Artificial pathway emergence in central metabolism from three recursive phosphoketolase reactions

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Introduction
The evolutionary trajectory that gives rise to metabolic pathways is a central topic in evolutionary biology and biochemistry. Multiple theories have been suggested to explain how pathways emerge [1]. These include the classic retrograde evolution model [2], the forward, stepwise recruitment model [3], the pathway duplication–divergence model [4,5], and the patchwork model [6]. Despite their differences, most of these models share a common premise that new enzymatic activities—required to support the new pathway—originate from the promiscuous activity of generalist enzymes [7]. Especially interesting are cases where specialist enzymes of a common ancestry operate consecutively, indicating that a single generalist enzyme may have initially supported pathway activity; examples include heme and chlorophyll biosynthesis [8], tryptophan biosynthesis [9], methionine biosynthesis [10], and biosynthesis of peptidoglycan [11]. In this study, we use a synthetic biology approach to recreate this evolutionary setup in central metabolism and explore how cellular physiology adapts to accommodate and support a synthetic route that relies on three consecutive reactions of a single enzyme—phosphoketolase.

Phosphoketolase (PKT) is a unique enzyme that cleaves D-xylulose 5-phosphate (Xu5P) and D-fructose 6-phosphate. Xu5P is cleaved by PKT into D-fructose 6-phosphate (F6P) and D-ribulose 5-phosphate (Ru5P), which is then cleaved into D-sedoheptulose 7-phosphate (S7P) and D-ribulose 5-phosphate (Ru5P). This reaction sequence is known as the phosphoketolase reaction and is a key step in the pentose phosphate pathway (PPP). The promiscuous activities of a recursive, generalist enzyme provide raw material for the emergence of metabolic pathways. Here, we use a synthetic biology approach to recreate such an evolutionary setup in central metabolism and explore how cellular physiology adjusts to enable recursive catalysis. We generate an Escherichia coli strain deleted in transketolase and glucose 6-phosphate dehydrogenase, effectively eliminating the native pentose phosphate pathway. We demonstrate that the overexpression of phosphoketolase restores prototrophic growth by catalyzing three consecutive reactions, cleaving xylulose 5-phosphate, fructose 6-phosphate, and, notably, sedoheptulose 7-phosphate. We find that the activity of the resulting synthetic pathway becomes possible due to the recalibration of steady-state concentrations of key metabolites, such that the in vivo cleavage rates of all three phosphoketolase substrates are similar. This study demonstrates our ability to rewrite one of nature’s most conserved pathways and provides insight into the flexibility of cellular metabolism during pathway emergence.

Abbreviations
E4P, D-erythrose 4-phosphate; F6P, D-fructose 6-phosphate; GAP, D-glyceraldehyde 3-phosphate; R5P, D-ribose 5-phosphate; Ru5P, D-ribulose 5-phosphate; S7P, D-sedoheptulose 7-phosphate; Xu5P, D-xylulose 5-phosphate.
6-phosphate (F6P) to acetyl phosphate and the corresponding aldose phosphate, i.e., d-glyceraldehyde 3-phosphate (GAP) and d-erythrose 4-phosphate (E4P), respectively [12]. This enzyme was extensively used in metabolic engineering [13–17] and was shown to enable an alternative glycolytic structure, cleaving sugar phosphates into C2 moieties without the release of CO₂ or the production of reducing power [18,19]. In these metabolic engineering studies, as well as in all microbes that naturally utilize PKT, this enzyme is active alongside its catalytic ‘sibling’ transketolase [12]. Hence, the basic structure of the pentose phosphate pathway in these strains remains undisturbed.

We use several gene deletion strains—each auxotrophic to a different set of essential biomass precursors—to demonstrate high in vivo activity of PKT using the substrates Xu5P, F6P, and, notably, d-sedoheptulose 7-phosphate (S7P). We then show that the synthetic phosphoketolase shunt, which depends on three consecutive reactions of PKT (Fig. 1), restores the prototrophic growth of a strain in which the activity of the (oxidative and non-oxidative) pentose phosphate pathway is eliminated. We provide evidence that the activity of the new pathway is made possible by the accumulation of S7P, such that the in vivo cleavage rates of Xu5P, F6P, and S7P are similar. Our findings serve to demonstrate that the establishment of a novel pathway based on recursive chemistry can be straightforward, indicating that the emergence of pathways in a similar manner might have been common during evolution.

**Results and Discussion**

First, we selected for the primary reaction associated with PKT, that is, Xu5P cleavage. For this, we constructed a strain deleted in d-ribose 5-phosphate (R5P) isomerase (ΔripIAB). As shown in Fig. 2A,B, this strain could not grow when provided with either xylose or ribose as a sole carbon source, but growth was restored when both sugars were provided in the medium. Overexpression of PKT from *Bifidobacterium adolescentis* enabled the strain to grow on xylose but not ribose, as Xu5P is cleaved to GAP and acetyl phosphate, both serving as carbon sources for microbial growth. In this case, the production of the essential metabolite R5P—a building block in the biosynthesis of nucleotides and histidine—is supported by the activity of the pentose phosphate pathway.

Next, we explored the in vivo cleavage of F6P. We constructed a strain deleted in transketolase (ΔtktAB). This strain cannot synthesize E4P and hence must be supplemented with small amounts of essential biomass building blocks whose biosynthesis is dependent on

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**Fig. 1.** The phosphoketolase shunt. Phosphoketolase (PKT) replacing transketolase and the oxidative pentose phosphate pathway by enabling the production and recycling of all essential sugar phosphates by the cleavage of three compounds: d-xylulose 5-phosphate (blue arrow), d-fructose 6-phosphate (green arrow), and d-sedoheptulose 7-phosphate (purple arrow).

**Fig. 2.** Phosphoketolase effectively cleaves three sugar phosphates in vivo. (A) Selection scheme for the cleavage of Xu5P by PKT upon deletion of R5P isomerase (ΔripIAB) and feeding with xylose as sole carbon source—all biomass depends on the cleavage of Xu5P to GAP; (B) expression of PKT rescues the growth of a strain deleted in R5P isomerase (ΔripIAB) using 15 mM xylose as a carbon source; (C) selection scheme for the cleavage of F6P by PKT upon deletion of transketolase (ΔtktAB)—production of E4P is dependent on F6P cleavage; (D) expression of PKT rescues the growth of a strain deleted in transketolase (ΔtktAB) using 20 mM glycerol, 10 mM glucose, 15 mM xylose, or 20 mM succinate as carbon sources; (E) selection scheme for the cleavage of S7P by PKT upon deletion of transketolase and R5P isomerase (ΔtktAB ΔripIAB)—production of R5P depends on S7P cleavage; (F) expression of PKT rescues the growth of a strain deleted in transketolase and R5P isomerase (ΔtktAB ΔripIAB) using 20 mM glycerol, 10 mM glucose, 15 mM xylose, or 20 mM succinate as carbon sources; (G) selection scheme for the activity of the PKT shunt upon deletion of transketolase and glucose 6-phosphate dehydrogenase (ΔtktAB ΔgapA)—production of E4P and pentose phosphates depends on PKT activity; (H) PKT rescues the growth of a strain in which the non-oxidative and oxidative pentose phosphate pathways were deleted (ΔtktAB ΔripIAB). In (A), (C), (E), and (G), carbon sources are shown in light brown, gene deletions in red, and PKT-dependent cleavage reactions in blue, green, and purple arrows. Ribose, when required, was added at 15 mM.
Three recursive phosphoketolase reactions

A. Selection for Xu5P cleavage

B. ΔrpiAB

C. Selection for Xu5P & F6P cleavage

D. ΔtktAB

E. Selection for Xu5P, F6P & S7P cleavage

F. ΔtktAB ΔrpiAB

G. Selection for phosphoketolase shunt

H. ΔtktAB Δzwf

Legend:
- +PKT, xylose
- +PKT, ribose
- -PKT, xylose
- -PKT, ribose
- +PKT, succinate
- -PKT, succinate
- +PKT, glucose
- -PKT, glucose
- +PKT, xylose
- -PKT, xylose
- +PKT, glycerol
- -PKT, glycerol
- +PKT, E4Psup
- -PKT, E4Psup
- +PKT, ribose
- -PKT, ribose
- +PKT, glycerol, E4Psup
- -PKT, glycerol, E4Psup
- +PKT, ribose
- -PKT, ribose

Selection for Xu5P cleavage

Selection for Xu5P, F6P & S7P cleavage

Selection for phosphoketolase shunt
E4P: phenylalanine, tyrosine, tryptophan, shikimate, pyridoxine, 4-aminobenzoate, 4-hydroxybenzoate, and 2,3-dihydroxybenzoate (referred to as E4P-supplements). Pentose phosphates can be produced in this strain by the activity of the oxidative pentose phosphate pathway (Fig. 2C). As shown in Fig. 2D, growth of the ΔtktAB strain was indeed possible only when E4P-supplements were added to a medium in which glycerol serves as a primary carbon source. When PKT was expressed, prototrophic growth was restored by using glycerol, glucose, xylose, or succinate as a sole carbon source (E4P-supplements were not needed), demonstrating efficient cleavage of F6P to provide E4P for cell growth. Of particular interest is the growth of the strain on xylose, which requires two sequential cleavage reactions: Xu5P cleavage to provide the cell with biomass precursors (blue arrow in Fig. 2C) and F6P cleavage to generate E4P (green arrow).

We then turned our attention to S7P as a potential substrate for PKT. While sporadic reports indicate that PKT can cleave S7P to R5P and acetyl phosphate [20,21], it remained unclear whether this reaction could be of metabolic significance within the cellular environment. To test this reaction in vivo, we constructed a ΔrpiAB ΔtktAB strain. As shown in Fig. 2E, F, this strain could grow only when E4P-supplements and ribose were added to a minimal medium in which glycerol served as a carbon source. Expression of PKT restored prototrophic growth, demonstrating an efficient sequence of PKT-dependent conversions: F6P is cleaved to E4P, which is used to generate S7P via the activity of transaldolase; S7P is then cleaved to provide ribose 5-phosphate. Growth on xylose is especially impressive as it requires all three PKT activities: cleavage of Xu5P to provide the cells with biomass precursors (blue arrow in Fig. 2E), cleavage of F6P for the biosynthesis of E4P and R5P (green arrow) and cleavage of S7P to produce R5P (purple arrow). As R5P provides 10% of the carbons in biomass [22], the cleavage of all three phosphosugars must proceed at high rate to enable the growth shown in Fig. 2F.

After confirming high rates of PKT-dependent cleavage of Xu5P, F6P, and S7P, we aimed to replace the pentose phosphate pathway with the phosphoketolase shunt. We generated a strain in which both transketolase and glucose 6-phosphate dehydrogenase were deleted (ΔtktAB Δzwf), effectively eliminating the activity of the (oxidative and non-oxidative) pentose phosphate pathway (Fig. 2G). As shown in Fig. 2H, this strain could grow only when ribose and E4P-supplements were added to a minimal medium with glycerol as a carbon source. Upon expression of PKT, prototrophic growth was restored on a wide array of carbon sources, demonstrating high activity of the phosphoketolase shunt (Fig. 1).

To confirm that the metabolic flux is in line with the activity of the phosphoketolase shunt we conducted carbon labeling experiments. We fed the ΔtktAB Δzwf strain overexpressing PKT with glucose labeled either in the first, second, third, or sixth position, i.e., Glu-1-13C, Glu-2-13C, Glu-3-13C, and Glu-6-13C. We followed the labeling pattern within proteinogenic histidine: five of the histidine carbons originate from R5P and one carbon originates from the C1 carbon of formyl-THF [23]. Formyl-THF is mainly derived from the third carbon of 3-phosphoglycerate (3PG) via serine biosynthesis and cleavage (a small amount of formyl-THF originates from the second carbon of 3PG, via glycine cleavage) [24]. Hence, we can predict the labeling pattern of histidine when the phosphoketolase shunt replaces the pentose phosphate pathway and upon feeding with the differentially labeled glucose molecules. As shown in Fig. 3A, feeding with Glu-1-13C should result in unlabeled R5P and half-labeled formyl-THF (F6P cleavage generates two 3PG molecules, only one is labeled); hence, half of the histidine molecules should be labeled once and half unlabeled. Feeding with Glu-2-13C should result in unlabeled R5P and formyl-THF such that histidine should be completely unlabeled.

![Fig. 3. 13C-labeling of histidine confirms the activity of the phosphoketolase shunt. Predicted and observed labeling of histidine upon feeding with glucose labeled at the (A) first carbon (Glu-1-13C); (B) second carbon (Glu-2-13C); (C) third carbon (Glu-3-13C); and (D) sixth carbon (Glu-6-13C). The carbon atoms of histidine are derived from those of R5P and the one-carbon unit carried by formyl-THF. The labeling of histidine in the PKT shunt-dependent strain, i.e., ΔtktAB Δzwf overexpressing PKT, is compared to the labeling within control strains in which the biosynthesis of histidine is not dependent on the activity of the PKT shunt. We note that, generally speaking, we expect half of the GAP molecules produced via glycolysis to be labeled once regardless of the identity of the labeled carbon in glucose (one labeled carbon in a C6 compound is converted to half a label in a C3 compound). Yet, the activity of transaldolase changes this 1:1 labeled-unlabeled ratio: if glucose is labeled in positions 1, 2, or 3, transaldolase will generate an unlabeled GAP, and vice versa, glucose labeled in positions 4, 5, or 6 results in transaldolase producing labeled GAP. This explains why the labeling of histidine upon feeding with Glu-1-13C (in ΔtktAB Δzwf strain overexpressing PKT) is somewhat lower than 50%, while feeding with Glu-6-13C results in somewhat more than 50% of the histidine molecules labeled twice. We further note that the slight labeling of histidine when Glu-2-13C served as a carbon source is attributed to the small amount of labeled glycine cleaved to produce formyl-THF.](image-url)
Three recursive phosphoketolase reactions
(Fig. 3B). With Glu-3-13C as a carbon source, R5P should be labeled twice and formyl-THF unlabeled, resulting in histidine labeled twice (Fig. 3C). Finally, feeding with Glu-6-13C should result in R5P labeled once and formyl-THF half-labeled, leading to half of the histidine molecules labeled once and half-labeled twice (Fig. 3D). As shown in Fig. 3, the measured labeling of histidine matches the expected patterns, confirming the activity of the phosphoketolase shunt.

We speculated that the efficient cleavage of S7P within the ΔtktAB strains is possible due to the accumulation of this intermediate, which usually exists at low intracellular concentrations (~0.2 mM [25]). Specifically, as the cleavage of F6P to E4P is irreversible, it is expected that E4P and S7P — linked by the transaldolase reaction — will accumulate, until the concentration of S7P is high enough to support cleavage by PKT. To test this hypothesis, we measured and compared the concentrations of sugar phosphates in the WT strain and in the ΔtktAB Δzwf strain overexpressing PKT by LC-MS/MS. As shown in Fig. 4, the steady-state concentration of S7P showed a dramatic increase in concentration from ~0.1 mM (WT) to almost 3 mM (ΔtktAB Δzwf overexpressing PKT), confirming our hypothesis. While the concentration of E4P cannot be directly measured, if we assume that transaldolase and triose phosphate isomerase work close to equilibrium, we can estimate that the concentration of this tetrose phosphate increased from ~7 μM (WT) to almost 200 μM (ΔtktAB Δzwf overexpressing PKT). Correspondingly, the concentrations of the pentose phosphates Xu5P and D-ribulose 5-phosphate (Ru5P) fell dramatically in the engineered strain.

We measured the kinetic parameters of PKT with its three substrates Xu5P, F6P, and S7P. As shown in Table 1, the $k_{cat}$ and $K_M$ values associated with S7P—3.5 s$^{-1}$ and 68 mM, respectively—are considerably worse than with F6P (10.5 s$^{-1}$ and 22.7 mM) or Xu5P (17.8 s$^{-1}$ and 6.7 mM). Considering the concentrations of the metabolites in a WT strain, the rate of the PKT reaction with S7P would be ~150-fold lower than with Xu5P and ~80-fold lower than with F6P (Table 1). In this case, S7P cleavage would be virtually negligible. However, in the ΔtktAB Δzwf strain, due to the dramatic increase in the concentration of S7P and the drop in the concentration of Xu5P, the rate of PKT with S7P should be practically identical to that with Xu5P, and only threefold lower than with F6P, the entry point of the synthetic pathway (Table 1). This
confirms our hypothesis that, in the ΔtktAB Δzwf strain, S7P accumulates to a level high enough to make its cleavage sufficiently fast to support the activity of the phosphoketolase shunt.

This study demonstrates that the function of the pentose phosphate pathway—production of essential sugar phosphates—can be taken over by a synthetic shunt dependent on the triple activity of a single enzyme. This was established without global rewiring of endogenous metabolism or its regulatory network, suggesting that cellular physiology can easily adapt to accommodate recursive catalysis. This further indicates that recursive chemistry could have been a prominent evolutionary mechanism to smoothly facilitate the emergence of new pathways. In particular, such recursive chemistry fits nicely with Granick’s ‘Forward Pathway Evolution’ model [1,3] where a generalist enzyme catalyzing the same chemical modification on different pathway intermediates gives rise to a set of useful metabolites, each with a concrete physiological significance; in our case, phosphoketolase first produces E4P for the biosynthesis of aromatic amino acids and cofactors and then R5P for nucleotide and histidine biosynthesis. In many cases, following gene duplication, a generalist enzyme gives rise to specialist enzymes, as is the case for MetB and MetC that operate sequentially in the methionine biosynthesis pathway [10] and for MurC, MurD, MurE, and MurF, the sequential activity of which support peptidoglycan biosynthesis [11]. Yet, in some cases, a generalist enzyme could have sufficiently high activity with all relevant substrates and thus retain its key metabolic role without further specialization; the best example for this is the catalytic sibling of phosphoketolase, i.e., transketolase, which catalyzes two essential reactions in the pentose phosphate pathway. Our study demonstrates this latter option using a synthetic biology approach, where a generalist enzyme that supports consecutive reactions at high enough activity is able to modify central metabolism without hampering growth.

**Methods**

**Escherichia coli strains and gene deletions**

*Escherichia coli* strain SI488 was used as a background for gene deletions. In the SI488 strain, a MG1655 derivative, arabinose-inducible lambda Red recombinering genes and a rhamnose-inducible flippase gene are integrated into the genome [26]. This strain allows plasmid free and therefore fast deletion of multiple genes. Gene deletions using kanamycin (FRT-PGK-gb2-neo-FRT (KAN), Gene Bridges, Germany) or chloramphenicol cassettes (pKD3 [27]) were carried out as described by Baba et al. [28]. All strains used in this study are listed in Table 2. For gene deletions, antibiotic resistance cassettes were amplified with 50 bp overhangs to enable homologous recombination. Oligonucleotides used for amplification of gene deletion cassettes and verification of gene deletions are listed in Table S1.

**Construction of PKT overexpression construct**

Cloning and plasmid propagation were carried out in *E. coli* DH5α. An *E. coli* codon adapted version of the phosphoketolase gene from *Bifidobacterium adolescentis* (pkt, UniProt: A1A185, previously used in multiple metabolic engineering studies [18,19,29]) was synthesized by Life Technologies (Thermo Fisher Cambridge, MA, USA). Optimization of codon usage was done using JCat [30], while excluding recognition sites of restriction enzymes involved in the cloning process: *EcoRI*, *NheI*, *Mph1103I*, *PsI*, *SalI*, *BcaI*, *XhoI*. The codon optimized sequence is shown in the Supporting Information. A HIS-tag was added after the start codon. The PKT gene was amplified by PCR using primers pkt-F and pkt-R and cloned into cloning vector pJet (CloneJet PCR Cloning Kit, Thermo Scientific). The resulting plasmid was cut with enzymes *Mph1103I* and *NheI* in order to ligate the gene into a *Mph1103I* and *NheI*-digested pNivC vector (with ampicillin resistance marker), where it was attached to ribosome binding site (RBS) ‘C’ (AAGTTAAGAGGCAAGA) which allows a moderate translation rate [31]. The RBS₉-PKT segment was cloned into vector pZ-ASS using *EcoRI* and *PsI*. pZ-ASS replicates under p15A, a medium-copy

### Table 2. Strains used in this study. KAN corresponds to kanamycin and CAP to chloramphenicol resistance markers.

| Strain name | Deletions/Genotype | Description | Source |
|-------------|-------------------|-------------|--------|
| SIJ488      | E. coli K-12 MG1655Tn7::para-exo-beta-gam; prha-FLP; xyISpm-lscl | MG1655 derivative with genome integrated recombinase and flippase genes | [26] |
| ΔrpiAB      | SIJ488 ΔrpiB, rpiA::KAN | Ribose-5-P isomerase deletion strain | This study |
| ΔtktB       | SIJ488 ΔtktA, ΔtktB | Transketolase deletion strain | This study |
| ΔtktAB ΔrpiAB | SIJ488 ΔtktA, ΔtktB, ΔrpiB, ΔrpiA::CAP | Ribose-5-P isomerase deletion strain, Transketolase deletion strain | This study |
| ΔtktAB Δzwf | SIJ488 Δzwf, ΔtktB, ΔtktA::KAN | Transketolase, glucose-6-phosphate dehydrogenase deletion strain | This study |
number origin of replication, while gene expression is controlled by the constitutive ‘strong promoter’ pgi-20 [32]. This ‘default’ combination of regulatory elements is a normal practice in our lab [33]; furthermore, a promoter with lower expression strength (pgi-10) was found to hinder PKT-dependent growth, indicating insufficient activity of the heterologously expressed enzyme. A streptomycin resistance cassette allowed for selection. All restriction enzymes used were FastDigest (Thermo Scientific).

Media and growth conditions

LB medium (1% NaCl, 0.5% yeast extract, 1% tryptone) was used for strain maintenance, cloning, and genomic modifications. Antibiotics were used at the following concentrations: kanamycin, 50 µg·mL⁻¹; ampicillin, 100 µg·mL⁻¹; streptomycin, 100 µg·mL⁻¹; chloramphenicol, 30 µg·mL⁻¹.

Growth experiments and pre-cultures were carried out in M9 minimal media (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, 13 µM FeCl3·6H2O, 6.2 µM ZnCl2, 0.76 µM CuCl2·2H2O, 0.42 µM CoCl2·2H2O, 1.62 µM H3BO3, 0.81 µM MnCl2·4H2O). Carbon sources were added according to the strain requirements and specific selection conditions. In pre-cultures, cells were incubated in 4 mL M9 medium containing nonselective carbon sources at 37 °C and 230 rpm. When the transketolase genes (tktA, tktB) were deleted, the medium was supplemented with compounds derived from erythrose 4-phosphate (E4P-supplements): 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 [34]. When ribose 5-phosphate isomerase genes (rpiA, rpiB) were deleted, 5 mM d-ribose was added to the medium. Strains lacking glucose 6-phosphate dehydrogenase (zwf) and transketolase genes were supplemented with 5 mM of xylose in addition to the E4P-supplements. When transketolase and ribose 5-phosphate isomerase genes were deleted, the medium was supplemented with 5 mM of d-ribose and E4P-supplements.

Prior to growth experiments, cells from the pre-cultures were harvested by centrifugation (3 min, 4500 g, RT) and washed three times with M9 medium without any carbon source. M9 media containing the indicated carbon sources were inoculated with a starting OD₆₀₀ of 0.01. 150 µL of culture was added to each well of a sterile 96-well microtiter plate (Nuncolon Delta Surface, Thermo Scientific) and, to avoid evaporation, covered with 50 µL mineral oil (Sigma-Aldrich, St. Louis, MO, USA). (We note that this oil cover enables gas transfer thus maintaining aerobic conditions.) Microtiter plates were incubated at 37 °C in a plate reader (Infinite M200 pro, Tecan). Cultures were shaken (controlled by Tecan I-control v1.11.1.0) in 12-step cycles, each lasting 60 s with 3 mm amplitude linear shaking, 3 mm amplitude orbital shaking, 1 mm amplitude linear shaking, 1 mm amplitude orbital shaking, repeating three times.

OD₆₀₀ measurements were taken every 12.5 minutes. OD₆₀₀ values were calibrated to normal cuvette OD₆₀₀ values: ODcuvette=ODplate/0.23. Growth curves and rates were generated and calculated using MATLAB. All measurements were done in triplicates; the variability between triplicate measurements was less than 5%.

Isotopic-labeling experiments

For sample preparation for ¹³C isotope tracing of proteinogenic amino acids, strains grew in M9 containing the indicated carbon sources. Glucose with ¹³C on carbon atom 1, 2, 3, and 6 was used under the conditions mentioned in the text. After reaching stationary phase the equivalent to 1 mL of OD₆₀₀ = 1 was harvested and washed in H₂O by centrifugation. Hydrolysis of protein was carried out with 6 M HCl, at 95 °C for 24 h [35]. HCl was removed overnight by incubation at 95 °C under an air stream. Dried samples were resuspended in 1 mL H₂O, centrifuged (5 min, 16 000 g) to remove insoluble compounds, and supernatants used for analysis. Proteinogenic amino acids were analyzed by UPLC–ESI–MS described previously [36] with a Waters Acquity UPLC system (Waters, Milford, MA, USA) using a HSS T3 C₁₈ reversed phase column (100 mm × 2.1 mm, 1.8 µm; Waters). The mobile phase was 0.1% formic acid in H₂O (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.4 mL·min⁻¹ with a gradient of 0 to 1 min – 99% A; 1 to 5 min – linear gradient from 99% A to 82%; 5 to 6 min – linear gradient from 82% A to 1% A; 6 to 8 min – kept at 1% A; 8.5–8.85 min – linear gradient to 99% A; 8.5–11 min – re-equilibrate. Mass spectra were acquired using an Exactive mass spectrometer (Thermo Scientific) in positive ionization mode, with a scan range of 50.0 to 300.0 m/z. The spectra were recorded during the first 5 min of the LC gradients. Data analysis was performed using Xcalibur (Thermo Scientific). Determination of retention times was performed by analyzing amino acid standards (Sigma-Aldrich) under the same conditions.

Extraction of metabolites for LC-MS/MS measurements

Excessive chloride can interfere with LC-MS/MS measurements. Hence, we cultivated the strains in an altered minimal medium where NH₄Cl was replaced by 10 mM of (NH₄)₂SO₄. Cells at logarithmic phase (OD₆₀₀ ~ 0.8) were quenched and their metabolites extracted according to the method previously described [37]. Briefly, cells were quenched by tipping 2 mL of culture into 4 mL of 70% methanol precooled to ~70 °C. Three thaw–freeze cycles were performed by mixing 525 µL of the defrosted (~35 °C) cell material with 105 µL chloroform (on ice) and
letting the mix thaw and freeze for 1 h at −20 °C and −80 °C, respectively, while mixing in between. Afterward, metabolites were extracted from the defrosted material by adding 280 μL ddH2O and centrifuging at 4 °C and 18 500 g for 5 min before taking off the ddH2O/methanol fraction. This extraction step was repeated twice to increase the metabolite yield using 560 μL ddH2O each time. The resulting total volume of ~1920 μL was divided into two Eppendorf tubes and 280 μL ddH2O were added to prevent bubbling during freeze drying. Next, samples were lyophilized and filtered to avoid blockage and interferences during LC-MS/MS measurements. To prepare the sample for filtering, 125 μL ddH2O were added to the freeze-dried sample and centrifuged at 18 500 g and 4 °C for 10 min before transferring the supernatant to a filter plate placed on top of a 96-well microfilter plate. Samples were filtered by centrifuging for 2 h at 10 °C and 2300 g. The filtered samples were pipetted into Eppendorf tubes and stored at −80 °C. Metabolite concentrations were quantified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis as described in detail before [38,39].

**Protein purification and concentration**

The pellets from 1 L of cultured cells were thawed on ice and resuspended in 30 mL of 50 mM potassium phosphate pH 7.5 containing 50 mM NaCl, 5 mM MgCl2, 1 mM DTT and 10 mM Imidazole. 100 μL of lysonase (Merck) was added. Cells were incubated for 10 minutes at room temperature and then returned to ice for 20 minutes. Cell lysis was completed by sonication for 2 × 30 s. The bacterial extracts were then clarified by centrifugation at 4 °C, 13 000 g for 20 min. The clarified bacterial lysates were loaded on a PROTINO-2000 Ni-TED column (Macherey-Nagel) allowing adsorption of 6-His tagged proteins. Columns were washed and the enzymes of interest were eluted with 6 mL of 50 mM potassium phosphate pH 7.5 containing 500 mM NaCl and 250 mM imidazole. Eluates were then concentrated, desalted using an Amicon Ultra-4 10 kDa filter unit (Millipore) and enzymes were resuspended in 50 mM potassium phosphate pH 7.5 containing 100 mM NaCl. The enzyme preparation was complemented with 10% glycerol prior to long-term storage. Protein concentrations were quantified by direct UV 280 nm measurement on the NanoDrop 1000 spectrophotometer (Thermo Scientific).

**Kinetics analysis of PKT activity with phosphorylated C5, C6, and C7 substrates**

Phosphoketolase activity was measured spectrophotometrically as ferric acetyl-hydroxamate produced from the enzymatically generated acetyl phosphate, according to the procedure described by Racke [40]. The standard reaction mixture of 0.075 mL consists of 50 mM potassium phosphate pH 7.5, 0.6 mM thiamine pyrophosphate (TPP), 1 mM MgCl2, 1.9 mM L-cysteine hydrochloride, 23 mM NaF, and 8 mM NaI.

Kinetic parameters were determined using three substrates in a wide range of concentrations:

1. 0 to 100 mM for D-xylulose-5-phosphate (Carbosynth, Compton, UK).
2. 2 0 to 200 mM for D-fructose-6-phosphate (Sigma-Aldrich).
3. 0 to 500 mM for D-sedoheptulose-7-phosphate (Sigma-Aldrich and Carbosynth).

Each enzymatic reaction was started by adding 0.05 mg·mL⁻¹ of purified phosphoketolase and the samples were then incubated for 15 minutes at 30 °C. Acetyl phosphate concentration was determined through the detection of ferric acetyl-hydroxamate. About 25 μL of hydroxylamine hydrochloride (2 M, pH 6.5) was added to reaction mixtures. After 5-min incubation at 60 °C, the samples were acidified with 25 μL of 1.84 M trichloroacetic acid, and 25 μL of FeCl3 reagent (600 mM FeCl3 in 5 m HCl) were then added.

The samples were further clarified by centrifugation and transferred into a 96-well microplate. The absorbance of ferric acetyl-hydroxamate complex was measured at 505 nm (CLARIO star microplate reader). A calibration curve was prepared using commercial acetyl phosphate (Sigma-Aldrich).

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**Competing financial interests**

The authors declare no competing financial interests.

**Author contributions**

JLK, SNL, PM, and AB-E designed the experiments. JLK, SNL, SA, MD, MD, JW, HH, AK, RC, and MA performed the experiments. JLK, SNL, CARC, and AB-E wrote the manuscript.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Oligonucleotides used; bold: antibiotic cassette binding sequences, underlined: homologous sequences for recombination.