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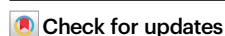
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Reprogramming energy system for growth and bioproduction from CO₂ and methanol in *Escherichia coli*

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Microbial CO₂ assimilation provides a strategy for sustainable biomanufacturing. However, CO₂ reduction requires substantial energy input, while native energy systems are tightly regulated and extensively consumed by endogenous metabolism, limiting assimilation efficiency. Here, we design and construct an orthogonal energy system in *Escherichia coli*, achieving an intracellular NUDH concentration exceeding 7.0 mM and an NUDH/NUD⁺ ratio above 55. Subsequently, we design a CO₂-formate-acetyl-CoA-pyruvate-malate pathway and reprogram key enzymes for NUDH-dependent operation. Integration of the OES-driven CAM* pathway with a rationally rewired native electron transport chain supports growth of the engineered *E. coli* on CO₂ and methanol, with a doubling time of 8.6 ± 0.3 h and a maximum OD₆₀₀ of 37.5 ± 5.8 . Moreover, the platform extends CO₂ assimilation to biosynthesis of multiple chemicals from distinct CAM* pathway nodes, including mevalonate, lactate, 2,3-butanediol, and succinate. Together, these results establish a framework for reprogramming microbial energy metabolism and expanding one-carbon biotransformation.

CO₂ is a primary greenhouse gas but also serves as the most abundant and renewable carbon resource^{1,2}. Therefore, developing sustainable technologies to convert CO₂ into value-added chemicals is crucial for the transition to a carbon-neutral economy^{3,4}. Currently, three main approaches are used for CO₂ resource utilization: chemical, physical, and biological methods^{5,6}. Among these, microbial fixation provides a green and sustainable pathway for chemical production. Heterotrophic microorganisms, particularly *Escherichia coli*, have become prominent industrial hosts due to their genetic tractability, well-characterized physiology, and extensive genetic tools^{7–9}. Although multiple strategies have been developed to enable CO₂ utilization in *E. coli*, including the reconstruction of natural carbon-fixation pathways, the design of artificial CO₂-fixation routes, adaptive laboratory

evolution, and the introduction of exogenous energy-supply modules, achieving high-efficiency carbon fixation that supports autonomous growth and bio-production remains a significant challenge. The performance of these engineered strains is primarily limited by two major bottlenecks: the substantial energy required to reduce highly oxidized CO₂ and the reliance on complex pathways to convert CO₂ into central metabolites, which imposes an additional metabolic burden on the host.

The primary barrier to efficient CO₂ fixation in heterotrophic hosts is the limited energy supply capability of native energy carriers, specifically ATP and NADH. To address this challenge, various strategies have been explored, including the co-metabolism of substrates (e.g., formate) and the introduction of exogenous

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energy modules, such as light-harvesting semiconductor biohybrids^{10,11}. For example, formate can be oxidized by many heterotrophic cells to generate NADH to drive CO₂ fixation¹². Recent studies have also demonstrated that the synthesis of cadmium sulfide nanoparticles and the anchoring of proteorhodopsin can enhance NADH and ATP supply¹³. However, these strategies face inherent limitations. Formate oxidation, for example, often outpaces fixation, leading to net CO₂ emission. Additionally, the efficacy of exogenous energy systems is constrained by the plasticity of the host's native cellular network. Notably, the diffuse distribution and tight regulation of native energy carriers across the metabolic network hinder the accumulation of high cofactor concentrations and the favorable ratios needed for efficient CO₂ utilization^{14,15}. Furthermore, excessive concentrations of these energy molecules could disrupt intracellular metabolic balance. To overcome these challenges, employing an orthogonal energy system (OES) offers significant advantages for engineering CO₂ fixation in non-native hosts. Such a system can minimize interference from the endogenous metabolic network and prevent the depletion of orthogonal energy carriers by native metabolic pathways¹⁴. This independence allows for precise tunability, facilitating the accumulation of reducing power to maximize the driving force for targeted CO₂ utilization pathways while minimizing disruption to overall cellular homeostasis¹⁶.

On the other hand, although natural CO₂ fixation pathways, including the Calvin-Benson-Bassham (CBB) cycle¹⁷, Wood-Ljungdahl (WL) pathway¹⁸, 3-hydroxypropionate (3HP) cycle¹⁹, and 3-hydroxypropionate-4-hydroxybutyrate (3HP-4HB) cycle¹⁹ have been introduced into heterologous hosts, their application is constrained by complex catalytic steps, low fixation efficiency, and the difficulty of expressing bulky multi-enzyme complexes. To address these limitations, artificial pathways have been designed as alternatives for CO₂ fixation, including the reductive tricarboxylic acid branch/4-hydroxybutyryl-CoA/ethylmalonyl-CoA/acetyl-CoA cycle²⁰, the crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA cycle²¹, and the artificial starch anabolic pathway²². However, their in vivo implementation has been restricted by a fundamental conflict: efficient microbial growth requires rapid assimilation of the fixed carbon into central metabolism. Because the native cellular energy environment provides limited driving force, synthetic pathways have often been forced to adopt extended cascades that consume multiple energy molecules for substrate activation to shift equilibria forward^{23,24}. These redundant steps, although more thermodynamically favorable under native conditions, have imposed substantial metabolic burden and then slowed pathway flux²⁵. Accordingly, the high driving force enabled by an orthogonal energy supply is expected to support a paradigm shift toward minimalist pathway design, in which complex activation bottlenecks are bypassed and CO₂ fixation is coupled to central metabolic hubs using minimal enzymatic steps.

To overcome limitations in native energy supply, we design and construct an OES in *E. coli* using methanol as the electron donor and achieve an intracellular NUDH concentration exceeding 7.0 mM and an NUDH/NUD⁺ ratio above 55. We then reprogram key enzymes in the CO₂-formate-acetyl-CoA-pyruvate-malate (CAM) pathway for NUDH-dependent catalysis, establishing the CAM* pathway for CO₂ assimilation. Integration of the OES-driven CAM* pathway with a rationally rewired native electron transport chain (ETC) supports growth of the engineered *E. coli* on CO₂ and methanol and the production of multiple chemicals, including mevalonate (4.05 ± 0.21 g/L), lactate (4.12 ± 0.26 g/L), 2,3-butanediol (3.15 ± 0.18 g/L), and succinate (3.35 ± 0.27 g/L). Together, these results establish a platform that decouples CO₂ fixation from native redox constraints and supports one-carbon biomanufacturing.

Results

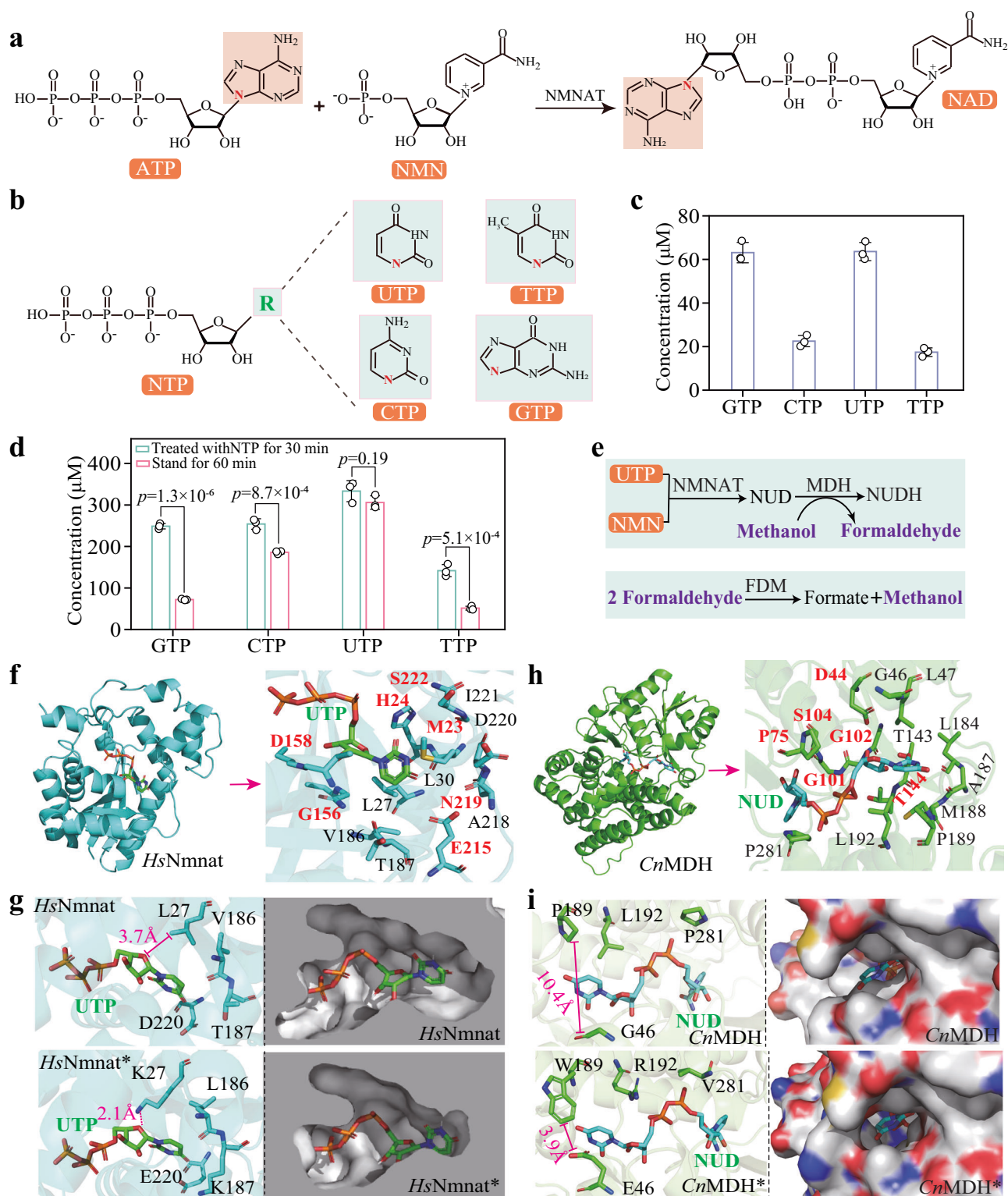
Design and construction of an orthogonal energy system (OES) in *E. coli*

To overcome limitations in endogenous energy supply, an orthogonal energy carrier must first be established that remains stable intracellularly while minimizing crosstalk with native metabolism. Nicotinamide mononucleotide adenyltransferase (NMNAT) has the potential to utilize non-ATP nucleoside triphosphates (NTPs), including GTP, UTP, CTP, and TTP, together with NMN to generate orthogonal energy molecules (NXDs), thereby providing a route for OES construction²⁶ (Fig. 1a, b). Among the tested NTPs, UTP showed both high intracellular availability and superior stability. Its basal intracellular concentration reached 63.6 ± 4.2 μM (Fig. 1c), and after intracellular loading, UTP decreased by only 8.2%, whereas GTP, CTP, and TTP showed much higher degradation rates of 71, 27, and 64%, respectively (Fig. 1d). Therefore, UTP was selected to design and construct the nicotinamide uracil dinucleotide (NUD/NUDH) system (Fig. 1e). In this system, UTP and NMN were condensed by NMNAT to form NUD, which was subsequently reduced to NUDH by methanol dehydrogenase (MDH) using methanol as the electron donor, with donor recycling mediated by formaldehyde dismutase (FDM) (Fig. 1e).

Guided by phylogenetic-based selection, twenty-seven NMNAT candidates were selected, sampling from distinct evolutionary clades across 106 bacterial families, and human-NMNAT (*HsNmnat*; PDB: 1KQN) exhibited the highest activity toward UTP but remained strongly biased toward its native substrate ATP (Supplementary Fig. 1). Apparent enzyme kinetic analysis performed at a fixed NMN concentration (2.0 mM) showed that *HsNmnat* displayed a $K_{M,app}^{ATP}$ of 56.1 μM (app indicates apparent kinetic parameters, 95% CI, 50.5 to 62.1 μM) and a $k_{cat,app}^{ATP}$ of 38.5 s⁻¹ (95% CI, 37.5 to 39.6 s⁻¹). By comparison, UTP utilization was inefficient, with a $K_{M,app}^{UTP}$ of 2.33 mM (95% CI, 2.04 to 2.66 mM) and $k_{cat,app}^{UTP}$ of 0.46 s⁻¹ (95% CI, 0.44 to 0.47 s⁻¹).

Accordingly, the apparent UTP preference, defined as $\frac{(k_{cat}/K_M)_{app}^{UTP}}{(k_{cat}/K_M)_{app}^{ATP}}$ (1), was only 2.85 × 10⁻⁴, indicating that *HsNmnat* remained highly selective for ATP despite its detectable UTP-dependent activity (Supplementary Fig. 2). Guided by the structural disparity between the uracil base of UTP and the adenine of ATP, non-conserved residues (ConSurf server: https://consurf.tau.ac.il/consurf_index.php, ConSurf score <9) within 6 Å of the uracil moiety were targeted to reprogram UTP preference, including L27, L30, V186, T187, A218, I221, and D220 (Fig. 1f and Supplementary Figs. 3 and 4). Initial screening identified L27K, V186L, and V186I as beneficial mutants, and to further broaden the sequence space, iterative site-saturation mutagenesis was extended to T187 and D220. After three rounds of optimization, the quadruple mutant *HsNmnat** (L27K/V186L/T187K/D220E) was obtained. Apparent kinetic analysis showed that $(k_{cat}/K_M)_{app}^{UTP}$ increased from 196 M⁻¹ s⁻¹ (95% CI, 177 to 218 M⁻¹ s⁻¹) to 4.03 × 10⁵ M⁻¹ s⁻¹ (95% CI, 3.72 × 10⁵ to 4.36 × 10⁵ M⁻¹ s⁻¹), and the UTP preference value increased to 3.57 × 10² (Supplementary Fig. 5). Structural analysis suggested that L27K introduced a hydrogen bond with UTP, while the combined mutations reshaped the catalytic cavity to improve steric complementarity with the uracil-containing substrate (Fig. 1g). These results established *HsNmnat** as an efficient and selective NUD synthase.

To enable sustained NUDH regeneration, methanol was selected as the electron donor because it is a green, abundant, and highly reduced feedstock. Accordingly, the engineered methanol dehydrogenase CT4-1 from *Cupriavidus necator* N-1 (*CnMDH*) was employed, as it has been shown to exhibit high substrate affinity and enable robust methanol utilization in *E. coli*²⁷ (Fig. 1h). However, apparent enzyme kinetic analysis showed that *CnMDH* remained strongly biased toward NAD, with a $(k_{cat}/K_M)_{app}^{NAD}$ of 244 M⁻¹ s⁻¹ (95% CI,



222 to 268 $\text{M}^{-1}\text{s}^{-1}$) compared with a $(k_{\text{cat}}/K_{\text{M}})_{\text{app}}^{\text{NAD}}$ of 23.3 $\text{M}^{-1}\text{s}^{-1}$ (95% CI, 20.9 to 26.1 $\text{M}^{-1}\text{s}^{-1}$). The substrate preference for NUD, defined as $\frac{(k_{\text{cat}}/K_{\text{M}})_{\text{app}}^{\text{NAD}}}{(k_{\text{cat}}/K_{\text{M}})_{\text{app}}^{\text{NAD}}}$ (2), reached only 0.09, motivating further engineering to improve NUD utilization (Supplementary Fig. 6). Structural analysis indicated that the enlarged binding pocket (10.4 Å) was poorly suited to accommodate the NUD^+ head group (Fig. 1i). To reshape the binding pocket, fifteen key residues within a 6 Å radius of the NUD moiety were

identified (Fig. 1h). Based on ConSurf server analysis, six highly conserved residues were excluded to minimize structural disruption (Fig. 1h and Supplementary Fig. 7). Consequently, nine variable residues (G46, L47, T143, L184, A187, M188, P189, L192, and P281) were selected for site-saturation mutagenesis (Fig. 1h and Supplementary Fig. 7). Then, a formaldehyde-responsive biosensor that linked intracellular formaldehyde formation to GFP fluorescence ($\text{GFP}/\text{OD}_{600}$) was used to select beneficial mutants G46E and P189W (Supplementary Fig. 7). Subsequent iterative mutagenesis and refinement of residues

Fig. 1 | Design and directed evolution of the orthogonal NUD/NUDH system. **a** Structures and natural enzymatic routes involved in native NAD(H) biosynthesis. **b** Chemical structures of various endogenous nucleoside triphosphates (NTPs), including uridine triphosphate (UTP), thymidine triphosphate (TTP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP). R represents a substitutable group. **c** Intracellular concentrations of the four representative NTPs in wild-type *E. coli*. Strains were first inoculated into LB medium and cultured at 37 °C for 12 h. **d** Determination of intracellular NTP concentrations and stability assessment. *E. coli* cells grown in LB medium for 6 h, and intracellular absolute concentrations were measured after cells permeabilized with 1 g/L Tween 80 were loaded with exogenous NTPs for 30 min (treated with 5 mM NTP), and after a subsequent 60-min static. **e** Schematic of the engineered NUDH biosynthetic module. UTP and NMN are condensed by NMNAT to generate NUD, which is subsequently reduced to NUDH by methanol dehydrogenase (MDH) using methanol as the electron donor. In this reaction, methanol is oxidized to formaldehyde while NUD⁺ is reduced to NUDH. Formaldehyde dismutase (FDM) catalyzes the disproportionation of

formaldehyde, converting two molecules of formaldehyde into formate and methanol. **f** Structural representation of the wild-type *HsNmnat* UTP-binding pocket. Fourteen key residues within a 6 Å radius of the UTP moiety are identified. These comprise seven variable residues and seven highly conserved residues shown in red. The conserved residues were excluded from the subsequent mutagenesis library. **g** Structural comparison of the UTP-binding pockets and cross-sectional surface views between wild-type *HsNmnat* and *HsNmnat*^{*}. **h** Structural representation of the wild-type *CnMDH* NUD-binding pocket. Fifteen key residues within a 6 Å radius of the NUD moiety are identified. These comprise nine variable residues and six highly conserved residues shown in red. **i** Structural comparison of the NUD-binding domains and spatial residue packing between wild-type *CnMDH* and *CnMDH*^{*}. For (**d**), statistical significance was assessed using a two-sided unpaired Student's *t*-test; exact *P* values are provided in the figure. All quantitative data are presented as mean ± s.d. from three biologically independent replicates (*n* = 3). Source data are provided as a Source Data file.

L192 and P281 yielded the improved variant *CnMDH*^{*} (G46E/P189W/L192R/P281V). Apparent kinetic analysis showed that $(k_{\text{cat}}/K_{\text{M}})_{\text{app}}^{\text{NUD}}$ increased to $3.55 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (95% CI, 3.05×10^3 to $4.15 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$), while $(k_{\text{cat}}/K_{\text{M}})_{\text{app}}^{\text{NAD}}$ decreased to $5.81 \text{ M}^{-1} \text{ s}^{-1}$ (95% CI, 5.08 to $6.66 \text{ M}^{-1} \text{ s}^{-1}$). Accordingly, the NUD preference reached 6.11×10^2 , demonstrating that *CnMDH*^{*} had been reprogrammed into a strongly NUD-prefering MDH (Supplementary Fig. 8). Structural modeling of *CnMDH*^{*} indicated that these mutations constricted the active site from 10.4 to 3.9 Å, and surface analysis revealed that the variant adopted a closed conformation, exhibiting a tight steric fit with the NUD molecule (Fig. 1i). This spatial confinement optimized steric complementarity for the NUD head group, thereby driving both the efficient utilization of NUD and a fundamental shift in specificity.

Building on these results, an in vivo NUDH biosynthesis module was established in *E. coli* W3110 through genomic integration of *HsNmnat*^{*}, *CnMDH*^{*}, and a formaldehyde dismutase from *Pseudomonas putida* (*PpFDM*) (Fig. 2a). The resulting strain K01 accumulated $15.9 \pm 2.2 \text{ μM}$ intracellular NUD⁺ and $94.6 \pm 12.3 \text{ μM}$ NUDH (Fig. 2b). To promote NUD⁺ reduction to NUDH, *HsNmnat*^{*} and *CnMDH*^{*} were fused via a (GSGSG)₄ linker, yielding strain K02, in which the intracellular NUDH concentration was increased to $264 \pm 22 \text{ μM}$ (Fig. 2b). To further boost the supply of UTP and NMN for enhanced NUDH accumulation, competing UTP/NMN consumption pathways were reduced by deleting *pyrG*, *pncC*, *ushA*, *pnuC*, and *purR*, generating K03 (Fig. 2c and Supplementary Fig. 9). This modification increased intracellular UTP and NMN to 568 ± 36 and $722 \pm 74 \text{ μM}$, respectively (Fig. 2c, d). The precursor pools were then further expanded in K04 by overexpressing uridine kinase (*SUMPK*) and nucleoside diphosphate kinase (*DaNDK*) to enlarge UTP supply and integrating nicotinamide phosphoribosyl transferase (*RsNAMPT*) and NMN synthase (*FtNadE*) to strengthen NMN biosynthesis, which raised intracellular UTP and NMN to 7.8 ± 0.5 and $3.3 \pm 0.3 \text{ mM}$, respectively, and enabled NUDH accumulation to $6.6 \pm 0.5 \text{ mM}$ (Fig. 2c, e, f). To correct the remaining imbalance between UTP and NMN, the promoters of *RsNAMPT* and *FtNadE* were replaced with the promoter PJ23111 instead of PJ23101, generating K05. This strain accumulated $7.3 \pm 0.4 \text{ mM}$ intracellular NUDH and achieved a NUDH/NUD⁺ ratio of 56 (Fig. 2f). In contrast, the intracellular NADH pool in actively growing *E. coli* is typically maintained at only 0.08–0.5 mM, with an NADH/NAD⁺ ratio generally ranging from 0.03 to 0.75 under physiological conditions^{28,29}. Thus, K05 established a robust OES chassis to power the reduction of highly oxidized substrates such as CO₂ and formate.

Design and construction of an OES-driven CAM^{*} pathway for efficient CO₂ fixation in *E. coli*

As illustrated in Fig. 3a, a CAM pathway was designed for CO₂ utilization. This seven-step cascade was organized into three modules: (i) a

CO₂ activation module, in which formate dehydrogenase (FDH), formaldehyde dehydrogenase (FADH) and glycolaldehyde synthase (GALS) were used to assemble CO₂ into glycolaldehyde; (ii) a C2 functionalization module, in which acetyl-phosphate synthase (ACPS) and phosphate acetyltransferase (PTA) were used to convert glycolaldehyde to acetyl-CoA; and (iii) an iterative carboxylation module, in which pyruvate: ferredoxin oxidoreductase (PFOR), ferredoxin reductase (FR), and malic enzyme (ME) were used to drive sequential reductive carboxylations for carbon chain elongation.

The CAM pathway was then assembled modularly under anaerobic in vitro conditions. Guided by literature mining, the CO₂ activation module was constructed using *TsFDH* (PDB: 3WR5), *BmFADH*, and the engineered high-activity *PrGALS*³⁰, the module produced $0.65 \pm 0.14 \text{ mM}$ glycolaldehyde with 3 mM NADH (Fig. 3b). Because NADH oxidation can lead to NAD⁺ accumulation, which inhibits CO₂ reduction and promotes the reverse oxidative reaction, NADH regeneration was introduced using *Pseudomonas stutzeri* phosphite dehydrogenase (*PspTDH*), thereby driving the formation of NADH and increasing the glycolaldehyde titer to $1.98 \pm 0.10 \text{ mM}$ (Fig. 3b). Subsequent incorporation of the C2-functionalization module (*BmACPS*, *BsPTA*) and the iterative carboxylation module (*DaPFOR*³¹, *CtFR*, *PcME*) supported pathway synergy, yielding $1.17 \pm 0.05 \text{ mM}$ acetyl-CoA and $0.92 \pm 0.11 \text{ mM}$ L-malate, respectively (Fig. 3c, d). Further in vitro optimization showed that increasing the NADH concentration to 5.0 mM shortened the reaction time from 9 to 6 h and increased the L-malate titer to $2.13 \pm 0.11 \text{ mM}$ (Fig. 3d). However, engineering the native NAD(H) system was insufficient to meet this high intracellular energy demand, yielding a maximum intracellular NADH concentration of only $1.14 \pm 0.09 \text{ mM}$ (Supplementary Fig. 10). To overcome this physiological limitation of native NAD(H) metabolism, we engineered the NUDH-driven CAM^{*} pathway and evaluated its ability to bypass native energetic constraints in *E. coli*.

For this, the cofactor specificity of *TsFDH*, *BmFADH*, and *PcME* in the pathway were reprogrammed from NADH to NUDH using a computer-aided IESE (Identification, Exclusion, Screening, and Evaluation) protein directed evolution framework (Fig. 3e, Supplementary Fig. 11, and Supplementary Note 1). In this pipeline, mutational hotspots were first identified within an 8 Å radius of the NUDH uracil moiety, and residues were prioritized on the basis of computed binding free-energy changes ($\Delta\Delta G = \Delta G_{\text{mut}} - \Delta G_{\text{wild}}$) under stringent statistical criteria (*p*-values < 0.05, Benjamini–Hochberg false discovery rate (FDR) < 0.01). To preserve catalytic integrity, highly conserved residues (https://consurf.tau.ac.il/consurf_index.php, ConSurf score = 9) were excluded, and the remaining non-conserved hotspots were targeted using focused degenerate libraries (TDS, VYG, VWA, VRT) to maximize variant diversity (Supplementary Fig. 12). Guided by the IESE strategy, the following residues were prioritized for engineering: V198, T221, V256 in *TsFDH* (Fig. 3f and Supplementary

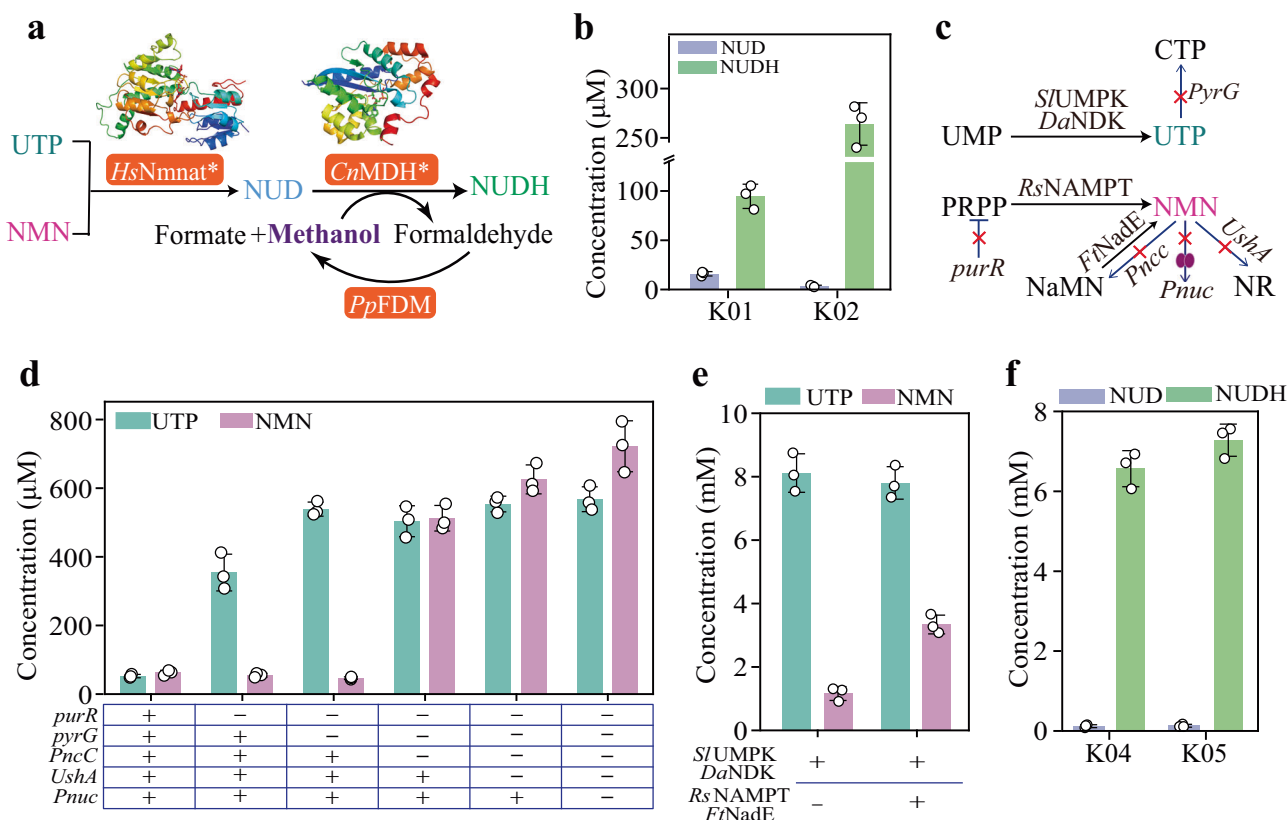


Fig. 2 | Systematic metabolic engineering of *E. coli* for high-level accumulation of orthogonal NUDH. For intracellular metabolite measurements, strains were cultured overnight in LB medium, then transferred into fresh LB medium at an initial OD₆₀₀ of 0.05 and cultivated at 37 °C for 6 h. **a** Schematic of the in vivo NUD(H) biosynthetic pathway utilizing UTP and NMN. **b** Intracellular NUD⁺ and NUDH concentrations in the initial strain K01 and strain K02. **c** Metabolic network diagram illustrating the targeted engineering strategies to enhance endogenous UTP and NMN supply. Red crosses indicate targeted gene deletions, and arrows indicate overexpression targets. PRPP phosphoribosyl pyrophosphate. *S/UMP*: uridine kinase from *Sulfurovum lithotrophicum*; *DaNDK*, Nucleoside diphosphate kinase from *Desulfurella amilii*; *RsNAMPT*, nicotinamide phosphoribosyl transferase from *Ralstonia solanacearum*; *FtNadE*, mutated NMN synthetase from *Francisella tularensis*³⁷. The genes and their encoded enzymes are as follows: *purR*,

DNA-binding transcriptional repressor; *pyrG*, CTP synthetase; *pncC*, Nicotinamide-nucleotide amidohydrolase; *ushA*, bifunctional UDP-sugar hydrolase and 5'-nucleotidase; *pnuC*, nicotinamide mononucleotide transporter. **d** Stepwise enrichment of basal UTP and NMN pools via multiplexed deletion of repressor and competing metabolic genes (*purR*, *pyrG*, *pncC*, *ushA*, and *pnuC*). **e** Massive expansion of the UTP and NMN pools into the millimolar range in strain K04 through the overexpression of key biosynthetic enzymes: uridine kinase (*S/UMP*), nucleoside diphosphate kinase (*DaNDK*), nicotinamide phosphoribosyltransferase (*RsNAMPT*), and NMN synthase (*FtNadE*). **f** Quantification of intracellular NUD and NUDH concentrations in strains K04 and K05. All quantitative data are presented as mean ± d. (*n* = 3 biologically independent replicates). Source data are provided as a Source Data file.

Figs. 13 and 14); A193, M219, L237, H268, V283 in *BmFADH* (Fig. 3g and Supplementary Figs. 15 and 16); and V300, I386, Q391, L394 in *PcME* (Fig. 3h and Supplementary Figs. 17 and 18). Parallel single-site screening followed by stepwise iterative recombination or accumulation was performed over three rounds for *TsFDH*, four rounds for *BmFADH* and three rounds for *PcME*, covering 470, approximately 1170 and 560 transformants, respectively (Supplementary Note 1). This iterative mutagenesis workflow yielded the optimized variants *TsFDH** (V198M/T221R/V256K), *BmFADH** (A193M/M219R/L237R/H268Q/V283L), and *PcME** (V300Q/Q391Y/L394K). Apparent kinetic profiling confirmed a pronounced inversion of cofactor specificity, with NUDH preference $\frac{(k_{cat}/K_M)_{app}^{NUDH}}{(k_{cat}/K_M)_{app}^{NADH}}$ (3), scores of 310, 447, and 94, respectively (Supplementary Fig. 19). Collectively, these results established that the IESE framework enabled rapid directed evolution of biocatalysts with high orthogonal cofactor specificity.

The corresponding enzymes were replaced with the NUDH-dependent variants *TsFDH**, *BmFADH**, and *PcME** to generate the CAM* pathway (Fig. 4a). To enable efficient NUDH-driven CAM* operation, the NUDH-supplying chassis strain K05 was further

optimized. First, the endogenous malic enzyme genes (*sfca* and *maeb*) were deleted to disrupt the native malate shunt, while a *Synechococcus*-derived CO₂-concentrating module consisting of a bicarbonate transporter and carbonic anhydrase was introduced to enhance intracellular CO₂ availability, generating strain K06 (Fig. 4a and Supplementary Fig. 20). Next, CRISPR-mediated genomic streamlining was used to disrupt competing pathways for formate (*fdhF*, *fdnG*), formaldehyde (*frmA*), glycolaldehyde (*fucO*, *aldA*), acetyl-phosphate (*ackA*), acetyl-CoA (*pta*, *adhE*), and pyruvate (*pflB*, *ldhA*, *dld*, *poxB*, *aceEF*), generating K07 (Fig. 4a and Supplementary Fig. 20). Assembly of the CAM* pathway in strain K07 generated K08, which was evaluated by whole-cell catalysis with CO₂ and methanol. During this assay, strain K08 produced an extracellular L-malate titer of 5.28 ± 0.58 mM; however, 7.22 ± 0.79 mM of formate was detected after 20 h (Fig. 4b, c). To reduce formate accumulation, a three-stage metabolic engineering strategy was employed. First, to minimize formate leakage from the CO₂ activation module, a synthetic GBD-SH3-PDZ scaffold was engineered to spatially co-localize the three formate-metabolizing enzymes³² (Fig. 4d). Optimization of the recruitment ratio to 1:2:3 for *TsFDH**, *BmFADH**, and *PnGALS* (strain K09) decreased the extracellular formate titer to 2.16 ± 0.14 mM, and increased the L-malate

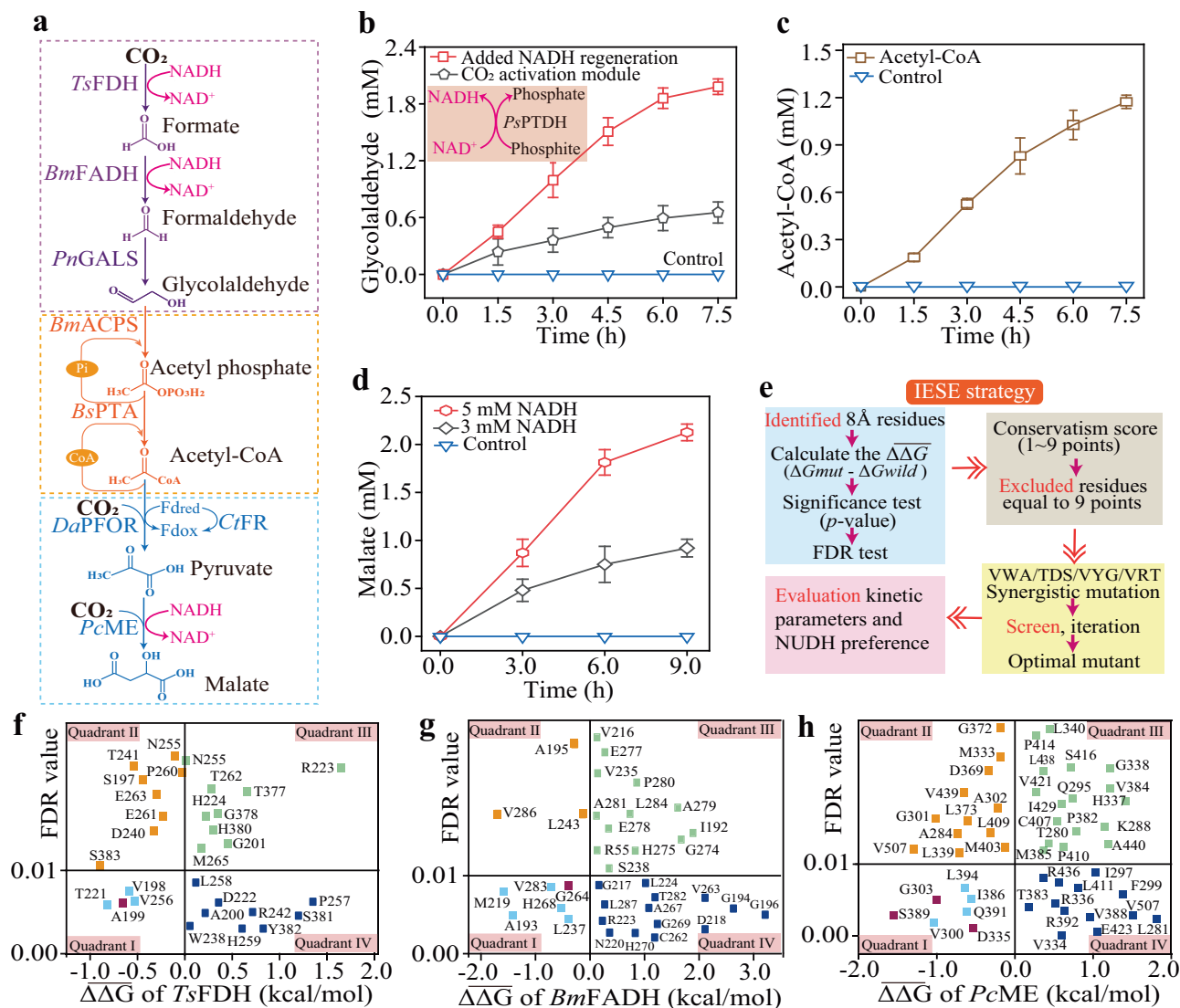


Fig. 3 | Modular in vitro assembly of the CAM pathway and IESE-guided directed evolution for orthogonal cofactor specificity. **a** Schematic of the minimalist CAM pathway designed for CO₂ fixation, organized into three functional modules.

TsFDH formate dehydrogenase from *Thiobacillus* sp. KNK65MA, *BmFADH* formaldehyde dehydrogenase from *Burkholderia multivorans*, *PnGALS* glycolaldehyde synthase from *Polynucleobacter necessarius*, *BmACPS* acetyl-phosphate synthase from *Bifidobacterium moukalabense*, *BsPTA* phosphate acetyltransferase from *Bacillus subtilis*, *DaPFOR* pyruvate: ferredoxin oxidoreductase from *Desulfovibrio africanus*, *CtfR* ferredoxin reductase from *Chlorobium tepidum*, *PcME* malic enzyme from *Pseudomonas citronellolis*. **b** In vitro time-course profiles of glycolaldehyde accumulation driven by the CO₂ activation module, with and without *PsPTDH*-mediated in situ NADH regeneration. **c** In vitro time-course of acetyl-CoA accumulation following the integration of the C2-functionalization module. **d** In vitro time-course of L-malate production by the complete CAM cascade supplemented with 3 or 5 mM initial NADH. **e** Workflow of the computer-aided IESE

(Identification, Exclusion, Screening, and Evaluation) framework used to reprogram cofactor specificity. **f** Distribution of mean $\Delta\Delta G$ and FDR values for 30 NUDH-contacting residues in *TsFDH*. **g** Distribution of mean $\Delta\Delta G$ and FDR values for 41 NUDH-contacting residues in *BmFADH*. **h** Distribution of mean $\Delta\Delta G$ and FDR values for 52 NUDH-contacting residues in *PcME*. For (**f**–**h**), statistical significance was assessed using a two-sided one-sample *t*-test ($n = 20$ in silico amino-acid states per residue), followed by Benjamini–Hochberg correction for multiple comparisons. Candidate residues were selected using mean $\Delta\Delta G < 0$ kcal mol^{−1}, $P < 0.05$ and FDR < 0.01. Exact *P* values were: **f** V198, 1.7×10^{-3} ; A199, 6.3×10^{-4} ; T221, 5.6×10^{-4} ; V256, 1.1×10^{-3} ; **g** A193, 6.2×10^{-7} ; M219, 5.1×10^{-9} ; L237, 1.3×10^{-3} ; G264, 9.7×10^{-4} ; H268, 6.0×10^{-4} ; V283, 6.8×10^{-5} ; and **h** V300, 1.1×10^{-4} ; G303, 2.7×10^{-3} ; D335, 3.0×10^{-3} ; I386, 1.4×10^{-4} ; S389, 7.5×10^{-4} ; Q391, 3.2×10^{-4} ; L394, 3.4×10^{-4} . For (**b**–**d**), data are presented as mean $\pm$ s.d. from three biologically independent replicates ($n = 3$). Source data are provided as a Source Data file.

titer to 16.7 ± 1.8 mM (Fig. 4b, c). Second, a formate salvage module was introduced by overexpressing pyruvate formate-lyase (*L/PFL*), enabling disproportionation-derived formate to be assimilated into the pyruvate node of CAM* and further increasing the L-malate titer to 21.7 ± 0.9 mM in K10 (Fig. 4b, e). Finally, iterative replacement of the *lscR* locus with the C2 functionalization and iterative carboxylation modules channeled more pyruvate toward L-malate biosynthesis, yielding K11 with a final extracellular L-malate titer of 24.5 ± 1.9 mM (Fig. 4b).

OES synergized with the electron transport chain enabling CO₂ and methanol-based growth of *E. coli*

Despite established OES-driven CAM* conversion of CO₂ to L-malate, strain K11 showed limited growth under CO₂ and methanol conditions (Fig. 5a). This limitation mainly reflected the orthogonality of OES: while OES drives CAM*-based CO₂ fixation, the conversion of L-malate into growth-essential precursors such as PEP, GAP, F6P, and G6P still depends on native energy-supported gluconeogenesis. To restore the native energy support required for gluconeogenesis and growth, the

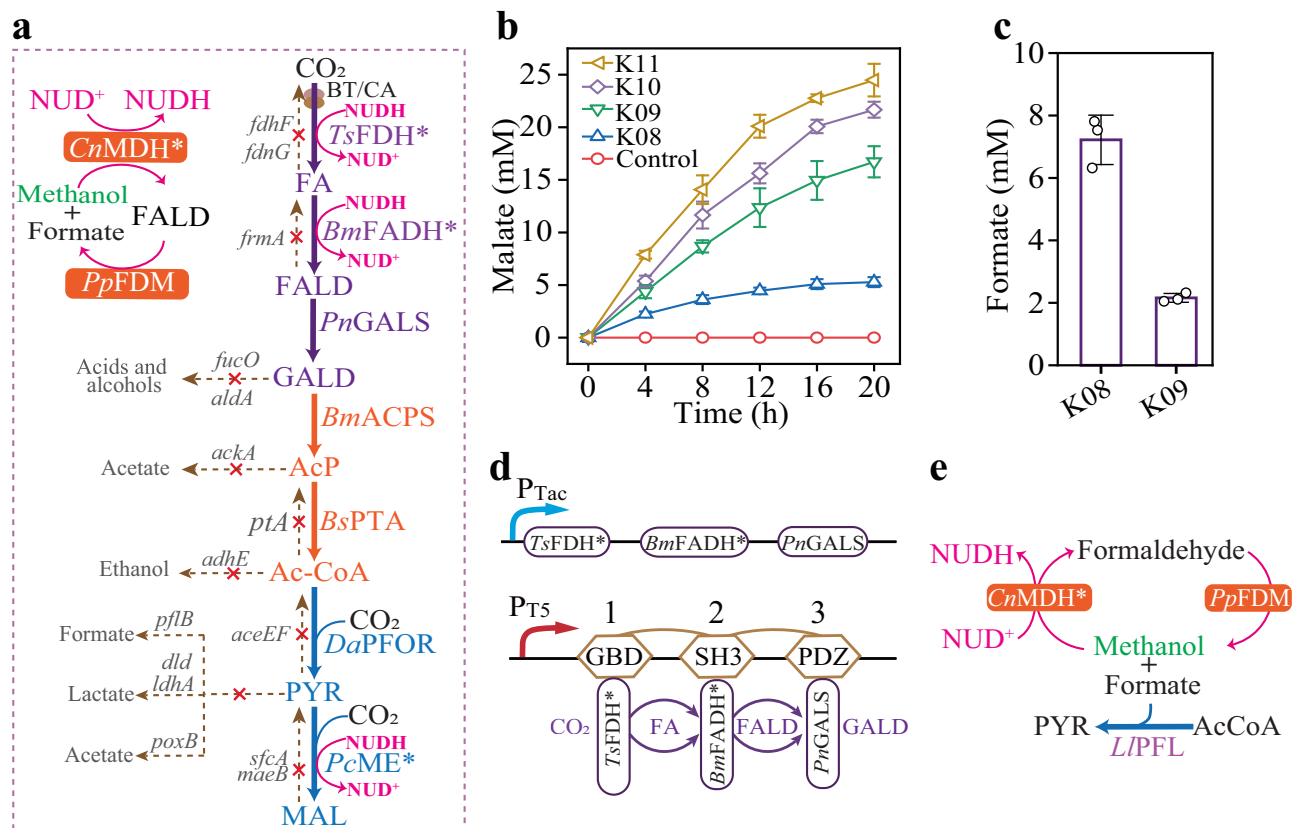


Fig. 4 | Resting-cell catalytic optimization of the orthogonal NUDH-driven CAM* pathway. Cells were cultivated in LB medium for 12 h, harvested, washed three times with M9 salt medium, and resuspended in fresh M9 medium to an OD₆₀₀ of 10. Resting-cell reactions were conducted in M9 medium supplemented with 400 mM NaHCO₃ and 400 mM methanol at 37 °C. **a** Systematic metabolic rewiring for in vivo implementation of the CAM* pathway. Red crosses indicate CRISPR-mediated disruptions of competing pathways to redirect carbon flux. The genes and their encoded enzymes are as follows: *fdhF* formate dehydrogenase-H, *fdnG* formate dehydrogenase-N, *frmA* formaldehyde dehydrogenase, *fucO* aldehyde oxidoreductase, *aldA* aldehyde dehydrogenase, *ackA* acetate kinase, *pta* phosphate acetyltransferase, *pflB* pyruvate formate lyase, *dld* D-lactate dehydrogenase, *adhE* bifunctional acetaldehyde-CoA/alcohol dehydrogenase, *poxB* pyruvate oxidase, *aceEF* pyruvate dehydrogenase, *sfcA* malate enzyme, *maeB* malic

enzyme. **b** Time-course profiles of L-malate production during the stepwise metabolic optimization from strain K08 to K11. **c** Extracellular formate accumulation in strains K08 and K09 at 20 h, demonstrating the mitigation of formate leakage via spatial enzyme co-localization. **d** Schematic of the synthetic GBD-SH3-PDZ protein scaffold designed to spatially co-localize the three formate-metabolizing enzymes (*TsFDH**, *BmFADH**, and *PnGALS*), thereby minimizing the leakage of the toxic intermediate formate. **e** Implementation of a formate salvage module. Pyruvate formate-lyase from *Lactococcus lactis* MG1363 (*L/PFL*) was overexpressed to recapture disproportionation-derived formate and direct it into the pyruvate node of the CAM* pathway. All quantitative data are presented as mean ± s.d. (*n* = 3 biologically independent replicates). Source data are provided as a Source Data file.

ETC was engineered at three strategic nodes: replenishing native reducing equivalents, enhancing ubiquinone-mediated electron transfer, and improving terminal electron transfer efficiency (Fig. 5b). First, endogenous reducing equivalents were replenished by introducing the strictly NAD-dependent MDH *CnMDH* into K11, yielding K12. Although this modification increased OD₆₀₀ by approximately three-fold, mortality reached 88.9% (Fig. 5a and Supplementary Fig. 21), likely due to formaldehyde accumulation caused by rapid methanol oxidation. To mitigate formaldehyde toxicity, *CnMDH* was fused with formaldehyde dismutase *PpFDM* to generate K13, which enhanced formaldehyde recycling, reduced mortality to 13.5% (Supplementary Fig. 21). This modification further supported biomass accumulation under CO₂ and methanol conditions, with K13 reaching an OD₆₀₀ of 0.21 ± 0.02 (Fig. 5a). Next, ubiquinone-mediated electron transfer was enhanced by overexpressing *ubil*, generating strain K14 with an OD₆₀₀ of 0.26 ± 0.02 (Fig. 5a, b). Finally, the membrane-targeted oxygen-binding protein *Vitreoscilla* hemoglobin (VHb)³³ was introduced to enhance terminal electron transfer, resulting in strain K15 with an OD₆₀₀ of 0.41 ± 0.03 (Fig. 5a, b).

Following restoration of the native energy system, quantitative profiling of key intracellular gluconeogenic metabolites indicated that

the primary growth limitation had shifted from energy supply to gluconeogenic capacity. Specifically, the key intermediates in strain K15, including PEP, GAP, F6P, and G6P, were severely depleted to <0.5 mM (Fig. 5d). This shortage created a critical bottleneck in downstream biosynthesis, thereby restricting precursor supply for lipid, cell-wall and nucleotide formation (Fig. 5d). To increase carbon flux, strain K16 was constructed by strengthening the gluconeogenic module through overexpression of malate dehydrogenase (*mdh*), phosphoenolpyruvate carboxykinase (*pck*) and fructose-1,6-bisphosphatase (*fbp*). This rewiring enabled efficient release of endogenous reducing equivalents during malate oxidation, thereby coupling gluconeogenic throughput with biomass accumulation and increasing OD₆₀₀ to 1.24 ± 0.16 (Fig. 5e). To enforce unidirectional flux and eliminate futile cycling, the competitive nodes encoded by *ppc*, *pykA*, and *pykF* were disrupted in strain K17 (Fig. 5d and Supplementary Fig. 22). This strategy replenished the anabolic precursor pools, restoring intracellular PEP, GAP, F6P, and G6P to millimolar level (1.17 ± 0.07–2.16 ± 0.12 mM) and increasing OD₆₀₀ to 2.23 ± 0.22 (Fig. 5d, e). To ensure that these restored intracellular resources were maximally directed toward biomass synthesis, a multiplex genome editing strategy was employed to delete four major flagella gene clusters (*fliE-R*, *flit-Z*, *flgA-L*, and *flhD-E*)

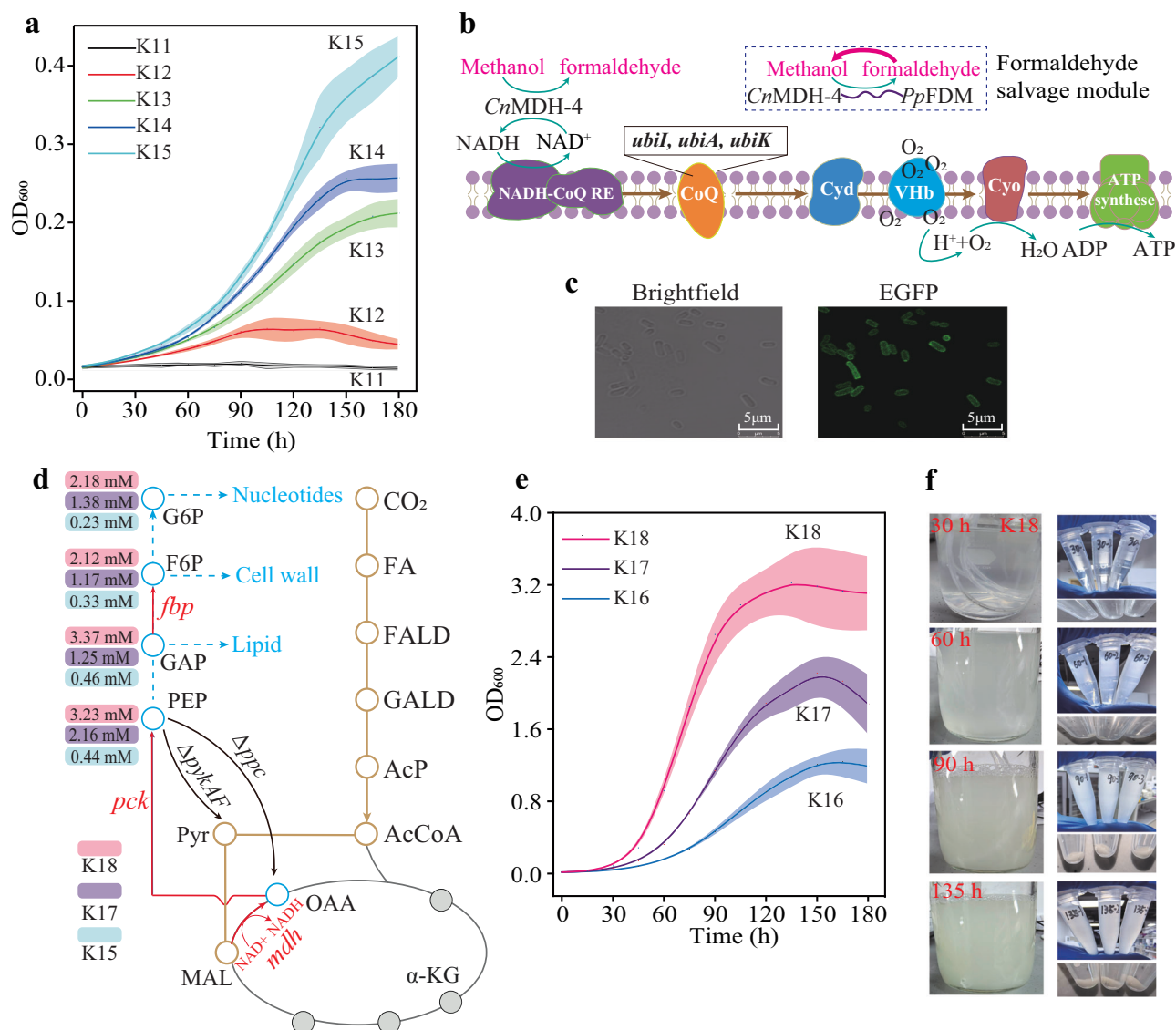


Fig. 5 | Modular optimization of the electron transport chain and metabolic flux rewiring for robust CO₂-based growth. Engineered *E. coli* strains were cultivated in NBS minimal medium with methanol as the electron donor under a gas mixture of 20% CO₂, 4% O₂, and 76% N₂. The pressure was maintained at 0.05 MPa, and the medium pH was readjusted to 7.0 after gas equilibration. **a** Stepwise improvement in cell biomass (OD₆₀₀) across engineered strains (K11 to K15), highlighting the cumulative physiological benefits of electron transport chain (ETC) optimization. **b** Schematic of the three-tiered strategy engineered to reinvigorate the native ETC, thereby synergizing with the orthogonal NUDH supply. *ubiI* 2-octaprenylphenol hydroxylase, *ubiK* ubiquinone biosynthesis accessory factor, *ubiA* 4-hydroxybenzoate octaprenyltransferase, CoQ coenzyme Q (ubiquinone), Cyd cytochrome bd ubiquinol oxidase, Cyo cytochrome bo₃ ubiquinol oxidase. **c** Membrane localization of the respiratory enhancer Vhb. Fluorescence microscopy images of strain K15 expressing a Vhb-EGFP fusion protein confirm its targeted anchoring to the cell membrane to facilitate oxygen transfer. The experiment was independently repeated three times with similar results. **d** De-bottlenecking

gluconeogenic flux. Schematic of the reconstructed central metabolic network and quantification of key intracellular intermediates (PEP, GAP, F6P, and G6P) in strains K15, K17, and K18, demonstrating the successful restoration of anabolic precursor pools. PEP phosphoenolpyruvate, GAP glyceraldehyde-3-phosphate, F6P fructose-6-phosphate, G6P glucose-6-phosphate, FA formate, FALD formaldehyde, GALD glycolaldehyde, AcP acetyl-phosphate, Ac-CoA acetyl-CoA, Pyr pyruvate, MAL malate, OAA oxaloacetate, α-KG α-ketoglutarate. **e** Comparative growth profiles of strains K16 (enhanced gluconeogenesis), K17 (futile cycle disrupted), and K18 (genome streamlining and CRP2* integrated), showcasing the progressive acceleration of CO₂-based growth. **f** Photographs of strain K18 cultures and cell pellets collected from 1.5 mL broth at 30, 60, 90, and 135 h, visually demonstrating the rapid accumulation of biomass. In (a and e), the solid line represents the mean and the shaded band represents $\pm$ s.d.; all other quantitative data are presented as mean $\pm$ s.d. with error bars. All data are based on three biologically independent replicates ($n = 3$). Source data are provided as a Source Data file.

(Supplementary Fig. 23). Building on this, the cAMP receptor protein (CRP), a global regulator of gluconeogenesis, was modified to prevent feedback-driven inactivation caused by intermediate accumulation. The feedback-resistant variant, CRP2* (G141D)³⁴, was introduced in strain K18, thereby sustaining gluconeogenic activation. As a result, intracellular PEP, GAP, F6P and G6P were increased by 7.3-, 7.3-, 6.5- and 9.5-fold, respectively (Fig. 5d). These elevated metabolite pools enabled strain K18 to achieve an OD₆₀₀ of 3.23 ± 0.36 after 135 h of

cultivation, with a doubling time of 8.6 ± 0.3 h, indicating robust growth (Fig. 5e, f).

OES drives the production of chemicals derived from the different CAM* pathway nodes

To evaluate the high-cell-density biomanufacturing potential of strain K18, fed-batch cultivation was initiated with CO₂ and methanol as substrates, enabling sustained growth to a maximum OD₆₀₀ of

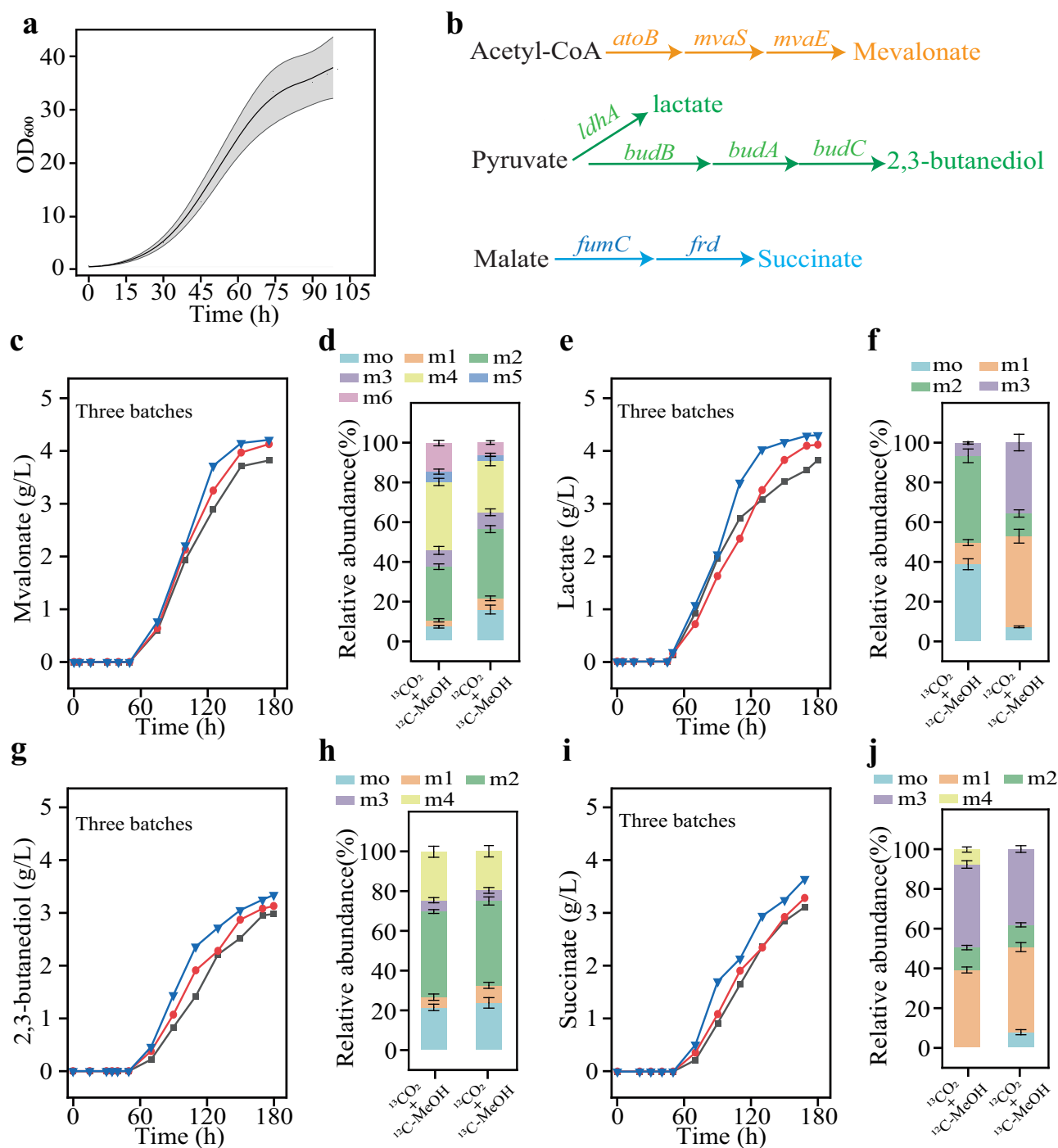


Fig. 6 | Biosynthetic versatility of the engineered CAM* pathway for chemical production. Engineered *E. coli* strains were cultivated in NBS minimal medium under 20% CO₂, 4% O₂, and 76% N₂ at 0.05 MPa, and methanol was maintained at approximately 100 mM by fed-batch addition. **a** Fed-batch fermentation profile of strain K18, demonstrating high-cell-density growth (OD₆₀₀). **b** Schematic of the modular metabolic pathways engineered for the production of mevalonate, lactate, 2,3-butanediol, and succinate from distinct CAM* nodes. Specifically, the acetyl-CoA node supplies the precursor for mevalonate biosynthesis via the heterologous *atoB-mvaS-mvaE* module; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; the pyruvate node is redirected to lactate and 2,3-butanediol via *ldhA* and the *budB-budA-budC* cascade, respectively; and the malate node is extended to succinate via overexpression of the reductive L-malate branch (*fumC-frd*). The genes and their encoded enzymes are as follows: *atoB* Acetyl-CoA acetyltransferase, *mvaS* Hydroxymethylglutaryl-CoA synthase, *mvaE* HMG-CoA reductase, *ldhA* lactate dehydrogenase, *budB* acetolactate synthase, *budA* acetolactate decarboxylase, *budC* 2,3-butanediol dehydrogenase, *fumC* fumarase, *frd* fumarate reductase.

c Time-course profile of mevalonate production in strain K19. **d** Relative abundances of mevalonate molecules containing different numbers of ¹³C atoms under the complementary ¹³CO₂ + ¹²C-methanol and ¹²CO₂ + ¹³C-methanol tracing conditions. **e** Time-course profile of lactate production in strain K20. **f** Relative abundances of lactate molecules containing different numbers of ¹³C atoms under the complementary ¹³CO₂ + ¹²C-methanol and ¹²CO₂ + ¹³C-methanol tracing conditions. **g** Time-course profile of 2,3-butanediol production in strain K21. **h** Relative abundances of 2,3-butanediol molecules containing different numbers of ¹³C atoms under the complementary ¹³CO₂ + ¹²C-methanol and ¹²CO₂ + ¹³C-methanol tracing conditions. **i** Time-course profile of succinate production in strain K22. **j** Relative abundances of succinate molecules containing different numbers of ¹³C atoms under the complementary ¹³CO₂ + ¹²C-methanol and ¹²CO₂ + ¹³C-methanol tracing conditions. In **(a)**, the solid line represents the mean and the shaded band represents ± s.d. from three biologically independent replicates (*n* = 3). In **(d, f, h, j)**, data are presented as mean ± s.d. from three biologically independent replicates (*n* = 3), with error bars representing s.d. Source data are provided as a Source Data file.

37.5 ± 5.8 (Fig. 6a). Strain K18 was then engineered to produce chemicals from three CAM* pathway nodes, including acetyl-CoA, pyruvate and L-malate. To quantify the relative carbon contributions from CO₂ and methanol, complementary isotope-tracing experiments were performed using $^{13}\text{CO}_2 + ^{12}\text{C}$ -methanol or $^{12}\text{CO}_2 + ^{13}\text{C}$ -methanol as co-substrates. The total labeled-carbon fraction was calculated from the full mass isotopologue distribution (MID) according to established isotope-tracing analysis methods¹³.

First, to validate flux redirection from the acetyl-CoA node, a terpenoid precursor pathway was established by assembling the *atoB-mvaS-mvaE* module, yielding strain K19. In three independent fed-batch cultivations, acetyl-CoA flux was redirected toward mevalonate, affording an average titer of 4.05 ± 0.21 g/L and a yield of 0.036 ± 0.004 mol mevalonate per mol methanol consumed (Fig. 6c). Isotope tracing further resolved the carbon origin of mevalonate. Under the $^{13}\text{CO}_2 + ^{12}\text{C}$ -methanol condition, the fully labeled mevalonate isotopologue *m* + 6 accounted for $14.5 \pm 1.4\%$, and weighted analysis of the complete MID showed that CO₂ contributed $55.7 \pm 1.3\%$ of the carbon atoms in mevalonate (Fig. 6d). Conversely, under the complementary $^{12}\text{CO}_2 + ^{13}\text{C}$ -methanol condition, the *m* + 6 fraction was $6.2 \pm 0.9\%$, corresponding to a $42.9 \pm 2.3\%$ contribution from methanol-oxidation-derived carbon (Fig. 6d). Together, these results demonstrate that mevalonate biosynthesis in K19 was supported by coordinated CO₂ assimilation and methanol oxidation.

Second, carbon-flux diversion at the pyruvate junction was evaluated by targeting downstream products of increasing metabolic complexity. For direct reductive conversion, lactate dehydrogenase (*ldhA*) was introduced to generate strain K20, resulting in an average lactate titer of 4.12 ± 0.26 g/L, corresponding to a yield of 0.105 ± 0.003 mol lactate per mol methanol consumed (Fig. 6e). To probe capacity for integration of a more complex pathway, flux was extended to 2,3-butanediol (2,3-BDO) by assembling acetolactate synthase (*budB*), acetolactate decarboxylase (*budA*) and acetoin reductase (*budC*). The resulting strain K21 produced 2,3-BDO at an average titer of 3.15 ± 0.18 g/L, corresponding to a yield of 0.075 ± 0.003 mol 2,3-BDO per mol methanol consumed (Fig. 6g). Although lactate and 2,3-BDO both originate from the pyruvate node, they retain different portions of the pyruvate carbon skeleton. In the CAM* pathway, pyruvate is formed by adding a C1 unit to an acetyl-CoA-derived C2 backbone through either *D*aPFL-mediated CO₂ fixation or *L*IPFL-mediated incorporation of methanol-derived formate generated by formaldehyde dismutase *Pp*FDM. Lactate retains the complete C3 skeleton, whereas 2,3-BDO formation removes the carboxyl carbons of two pyruvate molecules and retains their C2 moieties. Under the $^{13}\text{CO}_2 + ^{12}\text{C}$ -methanol condition, weighted analysis of the complete MIDs showed that CO₂-derived carbon accounted for $39.4 \pm 2.4\%$ of the carbon atoms in lactate and $51.8 \pm 2.1\%$ of those in 2,3-BDO (Fig. 6f, h). The lower CO₂-derived fraction in lactate is consistent with the incorporation of unlabeled methanol-derived formate into the pyruvate pool, whereas the labeling pattern of 2,3-BDO more closely resembled that of the acetyl-CoA node because its pyruvate-derived C1 carbons were removed during decarboxylation. Under the complementary $^{12}\text{CO}_2 + ^{13}\text{C}$ -methanol condition, methanol-derived carbon accounted for 58.8 ± 2.8 and $47.3 \pm 2.8\%$ of the carbon atoms in lactate and 2,3-BDO, respectively (Fig. 6f, h).

Finally, the biosynthetic potential of the malate node was evaluated using succinate as a representative C4 platform chemical. For succinate production, the malate-to-succinate branch, comprising *fumC* and *frd*, was overexpressed in strain K22, resulting in an average titer of 3.35 ± 0.27 g/L and a yield of 0.054 ± 0.004 mol succinate per mol methanol consumed (Fig. 6i). Under the $^{13}\text{CO}_2 + ^{12}\text{C}$ -methanol condition, weighted analysis of the complete MID showed that CO₂ contributed $54.5 \pm 0.5\%$ of the carbon atoms in succinate, which was 1.38-fold higher than the corresponding CO₂ contribution observed for lactate (Fig. 6j). This higher CO₂ contribution is consistent with

the reductive carboxylation of pyruvate to form malate, during which an additional CO₂-derived C1 unit is incorporated into the C4 backbone. Conversely, under the complementary $^{12}\text{CO}_2 + ^{13}\text{C}$ -methanol condition, methanol-oxidation-derived carbon contributed $45.1 \pm 0.7\%$ of the carbon atoms in succinate (Fig. 6j). Collectively, the successful production of these four chemically distinct products demonstrates the versatility of the K18-derived platform for biomanufacturing.

Discussion

In this study, an OES was constructed in *E. coli* to enable efficient intracellular accumulation of NUDH and to increase the NUDH/NUD ratio, with methanol used as the electron donor. A CAM pathway, powered by NUDH, was engineered to directly convert CO₂ into central carbon metabolites. By integrating the OES with the CAM* pathway, CO₂ and methanol based growth and the biosynthesis of diverse chemicals were achieved.

In this study, an OES was designed and constructed in *E. coli* to enable efficient intracellular accumulation of NUDH (> 7.0 mM) and a high ratio (NUDH/NUD > 55). In microbial cell factories, the native redox cofactors NAD(H) and NADP(H) function as central hubs that couple catabolism to anabolism and support the biosynthesis of high-value chemicals, including organic acids, alcohols, amino acids and their derivatives^{35–38}. However, both cofactor abundance and redox state are tightly constrained by cellular homeostasis. In wild-type *E. coli*, intracellular NADH is typically maintained at low levels (0.083–0.5 mM), and the NADH/NAD⁺ ratio remains between 0.03 and 0.75^{28,29}. By contrast, NADPH is maintained at a higher reduction ratio, but its intracellular pool is often limited and typically remains in the micromolar range^{39,40}. Moreover, natural energy carriers are broadly embedded in cellular metabolism. For example, NAD(H), NADP(H), and ATP participate in 1038, 1108, and 800 biochemical reactions, respectively, in the KEGG database (<https://www.kegg.jp/>). Accordingly, efforts to increase the pool size or redox ratio of natural cofactors through metabolic engineering often cause global metabolic perturbations. To bypass these constraints, several orthogonal redox systems have been developed based on chemically synthesized cofactors, including NpUD⁴¹, N¹⁵ADP⁺/N¹⁵ADPH⁴² and carba-NADP⁴³. However, these cofactors are not generated through endogenous metabolic routes and instead require external supplementation, which limits their deployment in industrial strains. Recently, Li and co-workers established NMN(H) as a biosynthesizable orthogonal redox cofactor by engineering an NMN⁺-specific glucose dehydrogenase that selectively channelled glucose-derived reducing power toward levodione synthesis in *E. coli*⁴⁴. The platform was further expanded by developing NMN⁺-reducing phosphite dehydrogenases for efficient cofactor regeneration⁴⁵, NMN⁺-dependent GapN variants that couple glycolysis to NMNH generation⁴⁶, and NMNH-specific oxidases for controlling the NMNH/NMN⁺ ratio⁴⁷. These advances also enabled growth-coupled selection platforms for evolving NMN(H)-utilizing enzymes and NMN(H)-driven stereoselective upgrading of 2,3-butanediol in *E. coli* whole cells^{47,48}. Together, they demonstrated that NMN(H) can be biosynthesized, recycled and functionally integrated into living cells to redirect reducing power with high specificity and programmability. To further enable the application of OES to C1-substrate utilization, methanol was introduced in this study as an independent electron donor for NUDH formation, together with a formaldehyde dismutation module that converted formaldehyde back to methanol. Formaldehyde toxicity was thereby mitigated, while methanol recycling was enabled. As a result, NUDH/NUD regeneration was effectively decoupled from central carbon metabolism, and intracellular controllability was improved. Consequently, the achieved intracellular NUDH concentration and NUDH/NUD ratio substantially exceeded those of endogenous NADH, thereby alleviating supply constraints imposed by the natural redox system.

In this study, a minimalist ATP-independent CO₂ fixation pathway was designed and constructed. Using only five, six, and seven biochemical reactions, CO₂ was directly converted into central metabolites, including acetyl-CoA, pyruvate, and malate, respectively. In addition, an IESE-based protein engineering strategy was developed to switch the cofactor preference of TsFDH*, BmFADH*, and PcME* in the CAM pathway from NADH to NUDH, thereby generating the CAM* pathway. This minimalist CO₂-fixation design was expected to reduce expression burden and intermediate loss while supplying diverse carbon skeletons for downstream biosynthesis. The previously described SACA pathway, capable of converting formaldehyde to acetyl-CoA, offered crucial insights for designing the C2 functional module in our study⁴⁹. Nevertheless, in contrast to the assimilation of highly reduced formaldehyde, the microbial utilization of highly oxidized CO₂ for bioproduction has long remained restricted. Although several CO₂-fixation pathways have been developed for specific products, many are reaction-intensive and are often centered on a single central metabolite. For example, acetyl-CoA formation through the 3HP-4HB cycle and the LATCH pathway requires 16 and 10 reactions, respectively^{50,51}; pyruvate formation through the 3HP bicycle and the WL-rGly pathway requires 15 and 9 reactions, respectively^{25,52}; and malate formation in *E. coli* through the synthetic HWLS pathway still requires 14 reactions¹³. Therefore, CO₂-fixation pathways with fewer biochemical reactions are crucial to support direct conversion of CO₂ into central metabolites. Such designs follow the principle of enzyme-cost minimization and are expected to reduce cellular burden while increasing metabolic flexibility. Beyond pathway length, most reported CO₂-fixation pathways require dual inputs of ATP and NAD(P)H⁵³. For each product molecule formed, the 3HP bicycle, 3HP-4HB cycle, and WL-rGly pathway consume NAD(P)H and additionally require 5, 4, and 2 ATP molecules, respectively^{25,52}. Because ATP availability is tightly constrained by cellular homeostasis, the excessive ATP demand imposed by CO₂ fixation can substantially increase the cellular metabolic burden⁵⁴. To increase intracellular ATP availability, three general strategies have been used: (i) expansion of external energy inputs through non-native energy systems (for example, light^{55,56}, electricity⁵⁷, or chemical energy⁵⁸) to raise ATP abundance; (ii) reduction of futile ATP dissipation through genome streamlining, including blockage of energy-intensive side processes⁵⁹; and (iii) construction of ATP-independent routes, such as recently reported chemo-enzymatic systems in which dithiothreitol is used as a reductant to drive CO₂-methanol condensation under ATP-independent conditions⁶⁰. However, such systems depend on costly exogenous reagents, which limits application in living cells. Moreover, even when ATP-independent fixation is achieved, reliance on natural reduced cofactors (NADH or NADPH) is expected to intensify competition with host metabolism, potentially causing global redox imbalance or depletion of reducing equivalents⁵⁵. To mitigate this limitation, previous efforts have mainly focused on redirecting cofactor supply or reprogramming enzyme cofactor preference. In the first approach, natural cofactor generation is adjusted to match pathway demand. For example, in NADPH-dependent lysine and cadaverine biosynthesis, transhydrogenase (*PntAB*) are often overexpressed to drive NADH-to-NADPH conversion, thereby increasing NADPH availability^{61,62}. In the second approach, protein engineering has been applied to shift cofactor preference at the enzyme or pathway level. At the single-enzyme level, MDH⁶³, FDH⁶⁴, and phosphite dehydrogenase⁶⁵ have been reprogrammed from NAD(H) to NADP(H) dependent, to reduce intracellular redox imbalance. At the pathway level, broader rewiring has also been demonstrated. For example, in isobutanol and 1,4-butanediol biosynthesis, all NADPH-dependent dehydrogenases were systematically reprogrammed into NADH-dependent variants, thereby increasing the titer of target product^{66,67}. Nevertheless, these strategies remain confined to natural carriers and therefore cannot eliminate competition between engineered pathways and endogenous metabolism for NAD(P)H. By

contrast, pathway-wide reprogramming toward NUDH preference resolves this constraint and provides a dedicated and efficient cofactor supply for intracellular CO₂ fixation.

By integrating the OES-driven CAM* pathway with a cyanobacterial CO₂-concentrating module and systematic metabolic engineering, the engineered *E. coli* achieved robust growth. It reached a doubling time of 8.6 ± 0.3 h with a maximum OD₆₀₀ of 37.5 ± 5.8, approaching the performance of some natural CO₂-fixing autotrophs. In addition, several chemicals derived from CAM* pathway nodes were produced, including mevalonate, lactate, 2,3-butanediol, and succinate. Through long-term evolution, natural autotrophs have developed one-carbon assimilation networks that are tightly matched to cellular physiology and often confer clear growth advantages. For example, natural *Clostridium* species that can use one-carbon gases (CO₂/CO) show doubling times of ~4–12 h and reach OD₆₀₀ values of ~2–10^{68,69}. Cyanobacteria can grow even faster, with doubling times of 2–5 h when utilizing light and CO₂⁷⁰. By contrast, growth of engineered autotrophic microbes often requires the introduction of heterologous pathways and adaptive evolution. For instance, expression of a metal-dependent formate dehydrogenase (*CnFDH*) coupled with the reductive glycine pathway (rGlyP) reduced the doubling time of *E. coli* on formate as the sole carbon source to 4.5 h⁷¹. Similarly, bacterial artificial chromosome with dynamic copy number variation has enabled *E. coli* to grow on methanol with a doubling time of 3.5 h, exceeding the performance of some natural methylotrophs⁷². However, when highly oxidized CO₂ is used as the primary carbon source, the growth of engineered autotrophic microbes is strongly constrained. For example, engineered CO₂ autotrophic *E. coli* strains based on the CBB cycle exhibit doubling times of ~18 h after 400 days of evolution, with formate oxidation providing the energetic driving force¹⁷. This limitation is more pronounced in eukaryotes such as *Pichia pastoris*, in which fully autotrophic growth on CO₂ has been reported with a doubling time of ~90 h⁷³. For chemical production from one-carbon substrates, engineered autotrophic *Clostridium* strains can convert syngas (CO, CO₂, and H₂) into single-cell protein, alcohols, and organic acids, and several processes have reached commercial operation⁷⁴. In addition, the methylotrophic yeast *Pichia pastoris*, which uses methanol as the sole carbon source and supports high-cell-density fermentation, has been widely used for production of sabinene (6.38 g/L)⁷⁵, dopamine (12.2 g/L)⁷⁶ and fatty acids (23.4 g/L)⁷⁷. By contrast, chemical production in engineered autotrophic *E. coli* remains at an early stage. For example, engineered *E. coli* has been reported to produce high-value chemicals from methanol, including poly 3-hydroxybutyrate (PHB, 0.32 mg/L), itaconate (1.0 g/L) and *p*-aminobenzoic acid (PABA; 199.9 mg/L)⁷⁸. In addition, when CO₂ is used as the primary substrate, product formation by engineered autotrophs is often limited by energy supply and CO₂ assimilation. For example, only 1.2 mM (~108 mg/L) lactate⁷⁹ and 0.172 mg/L PHB⁸⁰ were produced by *E. coli* from CO₂ and formate as carbon sources. Moreover, under fully autotrophic conditions without exogenous organic carbon, *S. cerevisiae* can hardly accumulate the target-product from CO₂.

In summary, an NUDH-dependent CO₂-assimilation pathway (CAM* pathway) was developed to decouple CO₂ fixation from native redox homeostasis, thereby alleviating a long-standing energetic constraint that limits efficient CO₂ utilization in *E. coli*. By assigning NUDH to CAM*-mediated CO₂ reduction, this orthogonal-synergistic strategy enabled CO₂/methanol-based growth and supported gram-scale biosynthesis of diverse chemicals. Notably, the intracellular NUDH concentration in the engineered strain K05 exceeded 7.0 mM, thereby establishing an OES chassis capable of providing dedicated reducing power for the reductive conversion of highly oxidized carbon substrates. However, compared with the unmodified wild-type *E. coli* W3110 strain, the maximum OD₆₀₀ of K05 decreased by 10.9 ± 0.3%, indicating that high-level NUDH biosynthesis and the associated metabolic rewiring imposed a certain physiological burden on the host

(Supplementary Fig. 24). This growth burden may arise from at least two factors. First, NUDH biosynthesis still depends on the endogenous UTP and NMN pools; therefore, systematic engineering of the corresponding precursor biosynthetic pathways may lead to precursor competition and redistribution of cellular resources. This also reflects the need to balance cofactor accumulation with host fitness in biosynthetically generated orthogonal cofactor systems. Second, at high intracellular NUDH concentrations, endogenous oxidoreductases may exhibit low-level cross-reactivity toward NUD(H), thereby causing minor perturbations to native redox metabolism. In our assays, after 10 h of incubation under resting-cell conditions, the intracellular NUDH concentration decreased from 7.33 ± 0.19 to 6.23 ± 0.21 mM, corresponding to a reduction of $15.0 \pm 1.5\%$; meanwhile, the intracellular NUD⁺ concentration increased from 0.14 ± 0.02 to 0.47 ± 0.04 mM (Supplementary Fig. 24). Therefore, future efforts should focus on further improving the insulation of orthogonal cofactor biosynthesis, minimizing cofactor crosstalk, and dynamically balancing NUDH accumulation with host physiology. With these refinements, OES-based energy rewiring is expected to become more robust and transferable for next-generation one-carbon biomanufacturing.

Methods

Strains, plasmids, and reagents

All bacterial strains, plasmids, and promoters used in this study are listed in Supplementary Data 1 and 2. *Escherichia coli* JM109 was used for plasmid construction, *E. coli* W3110 was used as the parental strain for strain engineering and bioproduction, and *E. coli* BL21(DE3) was used for heterologous expression and purification of *HsNmnat*, *CnMDH*, *TsFDH*, *BmFADH*, *PcME*, and their engineered variants. The plasmids used in this study included pCas, pTARGET, pTR, pET28a, pETac, pCDR, and p15A vectors. The pCas/pTARGET system was used for chromosomal editing, whereas the formaldehyde-responsive plasmid pTR was used for screening MDH variants. pET28a vectors were used for heterologous protein expression and enzyme engineering, pETac and pCDR vectors were used for modular expression of OES and CAM^{*} pathway components, and p15A vectors were used to express the biosynthetic pathways for mevalonate, lactate, 2,3-butanediol, and succinate. Information on the origin, references, and construction details of all plasmids used in this study is provided in Supplementary Data 2. The corresponding amino acid sequences and codon-optimized coding sequences are listed in Supplementary Data 3.

PrimeSTAR Max DNA polymerase and restriction endonucleases were purchased from Takara (Beijing, China). Homologous recombinases were purchased from Yeasen (Shanghai, China). Adenosine triphosphate, guanosine triphosphate, cytidine triphosphate, and uridine triphosphate were purchased from Sangon Biotech (Shanghai, China). Acetyl-CoA, antibiotics, and lysozyme were purchased from Yuanye Biotech (Shanghai, China). Thymidine triphosphate, nicotinamide mononucleotide, and nicotinamide adenine dinucleotide phosphate were purchased from Sigma-Aldrich (Shanghai, China). Nicotinamide uracil dinucleotide was purchased from Jena Bioscience (Dortmund, Germany). Other reagents were purchased from Biological Group (Shanghai, China).

Genetic manipulations

CRISPR-Cas9-based technology was used for chromosomal editing, including gene deletion, gene replacement, chromosomal integration, and strain streamlining, to construct the engineered *E. coli* strains⁸¹. All plasmids were constructed by one-step cloning kit procedure, Gibson Assembly with the NEBuilder kit and ligation cloning procedures. The genes were codon-optimized for *E. coli* and synthesized by Suzhou AZENTA Biotechnology. Engineered *HsNmnat*^{*}, *CnMDH*^{*}, *TsFDH*^{*}, *BmFADH*^{*}, and *PcME*^{*} variants were generated from the corresponding

parental genes by PCR-based mutagenesis. For CRISPR-Cas9-mediated editing, target-specific N2O sequences were designed using the ATUM gRNA Design Tool (<https://atum.bio/eCommerce/cas9/input>) and introduced into pTARGET by whole-plasmid PCR. Information on gene deletions and streamlined regions is provided in Supplementary Data 4, target loci and corresponding pTARGET N2O sequences are provided in Supplementary Data 5, and primers used for strain construction and verification are listed in Supplementary Data 6.

Protein expression and purification

His₆-tagged *HsNmnat*, *CnMDH*, *TsFDH*, *BmFADH*, *PcME*, and their engineered variants were heterologously expressed in *E. coli* BL21(DE3). Cells were cultivated in LB medium at 37 °C, and protein expression was induced with IPTG at 15–20 °C for 16 h. Cells were harvested and washed three times with 100 mM PBS before preparation of cell extracts. The extracts were loaded onto Ni-NTA columns equilibrated with binding buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10 mM imidazole at a flow rate of 0.5 mL min⁻¹. The columns were washed with buffer containing 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 50 mM imidazole, and the target proteins were subsequently eluted with 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 500 mM imidazole. Protein concentrations were determined by UV absorbance using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific), and purified proteins were stored at -40 °C in the presence of 15% glycerol.

Protein engineering and assays

HsNmnat was engineered to alter its nucleotide-substrate specificity from ATP toward UTP, whereas *CnMDH* was engineered to shift its cofactor preference from NAD toward NUD, thereby enabling biosynthesis and regeneration of the orthogonal NUD(H) system. For *HsNmnat*, residues surrounding the uracil-containing substrate-binding region were evaluated by structural and evolutionary conservation analyses, and non-conserved residues within the binding pocket were subjected to site-saturation mutagenesis. For activity assays, reactions were performed in a 200 µL mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM MnCl₂, 12 mM MgCl₂, 2.0 mM NMN, 2.5 mM UTP, and purified enzyme at 30 °C for 3 min. Reactions were terminated by addition of perchloric acid and subsequently neutralized with potassium carbonate. Residual UTP was quantified using a commercial UTP ELISA kit (Cloud-Clone Corp., Wuhan, China) according to the manufacturer's instructions, with absorbance measured at 450 nm. Variants exhibiting lower residual UTP levels, corresponding to increased UTP consumption under identical reaction conditions, were selected as candidates with enhanced UTP-dependent catalytic activity.

For *CnMDH*, residues surrounding the cofactor-binding pocket were evaluated by structural and evolutionary conservation analyses, and selected non-conserved residues were subjected to site-saturation mutagenesis. Variants were initially screened using a formaldehyde-responsive PfrmR-GFP biosensor in *E. coli* expression host supplied with *HsNmnat*^{*}. Following induced expression, cells were transferred to 96-well plates at a reaction volume of 200 µL per well, and methanol was supplied at final concentrations of 500 mM as the substrate for *CnMDH*-catalyzed oxidation. Reactions were conducted at 37 °C, and intracellular formaldehyde formation was monitored indirectly through PfrmR-dependent GFP expression, with fluorescence measured at excitation and emission wavelengths of 488 and 525 nm, respectively. Variants showing increased GFP signals in the NUD-supplying background while maintaining low signals in the corresponding control background were selected as candidates with enhanced NUD-dependent methanol oxidation activity. Candidate variants were subsequently evaluated by direct quantification of formaldehyde using the Nash colorimetric assay. A 200 µL aliquot of each reaction culture was collected and centrifuged, and 125 µL of the resulting supernatant was mixed with an equal volume of freshly

prepared Nash reagent containing 5 M ammonium acetate and 50 mM acetylacetone. After incubation at 37 °C in the dark to allow development of the yellow chromophore, absorbance was measured at 412 nm to quantify formaldehyde formation. Variants producing higher amounts of formaldehyde under the NUD-supplying condition were preferentially selected as beneficial variants with enhanced NUD-dependent methanol oxidation activity.

The cofactor preferences of *TsFDH*, *BmFADH*, and *PcME* were reprogrammed from NADH to NUDH using the IESE (Identification, Exclusion, Screening, and Evaluation) protein-engineering workflow. Candidate residues surrounding the NUDH-binding pocket were identified by molecular docking and binding-energy calculations, while highly conserved residues were excluded on the basis of ConSurf analysis. The remaining candidate positions were subjected to focused mutagenesis using TDS, VYG, VWA, and VRT degenerate codons. Mutations were introduced by PCR into the corresponding pET28a plasmids, followed by DpnI digestion of the parental templates and activity-based screening in 96-well plates. Beneficial mutations were accumulated through iterative rounds of screening to obtain engineered variants. Detailed computational procedures, target residues, library design, mutation combinations, and iterative screening strategies are provided in Supplementary Note 1.

Intracellular cofactor analysis

Intracellular concentrations were determined by dividing the total molar amount of each metabolite by the total intracellular aqueous volume. The intracellular volume was estimated assuming 1×10^9 cells per mL of culture per OD₆₀₀ unit and an average cell volume of 1×10^{-15} L per *E. coli* cell⁸². Based on the physicochemical properties of the target metabolites, two specific extraction and detection strategies were employed in this study. An acidic rapid quenching method was adopted for nucleoside triphosphate (NTP) metabolites. Cultures in the exponential growth phase, appropriately diluted to an OD₆₀₀ of 0.35, were rapidly collected by centrifugation. The cell pellets were immediately immersed in 2.5 mL of pre-cooled (−20 °C) acetonitrile/methanol/water (40:40:20, v/v/v) extraction solvent containing 0.1 M formic acid to achieve rapid protein denaturation and inactivation of metabolic enzymes. After a 15-min incubation for extraction, the cell pellets were rinsed again with an additional 1 mL of extraction solvent. The combined extracts were immediately neutralized using ammonium hydroxide or 5 M potassium carbonate. Following centrifugation at $12,000 \times g$ for 10 min at 4 °C, the supernatants were analyzed using a Waters ACQUITY UPLC system. Chromatographic separation was performed on a Waters ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm) with the column temperature set at 35 °C and a flow rate of 0.3 mL/min. Mobile phase A consisted of a buffer solution containing 10 mM tetrabutylammonium hydroxide and 10 mM potassium phosphate (pH 6.9), while mobile phase B was a 30% phosphate/methanol solution containing 5.6 mM tetrabutylammonium hydroxide (pH 7.0) to achieve separation and quantification of NTPs.

To prevent acid-induced degradation, a neutral cold-solvent extraction method was used for nicotinamide mononucleotide, nicotinamide adenine dinucleotide, and nicotinamide uracil dinucleotide^{83,84}. Approximately 2.0×10^9 cells were collected by rapid centrifugation at 4 °C and washed once with ice-cold PBS buffer to remove residual medium. The cell pellets were resuspended in 500 μL of pre-cooled (−20 °C) acetonitrile/methanol/water (40:40:20, v/v/v) mixture and incubated on ice for 20 min to ensure complete metabolite release. The resulting supernatants were analyzed using a UPLC system equipped with a Waters Atlantis PREMIER BEH C18 AX column (2.1 × 100 mm, 1.7 μm). Mobile phase A was a 10 mM ammonium formate solution (adjusted to pH 9.0 with ammonium hydroxide to balance the stability of NMN and reduced cofactors), and mobile phase B was 100% acetonitrile. The following elution gradient was applied:

0–2 min, 99% A; 2–4 min, 99–88% A; 4–5 min, 88–10% A; 5–6 min, 10% A; 6–7 min, 10–99% A; 7–12 min, 99% A.

Resting cell catalysis

Resting-cell bioconversions were performed as described previously with slight modifications^{6,49,85}. The reaction medium was based on a modified M9 salt medium supplemented with Na₂HPO₄ 6.78 g/L, 3.0 g/L KH₂PO₄, 1.0 g/L NH₄Cl, 0.5 g/L NaCl, 0.5 g/L cytidine, 0.3 g/L MgSO₄, 0.015 g/L CaCl₂⁸⁶, supplemented with 1% (v/v) trace-element solution. The trace-element solution contained 1.6 g/L FeCl₃, 0.1 g/L CuCl₂, 0.2 g/L Na₂MnO₄, 0.2 g/L CoCl₂·6H₂O, 0.05 g/L H₃BO₃, 0.2 g/L ZnCl₂·4H₂O dissolved in 0.1 M HCl. Cells were cultivated for 12 h in modified LB medium supplemented with 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 0.5 g/L cytidine. Cells were harvested by centrifugation at $4000 \times g$ for 5 min at 4 °C and washed three times with M9 salt medium to remove residual nutrients. The washed cells were resuspended in fresh M9 salt medium to an OD₆₀₀ of 10. Resting-cell reactions were conducted in 250-mL shake flasks containing 150 mL of cell suspension supplemented with 400 mM NaHCO₃ and 400 mM methanol as the inorganic carbon source and electron donor, respectively. The reactions were performed at 37 °C and 100 rpm. Samples were withdrawn at regular intervals to monitor substrate depletion and product formation, and the reaction was continued until no further increase in product concentration was observed.

Growth and bioproduction tests of engineered *E. coli* strains

Engineered *E. coli* strains were cultivated in NBS minimal medium containing 25.7 mM KH₂PO₄, 28.7 mM K₂HPO₄, 26.5 mM (NH₄)₂HPO₄, 2.06 mM cytidine, 2.0 mM MgSO₄·7H₂O, 0.15 mM FeSO₄, 102 μM CaCl₂·2H₂O, 50 μM vitamin B1, and 1.5% (v/v) trace elements stock solution²⁷. The trace-element stock solution contained 9.9 mM FeCl₃, 840.6 μM CoCl₂·6H₂O, 743.7 μM CuCl₂, 959.8 μM ZnCl₂·4H₂O, 1.4 mM Na₂MnO₄, 808.7 μM H₃BO₃, dissolved in 0.1 M HCl. IPTG was added to a final concentration of 0.1 mM to induce expression of the synthetic pathway enzymes. Because the engineered strains were deficient in acetyl-CoA biosynthesis due to deletion of *aceEF*, *poxB*, and *pflB*, seed cultures were prepared in LB medium and 15 mM acetate to support initial growth. To minimize carryover effects of these exogenous carbon sources in subsequent growth assays, seed cells were harvested by centrifugation, washed twice with sterile PBS, and resuspended before inoculation. For the growth assays described in Result 3, cultivations were carried out in a 500-mL reactor with a working volume of 220 mL and an initial inoculation density of approximately OD₆₀₀ 0.015. Cultures were maintained at 37 °C and pH 7.0 in a controlled-aeration shaking incubator operating at 200 rpm. For the growth assays described in Result 4, cultivations were performed in a 2-L controlled bioreactor with an initial working volume of 1.2 L and an initial inoculation density of approximately OD₆₀₀ 0.5. The bioreactor was maintained at 37 °C and pH 7.0 with agitation at 750 rpm. Methanol was supplied as the electron donor and maintained at approximately 100 mM by fed-batch addition. Methanol concentrations were determined by HPLC using an UltiMate 3000 system equipped with a Rezex ROA-Organic Acid H⁺ column (300 × 7.8 mm; Phenomenex) and a refractive index detector. The column was eluted isocratically with 2.5 mM H₂SO₄ at a flow rate of 0.6 mL/min. Cultures were grown under a gas mixture of 20% CO₂, 4% O₂, and 76% N₂, with the pressure maintained at 0.05 MPa. After gas equilibration, the medium pH was readjusted to 7.0. Samples were collected periodically to monitor cell growth by measuring OD₆₀₀. For bioproduction experiments, expression of the synthetic pathway enzymes was induced when the culture reached an OD₆₀₀ of approximately 10.0, and cultivation was continued in fed-batch mode until product accumulation reached a plateau.

Flow cytometry assay

Engineered *E. coli* cells were collected from cultures grown under the CO₂/methanol conditions described above and harvested by centrifugation at 4000 × *g* for 5 min at 4 °C. Cells were washed twice with pre-chilled PBS and resuspended in PBS. Cell suspensions were stained with propidium iodide (PI) for 15 min at room temperature in the dark. PI fluorescence was measured using a BD FACS Aria III flow cytometer (BD Biosciences, USA) with excitation at 561 nm and a 670/14-nm emission filter. All data was collected by FlowJo software. Flow cytometry data were analyzed using FlowJo v10.

Fluorescence microscopy

Engineered *E. coli* cells expressing GFP-tagged proteins were harvested by centrifugation, washed twice with PBS, and resuspended in PBS. A 2 µL aliquot of the cell suspension was placed on a glass slide, and fluorescence images were acquired using a Leica TCS SP8 confocal laser-scanning microscope equipped with a ×1000 oil-immersion objective. GFP was excited at 488 nm, and fluorescence emission was collected in the GFP channel.

Intracellular metabolite analysis and ¹³C-isotope tracing

Intracellular phosphoenolpyruvate (PEP), glyceraldehyde 3-phosphate (GAP), fructose 6-phosphate (F6P), and glucose 6-phosphate (G6P) were quantified in strains K15, K17, and K18 using three biologically independent cultures per strain (*n* = 3), with K15 serving as the reference strain. Approximately 25 mg of sample was transferred into a 2.0-mL centrifuge tube containing two small steel beads, followed by the addition of 500 µL of methanol–water extraction solvent (4:1, v/v), pre-cooled to −40 °C. The mixture was vortexed for 30 s, ground at 40 Hz for 4 min, and sonicated in an ice-water bath for 5 min. The grinding–sonication cycle was repeated three times. After incubation at −40 °C for 1 h, the extract was centrifuged at 12,000 × *g* for 15 min at 4 °C, and the supernatant was transferred into an autosampler vial for analysis. Chromatographic separation was performed using an Agilent 1290 Infinity II ultra-high-performance liquid chromatography system. Reversed-phase chromatography was conducted using an ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 × 150 mm), with ultrapure water containing 0.1% formic acid as mobile phase A and methanol–ultrapure water (95:5, v/v) containing 10 mmol L^{−1} ammonium formate as mobile phase B. Hydrophilic interaction liquid chromatography was performed using an Atlantis Premier BEH Z-HILIC column (1.7 µm, 2.1 × 150 mm), with ultrapure water–acetonitrile (90:10, v/v) containing 10 mmol L^{−1} ammonium acetate as mobile phase A and ultrapure water–acetonitrile (10:90, v/v) containing 10 mmol L^{−1} ammonium acetate as mobile phase B. The autosampler temperature was maintained at 6 °C, and the injection volume was 2 µL. Mass spectrometric detection was performed using a SCIEX Triple Quad 6500+ triple-quadrupole mass spectrometer equipped with an IonDrive Turbo V electrospray ionization source. Data were acquired in multiple reaction monitoring mode. The ion-source parameters were as follows: curtain gas, 35 psi; ion-spray voltage, +5500 V in positive-ion mode and −4500 V in negative-ion mode; source temperature, 400 °C; ion source gas 1, 50 psi; and ion source gas 2, 50 psi. Mass spectrometric data acquisition and quantitative analysis were performed using SCIEX Analyst Workstation Software v1.7.3 and BioBud v2.0.4, respectively.

For ¹³C-isotope tracing, two complementary labeling conditions were used to distinguish the carbon contributions from CO₂ and methanol: ¹³CO₂ + ¹²C-methanol and ¹²CO₂ + ¹³C-methanol. For the ¹³CO₂-labeling condition, ¹³CO₂ was supplied in place of unlabeled CO₂ while methanol remained unlabeled; for the complementary methanol-labeling condition, unlabeled CO₂ was supplied together with ¹³C-methanol. Cells were cultivated to the exponential growth phase, and 2 mL of culture was collected by

centrifugation at 8000 × *g* and 4 °C for 5 min. The cell pellets were resuspended in a pre-cooled extraction mixture containing 300 µL of −20 °C methanol, 300 µL of −20 °C chloroform, and 250 µL of 4 °C water. The aqueous metabolite-containing supernatant was transferred into a 1.5-mL microcentrifuge tube and freeze-dried at low temperature. The dried extracts were resuspended in 400 µL of acetonitrile/water (55:45, v/v), sonicated for 10 min, and centrifuged at 12,000 × *g* and 4 °C for 10 min to remove insoluble material. The resulting supernatant was transferred into an HPLC vial and stored at −80 °C until LC–MS/MS analysis. The total labeled-carbon fraction was calculated from the full MID by weighted averaging:

$$\text{Labelled fraction} = \frac{\sum_i^n m_i^* i}{\sum_i^n m_i^* n} \quad (4)$$

Statistics and reproducibility

All quantitative data are presented as mean ± s.d. unless otherwise stated. Data analysis and graphing were performed using Origin 2019. Statistical significance was assessed using appropriate two-tailed Student's *t*-tests in IBM SPSS Statistics R24.0.0, with correction for multiple comparisons where applicable, as specified in the corresponding figure legends. No statistical method was used to predetermine sample size. Unless otherwise stated, experiments were performed with at least three biologically independent replicates, and exact sample sizes are reported in the corresponding figure legends. No data were excluded from the analyses. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available within the Article, Supplementary Information and Source Data file. Source data are provided with this paper and are also deposited in Figshare under <https://doi.org/10.6084/m9.figshare.33264735> [<https://doi.org/10.6084/m9.figshare.33264735>]. The protein structures used for structural analyses are available from the Protein Data Bank under accession codes **1KQN** for *HsNmnat* and **3WRS** for *TsFDH*. Source data are provided with this paper.

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L.M.L., J.N., K.F.L., and C.G. conceived the project and wrote the paper. K.F.L., G.J.L., G.P.H., H.Q.C., X.M.L., W.Q.W., W.S., and J.L. designed and performed the experiments. K.F.L., G.J.L., G.P.H. and W.Q.W. analyzed the results. L.M.L. and C.G. supervised the project. L.M.L., K.F.L., and H.Q.C. acquired funding.

Competing interests

The authors declare no competing interests.

Additional information

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