Porting the synthetic D-glucaric acid pathway from Escherichia coli to Saccharomyces cerevisiae

Amita Gupta, Michael A. Hicks, Shawn P. Manchester, Kristala L. J. Prather
S.1. Supplementary Methods

S.1.1 Strain Construction

The *S. cerevisiae* strain (*CEN.PK2-1D*) was modified by deleting the *OPI1* gene to create strain *CEN.PK2-1Dopi1Δ* (Supplementary Figure S1). Deletion of *OPI1* was carried out by PCR amplification of the KanMX6 cassette from plasmid pFA6a-KanMX6 using primers PrKO-OPI1(+) and PrKO-OPI1(-). The PCR product was introduced into *CEN.PK2-1D* using the lithium acetate method [1] and deletions were isolated on YPD medium containing G418.

Deletion was confirmed by PCR amplification from genomic DNA of potential deletions using primers OPI1kochk(+) and prKOchk(-).

S.1.2. Phosphate-limited chemostat cultivation

In order to study pathway performance in conditions of D-glucose repression, a phosphate-limited study was conducted using the At strain under similar conditions as above but at lower dilution rates of 0.012, 0.024, 0.036 and 0.048 hr\(^{-1}\) to prevent washout. A phosphate-limiting medium [2] was modified to allow for increased cell densities that enabled detection of D-glucaric acid production. A limiting phosphate concentration of 50 mg/L of potassium phosphate monobasic was used to ensure extracellular D-glucose levels exceeded 14 mM at all dilution rates [3]. The culture medium contained the following (per liter): 250 mg calcium chloride, 250 mg sodium chloride, 1.25 g magnesium sulfate, 12.5 g ammonium sulfate, 2.5 g potassium chloride, 1.25 mg boric acid, 100 µg copper sulfate, 250 µg potassium iodide, 500 µg...
ferric chloride, 1 mg manganese sulfate, 500 µg sodium molybdate, 1 mg zinc sulfate, 5 µg biotin, 1 mg calcium pantothenate, 5 µg folic acid, 5 mg inositol, 1 mg niacin, 500 µg p-aminobenzoic acid, 1 mg pyridoxine, 500 µg riboflavin, 1 mg thiamine, 50 mg potassium phosphate, and 20 g D-glucose. Histidine, leucine and tryptophan were added at 20 mg/L, 100 mg/L and 40 mg/L due to the use of the auxotrophic CEN.PK2-1Dopi1Δ strain. Samples were analyzed and reported as for the D-glucose-limited chemostat.

S.1.3. Constructing His-tagged MIOX enzymes and quantifying protein abundance and MIOX expression in the Mm and At strains

In order to construct pOE521-3xHISmMioxUdh, primers SpeI-MmHIS(+) and NotI-MmMiox(-) were used to amplify Miox with an appended 6xHistag, and subsequently cloned as described in the main text. The construct pOE521-3xHISAtMioxUdh was created in an analogous manner with the primers SpeI-AtHIS(+) and LEU2t(-). Corresponding strains were made as described in the main text.

These N-terminal 6x-Histidine-tagged MIOX enzymes were used to assess the relative protein abundance of these enzymes in their respective host strains. Three mL of cells were collected by centrifugation 24 hours into the fermentation and were washed twice in 100 mM sodium phosphate buffer, pH 7.0. Cells were resuspended in sodium phosphate buffer, pH 7.0 supplemented with an EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), 0.5mm glass beads (USA Scientific, Ocala, FL) were added and samples were vortexed for 15 minutes. Cell debris was separated and lysates were quantified for total protein abundance by a modified Bradford assay [4] and for MIOX expression by Western Blot.
Cell lysates were treated with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and boiled at 90°C for 10 minutes. Equal volumes of treated cell lysate were loaded on SDS-PAGE gels and separated prior to transfer to nitrocellulose blotting membranes (Pall Life Sciences, Port Washington, NY). Membranes were blocked with a 5% solution of BSA in TBS for one hour and, after washing in TBS-Tween-20%, a 1:200 dilution of His-probe (H-3) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in TBS was added and incubated overnight. After washing in TBS-Tween-20%, a secondary antibody (goat-anti-mouse IgG1-HRP) was added at a 1:1000 dilution in TBS. After washes in TBS-Tween-20% and TBS, Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection according to the recommended manufacturer’s protocol.

S.2. Supplementary Results

S.2.1. Slow growth drives myo-inositol availability even during probable D-glucose repression

An aerobic phosphate-limited chemostat was used to study the same phenomenon of slow growth under conditions of probable D-glucose repression (Supplementary Figure S4). Glucose repression induces massive transcriptional changes such as inhibition of sugar transport and gluconeogenesis that could impact D-glucaric acid titers in S. cerevisiae. Glucose repression is thought to occur at extracellular glucose concentration above 14 mM (2.5 g/L) [3]. Extracellular D-glucose concentrations ranging between 16 mM (2.9 g/L) and 47 mM (8.5 g/L) were observed at the four dilution rates of 0.012, 0.024, 0.036 and 0.048 hr⁻¹. The necessary use of a synthetic medium led to decreases in cell densities compared to the D-glucose-limited chemostat. myo-Inositol was not detected at any dilution rate, but D-glucaric acid was detected at all dilution
rates. As in the D-glucose-limited chemostat, D-glucaric acid concentrations peaked at the lowest dilution rate and decreased with increasing dilution rate. OD$_{600}$ and D-glucose consumption were highest at the lowest dilution rate but increased at a smaller proportion than corresponding D-glucaric acid titers. The highest D-glucaric acid titer achieved in this case was 0.40 g/L.

S.2.2. The MIOX from the At strain expresses several times higher than the MIOX from the Mm strain

To assess the differences in expression of the MIOX enzyme between the two strains, a densitometry analysis of a Western Blot was undertaken. As can be seen in Supplementary Figure S6, the MIOX from the At strain is expressed at nearly 5 times the amount compared to the MIOX from the Mm strain at 24 hours. Total D-glucaric acid titers were comparable to the non-His-tagged versions (data not shown).

S.3. Supplementary Discussion

We confirmed that D-glucaric acid production can proceed even under conditions of probable D-glucose repression in a phosphate-limited chemostat (Supplementary Figure S4). Because no D-glucaric acid production was detected until D-glucose had been depleted in both shake-flasks and glucose-limited chemostat, it initially seemed that high extracellular D-glucose concentrations may have triggered catabolite repression and the subsequent inhibition of sugar transport, gluconeogenesis and other processes necessary for D-glucaric acid production. A previous study showed an extracellular D-glucose concentration of 14 mM was sufficient to induce catabolite repression in one S. cerevisiae strain [3]. The continued production of D-glucaric acid in a phosphate-limited chemostat indicated that high extracellular concentrations of
D-glucose (16 mM, 25 mM, 35 mM, 47 mM) are not inhibitory. However, catabolite repression may not have been fully induced due to the presence of phosphate limitation and may not have been induced at the lowest dilution rate where steady-state D-glucose concentrations reached just 16 mM.
Supplementary Figure S1. Comparison of D-glucaric production from CEN.PK2-1D and CEN.PK2-1D opi1Δ strains expressing previously-established pathway genes. Experiments used the *M. musculus* MIOX strain in YPD with and without supplementation of the *myo*-inositol intermediate.
Supplementary Figure S2. Comparison of D-glucaric acid yields on glucose (g/g) and glycerol (g/g) at the end of shake-flask fermentations (72 hours and 70 hours) for Figures 2 and 3, respectively.
Supplementary Figure S3. (A) No limitations in D-glucaric acid production or (B) growth defects are present in the At strain grown with up to 5 g/L D-glucaric acid added at the start of the fermentation.
Supplementary Figure S4. Comparison of glucaric acid yields on glucose (g/g) during batch and fed-batch modes for the shake-flask cultivations in Figure 5.
|                | Gel 1 |   | Gel 2 |   |
|----------------|-------|---|-------|---|
| 1              |       |   |       |   |
| 2              |       |   |       |   |
| 3              |       |   |       |   |
| 4              |       |   |       |   |
| 5              |       |   |       |   |
| 6              |       |   |       |   |

**Arabidopsis thaliana**

**Mus musculus**

|                          | Gel 1 | Gel 2 |
|--------------------------|-------|-------|
| Densitometry as measured by ImageJ | 20785.69 | 11843.81 |
|                          | 16938 | 12430.35 |
|                          | 19514.85 | 9431.953 |

|                          | Gel 1 | Gel 2 |
|--------------------------|-------|-------|
| Area normalized to Gel 2 using Mus musculus densitometry | 16152.59 | 9204.049 |
|                          | 13162.84 | 9659.857 |
|                          | 19514.85 | 9431.953 |

| Total protein loaded (mg/mL) | Gel 1 | Gel 2 |
|------------------------------|-------|-------|
| 2.7                          | 7.5   | 3.0   |
| 8.0                          | 3.8   | 9.1   |

| Normalized expression by total protein | Gel 1 | Gel 2 |
|---------------------------------------|-------|-------|
| 6000                                  | 1200  | 4400  |
| 1200                                  | 5100  | 1000  |
| MIOX homolog | Arabidopsis thaliana | Mus musculus |
|--------------|----------------------|--------------|
| Lanes        | 1  3  5              | 2  4  6      |
| Expression relative to Mus musculus | 4.6 +/- 0.7 | 1 +/- 0.1 |

Supplementary Figure S5. Western Blot showing differences in expression between the MIOX protein from *Arabidopsis thaliana* and *Mus musculus*. Equal volumes of cell lysates were loaded into each lane. Protein concentrations were determined by Bradford assay[4]. Densitometry was performed by ImageJ[5]. Relative expression was normalized to gel 2 using *Mus musculus* as a reference (values listed as “Area normalized to Gel 2 using *Mus musculus* densitometry”), and then normalized by total protein loaded (values listed as “Total protein loaded (mg/mL”)’). These values (“Normalized expression by total protein”) were averaged relative to *Mus musculus*, leading to a relative difference of expression of about 5-fold.
Supplementary Figure S6. Phosphate-limited chemostat experiment using the *A. thaliana* MIOX strain shows production of D-glucaric acid under conditions of probable glucose repression. Data points are the average of triplicate measurements with error bars indicating one standard deviation from the mean.
## Supplementary Tables

### Table S1. Strains and primers for strain construction and validation, plasmids and primers for plasmid construction.

| Name                     | Relevant Genotype                                                                 | Reference                                                                 |
|--------------------------|-----------------------------------------------------------------------------------|---------------------------------------------------------------------------|
| **Strains**              |                                                                                   |                                                                           |
| DH5α                     | F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ-thi-1 gyrA96 relA1 | Life Technologies (Carlsbad, CA)                                          |
| CEN.PK2-1D               | MAT α; ura3-52; trp1-289; leu2-3,112; his3 Δ1; MAL2-8C; SUC2                         | EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis (EUROSCARF) (Bad Homburg, Germany) |
| CEN.PK2-1D               | MAT α; ura3-52; trp1-289; leu2-3,112; his3 Δ1; MAL2-8C; SUC2; op1::KanR               | This study                                                                |
| **Plasmids/DNA source**  |                                                                                   |                                                                           |
| pRS306                   | Integrating plasmid, URA3 marker, MCS derived from pBLUESCRIPT (ColE1 (derivative) ori, f1 ori, AmpR) | Gift from Professor Narendra Maheshri                                       |
| pRS303                   | Integrating plasmid, HIS3 marker, MCS derived from pBLUESCRIPT (ColE1 (derivative) ori, f1 ori, AmpR) | Gift from Professor Narendra Maheshri                                       |
| pOE511-3xIno1Inm1        | Integrating plasmid, KIURA3 with repeated homology, UAS3xclb-GPDpr-Ino1-act1t, Tef1pr-Inm1-cyc1t | This study                                                                |
| pOE521-3xMmMioxUdh       | Integrating plasmid, KIURA3 with repeated homology, UAS3xclbGPDpr-MmMiox-act1t, Tef1pr-Udh-cyc1t | This study                                                                |
| pOE521-3xHISMMmMioxUdh   | Integrating plasmid, KIURA3 with repeated homology, UAS3xclbGPDpr-6xHistag-MmMiox-act1t, Tef1pr-Udh-cyc1t | This study                                                                |
| pOE521-3xAtMioxUdh       | Integrating plasmid, KIURA3 with repeated homology, UAS3xclbGPDpr-AtMiox-act1t, Tef1pr-Udh-cyc1t | This study                                                                |
| pOE521-3xHISAtMioxUdh | Integrating plasmid, KIURA3 with repeated homology, UAS3xclbGPDr-6xHistag-AtMiox-act1t, Tef1pr-Udh-cycl1t | This study |
|------------------------|-------------------------------------------------------------------------------------------------|-----------|
| pFA6a-kanMX6 | For deletion of OPI | Gift from Jürg Bähler & John Pringle (Addgene plasmid # 39296) [6] |
| pJ2-Miox | DNA source for M. musculus MIOX homolog | [7] |
| Genestring At-Miox | GeneArt® String™ for MIOX from A. thaliana codon-optimized for expression in S. cerevisiae | Life Technologies |
| pTrc-Udh | DNA source for Udh | [7, 8] |

**Oligonucleotides**

| 5’ $\rightarrow$ 3’ Sequence |
|-------------------------------|
| PrKO-OPI1(+) | TTAAAGCGTG TGTATCAGGA CAGTGTTTTT AACGAAGATA CTAGTCATTG GATCTGT TTAGCTTGCCCTCGTCCCC |
| PrKO-OPI1(-) | TATAATATTTAATTACTGTTGGTAATGCATGAAACACCTCAATCTCTCGG AACTGGATGCGCAGGCTTG |
| OPI1kochk(+) | ATTGCAGTACCTGCTTCTCTCTCTT |
| prKOchk(-) | GAGGCCCGAATACCCTCTCTTCTT |
| LEU2pr(+) | GGTCGCTGACGCACTATAC |
| KIU(-) | GCTTATCGCAATGGTTGTAATGACCTCAATCTCTCGG |
| KIU(+) | GTGATTCTGGGTAGAAGATCGA |
| LEU2t(-) | tgttcagtaatggctggttc |

**Plasmid construction**

| SpeI-Ino1(+) | actagt aacaaa atgacagaagataaatatgtgct |
| NotI-Ino1(-) | GCGGCAGC TTA TTACAACAAATCTCTCTCTGGAATCTTTAG |
| Nhel-Inm1(+) | GCTAGC AACAAA ATGACCATTGTCTAGCTTCTATCG |
| PacI-Inm(-) | ttaat tta ccgctcatatatttttatgcgc |
| SpeI-MmMiox(+) | gagga actagt aacaaa aagttatgtggatgtg |
| NotI-MmMiox(-) | aataat gcgcggtcc tta tta CCAGACAGGCTG |
| Nhel-Udh(+) | gagga gactag aacaaa ATGCCAATCGC |
| Xmal-Udh(-) | gagga cccggg aagctg gcaccaga ttaat tta ttatgcggcagtcggt |

**Strain construction and validation**

| PrKO-OPI1(+) | TTAAAGCGTG TGTATCAGGA CAGTGTTTTT AACGAAGATA CTAGTCATTG GATCTGT TTAGCTTGCCCTCGTCCCC |
| PrKO-OPI1(-) | TATAATATTTAATTACTGTTGGTAATGCATGAAACACCTCAATCTCTCGG AACTGGATGCGCAGGCTTG |
| OPI1kochk(+) | ATTGCAGTACCTGCTTCTCTCTCTT |
| prKOchk(-) | GAGGCCCGAATACCCTCTCTTCTT |
| LEU2pr(+) | GGTCGCTGACGCACTATAC |
| KIU(-) | GCTTATCGCAATGGTTGTAATGACCTCAATCTCTCGG |
| KIU(+) | GTGATTCTGGGTAGAAGATCGA |
| LEU2t(-) | tgttcagtaatggctggttc |
| SpeI-AtHIS(+) | gagga ACTAGT AACAAA atgggtcatctcaccatcaccatcacttcaggt ACCATCTCCGTTGAAAAAGCC |
|-------------|--------------------------------------------------------------------------------|
| SpeI-MmHIS(+) | gagga actagt aacaaa atgggtcatctcaccatcaccatcacttcaggt aaagttgatggtggtc  |

Table S2. Genetic elements, corresponding primers and DNA source for construction of pOE511.

| Order | Name          | 5’ primer             | 3’ primer             | Template DNA source          |
|-------|---------------|-----------------------|-----------------------|------------------------------|
| 1     | LEU2 promoter | gagga ctgcag GGTGCGCTTGCACGCAT ATAC | GAGGA gagctc GCTGAAAATGTAAAAG GTAAGAAAAG | CEN.PK2-1D |
| 2     | ACT1 terminator | cgcc cgagctc AATTTTTGAAATTTTC GTAGAAAAAGGG | ataat gtcgac gttcatgt gcggccgc TCTGCTTTTGTGCGCG TATG | CEN.PK2-1D |
| 3     | TDH3 promoter | gagga actagt TTGTGTTGTTATGTG TGTTTATTCG | gagga ctgtag CGAGTTTATCATTATC AATACTGCC | CEN.PK2-1D |
| 4     | UAS-CLB2 | gagga ctgtag GGGACAGGCACCGAA GTTC | gagga gcggcgc atctgtaa gcggcc gttcatcg cctaggg AATTTTGTGCGCG TATG | CEN.PK2-1D |
| 5     | UAS-CLB2 | gagga cctagg GGGACAGGCACCGAA GTTC | gagga gcggcc GCAGTTTATTAGTAGA ATGACCACTAC | CEN.PK2-1D |
| 6     | UAS-CLB2 | gagga gcggcgc GGGACAGGCACCGAA GTTC | gagga gcggcc GCAGTTTATTAGTAGA ATGACCACTAC | CEN.PK2-1D |
| 7     | TEF1 promoter | gagga gcggccgc ATAGCTTCAAATAATGT TCTACTCC | gagga gcggccgc gtttggc agtttgct gcgaattaggtgtg GTCTATGCTTTC | CEN.PK2-1D |
| 8     | CYC1 terminator | gagga TTAATTAA CATGTAATTAGTTATG TCACGCTTAC | GCGGC aagcct TAAAGCTCTTCAGCG TCCC | CEN.PK2-1D |
| 9     | Marker removal homology | CACCA GAATTC AACGTGGCGAGAAAG GAAG | GAGGA gggccc gttaccaatctgcc | pBluescriptII-SK(+) |
| 10    | KIURA3 marker | GAGGA gggccc ggagacaatcatatgg | GAGGA aagctt gtttgaatgtgtgtaatgg | K. lactis genomic DNA (gift of A. Regev) |
Table S3. Genetic elements, corresponding primers and DNA source for construction of pOE521.

| Order | Name                  | 5’ primer                          | 3’ primer                          | Template DNA source                      |
|-------|-----------------------|------------------------------------|------------------------------------|----------------------------------------|
| 1     | KlURA3 marker         | GAGGA gggccc gtagagacaatcatatggg    | GAGGA aagctt gttatagcagttgtatgg    | K. lactis genomic DNA (gift of A. Regev) |
| 2     | Marker removal homology| CACCA aagctt AACGTGGCGAGAAAG GAAG | CACCA gaacct ggtacccaattccgccc    | pBluescriptII-SK(+)                     |
| 3     | CYC1 terminator       | GCGGC aagctt TAAGCCTTCGAGCAG TCCC | gagga TTAATTAA CATGTAATTAGTTATG TCACGCTTAC | CEN.PK2-1D                             |
| 4     | TEF1 promoter         | gagga ggcgc gcctagct gcctag TAACCTGTATTAG ATGACCACCTAC | gagga ggcgc ATAGCTTTAAAAATGT TCTACTCC | CEN.PK2-1D                             |
| 5     | UAS-CLB2              | gagga ggcgc gctagct gctagc AGTGAATTATTAGA ATGACCACCTAC | gagga cctagc GGGACAGGCACCGAGA GTTC | CEN.PK2-1D                             |
| 6     | UAS-CLB2              | gagga ggcgc AGTGAATTATTAGA ATGACCACCTAC | gagga cctagc GGGACAGGCACCGAGA GTTC | CEN.PK2-1D                             |
| 7     | UAS-CLB2              | gagga ggcgc gctagct gctagc AGTGAATTATTAGA ATGACCACCTAC | gagga ggcgc GGGACAGGCACCGAGA GTTC | CEN.PK2-1D                             |
| 8     | TDH3 promoter         | gagga cttaca CGAGTTTATCATATC AATACGCC | gagga aactag TTTGTTGTTTATG TGTTATTCG | CEN.PK2-1D                             |
| 9     | ACT1 terminator       | ataat gtagac gttcagt gggctgctg TGCCGTTTTGGT TCTGACATGC | cgccg gacctc AAAAAATTTTTCTAGAAAAAGG | CEN.PK2-1D                             |
| 10    | LEU2 terminator       | GAGGA gagctc GAAACGACACGAAAT TACAAAATTG | gagga cttacg ctacatcaggtgctagg | CEN.PK2-1D                             |
Supplementary References

[1] Gietz, R.D., Schiestl, R.H., High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat. Protoc. 2007, 2, 31–4.

[2] Gresham, D., Desai, M.M., Tucker, C.M., Jenq, H.T., et al., The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. PLoS Genet. 2008, 4, e1000303.

[3] Meijer, M.M., Boonstra, J., Verkleij, A.J., Verrips, C.T., Glucose repression in Saccharomyces cerevisiae is related to the glucose concentration rather than the glucose flux. J. Biol. Chem. 1998, 273, 24102–7.

[4] Zor, T., Selinger, Z., Linearization of the Bradford Protein Assay Increases Its Sensitivity: Theoretical and Experimental Studies. Anal. Biochem. 1996, 236, 302–308.

[5] Schneider, C.A., Rasband, W.S., Eliceri, K.W., NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 2012, 9, 671–675.

[6] Bahler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., et al., Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. Yeast 1998, 14, 943–951.

[7] Moon, T.S., Yoon, S.H., Lanza, A.M., Roy-Mayhew, J.D., et al., Production of glucaric acid from a synthetic pathway in recombinant Escherichia coli. Appl. Environ. Microbiol. 2009, 75, 589–595.

[8] Yoon, S.-H., Moon, T.S., Iranpour, P., Lanza, A.M., et al., Cloning and characterization of uronate dehydrogenases from two pseudomonads and Agrobacterium tumefaciens strain C58. J. Bacteriol. 2009, 191, 1565–1573.