

# 1 Sulfur-oxidizing symbionts without canonical genes 2 for autotrophic CO<sub>2</sub> fixation

3 **Classification:** Biological Sciences – Environmental Sciences

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24 **Abstract**

25 Since the discovery of symbioses between sulfur-oxidizing (thiotrophic) bacteria and  
26 invertebrates at hydrothermal vents over 40 years ago, it has been assumed that autotrophic  
27 fixation of CO<sub>2</sub> by the symbionts drives these nutritional associations. In this study, we  
28 investigated *Candidatus Kentron*, the clade of symbionts hosted by *Kentrophoros*, a diverse  
29 genus of ciliates which are found in marine coastal sediments around the world. Despite  
30 being the main food source for their hosts, *Kentron* lack the key canonical genes for any of  
31 the known pathways for autotrophic fixation, and have a carbon stable isotope fingerprint  
32 unlike other thiotrophic symbionts from similar habitats. Our genomic and transcriptomic  
33 analyses instead found metabolic features consistent with growth on organic carbon,  
34 especially organic and amino acids, for which they have abundant uptake transporters. All  
35 known thiotrophic symbionts have converged on using reduced sulfur to generate energy  
36 lithotrophically, but they are diverse in their carbon sources. Some clades are obligate  
37 autotrophs, while many are mixotrophs that can supplement autotrophic carbon fixation with  
38 heterotrophic capabilities similar to those in *Kentron*. We have shown that *Kentron* are the  
39 only thiotrophic symbionts that appear to be entirely heterotrophic, unlike all other  
40 thiotrophic symbionts studied to date, which possess either the Calvin-Benson-Bassham or  
41 reverse tricarboxylic acid cycles for autotrophy.

42 **Keywords:** meiofauna, ectosymbiont, Gammaproteobacteria, protist, lithoheterotrophy

## 43 **Significance Statement**

44 Many animals and protists depend on symbiotic sulfur-oxidizing bacteria as their main food  
45 source. These bacteria use energy from oxidizing inorganic sulfur compounds to make  
46 biomass autotrophically from CO<sub>2</sub>, serving as primary producers for their hosts. Here we  
47 describe apparently non-autotrophic sulfur symbionts called Kentron, associated with marine  
48 ciliates. They lack genes for known autotrophic pathways, and have a carbon stable isotope  
49 fingerprint heavier than other symbionts from similar habitats. Instead they have the potential  
50 to oxidize sulfur to fuel the uptake of organic compounds for heterotrophic growth, a  
51 metabolic mode called chemolithoheterotrophy that is not found in other symbioses.  
52 Although several symbionts have heterotrophic features to supplement primary production, in  
53 Kentron they appear to supplant it entirely.

## 54 **Introduction**

55 Chemosynthetic symbioses between heterotrophic, eukaryotic hosts and bacteria that use the  
56 oxidation of inorganic chemicals or methane to fuel growth are common in marine  
57 environments. They occur in habitats ranging from deep sea vents and seeps, where they are  
58 responsible for much of the primary production, to the shallow water interstitial, where the  
59 hosts are often small and inconspicuous meiofauna. Among the energy sources for  
60 chemosynthesis are reduced sulfur species like sulfide and thiosulfate, and such sulfur-  
61 oxidizing (thiotrophic) symbioses have convergently evolved multiple times (1). They are  
62 commonly interpreted as nutritional symbioses where the symbionts fix CO<sub>2</sub> autotrophically  
63 into biomass with the energy from sulfur oxidation and eventually serve as food for their  
64 hosts (1, 2). Indeed, several host groups have become so completely dependent on their  
65 symbionts for nutrition that they have reduced digestive systems. All sulfur-oxidizing  
66 symbioses investigated thus far possess a primary thiotrophic symbiont with genes of either  
67 the Calvin-Benson-Bassham (CBB) (3–10) or reverse tricarboxylic acid (rTCA) (11, 12)  
68 cycles for CO<sub>2</sub> fixation, and the different pathways may relate to different ecological niches  
69 occupied by the symbioses (13). The symbionts of the vestimentiferan tubeworms are  
70 additionally able to encode both the CBB and rTCA cycles, which may be active under  
71 different environmental conditions (14–16). Beyond sulfur oxidation and carbon fixation,  
72 several thiotrophic symbionts have additional metabolic capabilities such as the uptake of  
73 organic carbon (17), the use of carbon monoxide (18) and hydrogen (19) as energy sources,  
74 and the ability to fix inorganic nitrogen (4, 5).

75 The thiotrophic ectosymbionts of the ciliate genus *Kentrophoros* constitute a distinct clade of  
76 Gammaproteobacteria named “*Candidatus Kentron*” (hereafter Kentron) (20). Kentron has  
77 previously been shown to oxidize sulfide and fix CO<sub>2</sub> (21), and to be consumed and digested

78 by its hosts (21, 22). Unlike most ciliates, which consume their food at a specific location on  
79 the cell that bears feeding structures composed of specialized cilia, *Kentrophoros* has only  
80 vestiges of such cilia, and instead directly engulfs its symbionts along the entire cell body  
81 (23), suggesting that Kentron bacteria are its main food source.

82 Given that all previous studies of thiotrophic symbionts, including Kentron, have  
83 characterized them as autotrophic, we expected that the pathways of energy and carbon  
84 metabolism used by Kentron would resemble those in other thiotrophic bacteria involved in  
85 nutritional symbioses. In this study, we used metagenomic and transcriptomic analyses of  
86 single host individuals to show that the Kentron clade lacks the canonical pathways of  
87 autotrophic CO<sub>2</sub> fixation. Based on a metabolic reconstruction of the core genome from  
88 eleven Kentron phylotypes collected from three different sites, and results from direct protein  
89 stable isotope fingerprinting, we propose that it is a lithoheterotrophic nutritional symbiont,  
90 relying on assimilation of organic substrates rather than fixation of inorganic carbon to feed  
91 its hosts.

## 92 **Results**

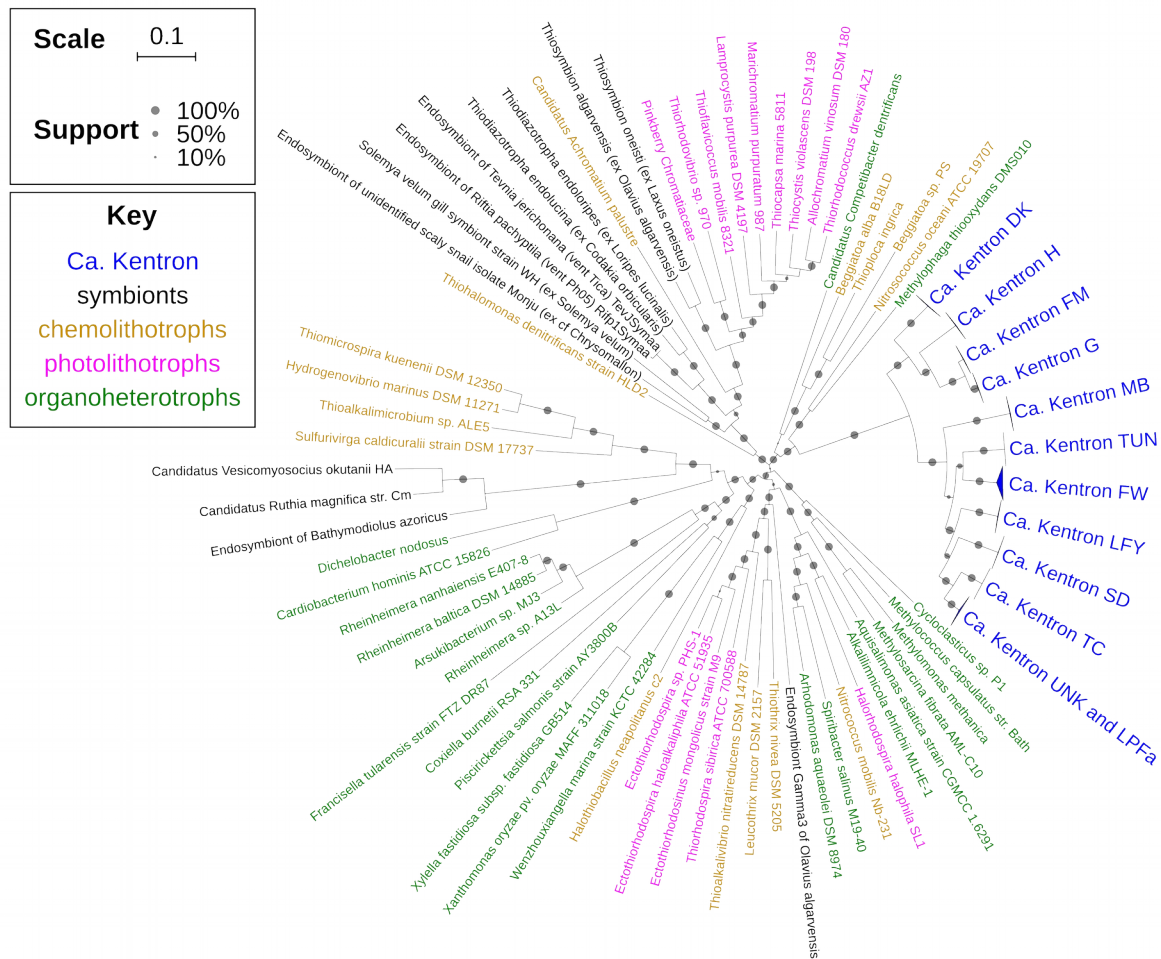
### 93 ***Symbiont genome assemblies have high coverage and*** 94 ***completeness, and represent eleven phylotypes***

95 Genomes of Kentron symbionts were binned from 34 metagenome assemblies, each  
96 corresponding to a single *Kentrophoros* host ciliate individual. These samples represented 12  
97 host morphospecies from three different geographical locations: the Mediterranean,  
98 Caribbean, and Baltic Seas (Supplementary Table 1). The symbiont genome assemblies had  
99 total lengths between 3.31 to 5.02 Mbp (median 3.91 Mbp), although they were relatively  
100 fragmented (N50: 3.52 to 37.5 kbp, median 21.4 kbp). Genome sizes and assembly  
101 fragmentation appeared to be species/phylotype-dependent (Supplementary Figure 1).

102 Nonetheless, the genome bins were relatively complete (91.4 to 94.9%, median 93.8%) and  
103 had low contamination (0.75 to 3.56%, median 1.87%) (Supplementary Table 2). The core  
104 genomic diversity in the clade was well-sampled: 1019 protein-coding gene orthologs were  
105 found in all 34 genomes, and the core genome accumulation curve reached a plateau  
106 (Supplementary Figure 2). Kentron genome sizes were relatively large for thiotrophic  
107 symbionts, and were comparable to values for *Ca. Thiodiazotropha* spp. (4.5 Mbp) and the  
108 Gamma3 symbiont of *Olavius algarvensis* (4.6 Mbp).

109 Kentron formed a well-supported clade (100% SH-like support value) within the  
110 Gammaproteobacteria, in a phylogenetic analysis using conserved protein-coding marker  
111 genes (Figure 1). Their closest relatives in the set of basal Gammaproteobacteria analysed  
112 were *Nitrosococcus oceani*, *Methylophaga thiooxydans*, *Thioploca ingrlica*, *Ca.*  
113 *Competibacter denitrificans*, and *Beggiatoa* spp. (100% support), which differed from the  
114 16S rRNA gene phylogeny, where Kentron was sister to the Coxiellaceae (20). Symbionts  
115 from different host morphospecies formed separate, well-supported phylotype clusters, with  
116 the exception of Kentron from *Kentrophoros* sp. UNK and *K. sp.* LPFa, where a single  
117 symbiont phylotype was associated with two different host phylotypes, as previously  
118 observed with 16S and 18S rRNA sequences. Among genomes of the same phylotype,  
119 average nucleotide identities (ANI) were 93.0–100% and average amino acid identities (AAI)  
120 were 93.2–100%, whereas between different phylotypes, these values were 83.2–93.8% and  
121 70.6–91.3% respectively, which supports them being different species in the same genus (24).

122 Kentron phylotypes will therefore be referred to here with their corresponding host  
123 morphospecies identifiers, except for Kentron UNK/LPFa.



124 **Figure 1.** Maximum-likelihood phylogeny of Kentron and basal Gammaproteobacteria from concatenated  
 125 alignment of 30 conserved protein-coding marker genes. Support values: SH-like aLRT. Branch lengths:  
 126 Substitutions per site.

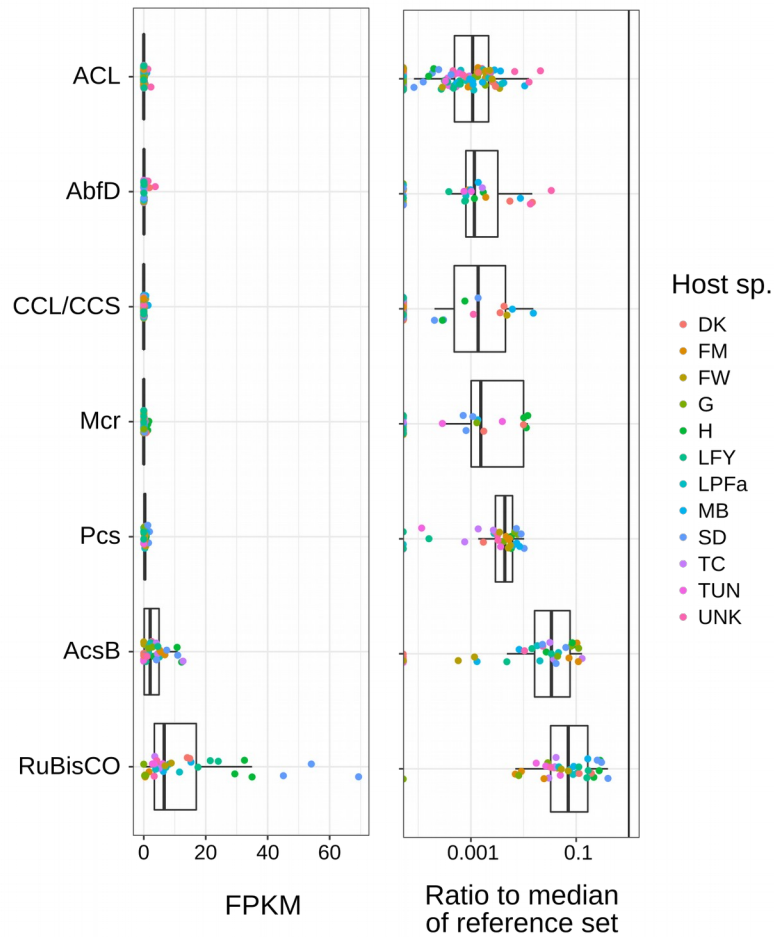
127 **Genes for key enzymes in known autotrophic pathways are absent**

128 Unlike other investigated thiotrophic symbionts, the genes for ribulose-1,5-bisphosphate  
 129 carboxylase/oxygenase (RuBisCO) and other key enzymes in known autotrophic CO<sub>2</sub>  
 130 fixation pathways (Supplementary Table 3) were not predicted in the binned Kentron  
 131 genomes by standard annotation pipelines. A gene annotated as RuBisCO in Kentron sp. H  
 132 fell within Group IV of the RuBisCO family (Supplementary Figure 3). Group IV RuBisCOs,  
 133 also known as RuBisCO-like proteins (RLPs), are not known to play a role in carbon fixation

134 but participate in a variety of other pathways such as thiosulfate metabolism (25).

135 To rule out the possibility that genes for these enzymes were not found because of  
136 misannotation, incomplete genome binning, or problems with genome assembly, we aligned  
137 raw, unassembled reads from *Kentrophoros* metagenome libraries to the curated SwissProt  
138 database of protein sequences. Key autotrophy proteins had coverage values (median 0.00,  
139 max 69.3 FPKM) that were always lower than the median coverage of reference proteins  
140 from the TCA and partial 3-hydroxypropionate (3HPB) pathways (Figure 2a). In 89% of  
141 cases, the coverage was at least 50-fold lower than the reference median, and if not, the  
142 majority of reads could be attributed either to other microbial genome bins in the  
143 metagenome (mostly RuBisCO or AcsB), or to to the RuBisCO-like protein in Kentron H  
144 (Supplementary Figure 4). Metatranscriptomes of two phylotypes (H and SD) were also  
145 screened with the same pipeline, and key autotrophy proteins again had coverages that were  
146 always below the median of the reference set (median 0.00, max 1.62 FPKM)  
147 (Supplementary Figure 5). We interpret this to mean that canonical autotrophy genes were  
148 indeed absent from Kentron genomes, and not merely misassembled or mispredicted.

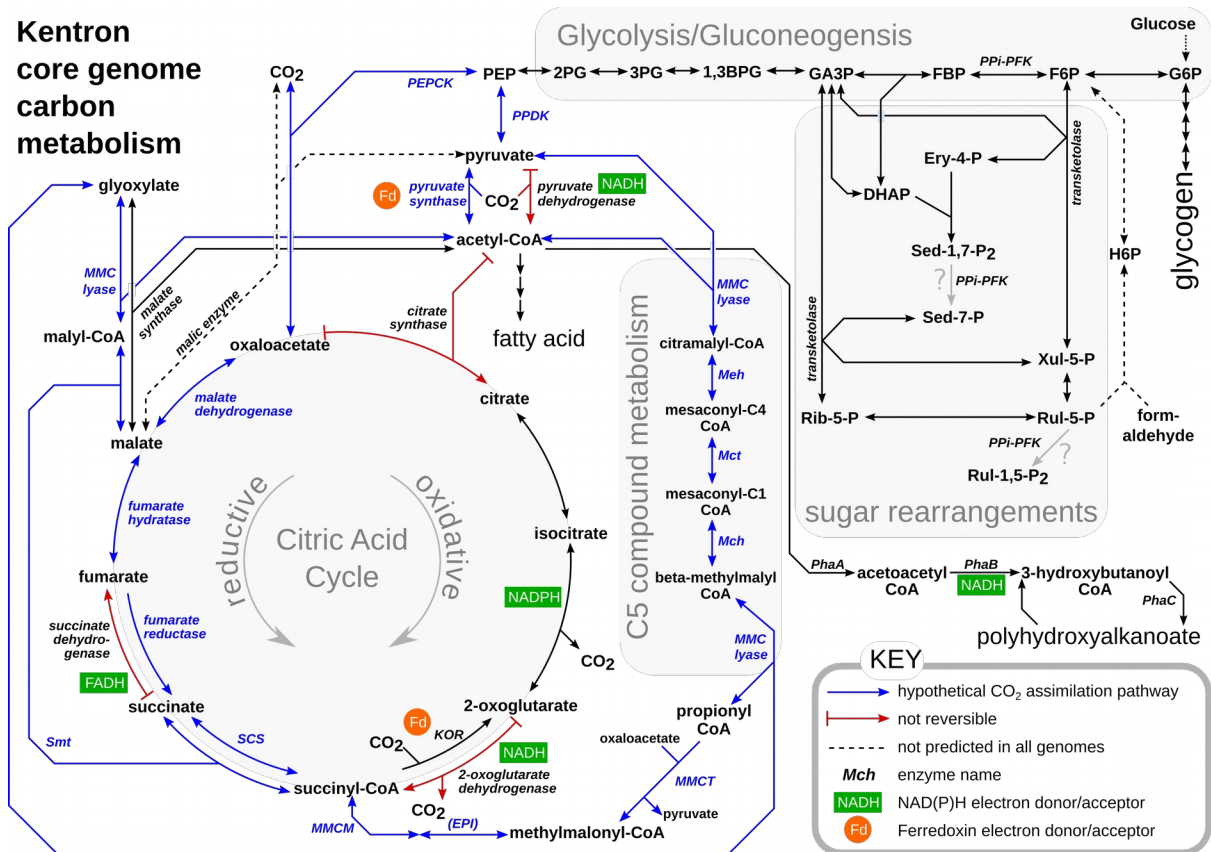




149 **Figure 2.** Read coverage (individual values and boxplots) in *Kentrophoros* metagenomes for key enzymes of  
150 autotrophic CO<sub>2</sub>-fixation pathways, expressed as FPKM values (*left*) and as a fraction of the median coverage of  
151 a reference set of proteins that are expected to be present in all *Kentron* species (*right*) (Supplementary Table 3).  
152 Each point represents a separate metagenome library, colored by *Kentrophoros* host morphospecies. Box  
153 midline represents median, hinges the interquartile range (IQR), whiskers are data within 1.5× IQR of hinges.  
154 *Abbreviations:* ACL, ATP citrate lyase; AbfD, 4-hydroxybutanoyl-CoA dehydratase; CCL/CCS, citryl-CoA  
155 lyase/citryl-CoA synthase; Mcr, malonyl-CoA reductase; Pcs, propionyl-CoA synthase; AcsB, CO-methylating  
156 acetyl-CoA synthase;. RuBisCO, ribulose-1,6-bisphosphate carboxylase/oxygenase.

157 **Evidence for lithoheterotrophic metabolism in Kentron**

158 Kentron genome annotations suggested a lithoheterotrophic metabolism, in which energy is  
 159 produced by oxidation of reduced sulfur, and carbon is assimilated in the form of organic  
 160 compounds (Figure 3).



161 **Figure 3.** Schematic reconstruction of carbon and central metabolism of Kentron clade, focussing on pathways  
 162 discussed in the text. *Compound name abbreviations:* 1,3BPG, 1,3-bisphosphoglycerate; 2PG, 2-  
 163 phosphoglycerate; 3PG, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; Ery-4-P, erythrose-4-  
 164 phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; G6P, glucose-6-phosphate; GA3P,  
 165 glyceraldehyde-3-phosphate; H6P, hexose-6-phosphate; PEP, phosphoenolpyruvate; Rib-5-P, ribose-5-  
 166 phosphate; Rul-1,5-P<sub>2</sub>, ribulose-1,5-bisphosphate; Rul-5-P, ribulose-5-phosphate; Sed-1,7-P<sub>2</sub>, sedoheptulose-  
 167 1,7-bisphosphate; Sed-7-P, sedoheptulose-7-phosphate; Xul-5-P, xylulose-5-phosphate. *Enzyme name*  
 168 *abbreviations:* EPI, methylmalonyl-CoA epimerase; KOR, alpha-ketoglutarate oxidoreductase; Mch,  
 169 mesaconyl-C1-CoA hydratase; Mct, mesaconyl-CoA C1-C4 CoA transferase; Meh, mesaconyl-C4-CoA

170 hydratase; MMC lyase, (S)-malyl-CoA/beta-methylmalyl-CoA/(S)-citramalyl-CoA lyase; MMCM  
171 methylmalonyl-CoA mutase; MMCT, methylmalonyl-CoA carboxytransferase; PEPCK, phosphoenolpyruvate  
172 carboxykinase; PPDK, pyruvate phosphate dikinase; PPI-PFK, pyrophosphate-dependent phosphofructokinase;  
173 Smt, succinyl-CoA:(S)-malate-CoA transferase.

## 174 **Electron donors and energetics**

175 Kentron genomes encoded a hybrid Sox-reverse Dsr pathway for sulfur oxidation, similar to  
176 other symbiotic and free-living thiotrophs (e.g. *Allochromatium vinosum*), which would allow  
177 the use of thiosulfate, elemental sulfur, and sulfide as energy sources (26, 27). They had a  
178 complete electron transport chain for oxidative phosphorylation and an F<sub>0</sub>F<sub>1</sub>-type ATP  
179 synthase. The only terminal oxygen reductase predicted was cbb3-type cytochrome c oxidase  
180 (complex IV), which has a high oxygen affinity and is typically expressed under micro-oxic  
181 conditions (28, 29). In the two Kentron phylotypes for which expression profiles were  
182 available, this set of functions was among the most highly-expressed genes (Supplementary  
183 Figure 6).

184 Four Kentron phylotypes (H, SD, FW, G) encoded anaerobic-type Ni-dependent CO  
185 dehydrogenase precursors, adjacent to CO dehydrogenase Fe-S subunits (in FW and SD) or a  
186 CO dehydrogenase maturation factor (in G). In addition, H<sub>2</sub> may serve as an electron donor  
187 for Kentron TC, TUN, G, and FW (one genome), which encoded genes related to the  
188 oxidative-type [Ni-Fe] hydrogenase Mvh (A and G subunits), as well as auxiliary proteins for  
189 hydrogenase maturation and Ni incorporation, although they did not all occur in a single gene  
190 cluster. Both CO and H<sub>2</sub> are known to be potential electron donors for symbiotic thiotrophs,  
191 and have been measured in their habitat in Sant' Andrea, Elba (18), where one of these  
192 *Kentrophoros* phylotypes (H) was collected.

193 Oxidoreductases for anaerobic respiration were not predicted, except for subunits NapA and

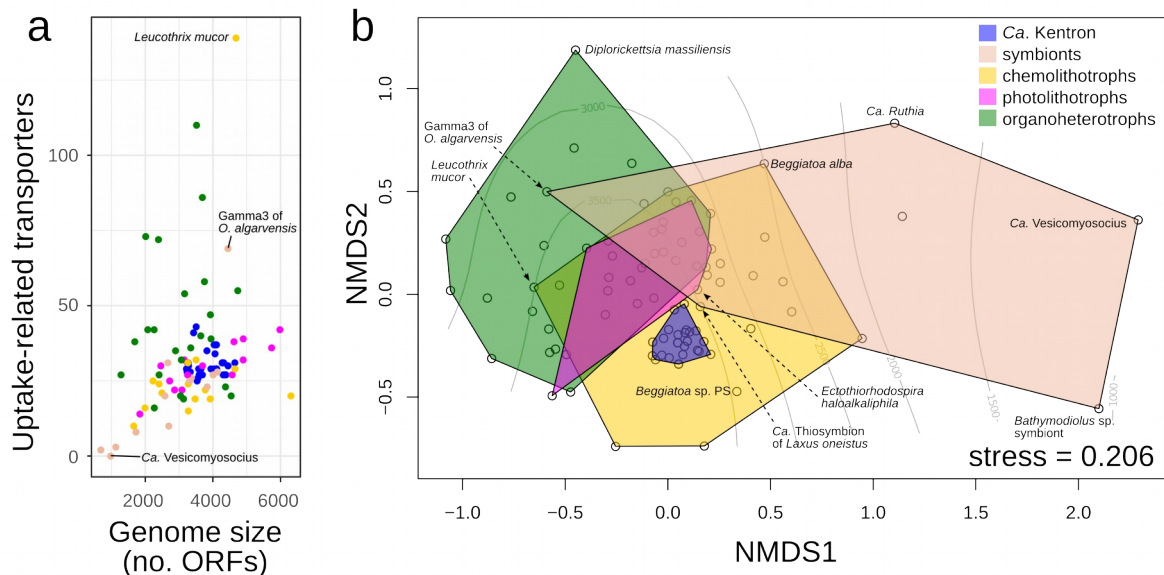
194 B of periplasmic nitrate reductase (in 28 and 25 genomes respectively). However, the rest of  
195 the dissimilatory nitrate reduction to ammonia pathway was absent. Na<sup>+</sup>-translocating  
196 ferredoxin:NAD<sup>+</sup> (Rnf) and NADH:ubiquinone (Nqr) oxidoreductases, which can couple  
197 reducing equivalents to the Na<sup>+</sup> membrane potential, were also predicted.

## 198 **Uptake transporters for organic substrates**

199 Genes encoding uptake transporters for organic substrates were abundant in *Kentron* genomes  
200 and were also expressed in the transcriptomes (Supplementary Figure 7, Supplementary File  
201 2). An average of 54.1 of such genes were predicted per genome (representing 18.1% of all  
202 genes with TCDB hits), of which more than half had transmembrane (TM) domains (mean  
203 30.5 per genome). The families with the highest mean counts per genome were the ATP-  
204 binding cassette (ABC) superfamily (33.9 total, 16.4 transmembrane, counting only uptake-  
205 related subfamilies), tripartite ATP-independent periplasmic transporter (TRAP-T) family  
206 (7.2 total, 5.1 TM), and the solute:sodium symporter (SSS) family (1.6 total, 1.3 TM). Three  
207 other families – concentrative nucleoside transporter (CNT), dicarboxylate/amino acid cation  
208 symporter (DAACS), and neurotransmitter/sodium symporter (NSS) – were represented by a  
209 single gene in all *Kentron* genomes. Most of these families are known to target organic acids,  
210 amino acids, or small peptides. In comparison, sugar uptake transporter families were less  
211 numerous and present in only a subset of genomes (e.g. ABC subfamilies CUT 1 and CUT2),  
212 or not predicted in *Kentron* at all (e.g. phosphotransferase system family).

213 The number of organic uptake transporters in *Kentron* was high when compared to other  
214 symbiotic thiotrophs, which had counts ranging from 2 (0 TM) in *Ca. Vesicomysocius*  
215 *okutanii* to 134 (69 TM) in the Gamma3 symbiont of *Olavius algarvensis*. However, larger  
216 genomes tend to have more transporters, and *Kentron* genomes were also relatively large  
217 (Figure 4a). We therefore compared the content of organic-uptake-related TCDB family

218 members per genome between Kentron and other basal Gammaproteobacteria by non-metric  
219 multidimensional scaling. Kentron overlapped with the range of variation for both  
220 phototrophs and chemolithotrophs (both free-living and symbiotic), but were most distant  
221 from pathogenic organoheterotrophs, and from the thiotrophic symbionts of deep-sea  
222 bivalves (which have few uptake transporters) (Figure 4b).



223 **Figure 4.** Comparison of organic substrate transporters in genomes of Kentron and other basal  
224 Gammaproteobacteria. (a) Counts of uptake-related transporters (transmembrane only) vs. genome size  
225 (expressed in no. of open reading frames). (b) 2-dimensional ordination plot (non-metric multidimensional  
226 scaling) of genomes based on counts of uptake-related TC families and subfamilies per genome. Bray-Curtis  
227 distance metric; stress = 0.206. Contour lines indicate approximate genome size. Colors in both plots share the  
228 same legend and represent type of metabolism.

## 229 Heterotrophic carbon metabolism

230 Kentron genomes encoded both glycolysis (Embden-Meyerhoff-Parnas pathway) and the  
231 oxidative tricarboxylic acid (TCA) cycle. The canonically irreversible reactions of glycolysis,  
232 pyruvate kinase and phosphofructokinase, were replaced in Kentron by pyrophosphate-  
233 dependent alternatives pyruvate phosphate dikinase and P<sub>Pi</sub>-dependent phosphofructokinase

234 (P<sub>Pi</sub>-PFK) respectively. These catalyze reversible reactions that could also function in the  
235 direction of gluconeogenesis. These reversible alternatives have been found in other  
236 thiotrophic symbioses, where they have been proposed to function in a more energy-efficient  
237 version of the CBB cycle (30).

238 Genes for pyruvate dehydrogenase and the complete oxidative TCA cycle were present,  
239 including 2-oxoglutarate dehydrogenase, which is often missing in obligate autotrophs (31).  
240 The reductive equivalents for the key steps of the oxidative TCA cycle were also present,  
241 namely ferredoxin-dependent pyruvate synthase, ferredoxin-dependent 2-oxoglutarate  
242 synthase, and fumarate reductase. However, because neither ATP citrate lyase (ACL) nor  
243 citryl-CoA lyase/citryl-CoA synthase (CCL/CCS) were predicted, a canonical autotrophic  
244 reductive TCA cycle was not predicted.

245 Heterotrophic carboxylases also had relatively high expression levels. Ferredoxin-dependent  
246 pyruvate synthase was present in multiple copies per genome, of which the highest-expressed  
247 were at the 98.4 and 93.0 percentiles in Kentron H and SD respectively (Supplementary  
248 Figure 6). GDP-dependent PEP carboxykinase, which can replenish oxaloacetate  
249 anaplerotically, was also highly expressed (93.0 and 96.7 percentiles) (Supplementary Figure  
250 6). Unlike PEP carboxylase, which was not predicted, PEP carboxykinase catalyzes a  
251 reversible reaction.

252 The glyoxylate shunt, which enables growth solely on acetate as the only energy and carbon  
253 source, appeared to be incomplete, as malate synthase was predicted but not isocitrate lyase.  
254 Other pathways for growth on acetate, namely the ethylmalonyl-CoA pathway and  
255 methylaspartate cycle, were not predicted either.

## 256 **Partial 3-hydroxypropionate bi-cycle**

257 Genes encoding most enzymes of the 3-hydroxypropionate bi-cycle (3HPB), which is the  
258 autotrophic pathway used by members of the distant bacterial phylum Chloroflexi, were  
259 predicted in Kentron. These genes had expression levels in the 64.1–83.0 and 48.2–96.4  
260 percentile ranges for Kentron H and SD respectively (Supplementary Figure 6). The key  
261 enzymes malonyl-CoA reductase and propionyl-CoA synthase were absent, hence the bi-  
262 cycle was not closed and would not function autotrophically. However, the remainder of the  
263 pathway could function in the assimilation of organic substrates (e.g. acetate and succinate),  
264 or to connect metabolite pools (acetyl-CoA, propionyl-CoA, pyruvate, and glyoxylate) (32),  
265 as previously proposed for *Chloroflexus* (33) and the *Ca.* Thiosymbion symbionts of gutless  
266 oligochaetes (3).

267 These enzymes are unusual because their genes are uncommon and have a disjunct  
268 phylogenetic distribution: Chloroflexi, at least four clades in Gammaproteobacteria (Kentron,  
269 *Ca.* Thiosymbion, *Ca.* Competibacter, “Pink Berry” Chromatiaceae), and Betaproteobacteria  
270 (*Ca.* Accumulibacter). While they were previously thought to have been horizontally  
271 transferred from Chloroflexi to the other groups (33), gene phylogenies show that the  
272 Chloroflexi probably also gained the 3HPB by horizontal transfer (34), which was supported  
273 by our analysis when Kentron homologs were also included (Supplementary Figure 8).

## 274 **Storage compounds**

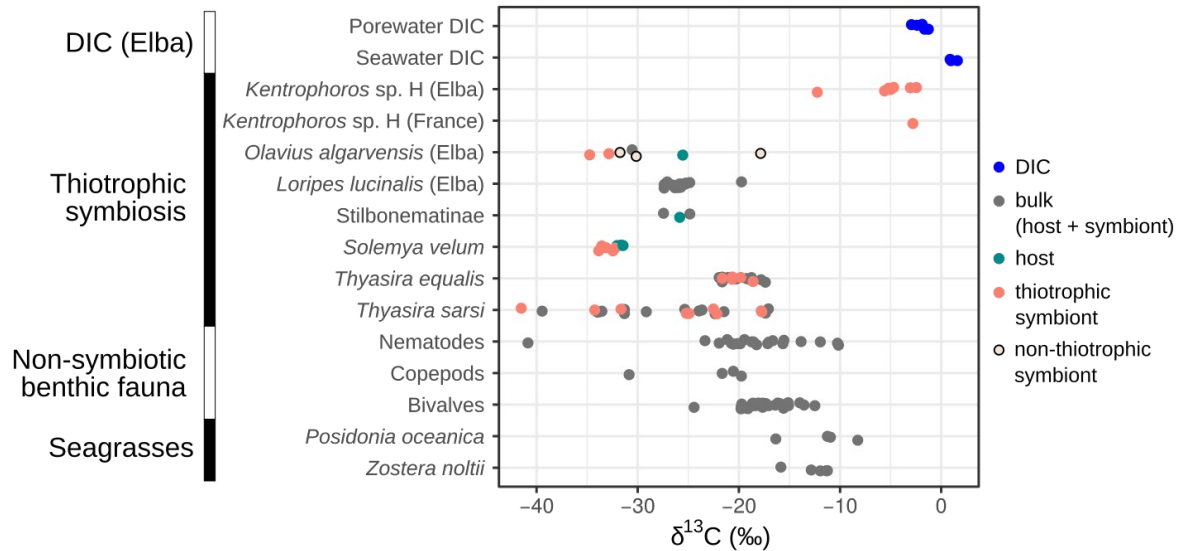
275 In addition to elemental sulfur, Kentron also have the potential to store and mobilize carbon  
276 (as polyhydroxyalkanoates (PHA) and starch/glycogen) and phosphorus (as polyphosphate).  
277 Genes related to PHA synthesis were among the most highly-expressed, namely those  
278 encoding phasin, a protein associated with the surface of PHA granules, and putative  
279 acetoacetyl-CoA reductase (*phaB*) (Supplementary Figure 6). Trehalose was detected in

280 *Kentrophoros* sp. H but is probably produced and accumulated by the host ciliate rather than  
281 the symbionts (Supplementary Results).

### 282 **Carbon stable isotope fingerprinting (SIF) of *Kentron***

283 Measuring the natural abundance ratio of carbon stable isotopes  $^{13}\text{C}/^{12}\text{C}$ , also known as the  
284 stable isotope fingerprint (SIF), is a challenge in *Kentrophoros* because of its small biomass  
285 ( $\sim 10^6$  symbionts and  $\sim 10$   $\mu\text{g}$  wet weight per ciliate in the largest species). Sensitive  
286 applications of isotope ratio mass spectrometry (IRMS) for a bulk (combined host and  
287 symbionts) measurement would require at least  $\sim 10^7$  bacterial cells (35), and compound-  
288 specific IRMS for signatures of specific pathways like  $^{13}\text{C}$  enrichment in fatty acids in the  
289 rTCA cycle (11) would require considerably more. We therefore used a newly-developed  
290 metaproteomics method that could distinguish the SIF of the symbiont from other biomass in  
291 the sample (36). The protein-based carbon SIF for *Kentron* sp. H from Elba and France  
292 ranged from -12.3 to -2.5 ‰ (n = 8), expressed as  $\delta^{13}\text{C}$  values which report deviation from the  
293 V-PDB standard (Figure 5, Supplementary Table 11). In comparison, published  $\delta^{13}\text{C}$  values  
294 for other shallow-water thiotrophic symbioses were  $< -17$  ‰, and the  $\delta^{13}\text{C}$  of dissolved  
295 inorganic carbon (DIC) in porewater from Elba was between -2.99 and -1.32 ‰ (Figure 5,  
296 Supplementary Table 12).





297 **Figure 5.** Carbon stable isotope  $\delta^{13}\text{C}$  composition values in *Kentrophoros* sp. H (this study) and published  
298 values for other shallow-water thiotrophic symbioses (5, 36, 43–45), non-symbiotic benthic animals (5, 58, 59),  
299 and two Mediterranean seagrass species (58–61), compared with dissolved inorganic carbon (DIC) from  
300 porewater and seawater at Elba (this study). Values for *Kentrophoros* and *Olavius algarvensis* (except the “bulk”  
301 value) are from direct protein-SIF, others are from isotope ratio mass spectrometry (IRMS). Values for  
302 symbiont-bearing tissue (e.g. gills) are also included under “symbiont”.

## 303 Discussion

304 In this study, we have presented evidence that the Kentron symbionts of *Kentrophoros* ciliates  
305 are unique among thiotrophic symbionts because they do not encode canonical pathways for  
306 autotrophic carbon fixation, despite being a food source for their hosts. Their carbon stable  
307 isotope fingerprints are also substantially heavier than other thiotrophic symbioses from  
308 similar habitats. Their genomes encode heterotrophic features, including abundant uptake  
309 transporters for organic substrates, and the ability to store and mobilize organic carbon in  
310 storage polymers. We therefore propose that Kentron are chemolithoheterotrophs (37),  
311 oxidizing inorganic compounds (in this case reduced sulfur species) to provide energy for  
312 assimilating organic carbon as the main carbon source for growth.

### 313 ***Role of heterotrophic CO<sub>2</sub> fixation***

314 Our results conflict with the previous interpretation of Kentron as an autotrophic symbiont,  
315 based on experiments with <sup>14</sup>C-labeled bicarbonate that showed inorganic carbon fixation by  
316 Kentron at a maximum rate of 0.11 bacterial cell carbon h<sup>-1</sup> (21). To rule out the possibility  
317 that only some species are autotrophs, we collected *Kentrophoros* matching the described  
318 morphology from the same site (Nivå Bay, Denmark), but their symbionts (phylotype DK)  
319 lacked canonical autotrophic pathways like all other Kentron phylotypes examined in this  
320 study.

321 However, the ability to fix CO<sub>2</sub> alone is insufficient evidence for autotrophy, which is defined  
322 as the ability to grow with inorganic carbon as the sole or major carbon source (38), because  
323 heterotrophs can also fix CO<sub>2</sub> to some extent, e.g. via anaplerotic reactions in the oxidative  
324 TCA cycle (39). Such heterotrophic fixation can account for 10% or more of total cell carbon  
325 in some bacteria (40, 41). The strictest standard of evidence for autotrophy requires  
326 cultivation to show growth in the absence of organic substrates or to measure growth rates  
327 and carbon stoichiometry, but *Kentrophoros* and its symbionts remain unculturable.

328 Kentron had two heterotrophic carboxylases in the central carbon metabolism, ferredoxin-  
329 dependent pyruvate synthase and PEP carboxykinase, that were both highly expressed. The  
330 former is involved in carboxylating acetyl-CoA to pyruvate, which can occur when the  
331 storage polymer PHA is mobilized. The experiments of Fenchel & Finlay (21) were  
332 performed with freshly-collected organisms that had visible cellular inclusions, and were  
333 conducted with filtered coastal seawater, which typically has more dissolved organic carbon  
334 than oceanic seawater. It is therefore likely that storage polymers and organic substrates were  
335 present in the symbiosis that were mobilized or assimilated, and that the measured CO<sub>2</sub>  
336 assimilation was due to heterotrophic carboxylation.

337 ***Could Kentron use a novel autotrophic CO<sub>2</sub> fixation pathway?***

338 Different autotrophic carbon fixation pathways each have characteristic degrees of isotope  
339 fractionation discriminating against the heavier isotope <sup>13</sup>C, resulting in biomass that is  
340 relatively depleted in <sup>13</sup>C (i.e. more negative δ<sup>13</sup>C values) (42). Kentron were more enriched  
341 in <sup>13</sup>C than other shallow-water thiotrophic symbioses collected at the same locality or  
342 elsewhere, which primarily use the CBB cycle, and which have δ<sup>13</sup>C values in the range of  
343 -30 to -20 ‰ (Figure 5) (5, 36, 43–45). Kentron showed only a modest <sup>13</sup>C depletion relative  
344 to DIC from the same site (Figure 5), which ruled out the possibility that they use a pathway  
345 with strong isotope fractionation (ε), such as the CBB cycle (ε = 10 to 22 ‰) or the reductive  
346 acetyl-CoA pathway (ε = 15 to 36 ‰) (46). Other pathways such as the reverse TCA cycle (ε  
347 = 4 to 13 ‰) or 3-hydroxypropionate bicycle (ε ≈ 0 ‰) may still fall in this range, but given  
348 that the key genes for these pathways were not detected, this possibility would require the  
349 postulation of hitherto unknown enzymes.

350 In two different thermophilic bacteria, the oxidative TCA cycle has recently been found to  
351 function in the autotrophic direction without using ACL or CCL/CCS, but instead by  
352 reversing the citrate synthase reaction. Such a “reversed oxidative TCA” (roTCA) cycle  
353 would not be distinguishable from the oxidative TCA by genome sequences alone (47, 48).  
354 However, both roTCA bacteria require anoxic conditions with hydrogen as the energy source  
355 for autotrophic growth. They are also facultative autotrophs, and switch to heterotrophic  
356 growth when suitable substrates like acetate are available. Citrate synthase is also highly  
357 expressed in the roTCA, whereas in Kentron the gene has only moderate expression  
358 (Supplementary Figure 6, 51.2 and 56.3 percentiles in Kentron H and SD respectively). For  
359 these reasons it is unlikely that a microaerophilic sulfur oxidizer like Kentron uses the roTCA  
360 for autotrophic growth.

361 Alternatively, a set of reactions that could allow autotrophic CO<sub>2</sub> fixation by Kentron can be  
362 reconstructed by combining elements of the partial 3HPB and another previously proposed  
363 hypothetical pathway (49), without proposing any novel enzymes or biochemical reactions  
364 (Figure 3, Supplementary Discussion). Like the canonical 3HPB in *Chloroflexus* (33), this  
365 hypothetical pathway would allow co-assimilation of organic substrates if available, while  
366 fixing CO<sub>2</sub> with ferredoxin-dependent pyruvate synthase and PEP carboxykinase, the  
367 aforementioned heterotrophic carboxylases. Although it is stoichiometrically and  
368 energetically feasible for Kentron to fix CO<sub>2</sub> purely autotrophically through this hypothetical  
369 pathway, it is more likely that the involved enzymes function lithoheterotrophically or  
370 mixotrophically, enabling them to exploit different carbon sources at the same time  
371 (Supplementary Discussion).

372 **Table 1.** Comparison of metabolic features predicted in thiotrophic symbiont genomes. Key: +, present; (+), not  
 373 in all genomes. Abbreviations: Bathy, *Bathymodiolus*; CBB, Calvin-Benson-Bassham cycle; Cyt c, Cytochrome  
 374 c; Frd, fumarate reductase; Kor, 2-oxoglutarate:ferredoxin oxidoreductase; PEP, phosphoenolpyruvate; P*P*<sub>i</sub>,  
 375 pyrophosphate; rTCA, reverse TCA cycle.

Feature	Bathy symbiont	<i>Ruthia</i>	<i>Endoriftia</i>	<i>Thio- diazotropha</i>	<i>Solemya</i> symbiont	<i>Thio- symbion</i>	Kentron
Host habitat	Hydrothermal vents and seeps			Shallow water sediment interstitial			
<b>Autotrophy</b>							
CBB cycle (RuBisCO, phosphoribulokinase)	+	+	+	+	+	+	
PPi-Phosphofructokinase	+	+	+	+	+	+	+
rTCA (Citrate cleavage)			+				
<b>Diazotrophy</b>							
Nitrogenase				+		(+)	
<b>Tricarboxylic acid (TCA) cycle</b>							
Oxidative TCA			+	+	+	+	+
Kor & Frd (reductive TCA)			+	+		+	+
Glyoxylate shunt				+	+	+	
<b>Central metabolism</b>							
Pyruvate phosphate dikinase			+	+	+	+	+
PEP synthase			+	+	+	+	
Pyruvate synthase			+	+	+	+	+
Pyruvate carboxylase				+		+	
PEP carboxylase				+	+		
PEP carboxykinase (GTP)				+		+	+
Malic enzyme	+	+	+	+	+	+	(+)
<b>C5 reactions of 3-hydroxypropionate bi-cycle (p3HPB)</b>							
p3HPB						+	+
<b>Energy</b>							
Rnf transporter	+	+	+	+	+	+	+
V-type ATPase			+	+	+		
Cyt c oxidase cbb3 type	+	+	+	+	+	+	+
Cyt c oxidase aa3 type	+	+		+	+	+	
<b>Storage compounds</b>							
Glycogen			+	+	+	+	+
Polyhydroxyalkanoates				+	+	+	+
Polyphosphate synthesis				+	+	+	+

376 ***The autotrophy-heterotrophy spectrum in thiotrophic symbiosis***  
377 Thiotrophic symbioses are most commonly found in nutrient-limited environments, and their  
378 symbionts are assumed to provide the hosts with nutrition through the autotrophic fixation of  
379 CO<sub>2</sub>. Indeed, the symbionts of deep-sea bivalves *Bathymodiolus* and *Calyptogena* show  
380 characteristic features of obligate autotrophy in their genomes, namely an incomplete TCA  
381 cycle and the lack of organic uptake transporters (Table 1) (19, 50–52). This appears to be the  
382 exception, however, as other symbiont clades possess heterotrophic features to varying  
383 degrees (Table 1). Some features, e.g. glycolysis, are involved in the mobilization of storage  
384 compounds, but abundant presence and expression of organic uptake transporters, as we  
385 observed in Kentron in this study, are a clearer marker of heterotrophic assimilation (53).  
386 Mixotrophic potential in other symbionts has been variously suggested to be a strategy to  
387 cope with carbon limitation by recycling host waste, as a nutritional supplement to  
388 autotrophy, or to be retained for a hypothetical free-living stage of the symbiont life cycle (3,  
389 7, 30). Thus, there is a spectrum among thiotrophic symbionts between obligate autotrophs  
390 and the possibly heterotrophic Kentron, with various degrees of mixotrophy in between.  
391 Symbiotic thiotrophs that lack the canonical CBB and rTCA pathways, as Kentron does, have  
392 not been previously described. Among free-living thiotrophic bacteria, lithoheterotrophy  
393 appears to be more common among those that have the Sox pathway (i.e. thiosulfate  
394 oxidizers) than those with the rDsr/Sox pathway (i.e. thiotrophs that can store and oxidize  
395 elemental sulfur) (Supplementary Discussion). Of the latter, we are aware of two isolates –  
396 *Ruegeria marina* CGMCC 1.9108 and *Thiothrix flexilis* DSM 14609 – whose genomes lack  
397 CBB and rTCA. Moreover, some free-living thiotrophs that possess the CBB cycle may  
398 nonetheless grow only when supplied with organic substrates, e.g. freshwater *Beggiatoa* (54).  
399 Functional heterotrophy may therefore be underestimated as it is not necessarily apparent

400 from genomic predictions.

401 Host biology constrains the feasibility of autotrophy for a thiotrophic symbiont. To meet  
402 nutritional requirements by chemoautotrophy alone, the host must provide high O<sub>2</sub> flux to its  
403 symbionts, beyond what it requires itself (55). This is metabolically demanding, and it is  
404 telling that the bathymodioline and vesicomid bivalves, whose symbionts have the most  
405 autotrophic features, are relatively large animals with intracellular symbionts that are located  
406 in their gill tissues, which can better maintain ventilation and homeostasis than smaller hosts  
407 that have extracellular symbionts. Specialization for high autotrophic production rates is also  
408 seen in the pre-concentration of CO<sub>2</sub> by the bivalve *Bathymodiolus azoricus* for its  
409 symbionts, and in its thiotrophic symbiont's metabolic dependence on the animal to replenish  
410 TCA cycle intermediates (56).

411 Meiofaunal hosts like *Kentrophoros* and stilbonematine nematodes, in contrast, are much  
412 smaller, cannot span substrate gradients, and must be able to tolerate fluctuating anoxia.  
413 Given that shallow-water coastal environments also receive more organic input, for example  
414 from land or from seagrass beds, than deep-sea hydrothermal environments, it is not  
415 surprising that the shallow-water meiofaunal symbioses have more heterotrophic features  
416 than the deep-sea ones (Table 1).

### 417 ***Ecophysiological model of the Kentrophoros symbiosis***

418 Based on our results and previous descriptions of morphology and behavior in *Kentrophoros*  
419 and other thiotrophic symbioses, we propose the following model for the ecophysiology of  
420 this symbiosis:

421 *Kentrophoros* fuels its growth by the phagocytosis and digestion of its symbionts, which was  
422 previously observed by electron microscopy (22). There has to be a net input of energy and

423 organic carbon from environmental sources for the overall growth of the host-symbiont  
424 system, and heterotrophic carboxylation may also be a substantial carbon source. To give its  
425 symbionts access to these substrates, *Kentrophoros* likely shuttles between oxic and anoxic  
426 zones in marine sediment, like other motile, sediment-dwelling hosts with thiotrophic  
427 symbionts (43). In anoxic sediment, both the predicted energy and carbon sources, namely  
428 sulfide and organic acids, are produced by microbial activity (57). Many organic acids, such  
429 as acetate and succinate, are more oxidized than average biomass (Supplementary Table 4),  
430 hence Kentron needs reducing equivalents to assimilate them, which could come from  
431 sulfide. As the complete oxidation of sulfide to sulfate requires oxygen, the partly-oxidized  
432 sulfur can be stored by the symbionts as elemental sulfur when under anoxic conditions, until  
433 the symbionts are again exposed to oxygen. The synthesis of PHA from small organic acids  
434 like acetate can also function as both an additional electron sink for sulfide oxidation and as a  
435 carbon store. Hydrolysis of polyphosphate and mobilization of glycogen are also potential  
436 sources of energy in the absence of oxygen.

437 Under oxic conditions, elemental sulfur inclusions in Kentron can be further oxidized to  
438 sulfate to yield energy, and PHA can be mobilized for biosynthesis. Glycogen and  
439 polyphosphate reserves can also be regenerated. The various storage inclusions in Kentron,  
440 namely elemental sulfur, PHA, glycogen, and polyphosphate, hence, represent pools of  
441 energy, reducing equivalents, and carbon that function as metabolic buffers for the symbiont  
442 living in a fluctuating environment.

443 The symbionts may also bring a syntrophic benefit to their hosts under anoxic conditions,  
444 when the ciliates can only yield energy by fermentation. By assimilating fermentation waste  
445 products and keeping their concentrations low in their host, the symbionts can improve the  
446 energy yields for their hosts and allow them to better tolerate periods of anoxia. This could



447 also be a form of resource recycling under carbon-limited conditions, which has been  
448 proposed for other thiotrophic symbionts with the potential to assimilate organic acids (3, 7).  
449 Kentron is relatively enriched in  $^{13}\text{C}$  compared to non-symbiotic shallow-water benthic fauna,  
450 such as nematodes and bivalves ( $\delta^{13}\text{C} \sim -20$  to  $-10$  ‰) (5, 58, 59), and to the seagrasses ( $\delta^{13}\text{C}$   
451  $\sim -15$  to  $-10$  ‰) (58–61) that are the main primary producers in the habitat of *Kentrophoros*  
452 (Figure 5). The higher values in Kentron could be partly caused by preferring specific  
453 substrates with higher  $^{13}\text{C}$  content, such as acetate, which has a wide range of  $\delta^{13}\text{C}$  ( $-2.8$  to  
454  $-20.7$  ‰) in marine porewaters depending on the dominant microbial processes at the site  
455 (62). Given how close the  $\delta^{13}\text{C}$  of Kentron is to DIC, it is possible that heterotrophic  $\text{CO}_2$   
456 fixation contributes to this  $^{13}\text{C}$  signature, but the isotope fractionation values of the  
457 heterotrophic carboxylases have not been characterized, to our knowledge. Repeated internal  
458 recycling of host waste products, as we postulate, could also cause accumulation of  $^{13}\text{C}$  in the  
459 host-symbiont system.

460 Our metabolic model has parallels to free-living thiotrophs (63, 64) and to heterotrophic  
461 bacteria involved in enhanced biological phosphorus removal (EBPR) from wastewater (65).  
462 What they have in common is their use of storage inclusions as metabolic buffers for  
463 fluctuating oxygen and nutrient conditions. For example, lithomixotrophic giant sulfur  
464 bacteria like *Thiomargarita* and *Thioploca* survive anoxia by using nitrate stored in vacuoles  
465 as an alternative electron acceptor to partially oxidize sulfide to elemental sulfur. They also  
466 use polyphosphate for energy and can store assimilated carbon as glycogen or PHA (64, 66).

## 467 **Conclusion**

468 We have shown that a diverse and widespread clade of symbiotic sulfur bacteria lacks genes  
469 encoding canonical enzymes for autotrophic  $\text{CO}_2$  fixation, despite being a food source for

470 their hosts. This is unlike all other thiotrophic symbionts sequenced to date, which possess  
471 the CBB or rTCA cycles for autotrophy. We propose a lithoheterotrophic model for the  
472 *Kentrophoros* nutritional symbiosis, which challenges the chemoautotrophic paradigm  
473 usually applied to thiotrophic symbiosis. Uptake of organic substrates from the environment,  
474 heterotrophic carboxylation, and recycling of host waste may play a bigger part in thiotrophic  
475 symbioses than previously thought. Our results suggest that nutritional symbioses can also be  
476 supported by chemolithoheterotrophy, and that thiotrophic symbioses fall on a spectrum  
477 between autotrophy and heterotrophy.

## 478 **Materials and Methods**

### 479 ***Sample collection***

480 Specimens of *Kentrophoros* were collected in 2013 and 2014 from Elba, Italy (Mediterranean  
481 Sea), in 2015 from Twin Cayes, Belize (Caribbean Sea), and in 2016 from Nivå Bay,  
482 Denmark (Øresund Strait between Baltic and North Sea), as previously described (20).  
483 Sampling localities and dates, as well as the number of specimens and phylotypes that were  
484 sequenced are given in Supplementary Table 1.

### 485 ***DNA/RNA extraction and sequencing***

486 Samples for DNA and RNA extraction, comprising single ciliate cells and their symbionts,  
487 were fixed in RNAlater (Ambion) and stored at 4 °C. Before DNA extraction, samples were  
488 centrifuged (8000 g, 5 min) and excess RNAlater was removed by pipetting. DNA was  
489 extracted with the DNeasy Blood and Tissue kit (Qiagen) following manufacturer's  
490 instructions, and eluted in 50 µL of buffer AE. DNA concentration was measured  
491 fluorometrically with the Qubit DNA High-Sensitivity kit (Life Technologies). Each DNA  
492 sample was screened by PCR with eukaryotic 18S rRNA primers EukA/EukB (67) followed

493 by capillary sequencing to identify the *Kentrophoros* phylotype, as previously described (20).  
494 Libraries for metagenomic sequencing were prepared with the Ovation Ultralow Library  
495 System V2 kit (NuGEN) following manufacturer's protocol. Libraries were sequenced as  
496 either 100 or 150 bp paired-end reads on the Illumina HiSeq 2500 platform.  
497 RNA was extracted with the RNeasy Plus Micro Kit (Qiagen) following manufacturer's  
498 protocol, and eluted in 15  $\mu$ L RNase-free water. cDNA was synthesized with the Ovation  
499 RNASeq System v2 (NuGEN) following manufacturer's protocol, sheared to 350 bp target  
500 size with Covaris microTUBE system, cleaned up with Zymo Genomic DNA Clean &  
501 Concentrator Kit. Sequencing library was prepared from cDNA with NEBNext Ultra DNA  
502 library preparation kit for Illumina, and sequenced on the Illumina HiSeq 2500 platform as  
503 100 bp single-end reads.  
504 Library preparation and sequencing were performed at the Max Planck Genome Centre  
505 Cologne, Germany (<http://mpgc.mpiiz.mpg.de/home/>).

### 506 ***Assembly, binning, and annotation of symbiont genomes***

507 Reads were trimmed from both ends to remove fragments matching Truseq adapters, and to  
508 remove bases with Phred quality score  $< 2$ , using either Nsoni v0.111  
509 (<https://github.com/Victorian-Bioinformatics-Consortium/nsoni>) or BBmap v34+  
510 (<https://sourceforge.net/projects/bbmap/>). Trimmed reads were error-corrected with  
511 BayesHammer (68). Error-corrected reads were assembled with IDBA-UD v1.1.1 (69) or  
512 SPAdes v3.5.0+ (68) to produce the initial assembly. The reference coverage of each contig  
513 was obtained by mapping the error-corrected read set against the assembly with BBmap  
514 (“fast” mode). Conserved marker genes in the assembly were identified and taxonomically  
515 classified with Amphora2 (70) or Phyla-Amphora (71). 16S rRNA genes were identified with

516 Barrnap v0.5 (<https://github.com/tseemann/barrnap>) and classified by searching against the  
517 Silva SSU-Ref NR 119 database (72) with Usearch v8.1.1831 (73). Differential coverage  
518 information (74) was obtained by mapping reads from other samples of the same host  
519 morphospecies onto the assembly with BBmap. Contigs belonging to the primary  
520 *Kentrophoros* symbiont (the “primary symbiont bin”) were heuristically identified by a  
521 combination of differential coverage, assembly graph connectivity, GC%, affiliation of  
522 conserved marker genes, and affiliation of 16S rRNA sequence using gbtools v2.5.2 (75).  
523 Reads mapping to the primary symbiont bin were reassembled with SPAdes. Binning and  
524 reassembly of the primary symbiont genome was iteratively repeated for each metagenome  
525 sample until the primary symbiont bin appeared to contain only a single genome, based on  
526 the number and taxonomic affiliation of conserved marker genes and 16S rRNA. For final  
527 genome bins, summary statistics were computed with Quast v4.4 (76), and completeness and  
528 contamination were estimated with CheckM v1.0.11 (77) using the Gammaproteobacteria  
529 taxonomy workflow. Average amino-acid identity (AAI) and average nucleotide identity  
530 (ANI) values between genomes were calculated with CompareM v0.0.21 ([https://github.com/](https://github.com/dparks1134/CompareM)  
531 [dparks1134/CompareM](https://github.com/dparks1134/CompareM)) and jSpecies v1.2.1 respectively (78).  
532 Genome bins were annotated with the IMG/M pipeline for downstream analyses (79).  
533 Metabolic pathways were predicted from the annotated proteins with the PathoLogic module  
534 (80) of Pathway Tools v20.5 (81), followed by manual curation. Metabolic modules from  
535 KEGG (82) were also predicted with the KEGG Mapper tool  
536 (<http://www.kegg.jp/kegg/mapper.html>, accessed Jan 2017) from KEGG Orthology terms in  
537 the IMG annotation.

### 538 ***Core- and pan-genome analysis***

539 Ortholog clusters of Kentron protein sequences were predicted by first performing a

540 reciprocal Blastp (version 2.2.29+) search (83) of all translated open reading frames (ORFs)  
541 annotated by the IMG pipeline (E-value cutoff  $10^{-5}$ ), and then identifying clusters in the  
542 search results with the Markov cluster algorithm (84) using FastOrtho (inflation value 1.5),  
543 which is a reimplementation of OrthoMCL (85) by the PATRIC project (86). Accumulation  
544 curves and uncertainty estimates for the core and pan genome size were generated by random  
545 resampling ( $n = 200$ ) of genome memberships for the predicted orthologs.

### 546 ***Transcriptome analysis***

547 Metatranscriptome reads for *Kentrophoros* sp. H and SD were mapped on to symbiont  
548 genome assemblies from the respective species (IMG genome IDs 2609459750 and  
549 2615840505) using BBmap (minimum identity 0.97). Read counts per genomic feature were  
550 calculated with featureCounts v1.5.2 (87), and transformed into FPKM values (fragments per  
551 kbp reference per million reads mapped).

### 552 ***Verifying absence of key genes for autotrophic pathways***

553 Key enzymes that are diagnostic for known autotrophic pathways were identified from the  
554 literature (42, 88–90) (Supplementary Table 3). These were absent from Kentron genome  
555 annotations, with the exception of a RuBisCO-like protein (RLP) in Kentron sp. H (see  
556 below). To verify that the absence of autotrophy-related sequences was not caused by  
557 incomplete genome bins, misprediction of open reading frames, or misassembly of the reads,  
558 we aligned raw reads from host-symbiont metagenomes and metatranscriptomes against the  
559 UniProt SwissProt database (release 2017\_01) (91) using diamond blastx (v0.8.34.96,  
560 “sensitive” mode) (92). Sequences for certain key enzymes were absent from SwissProt, so  
561 representative sequences from UniProtKB were manually added to the database  
562 (Supplementary Table 3, Supplementary File 4). Reads with hits to target enzymes (identified

563 by EC number or from the list of additional sequences) were counted, extracted, and mapped  
564 against the initial metagenomic assembly for the corresponding library. Raw counts of reads  
565 were transformed to FPKM values using three times the mean amino acid length of the target  
566 proteins as the reference length. As a comparison, FPKM values were also calculated for a  
567 reference set of enzymes of the TCA cycle and partial 3HPB pathway (Supplementary Table  
568 3), which were annotated in Kentron genomes and thus expected to have much higher  
569 coverage than the putatively absent genes.

### 570 ***Identification of transporter genes for substrate uptake***

571 Families and subfamilies of transporter proteins from the Transporter Classification Database  
572 (TCDB, accessed 2 Feb 2017) (93) that were described as energy-dependent uptake  
573 transporters for organic substrates were shortlisted (Supplementary Table 5). Translated ORFs  
574 for Kentron and selected genomes of other symbiotic and free-living basal  
575 Gammaproteobacteria (Supplementary Table 6) were aligned with Blastp (83) (best-scoring  
576 hit with E-value  $< 10^{-5}$ ,  $>30\%$  amino acid sequence identity, and  $>70\%$  coverage of reference  
577 sequence, parameters from (53)) against TCDB. As TCDB also includes non-membrane  
578 proteins that are involved in transport (e.g. ATPase subunit of ABC transporters), we also  
579 counted how many hits contained transmembrane domains, predicted with tmhmm v2.0c  
580 (94). To compare the transporter content between genomes, the tabulated counts of organic  
581 substrate uptake TC family hits per genome were analyzed by non-metric multidimensional  
582 scaling (NMDS) with the metaMDS function in the R package vegan v2.5.1  
583 (<https://CRAN.R-project.org/package=vegan>) (Bray-Curtis distance, 2 dimensions, 2000  
584 runs).

585 **Phylogenetic analyses**

586 Maximum-likelihood phylogenetic trees were inferred from the following alignments with  
587 Fasttree v2.1.7 (95), using the JTT model with CAT approximation (20 rate categories) and  
588 SH-like support values.

589 **Kentron and related Gammaproteobacteria.** Conserved marker genes from Kentron and  
590 selected basal Gammaproteobacteria (Supplementary Table 6) were extracted by the  
591 Amphora2 pipeline. Amino acid sequences of 30 markers were aligned with Muscle v3.8.31  
592 (96) and concatenated.

593 **RuBisCO-like protein from Kentron sp. H.** RuBisCO superfamily protein accessions and  
594 their classification were obtained from (25). These were aligned with RuBisCO-like protein  
595 sequences from Kentron sp. H and RuBisCO from selected sulfur-oxidizing symbiotic  
596 Gammaproteobacteria, using Muscle.

597 **Proteins of partial 3-hydroxypropionate bi-cycle.** Homologs to proteins of the 3-  
598 hydroxypropionate bi-cycle in *Chloroflexus aurantiacus* were obtained from the UniRef50  
599 clusters containing the *C. aurantiacus* sequences in the UniProt database. These were aligned  
600 with the Kentron homologs with Muscle.

601 **Protein extraction and peptide preparation**

602 Samples of *Kentrophoros* sp. H for proteomics were collected by decantation from sediment  
603 adjacent to seagrass meadows at Sant' Andrea, Isola d'Elba, Italy on 3 June 2014, and from  
604 Pampelonne Beach, Provence-Alpes-Côte d'Azur, France in July 2018. Ciliates were  
605 individually fixed in RNAlater, and subsequently stored at 4 °C and then at -80 °C. One  
606 individual *Kentrophoros* sp. H specimen and nine pooled samples of four or five individuals  
607 each (Supplementary Table 11) were used to prepare tryptic digests following the filter-aided

608 sample preparation (FASP) protocol (97) with minor modifications (98). Samples were lysed  
609 in 30  $\mu$ l of SDT-lysis buffer (4% (w/v) SDS, 100 mM Tris-HCl pH 7.6, 0.1 M DTT) by  
610 heating to 95 °C for 10 min. To avoid sample losses we did not clear the lysate by  
611 centrifugation after lysis. Instead, we loaded the whole lysate on to the 10 kDa filter units  
612 used for the FASP procedure. The Qubit Protein Assay Kit (Thermo Fisher Scientific, Life  
613 Technologies) was used to determine peptide concentrations, following the manufacturer's  
614 instructions. Peptide concentrations were below the detection limit in all samples.

### 615 **1D-LC-MS/MS**

616 All peptide samples were analyzed by 1D-LC-MS/MS as previously described (99), with the  
617 modification that a 75 cm analytical column was used. Briefly, an UltiMate 3000 RSLCnano  
618 Liquid Chromatograph (Thermo Fisher Scientific) was used to load peptides with loading  
619 solvent A (2% acetonitrile, 0.05% trifluoroacetic acid) onto a 5 mm, 300  $\mu$ m ID C18 Acclaim  
620 PepMap100 pre-column (Thermo Fisher Scientific). Since peptide concentrations were very  
621 low, complete peptide samples (80  $\mu$ L) were loaded onto the pre-column. Peptides were  
622 eluted from the pre-column onto a 75 cm  $\times$  75  $\mu$ m analytical EASY-Spray column packed  
623 with PepMap RSLC C18, 2  $\mu$ m material (Thermo Fisher Scientific) heated to 60° C.  
624 Separation of peptides on the analytical column was achieved at a flow rate of 225 nL min<sup>-1</sup>  
625 using a 460 min gradient going from 98% buffer A (0.1% formic acid) to 31% buffer B  
626 (0.08% formic acid, 80% acetonitrile) in 363 min, then to 50% B in 70 min, to 99% B in 1  
627 min and ending with 26 min 99% B. Eluting peptides were analyzed in a Q Exactive Plus  
628 hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific). Carryover was  
629 reduced by running two wash runs (injection of 20  $\mu$ L acetonitrile) between samples. Data  
630 acquisition in the Q Exactive Plus was done as previously described (5).



631 ***Protein identification and quantification***

632 A database containing protein sequences predicted from the *Ca. Kentron* genomes described  
633 above and predicted protein sequences from a preliminary host transcriptome was used for  
634 protein identification. The *Ca. Kentron* protein sequences were clustered at 98% identity with  
635 CD-HIT v4.7 (100), and only the representative sequences were used for the protein  
636 identification database. The cRAP protein sequence database (<http://www.thegpm.org/crap/>),  
637 which contains sequences of common lab contaminants, was appended to the database. The  
638 final database contained 5,715 protein sequences. For protein identification, MS/MS spectra  
639 were searched against this database using the Sequest HT node in Proteome Discoverer  
640 version 2.2 (Thermo Fisher Scientific) as previously described (5).

641 ***Direct Protein-SIF***

642 Stable carbon isotope fingerprints (SIFs =  $\delta^{13}\text{C}$  values) for *Ca. Kentron* symbiosis were  
643 determined using the proteomic data (36). Briefly, human hair with a known  $\delta^{13}\text{C}$  value was  
644 used as reference material to correct for instrument fractionation. A tryptic digest of the  
645 reference material was prepared as described above and analyzed with the same 1D-LC-MS/  
646 MS method as the samples. The peptide-spectrum match (PSM) files generated by Proteome  
647 Discoverer were exported in tab-delimited text format. The 1D-LC-MS/MS raw files were  
648 converted to mzML format using the MSConvertGUI available in the ProteoWizard tool suite  
649 (101). Only the MS<sup>1</sup> spectra were retained in the mzML files and the spectra were converted  
650 to centroided data by vendor algorithm peak picking. The PSM and mzML files were used as  
651 input for the Calis-p software (<https://sourceforge.net/projects/calis-p/>) to extract peptide  
652 isotope distributions and to compute the direct Protein-SIF  $\delta^{13}\text{C}$  value for *Ca. Kentron* and  
653 the human hair reference material (36). The direct Protein-SIF  $\delta^{13}\text{C}$  values were corrected for  
654 instrument fragmentation by applying the offset determined by comparing the direct Protein-

655 SIF  $\delta^{13}\text{C}$  value of the reference material with its known  $\delta^{13}\text{C}$  value. We obtained between 50  
656 and 499 peptides with sufficient intensity for direct Protein-SIF from seven of the nine pooled  
657 samples (Supplementary Table 11). These samples were thus well above the necessary  
658 number of peptides needed to obtain an accurate estimate. Due to the low biomass of the  
659 individual *Kentrophoros* specimen (~ 10  $\mu\text{g}$ ) only 14 peptides with sufficient intensity for  
660 direct Protein-SIF were obtained for this sample. This lower number of peptides for the  
661 individual specimen can potentially lead to a lower accuracy of the respective SIF value,  
662 however, since the value fell in the same range as for the pooled samples we assume that the  
663 estimate is sufficiently accurate.

#### 664 ***Dissolved inorganic carbon $\delta^{13}\text{C}$***

665 Seawater and porewater samples were collected from the vicinity of seagrass meadows at  
666 Sant' Andrea, Elba, Italy in July 2017 to determine the  $\delta^{13}\text{C}$  of dissolved inorganic carbon  
667 (DIC). Seawater was sampled at the surface from a boat, whereas porewater was sampled at  
668 15 cm sediment depth with a steel lance. Samples were drawn into 20 mL plastic syringes;  
669 6 mL of each was fixed with 100  $\mu\text{L}$  of 300 mM  $\text{ZnCl}_2$ , and stored at 4  $^\circ\text{C}$  until processing.  
670  $\delta^{13}\text{C}$  was measured with a Finnigan MAT 252 gas isotope ratio mass spectrometer with  
671 Gasbench II (Thermo Scientific), using Solnhofen limestone as a standard and 8 technical  
672 replicates per sample.

#### 673 ***Data availability***

674 Annotated genomes are available on the Joint Genome Institute GOLD database  
675 (<https://gold.jgi.doe.gov/>) under study Gs0114545. Metagenomic and metatranscriptomic  
676 sequence libraries are deposited in the European Nucleotide Archive under study accessions  
677 PRJEB25374 and PRJEB25540 respectively. The mass spectrometry metaproteomics data,

678 direct Protein-SIF relevant files, and protein sequence database have been deposited to the  
679 ProteomeXchange Consortium via the PRIDE partner repository  
680 (<https://www.ebi.ac.uk/pride/archive/>) with the dataset identifier PXD011616. [Reviewer  
681 access: username – reviewer32857@ebi.ac.uk, password – eLmqKA0b.] Supplementary files  
682 are available via Zenodo at doi:10.5281/zenodo.2555833.

### 683 **Code availability**

684 Scripts used to screen for autotrophy-related genes in metagenome libraries, to classify  
685 transporter families, and to calculate phylogenetic trees are available at  
686 <https://github.com/kbseah/mapfunc>, [https://github.com/kbseah/tcdbparse\\_sqlite](https://github.com/kbseah/tcdbparse_sqlite), and  
687 <https://github.com/kbseah/phylogenomics-tools> respectively.

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## 713 **Author contributions**

714 BS, ND, HGV designed study. BS, HGV performed field work. BH prepared sequencing  
715 libraries with BS and coordinated sequencing. ML performed metabolomics mass  
716 spectrometry analyses. AK prepared samples and generated data for proteomics. MK and AK  
717 processed and analyzed proteomics data. MK performed protein-SIF analysis. BS, CPA, JZ,  
718 LSvB, TJE, ML, HGV analyzed genomics and transcriptomics data. BS wrote manuscript  
719 draft. All authors participated in revising manuscript.

## 720 **Competing interests**

721 None declared.

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