Supplementary Data

Chapter 3

What's wrong with SwYG? Reaching the limits of S. cerevisiae molecular and analytical toolbox

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Figure S1: Specific growth rates of yeast strains with Minimal Glycolysis background after integration of the single locus glycolysis

Reconstruction of strain IMX382 (SinLoG integration in *sga1* in IMX370) led to a similar growth rate defect. All growth rates are normalized to the CEN.PK113-7D control strain from the same experiment, prototrophic strains are shown as filled bars, uracil auxotrophic strains with dashed fill. Statistically significant differences relative to the auxotrophic MG strain reference strain are indicated (* $P<0.05$, t-test 2-tailed, homoscedastic).
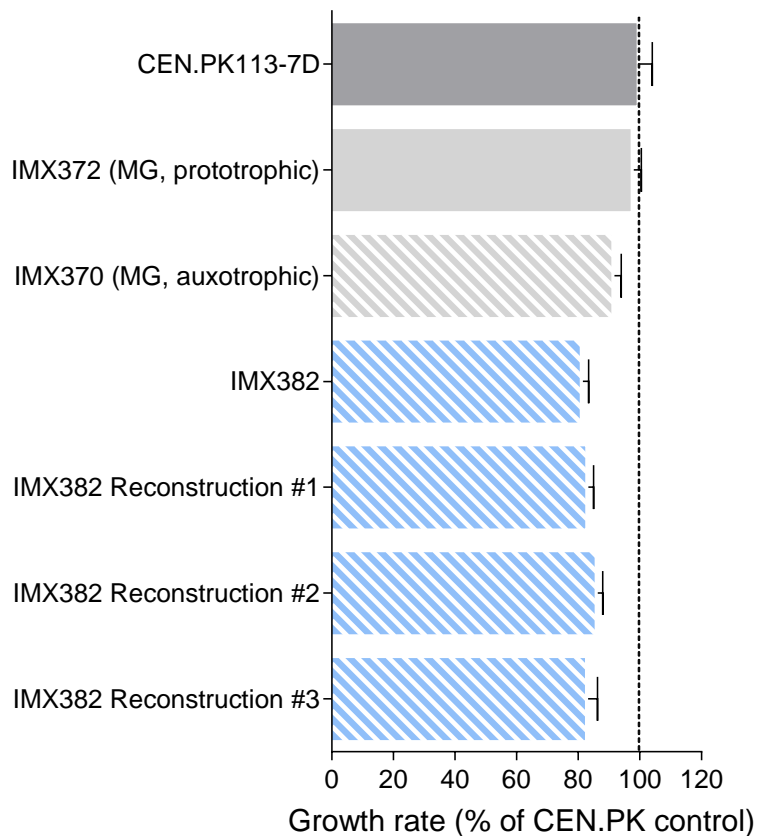
Figure S2: Strain lineage of the single locus glycolysis strains

The lineage of strain construction is indicated for the main single locus glycolysis strains used in this study. Various designs of SinLoG with changes in promotors and terminators, watermarking, orientation of genes, and presence of ARS sequences are indicated by color.
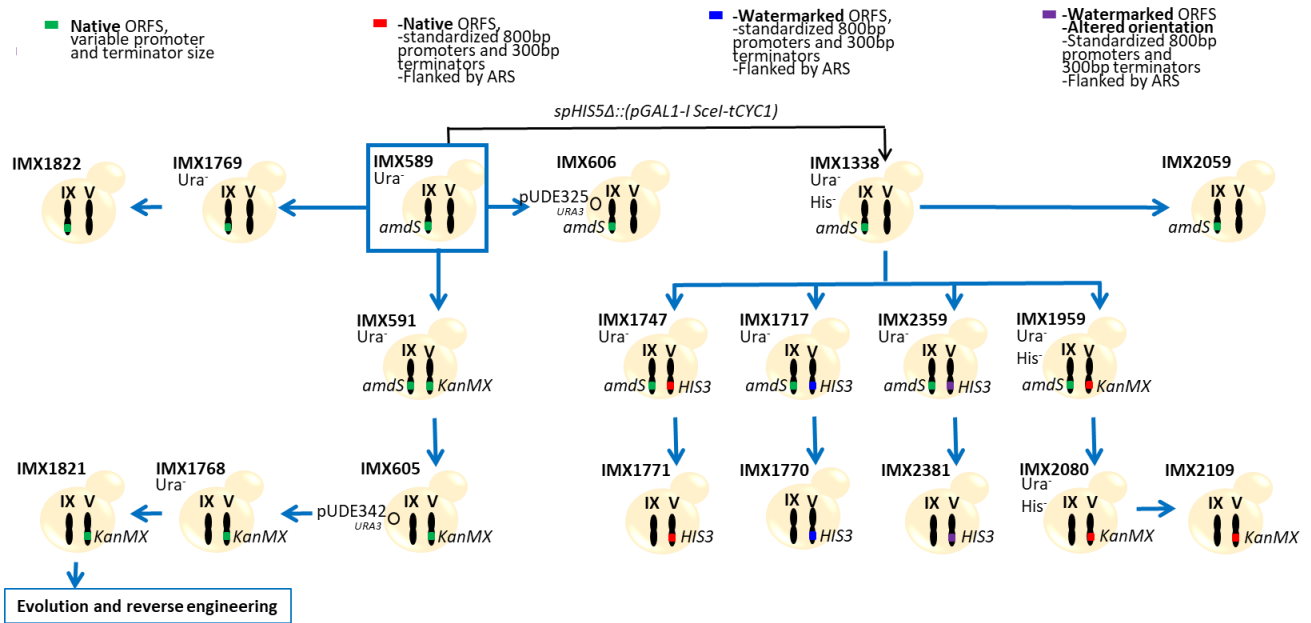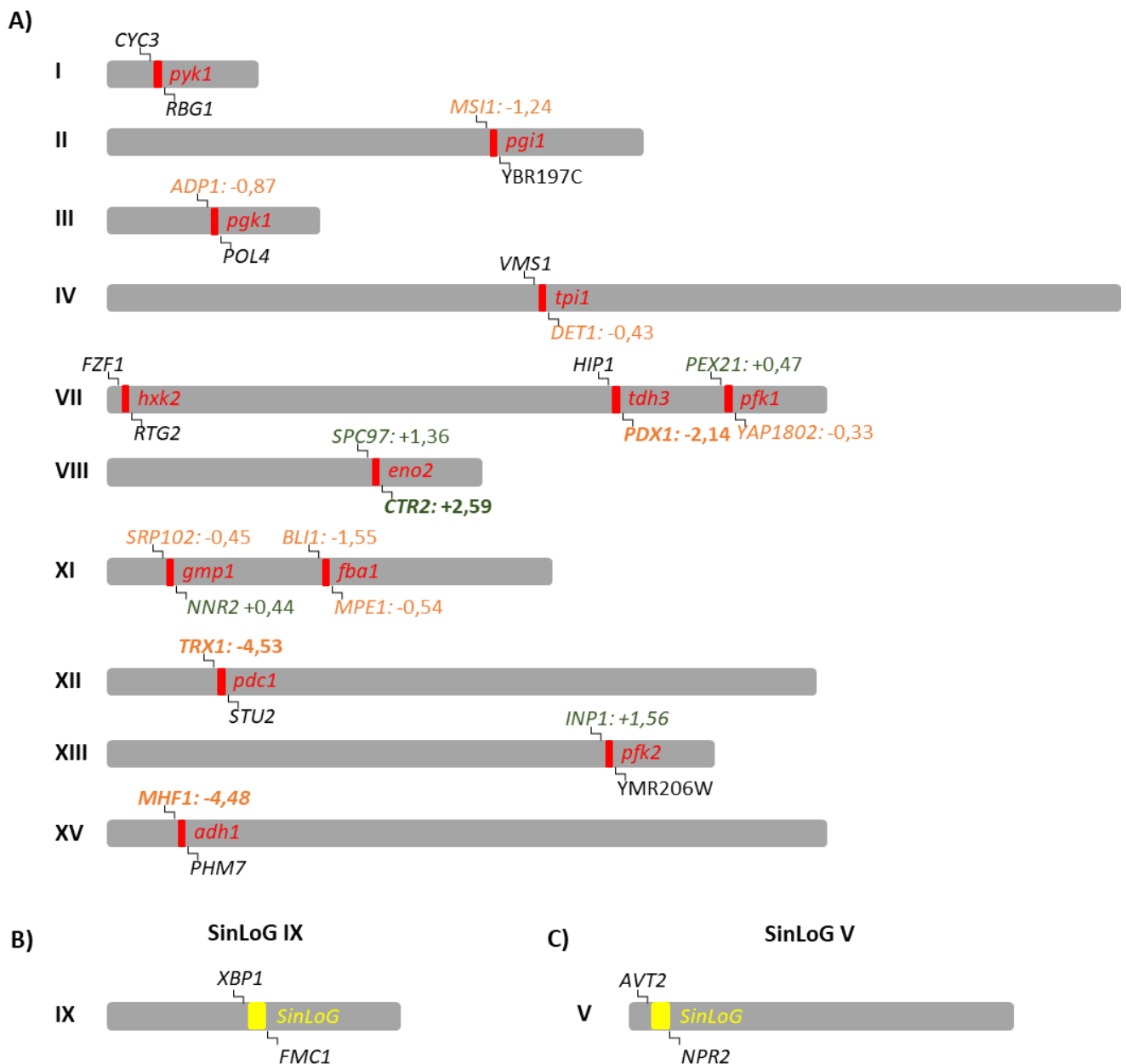
Figure S3: Relative transcription of genes neighbouring the deletion loci of native glycolytic genes

A) Relative expression in strain IMX606 (SinLoG-Chr IX) relative to the MG strain IMX372 of the genes neighbouring the deleted glycolytic loci is indicated. Genes which are decreased in expression are marked in orange, genes which are increased in expression are marked in green and genes which are not expressed or did not change in expression are marked black (Mean PFKM_MG>10 or Mean PFKM IMX606>10, padj<0.05). Genes with $\log_2$FoldChange>2 are marked in bold. B,C) Expression of genes neighbouring the SinLoG loci on Chr. IX (IMX606) and Chr. V (IMX605). D) Function and PFKM count of IMX372 (MG) of genes that are changed in expression. Genes were considered not expressed if PFKM<10 in all strains (1); high expression was considered for PFKM>300.

D)

Gene	Function	PFKM normalized counts
MSI1	Subunit of chromatin assembly factor I (CAF-1); chromatin assembly by CAF-1 affects multiple processes	53
ADP1	Putative ATP-dependent permease of the ABC transporter family	32
DET1	Acid phosphatase; involved in the non-vesicular transport of sterols in both directions between the endoplasmic reticulum and plasma membrane; deletion confers sensitivity to nickel	89
PDX1	E3-binding protein of the mitochondrial pyruvate dehydrogenase complex	105
PEX21	Peroxin required for peroxisomal matrix protein targeting	125
YAP1802	Protein of the AP180 family, involved in clathrin cage assembly	53
SPC97	Component of the microtubule-nucleating Tub4p (gamma-tubulin) complex	18
CTR2	Low-affinity copper transporter of the vacuolar membrane	148
SRP102	Signal recognition particle (SRP) receptor beta subunit; involved in SRP-dependent protein targeting	125
NNR2	Widely-conserved NADHX dehydratase; converts (S)-NADHX to NADH in ATP-dependent manner	45
BLI1	Subunit of the BLOC-1 complex involved in endosomal maturation	61
MPE1	Subunit of CPF cleavage and polyadenylation factor and E3 Ub-ligase; involved in 3' end formation of mRNA via cleavage and polyadenylation of pre-mRNA	51
TRX1	Cytoplasmic thioredoxin isoenzyme; part of the thioredoxin system that protects cells from oxidative and reductive stress	1580
INP1	Peripheral membrane protein of peroxisomes; involved in peroxisomal inheritance	16
MHF1	Component of the MFH histone-fold complex	115

Figure S4: Ploidy and cell cycle distribution estimation by flow cytometric analysis

A) Ploidy measurement based on DNA staining and flow cytometry. All analysed single locus glycolysis strains match the haploid reference. B) Cell cycle distribution based on the DNA content shown in panel A. The estimated percentage of cells in each cell cycle phase is shown. Error bars represent standard deviation between duplicate samples. The single locus glycolysis strain IMX589 (Chr IX) shows a slight increase in cells in G1 compared to the CEN.PK113-7D and IMX372 (minimal glycolysis) control strains.
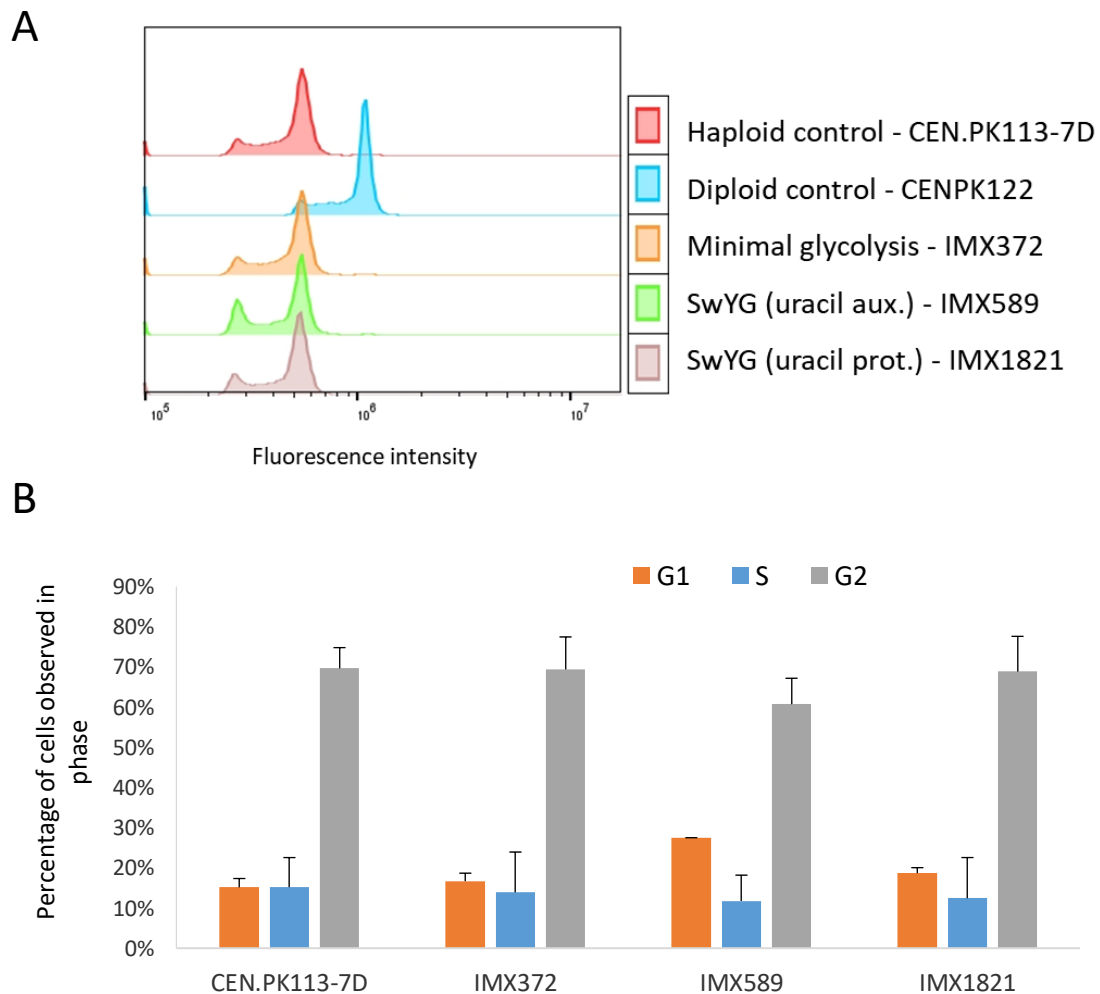
A

B

Figure S5: Global transcription effects from the single locus glycolysis

Global transcriptional changes in strains IMX605 (SinLoG on Chr V) and IMX606 (SinLoG on Chr IX) compared to the minimal glycolysis strain IMX372. Since both strains carrying a SinLoG share a similar growth rate enrichment analysis was performed on significantly up and down regulated genes in both strains to identify possible causal factors for this growth defect. Up- and down regulated genes with an adjusted p-value below 0.05 and a fold change greater than 2 were selected and doublons were removed. Enrichment analysis was performed with FunSpec (http://funspec.med.utoronto.ca/cgi-bin/funspec), GO terms with a Bonferroni-corrected p-value $< 10^{-5}$ are reported. Although several categories are significantly enriched, no link to processes related to the integration of the SinLoG were

UP

IMX606

12

210

IMX605

50

GO category	Genes UP/ genes in category	Adjusted P-value
Response to stress GO:0006950	30/152	2.9×10^{-13}
Thiamine biosynthesis GO:0009228	13/68	2.9×10^{-6}
Cell wall GO:0005618	9/17	5.1×10^{-9}

Many genes from families with high homology

SNO1
SNO3
SNZ1
SNZ2
SNZ3

Redundant gene families with high homology, transcriptionally co-regulated

THI5
THI11
THI12
THI13

Large overlap with 'stress' category

DOWN

IMX606

11

108

IMX605

21

GO category	Genes DOWN/ genes in category	Adjusted P-value
Sporulation GO:0030435	15/103	2.5×10^{-9}
Meiosis GO:0007126	13/134	3.7×10^{-6}
Transporter activity GO:0005215	11/90	2×10^{-6}

Haploid, not relevant

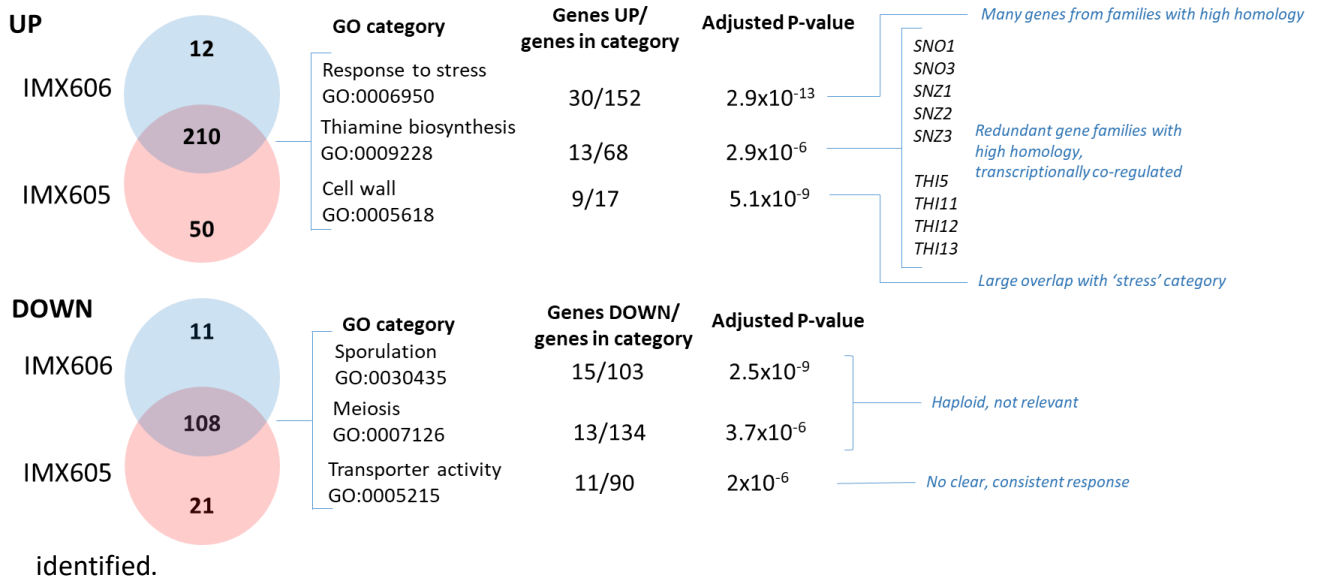*No clear, consistent response*

identified.

Figure S6: Strain lineage of evolved isolates of the SwYG strain and reverse engineered strains

A) Mutations in *ATG41*, *SUR2* and *CNB1* in evolved strains IMS1022, IMS1026 and IMS1031. B) Reverse engineering strategy of the *SUR2*, *ATG41* and *CNB1* mutations in the SwYG strain IMX1821.

A)

IMS1022
ATG41^{W132C}
SUR2^{E192STP}
CNB1^{L82F}

IMS1026
ATG41^{W132STP}
SUR2^{M179K}
CNB1^{L82F}

IMS1031
ATG41^{Disrupted}
SUR2^{G251R}
CNB1^{L82F}

B)

IMX1821

IMX2542
sur2Δ

IMX2543
atg41Δ

IMX2544
cnb1Δ

IMX2570
sur2Δ
atg41Δ

IMX2567
sur2Δ
atg41Δ
cnb1Δ

IMX2605
SUR2^{E192STP}

IMX2589
ATG41^{W132C}

IMX2594
CNB1^{L82F}

IMX2591
ATG41^{W132C}
SUR2^{E192STP}

IMX2606
SUR2^{M179K}

IMX2590
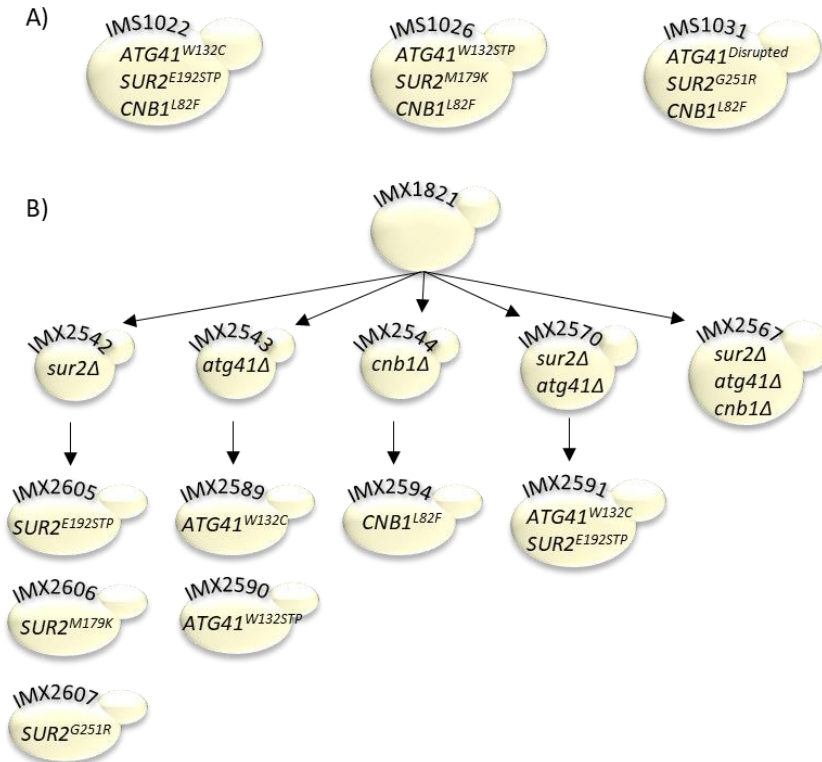ATG41^{W132STP}

IMX2607
SUR2^{G251R}

Figure S7: Physiological and genetic characterization of populations and single colony isolates after evolution of the CEN.PK113-7D reference strain

A) Specific growth rate CEN.PK113-7D and derived strains after evolution, represented as evolved population and single colony isolates. Growth rates are shown as percentage of the unevolved reference strain. No significant increase in growth rate was found except a slight increase for the population of line 1. Bars represent the average growth rate and standard deviation of duplicate shake flask cultures. Significant differences as compared to unevolved control are indicated (*, P<0.05, T test, homoscedastic). B) Venn diagram showing genes in which mutations were identified in the evolved strains derived from CEN.PK113-7D.
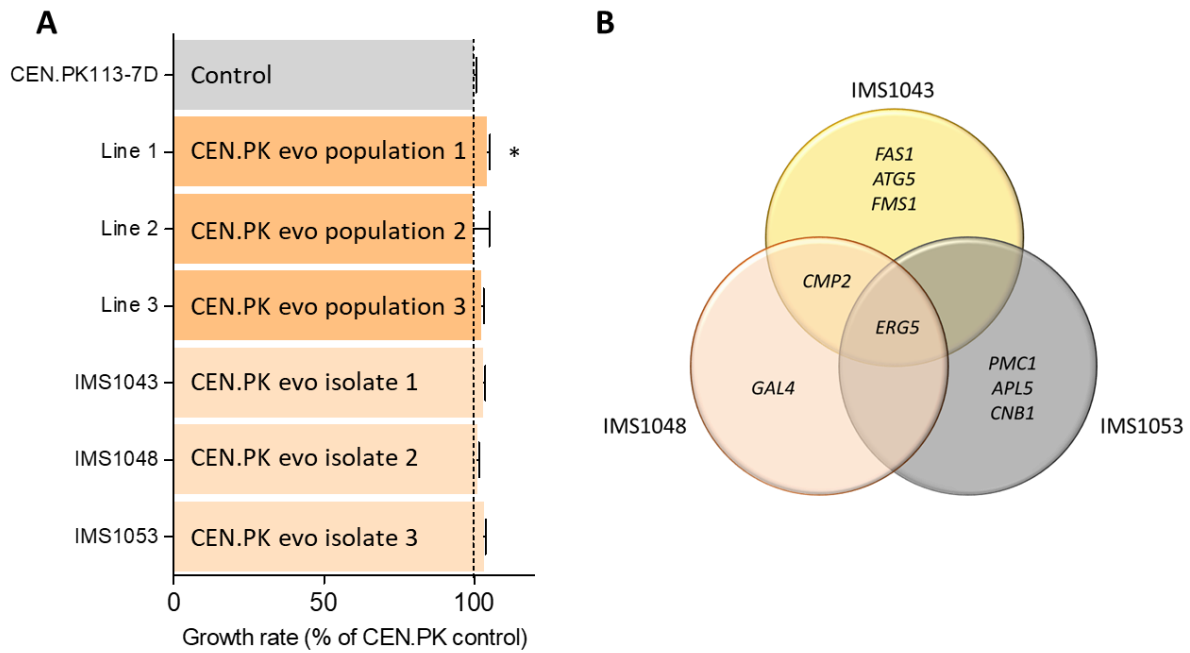
Table S1 Single nucleotide polymorphisms in the auxotrophic SwYG strain (IMX589) compared to MG strain (IMX372)

Information from Kuijpers *et al.* (2)

Mutations (SNP)	Genome	SinloG
All	15	9
In ORF	6#	1*
MisSense	3 (*VPS15, OPT1, CWC25*)	0

 # Information on the 6 in ORF SNPs in the genome

Systematic name	Name	Type	Amino Acid change
YBR079W	*VPS15*	Non-synonymous	E474K
YJL212C	*OPT1*	Non-synonymous	I463T
YNL245C	*CWC25*	Non-synonymous	P62L
YDL079C	*MRK1*	Synonymous	I190I
YLR180W	*SAM1*	Synonymous	V217V
YNL262W	*POL2*	Synonymous	F1536F

* Information on the single SNP in the SinLoG

Systematic name	Name	Type	Amino Acid change
YOL086C	*ADH1*	Synonymous	A180A

Table S2: List of strains used in this study

SHRs are differently annotated than in Kuijpers *et al.* (2) and Boonekamp *et al.* (3). SHRs are annotated in subscript between the genetic fragments that they join together. Watermarked genes are marked with a *.

Strain	Description	Relevant genotype	Reference
CEN.PK113-7D	Prototrophic reference	*MATα URA3 TRP1 LEU2 HIS3 MAL2-8c SUC2*	(4)
CEN.PK113-5D	Auxotrophic reference	*MATa URA3-52 TRP1 LEU2 HIS3 MAL2-8c SUC2*	(4)
CEN.PK122	Diploid reference	*MATα/MATα URA3/URA3 TRP1/TRP1 LEU2/LEU2 HIS3/HIS3 MAL2-8c/MAL2-8c SUC2/SUC2*	(4)
IMX372	Prototrophic minimal glycolysis (MG) strain	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ*	(5)
IMX370	Auxotrophic minimal glycolysis (MG) strain	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4*	(5)
IMX377	Insertion *I-SceI* expression cassette at *sga1* locus of MG	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5, hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(TagG-KlURA3- pGAL1-SCEI-tCYC1-TagF)*	(2)
IMX382	Insertion SinLoG in IMX377	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$ FBA1 $_H$ TPI1 $_P$ PGK1 $_Q$ ADH1 $_N$ PYK1 $_O$ TDH3 $_A$ amdSYM $_B$ HXK2 $_C$ PGI1 $_D$ PFK1 $_J$ PFK2 $_K$ ENO2 $_L$ GPM1 $_M$ PDC1-SYN $_F$)*	(2)
IMX457	Deletion *PYK1* from IMX382	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$ FBA1 $_H$ TPI1 $_P$ PGK1 $_Q$ ADH1 $_N$ PYK1 $_O$ TDH3 $_A$ amdSYM $_B$ HXK2 $_C$ PGI1 $_D$ PFK1 $_J$ PFK2 $_K$ ENO2 $_L$ GPM1 $_M$ PDC1-SYN $_F$) pyk1::natNT1*	(2)
IMX493	Deletion *PGI1* and *TPI1* from IMX457	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$ FBA1 $_H$ TPI1 $_P$ PGK1 $_Q$ ADH1 $_N$*	(2)

		PYK1 $_O$ TDH3 $_A$ amdSYM $_B$ HXK2 $_C$ PGI1 $_D$ PFK1 $_J$ PFK2 $_K$ ENO2 $_L$ GPM1 $_M$ PDC1-SYN $_F$) pyk1::natNT1 pgi1::kanMX tpi1::hphNT1	
IMX509	Introduction of I-SceI expression plasmid pUDC073 in IMX493	MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$ FBA1 $_H$ TPI1 $_P$ PGK1 $_Q$ ADH1 $_N$ PYK1 $_O$ TDH3 $_A$ amdSYM $_B$ HXK2 $_C$ PGI1 $_D$ PFK1 $_J$ PFK2 $_K$ ENO2 $_L$ GPM1 $_M$ PDC1-SYN $_F$) pyk1::natNT1 pgi1::kanMX tpi1::hphNT1 pUDC073	(2)
IMX510	Removal of markers and the pUDC073 plasmid from IMX509	MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$ FBA1 $_H$ TPI1 $_P$ PGK1 $_Q$ ADH1 $_N$ PYK1 $_O$ TDH3 $_A$ amdSYM $_B$ HXK2 $_C$ PGI1 $_D$ PFK1 $_J$ PFK2 $_K$ ENO2 $_L$ GPM1 $_M$ PDC1-SYN $_F$) pyk1 pgi1 tpi1	(2)
IMX511	Deletion TDH3 from IMX510	MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$ FBA1 $_H$ TPI1 $_P$ PGK1 $_Q$ ADH1 $_N$ PYK1 $_O$ TDH3 $_A$ amdSYM $_B$ HXK2 $_C$ PGI1 $_D$ PFK1 $_J$ PFK2 $_K$ ENO2 $_L$ GPM1 $_M$ PDC1-SYN $_F$) pyk1 pgi1 tpi1 tdh3::kanMX	(2)
IMX535	Deletion of PFK2 from IMX511 by insertion of Cas9	MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$ FBA1 $_H$ TPI1 $_P$ PGK1 $_Q$ ADH1 $_N$ PYK1 $_O$ TDH3 $_A$ amdSYM $_B$ HXK2 $_C$ PGI1 $_D$ PFK1 $_J$ PFK2 $_K$ ENO2 $_L$ GPM1 $_M$ PDC1-SYN $_F$) pyk1 pgi1 tpi1 tdh3::kanMX pfk2::(pTEF-cas9-tCYC1 natNT1)	(2)
IMX557	Deletion of PGK1 from IMX535	MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$ FBA1 $_H$ TPI1 $_P$ PGK1 $_Q$ ADH1 $_N$ PYK1 $_O$ TDH3 $_A$ amdSYM $_B$ HXK2 $_C$ PGI1 $_D$ PFK1 $_J$ PFK2 $_K$ ENO2 $_L$ GPM1 $_M$ PDC1-SYN $_F$) pyk1 pgi1 tpi1 tdh3::kanMX pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1::KlURA3	(2)

IMX561	Removal of *KanMX* and *KlURA3* marker from IMX557	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$FBA1 $_H$TPI1 $_P$PGK1 $_Q$ADH1 $_N$PYK1 $_O$TDH3 $_A$amdSYM $_B$HXK2 $_C$PGI1 $_D$PFK1 $_J$PFK2 $_K$ENO2 $_L$GPM1 $_M$PDC1-SYN $_F$) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1*	(2)
IMX566	Deletion of *GPM1* from IMX561	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$FBA1 $_H$TPI1 $_P$PGK1 $_Q$ADH1 $_N$PYK1 $_O$TDH3 $_A$amdSYM $_B$HXK2 $_C$PGI1 $_D$PFK1 $_J$PFK2 $_K$ENO2 $_L$GPM1 $_M$PDC1-SYN $_F$) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1 gpm1*	(2)
IMX568	Deletion of *FBA1* from IMX566	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$FBA1 $_H$TPI1 $_P$PGK1 $_Q$ADH1 $_N$PYK1 $_O$TDH3 $_A$amdSYM $_B$HXK2 $_C$PGI1 $_D$PFK1 $_J$PFK2 $_K$ENO2 $_L$GPM1 $_M$PDC1-SYN $_F$) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1 gpm1 fba1*	(2)
IMX570	Deletion of *HXK2* from IMX568	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$FBA1 $_H$TPI1 $_P$PGK1 $_Q$ADH1 $_N$PYK1 $_O$TDH3 $_A$amdSYM $_B$HXK2 $_C$PGI1 $_D$PFK1 $_J$PFK2 $_K$ENO2 $_L$GPM1 $_M$PDC1-SYN $_F$) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1 gpm1 fba1 hxk2*	(2)
IMX571	Deletion of *PFK1* from IMX570	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::($_G$FBA1 $_H$TPI1 $_P$PGK1 $_Q$ADH1 $_N$PYK1 $_O$TDH3 $_A$amdSYM $_B$HXK2 $_C$PGI1 $_D$PFK1 $_J$PFK2 $_K$ENO2 $_L$GPM1 $_M$PDC1-SYN $_F$) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1 gpm1 fba1 hxk2 pfk1*	(2)
IMX583	Deletion of *ADH1* and *PDC1* from IMX571	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5*	(2)

		hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(ₒFBA1 ₕTPI1 ₚPGK1 Q ADH1 N PYK1 O TDH3 A amdSYM B HXK2 C PGI1 D PFK1 J PFK2 K ENO2 L GPM1 M PDC1-SYN F) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1	
IMX586	Insertion of an extra copy of *ENO2* with a longer promoter integrated at the AmdSYM locus of IMX583	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(ₒFBA1 ₕTPI1 ₚPGK1 Q ADH1 N PYK1 O TDH3 A ENO2 B HXK2 C PGI1 D PFK1 J PFK2 K ENO2 L GPM1 M PDC1-SYN F) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1*	(2)
IMX587	Deletion of *ENO2* from IMX586	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1::Sphis5 hxk1::KlLEU2 tdh1 tdh2 gpm2 gpm3 eno1 pyk2 pdc5 pdc6 adh2 adh5 adh4 sga1::(ₒFBA1 ₕTPI1 ₚPGK1 Q ADH1 N PYK1 O TDH3 A ENO2 B HXK2 C PGI1 D PFK1 J PFK2 K ENO2 L GPM1 M PDC1-SYN F) pyk1 pgi1 tpi1 tdh3 pfk2::(pTEF-cas9-tCYC1 natNT1) pgk1 gpm1 fba1 hxk2 pfk1 adh1 pdc1 eno2*	(2)
IMX589	Insertion of AmdSYM at *ENO2* with short promoter in SinLoG of IMX587 **Original SwYG strain**. -SinLoG in Chr. IX -*amdS* marker in SinLoG -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Uracil auxotroph	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(ₒtFBA1-FBA1-pFBA1 ₕpTPI1-TPI1-tTPI1 ₚtPGK1-PGK1-pPGK1 Q tADH1-ADH1-pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-TDH3-pTDH3 A pENO2-ENO2-tENO2 B pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1 D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-pPFK2 K pAgTEF1-AmdSYM-tAgTEF1 L tGPM1-GPM1-pPGM1 M pPDC1-PDC1-tPDC1-SYN F) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1 eno2Δ*	(2)
IMX591	Derived from IMX589. -2 SinLoGs in Chr. IX and in Chr. V,	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ*	(2)

	-Chr. IX SinLoG with *amdS* marker and Chr. V SinLoG with *KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Uracil auxotroph	*tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(_G tFBA1-FBA1-pFBA1 _H pTPI1-TPI1-tTPI1 _P tPGK1-PGK1-pPGK1 _Q tADH1-ADH1-pADH1 _N pPYK1-PYK1-tPYK1 _O tTDH3-TDH3-pTDH3 _A pENO2-ENO2-tENO2 _B pHXK2-HXK2-tHXK2 _C pPGI-PGI1-tPGI1 _D pPFK1-PFK1-tPFK1 _J tPFK2-PFK2-pPFK2 _K pAgTEF1-AmdSYM-tAgTEF1 _L tGPM1-GPM1-pPGM1 _M pPDC1-PDC1-tPDC1-SYN _F) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 _H pTPI1-TPI1-tTPI1 _P tPGK1-PGK1-pPGK1 _Q tADH1-ADH1-pADH1 _N pPYK1-PYK1-tPYK1 _O tTDH3-TDH3-pTDH3 _A pENO2-ENO2-tENO2 _B pHXK2-HXK2-tHXK2 _C pPGI-PGI1-tPGI1 _D pPFK1-PFK1-tPFK1 _J tPFK2-PFK2-pPFK2 _K pAgTEF-KanMX-tAgTEF _L tGPM1-GPM1-pPGM1 _M pPDC1-PDC1-tPDC1)*	
IMX605	Derived from IMX591; -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (uracil complemented from an episomal plasmid).	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 _H pTPI1-TPI1-tTPI1 _P tPGK1-PGK1-pPGK1 _Q tADH1-ADH1-pADH1 _N pPYK1-PYK1-tPYK1 _O tTDH3-TDH3-pTDH3 _A pENO2-ENO2-tENO2 _B pHXK2-HXK2-tHXK2 _C pPGI-PGI1-tPGI1 _D pPFK1-PFK1-tPFK1 _J tPFK2-PFK2-pPFK2 _K pAgTEF-KanMX-tAgTEF _L tGPM1-GPM1-pPGM1 _M pPDC1-PDC1-tPDC1) pUDE342*	(2)
IMX606	Derived from IMX589. -SinLoG in Chr. IX -*amdS* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(_G tFBA1-FBA1-pFBA1 _H pTPI1-TPI1-tTPI1 _P tPGK1-PGK1-pPGK1 _Q*	(2)

	-Prototroph (uracil complemented from an episomal plasmid)	tADH1-ADH1-pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-TDH3-pTDH3 A pENO2-ENO2-tENO2 B pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1 D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-pPFK2 K pAgTEF1-AmdSYM-tAgTEF1 L tGPM1-GPM1-pPGM1 M pPDC1-PDC1-tPDC1-SYN F) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ pUDE325	
IMX1338	Derived from IMX589; -SinLoG in Chr. IX - *amdS* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Uracil and histidine auxotroph	MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(G tFBA1-FBA1-pFBA1 H pTPI1-TPI1-tTPI1 P tPGK1-PGK1-pPGK1 Q tADH1-ADH1-pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-TDH3-pTDH3 A pENO2-ENO2-tENO2 B pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1 D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-pPFK2 K pAgTEF1-AmdSYM-tAgTEF1 L tGPM1-GPM1-pPGM1 M pPDC1-PDC1-tPDC1-SYN F) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ	(3)
IMX1717	Derived from IMX1338 -2 SinLoGs in Chr. IX and Chr. V - Chr. IX SinLoG with *amdS* marker and Chr. V SinLoG with *HIS3* marker -Chr. V SinLoG flanked by ARS sequences -non-standardised promoter and terminator lengths for Chr. IX SinLoG Standardised promoter (800 bp) and terminator (300 bp) lengths for Chr. V SinLoG -Chr. V SinLoG is watermarked -random orientation of glycolytic genes -Uracil auxotroph	MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(G tFBA1-FBA1-pFBA1 H pTPI1-TPI1-tTPI1 P tPGK1-PGK1-pPGK1 Q tADH1-ADH1-pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-TDH3-pTDH3 A pENO2-ENO2-tENO2 B pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1 D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-pPFK2 K pAgTEF1-AmdSYM-tAgTEF1 L tGPM1-GPM1-pPGM1 M pPDC1-PDC1-tPDC1-SYN F) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(ARS418 AH tFBA1-FBA1*-pFBA1 H pTPI1-TPI1*-tTPI1 P tPGK1-PGK1*-pPGK1 Q tADH1-	(3)

		ADH1*-pADH1 _N_ pPYK1-PYK1*-tPYK1 _O_ tTDH3-TDH3*-pTDH3 _A_ pENO2-ENO2*-tENO2 _B_ pHXK2-HXK2*-tHXK2 _C_ pPGI-PGI1*-tPGI1 _D_ pPFK1-PFK1*-tPFK1 _J_ tPFK2-PFK2*-pPFK2 _BP_ pHIS3-HIS3-tHIS3 _L_ tGPM1-GPM1*-pPGM1 _M_ pPDC1-PDC1*-tPDC1 _AR_ ARS1211)	
IMX1747	Derived from IMX1338 -2 SinLoGs in Chr. IX and Chr. V - Chr. IX SinLoG with *amdS* marker and Chr. V SinLoG with *HIS3* marker -Chr. V SinLoG flanked by ARS sequences -non-standardised promoter and terminator lengths for Chr. IX SinLoG Standardised promoter (800 bp) and terminator (300 bp) lengths for Chr. V SinLoG -random orientation of glycolytic genes -Uracil auxotroph	MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(_G_ tFBA1-FBA1-pFBA1 _H_ pTPI1-TPI1-tTPI1 _P_ tPGK1-PGK1-pPGK1 _Q_ tADH1-ADH1-pADH1 _N_ pPYK1-PYK1-tPYK1 _O_ tTDH3-TDH3-pTDH3 _A_ pENO2-ENO2-tENO2 _B_ pHXK2-HXK2-tHXK2 _C_ pPGI-PGI1-tPGI1 _D_ pPFK1-PFK1-tPFK1 _J_ tPFK2-PFK2-pPFK2 _K_ pAgTEF1-AmdSYM-tAgTEF1 _L_ tGPM1-GPM1-pPGM1 _M_ pPDC1-PDC1-tPDC1-SYN _F_) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(ARS418 _AH_ tFBA1-FBA1-pFBA1 _H_ pTPI1-TPI1-tTPI1 _P_ tPGK1-PGK1-pPGK1 _Q_ tADH1-ADH1-pADH1 _N_ pPYK1-PYK1-tPYK1 _O_ tTDH3-TDH3-pTDH3 _A_ pENO2-ENO2-tENO2 _B_ pHXK2-HXK2-tHXK2 _C_ pPGI-PGI1-tPGI1 _D_ pPFK1-PFK1-tPFK1 _J_ tPFK2-PFK2-pPFK2 _BP_ pHIS3-HIS3-tHIS3 _L_ tGPM1-GPM1-pPGM1 _M_ pPDC1-PDC1-tPDC1 _AR_ ARS1211)	(3)
IMX1768	Derived from IMX605. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Uracil auxotroph	MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 _H_ pTPI1-TPI1-tTPI1 _P_ tPGK1-PGK1-pPGK1 _Q_ tADH1-ADH1-pADH1 _N_ pPYK1-PYK1-tPYK1 _O_ tTDH3-TDH3-pTDH3 _A_ pENO2-ENO2-tENO2 _B_ pHXK2-HXK2-tHXK2 _C_ pPGI-PGI1-tPGI1 _D_ pPFK1-PFK1-tPFK1 _J_ tPFK2-PFK2-	(6)

		pPFK2 $_K$ pAgTEF-KanMX-tAgTEF $_L$ tGPM1-GPM1-pPGM1 $_M$ pPDC1-PDC1-tPDC1)	
IMX1769	Derived from IMX589 -SinLoG in Chr. IX -No marker in SinLoG -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Uracil auxotroph	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::($_G$ tFBA1-FBA1-pFBA1 $_H$ pTPI1-TPI1-tTPI1 $_P$ tPGK1-PGK1-pPGK1 $_Q$ tADH1-ADH1-pADH1 $_N$ pPYK1-PYK1-tPYK1 $_O$ tTDH3-TDH3-pTDH3 $_A$ pENO2-ENO2-tENO2 $_B$ pHXK2-HXK2-tHXK2 $_C$ pPGI-PGI1-tPGI1 $_D$ pPFK1-PFK1-tPFK1 $_J$ tPFK2-PFK2-pPFK2 $_K$ $_L$ tGPM1-GPM1-pPGM1 $_M$ pPDC1-PDC1-tPDC1-SYN $_F$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ*	(6)
IMX1770	Derived from IMX1717 -SinLoGs in Chr. V -SinLoG with *HIS3* marker -SinLoG flanked by ARS sequences -Standardised promoter (800 bp) and terminator (300 bp) lengths -SinLoG is watermarked -random orientation of glycolytic genes -Prototroph (*KlURA3* at *sga1* locus)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(pKlURA3-KlURA3-tKlURA3) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(ARS418 $_{AH}$ tFBA1-FBA1*-pFBA1 $_H$ pTPI1-TPI1*-tTPI1 $_P$ tPGK1-PGK1*-pPGK1 $_Q$ tADH1-ADH1*-pADH1 $_N$ pPYK1-PYK1*-tPYK1 $_O$ tTDH3-TDH3*-pTDH3 $_A$ pENO2-ENO2*-tENO2 $_B$ pHXK2-HXK2*-tHXK2 $_C$ pPGI-PGI1*-tPGI1 $_D$ pPFK1-PFK1*-tPFK1 $_J$ tPFK2-PFK2*-pPFK2 $_{BP}$ pHIS3-HIS3-tHIS3 $_L$ tGPM1-GPM1*-pPGM1 $_M$ pPDC1-PDC1*-tPDC1 $_{AR}$ ARS1211)*	(3)
IMX1771	Derived from IMX1747 -SinLoG in Chr. V -SinLoG with *HIS3* marker -SinLoG flanked by ARS sequences -Standardised promoter (800 bp) and terminator (300 bp) lengths	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(pKlURA3-KlURA3-tKlURA3) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1)*	(3)

	-random orientation of glycolytic genes -Prototroph (*KlURA3* at *sga1* locus)	*pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(ARS418* $_{AH}$ *tFBA1-FBA1-pFBA1* $_H$ *pTPI1-TPI1-tTPI1* $_P$ *tPGK1-PGK1-pPGK1* $_Q$ *tADH1-ADH1-pADH1* $_N$ *pPYK1-PYK1-tPYK1* $_O$ *tTDH3-TDH3-pTDH3* $_A$ *pENO2-ENO2-tENO2* $_B$ *pHXK2-HXK2-tHXK2* $_C$ *pPGI-PGI1-tPGI1* $_D$ *pPFK1-PFK1-tPFK1* $_J$ *tPFK2-PFK2-pPFK2* $_{BP}$ *pHIS3-HIS3-tHIS3* $_L$ *tGPM1-GPM1-pPGM1* $_M$ *pPDC1-PDC1-tPDC1* $_{AR}$ *ARS1211)*	
IMX1821	Derived from IMX1768. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1* $_H$ *pTPI1-TPI1-tTPI1* $_P$ *tPGK1-PGK1-pPGK1* $_Q$ *tADH1-ADH1-pADH1* $_N$ *pPYK1-PYK1-tPYK1* $_O$ *tTDH3-TDH3-pTDH3* $_A$ *pENO2-ENO2-tENO2* $_B$ *pHXK2-HXK2-tHXK2* $_C$ *pPGI-PGI1-tPGI1* $_D$ *pPFK1-PFK1-tPFK1* $_J$ *tPFK2-PFK2-pPFK2* $_K$ *pAgTEF-KanMX-tAgTEF* $_L$ *tGPM1-GPM1-pPGM1* $_M$ *pPDC1-PDC1-tPDC1)*	(6)
IMX1822	Derived from IMX1769 -SinLoG in Chr. IX -No marker in SinLoG -non-standardised promoter and terminator lengths -random orientation of glycolytic genes - Prototroph (*URA3* in *tdh1* locus)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(* $_G$ *tFBA1-FBA1-pFBA1* $_H$ *pTPI1-TPI1-tTPI1* $_P$ *tPGK1-PGK1-pPGK1* $_Q$ *tADH1-ADH1-pADH1* $_N$ *pPYK1-PYK1-tPYK1* $_O$ *tTDH3-TDH3-pTDH3* $_A$ *pENO2-ENO2-tENO2* $_B$ *pHXK2-HXK2-tHXK2* $_C$ *pPGI-PGI1-tPGI1* $_D$ *pPFK1-PFK1-tPFK1* $_J$ *tPFK2-PFK2-pPFK2* $_K$ $_L$ *tGPM1-GPM1-pPGM1* $_M$ *pPDC1-PDC1-tPDC1-SYN* $_F$ *) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ*	(6)

IMX1959	Derived from IMX1338 -2 SinLoGs in Chr. IX and Chr V - Chr. IX SinLoG with *amdS* marker and Chr. V SinLoG with *KanMX* marker - SinLoG in Chr. V flanked by ARS sequences -non-standardised promoter and terminator lengths for Chr. IX SinLoG Standardised promoter (800 bp) and terminator (300 bp) lengths for Chr. V SinLoG -random orientation of glycolytic genes -Uracil and histidine auxotroph	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::($_G$ tFBA1-FBA1-pFBA1 $_H$ pTPI1-TPI1-tTPI1 $_P$ tPGK1-PGK1-pPGK1 $_Q$ tADH1-ADH1-pADH1 $_N$ pPYK1-PYK1-tPYK1 $_O$ tTDH3-TDH3-pTDH3 $_A$ pENO2-ENO2-tENO2 $_B$ pHXK2-HXK2-tHXK2 $_C$ pPGI-PGI1-tPGI1 $_D$ pPFK1-PFK1-tPFK1 $_J$ tPFK2-PFK2-pPFK2 $_K$ pAgTEF1-AmdSYM-tAgTEF1 $_L$ tGPM1-GPM1-pPGM1 $_M$ pPDC1-PDC1-tPDC1-SYN $_F$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ:: (ARS418 $_G$ pAgTEF-KanMX-tAgTEF $_{AA}$ tFBA1-FBA1-pFBA1 $_H$ pTPI1-TPI1-tTPI1 $_P$ tPGK1-PGK1-pPGK1 $_Q$ tADH1-ADH1-pADH1 $_N$ pPYK1-PYK1-tPYK1 $_O$ tTDH3-TDH3-pTDH3 $_A$ pENO2-ENO2-tENO2 $_B$ pHXK2-HXK2-tHXK2 $_C$ pPGI-PGI1-tPGI1 $_D$ pPFK1-PFK1-tPFK1 $_J$ tPFK2-PFK2-pPFK2 $_L$ tGPM1-GPM1-pPGM1 $_M$ pPDC1-PDC1-tPDC1 $_{AR}$ ARS1211)*	(1)
IMX2059	Derived from IMX1338; -SinLoG in Chr. IX - *amdS* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* and *HIS3* integrated at the X2 locus)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::($_G$ tFBA1-FBA1-pFBA1 $_H$ pTPI1-TPI1-tTPI1 $_P$ tPGK1-PGK1-pPGK1 $_Q$ tADH1-ADH1-pADH1 $_N$ pPYK1-PYK1-tPYK1 $_O$ tTDH3-TDH3-pTDH3 $_A$ pENO2-ENO2-tENO2 $_B$ pHXK2-HXK2-tHXK2 $_C$ pPGI-PGI1-tPGI1 $_D$ pPFK1-PFK1-tPFK1 $_J$ tPFK2-PFK2-pPFK2 $_K$ pAgTEF1-AmdSYM-tAgTEF1 $_L$ tGPM1-GPM1-pPGM1 $_M$ pPDC1-PDC1-tPDC1-SYN $_F$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ X2::pURA3-URA3-tURA3 $_{DT}$ pHIS3-HIS3-tHIS3*	(1)
IMX2080	Derived from IMX1959 -SinLoGs in Chr. V -*KanMX* marker	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-*	(1)

	- SinLoG flanked by ARS sequences -Standardised promoter (800 bp) and terminator (300 bp) lengths -random orientation of glycolytic genes -Uracil and histidine auxotroph	*tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ:: (ARS418 G pAgTEF-KanMX-tAgTEF AA tFBA1-FBA1-pFBA1 H pTPI1-TPI1-tTPI1 P tPGK1-PGK1-pPGK1 Q tADH1-ADH1-pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-TDH3-pTDH3 A pENO2-ENO2-tENO2 B pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1 D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-pPFK2 L tGPM1-GPM1-pPGM1 M pPDC1-PDC1-tPDC1 AR ARS1211)*	
IMX2109	Derived from IMX2080 -SinLoGs in Chr. V -*KanMX* marker - SinLoG flanked by ARS sequences -Standardised promoter (800 bp) and terminator (300 bp) lengths -random orientation of glycolytic genes -Prototroph (*URA3* and *HIS3* integrated at the X2 locus)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ:: (ARS418 G pAgTEF-KanMX-tAgTEF AA tFBA1-FBA1-pFBA1 H pTPI1-TPI1-tTPI1 P tPGK1-PGK1-pPGK1 Q tADH1-ADH1-pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-TDH3-pTDH3 A pENO2-ENO2-tENO2 B pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1 D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-pPFK2 L tGPM1-GPM1-pPGM1 M pPDC1-PDC1-tPDC1 AR ARS1211) X2::pURA3-URA3-tURA3 DT pHIS3-HIS3-tHIS3*	(1)
IMX2359	Derived from IMX1338 -2 SinLoGs in Chr. IX and Chr V - Chr. IX SinLoG with *amdS* marker and Chr. V SinLoG with *HIS3* marker - SinLoG in Chr. V flanked by ARS sequences -non-standardised promoter and terminator lengths for Chr. IX SinLoG	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(G tFBA1-FBA1-pFBA1 H pTPI1-TPI1-tTPI1 P tPGK1-PGK1-pPGK1 Q tADH1-ADH1-pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-TDH3-pTDH3 A pENO2-ENO2-tENO2 B pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1 D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-pPFK2 K pAgTEF1-AmdSYM-tAgTEF1 L*	This study

	Standardised promoter (800 bp) and terminator (300 bp) lengths for Chr. V SinLoG -Chr. V SinLoG is watermarked -SinLoG in Chr. V: Orientation of glycolytic genes such that transcription and replication are aligned -Uracil auxotroph	*tGPM1-GPM1-pPGM1 $_M$ pPDC1-PDC1-tPDC1-SYN $_F$) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(ARS418 $_{DL}$ pFBA1-FBA1*-tFBA1 $_H$ pTPI1-TPI1*-tTPI1 $_P$ pPGK1-PGK1*-tPGK1 $_Q$ pADH1-ADH1*-tADH1 $_N$ pPYK1-PYK1*-tPYK1 $_O$ pTDH3-TDH3*-tTDH3 $_A$ pENO2-ENO2*-tENO2 $_B$ tHXK2-HXK2*-pHXK2 $_C$ tPGI-PGI1*-pPGI1 $_D$ tPFK1-PFK1*-pPFK1 $_J$ tPFK2-PFK2*-pPFK2 $_{BU}$ tHIS3-HIS3-pHIS3 $_L$ tGPM1-GPM1*-pPGM1 $_M$ tPDC1-PDC1*-pPDC1 $_{AR}$ ARS1211)*	
IMX2381	Derived from IMX2359 -SinLoGs in Chr. V -SinLoG with *HIS3* marker -SinLoG flanked by ARS sequences -Standardised promoter (800 bp) and terminator (300 bp) lengths -SinLoG is watermarked -Orientation of glycolytic genes such that transcription and replication are aligned -Prototroph (integration of *KlURA3* in *sga1* locus)	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ::(pAgTEF1-SpHIS5-tAgTEF1)Δ::(pGAL1-I SceI-tCYC1) hxk1Δ::KlLEU2 tdh1Δ tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ::(pKlURA3-KlURA3-tKlURA3) pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(ARS418 $_{DL}$ pFBA1-FBA1*-tFBA1 $_H$ pTPI1-TPI1*-tTPI1 $_P$ pPGK1-PGK1*-tPGK1 $_Q$ pADH1-ADH1*-tADH1 $_N$ pPYK1-PYK1*-tPYK1 $_O$ pTDH3-TDH3*-tTDH3 $_A$ pENO2-ENO2*-tENO2 $_B$ tHXK2-HXK2*-pHXK2 $_C$ tPGI-PGI1*-pPGI1 $_D$ tPFK1-PFK1*-pPFK1 $_J$ tPFK2-PFK2*-pPFK2 $_{BU}$ tHIS3-HIS3-pHIS3 $_L$ tGPM1-GPM1*-pPGM1 $_M$ tPDC1-PDC1*-pPDC1 $_{AR}$ ARS1211)*	This study
IMX2542	Derived from IMX1821. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) -*sur2Δ::(synthetic gRNA)*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 $_H$ pTPI1-TPI1-tTPI1 $_P$ tPGK1-PGK1-pPGK1 $_Q$ tADH1-ADH1-pADH1 $_N$ pPYK1-PYK1-tPYK1 $_O$ tTDH3-TDH3-pTDH3 $_A$ pENO2-ENO2-tENO2 $_B$ pHXK2-HXK2-tHXK2 $_C$ pPGI-PGI1-tPGI1*	This study

		_D_ *pPFK1-PFK1-tPFK1* _J_ *tPFK2-PFK2-pPFK2* _K_ *pAgTEF-KanMX-tAgTEF* _L_ *tGPM1-GPM1-pPGM1* _M_ *pPDC1-PDC1-tPDC1) sur2Δ::(synthetic gRNA)*	
IMX2543	Derived from IMX1821. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) -*atg41Δ::(synthetic gRNA)*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1* _H_ *pTPI1-TPI1-tTPI1* _P_ *tPGK1-PGK1-pPGK1* _Q_ *tADH1-ADH1-pADH1* _N_ *pPYK1-PYK1-tPYK1* _O_ *tTDH3-TDH3-pTDH3* _A_ *pENO2-ENO2-tENO2* _B_ *pHXK2-HXK2-tHXK2* _C_ *pPGI-PGI1-tPGI1* _D_ *pPFK1-PFK1-tPFK1* _J_ *tPFK2-PFK2-pPFK2* _K_ *pAgTEF-KanMX-tAgTEF* _L_ *tGPM1-GPM1-pPGM1* _M_ *pPDC1-PDC1-tPDC1) atg41Δ::(synthetic gRNA)*	This study
IMX2544	Derived from IMX1821. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) -*cnb1Δ::(synthetic gRNA)*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1* _H_ *pTPI1-TPI1-tTPI1* _P_ *tPGK1-PGK1-pPGK1* _Q_ *tADH1-ADH1-pADH1* _N_ *pPYK1-PYK1-tPYK1* _O_ *tTDH3-TDH3-pTDH3* _A_ *pENO2-ENO2-tENO2* _B_ *pHXK2-HXK2-tHXK2* _C_ *pPGI-PGI1-tPGI1* _D_ *pPFK1-PFK1-tPFK1* _J_ *tPFK2-PFK2-pPFK2* _K_ *pAgTEF-KanMX-tAgTEF* _L_ *tGPM1-GPM1-pPGM1* _M_ *pPDC1-PDC1-tPDC1) cnb1Δ::(synthetic gRNA)*	This study
IMX2567	Derived from IMX2544. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ*	This study

	-Prototroph (*URA3* in *tdh1* locus) -*cnb1Δ::(synthetic gRNA)* *atg41Δ::(synthetic gRNA)* *sur2Δ::(synthetic gRNA)*	*pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 $_H$ pTPI1-TPI1-tTPI1 $_P$ tPGK1-PGK1-pPGK1 $_Q$ tADH1-ADH1-pADH1 $_N$ pPYK1-PYK1-tPYK1 $_O$ tTDH3-TDH3-pTDH3 $_A$ pENO2-ENO2-tENO2 $_B$ pHXK2-HXK2-tHXK2 $_C$ pPGI-PGI1-tPGI1 $_D$ pPFK1-PFK1-tPFK1 $_J$ tPFK2-PFK2-pPFK2 $_K$ pAgTEF-KanMX-tAgTEF $_L$ tGPM1-GPM1-pPGM1 $_M$ pPDC1-PDC1-tPDC1) cnb1Δ::(synthetic gRNA) atg41Δ::(synthetic gRNA) sur2Δ::(synthetic gRNA)*	24
IMX2570	Derived from IMX2543. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) -*atg41Δ::(synthetic gRNA)* *sur2Δ::(synthetic gRNA)*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 $_H$ pTPI1-TPI1-tTPI1 $_P$ tPGK1-PGK1-pPGK1 $_Q$ tADH1-ADH1-pADH1 $_N$ pPYK1-PYK1-tPYK1 $_O$ tTDH3-TDH3-pTDH3 $_A$ pENO2-ENO2-tENO2 $_B$ pHXK2-HXK2-tHXK2 $_C$ pPGI-PGI1-tPGI1 $_D$ pPFK1-PFK1-tPFK1 $_J$ tPFK2-PFK2-pPFK2 $_K$ pAgTEF-KanMX-tAgTEF $_L$ tGPM1-GPM1-pPGM1 $_M$ pPDC1-PDC1-tPDC1) atg41Δ::(synthetic gRNA) sur2Δ::(synthetic gRNA)*	This study
IMX2589	Derived from IMX2543. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) -*ATG41^{W132C}*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 $_H$ pTPI1-TPI1-tTPI1 $_P$ tPGK1-PGK1-pPGK1 $_Q$ tADH1-ADH1-pADH1 $_N$ pPYK1-PYK1-tPYK1 $_O$ tTDH3-TDH3-pTDH3 $_A$ pENO2-ENO2-tENO2 $_B$ pHXK2-HXK2-tHXK2 $_C$ pPGI-PGI1-tPGI1*	This study

		_D pPFK1-PFK1-tPFK1 _J tPFK2-PFK2-pPFK2 _K pAgTEF-KanMX-tAgTEF _L tGPM1-GPM1-pPGM1 _M pPDC1-PDC1-tPDC1) ATG41^{W132C}	25
IMX2590	Derived from IMX2543. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) -*ATG41^{W132stop}*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 _H pTPI1-TPI1-tTPI1 _P tPGK1-PGK1-pPGK1 _Q tADH1-ADH1-pADH1 _N pPYK1-PYK1-tPYK1 _O tTDH3-TDH3-pTDH3 _A pENO2-ENO2-tENO2 _B pHXK2-HXK2-tHXK2 _C pPGI-PGI1-tPGI1 _D pPFK1-PFK1-tPFK1 _J tPFK2-PFK2-pPFK2 _K pAgTEF-KanMX-tAgTEF _L tGPM1-GPM1-pPGM1 _M pPDC1-PDC1-tPDC1) ATG41^{W132stop}*	This study
IMX2591	Derived from IMX2570. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) - *ATG41^{W132C} SUR2^{E192stop}*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 _H pTPI1-TPI1-tTPI1 _P tPGK1-PGK1-pPGK1 _Q tADH1-ADH1-pADH1 _N pPYK1-PYK1-tPYK1 _O tTDH3-TDH3-pTDH3 _A pENO2-ENO2-tENO2 _B pHXK2-HXK2-tHXK2 _C pPGI-PGI1-tPGI1 _D pPFK1-PFK1-tPFK1 _J tPFK2-PFK2-pPFK2 _K pAgTEF-KanMX-tAgTEF _L tGPM1-GPM1-pPGM1 _M pPDC1-PDC1-tPDC1) ATG41^{W132C} SUR2^{E192stop}*	This study
IMX2594	Derived from IMX2544. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ*	This study

	-Prototroph (*URA3* in *tdh1* locus) -*CNB1*^{L82F}	*pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1)* *pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ* *adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-* *FBA1-pFBA1 H pTPI1-TPI1-tTPI1 P* *tPGK1-PGK1-pPGK1 Q tADH1-ADH1-* *pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-* *TDH3-pTDH3 A pENO2-ENO2-tENO2 B* *pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1* *D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-* *pPFK2 K pAgTEF-KanMX-tAgTEF L* *tGPM1-GPM1-pPGM1 M pPDC1-PDC1-* *tPDC1) CNB1*^{L82F}	
IMX2605	Derived from IMX2542. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) - *SUR2*^{E192stop}	*MATa ura3-52 his3-1 leu2-3,112* *MAL2-8c SUC2 glk1Δ:: (pAgTEF1-* *SpHIS5-tAgTEF1) hxk1Δ::KlLEU2* *tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ* *gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ* *pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ* *pyk1Δ pgi1Δ tpi1Δ tdh3Δ* *pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1)* *pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ* *adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-* *FBA1-pFBA1 H pTPI1-TPI1-tTPI1 P* *tPGK1-PGK1-pPGK1 Q tADH1-ADH1-* *pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-* *TDH3-pTDH3 A pENO2-ENO2-tENO2 B* *pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1* *D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-* *pPFK2 K pAgTEF-KanMX-tAgTEF L* *tGPM1-GPM1-pPGM1 M pPDC1-PDC1-* *tPDC1) SUR2*^{E192stop}	This study
IMX2606	Derived from IMX2542. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) - *SUR2*^{M179K}	*MATa ura3-52 his3-1 leu2-3,112* *MAL2-8c SUC2 glk1Δ:: (pAgTEF1-* *SpHIS5-tAgTEF1) hxk1Δ::KlLEU2* *tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ* *gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ* *pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ* *pyk1Δ pgi1Δ tpi1Δ tdh3Δ* *pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1)* *pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ* *adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-* *FBA1-pFBA1 H pTPI1-TPI1-tTPI1 P* *tPGK1-PGK1-pPGK1 Q tADH1-ADH1-* *pADH1 N pPYK1-PYK1-tPYK1 O tTDH3-* *TDH3-pTDH3 A pENO2-ENO2-tENO2 B* *pHXK2-HXK2-tHXK2 C pPGI-PGI1-tPGI1* *D pPFK1-PFK1-tPFK1 J tPFK2-PFK2-* *pPFK2 K pAgTEF-KanMX-tAgTEF L*	This study

		tGPM1-GPM1-pPGM1 ₘ pPDC1-PDC1-tPDC1) SUR2^{M179K}	
IMX2607	Derived from IMX2542. -SinLoG in Chr. V -*KanMX* marker -non-standardised promoter and terminator lengths -random orientation of glycolytic genes -Prototroph (*URA3* in *tdh1* locus) - *SUR2^{G251R}*	*MATa ura3-52 his3-1 leu2-3,112 MAL2-8c SUC2 glk1Δ:: (pAgTEF1-SpHIS5-tAgTEF1) hxk1Δ::KlLEU2 tdh1Δ::(pURA3-URA3-tURA3) tdh2Δ gpm2Δ gpm3Δ eno1Δ pyk2Δ pdc5Δ pdc6Δ adh2Δ adh5Δ adh4Δ sga1Δ pyk1Δ pgi1Δ tpi1Δ tdh3Δ pfk2Δ::(pTEF1-Spcas9-tCYC1 natNT1) pgk1Δ gpm1Δ fba1Δ hxk2Δ pfk1Δ adh1Δ pdc1Δ eno2Δ can1Δ::(tFBA1-FBA1-pFBA1 _H pTPI1-TPI1-tTPI1 _P tPGK1-PGK1-pPGK1 _Q tADH1-ADH1-pADH1 _N pPYK1-PYK1-tPYK1 _O tTDH3-TDH3-pTDH3 _A pENO2-ENO2-tENO2 _B pHXK2-HXK2-tHXK2 _C pPGI-PGI1-tPGI1 _D pPFK1-PFK1-tPFK1 _J tPFK2-PFK2-pPFK2 _K pAgTEF-KanMX-tAgTEF _L tGPM1-GPM1-pPGM1 _M pPDC1-PDC1-tPDC1) SUR2^{G251R}*	This study
IMS1022	Single colony isolate from IMX1821 evolved on SMD, Evolution line 1		This study
IMS1026	Single colony isolate from IMX1821 evolved on SMD, Evolution line 2		This study
IMS1031	Single colony isolate from IMX1821 evolved on SMD, Evolution line 3		This study
IMS1043	Single colony isolate from CEN.PK113-7D evolved on SMD, Evolution line 1		This study
IMS1048	Single colony isolate from CEN.PK113-7D evolved on SMD, Evolution line 2		This study
IMS1053	Single colony isolate from CEN.PK113-7D evolved on SMD, Evolution line 3		This study

Table S3 List of plasmids used in this study

Table S3A gRNA plasmids

Plasmid	Relevant characteristics	Primer(s) used for gRNA	Source
pMEL10	2μm ampR *KlURA3* gRNA-*CAN1.Y*	N.A.	(7)
pROS10	2μm ampR *KlURA3* gRNA-*CAN1.Y* gRNA-*ADE2.Y*	N.A.	(7)
pROS12	2μm ampR *hphNT1* gRNA-*CAN1.Y* gRNA-*ADE2.Y*	N.A.	(7)
pROS13	2μm ampR *kanMX* gRNA-*CAN1.Y* gRNA-*ADE2.Y*	N.A.	(7)
p426-SNR52p-gRNA.CAN1.Y-SUP4t	2 μm, ampR, *URA3*, gRNA.*CAN1*.Y	N.A.	(8)
pUDR114	2μm ampR *hphNT1* gRNA- *Synthetic gRNA*	N.A.	(9)
pUDR413	2μm ampR *kanMX* gRNA- *RECYCLE SinLoG*	N.A.	(3)
pUDR748	2 μm, ampR, *hphNT1*, gRNA - *ATG41* (2x)	17545	This study
pUDR749	2 μm, ampR, *hphNT1*, gRNA - *CNB1* (2x)	17554	This study
pUDR750	2 μm, ampR, *hphNT1*, gRNA - *SUR2* (2x)	17563	This study
pUDR751	2 μm, ampR, *hphNT1*, gRNA - *ATG41* gRNA-*SUR2*	17545 & 17563	This study

Table S3B Expression plasmids of watermarked glycolytic genes.

*Gene is watermarked

Plasmid	Relevant characteristics	Source
pUDC212	CEN6/ARS4 *ampR natNT2 pFBA1-FBA1*-tFBA1*	(3)
pUDC213	CEN6/ARS4 *ampR natNT2 pPGM1-PGM1*-tPGM1*	(3)
pUDC214	CEN6/ARS4 *ampR natNT2 pHXK2-HXK2*-tHXK2*	(3)
pUDC215	CEN6/ARS4 *ampR natNT2 pPDC1-PDC1*-tPDC1*	(3)
pUDC216	CEN6/ARS4 *ampR natNT2 pPFK1-PFK1*-tPFK1*	(3)
pUDC217	CEN6/ARS4 *ampR natNT2 pPFK2-PFK2*-tPFK2*	(3)
pUDC219	CEN6/ARS4 *ampR natNT2 pPGK1-PGK1*-tPGK1*	(3)
pUDC220	CEN6/ARS4 *ampR natNT2 pPYK1-PYK1*-tPYK1*	(3)
pUDC222	CEN6/ARS4 *ampR natNT2 pTPI1-TPI1*-tTPI1*	(3)
pUDC229	CEN6/ARS4 *ampR natNT2 pADH1-ADH1*-tADH1*	(3)
pUDC230	CEN6/ARS4 *ampR natNT2 pTDH3-TDH3*-tTDH3*	(3)
pUDC231	CEN6/ARS4 *ampR natNT2 pENO2-ENO2*-tENO2*	(3)
pUDC232	CEN6/ARS4 *ampR natNT2 pPGI1-PGI1*-tPGI1*	(3)

Table S4 List of primers used in this study

Table 4A: List of gRNA Primers

Primer number	Primer name	Sequence (5' to 3')
5974	2mu inside fw	TACTTTTGAGCAATGTTTGTGGA
5975	2mu inside rv	AACGAGCTACTAAAATATTGCGAA
6005	p426 CRISP rv	GATCATTTATCTTTCACTGCGGAGAAG
17545	ATG41_targetRNA_FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAA ATGATCAAAGGCATCGTTACTTGCAAGTTTTAGAGCTAGAAATA GCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
17554	CNB1_targetRNA_FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAA ATGATCAATGAATTTATGAGCATTCCGTTTTAGAGCTAGAAATA GCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
17563	SUR2_targetRNA_FW	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATAA ATGATCTGTTCCAAACTAACTTTAAAGTTTTAGAGCTAGAAATAG CAAGTTAAAATAAGGCTAGTCCGTTATCAAC

Table 4B: List of primers to confirm gRNA plasmids

Primer number	Primer name	Sequence (5' to 3')
2918	F_RV_2mu (PDH construct ctrl)	ATGAAGCACAGATTCTTCGTTG
3235	FK164	GAAAGGTGGATGGGTAGG
4672	Fus Tag B fw	CACCTTTCGAGAGGACGATG
5941	LP crRNA rv	GCTGGCCTTTTGCTCACATG
17547	ATG41_gRNA_check	TTGCAAGTAACGATGCCTTTG
17556	CNB1_gRNA_check	ATCAATGAATTTATGAGCATTCCG
17565	SUR2_gRNA_check	TCTGTTCCAAACTAACTTTAAAG

Table 4C: Primers for amplification of SinLoG in strain IMX2359

Primer Number	Primer Name	Sequence (5' to 3')
13161	ARS418 + Tag CAN1 Fw	GGTGTATGACTTATGAGGGTGAGAATGCGAAATGGCGTGGGAATGTG ATTAAAGGTAATATGAAAGTTTATGTTTTTTCACTGGA
16828	DL_ARS418_rv	CGCAAATGTCCCATCGTATTTCAGAACCTTGTCACTCATGCGAGCAAGT GTGACAGCTATTTGAAAAGCAATATTAAAATATAGA
16561	DL_pFBA1_FW	ATAGCTGTCACACTTGCTCGCATGAGTGACAAGGTTCTGAAATACGATG GGACATTTGCGTGAACAACAATACCAGCCTTCC
16562	H_tFBA1_RV	GTCACGGGTTCTCAGCAATTCGAGCTATTACCGATGATGGCTGAGGCGT TAGAGTAATCTAATGAGCTATCAAAAACGATAGATC
12957	TPI FW + H	AGATTACTCTAACGCCTCAGCCATCATCGGTAATAGCTCGAATTGCTGA GAACCCGTGACAACGAAGACCCAGAGATGTTGTTGT
12958	TPI Rv + P	CTGATAGTGCTGTAAGTCGCCTCCATCTTAGCAGAGCTGTCCCTGAATG CGTACTCGTGATGAGTAACCCATATAGAGATCGTAC
16563	P_pPGK1_FW	TCACGAGTACGCATTCAGGGACAGCTCTGCTAAGATGGAGGCGACTTA CAGCACTATCAGTCTTTTTATTAACCTTAATTTTTAT
16564	Q_tPGK1_RV	GAGCTGAATGTATATGCTGCGGGATCATTGCACAGCTCTGAGAGCCCTG CAACGCGATATAAATAATATCCTTCTCGAAAGC

16565	Q_pADH1_FW	ATATCGCGTTGCAGGGCTCTCAGAGCTGTGCAATGATCCCGCAGCATATACATTCAGCTCAAGTCCAATGCTAGTAGAGAAG
16566	N_tADH1_RV	TTCTAGGCTTTGATGCAAGGTCCACATATCTTCGTTAGGACTCAATCGTGGCTGCTGATCTTGTCCTCTGAGGACATAAAATAC
12963	PYK1 Fw + N	GATCAGCAGCCACGATTGAGTCCTAACGAAGATATGTGGACCTTGCATCAAAGCCTAGAAAACGTGGTCAAACTTCAGAACTAAG
12964	PYK1 Rv + O	ATACTCCCTGCACAGATGAGTCAAGCTATTGAACACCGAGAACGCGCTGAACGATCATTCATAATCATGATAACCTTGAGGGAAG
16567	O_pTDH3_FW	GAATGATCGTTCAGCGCGTTCTCGGTGTTCAATAGCTTGACTCATCTGTGCAGGGAGTATATACTAGCGTTGAATGTTAGCGTC
16568	A_tTDH3_RV	GTGCCTATTGATGATCTGGCGGAATGTCTGCCGTGCCATAGCCATGCCTTCACATATAGTATCCTGGCGGAAAAAATTCATTTG
12967	ENO2 Fw + A	ACTATATGTGAAGGCATGGCTATGGCACGGCAGACATTCCGCCAGATCATCAATAGGCACAACGGATGATGAAAACACTAAACGA
13227	ENO2 Rv + B	GTTGAACATTCTTAGGCTGGTCGAATCATTTAGACACGGGCATCGTCCTCTCGAAAGGTGTAACGAAGACGTTACCAGCTGATTG
16569	B_tHXK2_FW	CACCTTTCGAGAGGACGATGCCCGTGTCTAAATGATTCGACCAGCCTAAGAATGTTCAACACTTGAACAATAAATACGAAATCC
16570	C_pHXK2_RV	CTAGCGTGTCCTCGCATAGTTCTTAGATTGTCGCTACGGCATATACGATCCGTGAGACGTACGCTGGTAAAGTACAGCTA
12971	PGI1 Fw + D	AATCACTCTCCATACAGGGTTTCATACATTTCTCCACGGGACCCACAGTCGTAGATGCGTAACGTATTCTTAGTGGATAACATGC
12972	PGI1 Rv + C	ACGTCTCACGGATCGTATATGCCGTAGCGACAATCTAAGAACTATGCGAGGACACGCTAGTTTTAAACAGTTGATGAGAACCTTT
16571	D_tPFK1_FW	ACGCATCTACGACTGTGGGTCCCGTGGAGAAATGTATGAAACCCTGTATGGAGAGTGATTATTCCATAGCTTAGTTTAATCAAGG
16572	J_pPFK1_RV	CGACGAGATGCTCAGACTATGTGTTCTACCTGCTTGGACATCTTCGCGTATATGACGGCCCGGCTAGTAAAAAAGAAAATTAATA
12976	PFK2 Rv + J	GGCCGTCATATACGCGAAGATGTCCAAGCAGGTAGAACACATAGTCTGAGCATCTCGTCGAAATCGTCTATATCACATATTCCAG
13900	PFK2_fw + BU	AATCATGTGACCCAGGCTTGCGCATACATGATCCTTCTTGCGCTGCATGGGCGACTATATAACGATTCTCTGCTGCTTTGTTGCA
16573	BU_tHIS3_FW	ATATAGTCGCCCATGCAGCGCAAGAAGGATCATGTATGCGCAAGCCTGGGTCACATGATTGCATCTGTGCGGTATTTCACAC
16574	L_pHIS3_RV	GCCGTAGCTTCCGCAAGTATGCCGTAGTTGAAGAGCATTTGCCGTCGGTTCAGGTCATATTGCGGCATCAGAGCAGATTG
12980	GPM1 Rv + L	ATATGACCTGAACCGACGGCAAATGCTCTTCAACTACGGCATACTTGCGGAAGCTACGGCTATTGCTATAACATGTCATGTCACC
12979	GPM1 Fw + M	ACGAGAGATGAAGGCTCACCGATGGACTTAGTATGATGCCATGCTGGAAGCTCCGGTCATAACGGTGATACTTTGACAGGAGCTA
16575	M_tPDC1_FW	ATGACCGGAGCTTCCAGCATGGCATCATACTAAGTCCATCGGTGAGCCTTCATCTCTCGTACAGTGTTCCTTAATCAAGGATAC
16576	AR_pPDC1_RV	TGACGAGATTTGAGAAGTCCCCAATATCGACTCGTGATGTGCCATGCGTGCTGTCAGTATCATGCGACTGGGTGAGCATA

| 12983 | ARS1211_fw +AR | ATACTGACAGCACGCATGGCACATCACGAGTCGATATTGGGGACTTCTC AAATCTCGTCAGACATAGTATTTCGCAACCTTTCAG |
| 13745 | ARS1211 Rv+Tag CAN1 | GATGAGAAAAGTAAAGAATTGTATCCATTGCGCTCTTTCCCGACGAGAG TAAATGGCGAGGACAGGCGTTTCTGTACCGCTGTTA |

Table 4D: Primers for verification of SinLoG in strain IMX2359

Verify	Primer Number	Primer Name	Sequence (5' to 3')
can1-ARS418-FBA1	2496	CAN1 FW-conf-upstrm	CGGGAGCAAGATTGTTGTG
	13738	pFBA1_rev	TTAGGGCTTGCGTCAGC
FBA1-TPI1	5025	FBA1 Flank left RVFw	CCACTGCGGAGTCATTTCAAAGTC
	2909	D_RV (PDH construct ctrl)	CCCGCTCACACTAACGTAGG
TPI1-PGK1	2375	I-forward	TGAGCCACTTAAATTTCGTGAATG
	5033	PGK1 Flank left RV	GCGATAGTTCCTCACTCTTTCC
PGK1-ADH1	14513	dg_PGK1_rv_tagP	CTATGTTCGGGTTCAGCGTA
	7496	YAT2_2	CAGCTCTGGAACAACGACATCTG
ADH1-PYK1	2620	Nat Ctrl Fw	GCCGAGCAAATGCCTGCAAATC
	9602	Wan5543 Seq Primer RV 3	TGTGGAAGCCCGATGTCTGG
PYK1-TDH3	2914	J_FW (PDH construct ctrl)	GTCGTCATAACGATGAGGTGTTGC
	6714	GDP_seq_rev	GCATGTACGGGTTACAGCAGAATTAAAG
TDH3-ENO2	5029	TDH3 Flank left RV	GGCGCTCTTAATAATTTTGGGG
	5013	ENO2 Flank left RV	GGCGTGCGGTGTGTAGATGTATC
ENO2-HXK2	10132	AM_ctrl_fw	ACTGGAAGGGGAAAAAAAGG
	2025	HXK2FW3	GAAATTCACGGGATTTATTCGTGAC
HXK2-PGI1	5001	HXK2 Flank left RV	CCAATGTGCGAGGAGGTTCAG
	4664	c MDH3 fw	CATTATTGAACAGCGTCCAAGTAACTAC
PGI1-PFK1	5006	PGI1 Flank right FW	CTACCCACATCCGAACATTGC
	11621	FW_PFK1_seq2	CAATTGCGTGACGGTAGAAC
PFK1-PFK2	2368	D-reverse	AATCATGTTGATGACGACAATGG
	15357	tPFKI1 - FW	ATCCAAGAAAATGACCTTTTAT
PFK2-HIS3	2433	Tag-F forward	GACGCCATTTGGAACGAAAAAAAG
	10582	HIS3t-fw	TGACACCGATTATTTAAAGCTGC
HIS3-GPM1	5897	diag primer 2rv	GGGCTTTCTGCTCTGTCATC
	5757	tgpm1- Tag L rv	CGTCAGGGACAGTATGTTGGAATG
GPM1-PDC1	5036	GPM1 Flank right FW	GGTTACTTAGACATCACTATGGC
	13741	tPDC1-fwd	GAATTGGCTTAAGTCTGGGTCC
PDC1-ARS1211-can1	2851	Rv-ScPDC1	TTGCGTGAGGTTATGAGTAG
	7319	SOL1promDiagfw	CCACAGACGTGGGTCAATAC
outside-outside	2496	CAN1 FW-conf-upstrm	CGGGAGCAAGATTGTTGTG
	7319	SOL1promDiagfw	CCACAGACGTGGGTCAATAC

Table 4E: Primers for amplification of Kl*URA3* for construction of IMX2381

Primer number	Primer name	Sequence (5' to 3')
Amplification of *ScURA3* fragment		
13273	URA3 repair SGA1 Fw	TTTTTCTCATCTCTTGGCTCTGGATCCGTTATCTGTTCTGTTACAC AAGAAATCGTACATAACTGTCATCCTGCGTGAAGATTAA
13274	URA3 repair SGA1 Rv	TCTCGCTTTTCTTTTATTTTTTTTTGTCTACAAACTCTGTAAAACTT CTTGTCTTATTTGAGTGTTGCACCGTGCCAATGCAGGT
Confirmation of *KlURA3* integration at the *sga1* locus		
3751	sequence primer right - fw	GGTCAGCAGTACAGAACCGTCG
4229	Sequence SGA1 2 rv	TGGTCGACAGATACAATCCTGG
4880	c I-SceI inside rv	GCCAATCAAACCCTTCTTCTC
7298	FW_sga1u_check	TTGTTCAATGGATGCGGTTC

Table 3F: Primers to make repair fragments for integration of synthetic gRNA and PAM at *SUR2*, *ATG41* and *CNB1*.

Primer number	Primer name	Sequence (5' to 3')
17539	ATG41_rep_FW_SYNgRNA	ATGCGATGCAAGGGGTCGTGAGAACAAGTAGAGATGTGGTAAA ATGCGTGTAGAATTTCACCTAGACGTGGTGTAAATAATTGTAAT TGTATATTATTGTGTGTAAGTTTGGTTGGTTT
17540	ATG41_rep_RV_SYNgRNA	AAACCAACCAAACTTACACACAATAATATACAATTACAATTATTT ACACCACGTCTAGGTGAAATTCTACACGCATTTTACCACATCTCT ACTTGTTCTCACGACCCCTTGCATCGCAT
17548	CNB1_rep_FW_SYNgRNA	GTAACTCAATGGTGATCAGAATCCATAGAAGCATTTTTATTTCTT AAATGTAGAATTTCACCTAGACGTGGGACTAGGGGACACTTCAT TCATTTATGGTATGCCAATATTTTTAAGAA
17549	CNB1_rep_RV_SYNgRNA	TTCTTAAAAATATTGGCATACCATAAATGAATGAAGTGTCCCCTA GTCCCACGTCTAGGTGAAATTCTACATTTAAGAAATAAAAATGC TTCTATGGATTCTGATCACCATTGAGTTAC
17557	SUR2_rep_FW_SYNgRNA	TTCTAACATTCTAGTCCGAAGAGGGTGTATACGAAAAGAAAATA TACGTGTAGAATTTCACCTAGACGTGGCCCTTTTGCATACCTCCC GTTCAATTGCTGGGTAAAGGCAATGTCTCC
17558	SUR2_rep_RV_SYNgRNA	GGAGACATTGCCTTTACCCAGCAATTGAACGGGAGGTATGCAA AAGGGCCACGTCTAGGTGAAATTCTACACGTATATTTTCTTTTCG TATACACCCTCTTCGGACTAGAATGTTAGAA

Table 4G: Primers for amplification of *SUR2*, *ATG41* and *CNB1* repair fragments from the IMX1821 evolution lines.

Primer number	Primer name	Sequence (5' to 3')
17541	ATG41_FW_outs_rep	GGCTACACCAAACCAACCAAAC
17542	ATG41_RV_outs_rep	GTGTAGGATATGCGATGCAAGG
17550	CNB1_FW_outs_rep	GGTGTGGTTTACACGGAACATC
17551	CNB1_RV_outs_rep	TCTAACTGGGCGAGTGTGC
17559	SUR2_FW_outs_rep	TCCGGGTCTTCGTCTTTACTG
17560	SUR2_RV_outs_rep	GTGGGAAGTCGGAGACATTG

Table 4H: Diagnostic primers to check integration/deletion of *SUR2*, *ATG41* and CNB1.

Primer number	Primer name	Sequence (5' to 3')
17543	ATG41_outs_FW	TTTCGGAAAGGGTCTGGAGC
17544	ATG41_outs_RV	TGTGACCTTGAGCGATGTCC
17552	CNB1_outs_FW	GCATCAGCACTGCAGAATCG
17553	CNB1_outs_RV	GATCCCCTTTGTGCATTGC
17561	SUR2_outs_FW	TCCCTTGCATTTACTCCGGG
17562	SUR2_outs_RV	TTTGAGCGCCTCCAAATTGG
17883	VPS15_FW2	TCCACGAGACAGTACCATTG
17884	VPS15_RV2	GCGTTGCAAGCCCTTTC
18461	VPS15_ins_fw	GGGTGAGATTGGTTCAGACCC
18462	VPS15_ins_rv	CTCGCAATGCTGGGTCCTTATC
17570	OPT1_outs_FW	GGCACCAAGGTTTTCAGCAG
17571	OPT1_outs_RV	GGGTCGCTTTGAATGTTTCG
18463	OPT1_ins_fw	CTTTGCCGTCCAAATCTATGCC
17861	CWC25_dg_fw	ACACAGGAGACGACGAAACC
18245	CWC25_outs_rv	TTACAGGCGGTGTTGCAGG

Table S5 Configuration of SinLoG in IMX2359 and IMX2381

[#]The +, - or 0 signifies the orientation of the part with respect to the chromosome

* Size of the fragments does not include the SHR sequences.

SHR Fw	Component[#]	SHR Rv	Size*	Template	Primer Fw	Primer Rv
can1	ARS418 (0)	DL	165	*S. cerevisiae* CEN.PK113-7D	13161	16828
DL	FBA1 (+)	H	2185	pUDC212	16561	16562
H	TPI1 (+)	P	1856	pUDC222	12957	12958
P	PGK1 (+)	Q	2356	pUDC219	16563	16564
Q	ADH1 (+)	N	2152	pUDC229	16565	16566
N	PYK1 (+)	O	2612	pUDC220	12963	12964
O	TDH3 (+)	A	2104	pUDC230	16567	16568
A	ENO2 (+)	B	2423	pUDC231	12967	13227
B	HXK2 (-)	C	2566	pUDC214	16569	16570
C	PGI1 (-)	D	2774	pUDC232	12972	12971
D	PFK1 (-)	J	4069	pUDC216	16571	16572
J	PFK2 (-)	BU	3989	pUDC217	12976	13900
BU	HIS3 (-)	L	1254	pLM092	16573	16574
L	GPM1 (-)	M	1853	pUDC213	12980	12979
M	PDC1 (-)	AR	2797	pUDC215	16575	16576
AR	ARS1211 (0)	can1	251	*S. cerevisiae* CEN.PK113-7D	12983	13745

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