

Awakening a latent carbon fixation cycle in *Escherichia coli*

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Abstract

Carbon fixation is one of the most important biochemical processes. Most natural carbon fixation pathways are thought to have emerged from enzymes that originally performed other metabolic tasks. Can we recreate the emergence of a carbon fixation pathway in a heterotrophic host by recruiting only endogenous enzymes? In this study, we address this question by systematically analyzing possible carbon fixation pathways composed only of *Escherichia coli* native enzymes. We identify the GED (Gnd-Entner-Doudoroff) cycle as the simplest pathway that can operate with high thermodynamic driving force. This autocatalytic route is based on reductive carboxylation of ribulose 5-phosphate (Ru5P) by 6-phosphogluconate dehydrogenase (Gnd), followed by reactions of the Entner-Doudoroff pathway, gluconeogenesis, and the pentose phosphate pathway. We demonstrate the *in vivo* feasibility of this new-to-nature pathway by constructing *E. coli* gene deletion strains whose growth on pentose sugars depends on the GED shunt, a linear variant of the GED cycle which does not require the regeneration of Ru5P. Several metabolic adaptations, most importantly the increased production of NADPH, assist in establishing sufficiently high flux to sustain this growth. Our study exemplifies a trajectory for the emergence of carbon fixation in a heterotrophic organism and demonstrates a synthetic pathway of biotechnological interest.

Introduction

The ability to assimilate inorganic carbon into biomass sets a clear distinction between autotrophic primary producers and the heterotrophs depending on them for the supply of organic carbon. Most primary production occurs via the ribulose bisphosphate (RuBP) cycle – better known as the Calvin-Benson cycle – used in bacteria, algae, and plants ¹. Five other carbon fixation pathways are known to operate in various bacterial and archaeal lineages ^{2, 3} and also contribute to primary production ¹. Recent studies have made considerable progress in establishing carbon fixation pathways in heterotrophic organisms, with the long-term goal of achieving synthetic autotrophy, which could pave the way towards sustainable bioproduction schemes rooted in CO₂ and renewable energy ^{4, 5}. Most notably, overexpression of phosphoribulokinase and Rubisco, followed by long-term evolution, enabled the industrial hosts *Escherichia coli* ^{6, 7} and *Pichia pastoris* ⁸ to synthesize all biomass from CO₂ via the RuBP cycle. Also, overexpression of enzymes of the 3-hydroxypropionate bicycle established the activity of different modules of this carbon fixation pathway in *E. coli* ⁹. While such studies help us to gain a deeper understanding of the physiological changes required to adapt a heterotrophic organism to autotrophic growth, they do not, however, shed light on the origin of the carbon fixation pathways themselves.

Besides the reductive acetyl-CoA pathway and the reductive TCA cycle – both of which are believed to have originated early in the evolution of metabolism ¹⁰ – the other carbon fixation routes are thought to have evolved by recruiting enzymes from other metabolic pathways. For example, Rubisco – the carboxylating enzyme of the RuBP cycle – probably evolved from a non-CO₂-fixing ancestral enzyme, thus emerging in a non-autotrophic context ¹¹. Similarly, acetyl-CoA carboxylase likely originated as a key component of fatty acid biosynthesis before being recruited into carbon fixation pathways in several prokaryotic lineages ^{2, 3}. The limited number of natural carbon fixation pathways might indicate that the recruitment of endogenous enzymes to support carbon fixation is a rather exceptional event. To understand this process better we aimed to recreate it in a heterotrophic bacterium.

Here, we use a computational approach to comprehensively search for all thermodynamically feasible carbon fixation pathways that rely solely on endogenous *E. coli* enzymes. We identify a promising candidate route – the GED cycle – that is expected to enable carbon fixation with minimal reactions and with a high thermodynamic driving force. This synthetic route combines reductive carboxylation of ribulose 5-phosphate (Ru5P) with the Entner–Doudoroff (ED) pathway, gluconeogenesis, and the pentose phosphate pathway. The GED cycle mirrors the structure of the RuBP cycle and is expected to outperform it in terms of ATP-efficiency. We demonstrate that overexpression of key pathway enzymes together with small modifications of the endogenous metabolic network enable growth via the “GED shunt” – a linear route that requires the key reactions of the GED cycle, including the carboxylation step, for the biosynthesis of (almost) all biomass building blocks. Our findings indicate the feasibility of recruiting endogenous enzymes to establish a novel carbon fixation pathway and pave the way for future establishment of synthetic autotrophy based on new-to-nature pathways.

Results

Systematic search for latent carbon fixation pathways in *E. coli*

To identify possible carbon fixation pathways that can be established using only native *E. coli* enzymes¹², we used the genome-scale metabolic model of this bacterium¹³. We assumed that all reactions are reversible and then used an algorithm to systematically uncover all possible combinations of enzymes, the net reaction of which use CO₂ and cofactors (e.g., ATP, NAD(P)H) as sole substrates to produce pyruvate, a reference product commonly used to compare carbon fixation pathways²⁻⁴ (Methods and Supplementary Text). The pathways were then analyzed thermodynamically: for each pathway, we calculated the Max-min Driving Force (MDF), representing the smallest driving force among all pathway reactions after optimizing metabolite concentrations within a physiological range (Methods and Supplementary Text)¹⁴. We assumed an elevated CO₂ concentration of 20% (200 mbar), which is easily attainable in microbial cultivation within an industrial context and further characterizes the natural habitats of *E. coli*, e.g., the mammalian gut^{15, 16}. The MDF criterion enabled us to discard thermodynamically infeasible routes (having MDF<0) and to compare the feasible pathways according to their energetic driving force, which directly affects their kinetics¹⁴.

Using this approach, we identified multiple carbon fixation pathways based on endogenous *E. coli* enzymes (see Supplementary Text for a full list). We ranked the pathways according to two key criteria that can be calculated for each of them in a straightforward manner: their MDF and the number of enzymes they require (preferring fewer enzymes, see Methods and Supplementary Text). Pathways ranked high in terms of these criteria are expected to be simpler to establish and to operate more robustly under fluctuating physiological conditions. The pathway that was ranked highest (Fig. 1A) had the lowest number of enzymes while still supporting a high MDF (> 3 kJ/mol, such that reverse enzyme flux is minimal¹⁴). This cycle is based on the reductive carboxylation of ribulose 5-phosphate (Ru5P) by 6-phosphogluconate (6PG) dehydrogenase (Gnd). The carboxylation product, 6PG, is then metabolized by the enzymes of the Entner–Doudoroff (ED) pathway – 6PG dehydratase (Edd) and 2-keto-3-deoxygluconate 6-phosphate aldolase (Eda) – to produce glyceraldehyde 3-phosphate (GAP) and pyruvate (Fig. 1A). Pyruvate is subsequently converted to GAP via native gluconeogenesis, and GAP is metabolized via the pentose phosphate pathway to regenerate Ru5P, thus completing the cycle. We termed this pathway the GED (Gnd-Entner-Doudoroff) cycle, according to its key enzymes that serve to connect CO₂ fixation to central metabolism.

Our computational analysis identified multiple variants of the GED cycle (Supplementary Text). However, as these are unnecessarily more complex than the simple GED cycle design, we decided not to consider them further. We also identified numerous pathways that do not require Gnd. These Gnd-independent pathways share a common carbon fixation strategy in which a sub-cycle converts CO₂ to formate, where the release of formate is catalyzed by an oxygen-sensitive oxoacid formate-lyase¹⁷ (Supplementary Text). Formate is then assimilated via one of several variants of the reductive glycine pathway^{18, 19} (see Fig. 1B for an example),

the activity of which was recently demonstrated in *E. coli*^{20, 21}. Alternatively, formate is assimilated via variants of the serine cycle, or, more precisely, the previously suggested serine-threonine cycle²² (see Fig. 1C for an example). While these formate-dependent pathways are interesting, their high oxygen sensitivity, as well as general complexity, make them less attractive. Therefore, for further investigation, we decided to focus on the GED cycle.

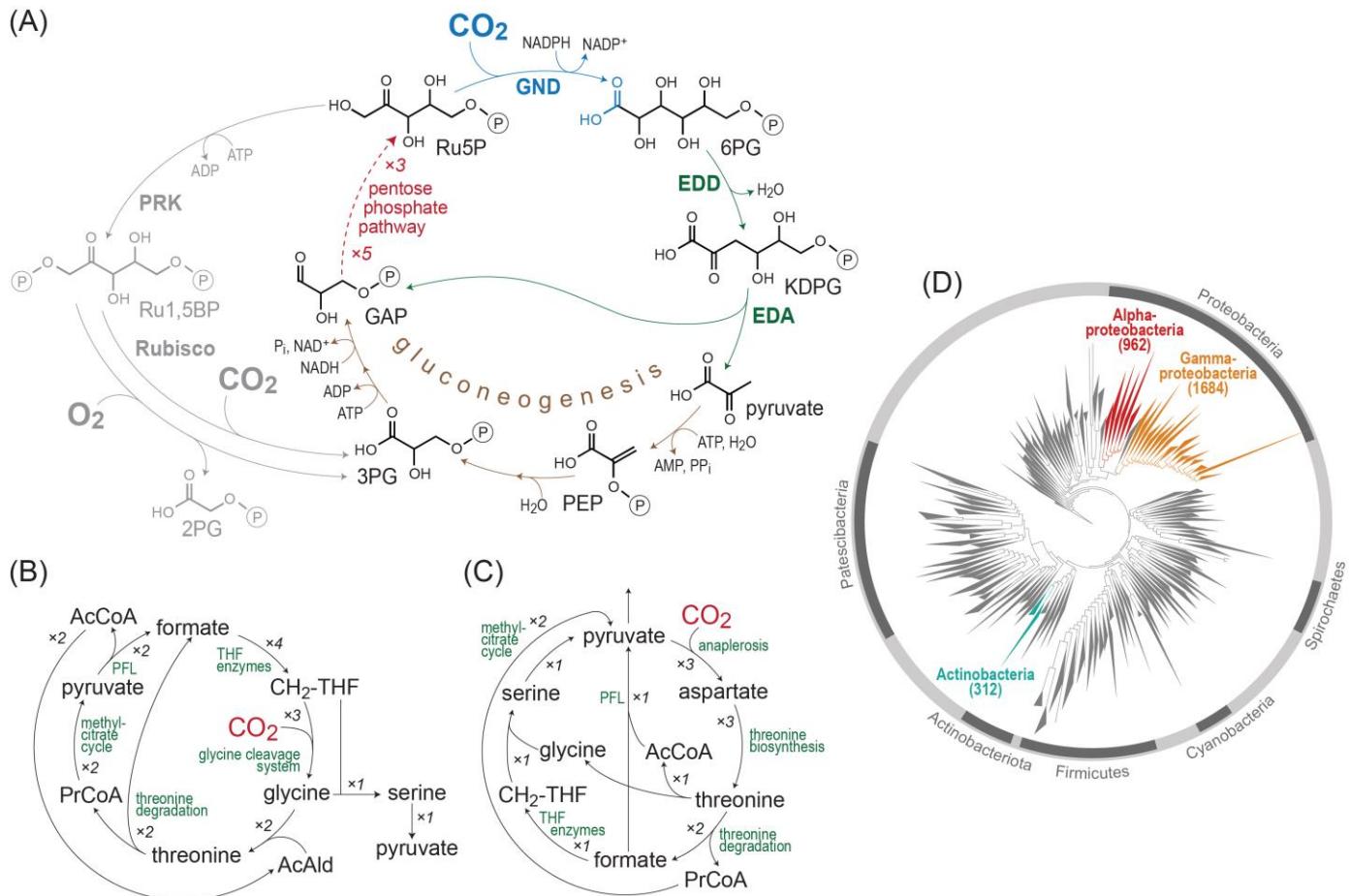


Figure 1. *In silico* identification of latent carbon fixation pathways. (A) The GED cycle, the simplest carbon fixation pathway that can be generated from *E. coli* endogenous enzymes. The pathway is based on the reductive carboxylation of ribulose 5-phosphate (Ru5P) to 6-phosphogluconate (6PG) by Gnd, followed by the activity of the Entner–Doudoroff pathway (enzymes Edd and Eda) to produce pyruvate and glyceraldehyde 3-phosphate (GAP). Pyruvate is converted to GAP via gluconeogenesis and the intermediates phosphoenolpyruvate (PEP) and 3-phosphoglycerate (3PG). To close the cycle, GAP is recycled to Ru5P via the pentose phosphate pathway. The GED cycle mirrors the ribulose bisphosphate cycle (i.e., Calvin–Benson cycle), which is shown in grey. (B,C) Two other general archetypes of carbon fixation pathways that were computationally uncovered. Both are based on integrated cycles, which together reduce CO₂ to formate and then assimilate formate into pyruvate. AcCoA corresponds to acetyl-CoA and PrCoA corresponds to propionyl-CoA. CH₂-THF corresponds to methylene-THF. (D) A phylogenetic tree of bacteria, showing the three phyla that harbor all key enzymes of the GED cycle (shown in color). Numbers in parentheses correspond to the number of species in which the key pathway enzymes were found.

Properties of the GED cycle and its enzymes

The GED cycle mirrors the structure of the canonical RuBP cycle, where phosphoribulokinase and Rubisco are replaced with Gnd, Edd, Eda, and gluconeogenic enzymes (Fig. 1). Similarly to the RuBP cycle, the GED cycle is autocatalytic and any one of its intermediates can be used as a product to be diverted towards

biosynthesis of cellular building blocks²³. Production of pyruvate, a key biosynthetic building block, is more ATP-efficient via the GED cycle than via the RuBP cycle: while the former pathway requires 6 ATP molecules to generate pyruvate, the latter pathway needs 7 ATP molecules (not accounting for further losses due to Rubisco's oxygenation reaction and the resulting photorespiration). Indeed, using Flux Balance Analysis, we found that, compared to the RuBP cycle, the GED cycle is consistently expected to support higher yields of biomass and multiple products that are derived from pyruvate or acetyl-CoA, including ethanol, lactate, isobutanol, 2,3-butanediol, acetone, butyrate, n-butanol, citrate, itaconate, 2-ketoglutarate, and levulinic acid (Supplementary Fig. S1 and Methods).

All enzymes of the GED cycle are known to carry flux in the direction required for pathway activity, with the exception of Gnd. The Gibbs energy of the Gnd reaction indicates that it should be fully reversible under elevated CO₂: $\Delta_r G^m \approx -1.5$ kJ/mol in the oxidative decarboxylation direction (pH 7.5, ionic strength of 0.25 M, [CO₂] = 200 mbar, and 1 mM concentration of the other reactants²⁴). Indeed, similar oxidative decarboxylation enzymes are known to support reductive carboxylation, for example, the malic enzyme²⁵⁻²⁷ and isocitrate dehydrogenase^{28, 29}. While sporadic studies have reported that some Gnd variants support the reductive carboxylation of Ru5P *in vitro*³⁰⁻³⁴, a comprehensive kinetic characterization of this activity in bacterial Gnd variants is lacking. More importantly, it remains unclear whether this reaction could operate under physiological conditions, where the concentrations of substrates and products are constrained; that is, substrate concentrations are not necessarily saturating and product concentrations are non-negligible.

First, we measured the kinetics of *E. coli* Gnd. We found Gnd to have a rather high k_{cat} in the reductive carboxylation direction, approaching 6 s⁻¹ (5.9 ± 0.2 s⁻¹ with Ru5P as substrate, Table 1 and Supplementary Fig. S2), about twice as high as the k_{cat} of most plant Rubisco variants³⁵. The affinity of Gnd towards CO₂ is high enough to enable saturation under elevated CO₂ concentrations: K_M = 0.9 ± 0.1 mM (Table 1 and Supplementary Fig. S2) which is equivalent to ~3% CO₂ in the headspace (at ambient pressure). Notably, these kinetic parameters are substantially better than those previously reported for a eukaryotic Gnd variant (k_{cat} ~ 1 s⁻¹ and K_M(CO₂) ≥ 15 mM^{30, 31}).

Table 1. Kinetic parameters of *E. coli* Gnd in the reductive and oxidative directions. Fitted Michaelis-Menten curves are shown in Supplementary Fig. S2. See Methods for a detailed description of the kinetic characterization.

	k _{cat} (s ⁻¹)	K _M (mM)
<i>Reductive carboxylation</i>		
ribulose 5-phosphate	5.9 ± 0.2	2.8 ± 0.2
NADPH	5.2 ± 0.2	1.0 ± 0.1
CO ₂	4.7 ± 0.2	0.9 ± 0.1
<i>Oxidative decarboxylation</i>		
6-phosphogluconate	49 ± 1	0.035 ± 0.002
NADP ⁺	46 ± 1	0.012 ± 0.003

To check how prevalent the potential of carbon fixation via the GED cycle is, we performed a phylogenetic analysis to identify bacteria that harbor its key enzymes (Methods). We found that the enzymes of the GED cycle are ubiquitous in alpha-proteobacteria (962 species), gamma-proteobacteria (1684), and

actinobacteria (312) (Fig. 1D). The species of these phyla might therefore be prime candidates to search for the carbon fixation activity of the GED cycle. However, in other bacterial lineages, the combined occurrence of the GED cycle enzymes is quite rare, with only 10 other species harboring all key enzymes.

Selection for the activity of the GED shunt within a Δrpe context

Engineering *E. coli* for autotrophic growth via the GED cycle would be a challenging task requiring considerable metabolic adaptation of the host; for example, establishing a delicate balance between the metabolic fluxes within the cycle and those diverging out of the cycle (as found in previous efforts to establish the RuBP cycle in *E. coli*^{6, 7}). Hence, to check the feasibility of the cycle, we focused on establishing growth via the “GED shunt”, representing a segment of the full cycle which consists of reductive carboxylation by Gnd and the subsequent ED pathway (blue reactions in Fig. 2A; for a similar approach see ref.^{36, 37}). As we show below, growth via this linear shunt requires the activity of most enzymes of the GED cycle, but relies on a pentose substrate rather than regeneration of Ru5P.

First, we generated an *E. coli* strain deleted in the gene encoding for ribulose 5-phosphate 3-epimerase (Δrpe). This strain cannot grow on ribose as a sole carbon source, as ribose 5-phosphate (R5P) cannot be converted to xylulose 5-phosphate, thus blocking the pentose phosphate pathway (Fig 2A)^{38, 39}. Activity of Gnd, Edd, and Eda should restore growth by enabling conversion of R5P to GAP and pyruvate, from which all cellular building blocks can be derived (Fig. 2A). This would enable direct selection for the activity of the GED shunt.

We found that overexpression of Gnd, Edd, and Eda from a plasmid (pGED) enabled the growth of the Δrpe strain on ribose only under elevated CO₂ concentration (green lines in Fig. 2B). The observed growth rate via the GED shunt is almost half of that obtained with gluconate, which requires no carboxylation by Gnd and thus serves as a positive control (doubling times 6.9 h and 2.8 h, respectively). While Gnd, Edd, and Eda are all present in the genome of *E. coli*, their native expression level is too low to enable sufficient activity of the GED shunt: overexpression of Gnd alone (pG) or of Edd and Eda alone (pED) did not support growth (less than two doublings, brown lines in Fig. 2B).

To confirm that growth indeed proceeds via the GED shunt, we performed a ¹³C-labeling experiment. We cultivated the Δrpe +pGED strain with unlabeled ribose and ¹³CO₂, and measured the labeling pattern of five proteinogenic amino acids – serine, glycine, alanine, valine, and histidine. The results confirm the activity of the GED shunt (Fig. 2C): (i) since ¹³CO₂ is incorporated as the carboxylic carbon of 6PG, GAP is completely unlabeled and hence serine and glycine that are derived from it are unlabeled; (ii) pyruvate is generated both from GAP and directly from Eda activity (Fig. 2C), such that about half of the pyruvate molecules are unlabeled and half are labeled once at their carboxylic carbon – as a result, half of the alanine and valine molecules are labeled (during valine biosynthesis two pyruvate molecules are condensed and one carboxylic carbon is lost as CO₂).

These results confirm that, upon overexpression of Gnd, Edd, and Eda, the GED shunt is sufficiently active to provide the cell with almost all cellular building blocks as well as energy (by complete oxidation of pyruvate via the TCA cycle). As mentioned above, the growth of this strain requires the simultaneous activity of most enzymes on the GED cycle, including those of glycolysis and the pentose phosphate pathway; for example, net production of erythrose 4-phosphate (E4P) from ribose requires the combined activity of Gnd, the ED pathway, and enzymes of the pentose phosphate pathway.

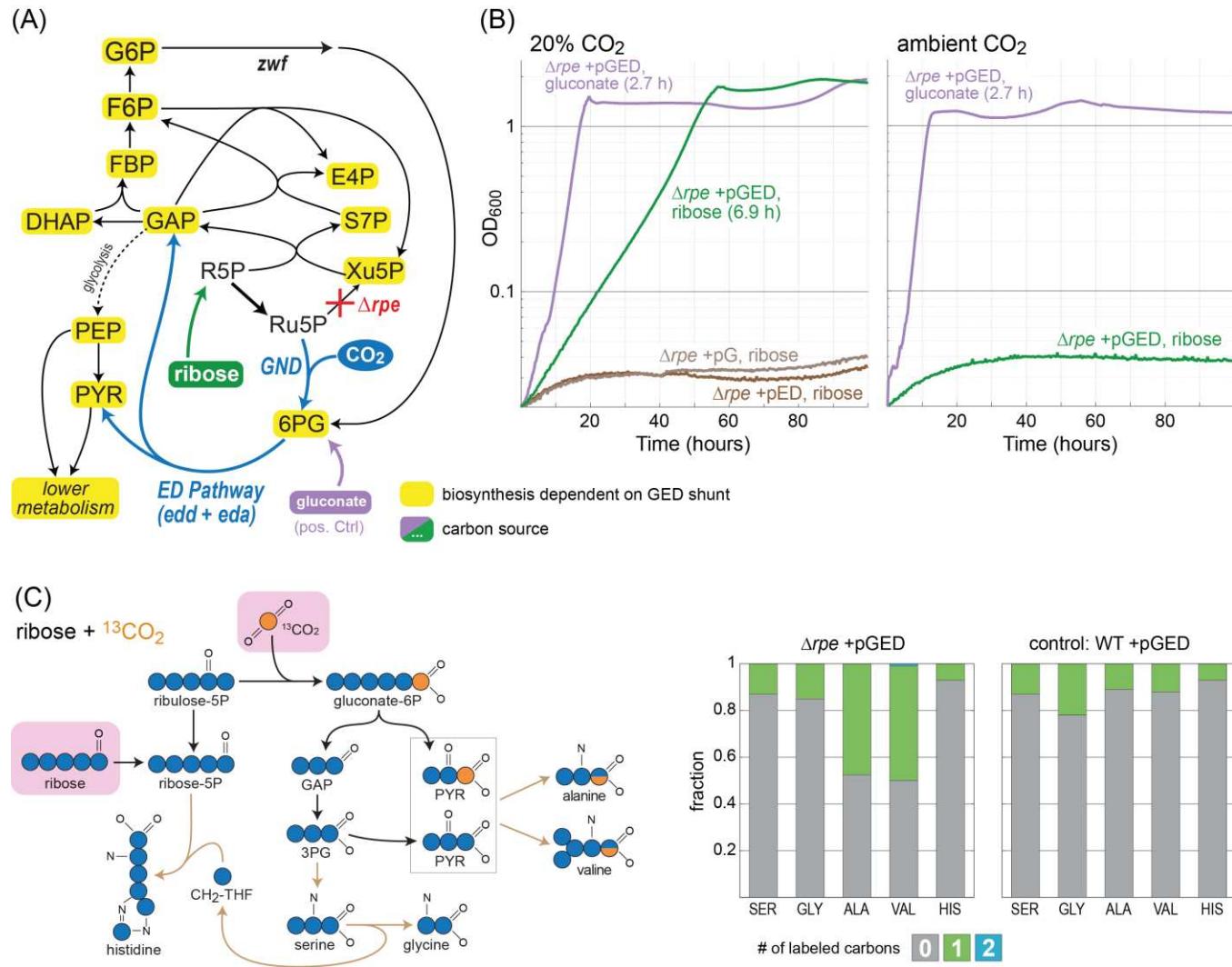


Figure 2. Activity of the GED shunt in a Δrpe strain. (A) Design of the Δrpe selection scheme. Ribose can be assimilated only via the activity of the GED shunt, where the biosynthesis of almost all biomass building blocks is dependent on the pathway (marked in yellow). Growth on gluconate (violet) is not dependent on reductive carboxylation via Gnd and thus serves as a positive control. Reaction directionalities are shown as predicted by Flux Balance Analysis. (B) Growth of a Δrpe strain on ribose (20mM) as sole carbon source is dependent on elevated CO₂ concentration (20%, i.e., 200 mbar) and overexpression of *gnd*, *edd*, and *eda* (pGED). Overexpression of only *gnd* (pG) or only *edd* and *eda* (pED) failed to establish growth (less than two doublings). Cultivation at ambient CO₂ also failed to achieve growth. Values in parentheses indicate doubling times. Curves represent the average of technical duplicates, which differ from each other by less than 5%. Growth experiments were repeated independently three times to ensure reproducibility. (C) Cultivation on ¹³CO₂ confirms operation of the GED shunt. On the left, a prediction of the labeling pattern of key amino acids is shown. The observed labeling fits the prediction and differs from the WT control cultivated under the same conditions. Labeling of amino acids in the WT strain stems from the natural occurrence of ¹³C as well as from reactions that exchange cellular carbon with CO₂, e.g., the glycine cleavage system and anaplerotic/cataplerotic cycling. Values represent averages of two independent cultures that differ from each other by less than 10%. Abbreviations: 3PG, 3-phosphoglycerate; ALA, Alanine; GAP, glyceraldehyde-3-phosphate; GLY, Glycine; HIS, Histidine; PYR, pyruvate; SER, Serine; VAL, Valine.

A $\Delta tktAB$ context requires additional metabolic adaptations to enable growth via the GED shunt

To check whether the operation of the GED shunt is robust, we decided to use another metabolic background to select for its activity. We deleted the genes encoding for both isozymes of transketolase ($\Delta tktAB$). This strain, in which the non-oxidative pentose phosphate pathway is effectively abolished, cannot grow when provided with a pentose as sole carbon source^{36, 40}. Furthermore, as E4P cannot be synthesized in this strain, small amounts of essential cellular components, the biosynthesis of which is E4P-dependent, need to be added to the media^{36, 40}: phenylalanine, tyrosine, tryptophan, shikimate, pyridoxine, 4-aminobenzoate, 4-hydroxybenzoate, and 2,3-dihydroxybenzoate (referred to as E4P-supplements, see Methods).

As with the Δrpe strain, we expected overexpression of Gnd, Edd, and Eda to enable growth on a pentose substrate such as xylose (supplemented with E4P-supplements) (Fig. 3A). However, we failed to obtain growth even at an elevated CO₂ concentration (less than two doublings, green lines in Fig. 3B). This is in line with previous findings that seemingly small differences in the design of metabolic growth selection schemes (e.g. the choice of deleted enzymes) can lead to substantially dissimilar metabolic behaviors^{36, 41}.

Following the failure to obtain GED shunt-dependent growth within the $\Delta tktAB$ context, we sought to harness adaptive evolution to provide us with information on further cellular adaptations required for the activity of the synthetic route. Towards this aim, we inoculated the $\Delta tktAB$ +pGED strain into multiple test-tubes with xylose and E4P-supplements and incubated them for an extended period of time at 37°C and 20% CO₂. After ~2 weeks, the culture in several of these test-tubes showed apparent growth. When the cells from the growing cultures were transferred to fresh selective medium, we observed immediate growth, indicating that genetic adaptation had occurred. Yet, only one of these cultures showed robust growth on the selective medium, whereas that of the others was less reproducible and highly sensitive to the exact conditions, preculture, and inoculation. An isolated single clone from this robust culture grew, under elevated CO₂ concentration, with a doubling time of 5.3 h (red lines in Fig. 3B).

We sequenced the genome of the mutated strain and identified a single mutation (compared to the parental strain): the mobile element IS5⁴² was inserted 104 base pairs upstream of the *pntAB* operon (Supplementary Data), which encodes for the membrane-bound transhydrogenase that plays a key role in supplying the cell with NADPH^{43, 44}. Insertion of the IS5 mobile element is well-known to occur in adaptive evolution experiments, increasing the expression levels of the downstream genes^{45, 46}. Indeed, we found that the transcription of *pntA* increased ~3-fold in the mutated strain compared to the non-mutated parent and WT strains (Fig. 3C). The contribution of this mutation to the activity of the GED shunt can be easily explained, as it increases the generation of NADPH required for the reductive carboxylation of Ru5P by Gnd (Fig. 1).

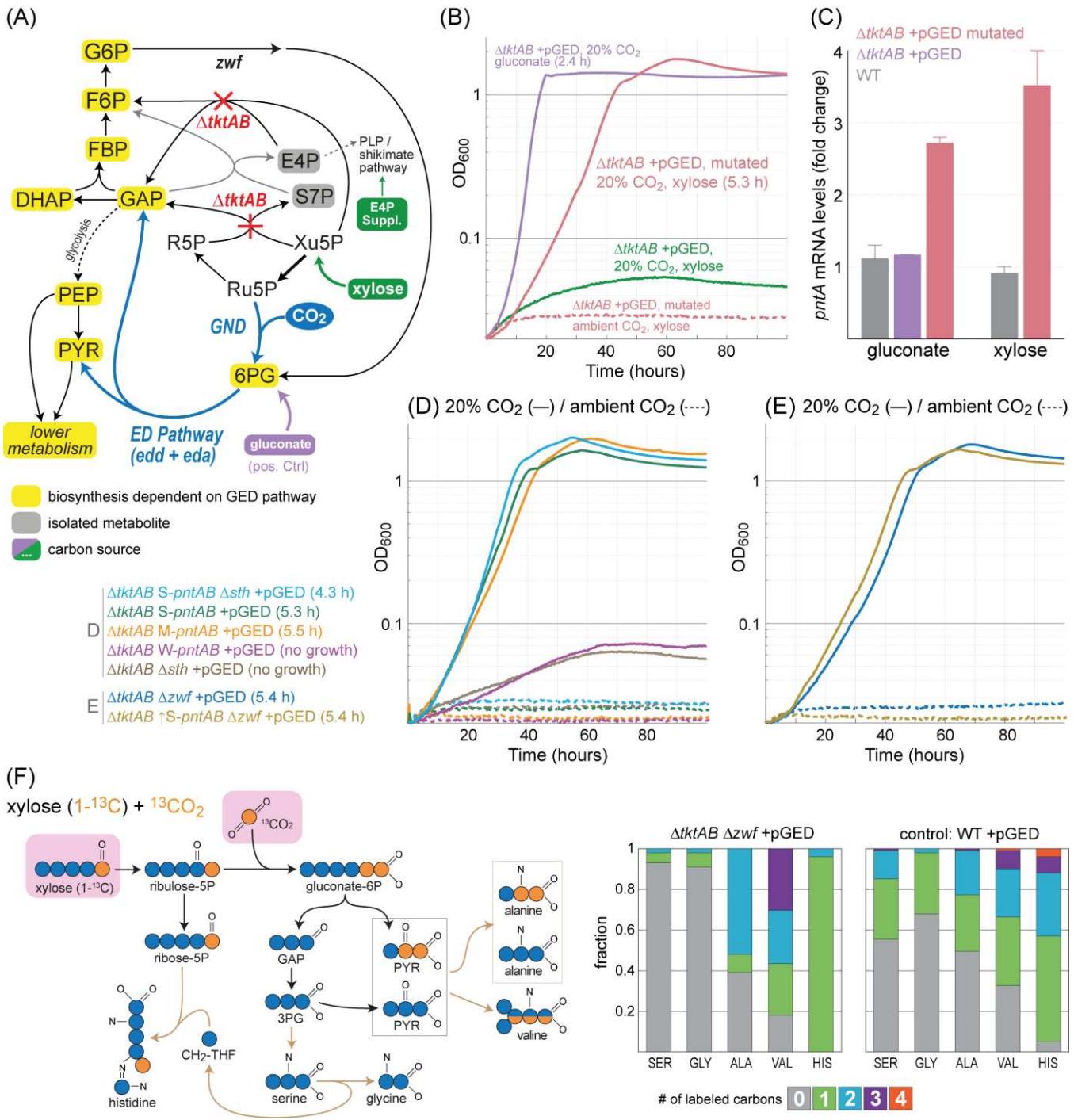


Figure 3. Activity of the GED shunt in a $\Delta tktAB$ strain. (A) Design of the $\Delta tktAB$ selection scheme. Xylose can be assimilated only via the GED shunt. E4P supplements are provided as the $\Delta tktAB$ strain cannot synthesize erythrose 4-phosphate. Growth on gluconate is not dependent on reductive carboxylation by Gnd and thus serves as a positive control. Reaction directionalities are shown as predicted by Flux Balance Analysis. (B) Growth on xylose upon overexpression of *gnd*, *edd*, and *eda* (pGED) was achieved only after mutation and was still dependent on elevated CO₂ concentration. Values in parentheses indicate doubling times. Curves represent the average of technical duplicates, which differ from each other by <5%. Growth experiments were repeated independently three times to ensure reproducibility. (C) Expression analysis by quantitative RT-PCR revealed that the transcript level of *pntA* increased ~3 fold in the mutated strain. Values correspond to the average of two independent experiments; error bars indicating the standard errors. Gluconate and xylose indicate carbon sources. (D) Genomic overexpression of *pntAB* using medium (M) or strong (S) promoter, but not weak (W) promoter, supported growth of a $\Delta tktAB$ pGED strain on xylose (legend to the left). (E) Deletion of glucose 6-phosphate dehydrogenase (Δzwf) supported growth of a $\Delta tktAB$ pGED strain on xylose (legend to the left). (F) ¹³C-labeling experiments confirm the operation of the GED shunt. Cells were cultivated with xylose (1-¹³C) and ¹³CO₂. Observed labeling fits the expected pattern and differs from that of a WT strain cultured under the same conditions. Results from additional labeling experiments are shown in Supplementary Fig. S3. Abbreviations: 3PG, 3-phospho-glycerate; ALA, Alanine; GAP, glyceraldehyde-3-phosphate; GLY, Glycine; HIS, Histidine; PYR, pyruvate; SER, Serine; VAL, Valine.

To confirm that increased *pntAB* expression indeed enables GED shunt-dependent growth, we replaced the native promoter of *pntAB* (within the unmutated strain) with three previously characterized constitutive promoters: weak (W-*pntAB*), medium (M-*pntAB*), and strong (S-*pntAB*), with relative strengths of 1:10:20, respectively⁴⁷. We found that while the weak promoter failed to support growth (less than two doublings, purple line in Fig. 3D), the medium and strong promoters supported growth with a similar doubling time to the mutated strain, ~5.4 h, at elevated CO₂ concentrations (orange and green lines in Fig. 3D). This indicates that sustaining sufficiently high expression of *pntAB* suffices to enable the activity of the GED shunt within a $\Delta tktAB$ metabolic context.

We wondered whether other metabolic manipulations that target NADPH homeostasis could also enable the growth of the $\Delta tktAB$ +pGED strain. We tested the deletion of the gene encoding for the soluble transhydrogenase (*asth*), as this enzyme is known to provide a strong sink for NADPH^{43, 44}. Yet, the $\Delta tktAB$ *asth* +pGED strain did not grow on xylose even at an elevated CO₂ concentration (less than two doublings, brown lines in Fig. 3D). Furthermore, deletion of *sth* in the $\Delta tktAB$ S-*pntAB* +pGED strain improved its growth only marginally (light blue line in Fig. 3D). Taken together, it seems that the soluble transhydrogenase has only a minor effect on NADPH availability within this metabolic context.

We hypothesized that alongside NADPH availability, competing sources of 6PG could play a key role in determining the feasibility of the GED shunt. Specifically, 6PG is natively produced by the oxidative pentose phosphate pathway, which thus provides a metabolic push against the reductive activity of Gnd. Moreover, the activity of glucose 6-phosphate 1-dehydrogenase (encoded by *zwf*), the first enzyme of the oxidative pentose phosphate pathway, has been reported to increase under conditions of high NADPH demand (i.e. upon depletion of cellular NADPH levels)^{48, 49}. Hence, we wondered whether deletion of *zwf* could remove a barrier for reductive carboxylation by Gnd and thus assist the activity of the GED shunt. We found that this is indeed the case, where the $\Delta tktAB$ *Δzwf* +pGED strain was able to grow under elevated CO₂ concentrations with a doubling time of 5.4 h (blue line in Fig. 3E). Deleting *zwf* in the $\Delta tktAB$ S-*pntAB* +pGED strain did not improve growth (dark yellow line in Fig. 3E), suggesting that the effects of NADPH and 6PG availability are not additive or that a different bottleneck is limiting growth.

To provide an unequivocal confirmation for the activity of the GED shunt within the $\Delta tktAB$ metabolic context we performed several ¹³C-labeling experiments (Fig. 3F and Supplementary Fig. S3). When the $\Delta tktAB$ *Δzwf* +pGED strain was fed with both ¹³CO₂ and 1-¹³C-xylose, we expected the GED shunt to produce unlabeled GAP and twice labeled pyruvate (Fig. 3F). Hence, serine and glycine, which are derived directly from GAP, should be unlabeled while about half of pyruvate (derived from GAP metabolism) should be unlabeled while the other half (generated directly by Eda activity) should be twice labeled. This should lead to half of the alanine being unlabeled and half twice labeled while the labeling of valine should roughly follow a 1:1:1:1 pattern (unlabeled: one labeled: twice labeled: thrice labeled). The observed labeling confirms these expected patterns (Fig. 3F). Histidine, the carbons of which originate from R5P and the β -carbon of serine, is labeled once as expected (Fig. 3F). The labeling patterns we observe upon feeding with unlabeled xylose

and $^{13}\text{CO}_2$ (Supplementary Fig. S3A) as well as upon feeding with 5- ^{13}C -xylose and unlabeled CO_2 (Supplementary Fig. S3B) further confirm that growth of the $\Delta tktAB \Delta zwf$ +pGED strain indeed takes place exclusively via the GED shunt.

Growth via the GED shunt in a strain that could support cyclic flux

While the Δrpe and $\Delta tktAB$ strains were useful selection platforms to test the activity of the GED shunt, they are, in a sense, ‘metabolic dead-ends’. This is because the activities of both ribulose-phosphate 3-epimerase (Rpe) and transketolase (Tkt) are essential for the operation of the full GED cycle, that is, for the regeneration of Ru5P from GAP. To address this problem, we aimed to construct a strain which keeps all necessary enzymes of the GED cycle intact, while still allowing to select for the activity of the GED shunt, i.e., preventing utilization of a pentose substrate as sole carbon source via the canonical pentose phosphate pathway. Such a strain would enable a smooth transition from GED shunt-dependent growth on a pentose substrate towards autotrophic growth via the GED cycle.

We therefore constructed a strain deleted in all enzymes that can metabolize fructose 6-phosphate, directly or indirectly, into a downstream glycolytic intermediate ($\Delta pfkAB \Delta fsaAB \Delta fruK$) or channel it into the oxidative pentose phosphate pathway (Δzwf). The latter gene deletion should also support the activity of the GED shunt, as was shown above within the $\Delta tktAB$ context. The strain containing all of these deletions, which we term ΔPZF , establishes a uni-directional block within the pentose phosphate pathway. That is, growth on a pentose substrate is not possible due to the accumulation of fructose 6-phosphate that prevents further conversion of pentose phosphates into GAP (Fig. 4A) ⁷. In contrast, flux in the opposite direction, as required for the GED cycle, can still occur, since fructose 1,6-bisphosphate can be dephosphorylated to fructose 6-phosphate which is then used to regenerate Ru5P.

To establish growth of the ΔPZF strain on xylose via the GED shunt, we overexpressed Gnd, Edd, and Eda. However, transforming the ΔPZF strain with pGED failed to support growth on xylose even at elevated CO_2 (less than two doublings, red line in Fig. 4B). Hence, we again harnessed natural selection and performed short-term evolution by incubating the strain in multiple test-tubes for an extended period of time in xylose minimal medium at 37°C and 20% CO_2 . Within 6-8 days, three parallel cultures started growing. Isolated clones from two of these three mutant cultures displayed a fairly high growth rate (doubling time of 8-10 hours, green and blue lines in Fig. 4B), while clones from the third culture showed considerably slower growth (doubling time > 50 h, orange line in Fig. 4B). We conducted ^{13}C -labeling experiments using the fastest-growing strain (ΔPZF +pGED mutant “C”) and found that it displayed a labeling pattern almost identical to that of the $\Delta tktAB \Delta zwf$ +pGED strain described above, thus confirming growth via the GED shunt (Fig. 4C and Supplementary Fig. S4). We sequenced the genomes of the mutant strains, compared them to the parental strain, and discovered several mutations (Supplementary Table 1). All isolated colonies from the two fast-growing cultures shared an identical mutation at the start of an L-leucyl-tRNA (*leuX*) and, in most colonies, *avtA*, encoding for valine-pyruvate aminotransferase, had mutated. While the exact

contribution of these mutations to the growth phenotype remains elusive and could be further investigated, the isolated strains provide a promising starting point for evolution of the full GED cycle.

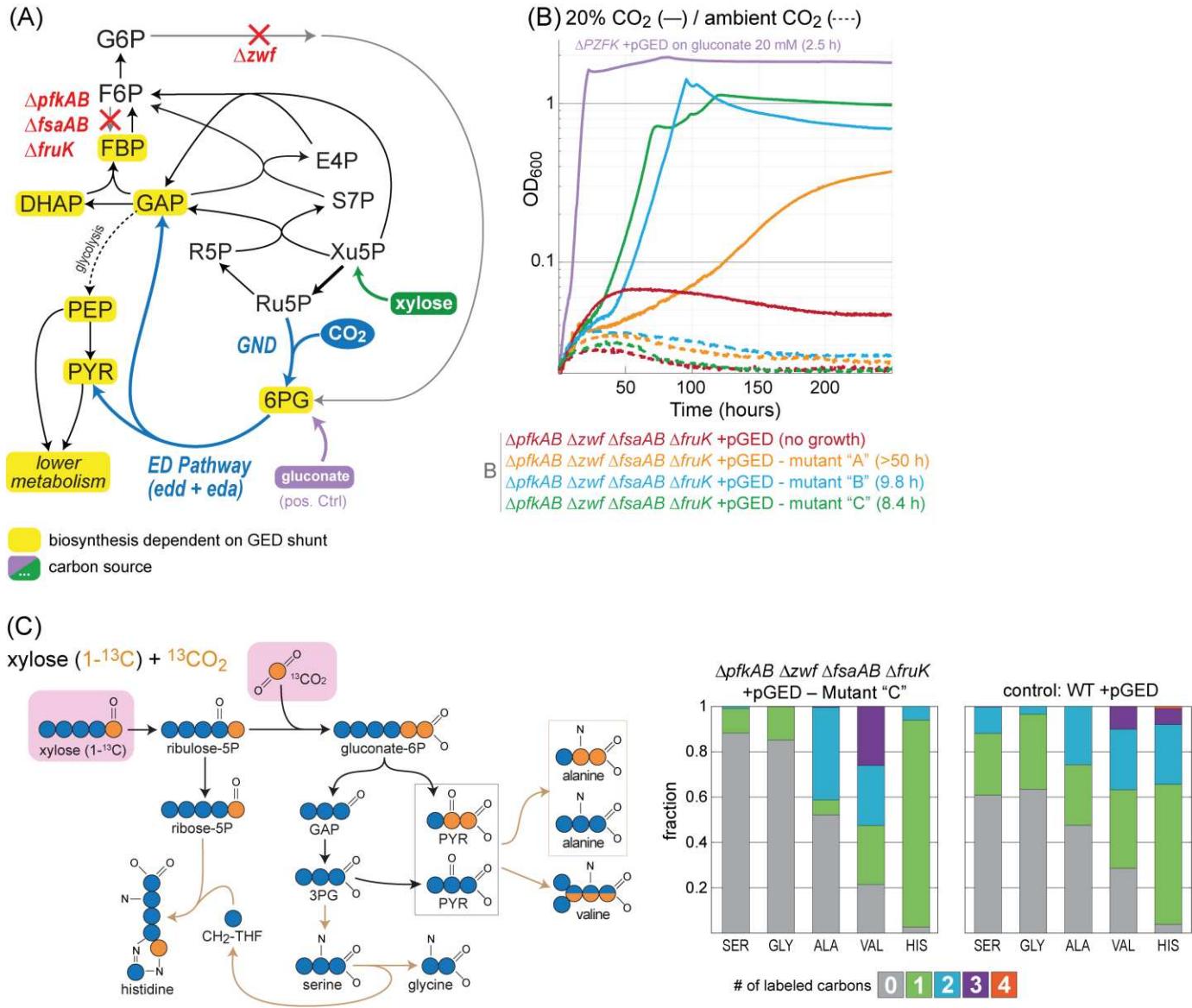


Figure 4. Activity of the GED shunt in a ΔPZF strain which enables a smooth transition into a GED cycle. (A) Design of the ΔPZF ($\Delta pfkAB$ Δzwf $\Delta fsaAB$ $\Delta fruK$) selection scheme. Xylose can be assimilated only via the activity of the GED shunt, where the biosynthesis of most biomass building blocks is dependent on the pathway (marked in yellow). Growth on gluconate (violet) is not dependent on reductive carboxylation via Gnd and thus serves as a positive control. Reaction directionalities are shown as predicted by Flux Balance Analysis. (B) Growth on xylose upon overexpression of *gnd*, *edd*, and *eda* (pGED) was achieved only after adaptive evolution and was dependent on elevated CO₂ concentration. Values in parentheses indicate doubling times. Curves represent the average of technical quadruplicates, which differ from each other by <5%. Growth experiments were repeated independently three times to ensure reproducibility. (C) ¹³C-labeling experiments confirm the operation of the GED shunt (mutant 'C'). Cells were cultivated with xylose (1-¹³C) and ¹³CO₂. Observed labeling fits the expected pattern and differs from that of a WT strain cultured under the same conditions. Results from additional labeling experiments are shown in Supplementary Fig. S4. Abbreviations: 3PG, 3-phosphoglycerate; ALA, Alanine; GAP, glyceraldehyde-3-phosphate; GLY, Glycine; HIS, Histidine; PYR, pyruvate; SER, Serine; VAL, Valine.

Discussion

Our computational analysis identified multiple carbon fixation pathways that are based solely on endogenous *E. coli* enzymes. A key factor for the success of this analysis was to ignore the rather arbitrary dichotomic classification of reactions as reversible or irreversible as suggested by metabolic models. Instead, we first identified potential pathways based on pure stoichiometric analysis, and then calculated the thermodynamic feasibility and driving force of each of the candidate routes. This enabled us to uncover potential carbon fixation pathways that were not identified before ¹². Indeed, the GED cycle itself was previously ignored as Gnd was considered to be an irreversible decarboxylating enzyme. As we have shown here, however, Gnd can catalyze the carboxylation reaction quite efficiently, with a k_{cat} almost double that of a typical plant Rubisco ³⁵. This finding is similar to a recent study that found that citrate synthase – which is usually thought to be irreversible – can catalyze citrate cleavage, thus enabling carbon fixation via a unique variant of the reductive TCA cycle ^{50, 51}. These examples indicate that we should revise our dogmatic interpretation of enzyme reversibility and instead adopt a more quantitative approach to understand reaction directionality.

Previous studies have suggested multiple synthetic carbon fixation pathways that could surpass the natural routes in terms of resource use efficiency, thermodynamics, and/or kinetics ^{52, 53}. The most advanced of these pathways is the CETCH cycle ⁵³ that combines segments of the 3-hydroxypropionate/4-hydroxybutyrate cycle ⁵⁴ and the ethylmalonyl-CoA pathway ⁵⁵. The CETCH cycle was assembled *in vitro* using enzymes from nine organisms and optimized in several rounds of enzyme engineering ^{53, 56}. However, the *in vivo* implementation of this synthetic pathway, as well as of other previously suggested routes, is highly challenging due to its complexity and requirement for considerable rewiring of central metabolic fluxes. The GED cycle provides a favorable alternative to these routes, as the fluxes it requires mostly correspond to native gluconeogenesis and the pentose phosphate pathway. Hence, the establishment of carbon fixation via the GED cycle might be less demanding and more likely to succeed.

We demonstrated the feasibility of carbon fixation via the GED cycle by establishing growth via the GED shunt – a linear pathway variant that requires the key pathway reactions to provide (almost) all biomass building blocks and cellular energy without regenerating the substrate Ru5P (Fig. 2A and 3A). In line with previous studies, we found that changing the metabolic context can have a dramatic effect on the activity of a metabolic module under selection: the GED shunt was able to directly support growth of a Δrpe strain but not of a $\Delta tktAB$ strain or a ΔPZF strain, even though in all of them the pentose phosphate pathway is disrupted. Despite this, a short-term adaptation was able to restore the growth of the latter two strains. Within the $\Delta tktAB$ strain, we demonstrated that either an increase in the supply of the substrate (e.g., NADPH, via *pntAB* overexpression) or a decrease in the availability of the product (e.g., 6PG, via *zwf* deletion) is sufficient to enable Gnd-dependent carboxylation and growth via the GED shunt.

The GED shunt might have biotechnological advantages on its own. By using Flux Balance Analysis, we found that rerouting the utilization of sugar substrates via the GED shunt is expected to increase the yield of various commercially interesting products, such as acetate, pyruvate, acetone, citrate, itaconate, and levulinic acid (Fig. 5, Supplementary Fig. S5, and Methods). This is attributed to the fact that the biosynthesis of these compounds from sugar feedstocks results in the production of excess reducing power, which the GED shunt can utilize to fix CO_2 and generate more product. We note that this assimilation of CO_2 also serves to compensate for the carbon released during the oxidation of pyruvate to acetyl-CoA, thus addressing a common challenge for the production of value-added chemicals derived from acetyl-CoA^{57, 58}. Further supply of reducing power by adding auxiliary substrates such as hydrogen or formate⁵⁹ can make the GED shunt advantageous over glycolysis for even more reduced products, such as ethanol, lactate, n-butanol, and isobutanol (Fig. 5, Supplementary Fig. S5, and Methods). While the RuBP shunt – a linear version of the RuBP cycle, which channels Ru5P via Rubisco – can also increase fermentative yield of some products⁶⁰⁻⁶², the GED shunt always outperforms it due to a lower ATP requirement (Fig. 5 and Supplementary Fig. S5).

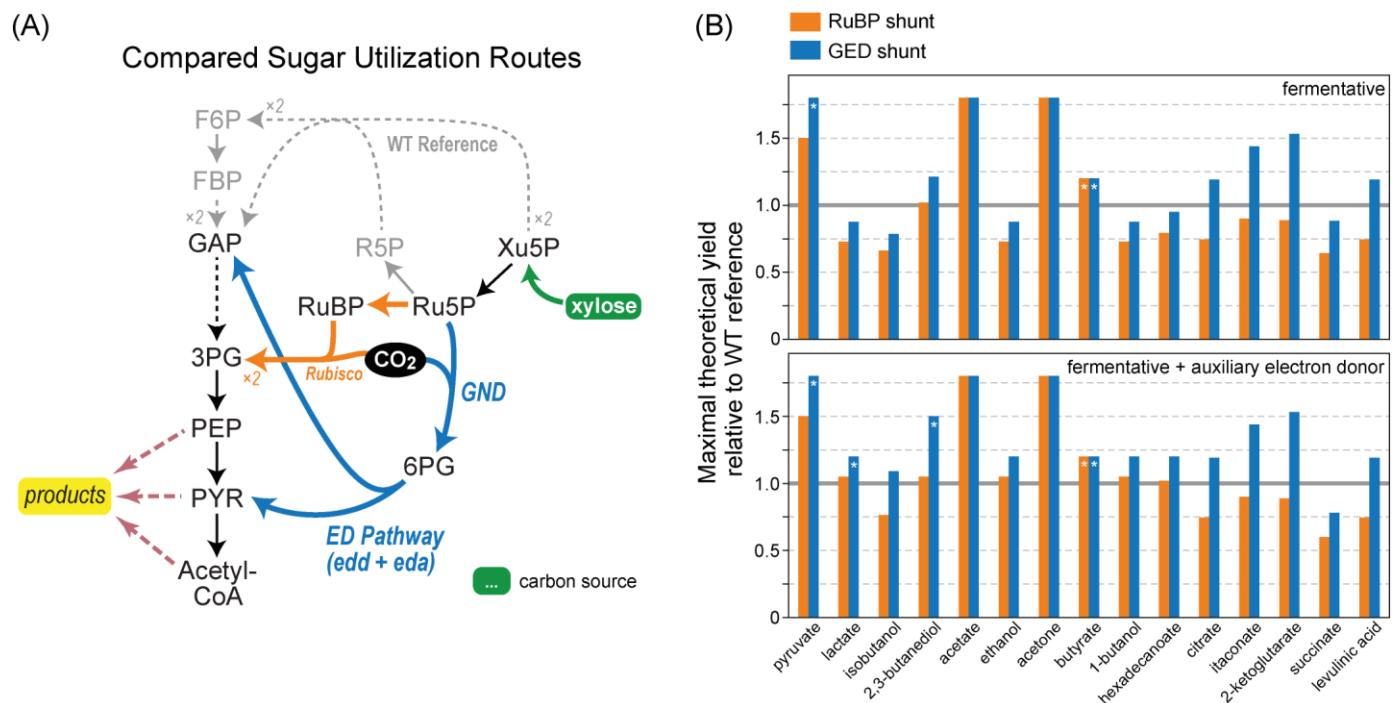


Figure 5. Rerouting sugar fermentation via the GED shunt can increase product yields. In this example, we chose xylose as the sugar substrate, due to its growing relevance as a renewable feedstock derived from lignocellulosic wastes. An additional analysis for glucose as the fermentation feedstock is shown in Supplementary Fig. S5. (A) An overview comparing xylose utilization routes: Canonical utilization (gray) via the pentose phosphate pathway and glycolysis; the GED shunt (blue); and a linear pathway based on carboxylation by Rubisco (“RuBP shunt”, orange). (B) Maximal theoretical yields of 15 fermentation products are shown relative to the WT reference, as calculated by Flux Balance Analysis. Presented values are normalized to the product yield from canonical sugar utilization (pentose phosphate pathway and glycolysis). Most products required the secretion of other organic compounds (e.g. acetate, formate) to achieve balancing of reducing equivalents or support ATP biosynthesis. Asterisks denote products that could be produced without byproducts.

A previous study has established the RuBP cycle in *E. coli*, demonstrating that this heterotrophic bacterium can be modified to grow autotrophically with CO₂ as a sole carbon source ^{6, 7}. However, to our knowledge, the current study is the first one in which the capacity for net carbon fixation was explored *in vivo* using only endogenous enzymes of a heterotrophic host, thus shedding light on the emergence of novel carbon fixation pathways. Importantly, the establishment of the RuBP cycle in *E. coli* required long-term adaptive evolution of the microbe under selective conditions, which modulated the partitioning of metabolic fluxes between carbon fixation and biosynthetic pathways ^{23, 63}. We expect that autotrophic growth via the GED cycle can be achieved in a similar manner. The ΔPZF strain serves as an ideal starting point for such a future evolution experiment, as its growth is dependent on the activity of the GED shunt while it still harbors all necessary enzymes to run the GED cycle. The gradual evolution of autotrophic growth via the GED cycle would be achieved via the additional expression of formate dehydrogenase as an energy-supplying module and long-term cultivation with limiting amounts of xylose and saturating amounts of CO₂ and formate ^{6, 7}.

As the GED cycle is composed of ubiquitous enzymes that are widespread throughout major bacterial phyla, it is tempting to speculate that this route naturally operates in yet unexplored microorganisms. Especially promising are α-proteobacteria, γ-proteobacteria, and actinobacteria, which contain many species that harbor all key enzymes of the pathway (Fig. 1D). Such bacteria could evolve autotrophic growth by recruiting the enzymes of the GED cycle if exposed to the appropriate selective conditions – for example, lack of organic carbon sources and availability of energy sources such as inorganic electron donors (e.g., hydrogen).

The GED cycle could be used to replace the RuBP cycle in plants, algae, and bacteria ⁶⁴, requiring relatively modest changes to the endogenous metabolic structure of carbon fixation. Replacing the RuBP cycle in chemolithotrophic bacteria of biotechnological significance, e.g., *Cupriavidus necator* ⁶⁵, would be relatively straightforward as cultivating these microorganisms on elevated CO₂ is a common practice, thus avoiding the rate limitation associated with the low affinity of Gnd to CO₂. Such an engineered microorganism could support higher product yields when cultivated under autotrophic conditions as pyruvate and acetyl-CoA, from which most value-added chemicals are derived, are produced more efficiently via the GED cycle than via the RuBP cycle (Supplementary Fig. S1). Furthermore, as the carboxylating activity of Gnd would be enhanced by the carbon concentrating mechanisms of algae ⁶⁶ and cyanobacteria ⁶⁷, engineering these organisms to use the GED cycle could be advantageous. However, to facilitate the establishment of the GED cycle in higher plants, the affinity of Gnd towards CO₂ would have to be improved, e.g., via the rational engineering of CO₂ binding sites ⁶⁸, as successfully demonstrated recently in a proof-of-principle study ⁶⁹. Alternatively, replacing *E. coli* Gnd with a variant that has a considerably higher k_{cat} (~ 100 s⁻¹) could compensate for the low affinity towards CO₂ (i.e., achieving k_{cat}/K_M at least as high as that of plant Rubisco). As some variants of similar reductive carboxylation enzymes – isocitrate dehydrogenase and the malic enzyme – incorporate CO₂ with k_{cat} surpassing 100 s⁻¹ ⁷⁰, identifying a Gnd variant supporting such a high carboxylation rate might be feasible. Engineering such an optimized GED cycle into crop plants could boost agricultural productivity, thus addressing one of our key societal challenges ⁶⁴.

Methods

Identifying carbon fixation cycles in *E. coli* using a constraint-based model

In order to find all possible carbon fixation cycles using *E. coli* endogenous enzymes, we used an approach similar to the one we have previously developed⁵². In this previous study, all reactions found in the KEGG database⁷¹ (denoted the universal stoichiometric matrix) were used to design potential CO₂ fixating pathways using a Mixed-Integer Linear Programming (MILP) approach. Here, we focused only on enzymes present in the most recent genome-scale metabolic reconstruction of *E. coli*: iML1515¹³. We further added thermodynamic constraints in order to exclude infeasible pathways and to rank the feasible ones based on their Max-min Driving Force (MDF)¹⁴. The source code and all input and output files required for repeating this analysis can be found on GitLab (<https://gitlab.com/elad.noor/path-designer/>) and are available under an open-source license (MIT).

We used the COBRApy package to identify carbon fixation pathways⁷². First, we removed all exchange and transport reactions and kept only the strictly cytoplasmic ones. Then, we added reactions that allow the free flow of electrons and energy (by regenerating ATP, NADH, and NADPH) as well as inorganic compounds (protons, water, oxygen, and ammonia). Finally, we defined an optimization problem, where the set of reactions in each pathway should overall convert 3 moles of CO₂ to one mole of pyruvate. The objective function of this optimization is a combination of the MDF¹⁴ and the minimum number of reactions in each pathway. The standard approach for multi-objective optimization is maximization of a linear combination of the two functions. Here, we maximized the MDF (in units of RT) minus the number of reactions. We note that changing the relative weight between these two objectives did not change our main result, namely that the GED pathway is Pareto-optimal. A more detailed list of changes to the model, and the formal description of the optimization problem can be found in the Supplementary Text.

We used the framework described above to iterate the space of possible solutions, i.e., all pathways that have a positive MDF. This requirement ensures that the pathway is thermodynamically feasible given our constraints, i.e. all of its reactions can operate simultaneously without violating the second law of thermodynamics. Covering this space exhaustively would require many days of computer time and is not useful since most of these solutions are unnecessarily complicated. The Pareto-optimal solutions are those that are not dominated by any other pathway in both objectives simultaneously (i.e. compared to any other pathways, they either have either a higher MDF or a lower number of reactions). We found only two such pathways: the GED cycle and a pathway which uses the reverse glycine cleavage system as its carboxylating mechanism (similar to the one shown in Fig. 1B). If we remove all the oxygen-sensitive reactions (specifically, PFL and OBTFL) then the GED cycle is the only Pareto-optimal pathway, i.e. it has the minimum number of steps and the highest MDF possible. For more details on the method, and for the list of all identified pathways, see the Supplementary Text.

Phylogenetic analysis

In order to assess how many bacterial genomes contain the genes necessary for GED, we used AnnoTree - a web tool for visualization of genome annotations in prokaryotes (<http://annotree.uwaterloo.ca/>)⁷³. AnnoTree generates a phylogenetic tree and highlights genomes that include all of the KEGG orthologies⁷¹ selected in the query. Since most annotations are homology based, and therefore not always precise, we restricted our search to the enzymes that are most indicative of the GED pathway and cannot be easily replaced by a metabolic bypass (e.g., transaldolase could be replaced by sedoheptulose 1,7-bisphosphate aldolase and phosphatase):

- K00033 – 6-phosphogluconate dehydrogenase (EC:1.1.1.44 1.1.1.343): the enzymatic keystone of the GED cycle.
- K01690 – 6-phosphogluconate dehydratase [EC:4.2.1.12]: indicative of the Entner–Doudoroff pathway.
- K00615 – transketolase [EC:2.2.1.1]: indicative of the pentose phosphate pathway.
- K00927 – phosphoglycerate kinase [EC:2.7.2.3]: indicative of gluconeogenesis (as glycolysis can operate irreversibly via a non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase).

We chose the family level as the tree resolution, since it provides a good balance between number of leaves and branching diversity. For the sake of readability of Fig. 1D, we applied the *auto collapse clades* option in iTOL with the setting BRL < 1.

Yield estimation via Flux Balance Analysis

Flux Balance Analysis was conducted in Python with COBRApy⁷². We used the most updated *E. coli* genome-scale metabolic network iML1515¹³ with several curations and changes: (i) transhydrogenase (THD2pp) translocates one proton instead of two⁷⁴; (ii) homoserine dehydrogenase (HSDy) produces homoserine from aspartate-semialdehyde irreversibly instead of reversibly⁷⁵; (iii) GLYCK (glycerate-3P producing glycerate kinase) and POR5 (pyruvate synthase) were removed from the model as their existence in *E. coli* is highly disputable; (iv) the exchange reactions of Fe2+, H2S, methionine and cysteine were removed to prevent respiration with inorganic Fe or S as electron acceptors in simulated anaerobic conditions; (v) the threonine cleavage routes were blocked by deleting threonine aldolase (THRA), threonine dehydrogenase (THRD), and glycine C-acetyltransferase (GLYAT); (vi) two unrealistic pathways for the conversion of pentoses to acetyl-CoA were removed by deleting reactions DRPA and PAI2T; (vii) the pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase (GLCDpp) was removed from the model; (viii) pyruvate formate lyase (PFL) and 2-oxobutanoate formate lyase (OBTFL) were deactivated only under aerobic conditions. We further removed the ATP maintenance reaction (ATPM) due to the fact that, rather than estimating growth rate, we used FBA to estimate the maximal yield. We used the model with these modifications as a ‘wild-type’ reference.

The Gnd reaction was changed to be reversible as part of the GED cycle/shunt; phosphoribulokinase (PRK) and Rubisco (RBPC) reactions were added to create the RuBP cycle/shunt. In order to use hydrogen as a proxy electron donor, hydrogen dehydrogenase reaction (H2DH, irreversible) was added to the model. The GED cycle and RuBP cycle were analyzed by assuming CO₂ as the sole (unconstrained) carbon source, with hydrogen import as a constrained source of electrons and energy. Given that the GAP dehydrogenase (GAPDH) of the RuBP cycle is canonically considered NADPH-dependent, the according reaction of the model was modified to utilize this co-factor (only for analysis of the cycles, not the shunts). We note that this removes a positive bias, which would have caused yields of the GED cycle to be estimated higher. To analyze yields of the GED shunt and RuBP shunt, these linear variants were created by blocking the reactions of phosphofructokinase (PFK), S7P-reacting phosphofructokinase (PFK_3), fructose 6-phosphate aldolase (F6PA), glucose 6-phosphate dehydrogenase (G6PDH2r), and fructose-bisphosphatase (FBP). Then, xylose or glucose were assumed as constrained carbon source together with unconstrained CO₂ (similar to ref. ¹²). The full code, including changes to the model and the new reactions of each production route can be found at https://gitlab.com/elad.noor/path-designer/-/tree/master/co2_fixation/FBA.

Strains and genomic modifications

All strains used in this study are listed in Table 2. Gene deletions and growth experiments were performed in strains derived from *E.coli* SIJ488 ⁷⁶, a strain derived from wild-type *E.coli* MG1655. The SIJ488 strain contains inducible genes for λ-Red recombineering (Red-recombinase and flippase) integrated into its genome to increase ease-of-use for multiple genomic modifications ⁷⁶. Gene deletion strains were either taken from previous studies (Table 2) or generated by P1 phage transduction as described before ⁷⁷. Strains from the Keio collection carrying single gene deletions with a kanamycin-resistance gene (KmR) as selective marker were used as donor strains ⁷⁸. Strains that had acquired the desired deletion were selected by plating on appropriate antibiotics (Kanamycin, Km) and confirmed by determining the size of the respective genomic locus via PCR (oligonucleotide sequences shown in Supplementary Table 2). To remove the selective marker, flippase was induced in a fresh culture grown to OD₆₀₀ ~ 0.2 by adding 50mM L-Rhamnose and cultivating for ~4h at 30°C. Loss of the antibiotic resistance was confirmed by identifying individual colonies that only grew on LB in absence of the respective antibiotic and by PCR of the genomic locus.

For genomic overexpression of the *pntAB* operon, its promoter region was edited using a method based on λ-Red recombineering ⁷⁷. We replaced the native *pntAB* promoter region (spanning 443bp upstream of the *pntA* start codon) by a weak (pgi#1), moderate (pgi#10), or strong (pgi#20) constitutive promoter ^{42, 78} and a medium-strength ribosome binding site (RBS “C”: AAGTTAAGAGGCAAGA ⁷⁹), downstream of a CmR-cassette for selection (for resulting sequence, see Supplementary Table 2). For this purpose, the CmR cassette was amplified from plasmid pKD3 ⁷⁷ with the primers CmR-1 and CmR-2 followed by overlap extension PCR to combine it with the promoter and RBS amplified from plasmid pGED with the primers

PromW-Fwd, PromM-Fwd or PromS-Fwd, respectively, and pntA-Prom2. The construct was inserted into a pJET1.2 cloning-vector (Thermo Scientific, Dreieich, Germany) and confirmed by Sanger sequencing. Linear dsDNA donors for λ -Red recombineering were generated by amplification with primers pntA-Prom1 and pntA-Prom2. DNA (400ng) was transformed into the desired strains by electroporation after fresh culturing to $OD_{600} \sim 0.3$ and induction of recombinase enzymes by addition of 15mM L-Arabinose for 45 minutes. Confirmation of the engineered promoter region, ancestral background deletions and removal of the selective marker were performed as described above for the P1 transduction method. The *pntAB* promoter locus was additionally verified by Sanger sequencing (LGC Genomics, Berlin, DE) after amplification with pntA-V1 and pntA-V2.

Table 2. List of strains and expression plasmids used in this study. KmR and CmR denote kanamycin or chloramphenicol resistance markers, respectively.

Name	Genotype	Source
Plasmid pGED	pZ-ASS-gnd-eda-edd-CmR	this study
Plasmid pG	pZ-ASS-gnd-CmR	this study
Plasmid pED	pZ-ASS-eda-edd-CmR	this study
DH5 α	F - endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169, hsdR17(rK - mK +), λ -	Lab collection.
MG1655	E.coli K-12 F-, λ -, rph-1	Lab collection.
SIJ488	MG1655 Tn7::para-exo-beta-gam; prha-FLP; xyISpm-lscel	⁷⁶
Δ rpe donor	BW25113 Δ rpe::KmR	Keio collection ⁷⁸
Δ rpe	SIJ488 Δ rpe	this study
Δ tktAB	SIJ488 Δ tktA Δ tktB	⁷⁹
Δ tktAB +pGED mutated	SIJ488 Δ tktA Δ tktB; pZ-ASS-gnd-eda-edd-CmR; Insertion of mobile element (IS5) 104bp upstream of pntA ORF	this study
Δ tktAB pntAB*	SIJ488 Δ tktA Δ tktB; pntAB-Prom::CmR-Prom"pgi#20"-rbs"C" (see methods)	this study
Δ ssthA donor	BW25113 Δ ssthA::KmR (synonym: udhA)	Keio collection ⁷⁸
Δ tktAB Δ ssth	SIJ488 Δ tktA Δ tktB Δ ssthA	this study
Δ tktAB Δ ssth W-pntAB	SIJ488 Δ tktA Δ tktB Δ ssthA; pntAB-Prom::CmR-Prom"pgi#1"-rbs"C" (see methods)	this study
Δ tktAB Δ ssth M-pntAB	SIJ488 Δ tktA Δ tktB Δ ssthA; pntAB-Prom::CmR-Prom"pgi#10"-rbs"C" (see methods)	this study
Δ tktAB Δ ssth S-pntAB	SIJ488 Δ tktA Δ tktB Δ ssthA; pntAB-Prom::CmR-Prom"pgi#20"-rbs"C" (see methods)	this study
Δ tktAB Δ zwf	SIJ488 Δ tktA Δ tktB Δ zwf	⁷⁹
Δ tktAB Δ zwf pntAB*	SIJ488 Δ tktA Δ tktB Δ zwf; pntAB-Prom::CmR-Prom"pgi#20"-rbs"C" (see methods)	this study
Δ pfkAB Δ zwf Δ fsaAB Δ fruK (Δ PZF)	SIJ488 Δ pfkA Δ pfkB Δ zwf Δ fsaA Δ fsaB Δ fruK::KmR	this study
Δ PZF +pGED – mutated strains	SIJ488 Δ pfkA Δ pfkB Δ zwf Δ fsaA Δ fsaB Δ fruK::KmR – various mutations – see Supplementary Table 1	this study

Construction of pGED, pG, and pED vectors

Cloning was carried out in *E. coli* DH5 α . The native *E. coli* genes encoding 6-phosphogluconate dehydrogenase (*gnd*, UniProt: P00350), KHG/KDPG aldolase (*eda*, Uniprot: P0A955) and phosphogluconate dehydratase (*edd*, UniProt: P0ADF6) were amplified from *E. coli* MG1655 genomic DNA with high-fidelity Phusion Polymerase (Thermo Scientific, Dreieich, Germany) using primers listed in Supplementary Table 2. Silent mutations were introduced to remove relevant restriction sites in *gnd* (C292T to remove a PstI site) and *eda* (G196A to remove a Pvul site and C202T to remove a PstI site). Assembly of synthetic operons and expression plasmids was performed as described before ^{47, 80}. In brief, genes were

first inserted individually into a “pNivC” vector⁸⁰ downstream of a ribosomal binding site (RBS “C”, AAGTTAAGAGGCAAGA). For synthetic operons, multiple genes were assembled in pNivC vectors using BioBrick restriction enzymes (Fast-Digest: BcuI, Xhol, Sall, NheI; Thermo Scientific, Dreieich, Germany). The generated operons were excised from the pNivC vector by restriction with EcoRI and NheI (Fast Digest, Thermo Scientific, Dreieich, Germany) and inserted into a pZ-ASS vector⁴⁷ (p15A medium-copy origin of replication, streptomycin resistance for expression under the control of the constitutive strong promoter “pgi #20⁸¹). The order of genes in the operons was *gnd*, *eda*, *edd* for pGED; and *eda*, *edd* for pED. Constructed vectors were confirmed by Sanger sequencing (LGC Genomics, Berlin, DE). The software Geneious 8 (Biomatters, New Zealand) was used for *in silico* cloning and sequence analysis.

Culture conditions & growth experiments

For routine culturing of *E. coli* strains, LB medium was used (5 g/l yeast extract, 10 g/l tryptone, 10 g/l NaCl). Antibiotics were added when appropriate at the following concentrations: Kanamycin 50 µg/ml; Chloramphenicol 30 µg/ml; Ampicillin 100 µg/ml; Streptomycin 100 µg/ml. Growth assays were performed in M9 minimal medium (47.8 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.6 mM NaCl, 18.7 mM NH₄Cl, 2 mM MgSO₄ and 100 µM CaCl₂), supplemented with trace elements (134 µM EDTA, 31 µM FeCl₃·6H₂O, 6.2 µM ZnCl₂, 0.76 µM CuCl₂·2H₂O, 0.42 µM CoCl₂·2H₂O, 1.62 µM H₃BO₃, 0.081 µM MnCl₂·4H₂O). Carbon sources were added as described in the text at a concentration of 20 mM. No antibiotics were used in growth experiments, except in precultures. When elevated CO₂ was required, cultures were grown in an orbital shaker set to maintain 37°C and an atmosphere of 20% CO₂ mixed with air. Growth on strain deleted in *tktAB* required further supplementation of “E4P Supplements”^{36, 40}: 1 mM shikimic acid, 1 µM pyridoxine, 250 µM tyrosine, 500 µM phenylalanine, 200 µM tryptophan, 6 µM 4-aminobenzoic acid, 6 µM 4-hydroxybenzoic acid and 50 µM 2,3-dihydroxybenzoic acid.

Precultures for growth experiments were generally grown in M9 medium with 20 mM gluconate as carbon source (“relaxing conditions”). Antibiotics were added to the precultures if appropriate but omitted for growth experiments. Cells from the preculture were washed three times in M9 medium without carbon source and inoculated to a starting OD₆₀₀ of 0.02 into M9 media with the final carbon sources as detailed in the text. 96-well plates (Nunclon Delta Surface, Thermo Scientific, Dreieich, Germany) were filled with 150 µl culture and covered with 50 µl mineral oil (Merck, Darmstadt, Germany) to avoid evaporation while allowing gas exchange. Aerobic growth was monitored in technical duplicates or triplicates at 37°C in a BioTek Epoch 2 Microplate Spectrophotometer (BioTek, Bad Friedrichshall, Germany) by absorbance measurements (600nm) of each well every ~10 minutes with intermittent orbital and linear shaking. Blank measurements were subtracted and OD₆₀₀ measurements were normalized to “cuvette OD₆₀₀” values with the following equation previously established empirically for the instruments: OD_{cuvette} = OD_{plate}/0.23. When elevated CO₂ was required, the atmosphere was maintained at 20% CO₂ mixed with 80% air by placing the plate reader inside a Kuhner ISF1-X incubator shaker (Kuhner, Birsfelden, Switzerland).

Isolation and sequencing of a $\Delta tktAB$ +pGED mutant capable of growing via the GED shunt

Tube cultures (batch growth) of 4 mL selective minimal medium (M9 + E4P supplements + 20mM xylose) were inoculated to an OD_{600} of 0.05 ($\sim 1.5 \times 10^7$ cells) and monitored during prolonged incubation at 37° and 20% CO_2 (up to 3 weeks). Several cultures reached OD_{600} values above 1.0 after ~ 2 weeks. Single colonies were isolated from these cultures by dilution streak from liquid cultures onto LB medium with chloramphenicol (to maintain the pGED plasmid). Individual clones were then re-assayed for immediate growth (observable OD_{600} increase within 48h) on selective liquid minimal medium (M9+E4Ps+Xylose+20% CO_2).

Genomic DNA was extracted using the GeneJET genomic DNA purification Kit (Thermo Scientific, Dreieich, Germany) from 2×10^9 cells of an overnight culture in LB medium supplied with chloramphenicol (to maintain the pGED plasmid). Construction of PCR-free libraries for single-nucleotide variant detection and generation of 150 bp paired-end reads on an Illumina HiSeq 3000 platform were performed by the Max-Planck Genome Centre (Cologne, DE). Reads were mapped to the reference genome of *E.coli* MG1655 ⁸² (GenBank accession no. U00096.3) using the software Geneious 8 (Biomatters, New Zealand). Using algorithms supplied by the software package, we identified single-nucleotide variants (with $>50\%$ prevalence in all mapped reads) and searched for regions with coverage deviating more than 2 standard deviations from the global median coverage. Confirmation of the *pntA* promoter locus in the Δtkt + pGED mutant was performed by Sanger Sequencing of a PCR product from amplification of the respective locus with high-fidelity Phusion Polymerase (Thermo Scientific, Dreieich, Germany). Sanger sequencing was performed by LGC Genomics (Berlin, DE).

Expression analysis by reverse transcriptase quantitative PCR (RT-qPCR)

In order to determine mRNA levels, total RNA was extracted from growing cells in exponential phase (OD_{600} 0.5-0.6) on M9 minimal medium with 20mM carbon source (gluconate or xylose, and E4P supplements) in presence of 20% CO_2 . Total RNA was purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany) as instructed by the manufacturer. In brief, $\sim 5 \times 10^8$ cells (1 ml of OD_{600} 0.5) were mixed with 2 volumes of RNAProtect Bacteria Reagent (Qiagen, Hilden, Germany) and pelleted, followed by enzymatic lysis, on-column removal of genomic DNA with RNase-free DNase (Qiagen, Hilden, Germany) and spin-column-based purification of RNA. Integrity and concentration of the isolated RNA were determined by NanoDrop and gel electrophoresis. Reverse transcription to synthesize cDNA was performed on 1 μ g RNA with the qScript cDNA Synthesis Kit (QuantaBio, Beverly, MA USA). Quantitative real-time PCR was performed in technical triplicates using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Dreieich, Germany). An input corresponding to 3.125 ng total RNA was used per reaction. Non-specific amplification products were excluded by melting curve analysis. The gene encoding 16S rRNA (*rrsA*) was chosen as a well-established reference transcript for expression normalization ⁸³. Two alternative primer pairs for amplification of *pntA* were tested and the primer pair showing highest specificity and amplification efficiency

was chosen (Supplementary Table 2). Negative control assays with direct input of RNA (without previous reverse transcription) confirmed that residual genomic DNA contributed to less than 5% of the signal (ΔCt between +RT/-RT samples >4 for all). Differences in expression levels were calculated according to the $2^{-\Delta Ct}$ method^{84, 85}. Reported data represents the $2^{-\Delta Ct}$ value that was calculated for each sample individually relative to the first biological wild-type replicate.

Stationary ^{13}C isotopic labelling of proteinogenic amino acids

For isotope tracing, cells were cultured in 3 mL M9 medium supplied with the labeled/unlabeled carbon sources described in the main text. For $^{13}\text{CO}_2$ labeling, the experiment was performed in a 10 L desiccator that was first purged twice of the contained ambient air with a vacuum pump and refilled with an atmosphere of 80% air and 20% $^{13}\text{CO}_2$ (Cambridge Isotope Laboratories Inc., MA USA). All cultures were inoculated to an OD_{600} 0.02 and grown at 37°C until stationary phase. Then, $\sim 10^9$ cells (1 mL of culture with $OD_{600} = 1$) were pelleted, washed once with ddH₂O and hydrolyzed in 1 mL hydrochloric acid (6M) at 95°C for a duration of 24h. Subsequently, the acid was evaporated by heating at 95°C and the hydrolyzed biomass was re-suspended in ddH₂O.

Hydrolyzed amino acids were separated using ultra-performance liquid chromatography (Acquity, Waters, Milford, MA, USA) using a C18-reversed-phase column (Waters, Eschborn, Germany) as previously described⁸⁶. Mass spectra were acquired using an Exactive mass spectrometer (Thermo Scientific, Dreieich, Germany). Data analysis was performed using Xcalibur (Thermo Scientific, Dreieich, Germany). Prior to analysis, amino-acid standards (Merck, Darmstadt, Germany) were analyzed under the same conditions in order to determine typical retention times.

Purification and kinetic characterization of *E.coli* Gnd

Proteins were expressed from *E.coli* BL21-AI strains (Invitrogen) carrying appropriate plasmids for expression of *E.coli* Gnd or *E.coli* RpiA (ribose-5-phosphate isomerase) which were taken from the ASKA collection⁸⁷. Expression was induced overnight at 30°C in TB medium (24 g/L yeast extract, 12 g/L tryptone, 4 mL/L glycerol, 17 mM KH₂PO₄, 72 mM K₂HPO₄) by addition of 0.5 mM IPTG and 2.5 mM arabinose upon reaching an OD_{600} of 1. Cells were lysed in 500 mM NaCl, 20 mM Tris-HCl pH 6.9 by sonication. After centrifugation (1h at 30,000 g), proteins were purified on an ÄKTA start system (GE Healthcare) by HisTrap Purification (GE Healthcare, Illinois, USA) as instructed by the manufacturer, using a wash step with 18% Buffer B (500mM NaCl, 20mM TrisHCl pH 6.9, 500mM imidazole). Desalting was performed in 100mM NaCl, 20 mM Tris-HCl pH 6.9 and enzymes were stored at -20°C in desalting buffer with 20% glycerol.

Kinetic assays were carried out on a Carry-60 UV-vis spectrometer (Agilent, Ratingen, Germany) at 30°C using a 1 mm quartz cuvette (Hellma). All assays were carried out in 100 mM TrisHCl buffer at pH 8 following consumption or production, respectively, of NADPH at 340 nm ($\epsilon_{340\text{ nm}} = 6.2\text{ cm}^{-1}\text{ mM}^{-1}$). The reductive carboxylation parameters for Gnd were determined with assays containing 2.4 mM NADPH,

16 mM ribose 5-phosphate, 1.5 M KHCO₃ and 140 μ M RpiA (with varying concentrations of the substrate under investigation). The assays were preincubated for 2 min and started with the addition of 750 nM of freshly diluted Gnd. Carbonic anhydrase was used to confirm that CO₂ equilibration was not rate-limiting in these assays. Isomerization of ribose 5-phosphate to ribulose 5-phosphate was confirmed not to be rate-limiting. The concentration of ribulose 5-phosphate was calculated from the equilibrium constant of the isomerization reaction: $K_{eq} = 0.458$ (eQuilibrator ²⁴). The kinetic parameters of the oxidative decarboxylation were determined with assays containing either 800 μ M NADP⁺ (for 6-phosphogluconate parameters) or 200 μ M 6-phosphogluconate (for NADP⁺ parameters). Assays were started with the addition of 7.5 nM Gnd. All points were measured in triplicates and each Michaelis-Menten curve was determined using at least 15 measurements.

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Author Contributions

S.N.L. and A.B.-E. conceived the study.

A.S., B.D., S.N.L., and A.B.-E. designed the experiments.

A.S., B.D., P.W., and S.N.L. performed the *in vivo* experiments.

E.N. and H.H. performed the *in silico* experiments.

B.V., and T.E. performed the *in vitro* experiments.

A.S., B.D., S.N.L., and A.B.-E. analyzed the results.

A.S., B.D., E.N., S.N.L., and A.B.-E. wrote the manuscript with contributions from all authors.

Competing Financial Interests

The authors declare no competing interests.

Data Availability

Accession codes for protein sequences and structures are shown in the text. The data that support the findings of this study are available from the corresponding authors upon request.

Code Availability

The code used in this study can be found at <https://gitlab.com/elad.noor/path-designer/>.

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