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ARTICLE

The hidden chemolithoautotrophic metabolism of *Geobacter sulfurreducens* uncovered by adaptation to formate

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Abstract

Multiple Fe(III)-reducing *Geobacter* species including the model *Geobacter sulfurreducens* are thought to be incapable of carbon dioxide fixation. The discovery of the reversed oxidative tricarboxylic acid cycle (roTCA) for CO_2 reduction with citrate synthase as key enzyme raises the possibility that *G. sulfurreducens* harbors the metabolic potential for chemolithoautotrophic growth. We investigate this hypothesis by transferring *G. sulfurreducens* PCA serially with Fe(III) as electron acceptor and formate as electron donor and carbon source. The evolved strain T17-3 grew chemolithoautotrophically with a 2.7-fold population increase over 48 h and a Fe(III) reduction rate of 417.5 μM h^{-1}. T17-3 also grew with CO_2 as carbon source. Mutations in T17-3 and enzymatic assays point to an adaptation process where the succinyl-CoA synthetase, which is inactive in the wild-type, became active to complete the roTCA cycle. Deletion of the genes coding for the succinyl-CoA synthetase in T17-3 prevented growth with formate as substrate. Enzymatic assays also showed that the citrate synthase can perform the necessary cleavage of citrate for the functional roTCA cycle. These results demonstrate that *G. sulfurreducens* after adaptation reduced CO_2 via the roTCA cycle. This previously hidden metabolism can be harnessed for biotechnological applications and suggests hidden ecological functions for *Geobacter*.

Introduction

Biological CO_2 reduction is an essential reaction for life on Earth responsible for the synthesis of most organic carbon molecules. Until recently, six CO_2 fixation metabolic pathways were known [1, 2]. One of these pathways is the reductive tricarboxylic acid cycle (rTCA), which shares multiple enzymatic reactions with the oxidative TCA cycle (oTCA) of anaerobic microorganisms, but operating in reverse for CO_2 reduction [3–6]. The oTCA cycle is a common metabolic pathway performing the oxidation of acetyl-CoA for the generation of energy and redox equivalents. The only key enzyme of the oTCA cycle that is not involved in the rTCA cycle is the citrate synthase (CS), which catalyzes the condensation of acetyl-CoA and oxaloacetate into citrate. In the rTCA cycle, citrate is converted into acetyl-CoA and oxaloacetate by an ATP-dependent citrate lyase (ACL) or by an ATP-dependent two-step pathway catalyzed by a citryl-CoA synthetase and a citryl-CoA lyase (CCS/CCL) [7–11].

Until recently, the CS reaction was regarded as irreversible under physiological conditions [1, 12–14]. Two studies have demonstrated that it is not always the case and that the oTCA cycle can operate in reverse with CS instead of ACL or CCS/CCL as key enzyme for CO_2 reduction in the hydrogen or acetate-oxidizing and sulfur-reducing deltaproteobacterium *Desulfurella acetivorans* and hydrogen-oxidizing and sulfur-reducing Aquificae bacterium *Thermosulfidibacter takaii* [15, 16]. This carbon fixation

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pathway has been named reversed oxidative TCA cycle (roTCA) since it involves the same series of enzymes as the oTCA cycle but operating the opposite direction. Based on this new discovery and due to the widespread presence of the oTCA cycle in microbial community, it is likely that the capacity of chemolithotrophic inorganic carbon fixation in many microbes cannot be investigated only with genome annotation [17]. Likewise, microbial species with oTCA cycle isolated via laboratory cultivation with organic carbon substrates may have a hidden capability of inorganic carbon fixation encoded in their genomes, which could be revealed if put under the right selection pressure.

Fe(III)-reducing anaerobic *Geobacter* species are major contributor to the carbon and mineral cycles in iron-rich habitats including soils, aquatic sediment, and subsurface environments where they are often found as the dominant population [18, 19]. Because of their capacity to couple acetate oxidation via the oTCA cycle with Fe(III) reduction, one of the main ecological functions of *Geobacter* spp. is the degradation of organic acids generated by fermentative microbes [20–22]. Besides Fe(III)-based acceptors, *Geobacter* spp. can also transfer electrons directly to methanogens for methane production in diverse environments such as wetlands and anaerobic digesters [23–25]. Another widely investigated attribute of *Geobacter* spp. is their capacity to either transfer electrons to an electrode for the production of electricity in microbial fuel cell or to use an electrode as electron donor [26–28]. The most commonly studied *Geobacter* is *Geobacter sulfurreducens* PCA because it is the first one to be sequenced in the genus and gene recombination is easy to be applied [29, 30]. *G. sulfurreducens* was isolated as an obligately heterotroph with acetate as an electron donor and carbon source [31]. On the other hand, the functional rTCA cycle *in G. sulfurreducens* was developed with expressing ACL from *Chlorobium limicola* [26].

Besides genetic engineering, the metabolism and cell functions of *G. sulfurreducens* have been investigated and modified via adaptive laboratory evolution (ALE). ALE can serve to study cell metabolism and response to stresses as well as to develop more efficient and robust industrial strains [32–36]. During ALE, a microbe is cultivated under a selective pressure over many generations, which can lead to the acquisition of mutations conferring or altering metabolic capacities [37–39]. For *G. sulfurreducens*, ALE has been used to investigate and improve extracellular electron transfer to insoluble electron acceptor as well as the metabolic pathway involved in the oxidation of the organic substrate lactate [40–42].

Here, *G. sulfurreducens* PCA wild-type was serially transferred into a chemolithoautotrophic growth medium with formate as electron donor and carbon source and Fe (III) as electron acceptor (FcF). The capability of chemolithoautotrophic growth of the FcF-evolved strain was further evaluated in a medium, where H_2 was the electron donor, CO_2 was the carbon source, and Fe(III) was the electron acceptor. Genome of FcF-evolved clones was sequenced to identify the mutations that may be responsible for the observed metabolic changes. Enzymatic assays, quantitative PCR, functional genetic, and isotopologue analysis of proteinogenic amino acids were employed to investigate the carbon fixation pathway responsible for chemolithoautotrophic growth.

Materials and methods

Strain and growth condition

Strains used in this study are listed in Table S1. *G. sulfurreducens* PCA was routinely cultured in 25 ml anaerobic pressure tubes (15 ml headspace) with a N_2:CO_2 (80:20; 1 atm) atmosphere at 30 °C in either acetate-fumarate (NBAF) or acetate-Fe(III) citrate medium (FcA) at pH 6.8 as described previously [29, 31]. Formate-adapted strains were routinely maintained in FcF medium, which has the same composition as FcA but with formate replacing acetate. Heterotrophic growth experiments were conducted with NBAF (acetate 10 mM, fumarate 40 mM) and FcA (acetate 10 mM, Fe(III) citrate 50 mM) media. Chemolithoautotrophic growth experiments were carried out with FcF (formate 40 mM, Fe(III) citrate 50 mM) and FnF (formate 20 mM, Fe(III)-NTA 10 mM) media. FnF medium has the same composition as FcF but with Fe(III)-NTA instead of Fe(III) citrate as electron acceptor. Chemolithoautotrophic growth was also assessed in FcHC medium, which is similar to FcF but without formate, and the N_2:CO_2 atmosphere replaced by H_2:CO_2 (80:20; 1.3 atm). Additional growth experiments were conducted with an inorganic carbon-free FcH medium (H_2–Fe(III) citrate 50 mM), where the CO_2/HCO_3^- buffer system was replaced by 20 mM MOPS pH 7.0 buffer in the presence or not of 1 mM acetate. In FHC medium, the atmosphere is pure H_2 (1.3 atm). The concentration of Fe(II) in Fe(III)-reducing cultures was measured with the ferrozine assay [43].

Adaptive laboratory evolution with formate

The ALE experiment with formate as electron donor and carbon source was started by transferring 15% of an FcA-grown *G. sulfurreducens* PCA culture into 10 ml sterile FcF medium. Subsequent 15% transfers were done each time when FcF cultures grown anaerobically at 30 °C reached 15 mM Fe(II). The ALE experiment was stopped after 23 transfers as Fe(III) reduction rate stabilized and did not accelerate further. Culture from transfer #17, which was the

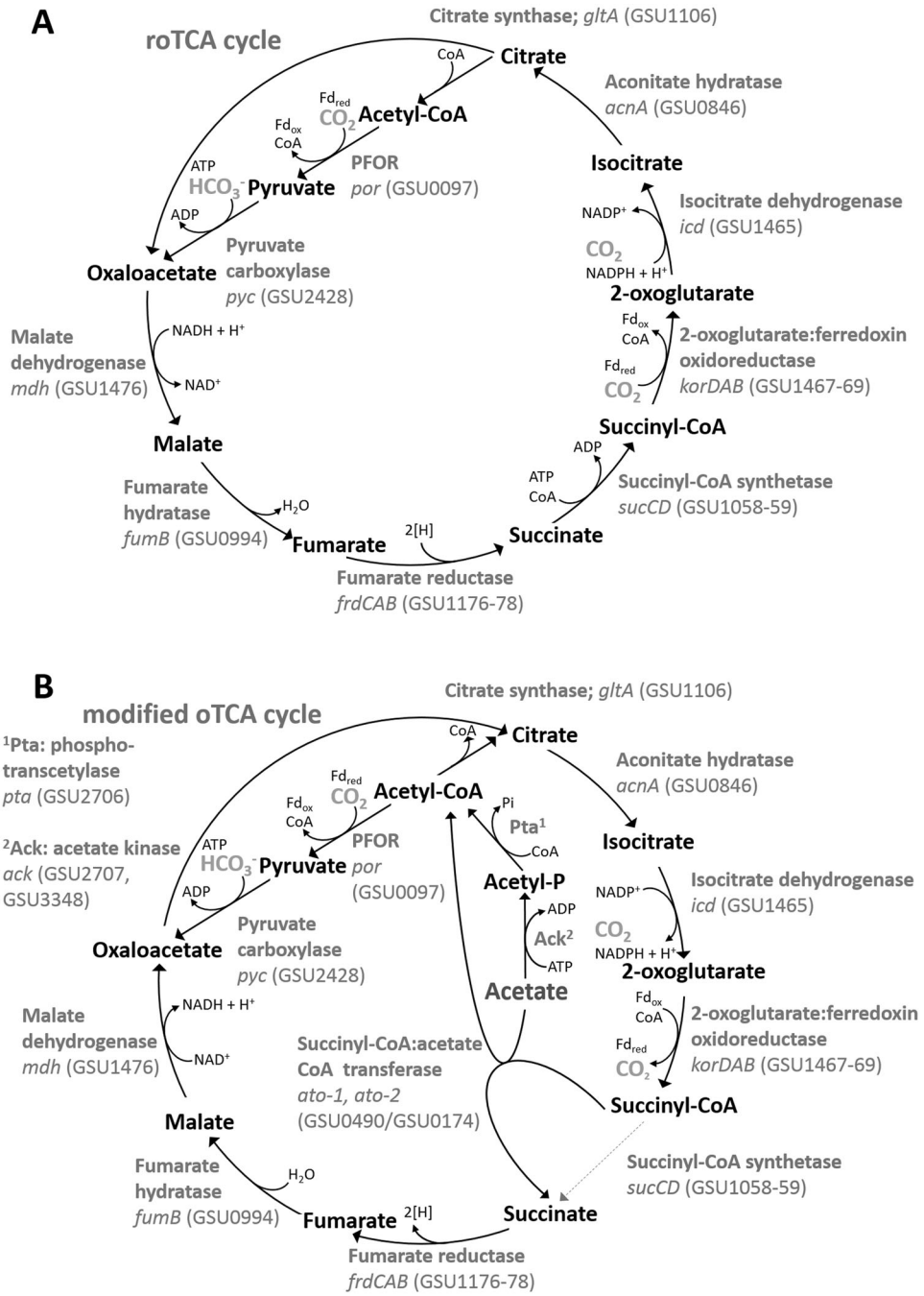

Fig. 1 The TCA cycle of *G. sulfurreducens* in both directions. a roTCA cycle for carbon fixation. All the enzymes required for roTCA cycle are present in *G. sulfurreducens*. Genes coding for ACL or CCS/CCL required for carbon fixation rTCA cycle are not found in *G. sulfurreducens*. **b** oTCA cycle for organic substrate oxidation. When growing with acetate as substrate, *G. sulfurreducens* has a modified oTCA cycle where succinyl-CoA synthetase has minimal activity and is mostly replaced by the succinyl-CoA: acetate CoA-transferase [21, 54].

fastest to reach 15 mM Fe(II), was streaked on plates and several colonies were picked for cultivation into FcF liquid medium. PCR was used to confirm that the isolated clones were *G. sulfurreducens*.

Whole-genome DNA sequencing

Genomic DNA of formate-adapted clones T17-3 and T17-4 was extracted with the M5 Bacteria Genomic DNA Kit (Mei5 Biotechnology, China). The NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, MA, USA) was used to generate genomic DNA libraries. Adapters were ligated to DNA fragments ranging from 300 to 400 bp, which were then recovered by beads purification. A PCR of 12 cycles was used to enrich the adapter-modified DNA before high-throughput sequencing with an Illumina HiSeq 4000 (Illumina, CA, USA) with a paired-end protocol and read lengths of 150 nt. The sequencing reads

were trimmed with PRINSEQ and aligned for variant calling with the Picard DNA-seq analysis pipeline and BBTools [44–46]. The reference genome for the analysis was *G. sulfurreducens* PCA (NCBI reference sequence NC_002939.5). All the samples have an average coverage of at least 30×.

Quantitative reverse transcription PCR (RT-qPCR)

Sequences of all primers used for RT-qPCR are listed in Table S2. Total RNA was extracted with the TRIzol Max Bacterial RNA Isolation Kit (Thermo Fisher Scientific, MA, USA) from mid-log FcF or FcA cultures. RNA samples were then treated with DNase I (RNase-free) (Thermo Fisher Scientific) to remove DNA contamination. cDNA was generated from treated RNA samples with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The SYBR green Real-Time PCR supermix (Mei5 Biotechnology) and the CFX Connect Real-Time PCR Detection System (Bio-Rad, CA, USA) were used to amplify and quantify PCR products for GSU0514, *sucC*, and *gltA*. The expression of these three genes was normalized with *proC* expression, a gene expressed constitutively in *G. sulfurreducens* [47]. Relative levels of expression of GSU0514, *sucC*, and *gltA* were calculated by the $2^{-\Delta\Delta CT}$ method [48].

sucCD^{-} mutant construction

Sequences of all primers used for construction of *ΔsucCD*::Km^{r} mutant allele are listed in Table S2. The mutant construction was done as previously described [49]. The coding sequence of *sucCD* was replaced by a kanamycin resistance cassette. Primer pair sucCDUP/sucCDfusUP was used to amplify 508 bp upstream from *sucC* (GSU1058) and sucCDfusDN/sucCDDN 582 bp downstream from *sucD* (GSU1059) with *G. sulfurreducens* T17-3 genomic DNA as template. The KmRXhoIUp/KmRXbaIDn primer pair was used to amplify the kanamycin resistance cassette from pBBR1MCS-2 [50]. UP and DN PCR products were combined by overlapping extension PCR resulting into the *ΔsucCD* allele, and then cloned with a Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher Scientific). The kanamycin resistance cassette was digested by XhoI–XbaI and ligated with the T4 DNA ligase into the *ΔsucCD* allele resulting into a *ΔsucCD*::Km^{r} allele. The vector carrying *ΔsucCD*::Km^{r} was linearized by digesting with ClaI and concentrated by ethanol precipitation [40]. Electroporation into the adapted strain T17-3 of the linearized vector carrying *ΔsucCD*::Km^{r} and mutant selection was performed as previously described [29, 51]. The *sucCD*$^{-}$ mutant was verified by PCR and Sanger sequencing.

Cell counting, high performance liquid chromatography, isotopologue analysis, and enzymatic assays

The methods for cell counting, high performance liquid chromatography, isotopologue analysis, succinyl-CoA synthetase, and CS assays have been included in the Supplementary Information.

Results

Adaptation of *G. sulfurreducens* with formate as electron donor and carbon source

It has been assumed that *G. sulfurreducens* PCA has a heterotrophic metabolism and cannot grow with CO_2 or other C1 compounds such as formate as sole source of carbon. In previous studies investigating the metabolism of *G. sulfurreducens* with H_2, CO, or formate as electron donor, acetate and/or fumarate were always provided as carbon sources [52, 53]. The genome sequence of *G. sulfurreducens* also supported the claim that this bacterium is possibly an obligate heterotroph since genes coding for the essential enzymes of the six previously known carbon fixation pathways are absent (Table S3) [30].

With the recent discovery of the CS-dependent carbon fixation pathway roTCA cycle in anaerobic bacteria [15, 16], it became interesting to reassess more carefully the chemolithoautotrophic potential of *G. sulfurreducens* PCA. Like multiple other bacteria, *G. sulfurreducens* PCA's genome codes for the full complement of enzymes necessary for TCA cycles in anaerobes (Fig. 1a). Furthermore, the activity of most of these enzymes has already been demonstrated (Table S3) [21, 54]. The complete components of TCA cycle and the capability of hydrogen-oxidizing metabolism in *G. sulfurreducens* PCA suggest the genomic capability of roTCA cycle while chemolithoautotrophic growth was not observed in this strain.

To assess if *G. sulfurreducens* PCA has a chemolithoautotrophic capability harbored in its genome, an ALE experiment was initiated where the bacterial culture was transferred serially into FcF medium. The medium contains the C1 compound formate as the electron donor and carbon source and Fe(III) citrate as the electron acceptor. Fe(III), an electron acceptor of *Geobacter* spp., is commonly supplemented as Fe(III) citrate, and growth and isotope tracing experiments have established that citrate is not used as a carbon source of *G. sulfurreducens* [26, 55, 56]. These results are to be expected since no bacterial transporter demonstrated experimentally to be capable of citrate uptake are encoded on the genome of *G. sulfurreducens* [30]. Furthermore, *G. sulfurreducens* reduces Fe(III) outside of

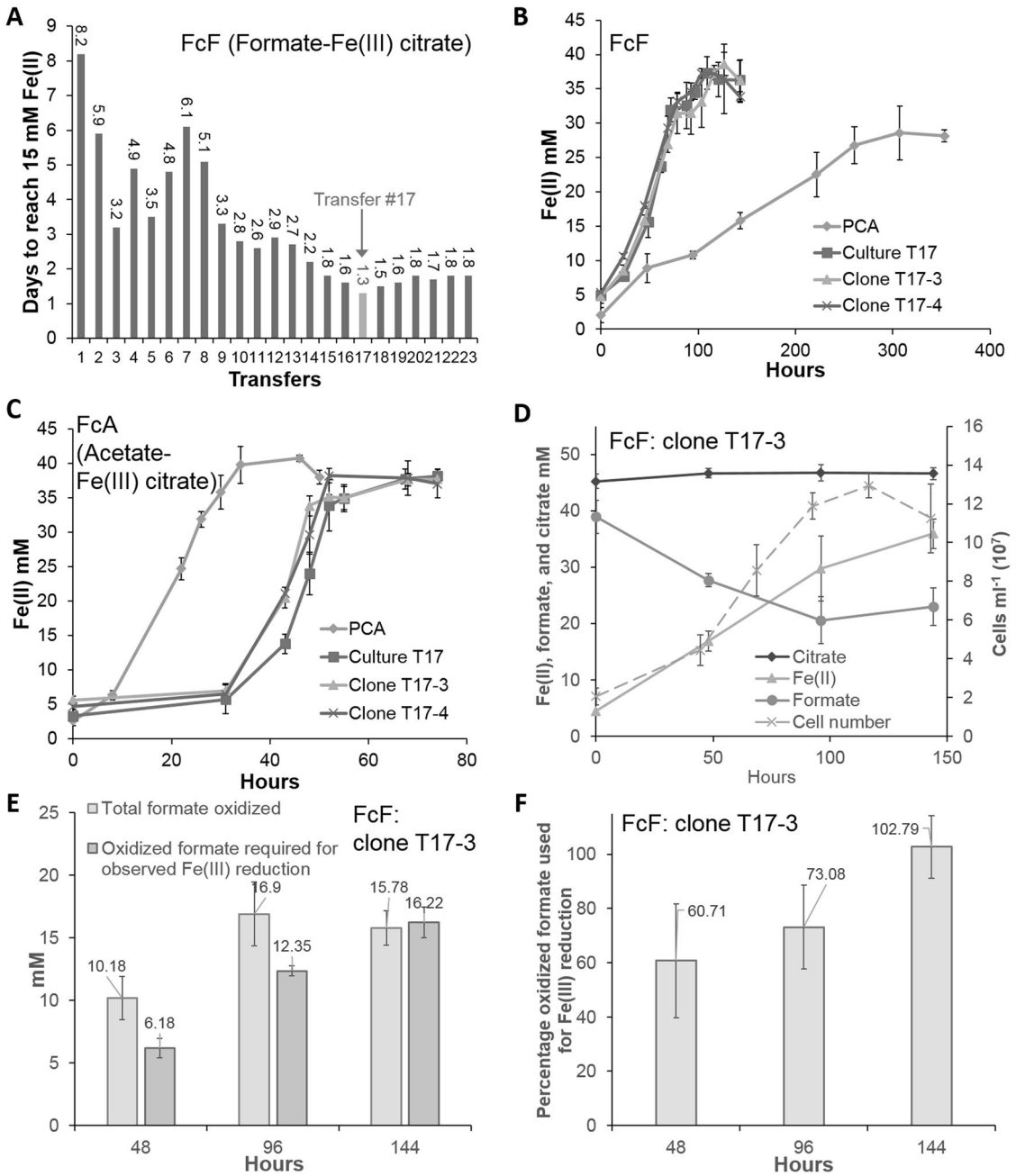

Fig. 2 Adaptation, growth, and metabolism of *G. sulfurreducens* PCA with formate as carbon source and electron donor as well as Fe(III) citrate as electron acceptor. a Number of days to reach 15 mM Fe(II) for each transfer during the ALE experiment with FcF medium. **b** Growth of PCA, the FcF-adapted culture T17, the clones T17-3, and T17-4 on FcF medium and **c** FcA medium. **d** Fe(III) reduction, formate oxidation, citrate concentration, and cell count increase when clone T17-3 grew in FcF medium. **e** Total formate consumed, oxidized formate equivalent required for observed Fe(III) reduction and **f** percentage of oxidized formate used for Fe(III) reduction at 48, 96, and 144 h by T17-3 in FcF medium. Formate is oxidized in *G. sulfurreducens* according to the reaction $HCOO^- \rightarrow CO_2 + H^+ + 2e^-$. Fe(III) is reduced in *G. sulfurreducens* according to the reaction $Fe^{3+} + e^- \rightarrow Fe^{2+}$. Overall, 1 mol formate reduces 2 mol Fe(III). Each curve and bar for **b**–**f** is the mean of three replicates with standard deviation.

the cell at the outer surface and does not have homologs for the Fe(III) citrate transporter complex FecABCDE found in other Gram-negative bacteria such as *E. coli* [57, 58].

The first transfer into FcF was from an acetate-grown PCA culture in an Fc medium with acetate as electron donor and organic carbon source and Fe(III) as electron acceptor. This 15% transfer required 8.2 days to reach 15 mM Fe(II) in FcF medium (Fig. 2a). Subsequently, a 15% inoculum from the first FcF culture was transferred into fresh FcF medium for the second transfer, which took 5.9 days to

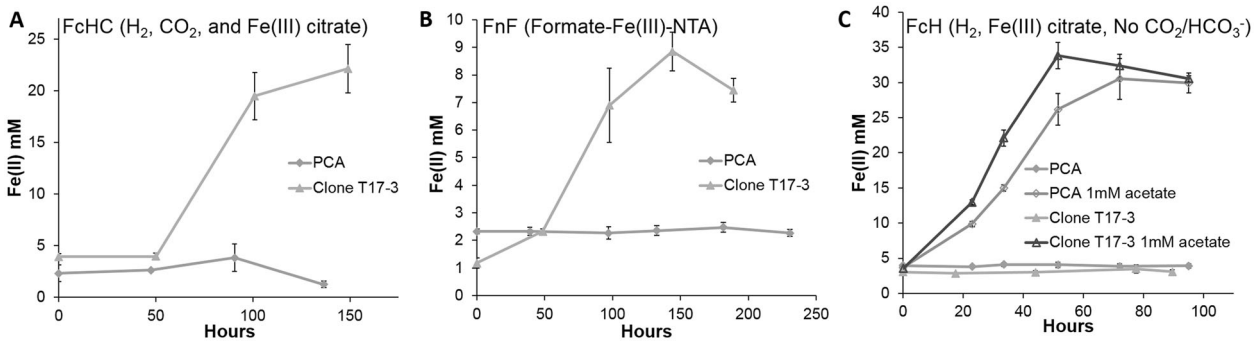

Fig. 3 *G. sulfurreducens* T17-3 growth with alternative carbon sources, electron donors, and acceptors. a Fe(III) reduction by PCA and T17-3 in an FcHC medium with H_2 as electron donor, CO_2 as carbon source, and Fe(III) citrate as electron acceptor. **b** Fe(III) reduction by PCA and T17-3 in an FnF medium with formate as electron donor and carbon source and Fe(III)-NTA as electron acceptor. **c** Fe(III) reduction by PCA and T17-3 in an FcH medium without inorganic carbon CO_2/HCO_3^-, with H_2 as electron donor, Fe (III) citrate as electron acceptor, and MOPS as buffer in the presence or not of 1 mM acetate. Each curve is the mean of three replicates with standard deviation.

reach 15 mM Fe(II). After five transfers in FcF, acetate concentration was measured at the beginning of transfer 6 and no residual acetate from the initial FcA culture was detected. After ten transfers in FcF fresh medium, the PCA culture was clearly adapting and required only 2.8 days to reach 15 mM Fe(II). The Fe(III) reduction rate of the adapted PCA culture became stabilized after transfer 15 and did not improve significantly afterward. The ALE experiment was stopped at transfer 23. Culture from transfer 17, which reached 15 mM Fe(II) after 1.3 days, was used for further experiments.

To obtain individual clonal population, colonies were isolated from the T17 culture and tested for Fe(III) reduction in FcF medium. Culture T17, clones T17-3 and T17-4 reduced Fe(III) at a rate of 490.3 ± 23.0, 417.5 ± 54.0, and 414.3 ± 21.5 μM h^{-1}, respectively (Fig. 2b). In comparison, unadapted PCA after one transfer from FcA to FcF reduced Fe(III) at a rate of 94.3 ± 23.0 μM h^{-1}, which was 5.2 times slower than culture T17. When grown on FcA, culture T17 and derived clones had significantly longer lag phase than PCA, which showed that gain of fitness when growing chemolithoautotrophically with formate was accompanied with a loss of fitness for heterotrophic growth with acetate as substrate (Fig. 2c).

Cell count, formate oxidation, citrate concentration, and Fe(III) reduction

To confirm that adapted bacteria grow in FcF medium and not only reduce Fe(III), T17-3 cells were counted at different time points over a period of 143 h after inoculation (Fig. 2d). From 45 to 93 h, the cell number in the FcF medium increased by $7.45 \times 10^7 \pm 1.05 \times 10^7$ cells ml^{-1} ($n = 3$). In comparison, increase in T17-3 cell number was negligible when no formate was provided (Fig. S1). The results demonstrated that Fe(III) reduction with formate as energy and carbon sources was accompanied with cell biosynthesis, division, and growth. When formate consumption ceased, T17-3 cell number stopped increasing and eventually decreased, which indicates the end of biosynthesis and the beginning of cell death.

Besides Fe(III) reduction and cell number increase, formate concentration was also monitored during growth of the T17-3 clone (Fig. 2d). During exponential growth phase, more formate than required for the observed Fe(III) reduction was consumed by the T17-3 clone (Fig. 2e, f). Only 60.7 ± 21.0% and 73.1 ± 15.5% of all the electrons derived from formate consumed by cells after 48 and 96 h of growth, respectively, were used for energy conservation via Fe(III) reduction. During the stationary growth phase from 96 to 144 h, formate consumption ceased while Fe(III) reduction continued at a slower rate compared with 0 to 96 h. After 144 h, all the electrons derived from oxidized formate have been employed for Fe(III) reduction (Fig. 2e, f). In the absence of formate oxidation in the stationary growth phase, it is highly possible that T17-3 cell starts reducing Fe (III) with electrons coming from substrates excreted by cells, from metabolites released by dead cells or from compounds stored beforehand in cell reserve.

In *G. sulfurreducens*, formate is oxidized (formate → $CO_2 + H^+ + 2e^-$, $E_0' = -420$ mV) by the periplasmically oriented membrane-bound formate dehydrogenase FdnGHI [59]. For chemolithoautotrophic growth on formate as substrate to be feasible, part of the electrons generated by the oxidation reaction catalyzed by the formate dehydrogenase must be carried by the electron transport chain to the final electron acceptor Fe(III), while protons released in the periplasm form the proton gradient necessary for energy conservation. Concomitantly, another fraction of electrons coming from formate oxidation must be used for the metabolic reduction of CO_2 required for the synthesis of cell components. Both formate consumption and Fe(III)

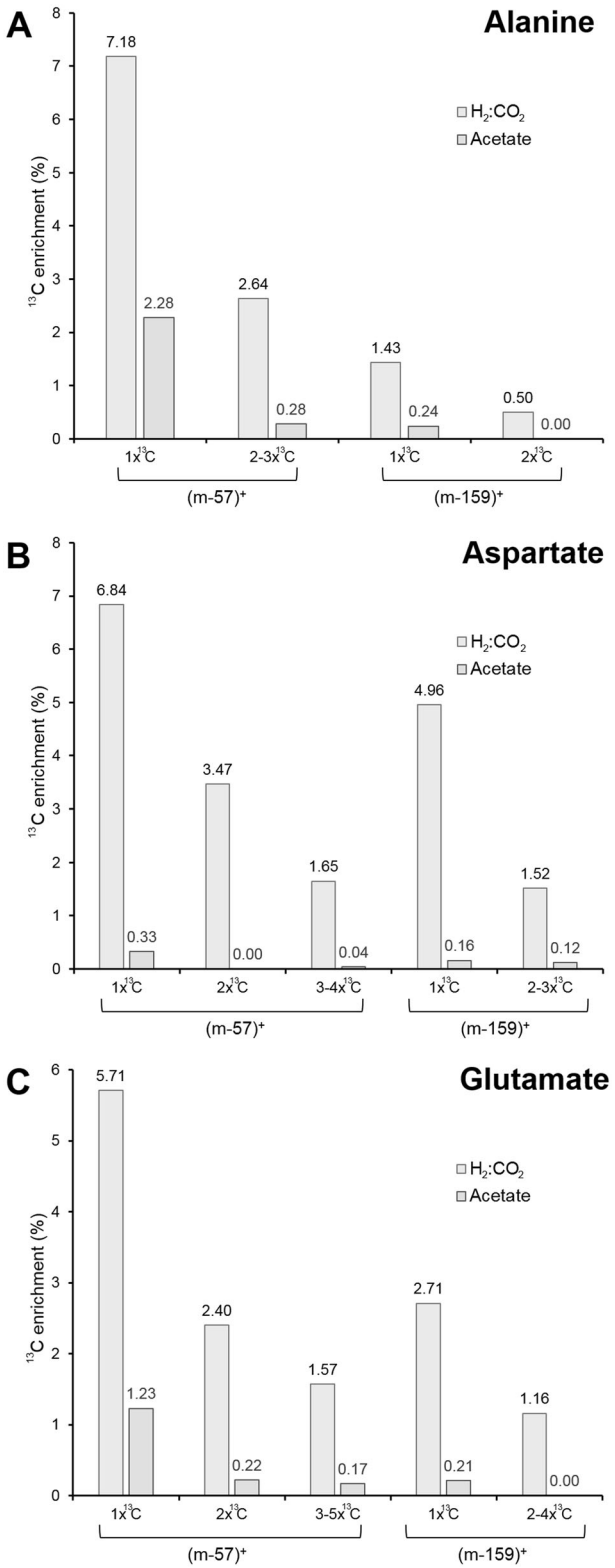

Fig. 4 Isopotomer analysis of *G. sulfurreducens* T17-3. ^{13}C enrichments of **a** alanine, **b** aspartate, and **c** glutamate mass fragments from *G. sulfurreducens* T17-3 grown either chemolithoautrophically or heterotrophically with $H^{13}CO_3^-$. $(M\text{-}57)^+$ is the ^{13}C enrichment of the entire amino acids while $(m\text{-}159)^+$ is the amino acid without the α-carboxyl group. $n \times {}^{13}C$ or $n–y \times {}^{13}C$ indicates the presence of n ^{13}C or n to y ^{13}C in the amino acid, respectively.

reduction results during the exponential growth phase of strain T17-3 support this dual metabolism where formate is used simultaneously as a substrate for biosynthesis and as an electron donor for energy conservation.

Besides formate, the C6 organic carbon compound citrate is also present in the FcF medium under the form of Fe(III) citrate. Although a large number of evidences demonstrated that Fe(III) citrate is not used as a carbon source by *G. sulfurreducens* [26, 55, 56], the citrate concentration was monitored during growth (Fig. 2d). From time 0 to 144 h, citrate concentration did not change, which suggested that it was not used by strain T17-3.

Chemolithoautotrophic growth of *G. sulfurreducens* T17-3 with alternative substrates

To provide additional evidences of chemolithoautotrophic metabolism, adapted strain T17-3 was also cultivated with H_2 as electron donor, CO_2 as carbon source and Fe(III) citrate as electron acceptor (FcHC), as well as with formate as electron donor and carbon source and Fe(III)-NTA as electron acceptor (FnF) (Fig. 3a, b). Before collecting data for Fe(III) reduction curves with T17-3, four prior transfers on either FcHC or FnF were carried out from an original FcF inoculum to ensure complete depletion of carbon sources, electron donors, and acceptors found in FcF medium. Fe(III) reduction curves with PCA were done with the second transfer on FcHC or FnF from an FcA culture. T17-3 grew in FcHC at a Fe(III) reduction rate of 346.9 ± 57.3 μM h^{-1} between 50 and 100 h and significant increase of cell number while PCA did not reduce Fe(III) over an incubation period of 144 h (Fig. S1). In addition, citrate concentration was also assessed during chemolithoautotrophic growth of T17-3 in FcHC medium (Fig. S2). Similar to the results from FcF, citrate concentration did not vary in FcHC medium. For FnF growth, T17-3 had a Fe(III) reduction rate of 95.7 ± 29.7 μM h^{-1}, while G. *sulfurreducens* PCA did not reduce Fe(III)-NTA over 230 h (Fig. 3b). These results demonstrated that chemolithoautotrophic growth by *G. sulfurreducens* T17-3 is not specific to formate and Fe(III) citrate, but is also observed with CO_2 as carbon source, H_2 as electron donor, and Fe(III)-NTA as electron acceptor.

Removal of CO_2 and HCO_3^- prevents H_2-driven growth of T17-3

Chemolithoautotrophic capability of *G. sulfurreducens* T17-3 was further investigated by growing cells in an FcH medium with H_2 as the electron donor and Fe(III) citrate as the electron acceptor, but where CO_2 and HCO_3^- had been removed from the gas phase and the liquid phase (Fig. 3c). An MOPS buffer was added in this growth medium to

replace the bicarbonate buffer system and maintain pH to 7.0. Neither PCA nor T17-3 could grow in the absence of CO_2/HCO_3^- providing further evidence that the formate adaptation process activated a chemolithoautotrophic metabolism in T17-3 dependent on the presence of inorganic carbon sources. When 1 mM acetate was provided as carbon source with the MOPS-based FcH medium, both PCA and T17-3 reduced Fe(III) at a rate of 577.3 ± 73.8 and 724.3 ± 56.8 μM Fe(II) h^{-1}, respectively.

Isopotomer analysis of chemolithoautotrophic growth with $H^{13}CO_3^-$

To investigate if the roTCA cycle was active in the adapted strain T17-3, 6 mM $H^{13}CO_3^-$ was added to mid-log phase cultures growing either chemolithoautotrophically with FcHC or heterotrophically with FcA. Sixteen hours after the addition of ^{13}C, cells were harvested, hydrolyzed, and proteinogenic amino acids were derivatized for isotopologue profiling by GC-MS (Fig. S3). ^{13}C enrichments of entire amino acids $(m\text{-}57)^+$ and amino acids lacking the α-carboxyl group $(m\text{-}159)^+$ were analyzed to determine ^{13}C position [60]. Alanine, aspartate, and glutamate were selected for the isopotologue analysis since they are synthesized from precursors generated by the TCA cycles [56].

^{13}C enrichment of all three amino acids in H_2:CO_2-grown cells compared with acetate-grown cells indicated that the roTCA cycle was functional in T17-3 (Figs. 4 and S4). For alanine, ^{13}C labeling of C1 indicated carboxylation of acetyl-CoA by PFOR for both acetate-grown and H_2:CO_2-grown cells. Carboxylation of acetyl-CoA by PFOR is a contributing gluconeogenetic reaction for the biosynthetic metabolism of *G. sulfurreducens* during growth with acetate as carbon source and Fe(III) as electron acceptor [56]. However, it was only with H_2:CO_2-grown cells that a significant fraction of alanine was labeled with two to three ^{13}C (Fig. 4a). This result shows that pyruvate molecules, the precursor of alanine, were synthesized with at least two inorganic carbon molecules probably via the roTCA cycle.

Aspartate and glutamate molecules with ^{13}C were also significantly more frequent when T17-3 was grown chemolithoautotrophically (Fig. 4b). Precursors for aspartate and glutamate are oxaloacetate and 2-oxoglutarate, respectively. The isotopologue profile for aspartate showed that both PFOR and the pyruvate carboxylase carboxylated acetyl-CoA into oxaloacetate. Furthermore, the detection of aspartate molecules labeled with three or four ^{13}C indicated that oxaloacetate underwent multiple turns in the roTCA cycle. ^{13}C enrichment and position in glutamate molecules from T17-3 grown with H_2:CO_2 versus acetate provides additional evidences of the activity of the roTCA cycle in the adapted strain (Fig. 4c).

Mutations in *G. sulfurreducens* T17-3 and T17-4

The genomes of formate-adapted clones T17-3 and T17-4 were sequenced to gain insights on how *G. sulfurreducens* evolved to grow chemolithoautotrophically in FcF (Table S4). T17-3 and T17-4 had a total of 15 single-nucleotide polymorphisms (SNP), 1 insertion, and 2 deletions in common. One SNP was exclusive to T17-4. Mutations were located in or near genes coding for hypothetical proteins, membrane proteins, *c*-type cytochromes, and enzymes involved in RNA methylation, oxidoreduction, glycerophospholipid metabolism, nucleotide salvage, and branched-chain amino acid catabolism. *dcuB*, the gene coding for the anaerobic C4-dicarboxylate transporter essential for the growth of *Geobacter* spp. with fumarate as electron acceptor [61], was mutated with an SNP resulting in the substitution of tyrosine-385 for a histidine. However, this mutation had no major impact since the adapted strains T17-3 and T17-4 could still grow with fumarate as electron acceptor (Fig. S5).

rpoB, the gene coding for the β-subunit of the RNA polymerase, had an SNP resulting in the substitution of glutamine-1089 by an arginine in the C-terminal part of the protein (Table S4) [62]. Mutations in *rpoB* or in other global transcription-related genes have been observed in multiple ALE experiments where they have major beneficial impacts on fitness by affecting simultaneously the expression of hundreds to thousands of genes [63–65]. These important changes in the expression profile usually lead to pleiotropic effects on cell's function [66, 67]. Here, genome sequencing results suggest that *rpoB* mutation is involved in the capacity of *G. sulfurreducens* to adapt and grow chemolithoautotrophically on formate.

Role of the succinyl-CoA synthetase in the adapted strain

Among the 19 mutations found by whole-genome sequencing, one SNP in T17-3 and T17-4 was located upstream of GSU0514, which is a gene that has been shown to have a direct regulatory impact on the oTCA cycle of *G. sulfurreducens*. GSU0514 is a transcriptional regulator of the IclR family involved in the repression of the transcription of *sucCD*, the two genes coding for the succinyl-coA synthetase, which is a central enzyme of the oTCA cycle [42] as well as of the roTCA and rTCA cycles [1, 15]. In *G. sulfurreducens* PCA grown with acetate as substrate, the conversion of acetate into acetyl-CoA prior to oxidation via oTCA is achieved by the succinyl-CoA:acetate CoA-transferase, which does not require ATP hydrolysis contrary to the alternative pathway catalyzed by an acetate kinase and a phosphotransacetylase (Fig. 1b) [21, 54]. When the succinyl-CoA:acetate

CoA-transferase is active, the succinyl-CoA synthetase is not required for the completion of the oTCA cycle. This has been highlighted by the absence of succinyl-coA synthetase activity in *G. sulfurreducens* growing heterotrophically [21, 54]. Since *G. sulfurreducens* is usually maintained with acetate as substrate in laboratory conditions, the bacterium has adapted and optimized its acetate metabolism by repressing the expression of succinyl-CoA synthetase via a GSU0514-dependent regulatory mechanism.

However, when acetate is not available in sufficient quantity for the succinyl-CoA:acetate CoA-transferase, *G. sulfurreducens* must adapt and reoptimize its metabolism to ensure efficient growth. This has been demonstrated by adapting *G. sulfurreducens* to grow on lactate as sole substrate [42]. After serial transfer of *G. sulfurreducens* PCA on lactate, mutations inactivating GSU0514 appeared and the expression of the genes coding for the succinyl-coA synthetase had a multifold increase. For chemolithoautotrophic growth of *G. sulfurreducens* with formate or other C1 compounds, the succinyl-CoA synthetase would be required for the completion of a functional roTCA cycle, which may explain the mutation found upstream of GSU0514 in T17-3 and T17-4.

The SNP A → G was found in position −161 in relation to the start of the coding sequence of GSU0514 and was localized in the −35 box of the promoter of GSU0514 as predicted by BPROM (Fig. S6) [68]. Quantitative PCR showed that GSU0514 was downregulated 3.6-fold (3.4–3.8) in T17-3 grown with formate compared with PCA grown with acetate (Fig. 5a). Concomitantly, *sucC*, the gene coding for the β-subunit of the succinyl-CoA synthetase, was upregulated fivefold (4.2–6.0) in T17-3 grown in FcF compared with PCA grown in FcA. The enzymatic activity of succinyl-CoA synthetase was also measured by monitoring the formation of succinyl-CoA from succinate, ATP, and CoA in whole-cell lysates of T17-3 grown in FcF and PCA grown in FcA (Fig. 5b) [69]. A barely detectable succinyl-CoA synthetase activity of 4.81 ± 2.69 nmol succinyl-CoA min^{-1} mg per protein was measured in PCA grown in FcA. In comparison, succinyl-CoA synthetase activity in T17-3 grown in FcF was 3.4-fold higher at 16.41 ± 2.86 nmol succinyl-CoA min^{-1} mg per protein.

Furthermore, deletion in the adapted strain T17-3 of both *sucC* and *sucD*, the gene coding for the α-subunit of the succinyl-CoA synthetase, prevented growth in FcF but not in FcA medium (Fig. 5c). The absence of a functional succinyl-CoA synthetase appears to disrupt the roTCA cycle, which inhibits chemolithoautotrophic growth by *G. sulfurreducens*. This result confirms the importance of the SNP upstream of GSU0514 for the adaptation of *G. sulfurreducens* to grow with formate as substrate.

Activity of the citrate synthase in the reductive direction

The CS is a key enzyme catalyzing an essential step of the oTCA cycle in the oxidative direction and of the roTCA cycle in the reductive direction (Fig. 1). In *G. sulfurreducens* PCA, activity of the CS GltA in the oxidative direction where free CoA and citrate are formed by the condensation of acetyl-CoA and oxaloacetate has already been observed [54]. However, GltA must catalyze the reverse ATP-independent conversion of citrate and CoA into acetyl-CoA and oxaloacetate for the roTCA cycle to be functional. This activity has never been demonstrated before in *G. sulfurreducens*. Here, GltA activity in the reductive direction was coupled with porcine malate dehydrogenase and NADH oxidation was monitored [15]. The reductive CS activity in the whole-cell lysate of PCA grown in FcA was 102.87 ± 1.97 nmol NAD^+ min^{-1} mg per protein (Fig. 5d). In the adapted strain T17-3 grown in FcF, CS activity was still detected but lower at 40.19 ± 1.97 nmol NAD^+ min^{-1} mg per protein. Quantitative PCR results are in line with enzymatic activity. *gltA* was expressed 5.1 (4.0–6.4) times less in T17-3 grown in FcF compared with PCA grown in FcA (Fig. 4a).

The lower activity and transcription of CS in T17-3 grown in formate is to be expected if we consider that CS participates into the roTCA cycle during chemolithoautotrophic growth with formate, and its main function is the biosynthesis of cell components from CO_2. While during heterotrophic growth on acetate, CS participates into the oTCA cycle, and its principal function is to release energy stored in organic carbon substrate for respiration. Thus, metabolic flux is likely to be more important through CS in an oxidative context for respiration than in a reductive context for biosynthesis and, under these circumstances, the cell may limit energy expense by decreasing CS expression.

Discussion

Genome annotation have led to the suggestion that *Geobacter bemidjiensis* has a chemolithoautotrophic metabolism via the rTCA cycle [70]. Growth experiment showed that another *Geobacter* species, *Geobacter metallireducens*, can grow chemolithoautotrophically, and based on genome annotation, it has been suggested that either the rTCA or the dicarboxylate–4-hydroxybutyrate cycles are involved [71]. The discovery of the roTCA cycle [15, 16] as well as experimental evidences presented here demonstrating chemolithoautotrophic growth via roTCA cycle by *G. sulfurreducens* raise the possibility that carbon fixation capability is more widespread than previously thought in the *Geobacteraceae* family. Genes coding for CS and other enzymes of the o/roTCA cycles are present in the vast

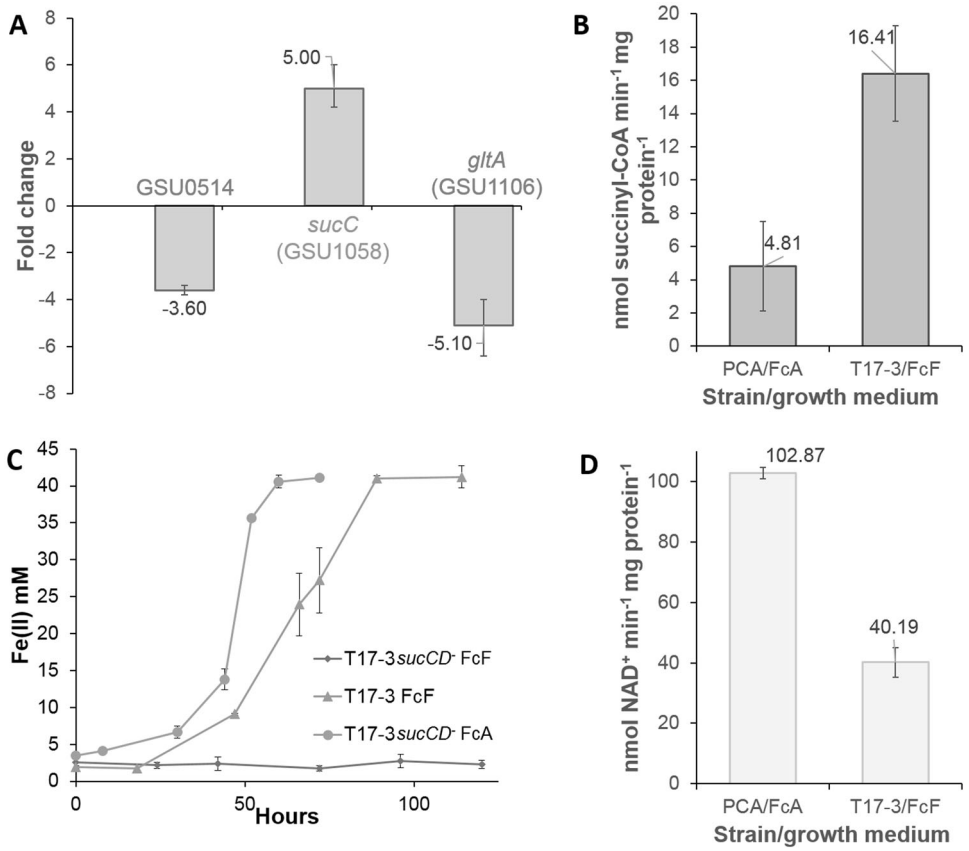

Fig. 5 Transcript abundance fold change of succinyl-CoA synthetase and citrate synthase genes, enzymatic activities, and growth of the T17-3 *sucCD*⁻ mutant. a Transcript abundance fold change of GSU0514, *sucC*, and *gltA* in strain T17-3 grown in FcF medium compared with PCA grown in FcA medium. **b** Enzymatic activity of the succinyl-CoA synthetase (SucCD). Succinyl-CoA synthetase activity was measured via the formation of succinyl-CoA from succinate according to the reaction succinate + CoA + ATP → succinyl-CoA + ADP. **c** Growth of the T17-3 *sucCD*⁻ mutant on FcF and FcA medium. **d** Enzymatic activity of CS (GltA) in whole-cell lysates of PCA grown in FcA and of T17-3 grown in FcF. CS activity was measured via oxaloacetate formation coupled with porcine malate dehydrogenase for NADH oxidation according to the following reactions: (1) citrate + CoA → acetyl-CoA + oxaloacetate and (2) oxaloacetate + NADH + H^+ → malate + NAD^+. The results shown are from at least three replicates for each experiment with standard deviation.

majority of *Geobacter* species genomes sequenced until now and this includes *G. bemidjiensis* and *G. metallireducens*. When compared with the rTCA and the dicarboxylate–4-hydroxybutyrate cycles, the roTCA cycle appears to be more efficient bioenergetically [15]. Thus, *G. bemidjiensis* and *G. metallireducens* thought to reduce CO_2 via alternative carbon fixation pathways may also use the roTCA cycle concomitantly or preferentially.

Several recent studies show that *Geobacter* spp. have a chemolithoautotrophic metabolism [70, 71]. Based on these developments, the ecological niche of *Geobacter* species as degraders of organic acid substrates released by fermentative microbes may have to be reconsidered. At the very least, the chemolithoautotrophic capacity of *Geobacter* spp. confers a competitive advantage over other species and may explain why this family can maintain itself in habitats where organic carbon substrates are likely to fluctuate [18, 19].

In addition, the chemolithoautotrophic metabolism of *G. sulfurreducens* is intriguing for potential industrial applications since this bacterium can synthesize bioelectronic compounds such as electrically conductive pili and can be employed as microbial catalyst in bioelectrochemical systems [26, 72]. Thus, bioproduction processes catalyzed by *G. sulfurreducens* could be driven by microbial electrosynthesis or could be optimized for the synthesis of bioelectronic parts from the greenhouse gas CO_2.

Furthermore, another interesting observation reported here is that an anaerobic bacterial species previously thought to be a strict heterotroph has a chemolithoautotrophic metabolic capacity that was revealed by ALE. *G. sulfurreducens* PCA was isolated in the laboratory with acetate as electron donor and carbon source [31]. This isolation method might have introduced metabolic biases and adaptive mutations hiding physiological functions with major role in the natural habitat of the isolated microbe. Based on the observation here, it is possible that other laboratory-isolated anaerobic microbes with genes for the roTCA cycle have been characterized prematurely as strict heterotroph.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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References

1. Fuchs G. Alternative pathways of carbon dioxide fixation: insights into the early evolution of life? Annu Rev Microbiol. 2011;65:631–58.
2. Hügler M, Sievert SM. Beyond the Calvin cycle: autotrophic carbon fixation in the ocean. Ann Rev Mar Sci. 2011;3:261–89.
3. Evans MC, Buchanan BB, Arnon DI. A new ferredoxin-dependent carbon reduction cycle in a photosynthetic bacterium. Proc Natl Acad Sci USA. 1966;55:928–34.
4. Fuchs G, Stupperich E, Eden G. Autotrophic CO_2 fixation in *Chlorobium limico*la. Evidence for the operation of a reductive tricarboxylic acid cycle in growing cells. Arch Microbiol. 1980;128:64–71.
5. Schauder R, Widdel F, Fuchs G. Carbon assimilation pathways in sulfate-reducing bacteria II. Enzymes of a reductive citric acid cycle in the autotrophic *Desulfobacter hydrogenophilus*. Arch Microbiol. 1987;148:218–25.
6. Shiba H, Kawasumi T, Igarashi Y, Kodama T, Minoda Y. The CO_2 assimilation via the reductive tricarboxylic acid cycle in an obligately autotrophic, aerobic hydrogen-oxidizing bacterium, *Hydrogenobacter thermophilus*. Arch Microbiol. 1985;141:198–203.
7. Ivanovsky RN, Sintsov NV, Kondratieva EN. ATP-linked citrate lyase activity in the green sulfur bacterium *Chlorobium limicola forma thiosulfatophilum*. Arch Microbiol. 1980;128:239–41.
8. Aoshima M, Ishii M, Igarashi Y. A novel enzyme, citryl-CoA synthetase, catalysing the first step of the citrate cleavage reaction in *Hydrogenobacter thermophilus* TK-6. Mol Microbiol. 2004;52:751–61.
9. Aoshima M, Ishii M, Igarashi Y. A novel enzyme, citryl-CoA lyase, catalysing the second step of the citrate cleavage reaction in *Hydrogenobacter thermophilus* TK-6. Mol Microbiol. 2004;52:763–70.
10. Kanao T, Fukui T, Atomi H, Imanaka T. ATP-citrate lyase from the green sulfur bacterium *Chlorobium limicola* is a heteromeric enzyme composed of two distinct gene products. Eur J Biochem. 2001;268:1670–8.
11. Beh M, Strauss G, Huber R, Stetter K-O, Fuchs G. Enzymes of the reductive citric acid cycle in the autotrophic eubacterium *Aquifex pyrophilus* and in the archaebacterium *Thermoproteus neutrophilus*. Arch Microbiol. 1993;160:306–11.
12. Berg IA. Ecological aspects of the distribution of different autotrophic CO_2 fixation pathways. Appl Environ Microbiol. 2011;77:1925–36.
13. Thauer RK, Möller-Zinkhan D, Spormann AM. Biochemistry of acetate catabolism in anaerobic chemotrophic bacteria. Annu Rev Microbiol. 1989;43:43–67.
14. Ito S, Koyama N, Osanai T. Citrate synthase from *Synechocystis* is a distinct class of bacterial citrate synthase. Sci Rep. 2019;9:6038.
15. Mall A, Sobotta J, Huber C, Tschirner C, Kowarschik S, Bačnik K, et al. Reversibility of citrate synthase allows autotrophic growth of a thermophilic bacterium. Science. 2018;359:563–7.
16. Nunoura T, Chikaraishi Y, Izaki R, Suwa T, Sato T, Harada T, et al. A primordial and reversible TCA cycle in a facultatively chemolithoautotrophic thermophile. Science. 2018;359:559–63.
17. Ragsdale SW. Stealth reactions driving carbon fixation. Science. 2018;359:517–8.
18. Lovley DR, Ueki T, Zhang T, Malvankar NS, Shrestha PM, Flanagan KA, et al. *Geobacter*: the microbe electric's physiology, ecology, and practical applications. Adv Micro Physiol. 2011;59:1–100.
19. Röling WFM. The family *Geobacteraceae*. In: Rosenberg E, DeLong EF, Lory S, Stackebrandt E, Thompson F, editors. The prokaryotes: *Deltaproteobacteria* and *Epsilonproteobacteria*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2014. p. 157–72.
20. Mahadevan R, Bond DR, Butler JE, Esteve-Nuñez A, Coppi MV, Palsson BO, et al. Characterization of metabolism in the Fe(III)-reducing organism *Geobacter sulfurreducens* by constraint-based modeling. Appl Environ Microbiol. 2006;72:1558–68.
21. Segura D, Mahadevan R, Juárez K, Lovley DR. Computational and experimental analysis of redundancy in the central metabolism of *Geobacter sulfurreducens*. PLoS Comput Biol. 2008;4:e36.
22. Lovley DR, Chapelle FH. Deep subsurface microbial processes. Rev Geophys. 1995;33:365–81.
23. Morita M, Malvankar NS, Franks AE, Summers ZM, Giloteaux L, Rotaru AE, et al. Potential for direct interspecies electron transfer in methanogenic wastewater digester aggregates. MBio. 2011;2:e00159–11.
24. Rotaru A-E, Shrestha PM, Liu F, Shrestha M, Shrestha D, Embree M, et al. A new model for electron flow during anaerobic digestion: direct interspecies electron transfer to *Methanosaeta* for the reduction of carbon dioxide to methane. Energy Environ Sci. 2013;7:408–15.
25. Holmes DE, Shrestha PM, Walker DJF, Dang Y, Nevin KP, Woodard TL, et al. Metatranscriptomic evidence for direct interspecies electron transfer between *Geobacter* and *Methanothrix* species in methanogenic rice paddy soils. Appl Environ Microbiol. 2017;83:e00223–17.
26. Ueki T, Nevin KP, Woodard TL, Aklujkar MA, Holmes DE, Lovley DR. Construction of a *Geobacter* strain with exceptional growth on cathodes. Front Microbiol. 2018;9:1512.
27. Bond DR, Lovley DR. Electricity production by *Geobacter sulfurreducens* attached to electrodes. Appl Environ Microbiol. 2003;69:1548–55.
28. Gregory KB, Bond DR, Lovley DR. Graphite electrodes as electron donors for anaerobic respiration. Environ Microbiol. 2004;6:596–604.
29. Coppi MV, Leang C, Sandler SJ, Lovley DR. Development of a genetic system for *Geobacter sulfurreducens*. Appl Environ Microbiol. 2001;67:3180–7.
30. Methé BA, Nelson KE, Eisen JA, Paulsen IT, Nelson W, Heidelberg JF, et al. Genome of *Geobacter sulfurreducens*: metal reduction in subsurface environments. Science. 2003;302:1967–9.
31. Caccavo F, Lonergan DJ, Lovley DR, Davis M, Stolz JF, McInerney MJ. *Geobacter sulfurreducens* sp. nov., a hydrogen- and acetate-oxidizing dissimilatory metal-reducing microorganism. Appl Environ Microbiol. 1994;60:3752–9.
32. Caspeta L, Chen Y, Ghiaci P, Feizi A, Buskov S, Hallström BM, et al. Biofuels. Altered sterol composition renders yeast thermotolerant. Science. 2014;346:75–78.
33. Tremblay P-L, Höglund D, Koza A, Bonde I, Zhang T. Adaptation of the autotrophic acetogen *Sporomusa ovata* to methanol accelerates the conversion of CO_2 to organic products. Sci Rep. 2015;5:16168.
34. Atsumi S, Wu T-Y, Machado IMP, Huang W-C, Chen P-Y, Pellegrini M, et al. Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*. Mol Syst Biol. 2010;6:449.

35. Conrad TM, Lewis NE, Palsson BØ. Microbial laboratory evolution in the era of genome-scale science. Mol Syst Biol. 2011;7:509.
36. Zhang T, Shi X-C, Xia Y, Mai L, Tremblay P-L. *Escherichia coli* adaptation and response to exposure to heavy atmospheric pollution. Sci Rep. 2019;9:10879.
37. Dragosits M, Mattanovich D. Adaptive laboratory evolution - principles and applications for biotechnology. Micro Cell Fact. 2013;12:64.
38. Elena SF, Lenski RE. Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. Nat Rev Genet. 2003;4:457–69.
39. Wiser MJ, Ribeck N, Lenski RE. Long-term dynamics of adaptation in asexual populations. Science. 2013;342:1364–7.
40. Tremblay P-L, Summers ZM, Glaven RH, Nevin KP, Zengler K, Barrett CL, et al. A *c*-type cytochrome and a transcriptional regulator responsible for enhanced extracellular electron transfer in *Geobacter sulfurreducens* revealed by adaptive evolution. Environ Microbiol. 2011;13:13–23.
41. Smith JA, Tremblay P-L, Shrestha PM, Snoeyenbos-West OL, Franks AE, Nevin KP, et al. Going wireless: Fe(III) oxide reduction without pili by *Geobacter sulfurreducens* strain JS-1. Appl Environ Microbiol. 2014;80:4331–40.
42. Summers ZM, Ueki T, Ismail W, Haveman SA, Lovley DR. Laboratory evolution of *Geobacter sulfurreducens* for enhanced growth on lactate via a single-base-pair substitution in a transcriptional regulator. ISME J. 2012;6:975–83.
43. Lovley DR, Phillips EJ. Organic matter mineralization with reduction of ferric iron in anaerobic sediments. Appl Environ Microbiol. 1986;51:683–9.
44. Picard Tools - By Broad Institute. http://broadinstitute.github.io/picard/. Accessed 29 Aug 2019.
45. Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets. Bioinformatics. 2011;27:863–4.
46. Bushnell B, Rood J, Singer E. BBMerge - accurate paired shotgun read merging via overlap. PLoS ONE. 2017;12:e0185056.
47. Holmes DE, Nevin KP, O'Neil RA, Ward JE, Adams LA, Woodard TL, et al. Potential for quantifying expression of the *Geobacteraceae citrate synthase gene to assess the activity of* Geobacteraceae in the subsurface and on current-harvesting electrodes. Appl Environ Microbiol. 2005;71:6870–7.
48. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25:402–8.
49. Nevin KP, Kim B-C, Glaven RH, Johnson JP, Woodard TL, Methé BA, et al. Anode biofilm transcriptomics reveals outer surface components essential for high density current production in *Geobacter sulfurreducens* fuel cells. PLoS ONE. 2009;4:e5628.
50. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 1995;166:175–6.
51. Lloyd JR, Leang C, Hodges Myerson AL, Coppi MV, Cuifo S, Methe B, et al. Biochemical and genetic characterization of PpcA, a periplasmic *c*-type cytochrome in *Geobacter sulfurreducens*. Biochem J. 2003;369:153–61.
52. Geelhoed JS, Henstra AM, Stams AJM. Carboxydotrophic growth of G*eobacter sulfurreducens*. Appl Microbiol Biotechnol. 2016;100:997–1007.
53. Speers AM, Reguera G. Electron donors supporting growth and electroactivity of *Geobacter sulfurreducens* anode biofilms. Appl Environ Microbiol. 2012;78:437–44.
54. Galushko AS, Schink B. Oxidation of acetate through reactions of the citric acid cycle by *Geobacter sulfurreducens* in pure culture and in syntrophic coculture. Arch Microbiol. 2000;174:314–21.
55. Coppi MV, O'Neil RA, Lovley DR. Identification of an uptake hydrogenase required for hydrogen-dependent reduction of Fe(III) and other electron acceptors by *Geobacter sulfurreducens*. J Bacteriol. 2004;186:3022–8.
56. Yang TH, Coppi MV, Lovley DR, Sun J. Metabolic response of *Geobacter sulfurreducens* towards electron donor/acceptor variation. Micro Cell Fact. 2010;9:90.
57. Coppi MV, O'neil RA, Leang C, Kaufmann F, Methé BA, Nevin KP, et al. Involvement of *Geobacter sulfurreducens* SfrAB in acetate metabolism rather than intracellular, respiration-linked Fe (III) citrate reduction. Microbiology. 2007;153:3572–85.
58. Härle C, Kim I, Angerer A, Braun V. Signal transfer through three compartments: transcription initiation of the *Escherichia coli* ferric citrate transport system from the cell surface. EMBO J. 1995;14:1430–8.
59. Coppi MV. The hydrogenases of *Geobacter sulfurreducens*: a comparative genomic perspective. Microbiology. 2005;151:1239–54.
60. You L, Page L, Feng X, Berla B, Pakrasi HB, Tang YJ. Metabolic pathway confirmation and discovery through (13)C-labeling of proteinogenic amino acids. J Vis Exp. 2012;59:3583.
61. Butler JE, Glaven RH, Esteve-Núñez A, Núñez C, Shelobolina ES, Bond DR, et al. Genetic characterization of a single bifunctional enzyme for fumarate reduction and succinate oxidation in *Geobacter sulfurreducens* and engineering of fumarate reduction in Geobacter metallireducens. J Bacteriol. 2006;188:450–5.
62. Ishihama A. Functional modulation of *Escherichia coli* RNA polymerase. Annu Rev Microbiol. 2000;54:499–518.
63. LaCroix RA, Sandberg TE, O'Brien EJ, Utrilla J, Ebrahim A, Guzman GI, et al. Use of adaptive laboratory evolution to discover key mutations enabling rapid growth of *Escherichia coli* K-12 MG1655 on glucose minimal medium. Appl Environ Microbiol. 2015;81:17–30.
64. Tenaillon O, Rodríguez-Verdugo A, Gaut RL, McDonald P, Bennett AF, Long AD, et al. The molecular diversity of adaptive convergence. Science. 2012;335:457–61.
65. Rodríguez-Verdugo A, Carrillo-Cisneros D, González-González A, Gaut BS, Bennett AF. Different tradeoffs result from alternate genetic adaptations to a common environment. Proc Natl Acad Sci USA. 2014;111:12121–6.
66. Rodríguez-Verdugo A, Tenaillon O, Gaut BS. First-step mutations during adaptation restore the expression of hundreds of genes. Mol Biol Evol. 2016;33:25–39.
67. Rodríguez-Verdugo A, Gaut BS, Tenaillon O. Evolution of *Escherichia coli* rifampicin resistance in an antibiotic-free environment during thermal stress. BMC Evol Biol. 2013;13:50.
68. Solovyev V, Salamov A. Automatic annotation of microbial genomes and metagenomic sequences. In: Li RW, editor. Metagenomics and its applications in agriculture, biomedicine and environmental studies. Nova Science Publishers are based in Hauppauge, New York, United States; 2011. p. 61–78.
69. Cha S. [13] Succinate thiokinase from pig heart: [EC 6.2.1.4. Succinate: CoA ligase (GDP)]. Method Enzymol. 1969;13:62–69.
70. Aklujkar M, Young ND, Holmes D, Chavan M, Risso C, Kiss HE, et al. The genome of *Geobacter bemidjiensis*, exemplar for the subsurface clade of *Geobacter* species that predominate in Fe(III)-reducing subsurface environments. BMC Genomics. 2010;11:490.
71. Feist AM, Nagarajan H, Rotaru A-E, Tremblay P-L, Zhang T, Nevin KP, et al. Constraint-based modeling of carbon fixation and the energetics of electron transfer in *Geobacter metallireducens*. PLoS Comput Biol. 2014;10:e1003575.
72. Lovley DR. Electrically conductive pili: Biological function and potential applications in electronics. Curr Opin Electrochem. 2017;4:190–8.