bio-protocol

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Tracking the Reversed Oxidative Tricarboxylic Acid Cycle in Bacteria

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Abstract

Different pathways for autotrophic CO_2 fixation can be recognized by the presence of genes for their specific key enzymes. On this basis, (meta)genomic, (meta)transcriptomic, or (meta)proteomic analysis enables the identification of the role of an organism or a distinct pathway in primary production. However, the recently discovered variant of the reductive tricarboxylic acid (rTCA) cycle, the reverse oxidative tricarboxylic acid (roTCA) cycle, lacks unique enzymes, a feature that makes it cryptic for bioinformatics analysis. This pathway is a reversal of the widespread tricarboxylic acid (TCA) cycle. The functioning of the roTCA cycle requires unusually high activity of citrate synthase, the enzyme responsible for citrate cleavage, as well as elevated CO_2 partial pressures. Here, we present a detailed description of the protocol we used for the identification of the roTCA cycle in members of *Desulfurellaceae*. First, we describe the anaerobic cultivation of *Desulfurellaceae* at different CO_2 concentrations with a method that can be adapted to the cultivation of other anaerobes. Then, we explain how to measure activities of enzymes responsible for citrate cleavage, malate dehydrogenase reaction, and the crucial carboxylation step of the cycle catalyzed by pyruvate synthase in cell extracts. In conclusion, we describe stable isotope experiments that allow tracking of the roTCA cycle *in vivo*, through the position-specific incorporation of carbon-13 into amino acids. The label is provided to the organism as $^{13}CO_2$ or [1-^{13}C]glutamate. The same key methodology can be used for the reliable evaluation of the functioning of the roTCA cycle in any organism under study. This pathway is likely to participate, completely unseen, in the metabolism of various microorganisms.

Keywords: Autotrophy, CO_2 fixation, roTCA cycle, Citrate synthase, Isotopologue profiling, Enzyme activity assays

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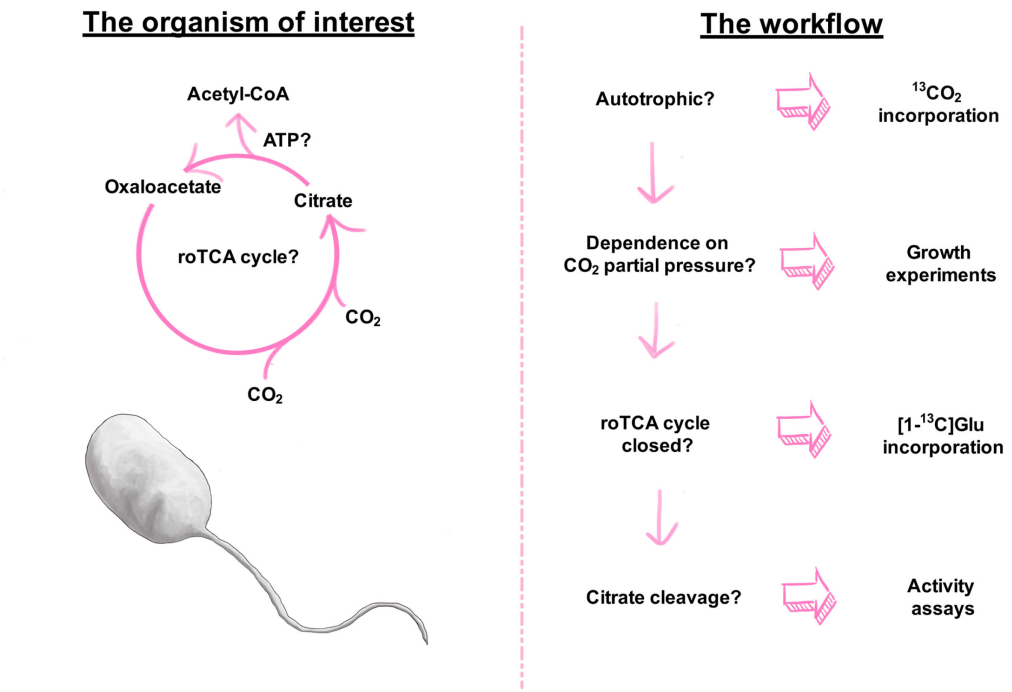
Graphic abstract:



Background

The study of metabolic pathways responsible for the fixation of inorganic carbon is important for the understanding of the evolution of early cellular life (Fuchs, 2011; Weiss *et al.*, 2016) and the investigation of current global issues, such as climate change. Being responsible for the net fixation of 7×10^{16} g carbon annually (Field *et al.*, 1998), this biological process is critical for the sustainment of life on Earth. Until today, seven different autotrophic CO_2 fixation pathways have been described (Berg, 2011; Fuchs, 2011; Sánchez-Andrea *et al.*, 2020). In most cases, the detection of genes that encode enzymes catalyzing key steps in a specific pathway in (meta)genomes and (meta)transcriptomes allows us to estimate the contribution of the corresponding organisms or communities to primary production. However, the recent discovery of the reversed oxidative tricarboxylic acid (roTCA) cycle in the obligate anaerobic sulfur reducers, *Desulfurella acetivorans* (Mall *et al.*, 2018) and *Thermosulfidibacter takaii* (Nunoura *et al.*, 2018), challenges this view. The roTCA cycle is the reversal of the widespread (Krebs) tricarboxylic acid (TCA) cycle, and its key features are high energetic efficiency and the lack of any unique enzyme. Being a variant of the reductive TCA (rTCA) cycle, the roTCA cycle utilizes citrate synthase, instead of ATP-citrate lyase, to catalyze the citrate cleavage reaction (citrate + CoA → acetyl-CoA + oxaloacetate + H_2O). This step is thermodynamically and kinetically unfavorable—it requires an elevated amount of catalyst (*i.e.*, citrate synthase represents 7% of the total protein content in *D. acetivorans* [Steffens *et al.*, 2021]), and also an unusually high substrate to product ratio (*i.e.*, CoA/acetyl CoA is 93 in *D. acetivorans*, using the roTCA cycle [Mall *et al.*, 2018], and only 2.3 in *Escherichia coli*, using the classical TCA cycle [Bennett *et al.*, 2009]).

Considering the almost universal distribution of citrate synthase in bacteria, this pathway may potentially function unnoticed in many autotrophs, making bioinformatical predictions challenging and raising the question on how to identify it (Mall *et al.*, 2018). Our recent study (Steffens *et al.*, 2021), from which this protocol originates, helped to resolve this issue, but also revealed physiological constraints governing the functioning of the roTCA cycle. Therein, for several representatives of the family *Desulfurellaceae* (*i.e.*, *Hippea maritima*, *D. acetivorans*, *D. multipotens*, *D. propionica*), we demonstrated that the usage of the roTCA cycle requires high partial pressure of

CO_2. The growth of these bacteria showed a clear dependence on CO_2 concentration, with cells barely growing at partial pressure of CO_2 below 10 kPa. In contrast, *Desulfobacter hydrogenophilus,* which uses the ATP-citrate lyase-dependent variant of the cycle (Schauder *et al.*, 1987), grew equally well at all tested CO_2 concentrations (from 2 to 80 kPa) (Steffens *et al.*, 2021). Our data revealed that the pyruvate synthase reaction (acetyl-CoA + CO_2 + reduced ferredoxin → pyruvate + CoA + oxidized ferredoxin) was responsible for this CO_2 dependence, as this thermodynamically challenging reaction requires low CoA/acetyl-CoA ratio. As already mentioned, the reversal of the citrate synthase reaction (and thus the functioning of the roTCA cycle) is only possible at high CoA and low acetyl-CoA concentrations, making the following acetyl-CoA carboxylation with pyruvate synthase difficult. This unfavorable condition is compensated by the increase in the concentration of CO_2, another substrate of the pyruvate synthase reaction, making the assimilation of acetyl-CoA possible, as the product of the roTCA cycle.

With these features in mind, we can now streamline different techniques in a protocol that will permit the verification of the functioning of the roTCA in promising candidate organisms. Anaerobic cultivation experiments prove the growth dependence on different CO_2 concentrations. Extremely high activities of the citrate synthase and malate dehydrogenase reactions in cell extracts are not necessary for other metabolic pathways, thus proving the requirement for an increased amount of catalysts to perform the ATP-independent citrate cleavage. The presence in cell extracts of the critical carboxylases of the pathway (pyruvate and 2-oxoglutarate synthases) further confirms the functioning of the roTCA cycle. Finally, isotopologue profiling of amino acids show position-specific incorporations of carbon-13 and prove the operation of the roTCA cycle in growing bacteria. Cultures grown with $^{13}CO_2$ show an enrichment of fully labelled amino acids, especially of glutamate, thus proving the existence of a functional CO_2 fixation pathway. Cultures grown in the presence of traces of [1-^{13}C]glutamate show the specific formation and accumulation of [4-^{13}C]aspartate, evidencing that the roTCA cycle is closed. However, some bacteria are not capable to transport glutamate into the cells; in this case, the glutamate labelling experiment will not be applicable and alternatives might be considered, such as ^{13}C-labelled pyruvate, glutamine, or citrate.

The identification of promising candidate organisms can be carried out with the help of bioinformatics tools; analysis of (meta)genomes could help to exclude assuredly autotrophic organisms *a priori*, due to the presence of key enzymatic genes of known CO_2 fixation pathways. Nevertheless, the presence of other autotrophic enzymes does not make the functioning of the roTCA cycle impossible. For example, *D. multipotens* uses the roTCA cycle while possessing an apparently functional gene for ATP-citrate lyase (Mall *et al.*, 2018; Steffens *et al.*, 2021). The usage of software Interactive Codon Analysis (INCA) (Supek *et al.*, 2004, 2005) could help to screen organisms in advance. Indeed, codon usage patterns could be used for the prediction of highly expressed genes, and high expression of the citrate synthase gene is crucial for the functioning of this cycle. However, the results of the codon usage analysis are not decisive and may lead to false-positives or false-negatives, requiring biochemical assays with cell extracts, or perhaps, quantitative proteomics/transcriptomics data for further proof. Accordingly, every case has to be necessarily validated experimentally.

Being an energetically efficient autotrophic pathway, the roTCA cycle may be widespread in natural environments at elevated partial pressures of CO_2 (*e.g.*, deep-sea hydrothermal vents) (Steffens *et al*., 2021). Its elusiveness compromises the reliability of bioinformatics analysis, and the possibility to make solid predictions about the capability of microorganisms for autotrophic growth or primary production. The protocol presented here allows an unambiguous identification of the roTCA cycle in organisms under study.

Materials and Reagents

A. Cultivation experiments of anaerobic organisms
   1. Serum bottle N20, 120 mL (MediPac, catalog number: 10113100) or equivalent
   2. Infusion bottles, 1 L (VWR, catalog number: 215-9243) or equivalent
   3. Butyl rubber stopper N20 (Glasgerätebau Ochs, catalog number: 102049) or equivalent
   4. Rubber stoppers for 1 L infusion bottles (ERIKS)
   5. Crimp aluminum caps (WICOM, catalog number: WIC 44580) or equivalent
   6. Crimper (VWR, catalog number: 548-0073)
   7. Decapper (VWR, catalog number: 548-0074)

8. Screw top, with hole Ø 19 mm (VWR, catalog number: MUEL14.076.04)
9. Duran® flasks, 1 L (VWR, catalog number: SCOT818015403)
10. Rubber stoppers for Duran® flasks (Glasgerätebau Ochs, catalog number: 444704)
11. Lecture-bottle control valve (Sigma, catalog number: Z146951)
12. Sterile syringes, male Luer-Lock (Fisher Scientific, catalog number: 14955461) or equivalent
13. Luer-to-tubing connector, female Luer-Lock (Sigma, catalog number: Z261580)
14. Sterile ERSTA syringes for single use, 1 mL (Th. Geyer, catalog number: 6075936) or equivalent
15. Disposable needles (Carl Roth, catalog number: X132.1) or equivalent
16. Sterile syringe filters, 0.2 µm pore size (VWR, catalog number: 514-0061)
17. $Na_2S \cdot 9H_2O$ (Sigma, catalog number: 1313-84-4); store at 4°C
18. Sulfur powder (Carl Roth, catalog number: 9304.1); store at room temperature (RT)
19. Resazurin sodium salt (Sigma, catalog number: R7017); store at RT
20. Hydrogen gas (Air Liquide Deutschland, catalog number: P0231L50R2A001)
21. Carbon dioxide gas (Air Liquide Deutschland, catalog number: P0760L50R0A001)
22. Carbon-13 dioxide gas, 99% ^{13}C (Sigma, catalog number: 364592)
23. L-Glutamic acid-1-^{13}C, 99% ^{13}C (Sigma, catalog number: 604968)
24. *H. maritima* medium (modified from DSMZ 854) (see Recipe 1)
25. *D. acetivorans, D. multipotens,* and *D. propionica* medium (modified from DSMZ 480) (see Recipe 1)
26. SL-10 trace element solution (see Recipe 2)
27. Wolfe vitamin solution (see Recipe 3)
28. Trace element solution (DSMZ 141) (see Recipe 4)

B. Harvesting of biomass and preparation of cell extracts
1. Folded filters, Ø 240 mm (Whatman Schleicher & Schuell, catalog number: 10311651) or equivalent
2. Funnels with short stem (VWR, catalog number: 221-0180) or equivalent
3. Centrifuge bottles with sealing closure, 1 L (Thermo Fisher Scientific, catalog number: 05-564-26) or equivalent
4. Centrifuge tubes, 50 mL (VWR, catalog number: 21008-940) or equivalent
5. Microcentrifuge tubes, 1.5 and 2 mL (VWR, catalog number: 72.690.001, 72.689) or equivalent
6. Serological pipettes (Thermo Fischer Scientific, catalog number: 170350) or equivalent
7. Liquid nitrogen
8. 0.9% [w/v] NaCl solution (saline)
9. Aluminum cooling block (Sigma, catalog number: Z743486)
10. Cell lysis buffer (see Recipe 5)

C. Enzyme assays

Reaction mixtures are listed. While working, we recommend keeping all chemicals and helping enzymes on ice. All listed components were solved or diluted with dH_2O, unless mentioned otherwise. When special attention is required due to their stability, recommended storage conditions or special notes are reported (*e.g.*, freshly solved before the assays). The pH of organic acids and ATP solutions should be adjusted to neutral; the pH of Tris-HCl buffers can be adjusted at RT, but please note that when the final test temperature differs from RT, the pH also changes according to the following formula: $\Delta pKa/°C \approx -0.028$ (*e.g.*, the pH of Tris solutions decreases 0.028 pH units for each 1°C increase in temperature). For long-term storage, organic compounds and CoA esters solutions should be kept at -20°C or -80°C, if not mentioned otherwise; all buffers and salt solutions could be kept at RT. Acetyl-CoA was chemically synthesized from the corresponding anhydrides and CoA, according to a previously described method (Simon *et al.*, 1953).

In our case, the concentrations of stock solutions were as follows:

1. Citrate synthase (forward reaction, spectrophotometric continuous assay)
   a. 1 M Tris-HCl (pH 7.5) (Carl Roth, catalog number: 9090.4)
   b. 50 mM oxaloacetate, store at -20°C or -80°C for ≤2 months (Sigma, catalog number: 04126)
   c. 10 mM acetyl-CoA

   d. 10 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB), freshly solved in 100 mM Tris-HCl (pH 7.5) (Sigma, catalog number: D8130)
2. Citrate synthase (backward reaction, spectrophotometric continuous assay)
   a. 1 M Tris-HCl (pH 7.8) (Carl Roth, catalog number: 9090.4)
   b. 50 mM $MgCl_2$
   c. 50 mM dithioerythritol (DTE), freshly solved (Sigma, catalog number: D8255)
   d. 5 mM CoA (Neofroxx, catalog number: 2100GR005)
   e. 200 mM citrate (Sigma, catalog number: C8532)
   f. 5 mM NADH, freshly solved (Acros organics, catalog number: 10711911)
   g. 200 U/mL porcine malate dehydrogenase, store at 4°C, freshly diluted (Sigma, catalog number: M1567)
3. Citrate synthase (backward reaction, UHPLC discontinuous assay)
   a. 1 M Tris-HCl (pH 7.8) (Carl Roth, catalog number: 9090.4)
   b. 50 mM $MgCl_2$
   c. 50 mM DTE, freshly solved (Sigma, catalog number: D8255)
   d. 5 mM CoA (Neofroxx, catalog number: 2100GR005)
   e. 200 mM citrate (Sigma, catalog number: C8532)
   f. 5 mM NADH, freshly solved (Acros organics, catalog number: 10711911)
   g. 200 U/mL porcine malate dehydrogenase, store at 4°C, freshly diluted (Sigma, catalog number: M1567)
   h. Vial insert, 250 µL, pulled point glass (Agilent, catalog number: 5183-2085)
   i. Screw top vials, 2 mL, and screw caps (Agilent) or equivalent
   j. Microplate, sterile, 96 well (Greiner Bio-One, catalog number: 655161) or equivalent
   k. Membrane filter, 0.22 µm pore size (Sigma, catalog number: GSWP04700)
   l. Acetonitrile anhydrous, 99.8% (Sigma, catalog number: 271004)
   m. Stop solution (see Recipe 6)
   n. UHPLC-grade double distilled water (see Recipe 7)
   o. Potassium phosphate buffer (see Recipe 7)
4. ATP-citrate lyase
   a. 1 M Tris-HCl (pH 7.5) (Carl Roth, catalog number: 9090.4)
   b. 100 mM $MgCl_2$
   c. 10 mM ATP, freshly solved (Sigma, catalog number: A26209)
   d. 50 mM DTE, freshly solved (Sigma, catalog number: D8255)
   e. 50 mM CoA (Neofroxx, catalog number: 2100GR005)
   f. 100 mM citrate (Sigma, catalog number: C8532)
   g. 5 mM NADH, freshly solved (Acros Organics, catalog number: 10711911)
   h. 200 UF/mL porcine malate dehydrogenase, store at 4°C, freshly diluted (Sigma, catalog number: M1567)
5. Citrate lyase
   a. 1 M Tris-HCl (pH 7.5) (Carl Roth, catalog number: 9090.4)
   b. 100 mM $MgCl_2$
   c. 50 mM DTE, freshly solved (Sigma, catalog number: D8255)
   d. 100 mM citrate (Sigma, catalog number: C8532)
   e. 5 mM NADH, freshly solved (Acros Organics, catalog number: 10711911)
   f. 200 U/mL porcine malate dehydrogenase, store at 4°C, freshly diluted (Sigma, catalog number: M1567)
6. Malate dehydrogenase
   a. 1 M Tris-HCl (pH 8) (Carl Roth, catalog number: 9090.4)
   b. 50 mM $MgCl_2$
   c. 50 mM DTE, freshly solved (Sigma, catalog number: D8255)
   d. 25 mM oxaloacetate, store at -20°C or -80°C for ≤2 months (Sigma, catalog number: 04126)
   e. 5 mM NADH, freshly solved (Acros organics, catalog number: 10711911)

7. Pyruvate synthase
   a. 1 M Tris-HCl (pH 7.5) (Carl Roth, catalog number: 9090.4)
   b. 100 mM $MgCl_2$
   c. 50 mM DTE, freshly solved (Sigma, catalog number: D8255)
   d. 50 mM CoA (Neofroxx, catalog number: 2100GR005)
   e. 100 mM pyruvate (Sigma, catalog number: P2256)
   f. 10 mM methyl viologen (Sigma, catalog number: 856177)
8. 2-Oxoglutarate synthase
   a. 1 M Tris-HCl (pH 7.5) (Carl Roth, catalog number: 9090.4)
   b. 100 mM $MgCl_2$
   c. 50 mM DTE, freshly solved (Sigma, catalog number: D8255)
   d. 50 mM CoA (Neofroxx, catalog number: 2100GR005)
   e. 100 mM 2-oxoglutarate (Sigma, catalog number: 75890)
   f. 20 mM benzyl viologen (Sigma, catalog number: D8130)

D. Isotopologue profiling (based on GC-MS analysis)
1. Glass vials, 1.5 mL (VWR, catalog number: 548-0018)
2. Screw caps for GC/MS vials (VWR, catalog number: 548-0814)
3. Glass inlets, 200 µL (VWR, catalog number: 548-0780)
4. Microcentrifuge tubes, 2 mL (VWR, catalog number: 525-0228)
5. Glass Pasteur pipettes, short form (Glasswarenfabrik Karl Hecht GmbH & Co. KG, catalog number: 40567001)
6. Glass wool, superfine (Glasswarenfabrik Karl Hecht GmbH & Co. KG, catalog number: 41408003)
7. Hydrochloric acid, 37% (Sigma, catalog number: 320331)
8. Acetic acid, ≥99% (Sigma, catalog number: A6283)
9. Dowex 50WX8 200-400 (H^+) (Alfa Aesar, catalog number: L13922.18)
10. Methanol, 99.8% (Sigma, catalog number: 322415)
11. Ammonia solution in water, 25% (Merck, catalog number: 1054321000)
12. Acetonitrile anhydrous, 99.8% (Sigma, catalog number: 271004)
13. *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide ≥95% containing 1% *tert*-butyldimethylchlorsilane (MTBSTFA) (Sigma, catalog number: 375934), store at -20°C

Equipment

A. Cultivation experiments of anaerobic organisms
1. Scales
2. Gas piping system
3. Membrane vacuum pump (Vacuubbrand, catalog number: 19137003)
4. Vinyl anaerobic chamber (Coy laboratory products) or equivalent
5. Bottle-top dispensers (Brand, catalog number: 4600360) or equivalent
6. pH Meter SevenCompact S210 (Mettler Toledo, catalog number: 30130862) or equivalent
7. Incubator shaker New Brunswick Innova® 44R (Eppendorf, catalog number: M1282-006) or equivalent temperature-controlled shaker
8. Optical microscope (Zeiss, model: Axiostar) or equivalent
9. Thoma cell counting chamber (VWR, catalog number: 631-0697)

B. Harvesting of biomass and preparation of cell extracts
1. Sorvall centrifuge RC6+ (Thermo Fisher Scientific, catalog number: 46915) or equivalent
2. Fixed-angle rotor F9-4x1000y (Thermo Fisher Scientific, catalog number: 096-041032)
3. Table centrifuge 5810/5810 R (Eppendorf, catalog number: 5811000015) or equivalent

4. Rotor A-4-81 (Eppendorf, catalog number: 5810718007)
5. Table microcentrifuge 5417R (Eppendorf) or equivalent
6. Rotor F45-30-11 (Eppendorf)
7. Electronic pipette (Eppendorf, model: Easypet) or equivalent
8. Vinyl anaerobic chamber (Coy laboratory products) or equivalent
9. Optical microscope (Zeiss, model: Axiostar or equivalent)
10. Ultrasonic homogenizer Sonopuls (BANDELIN electronic, catalog number: 2451) or equivalent
11. Aluminum cooling block
12. -80°C Freezer

C. Enzyme assays
1. Cary 3500 UV-Vis spectrophotometer (Agilent) or equivalent; please note that working with thermophiles requires a temperature-controlled cuvette holder (air-cooled Peltier temperature control in our case)
2. Cuvettes, 400 µL, light path 10 mm (Hellma, catalog number: 115-10-40) or equivalent
3. Syringe Hamilton, 50 µL (Carl Roth, catalog number: X048.1) or equivalent
4. Rubber stoppers for cuvettes
5. Vinyl anaerobic chamber (Coy laboratory products) or equivalent
6. Thermomixer (Eppendorf, model: comfort) or equivalent
7. Vacuum pump
8. Ultrasonic bath (BANDELIN electronic, model: DT 100) or equivalent
9. Ultra-high-performance liquid chromatography instrument (UHPLC) (Agilent, model: 1290 Infinity II LC System) or equivalent
10. Column for reversed-phase LC separations (phase EC-C18) (Agilent, catalog number: 699675-902) or equivalent
11. Pipette set (RAININ) or equivalent

D. Isotopologue profiling (based on GC-MS analysis)
1. Freeze-dryer Alpha 2-4 LDplus (Martin Christ, Osterode, Germany) or equivalent
2. Oven (Binder, model E28, Tuttlingen, Germany,) or equivalent
3. Heating block Techne® DRI Block DB 2A (Merck, Darmstadt, Germany) or equivalent
4. GCMS-QP 2010 plus with autosampler AOC20i (Shimadzu, Duisburg, Germany) or equivalent
5. Silica capillary column (equity TM-5; 30 m by 0.25 mm; 0.25 µm film thickness; Sigma-Aldrich, Supelco, catalog-number 28098-U)

Software

1. Interactive Codon Analysis INCA software (Supek and Vlahovicek, 2005; http://bioinfo.hr/software-tools/)
2. National Center for Biotechnology Information (NCBI) BLAST (http://www.ncbi.nlm.nih.gov/BLAST/)
3. Labsolutions (Shimadzu, https://www.shimadzu.de/labsolutions%E2%84%A2-lcms-software-simple-analytical-platform)
4. Isotopo (Ahmed *et al*., 2014, https://www.uni-wuerzburg.de/tr34/software-developments/isotopo/)
5. Microsoft Excel (Microsoft, 2016, https://www.microsoft.com/en-us/microsoft-365/excel)

Procedure

A. Cultivation experiments with anaerobic organisms

Please note that the use of gases is described according to the specifications of our piping system. In particular,

the manual addition of CO_2 required a sterile syringe (male Luer-lock), a Luer-to-tubing connector (female Luer Lock), and, specifically for the $^{13}CO_2$ gas, a lecture-bottle control valve to connect to the carbon steel lecture bottle (see Materials and Reagents A).

1. Growth with different CO_2 concentrations
   a. Here we describe the cultivation of anaerobic microorganisms in serum bottles (117 mL total volume; 20 mL, 5 mL, and 2 mL medium).
   b. Solve the ingredients (see Recipe 1 for cultivation of *Desulfurellaceae* spp). Please remember that the reported list of ingredients may differ from the one suited for the cultivation of other autotrophs.
   c. Make the solution anoxic by performing vacuum-gas (usually 1 min each) cycles with 100% N_2 (50–100 kPa overpressure) for approximately 40 min (see Note 5).
   d. Reduce the solution by the addition of $Na_2S{\cdot}9H_2O$ at a final concentration of 0.05% (w/v).
   e. Adjust the pH of the medium to the growth optimum of the organism in question. Indeed, since the addition of sodium sulfide makes the medium pH alkaline (pH of ~8 in our case), it is necessary to adjust it by addition of an anoxic HCl solution (see Note 5). Other acids could be used, if compatible with the growth medium. Please consider that different medium compositions might influence the resulting pH and differ from our experience.
   f. Get rid of the overpressure in the medium bottle, then transfer it to the anaerobic chamber (see Note 3 for an alternative way of aliquoting without using the anaerobic chamber).
   g. Aliquot the medium (20 mL, 5 mL, and 2 mL) with the help of a bottle-dispenser. When cultivating sulfur reducers, distribute the elemental sulfur powder (10 g L^{-1}) in advance into the serum bottles.
   h. Close the serum bottles with butyl-rubber stoppers, then transfer them out of the anaerobic chamber, and seal them with crimp aluminum caps.
   i. Autoclave sulfur-containing media at 110°C for 40 min. The autoclaved media can be stored at RT long-term. Please remember that the medium for the cultivation of other autotrophic microbes may require different sterilization temperatures.
   j. Prior to inoculation, add the sterile filtered vitamins (see Recipe 3) to the cultures and replace the gas phase. Due to the heat sensitivity, vitamins are usually not added to the medium before sterilization. Transfer 40 mL of vitamin solution to a serum bottle, make it anoxic (see Note 5), then add the vitamins to the culture medium anaerobically using sterile disposable syringes and needles, to a final concentration of 10 mL L^{-1}.
   k. Replace the gas phase of fresh cultures by performing vacuum-gas cycles with 100% H_2. Before adding the CO_2, release any overpressure present in the cultures.
   l. Add CO_2 manually with a syringe at the respective final partial pressures: 80 kPa or 40 kPa for 20 mL cultures, 10 kPa for 5 ml cultures, 4 kPa for 5 mL cultures, and 2 kPa for 2 mL cultures.
   m. Fill up with H_2 to 100 kPa overpressure.
   n. If necessary, adjust the pH to the organism optimum with anoxic 1 M NaOH (see Note 5). This is important because the addition of CO_2 acidifies the medium ($CO_2 + H_2O \leftrightharpoons H_2CO_3 \leftrightharpoons HCO_3^- + H^+$).
   o. For the inoculation, use actively growing cultures during late exponential phase, to minimize the lag phase. In our case, the starting cell density was 10^6 [cells/mL]. Incubate the main cultures (Figure 1A) at the optimum growth temperature with agitation.

2. Carbon-13 labelling experiments
   a. Here, we describe the cultivation of anaerobic microorganisms in the presence of carbon-13 compounds in 1 L infusion bottles (200 mL medium).
   b. Solve the ingredients (see Recipe 1 for cultivation of *Desulfurellaceae* spp). Please remember that the reported list of ingredients may differ from the one suited for the cultivation of other autotrophic microbes.
   c. Aliquot the medium (200 mL) with the help of a measuring cylinder. When cultivating sulfur reducers, distribute the elemental sulfur powder (10 g L^{-1}) into the 1 L infusion bottles in advance. Close the bottles with rubber stoppers and aluminum caps.
   d. Make the cultures anoxic by performing vacuum-gas cycles (usually 1 min each) with 100% N_2 (50–100 kPa overpressure) for approximately 15–20 min (see Note 5).

e. Reduce the cultures by the addition of $Na_2S·9H_2O$ at a final concentration of 0.05% (w/v).

p. Adjust the pH of the medium to the growth optimum of the organism in question. Indeed, since the addition of sodium sulfide makes the medium pH alkaline (pH of ~8 in our case), it is necessary to adjust it by addition of an anoxic HCl solution (see Note 5). Other acids could be used, if compatible with the growth medium. Please consider that different medium compositions might influence the resulting pH and differ from our experience.

f. Autoclave sulfur-containing media at 110°C for 40 min. The autoclaved media can be stored at RT long-term. Please remember that the medium for the cultivation of other autotrophic microbes may require different sterilization temperatures.

g. Prior to inoculation, add the sterile filtered vitamins (see Procedure A1j) to the cultures.

h. Add the tracer (i: $^{13}CO_2$, or ii: [1-^{13}C]glutamate), according to the following instructions.

   i. $^{13}CO_2$:

      Replace the gas phase of fresh cultures by performing vacuum-gas cycles with 100% H_2. Before adding the $^{13}CO_2$, release any overpressure present in the cultures. Manually add $^{13}CO_2$ (99% carbon-13) with a syringe to a final partial pressure of 40 kPa, and fill up with H_2 to 100 kPa overpressure.

   ii. [1-^{13}C]glutamate:

      Replace the gas phase with H_2:CO_2 (80:20; carbon-12) and add [1-^{13}C]glutamate to the medium to a final concentration of 14 mg L^{-1}.

q. If necessary, adjust the pH to the organism optimum with anoxic 1 M NaOH (see Note 5). It is important because the addition of CO_2 acidifies the medium ($CO_2 + H_2O \leftrightharpoons H_2CO_3 \leftrightharpoons HCO_3^- + H^+$).

i. For the inoculation, use actively growing cultures during late exponential phase to minimize the lag phase. Please note that in our case the starting cell density was 10^6 [cells/mL]. Incubate the main cultures (Figure 1B) at the optimum growth temperature with agitation.

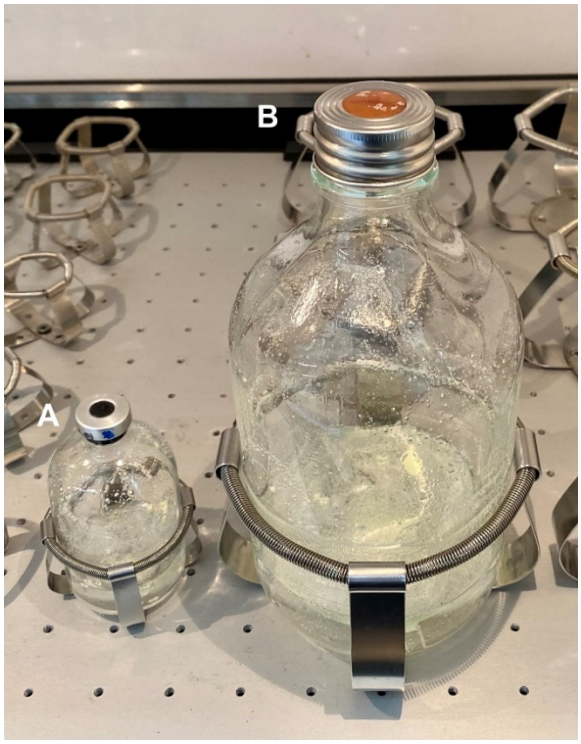


Figure 1. Anaerobic cultures in the incubator shaker.
A. Serum bottle with 20 mL of medium. **B.** Infusion bottle with 200 mL of medium. Both cultures contain a suspension of elemental sulfur.

B. Harvest of biomass and preparation of cell extracts

1. Harvest the cells during mid-exponential growth for enzyme activity measurements, and during late-exponential growth for the GC-MS analysis. To identify the late-exponential growth phase, we counted the cells in the medium using a Thoma cell counting chamber (see Note 7 and Data analysis A2) and compared the results with the growth curves measured for the corresponding organism. In our case, the cultures typically reached the late-exponential growth phase after 48–96 h of growth.
2. To separate elemental sulfur and cells before centrifugation, release the overpressure, open the bottle, and filter the medium with a folded filter and a funnel under aerobic conditions. Please note that performing this procedure under the fume hood is preferable, especially in the case of sulfur reducers producing H_2S as a metabolic by-product.
3. Centrifuge the filtered culture (15,000 × *g*, 4°C, 20 min) and carefully discard the supernatant. Please note that cell pellets of some organisms might get resuspended very easily. For better results, it is possible to use electronic pipettes to discard the supernatant in this procedure.
4. Resuspend the cell pellet with approximately 20 mL of leftover medium and transfer the cell suspension to a 50 mL centrifuge tube.
5. Centrifuge the cell suspension (3,000 × *g*, 4°C, 40 min) and carefully discard the supernatant.
6. Resuspend the cell pellet with 1 mL of 0.9% [w/v] NaCl solution (saline) and transfer the cell suspension to a 2 mL microcentrifuge tube.
7. Centrifuge the cell suspension (21,000 × *g*, 4°C, 20 min) and carefully discard the supernatant.
8. Freeze the cell pellet with liquid nitrogen and store it at -80°C, or disrupt the cells for further tests.
9. In the latter case, transfer the microcentrifuge tube with the cell pellet to the anaerobic chamber.
10. Resuspend the cell pellet with 500 µL of anoxic cell lysis buffer (see Recipe 5). Keep the solution cool with a cooled aluminum block.
11. Lyse the cells with an ultrasonic homogenizer (60% amplitude, 4 min run, 1-s pulse, 2-s break; total energy input of 2,000 kJ). Especially for oxygen sensitive enzymes (*i.e.*, pyruvate synthase, and 2-oxoglutarate synthase), it is crucial to perform the cell opening anoxically, either in the anaerobic chamber or in an anaerobic environment.
12. Remove the insoluble cell debris by centrifugation (21,000 × *g*, 4°C, 20 min).
13. Determine the protein concentration (Bradford, 1976), and handle on ice. Cell extracts are suitable for approximately 3–4 h of work. Long-term storage at -20°C compromises enzyme stability.

C. Enzyme assays

In the following procedures, the final concentrations of the reaction components are given. The suggested stock concentrations are listed in the Materials and Reagents section C. Before performing the assays, please take into account the following practical recommendations: start the enzymatic reactions with the substrates; choose the test temperature in accordance with the optimum growth temperature of the organism in question, and assume that a 10°C rise in temperature doubles the reaction rate; high temperatures might compromise the stability of the chemicals present in the reaction mixture; in case of spectrophotometric measurements, notice the maximum absorbance limit of the device in use.

Test at least two different concentrations of cell extract to obtain a linear dependence of activity on the amount of the added enzyme. For the measurement of highly active enzymes of the roTCA cycle (*i.e.*, citrate synthase and malate dehydrogenase), only small amounts of cell extract should be added to the reaction mixture, if studied organisms possess this cycle. In our case, 0.5–1 (µg protein·mL^{-1}) of cell extract was sufficient to obtain a linear activity. This corresponded to a difference of 0.3 to 0.7 absorption units per minute (Δabs ·min^{-1}) in the assays of citrate synthase in the direction of citrate synthesis (oxaloacetate + acetyl-CoA + H_2O → citrate + CoA), or malate dehydrogenase in the direction of oxaloacetate reduction (oxaloacetate + NADH + H^+ → malate + NAD^+). However, citrate synthase is much less active in the direction of citrate cleavage (citrate + CoA → acetyl-CoA + oxaloacetate + H_2O), thus requiring high protein concentration in the mixture (in our case, at least 20-fold higher). If the results of the assays are ambiguous, it may be useful to perform additional

negative controls, by replacing the cell extract or the substrates with water.
In our case, the final volume of the reaction mixture was 300 µL.

1. Citrate synthase (forward reaction, spectrophotometric continuous assay, λ=412 nm)
   a. Activity of citrate synthase was measured in a reaction mixture consisting of:
      i. 100 mM Tris-HCl (pH 7.5)
      ii. 1 mM DTNB
      iii. 0.5 mM acetyl-CoA
      iv. 5 mM oxaloacetate
      v. Cell extract
      vi. dH_2O
   b. Preheat the cuvette holder of the spectrophotometer to the test temperature.
   c. Add all the reaction mixture components to the cuvette, except oxaloacetate, to reach a final volume of 300 µL.
   d. Mix the reaction mixture thoroughly with a pipette.
   e. Start the measurement and get a stable baseline.
   f. As the baseline is stabilized, wait 1 min to record the background activity, and then start the reaction by adding 5 mM oxaloacetate. Monitor the oxaloacetate-dependent formation of free CoA from acetyl-CoA with DTNB (ε_{412}=14.2 mM^{-1} cm^{-1} [Riddles *et al*., 1979]) as reagent.
2. Citrate synthase (backward reaction, spectrophotometric continuous assay, λ=365 nm)
   a. Activity of citrate synthase in the backward reaction was measured in a reaction mixture consisting of:
      1) 100 mM Tris-HCl (pH 7.5)
      2) 5 mM DTE
      3) 5 mM $MgCl_2$
      4) 0.5 mM CoA
      5) 0.5 mM NADH
      6) 20 mM citrate
      7) 20 U/mL porcine malate dehydrogenase
      8) Cell extract
      9) dH_2O
   b. Preheat the cuvette holder of the spectrophotometer to the test temperature.
   c. Add all the reaction mixture components to the cuvette, except citrate, to reach a final volume of 300 µL.
   d. Mix the reaction mixture thoroughly with a pipette.
   e. Start the measurement and get a stable baseline.
   f. As the baseline is stabilized, wait 1 min to record the background activity, and then start the reaction by adding 20 mM citrate. Monitor the citrate-dependent oxidation of NADH (ε_{365}=3.4 mM^{-1} cm^{-1} [Bergmeyer, 1975]) due to the activity of the helping enzyme malate dehydrogenase, with oxaloacetate forming in the citrate synthase backward reaction.
3. Citrate synthase (backward reaction, UHPLC discontinuous assay, λ=260 nm)
   a. Performing the assay
      i. Activity of citrate synthase in the backward reaction was measured in a reaction mixture consisting of:
         1) 100 mM Tris-HCl (pH 7.5)
         2) 5 mM DTE
         3) 5 mM $MgCl_2$
         4) 0.5 mM CoA
         5) 0.5 mM NADH
         6) 20 mM citrate
         7) 20 U/mL porcine malate dehydrogenase
         8) Cell extract
         9) dH_2O

ii. Preheat the thermomixer for 1.5 mL microcentrifuge tubes to the test temperature.
iii. In this case, mix all the reaction mixture components except citrate. The final volume should ensure the correct sampling of four time points (*e.g.*, final volume of 100 µL for four 20 µL samples).
iv. Mix the reaction mixture thoroughly and spin it down.
v. Preincubate the reaction mixture for 1 min at the test temperature, then start the reaction with 20 mM citrate.
vi. Take a sample (t0) and stop the reaction immediately (sample:stop solution, 1:1 [v/v]).
vii. Similarly, take samples after 1 min (t1), 2 min (t2), and 10 min (t10), and each time stop the reaction immediately (sample:stop solution, 1:1 [v/v]).
viii. During the assay, keep the samples cooled, then freeze them with liquid nitrogen and store at -20°C.

b. UHPLC run
i. Centrifuge the samples twice (21,000 × *g*, 4°C, 5 min) to get rid of insoluble proteins and particles.
ii. Prepare the samples for the UHPLC in 2 mL screw top vials or in 96-well microplates. If necessary, dilute the samples only with UHPLC-grade ddH_2O.
iii. In our case, the CoA-esters were analysed with a 1290 Infinity II UHPLC system equipped with a EC-C18 column for reversed-phase LC separations (Agilent InfinityLab Poroshell 120 EC-C18 1.9 µm 2.1 × 50 mm). The diode array detector (Agilent, lamp catalog number: G7117B) allowed the UV detection of CoA and of different CoA-esters at 260 nm.
iv. Before the run, wash the pumps with UHPLC-grade ddH_2O and potassium phosphate buffer, and equilibrate the column to the starting conditions (2% acetonitrile – 98% potassium phosphate buffer).
v. For the actual sample analysis, use the following chromatographic solvents: solvent A, acetonitrile, and solvent B, potassium phosphate buffer in water (pH 7) (see Recipe 7). Perform chromatography using a gradient elution: after injection, increase the level of acetonitrile from 2 to 8% over 2.66 min, up to 30% in 3.33 min, then reduce it to 2% in 3.68 min, and maintain it isocratic up to 5.00 min. Keep the flow rate constant at 0.55 mL min^{-1}.

4. ATP-citrate lyase (spectrophotometric continuous assay, λ=365 nm)
a. Activity of ATP-citrate lyase was measured in a reaction mixture consisting of:
1) 100 mM Tris-HCl (pH 7.5)
2) 5 mM $MgCl_2$
3) 5 mM DTE
4) 0.5 mM CoA
5) 0.4 mM NADH
6) 20 mM citrate
7) 0.5 mM ATP
8) 20 U/mL porcine malate dehydrogenase
9) Cell extract
10) dH_2O
b. Preheat the cuvette holder of the spectrophotometer to the test temperature.
c. Add all the reaction mixture components except ATP to the cuvette, to reach a final volume of 300 µL.
d. Mix the reaction mixture thoroughly with a pipette.
e. Start the measurement and get a stable baseline.
f. As the baseline is stabilized, wait 1 min to record the background activity, and then start the reaction by adding 0.5 mM ATP. Measure oxaloacetate formation by monitoring NADH oxidation (ε_{365}=3.4 mM^{-1} cm^{-1} [Bergmeyer, 1975]) due to the activity of the helping enzyme malate dehydrogenase that is present in the mixture.

5. Citrate lyase (spectrophotometric continuous assay, λ=365 nm)
a. Activity of citrate lyase was measure in a reaction mixture consisting of:

1) 100 mM Tris-HCl (pH 7.5)
2) 5 mM DTE
3) 5 mM $MgCl_2$
4) 0.5 mM NADH
5) 20 mM citrate
6) 20 U/mL porcine malate dehydrogenase
7) Cell extract
8) dH_2O

b. Preheat the cuvette holder of the spectrophotometer to the test temperature.
c. Add all the reaction mixture components except citrate to the cuvette, to reach a final volume of 300 µL.
d. Start the measurement and get a stable baseline.
e. As the baseline is stabilized, wait 1 min to record the background activity, and then start the reaction by adding 20 mM citrate. Measure oxaloacetate formation monitoring NADH oxidation (ε_{365}=3.4 mM^{-1} cm^{-1} [Bergmeyer, 1975]) due to the activity of the helping enzyme malate dehydrogenase that is present in the mixture.

6. Malate dehydrogenase (spectrophotometric continuous assay, λ=365 nm)
   a. Activity of malate dehydrogenase was measured in a reaction mixture consisting of:
      1) 100 mM Tris-HCl (pH 8)
      2) 5 mM DTE
      3) 5 mM $MgCl_2$
      4) 0.5 NADH
      5) 2.5 mM oxaloacetate
      6) Cell extract
      7) dH_2O
   b. Preheat the cuvette holder of the spectrophotometer to the test temperature.
   c. Add all the reaction mixture components except oxaloacetate to the cuvette, to reach a final volume of 300 µL.
   d. Mix the reaction mixture thoroughly with a pipette.
   e. Start the measurement and get a stable baseline.
   f. As the baseline is stabilized, wait 1 min to record the background activity, and then start the reaction by adding 2.5 mM oxaloacetate. Measure the oxidation of NADH (ε_{365}=3.4 mM^{-1} cm^{-1} [Bergmeyer, 1975]).
7. Pyruvate synthase (spectrophotometric anaerobic continuous assay, λ=578 nm)
   The pyruvate synthase reaction requires reduced conditions. Add dithionite to reduce the viologens until the solution is slightly blue (absorbance at 578 nm of ~0.8), then proceed. There is an alternative way to perform this test without the use of the anaerobic tent (see Note 6).
   a. Activity of pyruvate synthase was measured in a reaction mixture consisting of:
      1) 50 mM Tris-HCl (pH 7.5)
      2) 5 mM $MgCl_2$
      3) 2.5 mM DTE
      4) 0.5 mM CoA
      5) 1 mM methyl viologen
      6) 10 mM pyruvate
      7) Cell extract
      8) dH_2O
   b. Preheat the cuvette holder of the spectrophotometer to the test temperature.
   c. While working in an anaerobic tent, add all the anoxic reaction mixture components except pyruvate to the cuvette, to reach a final volume of 300 µL.
   d. Mix the reaction mixture thoroughly with a pipette.
   e. Close the cuvette with a rubber plug and transfer it out of the tent.
   f. Replace the gas phase of the cuvette with N_2.

g. Start the measurement and get a stable baseline.
h. As the baseline is stabilized, wait 1 min to record the background activity, and then start the reaction by adding 10 mM pyruvate using a Hamilton syringe. Monitor the pyruvate-dependent reduction of methyl viologen (ε_{578} nm=9.7 mM^{-1} cm^{-1} [Dawson *et al*., 1986]).

8. 2-oxoglutarate synthase (spectrophotometric anaerobic continuous assay, λ=578 nm)
The 2-oxoglutarate synthase reaction requires reduced conditions. Add dithionite to reduce the viologens until the solution is slightly blue (absorbance at 578 nm of ~0.8), then proceed. Please note that there is an alternative way to perform this test without the use of an anaerobic tent (see Note 6).
a. Activity of 2-oxoglutarate synthase was measured in a reaction mixture consisting of:
1) 100 mM Tris-HCl (pH 7.5)
2) 5 mM $MgCl_2$
3) 2.5 mM DTE
4) 0.5 mM CoA
5) 1 mM benzyl viologen
6)10 mM 2-oxoglutarate
7) Cell extract
8) dH_2O
b. Preheat the cuvette holder of the spectrophotometer to the test temperature.
c. While working in the anaerobic tent, add all the anoxic reaction mixture components except 2-oxoglutarate to the cuvette, to reach a final volume of 300 µL.
d. Mix the reaction mixture thoroughly with a pipette.
e. Close the glass cuvette with a rubber plug and transfer it out of the tent.
f. Replace the gas phase of the cuvette with N_2.
g. Start the measurement and get a stable baseline.
h. As the baseline is stabilized, wait 1 min to record the background activity, and then start the reaction by adding 10 mM 2-oxoglutarate. Monitor 2-oxoglutarate-dependent reduction of benzyl viologen (ε_{578} nm=9.7 mM^{-1} cm^{-1} [Dawson *et al*., 1986]).

D. Isotopologue profiling (based on GC-MS analysis)
1. Lyophilize the bacterial cell pellet using a freeze-dryer. To ensure dryness, the sample is typically dried overnight.
2. Transfer 2 mg of the dried sample into a 1.5 mL glass vial.
3. Add 500 µL of 6 M HCl (aqueous) and incubate the sample at 105°C for 15 h, to perform acidic hydrolysis of cellular proteins.
4. Dry the sample under a stream of nitrogen in a heating block at 70°C for approximately 45 min.
5. Add 200 µL of 50% acetic acid (aqueous) to the dried residue and resolubilize the sample by vigorously vortexing the vial for 10 s.
6. Prepare a cation-exchange column for amino acid isolation.
a. Fill the lower fifth of a 1 mL pipette tip with glass wool.
b. Add 200 µL of Dowex on top of the glass wool.
c. Elute the column with 1 mL of 70% MeOH (aqueous) and 1 mL of ddH_2O.
7. Place a 2 mL microcentrifuge tube underneath the column to collect the aqueous phase.
8. Carefully apply the sample to the column using a 150 mm Pasteur pipette, without elutriating the column material.
9. After the sample has seeped through the column, fill 800 µL of H_2O into the initial sample vial and add this portion of water to the column, using the Pasteur pipette from step 8.
10. Add another 800 µL of ddH_2O after that the amount applied first (step 9) has seeped through to the column.
11. After the whole aqueous eluate has seeped through the column, place a 1.5 mL glass vial under it to collect the alkalic eluate.
12. Apply 1 mL of 4 M ammonia solution (aqueous) to the column.
13. Dry the alkalic eluate under a stream of nitrogen in a heating block at 70 °C for approximately 50 min.
14. For the derivatization of the amino acids, add 50 µL of anhydrous acetonitrile and 50 µL of MTBSTFA to

the dried alkalic residue.

15. Incubate the mixture at 70°C for 30 min in a heating block, and then transfer it to a 200 µL glass inlet.
16. For GC/MS measurement, inject an aliquot (0.1–5 µL) of the sample in split mode (1:5) at an injector with interface temperature of 260°C.
17. Maintain the column at 150°C for 3 min, then increase the temperature with a gradient of 7°C/min to a final 280°C. Hold this temperature for a further 3 min.
18. Measure the samples in triplicates, using selected ion monitoring (SIM). For each fragment (mass M, containing only ^{12}C), measure the intensities for ion traces from M-1 to M+X+4, where X is the number of carbons in the respective fragment.
19. Using Labsolutions, integrate the respective signals.
20. Calculate the overall ^{13}C-excess, as well as the isotopologue composition in each fragment, by comparison with unlabeled samples, according to Ahmed *et al.* (2014).
21. The ^{13}C-excess or the percentage of labelled molecules can be represented with column charts, while the isotopologue composition is ideally presented as stacked 100% columns using Excel 2016.

Data analysis

A. Different CO_2 concentration growth experiment
   1. Perform the growth experiments with at least three replicate cultures per each tested CO_2 concentration and display your results as mean ± S.E.M.
   2. Starting from the inoculation, count the cells every 24 h until the cultures reach the stationary phase. Please remember that other species may differ in their generation times; consequently, other intervals for cell counting should be chosen.
   3. In the case of organisms using the roTCA cycle, the growth should strongly depend on the CO_2 concentration. In our case, the tested *Desulfurellaceae* showed approximately 1.5–2 times extended generation times $\{gt= (t_2-t_1)/(3.3*lg [cell\ density_2/cell\ density_1)]\}$ with the CO_2 decreasing from 40% to 5%. The generation times for *H. maritima* at these CO_2 concentrations were 17 and 30 h, respectively, for *D. acetivorans* 8 and 13.5 h, for *D. propionica* 9 and 18 h, and for *D. multipotens* 10 and 13.5 h (Steffens *et al.*, 2021).
   4. The final cell densities should also show a similar dependence in accordance with the CO_2 partial pressure, finally reaching a moderate or not detectable growth with 2% and 1% CO_2.

B. Enzyme assays
   1. Perform at least two biological repetitions for the activity assays of enzymes of the central metabolism, and carry out at least three technical replicates for each biological repetition. The technical replicates should not be identical assays, but rather proofs of the dependency of the reaction on the amount of enzyme present in the reaction mixture. To do so, test different concentrations of cell extract. The resulting measurements should show a higher activity rate [$\Delta Abs \cdot min^{-1}$] increasing with the concentration of enzyme but retain about the same specific activity [$\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein].
   2. The specific activity of an enzyme is usually reported as µmol of substrate converted per minute per mg of protein in the reaction mixture (*i.e.*, specific activity [$\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein]).
      a. In the case of spectrophometric enzyme assays, calculate the activity using the Lambert-Beer law: $c = A \cdot \varepsilon^{-1} \cdot d^{-1}$, where ε is the molar extinction coefficient [$M^{-1} \cdot cm^{-1}$], d is the optical path length [cm], and c is the concentration of the attenuating species, usually substrate consumed or product formed.
         i. First, determine the absorbance change per minute [$\Delta A \cdot min^{-1}$].
         ii. Divide the absorbance change by the corresponding extinction coefficient and the optical path length (usually 1 cm), to obtain the concentration change per minute [$M \cdot min^{-1}$]. Recalculate it for the millimolar concentration (*i.e.*, divide by 1,000), which is equivalent to the enzyme activity in [$\mu mol \cdot ml^{-1} \cdot min^{-1}$].
         iii. Multiply by the dilution factor of the tested enzyme preparation/cell extract (*e.g.*, a test with 5%

[v/v] cell extract represents a dilution factor of 20). If the enzyme solution was diluted, consider this dilution as well.

iv. Finally, calculate the specific activity in [µmol·min^{-1}·mg^{-1} protein] by dividing the obtained total activity in the reaction mixture [µmol·mL^{-1}·min^{-1}] by the protein concentration [mg protein·mL^{-1}], determined by the Bradford method (see Procedure B13).

b. In the case of citrate cleavage discontinuous assay (UHPLC; citrate + CoA → acetyl-CoA + oxaloacetate + H_2O), the peaks were correlated with standards of acetyl-CoA and CoA by retention time (Figure 2A and 2B). The spectra at 260 nm of the two species can be used to distinguish free CoA and CoA thioesters (Figure 2C).

i. Here, the specific activity was calculated by considering the integrated areas of CoA and acetyl-CoA peaks. As CoA and acetyl-CoA have about the same extinction coefficient (16.3 and 16.4 mM^{-1} cm^{-1}, respectively), it is possible to multiply the initial CoA concentration by the relative abundance [%] of the formed acetyl-CoA, to obtain its concentration in the reaction mixture at the corresponding time points.

ii. Once calculated the concentration [µmol·mL^{-1}] of acetyl-CoA, divide it by the minute at which the reaction was stopped [min^{-1}].

iii. Multiply the total activity by the dilution factor [µmol·mL^{-1}·min^{-1}]. If the enzyme solution was diluted, consider this dilution as well.

iv. To finally get the specific activity [µmol·min^{-1}·mg^{-1} protein], divide the total activity by the enzyme concentration [mg·ml^{-1}], determined by the Bradford method (see Procedure B13).

3. For organisms using the roTCA cycle, expect citrate synthase and malate dehydrogenase activities >10 [µmol·min^{-1}·mg^{-1} protein].

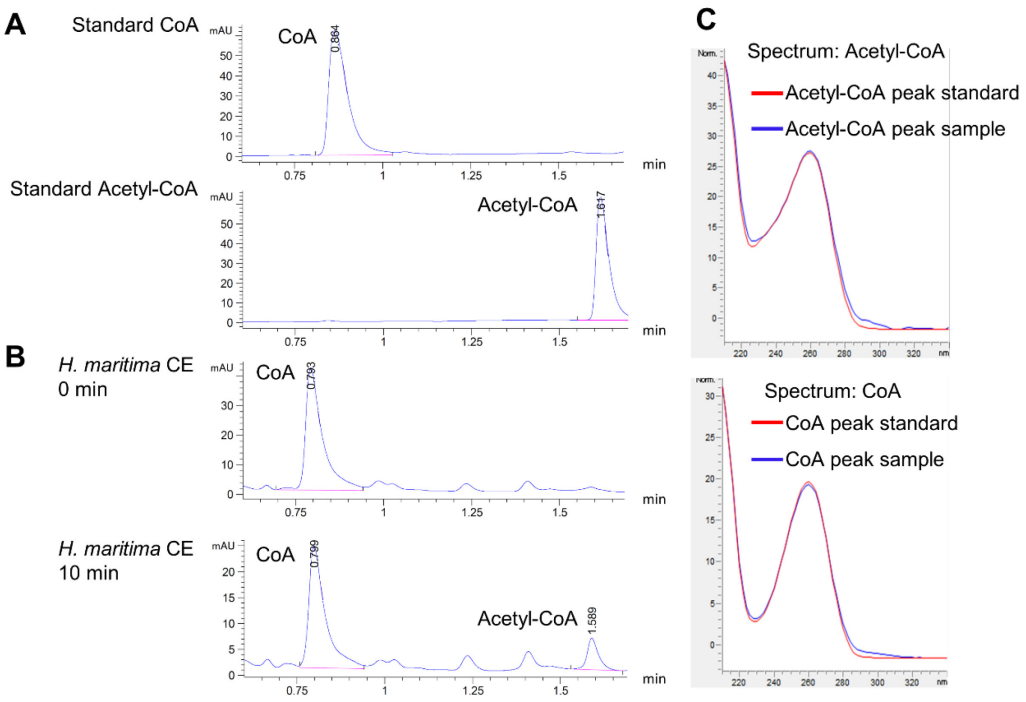


Figure 2. The citrate cleavage reaction performed by citrate synthase (discountinous assay).
A. The UHPLC chromatograms of two standards (free CoA and acetyl-CoA) are reported with the corresponding retention times (0.864 and 1.617 min, respectively). **B.** The UHPLC chromatograms of two time points: samples were taken immediately at the start (0 min) of the reaction (citrate + CoA → acetyl-CoA + oxaloacetate + H_2O), at the addition of cell extracts of autotrophic grown *H. maritima,* and after 10 min. **C.** The comparison of absorbance spectra at 260 nm of acetyl-CoA and CoA peaks between standards (red) and samples (blue).

C. Isotopologue profiling (based on GC-MS analysis)

1. Examine Figure 3 to have an overview of the analysis of labeling experiments performed to track the roTCA cycle activity. Aspartate and glutamate work as surrogates for the direct roTCA cycle intermediates oxaloacetate and 2-oxoglutarate.
2. In experiments using $^{13}CO_2$ as a tracer (Figure 3A), the analysis of the isotopologue composition of aspartate and glutamate yields direct insights into the roTCA cycle activity; while lighter isotopologues (<M+3) could also be produced when only selected parts of the cycle are incorporating $^{13}CO_2$, the presence of heavier isotopologues (M+4 in aspartate, and M+5 in glutamate) is a clear indication of autotrophy or mixotrophy and, in our case, of the activity of the fully closed roTCA cycle.
3. When looking at experiments with [1-^{13}C]glutamate as a tracer (Figure 3B), comparison of the percentage of labelled molecules in different fragments of aspartate and glutamate is used as a read-out for the roTCA cycle activity. In case of an active roTCA cycle, the label is transferred from C1 of glutamate into C4 of aspartate. Subsequently, fragments of aspartate containing either C1-4 or C2-4 show the same percentage of labelled molecules. Further propagation through the roTCA cycle induces a label in position C2 or C5 of glutamate, which was initially only labelled at the C1 position.

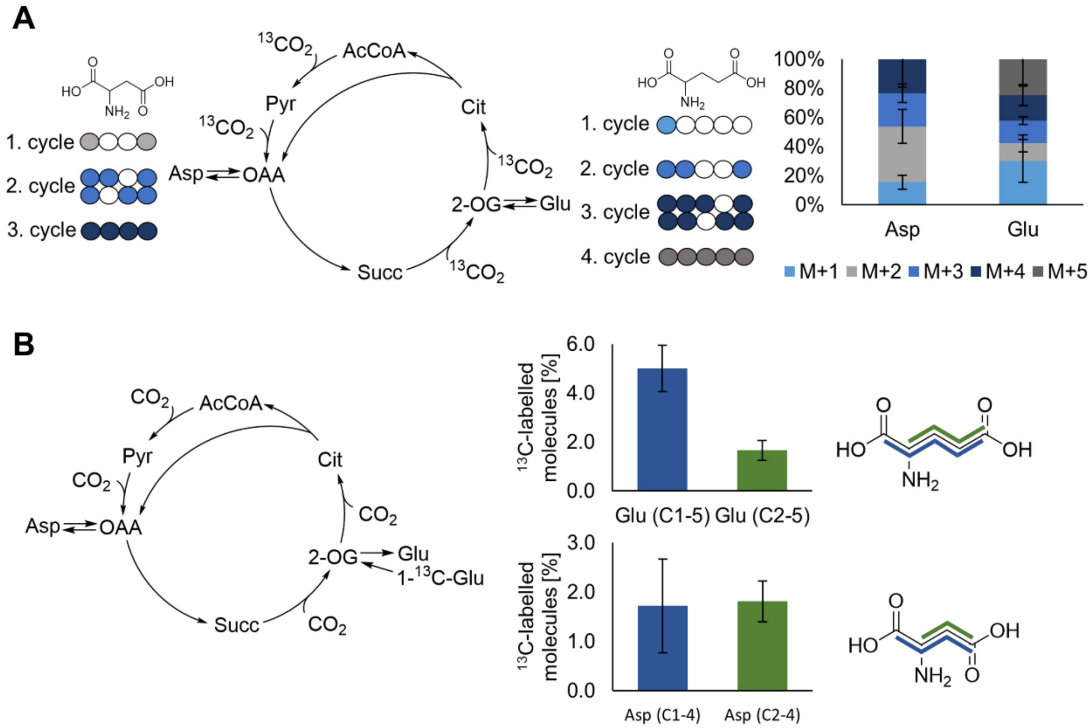


Figure 3. Analysis of labelling experiments to track the roTCA cycle activity.
A. The incorporation of $^{13}CO_2$ into aspartate and glutamate after three and four rounds of the roTCA cycle, respectively, produces diverse isotopologues. The histogram plot shows the relative abundances of the detected isotopologues. The labelled carbons are schematically represented as colored circles, in accordance with the legend of the histogram plot. **B.** Incorporation of [1-^{13}C]glutamate through the roTCA cycle is followed by comparing the percentage of labelled molecules in different fragments of glutamate (blue: C1-5; green: C2-5) and aspartate (blue: C1-4; green: C2-4). The colored bars in the chemical structures indicate the portion of the molecule in the respective fragment. AcCoA: acetyl-CoA; Pyr: pyruvate; OAA: oxaloacetate; Asp: aspartate; Succ: succinate; 2-OG: 2-oxoglutarate; Glu: glutamate; Cit: citrate. Data are mean ± S.E.M. of at least three biological replicates.

Notes

1. Autotrophic organisms using other CO_2 fixation pathways might not show the same concentration dependence in growth experiments. Therefore, they might be excluded from further analysis. For example, as already mentioned, *Desulfobacter hydrogenophilus* using the ATP-citrate lyase variant of this cycle (rTCA cycle) grew similarly well at all tested growth conditions. The independence of the growth from the CO_2 concentration of bacteria using the rTCA cycle was not systematically studied for other autotrophic organisms.
2. Please note that before the actual sterile filtration, the filters (sterile syringe filters; membrane filters) should undergo a washing step with 20 mL 0.9% [w/v] NaCl solution (saline) and 20 mL of ddH_2O, to remove any possible contaminants present in the filter material (*e.g.*, glycerine).
3. It is possible to aliquot the medium without the use of the anaerobic tent, by transferring the media with disposable syringes and needles from the anoxic medium to the anaerobic serum bottles.
4. Cultures turning pink (resazurin oxidation) often indicate an oxygen contamination or a strong pH shift. Resofurin, the intermediate compound of the reaction responsible for the pink coloring, is usually cytotoxic and that the cultures that turned pink are probably dead.
5. In our case, the term *anoxic* indicates a solution free of molecular oxygen. To make a solution anoxic, apply vacuum-nitrogen gas cycles (1–3 min each, depending on the volume) under agitation. As a general rule, 40–45 min per liter of solution is the minimum time required for this procedure. The subsequent addition of reducing agents (in this protocol, only in the case of cultivation medium) ensures that the solution is completely free of molecular oxygen.
6. Performing these anaerobic enzyme assays does not necessarily require the anaerobic glove box. It is possible to aerobically mix all the components of the reaction mixture, close the cuvette with a rubber stopper, then flush the gas space with N_2. After having reduced the viologens with dithionite until the solution is slightly blue (absorbance of ~0.8 at 578 nm), add the cell extract and proceed. Alternatively, it is also possible to make each solution component anoxic, as described in Note 5, and mix them anaerobically in the cuvette, made anoxic by flushing N_2 (~1 min). Nevertheless, his method is difficult to use for the measurements of enzymes with low activities, as oxygen solved in the stoppers may compromise such assays.
7. The cell density (cells/mL) of a culture can be calculated using a Thoma counting chamber. It usually has two counting areas located at a defined depth (0.1 mm) that consists of 16 large squares bordered by three parallel lines. Each large square is composed of 16 small squares (0.0025 mm^2), used for the actual counting.

   Before the sample application, put the cover glass on a clean Thoma chamber, applying a gentle pressure on the outer bars until the formation of Newton's rings. If necessary, dilute the sample, then slowly load it with a pipette in proximity of the counting areas. The water surface tension will help the proper loading of the sample. Find the counting areas with a PH1 10× objective, then move to a PH2 40× objective. Remember that the chamber is too thick to be used with immersion oil. After each use, the chamber must be thoroughly cleaned with 70% [v/v] EtOH.

   Start the counting in the small square located in the upper left corner and proceed right in a serpentine pattern. Count the cells laying inside a small square and onto adjacent sides (either left and upper or right and lower). Dividing cells counts as two. To spot all the cells located on different planes, change the focus (out of focus-sharp-out of focus). Count four large squares; the final difference among the single large squares' counts should not be higher than 10 cells/square.

   Determine the average of the cell counts and calculate the cell density (cells/mL). The actual volume of the counting chamber is 0.004 mm^3, which is equal to 0.004 µL (0.1 mm·0.0025 mm^2·16 small squares). Finally, multiply the volume by 250, to get cells/µL, then by 1,000, to get cells/mL.

   cells/mL=average cell count·dilution factor·$2.5*10^5$

Recipes

1. *Hippea maritima*, *Desulfurella acetivorans*, *D. multipotens*, and *D. propionica* cultivation media

Dissolve the listed ingredients, except sulfur and vitamins, in 700 mL of dH_2O, then fill up to 1 L.

a. List of ingredients for *H. maritima* (modified from DSMZ medium 854, pH 5.5–6.0):
   0.33 g L^{-1} NH_4Cl
   0.33 g L^{-1} $CaCl_2 \cdot 2H_2O$
   0.33 g L^{1} $MgCl_2 \cdot 6H_2O$
   0.33 g L^{-1} KCl
   0.33 g L^{-1} KH_2PO_4
   25 g L^{-1} NaCl
   3 g L^{-1} MOPS buffer
   200 mg L^{-1} yeast extract
   1 mg L^{-1} resazurin
   10 g L^{-1} sulfur powder
   10 ml L^{-1} trace element solution (DSMZ 141)
   10 ml L^{-1} Wolfe's vitamin solution

b. List of ingredients for *D. acetivorans* (pH 6.5–7.0), *D. multipotens* (pH 6.8), and *D. propionica* (pH 6.9–7.2) (modified from DSMZ 480):
   0.33 g L^{-1} NH_4Cl
   0.33 g L^{-1} $CaCl_2 \cdot 2H_2O$
   0.33 g L^{-1} $MgCl_2 \cdot 6H_2O$
   0.33 g L^{-1} KCl
   0.33 g L^{-1} KH_2PO_4
   3 g L^{-1} MOPS
   10 g L^{-1} sulfur powder
   1 mg L^{-1} resazurin
   1 mL L^{-1} SL-10 trace element solution
   10 mL L^{-1} Wolfe's vitamin solution

2. SL-10 trace element solution

a. Dissolve the ingredients in dH_2O.
   3 g L^{-1} $FeCl_2 \cdot 4H_2O$
   70 mg L^{-1} $ZnCl_2$
   100 mg L^{-1} $MnCl_2 \cdot 4H_2O$
   4 mg L^{-1} $CuCl_2 \cdot 2H_2O$
   24 mg L^{-1} $NiCl_2 \cdot 6H_2O$
   36 mg L^{-1} $NaMoO_4 \cdot 2H_2O$
   30 mg L^{-1} H_3BO_3
   224 mg L^{-1} $CoCl_2 \cdot 6H_2O$
b. Fill up to 1,000 mL dH_2O.
c. Sterile filter (see Note 2) and store at 4°C.

3. Wolfe's vitamin solution (DSMZ 141)

a. Dissolve the ingredients in dH_2O.
   2 mg L^{-1} biotin
   2 mg L^{-1} folic acid
   10 mg L^{-1} pyridoxine-HCl
   5 mg L^{-1} thiamine-HCl
   5 mg L^{-1} riboflavin

5 mg L^{-1} nicotinic acid
5 mg L^{-1} D-Ca-pantothenate
0.1 mg L^{-1} cyanocobalamin
5 mg L^{-1} p-aminobenzoic acid
5 mg L^{-1} lipoic acid

b. Fill up to 1,000 mL dH_2O.
c. Sterile filter (see Note 2) and store at 4°C.

4. Trace element solution (DSMZ 141)

a. First, dissolve 1.5 g of nitrilotriacetic acid (NTA) in 200 mL of distilled water.
b. Adjust pH to 6.5 with KOH, then dissolve the following mineral salts:
3 g L^{-1} $MgSO_4 \cdot 7H_2O$
0.5 g L^{-1} $MnSO_4 \cdot H_2O$
1 g L^{-1} NaCl
0.1 g L^{-1} $FeSO_4 \cdot 7H_2O$
0.18 g L^{1} $CoSO_4 \cdot 7H_2O$
0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$
0.18 g L^{-1} $ZnSO_4 \cdot 7H_2O$
0.01 g L^{-1} $CuSO_4 \cdot 5H_2O$
0.02 g L^{-1} $KAl(SO_4)_2 \cdot 12H_2O$
0.01 g L^{-1} H_3BO_3
0.01 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$
0.03 g L^{-1} $NiCl_2 \cdot 6H_2O$
0.3 mg L^{-1} $Na_2SeO_3 \cdot 5H_2O$
0.4 mg L^{-1} $Na_2WO_4 \cdot 2H_2O$

c. Finally, readjust pH to 7.0 with KOH, and fill up to 1,000 mL dH_2O.
d. Sterile filter (see Note 2) and store at 4°C.

5. Cell lysis buffer (20 mM Tris-HCl, pH 7.8, 5 mM DTE)

a. Weight 242 mg of Tris-HCl and 77 mg of DTE, and solve them in 70 mL of dH_2O.
b. Adjust the pH to 7.8 at the test temperature (*e.g.*, the optimum growth temperature of the organism in question) with HCl and NaOH.
c. Fill up to 100 mL of dH_2O.
d. Make the solution anoxic by performing vacuum-gas cycles with N_2 100% (50–100 kPa overpressure; see Note 5) for 15 min.

6. Stop solution (10% acetonitrile, 1 M HCl)

a. Carefully mix 2 mL of acetonitrile (99.8%) and 2 mL of 10 M HCl in 18 mL of dH_2O.
b. Store at RT.

7. Potassium phosphate buffer (10 mM KH_2PO_4/K_2HPO_4, pH 7.0) and UHPLC-grade water

a. Solve 0.34 g of KH_2PO_4 and 0.57 g of K_2HPO_4 in 500 mL of ddH_2O to reach a final concentration of 10 mM phosphate buffer.
b. Filter 500 mL of ddH_2O, then 500 mL of potassium phosphate buffer (0.22 µm membrane filter).
c. Degas the solutions for 5 min with an ultrasonic bath or a vacuum pump.
d. Prepare fresh UHPLC-grade solutions before each run.

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Competing interests

The authors have no conflicts of interest or competing interests.

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