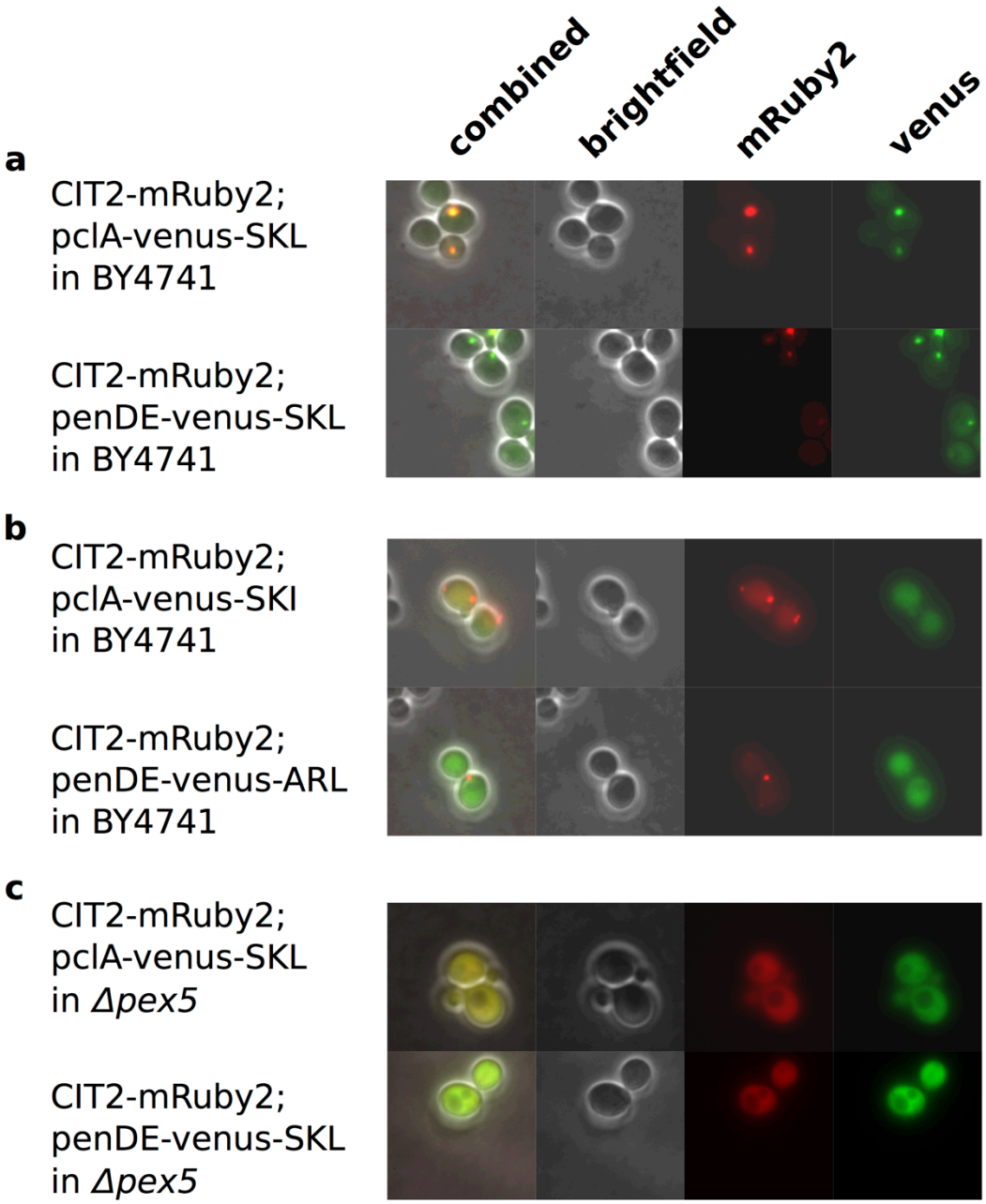
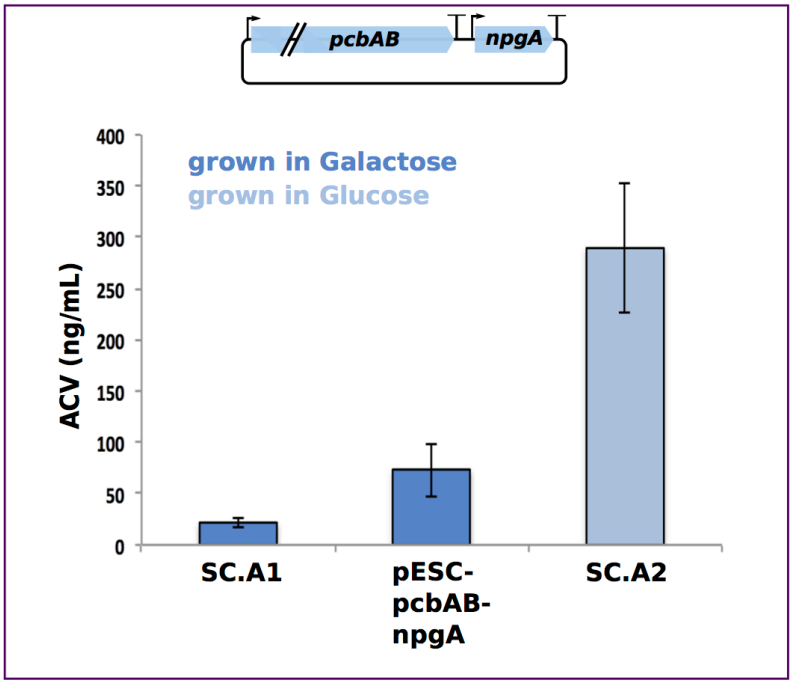


Supplementary Figure 1. *S. cerevisiae* PTS1 tags are necessary to localise pclA and penDE to peroxisomes. (a) *S. cerevisiae* peroxisomal protein CIT2 with an mRuby2 fluorescence tag co-localises with venus-fluorescence-tagged *pclA* and *penDE* proteins additionally tagged with the *S. cerevisiae* Peroxisome Targeting Sequence 1 (PTS1) tripeptide SKL at the C-terminus. For this and subsequent parts, the promoter driving expression of *pclA* and *penDE* with the venus fluorescence tag is the strong constitutive promoter pTDH3. **(b)** Venus-fluorescence-tagged *pclA* and *penDE* proteins without the *S. cerevisiae* PTS1 but tagged with the native *P. chrysogenum* PTS1 tripeptides SKI and ARL do not co-localise with mRuby2-tagged CIT2. **(c)** Plasmids from part (a) transformed into an *S. cerevisiae* strain harbouring a delete of the peroxisomal importer *pex5* prevent peroxisomal localisation of CIT2 and the *S. cerevisiae* PTS1-tagged *pclA* and *penDE* proteins.



Supplementary Figure 2. Optimising ACV yields. Strong, constitutive promoters driving the ACV-producing part of the benzylpenicillin pathway on a low-copy plasmid (Sc.A2) gives higher maximal ACV yields than the same genes under galactose inducible promoters either integrated into a chromosome (Sc.A1) or on a high-copy plasmid (pESC-pcbAB-npgA). Sc.A2 was grown using glucose as the carbon source, while Sc.A1 and pESC-pcbAB-npgA were grown using galactose as the carbon source. Error bars represent standard deviation from three biological replicates.

pcbAB	npgA	GAL 1	GAL 2	GAL 3	mean GAL	GAL s.d.	Glu 1	Glu 2	Glu 3	mean GLU	Glu s.d.
GAL1	GAL7	502212.0	633534.0	217523.0	451089.7	212665.0	Not detected	Not detected	Not detected	0.0	0.0
GAL1	TDH3	786826.0	943928.0	391730.0	707494.7	284518.5	Not detected	Not detected	Not detected	0.0	0.0
GAL1	HHF2	561543.0	649935.0	279805.0	497094.3	193298.4	Not detected	Not detected	Not detected	0.0	0.0
GAL1	RPL18B	545298.0	565738.0	349162.0	486732.7	119577.2	Not detected	Not detected	Not detected	0.0	0.0
RPL18B	GAL1	100022.0	116057.0	51593.0	89224.0	33561.1	56568.0	53651.0	43446.0	51221.7	6890.1
RPL18B	PGK1	138596.0	110451.0	58385.0	102477.3	40695.6	197598.0	109863.0	157372.0	154944.3	43917.9
RPL18B	CCW12	144395.0	116449.0	59786.0	106876.7	43109.1	211374.0	150211.0	87174.0	149586.3	62102.4
RPL18B	ALD6	146092.0	68694.0	56735.0	90507.0	48508.0	131432.0	100424.0	107881.0	113245.7	16185.1
TDH3	PGK1	193113.0	86218.0	103581.0	127637.3	57364.3	404773.0	277704.0	284469.0	322315.3	71490.5
TDH3	CCW12	178619.0	138064.0	73651.0	130111.3	52934.0	361186.0	331388.0	218401.0	303658.3	75323.2
TDH3	ALD6	168824.0	74255.0	60426.0	101168.3	58998.1	369769.0	201340.0	218023.0	263044.0	92802.2
HHF2	PGK1	87157.0	40076.0	36120.0	54451.0	28393.2	56687.0	40722.0	28941.0	42116.7	13925.5
HHF2	CCW12	76374.0	72364.0	46495.0	65077.7	16217.5	34133.0	42551.0	21065.0	32583.0	10826.5
HHF2	ALD6	84088.0	32408.0	24310.0	46935.3	32428.9	65046.0	31137.0	45320.0	47167.7	17029.8

ACV Yields

Strain	pcbAB	npgA	sugar	reading 1	reading 2	reading 3	mean conc. (ng/uL)	conc. s.d.
Sc.A1	GAL10	GAL1	gal	26.868	20.011	17.482	21.454	4.857
pESC-npgA-pcbAB	GAL10	GAL1	gal	102.938	53.643	63.204	73.262	26.142
Sc.A2	TDH3	PGK1	glu	362.586	249.952	255.949	289.495	63.369

Supplementary Table 1. LCMS peak areas and yields for ACV for the pcbAB, npgA promoter screen

Name (X = sample from screened strain)	Area
Water	
Water	
benzylpenicillin; 10 pg/uL	
benzylpenicillin; 100 pg/uL	2233
benzylpenicillin; 1 ng/uL	18103
benzylpenicillin; 10 ng/uL	165306
Water	981629
Water	
X1	
X2	
X3	
X4	
X5	
X6	
X7	
X8	
X9	
X10	
X11	
X12	318
X13	
X14	
X15	
X16	
X17	
X18	
X19	2761
X20	
X21	2709
X22	1062
X23	
X24	
X25	
X26	
X27	1292
X28	
X29	
X30	
X31	
X32	
X33	
X34	
X35	
X36	
X37	527
X38	
X39	
X40	
X41	
X42	
X43	359
X44	
X45	
X46	
X47	
X48	

Name (X = sample from screened strain)	Area
X49	
X50	
X51	
X52	
X53	
X54	
X55	
X56	
X57	1506
X58	
X59	
X60	
X61	
X62	
X63	
X64	
X65	
X66	
X67	
X68	
X69	
X70	
X71	385
X72	
X73	1285
X74	
X75	
X76	295
X77	
X78	
X79	
X80	411
X81	
X82	
X83	
X84	665
X85	
X86	
X87	
X88	
X89	
X90	467
X91	
X92	
X93	
X94	
X95	
X96	
X97	
X98	2193
X99	
X100	
X101	
X102	
X103	
X104	

Name (X = sample from screened strain)	Area
X105	
X106	
X107	
X108	
X109	
X110	
X111	
X112	
X113	
X114	
X115	
X116	
X117	
X118	586
X119	
X120	
X121	
X122	
X123	
X124	
X125	
X126	
X127	
X128	
X129	
X130	
X131	
X132	
X133	
X134	
X135	
X136	
X137	
X138	171
X139	
X140	
X141	
X142	
X143	
X144	
X145	
X146	692
X147	
X148	1648
X149	
X150	1060
X151	
X152	
X153	
X154	
X155	
X156	
X157	
X158	
X159	
X160	

Supplementary Table 2. LCMS peak areas and yields for ACV for the pcbAB, npgA promoter screen

Summed nanopore read count data for single assemblies

	pcbC	pclA	penDE
pGAL1	58	79	25
pGAL7	216	139	33
pTDH3	40	107	33
pCCW12	39	59	42
pPGK1	209	89	32
pHHF2	317	91	52
pRPL18B	105	71	39
pALD6	190	125	42
pPSP2	103	44	73
pREV1	78	67	26
TOTAL	*1355*	*871*	*397*

Summed nanopore read count data for multiegene level assembly

	pcbC	pclA	penDE
pGAL1	16	15	12
pGAL7	19	24	7
pTDH3	12	10	10
pCCW12	17	11	13
pPGK1	7	28	13
pHHF2	17	27	25
pRPL18B	21	4	12
pALD6	3	3	14
pPSP2	13	5	26
pREV1	10	8	3
TOTAL	*135*	*135*	*135*

Summed Sanger read count data from all 10 benzylpenicillin producers

	pcbC	pclA	penDE
pGAL1	0	0	0
pGAL7	1	0	0
pTDH3	0	2	2
pCCW12	1	5	0
pPGK1	0	3	2
pHHF2	2	0	2
pRPL18B	5	0	1
pALD6	0	0	0
pPSP2	1	0	3
pREV1	0	0	0
TOTAL	*10*	*10*	*10*

one-sided Fisher's exact test for enrichment of pclA strong promoters in multigene assemblies vs. benzylpenicillin producers

	strong	non-strong
multigene	44	938
producers	10	0

p < 0.005 (Fisher's exact test)

Observed nanopore read count data by promoter category for single assemblies

	pcbC	pclA	penDE
Strong	605	346	159
Medium	295	196	81
Weak	181	111	99
Inducible	274	218	58

Expected nanopore read counts for single assemblies for Chi square test based on promoter proportions used in library construction

	pcbC	pclA	penDE
Strong	542	348	159
Medium	271	174	79
Weak	271	174	79
Inducible	271	174	79

Observed nanopore read count data by promoter category for multigene assemblies

	pcbC	pclA	penDE
Strong	53	76	61
Medium	24	7	26
Weak	23	13	29
Inducible	35	39	19

Expected nanopore read count data by promoter category for multigene assemblies based on single assembly observed distributions

	pcbC	pclA	penDE
Strong	60	54	54
Medium	29	30	28
Weak	18	17	34
Inducible	27	34	20

Observed nanopore read count data by promoter category for producers

	pcbC	pclA	penDE
Strong	3	10	6
Medium	5	0	1
Weak	1	0	3
Inducible	1	0	0

p-values for Pearson's Chi-squared tests comparing single to multigene level assembly, and Fisher's exact test comparing multigene level assembly to producer for promoter category distributions

	pcbC	pclA	penDE
uniform dist vs. single	p < 0.27	p < 0.27	p < 0.27
single vs. multigene	p < 0.22	p < 0.22	p < 0.22
multigene vs. producers	p < 0.15	p < 0.005	p < 0.52

Supplementary Table 3. Read counts and statistics for nanopore and Sanger sequencing

Experiment	Strain name(s)	Background Strain	plasmid(s)
Fig 1c, 2a	Sc.A1	BY4741	-
Fig 1c, d	Sc.P1	Sc.A1	pAA056
Fig 1c	Sc.P1x		
Fig 2b, 3	Sc.P2	Sc.A2	pAA179
Fig 3	Sc.P2x	BY4741	pAA180, pAA171
Fig 3	Sc.S1 – Sc.S12	BY4741	variants of pAA179*
Fig S1	-	BY4741	pAA210
Fig S1	-	BY4741	pAA211
Fig S1	-	BY4741	pAA212
Fig S1	-	BY4741	pAA213
Fig S1	-	BY4741*Δpex5*	pAA210
Fig S1	-	BY4741*Δpex5*	pAA211
Fig S2	pESC-npgA-pcbAB	BY4741	pESC-npgA-pcbAB
Fig S2	Sc.A2	BY4741	pAA145

Primers used to create the *Δpex5* strain

Purpose	Orientation	Sequence
to amplify and retarget gRNA vector	F	CGTTTCTATTTTGAATACTAaaagtcccattcgccacccg
to amplify and retarget gRNA vector	R	/5Phos/gttttagagctagaaatagcaagttaaaataaggctag
Overlap extension PCR 1	F	GTGAAGGAAATCGCAGCTCC
Overlap extension PCR 1	R	AATGCTAATGAATTTGGGCAGTGATGCGAGCTCCGCCGGAAGAGGGTACCGACTTGTCGG
Overlap extension PCR 2	F	CCGACAAGTCGGTACCCTCTTCCGGCGGAGCTCGCATCACTGCCCAAATTCATTAGCATT
Overlap extension PCR 2	R	CCTCTCTGTATTCGTGAGAGTATC

Supplementary Table 4. Strains and plasmids used in this paper

* same as pAA179, but with promoters driving pcbC, pclA and penDE as specified by Table S12

Experiment	Molecule of interest	Sugar	Dropouts	Supplements	Volume
Fig 1c, d	ACV	galactose	uracil	5 mM AAA	30 mL
Fig 1c, d	benzylpenicillin	glucose	uracil, histidine	5 mM AAA, 0.25 mM PAA	30 mL
Fig S2	ACV	galactose/ glucose	uracil	5 mM AAA	600 µL
Fig 2b, 3, S3, S4	benzylpenicillin	glucose	uracil, histidine	5 mM AAA, 0.25 mM PAA	600 µL

AAA = alpha-amino adipic acid (VWR L13924.0
PAA = phenylacetic acid (Sigma P16621)

Supplementary Table 5. ACV and benzylpenicillin production media composition

The LC gradient elution method for the separation of compounds in the penicillin-G pathway

Time (minutes)	% Solvent A	% Solvent B	Flow rate (mL/min)
0	98	2	0.2
2	98	2	0.2
8	35	65	0.2
8.01	5	95	0.2
9	5	95	0.2

The MS/MS fragment ions used for the measurement of benzylpenicillin and other materials in the pathway

Analyte	Formula	Precursor ion [M+H]+	Product ion	Collision energy (eV)
benzylpenicillin	C16H18N2O4S	335.106	176.0707	16
LLD-ACV	C14H25N3O6S	364.1537	144.0655	16

Supplementary Table 6. LCMS specifications

Promoter	Category	Relative Expression*
pTDH3	Strong	820
pCCW12	Strong	575
pPGK1	Strong	291
pHHF2	Strong	230
pRPL18B	Medium	60
pALD6	Medium	39
pPSP2	Weak	2.2
pREV1	Weak	1.7
pGAL1	Inducible	1.3
pGAL7	Inducible	**

Supplementary Table 7. Promoters used in first promoter screen to optimise conversion of ACV to benzylpenicillin.

* Based on fold-change expression over background of fluorescent reporter Venus protein under each promoter, cells grown in glucose.
Taken from Lee, M.E., DeLoache, W.C., Cervantes, B. & Dueber, J.E.
ACS Synth Biol 4, 975-986 (2015).

** Uncharacterised in the above study, but should be similar to value from pGAL1, as these promoters naturally drive similar levels of expression when uninduced.

Sample	pcbC	pclA	penDE	LCMS	pclA promoter Strength	penDE promoter Strength	Geometric mean of promoter strength pclA + penDE	[benzylpenicillin] ng/mL
X19	pRPL18B	pTDH3	pCCW12	2761	820	575	686.659	3.204
X148	pRPL18B	pTDH3	pPGK1	1648	820	291	488.487	1.845
X98	pRPL18B	pCCW12	pPGK1	2193	575	291	409.054	2.505
X80	pRPL18B	pTDH3	pGAL1	411	820	1.3	32.650	0.418
X84	pRPL18B	pPGK1	pPGK1	665	291	291	291.000	0.699
X37	pRPL18B	pPGK1	pHHF2	527	291	230	258.708	0.545
X90	pRPL18B	pALD6	pHHF2	467	39	230	94.710	0.479

Supplementary Table 8. Relationship between promoter strength and benzylpenicillin yield in first promoter screen

Carbon source for these experiments is glucose, with promoter strengths that are indicated those for growth in glucose. Blue = strains featured in Figure 2. Red = strains with low penicillin production, not featured in Figure 2.

Single Gene Assemblies

Reaction	Type 2	Type 3	Type 4	Pre-assembled Type 1;Type 5;Type 8
Single 1	promoter mix	pcbC	tPGK1	ConLS;ConR1;AmpR-ColE1
Single 2	promoter mix	pclA	tENO2	ConL1;ConR2;AmpR-ColE1
Single 3	promoter mix	penDE	tTDH1	ConL2;ConRE;AmpR-ColE1

Multigene Assembly

Gene 1	Gene 2	Gene 3	Pre-assembled Type 6;Type7;Type 8
Single 1	Single 2	Single 3	His3;2micron;KanR-ColE1

Supplementary Table 9. Golden Gate Assembly reaction parts for promoter screen to optimise conversion of ACV to benzylpenicillin and for nanopore sequencing

Promoter	Category	Relative Expression*
pTDH3	Strong	820
pCCW12	Strong	575
pPGK1	Strong	291
pTEF1	Strong	230
pHHF1	Medium	81
pRPL18B	Medium	60

Supplementary Table 10. Promoters used in second promoter screen to optimise conversion of ACV to benzylpenicillin

* Based on fold-change expression over background of fluorescent reporter Venus protein under each promoter, cells grown in glucose.
Taken from Lee, M.E., DeLoache, W.C., Cervantes, B. & Dueber, J.E.
ACS Synth Biol 4, 975-986 (2015).

Single Gene Assemblies

Reaction	Type 2	Type 3	Type 4	Pre-assembled Type 1;Type 5;Type 8
Single 1	pTDH3	pcbC	tPGK1	ConLS;ConR1;AmpR-ColE1
Single 2	pCCW12	pcbC	tPGK1	ConLS;ConR1;AmpR-ColE1
Single 3	pPGK1	pcbC	tPGK1	ConLS;ConR1;AmpR-ColE1
Single 4	pTEF1	pcbC	tPGK1	ConLS;ConR1;AmpR-ColE1
Single 5	pHHF1	pcbC	tPGK1	ConLS;ConR1;AmpR-ColE1
Single 6	pRPL18B	pcbC	tPGK1	ConLS;ConR1;AmpR-ColE1
Single 7	pTDH3	pclA	tENO2	ConL1;ConR2;AmpR-ColE1
Single 8	pCCW12	pclA	tENO2	ConL1;ConR2;AmpR-ColE1
Single 9	pPGK1	pclA	tENO2	ConL1;ConR2;AmpR-ColE1
Single 10	pTEF1	pclA	tENO2	ConL1;ConR2;AmpR-ColE1
Single 11	pHHF1	pclA	tENO2	ConL1;ConR2;AmpR-ColE1
Single 12	pRPL18B	pclA	tENO2	ConL1;ConR2;AmpR-ColE1
Single 13	pTDH3	penDE	tTDH1	ConL2;ConRE;AmpR-ColE1
Single 14	pCCW12	penDE	tTDH1	ConL2;ConRE;AmpR-ColE1
Single 15	pPGK1	penDE	tTDH1	ConL2;ConRE;AmpR-ColE1
Single 16	pTEF1	penDE	tTDH1	ConL2;ConRE;AmpR-ColE1
Single 17	pHHF1	penDE	tTDH1	ConL2;ConRE;AmpR-ColE1
Single 18	pRPL18B	penDE	tTDH1	ConL2;ConRE;AmpR-ColE1

Multigene Assebmly

Gene 1	Gene 2	Gene 3	Pre-assembled Type 6;Type7;Type 8
Single 1 – Single 6	Single 7 – Single 12	Single 13 – Single 18	His3;2micron;KanR-ColE1

Supplementary Table 11. Golden Gate Assembly reaction parts for second promoter screen to optimise conversion of ACV to benzylpenicillin

Strain	pcbC	pclA	penDE	pcbC promoter Strength	pclA promoter Strength	penDE promoter Strength
Sc.P2	HHF1	TEF1	PGK1	81	230	291
S7	TDH3	CCW12	TEF1	820	575	230
S2	PGK1	TDH3	TEF1	291	820	230
S3	RPL18B	TEF1	TEF1	60	230	230
S1	RPL18B	TEF1	TEF1	60	230	230
S6	RPL18B	TDH3	CCW12	60	820	575
S4	PGK1	TEF1	PGK1	291	230	291
S5	TEF1	TDH3	PGK1	230	820	291
S10	CCW12	PGK1	CCW12	575	291	575
S11	RPL18B	HHF1	RPL18B	60	81	60
S8	TEF1	TDH3	CCW12	230	820	575
S12	TDH3	PGK1	TEF1	820	291	230
S9	PGK1	RPL18B	TEF1	291	60	230

Supplementary Table 12. Promoter strengths for strains shown in Figure 3d