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Dietary change enables robust growth-coupling of heterologous methyltransferase activity in yeast

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ABSTRACT

Genetic modifications of living organisms and proteins are made possible by a catalogue of molecular and synthetic biology tools, yet proper screening assays for genetic variants of interest continue to lag behind. Synthetic growth-coupling (GC) of enzyme activities offers a simple, inexpensive way to track such improvements. In this follow-up study we present the optimization of a recently established GC design for screening of heterologous methyltransferases (MTases) and related pathways in the yeast *Saccharomyces cerevisiae*.

Specifically, upon testing different media compositions and genetic backgrounds, improved GC of different heterologous MTase activities is obtained. Furthermore, we demonstrate the strength of the system by screening a library of catechol O-MTase variants converting protocatechuic acid into vanillic acid. We demonstrated high correlation ($R^2 = 0.775$) between vanillic acid and cell density as a proxy for MTase activity. We envision that the improved MTase GC can aid evolution-guided optimization of biobased production processes for methylated compounds with yeast in the future.

Keywords growth-coupling, methyltransferase, yeast, synthetic biology, natural products, screening

To facilitate the transition into a biobased society, a myriad of tools and strategies have been developed to engineer both proteins and microbes into biological catalysts to sustain production of natural and heterologous compounds^{1,2}. Biochemical production or enzyme activity, whether improved or established *de novo*, rarely confers a beneficial, easily selectable phenotype, yet assays and tools to screen for improved variants are needed^{3,4}. Synthetic growth-coupling (GC) offers a valuable complementary tool to other high-throughput screens depending on more advanced laboratory equipment like fluorescence-activated cell sorting or state-of-the-art analytical instrumentation^{5,6}, due to growth being a simple read-out. In addition, GC overcomes crucial bottlenecks in the current bioengineering workflow as no knowledge is needed *a priori* about the limitations underpinning the trait in question. Indeed, properly designed GC allows screening for any edit, genome-wide or targeted to defined open reading frames, giving rise to improved growth, such as recently exemplified *in silico* and experimentally for vanillin and succinic acid production, respectively^{7,8}. Despite this, the number of successful implemented GC designs are limited⁹. Additionally, many current GC

strategies only target optimization of a single enzyme or product, making the return-of-investment low regarding effort and time spent on establishing the GC design.

We have previously presented a universal GC design of SAM-dependent methyltransferases (MTase) and associated pathways in *Escherichia coli* and *Saccharomyces cerevisiae* by taking advantage of coupled methionine and cysteine biosynthesis in sulfur amino acid auxotrophic strains¹⁰. MTases form an abundant class (EC 2.1.1.-) of enzymes catalyzing key steps in many biosynthetic pathways towards natural products of commercial and societal interests^{11–14} and are promising biocatalysts¹¹. Uniquely targeting a large and substrate-diverse enzyme class, our GC design allows screening for and optimization of the biosynthesis of a broad range of products via simple assessment of growth. In yeast, GC is only obtained when abundant competing native MTases are deleted. However, a substantial growth-leakiness is still observed in strains expressing a heterologous MTase, even without any substrate to methylate¹⁰. Given the interest in biobased production of bioactive compounds in yeast¹⁵, we therefore sought to improve the MTase GC.

Here we present results from our efforts to robustly growth-couple diverse heterologous MTase activities in yeast. By testing different cultivation conditions and genetic backgrounds we successfully abolish the previously observed leakiness. We then demonstrate GC of two additional heterologous MTases, thus adding further evidence for this robust and generic GC as a synthetic biology tool for development and identification of superior enzymes and yeast cell factories for biobased manufacturing of methylated compounds.

Results and Discussion

The recently published functional MTase GC in *S. cerevisiae* consists of four gene knock-outs (*S. cerevisiae* SCAH124)¹⁰: *met17Δ* and *met2Δ* for establishing sulfur amino acid

auxotrophy, as well as *cho2Δ* and *opi3Δ*, encoding two endogenous MTases, to reduce native conversion capacity from SAM to S-adenosylhomocysteine (SAH) (Fig. 1A)¹⁶. As *S. cerevisiae* harbors > 90 endogenous MTases that make up a large intertwined genetic interaction network, the methyltransferome¹⁷, we speculated that the leakiness of the minimal MTase GC design was caused by (hyper-)activity of remaining native MTases. In line with this hypothesis, it has been reported that the activity of yeast histone MTases increases in *cho2Δ* strains when cultivated in lactate-based medium¹⁸. Accordingly, we investigated the effect on MTase GC of 1) replacing the previously applied fermentable carbon and energy source glucose with the non-fermentable alternative lactate, and 2) deleting three histone MTases Set2p, Set1p and Dot1p (Fig. 1A).

First, we developed a simplified and more easily manageable workflow without sulfur amino acid starvation compared to the previous cultivation scheme¹⁰. We also adjusted the medium, and left out the buffer system and excess amounts of inorganic sulfate, since the MTase GC strains can not assimilate it due to the *MET17* deletion. Both methionine and choline (to enable phosphatidylcholine synthesis) were maintained supplemented at a concentration of 1 mM. In these conditions, the modest GC of the quadruple knock-out GC strain SCAH124 was recapitulated (Fig. 1B). Upon expression of heterologous N-MTase caffeine synthase 1 from *Coffea arabica* (*CaCcs1p*) in the SCAH124 background (SCAH370) we observed GC of *CaCcs1p* activity on glucose in the presence of the MTase substrate theobromine (1 mM). Yet, as observed previously¹⁰, strain SCAH370 also grew in the absence of theobromine and reached similar cell density by stationary phase compared to growth with theobromine (Fig. 1B).

Next, upon replacing glucose with lactate, we observed that GC dramatically increased (Fig. 1B). Indeed, in the presence of theobromine, strain SCAH370 reached approximately 35-fold higher cell density compared to growth in the absence of theobromine ($OD_{600}=4.19\pm0.21$

vs. $OD_{600}=0.12\pm0.03$, $n=3$), while reaching a titer of $105.8 \pm 5.8 \mu\text{M}$ caffeine upon sampling compared to no measurable production for strain SCAH362 without *CaCcs1p* expression in lactate (Fig. 1C).

Deleting the genes encoding the histone MTases Set2p, Set1p and Dot1p in the SCAH124 background (SCAH354) and introducing *CaCCS1* (SCAH371) caused an increase in lag-phase and similar modest GC on glucose as observed for SCAH370 (Fig. 1D). Yet, even though GC for the septuple knock-out strain SCAH371 was also improved on lactate ($OD_{600}=0.82\pm0.18$ with theobromine vs. $OD_{600}=0.04\pm0.04$ without theobromine), and a caffeine titer of $40.6\pm1.7 \mu\text{M}$ was observed, SCAH371 grew to lower OD_{600} compared to the quadruple knock-out strain SCAH370 ($OD_{600}=0.82\pm0.18$ vs. $OD_{600}=4.19\pm0.21$)(Fig. 1D-E).

Extending from the improved GC when using lactate, we next sought to further investigate the effect of the cultivation medium. Lactic acid is a weak short-chain monocarboxylic acid ($pK_a = 3.86$), which in the presence of glucose passively diffuses into the cell in its undissociated form, while at the same time posing a growth-inhibitory effect on yeast among other things due to the energy-intensive mechanism for maintaining proton homeostasis^{19,20} (Fig. 1F). However, in the absence of glucose, lactate is primarily imported via glucose-repressed permease Jen1p, a proton symporter^{21,22} (Fig. 1F). Acknowledging the potential importance of the pH of the cultivation medium for lactate/lactic acid uptake, its growth inhibitory effect and the effect on general cell physiology of pH when feeding an acid, we investigated the effect on growth and GC of strains SCAH124 and SCAH354 transformed with either empty plasmids (SCAH362 and SCAH368) or a plasmid with *CaCCS1* (SCAH370 and SCAH371) in lactate based medium adjusted to pHs 3.5, 4.5 or 5.5 (Fig. 1G). Here, the GC, as inferred by growth in medium with theobromine, was best at pH 4.5, as also observed for a wild-type *S. cerevisiae* strain grown in the same media (Fig. 1G, Suppl. Fig. S1). For the entire pH range, we observed clear separation between growth of strains expressing *CaCcs1p*

compared to empty plasmid controls, indicating that pH of the cultivation media can be adjusted as needed without affecting GC (Fig. 1G).

Taken together, we have established a significantly improved GC of the platform quadruple knock-out GC strain SCAH124 expressing a heterologous MTase when feeding its substrate by replacing glucose with lactate as a carbon source. In addition, we have developed a simplified workflow enabling high-throughput screening and demonstrated that GC is functional at various pHs.

Next, we set out to confirm that the GC was applicable for other SAM-dependent MTases as well. Thus, in addition to the N-MTase *CaCcs1p*, we tested two O-MTases in MTase GC strain *S. cerevisiae* SCAH124. First, we tested the plant loganic acid O-MTase from *Catharanthus roseus* (*CrLamtp*) methylating loganic acid into loganin, a precursor for the scaffold strictosidine branching out to >2,000 different monoterpene indole alkaloids, several with medicinal properties (SCAH136, Fig. 2A)^{12,23}. Here, feeding different concentrations of loganic acid to SCAH136 expressing *CrLamtp* enabled titratable growth with a maximum of approximately 57-fold and 25-fold higher cell densities compared to the strain without expression of *CrLamtp* (SCAH138) or loganic acid feeding (Fig. 2B), respectively ($OD_{600}=3.98\pm0.14$ (SCAH136) vs. $OD_{600}=0.07\pm0.04$ (SCAH138) in 2 mM loganic acid, and $OD_{600}=0.16\pm0.02$ (SCAH136 without loganic acid)) (Fig. 2B). MTase activity was confirmed to correlate with cell densities enabling loganin titers of $95.12\pm11.62\ \mu\text{M}$ following 2.0 mM loganic acid feeding for strain SCAH136 (Fig. 2C).

Next, motivated by the improvement and robustness of the yeast MTase GC, we sought to apply the GC system as a simple screening tool for MTase product formation of relevance for semi-throughput workflows. For this test we first applied GC of human catechol O-MTase (*HsOmtp*) activity for methylating protocatechuic acid thereby forming vanillic acid

(SCAH344)²⁴. Here, feeding the *Hs*OMT substrate protocatechuic acid (1 mM) to SCAH344 cultivated in lactate-based medium conferred a significant growth advantage over the absence of *Hs*OMT (SCAH138) or protocatechuic acid ($OD_{600}=6.16\pm0.17$, $OD_{600}=1.01\pm0.02$, and $OD_{600}=0.67\pm0.17$ after cultivation for 5492.25 min, $n=3$, respectively) (Fig. 3A-B). Next, we tested a library of 16 previously characterized OMT variants of different origins²⁵ (Fig. 3A) together with the *Hs*OMT and empty plasmid control in the SCAH124 background (SCAH138 and SCAH328-SCAH344, Fig. 3C). The library has previously been found to possess different activities by others²⁵. Each yeast strain expressing an OMT variant was cultivated individually in lactate-based medium with protocatechuic acid (1 mM), from which growth and vanillic acid formation was quantified. From this screen, OMT variants were observed to give rise to a range of different cell densities from 0.05 ± 0.02 to 4.66 ± 0.08 (OD_{600}), as well as vanillic acid titers from 0 to 135.8 ± 7 μ M by the end of the cultivation (Fig. 3C, Supplementary Table S1). This illustrates the applicability of the system for semi-throughput screening. Indeed, with a correlation coefficient of $R^2 = 0.775$ from linear regression between reached cell density and produced vanillic acid for OMT library variants possessing vanillic acid production, the system holds promise as a tool for easy discrimination of variants within large libraries (Fig. 3D).

In summary, in this study a noticeable improvement of the resolution of the MTase GC of *S. cerevisiae* was obtained by simple medium optimization, with minimal leakiness (i.e. residual growth) observed in the absence of either MTase substrate or MTase activity. Conversely, deletion of proposed hyperactive histone MTases did not have an impact on GC leakiness. While this study does not address the mechanism for the strengthened coupling phenotype upon change of carbon source, the effect of lactate has previously been studied in relation to sulfur amino acid and phospholipid biosynthesis^{18,26,27}. Still, lactate is an unusual carbon source known to cause stress and abnormal growth, and we therefore have also tested

the more commonly applied carbon sources, galactose, glycerol and ethanol (Supplementary Figure S2). Here, we observed that only upon addition of 0.2% glucose were the strains able to grow on the non-fermentable alternatives ethanol and glycerol. Yet, although the resolution of the GC increased when using either galactose, glycerol and ethanol compared to using solely glucose, the alternative carbon sources also resulted in leaky growth of *S. cerevisiae* SCAH362 without MTase expression. While out of the scope for the present study, we foresee additional genetic and physiological studies in the near future to be needed in order to understand the mechanism behind the improved GC when using lactate as a carbon source.

The results on the GC of the activity of both N- and O-type MTases in *S. cerevisiae* underlines the expected broad applicability of the GC design to any sufficiently active SAM-dependent MTase that can i) be expressed in *S. cerevisiae*, and ii) for which the substrate can be presented endogenously or via feeding and is not lethal to the cells. Upon the optimization of the GC system we also demonstrated semi-throughput *in vivo* screening of MTase variants and proved correlation between titers and cell density. In the future, upon further testing and characterisation it might be possible to apply the yeast MTase GC for selection within high-throughput directed evolution and ALE workflows.

Beyond their abundance in biosynthetic pathways for natural products, MTase reactions are also found in many metabolic pathways and are implicated in genetic and metabolic diseases^{28,29}. We therefore also consider this optimized GC system of relevance for biomedical purposes outside the realm of biotechnology such as *in vivo* study of mutation effects within clinically interestingly human MTases.

Methods

Strains, media, and growth conditions

Saccharomyces cerevisiae CEN.PK102-5B (*MATa*) was used as a background strain in the study. A complete list of all yeast strains used in the study can be found in Supplementary Table S2. Gene deletions were facilitated by CRISPR/Cas9³⁰, and all yeast transformations were performed using the lithium acetate/single-stranded carrier DNA/PEG method³¹. *S. cerevisiae* SCAH100 derived from CEN.PK102-5B carried plasmid pRS415U. *S. cerevisiae* SCAH354, derived from SCAH124¹⁰, was constructed by CRISPR/Cas9-facilitated full ORF markerfree deletion mediated by homology-directed recombination of three open-reading frames (ORFs) *SET2*, *DOT1* and *SET1* using the guide-RNA (gRNA) expressing plasmids PL_01_E6 and PL_01_E3, together with plasmid PL_01_A9 with *SpCAS9* and two linear DNA fragments homologous to the up- and downstream regions flanking the targeted ORFs. The gRNA sequences were identified using the web service CRISPy adapted to the genome sequence of *S. cerevisiae* CEN.PK³²: *SET2* (5'-GAACCAAAGTGTGAGTGCG-3'), *DOT1* (5'-GGATATTGTAACCGTAGACA-3') and *SET1* (5'-AAGGCATAATTCACTCGCGT-3').

All chemicals used in the study were purchased from Sigma-Aldrich, unless otherwise stated. The basal minimal medium was modified from Boer *et al.*³³ by leaving out any inorganic sulfur source and consisted of per L: 4 g NH₄Cl, 3 g KH₂PO₄, 0.4412 g MgCl₂·6H₂O, 1 ml vitamin solution, 2 ml trace metal solution. In addition, choline chloride and L-methionine were supplemented to a final concentration to 1 mM each. As carbon sources either 2 % glucose or 3 % lactate (Sigma-Aldrich, W261106) were used. The trace metal solution consisted of per L: 4.5 g CaCl₂·2H₂O, 4.5 g ZnSO₄·7H₂O, 3 g FeSO₄·7H₂O, 1 g H₃BO₃, 1 g MnCl₂·4H₂O, 0.4 g Na₂MoO₄·2H₂O, 0.3 g CoCl₂·6H₂O, 0.1 g CuSO₄·5H₂O, 0.1 g KI, and 15 g EDTA. The vitamin solution consisted of per L: 50 mg biotin, 200 mg p-aminobenzoic acid, 1 g nicotinic acid, 1 g Ca-pantothenate, 1 g pyridoxine-HCl, 1 g thiamine-HCl, 25 mg myo-inositol. When stated, the media was supplemented with methyl acceptors protocatechuic acid (1 mM, PCA),

theobromine (1 mM, THEO) or loganic acid (0.5, 1 or 2 mM, loganic acid, (Carl Roth & CO). The pH of the media was adjusted with the base sodium hydroxide and the acid hydrogen chloride, and subsequently sterilized by filtration. For the carbon source experiment, the additional carbon sources were supplemented in the following concentrations: 2 % galactose, 3 % glycerol, and 3 % ethanol, and the media contained 14.4 g/L KH_2PO_4 functioning as a buffer system. When indicated, glucose was added to a concentration of 0.2 % to promote growth.

Oligonucleotides, synthetic genes and plasmids

Synthetic genes were commercially synthesized by Twist Biosciences and Integrated DNA Technologies, Inc. (IDT). The full sequence of the synthetic genes used in this study can be found in Supplementary Table S4. The OMT variant sequences of plasmid PL_02_F3-PL_02_G9 were codon optimized for *S. cerevisiae* in this study using the IDT algorithm.

Plasmid DNA assemblies were performed following the EasyClone method³⁴. Plasmids were propagated in *E. coli* DH5 α using 100 mg/L ampicillin. A full list of plasmids used can be found in Supplementary Table S3.

Cultivations

For plate-based cultivations, pre-cultures from three to six biological replicates per strain were performed in 96-round bottom deep well plates at 30 °C/300 rpm in a total volume of 500 μl for approximately 24-28 hours in synthetic complete medium without appropriate amino acids supplemented, and with 1 mM choline chloride. The pre-cultures were diluted (inoculum) in sterile Milli-Q H_2O (30 μl culture in 470 μl Milli-Q H_2O) prior to inoculation.

Growth was recorded in a Growth Profiler 960 (System Duetz, EnzyScreen, Heemstede, The Netherlands) (GP960) using 96-flat bottom microtiter plates sealed with a gas permeable

sandwich cover (Enzygscreen, Heemstede, The Netherlands). GP960 based cultivations were performed in a total volume of 250 μ l including 10 μ l inoculum at 30 °C/250 rpm. Growth was monitored at 30 min intervals by image scanning with GP960. Based on each image scan, integrated green values (G-values) for every well were light calibrated to account for position effects and converted into equivalent OD₆₀₀ values using a Monod function: $G\text{-value} = (a \cdot OD_{600}) / (b + OD_{600})$. The parameters a, 0.000566, and b, 2.202869, were determined by non-linear regression using averaged G-values for every plate position of the GP960 of a dilution series of known OD₆₀₀ values of a culture of strain *S. cerevisiae* SCAH100. Strain SCAH100 was grown overnight in the minimal medium described earlier, but with 14.4 g/L KH₂PO₄, 1 mM THEO and 1 mM ChoCl as well as 2 % glucose. The dilution series consisted of a medium blank, and seven OD₆₀₀ values from 0.08-24.7 (OD₆₀₀) in duplicate. All cell density values (OD₆₀₀) have been subtracted blank values.

Sampling of cultivation broth for chemical analysis was done after cultivation for 4776.5 min for strains SCAH362, SCAH368, SCAH370 and SCAH371, after 4079.25 min for strain SCAH136, and after 3899.5 min for strains SCAH138 and SCAH328-SCAH344. Cultivation of strain SCAH138 and SCAH344 for growth comparison was ended after 5492.25 min. For the carbon source experiments both with and without glucose addition, the data set was reduced to 5010.25 min for strains SCAH362 and SCAH370.

Chemical analyses

Samples for quantification of secreted vanillic acid were prepared by mixing 190 μ l cultivation broth and 190 μ l absolute ethanol carefully in a 96-round bottom deepwell plate, centrifuged at 4000 rpm for 2 min at 4 °C, and supernatant was transferred to a multiwell plate, sealed and stored at -20 °C until further analysis. For caffeine, the cultures were centrifuged in 96-round bottom deepwell plates at 4000 rpm for 2 min at room temperature, and 190 μ l

supernatant was carefully transferred to a multiwell plate and stored similarly to vanillic acid samples. Both vanillic acid and caffeine was quantified by HPLC on a Dionex Ultimate 3000 system (Thermo Fisher Scientific) with a Supelco Discovery HS G5-3 HPLC column (150 x 2.1 mm x 3 μ M) (Sigma Aldrich) held at 30 °C. For both compounds, a gradient of A: 10 mM ammonium formate, pH 3, and B: acetonitrile, at a constant flow rate of 0.7 mL/min was used as mobile phase. The elution profile for both vanillic acid and caffeine was as follows: 5 % B - 0.5 min, 5 % B to 60 % B - 5 min, 60 % B to 90 % B - 0.5 min, 90 % B - 2 min, 90 % B to 5 % B - 2 min (total 10 min/sample). Quantification against external standards diluted in medium without methyl acceptor substrate was performed using a DAD-3000 diode array detector at a wavelength of 277 nm (UV_Vis2) and a sample size of 10 μ l was injected for both vanillic acid and caffeine.

Samples for quantification of loganin production were prepared by mixing 200 μ l cultivation broth with 20 μ l 250 mg/L caffeine (internal standard) carefully in a 96-well flat bottom microtiter plate, 200 μ l was transferred to a filter plate (96-well, for multiplexing, AcroPrep Advance from VWR), and centrifuged twice at 2000 rpm for 30 seconds into a multiwell plate, sealed and stored similar to vanillic acid and caffeine samples. Data were acquired on an Advance UHPLC system (Bruker Daltonics, Fremont, CA, USA) equipped with a binary pump, degasser and PAL HTC-xt autosampler (CTC Analytics AG, Switzerland) coupled to an EVOQ Elite triple quadrupole MS (Bruker Daltonics, Fremont, CA, USA). The system was operated with MS Workstation 8.2.1 software (Bruker Daltonics, Fremont, CA, USA). The analytical column was a 100 mm C18 Acquity UPLC HSS T3 column 100Å, 1.8 μ m particle size, 2.1mm i.d. (Waters, Milford, MA, USA) with a KrudKatcher, HPLC in-line column filter, 0.5 μ m x 0.004 in i.d. (Phenomenex, Torrance, CA, USA). The column oven temperature was set to 35 °C with an injection volume of 1 μ L and a 2 μ L injection loop. The mobile phase consisted of 0.1 % formic acid (FA) in Milli-Q (solvent A) and 0.1 % FA in

acetonitrile (solvent B), delivered at a constant flow rate of 0.5 mL/min. The mobile phase gradient profile was as follows: starting condition 5 % eluent B, 0.0 – 0.5 min: 5% B, 0.5 – 3.0 min: 5% B to 60% B, 3.0 – 4.0 min: 60% B to 90% B, 4.0 – 5.0 min: 90% B, 5.0 – 5.3 min: 90% B to 5% B, 5.3 – 7.4 min: 5% B. Acquisition was carried out using electrospray ionization operated in positive ion mode. The following parameters were used to acquire Multiple Reaction Monitoring (MRM) data: spray voltage: 4.5 kV, cone temperature: 350 °C, cone gas flow 20, probe gas flow: 50, nebulizer gas flow: 50, heated probe temperature: 300 °C, exhaust gas: on, CID: 1.5 mTorr. The MRM scan time was set to 30 milliseconds with standard resolution for all transitions. The collision energy was optimized for each transition. Analyte stock solutions were prepared in 0.1 % FA and diluted with 0.1 % FA for eight calibration levels. Data analysis was performed in Thermo Xcalibur (version 3.0.63). Caffeine was added as an internal standard before injection to both samples and calibration standards.

Blank values were subtracted from all reported product concentrations. The linear regression algorithm within the python sub-package `scipy.stats` (of the python ecosystem SciPy³⁵) was used for calculation of the linear regression and the reported R^2 value. Only OMT variant strains with vanillic acid production has been included in the calculations, and an outlier of SCAH342 (-#1) has been excluded.

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Author contributions

A.S.L.H., M.H., J.D.K., M.D. and M.K.J. conceived the study. A.S.L.H. and D.A. conducted all experimental work related to strain designs, plasmid and strain constructions and cultivations. A.S.L.H. and J.Z. analysed data. A.S.L.H. and M.K.J. wrote the manuscript.

Conflicts of interest

A.S.L.H. has filed a patent application covering the design of the GC system (EP3504318A1). J.D.K has a financial interest in Amyris, Lygos, Demetrix, Maple Bio, Napigen, and Apertor Labs. The authors declare that they have no other competing interests.

Figure legends

Figure 1: Design and characterization of improved MTase GC in *S. cerevisiae*. (A) MTase GC design in *S. cerevisiae* SCAH124 (*met17Δ*, *met2Δ*, *cho2Δ*, *opi3Δ*) and SCAH354 (*met17Δ*, *met2Δ*, *cho2Δ*, *opi3Δ*, *set2Δ*, *set1Δ*, *dot1Δ*). *CaCCS1* methylation reaction is colored in purple. Red crosses indicate gene deletion. Byproducts, cofactors and substrates are left out. (B+D) Growth of MTase GC strains SCAH370 (B) and SCAH371 (D) expressing *CaCCS1* on GLC (left) and LAC (right) with MET and with (blue) and without (orange) the methyl group acceptor THEO (1 mM)(pH 5.5). (C+E) Caffeine titer for strain pairs (empty plasmid control and *CaCCS1* expressing strain), SCAH362 and SCAH370 (C), as well as SCAH368 and SCAH371 after 4776 min cultivation in LAC+MET+THEO (1 mM) medium (n=3) (pH 5.5). (F) Schematic of simplified LAC uptake mechanism in *S. cerevisiae* via diffusion or permease

JEN1. **(G)** Growth of strain pairs (empty plasmid control and *CaCCS1* expressing strain), SCAH362 and SCAH370 (left) and SCAH368 and SCAH371 (right) in LAC+MET+THEO (1 mM) medium at pH 3.5 (red and blue), 4.5 (purple and orange) and 5.5 (brown and green). Growth curves are based on data from one representative isolate (n=1). Curves for three replicates in Supplementary Figure S1. For all media, both MET and choline were supplemented to a final concentration of 1 mM each. Abbreviations, GLC: glucose, LAC: lactate/lactic acid, ME: methyl group, MET: methionine, THEO: theobromine, SAH: *S*-adenosylhomocysteine, SAM: *S*-adenosylmethionine, PDME: phosphatidyl dimethylethanolamine, PMME: phosphatidyl monomethylethanolamine.

Figure 2: Investigation of *CrLamtp* methylation within minimal *S. cerevisiae* MTase GC strain design. **(A)** *CrLamtp* catalyzed methylation of LA into loganin. **(B)** Growth of MTase GC strain (*met17Δ, met2Δ, cho2Δ, opi3Δ*) SCAH136 expressing *CrLAMT* and SCAH138 with an empty plasmid in LAC+MET medium with LA concentrations 2 (blue and purple), 1 mM (orange and brown), 0.5 mM (green and pink), or no feeding (red and grey) (pH 4.55). **(C)** Loganin titer upon 4,079 min cultivation of strain SCAH136 expressing *CrLAMT* in LAC+MET medium with either no, 0.5 mM, 1 mM, or 2 mM LA supplemented (n=3) (pH 4.55). For all media, both MET and choline were supplemented to a final concentration of 1 mM each. Abbreviations, LA: loganic acid, ME: methyl group.

Figure 3: Semi-throughput OMT variant screening using minimal *S. cerevisiae* MTase GC strain design. **(A)** OMT catalyzed methylation of PCA into VA. **(B)** Growth of MTase GC strain (*met17Δ, met2Δ, cho2Δ, opi3Δ*) SCAH344 expressing *HsOMT* from human and empty plasmid control SCAH138 in LAC+MET medium with no (brown and orange) or 1 mM PCA (blue and pink) (pH 4.3). **(C)** Growth of MTase GC strains expressing 17 different OMT

variant genes in LAC+MET medium with 1 mM PCA (left) or no feeding (right) (pH 4.3). **(D)** Correlation plot between of cell density (OD₆₀₀) on 1st axis, and VA (μM) titer on 2nd axis upon sampling after cultivation in LAC+MET+PCA (1 mM) medium for ~3,900 min of the 11 OMT variants with VA production (n=3, except for *Synechocystis* sp. PCC 6714 (n=2), and SCAH344 (n=6)). Bars represent standard deviation. Linear regression line is displayed in red. Growth curves are based on data from one representative isolate (n=1). Curves for three replicates in Supplementary Figure S1. Colors and nuances reflect OMT origin, archaeae: yellow, bacteria: purple, filamentous fungi: red, yeasts: blue, and higher eukaryotes: green, *Hs*OMT and empty plasmid control: grey. For all media, both MET and choline were supplemented to a final concentration of 1 mM each.

Associated content

Supporting information. Additional figures, tables and sequences can be found in the supporting material.

Table S1: Production and growth characteristics of *Saccharomyces cerevisiae* MTase GC strains expressing OMT variants.

Table S2: Strains of *S. cerevisiae* used in study.

Supplementary Table S3: List of plasmids used in the study. Sequences of the OMT variants of PL_02_F3-PL_02_E5 are listed in Supplementary Table S4.

Table S4: List of MTase sequences (originally ordered as synthetic gBlock sequences). The sequences have been codon optimized to *S. cerevisiae* using the IDT web service except

HsOMT and *CrLAMT*. Start and stop codon is underlined. All MTases were selected from Kunjapur & Prather, *ACS Synthetic Biology*, Sep 20;8(9):1958-1967 (2019).

Figure S1: Growth curves for three isolates (-#1, -#2, and -#3) of different strains with and without heterologous MTase cultivated in different growth media supporting Figures 1, 2 and 3. Curves for strains *S. cerevisiae* SCAH362 and SCAH370 in medium with GLC (A) and LAC (B) with and without THEO, and of SCAH368 and SCAH371 in with GLC (C) and LAC (D). (E) Growth of SCAH362 and SCAH370 isolates in media with and without THEO and pH 3.5, 4.5 and 5.5. (F) Growth of SCAH368 and SCAH371 isolates in media with LAC and with and without THEO, pH 5.5. (G) Growth of SCAH100 isolates in media with and without THEO and pH 3.5, 4.5 and 5.5. All media were supplied MET and choline chloride. Abbreviations, GLC: glucose, LAC: lactate, MET: methionine, MTase: methyltransferase, THEO: theobromine, PCA: protocatechuic acid.

Figure S2: Growth curves for three isolates (-#1, -#2, and -#3) of strains *S. cerevisiae* SCAH362 and SCAH370 in media (pH 4.5) with THEO, choline chloride and MET as well as fermentable carbon sources. (A) GAL, (B) GLC, and non-fermentable carbon sources (C) LAC, (D) GLY, and (E) ETH (upper figures without GLC, lower with GLC supplemented)

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