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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):

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Engineering energetically efficient transport of dicarboxylic acids in yeast *Saccharomyces cerevisiae*

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Edited by James C. Liao, Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan, and approved August 5, 2019 (received for review January 9, 2019)

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 divalent 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 Slac1(p) transporters involved in stomata closure. The conserved pherylalanine 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.

**Results**

**Selection of Candidate Dicarboxylic Acid Transporters.** We examined several candidates of carboxylic acid transporter genes from different organisms (Table 1). We included the *Mae1(p)* from *S. pombe*, previously shown to be effective for malate and succinate production in *S. cerevisiae* (21–23) and its homolog in the natural malic acid producer *Aspergillus oryzae* *AoMae1*. Interestingly, the *AoMae1* gene is colocaled with the succinyl-CoA ligase gene, involved in the tricarboxylic acid cycle (*SI Appendix*, Fig. S1). We also selected *S. cerevisiae* mitochondrial citrate transporter *Ctp1(p)*. From the bacterial transporters, we chose two dicarboxylate transporters *EcDcu(p) and EcDcb(p)* from *E. coli* (17) and a homolog of *EcDcb(p)*, a putative transporter called *AsDct(p)* from the succinate producer *Actinobacillus succinogenes*. The genes encoding *EcDcu(p) and EcDcb(p)* in *E. coli* are colocaled with the furmarase B gene and with genes involved in citrate utilization. Finally, we found a putative transporter *SLC13(p)* that was colocaled with succinyl-CoA synthetase and oxoglutarate dehydrogenase genes in *A. succinogenes*. The selected 7 transporter candidates were phylogenetically classified into 5 transporter families (Table 1). For *Mae1(p)* transporters, homologs could be found only in fungi and plant kingdoms, while for the DCU and DCUC families, the homologs were found in animals and fungi but not in plants. Several of the selected transporter genes were adjacent to related pathway genes, and these phenomena can be exploited for transporter function prediction as illustrated previously for secondary metabolites in bacteria and plants (24, 25).

**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.


The authors declare no conflict of interest.

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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 nontagged 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. *EcDcuB(p), EcDcuA(p), AsSliC13(p), and AsDct(p)* were able to export citrate (Fig. 1B). *SpMae1(p),* the most closely related transporter to the Dcu(p) transporters did not show citrate export capability (Fig. 1B). On the other hand, *SpMae1(p)* was able to export fumarate, succinate, and malate (Fig. 1B). *AsDct(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

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**Fig. 1.** Carboxylic acid transport assays in *Xenopus* oocytes and *S. cerevisiae*. (A) The expression of GFP, ScCtp1-GFP, and their N-terminal fusions with the HsOrai1 leader peptide was examined by confocal microscopy, scanning oocytes on the z axis from the most outer surface toward the inner cytoplasmic space. (B) Efflux of carboxylic acids from oocytes. The bars represent the carboxylic acid contents of the medium (means of 3 to 4 biological replicates each involving 20 oocytes with SDs shown as error bars) 3 h after injecting fumarate and citrate into the control (water injected) oocytes with no heterologous transporter and into the oocytes expressing individual candidate transporters. Asterisks mark significant changes in comparison with the control (***P < 0.01, *P < 0.05). The carboxylic acid concentrations in the medium were also examined before metabolite injection (before injection) and after 3 h incubation of oocytes without injecting metabolites (without injection). (C) Time course of metabolite concentrations in the fermentation broth of the transporter-expressing *S. cerevisiae* strains and the control strain. Error bars show the SDs of 3 biological replicates.
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**Table 1. Dicarboxylic acid membrane transporters**

<table>
<thead>
<tr>
<th>Transporter*</th>
<th>Transporter Family/Class</th>
<th>Homologs†</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em> Mae1</td>
<td>TDT/2.A.16</td>
<td>Plant</td>
</tr>
<tr>
<td><em>A. oryzae</em> Mae1</td>
<td>TDT/2.A.16</td>
<td>Plant</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> Ctp1</td>
<td>Mitochondrial Carrier/2.A.29</td>
<td>Animal, plant</td>
</tr>
<tr>
<td><em>A. succinogenes</em> Dct</td>
<td>DiCarboxylate Uptake/2.A.13</td>
<td>Animal</td>
</tr>
<tr>
<td><em>A. succinogenes</em> Slc13</td>
<td>Divalent anion:Na⁺ symporter/2.A.47</td>
<td>Animal, plant, Fungi</td>
</tr>
<tr>
<td><em>E. coli</em> Dcub</td>
<td>DiCarboxylate uptake/2.A.13</td>
<td>Animal</td>
</tr>
<tr>
<td><em>E. coli</em> Dccu</td>
<td>DiCarboxylate uptake/C2.A.61</td>
<td>Animal, ungi</td>
</tr>
</tbody>
</table>

*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.

*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]ASKL) 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), *A. succinogenes* Dct(p), and *A. succinogenes* Slc13(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(p) (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), *A. succinogenes* Dct(p), and *A. succinogenes* Slc13(p) affected the growth of yeast, resulting in a lower final OD₆₀₀ 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 improved redox balance. The other transporters that also increased the secretion of acids all inhibited the cellular growth. We, therefore, hypothesized that the SpMae1(p) transporter must have a different transport mechanism with less energetic expenditure. To investigate this, we performed a protein motif search on SpMae1(p) using the Pfam library version 31 with 16,712 models (available at http://ftp.ebi.ac.uk/pub/databases/Pfam/releases/ Pfas/releases/) and Gene3d models version 16 including 65,016 models (available at http://download.cathdb.info/gene3d/v16.0.0/ gene3d hmmsearch/). SpMae1(p) was annotated as a voltage-dependent slow activating (S-type) anion channel 1 (SLAC1) with e values below 10⁻⁷⁻ (Fig. 2). The closest homolog of SpMae1(p) in *S. cerevisiae* is sulfite pump ScSsu1(p), and it contains a SLAC1 Pfam motif. The phylogenetic relationship with other annotated Slac1(p) transporters from plants (33), bacteria (34), and with the closest homologs is illustrated in Fig. 2. The analysis also distinguished the aluminum-activated malate transporters (ALMTs) (Fig. 2), which are also voltage dependent (35, 36). It must be noted that malate transport by SpMae1(p) did not show any proton exchange in a previous study (21), which is in agreement with the SLAC1 family annotation. The predicted structure of SpMae1(p) is more similar (TM-score 0.704) to the experimentally determined structure of *Haemophilus influenza* HtTehAp(p) (34) as 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, Fig. S2).

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, F329 in transmembrane domain 3 and F329, F329 in transmembrane domain 9). These residues close the transporter tunnel with their phenyl rings (Fig. 3B 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 ArtSla1(p) and ArtSla4(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
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 *ArlSlac1(p)* from plant *A. thaliana*, *HipTeA(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 \( Sp\text{Mae1}(p) \)F329A. There were no significant changes in the activities of \( As\text{Slac1}(p) \) or \( Hi\text{TehA}(p) \). Expression of the mutant \( Ac\text{Dct}(p) \)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 \( Ac\text{Dct}(p) \)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 \( Sp\text{Mae1}(p) \) and \( Ac\text{Dct}(p) \) mutants was 40–50% lower than for the native transporters. The decreased expression of \( Sp\text{Mae1}(p) \) cannot explain the complete loss of activity by the mutated variant of \( Sp\text{Mae1}(p) \), 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, \( S.\text{cerevisiae} \) 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 undissociated form of acids (37). Channels, active pumps, permeases, and mitochondrial carriers are involved in the transport of carboxylic acids across the \( S.\text{cerevisiae} \) 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 \( S.\text{pombe} \) (21, 22, 32, 45). Recently, \( Ac\text{Dct}(p) \), the homolog of \( Sp\text{Mae1}(p) \), was found to boost C4-dicarboxylic acid production in \( A.\text{carbonarius} \) (46). Originally, \( Sp\text{Mae1}(p) \) 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 \( Sp\text{Mae1}(p) \) 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 \( Sp\text{Mae1}(p) \) did not affect the cellular growth, in contrast to \( Sc\text{Ctp1}(p) \), \( As\text{Dct}(p) \), and \( As\text{Slc13}(p) \) (Fig. 1C). Our phylogenetic and protein motif analyses annotated \( Sp\text{Mae1}(p) \) and \( Ac\text{Dct}(p) \) as members of the voltage-dependent \( Slac1(p) \) transporters (2). Together with the rapidly activated \( Alm1(p) \) 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, \( Sp\text{Mae1}(p) \) and \( Ac\text{Dct}(p) \) 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 \( Sp\text{Mae1}(p) \) has the potential for further improvements via engineering for higher transport efficiencies. There are two highly conserved phenylalanine residues in \( Sp\text{Mae1}(p) \) (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 \( Sp\text{Mae1}(p) \) (Fig. 3D). However, our data indicate that these phenylalanine residues of F107 and F329 in \( Sp\text{Mae1}(p) \), 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 participated in the structural changes that lead to the activation of the channel (49). To summarize, we showed that the \( Mae1(p) \) transporter from \( S.\text{pombe} \) had a very high activity toward C4-dicarboxylic acids (succinic, malic, and fumaric) in both \( Xenopus \) oocytes and yeast \( S.\text{cerevisiae} \) and that \( Sp\text{Mae1}(p) \) did not inhibit the growth of yeast cells both at neutral and at low pH. A homolog \( Ac\text{Dct}(p) \)
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 AcDct(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 transcribed 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 SPS-II confocal microscope was used for localization studies.

**Acknowledgments.** The authors acknowledge financial support from the Novo Nordisk 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 727687) and from the European Commission in the 7th Framework Pro- gramme (BioREFINE-2G Project, Grant Agreement FP7-613777). The authors also thank Sonnich Sunil Scholin Thacker and Kasper Ivert Hentzen Andersen for assistance with yeast experiments and Hanne Bjøre Christiansen for analytics. The authors thank Professor Jack Prong (TU Delft, The Netherlands) for the kind gift of the *S. cerevisiae* TAM strain.


**Supplementary Materials and Methods and Tables S1–S5**

**Acknowledgments.** The authors acknowledge financial support from the Novo Nordisk 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 727687), 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 Hentzen Andersen for assistance with yeast experiments and Hanne Bjøre Christiansen for analytics. The authors thank Professor Jack Prong (TU Delft, The Netherlands) for the kind gift of the *S. cerevisiae* TAM strain.