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Supplementary Information for

Hidden resources in the *Escherichia coli* genome restore PLP synthesis and robust growth after deletion of the essential gene *pdxB*

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Other supplementary materials for this manuscript include the following:

Datasets S1, S2 and S3

Supplementary Methods

Strains, primers and gBlock fragments used for mutation cassettes

Strains used in this work are listed in **Table S2**. PCR primers and sequences of fragments used for assembly of mutation cassette plasmids are listed in **Tables S3 – S6**. Primers for QuikChange site-directed mutagenesis are listed in **Table S7**.

Strain construction

ltaE, thrB, pdxA, serA and *gmhB* in JK1 were replaced with a chloramphenicol resistance gene amplified by PCR from pACYC184 as previously described (1). *gph* was replaced in JK1 with a kanamycin resistance gene amplified by PCR from the Δ*gph* mutant in the Keio collection (2). (The kanamycin resistance gene that replaced *pdxB* in the parental Δ*pdxB*::*kan* strain was removed prior to replacement of *gph* with *kan* using the method of Datsenko and Wanner (3).)

A mutation that renders SerA insensitive to serine inhibition was introduced into JK1 using the scarless genome editing protocol described by Kim et al. (4) (**Fig. S10**). A mutation cassette template plasmid was constructed by Gibson assembly (5) of a mutation cassette fragment with pHA_{1887} (a linear fragment of pUC18 amplified from pUC19 using primers pHAFor and pHArev that contains the replication origin and the *bla* gene conferring ampicillin resistance). The mutation cassette contained upstream and downstream homology arms targeted to *serA* flanking an I-SceI site followed by a chloramphenicol resistance gene. The upstream and downstream homology arms contained overlapping regions with the desired mutation. The mutation cassette was amplified from the plasmid by PCR and introduced into JK1 carrying pSLTS, a helper plasmid that encodes the Lambda Red recombinase enzymes and I-SceI (4). The Lambda Red enzymes were induced

with arabinose to catalyze recombination between the mutation cassette and the genome. Then I-SceI was induced with anhydrotetracycline to cleave the genome at the introduced I-SceI site. Healing of the break by recombination between the upstream and downstream homology arms eliminated the antibiotic resistance gene and incorporated the desired mutation into the genome. The resulting strain is designated *serA** JK1. *serC* in *serA** JK1 was replaced with a chloramphenicol resistance gene amplified by PCR from pACYC184 as previously described (1).

Mutations that change Gly210 to Val and Leu158 to Gln in GAPDH were introduced into the genome of wild-type *E. coli* K12 BW25113 using the method described in Kim et al. (4) for genome editing of essential genes. The mutation cassette in this case consisted of upstream and downstream homology arms flanking a fragment containing an I-SceI site, a chloramphenicol resistance gene, and a fragment of *gapA* recoded with synonymous codons. Recombination between the mutation cassette and the genome reconstituted an intact *gapA* (**Fig. S11**). Subsequent cleavage by I-SceI and recombination between HR3 regions accomplished elimination of the antibiotic resistance gene and incorporation of the target mutation into the genome. (Recoding the *gapA* fragment in the mutation cassette prevented homologous recombination in the recoded region after I-SceI cleavage, which would fail to introduce the desired mutation into the genome.)

The Δ*serA*::*cat* Δ*pdxB* (DE3) *E. coli* strain used for purification of wild-type and mutant versions of SerA was constructed by replacing *serA* with the chloramphenicol resistance gene *cat* in a Δ*pdxB*::*kan* BW25113 strain that had been lysogenized with the λ DE3 cassette (Novagen, EMD Chemicals Inc.) as described by Kim et al. (1), followed by removal of *kan* as described by Datsenko and Wanner (3). (Removal of *kan* was necessary to allow for selection of kanamycin resistance conferred by pEcPGDH, the expression plasmid encoding *serA*.)

Illumina sequencing and data analysis

The sequencing library was prepared as described by Turner et al. (6) according to the protocol of Baym et al. (7) using the Illumina Nextera kit (Illumina Inc., San Diego, CA) and sequenced using an Illumina NextSeq500 at the University of Pittsburgh Microbial Genome Sequencing Center. Coverage ranged from 80-fold to 255-fold for individual clones. BBTools (https://sourceforge.net/projects/bbmap/) were used to remove adapters and unbin the quality scores from the Illumina NextSeq results. Reads were mapped to the K12 BW25113 reference genome (GenBank CP009273.1) (8) using Breseq (9) with default parameters.

Purification of SerA, SerC and ThrB

pEcPGDH, which encodes SerA with a His_6-tag at the N terminus, was a generous gift from the laboratory of Gregory Grant. The wild-type *serA* in pEcPGDH was replaced with mutant *serA* alleles amplified by PCR from adapted strains containing *serA* mutations for expression of mutant versions of SerA. *thrB* was cloned into pET45b (EMD Biosciences Inc.), which results in incorporation of a His_6 tag at the N-terminus of the protein. *serC* was cloned into pET21b (Novagen) which results in incorporation of a His_6 tag at the C-terminus of the protein.

SerC and ThrB were expressed in *E. coli* BL21(DE3). SerA enzymes were expressed in Δ*serA* Δ*pdxB E. coli* DE3 to prevent contamination with wild-type SerA and PdxB.

Purification of SerC, ThrB and SerA was carried out as follows. A single colony from a fresh LB plate was inoculated into 3 mL LB containing 20 µg/mL kanamycin for expression of SerA and 100 µg/mL ampicillin for expression of ThrB and SerC. The culture was grown at 37 ºC overnight with shaking and then inoculated into 300 mL LB containing 20 µg/mL kanamycin or 100 µg/mL ampicillin. After growth with shaking at 37 ºC to an OD_{600} of 0.5, the culture was

cooled to room temperature, and 0.2 mM IPTG was added to induce protein expression. The culture was shaken at 22 ºC for 16 hours and then harvested by centrifugation at 5000 x g at 4 ºC for 15 minutes. The cell pellet was suspended in 5 volumes of Equilibration Buffer (20 mM sodium phosphate, pH 7.4, containing 300 mM NaCl and 10 mM imidazole). Protease inhibitor cocktail (Sigma P8849) (1 mL/g wet cell pellet) and lysozyme (1 mg/mL) were added and the cells were shaken gently at 22 ºC for 10 minutes. The cells were then lysed in an ice bath by 3-5 cycles of sonication using 20 pulses at a 50% pulse rate and intensity setting of 4/10. The lysed cells were centrifuged at 20,000 x g at 4 ºC for 15 minutes to remove cell debris. Proteins were purified using HisPur Ni-NTA Spin Columns (Thermoscientific, 88225) according to the manufacturer's instruction with the following modifications. Protein were eluted with four 1 mL aliquots of 20 mM sodium phosphate, 300 mM sodium chloride, pH 7.4, containing increasing concentrations of imidazole (75, 100, 250, and 500 mM). The final three fractions were combined and dialyzed against 20 mM Tris-HCl, pH 7.4, containing 200 mM NaCl at 4 ºC using a 10 kDa MWCO Slide-A-Lyzer dialysis cassette (ThermoFisher Scientific). Final protein concentrations were measured using a Qubit protein assay (Thermoscientific Q33211). Proteins were judged to be >95% pure by SDS-PAGE.

Purification of D-2-hydroxyglutarate dehydrogenase and electron transfer flavoprotein (ETF) AB

gBlocks (IDT) containing the sequences encoding D-2-hydroxyglutarate dehydrogenase and ETF AB from *Pseudomonas stutzeri* A150 (locus tags PST_0399, PST_2604 and PST_2605), each flanked by 5'-25 bp overhangs and 3'-18 bp overhangs overlapping the 6678-bp BamHI-HindIII fragment of pEcPGDH, were obtained from Integrated DNA Technologies. The gBlocks

were ligated into the BamHI-HindIII fragment of pEcPGDH by Gibson assembly (5) in order to install a His_6-Tev tag immediately upstream of the start codon for each gene. Sequences of the inserted genes were confirmed by Sanger sequencing.

The proteins were expressed in Δ*serA*::*cat* Δ*pdxB E. coli* cells. After introduction of the expression plasmid, transformants were grown overnight on plates containing LB and kanamycin (20 μg/mL). Several colonies were inoculated into 500 mL of 2XYT and the culture was incubated with shaking at 37 ºC. Two drops of a 1:10 dilution of autoclaved Antifoam B emulsion (Sigma) were added to prevent foaming. When the OD_{600} reached approximately 0.5, the cultures were moved to a room-temperature shaker and IPTG was added to a final concentration of 0.2 mM for expression of D-2-hydroxyglutarate dehydrogenase or 1 mM for expression of EFT AB. The cultures were incubated overnight. Cells were harvested by centrifugation at 4500 x g and 4 ºC for 20 minutes. The cell pellets were resuspended in Bugbuster containing lysozyme (1 mg/mL) and protease inhibitor cocktail II (Research Products International) diluted to give a final concentrations of 1.2 mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), 5 mM EDTA, 0.1 mM Bestatin, 15 μM E-64, and 15 μM Pepstatin A. After disruption of the cells by sonication in an ice bath using 20 cycles of 15 seconds of sonication separated by 45 second breaks, samples were subjected to centrifugation at 20,000 x g at 4 ºC. The supernatant was loaded onto a 1 mL Ni-NTA column (HisTrap-FF, GE Healthcare). The column was washed with 20 mL of PBS and the protein was eluted with 50 mM potassium phosphate, pH 7.0 containing 500 mM imidazole and 150 mM NaCl. The eluted sample was placed in a 3 kDa MWCO dialysis membrane and immersed in dry PEG Mv 8000 to reduce the volume. The sample was then dialyzed against PBS overnight at 4 ºC using a 10 kDa MWCO Slide-A-Lyzer cassette (ThermoFisher Scientific). Analysis of the purified proteins by SDS-PAGE indicated that they were approximately 80% pure.

Purification of wild-type and mutant GAPDH enzymes

Wild-type *gapA* was cloned into pET46 using a pET46/LIC vector kit (Novagen) so as to introduce an N-terminal His tag. Mutant *gapA* genes encoding G210V and L158Q GAPDH were generated using a QuikChange II Site-Directed Mutagenesis kit (Agilent) and the primers listed in Table S7.

His-tagged wild-type and variant GAPDH enzymes were expressed in E. cloni BL21(DE3) (Lucigen). A single colony was inoculated into 25 mL Terrific Broth supplemented with 62.5 µL filter-sterilized 20% glucose and 625 µL filter-sterilized 8% lactose and grown overnight at 30 ºC in a shaking incubator. Cells were harvested by centrifugation at 4,000 x g at 4 ºC. The cell pellets were resuspended in Bugbuster (Millipore) containing protease inhibitor cocktail II (Research Products International) diluted to give a final concentrations of 1.2 mM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride), 5 mM EDTA, 0.1 mM Bestatin, 15 µM E-64, and 15 µM Pepstatin A, and lysonase (EMDMillipore) according to the manufacturer's instructions. The suspension was incubated at room temperature for 30 min. After centrifugation at 20,000 x g at 4 ºC for 20 min, the supernatant was loaded onto a 5 mL Ni-agarose column (HisTrap-FF, GE Healthcare). The column was washed with 50 mL of 50 mM potassium phosphate, pH 7.0, containing 150 mM NaCl and 50 mM imidazole. Proteins were eluted with 50 mM potassium phosphate, pH 7.0, containing 150 mM NaCl and 500 mM imidazole. Fractions containing GAPDH were concentrated using a 10 kDa MWCO spin column (Millipore) and dialyzed twice against 4 L of 50 mM Tris-HCl, pH 7.0, containing 150 mM NaCl and then finally against 4 L of 50 mM Tris-HCl, pH 7.0, containing 150 mM NaCl and 1 mM dithiothreitol. Analysis of the purified proteins by SDS-PAGE indicated that they were approximately 95% pure.

Enzyme assays

GAPDH activity of purified enzymes was assayed at 37 ºC in 40 mM triethanolamine, pH 8.9, containing 50 mM potassium phosphate, 1 mM NAD^+ and variable amounts of glyceraldehyde 3-phosphate (0-0.5 mM). The reaction was initiated by addition of glyceraldehyde 3-phosphate and the initial absorbance change due to reduction of NAD^+ (ε_{340} = 6.2 mM^{-1} cm^{-1}) (10) was monitored. GAPDH activity was also assayed in crude lysates prepared by sonication of cells harvested from overnight cultures grown in LB medium. Protease inhibitor cocktail (Sigma P8849) was added prior to sonication (3 20-second pulses separated by one-minute cooling on ice). The cell lysates were subjected to centrifugation for 1 minute at 3900 x g. One mL of the supernatant was concentrated 10-fold in an Amicon® Ultra-4 Centrifugal Filter Units at 300 x g for 10 minutes and then diluted with 900 µL of 50 mM sodium phosphate, pH 7.2, and reconcentrated twice to 100 µL in order to exchange the buffer. GAPDH activity was assayed at 37 ºC in 50 mM sodium phosphate, pH 7.2, containing 1 mM NAD^+ and variable amounts of glyceraldehyde 3-phosphate (0-2 mM).

SerA activity was assayed at 37 ºC using the coupled enzymatic assay shown in **Fig. S12**. Reaction mixtures contained 200 mM Tris-HCl, pH 7.4, 1.9 µM D-2-hydroxyglutarate dehydrogenase, 3.6 µM electron transfer flavoprotein AB, 1 mM NAD^+, 1 mM α-ketoglutarate, 1 mM 2,6-dichlorophenol indophenol (DCIP), and varying amounts of 3-phosphoglycerate (0.25-16 mM) or erythronate (0.5-12 mM). Reaction mixtures were pre-incubated for 15 minutes at 37 ºC before addition of SerA (9.5-13.5 nM for reactions with 3-phosphoglycerate and 14-20 µM for reactions with erythronate). The initial absorbance change due to reduction of DCIP (ε_{600} = 22 mM^{-1} cm^{-1}) (10) was monitored in triplicate in a Thermoscientific Varioskan plate reader. A K-factor

of 0.187 was used to calculate the change in DCIP concentration from the observed change in OD_{600}. Inhibition of SerA by serine was assessed in a reaction mixture containing 4 mM 3-phosphoglycerate and variable concentrations of serine (2-200 μM). Data were fit in R (v 3.5.2) to the Michaelis-Menten equation to determine k_{cat} and K_M or to Equation 1 to determine $K_{i,\ serine}$, where v_{max} = velocity in absence of inhibitor, v_{min} = velocity in saturating concentrations of inhibitor, and L = concentration of inhibitor.

Eq. 1. $$v = v_{max} + (v_{min} - v_{max})\left[\frac{[L]}{[L] + K_i}\right]$$

SerC activity (**Fig. S13**) was assayed at 37 ºC in 200 mM Tris-HCl, pH 7.4, containing 50 mM NaCl, 40 μM SerC, 2 mg/mL bovine liver glutamate dehydrogenase (Sigma Aldrich), 1 mM ADP (to activate glutamate dehydrogenase), 2 mM α-ketoglutarate, 1.2 mM acetylpyridine adenine dinucleotide (APAD) and variable amounts of 4 HT (0 – 30 mM). Reaction mixtures were pre-incubated for 10 minutes before addition of SerC and then another 5 minutes before initiation of the reaction by addition of 4HT. Fluorescence of $APAD^+$ (excitation: 365 nm; emission: 480 nm) was followed for 3 hours in a Synergy H1 Hybrid Multi-Mode Reader plate reader.

Potential promiscuous activities of ThrB were assessed by following the formation of ADP by ^{31}P-NMR. Reaction mixtures contained 10% D_2O, 200 mM Tris-HCl, pH 7.4, 30 mM $MgSO_4$, 250 mM KCl, 10 mM NAD^+, 10 mM ATP, and 10 mM D-erythronate and 5 μM of SerA, SerC, and/or ThrB; 10 mM of L-glutamate was also added for the SerA/SerC/ThrB reaction. Reaction mixtures were incubated at 37 °C for 24 hours before ^{31}P-NMR analysis at room temperature using 4096 scans on a Bruker Avance-III 300 MHz spectrometer.

Proteomics

Freezer stocks were used to streak each strain on LB plates (wild-type and G210V *gapA* mutants) or LB plates containing 20 µg/mL kanamycin (JK1). Four parallel 2-mL aliquots of LB (containing 20 µg/mL kanamycin for JK1) were inoculated with individual colonies and the cultures were grown to mid-exponential phase at 37 °C with shaking. One mL of each culture was subjected to centrifugation at 16,000 x *g* for 1 min at room temperature. The cell pellets were resuspended in 1 mL PBS and washed twice more in PBS before resuspension and dilution to an OD of 0.001 in 5 mL of M9/glucose (0.4%). Cultures were grown to an OD_{600} of 0.2-0.3 at 37 °C with shaking and then chilled on ice for 10 min. Two mL of each culture was subjected to centrifugation at 4,000 x *g* at 4 °C. Cell pellets were frozen in liquid N_2 and stored at -70 °C.

Frozen cell pellets were thawed and lysed in 60 µL 50 mM Tris-HCl, pH 8.5, containing 4% (w/v) SDS, 10 mM tris(2-carboxyethylphosphine) (TCEP) and 40 mM chloroacetamide in a Bioruptor Pico sonication device (Diagenode) using 10 cycles of 30 seconds on, 30 seconds off, followed by boiling for 10 min, and then another 10 cycles in the Bioruptor. The lysates were subjected to centrifugation at 15,000 x *g* for 10 minutes at 20 °C and protein concentrations in the supernatant were determined by tryptophan fluorescence (11). Twenty µL of each sample (0.4-1.7 µg protein/µL) was digested using the SP3 method (12). Carboxylate-functionalized SpeedBeads (GE Life Sciences) were added to the lysates. Addition of acetonitrile to 80% (v/v) precipitated the proteins onto the beads. The beads were washed twice with 80% (v/v) ethanol and twice with 100% acetonitrile. Protein was digested and eluted from the beads with 19 µL of 50 mM Tris-HCl, pH 8.5, containing 1 µg endoproteinase Lys-C (Wako) for 4 hours with shaking at 1200 rpm at 37 °C in a thermomixer (Eppendorf). One µg of trypsin (Pierce) was then added and the suspension was incubated at 37 °C overnight with shaking at 1200 rpm. Tryptic peptides were precipitated

back onto the beads by addition of acetonitrile to a final concentration of 95% (v/v). The beads were then washed with acetonitrile. The desalted tryptic peptides were eluted with 1% (v/v) trifluoracetic acid containing 3% (v/v) acetonitrile. Peptide yields were determined by absorbance at 280nm using a nanospec (Thermo Scientific). The samples were dried using a SpeedVac and stored at -80 ºC.

Tryptic peptide samples were suspended in 0.1% (v/v) trifluoracetic acid containing 3% (v/v) acetonitrile and 1 µg of peptides was directly injected onto a C18 1.7 µm, 130 Å, 75 µm X 250 mm M-class column (Waters), using a Waters M-class UPLC. Peptides were eluted at 300 nL/minute using a gradient from 3% to 20% acetonitrile over 100 minutes into an Orbitrap Fusion mass spectrometer (Thermo Scientific). Precursor mass spectra (MS1) were acquired at a resolution of 120,000 from 380-1500 m/z with an AGC target of 2.0×10^5 and a maximum injection time of 50 ms. Dynamic exclusion was set for 20 seconds with a mass tolerance of +/– 10 ppm. Precursor peptide ion isolation width for MS2 fragment scans was 1.6 Da using the quadrupole, and the most intense ions were sequenced using Top Speed with a 3-second cycle time. All MS2 sequencing was performed using higher energy collision dissociation (HCD) at 35% collision energy and scanning in the linear ion trap. An AGC target of 1.0×10^4 and 35-second maximum injection time was used. Rawfiles were searched against the Uniprot *Escherichia coli* database using Maxquant version 1.6.3.4 with cysteine carbamidomethylation as a fixed modification. Methionine oxidation and protein N-terminal acetylation were searched as variable modifications. All peptides and proteins were thresholded at a 1% false discovery rate (FDR).

Metabolomics

Intracellular metabolites were extracted using a glycerol/saline quenching protocol (13, 14). Twenty-five mL cultures were grown in M9/glucose (0.4%) to an OD_{600} of 0.5. Using pipettes prewarmed to 37 °C, 10 mL of culture were transferred rapidly into 30 mL of Quenching Solution (60% v/v glycerol, 0.54% w/v NaCl) kept at -20 ºC with a 70% ethanol bath. The tube was inverted to mix. Samples were subjected to centrifugation at 29,000 x g for 20 min at -10 °C and the supernatant was removed. Cell pellets were immediately resuspended in 0.75 mL Saline Wash Solution (0.9% w/v NaCl) at 4 °C and transferred to a 2 mL tube on ice. The original tube was washed with an additional 0.75 mL Saline Wash Solution and the wash was added to the 2 mL tube. The samples were subjected to centrifugation at 15,100 x g for 1 minute at 4 °C. The supernatant was removed and the cell pellet was washed with an additional 0.9 mL Saline Wash Solution. The supernatant was removed and the cell pellet was immediately resuspended in 0.25 mL Extraction Solution (50% methanol) at -20 °C. The samples were returned to the -20 °C ethanol bath and stored at -70 °C until further processing. The samples were then subjected to three freeze-thaw cycles to lyse cells. The samples were thawed on wet ice for about 5 minutes and then vortexed vigorously for 1 minute before re-freezing on powdered dry ice (about 30 minutes). The lysed samples were subjected to centrifugation at 15,100 x g for 15 minutes at 4 °C. The supernatant was transferred to a new 2 mL Eppendorf tube on dry ice. The cell pellet was resuspended again in 0.15 mL Extraction Solution and vortexed for 30 seconds. After centrifugation at 15,100 x g for 15 minutes at 4°C, the supernatant was added to the supernatant from the previous step. All samples were dried completely using a SpeedVac and stored at -70 °C until processing.

To quantify total biomass in each sample, the cell pellet remaining after metabolite extraction was resuspended in 50 µL 0.2 M sodium hydroxide and incubated at 100 ºC for 20

minutes. Samples were cooled on ice, then subjected to centrifugation for 5 minutes at 2,500 x g to remove the precipitate. The protein concentration in the supernatant was measured using a Qubit protein assay (Thermoscientific Q33211) according to the manufacturer's instructions.

The metabolite extracts were resuspended in 100 µL MilliQ water and metabolites were analyzed on a LC-MS/MS mass spectrometer system consisting of a 1290 Infinity LC (Agilent Technologies) coupled to a 5500 QTRAP mass spectrometer (AB Sciex) in negative mode and with MRM scan type. Five-µL aliquots of metabolite extracts were injected onto an Agilent PoroShell 120 HILIC-Z column (150 x 2.1mm). A gradient of mobile phase A (10 mM ammonium acetate, pH 9, containing 5 µM medronic acid) and mobile phase B (90% acetonitrile containing 10 mM ammonium acetate and 5 µM medronic acid) was applied as follows: 0 min, 10% A 90% B; 2 min, 10% A 90% B; 12 min, 40% A 60% B ;15 min, 40% A 60% B; 16 min, 10% A 90% B; 24 min, 10% A 90% B. Flow rate was constant at 250 µL/minute and MRM settings were adapted from Yuan et al. (15). The raw data were processed and analyzed by custom software using Matlab (Mathworks).

Supplementary Figures

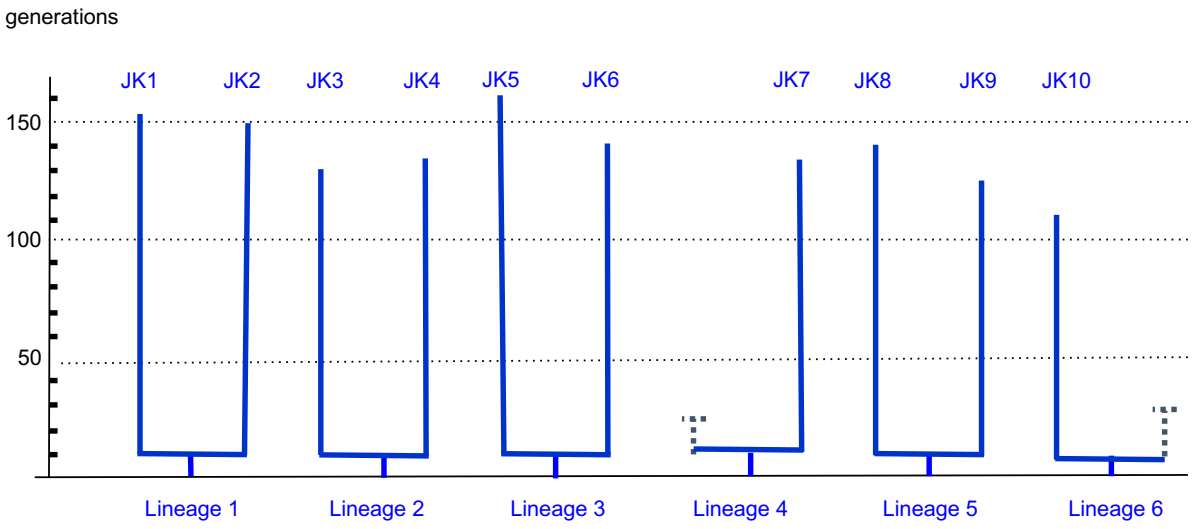

Fig. S1. Details of the process of adaptation of 12 parallel lineages of Δ*pdxB E. coli* in M9/glucose (0.4%).

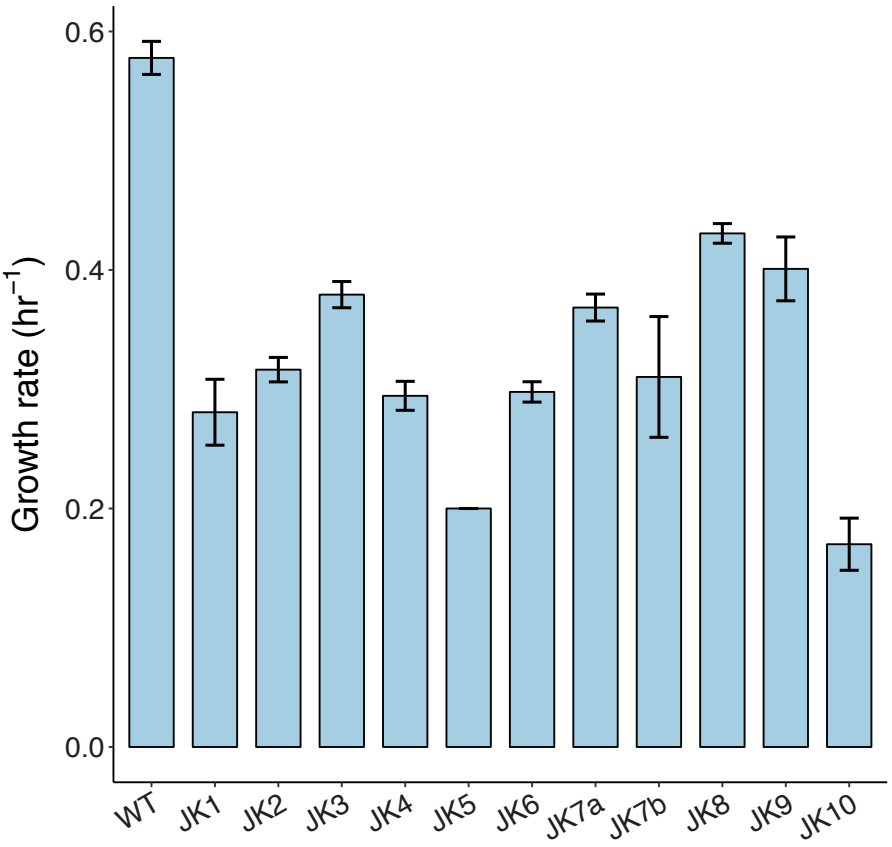

Fig. S2. Growth rates of wild-type (WT) and evolved strains of Δ*pdxB E. coli* in M9 glucose (0.4% w/v).

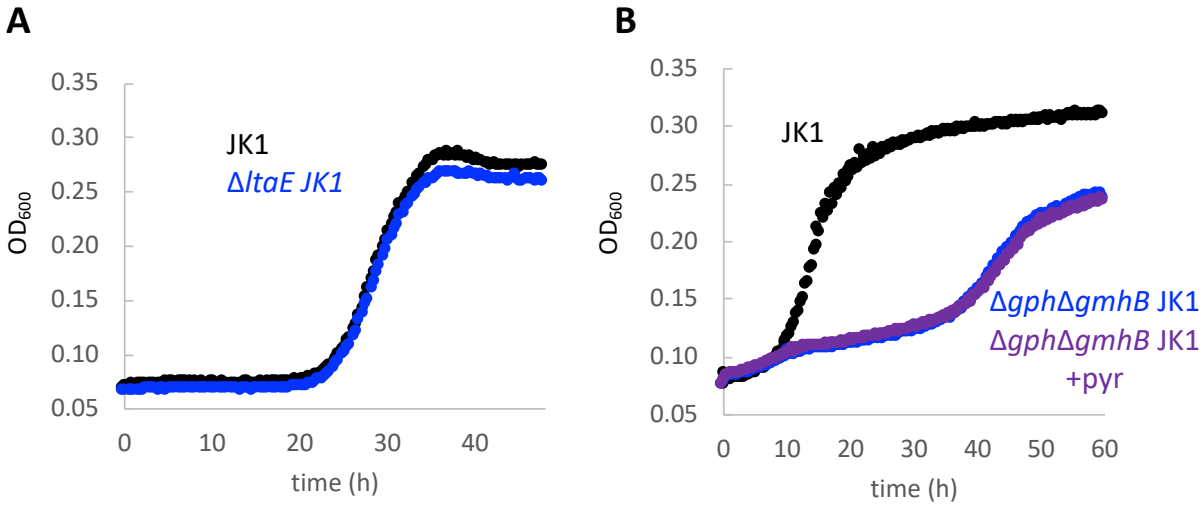

Fig. S3. LtaE, GmhB and Gph are not required for PLP synthesis on JK1. pyr, pyridoxine.

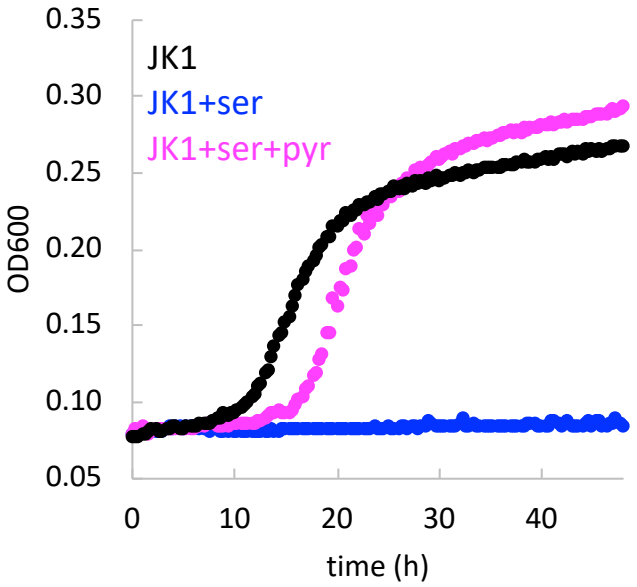

Fig. S4. Serine (ser) inhibits growth of JK1. Addition of pyridoxine (pyr) restores growth, indicating that serine inhibits production of PLP in JK1.

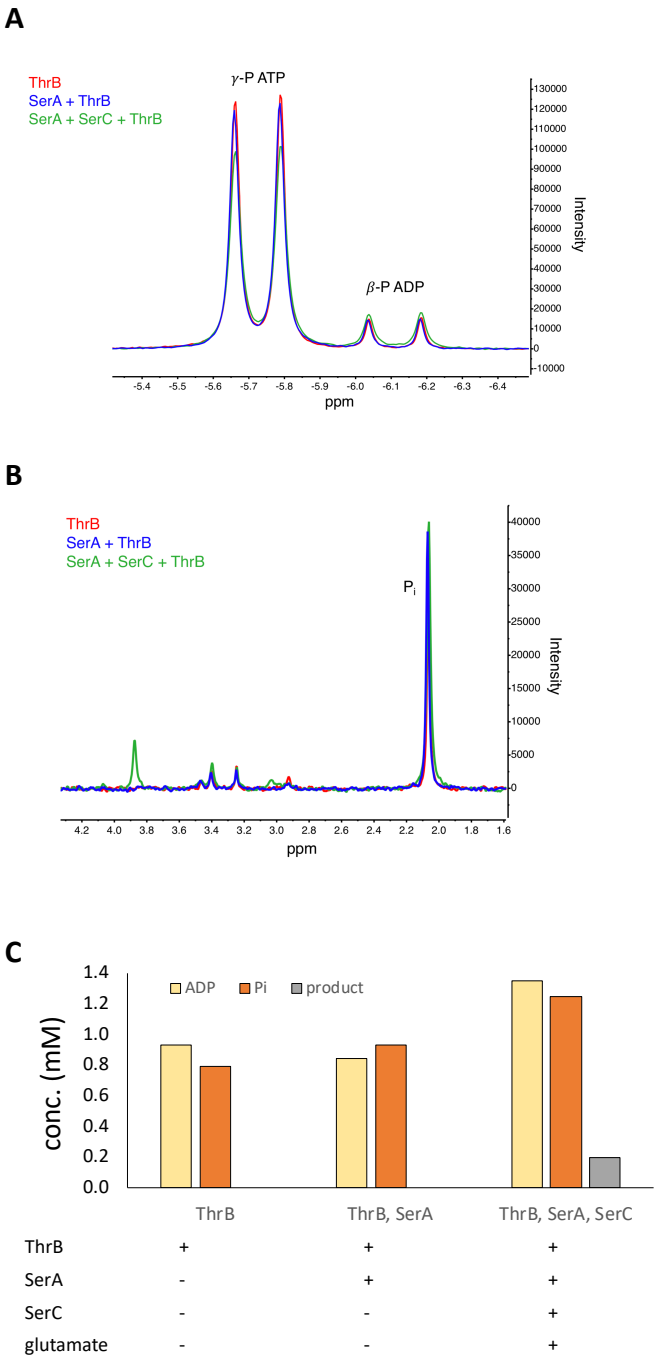

Fig. S5. ThrB does not phosphorylate (3*R*)-3,4-dihydroxy-2-oxobutanoate (DHOB). Reaction mixtures were incubated for 24 hours at 37 ºC prior to analysis by ^{31}P NMR. Reaction mixtures contained 200 mM Tris-HCl, pH 7.4, 10 mM ATP, 30 mM $MgSO_4$, 10 mM erythronate, 10 mM NAD+, 250 mM KCl, enzymes (5 μM) and glutamate (10 mM, to support the transamination

reaction catalyzed by SerC) as indicated. A) More ATP is converted to ADP when SerC is present to convert DHOB to 4HT. B) The yield of P_i was increased by 3% in the presence of SerC, suggesting that 4PHT produced from 4HT by ThrB may undergo hydrolysis during the experiment. An additional product peak at 3.88 ppm is seen only when SerC is present to convert DHOB to 4HT. This product accounts for 2% of the initial ATP. The position of the new peak does not correspond exactly to that of authentic 4PHT produced by ThrB-catalyzed phosphorylation of 4HT, suggesting that 4PHT may undergo decomposition to another phosphorylated product, possibly 3PHT, as well as hydrolysis, during the extended reaction time. C) Quantification of ADP, Pi and the new product based upon integration of the ^{31}P NMR signals.

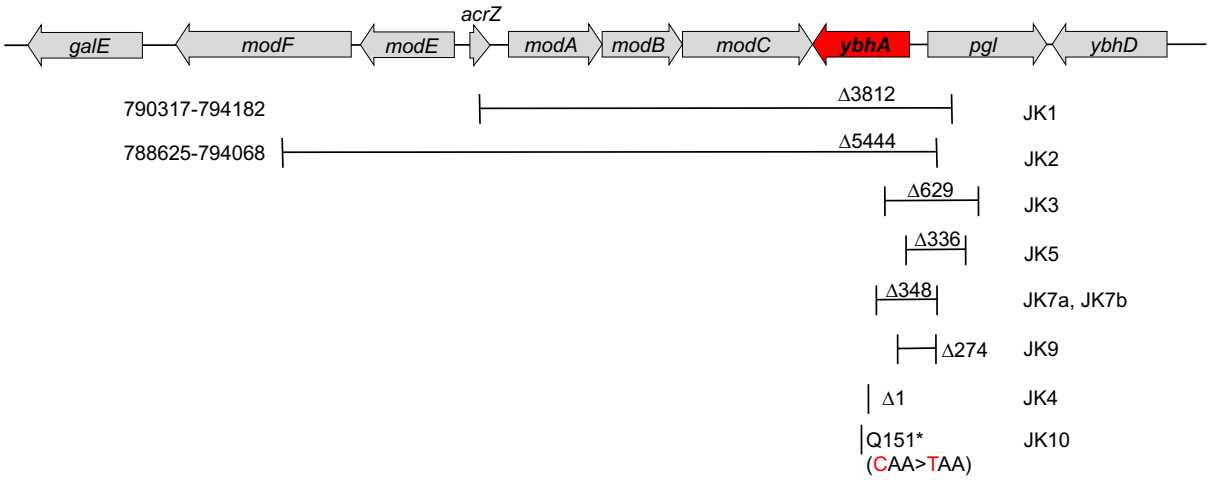

Fig. S6. Mutations affecting *ybhA* and *pgl* in adapted clones.

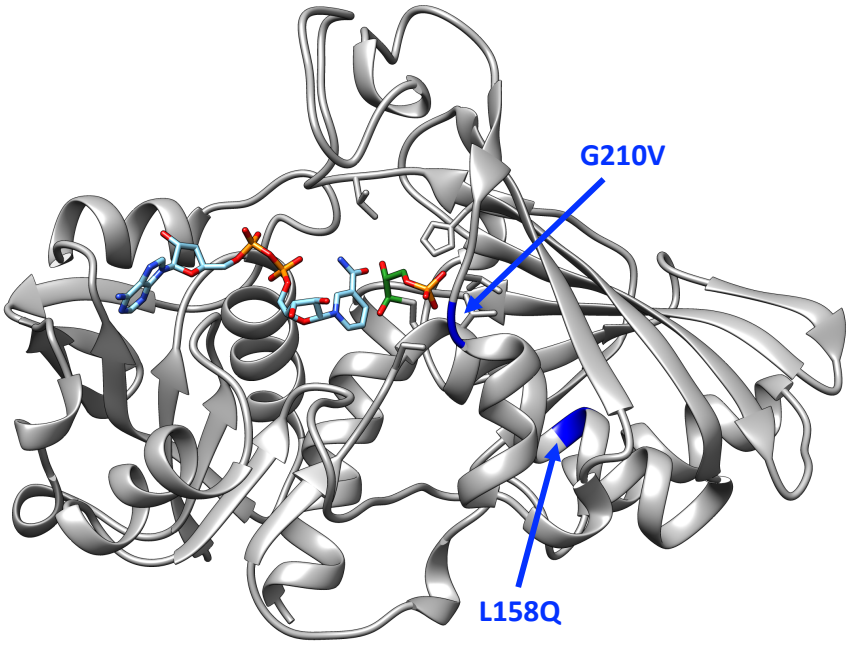

Fig. S7. Locations of mutations in GapA (glyceraldehyde 3-phosphate dehydrogenase) found in adapted clones. The figure shows an overlay of NAD^+ (cyan) from PDB 1DC6 onto the structure of PDB 1DC4, which has glyceraldehyde 3-phosphate (green) bound as the hemiacetal to Cys149 at the active site. Only one monomer of the homotetrameric enzyme is shown. Molecular graphics and analyses were performed with UCSF Chimera v. 1.13, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311 (16).

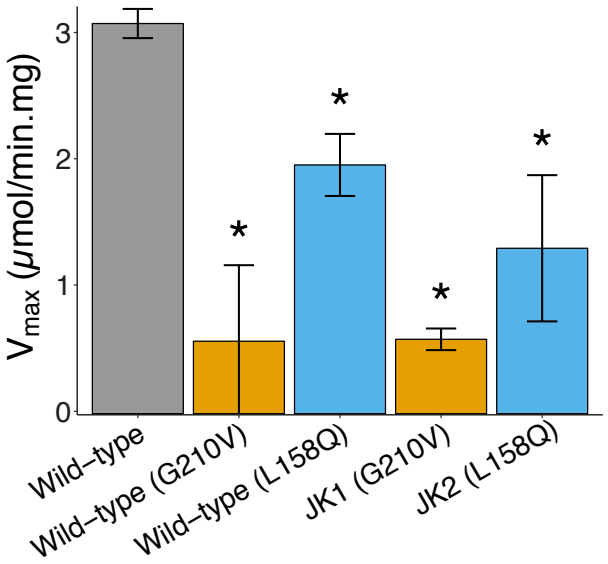

Fig. S8. GAPDH activity in cell lysates is decreased when changes in GAPDH (indicated in parentheses) are introduced into wild-type *E. coli* and in two adapted strains with changes in GAPDH indicated in parentheses. *, p-value < 0.01 (Student's t-test)

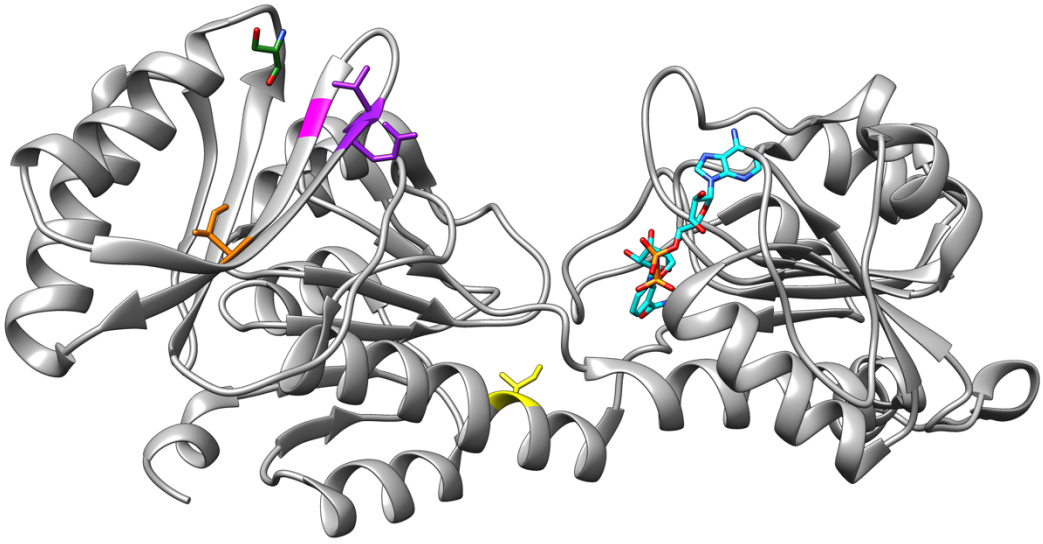

Fig. S9. Locations of mutations observed in adapted strains mapped onto the structure of G336V SerA (PDB 2PA3). Serine (green) and NAD^+ (cyan) are shown in the regulatory domain (left) and catalytic domain (right), respectively. Ile304, yellow; Ile381, orange; Gly377, magenta; Gln371 and Thr372, purple. Molecular graphics and analyses were performed with UCSF Chimera v. 1.13, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311 (16).

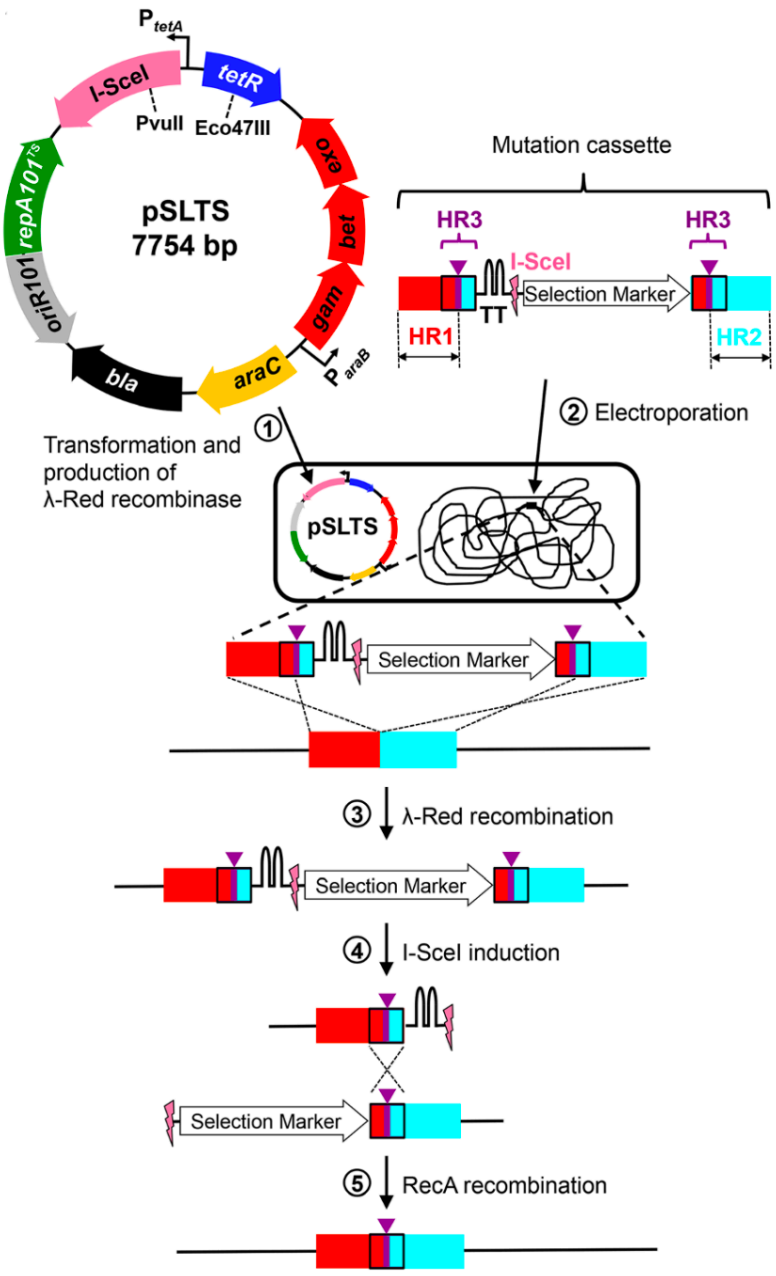

Fig. S10. Procedure for scarless genome editing used to introduce a mutation that renders SerA insensitive to feedback inhibition by serine. The purple bar in HR3 indicates the mutated sequence. 1, introduction of the helper plasmid pSLTS; 2, introduction of the mutation cassette; 3, recombination between the mutation cassette and the genome; 4, cleavage by I-SceI; 5, homologous recombination resulting in introduction of the desired mutation into the target gene. Reproduced from (4), published by Springer Nature.

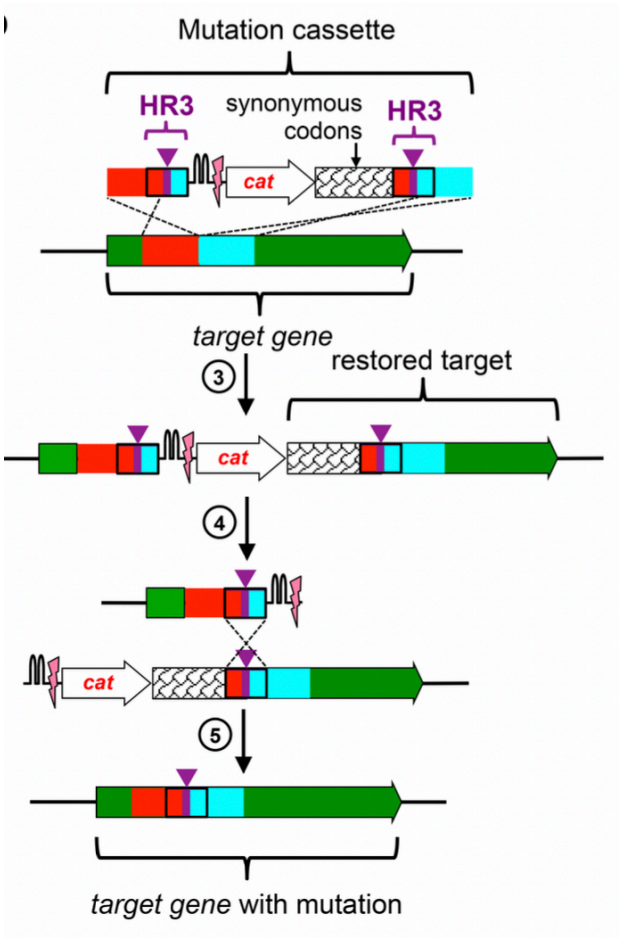

Fig. S11. Procedure for scarless genome editing used to introduce mutations into an essential gene such as *gapA*. The purple bar in HR3 indicates the mutated sequence. 3, recombination between the mutation cassette and the genome; 4, cleavage by I-SceI; 5, homologous recombination resulting in introduction of the desired mutation into the target gene. Reproduced from (4), published by Springer Nature.

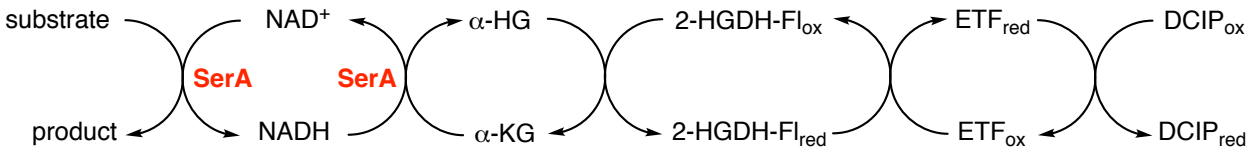

Fig. S12. Coupled assay for SerA activity. Reduction of DCIP was measured at 37 ºC in 200 mM Tris-HCl, pH 7.4, containing SerA, 1.9 µM D-2-hydroxyglutarate dehydrogenase (2-HGDH), 3.6 µM electron transfer flavoprotein (ETF) AB, 1 mM NAD^+, 1 mM α-ketoglutarate (α-KG), 1 mM 2,6-dichlorophenol indophenol (DCIP), and varying amounts of either 3-phosphoglycerate (0.25-16 mM), the native substrate, or erythronate (0.5-12 mM), the novel substrate in SP4. α-HG, α-hydroxyglutarate.

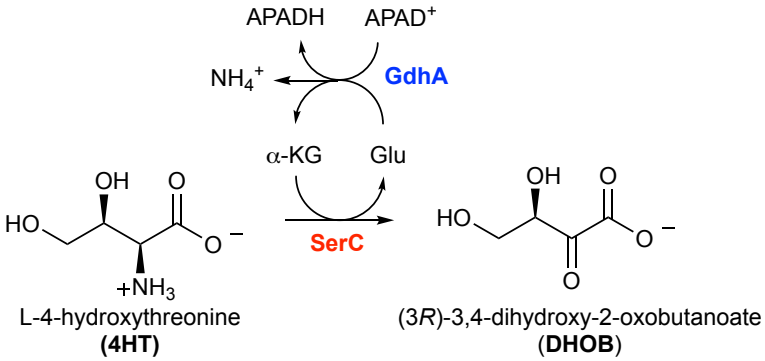

Fig. S13. Assay for the promiscuous 4HT transaminase activity of SerC. GdhA, glutamate dehydrogenase A.

Supplementary Tables

Table S1. Growth parameters for wild-type and evolved strains of Δ*pdxB E. coli* in M9/glucose (0.4%).

Strain	*growth rate* (hr^{-1})	*growth yield* (OD_{600})	*time to ½ final growth yield (hr)*
Wild-type	0.58 ± 0.01	1.41 ± 0.02	15.3 ± 0.1
JK1	0.28 ± 0.03	1.16 ± 0.04	14.8 ± 0.5
JK2	0.32 ± 0.01	1.59 ± 0.02	28.3 ± 0.7
JK3	0.38 ± 0.01	0.90 ± 0.01	25.2 ± 0.3
JK4	0.29 ± 0.01	1.51 ± 0.05	17.1 ± 0.3
JK5	0.20 ± 0.00	1.30 ± 0.08	47.7 ± 0.7
JK6	0.30 ± 0.01	1.34 ± 0.02	39.6 ± 1.5
JK7a	0.37 ± 0.01	1.01 ± 0.02	18.4 ± 0.3
JK7b	0.31 ± 0.05	1.22 ± 0.03	54.9 ± 1.7
JK8	0.43 ± 0.01	1.64 ± 0.05	21.7 ± 0.5
JK9	0.40 ± 0.03	1.17 ± 0.05	17.5 ± 0.4
JK10	0.17 ± 0.03	0.78 ± 0.08	96.4 ± 9.3

Table S2. Strains used in this work.

E. coli BW25113 (parental strain)
E. coli BW25113 G210V *gapA*
E. coli BW25113 L158Q *gapA*
E. coli BW25113 Δ*pdxB* (from the Keio collection)
E. coli BW25113 Δ*pdxB* Δ*serA*
JK1 Δ*ltaE*
JK1 Δ*thrB*
JK1 Δ*pdxA*
JK1 Δ*serA*
JK1 *serA** Δ*serC* (*serA** encodes the serine-insensitive N364A SerA)
JK1 Δ*gph*
JK1 Δ*gmhB*
JK1 Δ*gph* Δ*gmhB*

Table S3. Fragments used to assemble mutation cassette plasmids. These fragments were assembled into a mutation cassette plasmid by Gibson assembly with the vector backbone (pHA1887) and a fragment encoding a double transcription terminator followed by an I-SceI site and a chloramphenicol resistance gene (4). (MTC denotes mutation cassette plasmid.)

plasmid	purpose	fragment	source
pMTC-gapA-g629t	introduction of g629t mutation in *gapA* (generates G210V GAPDH)	gapA_g629t-up	amplified from JK1 genomic DNA with primers gapA_g629t_UpF and gapA_g629t_UpR
		gapA_syn_g629-down	gBlock (IDT) GGGAAGGTTTTGCCCGAGCTTAACGGAAAGTTGA CGGGCATGGCATTTCGGGTCCCCACGCCCAATGTG AGCGTCGTAGATCTTACGGTCCGCTTAGAGAAGGC GGCGACCTATGAACAAATAAAGGCAGCGGTGAAG GCGGCGGCGGAGGGTGAGATGAAGGGAGTGTTGG GGTATACGGAGGACGATGTGGTCAGCACGGACTT TAATGGTGAGGTGTGTACGAGTGTTTTTGACGCGA AGGCCGGGATTGCGTTGAATGATAATTTTGTTAAG TTAGTTAGTTGGTATGATAATGAGACTGGCTATTC AAATAAGGTGTTGGATCTTATTGCGCATATTTCGA AGTAAATCTCAAGAGTGGCAGCGGT
		gapA_g628t-down	amplified from JK1 genomic DNA with primers gapA_g629t_DownF and gapA_g629t_DownR
pMTC-gapA-t473a	introduction of t473a mutation in *gapA* (generates L158Q GAPDH)	gapA_t473a-up	amplified from JK2 genomic DNA with primers gapA_t473a_UpF and gapA_t473a_UpR
		gapA_syn_t473-down	gBlock (IDT) GACAATTTTGGAATTATAGAGGGCCTCATGACAAC TGTCCATGCCACAACGGCAACGCAAAAGACGGTA GACGGTCCATCACATAAGGATTGGCGGGGAGGTC GTGGAGCCTCACAAAATATTATTCCATCATCAACG GGCGCAGCAAAGGCGGTCGGCAAGGTCTTACCGG AGTTGAACGGTAAGCTCACAGGCATGGCCTTTCGT GTGCCCACACCCAATGTCTCAGTTGTCGATCTTAC GGTACGCCTCGAGAAGGCGGCCACCTATGAACAA ATTAAGGCCGCGGTCAAGGCCGCCGCGGAGGGTG AGATGAAGGGTGTACTTGGGTATACGGAGGACGA TGTCGTCAGTACAGACTTTAATGGGGAGGTCTGTA CGAGCGTTTTTGACGCGAAGGCGGGCATTGCCTTA AATGATAATTTTGTTAAGTTAGTGAGCTGGTATGA TAATGAGACAGGCTATAGCAATAAGGTCTTGGATT TGATAGCGCATATTAGCAAGTAAATCTCAAGAGT GGCAGCGGT
		gapA_t473a-down	amplified from JK2 genomic DNA with primers gapA_t473a_DownF) and gapA_t473a_DownR
pMTC-serA-N364A	introduction of mutation to generate serine-insensitive SerA	serA-N364A-up	gBlock (IDT) AGGCGTATCACGAGGCCCTTGAATATCGGCCTGG AAGTTGCGGGTAAATTGATCAAGTATTCTGACAAT GGCTCAACGCTCTCTGCGGTGAACTTCCCGGAAGT CTCGCTGCCACTGCACGGTGGGCGTCGTCTGATGC ACATCCACGAAAACCGTCCGGGCGTGCTAACTGC GCTGAACAAAATCTTCGCCGAGCAGGGCGTCGCC ATCGCCGCGCAATATCTCAAGAGTGGCAGCGGT

		serA-N364A-down	gBlock (IDT) GCAGGGCGGGGCGTAACCGAGCAGGGCGTCGCCA TCGCCGCGCAATATCTGCAAACTTCCGCCCAGATG GGTTATGTGGTTATTGATATTGAAGCCGACGAAGA CGTTGCCGAAAAAGCGCTGCAGGCAATGAAAGCT ATTCCGGGTACCATTCGCGCCCGTCTGCTGTACTA ATTCCCCTTCTCTGAAAATCAACGGGCAGGTCACT GACTTGCCCGCAGGAAAGAACATGTGAG

Table S4. Primers used to amplify fragments for assembly of mutation cassette plasmids.

fragment	template	primers
pHA1887 (backbone of mutation cassette plasmids)	pUC19	pHAFor: 5'-CGCAGGAAAGAACATGTG pHARev:5'-AAGGGCCTCGTGATACG
gapA_t473a_Up	M116B01	Forward: AGGCGTATCACGAGGCCCTTAACTGGTCTGTTCCTGACTG Reverse: TCTATAATTCCAAAATTGTCGTTGATAACTTTAGCC
gapA_t473a_Down	M116B01	Forward: GCAGGGCGGGGCGTAAACTGCCTGGCTCCGC Reverse: CTCACATGTTCTTTCCTGCGCAGTACTTTACCTACAGCTT
gapA_g629t_Up	JK1A01	Forward: AGGCGTATCACGAGGCCCTTTTCCTGCACCACCAACTGCC Reverse: AGCTCGGGCAAAACCTTCCCTACAGCTTTAGCAGCA
gapA_g629t_Down	JK1A01	Forward: GCAGGGCGGGGCGTAATCCCGTCCTCTACCG Reverse: CTCACATGTTCTTTCCTGCGAACGCCTTTCATTTCGCCTT

Table S5. Primers for amplification of mutation cassettes from mutation cassette plasmids.

Purpose	
amplification of a mutation cassette designed to introduce a mutation changing Leu158 to Gln in GAPDH from pMTC-gapA-t473a	Forward: GACAACACTCCGATGTTCGT Reverse: GTGAGACGGGCCATCAACGG
amplification of a mutation cassette designed to introduce a mutation changing Gly210 to Val in GAPDH from pMTC-gapA-g629t	Forward: CACGCTACTACCGCTACTCA Reverse: CAGGTCAACTACAGATACGT
amplification of a mutation cassette designed to introduce a mutation changing Gln364 to Ala in SerA from pMTC-serA-N364A	Forward: 5'CTCGCTGCCACTGCACGGTG Reverse: 5'CATTGCCTGCAGCGCTTTTT
amplification of the Δ*serA* mutation cassette containing *cat* flanked by sequences upstream and downstream of *serA*	Forward: 5'GCGGATGCAAATCCGCACACAACATTTCAAAA GACAGGATTGGGTAAATGCCTGGTGTCCCTGTTGATAC Reverse: 5'ACCTGCCCGTTGATTTTCAGAGAAGGGGAATT AGTACAGCAGACGGGCGCTTACGCCCCGCCCTGCCACT
amplification of the Δ*serC* mutation cassette containing *cat* flanked by sequences upstream and downstream of *serC*	Forward: 5'GTCACTGAATGATAAAACCG Reverse: 5'ATGCCGCCAGCAATAAAGCG
amplification of the Δ*ltaE* mutation cassette containing *cat* flanked by sequences upstream and downstream of *ltaE*	Forward: 5'GGTGTTGGTGGATGTGGTG Reverse: 5'AGCTGAGATCGACTTTATGG
amplification of the Δ*thrB* mutation cassette containing *cat* flanked by sequences upstream and downstream of *thrB*	Forward: 5'TATGTTGGCAATATTGATGAAGATGGCGTC Reverse: 5'ATTTCATCACCAATAAACGCCGAGAGGATC
amplification of the Δ*pdxA* mutation cassette containing *cat* flanked by sequences upstream and downstream of *pdxA*	Forward: 5'TTAATGGCAGACACAATACT Reverse: 5'TGCATACCGCATGCTGATGA
amplification of the Δ*gmhB* mutation cassette containing *cat* flanked by sequences upstream and downstream of *gmhB*	Forward: 5'TCCAGACGTCTAAATCAATC Reverse: 5'GGAGTCGGCTCAGGAAGA
amplification of the Δ*gph* mutation cassette containing kan^R flanked by sequences upstream and downstream of *gph*	Forward: 5'GCGGGCGCGGATATGTTCGT Reverse: 5'ATGGTAGTCATCCTGCATGT

Table S6. Miscellaneous PCR primers.

gene	purpose	
serA	confirming editing of *serA* codon 364 Asn → Ala	Forward: 5'TCAGGAAGCGCAGGAGAATATC Reverse: 5'GGAGAAGGGATAAAAAAACGGG
ltaE	confirming deletion of *ltaE*	Forward: 5'GTGGCGATGGAGATGAAA Reverse: 5'GCCTGCAACGAACTAAGA
thrB	confirming deletion of *thrB*	Forward: 5'GGTGATGTTGCCGCTTTTAT Reverse: 5'GGCTTATCACCCGCAATATG
serA	confirming deletion of *serA*	Forward: 5'TCCTCTAAACCAGCATATTCATCC Reverse: 5'CAGACTCGCAAAGTAGAAT
serC	confirming deletion of *serC*	Forward: 5'ACCAACGGGCAAATC Reverse: 5'AGGTAAGTGATCTTCGC
pdxA	confirming deletion of *pdxA*	Forward: 5'ATCGTCAGTTTCGGGCCTA Reverse: 5'CTTCATTCGGCTGGCATT
gph	confirming deletion of *gph*	Forward: 5' GCGGGCGCGGATATGTTCGT Reverse: 5' ATGGTAGTCATCCTGCATGT
gmhb	confirming deletion of *gmhB*	Forward: 5' TCCAGACGTCTAAATCAATC Reverse: 5' GGAGTCGGCTCAGGAAGA
serA	amplification of mutant *serA* alleles from adapted strains for cloning	Forward: TGAAAACCTGTACTTCCAGGGATCCGCAAAGGTATCGCTGGAGA Reverse: TCGAGTGCGGCCGCAAGCTTGGCTGCAGGTCGCAACGCGGCAACGGTG

Table S7. Primers for QuikChange site-directed mutagenesis of *gapA*.

mutation of *gapA* t473 to a to generate L158QV GAPDH	gapA_SDM_t473a_F	TGGCTCCGCaGGCTAAAGTTATC
	gapA_SDM_t473a_R	GGCAGTTGGTGGTGCAGGAA
mutation of *gapA* g629 to t to generate G210V GAPDH	gapA_SDM_g629t_F	CCTCTACCGtTGCTGCTAAAGCTG
	gapA_SDM_g629t_F	ACGGGATGATGTTCTGGGAA

Legends for Datasets S1 to S3

Dataset 1. Mutations found in evolved strains.

Dataset 2. Abundances of targeted metabolites in lysates of wild-type *E. coli*, JK1, and strains in which mutations generating G210V GAPDH had been introduced.

Dataset 3. Abundances of 1790 proteins in JK1 and wild-type *E. coli*.

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