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

Maxim Rubin-Blum[1,2*], Nicole Dubilier[1,3], Manuel Kleiner[4*]

[1]Max-Planck Institute for Marine Microbiology, Celsiusstrasse 1, 28359 Bremen, Germany

[2]Israel Limnology and Oceanography Research, Tel Shikmona, 3108000, Haifa, Israel

[3]MARUM, University of Bremen, 28359 Bremen, Germany

[4]Department of Plant & Microbial Biology, North Carolina State University, Raleigh, NC, USA

*Corresponding authors

mrubin@ocean.org.il

manuel_kleiner@ncsu.edu

Abstract: Very few bacteria are able to fix carbon via both the reverse tricarboxylic acid (rTCA) and the Calvin-Benson-Bassham (CBB) cycles, such as symbiotic, sulfur-oxidizing bacteria that are the sole carbon source for the marine tubeworm *Riftia pachyptila*, the fastest growing invertebrate. To date, this co-existence of two carbon fixation pathways had not been found in a cultured bacterium and could thus not be studied in detail. Moreover, 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. With comparative genomics, we show that *Thioflavicoccus mobilis,* a cultured, free-living gammaproteobacterial sulfur oxidizer, possesses the genes for both carbon fixation pathways. Here, we also show 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. 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 improve the efficiency of 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 most autotrophic organisms were assumed to have the genes for only one of these pathways. 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 pathways. Additionally, this will allow the investigation of the unusual, and potentially very efficient mechanism of electron flow that could drive the rTCA cycle in these autotrophs. Such studies 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). Only a handful of autotrophic organisms have more than one carbon
44 fixation pathway: The sulfur-oxidizing symbionts of marine tubeworms such as *Riftia, Escarpia,*
45 *Tevnia and Lamellibrachia* (the symbionts of these hosts are closely related to each other) have
46 and express both the oxygen-tolerant CBB cycle and the oxygen-sensitive reverse tricarboxylic
47 acid (rTCA) cycle (4–8). Only a few free-living bacteria may have the genes for both cycles, such
48 as the large sulfur bacteria, *Beggiatoa* and *Thiomargarita* spp., in which all CBB cycle genes and
49 some rTCA cycle genes were found to co-exist in their genomes (9–11). The CBB cycle in the
50 symbionts and the large sulfur bacteria is potentially more energy efficient than the classical
51 version of the CBB cycle based on the replacement of the fructose-1,6-bisphosphatase with a
52 pyrophosphate dependent enzyme (9, 10, 12, 13). 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 tubeworm symbioses (4, 5, 14), and consequently to the extremely
55 high growth rates of tubeworms, which grow faster than any other known invertebrate (15).

56 Given that tubeworm symbionts and large sulfur bacteria could not yet be cultivated, it was not
57 possible to investigate the co-occurrence of two carbon fixation cycles in detail to better
58 understand the biochemical and physiological mechanisms that enable the interplay between
59 these two pathways. In this study, we sequenced the genome and transcriptome of the symbiont
60 from the tubeworm *Escarpia laminata* and compared its genome to those of other tubeworm
61 symbionts and free-living microbes. These comparisons led us to discover the presence of co-
62 occurring CBB and rTCA 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* (16)), 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 *Thioflavicoccus mobilis* and a gammaproteobacterial genome from an environmental
71 metagenome(17), (**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 (18), 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**). If this is the case, the carbon fixation efficiency of the
115 rTCA cycle would be most likely considerably higher than the canonical rTCA cycle.

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

124 **Methods: Comparative genomics and transcriptomics.** Publically available genomes from
125 NCBI and JGI-IMG collections, as well as de-novo assembled genomes of *Escarpia laminata*
126 symbionts (estimated completeness 99.5%), were used for genomic comparison (see
127 Supplementary Methods). To verify presence/absence of target gene homologs in sequenced
128 organisms we used NCBI's BLAST against the nucleotide collection and non-redundant protein
129 database (19). *E. laminata* symbiont genomes were used as a template for genome-centered
130 transcriptomics (sequences available under the BioProject accession number PRJNA471406).

131 **Phylogenetic and phylogenomic analyses**. Phylogenomic treeing was performed using scripts
132 available at phylogenomics-tools (DOI:10.5281/zenodo.46122). Twenty-three marker proteins
133 that are universally conserved across the bacterial domain were extracted from genomes using
134 the AMPHORA2 pipeline (20). Twenty-three single-copy markers were used for alignment with
135 MUSCLE (21). The marker alignments were concatenated into a single partitioned alignment,

136 and poorly aligned regions were removed. Functional protein sequences were aligned with
137 MAFFT (22). Maximum Likelihood trees were calculated with IQ-tree (23) and MEGA7 (24),
138 using the best-fitting model.

139 **Author Contributions**

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

142 **Acknowledgments**

143 The authors thank all individuals who helped during the R/V Meteor research cruise
144 M114, including onboard technical and scientific personnel, the captain and crew, and the ROV
145 MARUM-Quest team. We thank the Max Planck-Genome-Centre Cologne
146 (http://mpgc.mpipz.mpg.de/home/) for generating the metagenomic and the metatranscriptomic
147 data used in this study. We thank Julie Reveillaud for providing genomes of Mid-Cayman Rise
148 tubeworm symbionts, and Matthias Winkel, Marc Mußmann and Jake V. Bailey for helpful and
149 supportive comments on a bioRxiv preprint version of this manuscript. The Campeche Knoll
150 cruise was funded by the German Research Foundation (DFG – Deutsche
151 Forschungsgemeinschaft). We are grateful to the Mexican authorities for granting permission to
152 conduct this research in the southern Gulf of Mexico (permission of DGOPA: 02540/14 from 5
153 November 2014). This study was funded by the Max Planck Society, the MARUM DFG-Research
154 Center / Excellence Cluster "The Ocean in the Earth System" at the University of Bremen, an
155 ERC Advanced Grant (BathyBiome, 340535), a Gordon and Betty Moore Foundation Marine
156 Microbial Initiative Investigator Award to ND (Grant GBMF3811) and the NC State Chancellor's
157 Faculty Excellence Program Cluster on Microbiomes and Complex Microbial Communities
158 (MK).

159 **Conflict of interest**

160 The authors declare no conflict of interest.

161 **References**

162 1. Figueroa IA, Barnum TP, Somasekhar PY, Carlström CI, Engelbrektson AL, Coates JD.
163 2018. Metagenomics-guided analysis of microbial chemolithoautotrophic phosphite
164 oxidation yields evidence of a seventh natural CO_2 fixation pathway. Proc Natl Acad Sci
165 115:E92–E101.

166 2. Erb TJ. 2011. Carboxylases in natural and synthetic microbial pathways. Appl Environ
167 Microbiol 77:8466–8477.

168 3. Hügler M, Sievert SM. 2011. Beyond the Calvin cycle: Autotrophic carbon fxation in the
169 Ocean. Ann Rev Mar Sci 3:261–289.

170 4. Gardebrecht A, Markert S, Sievert SM, Felbeck H, Thürmer A, Albrecht D, Wollherr A,
171 Kabisch J, Le Bris N, Lehmann R, Daniel R, Liesegang H, Hecker M, Schweder T. 2012.
172 Physiological homogeneity among the endosymbionts of Riftia pachyptila and *Tevnia*
173 *jerichonana* revealed by proteogenomics. ISME J 6:766–76.

174 5. Markert S, Arndt C, Felbeck H, Becher D, Sievert SM, Hugler M, Albrecht D, Robidart J,
175 Bench S, Feldman RA, Hecker M, Schweder T. 2007. Physiological Proteomics of the
176 Uncultured Endosymbiont of *Riftia pachyptila*. Science 315:247–250.

177 6. Markert S, Gardebrecht A, Felbeck H, Sievert SM, Klose J, Becher D, Albrecht D, Thürmer

178 A, Daniel R, Kleiner M, Hecker M, Schweder T. 2011. Status quo in physiological
179 proteomics of the uncultured *Riftia pachyptila* endosymbiont. Proteomics 11:3106–3117.

180 7. Robidart JC, Bench SR, Feldman RA, Novoradovsky A, Podell SB, Gaasterland T, Allen
181 EE, Felbeck H. 2008. Metabolic versatility of the *Riftia pachyptila* endosymbiont revealed
182 through metagenomics. Environ Microbiol 10:727–737.

183 8. Reveillaud J, Anderson R, Reves-Sohn S, Cavanaugh C, Huber JA. 2018. Metagenomic
184 investigation of vestimentiferan tubeworm endosymbionts from Mid-Cayman Rise reveals
185 new insights into metabolism and diversity. Microbiome 6:19.

186 9. Winkel M, Carvalho V, Woyke T, Richter M, Schulz-Vogt HN, Flood B, Bailey J,
187 Mußmann M. 2016. Single-cell sequencing of *Thiomargarita* reveals genomic flexibility for
188 adaptation to dynamic redox conditions. Front Microbiol 7:964.

189 10. MacGregor BJ, Biddle JF, Harbort C, Matthysse AG, Teske A. 2013. Sulfide oxidation,
190 nitrate respiration, carbon acquisition, and electron transport pathways suggested by the
191 draft genome of a single orange Guaymas Basin *Beggiatoa* (*Cand.* Maribeggiatoa) sp.
192 filament. Mar Genomics 11:53–65.

193 11. Flood BE, Fliss P, Jones DS, Dick GJ, Jain S, Kaster A-K, Winkel M, Mußmann M, Bailey J.
194 2016. Single-Cell (Meta-)Genomics of a dimorphic *Candidatus* Thiomargarita nelsonii
195 reveals genomic plasticity. Front Microbiol 7:1–17.

196 12. Kleiner M, Wentrup C, Lott C, Teeling H, Wetzel S, Young J, Chang Y-JY-J, Shah M,
197 VerBerkmoes NC, Zarzycki J, Fuchs G, Markert S, Hempel K, Voigt B, Becher D, Liebeke
198 M, Lalk M, Albrecht D, Hecker M, Schweder T, Dubilier N. 2012. Metaproteomics of a
199 gutless marine worm and its symbiotic microbial community reveal unusual pathways for
200 carbon and energy use. Proc Natl Acad Sci USA 109:E1173-82.

201 13. Kleiner M, Petersen JM, Dubilier N. 2012. Convergent and divergent evolution of
202 metabolism in sulfur-oxidizing symbionts and the role of horizontal gene transfer. Curr
203 Opin Microbiol 15:621–31.

204 14. Klatt JM, Polerecky L. 2015. Assessment of the stoichiometry and efficiency of CO_2
205 fixation coupled to reduced sulfur oxidation. Front Microbiol 6:484.

206 15. Bright M, Klose J, Nussbaumer AD. 2013. Giant tubeworms. Curr Biol 23:R224–R225.

207 16. Heim S, Kunkel A, Thauer RK, Hedderich R. 1998. Thiol : fumarate reductase (Tfr) from
208 *Methanobacterium thermoautotrophicum*. Identification of the catalytic sites for fumarate
209 reduction and thiol oxidation. Eur J Biochem 253:292–299.

210 17. Anantharaman K, Brown CT, Hug LA, Sharon I, Castelle CJ, Probst AJ, Thomas BC,
211 Singh A, Wilkins MJ, Karaoz U, Brodie EL, Williams KH, Hubbard SS, Banfield JF. 2016.
212 Thousands of microbial genomes shed light on interconnected biogeochemical processes
213 in an aquifer system. Nat Commun 7:13219.

214 18. Pasić L, Rodriguez-Mueller B, Martin-Cuadrado A-B, Mira A, Rohwer F, Rodriguez-

215 Valera F. 2009. Metagenomic islands of hyperhalophiles: the case of *Salinibacter ruber*.
216 BMC Genomics 10:570.

217 19. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL. 2008. NCBI
218 BLAST: a better web interface. Nucleic Acids Res 36:W5–W9.

219 20. Wu M, Scott AJ. 2012. Phylogenomic analysis of bacterial and archaeal sequences with
220 AMPHORA2. Bioinformatics 28:1033–1034.

221 21. Edgar RC. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high
222 throughput. Nucleic Acids Res 32:1792–1797.

223 22. Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7:
224 Improvements in performance and usability. Mol Biol Evol 30:772–780.

225 23. Nguyen LT, Schmidt HA, Von Haeseler A, Minh BQ. 2015. IQ-TREE: A fast and effective
226 stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol
227 32:268–274.

228 24. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis
229 version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874.

Figures

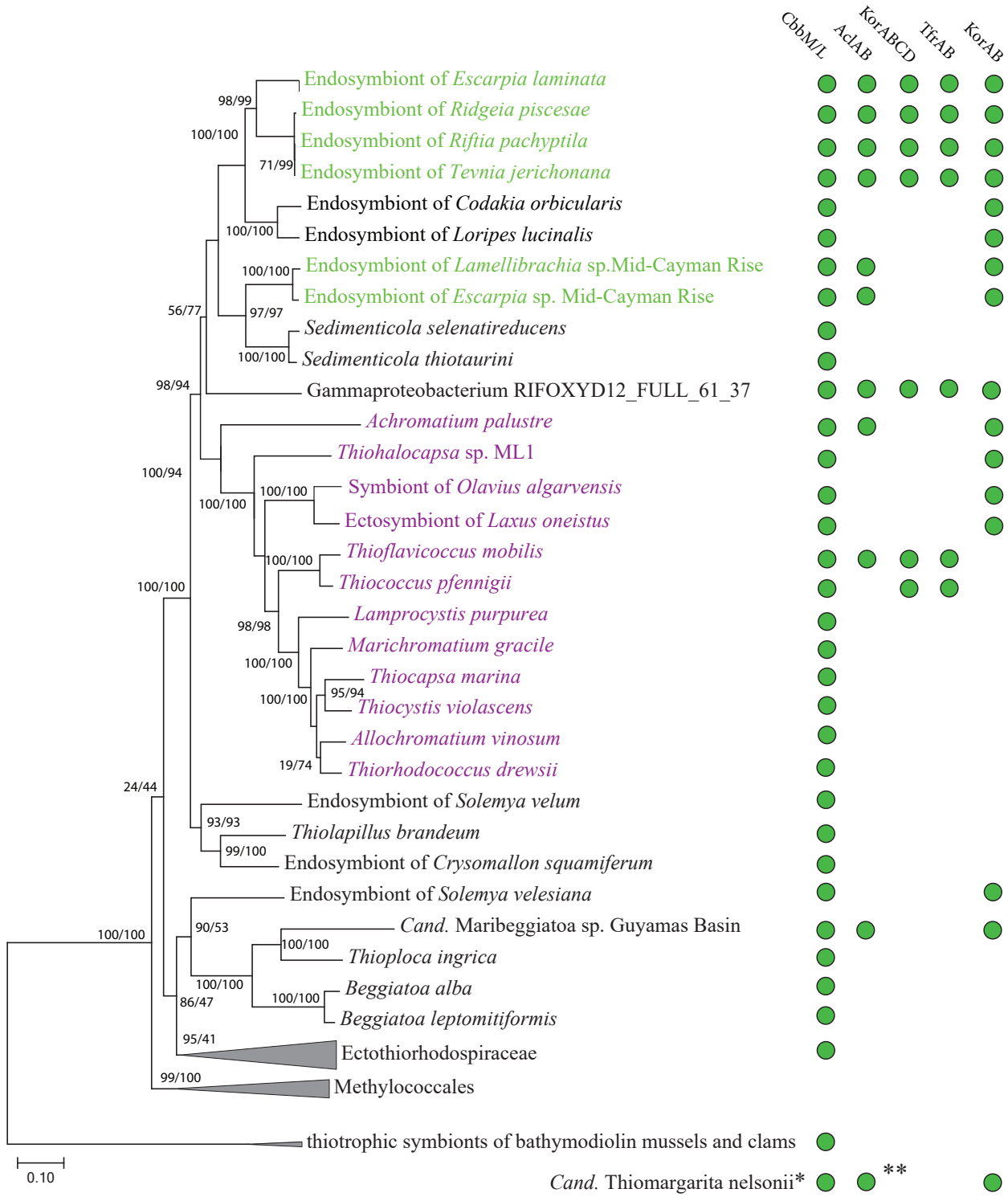

Figure 1: Phylogenomic tree showing occurrence of RuBisCO (CbbM/CbbL), ATP citrate lyase (AclAB), 4-subunit 2-oxoglutarate:ferredoxin oxidoreductase (KorABCD), putative thiol:fumarate reductase (TfrAB) and 2-subunit 2-oxoglutarate:ferredoxin oxidoreductase (KorAB) in the genomes of tubeworm symbionts (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.
* Was not included in the tree due to several missing single-copy marker genes or multiple versions of these genes, making an accurate phylogenomic placement challenging.
** Only the *aclB* gene was present.

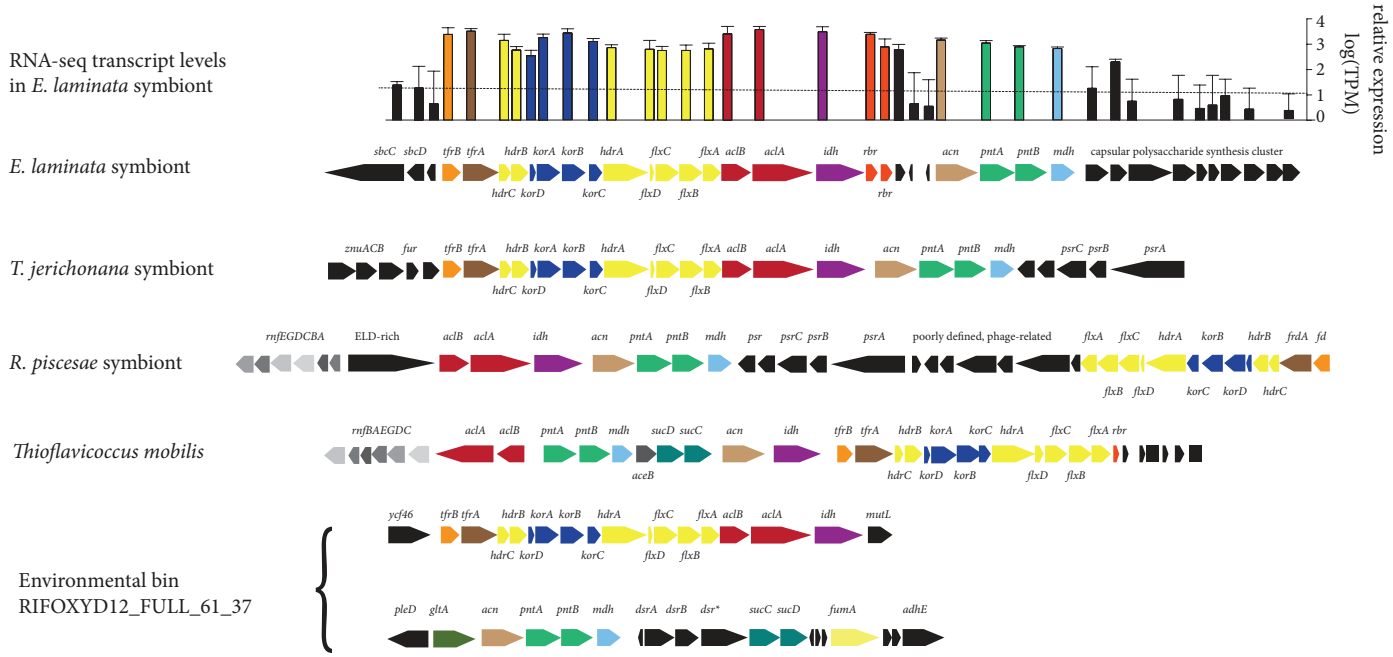

Figure 2: The rTCA cycle gene clusters in symbiotic and free-living bacteria, and the respective transcriptomic gene expression levels in the symbionts of *Escarpia laminata* tubeworm (*aclA*, log (TPM)=3.6; *korA*, log (TPM)=3.3; *hdrA*, log (TPM)=2.9; for comparison - *atpB*, log (TPM)=2.0; *cbbM*, log (TPM)=5.0. TPM, transcripts per kilobase million. *rbr*, rubrerythrin. *dsr**, oxidoreductase related to the NADPH-dependent glutamate synthase small chain, clustered with sulfite reductase. The dotted line is the median expression value for *E. laminata* genes.