Genetic evidence for two carbon fixation pathways in symbiotic and free-living bacteria: The Calvin-Bassham cycle and the reverse tricarboxylic acid cycle

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Abstract: Sulfide-oxidizing bacterial endosymbionts of marine tubeworms such as *Riftia pachyptila* are able to fix carbon via both the reverse tricarboxylic acid (rTCA) and the Calvin-Benson-Bassham (CBB) cycles. Until recently, this co-existence of two carbon fixation pathways was thought to be unique to tubeworm symbionts. However, it was not clear if these two pathways were encoded in the same symbiont individual, or if two symbiont populations, each with one of the pathways, co-existed within tubeworms. We show here that both the CBB and rTCA pathways are likely encoded in the genome of the sulfur-oxidizing symbiont of the tubeworm *Escarpia laminata* from deep-sea asphalt volcanoes in the Gulf of Mexico. Furthermore, with comparative genomics, we show that *Thioflavicoccus mobilis,* a cultured, free-living gammaproteobacterial sulfur oxidizer, also possesses the genes for both carbon fixation pathways. Finally, we provide genomic and transcriptomic data suggesting a potential electron flow towards the rTCA cycle carboxylase 2-oxoglutarate:ferredoxin oxidoreductase, via a rare variant of NADH dehydrogenase/heterodisulfide reductase. This electron bifurcating complex, together with NAD(P)+ transhydrogenase and Na+ translocating Rnf membrane complexes may drive the rTCA cycle in both the symbiotic and the free-living sulfur oxidizer.

Importance: Primary production on Earth is dependent on autotrophic carbon fixation, which leads to the incorporation of carbon dioxide into biomass. Multiple metabolic pathways have been described for autotrophic carbon fixation, but all known autotrophic organisms were assumed to have the genes for only one of these pathways. The only possible exception to this paradigm was the sulfur-oxidizing symbiont of tubeworms, but this could have been an artifact of strain diversity within the symbiont population. Our results revealed that two pathways for carbon fixation co-occur in both symbiotic and free-living bacteria. Our finding of a cultivable bacterium with two carbon fixation pathways in its genome opens the possibility to study the potential benefits of having two pathways and the interplay between these cycles. This will deepen our understanding of carbon fixation pathways and could provide new avenues for optimizing carbon fixation in biotechnological applications.

37 **Observation**

38 Primary production by autotrophic organisms drives the global carbon cycle. Currently, seven
39 naturally occurring pathways for inorganic carbon fixation are known in autotrophic organisms
40 (1). The dominant carbon fixation pathway used by plants, algae, and many bacteria is the
41 Calvin-Benson-Bassham (CBB) cycle. The six more efficient, alternative pathways are limited to
42 autotrophic microbes that live in reducing habitats, due to the oxygen sensitivity of these
43 alternative pathways (2, 3). All investigated autotrophic organisms have only one carbon fixation
44 pathway. The only known exception are intracellular, sulfur-oxidizing symbionts of marine
45 tubeworms such as *Riftia, Escarpia, Tevnia and Lamellibrachia* (the symbionts of these hosts are
46 closely related to each other*)*. These symbionts employ both the oxygen-tolerant CBB cycle and
47 the oxygen-sensitive reverse tricarboxylic acid (rTCA) cycle. However, it was not clear if both
48 pathways were encoded in the genome of a single symbiont individual, or if multiple strains with
49 very similar genomes that encoded either one or the other pathway co-occurred within
50 tubeworms (4–8). The CBB cycle in these symbionts is potentially more energy efficient than the
51 classical version of the CBB cycle based on the replacement of the fructose-1,6-bisphosphatase
52 with a pyrophosphate dependent enzyme (9, 10). In addition, it is likely that the interplay
53 between the CBB and rTCA cycle under fluctuating redox conditions contributes to the high
54 efficiency of carbon fixation in these symbioses (4, 5, 11), and consequently to the extremely high
55 growth rates of tubeworms, which grow faster than any other known invertebrate (12).

56 Given that tubeworm symbionts could not yet be cultivated, it was not possible to investigate the
57 co-occurrence of two carbon fixation cycles in detail to better understand the biochemical and
58 physiological mechanisms that enable the interplay between these two pathways. In this study,
59 we sequenced the genome and transcriptome of the symbiont from the tubeworm *Escarpia*
60 *laminata* and compared its genome to those of other tubeworm symbionts and free-living
61 microbes. These comparisons led us to discover the presence of co-occurring CBB and rTCA
62 cycles in the genome of a cultured bacterium.

63 **Co-occurrence of rTCA cycle genes with RuBisCo in symbiotic and free-living**
64 **gammaproteobacteria**. Genes for enzymes that are specific to the rTCA pathway, that is the ATP
65 citrate lyase (*aclAB* genes), 2-oxoglutarate:ferredoxin oxidoreductase (OGOR, *korABCD* genes),
66 and a putative fumarate reductase (*tfrAB* genes, homologs of genes encoding a thiol:fumarate
67 reductase from *Methanobacterium thermoautotrophicum*, (13)), were assumed to occur in only a
68 few symbiotic Gammaproteobacteria. We discovered, using comparative genomics, that these
69 rTCA cycle enzymes also occur in some Chromatiaceae, including the cultivated sulfur oxidizer
70 *Thioflacicoccus mobilis* and a gammaproteobacterial genome from an environmental
71 metagenome(14), (**Fig. 1**). The type II ATP citrate lyases of tubeworm symbionts and *T. mobilis*
72 were likely acquired via horizontal gene transfer from other bacterial clades (**Suppl. Fig. S1**), (6).
73 These gammaproteobacteria also encode either Form I or II RuBisCO, or both (**Suppl. Note 1**).

74 **Presence of the rTCA and the CBB pathways in the genome of a single bacterium:** Due to the
75 fragmented nature of the previously available genomes of tubeworm symbionts, past studies
76 could not determine whether the genes for both pathways are present in a single genome or if the
77 two pathways are distributed in a strain-specific manner, i.e. only one of the two pathways is
78 present in the genome of a single cell (3). Here, we provide two lines of evidence that both
79 pathways can co-occur in the genome of a single organism. First, sequencing coverage for the
80 genes of both pathways in the *E. laminata* symbiont was similar to that of single-copy marker
81 genes (**Suppl. Table 1**). Since genes that are strain specific are expected to have lower coverage
82 than the rest of the genome (15), the similar coverage of genes encoding the two pathways and
83 single-copy genes suggests that in the *E. laminata* symbiont both pathways are present in all cells.
84 Second, in the closed genome of the cultured *T. mobilis*, both the genes encoding the rTCA and
85 the CBB cycle co-occur, providing evidence that these genes co-exist in a single genome.

86 Our transcriptomic analyses of *E. laminata* tubeworm symbionts revealed high expression levels
87 of both the rTCA and the CBB cycle genes (Fig. 2). This observation is consistent with previous
88 proteomic analyses of the *Riftia* symbiont (4, 5). The high expression levels of genes from the

89 rTCA and the CBB cycle suggests that both pathways play an important metabolic role in these
90 symbionts. It is, however, not clear whether these cycles function simultaneously within single
91 symbiont cells, or are differentially expressed within the symbiont population (3).

92 **The rTCA gene clusters are conserved among the tubeworm symbionts and some**
93 **Chromatiaceae bacteria**. In the tubeworm symbionts, the cultivated *T. mobilis* and the
94 gammaproteobacterial genome from an environmental metagenome, there was a considerable
95 level of conservation of the rTCA gene clusters, at the sequence and synteny levels (**Fig. 2**). The
96 *aclAB* genes that encode the two subunits of the ATP citrate lyase were accompanied by those
97 that encode bidirectional TCA cycle enzymes, including *acn* (aconitase), *idh (*isocitrate
98 dehydrogenase), and *mdh* (malate dehydrogenase). The other rTCA specific genes *korABCD*
99 (four-subunit OGOR) and *tfrAB* (putative thiol:fumarate reductase), were also present in the
100 rTCA gene cluster. Similar to the ATP citrate lyase, the four-subunit OGOR, as well as the thiol:
101 fumarate reductase are very rare among Gammaproteobacteria, and were probably acquired via a
102 single horizontal gene transfer event from a distant bacterial clade (**Fig. 1**, **Suppl. Fig. S2 and**
103 **S3**). A dimeric OGOR (*korAB* genes), more common than the four-subunit enzyme among
104 gammaproteobacterial autotrophs, yet absent in *T. mobilis,* was located elsewhere in the genome
105 of the *E. laminata* symbiont. The *korAB* genes were co-localized with genes that encode
106 additional rTCA cycle enzymes (**Suppl. Note 2, Suppl. Fig. S4**).

107 An array of genes that encode several electron translocating complexes were integrated into the
108 rTCA cycle gene clusters. These complexes included an electron-bifurcating NADH
109 dehydrogenase/heterodisulfide reductase complex (*flxABCD-hdrABC* genes, **Suppl. Note 3**), a
110 NAD(P)+ transhydrogenase and Na+ translocating Rnf membrane complex (*pntAB* and
111 *rnfABCDGE* genes, **Suppl. Note 4**). Most interestingly, the conserved interspersing of the
112 *korABCD* and *tfrAB* genes with the *flxABCD-hdrABC* genes hints at the possibility that these
113 proteins form a complex that efficiently shuttles electrons directly to the OGOR and the thiol:
114 fumarate reductase (**Suppl. Figure S5**).

115 **Conclusions.** Until now, the only bacteria known to possess two carbon fixation pathways were
116 sulfur-oxidizing, tubeworm symbionts, which are currently not amenable to cultivation-based
117 studies. With the discovery of the co-existence of the CBB and rTCA cycle in the cultivable *T.*
118 *mobilis*, experimental studies are now feasible. Such studies would reveal if these pathways are
119 expressed under different physicochemical conditions, and potentially allow the biotechnological
120 optimization of efficiency and yield in production processes that rely on autotrophic carbon
121 fixers. To our knowledge, the use of organisms with multiple carbon fixation pathways has not
122 been used as a design principle for these applications.

123 **Methods: Comparative genomics and transcriptomics.** Publically available genomes from
124 NCBI and JGI-IMG collections, as well as de-novo assembled genomes of *Escarpia laminata*
125 symbionts (estimated completeness 99.5%), were used for genomic comparison (see
126 Supplementary Methods). To verify presence/absence of target gene homologs in sequenced
127 organisms we used NCBI's BLAST against the nucleotide collection and non-redundant protein
128 database (16). *E. laminata* symbiont genomes were used as a template for genome-centered
129 transcriptomics (sequences available under the BioProject accession number PRJNA471406).

130 **Phylogenetic and phylogenomic analyses**. Phylogenomic treeing was performed using scripts
131 available at phylogenomics-tools (DOI:10.5281/zenodo.46122). Twenty-three marker proteins
132 that are universally conserved across the bacterial domain were extracted from genomes using
133 the AMPHORA2 pipeline (17). Twenty-three single-copy markers were used for alignment with
134 MUSCLE (18). The marker alignments were concatenated into a single partitioned alignment,
135 and poorly aligned regions were removed. Functional protein sequences were aligned with

MAFFT (19). Maximum Likelihood trees were calculated with IQ-tree (20) and MEGA7 (21), using the best-fitting model.

Author Contributions

MRB, ND and MK conceived the study. MRB and MK analyzed the samples. MRB, ND and MK wrote the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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Figures

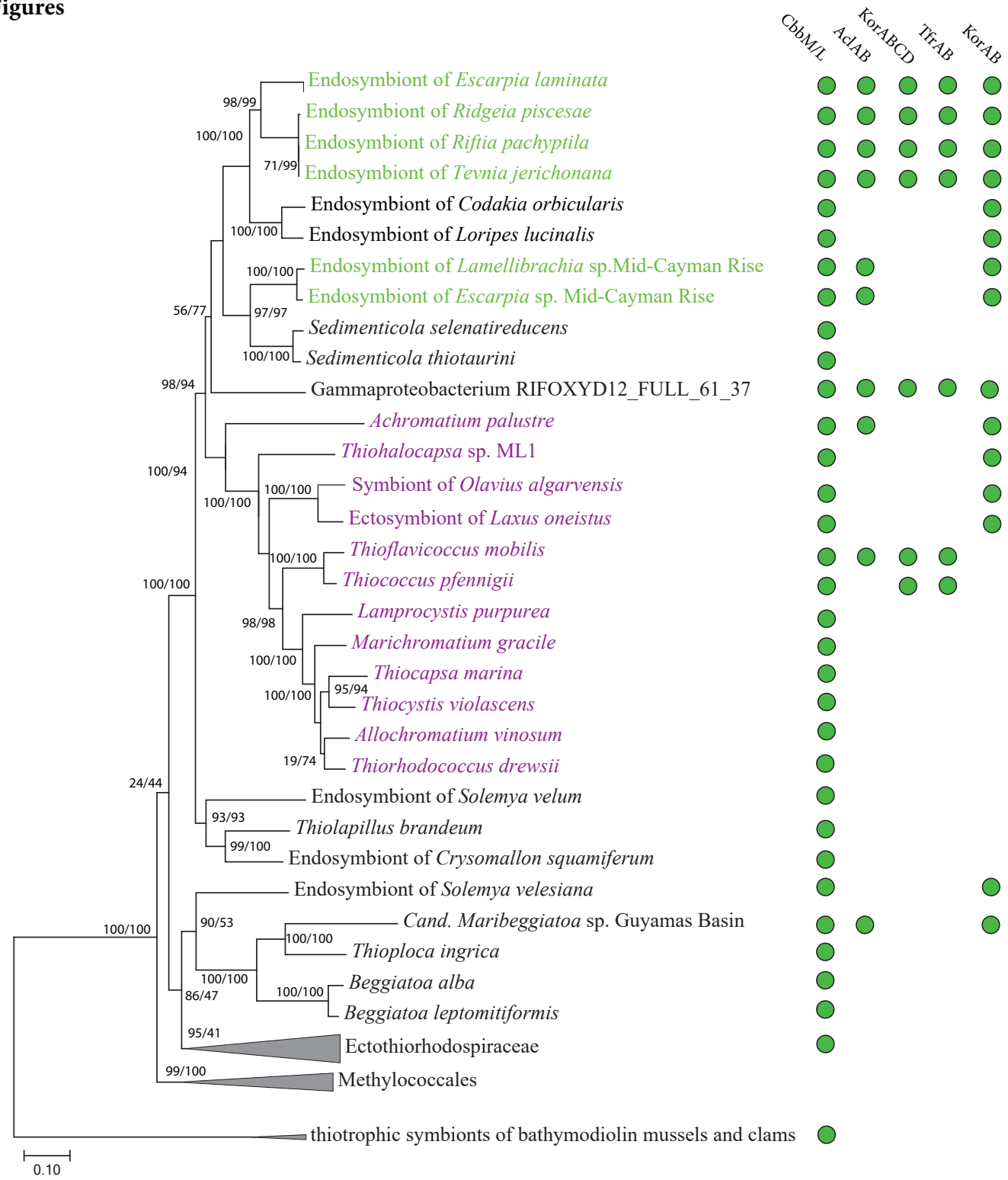

Figure 1: Phylogenomic tree showing occurrence of RuBisCO (CbbM/CbbL), ATP citrate lyase (AclAB), 4-subunit 2-oxoglutarate:ferredoxin oxidoreductase (KorABCD), putative thiol:fumarate reductase (TfrAB) and 2-subunit 2-oxoglutarate:ferredoxin oxidoreductase (KorAB) in the genomes of tubeworm symbionts (green), purple sulfur bacteria (purple) and other related bacteria (58 organisms total, alignment of 2526 amino-acid sites from 23 single-copy markers). The maximum likelihood tree was built with IQ-tree using the LG+R6 model of substitution (20). The tree is unrooted although outgroup "thiotrophic symbionts of bathymodiolin mussels and clams" is drawn at root. Branch labels are SH-aLRT support (%) / ultrafast bootstrap support (%). Accession numbers are provided in Supplementary Table 2.

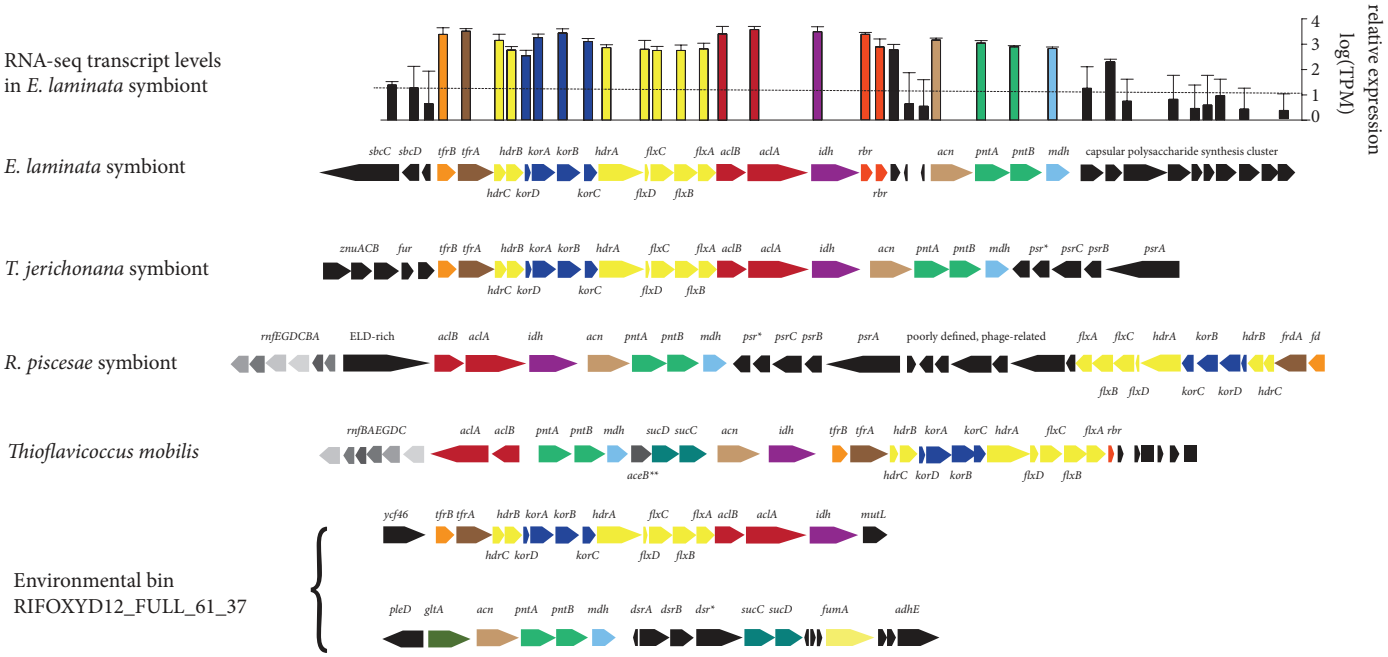

Figure 2: The rTCA cycle gene clusters in symbiotic and free-living bacteria, and the respective transcriptomic gene expression levels in the symbionts of *Escarpia laminata* tubeworm (*aclA*, log (TPM)=3.6; *korA*, log (TPM)=3.3; *hdrA*, log (TPM)=2.9; for comparison - *atpB*, log (TPM)=2.0); *cbbM*, log (TPM)=5.0. TPM, transcripts per kilobase million.