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Glycerol positive promoters for tailored metabolic engineering of the yeast *Saccharomyces cerevisiae*

Ping-Wei Ho[1], Mathias Klein[1], Matthias Futschik[2,3], Elke Nevoigt[1*]

[1] Department of Life Sciences and Chemistry, Jacobs University Bremen gGmbH, Campus Ring 1, 28759 Bremen, Germany

[2] School of Biomedical & Healthcare Sciences, Plymouth University Peninsula Schools of Medicine and Dentistry, Plymouth, Devon, PL4 8AA, UK

[3] Centre of Marine Sciences (CCMAR), Universidade do Algarve, Faro, 8005-139, Portugal

* Corresponding author: Mailing address: Jacobs University Bremen gGmbH, Department of Life Sciences and Chemistry, Campus Ring 1, 28759 Bremen, Germany; Phone: +49-421-2003541; Fax: +49-421-2003249; E-mail: e.nevoigt@jacobs-university.de

Email addresses

Ping-Wei Ho	p.ho@jacobs-university.de
Mathias Klein	m.klein@jacobs-university.de
Matthias Futschik	matthias.futschik@plymouth.ac.uk
Elke Nevoigt	e.nevoigt@jacobs-university.de

ABSTRACT

Glycerol offers several advantages as a substrate for biotechnological applications. An important step towards using the popular production host *Saccharomyces cerevisiae* for glycerol-based bioprocesses have been recent studies in which commonly used *S. cerevisiae* strains were engineered to grow in synthetic medium containing glycerol as the sole carbon source. For metabolic engineering projects of *S. cerevisiae* growing on glycerol, characterized promoters are missing. In the current study, we used transcriptome analysis and a yECitrine-based fluorescence reporter assay to select and characterize 25 useful promoters. The promoters of the genes *ALD4* and *ADH2* showed 4.2- and 3-fold higher activities compared to the well-known strong *TEF1* promoter. Moreover, the collection contains promoters with graded activities in synthetic glycerol medium and different degrees of glucose repression. To demonstrate the general applicability of the promoter collection, we successfully used a subset of the characterized promoters with graded activities in order to optimize growth on glycerol in an engineered derivative of CEN.PK, in which glycerol catabolism exclusively occurs via a non-native DHA pathway.

KEYWORDS:

Glycerol, *Saccharomyces cerevisiae*, metabolic engineering, promoter

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is an important production organism in industrial biotechnology. Its popularity can mainly be attributed to its robustness in industrial settings and the extensive array of available tools for the genetic engineering of this microorganism (Borodina & Nielsen, 2014). The developments in synthetic biology and systems metabolic engineering have led to the generation of numerous genetically modified *S. cerevisiae* strains for the production of valuable compounds, such as recombinant proteins (Egel-Mitani *et al*., 2000; Qin *et al*., 2015), bulk or fine chemicals (Brochado *et al*., 2010; Raab *et al*., 2010; Madsen *et al*., 2011; Hong & Nielsen, 2012), and bio-fuels (Chen *et al*., 2011; Schadeweg & Boles, 2016). So far, the majority of metabolic engineering endeavors in *S. cerevisiae* have been conducted using carbon sources such as glucose or other sugars resulting from the hydrolysis of oligo- and polysaccharides that are present in renewable feedstocks (Nevoigt, 2008). Yet, glycerol as a by-product from oil plant biorefineries (biodiesel production in particular) could be another attractive 'renewable' carbon source, provided that substrate price becomes competitive to sugar. In fact, the economic utilization of crude glycerol still poses several challenges which are associated with certain impurities (Yang *et al*., 2012). There are two main advantages of glycerol compared to common sugar-based substrates; each of them is associated with a particular type of *S. cerevisiae*-based bioprocesses. On one hand, processes aiming at high biomass production or biomass-associated products (e.g. heterologous proteins) might profit from the fact that glycerol does not exert a Crabtree effect in *S. cerevisiae*. In fact, the presence of glucose at concentrations higher than 1 g L^{-1} causes alcoholic fermentation and the production of toxic ethanol hampers the achievement of high cell densities (Crabtree, 1929; de Deken, 1966; Petrik *et al*., 1983).

Cultivations in glucose for yeast biomass production therefore require a feeding scheme to maintain the glucose concentration below the abovementioned threshold. On the other hand, processes aiming at the production of small molecules can profit from glycerol's higher degree of reduction per carbon compared to common sugars. The increased reducing power will allow higher maximum theoretical yields of certain chemicals such as succinic acid or propanediols (Clomburg & Gonzalez, 2013). However, this requires a fermentative mode of glycerol catabolism instead of the natural respiratory mode in wild-type *S. cerevisiae*. In addition, the liquid nature of glycerol may reduce dilution effects of the culture broth when used as a carbon source in bioprocesses run in fed-batch mode.

In spite of the assets of glycerol as a carbon source, its use in industrial *S. cerevisiae*-based bioprocesses has been neglected so far. One major reason is that most commonly used laboratory and industrial *S. cerevisiae* wild-type strains cannot grow in synthetic medium containing glycerol as the sole carbon source (Swinnen *et al.*, 2016; Klein *et al.*, 2017). In fact, glycerol utilization by these strains depends on the supplementation of the synthetic medium with amino acids and/or nucleic bases; a scenario that is not economically viable in industrial settings.

During recent years, our group has made large progress with regard to establishing and/or improving glycerol utilization of several *S. cerevisiae* strains in synthetic medium without any supplementation. A crucial milestone has been the establishment of glycerol-based growth of the commonly used laboratory strains from the CEN.PK family. Our results revealed that the inability of these strains to grow in synthetic glycerol medium is mainly linked to a truncated version of the Ubr2 protein (encodes for a ubiquitin ligase) and that allelic replacement of *UBR2* established growth (Swinnen *et al.*, 2016; Ho *et al.*, 2017). The same studies demonstrated that the additional replacement of the *GUT1* allele

in the respective CEN.PK strain further improved its growth on glycerol. A maximum specific growth rate (μ_{max}) of 0.13 h^{-1} was achieved in the study of Ho *et al*. (2017), in which the *UBR2* and *GUT1* alleles from the strain (JL1) previously evolved for glycerol utilization in synthetic medium (Ochoa-Estopier *et al*., 2011) were used for reverse engineering of CEN.PK113-7D.

In a parallel study, our group has demonstrated that the expression of a heterologous facilitator for glycerol uptake (*FPS1* from *Cyberlindera jadinii* referred to as *CjFPS1*) increased μ_{max} on glycerol of both the *S. cerevisiae* wild-type strain CBS 6412-13A (Klein *et al*., 2016b) and the reverse engineered derivative of CEN.PK113-1A bearing *UBR2* and *GUT1* alleles from strain CBS 6412-13A (Swinnen *et al*., 2016).

The endogenous glycerol catabolic pathway of *S. cerevisiae* is the so-called L-glycerol-3-phosphate (L-G3P) pathway as recently reviewed by Klein *et al*. (2017). Glycerol is first phosphorylated by a glycerol kinase (encoded by *GUT1*) followed by the oxidation of the resulting product L-G3P by an FAD-dependent glycerol-3-phosphate dehydrogenase (encoded by *GUT2*) to dihydroxyacetone phosphate (DHAP) (Rønnow & Kielland-Brandt, 1993) (Figure 1). The electrons from glycerol oxidation are directly transferred via $FADH_2$ to the mitochondrial respiratory chain thereby delivering ATP by oxidative phosphorylation.

As the L-G3P pathway does not support the formation of reduced fermentation products (small molecules) whose formation depends on NADH, we replaced it by an alternative glycerol catabolic pathway (the so-called DHA pathway, Figure 1). Apart from the deletion of *GUT1*, the pathway replacement involved the expression of an NAD^+-dependent glycerol dehydrogenase from *Ogataea parapolymorpha* (catalyzing the oxidation of glycerol to dihydroxyacetone (DHA)) and the overexpression of the

115 endogenous *DAK1* gene encoding a dihydroxyacetone kinase (converting DHA to DHAP).
116 This pathway replacement strategy has been demonstrated to be functional in several
117 *S. cerevisiae* strains (Klein *et al*., 2016a). Interestingly, the sole replacement of the
118 glycerol catabolic pathway (including the expression of the above-mentioned glycerol
119 facilitator from *C. jadinii*) allowed the non-growing *S. cerevisiae* strains CEN.PK113-1A
120 and Ethanol Red to grow in synthetic glycerol medium (even without further genetic
121 modifications such as *UBR2* allele replacement). The pathway replacement is supposed
122 to allow glycerol fermentation and an increase of the maximum theoretical yields of
123 reduced fermentation products compared to the use of sugars as explained above.
124 Currently, our laboratory is using the newly created strains for the development of novel
125 glycerol-based bioprocesses. The first results regarding the production of 1,2-propanediol
126 from glycerol have been recently published (Islam *et al*., 2017).

127 In metabolic engineering, promoters are still the main target for finely tuning the
128 activities of target enzymes (Hubmann *et al*., 2014), even though great advancements
129 have been made with regard to the understanding and use of posttranscriptional and
130 posttranslational control mechanisms (Babiskin & Smolke, 2011; Jensen & Keasling, 2015;
131 Borttcher & McManus, 2015; Redden *et al*., 2015). During our metabolic engineering
132 endeavors for glycerol-based bioprocesses, we have been increasingly realizing that
133 there is a need for promoters with predictable expression levels in synthetic glycerol
134 medium. In fact, the commonly used endogenous *S. cerevisiae* promoters such as *TEF1p,*
135 *PGK1p, TPI1p, ACT1p, TDH3p and ENO2p* have not been thoroughly characterized in
136 synthetic glycerol medium. However, most knowledge regarding the characteristics of
137 these promoters has been accumulated from using sugars as a carbon source (Partow *et*
138 *al*., 2010; Sun *et al*., 2012). In order to avoid unexpected results in metabolic engineering

139 approaches and to provide more reliable tools for fine-tuning, we decided to launch the
140 current study aiming at the identification and characterization of useful *S. cerevisiae*
141 promoters for metabolic engineering attempts in purely synthetic glycerol medium. The
142 first part of the work describes the search for promoters driving strong expression under
143 these conditions as well as the identification of promoters with graded strengths. The
144 second part demonstrates the utility of four selected promoters with graded, strong
145 activities for optimizing glycerol utilization via the above-mentioned 'synthetic' DHA
146 pathway.

MATERIAL AND METHODS

Strain and medium composition

All strains constructed in this study are listed in Table S1 (supplementary data). Yeast cells were routinely cultivated in a static incubator at 30°C and maintained on solid YPD medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose, and 15 g L^{-1} agar. For selection of transformed strains, media were supplemented with 100 µg mL^{-1} nourseothricin and 300 µg mL^{-1} hygromycin B. Solid YPG medium was prepared as described for solid YPD medium except for the replacement of glucose with 20 mL L^{-1} glycerol. Synthetic medium for batch cultivations was prepared according to Verduyn *et al*. (1992) and contained 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 15 mg L^{-1} EDTA, 4.5 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.84 mg L^{-1} $MnCl_2 \cdot 2H_2O$, 0.3 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 0.3 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 0.4 mg L^{-1} $NaMoO_4 \cdot 2H_2O$, 4.5 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 3 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 1 mg L^{-1} H_3BO_3, and 0.1 mg L^{-1} KI. After heat sterilization of the salts, 1 mL L^{-1} of the following vitamin stock solution was added: 0.05 mg L^{-1} D-(+)-biotin, 1 mg L^{-1} D-pantothenic acid hemicalcium salt, 1 mg L^{-1} nicotinic acid, 25 mg L^{-1} myo-inositol, 1 mg L^{-1} thiamine chloride hydrochloride, 1 mg L^{-1} pyridoxine hydrochloride, and 0.2 mg L^{-1} 4-aminobenzoic acid. The carbon source added to the medium was either 20 g L^{-1} glucose or 60 mL L^{-1} glycerol. The pH was adjusted to 6.5 with 4 M KOH for the synthetic glucose medium. The pH of the synthetic glycerol medium was adjusted to either 5.0 using 4 M KOH (for bioreactor cultivations) or to 4.0 using 2 M H_3PO_4 (for shake flask cultivations and growth analysis). *E. coli* DH5α was used for plasmid propagation and isolation. For its cultivation lysogeny broth (LB) was used containing 10 g L^{-1} peptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl (pH 7.0). For selection and maintenance of plasmids, 100 mg L^{-1} ampicillin was added. All cultivations were performed on an orbital shaker at 250 rpm and 37°C.

171 ***Transcriptome analysis***

172 In order to obtain a sample for transcriptome analysis strain CEN.PK113-7D *GUT1*$_{JL1}$
173 *UBR2*$_{JL1}$ was cultivated in synthetic glycerol medium using a Sartorious 1.6 L bioreactor
174 (Sartorious, Stedim Biotech, Göttingen, Germany) with 1 L working volume and equipped
175 with 2 Rushton six-blade disc turbines. The pH of the medium was controlled at 4.0 by
176 automatic addition of 2 M NaOH. The bioreactor was sparged with sterile atmospheric air
177 with the air flow set at 1 volume of air per volume of liquid per minute (vvm). The
178 temperature was maintained at 30°C and a stirring rate was set at 800 rpm throughout the
179 cultivations. When the cells reached the mid-exponential growth phase (OD_{600} of ~3.0), 1
180 mL culture (corresponding to ~6 x 10^7 cells) was pelleted at 3000 rpm for 5 mins and re-
181 suspended in 1 mL RNA*later*™ (Thermo Fisher Scientific, Waltham, MA, USA) for the
182 isolation of total RNA using the RiboPure-Yeast Kit (Thermo Fisher Scientific) according
183 to the manufacturer's instructions. 12 µg of total RNA was provided to GATC Biotech AG
184 (Konstanz, Germany) for RNA sequencing using the Illumina platform. Next generation
185 sequencing of the RNA sample resulted in more than 58 million single reads with an
186 average length of approximately 50 nt. For quality control of the generated reads, FastQC
187 (Andrews, 2010) was applied. The sequences showed a high quality score (>30) across
188 the full length and no obvious artifact was detected. Subsequently, the reads were
189 mapped to the yeast genome as provided by the Saccharomyces Genome Database
190 (sacCer3 assembly) using Bowtie (version 2.1.0) (Langmead *et al*., 2009). The overall
191 alignment rate was over 95%. To obtain expression values for genes, Cufflinks (version
192 2.1.1) (Trapnell *et al*., 2010) was applied using the annotation provided for the sacCer3
193 assembly of the yeast genome. RNA sequencing data can be accessed at the GEO
194 (Edgar *et al*., 2002), accession number GSE109036. FPKM values of the transcripts are

provided in Table S2 (supplementary data) and also in a separate CSV file (Supplementary data_Table S2).

General molecular biology and microbiological techniques

Genomic DNA extraction from yeast was performed using PCI (Phenol/Chloroform/Isoamyl-alcohol) according to the method described by Hoffman & Winston (1987). In more detail, approximately 25 mg of cells were first suspended in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) in a 1.5 mL microcentrifuge tube. Subsequently, 150 mg of acid-washed glass beads (diameter of 0.425 - 0.6 mm) and 100 µL of PCI (25:24:1) were added. The tubes were then vortexed at maximum speed for 2 min and centrifuged at 15,700 g for 10 min. The aqueous phase containing the isolated genomic DNA was directly used as template for conducting polymerase chain reactions (PCR). Phusion® High-Fidelity DNA polymerase (New England BioLabs, Frankfurt am Main, Germany) was used when the PCR products were required for genetic manipulation and sequencing purposes, otherwise *Taq* DNA polymerase (Thermo Fisher Scientific) was used. The purification of PCR products was carried out using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Plasmids were isolated from *E. coli* overnight cultures by using the Qiagen Miniprep Kit (Qiagen). Transformation of yeast cells with plasmids as well as PCR-amplified DNA fragments for genomic integration was performed using the LiAc/PEG method described by Gietz *et al*. (1995).

Strain construction

—For promoter characterization via the yECitrine-based fluorescence reporter assay—

All cassettes for the expression of yECitrine under the control of one of the selected promoters were assembled and integrated into the genome of CEN.PK113-7D $GUT1_{JL1}$

UBR2$_{JL1}$ via CRISPR-Cas9 mediated genome editing. For expression of Cas9, the strain was first transformed with plasmid p414-TEF1p-Cas9-CYC1t-nat1 (Klein *et al*., 2016a). All used promoter sequences are listed in Table S3 (supplementary data). The sequences of the selected candidate promoters were amplified from genomic DNA isolated from strain CEN.PK113-7D *GUT1$_{JL1}$ UBR2$_{JL1}$* using the primers listed in Table S3. If the exact region comprising the promoter elements was unknown, the primers were designed for the amplification of at least 1 kb of the genomic region upstream of the annotated translation start of the respective gene. All sequences of the traditional `standard promoters´ were amplified from genomic DNA isolated from strain S288C. The yECitrine coding sequence and the *CYC1* terminator were PCR-amplified as a single fragment from plasmid p416-TEF-yECitrine (Alper *et al*., 2006). The used primers were designed to generate 50-60 bp extensions homologous to regions up- and downstream of the target locus, or to the respective adjacent DNA fragment. The two fragments (one of the selected promoter sequences as well as the fragment comprising the yECitrine coding sequence and the *CYC1* terminator) were co-transformed together with p426-SNR52p-gRNA.YGLCτ3-SUP4t-hphMX (Islam *et al*., 2017) into strain CEN.PK113-7D *GUT1$_{JL1}$ UBR2$_{JL1}$* resulting in assembly and integration of the respective yECitrine expression cassette at the *YGLCτ3* locus (Flagfeldt *et al*., 2009). The correct integration of the yECitrine expression cassette for each of the 25 generated strains was verified by diagnostic PCR using the primers listed in Table S3. In addition, the sequences of all expression cassettes were validated by Sanger sequencing.

—For optimization of the DHA pathway for glycerol catabolism—

All *S. cerevisiae* strains for optimization of the DHA pathway for glycerol catabolism were constructed on CEN.PK113-1A *UBR2*$_{CBS}$ *CjFPS1* (Swinnen *et al*., 2016) via CRISPR-

242 Cas9 mediated genome editing. For genomic integration of the two expression cassettes
243 for the synthetic DHA pathway (*Opgdh* and *ScDAK1*), the coding region of the *GUT1* locus
244 was used as the target site. In all strains the same expression cassette for *Opgdh* (under
245 the control of *TEF1* promoter and *CYC1* terminator) was used while the *ScDAK1*
246 expression cassette was *de novo* assembled using one of the selected promoters (*TDH3p,*
247 *JEN1p, ADH2p or ALD4p*) during the integration process. The *Opgdh* expression cassette
248 was amplified from plasmid p41bleTEF-*Opgdh* (Klein *et al*., 2016a). The selected
249 promoter sequences were amplified from genomic DNA isolated from strain CEN.PK113-
250 7D $GUT1_{JL1}$ $UBR2_{JL1}$ as described above. The fragment comprising the *DAK1* coding
251 sequence and the *TPS1* terminator was PCR-amplified from plasmid pUC18-*DAK1* (Klein
252 *et al*., 2016a). The control strain carries the DHA pathway consisting the *Opgdh*
253 expression cassette (amplified from plasmid p41bleTEF-*Opgdh*) and the *ScDAK1*
254 expression cassette under the control of the *ACT1* promoter amplified from pUC18-*DAK1*.
255 Both of the amplified cassettes were integrated together at the *GUT1* locus. The primers
256 (Table S4, supplementary data) were designed to generate PCR products with flanking
257 sequences (30-60 bp) homologous to the regions upstream or downstream of the target
258 *GUT1* locus or to the adjacent fragments to be integrated. For CRISPR-Cas9 mediated
259 genomic integration, the strain CEN.PK113-1A $UBR2_{CBS}$ *CjFPS1* was first transformed
260 with plasmid p414-TEF1p-Cas9-CYC1t-nat1. Next, the aforementioned amplified DNA
261 fragments were co-transformed with plasmid p426-SNR52p-gRNA.GUT1-SUP4t-hphMX
262 (Klein *et al*., 2016a), resulting in assembly and integration of the respective two expression
263 cassettes into the *GUT1* locus. Correct constructs were verified by diagnostic PCR and
264 validated by Sanger sequencing.

265 ***Flow cytometry***

The fluorescence of the constructed strains expressing the yECitrine encoding gene from the selected promoters was measured using the CyFlow Space (Sysmex Partec, Goerlitz, Germany). To obtain samples for the measurement, cells from a single colony grown on either YPD or YPG were used to inoculate 3 mL of either synthetic glucose or synthetic glycerol medium in a 10 mL glass tube (referred to as pre-culture hereafter). The pre-culture was incubated in an orbital shaker at 200 rpm at 30^{o}C until an optical density (OD_{600}) of approximately 4.0 was reached. An appropriate amount of pre-culture was used for inoculation in 3 mL of the respective synthetic medium adjusting an OD_{600} of 0.2 (corresponding to ~4.0 x 10^6 cells mL^{-1}). This culture (referred to as intermediate culture hereafter) was subsequently incubated under the same conditions as the pre-culture for 16 hours. An appropriate amount of cells from the intermediate culture was then used to inoculate 10 mL fresh synthetic medium in 100 mL shake flasks adjusting to an OD_{600} of 0.15 (corresponding to ~3.0 x 10^6 cells mL^{-1}). This culture was grown under the same conditions as described above until mid-exponential growth phase was reached (OD_{600} of ~1.0; corresponding to 2.0 x 10^7 cells mL^{-1}). An aliquot of this final culture (275 µL each) was pelleted by centrifugation at 3000 rpm for 3 min, and washed once in 800 µL synthetic medium without any carbon source. After an additional centrifugation step, the cell pellet was re-suspended in 4 mL of the same synthetic medium used for the washing step. Flow cytometry using FloMax (Sysmex Partec) was performed for each tested sample. 50,000 gated events were analyzed for their yECitrine expression levels by exciting with the 488 nm laser. The geometric mean fluorescence value of each construct was first calculated across the biological triplicates and then subtracted by the mean autofluorescence value obtained from the control strain where yECitrine was not expressed.

Growth analysis and physiological characterization

290 Growth analysis of *S. cerevisiae* was performed according to the procedure described by
291 Swinnen *et al*. (2013). For the growth and physiological characterization of each strain,
292 cells taken from a single cell colony were inoculated in 3 mL synthetic glucose medium
293 and incubated at 30^{o}C for 16 h in an orbital shaker set at 200 rpm. This pre-culture was
294 used to prepare an intermediate culture consisting of 3 mL fresh synthetic glucose medium
295 and OD_{600} adjusted to 0.2. The intermediate culture was subsequently incubated for 48
296 hours under the same conditions used for the pre-culture. The amount of cells from the
297 intermediate culture required to obtain an OD_{600} of 0.2 in 5 mL medium (100 mL for
298 physiological analysis) was calculated and pelleted by centrifugation at 3000 rpm for 5
299 min. The cell pellets were then re-suspended in 800 µL of synthetic glycerol and pelleted
300 again by centrifugation to remove the supernatant. The washed cell pellets were then re-
301 suspended in 5 mL synthetic glycerol medium for subsequent growth analysis. Two
302 aliquots (750 µL each) of this cell suspension were transferred into separate wells of a
303 White KrystalTM 24-microplate (Porvair Sciences, Leatherhead, United Kingdom) and
304 cultivated in the Growth Profiler 1152 (Enzyscreen, Haarlem, The Netherlands) at 30°C
305 with orbital shaking at 200 rpm. The Growth Profiler 1152 allowed determining the density
306 of the culture (expressed as green value or *G*-value) in each single well of the microplate
307 up to OD_{600} values of ~2.5 at intervals of 40 minutes. The obtained *G*-values were
308 afterwards converted into OD_{600} values (referred to as OD_{600} equivalents) using a
309 calibration curve with the equation of the best fit line: OD_{600} equivalent = $6.1761.10^{-8}$ x *G*-
310 value$^{3.4784}$.

RESULTS AND DISCUSSION

RNA sequencing as a guide for quantifying the expression strengths of endogenous S. cerevisiae promoters in synthetic glycerol medium

As a first step towards the identification of useful promoters for driving strong expression of target genes in pure synthetic glycerol medium with predictable quantitative outcome, a transcriptome analysis (RNA sequencing) experiment was performed by analyzing RNA abundances in cells exponentially growing under these conditions in a pH-controlled bioreactor. A prerequisite for such an experiment was a *S. cerevisiae* strain able to grow in synthetic glycerol medium without supplements. This capability is only found in relatively few wild-type *S. cerevisiae* strains, but not in well-characterized laboratory strains such as S288C or the CEN.PK family (Swinnen *et al*., 2013). We therefore used the above-mentioned reverse-engineered strain CEN.PK113-7D $GUT1_{JL1}$ $UBR2_{JL1}$ (Ho *et al*., 2017) for our transcriptome analysis. The genome sequence of wild-type CEN.PK113-7D is publicly available (Nijkamp *et al*., 2012) and the strain's physiology is well-characterized (van Dijken *et al*., 2000). Compared to the wild-type CEN.PK113-7D (which does not grow at all in synthetic glycerol medium), the alleles of *GUT1* and *UBR2* both contain only a single point-mutation in this reverse-engineered strain, enabling the latter to grow in synthetic glycerol medium with a μ_{max} of ~0.13 h^{-1}.

Total RNA was collected from strain CEN.PK113-7D $GUT1_{JL1}$ $UBR2_{JL1}$ during the exponential growth phase in batch cultivation and subjected to Illumina single-end sequencing (see Materials and Methods). Mapping of the generated single reads to the annotated S288C genome (sacCer3 assembly) provided a comprehensive representation of RNA abundances (Figure 2). As the number of mapped reads covering a specific gene tends to be proportional to the gene's length, we used the number of fragments per

kilobase of exon per million reads mapped (FPKM) as a normalized measure for expression (Trapnell *et al*., 2010). The use of FPKM as a normalized measure enabled us to directly compare the expression intensity of different genes within the sample.

As we were mainly interested in relatively strong promoters, we set an FPKM > 1,000 as a minimum expression level for guidance during data analysis. This cut-off resulted in 322 genes with relatively high expression in synthetic glycerol medium. As expected, a considerable number (N=103) of the highly expressed genes corresponded to ribosomal proteins. The transcript abundances for the above-mentioned commonly used promoters (see Introduction) varied between FPKM values of 8,000 (*TDH3p*) and 1,500 (*ACT1p*) in the following order: *TDH3p* > *TEF1p* > *TPI1p* > *PGK1p* > *ENO2p* > *ACT1p*. Notably, several transcripts were found to exert FPKM values that were by orders of magnitudes higher than that of *TDH3*, such as those for the ribosomal proteins L41-B (320,000) and L41-A (250,000) (Figure 2 and Table S2). The FPKM values were subsequently used as a guideline for selecting 25 candidate promoters for a more thorough characterization. This short-list contained the 6 above-mentioned 'traditional' promoters as well as 19 'new' promoters that were selected based on graded FPKM values between those of *ACT1* (FPKM ≈ 1,500) and of *RPL41B* (FPKM > 320,000) (Figure 2).

Characterization of 25 selected promoters in synthetic glycerol medium based on yECitrine reporter gene expression

In this work and in most other promoter studies in the field of metabolic engineering and synthetic biology, a 'promoter' sequence is considered as the non-coding DNA sequence situated directly upstream of a gene's translation start codon, i.e. including its 5' untranslated regions (5' UTR). For most genes, the exact length of the 5' UTR has not

359 been identified yet. However, it has been shown that mRNA stability and protein
360 translation can be greatly influenced by structural elements in the 5' UTR (McCarthy,
361 1998). As transcriptome analysis solely provides information regarding the abundance of
362 transcripts, quantifying the expression of a reporter gene at the protein (activity) level
363 therefore reflects promoter activity in a more realistic way. Thus, each of the selected 25
364 candidate promoters was fused to the coding sequence of the yeast codon-optimized
365 yellow fluorescent protein yECitrine (Sheff & Thorn, 2004). Downstream of the yECitrine
366 coding sequence, the *CYC1* terminator was used in all constructs in order to allow precise
367 comparison of all selected promoters. Using the CRISPR-Cas9 system, all 25 reporter
368 expression cassettes were assembled and integrated into the same genomic location of
369 the strain CEN.PK113-7D $GUT1_{JL1}$ $UBR2_{JL1}$ (see Materials and Methods). All constructed
370 strains (Table S1) were validated by sequencing the relevant locus and afterwards
371 assayed for their growth performance in synthetic glycerol medium using the Growth
372 Profiler 1152. The results showed that reporter gene expression did not influence the
373 growth properties of the constructed strains under the tested conditions (data not shown).
374 In order to quantify the expression of the reporter gene yECitrine, the 25
375 constructed strains were grown in Erlenmeyer flasks with synthetic medium containing
376 glucose or glycerol as the carbon source. We included experiments using glucose
377 containing medium to also obtain information with regard to the level of glucose repression
378 of the selected promoters. The specific fluorescence of 50,000 cells harvested during the
379 mid-exponential growth phase was determined by flow cytometry for each culture
380 (Figure 3). We decided to use *TEF1p* as a reference for quantifying promoter activities
381 throughout this study since it is one of the strongest and most thoroughly characterized
382 endogenous *S. cerevisiae* promoters. Moreover, it has been considered as a true

383 ‘constitutive’ promoter, i.e. it leads to similar expression levels even under different
384 conditions such as shifting the carbon source from glucose to ethanol/glycerol (Nevoigt *et*
385 *al*., 2006; Partow *et al*., 2010). Its ‘constitutive’ nature was confirmed by the current data
386 showing that *TEF1p* led to remarkably similar expression levels in both tested carbon
387 sources (Figure 3). Other promoters exerting similar expression strength in both carbon
388 sources were *ACT1p, HHF2p*, *CYC1p*, *PMP1p*, and *RPL41Bp* (Figure 3C). In contrast,
389 *TDH3p* in glycerol medium showed only 56 % of its activity in glucose medium.

390 In general, the results obtained from cells grown in glycerol-containing medium
391 were in good agreement with the results from our transcriptome analysis. Most of the
392 shortlisted promoters indeed exerted an expression capacity higher than *ACT1p* (Figure
393 3A). Moreover, the ranking with regard to the expression strength of the characterized
394 `standard promoters´ was similar to that observed for the transcriptome data even though
395 the sequence of *TPI1p*, *PGK1p* and *ENO2p* slightly changed (*TDH3p* > *TEF1p* > *ENO2p*
396 > *TPI1p* > *PGK1p* > *ACT1p*). Nevertheless, several promoters showed surprising
397 deviations as we compared their yECitrine-based expression levels to those deduced from
398 the transcriptome data. For example, *RPL41Ap*, *RPL41Bp* and *DDR2p* showed 44, 55
399 and 2 fold higher FPKM values than *TEF1p* in the transcriptome experiment, but were
400 found to result in lower expression levels (25, 52 and 23 % compared to *TEF1p*) in the
401 yECitrine-based fluorescence reporter assay.

402 In a previous study, Keren *et al*. (2013) characterized almost 900 yeast promoters
403 by using yellow fluorescent protein (YFP) as the reporter gene in different carbon sources,
404 including glucose, galactose, fructose, ethanol, and glycerol. However, the glycerol
405 medium used was yeast nitrogen base supplemented with 1.6 g L^{-1} amino acids which is
406 known to have at least a strong influence on growth (Swinnen *et al*., 2013). An influence

of adding amino acids to the medium on both growth and average promoter activities has also been demonstrated by Keren *et al*. (2013) when using glucose as the carbon source. When simply comparing the sorting of the 6 commonly used promoters between the current study and the one of Keren *et al*. (2013), only slight differences can be recognized. In the latter study, the sequence was as follows: *TDH3p* > *TEF1p* > *ENO2p* > *PGK1p* > *ACT1p* > *TPI1p*. However, the comparison of the two studies also revealed obvious differences, particularly concerning the comparison of promoter activities between glycerol- and glucose-containing media. For example, the promoter activity of *TEF1p* was reduced to 30 % when glycerol was used as the carbon source instead of glucose in the study of Keren *et al*. (2013), while our data suggests a relatively similar strength of *TEF1p* under both growth conditions (Figure 3A and B). A similar difference was observed for *FBA1p*: Keren *et al*. (2013) reported an almost 10-fold reduction of the expression strength in glycerol-containing medium, while the levels for the two carbon sources were nearly the same in the current study (Figure 3C). However, one has to consider that the two studies used different approaches for the approximation of the promoter strength. Keren *et al*. (2013) determined the rate of the total fluorescence increase between two time points during the exponential growth phase, whereas we measured cumulative fluorescence of 50,000 cells at a single time point during the mid-exponential growth phase. As both methods discussed here did not take transcript and/or protein turnover into account, which also contribute to transcript and protein abundance (Pratt *et al*., 2002; Huch & Nissan, 2014), the results should be considered as an approximation of the actual (real) rate of transcription. It should also be noted that the influence of the aforementioned factors might change over the course of a batch shake flask cultivation, a fact that potentially further affects the results determined by different

sampling and promoter activity quantification approaches. Nevertheless, our goal was to provide tools for adjusting protein abundance in metabolic engineering approaches rather than characterizing the promoter activity *per se*. In this context, quantification of the cumulative abundance of the reporter protein should be legitimate as only the protein molecules present at a certain point in time will contribute to the metabolic flux of interest.

It is visible from Figure 3A that the 25 characterized promoters represent a collection that exerts a continuous range of expression levels in synthetic glycerol medium which offers the opportunity of fine-tuning gene expression under these conditions. Among the selected promoters with very strong activities in glycerol medium, *ADH2p* and *ALD4p* showed by far the highest expression strengths, i.e. their yECitrine-based specific fluorescence levels are 3- and 4.2-fold stronger compared to *TEF1p*, respectively (Figure 3A). In contrast, *HOR7p* and *JEN1p* only led to slightly higher fluorescence levels compared to *TEF1p* (Figure 3A). The promoters of *ADH2* and *ALD4* also showed the strongest level of glucose repression as visible by fold changes of 17.5 and 16.0 in expression strength, respectively, when levels in glucose and glycerol were compared (Figure 3C). *ADH2* encodes for an alcohol dehydrogenase which catalyzes the conversion of ethanol to acetaldehyde (Ciriacy, 1975), whereas *ALD4* encodes for an aldehyde dehydrogenase, converting acetaldehyde to acetate (Navarro-Aviño *et al*., 1999). The respective enzymes play a central role for the catabolism of ethanol after the diauxic shift which naturally occurs when *S. cerevisiae* grows on glucose. The fermentative glucose catabolism leads to the accumulation of ethanol and glycerol that are used as carbon sources by *S. cerevisiae* in a respiratory manner upon glucose depletion. It has been demonstrated that a large set of genes including those for the respiratory ethanol and glycerol utilization are subject to glucose repression and that they are derepressed during

455 the diauxic shift (Trumbly, 1992; Roberts & Hudson, 2006; Derisi *et al*., 2007). Without
456 exception, the expression of both genes have been shown to be highly induced in the
457 absence of glucose (Navarro-Aviño *et al*., 1999; Walther & Schüller, 2001). It seems that
458 the strong activity of the promoters in medium containing glycerol as the sole carbon
459 source is rather associated with the absence of glucose than with the presence of glycerol.
460 Nevertheless, both promoters should provide metabolic engineering tools for driving
461 protein expression to an extraordinary high level in glycerol-based conditions.

462 The use of flow cytometry for quantifying specific fluorescence also allowed us to
463 obtain an insight into population heterogeneity with regard to yECitrine expression. Cell-
464 to-cell heterogeneities in an isogenic cell population may originate from spatio-temporal
465 variations in the environment, from the randomness of intrinsic biochemical reactions, or
466 from the differences in cell phenotypes such as age, cell size, or growth rates (Llamosi *et*
467 *al*., 2016). Such deviations in protein expression levels in isogenic populations may have
468 a fundamental impact on the productivity of a bioprocess (Balázsi *et al*., 2011). As visible
469 from supplementary data S5, the noise level of our selected promoters (as shown by the
470 coefficient of variation (CV)) decreased with increasing promoter strength. This
471 observation is in line with the finding of Newman *et al*. (2006), who demonstrated that the
472 global noise level conversely correlates to the expression level. The relatively low noise
473 levels exerted from promoters that are strongly upregulated in glycerol medium certainly
474 represent an asset when using them in industrial applications.

475 ***Optimizing glycerol metabolism using the new promoter set***

476 Limitation of the traditionally employed set of common endogenous *S*. *cerevisiae*
477 promoters became first apparent during our above-mentioned study (Klein *et al*., 2016a;
478 Figure 1). The integration of an overexpression cassette for endogenous *DAK1* (under the

control of the *ACT1* promoter) enabled growth of a *gut1*Δ mutant expressing the NAD^+-dependent GDH from *O. parapolymorpha* (under the control of the *TEF1* promoter) (Klein *et al*., 2016a). However, a slight accumulation of the pathway intermediate DHA (the substrate for Dak1p) could still be detected in the culture supernatant, indicating that the overexpression of *DAK1* from the *ACT1* promoter was still not optimal to balance the flux through the established pathway. In order to further increase Dak1p activity, we included a second *DAK1* overexpression cassette (under the control of the *TDH3* promoter) (Klein *et al*., 2016a). This modification (referred to as module II in that study) had been implemented in four different strains. In particular, a remarkable improvement of μ_{max} (from 0.12 to 0.26 h^{-1}) was observed in the CEN.PK derivative carrying the *UBR2* allele replacement from CBS 6412-13A (CEN.PK113-1A *UBR2*$_{CBS}$) (Klein *et al*., 2016a). The question arose whether the same improvement in μ_{max} could be obtained with a single expression cassette using a promoter strong enough to drive a sufficient level of *DAK1* expression. Such a strategy might be superior to using several expression cassettes of the same coding sequence, as the extensive homology available increases the chance of DNA excision through direct-repeat recombination events (Thomas & Rothstein, 1989). Apart from the *ACT1* promoter (reference), four different promoters of varying strength were selected from our collection (*TDH3p*, *JEN1p*, *ADH2p,* and *ALD4p*) to construct strains with graded levels of *DAK1* overexpression. We decided to use strain CEN-PK113-1A *UBR2*$_{CBS}$ *CjFPS1* (Swinnen *et al*., 2016) as a starting point. Besides the *UBR2* allele from strain CBS 6412-13A, this strain carried the expression cassette for the glycerol facilitator from *C. jadinii*. The expression cassettes for *Opgdh* and *DAK1* were integrated together at the *GUT1* locus via CRISPR-Cas9 mediated genome editing. The only difference between the constructed strains was the promoter used for *DAK1* expression.

503 The resulting strains were analyzed for their μ_{max} in synthetic glycerol medium
504 (Figure 4). Notably, the growth rates of the recombinant strains increased from ~0.10
505 (*ACT1p-DAK1*) to ~0.24 h^{-1} (*ADH2p-DAK1*) in obvious analogy to the expression
506 strengths of the employed promoters. Interestingly, the highest μ_{max} was obtained for the
507 *ADH2* promoter (~0.24 h^{-1}), which only showed the second highest expression level from
508 the yECitrine-based reporter assay, while the strongest promoter (*ALD4p*) only resulted
509 in a μ_{max} of ~0.17 h^{-1}. It seems that too high *DAK1* expression levels were rather
510 detrimental for cellular growth based on the DHA pathway. A possible explanation may
511 stem from the ATP dependence of the DAK catalyzed reaction, leading to an ATP
512 shortage for other essential cellular functions.
513

CONCLUSION

The current study aimed at the identification and characterization of promoters suitable for metabolic engineering of *S. cerevisiae* in synthetic glycerol-containing media. The 25 promoters selected and characterized in this study can be useful tools for engineering *S. cerevisiae* strains. The observed variations in promoter activity under the applied conditions will allow a flexible design of expression cassettes for the control of the expression strengths of target proteins as well as whole metabolic pathways in glycerol-based media including fine-tuning of metabolic fluxes. To demonstrate the general applicability of the promoter collection, a subset of the characterized promoters was used for optimizing glycerol metabolism in an engineered CEN.PK derivative aiming at increasing the strains' maximum specific growth rate in synthetic glycerol medium.

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CONFLICT OF INTEREST

The authors declare no competing interest.

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FIGURE LEGENDS

Figure 1. The native FAD-dependent glycerol catabolic pathway in *S. cerevisiae* via L-glycerol-3-phosphate (L-G3P; left) and the synthetic NAD^+-dependent pathway via dihydroxyacetone (DHA; right). While the wild-type strain CEN.PK113-13D and the reverse engineered strain CEN.PK113-7D $GUT1_{JL1}$ $UBR2_{JL1}$ carry the native L-G3P pathway and the native (active) transporter for glycerol uptake Stl1p, the newly generated strains for testing the selected promoters have the DHA pathway plus both Stl1p and a heterologous glycerol facilitator (encoded by *CjFPS1*). For establishing glycerol utilization via the DHA pathway, the native L-G3P pathway was abolished by the deletion of *GUT1*. Endogenous catabolic steps are indicated by solid lines, in which the involved genes are given. The synthetic catabolic steps are shown in broken lines, with which the associated genes from the heterologous species are indicated. *Opgdh*: *Ogataea parapolymorpha gdh, CjFPS1*: *Cyberlindera jadinii FPS1*.

Figure 2. Transcript abundances (FPKM) of annotated genes in cells of the *S. cerevisiae* strain CEN.PK113-7D $GUT1_{JL1}$ $UBR2_{JL1}$ harvested during exponential growth in synthetic medium containing 6% (v/v) glycerol. The 25 genes selected for promoter characterization by the yECitrine reporter are highlighted.

Figure 3. Activity of 25 selected native *S. cerevisiae* promoters in synthetic medium containing 6% (v/v) glycerol (A) or 2% (w/v) glucose (B). The quantification of promoter activity is based on the specific fluorescence of the cells after fusing the respective promoter to the coding sequence of the reporter gene yECitrine. Cells for the measurement were harvested from mid-exponential growth phase and analyzed by flow cytometry (50.000 gated cells). (C) Log_2 transformation of fold-change of the fluorescence

723 intensities of the 25 promoters when glycerol was compared to glucose as the carbon
724 source. Fold changes were ranked from the lowest (left) to the highest (right). Mean values
725 and standard deviations were obtained from three biological replicates. Values for the
726 included set of commonly used promoters for metabolic engineering in glucose-based
727 media are highlighted in gray.

728 **Figure 4.** Effect of different promoter strengths for *DAK1* expression on growth in synthetic
729 glycerol medium. The native FAD-dependent glycerol catabolic pathway (L-G3P pathway)
730 of strain CEN *UBR2$_{CBS}$ CjFPS1* (CEN RE) was replaced by the synthetic NAD$^+$-dependent
731 pathway (DHA pathway), in which the *DAK1* expression was under the control of five
732 promoters of increasing strength (*ACT1p, TDH3p, JEN1p, ADH2p and ALD4p*) selected
733 in this study. The constructed strains are named after the promoter employed for
734 assembling the *DAK1* expression cassette, e.g. strain *ACT1p* stands for CEN RE
735 *gut1*::*Opgdh ACT1p-DAK1*. Growth performance of strain CEN RE is provided as a
736 reference. Standard deviations were obtained from at least three biological replicates.