Reconciling a Model of Core Metabolism with Growth Yield Predicts Biochemical Mechanisms and Efficiency for a Versatile Chemoautotroph

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Abstract:

Obligately chemoautotrophic *Campylobacteria* dominate productivity in dark, sulfidic, and oxygen-depleted environments. However, biochemical mechanisms underlying their growth remain poorly known, limiting understanding of their physiology, ecology, and biogeochemical impact. In this study, we used comparative genomics, conceptual modeling of core metabolism, and chemostat growth yields to derive a model of energy conservation consistent with experimental data for the versatile chemoautotroph *Sulfurimonas denitrificans*. Our model rests on three core mechanisms: Firstly, to allow electrogenic sulfur-based denitrification, we predict that the campylobacterial-type sulfur oxidation enzyme complex must donate electrons to the membrane quinone pool, possibly via a sulfide:quinone oxidoreductase. Secondly, to account for the unexpectedly low growth efficiency of aerobic sulfur oxidation compared to denitrification, we posit the high-affinity campylobacterial-type *cbb₃* cytochrome c oxidase has a relatively low H⁺/e⁻ of 1, likely due to a lack of proton pumping under physiological conditions. Thirdly, we hypothesize that reductant for carbon fixation by the reverse tricarboxylic acid cycle is produced by a non-canonical complex I that reduces both ferredoxin and NAD(P)H. This complex is conserved among related *Campylobacteria* and may have allowed for the radiation of organisms like *S. denitrificans* into sulfur-rich environments that became available after

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30 the great oxidation event. Our theoretical model has two major implications. Firstly, it sets the stage for
 31 future experimental work by providing testable hypotheses about the physiology, biochemistry, and
 32 evolution of chemoautotrophic *Campylobacteria*. Secondly, it provides constraints on the carbon
 33 fixation potential of chemoautotrophic *Campylobacteria* in sulfidic environments worldwide by
 34 predicting theoretical ranges of chemosynthetic growth efficiency.

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36 Significance:

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38 Chemoautotrophic *Campylobacteria* are abundant in many low-oxygen, high-sulfide
 39 environments where they contribute significantly to dark carbon fixation. Although the overall redox
 40 reactions they catalyze are known, the specific biochemical mechanisms that support their growth are
 41 mostly unknown. Our study combines conceptual modeling of core metabolic pathways, comparative
 42 genomics, and measurements of physiological growth yield in a chemostat to infer the most likely
 43 mechanisms of chemoautotrophic energy conservation in the model organism *Sulfurimonas*
 44 *denitrificans*. The hypotheses proposed herein are novel, experimentally falsifiable, and will guide
 45 future biochemical, physiological, and environmental modelling studies. Ultimately, investigating the
 46 core mechanisms of energy conservation will help us better understand the evolution and physiological
 47 diversification of chemoautotrophic *Campylobacteria* and their role in modern ecosystems.

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49 Introduction:

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51 At deep-sea hydrothermal vents, chemoautotrophic microbes support entire food webs by fixing
 52 inorganic carbon into biomass (1, 2). Chemoautotrophs from the class *Campylobacteria* often dominate
 53 the microbial communities at deep-sea vents and in other sulfidic environments, coupling the oxidation
 54 of hydrogen and reduced sulfur compounds to the reduction of nitrate, oxygen and sulfur to support
 55 autotrophic CO₂ fixation via the reverse tricarboxylic acid (rTCA) cycle (3–6). Aside from their
 56 important environmental role, the *Campylobacteria* are of interest from an evolutionary perspective.
 57 Long known as the epsilon subdivision of the *Proteobacteria*, this group has been recently reclassified
 58 as the class *Campylobacteria* in the new phylum *Campylobacteraeota* to reflect their divergent

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59 evolutionary heritage (6). Recent data suggest that *Campylobacteria* possess a number of unique
60 biochemical characteristics that may be essential for their success as chemoautotrophs in sulfidic
61 environments (7–11). Despite these data, we still lack information on the specific mechanisms of
62 chemoautotrophic energy conservation for *Campylobacteria*. One major reason for this knowledge gap
63 is that their fastidious microaerophilic/anaerobic growth phenotype makes them difficult to manipulate
64 experimentally.

65 In this study, we addressed these unknowns by developing an experimentally-consistent
66 theoretical model of core metabolism for the campylobacterial chemoautotroph *Sulfurimonas*
67 *denitrificans* (12, 13). This organism is metabolically-flexible, capable of using sulfide, sulfur,
68 thiosulfate, and hydrogen as electron donors as well as oxygen or nitrate as terminal electron acceptors.
69 In addition, *S. denitrificans* is comparatively well-studied from a physiological and genomic
70 perspective among chemoautotrophic *Campylobacteria* (12, 14–19). This makes *S. denitrificans* an
71 ideal candidate to investigate multiple different energy-conserving processes and their potential
72 biochemical underpinnings.

73 To construct our conceptual model, we first identified potential pathways that support energy
74 conservation in *S. denitrificans* using comparative genomics and biochemical knowledge from related
75 organisms (15, 20). This resulted in a number of possible mechanisms and/or proton translocation
76 stoichiometries for core energy-conserving protein complexes. Next, biomass yields measured in
77 continuous culture (this study, (14) were compared with yields predicted from these putative models to
78 determine the most parsimonious biochemical mechanism for chemoautotrophic growth in *S.*
79 *denitrificans*. Below, we discuss the logic of model construction and how it led us to predict novel
80 mechanisms of energy conservation. We also propose direct experimental tests and discuss the
81 implications of these hypothetical mechanisms for understanding the bioenergetics, evolution, and
82 niche differentiation of diverse chemoautotrophic *Campylobacteria*.

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84 Results and Discussion:

85 We developed a theoretical model of energy conservation coupled to carbon fixation by the
86 rTCA cycle for the chemoautotrophic bacterium *Sulfurimonas denitrificans* (Table 1; Figure 1). To
87 determine the most parsimonious model, we compared predicted relative growth yield ratios (RGY) of

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hypothetical mechanisms to substrate growth yield ratios measured in a chemostat (Table 2; 14). Using this approach, we eliminated implausible mechanisms and converged on a model that accurately reproduced RGY in *S. denitrificans* under three distinct growth conditions (Tables 1 and 2; see discussion below and in SI). Our model also allowed us to constrain the chemosynthetic growth efficiency (CGE) of *S. denitrificans*. CGE is a measure of the overall efficiency of metabolism, representing the fraction of total electrons from electron donors used to fix CO₂ (equivalent to 1 - γ in reference (21); Table 1). While the biochemical reactions that we inferred have explicit stoichiometry, they could not directly be used to calculate CGE. This is because CGE is also dependent on the amount of proton-motive force (PMF) required for growth in addition to carbon fixation (e.g. ATP/PMF for other assimilatory/biosynthetic reactions or for motility/transport). This quantity, hereafter referred to as “non-rTCA-PMF” was also inferred from our model and RGY data as follows.

Assuming that the amount of non-rTCA-PMF is identical between growth conditions, both RGY ratios (H/N : T/N and T/O : T/N) will approach 2 as the amount of non-rTCA-PMF relative to that needed for carbon fixation increases (Figures S1 and S2). Using this information, we inferred the proportion of non-rTCA-PMF and a range of theoretical CGE (Table 1; see SI for more detail). These theoretical values were very close to chemostat yield measurements previously reported for *S. denitrificans* and other members of the genus *Sulfurimonas* (0.167 and 0.098 - 0.148, for microaerobic and denitrifying growth respectively; summarized in 21). Although CGE will ultimately be dependent on the fraction of non-rTCA-PMF required under a given growth condition, our theoretical approach allows us to calculate explicit bounds on CGE and therefore constrain maximal primary productivity in ecosystems dominated by chemoautotrophic *Campylobacteria*. Below, we describe the three biochemical mechanisms predicted from our approach and their implications for understanding the evolution and ecology of chemoautotrophic *Campylobacteria*.

Thiosulfate-Dependent Denitrification:

S. denitrificans was isolated in a nitrate-limited chemostat with thiosulfate as the sole electron donor (12). We initially based our model of sulfur oxidation on previously-validated biochemical mechanisms for the SO_x sulfur oxidation system, whereby it donates electrons at the level of soluble cytochrome c (22, 23). However, this canonical SO_x mechanism cannot support electrogenic

thiosulfate-dependent denitrification since electrons would have to be transferred endergonically (“uphill”) to the quinone-dependent Nap/QcrABC nitrate reductase supercomplex (24) and the nitrous-oxide reducing Nos/QcrABC supercomplex (25). Although the intervening enzymatic steps in denitrification (nitrite and nitric oxide reduction) likely accept electrons at the level of cytochrome c, these complexes are thought to be non-electrogenic, meaning no energy could be generated with thiosulfate/nitrate in the absence of QcrABC (24–26). We therefore concluded that the most likely explanation is the existence of an alternative “campylobacterial-SOx” system that donates electrons from sulfur oxidation to the membrane quinone pool (Fig 1a).

Biochemical data tentatively support the existence of a membrane-bound SOx complex in chemoautotrophic *Campylobacteria* and other sulfur-oxidizing bacteria. In *Sulfurovum* NBC37-1, both membrane-bound and soluble protein fractions are necessary for thiosulfate oxidation (8); similar data have been reported for the alphaproteobacterium *Starkeya novella* (27, 28). What protein(s) potentially serve as a membrane anchor for the SOx system in *S. denitrificans* remains unknown. Yamamoto et al. (2010) identified several proteins involved in thiosulfate oxidation that have putative membrane-binding motifs, including SoxH, a cytochrome c, and a sulfide:quinone oxidoreductase (SQR)-like protein. SQR proteins are an attractive candidate for an campylobacterial-SOx anchor as they are known to be membrane-bound and capable of sulfide-dependent quinone reduction in *S. denitrificans* and other bacteria (17, 29). Consistent with its possible role in thiosulfate oxidation, RT-qPCR shows that a putative SQR in *S. denitrificans* (Suden_0619) is highly expressed in the absence of sulfide (Figs S3 and S4).

By including the campylobacterial-SOx complex, our model was able to closely reproduce experimental RGY data between hydrogen and thiosulfate-based denitrification conditions (Table 2) (16). We predict higher growth yields with hydrogen because the membrane-spanning hydrogenase in *S. denitrificans* (15) abstracts protons from the cytoplasm during hydrogen-dependent reduction of menaquinone. When menaquinol is reoxidized, protons are released into the periplasm (Fig 1b), thus generating a proton-motive force via a redox-loop mechanism (20). In contrast, periplasmic sulfur-oxidizing enzymes likely do not span the membrane (8, 30). Therefore, thiosulfate-dependent quinone reduction itself is probably electroneutral, although a proton-motive force is still generated by the action of complex III (QcrABC). QcrABC oxidizes quinol and either transfers electrons to soluble

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146 cytochrome c or acts together as a supercomplex with the Nap/Nos complexes that reduce nitrate and
147 nitrous oxide respectively (Fig 1). The overall effect is that QcrABC makes electron transport to nitrite,
148 nitric oxide and nitrous oxide an electrogenic process (25). Complex III will also conserve energy with
149 hydrogen oxidation, which is in addition to the energy from the aforementioned redox loop.

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151 Aerobic Respiration vs. Denitrification:

152 *S. denitrificans* is able to grow microaerobically with thiosulfate, which yields ~ 60 % more
153 biomass than with thiosulfate/nitrate (Table 2; (14)). Microaerobic respiration is most likely sustained
154 by a high-affinity cytochrome c oxidase (cbb₃; complex IV) as no other putative oxygen-reducing
155 complexes are present in the genome. The number of protons pumped by cbb₃ is controversial, but
156 some have suggested that it has the potential to equal low-affinity forms of complex IV (i.e.
157 mitochondrial aa₃; (31)). However, modelling *S. denitrificans*' growth using the same proton-pumping
158 stoichiometry as aa₃ coupled to complex III (3 H⁺/e⁻; (20)) presented a problem - both CGE and the
159 RGY between aerobic and denitrifying conditions were far higher than observed in this study (Tables 1
160 and 2) and in (14).

161 To reconcile our model with growth yield data, the most parsimonious explanation is that
162 complex IV in *S. denitrificans* has an overall H⁺/e⁻ of 1 (in contrast to the canonical aa₃ complex which
163 both pumps protons and consumes cytoplasmic protons for a total of a H⁺/e⁻ of 2). This could occur if
164 either no protons are pumped during aerobic respiration or if the protons used in the reduction of
165 oxygen (hereafter "chemical protons") are abstracted from the periplasm instead of the cytoplasm as is
166 known for the aa₃ complex (Fig 1c). We believe the former explanation is more likely, as experiments
167 have shown that cbb₃-type cytochrome c oxidases behave differently during *in vitro* biochemical assays
168 (32) and cannot sustain proton pumping at physiologically-relevant membrane potentials ((33); see also
169 SI). This is due to the fact that high-affinity cytochrome c oxidases use more free energy in binding
170 their substrate, resulting in less energy available to pump protons against a membrane gradient (33).
171 We also note several differences exist between the "campylobacterial-type cbb₃" found in *S.*
172 *denitrificans* and other bacteria. For example, the *fixGHIS* genes are missing (15), as well as a number
173 of histidine residues conserved elsewhere (34) - one of which has been shown to have an important
174 catalytic role (35).

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176 Reversed Electron Transport:

177 Another pathway essential to the growth of *S. denitrificans* is the chemoautotrophic fixation of
 178 CO₂ via the rTCA cycle (36). In order to support carbon fixation, reduced ferredoxin is required as an
 179 electron donor for reductive carboxylation of succinyl-CoA to alpha-ketoglutarate and acetyl-CoA to
 180 pyruvate (36, 37). However, how reduced ferredoxin is produced in chemoautotrophic
 181 *Campylobacteria* is unclear (38). Sievert et al. (2008) suggested that hydrogen-dependent electron
 182 bifurcation could generate both NAD(P)H and reduced ferredoxin. However, since this mechanism
 183 depends on hydrogen, it cannot reduce ferredoxin with sulfur compounds as an electron donor. In
 184 addition, the soluble hydrogenase hypothesized to be responsible by Sievert et al. (2008) is not
 185 expressed at high levels in mRNA under a variety of conditions (Figs S3 and S4; (19)). No known
 186 electron-bifurcating complexes are present in the *S. denitrificans* genome (38), nor is there evidence for
 187 complexes that couple endergonic ferredoxin reduction to the proton motive force (i.e. the Rnf
 188 complex; (39)).

189 A more plausible mechanism for ferredoxin production in *Campylobacteria* was uncovered by
 190 Weerakoon and Olson (2008) in a study of the heterotroph *Campylobacter jejuni*. These authors report
 191 that the NADH:ubiquinone oxidoreductase (complex I) in *C. jejuni* does not accept electrons from
 192 NADH. Instead, reduced flavodoxin serves as the physiological electron donor during heterotrophic
 193 growth. Since flavodoxin can substitute for ferredoxin (40), such a non-canonical complex I run in
 194 reverse has the potential to reduce ferredoxin. Weerakoon and Olson (2008) demonstrated that this
 195 unusual mechanism is due to differences in the NuoE/F subunits that interact with NADH in canonical
 196 complex I. They also note that *S. denitrificans* and other chemoautotrophic *Campylobacteria* possess
 197 non-canonical NuoEF subunits and two copies of NuoG. Based on currently available genomes, this
 198 non-canonical complex I subunit composition is universally conserved among the chemoautotrophic
 199 genera *Sulfurimonas* and *Sulfurovum* (15, 41–45)(NZ_CP011308). Considering this significant and
 200 evolutionarily-conserved structural difference in complex I among chemoautotrophic *Campylobacteria*,
 201 it appears likely to have an essential role in core metabolism. However, NAD(P)H is also needed as a
 202 reductant for the rTCA cycle, and the mechanism demonstrated by Werakoon and Olson does not
 203 involve NAD(P)H. In *Hydrogenobacter thermophilus*, a “plant-like” ferredoxin:NADP⁺ reductase

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transfers electrons from ferredoxin to NADPH (46), whereas in *Nitrospina gracilis* genomic evidence suggests two distinct complexes may be specialized for NAD(P)H or ferredoxin reduction, respectively (47). However, there is no evidence for either of these mechanisms in *S. denitrificans*.

How then does *S. denitrificans* then produce NAD(P)H? An important clue comes from comparison of the protein replacing NuoF in *S. denitrificans* (Suden_1824) to known electron-bifurcating complexes. We note that Suden_1824 is homologous to the NfnB subunit of the NfnAB complex of *Thermotoga maritima* (33% AA ID; 88% coverage; Fig S5) which transfers electrons from NADPH to both reduced ferredoxin/NADH during flavin-based electron bifurcation (48); this protein was also recently shown to bind NADPH and probably also ferredoxin (49). Relevant residues for binding the phosphate group of NADP(H) (Tyr310, Arg311-312 in *T. maritima*) and cysteines ligating the iron-sulfur clusters that putatively pass electrons to ferredoxin are conserved in Suden_1824, as well as most residues involved in binding FAD ((49); Fig S5). Therefore, we propose that the function of complex I in *S. denitrificans* is to reduce NAD(P)⁺ and ferredoxin simultaneously during reversed electron transport. In this hypothetical reaction, 4 electrons derived from two menaquinol molecules reduce one molecule of NAD(P)⁺ and two molecules of ferredoxin via an electron-bifurcation mechanism (Fig 1d). We hypothesize that this endergonic reaction is driven by the transport of eight protons from the periplasm into the cytoplasm. A similar mechanism has been proposed in anammox bacteria to produce both NADH and formate simultaneously based on a similar non-canonical complex I structure (50). We also note recent work showing that modifications to this core bioenergetic complex are commonly found in other taxa, presumably to accommodate different electron carriers or increase proton-pumping efficiency (51).

If our hypothesis is true, then a site for electron bifurcation must exist in *S. denitrificans*' complex I. One possible location is in the NuoG subunit, which contains an iron-sulfur cluster (N7) that is separated from the electron transfer pathway and thought to be an evolutionary relic in organisms such as *E. coli* (52). However, NuoG in *Campylobacteria* and *Aquifex aeolicus* contain four conserved cysteine residues that have been shown to ligate an additional iron-sulfur cluster in *A. aeolicus* (34, 53, 54). This additional cluster may connect the N7 “relic” iron-sulfur cluster to electron transport, allowing electron bifurcation to occur at N4. In addition, *S. denitrificans* and close relatives contain two distinct copies of NuoG, both of which are expressed at a similar level in transcriptomes of *S.*

233 *denitrificans* (19). These two copies have similar sequences (36 % amino acid identity), but differ in
 234 terms of length. The short subunit (Suden_1823) is “missing” two main regions (Fig S6). The first (AA
 235 466-532 of Suden_1822) contains no obvious functional motifs. The second missing region (AA 175-
 236 204 of Suden_1822) is found between two cysteine residues that putatively bind an additional iron-
 237 sulfur cluster (34). Without this stretch of sequence, the distance between these two cysteines is
 238 shortened to 29 amino acids which is very similar to the *A. aeolicus* NuoG (53). Therefore,
 239 Suden_1823 may be a potential location for electron bifurcation. What then could be the function of the
 240 other NuoG subunit? Perhaps it is involved in shuttling electrons to the non-canonical NuoF subunit, or
 241 maybe it is itself involved in binding ferredoxin (55).

242 The ability to use complex I to produce cellular reductant would have several advantages for *S.*
 243 *denitrificans*. Since this mechanism is dependent on the proton motive force, it would be linked to (and
 244 regulated by) energy generation processes and independent of the electron donor (Fig 1d).
 245 Additionally, it provides both reductants in a 1:1 ratio. Since the rTCA cycle uses electrons from
 246 ferredoxin and NAD(P)H in a 1:1 ratio if fumarate reduction occurs with quinol as the electron donor
 247 (15, 56), this balanced stoichiometry would be important for balancing the redox state of the cell.

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249 Environmental Implications:

250 Our model and experimental data show how different electron donor/acceptor combinations
 251 greatly affect the CGE of *S. denitrificans*. For example, under denitrifying conditions, the use of
 252 hydrogen allows for an approximate doubling of CGE versus the use of thiosulfate (Table 1). Similarly,
 253 since aerobic respiration pumps twice as many protons as denitrification, it is the most efficient
 254 electron-accepting process for *S. denitrificans*. Our model therefore predicts that hydrogen-dependent
 255 aerobic respiration is the most efficient dissimilatory pathway for *S. denitrificans* - approximately 3
 256 times greater than growth under the T/N condition.

257 Future work should include both biochemical and physiological studies to test our hypotheses
 258 (Table S1). These physiological experiments may be challenging to conduct. For example, measuring
 259 the CGE of *S. denitrificans* with hydrogen and oxygen as a donor/acceptor pair is complicated by the
 260 obligate microaerobic nature of *S. denitrificans*. Providing a continuous, non-limiting supply of low
 261 levels of oxygen while simultaneously measuring low levels of consumption will require development

of novel experimental procedures. With regards to biochemical validation, although we have directly identified some biochemical targets (e.g. complex I and IV), others are currently unknown (e.g. the posited membrane anchor of the SOX complex). To address this uncertainty, techniques such as transposon-mutagenesis sequencing (57) may allow for the identification of putative target genes without relying on genetic approaches that are difficult to apply to organisms such as *S. denitrificans*.

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Conclusions:

Previous work has used thermodynamics to estimate the amount of energy available for microbial life at hydrothermal vents and thus their potential for generating new organic carbon (58–60). Our recent field measurements have complemented these theoretical approaches by directly measuring the rates and efficiency of carbon fixation by chemoautotrophs in hydrothermal fluids under natural temperature/pressure conditions (2, 61). Here, we aimed to better understand the mechanistic basis behind these processes by applying a theoretical framework relying on advances in genomics and recent discoveries of bioenergetic mechanisms. Equally important, we also used classical cultivation and growth yield techniques that were last applied to *S. denitrificans* nearly 45 years ago (12, 14). It was only by incorporating these physiological data that we could justify our predictions of novel mechanisms putatively underlying the growth of *S. denitrificans* and similar chemoautotrophic *Campylobacteria*. Not only are our hypotheses parsimonious and consistent with experimental data, they also provide a theoretical framework for designing biochemical experiments (Table S1) and modelling natural ecosystems.

In a broader sense, studying the function and mechanisms of core energy-conserving enzymes will also help understand the evolutionary trajectory of the *Campylobacteria*. This point can be best illustrated by the non-canonical complex I found among the *Campylobacteria*. In contrast to *S. denitrificans*, deep-branching representatives possess a much simpler complex I that completely lacks the NuoEFG subunits that probably interact with ferredoxin and/or NAD(P)H (7, 62). Such a truncated complex resembles so-called energy-converting hydrogenases (Ech) that are thought to be the evolutionary precursors of complex I (63). Since this form of complex I is only found in hydrogen-dependent and oxygen-sensitive taxa, it may represent the ancestral form of this core bioenergetic complex in *Campylobacteria*. In contrast, for oxygen-tolerant organisms such as *S. denitrificans* that do

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291 not rely on hydrogen for growth, complex I is larger and contains additional subunits that were
 292 probably acquired at a later time. We speculate that these additional subunits may have allowed for the
 293 adaptive radiation of chemoautotrophic *Campylobacteria* into oxic, sulfide-rich niches that became
 294 available during the rise of atmospheric oxygen on the ancient Earth (6, 64). Therefore, by studying the
 295 physiology and biochemistry of chemoautotrophic *Campylobacteria*, we can gain insight into key
 296 evolutionary changes that occurred in tandem with the biogeochemical evolution of the biosphere.

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298 Materials and Methods:

299 Development of conceptual model:

300 Based on knowledge from the initial genome characterization of *S. denitrificans* (15), curation
 301 of core energy-conserving metabolic reactions was carried out to reproduce *S. denitrificans*' observed
 302 physiology (14–16). Reactions were based on Fig. 2 from Sievert et al. (2008) with the following
 303 modifications. Cytochrome *cbb*₃ uses only oxygen (not nitric oxide as proposed in Sievert et al. (2008)
 304 since this is currently unsupported by experimental evidence), NorBC is the only nitric oxide reductase
 305 (19), and NosZ accepts electrons from menaquinol via complex III (25). Both thiosulfate and sulfide
 306 oxidation were modeled as complete oxidation to sulfate, transferring electrons to menaquinone instead
 307 of cytochrome *c* as previously described for the SOx complex (23). Neither polysulfide reduction nor
 308 formate oxidation were included as experimental evidence does not currently support these metabolic
 309 pathways, and reverse electron transport was modelled as discussed below.

310 Several postulated functions are not supported by direct biochemical evidence. Even for those
 311 protein complexes whose function has been identified in other organisms (e.g. cytochrome *cbb*₃
 312 oxidase), the stoichiometry of proton translocation *in vivo* is unknown for *S. denitrificans*. In these
 313 cases, several hypothetical mechanisms and/or proton pumping stoichiometries were considered. To
 314 identify which was most realistic, predicted relative growth efficiency (RGY; see Supplemental
 315 Methods for detailed derivation) was compared with yields of *S. denitrificans* grown in continuous
 316 culture (Table 2, (14)). If the predicted growth efficiency was close to the experimental data, the
 317 mechanism was considered plausible and vice-versa. We also inferred maximal chemosynthetic growth
 318 efficiency (CGE) by estimating the fraction of electrons required for carbon fixation via the reverse
 319 tricarboxylic acid (rTCA) cycle vs. other forms of metabolism (non-rTCA-PMF). A value for non-

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320 rTCA-PMF was obtained by comparing RGY between the hydrogen/nitrate condition and
321 thiosulfate/nitrate condition in consideration of the overall metabolic model framework (Figure 1; see
322 supplementary methods and Figs S1 & S2). Once this additional energy required for biosynthesis was
323 determined, we could then derive a range of theoretical CGE values (Table 1).

324 For additional annotation of the *Sulfurimonas denitrificans* genome to support genome-based
325 inferences, operons were predicted using OperonPredictor (65) and transmembrane domains were
326 predicted using the TMHMM program (66). Further details for justifying choices made for the
327 modeling are presented in the supplementary methods.

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329 Growth yield measurements in continuous culture:

330 A custom-built chemostat was used to cultivate *S. denitrificans* (part numbers and design are
331 available upon request). Triplicate temperature-controlled vessels with a liquid volume of 200 mL were
332 fed via a peristaltic pump at an average dilution rate of 0.57 d⁻¹ (the maximum growth rate of *S.*
333 *denitrificans* is 1.44 d⁻¹, (14)). Medium (see supplement) was kept anoxic by constant flushing with an
334 anoxic gas mixture (N₂/CO₂ or H₂/CO₂) at a slight overpressure (27.5 KPa). Cultures were stirred
335 constantly with teflon-coated magnetic stir bars, and all wettable surfaces were either glass, teflon,
336 PEEK tubing (IDEX Health & Science), or pharmed tubing (Cole-Parmer, USA). Light exposure was
337 minimized by wrapping glass cultivation vessels with aluminum foil.

338 For cultivation with H₂/NO₃⁻, thiosulfate was omitted and H₂/CO₂ (80:20) was the headspace
339 gas. Cultivation was carried out in a fume hood to prevent accumulation of flammable gas. An excess
340 of dissolved hydrogen in the medium vessel was confirmed during cultivation by headspace extraction
341 of a small volume of filtered culture and injection into a gas chromatograph. For cultivation with
342 S₂O₃²⁻/NO₃⁻, the headspace gas was N₂/CO₂ (80:20). For cultivation with S₂O₃²⁻/O₂, a microaerobic
343 environment was created by mixing N₂/CO₂ (80:20) with pure O₂ to a final concentration of ~1% air
344 saturation using a mass-flow controller (Tylan 260 series with RO-28 control box). Oxygen
345 concentrations in chemostat vessels were monitored in real time with Pts3 optodes (Presens, Germany).
346 The medium recipe and additional details are provided in the supplemental methods.

347 Cells fixed with borate-buffered formalin were stained with DAPI, filtered onto black 0.2 μM
348 polycarbonate filters, and counted with epifluorescence microscopy (67). Once numbers stabilized after

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349 5-8 days of cultivation, both source medium and chemostat vessels were sampled for cell carbon (CC)
350 and ash-free dry weight (AFDW; see SI for more detail).

351 For comparisons between hydrogen/nitrate and thiosulfate/nitrate, growth yield data were
352 normalized to total nitrate consumed as determined by chemiluminescent detection using a NoxBox
353 instrument (Teledyne, San Diego CA, USA) following the original protocol (68). For comparisons
354 between thiosulfate/nitrate and thiosulfate/oxygen, yields were normalized to total sulfate produced as
355 measured by ion chromatography with suppressed conductivity detection. *In silico* model results were
356 compared to ratios of growth yields measured in continuous culture (this study, (14)) and overall
357 growth yields (21).

358

359 Acknowledgements:

360 Funding was provided by NSF grants OCE-1136727, OCE-1559198, and the *WHOI Investment*
361 *in Science Fund* (SMS) and fellowships awarded to JM from the National Sciences and Engineering
362 Research Council of Canada (PGSM-405117-2011, PGSD-439487-2013) and the National Aeronautics
363 and Space Administration Earth Systems Science Fellowship (PLANET14F-0075), an award from the
364 Canadian Meteorological and Oceanographic Society, as well as funding from WHOI academic
365 programs. We thank Ying Zhang for a critical reading of an earlier version of this manuscript and for
366 advice on bioinformatics, François Thomas for invaluable help with qPCR, Florian Götz and Dali
367 Smolsky for help with chemostat cultivation, Jeff Seewald and Sean Sylva for assistance with GC/IC
368 measurements, Mak Saito for lending his lab's mass-flow controller for microoxic cultivation, Carl
369 Johnson for conducting the cell carbon measurements, and Scott Wankel and Carly Buchwald for
370 assistance in making nitrate measurements. We especially thank Jörg Simon, Mårten Wikström, and
371 Martin Klotz for helpful discussions regarding modelling and bioenergetics.

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Figure 1: Hypothetical model of electron transport and energy conservation in *Sulfurimonas denitrificans* during: A) Thiosulfate-dependent denitrification, B) Hydrogen-dependent denitrification, C) Thiosulfate-dependent aerobic respiration and, D) Reversed electron transport to produce reductant for the rTCA cycle. See text for explanation. Black arrows indicate electron transport, red arrows contributions to the proton-motive force and blue arrows are “chemical protons” used during reduction of O₂ to H₂O. Nap=Nap nitrate reductase complex; Sox=Sox sulfur oxidation complex; SQR=Sulfide:quinone oxidoreductase; Hyd=Hydrogenase; QcrABC=Complex III; MK/MKH₂=Oxidized/reduced menquinone; cyt c=Soluble cytochrome c; cbb₃=high-affinity cytochrome c oxidase; Fd=soluble ferredoxin; P=Periplasm; C=Cytoplasm.

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Table 1 - Hypothetical metabolic reactions for *S.denitrificans* inferred from comparative genomics and pathway logic, and calculated maximum chemosynthetic growth efficiency. See text for derivation and references.

<u>Half reactions</u>			
Dissimilatory oxidation		Description	Equation stoichiometry
1 (S2O3-OX)		Thiosulfate oxidation by SOX linked to menaquinone pool	S2O32- + 4 MK + 5 H2O + 8 H+ (p) => 2 SO42- + 10 H+ (p) + 4 MKH2
2 (H2-OX)		Hydrogen oxidation linked to menaquinone pool	H2 + 2 H+ (c) + 1 MK => 2 H+ + 1 MKH2
Dissimilatory reduction		Description	Equation stoichiometry
3 (QcrABC)		Electron transport to cytc by QcrABC (overall reaction)	MKH2 + 2 H+ (c) => MK + cytc2+ + 2 H+ (p)
4 (NO3-RED)		Nitrate reduction by Nap complex (with QcrABC)	NO3- + MKH2 + 2 H+ (c) + 2 H+ => 2 H+ (p) + H2O + NO2- + MK
5 (NO2-RED)		Nitrite reduction by Nir complex	2 H+ + NO2- + cytc2+ => H2O + cytc3+ + NO
6 (NO-RED)		Nitric oxide reduction by NorBC complex	2 H+ + 2 NO + 2 cytc2+ => 2 cytc3+ + 1 N2O + 1 H2O
7 (N2O-RED)		Nitrous oxide reduction by Nos complex (with QcrABC)	N2O + MKH2 + 2 H+ (c) + 2 H+ => N2 + MK + 2 H+ (p) + H2O
8 (O2-RED)		Oxygen reduction by cbb3 (PMF produced by chemical protons)	O2 (p) + 4 H+ (c) + 4 cytc2+ => 2 H2O + 4 cytc3+
<u>Reverse electron transport</u>		Description	Equation stoichiometry
8 (CPLX1)		NADPH and ferredoxin reduction via EPSI-type Complex I	NAD(P)+ + 2 Fd(ox) + 8 H+ (p) + 2 MKH2 => NAD(P)H + 11 H+ + 2 MKH2 + 2 Fd(red)
9 (CFIX-rTCA)		Overall reaction for rTCA cycle	4 CO2 + 4 Fd(red) + 2 NAD(P)H + MKH2 + 3 ATP => 1 Oxaloacetate + 4 Fd(ox) + 2 NAD(P)+ + MK + 3 ADP
<u>Coupled reactions and predicted chemosynthetic growth efficiency (CGE)</u>			
Pathway	Description	Reactions	Theoretical CGE*
T/N	S2O3-dependent denitrification	1 & 3-7	0.11 - 0.14
T/O	S2O3-dependent oxic respiration	1 & 8	0.20 - 0.25
H/N	H2-dependent denitrification	2 & 3-6	0.23 - 0.28
H/O	H2-dependent oxic respiration	2 & 8	0.31 - 0.38

*CGE was calculated by estimating the amount of proton-motive force/ATP required for biosynthesis in addition to that required for carbon fixation. This was in turn estimated from experimentally measured yield ratios between H/N and T/N conditions (Table 2; see text and SI for further explanation).

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Table 2 – Predicted relative growth yields compared with growth yields measured from chemostat experiments

Growth Condition	Modelled RGY [†]	Y _{SO4} [*] (this study)		Y _{S2O3/2} [*] (Timmer Ten-Hoor, 1981)		
		AFDW (n=3)	CC (n=3)	DW	CC	CP
S ₂ O ₃ ²⁻ /NO ₃ ⁻		0.89 ± 0.14	0.67 ± 0.14	2.58	1.18	0.89
S ₂ O ₃ ²⁻ /O ₂		1.49 ± 0.20	0.91 ± 0.12	3.87	2.00	1.95
Relative growth yield (RGY)	1.55 - 2 ‡	1.67	1.36	1.50	1.70	2.20

Growth Condition	Modelled RGY [†]	Y _{NO3} [*] (this study)		* = Biomass yield per mole substrate
		AFDW (n=3)	CC (n=3)	
S ₂ O ₃ ²⁻ /NO ₃ ⁻		1.01 ± 0.22	0.76 ± 0.21	AFDW = Ash-free dry weight (g • M ⁻¹)
H ₂ /NO ₃ ⁻		2.45 ± 0.73	1.75 ± 0.31	CC = Cell carbon (g C • M ⁻¹)
Relative growth yield (RGY)	2 - 3.33 ‡	2.43	2.30	DW = Dry weight (g • M ⁻¹)
				CP = Cell protein (g protein • M ⁻¹)

† = Predicted growth yield based on fixation of one oxaloacetate molecule (Reaction 9 in Table 1), and ranges (‡) are as a function of the fraction of “non-rTCA-ATP” used by the cell (see text, supplemental text, and Fig S1 and S2 for further explanation).

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Supplementary Information for

Reconciling a Model of Core Metabolism with Growth Yield Predicts Biochemical Mechanisms and Efficiency for a Versatile Chemoautotroph

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This PDF file includes:

Supplementary methods

Table S1: Hypotheses derived from the model and suggested tests for experimental validation.

Table S2 : CGE values determined from chemostat cultivation.

Figures S1 and S2: Plots of predicted growth yield ratios (RGY) versus non-rTCA-PMF for H/N:T/N and T/O:T/N ratios.

Figures S3 and S4: Expression of core metabolic genes in *S. denitrificans* under T/N and H/N conditions based on qPCR.

Figure S5: Sequence alignment of the *T. maritima* NfnB protein with SudeN_1824 (the subunit that substitutes for NuoF in *S. denitrificans*).

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429 **Figure S6:** Sequence alignment of both NuoG subunits in *S. denitrificans*, showing the missing
430 regions in one of the copies.

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432 **Figures S7, S8 and S9 :** Growth curves of *S. denitrificans* under chemostat cultivation.

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434 **Supplemental Dataset 1:** Descriptions of samples used for RNA extraction.

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436 **Supplemental Dataset 2:** Primers and targeted genes for qPCR analyses on batch cultures.

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438 **SI references**

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440 **Other supplementary material for this manuscript includes the following:**

441 1. qPCR sample and primer information excel sheet: “190619_supplemental_dataset_qPCR.xls”

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443 2. Code for generating plots, raw sequences for alignments, as well as example calculations for

444 CGE as a jupyter notebook can be found online here: <https://osf.io/x8e5j>

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Supplementary methods:

Further information on model construction/inference:

By only considering previously published mechanisms of sulfur oxidation, it was not possible to account for growth with thiosulfate and nitrate, despite being the original condition this organism was isolated under (1). This was because no electron transport pathway could link thiosulfate with the membrane quinone pool – necessary for the initial step of nitrate reduction. In the genome description of *S. denitrificans*, the SOx system was suggested to be responsible for thiosulfate oxidation (2). However, the SOx system is currently thought to be a soluble periplasmic membrane complex (3), and therefore cannot donate electrons to the quinone pool. Although a thiosulfate:quinone oxidoreductase responsible for partial oxidation of thiosulfate to tetrathionate has been described (4), neither this complex nor any other known thiosulfate-oxidizing enzymes are present in the genome of *S. denitrificans*. Therefore, we believe it is probable that a yet-undescribed complex serves to link the SOx system to the membrane quinone pool in *S. denitrificans*. Considering this, we employed a mechanism whereby the complete eight-electron oxidation of thiosulfate and sulfide to sulfate was linked to the reduction of menaquinone via the SOx system (Fig 1a).

To support carbon fixation, NAD(P)H and ferredoxin are both required for the reverse tricarboxylic acid (rTCA) cycle, yet no mechanism for ferredoxin production is known for *S. denitrificans* (5). However, the heterotrophic campylobacterium *Campylobacter jejuni* possesses a non-canonical NADH:ubiquinone oxidoreductase complex (complex I) that oxidizes reduced flavodoxin instead of NADH (6). Such a complex has the potential to reduce ferredoxin if run in reverse; indeed, *S. denitrificans* has a non-canonical complex I operon similar to *C. jejuni*. However, NAD(P)H is also needed, and not produced by such a mechanism. Therefore, for modeling purposes, we included a hypothetical mechanism that reduces both NAD(P)⁺ and ferredoxin simultaneously via an electron-bifurcation-like mechanism (Fig 1d). Further details on this mechanism are discussed in the main text.

Energy Conservation Efficiency of Enzymatic Complexes:

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474 We then considered growth under conditions previously described for *S. denitrificans* (2, 7, 8).
 475 Growth yields were estimated from combining core dissimilatory reactions, reversed electron transport,
 476 and an overall reaction for the rTCA cycle (Table 1). However, using canonical proton-pumping
 477 stoichiometries for these complexes resulted in several discrepancies. Firstly, growth efficiencies did
 478 not differ between thiosulfate and hydrogen oxidation, in contrast to data previously reported in batch
 479 culture (8) and chemostat growth data presented here (Table 2). Secondly, the *in silico* ratio between
 480 aerobic and denitrifying growth efficiency was much higher than what has previously been determined
 481 in chemostat experiments (7).

482 The first discrepancy – why modeled growth yields *in silico* were identical during sulfur and
 483 hydrogen oxidation but different in growth experiments - was resolved by considering the inferred
 484 structure of enzyme complexes. The hydrogenase in *S. denitrificans* likely has a transmembrane
 485 domain - allowing it to accept protons from the periplasm during quinone reduction (Fig 1b) (2). When
 486 coupled to a quinol-accepting reductase, this translocates protons via a redox-loop mechanism (9). In
 487 contrast, proteins catalyzing sulfur oxidation coupled to quinone reduction have not been demonstrated
 488 to translocate protons. This is because they either do not have transmembrane domains (10) or the
 489 quinone-accepting site and the redox module both face the cytoplasm and thus do not translocate
 490 protons (4, 9). Since thiosulfate oxidation likely occurs in the periplasm in *S. denitrificans*, we chose to
 491 model the SOx system with a periplasmic-facing non-transmembrane anchor similar to sulfide:quinone
 492 oxidoreductase (SQR; Fig. 1a). With this mechanism in place, the ratio between hydrogen oxidation
 493 and sulfur oxidation under denitrifying conditions was congruent with experimental data (Tables 1 and
 494 2).

495 The second inconsistency – large differences in growth yield ratios between aerobic and
 496 denitrifying respiration, but only a small difference observed during experiments - could be reconciled
 497 if the energy conservation efficiency of aerobic respiration was either lower or that of denitrification
 498 was higher. Although it was previously thought that proton translocation is not catalyzed by
 499 denitrification enzymes (11), recent studies have suggested that denitrification is net electrogenic
 500 through the concerted action of qcrABC (Complex III) and denitrification enzymes (12, 13). However,
 501 since this net reaction for denitrification is thought to have a H^+/e^- of 1, it would result in a much

502 higher RGY ratio than observed for *S. denitrificans* if aerobic respiration occurred with the same
503 efficiency as for typical aerobes ($H^+/e^- = 3$; (9)).

504 Therefore, the efficiency of aerobic respiration coupled to sulfur oxidation must be lower to
505 account for these observations. If the SOx system directly donated electrons to soluble cytochrome c
506 under aerobic conditions (14), this would reduce overall efficiency by bypassing the energy conserving
507 complex III. However, this would require that complex III operates in both the forward and reverse
508 direction. This occurs in the autotroph *Acidithiobacillus ferrooxidans*, but it possesses one dedicated
509 complex for each direction (15). Given that *S. denitrificans* has only one copy of complex III (2), we
510 rejected this possibility in the model.

511 The only plausible explanation remaining is that the complex predicted to catalyze aerobic
512 respiration in *S. denitrificans* (a cbb_3 high-affinity type cytochrome oxidase; (2)), has reduced
513 bioenergetic efficiency. While this complex is theoretically capable of proton transport, it has been
514 shown to be unable to pump protons effectively against membrane potentials typically encountered in
515 living cells (16–20). Therefore, under physiological conditions, it appears likely that *S. denitrificans'*
516 cbb_3 cytochrome oxidase does not pump protons.

517 Even without pumping protons, the cbb_3 complex would still conserve more energy than
518 denitrification enzymes because protons for oxygen reduction typically come from the cytoplasm,
519 contributing to the proton-motive force and a H^+/e^- of 1 (21). When coupled with Complex III, aerobic
520 respiration would have an overall H^+/e^- of 2, which would lead to predicted RGY yields that are
521 consistent with experimental observations (Table 2). An alternative (but in our opinion less likely)
522 explanation for these observations is that “chemical” protons for oxygen reduction are abstracted from
523 the periplasm. This has not yet been demonstrated for any cytochrome c oxidase, but it is known that
524 NO reductases obtain protons for NO reduction from the periplasm, and that they may be the
525 evolutionary precursor of complex IV (22, 23). If this is true, it follows that there may be some extant
526 cytochrome c oxidases that obtain their “chemical” protons from the periplasm. Indeed, such a
527 discovery would serve as a “missing link” between NO and O₂ reduction and reinforce this
528 evolutionary scenario (Mårten Wikström, pers. comm.). Only biochemical experiments that specifically
529 investigate these mechanisms can resolve this uncertainty (Table S1). With these hypothetical

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530 mechanisms in place (Fig 1), the model reproduced experimental growth yield data satisfactorily, and
531 was considered to be complete.

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533 Calculation of theoretical chemosynthetic growth efficiency (CGE):

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535 We also calculated CGE, which is a parameter that can be used for modelling chemosynthetic
536 primary productivity in natural environments (24, 25). CGE is the fraction of electrons that go to
537 assimilatory carbon fixation as a proportion of the total electrons oxidized. To do so, we exploited a
538 unique property of our conceptual model that allowed for the calculation of the proton-motive force
539 needed for cell growth, maintenance, motility etc., in addition to that needed for carbon fixation by the
540 rTCA cycle (hereafter “non-rTCA-PMF”). To estimate non-rTCA-PMF, we first assumed that the ratio
541 of non-rTCA-PMF to carbon fixed is consistent between different combinations of electron donors and
542 acceptors. In other words, we are assuming that cell composition and biosynthetic reactions are
543 constant between different growth conditions. Considering the equations in Table 1, we further assume
544 that to fix one molecule of oxaloacetate from CO₂, *S. denitrificans* needs to translocate 25 protons to
545 account for ATP and reductant required.

546 We then compared the RGY between T/N and H/N conditions to determine CGE. We chose not
547 to use the T/O : T/N ratio because of uncertainties with regards to the potential negative effects of
548 oxygen that may reduce CGE (25). The ratio of potential growth yields between H/N and T/N
549 conditions will vary as a function of the amount of non-rTCA-PMF needed (Fig S1). The maximum
550 ratio will be obtained where non-rTCA-PMF is zero. This is because for growth under T/N conditions,
551 our model predicts that the process of transferring electrons from thiosulfate to menaquinone and to
552 complex I is electroneutral. In contrast, under the H/N condition, electrons derived from H₂ oxidation
553 can generate a proton motive force by charge separation during reverse electron transport ((9); Fig 1d).
554 This does not mean that reverse electron transport is net electrogenic under H/N conditions, but rather
555 that the process of obtaining reductant for carbon fixation under H/N conditions does result in the
556 translocation of protons, which can be used for reversed electron transport. Because of this, carbon
557 fixation is much more efficient for the H/N condition relative to T/N, leading to a maximum theoretical
558 ratio of 3.33 (i.e. 7.5 electrons are needed to produce one molecule of oxaloacetate from CO₂ under the

56

57

559 H/N condition, whereas 25 are needed for the T/N condition). However, the actual value will always be
 560 below this value since the cell requires PMF/ATP for other biosynthetic reactions (what we term “non-
 561 rTCA-PMF”), which we predict will occur at a fixed efficiency ($H^+/e^- = 2$; Fig 1b).

562 In our experiments, the measured $Y_{H/N}:Y_{T/N}$ ratio was between 2.3-2.4. According to the
 563 conceptual model described above, this ratio can be written as follows (where the product of x and its
 564 coefficient represents the amount of “non-rTCA-PMF” needed, with a coefficient added to take into
 565 consideration the different proton-pumping efficiencies of the H/N and T/N conditions):

566

567 $(C_{\text{fixed}} / 7.5 + 0.5x) / (C_{\text{fixed}} / 25 + x) = \text{Yield ratio}$

568

569 which simplifies to:

570

571 $(25 + x) / (7.5 + 0.5x) = \text{Yield ratio}$

572

573 Solving for x by substituting both RGY values we measured (Table 2), we can calculate $f(2.3) =$
 574 35, $f(2.4) = 52$. This allows us to calculate theoretical CGE values based on the production of one
 575 molecule of oxaloacetate from CO_2 (requiring 10 electrons). An example is shown below for T/N
 576 conditions:

$$\text{where } x = 52, \quad CGE_{S_2O_3/NO_3} = \left(\frac{10}{(10 + 25 + 52)} \right) = 0.11$$

$$\text{where } x = 35, \quad CGE_{S_2O_3/NO_3} = \left(\frac{10}{(10 + 25 + 35)} \right) = 0.14$$

578 $CGE_{T/N} = 0.11(10/(10+25+52)) - 0.14(10/(10+25+35))$. The lowest value is slightly higher than
 579 Klatt and Polerecky’s estimates for *S. denitrificans* based on the data reported in (7) (0.098), but close
 580 to those estimated by the same authors for other *Sulfurimonas* cultures (0.148; 0.125; 0.113; (24)).

58

581 $CGE_{T/O} = 0.20 (10 / (10+12.5+26)) - 0.25 (10 / (10 + 12.5 + 17.5))$. These values are slightly
 582 higher than the Klatt and Polerecky value for *S. denitrificans* calculated from the data reported in (7)
 583 (0.167), but this difference could perhaps be attributed to oxidative damage caused by O₂ ((25) and
 584 references therein). No other CGE measurements are available for *Sulfurimonas* strains under
 585 microaerobic conditions (since our CGE measurements are likely underestimates; see discussion
 586 below).

587 $CGE_{H/N} = 0.23 (10 / (10 + 7.5 + 26)) - 0.28 (10 / (10 + 7.5 + 17.5))$. No current data is available
 588 for cultivars for CGE, but these values are congruent with highest CGE value reported for our
 589 previously published incubations of natural microbial communities dominated by *Sulfurimonas* (0.27,
 590 for incubations with H₂ and low oxygen; (25)).

591 $CGE_{H/O} = 0.31 (10 / (10 + 5 + 17.33)) - 0.375 (10 / (10 + 5 + 11.66))$. No current data is
 592 available for cultivars growing with hydrogen and oxygen, and is complicated by the negative effect
 593 that oxygen has on these organisms, meaning that optimal cultivation under this condition would
 594 require a more sophisticated experimental setup that could provide oxygen at low, but non-limiting
 595 concentrations.

596 We were unfortunately unable to compare these theoretical estimates to our chemostat data
 597 since our actual measurements of CGE were lower than previously reported (Table S2). We believe this
 598 is likely to be an experimental artefact that underestimated actual CGE. Nitrate limitation was not
 599 achieved at steady-state in our continuous culture experiments, despite an excess of
 600 thiosulfate/hydrogen. It is possible that constant flushing with gas during chemostat growth removed
 601 trace amounts of oxygen needed for biosynthesis (26) or some other limiting nutrient and accounted for
 602 lower CGE values we observed (approximately 1/2 of expected according to our model and previous
 603 observations). Other potential explanations for low CGE include the filters used to capture biomass
 604 (nominal pore size of 0.3 µm) not retaining all cells, excretion of fixed organic matter into the media.
 605 The latter two possibilities seem unlikely given that Timmer-Ten Hoor (1981) used filters with larger
 606 pore sizes and excretion would be expected to also occur in their experiments. Alternative explanations
 607 include non-optimal nutrient stoichiometry in the growth media, or the introduction of a toxic/nutrient-
 608 chelating substance into the medium from the gas or cultivation vessels/tubing. Another possibility is
 609 that differences in dilution rate affects yield. Little is known about this question, but the available data

61

610 (Fig 1 in (7)) suggest that absolute yield differences should be minor (~5%) between the dilution rate
611 employed in our study and in (7). Despite these limitations, our values for RGY are nearly identical to
612 those previously reported and consistent with the model.

613

614 Chemostat cultivation:

615 The chemostat was operated as described in the main text. The medium was made by first
616 dissolving and autoclaving the following components in MilliQ water (per L final volume): NaCl 10 g;
617 NH₄Cl 1 g, MgSO₄·7H₂O 3.5 g; CaCl₂·2H₂O 0.42 g; KCl 0.7 g; KNO₃ 2.0 g; Trace element solution
618 SL-6 (DSMZ 465) 1mL. After autoclaving, separately-sterilized anaerobic solutions of the following
619 components were added (per L final volume): NaHCO₃ 5 g; KH₂PO₄ 0.5 g; selenate/tungstate solution
620 (DSMZ 944a) 4 mL; 0.7 mL of 2 mg·mL⁻¹ FeCl₃ in 0.1 N H₂SO₄; Na₂S₂O₃·5H₂O 5 g. The thiosulfate
621 solution was sterilized by filtering through a 0.2 µm membrane and the bicarbonate solution was
622 autoclaved under a N₂/CO₂ (80:20) atmosphere.

623 For all incubation conditions, pH measured daily was 7.19 ± 0.04 for H₂/NO₃⁻, 7.06 ± 0.01 for
624 S₂O₃²⁻/NO₃⁻ and 7.02 ± 0.02 for S₂O₃²⁻/O₂. Upon sampling, cell numbers had not varied on average by
625 more than 11 % over at least 2 days of cultivation, indicating steady state had been reached (Fig. S7, S8
626 and S9). This occurred after 7, 8, and 5 days for H₂/NO₃⁻, S₂O₃²⁻/NO₃⁻, and S₂O₃²⁻/O₂ respectively.

627 Yield measurements (cell carbon, CC; ash-free dry-weight, AFDW) were conducted as follows.
628 Combusted and pre-weighed GF-75 filters (0.3 µm pore size; Advantec) were used for both
629 measurements. For AFDW, filters were first wetted with 0.5 M ammonium formate (27). Next, 20 mL
630 of medium or cells were filtered, followed by 10 mL of ammonium formate. Cells were dried overnight
631 at 80°C, placed in a vacuum desiccator, and weighed when cool. Then, filters were combusted at 480°C
632 for 8 h and weighed as before. Medium blanks were subtracted to determine AFDW. For cell carbon,
633 20 mL of both medium and cell culture were filtered using pre-combusted glassware. Filters were
634 stored in pre-combusted shell vials in -80°C prior to analysis. Total cell carbon was determined by
635 acidification and subsequent combustion of biomass on a Carlo Erba/Fisons 1107 Elemental Analyzer
636 as previously described (25).

637

638 Batch Cultivation for qPCR Experiments:

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63

639 Media was autoclaved for 20 min in a Widdel flask, cooled under anoxic N₂ gas, and transferred
640 to N₂-purged Hungate tubes, serum vials or nalgene flasks and sealed with butyl stoppers. pH was
641 adjusted to 7.0+/-0.05 before autoclaving by adding 3mL of a solution of 1N NaOH. Bicarbonate and
642 thiosulfate solutions were prepared in anoxic N₂-purged water and added after autoclaving through a
643 sterile 0.2 µm filter. In addition, 0.6 mL of a sterile 0.1N H₂SO₄ solution containing 2mg FeCl₃ x 5 H₂O
644 was added after autoclaving, and 1mL of trace metal solution SL-6 was added prior to autoclaving.

645 For hydrogen-grown cultures, thiosulfate was omitted from the medium, and the headspace was
646 evacuated using a vacuum pump and refilled with H₂/CO₂ (80/20%) to 28psi gauge pressure.
647 Thiosulfate-grown cultures were filled with 5psi gauge pressure N₂/CO₂ (80/20%). A solution of anoxic
648 sodium bicarbonate was used to buffer the pH at 7 where CO₂ was used in the headspace. For each
649 condition, quadruplicate cultures were grown, with an additional uninoculated control to account for
650 abiotic processes. Pressure was measured periodically in vessels using a pressure gauge fitted to a luer-
651 lok fitting to which a sterile needle was attached. pH was monitored by taking subsamples using
652 syringes and measuring using a desktop probe.

653 Cell growth was monitored by direct counts as described above. Cells were harvested in both
654 exponential and stationary phase, and 1 sample from each phase was taken from each treatment (S₂O₃²⁻/
655 NO₃⁻ and H₂/NO₃⁻) for RNA extraction. In addition, the S₂O₃²⁻/NO₃⁻ grown cells used to inoculate the
656 H₂/NO₃⁻ culture were sampled to observe changes in gene expression associated with adaptation to H₂
657 as an electron donor.

658

659 Culture Quality Control:

660 The presence of heterotrophic contamination was tested by incubating cells in anaerobic media
661 containing nitrate, 0.5% tryptone, 0.5% glucose and 0.5% yeast extract. Growth was also assayed with
662 formate (20mM) as sole electron donor with nitrate (20mM) as electron acceptor.

663 To test for contaminants that could not be detected by the method above, DNA was extracted
664 from 4 samples (1 frozen culture, and 3 growing, replicate cultures) using the UltraClean Microbial
665 DNA Isolation Kit (Mobio, Carlsbad, CA). 16S DNA was amplified using the 341F/907RC primer pair
666 (Schäfer et al., 2000) with a GC-clamp designed for DGGE using Fermentas Taq polymerase (Fisher

64

65

667 Fermentas, Vilnius, Lithuania) and the following conditions: (94°C for 5 min, 20 cycles of 94°C 1min,
668 65°C 1min (touchdown 0.5°C/cycle) and 72°C 1min, followed by 15 cycles of 94°C 1min, 55°C 1min
669 and 72°C 1min, followed by 7 min at 72°C). The PCR product was cleaned up using the Axyprep PCR
670 Clean-up kit (Axygen, Union City, CA). 300ng of DNA from each sample was loaded onto a
671 polyacrylamide gel as previously described (28) and stained using SYBR-safe DNA stain and
672 visualized under UV light.

673

674 RNA preservation, extraction and reverse transcription:

675 Quadruplicate samples taken during the growth experiments were preserved using RNAProtect
676 bacteria reagent (Qiagen, Hilden, Germany) as per manufacturer instructions, and frozen at -80°C until
677 RNA extraction, which was carried out within 2 weeks of preservation. RNA was extracted from
678 triplicate samples (one replicate was not used) using the RNeasy mini kit (Qiagen). Yield was
679 measured using a Nanodrop instrument (Fisher Scientific, Waltham, MA) and integrity was verified
680 using gel electrophoresis. Residual DNA was digested using the Turbo DNAase kit (Ambion, Austin,
681 TX) at 35°C for 1hr, keeping the RNA concentration constant between all digestions. RNA was then
682 purified with the RNeasy Minielute RNA cleanup kit (Qiagen) and re-quantified using a Nanodrop.
683 The absence of DNA contamination of the RNA was verified by conducting a PCR assay on the RNA
684 using Fermentas Taq polymerase (Fisher Fermentas, Vilnius, Lithuania) and the following conditions:
685 95°C:2min, 35 cycles of 95°C:30sec, 55°C:40sec, 72°C:30sec followed by a 5 minute hold at 72°C. A
686 no-RT control was also tested for amplification from two randomly selected RNA samples. Reverse
687 transcription was accomplished using the DyNAmo cDNA synthesis kit (Thermo Fisher, Waltham
688 MA) and the following thermocycling conditions: 25°C:10min, 37°C:60min, 85°C:5min. The amount of
689 RNA added to each reaction was 134.4 ng. cDNA was diluted 20-fold before qPCR.

690

691 qPCR primer design and assays:

692 PerlPrimer (29) was used to design primers for each specific gene (Supplemental Dataset 2)
693 according to the parameters described in (30). In-silico specificity of each primer pair was tested by
694 conducting BLAST searches against the *S. denitrificans* DSM 1251 genome. In-vitro specificity was
695 tested by conducting a PCR amplification on genomic DNA and gel electrophoresis. Single bands were

66

696 observed in all cases, indicating specific amplification. The linearity of response was tested by creating
697 a standard curve using *S. denitrificans* genomic DNA (10^6 copies to 10 copies per well). Efficiency was
698 between 85 and 103.6% for all primer pairs. Genomic DNA was isolated from pure cultures of *S.*
699 *denitrificans* using the UltraClean Microbial DNA Isolation Kit (Mobio, Carlsbad, CA).

700 qPCR assays were carried out on the Stratagene MX3005P instrument (Agilent, Santa Clara
701 CA) using Fermentas Maxima qPCR Master Mix (Fisher Fermentas, Vilnius, Lithuania) with the
702 following mixture per well: 7.5 μ L master mix, 1.5 μ L template, 1.5 μ L primer mix (5 μ M) and 4.5 μ L
703 H₂O. Thermocycling conditions were as follows: 95°C:10min, followed by 40 cycles of 95°C:15s,
704 60°C:30s, 72°C:30s, then by 55°C:30s and a slow ramp to 95°C to construct disassociation curves. Each
705 sample was run in duplicate, and standard curves were re-run with every plate. DNA amplification was
706 quantified using SYBR green with ROX as a reference dye.

707

708 qPCR quality control and normalization:

709 After thermocycling, wells were manually inspected for evaporation, and those that differed
710 significantly from their duplicate were removed from further analysis. Amplification plots were
711 manually inspected and baselines changed if necessary, and the critical cycle C_t was adjusted to make
712 sure it was within the log-linear portion of the amplification plot. Absolute expression values were
713 plotted with ggplot2 (code and raw data available at <https://osf.io/x8e5j/>).

714

715 Ion Chromatography:

716 Culture samples were centrifuged at 10,000g for 2 minutes to pellet cells, and the supernatant
717 decanted, and stored frozen at -20°C until analysis. Prior to analysis, samples were diluted 100-fold in
718 Milli-Q water using a 4-place balance, and for the H/N samples, shaken overnight at 35°C to
719 resolubilize any precipitate formed. Standards were prepared from commercially purchased standard
720 solutions to bracket expected concentrations (1000ppm Specpure standards for NO₂⁻, NO₃⁻, Cl⁻, SO₄²⁻:
721 Alfa Aesar; 11200ppm S₂O₃²⁻: Fisher Scientific).

722 Samples were diluted 100-fold, and run on a Dionex DX 500 Ion Chromatograph equipped with
723 a GP50 gradient pump, ED40 electrochemical detector, LC30 chromatography oven and a AS40
724 autosampler. Each chromatogram was manually adjusted to ensure peaks were integrated correctly.

69

725 Quantification of anions was accomplished by comparing the integrated peak size with a standard curve
726 generated from the above standards.

70

Table S1
Hypothetical biochemical mechanisms and experimental tests

Hypothesis	Predictions of hypothesis	Experimental test and expected result
SOx complex is attached to membrane and transfers electrons to quinone pool	QcrABC (complex III) solely responsible for energy conservation during sulfur oxidation; in contrast, hydrogen oxidation can conserve energy via redox loop mechanism	Knock out or inhibit complex III: Thiosulfate-nitrate dependent growth should stop; hydrogen-nitrate dependent growth may continue with lower yield if NapGH can act as a quinol:acceptor reductase independently (Kern and Simon 2008), but denitrification will not proceed beyond nitrite
cbb₃ cytochrome c oxidase does not pump protons under physiological conditions*	Proton-motive force during oxic respiration comes from the combined action of QcrABC and the abstraction of protons from the cytoplasm by cbb ₃	Uncouplers of cbb₃ proton pumping should not affect physiological growth yields
NADH:ubiquinone oxidoreductase is responsible for NAD(P)⁺ and ferredoxin reduction	Complex donates electrons to both NAD(P) ⁺ and ferredoxin during reverse electron transport	Use inverted vesicles with complex I and ATPase: Reduction of ferredoxin and NAD(P)⁺ should be possible with ATP present to generate pmf

*An alternative possibility is that “chemical protons” for cbb₃ are abstracted from the periplasm, which would result in the same growth yields.

Table S2 - Calculations of Chemosynthetic Growth Efficiency from chemostat growth experiments. TN=Thiosulfate/nitrate, HN=Hydrogen/nitrate, TO=Thiosulfate/oxygen.

Chemostat	Nitrate consumed (M)	Sulfate produced* (M)	Cell carbon (mg/L)	Cell carbon (M)	CGE (NO3, denominator corrected)	CGE (SO4, denominator not corrected)
TN4	6.8E-03	7.3E-03	3.8	3.2E-04	0.04	0.04
TN5	7.7E-03	9.4E-03	7.5	6.3E-04	0.06	0.07
TN6	7.7E-03	8.2E-03	5.7	4.8E-04	0.05	0.06
HN7	3.6E-03	NA	7.5	6.3E-04	0.12	NA
HN8	4.4E-03	NA	6.5	5.5E-04	0.09	NA
HN9	4.4E-03	NA	7.4	6.2E-04	0.10	NA
TO1	NA	3.8E-03	3.0	2.5E-04	NA	0.07
TO2	NA	4.7E-03	4.8	4.0E-04	NA	0.09
TO3	NA	4.9E-03	4.6	3.8E-04	NA	0.08

*Used instead of thiosulfate consumption since thiosulfate measurements were inconsistent, possibly reflecting incomplete sulfur oxidation known to occur in *Campylobacteria* (e.g. (33)).

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732

733 **Figure S1:** Predicted relative growth yields between H/N and T/N conditions depending on the fraction
734 of non-rTCA-PMF required per molecule of oxaloacetate fixed. See jupyter notebook at
735 <https://osf.io/x8e5j> for a more detailed explanation.

74

Figure S2: Predicted relative growth yields between T/O and T/N conditions depending on the fraction of non-rTCA-PMF required per molecule of oxaloacetate fixed. See jupyter notebook at <https://osf.io/x8e5j> for a more detailed explanation.

77

740 Figure S3: mRNA Expression Under Thiosulfate/Nitrate Growth Condition (n=6)

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Figures S3 and S4: RT-qPCR transcript abundance of selected genes in *S. denitrificans*. Values for absolute expression are derived from 6 independent measurements from both thiosulfate/nitrate and hydrogen/nitrate-grown cells at two separate time points (exponential and stationary phase) and normalized to total RNA (see Supplemental Dataset 1 for more detail). Genes are colored according to their predicted metabolic role as shown in the legend. Gene abbreviations correspond to the genes identified in Supplemental Dataset 1. The sulfide:quinone oxidoreductase mentioned in the main text corresponds to “squO” (the last gene in the boxplot). See jupyter notebook at <https://osf.io/x8e5j> for plotting code.

751

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81			
752	Sulfurimonas	1	MSKVYFSTWNGEFVNNINPTIEEWKESAYNLPAQYDSHRESKAFICGWDGV
	Thermotoga	1	M-----KNRKTPMKEQSPESRRR-----NFEFV
753	Sulfurimonas	51	ALFDKDVVDVIRLAMEYAAQYQYSEACGRCAPGRWGGRILYDQLDKIARG
754	Thermotoga	24	A-----LGYT-----LEEA-----
755	Sulfurimonas	101	EGSVADLDHLREIGKSMQITSKCEIGKTVPNPILDLMTHEHVFLECINE
756	Thermotoga	33	-----
757	Sulfurimonas	151	HKPSIHYSESIAYIAKITAPCTDACPSHVDIPAYIEGVRDLRMDDSLMAT
	Thermotoga	33	-----LEEAAQRCLQCPTHPCVSGCPVEIDIPGFIKRLRDGKLEESYRIL
758	Sulfurimonas	201	RQTMPLAHVCGRVCPHP--CEDACRRRTNL--DEPISIMAKKRLGADWETD
759	Thermotoga	77	KSYNNLPAAVCGRVCPQEQCESRCVVGKMKDSEPVAIGRIERFVADWAAE
760	Sulfurimonas	247	HGYDFFHPIQKKPNNGKRVAVIGAGPAGLTATAYYLAEGCIEVDVYELPV
	Thermotoga	127	NLEEDVKPLAG--SKKEKVAVVSGPAGLTAAADLAKMCGYHVDIFEAFHK
761	Sulfurimonas	297	LGGEVAVGVPEYRMPIDKYNKDIECVKDMGVNFIVNSKINAD-DMRRFES
762	Thermotoga	175	PGGVLVYGIPEFRLPKRIVEREVSYIRKLGVNFHLNTVVGKTVKVKELLS
763	Sulfurimonas	346	EYDAVMVATGTRISKKVYCNNERFEIKGYWGAIDFIDKVNLYDKYGYAIS
764	Thermotoga	225	EYDAVFIGTGAGTPKFM--GIPGTNLNGVYSANEFITRVNLMKAYLFP--
765	Sulfurimonas	396	KEDQEKNNLLTPYLDLTKGTLVCGGGFTSMDVVRCAIRANAKKVMIYR
	Thermotoga	271	EYD-----TPI--RVGKKVAVIGAGNTAMDARSALRLGAEEKVYIVYR
766	Sulfurimonas	446	RDEKTIKNTTYEYHEAVEEGVEFLFHSAVAEMT-DEEDILKSLLVDKF
767	Thermotoga	312	RTREMP--ARREYHHALEEGIEFLWLTLPYRIGDANGNVEAMECVRM
768	Sulfurimonas	495	ELVDPNGGRAELVKIEGASYVIEADYLIPAVSOSADLKLTPQEWETIEKT
	Thermotoga	360	ELKEADGSGRPRPVPIEGSNFVLEVDMVIEAIGOGPNRVLLSEFPGLELN
769	Sulfurimonas	545	SWATIKTNGKDYMTSRKGIFASGDCEYGPMITIVNAVGOAKRAASVMARYV
770	Thermotoga	410	ERGYIKADEDTGATSVKGVFAGGDIVTGAATVIKAMGAGKKAQFIHSYL
771	Sulfurimonas	595	QSGEVTLSDDEIMEDHLRKLRYDKKEKVTGWLAGVPROHSQVLEVEDRK
772	Thermotoga	460	T-GEWNPWQ-----
773	Sulfurimonas	645	YNNKEVNLGFTQEEAMSEAERCRCYIYIAMVQA
	Thermotoga	468	-----K

Figure S5: Sequence alignment of the *T. maritima* NfnB protein with Suden_1824 (the subunit that substitutes for NuoF in *S. denitrificans*). As mentioned in the main text, relevant residues for binding the phosphate group of NADP(H) (Tyr310, Arg311-312 in *T. maritima*) and cysteines ligating the iron-sulfur clusters that putatively pass electrons to ferredoxin are conserved in Suden_1824, as well as most residues involved in binding FAD (31). See <https://osf.io/x8e5j> for raw alignments.

83

779 Suden_1822_NuoG 1 MSK---TINIDGREIQTOEGEYILNAAARANDIEIPAIICYLTRCSPTLA
780 Suden_1823_NuoG 1 MGEMKKEINFKINGVSVVASRGETILNVARKNGIYIPTMCYISKTTPCAS

781 Suden_1822_NuoG 47 CRLCLVEADCKQ--VYACNAKSKEGMSVVTSTENIKERRAIMEVYDVNH
782 Suden_1823_NuoG 51 CRMCVVEASGMDGFVLSCNTPPVEGVEITQSAAALNHERVNIMKLYDVNH

783 Suden_1822_NuoG 95 PLECGVCDQSGECLELQNYTLEMGVDAQHYAVKDVNRKIDIDWGHVHNPGL
784 Suden_1823_NuoG 101 PLECGVCDKSGDCDLQNKTLFNFVGHQDFSARDQLRP IKHWGLIDYEASL

785 Suden_1822_NuoG 145 CIVCERCVTTCCKDMIGDNLAKTVARGADALEAEYKESMPKDAYAMWNKLN
786 Suden_1823_NuoG 151 CIMCEKCVHVCNEVIGDDAIEIKVGGYNSII-----

787 Suden_1822_NuoG 195 KSLIGLTSGEEMLDCTSCGECAAVCPVGALVDTHETIKSNAWELKQIPAT
788 Suden_1823_NuoG 182 -----VPKNSELLDCTFCGECTAVCPVGALTSSSEIRYRANAWELERVEST

789 Suden_1822_NuoG 245 CGHCSAGCQISYDVKHTSIDNTEAKIYRVNMENNYVALCCAGRYGFDYQN
790 Suden_1823_NuoG 227 CAHCSAGCPLEYETRHS SIN-GDDEIYRVKNNFEEFTSLCGAGRFGFDFDN

791 Suden_1822_NuoG 295 VVDAKDEVAEKKAVEAFKKADTLKFTSTITNEEAYLLQALKEKFGYKLVN
792 Suden_1823_NuoG 276 KA-KRDEGAENKAIEAIKSAKAIRESSMITNEEAHILELLKAKLGLKLFN

793 Suden_1822_NuoG 345 KEAKALQRELNSYSEISGKLYGYDLEATHNVNFVISVGTALKSDNPANAR
794 Suden_1823_NuoG 325 EDARKFASERMRSYSSVSGKLHHSGLDAIKQSDAVVVGISRIATDNPQVR

795 Suden_1822_NuoG 395 YALNNSMTVNVKGAGLYFHPLKDPVIEGLGKSTMTMYHAPLQEEVLYVIL
796 Suden_1823_NuoG 375 YALTTASKHNGAKIVYTHPMEDTLMQNVITQM--MKHEVGTEEGVMALLA

797 Suden_1822_NuoG 445 DLFADKEKLPSEIVEYLASFHSKKTITVEETIKKEEITEIVKVMKKNEESG
798 Suden_1823_NuoG 423 NALLSDAQDQDDDERAFFDELDLGYIYAETNIG-----

799 Suden_1822_NuoG 495 EEEEEVEEEKKKMVPKKISKEVEVDENALLEILGAKENEMEELEKNLAKKD
800 Suden_1823_NuoG 455 -----EEE-FSQMMKSFARAT

801 Suden_1822_NuoG 545 SFSLIAGPDLYTHPNSKNLARTLALIEKYSAFEVTMIPTLTNSLGVALLC
802 Suden_1823_NuoG 470 RRTLVIIGNDLIAHERSNNTAKLAALIEKYSDFSLVVVPREVNTLGVSLLN

803 Suden_1822_NuoG 595 ELD-DEIGSYAICYNTKADFTLSALGDGDLDMPALNQOECTLTSSINKRVN
804 Suden_1823_NuoG 520 SLNVDENISDVVGYNAGKSFVSSLEGADLAIAALNQOEGTFVSDNRVL

805 Suden_1822_NuoG 644 PTNAAIGYNGYELNDIANALGFDAQHVIEYTKMLPTKSGFISKDFDELPN
806 Suden_1823_NuoG 570 PTNVALTFDGYTLNDIAKAFDI IKDETIDYTKELSVSSGFKAIADFDDLEN

807 Suden_1822_NuoG 694 HYENDGTECRGYLLENIAVSTCGDESVEKFSDT-KLEGTLIYLANPVROF
808 Suden_1823_NuoG 620 FLSPLGEDNRGYILDEVECRV--DGKIQLDDMPEFNGTIIYQCDPVLOF

809 Suden_1822_NuoG 743 TPFTHKKTNDLDEVSGVYMSEELAKSDFNEGETVRVKSANGELVASIVSD

803 **Figure S6:** Sequence alignment of both NuoG subunits in *S. denitrificans*, showing the missing regions
804 in one of the copies. As noted in the main text, the shorter subunit (Suden_1823) is “missing” two main
805 regions. The first (AA 466-532 of Suden_1822) contains no obvious functional motifs. The second
806 missing region (AA 175-204 of Suden_1822) is found between two cysteine residues that putatively
807 bind an additional iron-sulfur cluster (32). See <https://osf.io/x8e5j> for raw alignments.

Figure S7: Growth curves for three replicate chemostat experiments grown with thiosulfate and nitrate. Arrows indicate time of sampling for growth yield measurements.

Figure S8: Growth curves for three replicate chemostat experiments grown with hydrogen and nitrate. Arrows indicate time of sampling for growth yield measurements.

Figure S9: Growth curves for three replicate chemostat experiments grown with thiosulfate and oxygen. Arrows indicate time of sampling for growth yield measurements.

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