Supporting Information

Refactoring the Embden-Meyerhof-Parnas pathway as a whole of portable GlucoBricks for implantation of glycolytic modules in Gram-negative bacteria

by

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SUPPLEMENTARY METHODS

Construction of mutant bacterial strains. Bacterial strains used in this study are listed in *Table S1*. A Δ*glk* derivative of *Pseudomonas putida* KT2440 was constructed by using the procedure described by Martínez-García and de Lorenzo[1], using the suicide pEMG vector (*Table S2*). Suitable oligonucleotides (*PP_1011*-TS1F and *PP_1011*-TS1R, and *PP_1011*-TS2F and *PP_1011*-TS2R, *Table S3*) were employed to amplify *ca*. 500-bp long flanking regions upstream and downstream of *glk* (*PP_1011*) by PCR using chromosomal DNA from strain KT2440 as the template. A 1-kbp amplification product (i.e., spanning the TS1-TS2 regions flanking *glk*) was obtained using the two 500-bp amplicons mentioned above as the template and the external oligonucleotides (i.e., *PP_1011*-TS1F and *PP_1011*-TS2R) by splicing-by-overlap extension (*SOEing*) PCR[2,3]. The TS1-TS2 DNA fragment was digested with *Eco*RI and *Bam*HI, cloned into the I-*Sce*I–bearing pEMG vector digested with the same enzymes (giving rise to plasmid pEMGΔ*glk*) and verified by enzyme restriction and Sanger DNA sequencing (Secugen SL, Madrid, Spain). The suicide pEMGΔ*glk* plasmid was transferred to *P. putida* KT2440 by electroporation. Kanamycin (Km)-resistant colonies (i.e., chromosomal co-integration events) were checked by colony PCR[1]. Successful co-integrates were transformed with plasmid pSW-I, which allows for the resolution of co-integration events by inducing the expression of the gene encoding the I-*Sce*I homing meganuclease from *Saccharomyces cerevisiae* with sodium 3-methylbenzonate at 15 mM. The resultant Km-sensitive colonies were checked by PCR to select for the correct deletion events. Plasmid pSW-I was finally cured by successive passages on LB medium[4] without antibiotics. The removal of the plasmid was tested by plating the cells onto LB plates containing 500 μg ml^{-1} ampicillin.

Preparation of bacterial cell-free extracts. All the enzyme activity determinations were carried out in cell-free extracts obtained from bacterial cells harvested during the mid-exponential phase of growth [i.e., corresponding to an optical density measured at 600 nm (OD_{600}) of *ca*. 0.5] or, in some cases, after 24 h of culture. Cell-free extracts were prepared starting from 50 ml of culture broth and centrifuging it at 4,000 r.p.m. for 15 min at 4ºC. Cell were washed twice with 25 ml of pre-cooled 67 mM potassium phosphate buffer (pH = 7.1) at 4ºC. The washed pellets were suspended in 1 ml of the same buffer and centrifuging in 2-ml Eppendorf tubes at 8,000 r.p.m. for 10 min at 4ºC. After carefully removing the supernatant, the cell wet weight was obtained for each pellet in order to calculate the volume of reagents needed for protein extraction using the Novagen BugBuster™ protocol (EMD Millipore Corp., Billerica, MA, USA). Pellets and cell-free extracts were kept on ice throughout the whole procedure. Bacterial lysis was achieved by using 5 ml of BugBuster™ Protein Extraction Reagent per gram of cell paste. Afterwards, 1 μl of Lysonase™ Bioprocessing Reagent was added per 1 ml of BugBuster™ Protein Extraction Reagent used for re-suspension of the cells. Bacteria were lysed by shaking for 20 min at room temperature in a Rotamax 120 orbital shaker (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) at 150 r.p.m. The insoluble cell debris was removed by centrifugation at 13,000 r.p.m. for 20 min at 4ºC and the supernatants (i.e., cell-free extracts) were stored at –20ºC until use.

***In vitro* enzymatic assays.** The activity of three key glycolytic enzymes (Glk, Pfk, and GA3P dehydrogenase) was assessed *in vitro* at 30ºC when using cell-free extracts from *P. putida* or at 37ºC when using cell-free extracts from *P. aeruginosa* and *Escherichia coli*, the remaining activities were measured at 25ºC as indicated by Sigma-Aldrich Co. (St. Louis, MO, USA) in the corresponding enzymatic assay protocols. All the enzymes were assessed under the optimal reported conditions for pH, substrate, and cofactor concentration[5-8].

In vitro assays were conducted in Nunc™ MicroWell™ 96-well microplates (Thermo Fisher Scientific Inc., Waltham, MA, USA) in a SpectraMax™ M2e multi-mode microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA). All the specific enzyme activities are reported as nmol substrate converted min^{-1} mg of $protein^{-1}$.

The protein concentration in cell-free extracts was obtained using the Bradford[9] Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA). All the accessory enzymes (with the exceptions indicated below) were from *Saccharomyces cerevisiae* and they were purchased from Sigma-Aldrich Co. An extinction coefficient ($\varepsilon_{NADP/H}$) of 6.22 mM^{-1} cm^{-1}, representing the difference between the extinction coefficients of NAD(P)H and $NAD(P)^+$, was used throughout. The specific protocols used for the determinations are detailed below.

Glk. *ATP–D-Hexose 6-phosphotransferase* or *hexokinase* (EC 2.7.1.1). The reaction mixture contained 67 μl of 120 mM Tris·HCl buffer (pH = 8.2), 26 μl of 500 mM glucose, 8 μl of 250 mM $MgCl_2$, 53 μl of 36 mM ATP, 10 μl of 20 mM $NADP^+$, 13 μl of glucose-6-*P* dehydrogenase (15 units ml^{-1}), 18 μl of water, and 5 μl of cell-free extract (or an appropriate dilution in 67 mM potassium phosphate buffer, pH = 7.1). The increase in the absorbance at 340 nm (A_{340}) was measured during the assay.

Pgi. *Phosphoglucose isomerase* (EC 5.3.1.9). The reaction mixture contained 33.3 μl of 240 mM glycylglycine Buffer (pH = 7.1), 6.7 μl of 100 mM D-fructose-6-*P*, 6.7 μl of 20 mM $NADP^+$, 6.7 μl of 100 mM $MgCl_2$, 6.7 μl of glucose-6-*P* dehydrogenase (50 units ml^{-1}), 133.2 μl of water, and 6.7 μl of cell-free extract (or an appropriate dilution in 67 mM potassium phosphate buffer, pH = 7.1). The increase in A_{340} was measured during the assay.

Pfk. *6-Phosphofructokinase* (EC 2.7.1.11). The reaction mixture contained 20 μl of 1 M Tris·HCl buffer (pH = 7.5), 2 μl of 100 mM fructose-6-*P*, 8 μl of 250 mM $MgCl_2$, 4 μl of 100 mM NH_4Cl, 4 μl of 10 mM NADH, 6 μl of 36 mM ATP, 4 μl of fructose-1,6-P_2 aldolase (50 units ml^{-1}), 1.5 μl of triosephosphate isomerase (500 units ml^{-1}), 1.5 μl of glycerol-3-*P* dehydrogenase from rabbit muscle (170 units ml^{-1}), 144 μl of water, and 5 μl of cell-free extract (or an appropriate dilution in 67 mM potassium phosphate buffer, pH = 7.1). The decrease in A_{340} was measured during the assay.

Aldolase. *Aldolase* (EC 4.1.2.13). The reaction mixture contained 17.3 μl of 1 M Tris·HCl buffer (pH = 7.4), 6.7 μl of 58 mM fructose-1,6-P_2, 1.9 μl of 14 mM NADH, 6.7 μl of α-glycerophosphate dehydrogenase/triosephosphate isomerase from rabbit muscle (50 units ml^{-1} each), 165.4 μl of water, and 2 μl of cell-free extract (or an appropriate dilution in 67 mM potassium phosphate buffer, pH = 7.1). The decrease in A_{340} was measured during the assay.

Tpi. *Triose phosphate isomerase* (EC 5.3.1.1). The reaction mixture contained 20 μl of 1 M triethanolamine buffer (pH = 7.9), 2 μl of 0.5 M EDTA, 4 μl of glycerol-3-*P* dehydrogenase from rabbit muscle (17 units ml^{-1}), 2 μl of 40 mM D,L-glyceraldehyde-3-*P*, 2 μl of 14 mM NADH, 168 μl of water, and 2 μl of cell-free extract (or an appropriate dilution in 67 mM potassium phosphate buffer, pH = 7.1). The decrease in A_{340} was measured during the assay.

GA3P dehydrogenase. *Glyceraldehyde-3-P dehydrogenase* [EC 1.2.1.12 (NAD^+ dependent)/EC 1.2.1.13 $NADP^+$ dependent)]. The reaction mixture contained 20 μl of 1 M Tris·HCl buffer (pH = 7.5), 5 μl of 250 mM D,L-glyceraldehyde-3-*P*, 40 μl of 50 mM cysteine·HCl, 20 μl of 150 mM NaH_2AsO_4, 34 μl of 120 mM NaF, 20 μl of 35 mM NAD^+ or $NADP^+$, 56 μl of water, and 5 μl of cell-free extract (or an appropriate dilution in 67 mM potassium phosphate buffer, pH = 7.1). The increase in A_{340} was measured during the assay.

SUPPLEMENTARY TABLES

Table S1. Bacterial strains used in this study.

Strain	Relevant characteristics[a]	Reference or source
Escherichia coli		
CC118	Cloning host; Δ(*ara-leu*) *araD* Δ*lacX174 galE galK phoA thiE1 rpsE rpoB*(Rif^R) *argE*(Am) *recA1*	Manoil and Beckwith[10]
DH5α λ*pir*	Cloning host; F$^-$ λ$^-$ *endA1 glnX44*(AS) *thiE1 recA1 relA1 spoT1 gyrA96*(Nal^R) *rfbC1 deoR nupG* Φ80(*lacZ*Δ*M15*) Δ(*argF-lac*)*U169 hsdR17*(r_K^- m_K^+), λ*pir* lysogen	Hanahan and Meselson[11]
BW25113[b]	Wild-type strain; F$^-$ λ$^-$ Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-3*) *rph-1* Δ(*rhaD-rhaB*)*568 hsdR514*	Datsenko and Wanner[12]
JW3887-1[b]	Same as BW25113, but Δ*pfkA775*::*aphA*; Km^R	Baba *et al.*[13]
JW5280-1[b]	Same as BW25113, but Δ*pfkB722*::*aphA*; Km^R	Baba *et al.*[13]
BPfkAB	Same as BW25113, but Δ*pfkA775*::*FRT* Δ*pfkB722*::*aphA*; Km^R	This study
JW2385-1[b]	Same as BW25113, but Δ*glk-726*::*aphA*; Km^R	Baba *et al.*[13]
JW2409-1[b]	Same as BW25113, but Δ*ptsI745*::*aphA*; Km^R	Baba *et al.*[13]
BPG	Same as BW25113, but Δ*glk-726*::*FRT* Δ*ptsI745*::*aphA*; Km^R	Nikel and de Lorenzo[2]
JW3890-2[b]	Same as BW25113, but Δ*tpiA778*::*aphA*; Km^R	Baba *et al.*[13]
W3110	Wild-type strain; F$^-$ λ$^-$ *IN*(*rrnD-rrnE*)*1 rph-1*	Jensen[14], Bachmann[15]
DS121[b]	Same as W3110, but Δ*epd-11*::*erm*; Ery^R	Seta *et al.*[16]
DS122[b]	Same as W3110, but Δ*gapA12*::*cat*; Cm^R	Seta *et al.*[16]
DS123[b]	Same as W3110, but Δ*gapA12*::*cat* Δ*epd-11*::*erm*; Cm^R Ery^R	Seta *et al.*[16]
DS123PK	Same as W3110, but Δ*gapA12*::*cat* Δ*epd-11*::*erm* Δ*ptsI745*::*aphA*; Cm^R Ery^R Km^R	This study
DS123P	Same as W3110, but Δ*gapA12*::*cat* Δ*epd-11*::*erm* Δ*ptsI745*::*FRT*; Cm^R Ery^R	This study
DF575 [b]	F$^-$ λ$^-$ *glnX44*(AS) *relA1 eno-2 rpsL104 malA malT1*(λ^R) *xyl-7 mtlA2 thiE1*	Thomson *et al.*[17]
DF576 [b]	F$^-$ λ$^-$ *glnX44*(AS) *relA1 pgk-2 rpsL104 malA malT1*(λ^R) *xyl-7 mtlA2 thiE1*	Thomson *et al.*[17]

Table S1 (cont.). Bacterial strains used in this study.

Strain	Relevant characteristics[a]	Reference or source
Pseudomonas putida		
KT2440	Wild-type strain, derived from *P. putida* mt-2[18] cured of the TOL plasmid pWW0	Bagdasarian *et al.*[19]
KT2440 Δ*glk*	Same as KT2440, but with an in-frame deletion of the *glk* gene (*PP_1011*)	This study
Pseudomonas aeruginosa		
PAO1	Wild-type strain (PAO1001), original stock conserved by P. V. Phibbs	Stover *et al.*[20]

[a] Antibiotic markers: *Ap*, ampicillin; *Cm*, chloramphenicol; *Ery*, erythromycin; *Gm*, gentamicin; *Km*, kanamycin; *Nal*, nalidixic acid; *Rif*, rifampicin; *Sm*, streptomycin; *Sp*, spectinomycin; and *Tc*, tetracycline.

[b] Strain obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT, USA).

Table S2. Plasmids used in this study.

Plasmids	Relevant characteristics[a]	Reference or source
pKD46	Ap^R; helper plasmid expressing the λ-Red recombination functions	Datsenko and Wanner[12]
pCP20	Ap^R Cm^R; helper plasmid used for excision of *FRT-aphA-FRT* (Km^R), *Saccharomyces cerevisiae FLP* λ*c*I857 λP_R *repA*(Ts)	Cherepanov and Wachernagel[21]
pEMG	Km^R; *oriV*(R6K), vector used for deletions, *lacZ*α with two flanking I-*Sce*I target sites	Martínez-García and de Lorenzo[1]
pEMGΔ*glk*	Km^R; pEMG derivative bearing a 1.0-kb TS1-TS2 *Eco*RI-*Bam*HI insert for deletion of the *glk* gene of *P. putida* KT2440	This study
pSW-I[b]	Ap^R; *oriV*(RK2), *xylS*, *Pm*→I-*Sce*I, transcriptional fusion of the gene encoding I-*Sce*I to the *Pm* promoter	Wong and Mekalanos[22]
pAeT41	Ap^R; derivative of cloning vector pUC18 (Thermo Fisher Scientific Inc.; Pittsburgh, PA, USA) bearing a *ca*. 5-kb *Sma*I/*Eco*RI DNA fragment spanning the *phaC1AB1* gene cluster from *Cupriavidus necator*	Peoples and Sinskey[23]
pSEVA224	Km^R; standard SEVA expression vector, *oriV*(RK2) $lacI^Q$, P_{trc}	Silva-Rocha *et al*.[24]
pS224·GPG	Km^R; pSEVA224 derivative bearing *glk* and *pfkA* from Module I, and *gapA* from Module II	This study
pS224·GBI	Km^R; pSEVA224 derivative bearing Module I as an *Avr*II-*Bam*HI insert	This study
pS224·GBII	Km^R; pSEVA224 derivative bearing Module II as a *Bam*HI-*Hin*dIII insert	This study
pSEVA234	Km^R; standard SEVA expression vector, *oriV*(pBBR1) $lacI^Q$, P_{trc}	Silva-Rocha *et al*.[24]
pS234·GBI	Km^R; pSEVA234 derivative bearing Module I as an *Avr*II-*Bam*HI insert	This study
pSEVA434	Sm^R/Sp^R; standard SEVA expression vector, *oriV*(pBBR1) $lacI^Q$, P_{trc}	Silva-Rocha *et al*.[24]
pS434·GBI	Sm^R/Sp^R; pSEVA434 derivative bearing Module I as an *Avr*II-*Bam*HI insert	This study
pSEVA424	Sm^R/Sp^R; standard SEVA expression vector, *oriV*(RK2) $lacI^Q$, P_{trc}	This study
pS424·*gapA*	Sm^R/Sp^R; pSEVA424 bearing *gapA* from Module II	This study
pS424·GBI	Sm^R/Sp^R; pSEVA424 derivative bearing Module I as an *Avr*II-*Bam*HI insert	This study
pSEVA438	Sm^R/Sp^R; standard SEVA expression vector, *oriV*(pBBR1) *xylS*, *Pm*	This study
pS438·GBI	Sm^R/Sp^R; pSEVA438 derivative bearing Module I as an *Avr*II-*Bam*HI insert	This study

[a] Antibiotic markers: *Ap*, ampicillin; *Cm*, chloramphenicol; *Km*, kanamycin; *Sm*, streptomycin; and *Sp*, spectinomycin. Ts, temperature-sensitive origin of replication.

[b] This plasmid is the same as pSW(I-*Sce*I)[22], renamed here as pSW-I for simplicity.

Table S3. Oligonucleotides used in this study.

Name	**Sequence** (5' → 3')[a]	**Tm** (ºC)	**Use**
PP_1011-TS1F	G**GA ATT C**GA GGC CCC GGC GCG GGT GTT CCA GGA CCA G	89.2	Construction of *P. putida* Δ*glk*
PP_1011-TS1R	*CAT CGG GGC CGC AAA GCG CCC CCC TCA GTG* GTG CTT CAT TTG AGG TGC TCC AGG GCC GAG	91.5	
PP_1011-TS2F	*CAC TGA GGG GGG CGC TTT GCG GCC CCG ATG*	86.5	
PP_1011-TS2R	CG**G GAT CC**C GCC AGT CGT CGA AGG CCA GCA CGG CGT TG	91.2	
pfkA·fbaA-Check-F	GAA AGG TAA AAA ACA CGC GAT C	58.7	Screening of recombinants carrying Module I[b]
pfkA·fbaA-Check-R	ACG CTG CGA TGG TGA AAC	59.2	
gpmA·eno-Check-F	ATT CTG CCG CGT ATG AAG AG	58.9	Screening of recombinants carrying Module II[c]
gpmA·eno-Check-R	CAG AGC TTC AGC GTT GGA AC	59.6	
phaC1-Check-F	GCC ACT GGA CTA ACG ATG CGC TG	68.7	Screening of recombinants carrying the *phaC1AB1* gene cluster[d]
phaA-Check-F	TCA CCG TGC CGG CCT TGT C	68.6	

[a] Bold letters indicate recognition site for the restriction enzymes and the complementary sequences used in splicing-by-overlap extension (*SOEing*[2,3]) PCR amplifications are shown in italics.

[b] A PCR amplification using these oligonucleotides yields a 1,015-bp amplicon in the junction of the *pfkA* and *fbaA* genes.

[c] A PCR amplification using these oligonucleotides yields a 938-bp amplicon in the junction of the *gpmA* and *eno* genes.

[d] A PCR amplification using these oligonucleotides yields a 1,017-bp amplicon in the junction of the *phaC1* and *phaA* genes.

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