Metaproteomics of a gutless marine worm and its symbiotic microbial community reveal unusual pathways for carbon and energy use

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Low nutrient and energy availability has led to the evolution of numerous strategies for overcoming these limitations, of which symbiotic associations represent a key mechanism. Particularly striking are the associations between chemosynthetic bacteria and marine animals that thrive in nutrient-poor environments such as the deep sea because the symbions allow their hosts to grow on inorganic energy and carbon sources such as sulfide and CO2. Remarkably little is known about the physiological strategies that enable chemosynthetic symbionts to colonize oligotrophic environments. In this study, we used metaproteomics and metabolomics to investigate the intricate network of metabolic interactions in the chemosynthetic association between Olavius algarvensis, a gutless marine worm, and its bacterial symbionts. We propose previously undescribed pathways for coping with energy and nutrient limitation, some of which may be widespread in both free-living and symbiotic bacteria. These pathways include (i) a pathway for symbiont assimilation of the host waste products acetate, propionate, succinate and malate; (ii) the potential use of carbon monoxide as an energy source, a substrate previously not known to play a role in marine invertebrate symbioses; (iii) the potential use of hydrogen as an energy source; (iv) the strong expression of high-affinity uptake transporters; and (v) as yet undescribed energy-efficient steps in CO2 fixation and sulfate reduction. The high expression of proteins involved in pathways for energy and carbon uptake and conservation in the O. algarvensis symbiosis indicates that the oligotrophic nature of its environment exerted a strong selective pressure in shaping these associations.

Growth in nutrient-limited environments presents numerous challenges to organisms. Symbiotic and syntrophic relationships have evolved as particularly successful strategies for coping with these challenges. Such nutritional symbioses are widespread in nature and, for example, have enabled plants to colonize nitrogen-poor soils and animals to thrive on food sources that lack essential amino acids and vitamins (1). Chemosynthetic symbioses, discovered only 35 years ago at hydrothermal vents in the deep sea, revolutionized our understanding of nutritional associations, because these symbioses enable animals to live on inorganic energy and carbon sources such as sulfide and CO2 (2, 3). The chemosynthetic symbionts use the energy obtained from oxidizing reduced inorganic compounds such as sulfide to fix CO2, ultimately providing their hosts with organic carbon compounds. Chemosynthetic symbioses thus are able to thrive in habitats where organic carbon sources are rare, such as the deep sea, and the symbionts often are so efficient at providing nutrition that many hosts have reduced their digestive systems (4).

The marine oligochaete Olavius algarvensis is a particularly extreme example of a nutritional symbiosis: These worms are dependent on their chemosynthetic symbionts for both their nutrition and their excretion, because they have reduced their mouth, gut, and nephridial excretory organs completely (5). O. algarvensis lives in coarse-grained coastal sediments off the island of Elba, Italy, and migrates between the upper oxidized and the lower reduced sediment layers (6). It hosts a stable and specific microbial consortium consisting of five bacterial endosymbionts in its body wall: two aerobic or denitrifying gammaproteobacterial sulfur oxidizers (γ1- and γ3-symbionts), two anaerobic deltaproteobacterial sulfate reducers (δ1- and δ4-symbionts), and a spirochete with an unknown metabolism (7, 8). The sulfate-reducing δ-symbionts provide the sulfur-oxidizing γ-symbionts with reduced sulfur compounds as an internal energy source for autotrophic CO2 fixation via the Calvin–Benson cycle, thus explaining how O. algarvensis can thrive in its sulfide-poor environment (6, 9). As in all living organisms, the symbiosis is dependent on external energy sources, but to date the identity of these sources has remained unclear.

Like the vast majority of symbiotic microbes, the O. algarvensis symbionts have defied cultivation attempts, making cultivation-independent techniques essential for their analysis. A metagenomic analysis of the O. algarvensis symbionts yielded initial insights into their potential metabolism (9), but the incomplete genome sequences hindered the reconstruction of complete metabolic pathways, leaving many questions unanswered (10). Further...
thermore, as in all genomic analyses, detailed insights into the physiology and metabolism of an organism are limited, because these analyses can predict only the metabolic potential of an organism, not its actual metabolism and physiology (11). This limitation is most apparent in a multimember community in which the interactions between the different members and between these members and their environment lead to a level of metabolic complexity that can greatly exceed the predictive ability of genomic reconstructions from single species.

Although metagenomic analyses reveal the metabolic potential of a microbial community, metaproteomic and metabolomic analyses provide evidence for the metabolic and physiological processes that actually are used by the community. In this study, we used metaproteomics and metabolomics as well as enzyme assays and in situ analyses of potential energy sources to gain an in-depth understanding of the intricate interactions between O. algarvensis and its microbial symbiont community and between the members of this community and their environment. Our goal was to identify the compounds that provide energy for the symbiosis, the functional roles of the different partners, and their interactions within the symbiosis.

Results and Discussion

High Coverage of the Symbiosis Metaproteome and Metabolome. We identified and quantified a total of 2,519 proteins and 97 metabolites in O. algarvensis and its symbiotic community (SI Appendix, Tables S1 and S2 and Datasets S1 and S2) using different methods for both the metaproteomic and the metabolomic analyses to overcome the intrinsic biases inherent in a single detection method (SI Appendix, SI Text). For host proteins, sequences from related annelids enabled the cross-species identification of 530 O. algarvensis proteins, thus providing insight into the metabolism of a marine oligochaete, a group of annelid worms for which no genomic data are available. For symbiont proteins, the published O. algarvensis symbiont metagenome, which contains only sequences assigned to specific symbionts through binning analyses (9), led to the identification of 1,586 proteins. The addition of unassigned sequences from the unbinned O. algarvensis symbiont metagenome allowed us to identify a total of 2,265 symbiont proteins, a 43% increase compared with the published metagenome alone. Because of the lack of metagenomic information for the spirochete, no proteins were found that could be assigned unambiguously to the spirochete symbiont of O. algarvensis (9).

To improve coverage of the metaproteome further, we developed a method using density-gradient centrifugation for physical separation of the O. algarvensis symbionts from each other and from host tissues (SI Appendix, SI Text and Fig. S1). This method greatly enhanced the number of identified symbiont proteins, particularly for those present in lower abundances (Fig. 1 and SI Appendix, Table S1). An additional advantage of symbiont enrichments was that we were able to assign proteins from the unbinned metagenomic sequences to a specific symbiont if they were detected in high abundances in enrichment fractions of the given symbiont (SI Appendix, SI Text). This proteomics-based binning allowed us to assign 544 previously unassigned proteins to a specific symbiont, thus significantly extending our understanding of the symbionts’ metabolism (SI Appendix, Table S3 and Dataset S3).

Energy Sources for the O. algarvensis Symbiosis. One of the major unresolved questions in the O. algarvensis symbiosis is the identity of the sources of energy from the environment that fuel the association. Earlier studies found that reduced sulfur compounds are supplied internally as an energy source to the aerobic sulfur-oxidizing γ-symbionts by the anaerobic sulfate-reducing δ-symbionts. In return, the δ-symbionts are supplied with oxidized sulfur compounds as electron acceptors (6, 9). Our metapro-
The CO₂/CO couple has a very negative redox potential, −520 mV (18), making CO an excellent electron donor whose electrons can be transferred to a variety of terminal electron acceptors such as oxygen, nitrate, elemental sulfur, and sulfate (12, 13, 19). Therefore CO could be used as an energy source by the O. algarvensis symbionts under all redox conditions as the worm
shuttles between sediment layers. In the reduced sediment layers, the δ-symbionts could use sulfate for the anaerobic oxidation of CO, thereby producing reduced sulfur compounds for the γ-symbionts; in the oxic and suboxic sediment layers, the γ3-symbiont could oxidize CO with nitrate as a terminal electron acceptor (SI Appendix, SI Text, SI Results and Discussion).

**Hydrogen may be used by the sulfate-reducing symbionts.** Our metaproteomic analyses revealed that hydrogen also may play an important role as an energy source in the *O. algarvensis* symbiosis, based on the abundant expression of periplasmic uptake [NiFeSe] hydrogenases in both δ-symbionts (61: SP088; δ4: SP089). These [NiFeSe] hydrogenases have high affinities for hydrogen (20), consistent with the low hydrogen concentrations reported for oligotrophic sediments (<10 nM) (21) and marine sediments in general (<60 nM) (22). Therefore we were surprised to measure unusually high concentrations of hydrogen, 438–2,147 nM, in the sediment pore waters at the *O. algarvensis* collection site (SI Appendix, Fig. S5). These high concentrations could be a result of biological H2 production by anaerobic CO oxidizers and are consistent with the elevated CO concentrations at the collection site. The hydrogen concentrations in the worms’ habitat are much higher than those needed by common hydroxidizing microorganisms for growth (23), indicating that the δ-symbionts could easily use the hydrogen present in the Elba sediment as an energy source.

The use of hydrogen as an energy source by chemoautotrophic sulfur-oxidizing symbions was shown recently for deep-sea *Bathylniodius* mussels from hydrothermal vents (24). Our study indicates that hydrogen also might play a role as an energy source in shallow-water chemosynthetic symbioses. As with CO, the use of externally supplied hydrogen might be another adaptation of the *O. algarvensis* symbiosis to life in the sulfide-depleted sediments of Elba.

**Highly abundant uptake transporters for organic substrates in the δ-symbionts.** The sulfate-reducing δ-symbionts expressed extremely high numbers and quantities of high-affinity uptake transport-related proteins, which enable them to take up organic substrates at very low concentrations (Datasets S2 and S4). In the δ1-symbiont, 89–116 transport proteins were detected per sample, corresponding to an average of 29% of all identified δ1-symbiont proteins. In terms of abundance, the δ1-symbiont transport proteins represent more than 38% of the total δ-symbiont protein (SI Appendix, Table S4). To our knowledge, higher abundances of these types of transporters have been found only in the metaproteome of the γ-proteobacterium *Pelagibacter ubique* (SAR11) from the Sargasso Sea during extreme low-nutrient conditions (SI Appendix, Table S4) (25).

Most of the identified transport proteins in the δ1-symbionts were periplasmic-binding proteins of high-affinity ATP-binding cassette (ABC)- or tripartite ATP-independent periplasmic (TRAP)-type transporters, which actively transport substrates against a large concentration gradient while using energy in the form of ATP or an ion gradient (26, 27). The great majority of the detected δ1-symbiont transport proteins are used for the uptake of a variety of substrates such as amino acids, peptides, di- and tricarboxylates, sugars, polyamines, and phosphonates, with amino acid and peptide transporters being the most dominant ones (Dataset S4). The abundance of transport-related proteins in the δ-symbionts suggests that these symbionts use organic substrates not only as an energy source but also as a source for preformed building blocks, thus saving resources by not having to synthesize these metabolic precursors de novo.

The organic substrates used by the δ-symbionts could be supplied internally from within the worms or externally from the environment. Our metabolomic analyses of whole worms revealed considerable amounts of dicarboxylates and some amino acids (in the low millimolar range), making an internal source of the organic substrates possible (SI Appendix, Fig. S7 and Table S2). However, the relatively high concentrations of these substrates are not consistent with the expression of energy-consuming high-affinity transporters by the δ-symbionts. In cultured bacteria (28–30) as well as in environmental communities (25, 31), ABC/TRAP transporters are induced at low substrate concentrations, and less energy-consuming transporters are used under nutrient-rich conditions. Most likely the metabolites that we measured in homogenized worms are not easily accessible to the δ-symbionts in situ, because the metabolites are enclosed in host or symbiont cells.

To examine if organic substrates are supplied externally from the *O. algarvensis* environment, we analyzed sediment pore waters from the worm’s collection site with GC-MS for the presence of a large range of di- and tricarboxylates, amino acids, and sugars. None of these metabolites was measurable with detection limits at about 10 nM (SI Appendix, Fig. S8). Such oligotrophic conditions are consistent with the high expression of ABC/TRAP transporters that have extremely high affinities for substrates at concentrations far below the detection limits of our method (32, 33). The worm’s cuticle is permeable for small, negatively charged compounds as well as substrates up to 70 kDa (5); thus the δ-symbionts would have access to both small organic compounds such as di- and tricarboxylates and larger organic substrates such as sugars and polyamines from the environment.

The expression of transporters for a very broad range of substrates would allow the δ-symbionts to respond quickly to and take up many different substrates that could be consistently present at low concentrations in their environment or that could fluctuate over time and space as the worm migrates through the sediment.

Regardless of whether the organic substrates come from the environment or internally from within the symbiosis, the high abundances of high-affinity uptake transporters in the δ-symbionts indicate that the symbionts experience nutrient limitation, forcing them to dedicate a major part of their resources to the acquisition of substrates. Despite their endosymbiotic location, the lifestyle of these bacteria thus appears to resemble most closely that of planktonic SAR11 bacteria from low-nutrient extremes in the Sargasso Sea (25).

**Recycling and Waste Management.** Given the extremely low concentrations of nutrients in the *O. algarvensis* habitat, the conservation of substrates and energy should be highly advantageous for the symbiosis. Our metaproteomic and metabolomic analyses revealed several pathways that could enable the symbionts to recycle waste products of their hosts and conserve energy.

**Proposed pathways for the recycling of host fermentative waste in multiple symbionts.** Cross-species identification of host proteins enabled us to gain insight into the metabolism of *O. algarvensis*. Our analyses revealed that, when living in deeper anoxic sediment layers, *O. algarvensis* expressed proteins for an anaerobic metabolism that produces large amounts of acetate, propionate, malate, and succinate as fermentative waste products (SI Appendix, SI Text and Fig. S6 and Dataset S2) (34, 35). Correspondingly, we detected considerable amounts (1–8 mM) of malate, succinate, and acetate in the worm metabolome (SI Appendix, Fig. S7 and Table S2). Aquatic invertebrates without symbionts must excrete these fermentative waste products to keep their internal pH stable, thereby losing large amounts of energy-rich organic compounds. In *O. algarvensis*, the ability of the sulfate-reducing δ-symbionts to use their host’s fermentative waste as substrates recycles and preserves considerable amounts of energy and organic carbon within the symbiotic system (SI Appendix, SI Text).

The dominant γ1-symbiont, previously assumed to fix only carbon autotrophically, also may function heterotrophically by assimilating acetate, propionate, succinate, and malate, thus also contributing to host waste recycling. We detected abundantly...
expressed enzymes for an almost complete 3-hydroxypropionate bi-cycle (3-HPB) in the γ1-symbiont (Fig. 3 and SI Appendix, Fig. S3B and Dataset S2). The 3-HPB is used for autotrophic CO₂ fixation in Chloroflexus aurantiacus, a filamentous anoxygenic phototroph (36), but parts of the 3-HPB pathway also can be used for the heterotrophic assimilation of acetate, propionate, succinate, and malate (37).

In retrospect, it is clear why the 3-HPB pathway was not discovered in the metagenomic analyses of the O. algarvensis symbionts: Many of its genes occurred on sequence fragments that could not be assigned to a specific symbiont and therefore were not included in the annotation analyses (9). Here, we used our proteomics-based binning method described above to assign abundantly expressed 3-HPB enzymes encoded on unassigned metagenomic fragments to the γ1-symbiont (SI Appendix, SI Text, Materials and Methods and Dataset S3). This method enabled us to identify nearly all enzymes required for the complete 3-HPB, with the exception of two diagnostic enzymes of the 3-HPB, malonyl-CoA reductase and propionyl-CoA synthase, that were missing in both the metagenome and the metaproteome (Fig. 3).

To understand better how the 3-HPB might function in the symbionts, we performed enzyme assays with extracts from whole worms and enriched γ1-symbionts. Activities of all 3-HPB enzymes were detected, except for the two diagnostic enzymes that also were absent from the metaproteome (SI Appendix, Table S5). We therefore propose a modified incomplete 3-HPB as shown in Fig. 3, which the γ1-symbiont could use to assimilate the host’s fermentative waste products acetate, propionate, succinate, and malate. The abundant expression of the modified 3-HPB suggests that it plays an important role in the central carbon metabolism of the γ1-symbionts. The net fixation of CO₂ is unlikely because of the absence of the two diagnostic enzymes and the low activities of the carboxylases involved in the 3-HPB (SI Appendix, Table S5). The pathway could also be linked to the synthesis and/or mobilization of the storage compound polyhydroxyalkanoate (PHA). A putative PHA synthase (2004222379) and a phasin protein (PHA granule protein, 6frame_RASTannot_14528) are highly expressed in the γ1-symbiont metaproteome, showing the importance of PHA synthesis for this symbiont. Under anaerobic conditions, PHA synthesis not only would produce a valu-

**Fig. 3.** Modified version of the 3-HPB in the γ1-symbiont. Reactions not needed for the assimilation of propionate and acetate are shown in the gray box; reaction 1 also can play a role in fatty acid metabolism. (1) Acetyl-CoA carboxylase (2004223475); (2) malonyl-CoA reductase; (3) propionyl-CoA synthase; (4) propionyl-CoA carboxylase (2004223080); (5) methylmalonyl-CoA epimerase (RASTannot_91923); (6) methylmalonyl-CoA mutase (RASTannot_20798); (7) succinyl-CoA-(S)-malate-CoA transferase (RASTannot_529, RASTannot_48547); (8) succinate dehydrogenase (2004223104, 2004223105); (9) fumarate hydratase (2004223692); (10 a,b,c) (S)-malyl-CoA/β-methylmalyl-CoA/(S)-citramalyl-CoA (MMC) lyase (RASTannot_91504); (11) mesaconyl-C1-CoA hydratase (β-methylmalyl-CoA dehydratase) (2004222675); (12) mesaconyl-CoA C1-C4 CoA transferase (RASTannot_38616); (13) mesaconyl-C4-CoA hydratase (S)-citramalyl-CoA dehydratase (RASTannot_6738).
able storage compound but also would relieve the symbiont of superfluous reducing equivalents.

Intriguingly, one of the closest free-living relatives of the γ1-symbiont, *Allochromatium vinosum*, whose genome was sequenced recently, does not possess the genes needed for the 3-HPB or its modified version (http://genome.jgi-psf.org/allvi/allvi.home.html). The absence of these genes suggests that the genes for the 3-HPB pathway were gained through lateral transfer. Certainly, there is a strong selective advantage for this pathway in the γ1-symbionts. The γ1-symbionts are present in almost all gutless oligochaete species and therefore are assumed to be the ancient primary symbionts that first established a mutualistic relationship with the oligochaetes (5). The ability to recycle organic host waste would have been a considerable advantage during the early stages of the symbiosis, before the establishment of associations with other bacteria such as the heterotrophic sulfate-reducing symbionts.

**Uptake and recycling of nitrogenous compounds.** Because sources of nitrogen are extremely limited in the habitat of *O. algarvensis* (38), efficient strategies for dealing with nitrogen limitation have a selective advantage. Our metaproteomic and metabolomic analyses of the *O. algarvensis* association indicate two major strategies for dealing with nitrogen limitation: (i) the use of high-affinity systems for the uptake of nitrogenous compounds from the environment, and (ii) conservation of nitrogen within the symbiosis through recycling.

Environmental nitrogen is most likely assimilated by the symbionts using glutamine synthetases as well as high-affinity uptake transporters. The γ1-, γ2-, and δ1-symbionts abundantly expressed glutamine synthetases (Dataset S2). This enzyme assimilates ammonia into glutamine with high affinity at very low ammonia concentrations and is expressed in cultured organisms only under low-nitrogen conditions (39, 40). Uptake of organic compounds from the environment presumably is a further source of nitrogen, given the abundant expression of high-affinity amino acid- and peptide-uptake transporters in the δ1-symbiont that enable it to acquire nitrogen-containing substrates at extremely low concentrations.

The second proposed strategy of the *O. algarvensis* association for dealing with low nitrogen availability is the internal recycling of nitrogenous host osmolytes and waste products by the symbionts. In many invertebrates, these compounds are removed through excretory organs called “nephridia.” Gutless oligochaetes are the only known annelid worms without nephridia, and their absence suggests that their symbionts have taken over the role of waste and osmolyte management. Our metabolomic analyses revealed high concentrations of two nitrogenous osmolyte and waste compounds in *O. algarvensis*, glycine betaine and urea (SI Appendix, Table S2), with glycine betaine being the most abundant metabolite detected in NMR measurements (~60 mM) (SI Appendix, Fig. S7). Glycine betaine is a well-known osmolyte in all kingdoms of life (41) and most likely also serves this function in *O. algarvensis*. The relatively high amounts of urea in *O. algarvensis* are unusual, because this nitrogenous waste compound and osmolyte is not commonly found in aquatic animals (41). The *O. algarvensis* symbionts abundantly expressed proteins for glycine betaine and urea uptake and for the pathways required to use them as carbon and nitrogen sources (Fig. 2 and SI Appendix, SI Text).

**Energy Conservation with Proton-Translocating Pyrophosphatases.**

We propose several pathways for energy conservation in the *O. algarvensis* symbiosis. Both the γ-symbionts and the δ1-symbiont expressed pyrophosphate-dependent enzymes that could conserve energy in as yet undescribed modifications of classical metabolic pathways. Our analyses of published genomes indicate that these pathways may be common in sulfate reducers and chemoheterotrophic bacteria.

The key enzyme for the proposed energy conservation pathways is a membrane-bound proton-translocating pyrophosphatase (H+-PPase), which is abundantly expressed in both the γ1- and the δ1-symbionts (SI Appendix, SI Text). H+-PPases are widespread in all three domains of life. Despite their pervasiveness, remarkably little is known about the metabolic pathways in which they are used (42). H+-PPases are proton pumps that use the hydrolysis of inorganic pyrophosphate (PPI) instead of ATP to generate a proton-motive force through the translocation of protons across biological membranes (Fig. 4). They also can work reversibly as proton-translocating pyrophosphate synthases (H+-PPI synthase) and produce PPI using a proton-motive force (42).

**H+-PPase energy conservation in sulfate reducers.** Sulfate-reducing bacteria produce large amounts of PPI as a by-product of the first step of sulfate reduction (Fig. 4). This PPI must be removed immediately to pull the reaction in the direction of sulfate reduction (43). For most sulfate reducers the mechanism of PPI removal is unknown. In some, it occurs through a wasteful hydrolysis of PPI by a soluble inorganic pyrophosphatase (44). In others, the energy from PPI hydrolysis may be conserved as an H+-PPase (45), but to date this process has not been proven. Our metaproteomic analyses support the conclusion that the sulfate-reducing δ1-symbiont uses the H+-PPase to conserve energy from PPI, based on the abundant expression of a H+-PPase and the absence of a soluble pyrophosphatase. The stoichiometry of the H+-PPase yields one ATP molecule per hydrolysis of three PPI molecules (46), providing the δ1-symbiont with a considerable energy gain of one additional ATP per three molecules of sulfate reduced.

Other sources of PPI besides sulfate reduction also appear to play an important role in the metabolism of the δ1-symbiont. In addition to expressing PPI-producing enzymes found in all organisms such as aminoacyl-tRNA synthetases and RNA and DNA polymerases, the δ1-symbiont abundantly expressed at
least two other PPI-producing enzymes: the acetate-CoA ligase (2004210485) and the propionate-CoA ligase (2004210481). Therefore, based on the abundant expression of numerous PPI-producing enzymes in the δ1-symbiont, we postulate that H+\textsuperscript{-}-PPase plays a key role in energy conservation in its metabolism (Fig. 4 and SI Appendix, Fig. S3A).

To examine how widespread H+\textsuperscript{-}-PPases are in sulfate reducers, we analyzed the genomes of sulfate reducers available in the databases. These analyses revealed H+\textsuperscript{-}-PPases in several sulfate reducers from two bacterial divisions, Desulfatibaculum alkenivorans AK-01 and Desulfofococcus oleovorans Hxd3 from the Deltaproteobacteria, and Candidatus Desulfonifrids audiavator MP104C and Desulfooctomodaclus reducans MI-1 from the division Clostridia. This finding suggests that the use of H+\textsuperscript{-}-PPases for energy conservation may be widely distributed among phylogenetically diverse sulfate-reducing bacteria.

Energy-efficient PPI-dependent pathways in sulfur oxidizers. We propose that the γ-symbionts use novel energy-saving modifications of the Calvin cycle, glycolysis, and gluconeogenesis pathways. The key enzymes for the proposed modifications are the H+\textsuperscript{-}-PPase and a closely coupled PPI-dependent 6-phosphofructokinase (PPI-PFK). We show that these enzymes could save as much as 30% of the energy used by the ATP-dependent pathways and that this energy-saving pathway may be widespread in chemoautotrophic bacteria.

Metagenomic analyses of the O. algarvensis consortium showed that the γ1-symbiont lacks two key enzymes of the classical Calvin cycle, fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase (the γ3-symbiont lacks only the latter) (Fig. 5C). Interestingly, the chemosynthetic symbionts of the hydrothermal vent tubeworm Riftia pachyptila and the vesicomyid clams Calyptogena okutanii also lack the genes for these two enzymes, even though all of them fix CO\textsubscript{2} via the Calvin cycle (47–49). Newton et al. (48) hypothesized that a PPI-PFK might replace fructose-1,6-bisphosphatase for the C. magnifica symbiont, but no enzyme was found that could replace sedoheptulose-1,7-bisphosphatase. Therefore it remained unclear how the Calvin cycle could function in these chemosynthetic symbionts.

We found that both γ-symbionts of O. algarvensis possess a gene for a PPI-PFK that is highly similar to that of the methanogenic Methanobacterium capsulatus: amino acid identities were 71% for γ1 and 69% for γ3. The M. capsulatus PPI-PFK catalyzes three reactions: (i) the reversible, phosphate-dependent transformation of fructose-1,6-bisphosphate to fructose-6-phosphate and PPI; (ii) the reversible, phosphate-dependent transformation of sedoheptulose-1,7-bisphosphate to sedoheptulose-7-phosphate and PPI; and (iii) the PPI-dependent phosphorylation of ribulose-5-phosphate to ribulose-1,5-bisphosphate (50). Thus, PPI-PFK can replace the enzymes involved in these three reactions (fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, and phosphoribulokinase) (Fig. 5B and C and SI Appendix, SI Text). The PPI-PFK was abundantly expressed in the γ1-symbiont (the low coverage of the γ3-symbiont proteome might explain why it was not detected in this symbiont). We propose that in the O. algarvensis γ-symbionts, and possibly in other chemosynthetic bacteria (see below), the PPI-PFK has multiple functions in the Calvin Cycle, glycolysis and gluconeogenesis, and that this leads to considerable energy savings as described below (Fig. 5A and B).

In the classical Calvin cycle, the reactions catalyzed by fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase produce phosphate ions that cannot be used for energy gain. In contrast, if PPI-PFK replaces these enzymes, both reactions produce energy-rich pyrophosphates. Interestingly, in the genomes of both γ-symbionts, the genes for PPI-PFK are located in the immediate neighborhood of H+\textsuperscript{-}-PPases, indicating a close metabolic relationship between these two enzymes and their cotranscription (Fig. 5D), as shown for M. capsulatus, in which these genes also occur (Fig. 5D) (50). We propose that the pyrophosphate produced by the PPI-PFK in the Calvin cycle is used to conserve energy via the proton-motive force generated by the H+\textsuperscript{-}-PPase (Fig. 5B). This metabolic coupling between the PPI-PFK and H+\textsuperscript{-}-PPase would lead to energy savings of at least 9.25% (1/7 fewer molecules of ATP per six molecules of fixed CO\textsubscript{2} in comparison with the classical Calvin cycle, in which 18 molecules of ATP are used for the fixation of six molecules of CO\textsubscript{2}). An even higher energy gain (31.5%) is possible if PPI-PFK also replaces ATP-dependent phosphoribulokinase in the last step of the Calvin cycle: The conversion of ribulose-5-phosphate to ribulose-1,5-bisphosphate could be energized with PPI from the two other Calvin-cycle reactions and/or the H+\textsuperscript{-}-PPase working in PPI synthesis direction, so that a total of 57/2 molecules of ATP (31.5%) would be saved per six molecules of CO\textsubscript{2} fixed.

In addition to their proposed role in the Calvin cycle, we hypothesize that the PPI-PFK and H+\textsuperscript{-}-PPase also provide considerable energy savings in glycolysis and gluconeogenesis through several additional enzymes (SI Appendix, SI Text). Thus we conclude that PPI-PFK and H+\textsuperscript{-}-PPase might play a key role in energy conservation in the γ1-symbiont and most likely also in the γ3-symbiont.

Widespread occurrence of colocalized H+\textsuperscript{-}-PPase/PPI-PFK genes in chemosynthetic bacteria. To examine if other microorganisms also use the PPI-PFK and H+\textsuperscript{-}-PPase for the pathways we propose above, we analyzed all bacterial (1,354) and archaeal (58) genomes available in the National Center for Biotechnology Information genomic database on January 29, 2009 (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). We discovered colocalized H+\textsuperscript{-}-PPase/PPI-PFK genes, indicating close metabolic coupling and cotranscription, in the chemosynthetic sulfur-oxidizing symbionts of C. magnifica and C. okutanii as well as in eight free-living bacterial species (Gamma- and Betaproteobacteria and Thermotogae), all of which possess ribulose-1,5-bisphosphate carboxylase/oxygenase genes for autotrophic CO\textsubscript{2} fixation (Fig. 5D). This broad distribution of colocalized H+\textsuperscript{-}-PPase/PPI-PFK genes in bacteria for which genomes are available suggests that H+\textsuperscript{-}-PPase/PPI-PFK–dependent pathways for energy conservation are widespread in both symbiotic and free-living chemosynthetic bacteria.

The discovery of these pathways in chemosynthetic bacteria is particularly interesting in light of evidence that H+\textsuperscript{-}-PPases may have an ancient origin (42). H+\textsuperscript{-}-PPase is the simplest known primary proton pump. It is the only known alternative to ATP synthases for the production of energy-rich phosphoanhydride bonds and is the only primary pump that is preserved in all three domains of life (42, 51). Given mounting evidence that the earliest forms of life were chemosynthetic bacteria (52), the apparent pervasiveness of energy-conserving H+\textsuperscript{-}-PPase pathways in chemosynthetic bacteria adds further weight to the hypothesis that PPI-PFK preceded ATP as the central energy carrier in the early evolution of life (51, 53, 54).

Conclusions

Our metaproteomic and metabolomic analyses of the O. algarvensis symbiosys provide strong indirect evidence for a number of unexpected and some as yet undescribed metabolic pathways and strategies that were not identified in the metagenomic analysis of the symbiotic consortium (9). We gained further functional insights by using proteomics-based binning. This method allowed us to include an additional 9 Mb of sequences in our analyses that could not be mined for genomic information by Woyke et al. (9) because their lengths were too short to enable a clear assignment to a specific symbiont.

One of the key questions in the metagenomic analyses of complex symbiotic consortia, including those of the human gut, is why there is so much functional redundancy (55, 56). The selective advantage for O. algarvensis of harboring two sulfur-oxi-
Fig. 5. Comparison of the classical Calvin cycle with a proposed version that is more energy efficient. (A) The textbook version of the Calvin cycle. (B) The more energy-efficient version of the Calvin cycle in the γ-symbionts through the use of PPI-dependent trifunctional 6-phosphofructokinase/sedoheptulose-1,7-bisphosphatase/phosphoribulokinase (green) and a proton-translocating pyrophosphatase/proton-translocating pyrophosphate synthase (H⁺-PPase/H⁺-PPi synthase) (red). The main differences between the cycles are highlighted in yellow. CM, cell membrane; DHAP, dihydroxyacetone phosphate; GAP, D-glyceraldehyde-3-phosphate; PPi, inorganic pyrophosphate; Sh-7-P, D-sedoheptulose-7-phosphate. (C) Overview of genes that are replaced by the trifunctional PPI-dependent enzyme in different organisms. (D) Colocalized H⁺-PPase/PPI-PFK genes in the γ-symbionts and other symbiotic and free-living bacteria.
dizing γ-symbionts with apparent functional redundancy was not clear. Our study shows that the only physiological traits shared by these two symbionts are their common use of reduced sulfur and carbon fixation via the Calvin cycle. Otherwise, they show very marked differences in their use of additional energy and carbon sources as well as electron acceptors. The γ3-symbionts may use CO and the host-derived osmolyte glycine betaine as additional energy and carbon sources, whereas the γ1-symbionts may use fermentative waste products from their hosts as additional carbon sources. Furthermore, the γ1-symbionts appear to rely heavily on storage compounds such as sulfur and polyhydroxyalkanoates, but storage compounds do not appear to play a dominant role in the metabolism of the γ3-symbionts. Resource partitioning also is visible in the differences in the electron acceptors used by the two symbionts. The γ1-symbionts may depend predominantly on oxygen for their respiration, but the γ3-symbionts apparently are not able to use this electron acceptor and instead use the energetically less favorable nitrate (SI Appendix, SI Text). Our metaproteomic analyses thus indicate functional differences in key metabolic pathways for chemosynthesis in the metabolism of these two symbionts, despite their genetic similarities. This theme appears to be a common one in microbial communities, because several recent proteomic and metaproteomic studies have shown that ecological differences between microorganisms with similar genomes are the result of major differences in their protein expression (57–59).

Although resource partitioning provides the association versatility and the ability to harvest a wide spectrum of energy and carbon sources, in one key aspect all four symbionts appear to share a remarkably similar metabolic strategy. They all express proteins involved in highly efficient pathways for the uptake, recycling, and conservation of energy and carbon sources. These pathways include (i) multiple strategies for the recycling of host waste products; (ii) the possible use of inorganic energy sources, such as hydrogen and CO, in addition to reduced sulfur compounds; (iii) the extremely abundant expression of high-affinity uptake transporters that would allow the uptake of a wide range of substrates at very low concentrations; and (iv) as yet undescribed energy-efficient steps in the pathways for sulfate reduction and CO2 fixation. Given the oligotrophic, nutrient-poor nature of the worm’s environment in which organic compounds were below detection limits and reduced sulfur compounds were barely detectable, the selective pressure for metabolic pathways that maximize energy and carbon acquisition and conservation appears to have been very strong in shaping these symbioses.

Materials and Methods

Sample Collection and Symbiont Enrichment. Worms were removed from the sediment via decantation and were frozen immediately, or symbionts were enriched via isopycnic centrifugation using a Histodenz-based (Sigma) density gradient before freezing (SI Appendix, SI Text). Symbiont abundance and composition in density-gradient fractions were analyzed with catalyzed reporter deposition-FISH using symbiont-specific probes (SI Appendix, SI Text, Fig. S1, and Table S6). Density-gradient fractions in which specific symbionts were enriched were chosen for subsequent analyses.

Protein Identification and Proteome Analyses. 1D PAGE followed by liquid chromatography (1D-PAGE-LC) and 2D-LC were used for protein and peptide separation as described previously (60, 61), with slight modifications (SI Appendix, SI Text). MS spectra and MS/MS spectra were acquired with a hybrid linear ion trap-Orbitrap (Thermo Fischer Scientific) as described previously (60, 62), with minor modifications (SI Appendix, SI Text). All MS/MS spectra were searched against two protein sequence databases composed of the symbiont metagenomes and the genomes of related organisms using the search algorithm (see SI Appendix, SI Text for details). For protein identification only peptides identified with high mass accuracy (maximum ±10 ppm difference between calculated and observed mass) were considered, and at least two different peptides were required to identify a protein. False-discovery rates were estimated with searches against a target-decoy database, as described previously (63, 64), and were determined to be between 0–3.27% (SI Appendix, SI Text and Table S7). For relative quantitation of proteins, normalized spectral abundance factor values were calculated for each sample according to the method of Flores et al. (65). All identified proteins and their relative abundance in different samples are shown in Datasets S1 and S2. Protein databases, peptide and protein identifications, and all MS/MS spectra are available from http://compmio.ornl.gov/lolavius_algarvensis_symbiont_metaproteome/.

Proteomics-Based Binning. Proteins encoded on metagenome fragments that were not assigned previously to a specific symbiont were assigned tentatively (binned) to a specific symbiont if they were detected repeatedly in higher abundances in enrichments of only one specific symbiont (SI Appendix, SI Text and Dataset S3). To validate this approach and to calculate a false-assignment rate, we also did proteomics binning with the proteins that already had been assigned to a specific symbiont in the metagenomic study (SI Appendix, SI Text and Table S3).

Enzyme Tests. Enzymatic activities were determined in cell extracts from whole worms or from enriched symbionts (SI Appendix, SI Text and Table S5). Detailed methods for all enzyme activity assays are provided in the SI Appendix, SI Text.

Measurement of Hydrogen and CO Concentrations in the O. algarvensis Habitat. Seawater and pore water samples from a sediment depth of 25 cm were collected by research divers using a stainless steel needle and capped syringes. A total of nine sites within an area of ~100 m2 at the O. algarvensis collection site were sampled. Hydrogen and CO concentrations were measured the same day using an RGA3 reduction gas analyzer (Trace Analytical Inc.) (SI Appendix, SI Text).

Metabolite Identification and Quantification in Whole Worms and Pore Water. Whole worms were extracted using ice-cold ethanol-based solvent mixture and ultrasonication. Metabolites were measured with GC-MS, LC-MS, and 1H-NMR as described previously (66), with minor modifications (SI Appendix, SI Text). Detected metabolites are shown in SI Appendix, Table S2. Relative quantification of metabolites was performed on the basis of complete spectrum/chromatogram intensities (SI Appendix, SI Text).

Pore water was sampled at different sediment depths in the O. algarvensis habitat by scuba divers with Rhizon MOM 10-cm soil water samplers (Rhizosphere Research Products, Wageningen, The Netherlands) and was measured using GC-MS as described in Liebeke et al. (66).

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