Supporting Information

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SI Materials and Methods

Construction of Mutant Strains. Kanamycin and chloramphenicol resistance genes (Km^R or Cm^R) carried by pKD4 or pKD3 plasmids, respectively, were amplified by PCR. Sense and antisense primers (Table S1) contained nucleotide sequences (80 bp) corresponding to the N- and C-terminus of each targeted gene followed by 20 bp (underlined) corresponding to the Flp recognition target site (FRT)-antibiotic resistance cassette. Single knock-out mutants were constructed by electroporation of amplified DNA fragments into *Escherichia coli* strains harboring pKD46 containing an arabinose-inducible Red recombinase. After the first gene replacement by an antibiotic resistance gene, additional chromosomal deletions and integrations were achieved by generalized P1 phage transduction from single mutant lysates (1). Removal of antibiotic resistance genes was by Flp recombinase expressed from pCP20 (2), leaving an FRT scar. Chromosomal deletions and/or integrations were tested for antibiotic resistance, PCR analysis, sequencing, and enzymatic activity assay.

Adaptive Evolution. Minimal synthetic medium contained 37.9 mM $(NH4)_2SO_4$, 46 mM K_2HPO_4, 14.1 mM Na_2HPO_4, 60.6 mM $(NH_4)_2HPO_4$, 2.4 mM NH_4Cl, 4.1 mM $MgSO_4$, 0.3 mM $CaCl_2$, 14 μM $ZnSO_4$, 12 μM $CuCl_2$, 118 μM $MnSO_4$, 34 μM $CoCl_2$, 16 μM H_3BO_3, 2 μM Na_2MoO_4, 0.01 g/L thiamine, 0.04 g/L $FeSO_4$, and 5 g/L glucose,. Glucose, thiamine, and $FeSO_4$ were sterilized separately by filtration. At the beginning of the evolutionary process, a preculture was grown in LB medium, harvested by centrifugation, washed in the same volume of fresh minimal medium (lacking carbon source, thiamine and $FeSO_4$), and used for inoculation to an optical density 600 nm (OD_{600}) of 0.1. Serial evolution subculture growth was regularly determined by OD_{600} measurement, and cells were diluted to an $OD_{600\,nm}$ of 0.1 into fresh medium before they entered stationary phase.

Determination of Intracellular Cofactor Concentrations. NAD(H) was measured in the presence of 0.093 mg/mL alcohol dehydrogenase, 130 mM TEA-HCl, pH 7.4, 5 mM Na_2EDTA, 2 mM phenazine ethosulphate (PES), 0.5 mM thiazolyl blue tetrazolium bromide (MTT), and 1 M ethanol. NADP(H) was measured in the presence of 1 units mL^{-1} ICD, 130 mM TEA-HCl, pH 7.4, 50 mM $MgCl_2$, 0.3 mM phenazine methosulphate (PMS), 0.5 mM MTT, and 10 mM isocitrate. For both cofactors, the kinetic of the cycling reaction were followed at 570 nm at 37 °C over one hour. The time corresponding to the best correlation factor to standard curves was used to calculate absorbance values.

Complex I Characterization. ***Expression, purification, and biochemical characterization of NuoEFG and NuoEF*G soluble fragments.*** All steps were at 4 °C. Cells were resuspended in an appropriate volume of 50 mM MES-NaOH, 50 mM NaCl, pH 6.6, 10 μg/mL DNase (Invitrogen), protease inhibitor (Complete Mini EDTA-free, Roche) buffer to a final concentration of 0.1 g wet cells per mL. Cells were disrupted by four sonication cycles of 30 seconds at 35 watts, and 2 min on ice. Cell debris and membranes were removed by centrifugation for 1 h at 250,000 × *g*. Supernatant treatment and soluble fragment purification were as previously described (3). The purity of the eluted NADH:ubiquinone oxidoreductase fragment was tested by SDS-PAGE gel before determination of NAD(P)H/ferricyanide reductase activity by spectrophotometric assay containing 100–200 ng of purified fragment, 35 mM MES-NaOH, 35 mM NaCl, pH 6.6 and 100 μM NAD(P)H (4). Km and Vmax values were determined by fluorometric measurements of the initial rates of NAD(P)H/ferricyanide reductase activity, as described previously (4), over a range of 1–50 μM NADH and 0.002–5 mM NADPH. Kinetics parameters were calculated using SigmaPlot (10.0.1) Enzymatic Kinetics Module 1.0 software (SPSS Inc.) by fitting to the Michaelis–Menten equation using a nonlinear regression equation $Vi = (Vmax * S)/(Km + S)$. Protein concentrations were determined using the Bradford method (5), with bovine serum albumin as the standard.

Construction of expression vectors. For construction of expression plasmids for the evolved soluble fragments, pET11a/*nuoB-G*/NuoF*(E183A)c and pET11a/*nuoB-G*/NuoF*(E183G)c, primers were synthesized to amplify the 5′-^{2888}XhoI/NheI^{4396}-3′ *nuoF* DNA fragment from the NA23 and NA24 evolved strains: i) 5′- TCCGTTATTGTGACAGCGTGG -3′ designed to amplify upstream sequence of *nuoE* 5′-end containing XhoI site, and ii) 5′- CTCCAGCTAGCCCAGCGCTCTTTCAGCAGG -3′ (underlining is the NheI site) introduced an NheI site between the 3′ end of *nuoF* and the Strep-tag II sequence. The resulting XhoI/NheI-digested *nuoF* DNA fragment was ligated into XhoI/NheI cut pET-11a/*nuoB-G*/NuoFc.

Preparation of inside-out vesicles. Cells were harvested at the logarithmic phase of growth (approximately 4 g of wet weight of cells), and were washed once in 50 mM potassium phosphate (KP_i; pH 7.5). Cell pellets were resuspended in 50 mM KP_i (pH7.5)/5 mM $MgSO_4$ (5 mL/g wet weight of cells) and benzonase (Sigma) was added to a final concentration of 50 U/mL to digest nucleic acids. Cell suspensions were passed once through French pressure cell at low shear forces (5,000 psi). Unbroken cells and cell debris were removed by centrifugation at 10,000 × *g* for 10 min at 4 °C, and the supernatant was centrifuged at 120,000 × *g* for 2 h at 4 °C so as to sediment the membrane fraction. The reddish brown precipitate was washed once with 50 mM KP_i (pH7.5)/5 mM $MgSO_4$. Finally, ISO vesicles were resuspended in 50 mM KP_i (pH7.5)/5 mM $MgSO_4$/1 mM dithiothreitol (DTT)/10% glycerol at a protein concentration of 1 mg/mL and were stored at −80 °C.

Enzyme assays on ISO vesicles. Reaction mixtures contained 10 μg of ISO vesicles, in 1 mL of 50 mM KP_i (pH7.5)/5 mM $MgSO_4$. Reactions were started by addition of 100 μM of NAD(P)H. NAD(P)H oxidase activities were measured at 340 nm, and were calculated by using a millimolar extinction coefficient of 6.22.

Determination of membrane potential (ΔΨ). The reaction mixture contained, in 1 mL, 50 mM KP_i (pH7.5)/5 mM $MgSO_4$, 1 μM oxonol V and 0.1 mg of ISO vesicles. NAD(P)H and potassium cyanide were added at a final concentration of 0.5 mM and 5 mM, respectively. The fluorescence emission of oxonol V was measured at 642 nm with excitation at 580 nm.

1. Miller JH (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichica coli and Related Bacteria* (Plainview, NY, Cold Spring Harbor Laboratory Press).

2. Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158:9–14.

3. Bungert S, Krafft B, Schlesinger R, Friedrich T (1999) One-step purification of the NADH dehydrogenase fragment of the *Escherichia coli* complex I by means of Strep-tag affinity chromatography. *FEBS Lett* 460:207–211.
4. Friedrich T, et al. (1989) A small isoform of NADH:ubiquinone oxidoreductase (complex I) without mitochondrially encoded subunits is made in chloramphenicol-treated *Neurospora crassa*. *Eur J Biochem* 180:173–180.
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254.

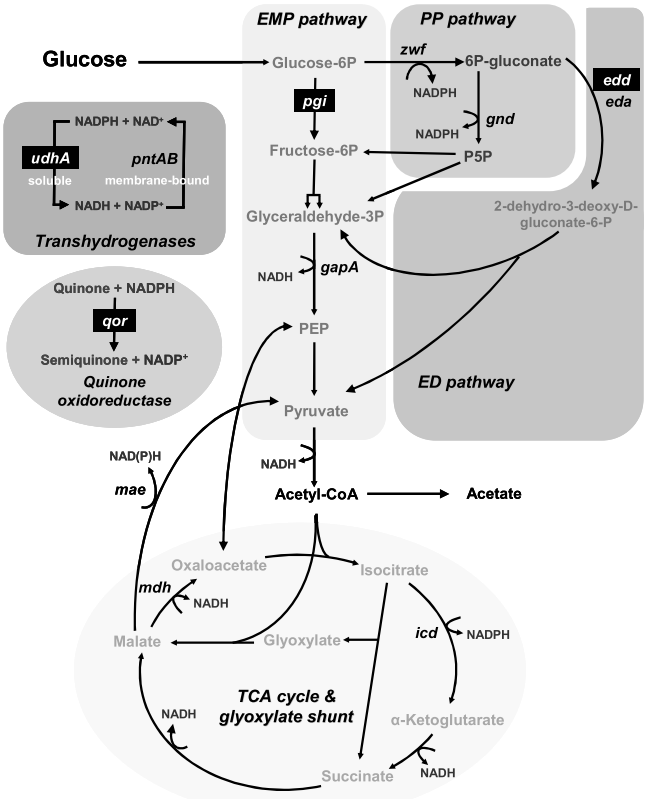

Fig. S1. Network of glucose metabolism reactions in *E. coli* MG1655 with NAD(P)H-producing reactions. Arrows indicate assumed reversibility. Key enzymes of central metabolism are indicated with their corresponding gene. Enzymes inactivated in the NADPH overproducing NA 23 mutant (MG1655 Δ*pgi*::*FRT* Δ*edd*::*FRT* Δ*qor*::*FRT* Δ*udhA*::*FRT*) are in white.

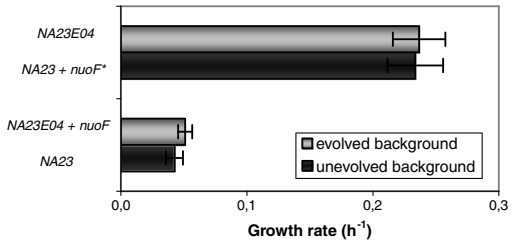

Fig. S2. Comparison of the maximum specific growth rate of evolved (NA23E04), unevolved (NA23), and reconstructed strains (*NA23*Δ*nuoF*::*nuoF**-Cm^R and *NA23E04*Δ*nuoF**::*nuoF*-Cm^R) Unevolved or evolved genotype background indicates the derivation of each reconstructed strain. Average and standard deviation are given for at least three independent experiments.

Table S1. *Escherichia coli* strains, plasmids, and primers

	Genotype and characteristics	Sources
Escherichia coli strains		
MG1655	*F⁻ λ⁻ ilvG- rfb-50 rph-1, from E. coli* K-12	(1)
NA23	MG1655 Δ*pgi*::*FRT* Δ*udhA*::*FRT* Δ*qor*::*FRT* Δ*edd*::*FRT*	This study
NA23E04	MG1655 Δ*pgi*::*FRT* Δ*udhA*::*FRT* Δ*qor*::*FRT* Δ*edd*::*FRT*, NA 23 strain evolved after 20 generations on glucose mineral medium	This study
WT*	MG1655 *nuoABCDEF*(E183A)GHIJKLMN-FRT*-Km^R-*FRT*	This study
NA24	MG1655 Δ*pfkAB*::*FRT* Δ*udhA*::*FRT* Δ*qor*::*FRT* Δ*edd*::*FRT*	This study
Plasmids		
pkD46	Ap^R, *araC-P*$_{araB}$ γ, β, et *exo*, overexpression of red recombinase system from bacteriophage λ, temperature-conditional replicon	(2)
pKD3	Cm^R, FRT—cat—FRT	(2)
pKD4	Km^R, FRT—kan—FRT	(2)
pCP20	Ap^R, *FLP*, λ, temperature-conditional replicon, overexpression of FLP recombinase from *Saccharomyces cerevisiae*	(3)
pET11a/nuoB-*G*/NuoF$_c$	Ap^R, overexpression of soluble fragment *nuoBCDEFG*, with a Strep-tag II at the C-terminus of NuoF	(4)
pET11a/nuoB-*G*/NuoF∗(E183A)$_c$	Ap^R, pET11a/nuoB-*G*/NuoF$_c$ with NuoF*(E183A) mutation	This study
pET11a/nuoB-*G*/NuoF∗(E183G)$_c$	Ap^R, pET11a/nuoB-*G*/NuoF$_c$ with NuoF*(E183G) mutation	This study
Primer sets		
pgi	5′-CCAACGCAGACCGCTGCCTGGCAGGCACTACAGAAACA CTTCGATGAAATGAAAGACGTTACGATCGCCGATCTTTTTGC TGTAGGCTGGAGCTGCTTCG-3′5′-GCGCCACGCTTTATAGCGGTT AATCAGACCATTGGTCGAGCTATCGTGGCTGCTGATTT CTTTATCATCTTTCAGCTCTGCATATGAATATCCTCCTTAG-3′	This study
udhA	5′-GGTGCGCGCGTCGCAGTTATCGAGCGTTATCAAAATGTTGGC GGCGGTTGCACCCACTGGGGCACCATCCCGTCGAAAGC CATATGAATATCCTCCTTAG-3′5′- CCCAGAATCTCTTTTGTTTCCCGATGGAACAAAATTTTCAGCGTGCCCACGTT CATGCCGACGATTTGTGCGCGTGCCAGTGTAGGCTGGAGCTGCTTCG-3′	This study
qor	5′-GGTGGCCCGGAAGTACTTCAAGCCGTAGAGTTCACTCC TGCCGATCCGGCGGAGAATGAAATCCAGGTCGAAAAT AAAGCCATATGAATATCCTCCTTAG-3′5′- CGCCCGGCTTTCCAGAATCTCATGCGCACGCTGCGCATCCTTCA GCGGATATTTCTGCTGCTCGGCGACATCGACCTTAATGTAGGCTGGAGCTGCTTCG-3′	This study
edd	5′-CGCGCGAGACTCGCTCTGCTTATCTCGCCCGGATAGAACAAGCGAAAA CTTCGACCGTTCATCGTTCGCAGTTGGCATGCGGTGTAGGCTGGAGCTGCTTCG-3′5′- CGCAAGGCGCTGAATAATTCACGTCCTGTTCCCACGCGTGACG CGCTCAGGTCAGGAATGTGCGGTTCGCGAGCAGCCCATATGAATATCCTCCTTAG-3′	This study
pfkA	5′-GGTGTGTTGACAAGCGGCGGTGATGCGCCAGGCATGAACGCCGCAA TTCGCGGGGTTGTTCGTTCTGCGCTGACAGAAGGTGTAGGCTGGAGCTGCTTCG-3′5′- TTCGCGCAGTCCAGCCAGTCACCTTTGAACGGACGCTTCATGTTTT CGATAGCGTCGATGATGTCGTGGTGAACCAGCTGCATATGAATATCCTCCTTAG-3′	This study
pfkB	5′-GCGCCCTCTCTCGATAGCGCAACAATTACCCCGCAAATTTATCCCGAA GGAAAACTGCGCTGTACCGCACCGGTGTTCGTGTAGGCTGGAGCTGCTTCG-3′5′- GCGGGAAAGGTAAGCGTAAATTTTTTGCGTATCGTCATGGGAGCACAGACGT GTTCCCTGATTGAGTGTGGCTGCACTCCCATATGAATATCCTCCTTAG-3′	This study
nuoABCDEFGHIJKLMN-FRT-Km^R-FRT	5′-GCAAAATTCAGGCAGTTATGTTTAGAGTATTTTGGCGATAGTCGTACA CAGTTCACTTAACGCTCTGCGTCTATCTACTATGTAGGCTGGAGCTGCTTCG-3′5′- CAATGCCGCTGATGTAATCTGTTACTTTGTAAGTCAGAAAGCCG CCGACATGCTCGGCGGTTTTTCTGAAAAAGAAGGGACATATGAATATCCTCCTTAG-3′	This study

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3 Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158:9–14.

4 Bungert S, Krafft B, Schlesinger R, Friedrich T (1999) One-step purification of the NADH dehydrogenase fragment of the *Escherichia coli* complex I by means of Strep-tag affinity chromatography. *FEBS Lett* 460:207–211.