

Supplementary Materials (Laboratory)

Culture conditions

Cultures of *Photobacterium angustum* S14 (wild type and Δicl) were initiated from a single colony grown on Marine Agar (Difco) at 20°C for 2 days. Cells were then transferred into artificial seawater AQUIL^{1,2} containing 1 mmol L⁻¹ D-glucose as the sole source of carbon, 350 μ mol L⁻¹ NaNO₃ and 10 μ mol L⁻¹ NaH₂PO₄. For cultures grown exclusively on acetate, 3 mmol L⁻¹ sodium acetate was added, corresponding to 72 mgC L⁻¹, the same amount of carbon supplied as D-glucose. Trace metal solutions were buffered with 100 μ mol L⁻¹ EDTA to obtain free-ion concentrations identical to those used previously^{3,4}. Cells were acclimated to two Fe conditions: +Fe (5.4 μ mol L⁻¹ Fe-EDTA) and -Fe (5.4 μ mol L⁻¹ Fe-EDTA plus DFOB), as described in detail elsewhere³.

MIMS experimental setups

Two complementary experimental setups were used for MIMS measurements.

Large-volume batch cultures. Prior to each experiment, cells were acclimated to the experimental medium by serial transfer in 100-mL batch cultures for approximately 15 generations. Acclimated cultures were then transferred to 500-mL flasks fitted with a membrane inlet connected to a Hiden Analytical HPR-40 DSA mass spectrometer. Cells were allowed to grow without further medium addition, while O₂ consumption and CO₂ production were monitored continuously throughout the growth trajectory. Measurements lasted between 13 to 32h and were stopped at the end of the exponential phase.

Exponential-phase-maintained cultures. A second, custom-built MIMS setup⁵ was used to measure respiration over short incubations while minimizing changes in growth phase during the measurement. Cultures were maintained in bioreactors at an optical density (wavelength 720nm) of 0.4, a setpoint selected from preliminary growth experiments to correspond to mid-exponential growth. Fresh medium was automatically supplied to maintain this optical density and thus keep cells in exponential growth phase. Three independent bioreactors were used for each bacterial strains (wild type and Δicl). Cultures were initially maintained under Fe-replete conditions; Fe limitation was then progressively imposed by adding DFOB to the fresh medium supplied to the bioreactors. At defined stages of Fe limitation, aliquots were withdrawn from the bioreactors and transferred to the 1.5-mL MIMS chamber for 30-min measurements of O₂ consumption and CO₂ production. For small-volume (1.5 mL) incubations, the medium was acidified to pH 6.2 with 10 mM MES (2-(N-morpholino)ethanesulfonic acid) just prior to measurement and directly in the measurement chamber to (1) minimize pH drift and (2) increase the proportion of dissolved CO₂ relative to bicarbonate and carbonate species and thereby improve MIMS sensitivity for CO₂ detection. Gas exchange rates were calculated using the MIMS analysis software⁵ (https://github.com/francoisBurlacot/MIMS_Analysis).

The two setups therefore served complementary purposes: the 500-mL batch setup captured the integrated dynamics of gas exchange over the growth cycle, whereas the exponential-phase-maintained setup provided short-term respiration measurements under a controlled and comparable growth state across Fe conditions. All MIMS experiments were stopped before O₂ concentrations reached 20% (approximately 60 μ mol L⁻¹) to avoid shift in bacterial metabolism (anaerobia).

Calculations of O₂ consumption (RO₂) and CO₂ production (RCO₂) rates were possible because of the pre-calibration of gazes using the same growth medium (sterile) as a baseline. The amperometric signal generated by the mass spectrometer for each m/z ratio is linearly

correlated to the gas concentration inside the reaction vessel⁵. Signal-to-concentration calibration was performed by equilibrating the medium with gas mixtures of known composition and relating the ion current to dissolved concentration through gas solubility coefficients at the experimental salinity ($S = 5.66$) and temperature ($T = 20\text{ }^{\circ}\text{C}$).

After calculating gas concentrations inside the reaction vessel, gas exchange rates are calculated by correcting from the mass spectrometer consumption. During MIMS measurements, the mass spectrometer vacuum continuously withdraws gas from the reaction vessel across the membrane inlet, so the measured decline in dissolved O_2 reflects both biological consumption and this instrumental removal. To recover the true biological rate, the instrumental term was quantified and subtracted. Because gas permeation across the membrane is proportional to the dissolved concentration in the vessel, this removal follows first-order kinetics, $dC/dt = -k \cdot C$, where C is the dissolved gas concentration and k (min^{-1}) is a consumption rate constant specific to the gas species, the membrane, and the sample-volume-to-membrane-surface ratio. k was determined from a cell-free blank (sterile medium, no cells) and re-measured for each session rather than assumed constant over time. This correction was applied to O_2 ; for CO_2 , instrumental removal was negligible and no correction was applied.

Calculations of respiratory rates

Volumetric rates. Volumetric RO_2 and RCO_2 were obtained by linear regression of the dissolved O_2 and CO_2 concentrations against time during the exponential phase.

Cell specific rates. Cell specific rates were calculated over the exponential growth phase only, identified from the linearity of the dissolved gas signals over time and confirmed by flow cytometry. In bacterial cultures, the cell number increases as

$$N(t) = N_0 \times e^{(\mu t)}$$

where μ is the specific growth rate and N_0 the cell number at the start of the measurement (t_0).

Cell-specific O_2 consumption and CO_2 production were therefore calculated as follows:

$$\text{Cell specific } \text{RO}_2(t) = \text{RO}_2(t) \div N(t)$$

And

$$\text{Cell specific } \text{RCO}_2(t) = \text{RCO}_2(t) \div N(t)$$

For clarity, the equations are written up to a unit-conversion factor, which reconciles the volumetric rates ($\mu\text{mol L}^{-1} \text{h}^{-1}$) with the per-cell units ($\text{fmol cell}^{-1} \text{h}^{-1}$).

To verify the linearity of gas evolution during the exponential growth phase, we reconstructed the expected gas concentrations from the observed increase in cell abundance as follows:

$$\text{O}_2(t) = (\text{O}_2)t_0 - (\text{RO}_2 \times N_0 / \mu) \times (e^{(\mu t)} - 1)$$

And

$$\text{CO}_2(t) = (\text{CO}_2)t_0 + (\text{RCO}_2 \times N_0 / \mu) \times (e^{(\mu t)} - 1)$$

These reconstructed concentrations were compared with the raw MIMS measurements over the same period to determine whether the observed gas trajectories were consistent with the increase in cell abundance expected during exponential growth. Because the respiratory quotient varies across growth phases⁶, the agreement between reconstructed and measured trajectories was used to identify the exponential-growth window over which per-cell rates remained constant. This ensured that the +Fe and -Fe conditions were compared over equivalent physiological states.

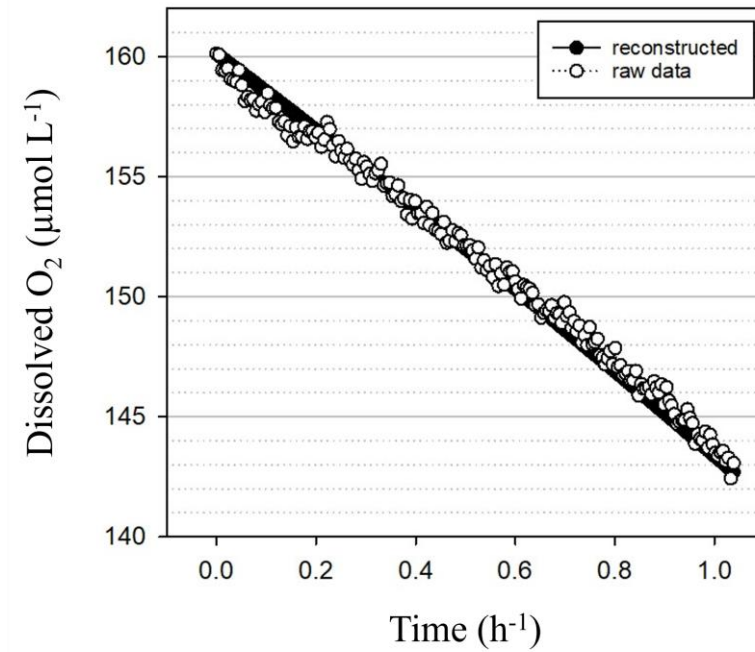


Figure S1 Comparison of measured (raw data) and reconstructed gas trajectories. Example of raw MIMS measurements of O₂ and CO₂ concentrations shown together with concentrations reconstructed from cell abundance. The comparison was used to identify the exponential-growth window over which gas evolution was consistent with changes in cell abundance and per-cell respiratory rates remained constant.

Respiratory quotient. The respiratory quotient was calculated as the ratio of the volumetric rates, which is independent of cell number:

$$RQ = RCO_2 / RO_2$$

Estimation of cellular carbon quota and biomass-to-respiratory carbon ratio

The cellular carbon quota (Q_c) was calculated from bacterial biomass measured at the end of the exponential growth phase and the corresponding bacterial cell abundance:

$$Q_c = POC_f / N_f$$

where POC_f is the bacterial particulate organic carbon concentration and N_f is the bacterial cell abundance at the end of the exponential growth phase. The resulting cellular carbon quota was expressed as $\mu\text{molC cell}^{-1}$.

Because qC was assumed to remain constant during exponential growth, bacterial biomass carbon production (BP) was derived as:

$$BP = qC \times \mu$$

where BP is bacterial biomass carbon production per cell ($\text{fmolC cell}^{-1} \text{h}^{-1}$), qC is the cellular carbon quota (fmolC cell^{-1}), and μ is the growth rate (h^{-1}).

The biomass-to-respiratory carbon ratio (BRC) was then calculated by comparing the carbon incorporated into newly produced biomass with cell-specific respiratory CO_2 production:

$$BRC = BP / RCO_2$$

where RCO_2 is the cell-specific CO_2 production rate ($\text{fmolCO}_2 \text{ cell}^{-1} \text{h}^{-1}$). The BRC therefore represents the ratio of carbon incorporation into bacterial biomass relative to respiratory carbon loss during exponential growth.

Theoretical Electron Balance

Iron limitation forces marine heterotrophic bacteria to remodel their central carbon metabolism, shifting flux away from the complete CAC toward the glyoxylate shunt. This metabolic switch has direct and quantifiable consequences for the energetic and carbon balance of the cell, which we formalize here through a simple stoichiometric model. To allow a direct comparison with the glyoxylate shunt, which requires two acetyl-CoA molecules per cycle, the balance is calculated on the basis of 2 acetyl-CoA.

In the complete CAC, 2 acetyl-CoA yields 6 NADH, 2 FADH_2 , 2 GTP, and 4 CO_2 . These reduced electron carriers are reoxidized by the respiratory chain, which uses the energy released by electron transfer to pump protons (H^+) across the inner membrane, generating a proton-motive force that drives ATP synthesis through ATP synthase. The efficiency of this coupling is described by the P/O ratio (the number of ATP molecules synthesized per atom of oxygen consumed) which equals approximately 2.5 for NADH and 1.5 for FADH_2 under standard conditions, reflecting the different number of protons pumped by each electron donor (Brand 1994, Hinkle 2005). At a P/O ratio of 2.5, 2 acetyl-CoA oxidized through the complete CAC yields approximately 20 mol ATP. In the glyoxylate shunt, the two oxidative decarboxylation steps catalyzed by isocitrate dehydrogenase (IDH) and α -ketoglutarate dehydrogenase (α KGDH) are bypassed by isocitrate lyase (ICL) and malate synthase (MS). As a result, the glyoxylate shunt yields only 1 NADH, 0 FADH_2 (if succinate is conserved), 0 GTP, and 0 CO_2 , producing approximately 2.5 mol ATP. Instead, it is complemented by other metabolic pathways, such as glycolysis and the Entner–Doudoroff pathway, which provide additional ATP and reducing equivalents.

Importantly, because NADH and FADH_2 are still oxidized via the respiratory chain regardless of which cycle is active, O_2 consumption decreases in proportion to the reduced electron donor pool, but far less steeply than CO_2 production, which drops to zero in the pure glyoxylate case. The respiratory quotient (RQ, $\Delta\text{CO}_2/\Delta\text{O}_2$) therefore declines sharply as the fraction of carbon flux routed through the shunt increases, consistent with the fourfold decrease in RQ we observe experimentally under Fe limitation. We note that changes in the P/O ratio itself, which can occur under Fe limitation through downregulation of Fe-S subunits in complex I and the bc1 complex, or through reduced expression of ATP synthase subunits β and γ (Fourquez et al. 2014) would affect ATP yield but leave the RQ unchanged, since the P/O ratio governs the H^+/ATP coupling efficiency but not the CO_2/O_2 stoichiometry. The observed collapse of RQ is therefore a direct and unambiguous stoichiometric signature of the glyoxylate shunt and cannot be explained by changes in respiratory chain architecture alone (Fig. S2, Table S1).

For a mixed metabolic state in which a fraction α of acetyl-CoA flux passes through the glyoxylate shunt and the remainder $(1 - \alpha)$ through the complete CAC, the stoichiometry is:

$$CO_2 = (1 - \alpha) \times 4$$

And

$$O_2 = (NADH + FADH_2) \times 0.5$$

The fraction entering the CAC is $(1 - \alpha)$ of the 2 acetyl-CoA molecules, i.e., $2(1 - \alpha)$ acetyl-CoA. Each acetyl-CoA completely oxidized in the CAC produces 6 NADH (via IDH, α KGDH, and MDH). Therefore, the CAC generates: $6(1 - \alpha)$ NADH. The fraction entering the glyoxylate shunt is α of the 2 acetyl-CoA molecules, i.e., 2α acetyl-CoA. However, the glyoxylate shunt consumes 2 acetyl-CoA per cycle and produces only 1 NADH. Thus, 2α acetyl-CoA correspond to α shunt cycles, yielding: $\alpha \times 1 = \alpha$ NADH. The total NADH production is therefore:

$$NADH = 6 - 5\alpha$$

$FADH_2$ is produced exclusively by succinate dehydrogenase (SDH), a reaction that occurs only in the complete CAC. This step is bypassed in the glyoxylate shunt because succinate is conserved rather than oxidized. Therefore, only the fraction of acetyl-CoA entering the CAC contributes to $FADH_2$ production:

$$FADH_2 = (1 - \alpha) \times 2$$

The same reasoning applies to GTP. GTP is generated by succinyl-CoA synthetase (SCS), another reaction that occurs only in the complete CAC and is bypassed by the glyoxylate shunt. Consequently, GTP production equals that of $FADH_2$.

This framework makes explicit that the disproportionate reduction in CO_2 relative to O_2 is a direct stoichiometric consequence of bypassing IDH and α KGDH and does not require invoking changes in the P/O ratio or respiratory chain architecture (Fig. S3).

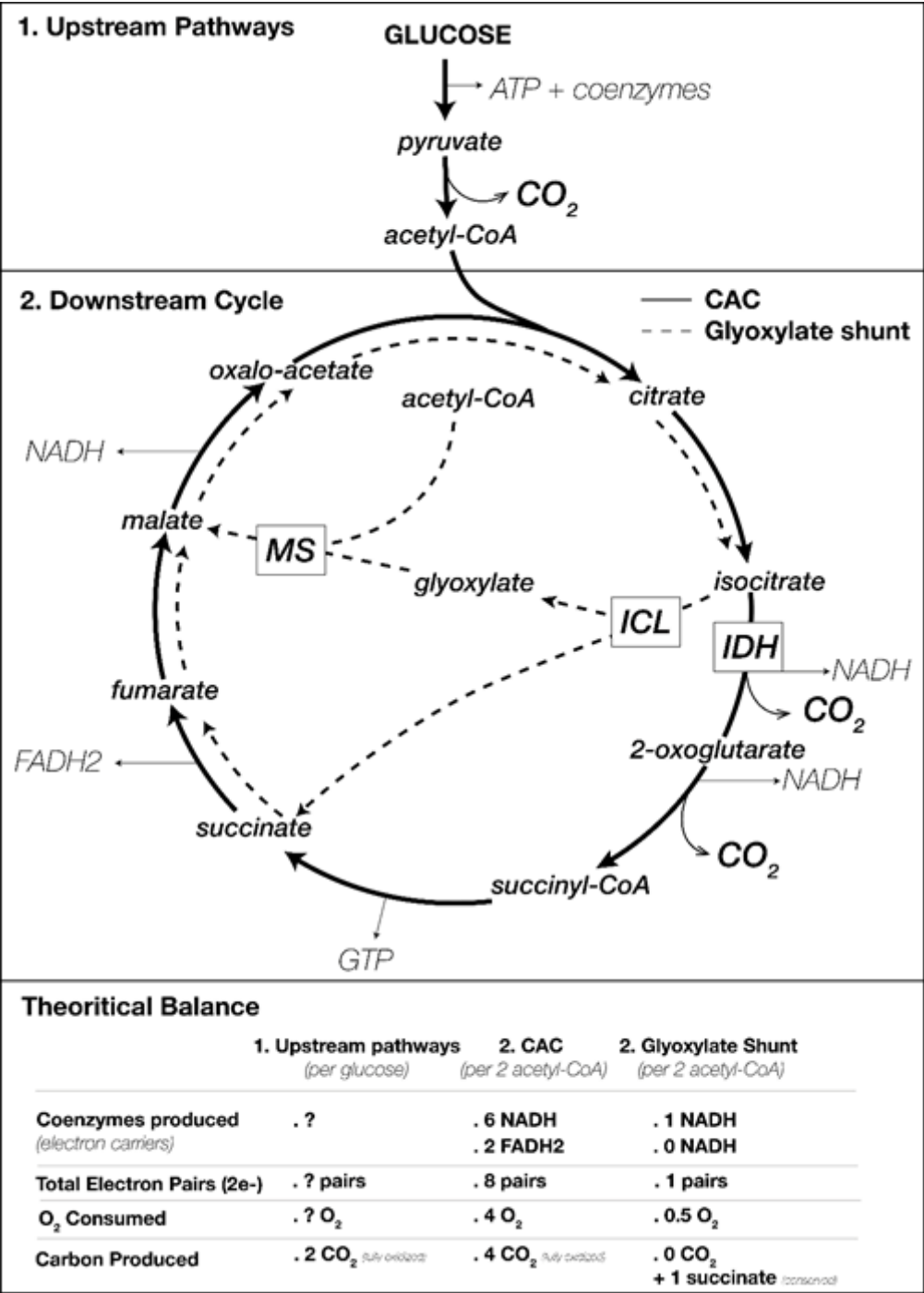


Figure S2 Stoichiometric model of carbon flux partitioning between the complete CAC and the glyoxylate shunt. Under Fe-replete conditions, each acetyl-CoA enters the complete CAC and releases 2 CO₂ and generates 3 NADH, 1 FADH₂, 1 GTP. Under Fe limitation, the two oxidative decarboxylation steps are bypassed, and carbon is rerouted through the glyoxylate shunt via yielding only 1 NADH, no CO₂ release. ILC, isocitrate lyase; IDH, isocitrate dehydrogenase; MS, malate synthase.

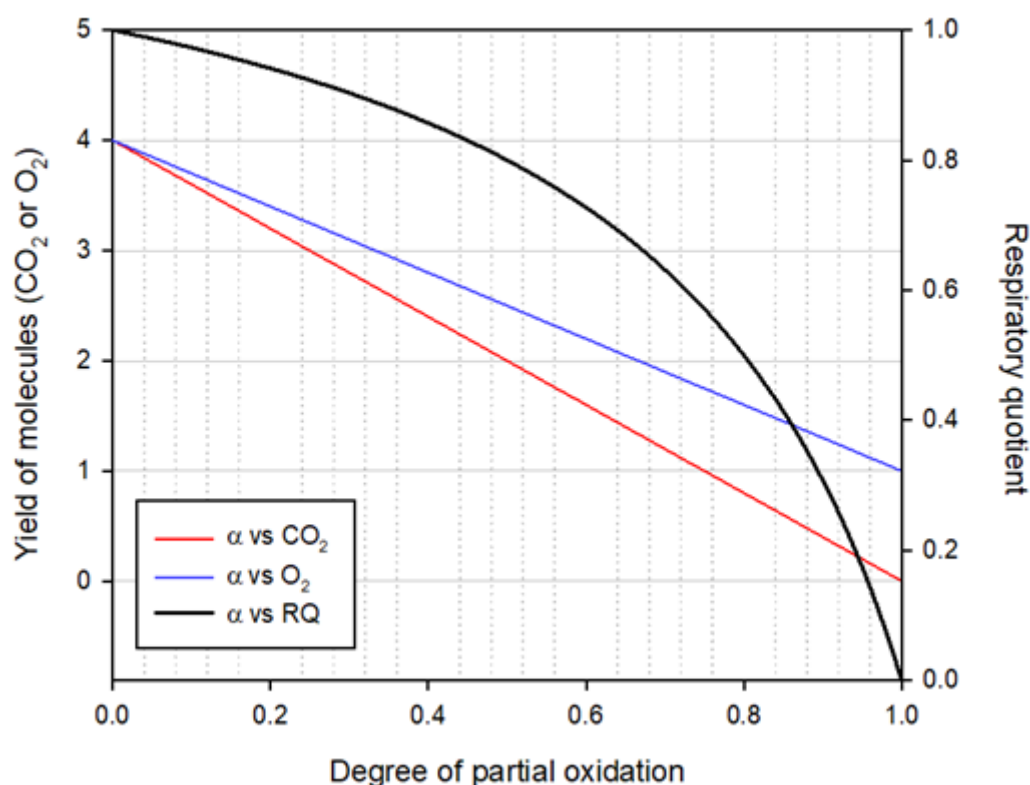


Figure S3 Predicted yields of O_2 and CO_2 per 2 acetyl-CoA as a function of the fraction of carbon flux routed through the glyoxylate shunt (α).

Theoretical Energetic Balance

When biosynthetic precursors are supplied directly by the shunt, the ATP cost of constructing them from more oxidized intermediates is avoided. In other words, the shunt reduces the ATP requirement per unit of biomass produced, partially compensating for the reduction in ATP supply. Our stoichiometric model is centered on the isocitrate branch point and is formulated from acetyl-CoA rather than glucose. Consequently, we do not include a fixed contribution from NADH generated upstream during the conversion of glucose to pyruvate. Although CO_2 is still released during these upstream reactions (Fig. S2), the associated production of NADH cannot be assigned a single stoichiometric value because it depends on the glycolytic pathway operating (e.g., the glycolysis or Entner–Doudoroff pathway) and on the fraction of reducing equivalents diverted to biosynthetic processes. Our previous studies showed that the expression of alternative pathways is strongly affected by Fe limitation^{4,7}. Because this upstream contribution to respiratory O_2 consumption is both variable and unconstrained, it is not explicitly included in the RQ calculation. This approach is consistent with the low RQ values (~ 0.3) measured in this study. The ATP budget is treated differently, and for a clear reason: whatever the glycolytic route, the breakdown of glucose to pyruvate yields ATP through substrate-level phosphorylation, and this ATP supports growth independently of the shunt. Accounting for it explains why growth declines only about twofold under Fe limitation, despite the much larger loss of the ATP normally generated by the complete CAC. The theoretical ATP yield per molecule of glucose (P/O explicit) is as follow :

<i>P/O ratios</i>		
<i>P/O — NADH (link & cycle)</i>	2.5	
<i>P/O — glycolytic NADH, low</i>	1.5	
<i>P/O — glycolytic NADH, high</i>	2.5	
<i>P/O — FADH2</i>	1.5	
<i>GTP → ATP</i>	1	
<i>Stage / Product</i>	Citric Acid Cycle	Glyoxylate Shunt
<i>Glycolysis — net ATP</i>	2	2
<i>Glycolysis NADH (2)</i>	3. to 5.	3. to 5.
<i>Link reaction (PDH) NADH (2)</i>	5	5
<i>Cycle yield (2 acetyl-CoA)</i>	20	2.5
<i>Net carbon products</i>	6 CO ₂ (fully oxidised)	2 CO ₂ + 1 succinate (conserved)
<i>Total net ATP per glucose</i>	30. to 32.	12.5 to 14.5

The RQ and the ATP budgets describe two complementary facets of a single metabolic reconfiguration and should be read at their respective levels. The RQ is used here as a qualitative tracer of carbon routing. The ATP yield, in contrast, is a theoretical value calculated for the complete breakdown of glucose (glycolysis, pyruvate oxidation and the cycle). Therefore, resolving its overall effect on ATP conservation will require further investigation.

Table S1 Theoretical stoichiometric model predicting the respiratory quotient ($RQ = \text{net CO}_2 / \text{net O}_2$) as a function of the fraction of carbon diverted through the glyoxylate shunt (α ; $\alpha = 0$, full CAC cycle; $\alpha = 1$, all isocitrate routed through the glyoxylate shunt). Per-acetyl-CoA (x2) yields of NADH, FADH₂, GTP, net CO₂ and net O₂, together with net ATP yields and the relative reduction in ATP yield (%), are given for each α . Values are theoretical predictions.

<i>fraction of glyoxylate shunt (α)</i>	<i>Net CO₂ /acetyl-CoA</i>	<i>NADH /acetyl-CoA</i>	<i>FADH₂ /acetyl-CoA</i>	<i>GTP /acetyl-CoA</i>	<i>Net e-released</i>	<i>Net O₂ consumed</i>	<i>RQ (CO₂/O₂)</i>	<i>Net ATP /acetyl-CoA</i>	<i>Net ATP /glucose (low estimate)</i>	<i>ATP reduction (%)</i>	<i>Net ATP/glucose (high estimate)</i>	<i>ATP reduction (%)</i>
0.00	4.00	6.00	2.00	2.00	16.00	4.00	1.000	20.0	30.0	0%	32.0	0%
0.05	3.80	5.75	1.90	1.90	15.30	3.83	0.993	19.1	29.1	3%	31.1	3%
0.10	3.60	5.50	1.80	1.80	14.60	3.65	0.986	18.3	28.3	6%	30.3	5%
0.15	3.40	5.25	1.70	1.70	13.90	3.48	0.978	17.4	27.4	9%	29.4	8%
0.20	3.20	5.00	1.60	1.60	13.20	3.30	0.970	16.5	26.5	12%	28.5	11%
0.25	3.00	4.75	1.50	1.50	12.50	3.13	0.960	15.6	25.6	15%	27.6	14%
0.30	2.80	4.50	1.40	1.40	11.80	2.95	0.949	14.8	24.8	18%	26.8	16%
0.35	2.60	4.25	1.30	1.30	11.10	2.78	0.937	13.9	23.9	20%	25.9	19%
0.40	2.40	4.00	1.20	1.20	10.40	2.60	0.923	13.0	23.0	23%	25.0	22%
0.45	2.20	3.75	1.10	1.10	9.70	2.43	0.907	12.1	22.1	26%	24.1	25%
0.50	2.00	3.50	1.00	1.00	9.00	2.25	0.889	11.3	21.3	29%	23.3	27%
0.55	1.80	3.25	0.90	0.90	8.30	2.08	0.867	10.4	20.4	32%	22.4	30%
0.60	1.60	3.00	0.80	0.80	7.60	1.90	0.842	9.5	19.5	35%	21.5	33%
0.65	1.40	2.75	0.70	0.70	6.90	1.73	0.812	8.6	18.6	38%	20.6	36%
0.70	1.20	2.50	0.60	0.60	6.20	1.55	0.774	7.8	17.8	41%	19.8	38%
0.75	1.00	2.25	0.50	0.50	5.50	1.38	0.727	6.9	16.9	44%	18.9	41%
0.80	0.80	2.00	0.40	0.40	4.80	1.20	0.667	6.0	16.0	47%	18.0	44%
0.85	0.60	1.75	0.30	0.30	4.10	1.03	0.585	5.1	15.1	50%	17.1	46%
0.90	0.40	1.50	0.20	0.20	3.40	0.85	0.471	4.3	14.3	53%	16.3	49%
0.95	0.20	1.25	0.10	0.10	2.70	0.68	0.296	3.4	13.4	55%	15.4	52%
1.00	0.00	1.00	0.00	0.00	2.00	0.50	0.000	2.5	12.5	58%	14.5	55%

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