Engineering energetically efficient transport of dicarboxylic acids in yeast Saccharomyces cerevisiae

Darbani, Behrooz; Stovicek, Vratislav; Van Der Hoek, Steven Axel; Borodina, Irina

Published in:
Proceedings of the National Academy of Sciences of the United States of America

Link to article, DOI:
10.1073/pnas.1900287116

Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Darbani, B., Stovicek, V., Van Der Hoek, S. A., & Borodina, I. (2019). Engineering energetically efficient transport of dicarboxylic acids in yeast Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America, 116(39), 19415-19420. https://doi.org/10.1073/pnas.1900287116
Engineering energetically efficient transport of dicarboxylic acids in yeast Saccharomyces cerevisiae

Behroz Darbani⁹, Vratislav Stovicke⁹, Steven Axel van der Hoek⁹, and Irina Borodina⁹,1

*The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

Biobased C4-dicarboxylic acids are attractive sustainable precursors for polymers and other materials. Commercial scale production of these acids at high titers requires efficient secretion by cell factories. In this study, we characterized 7 dicarboxylic acid transporters in Xenopus oocytes and in Saccharomyces cerevisiae engineered for dicarboxylic acid production. Among the tested transporters, the Mae1(p) from Schizosaccharomyces pombe had the highest activity toward succinic, malic, and fumaric acids and resulted in 3-, 8-, and 5-fold titer increases, respectively, in S. cerevisiae, while not affecting growth, which was in contrast to the tested transporters from the tellurite-resistance/dicarboxylate transporter (TDT) family or the Na⁺ coupled divergent anion–sodium symporter family. Similar to SpMae1(p), its homolog in Aspergillus carbonarius, AcDct(p), increased the malate titer 12-fold without affecting the growth. Phylogenetic and protein motif analyses mapped SpMae1(p) and AcDct(p) into the voltage-dependent slow-anion channel transporter (SLAC1) clade of transporters, which also include plant Slac(p) transporters involved in stomata closure. The conserved phylolalanine residue F329 closing the transport pore of SpMae1(p) is essential for the transporter activity. The voltage-dependent SLAC1 transporters do not use proton or Na⁺ motive force and are, thus, less energetically expensive than the majority of other dicarboxylic acid transporters. Such transporters present a tremendous advantage for organic acid production via fermentation allowing a higher overall product yield.

Expression of the Yeast Mitochondrial Membrane Transporter Ctp1(p) in the Plasma Membrane of Xenopus laevis Oocytes. We used the Xenopus oocytes for functional analysis of the transporters (24). To enable the study of a mitochondrial transporter, we designed a construct for targeting transporters into the plasma membrane.

Significance

The export of organic acids is typically proton or sodium coupled and requires energetic expenditure. Consequently, the cell factories producing organic acids must use part of the carbon feedstock on generating the energy for export, which decreases the overall process yield. Here, we show that organic acids can be exported from yeast cells by voltage-gated anion channels without the use of proton, sodium, or ATP motive force, resulting in more efficient fermentation processes.

Author contributions: B.D. and I.B. designed research; B.D., V.S., and S.A.v.d.H. performed research; B.D., V.S., S.A.v.d.H., and I.B. analyzed data; and B.D., V.S., S.A.v.d.H., and I.B. wrote the paper.

The authors declare no conflict of interest.

This open access article is distributed under the Creative Commons Attribution License 4.0 (CC BY).

To whom correspondence may be addressed. Email: irbo@biosustain.dtu.dk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1900287116/-/DCSupplemental.

First published August 29, 2019.

www.pnas.org/cgi/doi/10.1073/pnas.1900287116 PNAS | September 24, 2019 | vol. 116 | no. 39 | 19415–19420
Here, we took advantage of the N-terminal segment of the human calcium release-activated calcium channel Orai1(p) (amino acids 71–246; GenBank: NP_116179). The N-terminal segment is responsible for protein localization in plasma membrane both in native cells and upon expression in *Xenopus* oocytes (26, 27). We combined the Orai1(p) peptide segment with Gfp(p) or with ScCtp1-Gfp as N-terminal fusions and expressed these constructs in oocytes. The localization was studied by confocal laser scanning microscopy, scanning along the z axis from the surface toward the deep cytosolic space of oocytes (27, 28). The N-tagged variants of Gfp(p) and Ctp1(p) were shown to localize in the plasma membrane of oocytes, while their non-tagged variants were expressed in the cytosolic space (Fig. 1A).

**Functional Analysis of the Transporters in Oocytes.** The candidate membrane transporters (Table 1) were subjected to functional analysis upon expression in *Xenopus* oocytes. Oocytes expressing each of the candidate transporters were injected with citrate and fumarate (estimated internal concentrations of 2 and 1.5 mM, respectively), which could also be converted into succinate and malate through the TCA cycle within oocytes. After incubating the oocytes in a buffer for 3 h, the concentrations of exported dicarboxylic acid were measured by LC-MS. *Ec*Dcu-p, *Ec*Dcu-b, *As*Slc13-p, and *As*Dct(p) were able to export citrate (Fig. 1B). *Sp*MAe1(p), the most closely related transporter to the Dcu(p) transporters did not show citrate export capability (Fig. 1B). On the other hand, *Sp*MAe1(p) was able to export fumarate, succinate, and malate (Fig. 1B). *As*Dct(p) and ScCtp1(p) fused to the Orai1(p) peptide (Leader-ScCtp1) showed some detectable export of fumarate and malate.

**Effect of the Transporters on the Production of C4-Dicarboxylic Acids in *S. cerevisiae.*** To examine the performance of the carboxylic acid transporters in a yeast cell factory, we expressed them in a...
S. cerevisiae strain engineered for the production of C4-dicarboxylic acids. The strain was created based on the evolved pyruvate decarboxylase-deficient strain (29) in which we overexpressed the native cytosolic pyruvate carboxylases Pyc1(p) and Pyc2(p) and malate dehydrogenase without the peroxisomal targeting signal (Mdh3(p)) to retain it in the cytosol (30). This engineered strain was able to produce up to 0.5 and 0.8 g/L of extracellular malate and succinate, respectively (Fig. 1C). To enhance the efflux, we additionally expressed the most efficient transporters determined in the Xenopus oocyte screen: SpMae1(p), ScCtp1(p), AsDct(p), and AsSlc13(p) (Fig. 1B). In agreement with the oocyte assays, it was again the SpMae1(p) showing the highest transport rate (Fig. 1C). We found up to an 8-fold increase (4.3 g/L) in the malate titer upon expression of SpMae1 (Fig. 1C). The titer of succinate and fumarate also increased to 2.6 g/L (3-fold increase) and 0.33 g/L (5-fold increase), respectively (Fig. 1C). As expected, expression of ScCtp1(p), AsDct(p), and AsSlc13(p) affected the growth of yeast, resulting in a lower final OD800 and a slower glucose utilization. On the contrary, the expression of SpMae1(p) did not exhibit a negative effect on the growth of yeast cells, and the glucose consumption was similar to the control strain without a heterologous transporter (Fig. 1C).

SpMae1(p) Is a Member of the SLAC1 Family. The SpMae1(p) transporter was initially identified on the basis of a mutant defective in malate uptake (19, 21, 31, 32). However, other experiments have also shown SpMae1(p)’s ability to improve the export of carboxylic acids, including malate, from yeast cell factories (22, 23). In our experiments, the expression of SpMae1(p) did not impair the growth of yeast cells, while increasing the secretion of acids several fold. This was an indication that the production of acids was not coupled to growth, e.g., due to the secretion of acids all inhibited the cellular growth.

Conserved Phenylalanine Residue Found in the Transport Channel of Slac1(p) Transporters Is Critical for the Activity of SpMae1(p). Alignment of Slac1(p) transporters, including Mae1(p) transporters, uncovered 2 conserved phenylalanine residues located within the transport channel (F107 and F329) in transmembrane domain 5 and F329 in transmembrane domain 9. These residues close the transporter tunnel with their phenyl rings (Fig. 3 B and C). While the F329 of SpMae1(p) is 100% conserved in all of the investigated SLAC1 transporters, the F107 was substituted by similar amino acid tyrosine in AtrSlah1(p) and AtrSlah4(p) (Fig. 3C). To examine the essentiality of these residues, we created a single-residue mutant SpMae1(p)F107A and a double-residue mutant SpMae1(p)F107A,F329A and expressed them individually in S. cerevisiae. These changes removed the phenyl rings, respectively, from the middle or middle and cytosolic faces of the transport channel (Fig. 3B). While the in silico structure modeling predicted that mutations, particularly in combination, would a bacterial homolog of plant Slac1(p) from Arabidopsis thaliana than to the transporters from the DCU and ALMT families (Fig. 3A and SI Appendix, Table S2).

**Table 1. Dicarboxylic acid membrane transporters**

| Transporter* | Transporter Family/Class | Homologs† |
|--------------|--------------------------|-----------|
| S. cerevisiae Mae1 | TDT/2.A.16 | Plant |
| A. oryzae Mae1 | TDT/2.A.16 | Plant |
| S. cerevisiae Ctp1 | Mitochondrial Carrier/2.A.29 | Animal, plant |
| A. succinogenes Dct | DiCarboxylate Uptake/2.A.13 | Animal |
| A. succinogenes Slc13 | Divalent anion:Na+ symporter/2.A.47 | Animal, plant, Fungi |
| E. coli Dctb | DiCarboxylate uptake/2.A.13 | Animal |
| E. coli Dccu | DiCarboxylate uptake/2.A.61 | Animal, ungi |

*Sequence accession numbers are provided in SI Appendix, Table S6.†Homologs from other kingdoms, if any, retrieved as the immediate hit from National Center for Biotechnology Information protein blast.

![Fig. 2.](https://example.com/Fig2.png) SpMae1(p) harbors a SLAC1 protein domain responsible for voltage-dependent transport. The maximum likelihood phylogenetic tree was built on the Whelan and Goldman substitutional matrix. Bootstrap and branch lengths are shown in blue and green, respectively. Protein domains were predicted with e values below 10^-40. Sequence accession numbers are provided in SI Appendix, Table S6.
widen the channel (Fig. 3D), the single mutation of F329A and the double mutation of F107A and F329A abolished the effect of SpMae1(p) on malate secretion (Fig. 3E). We additionally found 2 groups of Mae1(p) transporter by comparing Mae1(p) transporters of the Aspergillus species with the Schizosaccharomyces species, the latter distinguished by an extended C-terminal peptide (Fig. 3F). To examine the possible role of this C-terminal peptide, we removed the last 46 amino acids from SpMae1(p) (Fig. 3F) and found up to a 40% decrease in malate secretion from S. cerevisiae (Fig. 3E). This decrease could, however, be explained by the lower expression level of SpMae1(p) without the C-terminal peptide, which was 40% lower than the native protein. The expression levels were determined using C-terminal fusion with a GFP protein (Fig. 4E).

**Fungal Mae1 Transporter from A. carbonarius Also Increases Malate Secretion at Neutral pH.** We then examined the effect of several other SLAC1 transporters on malate production in yeasts. We selected AtSlac1(p) from plant A. thaliana, HiTehA(p) from bacterium H. influenza, and AcDct(p) from fungus A. carbonarius. We also included an ALMT member, AtAlmt12(p) from A. thaliana. Among the examined transporters, AcDct(p) expression resulted in a 12-fold increase in malate titer, while the rest of the transporters lead to a smaller increase in 10–20% (Fig. 4A and B). These experiments were performed using calcium carbonate as the buffering agent in the medium as in the experiments described in the previous sections. To investigate the effect of transporters under low pH, we also performed the same experiment, now omitting the calcium carbonate from the medium. The initial pH of the medium was 4.8, and it rapidly declined to 2.4–2.6 during the cultivation. Overall, malic acid production and the growth were lower in the low-pH cultivation (Fig. 4C and D). SpMae1(p) increased malic acid titer 3-fold and improved the growth (Fig. 4C), but all of the other transporters had a negative effect on both the malic acid titer and the growth.
For the SLAC1 members, we also examined the effect of mutations corresponding to the $SpMae1(p)$F329A. There were no significant changes in the activities of $AtSlac1(p)$ or $HTchA1(p)$. Expression of the mutant $AcDct(p)$F354A resulted in a lower titer of malic acid than in the control strain not expressing a heterologous transporter at low pH (Fig. 4C). Curiously, the strain expressing $AcDct(p)$F354A had a severe growth defect at neutral pH (Fig. 4A), so we could not draw conclusions about the activity of the mutated transporter at neutral pH. To ensure that the observed effects were not just due to the different expression levels of the mutated transporters, we expressed C-terminal GFP fusions of transporters in yeast and measured the fluorescence (Fig. 4E and SI Appendix, Fig. S3). The GFP signal for $SpMae1(p)$ and $AcDct(p)$ mutants was 40–50% lower than for the native transporters. The decreased expression of $SpMae1(p)$ cannot explain the complete loss of activity by the mutated variant of $SpMae1(p)$, so we can conclude that phenylalanine residue F329 is essential for the transporter activity.

Discussion

Dicarboxylic acids, currently mainly produced from petroleum and gas, can be alternatively produced by fermentation of renewable feedstocks. Yeast cell factories are particularly attractive for these processes due to low-pH tolerance (37, 38). Carboxylates need membrane transporters to be secreted out of the cells (39, 40). Proton dissociation from carboxylic acids at neutral pH conditions releases membrane-nondiffusible anion carboxylates (37). The engineering of yeasts for malate production on carbon feedstock resulted in up to 70% of the maximum theoretical yield, and the malate was secreted into the fermentation medium (37, 41–43). Unlike bacterial succinic acid, producers that prefer neutral pH, $S. cerevisiae$ can grow in an acidic medium with a pH range of 3–6, which reduces the need for neutralization and allows direct recovery of an undisassociated form of acids (37). Channels, active pumps, permeases, and mitochondrial carriers are involved in the transport of carboxylic acids across the $S. cerevisiae$ membranes (44). Improvement of malate, succinate, and fumarate secretion in yeast was obtained by expression of the malate transporter gene $Mae1$ from the fission yeast $S. pombe$ (21, 22, 32, 45). Recently, $AcDct(p)$, the homolog of $SpMae1(p)$, was found to boost C4-dicarboxylic acid production in $A. carbonarius$ (46). Originally, $SpMae1(p)$ was annotated as a member of the TDT family and was believed to use a proton as the motive force (19). In agreement with the previous studies, we found that $SpMae1(p)$ is highly active for the export of malate, succinate, and fumarate in oocytes (Fig. 1B) and in yeast cells (Fig. 1C). It was surprising that expression of $SpMae1(p)$ did not affect the cellular growth, in contrast to $ScCtp1(p)$, $AcDct(p)$, and $AsSlc13(p)$ (Fig. 1C). Our phylogenetic and protein motif analyses annotated $SpMae1(p)$ and $AcDct(p)$ as members of the voltage-dependent Slac1(p) transporters (Fig. 2). Together with the rapidly activated $Almt(p)$ channels (Fig. 2), Slac1(p) transporters respond to the voltage changes (depolarization) and export osmolytes, such as malate, nitrate, and chloride anions, which lead to stomatal closure in plants (33, 36, 47). Therefore, $SpMae1(p)$ and $AcDct(p)$ are most likely equipped with mechanisms used by their evolutionary and structurally closely related transporters of the SLAC1 family (Table 1, Fig. 2, and SI Appendix, Fig. S2). This is in contrast to the TDT family where the activity of transporters is coupled with a proton or Na+ ions. We recently addressed the energetic evolution of transporters, both at the level of cellular transportome and also transporter family levels (48). It may be that the same energetic evolution has been playing a role within the proton motive force driven TDT family, giving rise to the voltage-dependent transporters. Unraveling the transport mechanism of $SpMae1(p)$ has the potential for further improvements via engineering for higher transport efficiencies. There are two highly conserved phenylalanine residues in $SpMae1(p)$ (Fig. 3). One of these residues has a phenyl ring at the cytosolic face of the transport pore and the other within the pore (Fig. 3 B and C). Replacing the inner phenylalanine with alanine in plant $Slac1(p)$ and bacterial $TehA(p)$ homologs has been shown to increase the chloride ion currents (34), which is in agreement with the structural changes, i.e., movements of the helices and widening of the channel, that we found should also happen in $SpMae1(p)$ (Fig. 3D). However, our data indicate that these phenylalanine residues of $F107$ and $F329$ in $SpMae1(p)$, while closing the transport channel in the substrate-free state, are also necessary for the transport of carboxylic acids (Figs. 3E and 4A and C). As a conserved motif, the phenylalanine $F107$ and the flanking amino acids have notably been reported as a part of the substrate binding pocket in plant Slac1(p) transporters (49).

To summarize, we showed that the $Mae1(p)$ transporter from $S. pombe$ had a very high activity toward C4-dicarboxylic acids (succinic, malic, and fumaric) in both Xenopus oocytes and yeast $S. cerevisiae$ and that $SpMae1(p)$ did not inhibit the growth of yeast cells both at neutral and at low pH. A homolog $AcDct(p)$...
from *A. carbonarius* could also increase the production of malate in yeast without inhibiting the growth, albeit only at neutral pH. We present evidence that SpMae1(p) and AcDec(p) belong to the voltage-gated anion channel family SLAC1 and their expression results in energetically efficient export of dicarboxylic acids. This finding is important for engineering efficient cell factories for the production of biobased organic acids.

**Materials and Methods**

All of the DNA constructs were built using the USER fusion technique. In vitro transcription of cRNAs were injected into the *X. laevis* oocytes by Roboinject (Multi Channel Systems, Germany). Candidates of membrane transporters were expressed in the *S. cerevisiae* cell factory designed to produce malic acid. A Leica TCS SLP-II confocal microscope was used for localization studies.

**ACKNOWLEDGMENTS.** The authors acknowledge financial support from the Nordoviski Foundation (Grant Agreement NNF10CC1016517), from the European Research Council under the European Union’s Horizon 2020 Research and Innovation Programme (YEAST-TRANS Project, Grant Agreement 727300) and from the European Commission in the 7th Framework Programme (BioREFINE-2G Project, Grant Agreement FP7-613777). The authors also thank Sonnich Sunil Scholin Thacker and Kasper Ivert Hentzer Andersen for assistance with yeast experiments and Hanne Bjerre Christensen for analytics. The authors thank Professor Jack Pronk (TU Delft, The Netherlands) for the kind gift of the *S. cerevisiae* TAM strain.

1. I. Borodina, Understanding metabolite transport gives an upper hand in strain development. Trends Biotechnol. 33, 237–246 (2015).
2. D. B. Kell, N. Swainston, P. Pir, S. G. Oliver, Membrane transporter engineering in industrial biotechnology and whole cell biocatalysis. Trends Biotechnol. 33, 237–246 (2015).
3. Z. Tzambanis et al., Efficient itaconic acid production from glycerol with *Ustilago virensiae* T21. Biotechnol. Biofuels 10, 131 (2017).
4. J.-F. Wang, Z.-Q. Xiong, S.-Y. Li, Y. Wang, Enhancing isoprenoid production through systematically assembling and modulating efflux pumps in Escherichia coli. Appl. Microbiol. Biotechnol. 97, 8057–8067 (2013).
5. S. Boyarsky, S. Davis López, N. Kong, D. Tullman-Ercek, Transcriptional feedback regulation of efflux pump expression for increased tolerance to and production of n-butanol. Metab. Eng. 33, 130–137 (2016).
6. J. L. Foo et al., Improving microbial biomass production in *Escherichia coli* cell using tolerance engineering. Mtbo 5, e01932 (2014).
7. M. J. McAnulty, T. K. Wood, YeeO from Escherichia coli exports flavins. Bioengineering 5, 386–392 (2019).
8. A.-Q. Yu, N. K. Pratomo Juwono, J. L. Foo, S. S. J. Leong, M. W. Chang, Metabolic engineering of *Saccharomyces cerevisiae* for the overproduction of short branched-chain fatty acids. Metab. Eng. 34, 36–43 (2016).
9. H. F. Lam, A. Ghaderi, G. R. Fink, G. Stephanopoulos, Biofuels. Engineering alcohol tolerance in yeast. Science 346, 71–75 (2015a).
10. M. C. Teixeira, C. P. Godinho, T. R. Cabrito, N. P. Mira, I. Sá-Correia, Increased expression of the *Saccharomyces cerevisiae* mdc1 gene modulates flux in the glycerol fermentation pathway. Appl. Environ. Microbiol. 80, 392 (2014).
11. N. Zhu, H. Xia, J. Yang, X. Zhao, T. Chen, Improved succinate production in *Corynebacterium glutamicum* CgynfM encodes a dicarboxylate transporter important for succinate production under aerobic and anaerobic conditions in Enterobacter aerogenes. Microb. Cell Fact. 11, 58 (2012).
12. C. M. Zehnder, B. E. Keller, Two types of anion channel currents in guard cells with distinct voltage regulation. Proc. Natl. Acad. Sci. U.S.A. 89, 5025–5029 (1992).
13. D. A. Abbott, R. M. Zelle, J. T. Pronk, A. J. A. van Maris, Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and challenges. FEMS Yeast Res. 9, 1123–1136 (2009).
14. D. A. Abbott, R. M. Zelle, J. T. Pronk, A. J. A. van Maris, Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and challenges. FEMS Yeast Res. 9, 1123–1136 (2009).
15. M. Bony, Metabolic analysis of *S. cerevisiae* strains engineered for malolactic fermentation. FEMS Lett. 410, 452–456 (1997).
16. H. Voloschenk et al., Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. Nat. Biotechnol. 15, 252–253 (1997).
17. J. Negi et al., CO2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. Nature 452, 483–486 (2008).
18. V. H. Chen et al., Homologue structure of the SLAC1 anion channel for closing stomata in leaves. Nature 467, 1074–1080 (2010).
19. P. Mumm et al., C-terminus-mediated voltage gating of Arabidopsis guard cell anion channel QUAC1. Mol. Plant 6, 1550–1563 (2013).
20. J. I. Schroeder, B. U. Keller, Two types of anion channel currents in guard cells with distinct voltage regulation. Proc. Natl. Acad. Sci. U.S.A. 89, 5025–5029 (1992).
21. D. A. Abbott, R. M. Zelle, J. T. Pronk, A. J. A. van Maris, Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and challenges. FEMS Yeast Res. 9, 1123–1136 (2009).
22. D. A. Abbott, R. M. Zelle, J. T. Pronk, A. J. A. van Maris, Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and challenges. FEMS Yeast Res. 9, 1123–1136 (2009).
23. D. A. Abbott, R. M. Zelle, J. T. Pronk, A. J. A. van Maris, Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and challenges. FEMS Yeast Res. 9, 1123–1136 (2009).
24. D. A. Abbott, R. M. Zelle, J. T. Pronk, A. J. A. van Maris, Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and challenges. FEMS Yeast Res. 9, 1123–1136 (2009).
25. D. A. Abbott, R. M. Zelle, J. T. Pronk, A. J. A. van Maris, Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and challenges. FEMS Yeast Res. 9, 1123–1136 (2009).
26. C. Y. Park, J. S. Park, K. Shin, Constitutive recycling of the store-operated Ca2
+ channel QUAC1. Biochim. Biophys. Acta 1857, 1123–1136 (2016).
27. F. Yu, L. Sun, K. Machaca, Constitutive recycling of the store-operated Ca2
+ channel Orai1 and its internalization during meiosis. J. Cell Biol. 191, 523–535 (2011).
28. V. S. Subramanian, S. M. Nabokina, Y. Lin-Moshier, J. S. Marchant, H. M. Said, Mito- \( \text{STIM1} \) clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. Cell 136, 876–890 (2009).
29. J. Chen et al., Directed evolution of pyruvate decarboxylase-negative *Sac- charomyces cerevisiae*, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. Appl. Environ. Microbiol. 70, 159–166 (2004).
30. A. J. van Maris et al., Metabolic engineering of *Saccharomyces cerevisiae* for the overproduction of short branched-chain fatty acids. Metab. Eng. 33, 19420 (2015).
31. J. Chen et al., Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. Appl. Environ. Microbiol. 70, 159–166 (2004).
32. J. Chen et al., Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. Appl. Environ. Microbiol. 70, 159–166 (2004).
33. J. Chen et al., Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. Appl. Environ. Microbiol. 70, 159–166 (2004).
34. J. Chen et al., Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproducing yeast. Appl. Environ. Microbiol. 70, 159–166 (2004).
35. J. Chen et al., Directed evolution of pyruvate decarboxylase-negative *Saccharomyces cerevisiae*, yielding a C2-independent, glucose-tolerant, and pyruvate-hyperproduc