene inactivation[34] (for symbiont transmission hypotheses see Supplementary Information).

From the metagenome to the environment

The oligochaete symbiosis is inseparably linked to the geochemical properties of the environment, needing access to both reduced and oxidized compounds for energy production and net carbon fixation (Fig 4). In the upper oxic sediment layers of the *O. algarvensis* habitat where no sulfide is present, both the γ1 and the γ3 symbiont can use reduced sulfur compounds produced internally by the sulfate-reducing symbionts in a syntrophic sulfur cycle. (We have shown previously that the sulfate-reducing symbionts can produce sulfide in *O. algarvensis* under microaerobic conditions comparable to those in the lower oxic zone at 2 – 5 cm sediment depth[14]). The γ1 symbiont can also gain energy independently of the sulfate-reducing symbionts by oxidizing the large supply of sulfur stored in its cytoplasm. For both gammaproteobacterial symbionts, oxygen would be the energetically most favorable electron acceptor (Fig. 4).

As the worm migrates downwards, it encounters sediment layers in which oxygen is no longer present. Under these conditions, the γ3 symbiont can use nitrate from the

environment for the oxidation of reduced sulfur compounds. Given the extremely low concentrations of sulfide in this layer as well as in the deeper reduced sediment layers of the Elba habitat (in the low nM range[14]), it is likely that the sulfate-reducing symbionts provide a large part of the reduced sulfur compounds for the γ-symbionts under most conditions.

In the deeper sediment layers characterized by reducing conditions and the absence of oxygen or nitrate, hydrogen oxidation by the sulfate-reducing symbionts may occur, in which hydrogen is used as an energy source for the autotrophic fixation of inorganic carbon. As hydrogen concentrations are commonly very low in most marine sediments, heterotrophic pathways should also play an important role for the deltaproteobacterial symbionts under these reducing conditions. Although energetically less favorable than nitrate or oxygen, organic electron acceptors including TMAO (host-derived) and fumarate (produced by the δ-symbionts) are provided internally within the symbiosis and may be used by the γ-symbionts for the oxidation of reduced sulfur compounds. This would enable the γ1 symbiont to replenish its sulfur stores, which could be fully oxidized using the energetically more favorable electron acceptor oxygen when the worm returns to the oxic zone. Fumarate respiration by the γ-symbionts would produce succinate, which could be used by the deltaproeobacterial symbionts as an energy source and reoxidized to fumarate, thus leading to a syntrophic cycling of reductants and oxidants.

Our analysis of the *O. algarvensis* microbial genomes has provided insights on how resources are used and shared among the different symbionts and with their host, and how different metabolic pathways are used by the symbionts to generate energy as the worm migrates through the chemocline. We have shown how the *O. algarvensis* symbiosis is unique among known chemosynthetic symbioses, as reductants and oxidants are not only supplied from the environment but also internally to drive energy production.

Thus, this comprehensive metagenomic analysis shows that these highly integrated synergistic assemblages of multiple bacterial partners provide their eukaryotic host with an optimal energy supply and waste management through resource partitioning and cooperation during syntrophic cycling of oxidized and reduced compounds.

Methods

O. algarvensis specimens were collected off Capo di Sant' Andrea, Elba, Italy. Metagenomic libraries were constructed from 200 pooled *O. algarvensis* specimens per library. A small insert **pMCL200** library was made from a nycodenz-separated, symbiont-enriched sample and two **pCC1Fos** fosmid libraries were constructed using the CopyControl^{TM} Fosmid Library Construction Kit (Epicentre). 16S rRNA PCR libraries were created from the DNA sources used for each of the libraries and approximately 384 clones sequenced and analyzed. From the shotgun libraries, we created 204 Mb of vector- and quality-trimmed sequence. This data was assembled using JAZZ, resulting in a set of 2,286 scaffolds, which were binned using a combinatory approach based on dimer to hexamer frequencies using the newly developed program[35]. Final clusters with 511 scaffolds were verified by phylogenetic affiliation of each scaffold based on the most common phylogeny of its predicted proteins, by a Bayesian classifier, and by checking for paralogs of 49 genes that typically occur with only one copy per genome (Kunin *et al*, unpublished). To assess nucleotide sequence variation within the bins, we analyzed the multiple alignment of the JAZZ assembly. Potential open reading frames (ORFs) were identified using "mORFind" (Waldmann, unpublished) and annotation performed with the GenDB v2.2 system[36] and MicHanThi[37]. The annotated symbiont metagenome was loaded into the metagenomics version of Integrated Microbial Genomes/M (IMG/M)[38] (http://img.jgi.doe.gov/m).

Details for all methods used are provided in the Supplementary Information.

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Author information Correspondence and requests for materials should be addressed to: N. D. (ndubilie@mpi-bremen.de) and E. M. R. (EMRubin@lbl.gov).

Figure 1

Clustering of the symbiont scaffolds. Visualization of the first three components of a principal component analysis, in which GC-content, net read depth, z-scores for all possible 64 trinucleotides and 256 tetranucleotides were incorporated with equal weight (z-scores calculated with TETRA[39], normalized by length). The colors represent the four clusters of scaffolds (calculated with MetaClust), that were binned based on GC-content, dinucleotide relative abundance, Markov model-based statistical evaluations of tri-, tetra and pentamer over- and under-representation and normalized chaos game representations for tri- to hexamers; sequences < 5 kb are not represented. Scaffolds containing 16S rRNA genes are tagged.

Figure 2

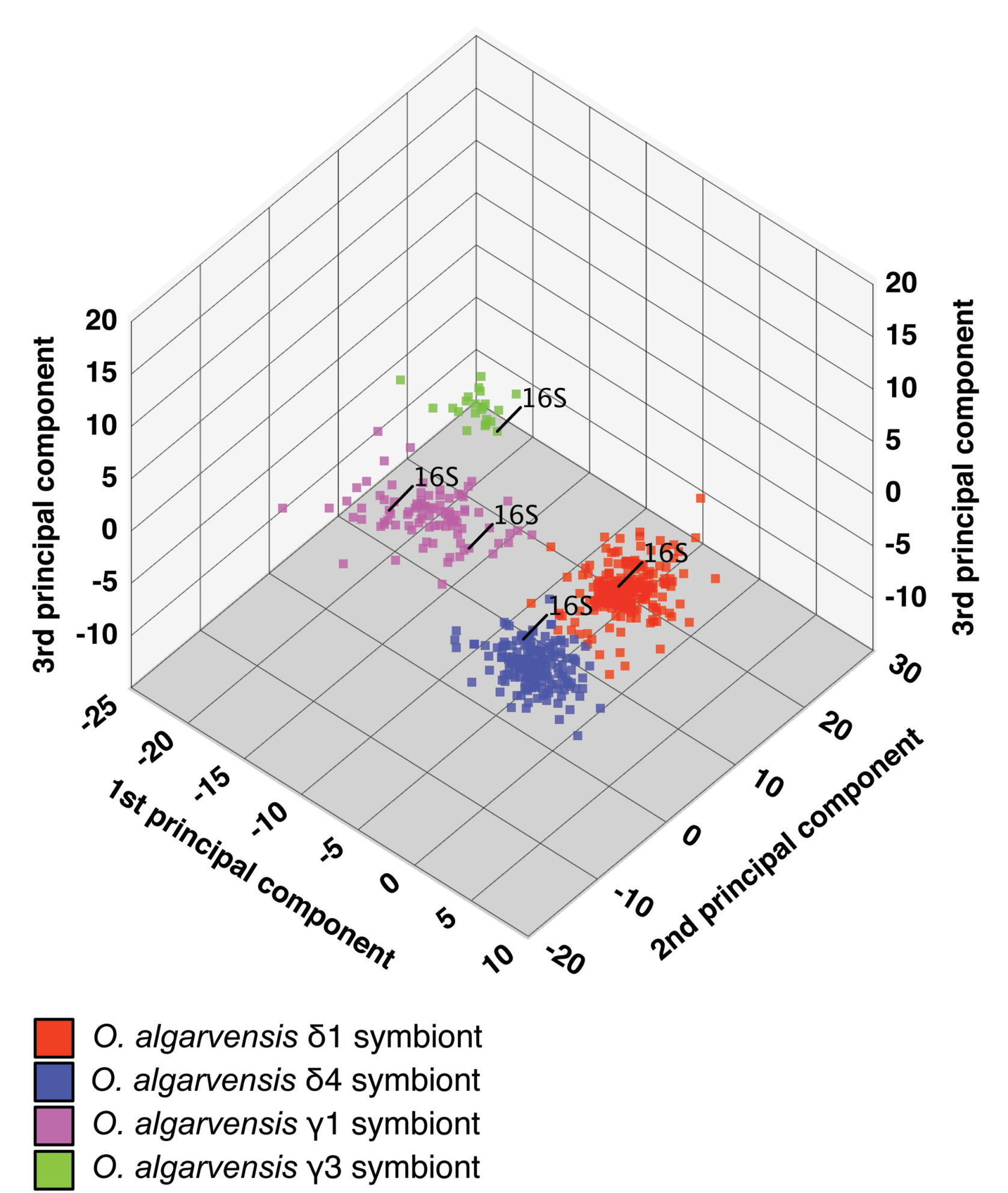
Reconstruction of the symbionts' physiology. PHA, polyhydroxyalkanoates. CM, cell material EMP, Embden-Meyerhof pathway. TCA, tricyclic acid. C-taxis, chemotaxis. APS, adenosine 5'-phosphosufate. G 3-P, glyceraldehyde 3-phosphate. Hmc, high-molecular-weight cytochrome c. TpI/II-c_3, tetrahaem type I/II tetrahaem cytochrome c_3. H_2ase, hydrogenase. PEP, phosphoenolpyruvatye. CoA, coenzymeA. ?, indicates the lack of nitric oxide reductase in the γ3 genome bin. TRAP, tripartite ATP-independent periplasmic. Numbers in parenthesis indicate the numbers of amino acids/vitamin biosynthesis pathways found (Supplementary Table S5).

Figure 3

Reconstruction of symbiont host interactions. The metagenomic data uncovered pathways for the uptake and recycling of organic osmolytes and excretion products of the worm by its symbionts. TMAO, trimethylamine *N*-oxide. TMA, trimethylamine.

Figure 4

Model for energy metabolism in the symbiosis. *O. algarvensis* inhabits shallow Mediterranean ocean sediments (5 - 15 cm depth). Electron acceptors and donors are available to the symbionts in all sediment layers, with some supplied from the environment (shown in the box and triangles) and some internally (shown next to the worms). Carbon is gained autotrophically using reduced sulfur compounds (γ-symbionts) or hydrogen (δ-symbionts) as well as heterotrophically (δ-symbionts). cms = cm below sea floor. SRS, sulfate-reducing symbionts. SOS, sulfur-oxidizing symbionts. TMAO, trimethylamine *N*-oxide. AM, anaerobic metabolites. S_{red}, reduced sulfur compounds. S_{ox}, oxidized sulfur compounds. OrgC, organic compounds.


3rd principal component
20
15
10
5
0
-5
-10
16S
16S
16S
16S
16S
-25
-20
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-10
-5
0
5
10
1st principal component
-20
-10
0
10
20
30
2nd principal component
3rd principal component
20
15
10
5
0
-5
-10
O. algarvensis δ1 symbiont
O. algarvensis δ4 symbiont
O. algarvensis γ1 symbiont
O. algarvensis γ3 symbiont

Uniporters
ammonium/ammonia (bi-directional)
arsenite/antimonite export

Antiporters
drugs (export) - H+ (import)
amino acids (export) - H+ (import)

Symporters
TRAP
betaine/carnitine/choline - H+
di-/tricarboxylates - Na+/H+
phosphate/sulfate - Na+/H+
aromatic acids - H+
folate - H+
benzoate - H+

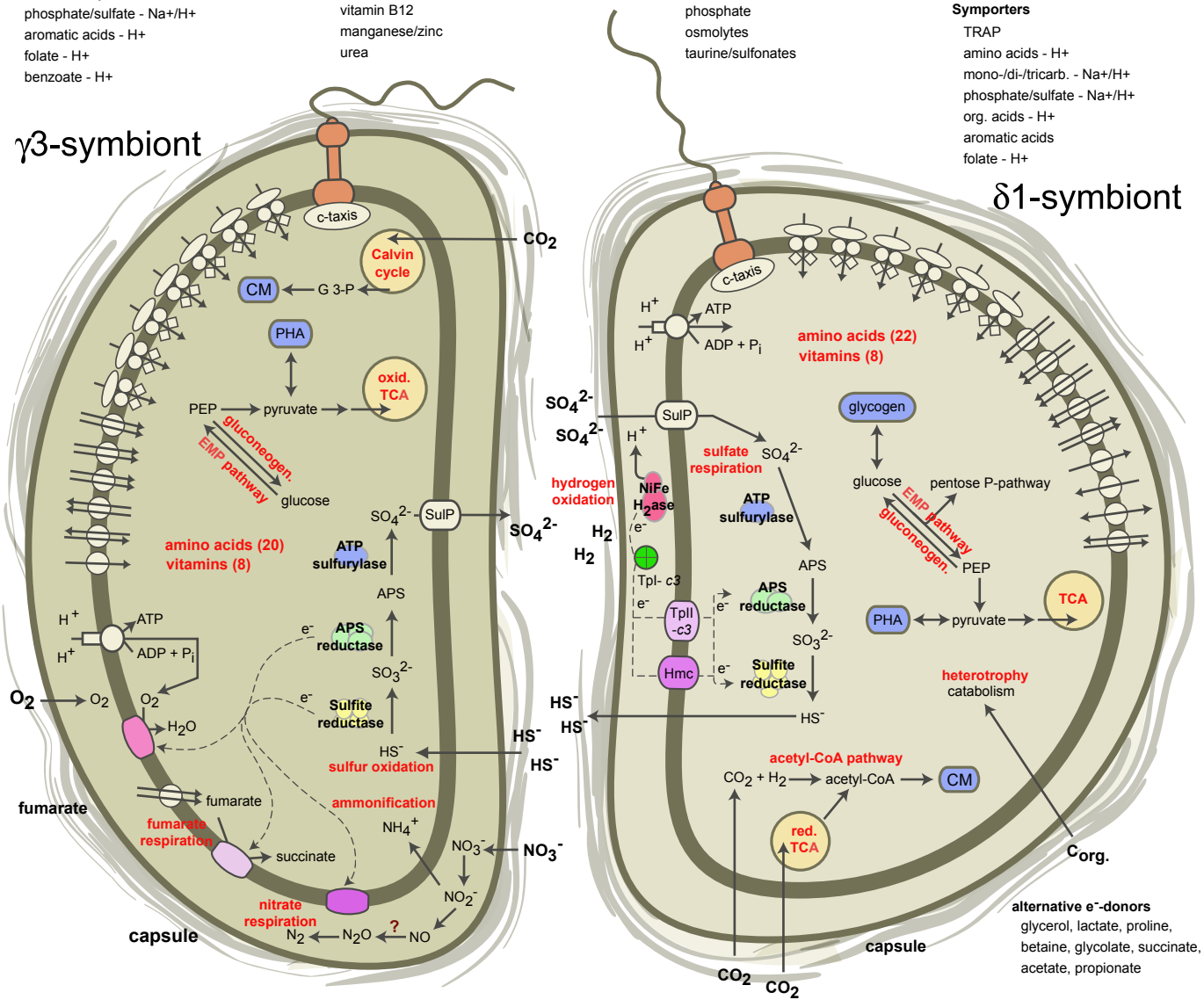
ABC-transporters
amino acids, amides
peptides/nickel/opines/sugars
polyamines/ opines
nitrate/cyanate/bicarbonate
osmolytes
taurine/sulfonates
phosphonate
phosphate
molybdate
iron
vitamin B12
manganese/zinc
urea

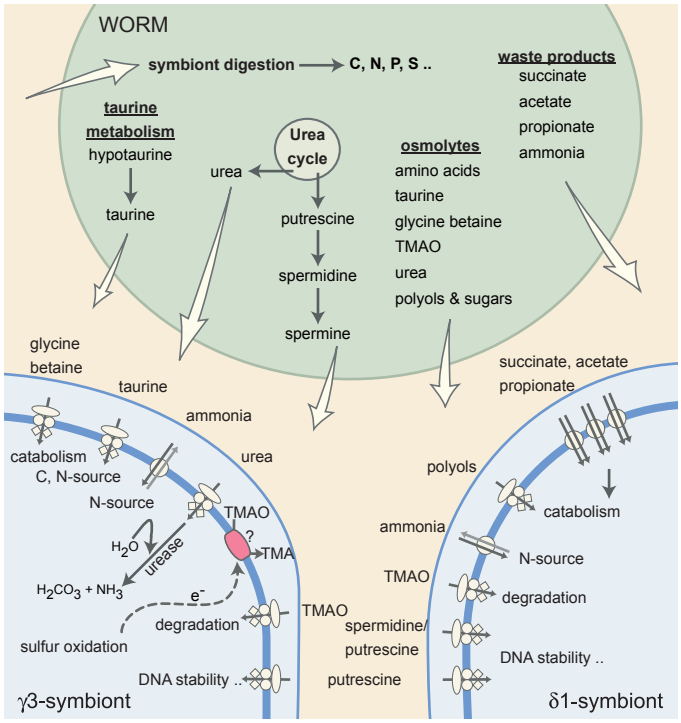
ABC-transporters
peptides/nickel/opines/sugars
amino acids, amides
polyamines/ opines
nitrate/cyanate/bicarbonate
mono-/disaccharides
phosphonate
iron
manganese/zinc
vitamin B12
cobalt
phosphate
osmolytes
taurine/sulfonates

Antiporters
drugs (export) - H+ (import)
formate (export) - oxalate (import)
aspartate - alanine (reversible)
ATP - ADP (reversible)
amino acids (export) - H+ (import)

Uniporters
ammonium/ammonia (bi-directional)
phenylpropionate import
arsenite/antimonite export

Symporters
TRAP
amino acids - H+
mono-/di-/tricarb. - Na+/H+
phosphate/sulfate - Na+/H+
org. acids - H+
aromatic acids
folate - H+




WORM
symbiont digestion
C, N, P, S ..
waste products
succinate
acetate
propionate
ammonia
taurine metabolism
hypotaurine
taurine
Urea cycle
urea
putrescine
spermidine
spermine
osmolytes
amino acids
taurine
glycine betaine
TMAO
urea
polyols & sugars
glycine betaine
taurine
ammonia
urea
succinate, acetate
propionate
catabolism
C, N-source
N-source
TMAO
?
TMA
H2O
urease
H2CO3 + NH3
e-
degradation
sulfur oxidation
TMAO
DNA stability ..
γ3-symbiont
polyols
catabolism
ammonia
N-source
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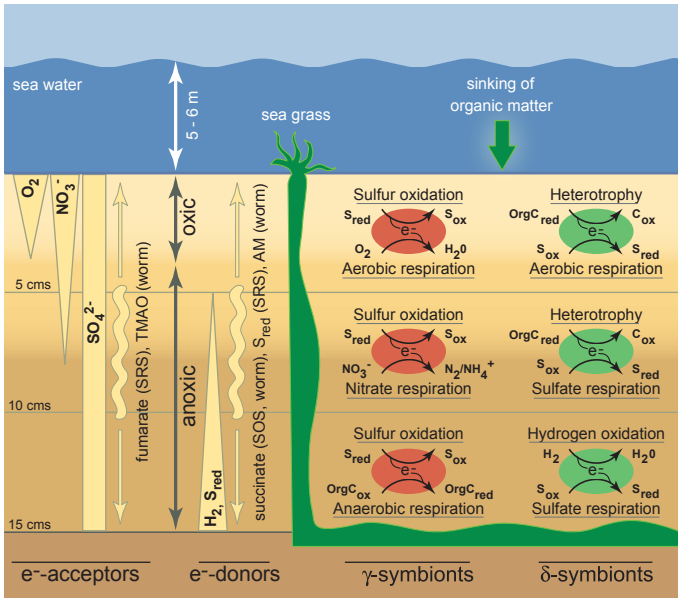
degradation
spermidine/
putrescine
DNA stability ..
putrescine
δ1-symbiont


sea water
5 - 6 m
sea grass
sinking of
organic matter
O2
NO3-
SO4 2-
oxic
anoxic
5 cms
10 cms
15 cms
fumarate (SRS), TMAO (worm)
succinate (SOS, worm), Sred (SRS), AM (worm)
H2, Sred
Sulfur oxidation
Sred
Sox
O2
H20
Aerobic respiration
Heterotrophy
OrgCred
Cox
Sox
Sred
Aerobic respiration
Sulfur oxidation
Sred
Sox
NO3-
N2/NH4+
Nitrate respiration
Heterotrophy
OrgCred
Cox
Sox
Sred
Sulfate respiration
Sulfur oxidation
Sred
Sox
OrgCox
OrgCred
Anaerobic respiration
Hydrogen oxidation
H2
H20
Sox
Sred
Sulfate respiration
e--acceptors
e--donors
γ-symbionts
δ-symbionts

Symbiosis insights through metagenomic analysis of a microbial consortium

Supplementary Information

1. Supplementary Methods

Specimen collection

Juvenile and adult *Olavius algarvensis* specimens were collected in May and September 2004 from 5.6 m water depth in silicate sediments around sea grass beds of *Posidonia oceanica* in a bay off Capo di Sant' Andrea, Elba, Italy (42°48'26"N, 010°08'28"E). The worms were removed from the sediment via decantation with seawater and identified under a dissection scope. Fresh samples collected in September 2004 were kept in the original sediment and seawater for 3 days until preparation. All other specimens were cleaned by successive washes in sterile seawater, snap-frozen on dry ice and stored at –80ºC until further processing. *O. algarvensis* is the dominant species at the collection site, however an additional gutless oligochaete species named *O. ilvae* co-occurs[1]. Identification at the species level requires careful examination of individual sexually mature worms by an expert and was thus not possible for this study.

Bacterial symbiont enrichment for the 3 kb library

Bacterial cells from approximately 200 live worms (~180 mg of sample containing ~2x 10^8 bacteria) were enriched using discontinuous nycodenz density gradient centrifugation. Briefly, fresh worms were removed from the sediment via decantation, cleaned by successive washes in sterile seawater and gently homogenized in 2 ml phosphate-buffered saline (PBS), pH 7.4 using a glass dounce homogenizer. A step gradient of 1.146-1.083 g/ml density was prepared with Histodenz™ (Sigma, St. Louis, MO) in 5 ml OpiSeal Polyallomer tubes (Beckman Coulter, Fullerton, CA) and the cell suspension loaded on top of the gradient. The overlain gradient was then centrifuged at 10,000 g in a Beckman L8-M ultracentrifuge and SW 55Ti swing rotor for 1 h at 4ºC. Following centrifugation, 200-300 µl fractions were withdrawn from the bottom of the

gradient tube and diluted with 10 vol of PBS to remove excess nycodenz. Cells were pelleted and resuspended in PBS and fractions evaluated semi-quantitatively for the enrichment of bacterial cells using real-time PCR amplification of 16S and 18S rRNA genes. As expected, the best enrichment of bacterial cells was found in higher density fractions, which were subsequently used for DNA extraction.

DNA extraction

For the fosmid libraries, metagenomic high molecular weight (HMW) DNA was extracted from approximately 200 pooled frozen worms for each library. The frozen worms were ground into fine powder under liquid nitrogen with mortar and pestle, transferred to a screw-cap tube and DNA extracted using the Qiagen Genomic-tip (Qiagen, Valencia, CA) according to the manufacturer's instructions. Approximately 60 μg of HMW metagenomic DNA was purified per 200 frozen specimens. For the 3 kb library, metagenomic DNA was extracted from nycodenz gradient enriched bacterial cells using Bactozol™ (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer. Fresh cells recovered from three combined nycodenz fractions yielded ~400 ng of DNA. DNA concentrations and purities were assessed by agarose gel electrophoresis and spectrophotometric analysis.

Shotgun library construction & end-sequencing

Three metagenomic shotgun libraries were constructed for this study: one 3 kb library from live worms collected in September 2004 and two fosmid libraries from frozen worms collected in April 2004 and September 2004.

3 kb library. A small insert library was constructed from DNA derived from the nycodenz-enriched sample collected in September 2004. Briefly, 300 ng of metagenomic DNA was randomly sheared to 2-4 kb fragments using a HydroShear (GeneMachines, San Carlos, CA). The sheared DNA was separated on an agarose gel, gel-purified using the QIAquick Gel Extraction Kit and end-repaired using the End-it™ DNA End-Repair kit (Epicentre, Madison, WI) according to the manufacturer's instructions. After an additional agarose gel separation, 2-4 kb DNA fragments were gel-purified once more.

The entire DNA extract was blunt-end ligated into 100 ng of **pMCL200** vector O/N at 16ºC using T4 DNA ligase (Roche Applied Science, Indianapolis, IN) and 10% (vol/vol) polyethylene glycol (Sigma). The ligation was phenol-chloroform extracted, ethanol precipitated and resuspended in 15 µl TE. According to the manufacturer's instructions, 1 µl of ligation product was electroporated into ElectroMAX DH10B™ Cells (Invitrogen, Carlsbad, CA) and plated on selective agar plates. Positive library clones were picked using the Q-Bot multitasking robot (Genetix, Dorset, U.K.) and grown in selective media for sequencing.

Fosmid libraries. Fosmid libraries were constructed using the CopyControl™ Fosmid Library Production Kit (Epicentre). Briefly, ~20 µg of metagenomic DNA derived from the frozen samples was randomly sheared using a HydroShear, blunt-end repaired as described above and separated on an agarose pulse-field gel O/N at 4.5 V/cm. The 35 kb fragments were excised, gel-purified using AgarACE™ (Promega, Madison, WI) digestion, followed by phenol-chloroform extraction, and ethanol precipitation. DNA fragments were ligated into the **pCC1Fos**™ Vector. The ligation was packaged using MaxPlax™ Lambda Packaging Extract and used to transfect TransforMax™ EPI300 *Escherichia coli*. Transfected cells were plated on selective agar plates and fosmid clones picked using the Q-Bot multitasking robot and grown in selective media for sequencing.

End-sequencing. Plasmids were amplified using the TempliPhi™ DNA Sequencing Template Amplification Kit (Amersham Biosciences, Piscataway, NJ) and sequenced using standard M13 –28 or –40 primers and the BigDye sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The reactions were purified using magnetic beads and run on an ABI PRISM 3730 (Applied Biosystems) capillary DNA sequencer (for research protocols, see www.jgi.doe.gov).

16S rRNA libraries & phylogenetic analysis

16S rRNA PCR libraries were created from DNA sources used for all three metagenomic libraries. Amplification of 16S rRNA genes was performed using the bacteria-specific universal primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-

GGTTACCTTGTTACGACTT-3')[2]. The following cycling conditions were used: 94°C for 5 min, followed by 20 cycles of 94°C for 30 sec, 55°C for 25 sec, 72°C for 90 sec, and an extension at 72°C for 7 min. To minimize heteroduplex formation, a reconditioning step was applied[3]. Briefly, PCR reactions were diluted 10-fold into fresh reaction mixtures of the same composition and cycled three more times using the above parameters. PCR products of five replicate reactions were combined, gel-extracted as described above and ligated into the pCR4-TOPO vector using the TOPO TA Cloning Kit (Invitrogen). Ligations were then electroporated into One Shot TOP10 Electrocomp™ *E. coli* cells and plated on selective media agar plates. Approximately 384 clones per library were picked and grown in selective media for sequencing (see above).

The bi-directional 16S rRNA gene sequence reads were end-paired and trimmed for PCR primer sequence and quality. Approximately 3% of the sequences were removed as putative chimeras by identification with Bellerophon[4]. The resulting chimera-free sequences were evaluated using BLAST analysis[5] against sequences in the NCBI database and the 16S rRNA sequences of the *O. ilvae* and *O. algarvensis* symbionts (unpublished data). Phylogenetic trees were calculated by neighbor joining analyses using the ARB software package (www.arb-home.de)[6]. Only sequences ≥ 1,400 bp were used for tree construction.

Processing, analysis & assembly of shotgun data

Initial data set. The initial data set was derived from the three shotgun libraries described above. We sequenced 279,157 reads from the 3 kb library, containing 279 Mb of raw sequence. 36,095 reads were sequenced from the two 35 kb libraries, containing 37 Mb of raw sequence. The reads were screened for vector using cross_match, then trimmed for vector and quality[7]. Reads < 100 bases after trimming were excluded. This reduced the amount of data to 250,034 reads (185 Mb) of 3 kb library end-sequence, and 31,414 reads (19 Mb) of 35 kb library end-sequence.

Analysis of unassembled shotgun sequences. Unassembled shotgun sequence reads (trimmed for vector sequence and quality) were evaluated using BLAST analysis[5] against the NCBI nr database (BLASTx, e-value 1e-3) and NCBI nt database (BLASTn), as well

as the 16S rRNA gene sequences of the *O. ilvae* and *O. algarvensis* symbionts (BLASTn).

Jazz assembly parameters. The data was assembled using release 2.8 of JAZZ, a WGS assembler developed at the JGI[7,8]. A word size of 13 was used for seeding alignments between reads. The unhashability threshold was set to 50, preventing words present in more than 50 copies in the data set from being used to seed alignments. A mismatch penalty of -30.0 was used, which will generally assemble sequences that are more than about 97% identical. As the different organisms in the data set were expected to be present at different sequence depths, the usual depth-based bonus/penalty system was turned off.

Post-assembly analysis. The initial assembly contained 5,016 scaffolds, with 42 Mb of sequence, of which 37% were gaps. As JAZZ links contigs into scaffolds based on fosmid paired-end information and pads these gaps with N's based on the known approximate insert fosmid size, many scaffolds are gapped. The scaffold N/L50 was 122/73 kb, while the contig N/L50 was 741/8.2 kb. If the scaffolds are sorted by total length in descending order, the scaffold N50 value is equal to the number of scaffolds one needs to go down the list before one has encompassed half of the total scaffold sequence in the set. The scaffold L50 value is then the total length of the smallest scaffold in the "top half" of this list. The Contig N50 and L50 are values analogous to the scaffold N50 and L50 values with the difference that the contig values are calculated using net, instead of total, scaffold lengths. Redundant scaffolds were identified by aligning all scaffolds with less than 5 kb of contig sequence against those with more than 5 kb of contig sequence using BLAST-like alignment tool[9]. Any scaffolds from the former set that matched any of the larger over more than 80% of their length were excluded. Short scaffolds (< 1 kb of contig sequence) were also excluded. The unassembled reads and short scaffolds largely represent the lower abundance species as well as worm DNA within this environmental sample, as suggested by the presence of the metagenomic reads encoding the *O. ilvae* symbionts 16S rRNA genes within this shrapnel. The filtering left 2,286 scaffolds, with 39 Mb of sequence, of which 40% was gap. The scaffold N/L50

was 106/80 kb, while the contig N/L50 was 606/9.6 kb. Approximately 61% of the reads fell into the filtered assembly. This filtered scaffold set served as the starting point of all downstream analyses.

Binning

Scaffolds from the *Olavius* spp. symbionts' metagenome were binned by a combinatory approach based on the following intrinsic DNA signatures: (a) GC-content (b) dinucleotide relative abundance[10] (c) Markov model-based statistical evaluations of tri-, tetra and pentamer over- and under-representation[11] and (d) normalized chaos game representations for tri- to hexamers Deschavanne[12,13]. Values for (c) and (d) were computed by ocount and cgr, two self-written C-programs that are available from www.megx.net/tetra.

A self-written Java program (MetaClust[14]) was used to automatically trigger the individual calculations and subsequently store them in a MySQL database. Seven different combinations of subsets of the individual methods were built for all scaffolds exceeding 50 kb and imported into Cluster 3.0[15]. The data was normalized and a hierarchical clustering was computed using complete linkage and the Euclidian distance as distance measure. The corresponding result files were analyzed using Java TreeView (http://genetics.stanford.edu/~alok/TreeView/) and merged into consensus clusters in a semi-automatic manner by parsing the Java TreeView result files. This procedure was repeated for all scaffolds exceeding 15 kb and thereafter for all scaffolds exceeding 5 kb. Shorter scaffolds were discarded because (a) the reliability of signature-based scaffold affiliation declines with decreasing sequence lengths and (b) the fraction of short unassembled scaffolds contain chimeric sequences. After each of these steps, the newer and the former clusters were compared and ambiguous scaffolds were sorted out. A Bayesian classifier[16] was trained with all scaffolds ≥ 50 kb and subsequently used to assign some of the much shorter scaffolds with ambiguous classifications. Final clusters were verified threefold, (a) by phylogenetic affiliation of each scaffold based on the most common phylogeny of its predicted proteins (BLASTp, e-value $\geq$ 1e-5, NCBI nr) (b) by a Bayesian classifier and (c) by checking for paralogs of 49 genes that typically occur with only one copy per genome (Kunin *et al*, unpublished).

Despite all the support for our binning, it is noteworthy that the approach is statistical by nature and thus the bins likely do contain false positive. It should also be stressed that the binning-approach benefits from a low diversity and a confined habitat of the to be separated organisms, since successful separation becomes the more problematic the higher the diversity of a sample gets. Low-diversity symbiotic communities are optimal samples in this regard, although their assembly and binning can be affected by an elevated proportion of lateral gene transfer among the symbionts.

Gene prediction and annotation

Potential open reading frames (ORFs) were identified using the meta gene prediction software "mORFind" (Waldmann, unpublished) developed at the MPI-Bremen. This system analyzes and combines the output of the three commonly used gene-finders CRITICA[17], GLIMMER3[18] and ZCURVE[19] to enhance sensitivity and specificity. To resolve conflicts, an iterative post-processing algorithm is used taking into account signal peptide[20] and transmembrane[21] predictions, ORF-length, and the number of gene-finders by which an ORF has been predicted. The system was adapted to deal with typical problems of community sequencing projects like ambiguities, stretches of Ns, and fragmented genes. Annotation was performed with the GenDB v2.2 system[22], seeking for each predicted ORF observations from similarity searches against sequence databases (nr, Swiss-Prot, Kegg-Genes, release December 2005) and protein family databases (Pfam (release 19.0), InterPro (release 12.0, InterProScan version 4.2)), and from predictive signal peptide- (SignalP v3.0[20]) and transmembrane helix-analysis (TMHMM v2.0[21]). tRNA genes were identified using tRNAScan-SE[23] and rRNA genes were detected by standard similarity searches (BLAST[5]) against public nucleotide databases and the 16S rRNA sequences of the *O. ilvae* and *O. algarvensis* symbionts. Predicted protein coding sequences were automatically annotated with the software MicHanThi[24] developed at the MPI Bremen. The system simulates the human annotation process using fuzzy logic. First, informative BLAST observations are selected taking into account several BLAST parameters. The gene product is then assembled by functional clustering of observations and by selection of the most supported one. Each annotation is labelled by the corresponding reliability value to support further human inspection. Once the gene

product is set, MicHanThi adds further information like gene name, EC, and GO numbers to each protein coding gene based on Swiss-Prot and InterPro observations, respectively. A functional classification was performed with similarity searches against COG v2[25]. All ORFs described in this publication were manually refined. All binned scaffolds were furthermore analyzed for the presence of clustered regularly interspaced short palindromic repeats (CRISPRs) using the CRISPR PILER-CR v1.0[26].

The annotated *Olavius* spp. symbionts' bins were incorporated into the metagenomics version of the U.S. Department of Energy Joint Genome Institute Integrated Microbial Genomes (IMG)[27], IMG/M (http://img.jgi.doe.gov/m), a data management and analysis platform for metagenomic data. This facilitates access and visualization and comparative analyses of the data in the context of other metagenomic datasets and all publicly available complete microbial genomes.

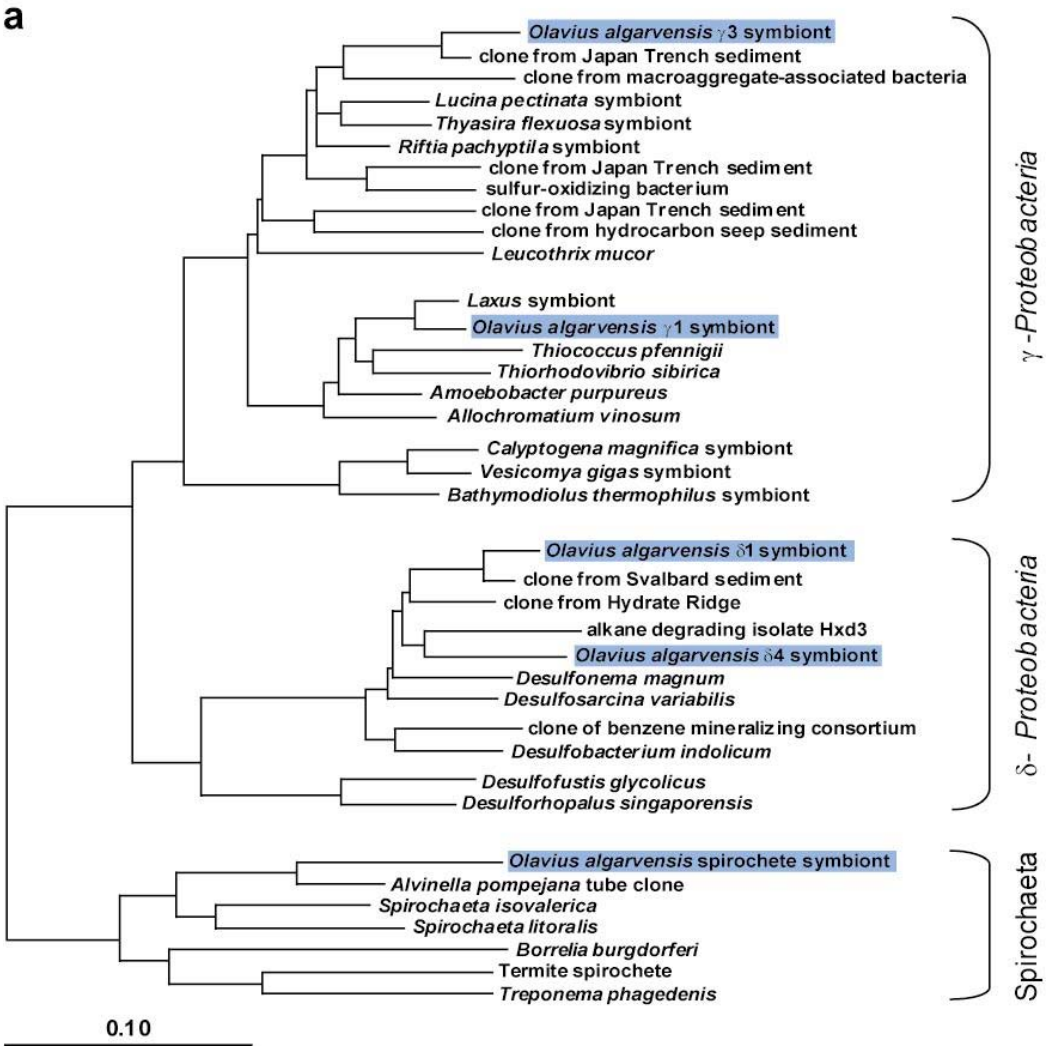
Community heterogeneity

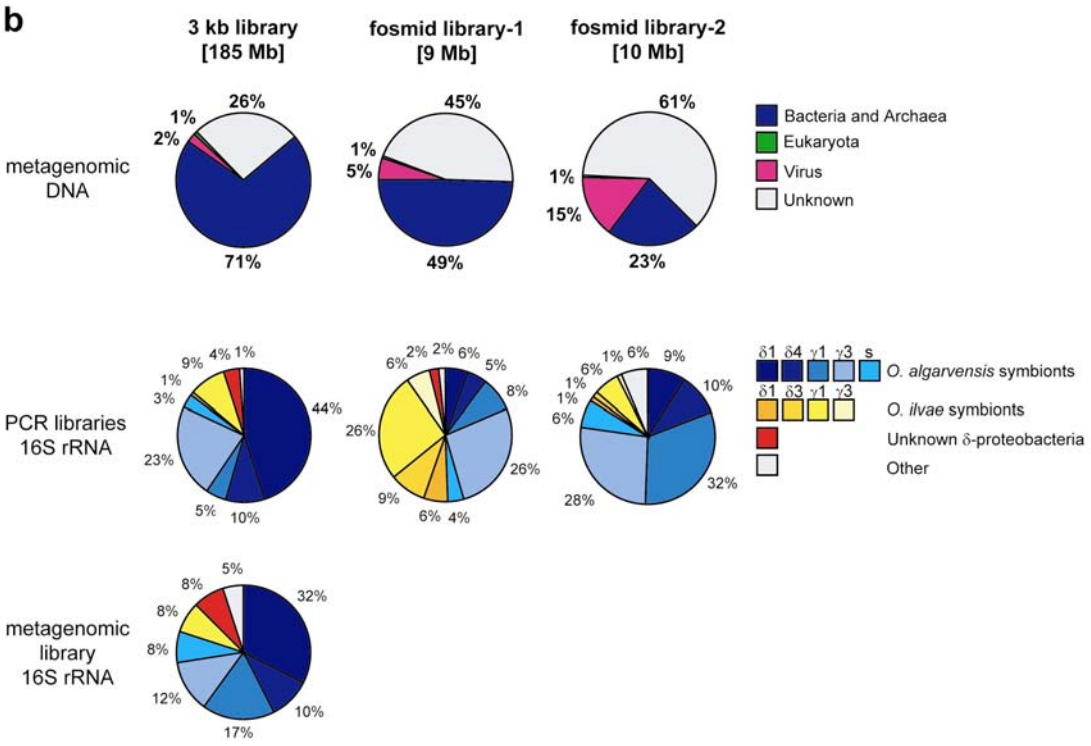
To assess nucleotide sequence variation within the binned scaffolds or bins, we analyzed the multiple alignment of the JAZZ assembly. A site was considered polymorphic, if at least two reads showed at least two different nucleotides (or gaps) in regions covered by 4-20 reads. Frequencies of polymorphic sites (the total number of polymorphic sites divided by the total number of nucleotide sites at 4-20X read depth) were averaged over all contigs assigned to a given bin.

Nucleotide sequence deposition

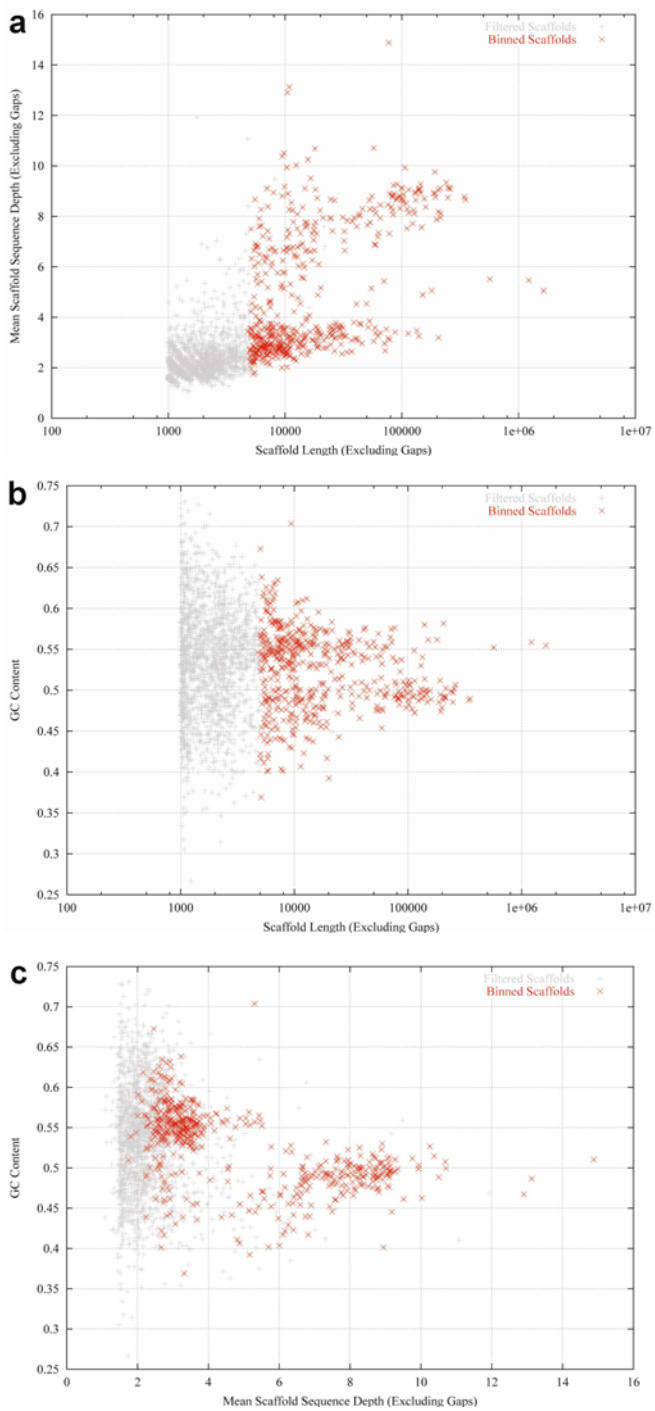
The metagenomic unassembled sequence reads have been deposited into the NCBI trace archive. Assembled sequences from the *Olavius* spp. symbionts' metagenome have been deposited into the NCBI database under the project accession AASZ00000000.

2. Supplementary Figures and Legends

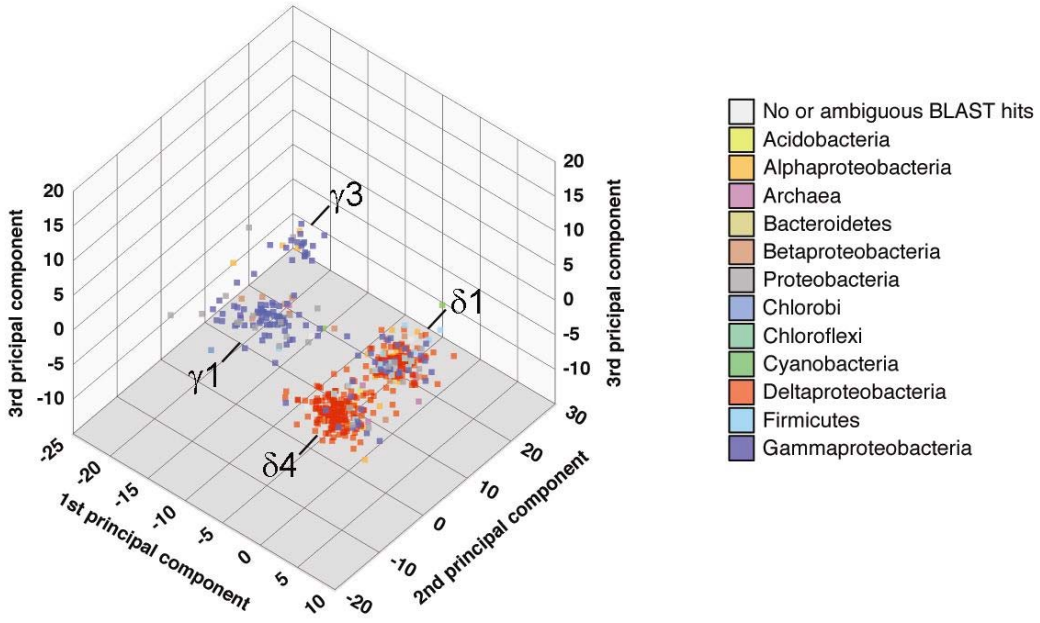




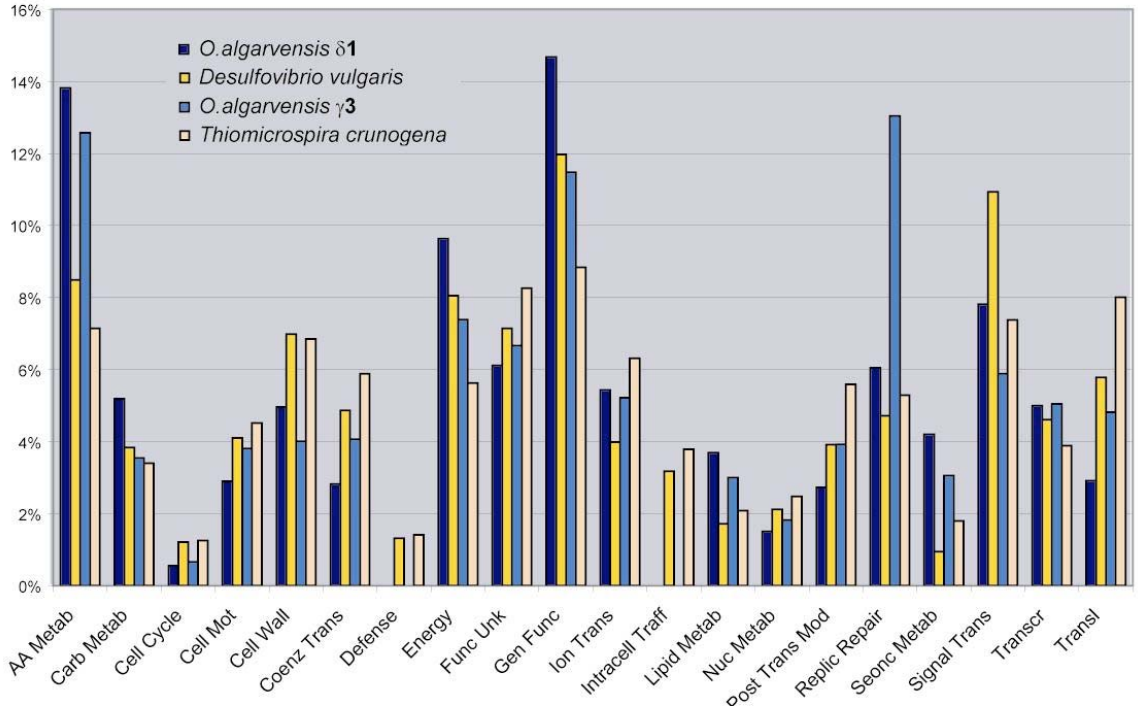
Supplementary Figure S1. (a) Phylogenetic relationship of the *O. algarvensis* endosymbionts (blue) based on parsimony analyses of 16S rRNA sequences. Bar, 10% estimated sequence divergence. **(b) Characterization of the metagenomic libraries and the 16S rRNA PCR libraries.** Percentage of 3 kb and fosmid library end reads (unassembled) with similarities to proteins of bacterial and archaeal, eukaryotic or viral origin (BLASTx, e-value 1e-3, NCBI nr) (top chart row). Unknown = reads with no similarity to proteins in the public databases. The middle chart row indicates the relative phylotype abundance in the DNA used for each library based on 16S rRNA PCR library sequences. For both fosmid libraries this phylotype comparison is a good indication of the natural bacterial abundance, as the DNA originated from pooled worms that were not density fractionated as were those for the 3 kb library. s = spirochete. Other includes 16S rRNA phylotypes of low abundance species. The relative phylotype abundance based on 16S rRNA gene representation within the metagenomic 3 kb library reads is shown in the bottom pie chart.



Supplementary Figure S2. Length, sequence depth and GC content distributions of the JAZZ assembled, filtered scaffold set. (a) Net scaffold length vs. mean scaffold sequence depth (excluding gaps). (b) Net scaffold length vs. GC content. (c) Mean scaffold sequence depth (excluding gaps) vs. GC content. The filtered set comprises 2,286 scaffolds with a combined net length of 23.7 Mb. The 511 scaffolds that were binned based on nucleotide signatures are shown in red (comprising a net length of 20.1 Mb).



Supplementary Figure S3. Phylogenetic scaffold affiliations within the nucleotide signature based symbiont clusters. Visualization of the first three components of a principal component analysis (PCA), in which GC-content, net read depth, z-scores for all possible 64 trinucleotides and 256 tetranucleotides were incorporated with equal weight (z-scores calculated with TETRA and normalized by length); sequences < 5 kb are not represented. Phylogenetic affiliation of each scaffold was based on the most common phylogeny of its predicted proteins and is indicated by color.



Supplementary Figure S4. Distribution of the genes identified for δ1 and γ3 bins by broad functional category of clusters of orthologous groups of proteins (COGs) (e-value 1e-5), as compared to the complete genomes of *Desulfovibrio vulgaris* Hildenborough and *Thiomicrospira crunogena* XCL-2. Categories which show gene representations below 0.2 % are excluded. Both symbiont genome bins show a higher incidence of genes involved in amino acid as well as lipid transport and metabolism as compared to the non-symbiotic bacteria, while genes involved in translation, ribosomal structure and biogenesis show a lower relative abundance. Genes involved in replication, recombination and repair are furthermore highly represented within the γ3 bin.

3. Supplementary Tables

Supplementary Table S1. General features of the *O. algarvensis* symbiont bins				
	δ_1	**δ_4**	**γ_1**	**γ_3**
Assembly statistics				
Genome bin size [bp]	13,536,737	6,382,161	5,317,000	4,647,793
Gaps filled with N's [%]	16	52	73	12
Number of scaffolds	226	172	91	22
GC content [%]	49.2	54.6	57.5	55.7
Mean total read depth*	7.1	1.6	0.8	4.6
Mean net read depth*	8.4	3.3	3.0	5.2
Mean total length [bp]	59,897	37,106	58,429	211,263
Mean net length [bp]	50,593	17,887	16,059	185,783
Gene predictions				
Protein coding genes	12,084	3,012	1,872	4,154
Genes with similarity to nr	7,505	2,399	1,302	3,778
Genes with similarity to COG	5,340	1,919	831	3,083
Number of rRNA operons	1	1	2**	1
Number of tRNA genes	49	23	17	33
Number of tRNA synthetases	26	22	12	26

*normalized with respect to length.

**one complete rRNA operon and one partial 16S rRNA gene.

nr, non-redundant Genbank.

Supplementary Table S2. General features of the *O. algarvensis* symbiont genome bins as compared to the genomes of other extracellular as well as intracellular endosymbionts

endosymbiont (class)	host organism	Genome (bin) size* [Mb]	GC content [%]	Number of protein coding genes	Number of rRNA operons	Number of tRNA genes
O. algarvensis δ1 symbiont (δ-Proteob.)	annelid *O. algarvensis*	13.5	49.2	12,084	1	49
O. algarvensis δ4 symbiont (δ-Proteob.)	annelid *O. algarvensis*	6.4	54.6	3,012	1	23
O. algarvensis γ1 symbiont (γ-Proteob.)	annelid *O. algarvensis*	5.3	57.5	1,872	2**	17
O. algarvensis γ3 symbiont (γ-Proteob.)	annelid *O. algarvensis*	4.6	55.7	4,154	1	33
Photorhabdus luminescens [28] (γ-Proteob.)	nematodes (Heterorhabditidae)	5.7	42.8	4,839	7	85
Vibrio fischeri ES114 [29] (γ-Proteob.)	bobtail squid *Euprymna scolopes*	4.3	38.3	3,647	12	119
Bradyrhizobium japonicum [30] (α-Proteob.)***	soybean *Glycine max*	9.1	64	8,317	1	50
Buchnera aphidicola [31] (γ-Proteob.)***	aphid *Baizongia pistacea*	0.6	25	504	1	32

* including plasmids.

**one complete rRNA operon and one partial 16S rRNA gene.

*** intracellular endosymbiont location.

Supplementary Table S3. Amino acyl tRNA synthetase genes in the symbiont bins			***O. algarvensis***	
	δ_1	**δ_4**	**γ_1**	**γ_3**
Glutamyl- and glutaminyl-tRNA synthetase	1	0	3	3
Alanyl--tRNA synthetase	1	1	0	1
Phenylalanyl-tRNA synthetase	1	1	0	1
Aspartyl/asparaginyl-tRNA synthetase	1	0	0	0
Arginyl-tRNA synthetase	1	1	1	1
Isoleucyl-tRNA synthetase	1	1	0	1
Phenylalanyl-tRNA synthetase beta subunit	1	1	0	2
Histidyl-tRNA synthetase	1	0	1	2
Tyrosyl-tRNA synthetase	0	0	0	1
Seryl-tRNA synthetase	1	1	0	2
Aspartyl-tRNA synthetase	1	1	1	1
Tryptophanyl-tRNA synthetase	1	0	1	0
Cysteinyl-tRNA synthetase	1	3	0	1
Threonyl-tRNA synthetase	2	1	0	1
Prolyl-tRNA synthetase	1	1	1	1
Leucyl-tRNA synthetase	1	1	2	1
Valyl-tRNA synthetase	1	2	0	1
Glycyl-tRNA synthetase beta subunit	1	0	0	2
Glycyl-tRNA synthetase alpha subunit	1	0	0	1
Lysyl-tRNA synthetase class II	2	2	0	2
Lysyl-tRNA synthetase class I	0	0	0	0
Pseudouridine-tRNA synthetase	3	2	0	1
Methionyl-tRNA synthetase	2	2	1	0
tRNA-dihydrouridine synthetase	0	1	0	0
tRNA(Ile)-lysidine synthetase	0	0	1	0

Supplementary Table S4. Repeats and polymorphic sites in the *O. algarvensis* symbiont bins				
	δ_1	δ_4	γ_1	γ_3
Number of transposases	276	17	389	313
Percent of transposases	2.3	0.6	20.5	7.5
Number of integrases	30	3	28	78
CRISPR elements	yes	no	no	no
Number of polymorphic sites*	7565	144	422	1280
Frequencies of polymorphic sites** [%]	0.08	0.01	0.1	0.04

*a site was considered polymorphic, if at least two reads showed at least two different nucleotides (or gaps) in regions covered by 4-20 reads.

**averaged over all contigs assigned to a given bin.

Supplementary Table S5. Amino acid and vitamin biosynthesis genes in the *O. algarvensis* symbiont bins

	δ1	δ4	γ1	γ3
amino acid biosynthesis				
histidine	+	+	±	+
phenylalanine	+	+	±	+
tyrosine	+	+	±	+
leucine	+	+	±	+
isoleucine	+	+	±	+
valine	+	+	±	+
tryptophan	+	+	-	+
arginine	+	+	±	+
lysine	+	+	±	+
methionine	+	+	±	+
threonine	±	+	±	±
serine	+	+	±	+
proline	+	+	±	+
glycine	+	+	±	+
cycteine	+	+	±	±
asparagine	+	+	±	±
glutamine	+	+	+	+
alanine	+	+	+	+
aspartic acid	+	+	+	+
glutamic acid	+	+	-	+
selenocysteine	+	+	-	-
pyrrolysine	+	+	-	-
coenzymes and cofactor biosynthesis				
biotin	-	+	+	+
cobalamin	+	+	+	-
coenzyme A	+	+	+	+
riboflavin and FAD	+	+	+	+
heme	+	+	+	+
NAD	+	+	+	+
pyridoxal phosphate	+	+	+	+
thiamin	+	+	+	+
ubiquinone	+	+	-	+

+ at least 80% of the required synthesis genes are present

Supplementary Table S6. Transposase genes in the symbiont bins	*O. algarvensis*			
	δ1	δ4	γ1	γ3
IS1	-	-	48	-
IS106	-	-	3	-
IS110	-	-	8	-
IS111A	-	-	3	-
IS111A/IS1328/IS1533	-	1	-	-
IS116	-	-	5	-
IS116/IS110/IS902	-	1	-	-
IS1249	10	-	-	-
IS1479	-	-	-	1
IS1480	-	-	1	-
IS1595	1	-	-	-
IS1663	-	-	1	-
IS180	-	-	3	-
IS186	13	-	-	-
IS200	-	-	5	2
IS21	2	-	-	-
IS298	-	-	1	-
IS3	-	-	-	4
IS3/IS911	1	-	12	59
IS3231	28	-	-	-
IS4	60	-	52	77
IS5	-	-	4	-
IS630	1	-	73	-
IS641	-	-	-	27
IS642	-	-	2	-
IS643	1	-	-	-
IS653	2	-	-	-
IS66	1	-	-	9
ISChy4	2	-	-	-
ISCps6	-	-	-	1
ISDvu4	2	6	-	-
ISEcp1	-	-	13	-
ISGsu4	5	-	-	7
ISGsu6	1	-	-	-
Iso-IS1	-	-	20	-
ISPpu8	3	-	-	-
ISPsy19	-	-	1	-
ISPsy5	-	-	-	1
ISR013	5	-	-	-
ISRm	-	-	4	-
ISRm22	-	-	-	18
ISRPsy14	5	-	-	-
ISSod13	5	8	-	-
ISSpo2	-	-	1	-
ISSpo3	1	-	-	-
ISSpo8	99	-	-	-
ISxac3	1	-	-	-
Tnp	3	-	-	-
TnpA	-	-	16	2
Transposase	10	-	75	13
Transposase & inactivated derivates	-	1	19	81

4. Supplementary Discussion

Sample characterization

O. algarvensis specimens were collected from shallow subtidal sediments off the island of Elba, Italy. *O. algarvensis* is the dominant gutless oligochaete species at the collection site, where a second gutless oligochaete species, *O. ilvae* co-occurs in low abundance[1]. Shotgun libraries were constructed from pooled fresh endosymbiont enriched sample (3 kb library), as well as pooled frozen worm specimens (fosmid libraries), due to the unavailability of large amounts of fresh sample. We generated 185 million bases (Mb) of 3 kb library end sequence and 19 Mb of fosmid end sequence, for a total of 204 Mb of high-quality shotgun sequence data. BLAST analysis[5] of the unassembled sequence reads as well as 16S rRNA gene analysis indicated that the 3 kb library largely consists of *O. algarvensis* symbiont DNA, supporting our choice of this library for in-depth sequencing (Supplementary Fig. S1b).

To estimate the relative abundance of the *O. algarvensis* symbionts within the libraries, we performed 16S rRNA PCR amplification of each source DNA using parallel PCR reaction aliquots, low PCR cycle numbers and a reconditioning step to minimize PCR bias as well as chimera and heteroduplex formation[3]. The 16S rRNA gene analysis of the DNA source used for the 3 kb library construction revealed that sequences derived from the *O. algarvensis* symbionts were highly represented (Supplementary Fig. S1b), dominated by δ1 and γ3. Additional 16S rRNA sequences derived from an unknown Deltaproteobacteria closely related to *O. algarvensis* δ1, *O. ilvae* γ1 and other bacterial species at low abundance. 16S rRNA gene sequences found within the metagenomic 3 kb library showed a similar distribution of phylotypes (Supplementary Fig. S1b). 16S rRNA gene analyses of DNA used for the fosmid libraries showed that all three samples differed in their symbiont species distribution and in contamination with *O. ilvae* symbionts (Supplementary Fig. S1b). This may be due to variations in oligochaete species distribution patterns.

Metagenomic data analysis and binning

The metagenomic shotgun sequences were assembled using the whole-genome shotgun assembler JAZZ[8], resulting in a total of 2,286 scaffolds with a combined total length of 39.3 Mb (net length not including gaps was 23.7 Mb; Supplementary Fig. S2) and a longest scaffold of 1.9 Mb (total length). Binning of the *Olavius* spp. symbionts' metagenome resulted in 511 scaffolds (Supplementary Fig. S2) forming four distinct clusters, identified as *O. algarvensis* symbionts δ1, δ4, γ1, and γ3, as based on 16S rRNA genes. Symbiont bin assignments were supported by phylogenetic analysis of predicted proteins within each cluster of scaffolds the distribution of 49 single-copy genes. Only one gene, *secG* encoding the preprotein translocase SecG subunit, was found in duplicate and only in the δ1 bin (sequence similarities between the two copies of *secG* were 95% at the nucleotide level and 98% at the amino acid level). This could indicate the presence of more than one strain in this bin and might explain its large size of 13.5 Mb (total length), although genomes of comparable size are known from the Deltaproteobacteria (e.g. *Polyangium cellulosum*) (http://genomesonline.org)[32]. The single occurrence of 48 out of 49 single-copy genes in the δ1 bin and the presence in single copy of other key genes, such as most ribosomal proteins, cell division genes, flagellum genes and amino acyl tRNA synthetases (Supplementary Table S3), is however indicative of the presence of a single dominant strain.

Carbon fixation in the δ-symbionts

Carbon fixation within both deltaproteobacterial symbionts is possible via the reductive acetyl-CoenzymeA (CoA) pathway and also likely via the reductive tricarboxylic acid (TCA) cycle. Both bins encode citrate lyase and oxoglutarate-ferrodoxin oxidoreductase, which catalyze two out of three potentially irreversible steps in the TCA. The third potentially irreversible step is catalyzed by succinate dehydrogenase (SDH). Depending on the specific implementation of this enzyme, it could be either reversible (as in *Bacillus subtilis*) or irreversible (as in *E. coli*, which has a separate succinate dehydrogenase and fumarate reductase). Succinate dehydrogenase is encoded in both proteobacterial symbionts, yet we are unable to determine in which direction their SDH catalyzes its reaction and whether it is reversible. The proteins highest percent identity is to the

actinobacterial succinate dehydrogenase. In actinobacteria, this enzyme strictly catalyzes succinate oxidation, while a second enzyme catalyzes fumarate reduction. However, in general the direction of reaction of SDH/QFR is dependent on the structure and type of cytochrome subunit; yet we were unable to find any cytochrome subunit in deltaproteobacterial bins (and no hydrophobic anchor subunit for that matter). The lack of subunit may either be due to the incompleteness of these genome bins, or the symbionts have a very unusual form of this enzyme, a soluble form. A soluble fumarate reductase is known in methanogenic archaea, where a soluble donor (coenzyme M) is used instead of a quinone.

Osmolyte breakdown

Bacterial[33] and archaeal[34] TMAO breakdown pathways are present in the *O. algarvensis* symbionts: several homologs of trimethylamine/dimethylamine dehydrogenase are found in the γ3 and δ1 symbiont bins and 22 proteins from the trimethylamine:corrinoid methyltransferase family were encoded in γ3, δ1 and δ4 symbionts, as well as on unassigned scaffolds. Six genes coding for dimethylamine:corrinoid methyltransferase and two genes encoding monomethylamine:corrinoid methyltransferase are present in the δ1 bin. Like their archaeal counterparts, at least two proteins from the δ1 symbiont coding for trimethylamine methyltransferases and all proteins encoding dimethylamine methyltransferases are interrupted by an amber stop codon UAG, which is most likely translated to pyrrolysine. This is supported by the presence of pyrrolysine-specific aminoacyl-tRNA synthetase and PylS-associated genes in the δ1 symbiont. Tetrahydrofolate appears to be the most likely acceptor of methyl groups in the symbionts due to the presence of at least three homologs of methylcobalamin:tetrahydrofolate methyltransferase MtvA from the vanillate demethylase complex of *Moorella thermoacetica*[35]. Some of the methylamine methyltransferase genes are found in chromosomal clusters with the genes encoding putative enzymes from the glycine oxidase/dimethylglycine dehydrogenase family and the molybdopterin dehydrogenase family, suggesting the existence of branched and possibly novel pathways for degradation of trimethylamine N-oxide.

Secondary metabolites

Many marine invertebrates are associated with endo- and epibiotic microorganisms producing secondary metabolites of large molecular diversity, which led us to evaluate the symbiont bins for genes indicative for the biosynthesis of such compounds. Both deltaproteobacterial bins encode genes likely involved in polyketide and non-ribosomal peptide synthesis, including non-ribosomal peptide synthases, polyketide synthases, and methyltransferases. The products of these genes could be toxins and antibiotics, used possibly by the hosts as a protection against predation, or signaling molecules involved in symbiont - host interactions.

None of the symbiont genome bins were found to encode any of the key enzymes involved in the biosynthesis of steroid hormones and catecholamines (dopamine, norepinephrine, epinephrine). Homoserine lactone synthases, involved in quorum sensing, were also not encoded in any of the symbionts. It is not possible to decipher which compounds produced by the symbionts could have an effect on host behavior based on gene content alone.

Symbiont transmission

Symbiotic bacteria without a free-living stage are transmitted vertically from one generation to the next. In gutless oligochaetes, at least some of the symbionts may be transmitted vertically in a smear-like infection as the eggs exit the worm and pass genital pads packed with the symbiotic bacteria[36]. However, the deposition of the eggs directly into the surrounding sediments would also offer free-living bacteria from the environment an opportunity to invade the egg. It is therefore possible that some of the symbionts are inherited vertically from the parents and some horizontally from the environment. It is also possible that the same symbiont is transmitted vertically as a rule, but can also be acquired from the environment as shown for the *Wolbachia* symbionts in arthropods[37,38]. The low levels of polymorphism in all four symbiont bins of *O. algarvensis* do not exclude horizontal transmission, as selection by the host for a single bacterial strain is well known from other marine symbioses in which the symbionts are acquired from the environment, such as the luminescence symbioses between squid and *Vibrio fischeri*[39,40].

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