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Identification and application of keto acids transporters in *Yarrowia lipolytica*Hongwei Guo^{1,3}, Peiran Liu¹, Catherine Madzak², Guocheng Du^{1,3}, Jingwen Zhou^{1,3} & Jian Chen^{1,3}

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Production of organic acids by microorganisms is of great importance for obtaining building-block chemicals from sustainable biomass. Extracellular accumulation of organic acids involved a series of transporters, which play important roles in the accumulation of specific organic acid while lack of systematic demonstration in eukaryotic microorganisms. To circumvent accumulation of by-product, efforts have been orchestrated to carboxylate transport mechanism for potential clue in *Yarrowia lipolytica* WSH-Z06. Six endogenous putative transporter genes, YALI0B19470g, YALI0C15488g, YALI0C21406g, YALI0D24607g, YALI0D20108g and YALI0E32901g, were identified. Transport characteristics and substrate specificities were further investigated using a carboxylate-transport-deficient *Saccharomyces cerevisiae* strain. These transporters were expressed in *Y. lipolytica* WSH-Z06 to assess their roles in regulating extracellular keto acids accumulation. In a *Y. lipolytica* T1 line over expressing YALI0B19470g, α -ketoglutarate accumulated to $46.7 \text{ g} \cdot \text{L}^{-1}$, whereas the concentration of pyruvate decreased to $12.3 \text{ g} \cdot \text{L}^{-1}$. Systematic identification of these keto acids transporters would provide clues to further improve the accumulation of specific organic acids with higher efficiency in eukaryotic microorganisms.

Light have been shed on economic feasibility of production of organic acids by microorganisms, especially considering the finiteness of fossil raw materials¹. Although organic acids occupy a key position among the building-block chemicals, economic production by microorganisms is still a big challenge to replace petroleum-derived commodity chemicals². In particular, bio-based production of organic acids is unharnessed by low titres, low yield and accumulation of by-products^{3–5}.

The metabolism and accumulation of α -ketoglutaric acid (α -KG) are subjected to a higher level of regulation than other organic acids involved in central carbon metabolism⁶, as α -KG occupies a key position in both carbon central metabolism⁷ and the regulation of the carbon–nitrogen balance⁸. Metabolic strategies concerning the following have been investigated for α -KG production: regulation of key enzymes, including pyruvate dehydrogenase complex⁹, pyruvate carboxylase¹⁰, fumarase³, aconitase¹¹, isocitrate lyase¹², isocitrate dehydrogenase¹³ and components of the α -ketoglutarate dehydrogenase complex¹⁴; and co-factor engineering of acetyl-CoA biosynthesis and regeneration¹⁵.

Extracellular accumulation of non-target carboxylates is a common problem for the production of carboxylates^{16,17}. Manipulation of transporters has been an efficient tool to improve the productivity for target carboxylates⁵. A successful metabolic engineering approach for the over-synthesis of organic acids also requires incorporation of an appropriate exporter to increase productivity¹⁸. A comparative study revealed that the highest malate yield was obtained once the malate transporter was recruited in the mutants¹⁹. Lactate production in *S. cerevisiae* could be significantly improved with the combined expression of lactate dehydrogenase and the lactate transporter²⁰. Although many studies have described the mechanisms of action and regulation for carboxylate transport in yeast^{20,21}, *Y. lipolytica* orthologous gene of the carboxylate transporter have not yet been identified.

In this study, a number of carboxylate transporters were identified from *Y. lipolytica* WSH-Z06, which exhibits flexibility in substrate specificity. The duplication of endogenous transporters might provide a powerful tool to ensure efficient carboxylate synthesis and maintain homeostasis of the intracellular environment. In this study, we observed that a competitive dual effect — a significant increase in α -KG production with a sharp decrease in pyruvate (PA) accumulation resulted from overexpression of YALI0B19470g. This result suggests a new and promising strategy for gene manipulation that can efficiently address α -KG accumulation. The identification of these transporters also have uncovered the mechanisms of extracellular accumulation of diverse organic acids in

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genome level, and provides new clues to orchestrate competition of extracellular accumulation between target and non-target carboxylates in yeast and other eukaryotic microorganisms.

Results

Bioinformatics analysis of potential carboxylate transporters in *Y. lipolytica*. To screen putative carboxylate transporters, 6611 proteins encoded by *Y. lipolytica* CLIB122 genome were obtained from the UniProt database. Of these, 1104 proteins (Supplementary S1) were predicted as transmembrane proteins by TMHMM v.2.0 (Fig. 1A). Subsequently, 117 proteins were excluded from this set, due to the presence of a possible signal peptide at the N terminus, as predicted by SignalP 4.1. A sequence search using a conserved carboxylate transporter signature of *JEN* family, NXXS/THXS/TQDXXXT, identified six putative proteins encoded by the YALI0B19470g, YALI0C15488g, YALI0C21406g, YALI0D24607g, YALI0D20108g, and YALIOE32901g genes, which exhibited high similarity to the signature sequence. A multiple sequence alignment of these sequences using homologous carboxylate transporter sequences from other fungi confirmed the presence of the conserved sequence (Fig. 1B).

In addition, a high level of sequence similarity among these putative transporters and the characterized carboxylate transporters was illustrated by two BLAST (<http://blast.ncbi.nlm.nih.gov/>) protocols, in which ScJen1p and KlJen2p were used as query sequences^{22–24}. In these cases, the identity to ScJen1p and to KlJen2p were prospected, respectively (Fig. 2). *In silico* analysis of these protein sequences using the TMRPres2D tool revealed that the conserved sequence was located in the predicted transmembrane helices (Fig. 3). Hence, these transmembrane proteins were suspected to be potential carboxylate transporters.

Exogenous keto acid treatment. To uncover the response of carboxylate transporter candidates for uptake of exogenous keto acids, glucose-grown (repression condition) *Y. lipolytica* WSH-Z06 cells were transferred at the exponential phase to YPK or YPP medium (A large amount exogenous organic acids were accumulated in yeast cells, Supplementary S2). Initially, neither α -KG nor PA was detected in these cultures. During the first hour, the intracellular concentration of both carboxylates increased quickly to maximums of $3.90 \mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1}$ for α -KG and $1.67 \mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1}$ for PA in α -KG- and PA-treated cells, respectively. After incubation in YPK or YPP medium for 1 h, the concentration of α -KG gradually decreased 1 h to 3 h, until the minimum of $0.85 \mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1}$ was reached. During this period, the content of PA also decreased to $0.23 \mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1}$ (Fig. 4A).

To assay the effects of exogenous carboxylates on the expression profiles of these potential transporters, the expression of these genes were examined by qRT-PCR, using RNA extracted at 0 h as the control. When intracellular α -KG levels increased, the expression of YALI0D20108g, YALI0C21406g, YALI0D24607g, and YALIOE32901g decreased by 3.72 ± 0.91 -fold, 3.88 ± 0.34 -fold, 2.47 ± 0.52 -fold, and 1.60 ± 0.18 -fold, respectively, whereas the expression of YALI0B19470g and YALI0C15488g increased by 9.39 ± 0.82 -fold and 4.32 ± 0.83 -fold, respectively. Exogenous PA had a similar effect on expression, albeit to different levels. When incubated with PA, expression of YALI0D20108g, YALI0C21406g, YALI0D24607g, and YALIOE32901g decreased by 5.83 ± 0.81 -fold, 4.04 ± 1.04 -fold, 6.44 ± 0.98 -fold, and 15.88 ± 1.94 -fold, respectively, whereas expression of YALI0B19470g and YALI0C15488g increased by 2.83 ± 0.21 -fold and 9.72 ± 0.22 -fold, respectively (Fig. 4B). Hence, we concluded that transport of both keto acids should be predominantly mediated by YALI0B19470p and YALI0C15488p.

Assessment of putative genes using heterologous expression in *S. cerevisiae*. To assess their possible roles in carboxylate transport, null

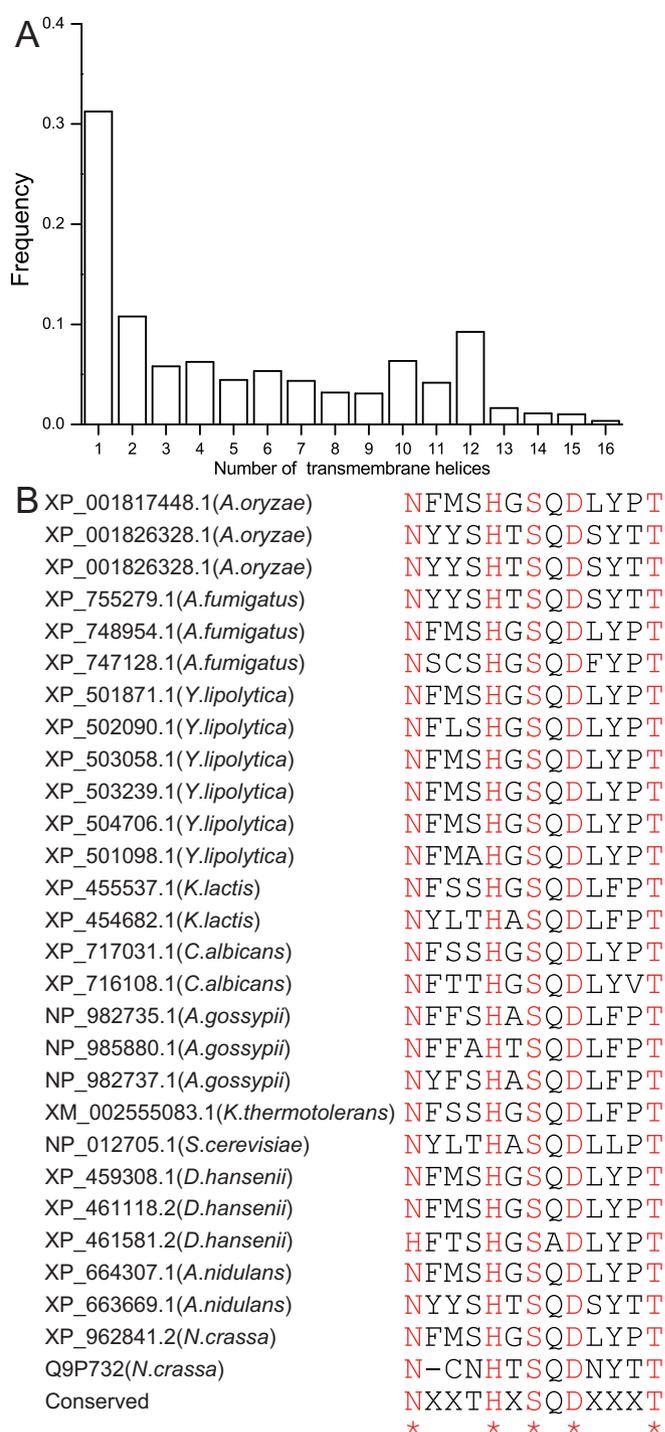


Figure 1 | Genome-wide prediction of transmembrane proteins in *Y. lipolytica*. (A) Number of predicted transmembrane helices in identified putative membrane proteins. (B) Multiple sequence alignment of amino acid sequences from available yeast carboxylate transporter sequences. The results showed that 28 orthologs of the *JEN* family from *Aspergillus oryzae*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Y. lipolytica*, *Kluyveromyces lactis*, *Candida albicans*, *Kluyveromyces thermotolerans*, *S. cerevisiae*, *Debaryomyces hansenii* and *Neurospora crassa* had highly conserved sequences. The name of these orthologs was based on protein ID in NCBI database, as one potential *JEN1* from *N. crassa* could not find in the database, it was named as Q9P732 based on protein ID from Uniprot.

mutants of endogenous transporters were constructed by gene disruption in *S. cerevisiae* CEN.PK2-1D. The *ScJEN1* deletion mutant displayed reduced growth on lactate, acetate, PA, malate,

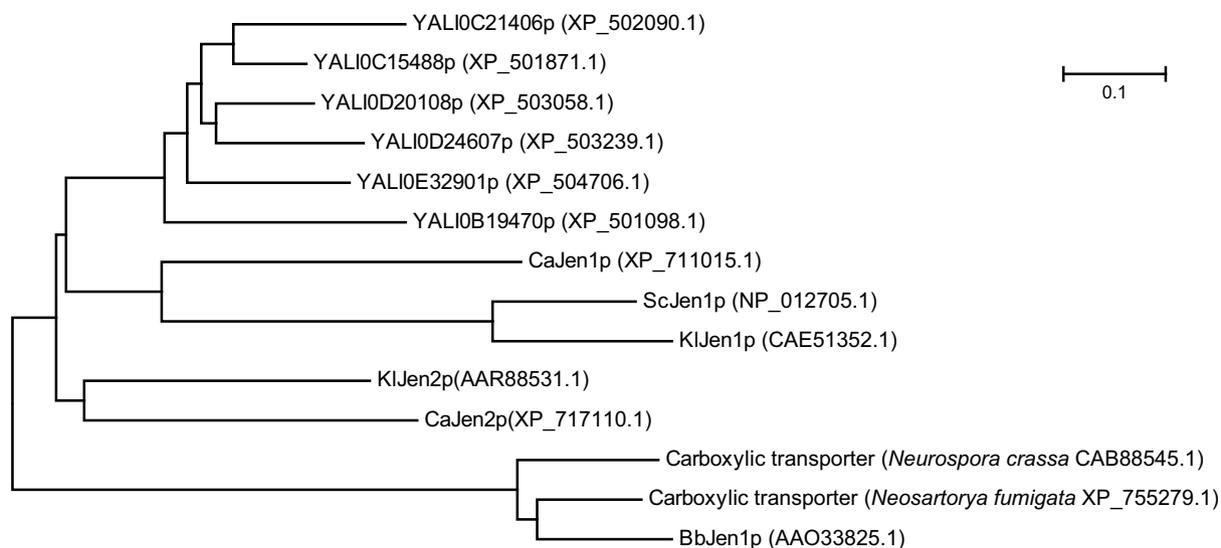


Figure 2 | Phylogenetic analysis of putative transporters. The phylogenetic tree of carboxylate proteins was constructed using the neighbor-joining method. Bootstrap values >50% are shown at the branch points. One thousand bootstrap replications were performed using the MEGA 5.0 software⁴⁸.

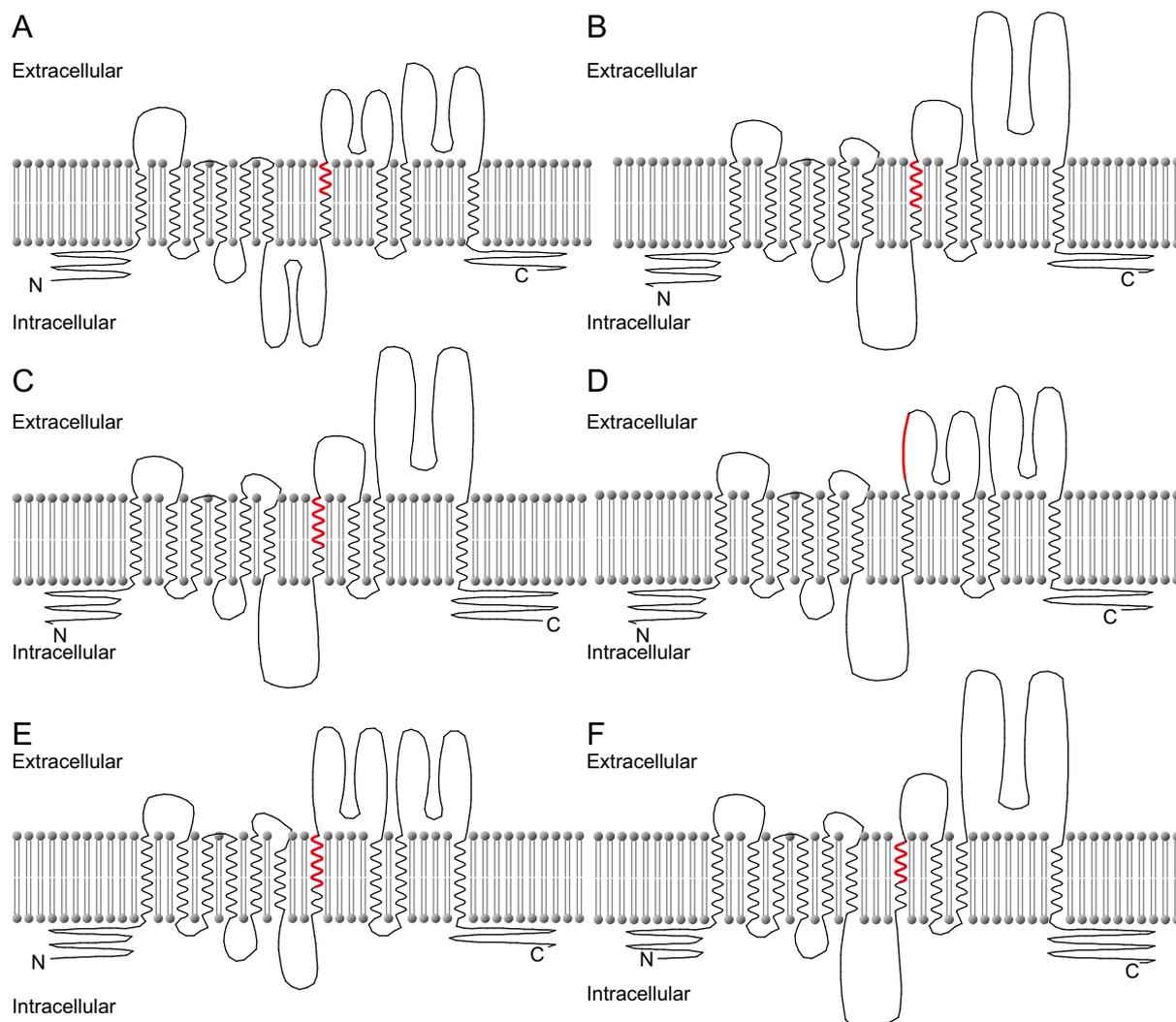


Figure 3 | Sequence-structure mapping of putative transporters. (A) YAL10B19470p, (B) YAL10C15488p, (C) YAL10C21406p, (D) YAL10D24607p, (E) YAL10D20108p, (F) YAL10E32901p. The conserved signature sequence was illustrated in red in every putative carboxylate transporter.

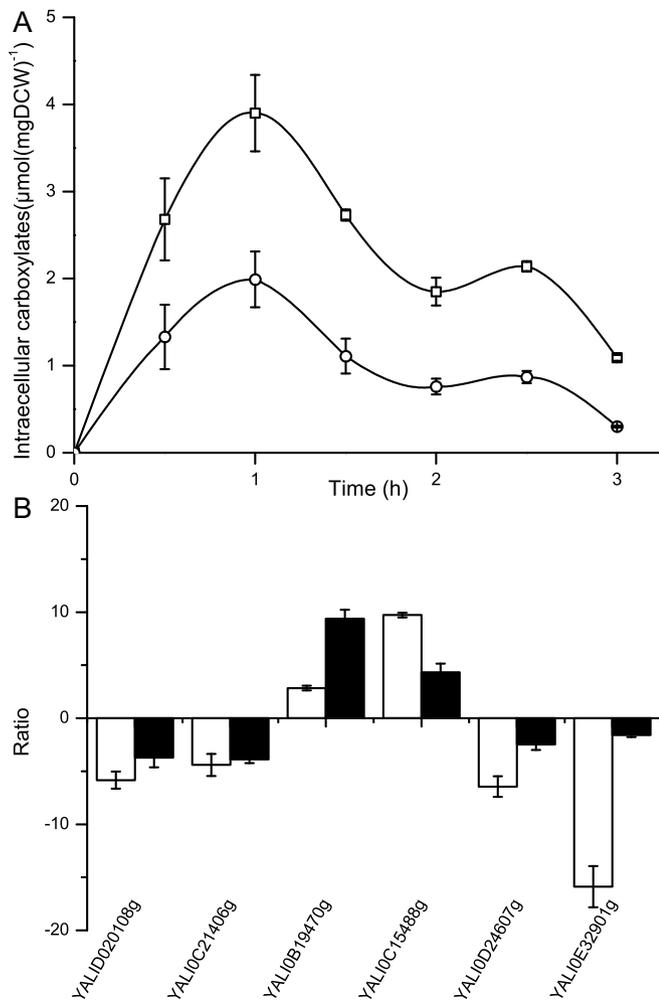


Figure 4 | Time courses of intracellular carboxylate content in *Y. lipolytica* WSH-Z06. (A) Open square: α -KG, open circle: PA. The change of concentration of α -KG and PA exhibited the same trend during throughout the treatment. (B) Expression profile of putative transporter genes at 1 h, at which time the intracellular contents of α -KG and PA were greatest. White: PA treatment; black: α -KG treatment.

and α -KG compared to the parental strain. To completely abolish the uptake by endogenous transporters, the *Ady2p* was also disrupted in W1 strain. In contrast, the W2 strain, in which both *ScJEN1* and *ScADY2* were disrupted, cannot grow on lactate, acetate, PA, malate, and α -KG containing media. Moreover, the parental strain as well as the W1 and W2 strains did not grow on citrate (Fig. 5). All putative transporter genes were introduced into the W2 strain by genetic transformation based on $\Delta his3$ complementation. The resulting strains grew well on all sources including citrate, supporting the conclusion that these genes encode carboxylate transporters (Fig. 5).

Substrate specificity assay. To investigate the substrate specificity of these proteins, glucose-grown *S. cerevisiae* CEN.PK2-1D and mutants were incubated in YPA, YPL, YPP, YPM, YPK, and YPC medium for 2 h after exhaustion of the endogenous carbon source in saline water. The intracellular carboxylate content in *S. cerevisiae* CEN.PK2-1D cells was measured (Table 1), revealing that $2.22 \pm 0.21 \mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1}$ lactate, $0.94 \pm 0.14 \mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1}$ PA, $3.14 \pm 0.24 \mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1}$ malate, and $1.29 \pm 0.12 \mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1}$ α -KG were present. In addition, acetate and citrate were not detected in cells incubated in the

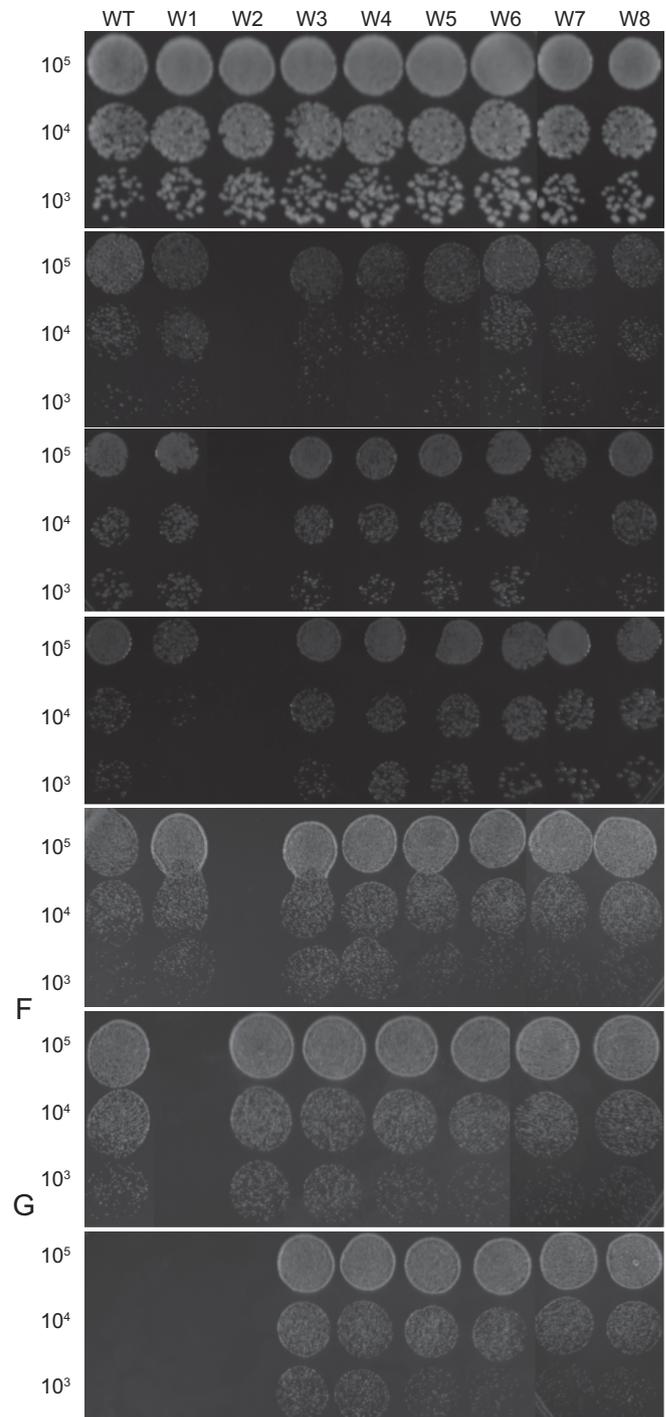


Figure 5 | Growth test of *S. cerevisiae* strains under different carboxylates. WT: *S. cerevisiae* CEN.PK2-1D; W1: *S. cerevisiae* CEN.PK2-1D $\Delta jen1$; W2: *S. cerevisiae* CEN.PK2-1D $\Delta jen1 \Delta ady2$; W3: *S. cerevisiae* CEN.PK2-1D $\Delta jen1 \Delta ady2$ (pY13-TEF1-470); W4: *S. cerevisiae* CEN.PK2-1D $\Delta jen1 \Delta ady2$ (pY13-TEF1-488); W5: *S. cerevisiae* CEN.PK2-1D $\Delta jen1 \Delta ady2$ (pY13-TEF1-406); W6: *S. cerevisiae* CEN.PK2-1D $\Delta jen1 \Delta ady2$ (pY13-TEF1-607); W7: *S. cerevisiae* CEN.PK2-1D $\Delta jen1 \Delta ady2$ (pY13-TEF1-108); W8: *S. cerevisiae* CEN.PK2-1D $\Delta jen1 \Delta ady2$ (pY13-TEF1-901). (A) YPD, (B) YPA, (C) YPL, (D) YPP, (E) YPM, (F) YPK, (G) YPC.

corresponding carboxylate-containing medium. As no carboxylates were detected in the double deletion cells, we concluded that *ScJen1p* and *ScAdy2p* deletions influenced the transport of PA, malate, and α -KG.

Table 1 | Intracellular carboxylate accumulation in *S. cerevisiae* CEN.PK2-1D and derivatives

Strain	Genotype	Acetate	Lactate	PA	Malate	α -KG	Citrate
WT	<i>S. cerevisiae</i> , CEN.PK2-1D	/*	2.22 \pm 0.21	0.94 \pm 0.12	3.14 \pm 0.24	1.29 \pm 0.12	/
W1	<i>S. cerevisiae</i> , CEN.PK2-1D Δ <i>jen1</i>	/	/	/	0.05 \pm 0.01	0.86 \pm 0.08	/
W2	<i>S. cerevisiae</i> , CEN.PK2-1D Δ <i>jen1Δ<i>ady2</i></i>	/	/	/	/	/	/
W3	<i>S. cerevisiae</i> CEN.PK2-1D, Δ <i>jen1Δ<i>ady2</i>(pY13-TEF1-470)</i>	/	/	/	0.15 \pm 0.01	0.09 \pm 0.01	0.04 \pm 0.01
W4	<i>S. cerevisiae</i> CEN.PK2-1D, Δ <i>jen1Δ<i>ady2</i>(pY13-TEF1-488)</i>	0.81 \pm 0.07	/	0.17 \pm 0.03	0.11 \pm 0.01	1.41 \pm 0.19	0.08 \pm 0.01
W5	<i>S. cerevisiae</i> CEN.PK2-1D, Δ <i>jen1Δ<i>ady2</i>(pY13-TEF1-406)</i>	/	0.53 \pm 0.04	/	0.09 \pm 0.01	0.77 \pm 0.06	0.04 \pm 0.01
W6	<i>S. cerevisiae</i> CEN.PK2-1D, Δ <i>jen1Δ<i>ady2</i>(pY13-TEF1-607)</i>	/	/	/	0.09 \pm 0.01	0.56 \pm 0.06	0.05 \pm 0.01
W7	<i>S. cerevisiae</i> CEN.PK2-1D, Δ <i>jen1Δ<i>ady2</i>(pY13-TEF1-108)</i>	/	0.67 \pm 0.09	/	/	0.38 \pm 0.04	0.09 \pm 0.01
W8	<i>S. cerevisiae</i> CEN.PK2-1D, Δ <i>jen1Δ<i>ady2</i>(pY13-TEF1-901)</i>	0.66 \pm 0.04	/	/	0.11 \pm 0.01	0.39 \pm 0.03	0.03 \pm 0.01

*: Carboxylate content less than 0.005 $\mu\text{mol}\cdot(\text{mg}\cdot\text{DCW})^{-1}$ or undetected. The intracellular carboxylate content was measured during substrate specificity tests and presented as $\mu\text{mol}\cdot(\text{mg}\cdot\text{DCW})^{-1}$.

The transport of α -KG was restored in all heterogeneous transporter-containing strains, with the highest accumulation (1.41 $\mu\text{mol}\cdot(\text{mg}\cdot\text{DCW})^{-1}$) occurring in the W4 strain. Among the set, only the W4 could accumulate PA intracellularly (0.17 $\mu\text{mol}\cdot(\text{mg}\cdot\text{DCW})^{-1}$). Based on these observations, we conclude that YALI0B19470g and YALI0D24607g encode proteins that transport dicarboxylates and tricarboxylates. In addition to PA, W4 cells (containing YALI0C15488p) also accumulated acetate, malate, α -KG, and citrate, indicating the corresponding protein also transported these carboxylates. In a similar manner, we conclude the proteins encoded by the corresponding genes transport the following carboxylates: YALI0C21406p for lactate, malate, α -KG, and citrate; YALI0D20108p for lactate, α -KG and citrate, and YALI0E32901p for acetate, malate, and citrate.

Copy number analysis of putative transporter genes in recombinant strains. In order to estimate copy number of each expression cassette that integrated in genome of recombinant strains, quantitative-PCR (qPCR) analysis was carried out. *ACT1* was utilized as an endogenous control²⁶. With comparison to *ACT1*, no obvious relative changes of these transporters were observed (Fig. 6). This indicated that these transporter genes were presented in a single copy in parental strain, respectively. After individual transportation of each endogenous transporters gene into cells of parental strain, relative changes of 2.54 \pm 0.22-fold for YALI0B19470g, 2.33 \pm 0.27-fold for YALI0C15488g, 2.62 \pm 0.33-fold for YALI0C21406g, 2.28 \pm 0.19-fold for YALI0D24607g, 2.39 \pm 0.26-fold for YALI0D20108g and 2.55 \pm 0.32-fold for YALI0E32901g were observed, which indicated one more copy of each transporter gene existed per genome in corresponding recombinant strain (Fig. 6).

The effects of transporter genes on carboxylate accumulation in *Y. lipolytica*. As previously reported carboxylate transporter possess bi-functions for carboxylate influx and efflux²⁵. To clarify the roles of these transporters on carboxylate extracellular accumulation, they were overexpressed in *Y. lipolytica* WSH-Z06. The *hp4d* promoter was used to increase the transcription of these genes, and YALI0B19470g, YALI0C15488g, YALI0C21406g, YALI0D24607g, YALI0D20108g and YALI0E32901g expression was improved by 23.34 \pm 2.67-fold, 8.53 \pm 0.90-fold, 9.32 \pm 0.82-fold, 11.79 \pm 1.32-fold, 3.37 \pm 0.49-fold and 10.50 \pm 0.97-fold, respectively (Fig. 7). Due to the flexible substrate specificity of these transporters, the accumulation of PA and α -KG increased in T5 cells, and the ratio of extracellular α -KG/PA decreased from 2.06 to 1.87 compared to the wild-type strain. This ratio also decreased in YALI0C15488g, YALI0C21406g,

YALI0D24607g, and YALI0E32901g overexpressing strains, as only extracellular PA increased for T2, T3, T4, and T6. A competitive dual effect was observed for strain T1: the transport of α -KG increased dramatically, whereas the concentration of PA dropped by 30.6%, resulting in an increase in the ratio of extracellular α -KG/PA from 2.06 to 3.79 (Fig. 8).

The intracellular carboxylate content, C_{in} ($\mu\text{mol}\cdot(\text{mg}\cdot\text{DCW})^{-1}$), was also determined (Table 2). The intracellular accumulation of α -KG decreased from 0.026 \pm 0.005 $\mu\text{mol}\cdot(\text{mg}\cdot\text{DCW})^{-1}$ to 0.014 \pm

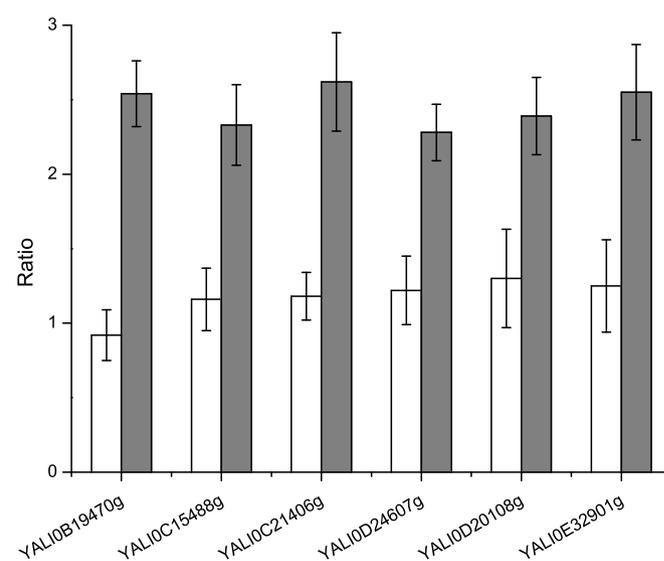


Figure 6 | Copy number analysis of putative transporter genes in recombinant strains. Copy number of YALI0B19470g in T1, YALI0C15488g in T2, YALI0C21406g in T3, YALI0D24607g in T4, YALI0D20108g in T5 and YALI0E32901g in T6 were determined through qPCR analysis. As *ACT1* existed in one copy per *Y. lipolytica* genome, copy number of putative transporter genes in wild-type strain (white) and recombinants (grey) was expressed as relative change. With comparison to *ACT1*, one more copy of each transporter gene was determined in corresponding recombinants.

0.002, 0.023 \pm 0.003, 0.014 \pm 0.002 and 0.020 \pm 0.002 $\mu\text{mol}\cdot(\text{mg}\cdot\text{DCW})^{-1}$ for strains T1, T4, T5 and T6. The intracellular accumulation of PA decreased from 0.034 \pm 0.006 $\mu\text{mol}\cdot(\text{mg}\cdot\text{DCW})^{-1}$

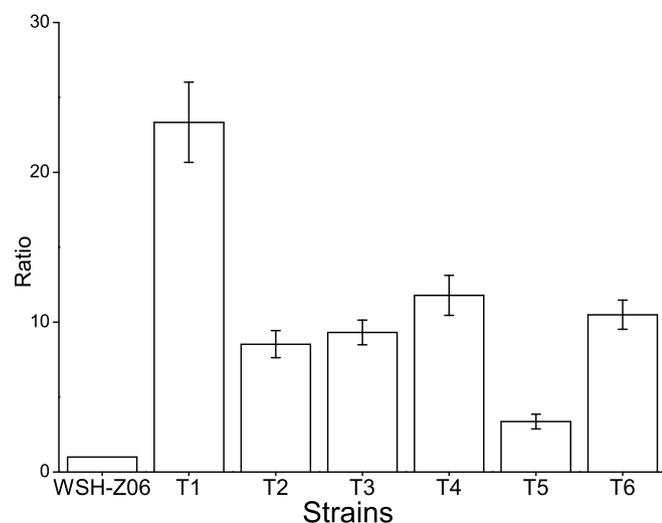


Figure 7 | Verification of transcription of putative carboxylate transporters in *Y. lipolytica* WSH-Z06. RNA extracted from glucose-grown cells of the T1, T2, T3, T4, T5, and T6 strains was subjected to qRT-PCR. The strains T1–T6 were mutant variants in which plasmid p0(hph)-470, p0(hph)-488, p0(hph)-406 p0(hph)-607, p0(hph)-108 and p0(hph)-901 were individually expressed in the wild-type strain. The transcription of YALIOB19470g, YALIOC15488g, YALIOC21406g, YALIOD24607g, YALIOD20108g, and YALIOE32901g increased by 23.34 ± 2.67 -fold, 8.53 ± 0.90 -fold, 9.32 ± 0.82 -fold, 11.79 ± 1.32 -fold, 3.37 ± 0.49 -fold and 10.50 ± 0.97 -fold, respectively.

to 0.009 ± 0.002 , 0.029 ± 0.003 , 0.017 ± 0.002 and $0.025 \pm 0.002 \mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1}$ in strains T1, T4, T5 and T6 respectively. Compared to the wild-type strain, the growth yield decreased from $11.17 \pm 1.08 \text{ g} \cdot \text{L}^{-1}$ to 8.45 ± 0.72 , 8.27 ± 0.89 , 8.34 ± 0.76 , 8.47 ± 0.61 , 8.36 ± 0.88 and $7.40 \pm 0.71 \text{ g} \cdot \text{L}^{-1}$ for strains T1, T2, T3, T4, T5 and T6, respectively, suggesting that enhanced carboxylate transportation caused the shift in carbon flux from cellular growth to carboxylate accumulation. The combination of increased carboxylate accumulation with decreased cell growth lead to a sharp increase in the yield of α -KG and PA, $Y_{\alpha\text{-KG}/\text{DCW}}$ and $Y_{\text{PA}/\text{DCW}}$. Finally, the ratio of extracellular carboxylate to intracellular carboxylate increased, and maximum values of 2399.71 ± 241.63 for α -KG and 1685.20 ± 208.56 for PA were observed for the T1 strain. Based on these observations, overexpression of YALIOA9470g was considered the best strategy to enhance α -KG transport and reduce PA accumulation.

Discussion

The object of the current work was to screen and identify carboxylate transporters, then determine whether the identified proteins regulate

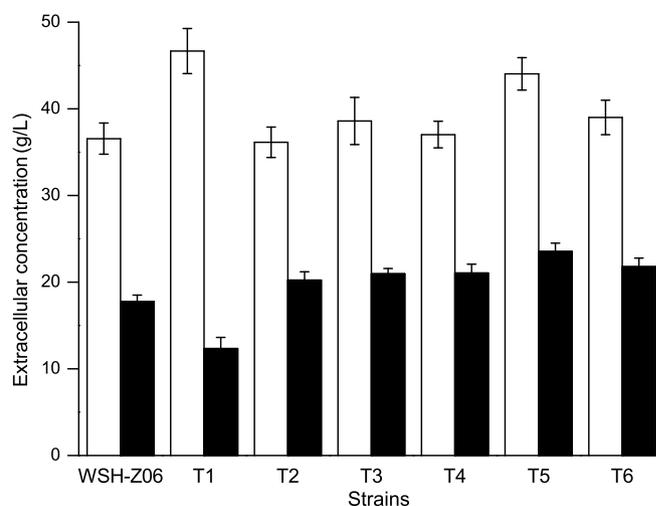


Figure 8 | Effects of potential transporters on keto acids accumulation. White: α -KG; black: PA. The strains T1–T6 were mutant variants in which plasmid p0(hph)-470, p0(hph)-488, p0(hph)-406 p0(hph)-607, p0(hph)-108 and p0(hph)-901 were individually expressed in the wild-type strain. The transport of PA was enhanced in all mutants except the T1 strain. Extracellular PA concentration increased from $17.77 \pm 0.72 \text{ g} \cdot \text{L}^{-1}$ to 20.22 ± 0.98 , 20.95 ± 0.62 , 21.06 ± 1.03 , 23.53 ± 0.98 , and $21.79 \pm 1.00 \text{ g} \cdot \text{L}^{-1}$ in cells of the T2, T3, T4, T5, and T6 strains, respectively, and decreased by 30.6% to the concentration of $12.32 \pm 1.31 \text{ g} \cdot \text{L}^{-1}$ for T1 strain. The content of α -KG varied slightly in strain T2, T3, T4 and T6, while the content of the dicarboxylate increased to 46.66 ± 2.59 - and $44.02 \pm 1.87 \text{ g} \cdot \text{L}^{-1}$ in strain T1 and T5.

accumulation of non-target carboxylates. The duplication of transporters and the flexible substrate specificity demonstrated by the identified carboxylate transporters facilitated extracellular accumulation of carboxylates. Moreover, knowledge of the examined carboxylate transport mechanism is a prerequisite for improving carboxylate synthesis through metabolic engineering. The results provide new insights for regulating extracellular carboxylate accumulation in similar eukaryotic microorganisms.

To circumvent the issue of PA accumulation, previous studies have focused mainly on the regulation of intrinsic forces that redistribute carbon flux from other intermediates to α -KG production. Expression of PA carboxylase¹⁰, malate dehydrogenase, and fumarase³ dramatically decreased the accumulation of PA. A strategy to regulate co-factor regeneration resulted in remarkable reduction of extracellular PA¹⁵. However, as PA has a pivotal role in the regulation of carbon metabolism²⁰, these modifications could not entirely overcome PA accumulation. As accumulation of carboxylate is believed to be a yeast defense response to severe environmental conditions²⁷,

Table 2 | The effects of transporter overexpression on carboxylate production in *Y. lipolytica* and derivatives

Strain	DCW($\text{g} \cdot \text{L}^{-1}$)	$C_{in}(\mu\text{mol} \cdot (\text{mg} \cdot \text{DCW})^{-1})$				Ratio _{ex/in}	
		α -KG	PA	$Y_{\alpha\text{-KG}/\text{DCW}}(\text{g} \cdot \text{g}^{-1})$	$Y_{\text{PA}/\text{DCW}}(\text{g} \cdot \text{g}^{-1})$	α -KG	PA
WSH-Z06	11.17 ± 1.08	0.026 ± 0.005	0.034 ± 0.006	3.28 ± 0.32	0.23 ± 0.03	860.35 ± 78.45	531.45 ± 48.97
T1	8.45 ± 0.72	0.014 ± 0.002	0.009 ± 0.002	4.94 ± 0.52	0.30 ± 0.04	2399.71 ± 241.63	1685.20 ± 208.56
T2	8.27 ± 0.89	0.027 ± 0.003	0.032 ± 0.004	3.90 ± 0.42	0.25 ± 0.03	975.27 ± 109.21	769.61 ± 84.21
T3	8.34 ± 0.76	0.030 ± 0.005	0.033 ± 0.005	4.13 ± 0.44	0.24 ± 0.02	938.78 ± 96.32	779.42 ± 94.38
T4	8.47 ± 0.61	0.023 ± 0.003	0.029 ± 0.003	3.91 ± 0.38	0.31 ± 0.04	1161.29 ± 125.76	865.52 ± 90.76
T5	8.36 ± 0.88	0.013 ± 0.002	0.017 ± 0.002	4.71 ± 0.46	0.59 ± 0.06	2358.20 ± 359.79	$1641.53 \pm 178/94$
T6	7.40 ± 0.71	0.020 ± 0.002	0.025 ± 0.002	5.27 ± 0.61	0.36 ± 0.04	1756.27 ± 193.1	1343.41 ± 147.89

The strains T1–T6 were mutant variants in which plasmid p0(hph)-470, p0(hph)-488, p0(hph)-406 p0(hph)-607, p0(hph)-108 and p0(hph)-901 were individually expressed in the wild-type strain. Ratio_{ex/in} is the mass ratio of extracellular carboxylate to intracellular carboxylate. The yield of $Y_{\alpha\text{-KG}/\text{DCW}}$ and $Y_{\text{PA}/\text{DCW}}$ is the mass ratio of α -KG to DCW and PA to DCW, respectively.



Table 3 | Strains used in this study

Strain	Genotype and remarks
<i>S. cerevisiae</i> CEN.PK2-1D	MAT α Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8 ^c ; SUC2
W1	MAT α Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8 ^c ; SUC2; Δ jen1::URA3
W2	MAT α Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8 ^c ; SUC2; Δ jen1::URA3; Δ ady2::LEU2
W3	MAT α Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8 ^c ; SUC2; Δ jen1::URA3; Δ ady2::LEU2(pY13-TEF1-470)
W4	MAT α Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8 ^c ; SUC2; Δ jen1::URA3; Δ ady2::LEU2(pY13-TEF1-488)
W5	MAT α Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8 ^c ; SUC2; Δ jen1::URA3; Δ ady2::LEU2(pY13-TEF1-406)
W6	MAT α Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8 ^c ; SUC2; Δ jen1::URA3; Δ ady2::LEU2(pY13-TEF1-607)
W7	MAT α Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8 ^c ; SUC2; Δ jen1::URA3; Δ ady2::LEU2(pY13-TEF1-108)
W8	MAT α Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8 ^c ; SUC2; Δ jen1::URA3; Δ ady2::LEU2(pY13-TEF1-901)
<i>Y. lipolytica</i> WSH-Z06	Wild-type
T1	Wild-type(p0(hph)-470)
T2	Wild-type(p0(hph)-488)
T3	Wild-type(p0(hph)-406)
T4	Wild-type(p0(hph)-607)
T5	wild-type(p0(hph)-108)
T6	Wild-type(p0(hph)-901)

regulation of carboxylate transportation process might be another potent route for enhancement of α -KG production.

The carboxylate transport process of yeast is an intensively investigated field²⁰. The *S. cerevisiae* *ScJEN1* and *ScADY2* genes were identified as key carboxylate transporters^{28,29}. The duplication of transporters has been strongly implicated in the utilization of organic acids as a carbon source²⁴. In *Kluyveromyces lactis*, the presence of two carboxylate transporters, Jen1p and Jen2p, guaranteed efficient uptake of lactic acid as a substrate from a lactic-acid-producing habitat³⁰. As the cells of *Y. lipolytica* harbored a powerful potential to use a wide range of substrates as a sources of carbon and energy³¹, these results indicated that powerful carboxylate transporters that maintain intracellular environment homeostasis. A previous study reported that reduction of by-product resulted in enhanced synthesis of target carboxylate by de-repression of the feedback inhibition³. It was speculated that the enhanced synthesis of α -KG could be achieved through de-repression of the feedback inhibition by efflux of intermediates.

In *Y. lipolytica*, efficient carboxylate transport was achieved by the duplication of iso-functional transporters. Evolution analysis and motif identification confirmed that a precursor form of Jen1p, preJen1p, arose from the duplication of an ancestral Jen2p³². In *S. cerevisiae*, the transport capacity and substrate affinity of Jen1p were determined by the conserved NXXS/THXS/TQDXXX sequence³³. Presence of this signature sequence also determined the flexibility of substrate specificity for these transporters. Previously, Jen1p was induced by lactate, PA, and propionate, whereas Ady2p and Jen2p were induced by acetate^{29,30}. Expression of transporters from *Y. lipolytica* displayed different responses to exogenous carbon source, and single carboxylate induced multiple transporters.

The roles of these carboxylate transporters were assayed via double their copy numbers in the genomic DNA of α -KG producer. One more copy of each endogenous carboxylate transporter was observed (Fig. 6). Our observation that overexpression of carboxylate transporters resulted in enhanced accumulation of extracellular carboxylates (Fig. 7). Previously, uncovered the mechanism for malate efflux was mediated by monoanionic malate concentration gradient, in which the proton symport was major force²⁵. Similar to the observations that production of carboxylate was benefited from overexpression of carboxylate transporter^{13,34}, the efflux of carboxylate accompanied by symport of proton was speculated²⁵. Based on measurement of total content of mixture of monoanionic, dianionic and undissociated form of the carboxylates, the intracellular content of carboxylates was not correlated with extracellular carboxylate content. While, the reported carboxylate transporter was specific for monoanionic form of carboxylate for efflux, the efflux specificity of this series of transporter would be the key to the contradictory observation in future studies.

Methods

Strains and plasmids. All strains used in this study were summarized in Table 3. *Y. lipolytica* WSH-Z06, an α -KG producing wild-type strain, was previously screened by our lab³⁵. The plasmid p0(hph) was previously constructed based on plasmid p0. The p0 could integrate a single copy of exogenous sequence via recombinant at the locus of *XRP2* in the genome of *Y. lipolytica*¹⁰. The hygromycin phosphotransferase encoded by *hph* was amplified from plasmid pUB4-CRE and was used to replace the *URA3* sequence in plasmid p0^{15,36}. Plasmids were propagated in *Escherichia coli* JM109. *S. cerevisiae* CEN.PK2-1D (MAT α , Δ ura3-52; Δ trp1-289; Δ leu2-3,112; Δ his3-1; MAL2-8^c; SUC2) from EUROSARF (Frankfurt, Germany) and the pY13-TEF1 plasmid was used for heterologous expression of potential transporters.

Table 4 | Oligonucleotide primers used for qRT-PCR

Gene	Primers	Sequence (5'-3')	Product size (bp)
YALIOB19470g	YALIOB19470-F YALIOB19470-R	CAACAAGGAAGACAACAG AGGTAGGTGAACATAAGC	153
YALIOC15488g	YALIOC15488-F YALIOC15488-R	GCAACCATCTCAGCCATTC GTAACCTCGCATCTTCAGC	199
YALIOC21406g	YALIOC21406-F YALIOC21406-R	GCAGACCTACCAGCAGTTC ACGACACAGAGCAAGTATCC	171
YALIOD20108g	YALIOD20108-F YALIOD20108-R	TGCTACAGGAAGGCTATGC GGAAGATGATGATGAGAACAGG	135
YALIOD24607g	YALIOD24607-F YALIOD24607-R	CTGCTTGTAGGTGGTGAC GAGTGCTGAGTGATAAATACG	104
YALIOE32901g	YALIOE32901-F YALIOE32901-R	TCTATGATTACGGTAAGGTTATG GACTCGCTCAAGGTTCTC	188
<i>ACT1</i>	ACT1-F ACT1-R	AAGTCCAACCGAGAGAAGATG ACCAGAGTCAAGAACGATACC	132

Table 7 | Oligonucleotides used for gene expression in *Y. lipolytica*

Primers	Sequence (5'-3')	Description
YALIOD20108g-F1	CGGGATCCATGAATTTTGACAACCTCCCAG	BamHI
YALIOD20108g-R1	GGAATTCCTTATCGAGTATCGCTCGAAGAAC	EcoRI
YALIOC21406g-F1	GGAATTCATGGATCTCGACAACCTACCTC	EcoRI
YALIOC21406g-R1	TTGCGGGCCGCTCACTTTTGGGATCCGGG	NotI
YALIOB19470g-F1	CGGGATCCATGCCCATCACAGTTTCACAAG	BamHI
YALIOB19470g-R1	GGAATTCCTTAACGAGTGAGATGGTGTCG	EcoRI
YALIOC15488g-F1	CGGGATCCATGGATTGGACAACCTCCC	BamHI
YALIOC15488g-R1	GGAATTCCTACTAGTAGCATTGGTGTCAACTC	EcoRI
YALIOD24607g-F1	CGGGATCCATGACCCAGTCGTACGAAGTC	BamHI
YALIOD24607g-R1	GGAATTCCTAATGAACACTTCCAACAGTGG	EcoRI
YALIOE32901g-F1	CGGGATCCATGGAAGCTCCTAATCTCTCGC	BamHI
YALIOE32901g-R1	GGAATTCCTACTTGGACTCGTAGGGGGA	EcoRI
VB-F	CGTTTGCCAGCCACAGATT	
V-YALIOD20108g-R	GCGTTTGCCAGCCACAGAT	
V-YALIOC21406g-R	GTAGATGCAGGCAGCACCG	
V-YALIOB19470g-R	AAGACAGAGGCGTTGATACCG	
V-YALIOC15488g-R	TGCGAGGTTACCAAGCTGAT	
V-YALIOD24607g-R	GACAAACGCCAGGGATAG	
V-YALIOE32901g-R	TGTCATCTGCTTGCCCTC	

Heterologous expression of putative transporter genes in *S. cerevisiae* W2. Six putative transporter genes, YALIOB19470g, YALIOC15488g, YALIOC21406g, YALIOD24607g, YALIOD20108g, and YALIOE32901g, were amplified from *Y. lipolytica* WSH-Z06 genomic DNA with the primers listed in Table 6. The amplified fragments were introduced into BamHI–EcoRI sites of the pY13-TEF1 expression vector, resulting in the pY13-TEF1-470, pY13-TEF1-488, pY13-TEF1-406, pY13-TEF1-607, pY13-TEF1-108, and pY13-TEF1-901 plasmids, respectively. These vectors were introduced into *S. cerevisiae* W2 using a previously described protocol, and the resulting lines are referred to as W3, W4, W5, W6, W7, and W8, respectively.

Overexpression of putative transporter genes in *Y. lipolytica*. The YALIOB19470g, YALIOC15488g, YALIOC21406g, YALIOD24607g, YALIOD20108g, and YALIOE32901g open reading frames were PCR-amplified from *Y. lipolytica* WSH-Z06 genomic DNA with the oligonucleotides listed in Table 7. The amplified fragments were digested with EcoRI and BamHI or NotI and were subsequently inserted into the integrative expression vector p0(hph), resulting in p0(hph)-470, p0(hph)-488, p0(hph)-406, p0(hph)-607, p0(hph)-108, and p0(hph)-901, respectively. These vectors were digested with AvrII, purified, and transformed into *Y. lipolytica* WSH-Z06 using a previously described protocol⁴⁷. The resulting transformants, *Y. lipolytica* T1, T2, T3, T4, T5, and T6, respectively, were screened on YPD plates containing 400 mg·L⁻¹ hygromycin B and verified with the oligonucleotides listed in Table 7.

Copy number analysis. In order to determine the copy number of the integrative expression cassettes in the recombinant strains, a qPCR analysis was performed on the genomic DNA template using *ACT1* as the internal control²⁶. Genomic DNA from parental strain (WSH-Z06) and six recombinant strains (T1, T2, T3, T4, T5 and T6) were isolated after disruption of yeast cells with glass beads (Sigma-Aldrich, St. Louis, MI) by FastPrep 24 (MP Biomedicals, Santa Ana, CA). qPCR was performed with the 5 ng genomic DNA and primers listed in Table 4 using the SYBR Premix Ex TaqTM Kit (Takara, Dalian, China) and a LightCycler 480 II Real-time PCR instrument (Roche Applied Science, Mannheim, Germany). All experiments were performed in triplicate and mean values were used for further calculations. Fold changes were determined by the 2^{-ΔΔC_T} method and normalized to the *ACT1* gene⁴⁵.

Shake flask culture. Shake flask culture was performed in 500 mL flasks containing 50 mL fermentation medium following the protocol stated previously³⁵. A yeast seed culture was inoculated from an agar slant and incubated in a 500 mL flask containing 50 mL medium for 18 h on a rotary shaker at 28°C. The culture was used to inoculate 500 mL flasks containing 50 mL fermentation medium. An inoculum volume of 10% (v/v) was used for α-KG accumulation assay. Flask cultures were incubated in a shaker at 200 r·min⁻¹ for 144 h at 28°C.

HPLC analysis. Samples taken from shake flask culture were centrifuged at 10,000 × g for 10 min. The supernatant was diluted 50 times and filtered through a membrane (pore size = 0.22 μm). α-KG, pyruvic acid, acetate, lactate, malate, and citrate present in the supernatant were simultaneously determined by HPLC (Agilent 1200 series, Santa Clara, CA, USA) with an Aminex HPLC-87H column (300 mm × 7.8 mm; Bio-Rad Laboratories Inc., Hercules, CA, USA). The mobile phase was 5 mmol L⁻¹ sulfuric acid in distilled, de-ionized water filtered to 0.22 μm. The mobile phase flow rate was 0.6 mL min⁻¹. The column temperature was maintained at 35°C, and the injection volume was 10 μL. α-KG, pyruvic acid, acetate, lactate, malate, and citrate were detected with a UV detector (wavelength at 210 nm)³⁷. To determine the intracellular carboxylates, cells taken from shake flask culture were disrupted and

lysates were centrifuged at 10,000 × g for 10 min. The carboxylate content in supernatant was determined by HPLC followed the protocol above.

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Author contributions

G.H.W. and Z.J.W. designed the experiments and wrote the paper. G.H.W. and L.P.R. performed the experiments. G.C.D. and J.C. conceived the project, analyzed the data and wrote the paper. C.M. analyzed the data.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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Sequence of six transporter genes, YALI0B19470g, YALI0C15488g, YALI0C21406g, YALI0D24607g, YALI0D20108g and YALI0E32901g, can be acquired in NCBI through GenBank (XP_503058.1, XP_503239.1, XP_504706.1, XP_501098.1, XP_501871.1 and XP_502090.1).

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