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Increasing galactose consumption by *Saccharomyces cerevisiae* through metabolic engineering of the *GAL* gene regulatory network

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Increasing the flux through central carbon metabolism is difficult because of rigidity in regulatory structures, at both the genetic and the enzymatic levels. Here we describe metabolic engineering of a regulatory network to obtain a balanced increase in the activity of all the enzymes in the pathway, and ultimately, increasing metabolic flux through the pathway of interest. By manipulating the *GAL* gene regulatory network of *Saccharomyces cerevisiae*, which is a tightly regulated system, we produced prototroph mutant strains, which increased the flux through the galactose utilization pathway by eliminating three known negative regulators of the *GAL* system: Gal6, Gal80, and Mig1. This led to a 41% increase in flux through the galactose utilization pathway compared with the wild-type strain. This is of significant interest within the field of biotechnology since galactose is present in many industrial media. The improved galactose consumption of the *gal* mutants did not favor biomass formation, but rather caused excessive respiro-fermentative metabolism, with the ethanol production rate increasing linearly with glycolytic flux.

Keywords: metabolic engineering, gene regulation, *Saccharomyces cerevisiae*, *GAL* system

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Within the last decade metabolic engineering has been successfully applied for optimization of several industrial fermentation processes (for reviews see refs 1–3). Recombinant DNA techniques have facilitated the ability to genetically modify suitable host systems. Most of the success stories in metabolic engineering have involved modification of only a single gene, for example, disruption of a pathway or expression of a particular gene for protein production. This has resulted in recombinant strains that have reduced by-product formation with a resulting increase in the overall yield of product. For many industrial processes—especially those involving low value-added products—the yield is certainly important, but it is also important to have a high rate of conversion of the substrate into the product. This requires optimization of the flux through the central carbon metabolism, which has been attempted for glycolysis in *Saccharomyces cerevisiae*[4] and in other microorganisms. These attempts have, however, largely failed for two major reasons. First, control of flux through the central carbon metabolism is distributed over many enzymes, so an increase in flux requires increased activity of many (or all) enzymes in the pathway[5–7]. Second, regulation of glycolysis—both at the genetic and at the enzymatic level—is very rigid, making it difficult to modulate flux through amplification of individual enzyme activities.

One way to solve these problems may be overexpression of many (or all) genes encoding the enzymes in a given pathway[6]. However, this may impose a physiological burden on the cell by draining pools of nucleotides or amino acids, or by slowing down transcriptional or translational efficiency. This may have metabolic consequences in other parts of metabolism, which negatively affects the overall performance of the cell. Furthermore, high levels of all the enzymes in the pathway may lead to significant changes in metabolite levels, which may result in downregulation of some enzymes.

A successful strategy for increasing the flux through a pathway therefore will require a balanced and similar degree of amplification of all enzyme activities in the pathway. This can be achieved through engineering of regulatory networks involved in control of gene expression. By reducing the levels of negative regulators or increasing the levels of positive activators of gene expression, it should be possible to obtain a uniform increase in the activity of all enzymes in the pathway. The flux through the pathway should thereby be increased without changing the metabolite levels, and therefore a minimum of physiological constraints is imposed on the microorganism. With the introduction of DNA array technology, new regulatory pathways will be discovered, and these constitute obvious targets for metabolic engineering in attempts to increase the flux through different pathways.

In this study we applied this strategy to improve the flux through the galactose utilization pathway of *Saccharomyces cerevisiae* (Fig. 1). The *GAL* genes are tightly regulated, being repressed by glucose and induced by galactose up to 1,000-fold[8]. *GAL* gene expression requires the well-studied transcriptional activator protein Gal4, which binds to the *GAL* gene promoters. Gal4 function is inhibited by Gal80, which binds directly to Gal4, and by Mig1, which represses expression of *GAL1* and *GAL4* in the presence of glucose (Fig. 2). *GAL6*, which was recently devoted as member of the *GAL* regulon[9], also exerts negative control over the *GAL* genes.

To determine the contribution each regulator of the *GAL* genes makes to control of flux through the galactose utilization pathway, we examined the physiology of *GAL* mutant strains. We obtained mutants with a significantly improved galactose consumption, and these

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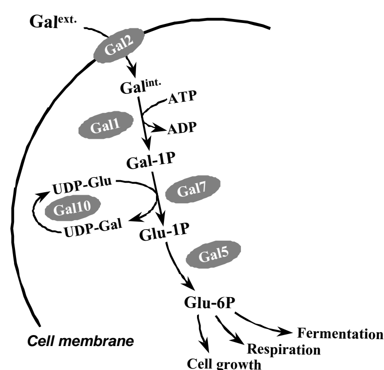
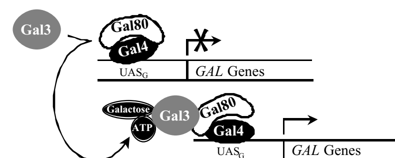
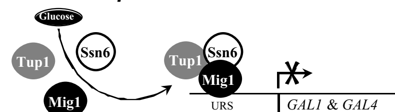


Figure 1. The galactose utilization pathway. Extracellular galactose is transported into the cell and subsequently converted to glucose-6-phosphate by several enzymatic steps. Glucose-6-phosphate may be directed toward respiratory metabolism, fermentative metabolism, or conversion of glucose-6-phosphate may lead to biosynthesis of various precursors required for cell growth. Gal2, galactose permease; Gal1, galactokinase; Gal7, galactose-1-phosphate uridylyltransferase; Gal10, UDP-glucose 4-epimerase; Gal5, phosphoglucomutase.

Galactose induction



Glucose repression



Gal6-mediated control

Figure 2. Various control mechanisms of the *GAL* system in *S. cerevisiae*. Gal4 acts as a positive transcriptional activator of the *GAL* genes by binding to specific upstream activating sites (UAS$_G$). During induction and repression of the *GAL* genes Gal80 is bound to Gal4 (ref. 24), but under repressed conditions it has been suggested that Gal80 is bound to Gal4 in two regions, one of them covering the transcriptional activation domain of Gal4 (ref. 25). Intracellular galactose plays a pivotal role, being the signal molecule that (by an ATP-dependent mechanism) is bound to Gal3, which acts as a transducer for galactose induction by interacting with Gal80 (refs 26,27). Furthermore, Mig1-mediated control prevents transcription of the *GAL1* gene and the *GAL4* gene in the presence of glucose by participating in a protein complex with Ssn6 and Tup1 that binds to specific upstream repression sites (URS)[28–30]. Gal6 also downregulates the *GAL* system by affecting production, stabilization, or degradation of *GAL1*, *GAL2*, and *GAL7* mRNA (ref. 9), but the exact mechanism is not well known.

mutants are valuable in industrial processes where organic material such as lignocellulose or beet molasses are used as raw material, since they contain galactose in the form of hemicellulose and raffinose, respectively[10,11]. Our experience with engineering of a regulatory network portends success for metabolic engineering of other pathways—either known or pathways discovered through whole-genome analysis.

Results and discussion

The *GAL* system of *S. cerevisiae* was metabolically engineered by construction of mutant strains with disrupted *GAL6* and/or *GAL80* and *MIG1* genes. To increase expression of the *GAL* genes that encode enzymes required for galactose utilization and thereby enable increased flux through the galactose utilization pathway, we constructed mutant strains missing *GAL6* and/or *GAL80* and *MIG1* (see Table 1). We also determined the impact on the flux through the galactose utilization pathway of increasing the level of Gal4.

The physiology of the recombinant strains was examined under controlled conditions in aerobic batch cultivations on galactose. This approach enabled estimation of the maximum specific galactose uptake rates without the influence of a growth limitation that could affect galactose uptake (for example, nitrogen-limited steady-state cultivations). Furthermore, evaluation of the strains in batch cultivations allowed precise estimation of the maximum specific growth rate and measurement of product formation. The major carbon fluxes into biomass and the metabolic products ethanol, acetate, and glycerol could be estimated precisely in terms of overall yield coefficients. All the strains characterized in this study were prototrophic, which avoids addition of various amino acids that could affect cellular physiology by interfering with galactose uptake and biomass formation.

Deletion of the genes encoding the transcriptional repressor Mig1 and the Gal4 regulator Gal80 (the SO3 strain) increased the flux through the galactose utilization pathway 15% compared with the wild-type strain. Surprisingly, deletion of *GAL6* (the SO15 strain), which encodes a protein that negatively regulates *GAL* gene expression by an unknown mechanism, increased the flux through the pathway by 24%. The maximum increase in flux (41%) was achieved by elimination of all three proteins that impose downregulation of the *GAL* gene regulatory network (SO16) (see Table 2).

Increasing the level of Gal4 by introduction of a high-copy plasmid containing *GAL4* (SO7), resulted in a 26% higher maximum specific galactose consumption rate compared with the wild-type strain, which is smaller than the increase in flux obtained by eliminating Gal6, Gal80, and Mig1. This may be due to increased expression of both *GAL6* (ref. 9) and *GAL80* (ref. 12) when the level of Gal4 is increased, and hereby the negative regulation exerted by Gal6 and Gal80 on expression of the Gal enzymes is intensified.

Table 1. Yeast strains used in this study

Strain	Genotype	Plasmid	Source/ reference
WT (CENPK113-7D)	*MAT**a** SUC2 MAL2-8*^{c}	–	SR&D[a]
SO2 (CENPK113-5D)	*MAT**a** SUC2 MAL2-8*^{c} *ura3-52*	–	SR&D[a]
SO3	*MAT**a** SUC2 MAL2-8*^{c} *gal80(41,1110): loxP mig1(100,1480):loxP*	–	Provided by P. Kötter
SO4	*MAT**a** SUC2 MAL2-8*^{c} *ura3-52 gal80(41,1110): loxP mig1(100,1480):loxP*	–	Provided by P. Kötter
SO7	*MAT**a** SUC2 MAL2-8*^{c}	p*GAL4* (2μ) (pBM959)	This study
SO15	*MAT**a** SUC2 MAL2-8*^{c} *gal6:KanMX*	–	This study
SO16	*MAT**a** SUC2 MAL2-8*^{c} *gal80(41,1110): loxP mig1(100,1480):loxP gal6:KanMX*	–	This study
SO17	*MAT**a** SUC2 MAL2-8*^{c} *ura3-52 gal80(41,1110): loxP mig1(100,1480):loxP gal6:KanMX*	–	This study
SO37	*MAT**a** SUC2 MAL2-8*^{c} *gal80(41,1110): loxP mig1(100,1480):loxP gal6:KanMX*	p*GAL4* (2μ) (pBM959)	This study

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Table 2. Data from the aerobic batch cultivations on galactose

Strain	Max. sp. galactose uptake[a] (mmol gal/g CDW/h)	Max. sp. growth rate (h^{-1})	Max. sp. ethanol prod.[b] (mmol EtOH/g CDW/h)	Biomass yield[c] (g CDW/ g gal)	Ethanol yield[c] (mmol EtOH/g gal)	Acetate yield[c] (mmol Ac/g gal)	Glycerol yield[c] (mmol gly/g gal)
WT (CEN.PK 113-7D)	3.01	0.13	2.71	0.24	5.00	0.60	0.23
SO3 (Δ*gal80* Δ*mig1*)	3.44	0.13	3.77	0.21	6.09	0.23	0.02
SO7 (p*GAL4*, 2μ)	3.80	0.13	5.35	0.19	7.83	0.27	0.02
SO15 (Δ*gal6*)	3.70	0.12	3.91	0.18	5.87	ND[d]	0.16
SO16 (Δ*gal6* Δ*gal80* Δ*mig1*)	4.25	0.13	6.48	0.17	8.48	ND[d]	0.01
SO37 (Δ*gal6* Δ*gal80* Δ*mig1* & p*GAL4*, 2μ)	3.57	0.09	4.19	0.14	6.52	0.25	0.03

[a]The maximum specific galactose uptake rate was calculated from the biomass yield and the maximum specific growth rate.
[b]The maximum specific ethanol production rate was calculated from the ethanol yield and the maximum specific galactose uptake rate.
[c]Obtained as the slope of the linear curve when plotting the biomass or metabolite concentration versus the galactose concentration during exponential growth.
[d]Not determined.

Introduction of the *GAL4* high-copy plasmid into a Δ*gal6* Δ*gal80* Δ*mig1* triple mutant strain (SO37) did not further increase flux through the galactose utilization pathway compared with SO16. In fact, the maximum specific galactose uptake rate of SO37 was lower than observed in both SO7 and SO16, but still 19% higher than the maximum specific galactose consumption rate of the wild-type strain. Presumably the level of Gal4 had already reached an upper limit in the Δ*gal6* Δ*gal80* Δ*mig1* triple mutant as a result of the lack of either Gal6 or Gal80, because too high a level of Gal4 has major physiological effects on cells. These effects include morphological modifications and alteration of cell size distribution, which lead to cell lysis[13,14], as was also observed for SO37. Additionally transcription of certain genes lacking *GAL4* binding sites may be inhibited—a phenomenon called "squelching"[15], which has a negative impact on cell growth, as illustrated by SO37 (Table 2).

Apart from SO37, the improved galactose consumption did not impose any physiological burden on the mutant strains, since they exhibited the same maximum specific growth rate on galactose as the wild-type strain. Improved flux through the galactose utilization pathway did not increase the rate of biomass production, but rather directed galactose toward ethanol formation (Table 2). Thus, *S. cerevisiae* exhibits strict respiro-fermentative metabolism not only on glucose, but also on galactose when the glycolytic flux is increased by genetic engineering, as illustrated in Figure 3, which shows the relation between the maximum specific galactose uptake rate and the maximum specific ethanol production rate. A least-square regression of data in Figure 3 reveals that 3.09 mol of ethanol are formed for each additional mole of galactose taken up. Thus, increasing galactose consumption has a positive impact on ethanol formation at the expense of the amount of galactose being oxidized aerobically, as the maximum

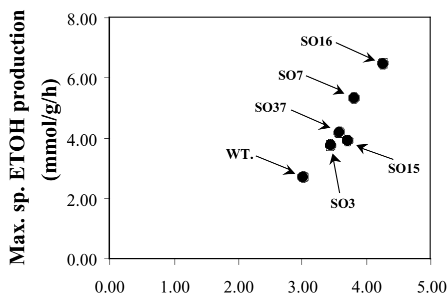

Figure 3. Correlation between the maximum specific ethanol production rate and the maximum specific galactose uptake rate for the wild-type strain (WT), SO3 (Δ*gal80* Δ*mig1*), SO7 (p*GAL4*, 2μ), SO15 (Δ*gal6*), SO16 (Δ*gal6* Δ*gal80* Δ*mig1*), and SO37 (Δ*gal6* Δ*gal80* Δ*mig1* & p*GAL4*, 2μ). The data were obtained from aerobic batch cultivations on galactose.

specific growth rates of the metabolically engineered strains are unaffected compared with the wild-type strain. Consequently, the galactose uptake exerts a negative control on the respiratory function of the cell. It is well established that the glucose uptake exerts a negative control on the respiratory function—often ascribed as a consequence of glucose repression, but this study demonstrates that also a nonrepressible carbon source such as galactose imposes constraints onto the oxidative metabolism when the glycolytic flux increases.

A minor part of the galactose consumed was directed toward acetate formation. The wild-type strain produced 0.60 mmol acetate per gram of galactose, and SO3, SO7, and SO37 produced 0.23–0.27 mmol acetate per gram of galactose. This difference is likely to be a result of the higher biomass yield of the wild-type strain, which then imposes a higher requirement for NADPH that can be supplied by acetate formation by the $NADP^+$-dependent acetaldehyde dehydrogenase. Furthermore, lower amounts of glycerol were detected in the galactose cultures of the mutant strains than for the wild-type strain (Table 2). Thus, the enhanced galactose consumption rates of the mutant strains caused a redirection of the carbon flux in favor of fermentative metabolism at the expense of biomass, acetate, and glycerol formation.

The growing knowledge concerning regulation of various genetic model systems will expand opportunities for genetic engineering to modulate flux through other pathways. Nevertheless, alteration of regulation of genetic systems may have unexpected metabolic consequences, since regulatory proteins may have functions beyond current knowledge.

Experimental protocol

Yeast strains. All *S. cerevisiae* strains used in this study (Table 1) were generated from the CEN.PK 113-7D wild-type strain. The two strains SO3 and SO4 were constructed by P. Kötter, Goethe Universität Frankfurt, of which the *MIG1* gene and the *GAL80* gene were deleted using the *loxP-kanMX-loxP* disruption cassette as described[16].

Deletion of the *GAL6* gene. The *GAL6* gene was deleted in the wild-type strain, SO3, and SO4, giving SO15, SO16, and SO17, respectively. The strains were transformed with a PCR product containing the Kan^r gene flanked with 45 bases on either side of the *GAL6* open reading frame. The 1.6 kb PCR product was obtained using the plasmid pBM3054 (pFA6-kanMX4 of ref. 17) containing the *kanMX4* module as template and the forward primer 5′-GGG CAC CCA TAA ATA AAC GAT AAA TAG CCC TTG CTC TTT TGT TAC CGT ACG CTG CAG GTC GAC-3′ (OM1582) and the backward primer 5′- AAC ATG TCG AGA TAC GTG TAT CTA CAT CAG AGC TGC TGA AAC TAT TCG ATG AAT TCG AGC TCG-3′ (OM1583). The deletion of *GAL6* in transformants growing on selective plates containing G418 was checked by PCR[18]. Chromosomal DNA was prepared[19] and used as template for the PCR reactions. A 0.9 kb fragment was amplified when using the forward primer 5′- CAG GCA AAT TTT GTC TCT TAA TCC -3′ (OM1584) upstream of *GAL6* from base −300 to −276 together with the backward primer 5′ -CGG GCT TCC CAT ACA ATC G -3′ (OM1117) from base 135 to 117 in the cod-

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ing region of the *Kan*[r] gene, which verified the existence of the *Kan*[r] gene in the *GAL6* locus. Furthermore, no PCR product was amplified when using OM1584 together with the backward primer 5′- CCA TGA ACA ACG ACA TCA ACC T -3′ (OM1585) from base 760 to 739 within the ORF of *GAL6*. To verify the functionality of the primers, a PCR product of 1.0 kb was amplified with OM1584, OM1585, and chromosomal DNA extracted from the parental strain of the *GAL6*-deleted strains. As was to be expected, no fragment was amplified when the two primers OM1584 and OM1117 were used in a PCR reaction with chromosomal DNA extracted from the parental strains.

Plasmid harboring strains. *GAL4* was overexpressed by introduction of the 4,728 bp *Hin*dIII/*Bam*HI fragment containing the *GAL4* gene and its own promoter into a YEp24 2μ high-copy vector giving pBM959 containing the *URA3* gene. Manipulation of plasmid DNA and transformation of plasmid into *Escherichia coli* DH5α were carried out according to standard procedures[20]. pBM959 was shown to complement the Δ*gal4* strain YM2268 (ref. 21) with growth on selective galactose plates, and on galactose plates containing bromthymol blue to confirm actual consumption of galactose. A color change from blue to yellow on the latter plates was observed, which confirmed a decrease in pH due to excretion of acidic metabolites produced when growing on galactose. Yeast transformations were performed as described[22]. SO7 was obtained by transformation of SO2 with the plasmid pBM959, and SO37 was obtained by transformation of SO17 with pBM959.

Media for strain development. Yeast strains were grown at 30°C on solid or in liquid rich media (YPD) or on solid synthetic media lacking uracil for selection of transformants[23]. Transformants carrying the *Kan*[r] gene were scored on YPD plates containing 250 mg/L G418 (Gibco BRL, Grand Island, NY). The galactose plates for the complementation test contained 33 mg/L bromthymol blue (Sigma, B-8630). Bacterial strains were grown at 37°C in Luria–Bertani medium with 100 μg ampicillin/ml[20].

Inoculum. The strains were stored at –80°C in Eppendorf tubes containing YPD medium[23] with 20% (vol/vol) glycerol. From the stock tubes, a sterile pipette was used to obtain single colonies on selective media for plasmid-bearing strains, and otherwise YPD plates were used. A single colony was picked to inoculate a shake flask.

Standard solutions for the media used. The standard solutions used for precultures was prepared as described[11].

Medium for precultures grown in shake flasks. Baffled, cotton-stopped, 500 ml Erlenmeyer flasks were used to obtain precultures for inoculation of the bioreactors. These flasks contained a volume of 100 ml medium with the following composition: 7.5 g/L $(NH_4)_2SO_4$; 14 g/L KH_2PO_4; 0.74 g/L $MgSO_4{\bullet}7H_2O$; 10 ml/L trace element solution; 1 ml/L vitamin solution; 50 μl/L synperonic antifoam (Sigma, A-5551); and 10 g/L galactose. The pH of the mineral medium was adjusted to 6.5 with NaOH and autoclaved separately from the galactose solution, which was added after autoclaving. The vitamin solution was added to the medium by filter sterilization. The shake flasks were grown at 30°C at 120 r.p.m. for ~24 h until the cell mass concentration reached 1–1.5 g cell dry wt (CDW)/L. Subsequently, the bioreactors were inoculated to give 1 mg CDW/L.

Medium for the batch cultivations. The medium for the batch cultivations had the following composition: 7.5 g/L $(NH_4)_2SO_4$; 3.5 g/L KH_2PO_4; 0.74 g/L $MgSO_4{\bullet}7H_2O$; 10 ml/L trace element solution[11]; 1 ml/L vitamin solution[11]; 50 μl/L synperonic antifoam (Sigma, A-5551); and 15 ± 1 g/L galactose. The sugar solution was autoclaved separately from the mineral medium, and subsequently added to the bioreactor. Likewise was the vitamin solution that was added by filter sterilization.

Cultivation conditions. The cells were grown as batch cultivations with galactose as the carbon source. The cultivations were carried out aerobically in well-controlled four-baffled 5 L in-house-manufactured bioreactors with working volumes of 4 L. The bioreactors were equipped with two disk turbine impellers rotating at 800 r.p.m., and the pH was kept constant at 5.0 by automatic addition of 2 M H_2SO_4 or 4 M NaOH. The air flow was 4 L/min, and the off gas passed through a condenser to avoid evaporation of ethanol from the bioreactor.

Cell mass determination. The cell mass concentration on a dry weight basis was determined by the use of nitrocellulose filters with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, MI). Initially the filters were predried in a microwave oven at 150 W for 10 min, and weighed. A known volume of cell culture was filtered, and the residue was washed with distilled water. Finally, the filter was dried in the microwave at 150 W for 15 min, and weighed.

Analysis of extracellular metabolites. For determination of the extracellular metabolites, 2 ml of sample was taken out of the bioreactor and immediately filtered through a 0.45 μm pore size cellulose acetate filter (Sartorius AG, Göttingen, Germany). The filtrate was frozen and kept at –20°C until analysis. Acetate, ethanol, galactose, and glycerol were separated on an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) at 65°C, using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min. Ethanol, glycerol, and galactose were detected refractometrically (Waters 410 Differential Refractometer Detector; Millipore Corp., Milford, MA), and acetate was determined spectrophotometrically with a Waters 486 Tunable Absorbance Detector set at 210 nm.

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1. Bailey, J.E. Lessons from metabolic engineering for functional genomics and drug discovery. *Nat. Biotechnol.* **17**, 616–618 (1999).
2. Cameron, D.C. & Tong, I.T. Cellular and metabolic engineering: an overview. *Appl. Biochem. Biotechnol.* **38**, 105–140 (1993).
3. Ostergaard, S., Olsson, L. & Nielsen, J. Metabolic engineering of *Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev.* **64**, 34–50 (2000).
4. Schaaff, I., Heinisch, J. & Zimmermann, F.K. Overproduction of glycolytic enzymes in yeast. *Yeast* **5**, 285–290 (1989).
5. Acerenza, L. & Kacser, H. Enzyme kinetics and metabolic control. *Biochem. J.* **269**, 697–707 (1990).
6. Niederberger, P., Prasad, R., Miozzari, G. & Kacser, H. A strategy for increasing an in vivo flux by genetic manipulations. *Biochem. J.* **287**, 473–479 (1992).
7. Small, R. & Kacser, H. Responses of metabolic systems to large changes in enzyme activities and effectors. *J. Biochem.* **213**, 613–624 (1993).
8. Johnston, M. & Carlson, M. In *The molecular and cellular biology of the yeast* Saccharomyces*: Gene expression.* (eds Jones, E.W., Pringel, J.R. & Broach, J.) 193–281 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; 1992).
9. Zheng, W., Xu, H.E. & Johnston, S.A. The cysteine–peptidase bleomycin hydrolase is a member of the galactose regulon in yeast. *J. Biol. Chem.* **272**, 30350–30355 (1997).
10. Wiselogel, A., Tyson, S. & Johnson, D. In *Handbook on bioethanol: production and utilization.* (ed. Wyman, C.E.) 105–118 (Taylor & Francis, Washington, DC; 1996).
11. Ostergaard, S., Roca, C., Rønnow, B., Nielsen, J. & Olsson, L. Physiological characterisation in aerobic batch cultivations of *Saccharomyces cerevisiae* strains harboring the *MEL1* gene. *Biotechnol. Bioeng.* **68**, 252–259 (2000).
12. Igarashi, M., Segawa, T., Nogi, Y., Suzuki, Y. & Fukasawa, T. Autogenous regulation of the *Saccharomyces cerevisiae* regulatory gene *GAL80. Mol. Gen. Genet.* **207**, 273–279 (1987).
13. Adam, A.C., Rubio-Texeira, M. & Polaina, J. Induced expression of bacterial β-glucosidase activity in *Saccharomyces. Yeast* **11**, 395–406 (1995).
14. Martegani, E., Brambilla, L., Porro, D., Ranzi, B.M. & Alberghina, L. Alteration of cell population structure due to cell lysis in *Saccharomyces cerevisiae* cells overexpressing the *GAL4* gene. *Yeast* **9**, 575–582 (1993).
15. Gill, G. & Ptashne, M. Negative effect of the transcriptional activator GAL4. *Nature* **334**, 721–724 (1988).
16. Güldener, U., Heck, S., Fiedler, T., Beinhauer, J. & Hegemann, J.H. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* **24**, 2519–2524 (1996).
17. Wach, A., Brachat, A., Pöhlmann, R. & Philippsen, P. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae. Yeast* **10**, 1793–1808 (1994).
18. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F. & Cullin, C. A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae. Nucleic Acids Res.* **21**, 3329–3330 (1993).
19. Hoffman, C.S. & Winston, F. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli. Gene* **57**, 267–272 (1987).
20. Sambrook, J., Fritsch, E.F. & Maniatis, T.M. *Molecular cloning: a laboratory manual.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; 1989).
21. Johnston, M. & Dover, J. Mutational analysis of the gal4-encoded transcriptional regulatory protein of *Saccharomyces cerevisiae. Genetics* **102**, 63–74 (1988).
22. Gietz, D., St. Jean, A., Woods, R.A. & Schiestl, R.H. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* **20**, 1425 (1992).
23. Rose, M.D., Winston, F. & Hieter, P. *Methods in yeast genetics: a laboratory course manual.* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; 1990).
24. Leuther, K.K. & Johnston, S.A. Nondissociation of GAL4 and GAL80 in vivo after galactose induction. *Science* **256**, 1333–1335 (1992).
25. Wu, Y., Reece, R.J. & Ptashne, M. Quantitation of putative activator–target affinities predicts transcriptional activating potentials. *EMBO J.* **15**, 3951–3963 (1996).
26. Suzuki-Fujimoto, T. *et al.* Analysis of the galactose signal transduction pathway in *Saccharomyces cerevisiae*: interaction between Gal3p and Gal80p. *Mol. Cell. Biol.* **16**, 2504–2508 (1996).
27. Yano, K. & Fukasawa, T. Galactose-dependent reversible interaction of Gal3p with Gal80p in the induction pathway of Gal4p-activated genes of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA* **94**, 1721–1726 (1997).
28. Keleher, C.A., Redd, M.J., Schultz, J., Carlson, M. & Johnson, A.D. Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell* **68**, 709–719 (1992).
29. Nehlin, J.O., Carlberg, M. & Ronne, H. Control of yeast *GAL* genes by MIG1 repressor: a transcriptional cascade in the glucose response. *EMBO J.* **10**, 3373–3377 (1991).
30. Treitel, M.A. & Carlson, M. Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. *Proc. Natl. Acad. Sci. USA* **92**, 3132–3136 (1995).

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