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DOI

[10.1093/femsyr/foab033](https://doi.org/10.1093/femsyr/foab033)

Publication date

2021

Document Version

Accepted author manuscript

Published in

FEMS Yeast Research

Citation (APA)

Baldi, N., de Valk, S. C., Sousa-Silva, M., Casal, M., Soares-Silva, I., & Mans, R. (2021). Evolutionary engineering reveals amino acid substitutions in Ato2 and Ato3 that allow improved growth of *Saccharomyces cerevisiae* on lactic acid. *FEMS Yeast Research*, 21(4), Article foab033. <https://doi.org/10.1093/femsyr/foab033>

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1 Evolutionary engineering reveals amino acid substitutions in Ato2 and Ato3 that allow improved
2 growth of *Saccharomyces cerevisiae* on lactic acid.

3

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17 Running title: Novel lactic acid transporters in yeast.

18 Manuscript for submission to FEMSYR

19

20 **ABSTRACT**

21 In *Saccharomyces cerevisiae*, the complete set of proteins involved in transport of lactic acid across the
22 cell membrane has not been determined. In this study we aimed to identify transport proteins not
23 previously described to be involved in lactic acid transport via a combination of directed evolution,
24 whole-genome resequencing and reverse engineering. Evolution of a strain lacking all known lactic acid
25 transporters on lactate led to the discovery of mutated Ato2 and Ato3 as two novel lactic acid transport
26 proteins. When compared to previously identified *S. cerevisiae* genes involved in lactic acid transport,
27 expression of *ATO3*^{T284C} was able to facilitate the highest growth rate ($0.15 \pm 0.01 \text{ h}^{-1}$) on this carbon
28 source. A comparison between (evolved) sequences and 3D models of the transport proteins showed
29 that most of the identified mutations resulted in a widening of the narrowest hydrophobic constriction
30 of the anion channel. We hypothesize that this observation, sometimes in combination with an
31 increased binding affinity of lactic acid to the sites adjacent to this constriction, are responsible for the
32 improved lactic acid transport in the evolved proteins.

33

34 1. INTRODUCTION

35 The yeast *Saccharomyces cerevisiae* is able to utilize a variety of compounds as carbon and
36 energy source, including monosaccharides, disaccharides, monocarboxylic acids, amino acids
37 and polyols (Kruckeberg and Dickinson, 2004; Lagunas, 1993). Assimilation and dissimilation
38 of these compounds inside cells is preceded by their transport across the plasma membrane.
39 A lot of research has been dedicated to the identification of proteins involved in the uptake
40 and the elucidation of their structure, function and mechanism of action, both to understand
41 cellular response to different conditions as well as for the application of metabolic engineer-
42 ing strategies to increase the efficiency of substrate usage and broaden the substrate range
43 of industrial cell factories (Agrimi and Steiger, 2021).

44 This research field has tremendously benefitted from engineered 'platform strains', in which
45 all transporters for a certain substrate have been knocked out. One of the most applied plat-
46 form strains is the so called '*hxt⁰* strain', in which the uptake of hexoses is completely abol-
47 ished by the knockout of all 21 genes involved in uptake of hexoses (Wieczorke et al., 1999).
48 This *hxt⁰* strain has been indispensable for studies where both endogenous and heterolo-
49 gous proteins were characterized for their ability to catalyze the uptake of various hexose
50 and pentose sugars (Anjos et al., 2013; Bueno et al., 2020; Chattopadhyay et al., 2020; Gao
51 et al., 2019; Huang et al., 2020; Li et al., 2015; Morii et al., 2020). The absence of all hexose
52 transporters in this strain renders it unable to grow on media in which a monosaccharide is
53 the sole carbon source, and therefore allows for screening of growth phenotypes that are
54 linked to the expression of an investigated transport protein. The application of the *hxt⁰*
55 strain is also preferred for *in vivo* transport assays in which the intracellular accumulation of
56 substrates is measured, since background signal caused by other transporters is minimized
57 (Nogueira et al., 2020; Paulsen et al., 2019; Schmidl et al., 2021). In addition, it has often

58 been used as background strain in directed evolution of (heterologous) transport proteins,
59 for which selection is based on growth on the transported substrate (Colabardini et al., 2014;
60 Li et al., 2016; Nijland and Driessen, 2020; Sloothaak et al., 2016; Wang et al., 2013; Zhang et
61 al., 2015). The benefit of a platform strain in an evolutionary engineering approach is that
62 the presence of other genes that could (upon mutation) provide a selective advantage is
63 minimized, and thus allows for improved selection of mutants of the gene under investiga-
64 tion. Similar platform strains have been constructed to study disaccharide transporters
65 (Riesmeier et al., 1992) and ABC transporters (Suzuki et al., 2011).

66 Transport of monocarboxylic acids across the yeast plasma membrane remains enigmatic
67 (Borodina, 2019) and therefore the establishment of specialized platform strains to study
68 transport of specific monocarboxylic acids could be an important next step to further our un-
69 derstanding. Whereas the undissociated, protonated form of carboxylic acids can cross bio-
70 logical membranes by passive diffusion, the charged anionic form that is predominant in pH
71 conditions well above the pK_a of the acid requires (a) protein(s) to mediate rapid transport
72 across the membrane (Gabba et al., 2020). These monocarboxylic acid transport proteins
73 play an important role in, for instance, food preservation, weak organic acid tolerance in sec-
74 ond generation bioethanol production, metabolic engineering strategies for industrial pro-
75 duction of carboxylic acids and in development of cancer therapies (Pinheiro et al., 2012;
76 Soares-Silva et al., 2020). Two genes encoding permeases for monocarboxylic acids have
77 been identified so far in *S. cerevisiae*: *JEN1* and *ADY2* (Casal et al., 2016). Jen1 is a member of
78 the Major Facilitator Superfamily which enables uptake of lactic, acetic and pyruvic acid
79 (Casal et al., 1999). Ady2 is an acetate transporter that belongs to the AceTr family, for which
80 two homologs have been described in *S. cerevisiae*, Ato2 and Ato3 (Paiva et al., 2004; Ribas
81 et al., 2019). A powerful strategy to identify more genes involved in a specific physiological

82 function is the use of adaptive laboratory evolution. Application of a selective pressure is
83 used to enrich for mutants with the phenotype of interest, which can subsequently be
84 analyzed by whole genome sequencing to identify mutated genes related to the evolved
85 phenotype (Mans et al., 2018). This concept was demonstrated in a previous study, in which
86 laboratory evolution of a *jen1Δ* strain in culture medium with lactic acid as sole carbon
87 source led to the identification of mutated *ADY2* alleles that had an increased uptake capac-
88 ity for lactic acid (de Kok et al., 2012). Lactic acid, which is produced on industrial scale using
89 biotechnological processes, is used as preservative in the dairy industry and as a precursor
90 for the production of bioplastic, with a demand of 1.220.000 tons in 2016 that is expected to
91 further increase by 16.2% before 2025 (Singhvi et al., 2018).

92

93 In this study, we use adaptive laboratory evolution to identify additional transporters, which
94 upon mutation can efficiently catalyze lactic acid uptake in *S. cerevisiae*. Subsequently, we
95 overexpress the complete suite of native and evolved lactic acid transporters in a strain
96 background devoid of all (putative) organic acid transporters, characterize the ability of the
97 resulting strains to grow on monocarboxylic acids and assess the uptake of labelled lactate
98 and acetate by the evolved transporters. Finally, we identify specific amino acid residues
99 playing a key role in the transport of lactic acid and provide a mechanistic explanation using
100 three-dimensional structure predictions combined with molecular docking analysis.

101

102 **2. MATERIALS AND METHODS**

103 **2.1 Strains and maintenance**

104 The *S. cerevisiae* strains used in this study (Table 2) share the CEN.PK113-7D or the
105 CEN.PK2-1C genetic backgrounds (Entian and Kötter, 2007). Stock cultures of *S. cerevisiae*
106 were grown aerobically in 500 mL round-bottom shake flasks containing 100 mL synthetic
107 medium (SM) (Verduyn et al., 1992) or YP medium (10 g L⁻¹ Bacto yeast extract, 20 g L⁻¹ Bacto
108 peptone) supplemented with 20 g L⁻¹ glucose. When needed, auxotrophic requirements were
109 complemented via addition of 150 mg L⁻¹ uracil, 100 mg L⁻¹ histidine, 500 mg L⁻¹ leucine and/or
110 75 mg L⁻¹ tryptophan (Pronk, 2002). For plate cultivation, 2% (w/v) agar was added to the
111 medium prior to heat sterilization. Stock cultures of *E. coli* XL1-Blue Subcloning Grade
112 Competent Cells (Agilent, Santa Clara, CA, USA) that were used for plasmid propagation were
113 grown in LB medium (5 g L⁻¹ Bacto yeast extract, 10 g L⁻¹ Bacto tryptone, 10 g L⁻¹ NaCl)
114 supplemented with 100 mg L⁻¹ ampicillin. Media were autoclaved at 121°C for 20 min and
115 supplements and antibiotics were filter sterilized and added to the media prior to use. Frozen
116 culture stocks were prepared by addition of sterile glycerol (to a final concentration of 30%
117 v/v) to exponentially growing shake flask cultures of *S. cerevisiae* or overnight cultures of
118 *E. coli* and 1 mL aliquots were stored at -80°C.

119

120 **2.2 Molecular biology techniques**

121 Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA)
122 was used for PCR amplification for cloning purposes. Diagnostic PCRs were performed using
123 DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific). In both cases, the manufacturer's
124 protocol was followed, with the exception of the use of lower primer concentrations (0.2 μM
125 each). Desalted (DST) oligonucleotide primers were used, except for primers binding to coding
126 regions, which were PAGE purified. Primers were purchased from Sigma Aldrich (Saint Louis,

127 MO, USA), with the exception of primers 17453 and 17453, which were purchased from Ella
128 Biotech (Planegg, Germany). For diagnostic PCR, yeast genomic DNA was isolated as described
129 by (Lööke et al., 2011). Commercial kits for DNA extraction and purification were used for
130 small-scale DNA isolation (Sigma Aldrich), PCR cleanup (Sigma Aldrich), and gel extraction
131 (Zymo Research, Irvine, CA, USA). Restriction analysis of constructed plasmids was performed
132 using FastDigest restriction enzymes (Thermo Scientific). Gibson assembly of linear DNA
133 fragments was performed using NEBuilder HiFi DNA Assembly Master Mix (New England
134 Biolabs, Ipswich, MA, USA) in a total reaction volume of 5 μ L. Transformation of chemically
135 competent *E. coli* XL1-Blue (Agilent) was performed according to the manufacturer's protocol.

136 **2.3 Plasmid construction**

137 The plasmids and oligonucleotide primers used in this study are listed in Table 1 and
138 Supplementary Table 1, respectively. All plasmids were constructed by Gibson assembly of
139 two linear fragments. With the exception of the fragments used for the construction of
140 plasmid pUDR420, all fragments were PCR-amplified from either a template plasmid or from
141 genomic DNA.

142 Plasmid pUDR405 was constructed by Gibson assembly of two linear fragments, both
143 obtained via PCR amplification of plasmid pROS13 using primers 8664 and 6262 (for the *JEN1*-
144 gRNA_2 μ _ADY2-gRNA insert) and 6005 (for the plasmid backbone), as previously described by
145 (Mans et al., 2015). Plasmid pUDR420 was constructed by Gibson assembly of a double-
146 stranded DNA fragment, obtained by annealing the complementary single-stranded
147 oligonucleotides 8691 and 13552, and a vector backbone amplified from plasmid pMEL13
148 using primers 6005 and 6006. Plasmid pUDR767 was constructed by Gibson assembly of two
149 linear fragments, both obtained via PCR amplification of plasmid pROS10 using primers 8688

150 (for the *ATO2*-gRNA_{2μ}*ATO2*-gRNA insert) and 6005 (for the plasmid backbone). For
151 construction of pUDE813, the linear p426-TEF plasmid backbone was amplified from plasmid
152 p426-TEF using primers 5921 and 10547 and the *ATO3* open reading frame (ORF) was
153 amplified from yeast strain CEN.PK113-7D genomic DNA using primers 13513 and 13514.
154 Subsequently, Gibson assembly of the linear p426-TEF plasmid backbone and the *ATO3* insert
155 yielded pUDE813. pUDE814, pUDE1001, pUDE1002, pUDE1003, pUDE1004, pUDE1021 and
156 pUDE1022 were constructed similar to pUDE813, using primers 5921 and 10547 to amplify
157 the linear p427-TEF plasmid backbone. The inserts were amplified from genomic DNA of strain
158 CEN.PK113-7D (for wildtype genes) or from genomic DNA of the corresponding evolved strain
159 (for mutated genes) using primers 13513 and 13514 (pUDE814), 17170 and 17171
160 (pUDE1001), 17168 and 17169 (pUDE1002, pUDE1003 and pUDE1004) or 17452 and 17453
161 (pUDE1021 and pUDE1022). For construction of pUDC319, plasmid p426-TEF was amplified
162 using primers 2949 and 17741 and the CEN6 origin of replication was amplified from pUDC156
163 using primers 17742 and 17743. Subsequently, Gibson assembly of the linear p426-TEF
164 plasmid fragment and the CEN6 fragment yielded pUDC319. pUDC320, pUDC321, pUDC322,
165 pUDC323, pUDC324, pUDC325, pUDC326 and pUDC327 were constructed in a similar way
166 using the same primers, but the linear plasmid fragment was amplified from pUDE813,
167 pUDE814, pUDE1001, pUDE1002, pUDE1003, pUDE1004, pUDE1021 and pUDE1022,
168 respectively.

169

170 Table 1: Plasmids used in this study.

Name	Relevant characteristic	Origin
pROS13	2μm ampR <i>kanMX</i> gRNA- <i>CAN1</i> gRNA-	(Mans et al., 2015)

	<i>ADE2</i>	
pROS10	2 μ m ampR <i>URA3</i> gRNA- <i>CAN1</i> gRNA- <i>ADE2</i>	(Mans et al., 2015)
pMEL13	2 μ m ampR <i>kanMX</i> gRNA- <i>CAN1</i>	(Mans et al., 2015)
pUDR405	2 μ m ampR <i>kanMX</i> gRNA- <i>JEN1</i> gRNA- <i>ADY2</i>	This study
pUDR420	2 μ m ampR <i>kanMX</i> gRNA- <i>ATO3</i>	This study
pUDR767	2 μ m ampR <i>URA3</i> gRNA- <i>ATO2</i>	This study
p426-TEF	2 μ m <i>URA3</i> pTEF1- <i>tCYC1</i>	(Mumberg et al., 1995)
pUDE813	2 μ m <i>URA3</i> pTEF1- <i>ATO3-tCYC1</i>	This study
pUDE814	2 μ m <i>URA3</i> pTEF1- <i>ATO3^{T284C}-tCYC1</i>	This study
pUDE1001	2 μ m <i>URA3</i> pTEF1- <i>JEN1-tCYC1</i>	This study
pUDE1002	2 μ m <i>URA3</i> pTEF1- <i>ADY2-tCYC1</i>	This study
pUDE1003	2 μ m <i>URA3</i> pTEF1- <i>ADY2^{C755G}-tCYC1</i>	This study
pUDE1004	2 μ m <i>URA3</i> pTEF1- <i>ADY2^{C655G}-tCYC1</i>	This study
pUDE1021	2 μ m <i>URA3</i> pTEF1- <i>ATO2-tCYC1</i>	This study
pUDE1022	2 μ m <i>URA3</i> pTEF1- <i>ATO2^{T653C}-tCYC1</i>	This study
pUDC156	<i>CEN6</i> <i>URA3</i> pTEF- <i>CAS9-tCYC1</i>	(Marques et al., 2017)
pUDC319	<i>CEN6</i> <i>URA3</i> pTEF- <i>tCYC1</i>	This study
pUDC320	<i>CEN6</i> <i>URA3</i> pTEF1- <i>ATO3-tCYC1</i>	This study
pUDC321	<i>CEN6</i> <i>URA3</i> pTEF1- <i>ATO3^{T284C}-tCYC1</i>	This study
pUDC322	<i>CEN6</i> <i>URA3</i> pTEF1- <i>JEN1-tCYC1</i>	This study
pUDC323	<i>CEN6</i> <i>URA3</i> pTEF1- <i>ADY2-tCYC1</i>	This study
pUDC324	<i>CEN6</i> <i>URA3</i> pTEF1- <i>ADY2^{C755G}-tCYC1</i>	This study
pUDC325	<i>CEN6</i> <i>URA3</i> pTEF1- <i>ADY2^{C655G}-tCYC1</i>	This study
pUDC326	<i>CEN6</i> <i>URA3</i> pTEF1- <i>ATO2-tCYC1</i>	This study
pUDC327	<i>CEN6</i> <i>URA3</i> pTEF1- <i>ATO2^{T653C}-tCYC1</i>	This study

171

172

173 2.4 Strain construction

174 *S. cerevisiae* strains were transformed with the LiAc/ssDNA method (Gietz and Woods,
175 2002). For transformations with a dominant marker, the transformation mixture was plated
176 on YP plates, containing glucose (20 g L⁻¹) as carbon source, and supplemented with 200 mg L⁻¹
177 G418 (Invitrogen, Carlsbad, CA, USA). Gene deletions were performed as previously
178 described (Mans et al., 2015). For transformation of plasmids harboring an auxotrophic
179 marker, transformed cells were plated on SM medium with glucose (20 g L⁻¹) as a carbon
180 source and when needed, appropriate auxotrophic requirements were supplemented.

181 The tryptophan auxotrophy of IMX1000 was the result of a single point mutation in
182 the *TRP1* gene (*trp1-289*) (Botstein et al., 1979) and was spontaneously reverted by plating
183 the strain on SM medium supplemented with uracil, histidine and leucine, and picking a
184 tryptophan prototrophic colony, yielding strain IMX2486. Strain IMX2487 was constructed by
185 transforming IMX2486 with a linear fragment, obtained by PCR amplification of the *LEU2* gene
186 from CEN.PK113-7D, using primers 1742 and 1743. Strain IMX2488 was constructed by
187 transforming IMX2487 with a linear fragment, obtained by PCR amplification of the *HIS3* gene
188 from CEN.PK113-7D, using primers 1738 and 3755. Strain IMK875 was constructed by
189 transforming the Cas9-expressing strain IMX585 with plasmid pUDR405 and two double
190 stranded repair oligonucleotides obtained by annealing oligonucleotides 8597 to 8598 and
191 8665 to 8666. Strain IMK876 was constructed by transforming the Cas9-expressing strain
192 IMX581 with plasmid pUDR405 and two double stranded repair oligonucleotides obtained by
193 annealing oligonucleotides 8597 to 8598 and 8665 to 8666. Strains IMK882 and IMK883 were
194 obtained by transforming strains IMK875 and IMK876, respectively, with plasmid pUDR420
195 and a double stranded repair oligonucleotide obtained by annealing oligonucleotides 14120

196 and 14121. Strain IMK982 was constructed by transforming strain IMK883 with plasmid
 197 pUDR767 and a double stranded repair oligonucleotide obtained by annealing
 198 oligonucleotides 8689 and 8690. Plasmids p426-TEF, pUDE813, pUDE814, pUDE1001,
 199 pUDE1002, pUDE1003, pUDE1004, pUDE1021, pUDE1022, pUDC319, pUDC320, pUDC321,
 200 pUDC322, pUDC323, pUDC324, pUDC325, pUDC326 and pUDC327 were transformed in strain
 201 IMX2488, yielding IME581, IME582, IME583, IME584, IME585, IME586, IME587, IME588,
 202 IME589, IMC164, IMC165, IMC166, IMC167, IMC168, IMC169, IMC170, IMC171 and IMC172,
 203 respectively.

204 Evolution of IMK341 and IMK882 was performed by inoculating duplicate shake flasks
 205 with 20 mL synthetic medium with lactic acid as the sole carbon source (SML, see section 2.5
 206 'Media and cultivation') with these strains to obtain a starting optical density (OD) of 0.1. Once
 207 the cultures grew and stationary phase was reached, a 1 mL aliquot of each culture was
 208 transferred to 20 ml fresh SML and grown until stationary phase again (in total approximately
 209 14 generations for IMK341 and 7 generations for IMK882). Single colony isolates from these
 210 evolution cultures ('IMS'-strains) were obtained by plating the cultures using an inoculation
 211 loop (~10 µl) on solid SML and restreaking a grown colony to a fresh plate three consecutive
 212 times, after which one colony was grown in liquid SML and stocked.

213 Table 2: *Saccharomyces cerevisiae* strains used in this study

Strain name	Relevant genotype ^a	Origin
CEN.PK113-7D	Prototrophic reference, <i>MATa</i>	(Entian and Kötter, 2007)
IMX581	<i>MATa ura3-52 can1::cas9-natNT2</i>	(Mans et al., 2015)
IMX585	<i>MATa can1::cas9-natNT2</i>	(Mans et al., 2015)
IMK341	<i>MATa ura3::loxP ady2::loxP-hphNT1-</i>	(de Kok et al.,

	<i>loxP jen1::loxP</i>	2012)
IMW004	<i>MATa URA3 ADY2^{C755G} jen1::loxP-KanMX4-loxP</i>	(de Kok et al., 2012)
IMW005	<i>MATa URA3 ADY2^{C655G} jen1::loxP-KanMX4-loxP</i>	(de Kok et al., 2012)
IMX1000	<i>MATa ura3-52 trp1-289 leu2-3112 his3Δ can1Δ::cas9-natNT2 mch1Δ mch2Δ mch5Δ aqy1Δ itr1Δ pdr12Δ mch3Δ mch4Δ yil166cΔ hxt1Δ jen1Δ ady2Δ aqr1Δ thi73Δ fps1Δ aqy2Δ yll053cΔ ato2Δ ato3Δ aqy3Δ tpo2Δ yro2Δ azr1Δ yhl008cΔ tpo3Δ</i>	(Mans et al., 2017)
IMK875	<i>MATa can1::cas9-natNT2 jen1Δ ady2Δ</i>	This study
IMK876	<i>MATa can1::cas9-natNT2 ura3-52 jen1Δ ady2Δ</i>	This study
IMK882	<i>MATa can1::cas9-natNT2 jen1Δ ady2Δ ato3Δ</i>	This study
IMK883	<i>MATa can1::cas9-natNT2 ura3-52 jen1Δ ady2Δ ato3Δ</i>	This study
IMK982	<i>MATa can1::cas9-natNT2 ura3-52 jen1Δ ady2Δ ato3Δ ato2Δ</i>	This study
IMS807	IMK341 evolved for growth on lactate, evolution line A	This study
IMS808	IMK341 evolved for growth on lactate, evolution line A	This study
IMS809	IMK341 evolved for growth on lactate, evolution line A	This study
IMS810	IMK341 evolved for growth on lactate, evolution line B	This study
IMS811	IMK341 evolved for growth on lactate, evolution line B	This study
IMS1122	IMK882 evolved for growth on lactate	This study
IMS1123	IMK882 evolved for growth on lactate	This study
IMS1130	IMK882 evolved for growth on lactate	This study
IMX2486	IMX1000 <i>ura3-52 TRP1, leu2-3112,</i>	This study

	<i>his3Δ</i>	
IMX2487	IMX1000 <i>ura3-52 TRP1, LEU2, his3Δ</i>	This study
IMX2488	IMX1000 <i>ura3-52 TRP1, LEU2, HIS3</i>	This study
IME581	IMX2488 p426-TEF (2μm)	This study
IME582	IMX2488 pUDE813 (2μm <i>ATO3</i>)	This study
IME583	IMX2488 pUDE814 (2μm <i>ATO3</i> ^{T284C})	This study
IME584	IMX2488 pUDE1001 (2μm <i>JEN1</i>)	This study
IME585	IMX2488 pUDE1002 (2μm <i>ADY2</i>)	This study
IME586	IMX2488 pUDE1003 (2μm <i>ADY2</i> ^{C755G})	This study
IME587	IMX2488 pUDE1004 (2μm <i>ADY2</i> ^{C655G})	This study
IME588	IMX2488 pUDE1021 (2μm <i>ATO2</i>)	This study
IME589	IMX2488 pUDE1022 (2μm <i>ATO2</i> ^{T653C})	This study
IMC164	IMX2488 pUDC319 (<i>CEN6</i>)	This study
IMC165	IMX2488 pUDC320 (<i>CEN6 ATO3</i>)	This study
IMC166	IMX2488 pUDC321 (<i>CEN6 ATO3</i> ^{T284C})	This study
IMC167	IMX2488 pUDC322 (<i>CEN6 JEN1</i>)	This study
IMC168	IMX2488 pUDC323 (<i>CEN6 ADY2</i>)	This study
IMC169	IMX2488 pUDC324 (<i>CEN6 ADY2</i> ^{C755G})	This study
IMC170	IMX2488 pUDC325 (<i>CEN6 ADY2</i> ^{C655G})	This study
IMC171	IMX2488 pUDC326 (<i>CEN6 ATO2</i>)	This study
IMC172	IMX2488 pUDC327 (<i>CEN6 ATO2</i> ^{T653C})	This study

214

215 2.5 Media and cultivation

216 Evolution experiments were performed in 500 mL shake-flask cultures containing 100
217 mL synthetic medium (Verduyn et al., 1992) with 84 mM L-lactic acid as sole carbon source.
218 The pH of the medium was set at 5.0 and the cultures were incubated at 30°C in an Innova
219 incubator shaker (New Brunswick Scientific, Edison, NJ, USA) set at 200 rpm. Auxotrophic
220 requirements were supplemented as needed.

221 Strains were characterized in SM supplemented with different carbon sources. To
222 achieve an initial carbon concentration of 250 mM, the culture media contained either 42 mM

223 D-glucose, 83 mM L-lactic acid, 125 mM acetic acid or 83 mM pyruvic acid. The characterization
224 was performed in a Growth-Profiler system (EnzyScreen, Heemstede, The Netherlands)
225 equipped with 96-well plates in a culture volume of 250 μ L, set at 250 rpm and 30°C. The
226 measurement interval was set at 30 minutes. Raw green values were corrected for well-to-
227 well variation using measurements of a 96-well plate containing a culture with an externally
228 determined optical density of 3.75 in all wells. Optical densities were calculated by converting
229 green values (corrected for well-to-well variation) using a calibration curve that was
230 determined by fitting a third-degree polynomial through 22 measurements of cultures with
231 known OD values between 0.1 and 20. Growth rates were calculated using the calculated
232 optical densities of at least 15 points in the exponential phase. Exponential growth was
233 assumed when an exponential curve could be fitted with an R^2 of at least 0.985.

234 **2.6 Analytical methods**

235 Culture optical density at 660 nm was measured with a Libra S11 spectrophotometer
236 (Biochrom, Cambridge, United Kingdom). In order to measure within the linear range of the
237 instrument (OD between 0.1 and 0.3), cultures were diluted in an appropriate amount of
238 demineralized water. Metabolite concentrations in culture supernatants and media were
239 analyzed using an Agilent 1260 Infinity HPLC system equipped with a Bio-rad Aminex HPX-87H
240 ion exchange column, operated at 60°C with 5 mM H_2SO_4 as mobile phase at a flow rate of
241 0.600 mL min^{-1} .

242 **2.7 DNA extraction and whole genome sequencing**

243 Strain IMK341 and the evolved single colony isolates (IMS-strains) were grown in 500 mL shake
244 flasks containing 100 mL YP medium supplemented with glucose (20 g L^{-1}) as a carbon source.
245 The cultures were incubated at 30°C until the strains reached stationary phase and genomic

246 DNA was isolated using the Qiagen 100/G kit (Qiagen, Hilden, Germany) according to the
247 manufacturer's instructions and quantified using a Qubit® Fluorometer 2.0 (Thermo Fisher
248 Scientific). The isolated DNA was sequenced in-house on a MiSeq (Illumina, San Diego, CA,
249 USA) with 300 bp paired-end reads using TruSeq PCR-free library preparation (Illumina). For
250 all the strains, the reads were mapped onto the *S. cerevisiae* CEN.PK113-7D genome (Salazar
251 et al., 2017) using the Burrows–Wheeler Alignment tool (BWA) and further processed using
252 SAMtools and Pilon for variant calling (Li and Durbin, 2010; Li et al., 2009; Walker et al., 2014).

253 2.8 Transport assays

254 The uptake of labelled carboxylic acids was assessed as previously described by (Ribas et al.,
255 2017), using [1-¹⁴C] acetic acid (Perkin Elmer, Massachusetts, USA) and [U-¹⁴C] L-lactic acid
256 (Perkin Helmer, Massachusetts, USA) with a specific activity of 2000 dpm/nmol. The data
257 shown are mean values of at least three independent experiments.

258 2.9 3D modelling and molecular docking of Ady2, Ato2 and Ato3

259 The three-dimensional modelling analysis was performed for the protein sequences of Ato1,
260 Ady2^{L219V}, Ady2^{A252G}, Ato2, Ato2^{L218S}, Ato3 and Ato3^{F95S}. The amino acid sequences were
261 retrieved from the *Saccharomyces* Genome Database (Cherry et al., 2012). To determine the
262 predicted transporter 3D structures, the amino acid sequences were threaded through the
263 PDB library using LOMETS (Local Meta-Threading-Server). The *Citrobacter koseri* succinate
264 acetate permease (CkSatP, PDB 5YS3) was the top ranked template threading identified in
265 LOMETS for Ato1, Ato2 and Ato3 (Qui et al., 2018). Since the CkSatP three-dimensional
266 modelling obtained the best score for protein structure prediction, it was further considered
267 for molecular docking analysis. CkSatP presents a protein identity of 35% with Ady2, 34% with
268 Ato2 and 28% with Ato3 and similarity of 0.566, 0.548 and 0.515, respectively. Molecular

269 docking simulations were performed as described by (Ribas et al., 2017). Ligand structures of
270 acetic, lactic and pyruvic acid for all target proteins in the study were downloaded from the
271 Zinc database (Sterling and Irwin, 2015). Structures used for docking were all confirmed in
272 Maestro v11.2 before ligand-protein simulations were performed using AutoDock Vina in the
273 PyRx software (Trott and Olson, 2010). The docking studies were performed with the
274 dissociated forms of each carboxylic acid. The protonation states were adjusted to match a pH
275 of 5.0-6.0 and exported in the mol2 format. Docking was performed with four docking-boxes
276 for each protein, containing top, bottom and middle-structure parts for a more robust use of
277 Autodock Vina program. The exhaustiveness parameter was set up for 1000 calculations for
278 each of the grid-zones defined for docking. The generated docking scores and 2-3D pose views
279 were evaluated for the establishment of molecular interactions and ligand binding affinities.

280

281

282 3. RESULTS

283 3.1 Laboratory evolution on lactic acid leads to point mutations in Ato2 or Ato3

284 In an attempt to identify additional transporters able to catalyze the uptake of lactic acid after
285 gaining point mutations, we incubated strains IMK341 and IMX1000 in duplicate shake flasks
286 cultures containing synthetic medium with lactic acid as the sole carbon source. In IMK341 the
287 known carboxylic acid transporters *JEN1* and *ADY2* are knocked out (*jen1Δ, ady2Δ*), whereas
288 IMX1000 contains a further 23 deletions in putative lactic acid transporter-encoding genes
289 (Table 2) (Mans et al., 2017). After 9 weeks, growth was observed in both cultures of IMK341
290 whereas no growth was observed after 12 weeks of incubation of IMX1000. Whole-genome
291 sequencing of evolved IMK341 (*jen1Δ, ady2Δ*) cell lines, named IMS807-811 which were
292 isolated after a transfer to fresh medium, revealed three to seven non-synonymous SNPs in
293 each mutant and no chromosomal duplications or rearrangements (Table 3). Strikingly, all
294 evolved isolates shared an identical mutation in *ATO3* (*ATO3^{T284C}*). To investigate the role of
295 *ATO3* in lactic acid uptake, we overexpressed both the native and evolved *ATO3* in IMK883
296 (*jen1Δ, ady2Δ, ato3Δ*) and tested the resulting strains for growth on SM lactic acid plates. After
297 5 days, only the reference strain CEN.PK113-7D and the strain carrying the *ATO3^{T284C}* allele
298 were able to grow (Supplementary Figure 1), indicating that the T284C mutation in *ATO3* was
299 responsible for the evolved phenotype. We then combined the deletion of *JEN1*, *ADY2* and
300 *ATO3* in strain IMK882 (*jen1Δ, ady2Δ, ato3Δ*) and repeated the evolution. After 5 and 12 days,
301 growth was observed in two independent cultures from which evolved strains IMS1122 and
302 IMS1123 were isolated after transfer to a flask with fresh medium. In both single colony
303 isolates, five SNPs were present (Table 3), including a common mutation in *ATO2*, (*ATO2^{T653C}*),
304 which has also been described as an ammonium transporter together with *ATO3* and *ADY2*

305 (Palková et al., 2002). Finally, the evolution was repeated with IMK982 (*jen1Δ, ady2Δ, ato3Δ,*
 306 *ato2Δ*), but no growth was observed after 12 weeks of incubation.

307 Table 3: Amino acid changes identified by whole-genome sequencing of single colony isolates
 308 evolved for growth in medium containing lactic acid as sole carbon source. Isolates IMS807 to
 309 IMS811 are derived from IMK341 (*jen1Δ, ady2Δ*) and IMS1122 and IMS1123 are derived from
 310 IMK882 (*jen1Δ, ady2Δ, ato3Δ*). IMS807, IM808 and IMS809 are isolates from evolution line
 311 #1 and IMS810 and IMS811 are isolates from evolution line #2. The mutation Sip5^{*490Q}
 312 indicates loss of the stop codon.

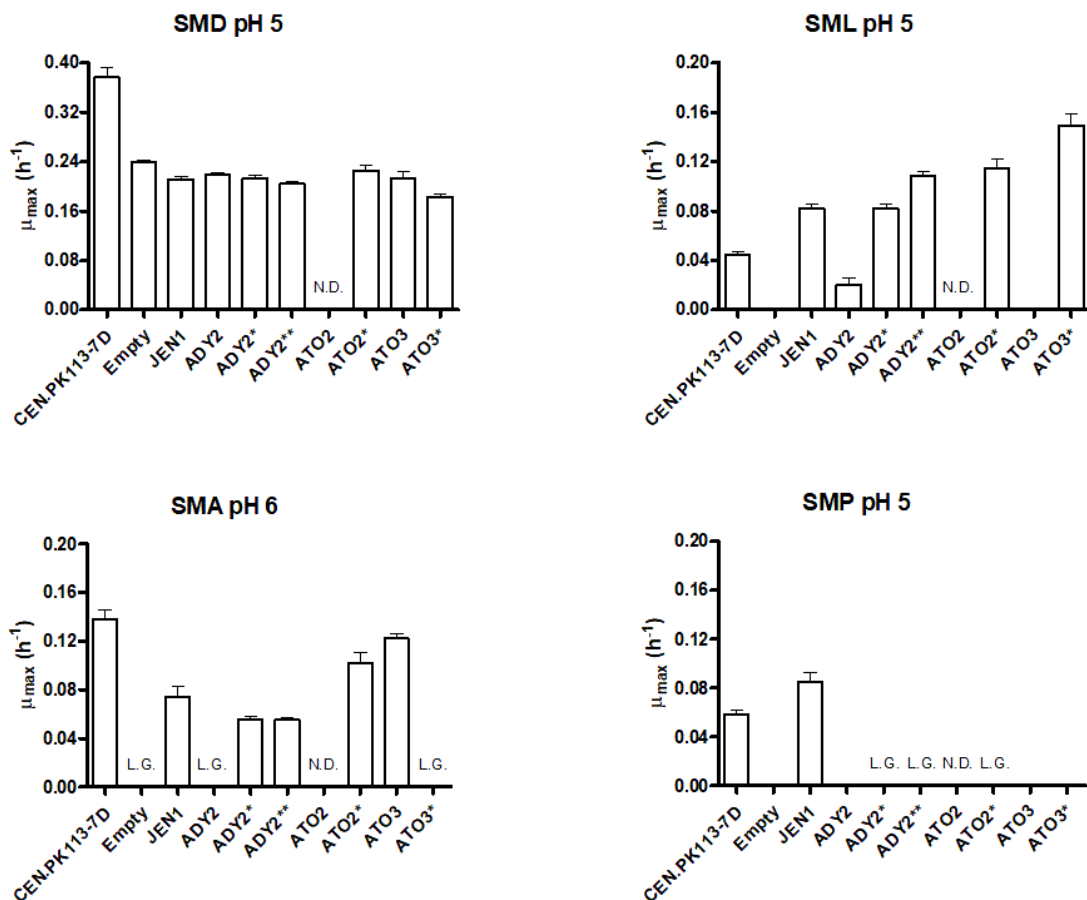
IMK341 evolution #1			IMK341 evolution #2		IMK822 evolution #1	IMK822 evolution #2
IMS807	IMS808	IMS809	IMS810	IMS811	IMS1122	IMS1123
Ato3 ^{F95S}	Ato3 ^{F95S}	Ato3 ^{F95S}	Ato3 ^{F95S}	Ato3 ^{F95S}	Ato2 ^{L218S}	Ato2 ^{L218S}
Mms2 ^{Y58C}	Mms2 ^{Y58C}	Mms2 ^{Y58C}	Sip5 ^{*490Q}	Sip5 ^{*490Q}	Lrg1 ^{H979N}	Whi2 ^{E119*}
Pih1 ^{D147Y}	Pih1 ^{D147Y}	Pih1 ^{D147Y}	Ssn2 ^{M1280R}	Lip5 ^{R4L}	Ykr051w ^{Y285H}	Ykr051w ^{Y285H}
Uba1 ^{L952F}		Drn1 ^{P213L}			Jjj1 ^{H356Q}	Jjj1 ^{H356Q}
Stv1 ^{L275F}					Trm10 ^{A49V}	Trm10 ^{A49V}
Whi2 ^{E187*}						
Vba4 ^{P198L}						

313

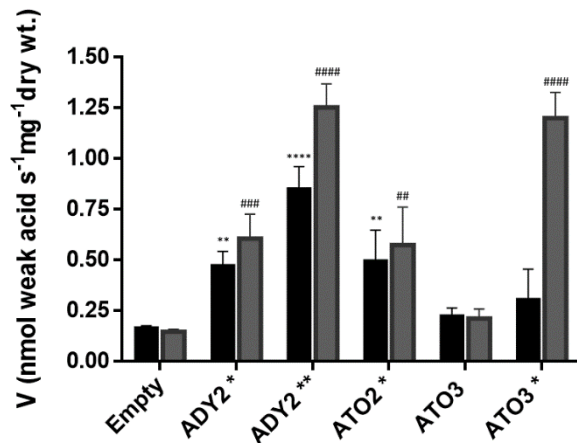
314 **3.2 Overexpression of mutated transporters enables rapid growth in liquid medium with** 315 **lactic acid as sole carbon source**

316 Strikingly, the evolved transporters able to catalyze the uptake of lactic acid (*ATO2* and
 317 *ATO3* in this study, and *ADY2* in work by de Kok et al., 2012) represent all members of the *S.*
 318 *cerevisiae* Acetate Uptake Transporter Family (TCDB 2.A.96). To characterize the impact of the
 319 mutations on the transport of organic acids, cellular growth was evaluated in strains
 320 individually expressing *JEN1*, *ADY2*, *ATO2* and *ATO3* and their mutated alleles under control
 321 of the strong *TEF1* promotor (Mumberg et al., 1995), via centromeric vectors in IMX2488, a
 322 strain background in which 25 (putative) organic acid transporters were deleted (Table 2). No
 323 viable cultures could be obtained with strains overexpressing wildtype *ATO2*, which suggests
 324 a severe toxic effect of the overexpression of *ATO2* on cellular physiology, and for this reason

325 no growth rate could be reported. All other IMX2488-derived transporter expressing strains
326 had similar growth rates in liquid medium with 42 mM glucose as carbon source compared to
327 the empty vector control (IMC164), indicating no major physiological effects caused by the
328 overexpression of the transporters when grown on glucose (Figure 1, top left panel).
329 Overexpression of the transporter variants from multicopy vectors resulted in a growth rate
330 reduction of up to 66% compared to the empty vector reference when grown on glucose and
331 were therefore not tested further (Supplementary Figure 2). In accordance with previous
332 research, strains overexpressing *ADY2*, *ADY2*^{C755G} and *ADY2*^{C655G} showed a maximum specific
333 growth rate of $0.02 \pm 0.01 \text{ h}^{-1}$, $0.08 \pm 0.01 \text{ h}^{-1}$ and $0.10 \pm 0.01 \text{ h}^{-1}$ when grown in medium
334 containing 83 mM lactic acid as carbon source, respectively (de Kok et al., 2012). Surprisingly,
335 strains expressing the evolved *ATO2*^{T653C} and *ATO3*^{T284C} alleles outperformed all the other
336 tested strains, with maximum specific growth rates of $0.11 \pm 0.01 \text{ h}^{-1}$ and $0.15 \pm 0.01 \text{ h}^{-1}$,
337 respectively (Figure 1, top right panel and Supplementary Figure 5). These represent the
338 highest reported growth rates reported for *S. cerevisiae* on this carbon source and indicate
339 that, similar to the role of evolved Ato3 in IMS807-811, the mutations in Ato2 are responsible
340 for the evolved phenotypes observed in IMS1122 and IMS1123 (Table 3). The transport of
341 labelled lactic acid in strains expressing native *ATO3* and evolved *ADY2*, *ATO2* and *ATO3* is in
342 accordance with the observed growth phenotypes (Figure 2). An increased uptake rate was
343 observed for all strains overexpressing evolved transporters compared to the empty vector
344 control strain, whereas expression of wildtype *ATO3* did not lead to a significant alteration in
345 lactic acid uptake (Figure 2).



347 Figure 1: Growth rates on different sole carbon sources of *S. cerevisiae* reference strain
 348 CEN.PK113-7D and the 25-transporter deletion strain IMX2488 expressing an empty vector or
 349 a vector carrying the indicated organic acid transporter. Bars and error bars represent the
 350 average and standard deviation of three independent experiments. SMD: synthetic medium
 351 with 42 mM glucose. SML: synthetic medium with 83 mM lactic acid. SMA: synthetic medium
 352 with 125 mM acetic acid. SMP: synthetic medium with 83 mM pyruvic acid. Empty: empty
 353 plasmid. ADY2*: *ADY2*^{C755G} allele. ADY2**: *ADY2*^{C655G} allele. ATO2*: *ATO2*^{T653C} allele.
 354 *ATO3*^{T284C} allele. For some experiments, a linear increase in optical density was observed,
 355 which impeded the determination of an exponential growth rate (indicated by L.G. for Linear
 356 Growth). N.D.: not determined.



358 Figure 2: Transport of acetate and lactate in *S. cerevisiae* IMX1000 cells expressing native and
 359 evolved *ADY2*, *ATO2* and *ATO3*. Black bars: uptake of 5 mM of ¹⁴C-acetic acid (pH 6.0). Grey
 360 bars: uptake of ¹⁴C-lactic acid (pH 5.0). Empty: empty plasmid. ADY2*: *ADY2*^{C755G} allele.
 361 ADY2**: *ADY2*^{C655G} allele. ATO2*: *ATO2*^{T653C} allele. ATO3*: *ATO3*^{T284C} allele. Cells were grown
 362 on YNB-glucose, washed and incubated in YNB-lactate (0.5 %, pH 5.0) for 4 hours at 30°C.
 363 Statistical significance was estimated by one-way ANOVA followed by a post hoc Tukey's
 364 multiple comparisons test as follows: ** p<0.01, *** p<0.001, **** p<0.0001, acetate uptake
 365 significantly different from cells transformed with empty plasmid; ## p<0.01, ### p<0.001, ####
 366 p<0.0001, lactate uptake significantly different from cells transformed with empty plasmid.
 367

368 3.3 Mutations in *ATO2* and *ATO3* alter the uptake capacity for acetate and pyruvate

369 After demonstrating that the point mutations increased the catalytic activity of Ato2,
 370 Ato3 and Ady2 for lactic acid transport, we also investigated their ability to transport acetic
 371 and pyruvic acid (Figure 1, bottom panels and Supplementary Figures 6 and 7). In liquid
 372 medium at pH 5.0 with 125 mM acetic acid (pK_a of 4.75), no growth was observed for any of
 373 the strains with the 25-deletion background, likely caused by acetic acid toxicity due to the
 374 absence of essential acetic acid exporters (Supplementary Figure 3). However, at pH 6.0
 375 different growth characteristics were observed. The empty vector control strain exhibited
 376 slow non-exponential growth, which was also observed for the strains expressing native *ADY2*
 377 and the evolved *ATO3* variant. On the other hand, expression of native *ATO3* and the evolved
 378 *ADY2* and *ATO2* variants improved growth performance on medium with acetic acid as sole
 379 carbon source. With the exception of native *ATO3*, these results are in accordance with

380 improved uptake rates observed in these strains, determined with labelled acetate (Figure 2).
381 In medium containing 83 mM pyruvic acid, no exponential growth was observed for any of the
382 strains expressing Ato2, Ato3 or Ady2 variants. However, slow, non-exponential growth was
383 observed for strains expressing *ATO2*^{T653C} or any variant of *ADY2* which could indicate a minor
384 change in affinity for this substrate caused by the point mutations.

385 **3.4 Protein modelling reveals mutations in the central hydrophobic constriction site as** 386 **important factor in determining substrate specificity**

387 In order to establish a link between the observed phenotypes and the structural alterations of
388 transporters carrying the mutated amino acid residues, the 3D protein structures of Ady2,
389 Ato2 and Ato3 were predicted based on the crystal structure of the *Citrobacter koseri* acetate
390 anion channel SatP (PDB 5YS3), a bacterial member of the AceTr family (Qui et al., 2018). When
391 combined with a sequence alignment of Ady2, Ato2 and Ato3, the 3D structures showed that
392 the Leu219Val mutation in Ady2, the Leu218Ser mutation in Ato2 and the Phe95Ser mutation
393 in Ato3 are amongst three amino acid residues that were previously identified to be essential
394 for the formation of the central narrowest hydrophobic constriction of the anion pathway in
395 *C. koseri* SatP (Qui et al., 2018) (Figure 3 and Figure 4). Specifically, these changes result in the
396 substitution of the amino acid side group with a smaller (and in the case of Ato2 and Ato3 a
397 more hydrophilic) alternative (Ato3 is shown in Figure 4 and the models for Ady2 and Ato2
398 can be found in Supplementary Figures 9 and 10). Based on these models, we estimated the
399 distance between these three hydrophobic residues. Since these distances are based on
400 model predictions and are, for instance, dependent on the rotation of the amino acid side
401 chains, they should not be interpreted as exact values. However, when comparing the relative
402 distances, we found an increased value for *ADY2*^{C655G}, *ATO3*^{T284C} and *ATO2*^{T653C} compared to

403 their corresponding wildtype protein, leading to a larger aperture in the center of the channel
404 (Table 4). We hypothesize that this increased size of the hydrophobic constriction may allow
405 larger substrates to pass through, possibly altering substrate specificity and transport
406 capacity.

407 To investigate if the mutations affected the presence and affinity of binding sites for acetate,
408 lactate and pyruvate, docking of ligands in the protein structures was simulated using
409 AutoDock Vina (Supplementary Figure 10, Supplementary Table 2). In all proteins, both
410 wildtype and mutated, four binding sites were identified for acetate, which is in accordance
411 with what has previously been reported for the homolog CkSatP (Qui et al., 2018). Of these
412 four binding sites, two, which are located closest to the hydrophobic constriction, also
413 consistently bind lactate and pyruvate. Strikingly, mutations in *Ady2*, *Ato2* and *Ato3* led to an
414 increased lactate affinity of at least one of these two sites closest to the hydrophobic
415 constriction, which might have contributed to the increased lactate transport capacity. No
416 clear correlation was found between the physiology observed for strains overexpressing the
417 different protein variants when grown on acetate and pyruvate and the corresponding binding
418 affinities of these two ligands (Supplementary Table 2).

419



421 Figure 3: Multiple sequence alignment of *Citrobacter koseri* SatP and *Saccharomyces*
 422 *cerevisiae* Ady2, Ato2 and Ato3. The multiple sequence alignment was built with ClustalOmega
 423 (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). Localization of transmembrane segments
 424 (TMSs) was predicted with PSI/TM-Coffee (<http://tcoffee.crg.cat/apps/tcoffee/do:tmcoffee>).
 425 Blue rectangles indicate residues of the narrowest constriction site F98-Y155-L219 (amino acid
 426 numbers refer to Ady2) (Qui et al., 2018). Bold, underlined letters indicate the mutated
 427 residue.
 428

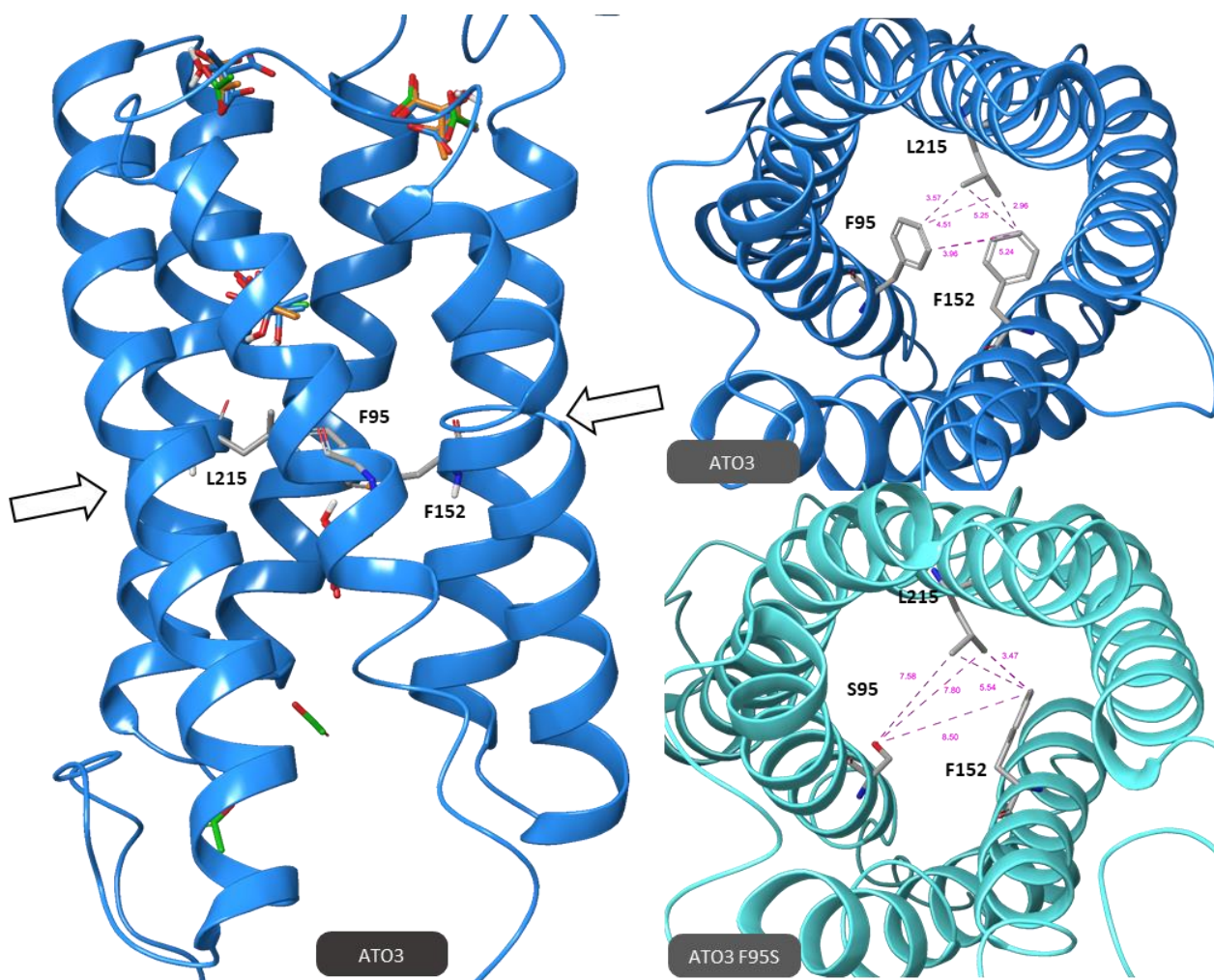
429 Table 4: Estimated average distances (in Å) between different amino acids (AA) in the
 430 constriction pore of Ady2, Ato2 Ato3 and mutated alleles, calculated using the
 431 corresponding protein model. Bold values in the table indicate distances which are at least 1
 432 Å larger than calculated in the reference structure.

Protein	Estimated distance between AA residues			Protein	Estimated distance between AA residues			Protein	Estimated distance between AA residues		
	219&98	98&155	155&219		218&97	97&154	154&218		215&95	95&152	152&215
Ady2	4.6	6.9	4.0	Ato2	4.2	5.7	4.4	Ato3	4.0	4.6	4.1
Ady2 L219V	4.4	6.9	5.3	Ato2 L218S	5.9	5.6	5.6	Ato3 F95S	7.7	8.5	4.5
Ady2 A252G	4.5	6.7	3.9								

433

434

435



436
 437 Figure 4: 3D models of the transporters Ato3 (dark blue) and Ato3^{F95S} (light blue). Left: side
 438 view of Ato3. Arrows indicate the hydrophobic constriction site, consisting of F95, L215 and
 439 F152. Binding sites for acetate (green ligand), lactate (blue ligand) and pyruvate (orange
 440 ligand) are presented. Right, top view of Ato3 (top) and Ato3^{F95S}(bottom). The amino acids
 441 involved in the constriction site are shown. Purple lines and values indicate estimated
 442 distances (in Å) between different anchor points of amino acids, calculated from the
 443 modelled protein structure.
 444

445

446 **4. DISCUSSION**

447 In this study, we report the identification and characterization of a family of transporter genes
448 which, upon mutation, are able to efficiently catalyze the import of lactic acid in *S. cerevisiae*.
449 As rational engineering to identify lactic acid transporters remains elusive (Borodina, 2019;
450 Mans et al., 2017), we used adaptive laboratory evolution to select for mutants capable of
451 consuming lactic acid, which led to the identification of mutations in *ATO3* (*ATO3*^{T284C}) and
452 *ATO2* (*ATO2*^{T653C}). Together with *ADY2*, *ATO2* and *ATO3* were previously described to code for
453 ammonium transporters (Ammonium Transport Outwards) based on two observations: the
454 high expression levels of these genes when *S. cerevisiae* exports ammonium, and the presence
455 of a motif associated with ammonium transport in the encoded proteins (Palková et al., 2002).
456 However, the function of *ADY2* has previously been assessed by (Rabitsch et al., 2001), who
457 identified it as a gene required for correct spore formation, and thus named it as *ADY2*
458 (Accumulation of DYads). In view of the observations in our study, where *ADY2*, *ATO2* and
459 *ATO3* and their evolved variants catalyzed uptake of lactic and in some cases acetic acid, and
460 the absence of mechanistic studies aimed at illustrating the phenomenon of ammonium
461 export, we support the recent proposition by (Alves et al., 2020) to rename these genes,
462 present in *S. cerevisiae* and other yeasts, as “Acetate Transporter Ortholog”.

463 For physiological studies focused on organic acid substrate uptake, a platform strain devoid of
464 organic acid importers is a useful tool as it enables characterization based on growth rate. No
465 growth was observed for IMC164 (25 deletions and empty vector) on medium containing
466 either lactic acid or pyruvate as sole carbon source (Figure 1), demonstrating that this is a
467 suitable strain background to test pyruvic and lactic acid transport capacity of transporter
468 variants. Strain IMK982 (*jen1Δ ady2Δ ato3Δ ato2Δ*) was also unable to grow on lactic acid, nor

469 could it evolve this trait, which suggests that this strain could also be employed as a platform
470 strain to investigate both endogenous and heterologous lactic acid transporters, without
471 requiring the additional 21 deletions. In contrast, when grown on acetic acid at pH 6.0, IMC164
472 exhibited non-exponential linear growth (Supplementary Figure 6), suggesting simple
473 diffusion of acetic acid, or the presence of at least one gene involved in acetate transport in
474 this strain background. The observed increase in the uptake rate of acetic acid for the evolved
475 *Ady2* and *Ato2* variants (Figure 2) corroborates with the increased growth rate on this carbon
476 source. For the strain expressing native *ATO3* an improved growth rate was observed, but no
477 increase in acetic acid uptake could be detected. This result led us to postulate the role of
478 *Ato3* as an exporter of acetic acid, thereby limiting the negative effects caused by the passive
479 diffusion of this monocarboxylic acid. The fact that the expression of native *ATO3*, besides
480 *ADY2*, results in an increased growth rate on acetate is in accordance with previous data
481 reporting that both genes are induced in cells shifted from glucose to acetic acid as sole carbon
482 source (Paiva et al., 2004).

483 It was reported by de Kok et al., (2012) that the overexpression of *ADY2*, under the control of
484 the strong glycolytic promoter *TEF1*, was sufficient to enable slow growth ($\mu_{\max} \sim 0.02 \text{ h}^{-1}$) in
485 medium containing lactic acid as sole carbon source. While the native alleles of *ATO3* and likely
486 *ATO2* were not able to sustain growth on lactic acid medium, their mutated versions
487 (*ATO2*^{T653C} and *ATO3*^{T284C}) enabled high growth rates, with the highest growth rate determined
488 at $0.15 \pm 0.01 \text{ h}^{-1}$ for the strain harboring *ATO3*^{T284C}. To the best of our knowledge this growth
489 rate represents the highest reported growth rate of *S. cerevisiae* expressing a single transport
490 protein on lactic acid and is close to the growth rate observed by de Kok et al., (2012) of 0.14
491 h^{-1} by a strain expressing *ADY2*^{C655G}. This 3-fold increase in growth rate of the engineered strain
492 compared to the reference strain CEN.PK113-7D indicates that, in non-engineered *S.*

493 *cerevisiae* strains, growth on lactic acid is likely limited by its transport into the cell, and not
494 the capacity to be further metabolized. Therefore, for future work that requires fast
495 consumption of lactic acid, overexpression of *ATO3*^{T284C} can be considered.

496 Based on the 3D structures of Ady2 (Ato1), Ato2 and Ato3 and the simulation of ligand docking
497 in the predicted protein structures, we postulate that an increased binding affinity upon
498 mutation may contribute to increased transport capacity by facilitating passage of the ligand
499 through the hydrophobic constriction, although the increased size of the hydrophobic
500 constriction is probably the main contributor to the evolved phenotype. Other mechanisms
501 may also contribute to an improved transport capacity, as observed for the A252G mutation
502 in Ady2, an amino acid residue located outside the constriction pore. These may include an
503 improved transition between the closed to open state of the transporter or increased stability
504 in the plasma membrane.

505 In this study, we show that laboratory evolution is a powerful tool for the identification of
506 genes involved in substrate transport and resulted in the identification of *Ato3*^{F95S}, which
507 enables the highest growth rate on lactic acid by *S. cerevisiae* reported in strains expressing a
508 single transport protein thus far. In addition, the presented data on transporter structure and
509 function led to the identification of important amino acid residues that dictate substrate
510 specificity of *S. cerevisiae* carboxylic acid transporters, which could potentially aid in future
511 rational engineering and annotation of additional proteins involved in organic acid transport.

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670

671 **6. Funding**

672 This work was supported by the BE-Basic R&D Program, which was granted an FES subsidy
673 from the Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I); the strategic
674 programme UID/BIA/04050/2019 funded by Portuguese funds through the FCT I.P.; the
675 projects: PTDC/BIAMIC/5184/2014, funded by national funds through the Fundação para a
676 Ciência e Tecnologia (FCT) I.P.; the European Regional Development Fund (ERDF) through the
677 COMPETE 2020–Programa Operacional Competitividade e Internacionalização (POCI);
678 EcoAgriFood: Innovative green products and processes to promote AgriFood BioEconomy
679 [grant number NORTE-01–0145-FEDER-000009]; Norte Portugal Regional Operational
680 Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the
681 European Regional Development Fund (ERDF); and UMINHO/BD/25/2016 PhD grant by the
682 Norte2020 [grant number NORTE-08–5369-FSE-000060] and a FEBS Short-Term Fellowship
683 to MSS.

684

1 **SUPPLEMENTARY INFORMATION**

2

3 **Evolutionary engineering for lactic acid uptake reveals key amino acid residues involved in**
4 **substrate specificity of *Saccharomyces cerevisiae* carboxylic acid transporters.**

5

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18 Running title: Novel lactic acid transporters in yeast.

19

20 Supplementary Figure 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10

21 Supplementary Table 1 and 2

Number	Name	Sequence (5' -> 3')	Purpose
8664	JEN1_target tRNA FW SspI	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCGTCACTCAATATTAATTTACGTTTTAGAGCTAGAAAT AGCAAGTAAAATAAGGCTAGTCCGTTATCAAC	Construction of pUDR405
6262	CrRNA insert ADY2 fw	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCCCACCGTAAGAACATAATGGTTTTAGAGCTAGAAA TAGCAAGTAAAATAAGGCTAGTCCGTTATCAAC	Construction of pUDR405
6005	p426 CRISP rv	GATCATTATCTTTCACTGCGGAGAAG	Construction of pUDR405 – pUDR420
8691	ATO3_target etRNA FW SspI	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCGAGTATATCTCTTGAATATTGTTTTAGAGCTAGAAAT AGCAAGTAAAATAAG	Construction of pUDR420
13552	ATO3_target etRNA_RV_ SspI	CTTATTTAACTTGCTATTCTAGCTCTAAAACAATATTCAAGAG ATATACTCGATCATTTATCTTTCACTGCGGAGAAGTTTCGAACG CCGAAACATGCGCA	Construction of pUDR420
8688	ATO2_target etRNA FW Eco52I	TGCGCATGTTTCGGCGTTCGAAACTTCTCCGCAGTGAAAGATA AATGATCCGGCCGTCAAAAATTTTAAGTTTTAGAGCTAGAAAT AGCAAGTAAAATAAG	Construction of pUDR767
6006	p426 CRISP fw	GTTTTAGAGCTAGAAATAGCAAGTAAAATAAGGCTAGTC	Construction of pUDR420 and pUDR767

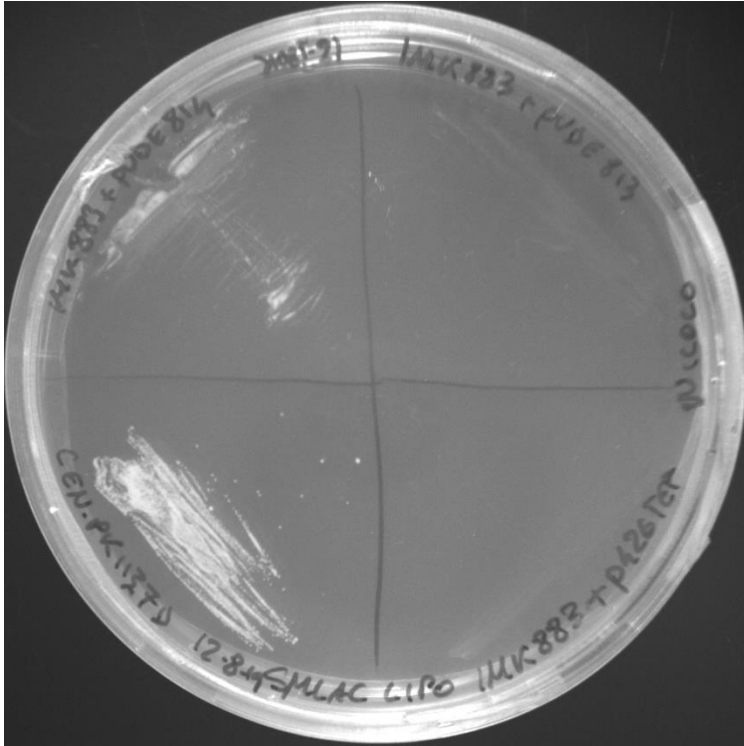
5921	Primer_pTE F1_rv	AAAACCTAGATTAGATTGCTATGCTTTCTTCTAATGAGC	Linear p426-TEF backbone amplification
10547	p426-GPD backbone rv	TCATGTAATTAGTTATGTCACGC	Linear p426-TEF backbone amplification
13513	pTEF1_ATO 3	TACAACCTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCA ATCTAATCTAAGTTTTATGACATCGTCTGCTTCTTC	Construction of pUDE813 and pUDE814
13514	tCYC1_ATO 3	CGGTTAGAGCGGATGTGGGGGAGGGCGTGAATGTAAGCGT GACATAACTAATTACATGATTAAGGAGCATTGGCATTG	Construction of pUDE813 and pUDE814
17168	pTEF1_ADY 2_fw	TACAACCTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCA ATCTAATCTAAGTTTTATGTCTGACAAGGAACAAACG	Construction of pUDE1002, pUDE1003 and pUDE1004
17169	tCYC1_ADY 2_rv	CGGTTAGAGCGGATGTGGGGGAGGGCGTGAATGTAAGCGT GACATAACTAATTACATGATTAAGATTACCTTTCAGTAG	Construction of pUDE1002, pUDE1003 and pUDE1004
17170	pTEF1_JEN 1_fw	TACAACCTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCA ATCTAATCTAAGTTTTATGTCTGTCGTCATTACAGATG	Construction of pUDE1001
17171	tCYC1_JEN1 _rv	CGGTTAGAGCGGATGTGGGGGAGGGCGTGAATGTAAGCGT GACATAACTAATTACATGATTAACGGTCTCAATATGCTCC	Construction of pUDE1001
17452	pTEF1_ATO 2_fw	TACAACCTTTTTTACTTCTTGCTCATTAGAAAGAAAGCATAGCA ATCTAATCTAAGTTTTATGTCTGACAGAGAACAAAGC	Construction of pUDE1021 and pUDE1022
17453	tCYC1_ATO 2_rv	CGGTTAGAGCGGATGTGGGGGAGGGCGTGAATGTAAGCGT GACATAACTAATTACATGATTAGAAGAACACCTTATCATTGC	Construction of pUDE1021 and pUDE1022
17742	p426_CENA RS_fw	TAGAAAAATAACAAATAGGGGTTCCGCGCACATTTCCCGGAA AAGTGCCACCTGAACGAACGGATCGCTTGCCTGTAAC	Amplification of CEN6 from pUDC156
17743	p426_CENA RS_rv	GATAATATCACAGGAGGTAAGTACCTTTTCATCTACATAA ATAGACGCATATAAGTTCCCGGAAAAGTGCCACCTG	Amplification of CEN6 from pUDC156

2949	F Tag Episomal Rev	CGTTCAGGTGGCACTTTTCG	Construction of pUDC319-pUDC327
17741	p426_origi nremoval	ACTTATATGCGTCTATTTATGTAGGATG	Construction of pUDC319-pUDC327
1742	LEU2 check fw	GGTCGCCTGACGCATATACC	<i>LEU2</i> amplification
1743	LEU2 check rv	TAAGGCCGTTTCTGACAGAG	<i>LEU2</i> amplification
1738	HIS3 check fw	GCAAGCAAGATAAACAAGG	<i>HIS3</i> amplification
3755	his3 outside rv (B)	CACTTGTTGCTCAGTTCAG	<i>HIS3</i> amplification
8597	JEN1_repai r oligo fw	AAGAAGAGTAACAGTTTCAAAGTTTTCTCAAAGAGATTAA ATACTGCTACTGAAAATTCACITTTTCATTGCTCTAGGGCGTG TTCGCTTCTCTATGTAAGTGCATTTACATATA	Deletion of <i>JEN1</i>
8598	JEN1_repai r oligo rv	TATATGTGAAATGCAGTTACATAGAGAAGCGAACACGCCCTAG AGAGCAATGAAAAGTGAATTTTCAGTAGCAGTATTTAATCTCTT TGAGGAAAACTTTTGAAGTGTACTCTTCTT	Deletion of <i>JEN1</i>
8665	ADY2_repai r oligo fw	CGACAGCTAACACAGATATAACTAAACAACCACAAAACAACTC ATATACAAACAATAATGAGCACGACCTACTAATAACGAGAAC TATTGAAATAAAAAAGAGTAGTTTTTATTTTC	Deletion of <i>ADY2</i>
8666	ADY2_repai r oligo rv	GAAAAATAAAAACTACTCTTTTTATTCAATAGTTCTCGTTAT TAGTAGGTCGTGCTCATTATTTGTTTGTATATGAGTTGTTTGT GGTTGTTTAGTTATATCTGTGTTAGCTGTCG	Deletion of <i>ADY2</i>

14120	ATO3_repair_syn_fw	ATTGAGACGCTCCCCAGCAGGGTTCGATTGCAGGCGTTTCGC AGGGCAGTAGAATTTACCTAGACGTGGCCTTCTTGATGTTGA TGTGTACATTGAAGAGCACGTGGGGTTTGTCT	Deletion of ATO3
14121	ATO3_repair_syn_fw	AGAACAAACCCACGTGCTCTTCAATGTACACATCAACATCAA GAAGGCCACGTCTAGGTGAAATTCTACTGCCCTGCGAAACGCC TGCAATCGAACCTGCTGGGGGAGCGTCTCAAT	Deletion of ATO3
8689	ATO2_repair_oligo_fw	TATGTAACATTCTACAGATCAATCAAAACAATCTTCAATCACA GAAAAAATAAAAGGCAACACAAAAGTGCAGGCTAAAATAA CTTTACCCCTATTATATATTCTTATGATCCATT	Deletion of ATO2
8690	ATO2_repair_oligo_rv	AATGGATCATAAGAATATATAATAGGGTAAAAGTTATTTAG CCTGCACTTTTGTGTTGCCTTTTTTTTTTCTGTGATTGAAGA TTGTTTTGATTGATCTGTAGAATGTTACATA	Deletion of ATO2

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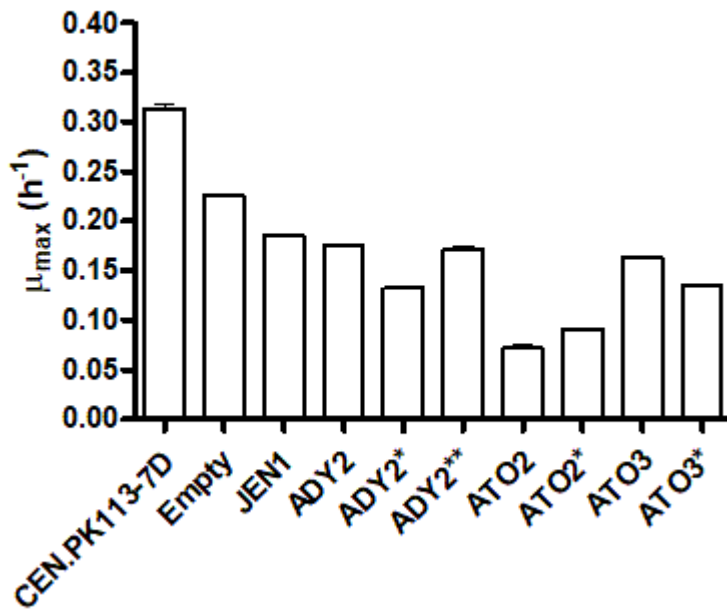


26

27 *Supplementary Figure 1:* Growth of different strains on SM media with lactic acid as the sole carbon source. Bottom left
28 quadrant: prototrophic strain CEN.PK113-7D. Bottom right quadrant: IMK883 (*ura3-52, jen1Δ, ady2Δ, ato3Δ*) carrying an
29 empty p426-pTEF plasmid. Top right quadrant: IMK883 carrying pUDE813 (p426-pTEF-ATO3). Top left quadrant: IMK883
30 carrying pUDE814 (p426-pTEF- *ATO3^{7284C}*). Cells were streaked from a single colony and the plate was incubated at 30 °C for
31 5 days.

32

multicopy SMD pH 5

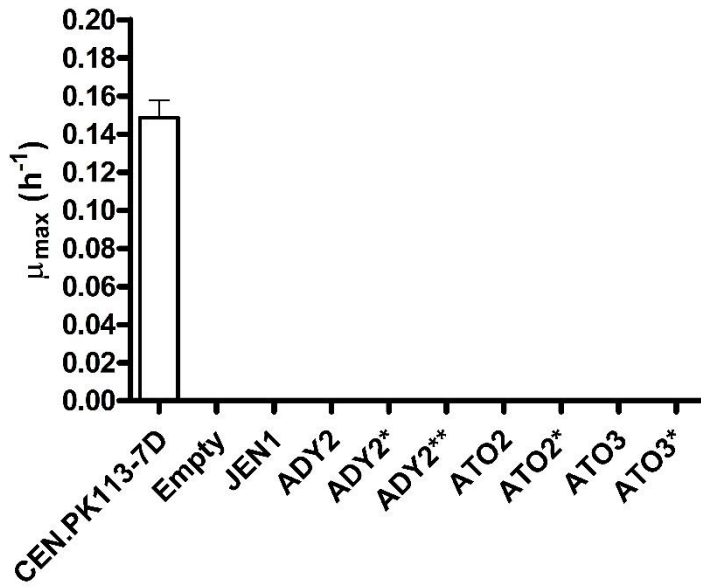


Supplementary Figure 2: Growth rates on SMD of *S. cerevisiae* reference strain CEN.PK113-7D and the 25-transporter deletion strain IMX2488 expressing an empty multicopy vector or a multicopy vector containing the indicated organic acid transporter gene. Bars and error bars represent the average and standard deviation of three independent experiments. Empty: empty plasmid. ADY2*: *ADY2*^{C755G} allele. ADY2**: *ADY2*^{C655G} allele. ATO2*: *ATO2*^{T653C} allele. ATO3*: *ATO3*^{T284C} allele.

33

34

SMA pH 5



Supplementary Figure 3: Growth rates of *S. cerevisiae* reference strain CEN.PK113-7D and the 25-transporter deletion strain IMX2488 expressing an empty centromeric vector or a centromeric vectors containing the indicated organic acid transporter gene. Growth on SMA medium set at pH5. Bars and error bars represent the average and standard deviation of three independent experiments. Empty: empty plasmid. ADY2*: *ADY2*^{C755G} allele. ADY2**: *ADY2*^{C655G} allele. ATO2*: *ATO2*^{T653C} allele. ATO3*: *ATO3*^{T284C} allele.

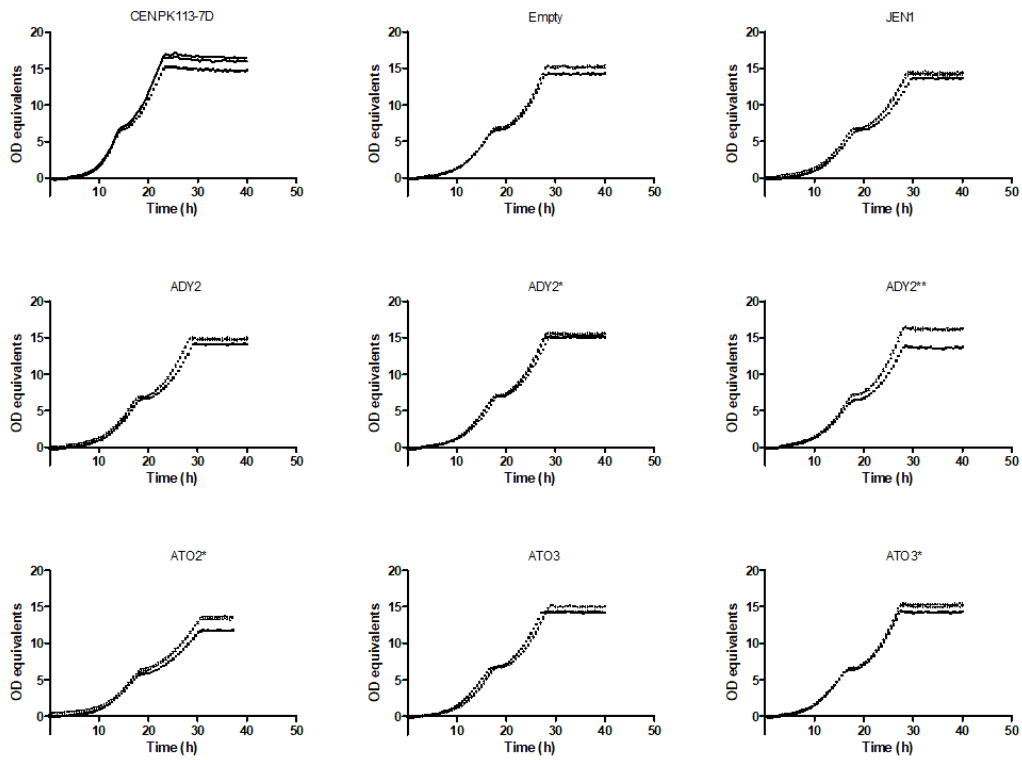
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SMD

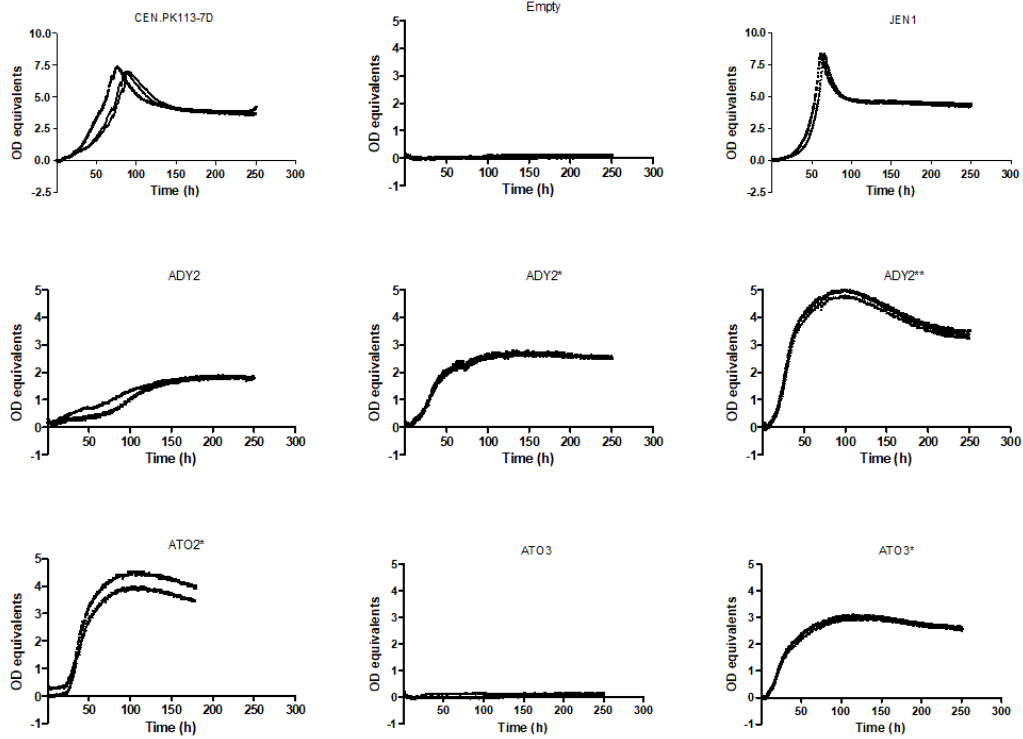


40

41 Supplementary Figure 4: Growth profiles in synthetic medium (pH 5.0) with glucose as the sole carbon source of
 42 CEN.PK113-7D and the 25-transporter deletion strain IMX2488 expressing an empty multicopy vector or a multicopy vector
 43 containing the indicated organic acid transporter gene. Empty: empty plasmid. ADY2*: *ADY2*^{C755G} allele. ADY2***: *ADY2*^{C655G}
 44 allele. ATO2*: *ATO2*^{T653C} allele. ATO3*: *ATO3*^{T284C} allele.

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SML

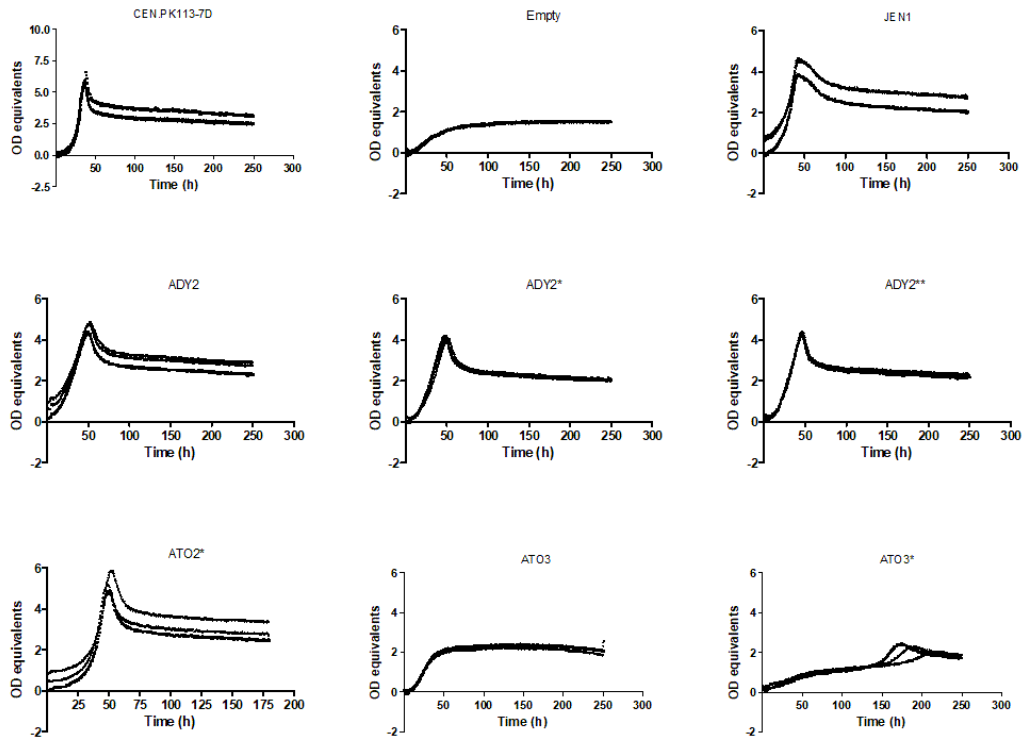


46

47 *Supplementary Figure 5: Growth profiles in synthetic medium (pH 5.0) with lactate as the sole carbon source of CEN.PK113-*
 48 *7D and the 25-transporter deletion strain IMX2488 expressing an empty multicopy vector or a multicopy vector containing*
 49 *the indicated organic acid transporter gene. Empty: empty plasmid. ADY2*: ADY2^{C755G} allele. ADY2**: ADY2^{C655G} allele.*
 50 *ATO2*: ATO2^{T653C} allele. ATO3*: ATO3^{T284C} allele.*

51

SMA



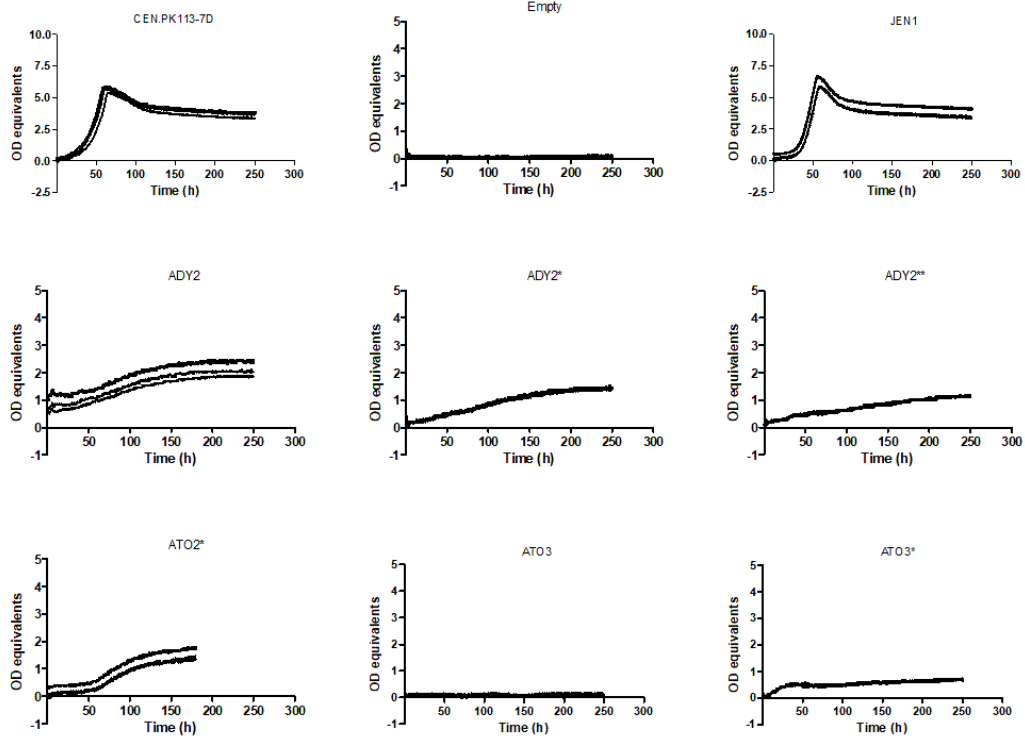
52

53 *Supplementary Figure 6: Growth profiles in synthetic medium (pH 6.0) with acetate as the sole carbon source of CEN.PK113-*
54 *7D and the 25-transporter deletion strain IMX2488 expressing an empty multicopy vector or a multicopy vector containing*
55 *the indicated organic acid transporter gene. Empty: empty plasmid. ADY2*: ADY2^{C755G} allele. ADY2**: ADY2^{C655G} allele.*
56 *ATO2*: ATO2^{T653C} allele. ATO3*: ATO3^{T284C} allele.*

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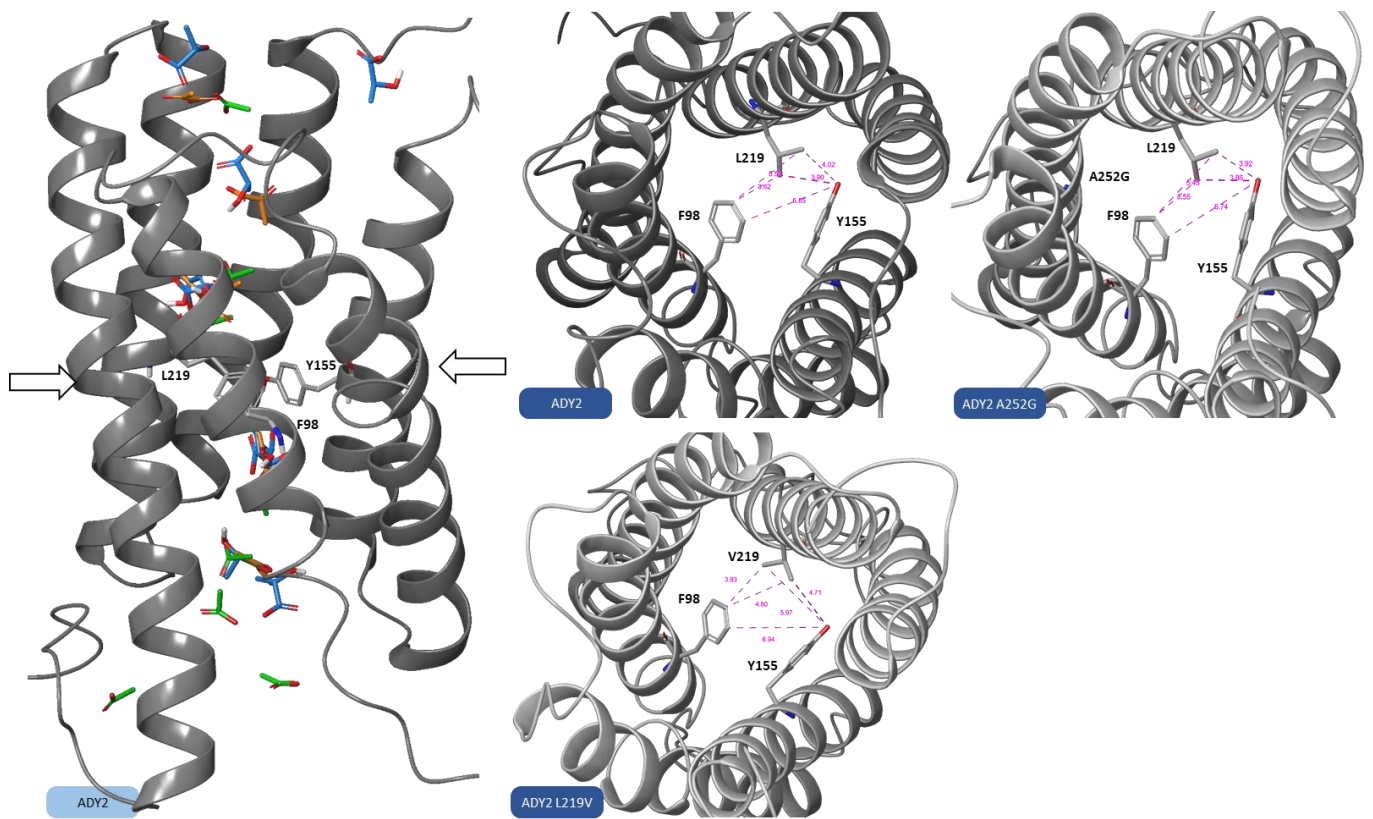
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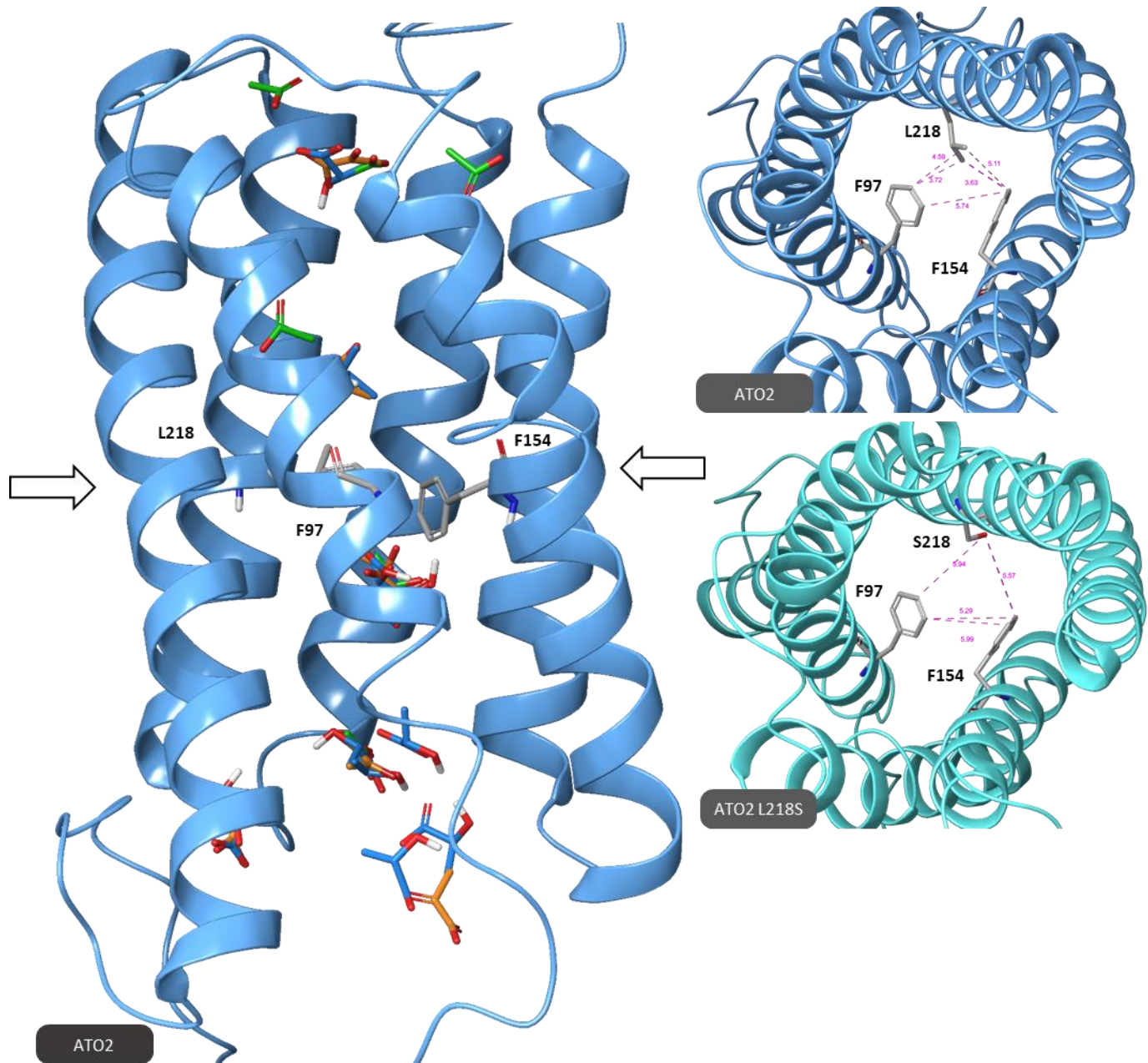
59

60 *Supplementary Figure 7: Growth profiles in synthetic medium (pH 5) with pyruvate as the sole carbon source of CEN.PK113-*
 61 *7D and the 25-transporter deletion strain IMX2488 expressing an empty multicopy vector or a multicopy vector containing*
 62 *the indicated organic acid transporter gene. Empty: empty plasmid. ADY2*: ADY2^{C755G} allele. ADY2**: ADY2^{C655G} allele.*
 63 *ATO2*: ATO2^{T653C} allele. ATO3*: ATO3^{T284C} allele.*

64



Supplementary Figure 8: 3D model of Ady2, Ady2^{C755G} and Ady2^{C655G} alleles. Left, side view of Ady2. Arrows indicate the hydrophobic constriction site. Binding sites for acetate (green ligand), lactate (blue ligand) and pyruvate (orange ligand) are presented. Right, top view of Ady2, Ady2^{C755G} and Ady2^{C655G} alleles. The amino acids involved in the hydrophobic constriction site are shown. Purple lines and values indicate distances (in Å) between different anchor points of amino acids.



68

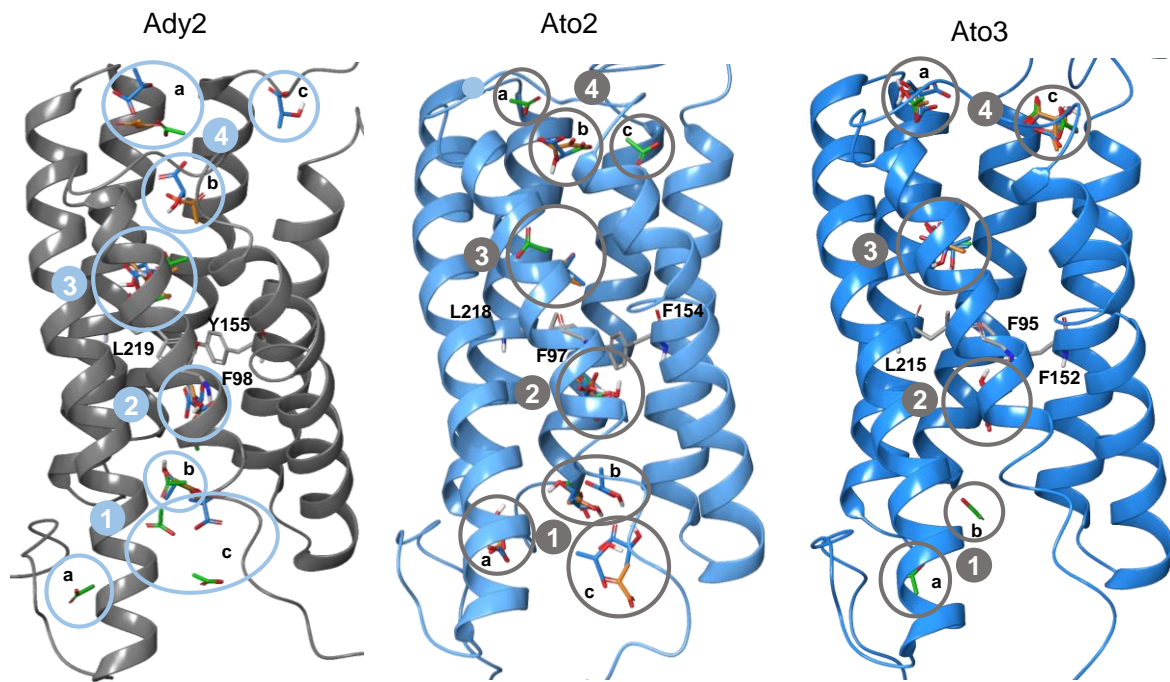
69 *Supplementary Figure 9: 3D model of Ato2 and Ato2^{L218S}. Left, side view of Ato2. Arrows indicate the constriction site.*
 70 *Binding sites for acetate (green ligand), lactate (blue ligand) and pyruvate (orange ligand) are presented. Right, top view of*
 71 *either Ato2 or Ato2^{L218S}. The amino acids involved in the constriction site are shown. Purple lines and values indicate*
 72 *distances (in Å) between different anchor points of different amino acids.*

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Supplementary Table 2: Average of the binding affinity values [kcal/mol] calculated with PyRx software for the docking of ligand in the predicted structures of wildtype and mutated Ady2, Ato2 and Ato3.

3D-Protein templates	Average of binding affinities (kcal/mol) at different binding sites								
	Acetate								
	1			2	3	4			
	a	b	c			a	b	c	
Ady2	-2,1	-2,4	-2,3	-2,7	-3,1	-2,7	-	-	78
Ady2 A252G	-2,3	-2,2	-2,1	-3,1	-3,1	-2,6	-2,7	-	
Ady2 L219V	-2,4	-	-	-3,0	-3,0	-2,5	-2,7	-	
Ato2	-2,5	-2,6	-	-3,1	-2,9	-2,8	-2,9	-	79
Ato2 L218S	-2,7	-	-	-2,7	-3,1	-3,3	-2,6	-2,5	
Ato3	-2,3	-2,2	-	-2,9	-3,0	-2,2	-	-2,4	
Ato3 F95S	-2,6	-2,4	-	-3,0	-2,9	-2,4	-2,4	-2,4	80
	Lactate								
	1			2	3	4			d
	a	b	c			a	b	c	
	Ady2	-	-3,1	-3,0	-3,6	-4,3	-3,5	-3,1	-3,1
Ady2 A252G	-2,9	-2,8	-2,7	-3,7	-4,4	-3,3	-3,4	-	82
Ady2 L219V	-3,4	-	-	-3,9	-4,4	-	-3,8	-	
Ato2	-3,2	-3,1	-3,2	-3,9	-3,8	-	-2,9	-	
Ato2 L218S	-3,5	-	-	-3,8	-4,2	-3,6	-	-3,4	83
Ato3	-	-	-	-3,4	-3,8	-3,1	-	-3,2	
Ato3 F95S	-3,3	-	-	-4,2	-4,0	-3,2	-3,2	-3,2	
	Pyruvate								
	1			2	3	4			
	a	b	c			a	b	c	
	Ady2	-	-3,1	-	-3,7	-4,2	-3,2	-3,3	-
Ady2 A252G	-3,0	-	-2,7	-3,9	-4,3	-3,3	-3,3	-	
Ady2 L219V	-3,2	-	-	-4,0	-4,2	-	-3,5	-	
Ato2	-3,3	-3,3	-3,1	-3,9	-4,1	-	-3,6	-	86
Ato2 L218S	-3,5	-	-	-3,9	-4,3	-	-	-3,3	
Ato3	-	-	-	-3,6	-4,0	-3,0	-	-3,2	
Ato3 F95S	-3,4	-	-	-4,2	-3,9	-	-3,4	-	87



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90 *Supplementary Figure 10: Molecular docking sites of acetate (green ligand), lactate (blue ligand) and pyruvate (orange*
 91 *ligand) in the predicted structure of Ady2, Ato2 and Ato3, identified using Autodock Vina.*

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