Universidade do Minho
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Characterization of carboxylate transporters: functional and structural studies for the improvement of cell factories

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Characterization of carboxylate transporters: functional and structural studies for the improvement of cell factories

Tese de Doutoramento
Doutoramento em Biologia Molecular e Ambiental

Trabalho efetuado sob a orientação da
Professora Doutora Margarida Casal
da
Professora Doutora Sandra Paiva
e do
Professor Doutor George Diallinas

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Declaração

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STATEMENT OF INTEGRITY

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"I will always choose a lazy person to do a difficult job
because a lazy person will find an easy way to do it…"
Bill Gates

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moldados e esculpidos
por aqueles que estão,
se aproximam,
ou se afastam.

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Resumo

À medida que a procura de fontes de energia renováveis aumenta, a produção de compostos químicos por fermentação microbiana, tais como os ácidos orgânicos, torna-se uma alternativa atrativa à síntese química a partir de derivados da indústria petrolífera. A biossíntese de ácidos orgânicos, em concentrações e rendimentos economicamente sustentáveis, requer a manipulação de vias metabólicas e da maquinaria enzimática dos microrganismos, assim como a exploração de transportadores de membrana. Estes são essenciais no efluxo dos ácidos orgânicos para o meio de fermentação, permitindo aumentar a tolerância da célula microbiana em relação a estes compostos e, concomitantemente, diminuir os custos de processamento pós-fermentação. De forma a garantir um progresso significativo em futuras aplicações que visem o aumento de produção de ácidos orgânicos, reveste-se de particular importância aprofundar o conhecimento sobre as proteínas transportadoras destes compostos. Os ácidos orgânicos têm um efeito tóxico nas células, associado à dissipação do potencial transmembranar, acidificação do citosol e acumulação de aniões. Devido à tolerância a condições acídicas, e à reduzida necessidade de manutenção e controlo do pH extracelular, a levedura *Saccharomyces cerevisiae* é considerada um dos microrganismos mais atrativos para a produção de ácidos orgânicos. Em leveduras, o transporte destes ácidos é realizado por diversas proteínas de membrana, nomeadamente as pertencentes às famílias Jen1 e Ady2.

Efectuámos uma caracterização funcional dos homólogos da proteína Jen1 através da expressão heteróloga na estirpe *S. cerevisiae jen1*Δ *ady2*Δ. Foram identificados transportadores de acetato (Dh27), de malato (Dh17) e de succinato (DH18 e Dh24) da levedura *Debaryomyces hansenii*. Estes estudos indicam ainda que o sacarato é substrato dos transportadores ScJen1-S271Q e KlJen2, o gluconato dos transportadores CaJen2 e KlJen2, e o xilarato e o mucato do transportador CaJen2.

Alguns análogos de ácidos orgânicos podem apresentar mais valias a nível terapêutico como é o caso do 3-Bromopiruvato (3BP), um composto com propriedades anticancerígenas e antimicrobianas. Desta forma, avaliou-se também o papel de homólogos de Jen1 pertencente ao microrganismo patogénico *Cryptococcus neoformans,* no transporte de 3BP. Verificou-se que a proteína Cn04 tem um papel relevante no transporte e sensibilidade das células de *C. neoformans* ao 3BP.

Neste trabalho foram caracterizados vários membros da família AceTr. Verificou-se que a proteína YaaH de *Escherichia coli* é um transportador ativo secundário de acetato e de sucinato por simporte com protões, e por esse motivo foi proposta a sua designação como SatP (succinate-acetate transporter); o gene *aceP* da arquea *Methanosarcina acetivorans* foi identificado como sendo específico para o transporte de acetato por cotransporte com protões; elucidámos ainda o papel da proteína Gpr1 de *Yarrowia lipolytica*, como sendo um transportador de acetato.

A análise filogenética da família AceTr revelou a presença de homólogos na maioria das espécies de fungos analisadas. Dados sobre o seu papel fisiológico sugerem a importância destes transportadores ao longo da evolução, associada à reprodução sexuada por esporulação. Os resíduos de aminoácidos do motivo conservado NPAPLGL(M/S) da família AceTr, foram identificados como essenciais para atividade destes transportadores, uma vez que a substituição de qualquer um destes resíduos de aminoácidos eliminou o transporte de acetato.

Globalmente, o presente estudo destacou o amplo número de aplicações para os transportadores de ácidos carboxílicos na biotecnologia industrial. Realça-se o aumento do conhecimento sobre transportadores de ácidos orgânicos e a contribuição sobre a evolução, filogenia e a estrutura dos membros da família AceTr.

Abstract

As the market demand for sustainable energy increases, production of platform chemicals, such as organic acids, from renewable feedstocks *via* microbial fermentation has become a more attractive alternative to petroleum-based chemicals. The biosynthesis of organic acids in high and sustainable yields requires the engineering of not only the microorganisms enzymatic and metabolic machinery, but also of plasma membrane transporters. These are able to export organic acids to the extracellular space, improving the host's tolerance and decreasing downstream processing costs.

Thus, to allow a significant impact in future applications envisaging the increase of organic acid production, the study of membrane transporters with specificity for these solutes is of utmost importance.

Organic acids are reported to be toxic to cell factories, being associated with trans-membrane potential disruption, cytosol acidification, and anion accumulation. Due to its tolerance to low pH, reducing the need for maintenance of neutral pH and being less vulnerable to contamination, *Saccharomyces cerevisiae* is taken as one of the most appealing hosts to produce these platform chemicals. In yeasts, the transport of organic acids is played by several membrane proteins, namely the ones belonging to the Jen1 and the Ady2 families.

In this study, we functionally characterized Jen1 homologues by heterologous expression in a *S. cerevisiae jen1*Δ *ady2*Δ strain. We identified an acetate (Dh27), malate (Dh17) and two succinate transporters (Dh18 and Dh24) from the yeast *Debaryomyces hansenii*. Additionally, we found evidences that saccharate is a substrate of the transporters ScJen1-S271Q and KlJen2, gluconate of CaJen2 and KlJen2, and xylarate and mucate of CaJen2.

Organic acid analogues may have therapeutic applications such as 3-Bromopyruvate (3BP) which is reported as an anticancer and antimicrobial drug. We evaluated the role of Jen1 homologues of the human pathogen *Cryptococcus neoformans* in the transport of 3BP. The Cn04 protein plays an important role in the 3BP uptake and sensitivity of *C. neoformans* cells.

In another approach, several members of the AceTr family were functionally characterized: we show that the YaaH protein from *Escherichia coli* is a secondary active organic acid/proton symporter, which led to its assignment as SatP, a succinate-acetate transporter; the *aceP* gene from the archaea *Methanosarcina acetivorans* was found to be an acetate/proton transporter; and we elucidated the role of Gpr1 from *Yarrowia lipolytica* as an acetate transport.

A phylogenetic analysis of the AceTr family uncovered the presence of homologues in almost all analyzed fungal organisms. Reports about the role of these homologues suggest the importance of these transporters during fungal evolution, associated with sexual reproduction by sporulation.

Moreover, we report the essential role of the NPAPLGL(M/S) “signature” motif of this family of transporter, since substitutions in any of the residues of this domain abolished acetate transport.
Overall, the present work underlines the wide range of applications of carboxylate transporters in the industrial biotechnology. It also contributed to the deepen the knowledge on microbial carboxylate transporters and provided insights into the evolution, phylogeny and structure of AceTr family members.

Table of contents

List of figures

Chapter I

Chapter II

a Reg1/Glc7 phosphatase and, then, imported into the nucleus [32]. In lactate, as sole carbon source, the transcriptional factors Cat8, Adr1, and Hap2/3/4/5 are able to activate *JEN1* expression. The inactivation of Mig1/2 by phosphorylation, through Snf1 protein kinase, leads to their export to the cytoplasm. Therefore, in these conditions, the formation and maturation of mRNA are unaffected which ultimately leads to the expression of the lactate transporter at the plasma membrane. Cat8, Adr1, and Hap2/3/4/5 are transcriptional activators associated to non-fermentable carbon sources growth [25, 33, 34], as lactate. Interestingly, *CAT8* is repressed at the DNA level by Mig1/2 proteins [35]. In formate, as sole carbon source, the transcription of *JEN1* is also active, however, the early synthesized mRNA is targeted for degradation, consequently, no Jen1 protein is produced. Dhh1 RNA helicase, Pat1, and Lsm are involved in *JEN1* mRNA degradation, in this condition cells, where the long-mRNA retained a half-life time of 17–20 min, independently of the addition of glucose to the culture medium. However, when CEN.PK2-1C cells were grown on ethanol, the *JEN1* long-mRNA half-life time was reduced from 26 to 7 min. upon the pulse of glucose. Mapping of the *JEN1* 5' and 3' UTR transcripts revealed multiple transcription start sites located at positions -51 (for the long transcript, translated), and C391 or C972 (for the short transcripts, not translated). It was demonstrated that when *JEN1*(C391) small transcript is present, it works as a glucose sensor, promoting *JEN1*(-51) protein-coding mRNA rapid decay [36]. The Dhh1 RNA helicase and the Pat1-Lsm decapping enhancers were found to play a crucial role in the mechanisms underlying the regulation of *JEN1* 5'3' mRNA decay pathway [37]. Dhh1 together with Pat1-Lsm complex controls the translation initiation, by targeting mRNAs to the P-bodies, contributing to the recruitment of the mRNA decapping machinery (reviewed by [38]). When cells were incubated in formic acid *JEN1* mRNA accumulates in *dhh1*, *pat1* or *lsm* mutants, contrarily to what was found in the wild-type strain [37]. In this condition, it was found that *JEN1* mRNA, although transcriptionally active, was targeted for degradation *via* Dhh1 and Pat1-Lsm complex (Fig. 1). Microarray analysis revealed that this mechanism of regulation is also shared by other genes involved in non-fermentable carbon source metabolism, as *ADY2*, *CAT8,* and *HAP4*. Besides its general effect in the global cytoplasmic mRNA decay, Dhh1 has additional roles in the post-transcriptional or transcriptional regulation of several genes in response to environmental stimuli [37].

Figure 2. Jen1 inactivation by glucose. In lactate, as only carbon source, Jen1 is expressed at the plasma membrane. In this condition, Rsp5 ubiquitin ligase is unable to target Jen1 for degradation *via* the arrestin-like protein Rod1. Rod1 phosphorylation is dependent on Snf1 protein kinase. Phosphorylated Rod1 interacts with 14-3-3 proteins, becoming inaccessible to Rsp5, thus impairing Jen1 degradation. Upon a pulse of glucose, Jen1 is rapidly removed from the plasma membrane. Reg1/Glc7 phosphatase is involved in Rod1 activation and Snf1 inactivation, through

dephosphorylation. As a consequence, Rod1 is released from a phospho-dependent interaction with 14-3-3 proteins and ubiquitylated by Rsp5. Finally, ubiquitylated-Rod1-Rsp5 complex ubiquitylates Jen1 resulting in its endocytosis and vacuolar degradation.

Figure 3. ScJen1 overall topology. The predicted transmembrane segments are coloured in rainbow spectrum, from the I TMS to XII TMS. Residues involved in substrate binding, specificity and translocation are shown as circles: II TMS (R188), V TMS (S266, F270, S271, A272, Y273, Y284) VII TMS (N379, H383, Q386, D387) and XI TMS (Q498, N501). Residues marked with squares (S4, S11, S81, S585, S606) are predicted targets for phosphorylation. Ubiquitylated residues (K9, K338) are shown as triangles. The hydrophilic lactate pore is designated by a white triangle between the II TMS and VII TMS, pointing the transport of lactate molecule from periplasm to cytoplasm. The N-terminus and C-terminus of the protein are annotated as N and C, respectively.

Figure 4. Jen1 predicted 3D structural model: (A) transversal, (B) cytoplasmic and (C) periplasmic view. Residues critical for substrate binding, specificity and trajectory are shown as stick molecular structures and identified by black arrows: II TMS (R188), V TMS (S266, F270, S271, A272, Y273, Y284) VII TMS (N379, H383, Q386, D387) and XI TMS (Q498, N501). The 12 TMS are coloured in the rainbow spectrum from the N-terminus in blue to the C-terminus in red. The model in ribbon representations was obtained with Modeller software (Version 9.14, Ben Web, CA, USA) based on GlpT transporter X-ray structure. The images were obtained with the PyMOL Molecular Graphics System (Version 1.5.0.4 Schrödinger, LLC).

Figure 5. Phylogenetic tree of AceTr family. A phylogenetic tree was constructed from alignments of bacteria and fungi AceTr homologous protein sequences, using the Nearest-NeighbourJoining algorithm. NCBI Protein accession numbers are shown next to the species name. Microbial species shown in the tree and protein clades are as follows: *Aspergillus sp.* (AcpA, AcpB, and AcpC clades); *Saccharomyces cerevisiae; Yarrowia lipolytica; Escherichia coli; Metanosarcina acetirovans*. The SatP transporter from *E. coli* was used as an outgroup. The scale bar indicates the number of amino acid substitution per site.

Figure 6. Mechanisms of carboxylic acids transport in*S. cerevisiae*. Jen1 and Ady2 are able to mediate the transport of carboxylates ($RCOO^-$, (a) lactate, pyruvate, acetate or propionate; (b) acetate, propionate, formate or lactate, respectively) into the cell, where they are used as carbon and energy sources. When the undissociated form of the acid (RCOOH) prevails, it enters by simple diffusion (c) and in the case of acetic acid (CH_3COOH) also through the Fps1 channel (d). The accumulation of protons (H^+) and anions (RCOO) may cause severe acidic conditions (low pH and high levels of

ROS), which eventually lead to programmed cell death. Acid stress triggers the efflux of H^+ by Pma1 (e) and of carboxylates by Pdr12 (f) sorbate, propionate, benzoate or levulinate). To increase the expression of Pdr12, cell activates the War1 transcription factor which induces the transcription of *PDR12*, through *WARE* cis-acting response element. Furthermore, the ubiquitylation and degradation of Fps1, promoted by Hog1 MAPK pathway, serves as a mechanism to avoid acetic acid facilitated diffusion.

Chapter III

Figure 1. Initial uptake rates of radiolabelled, ^{14}C-lactic acid (A), ^{14}C-acetic acid (B), ^{14}C-succinic acid (C) and ^{14}C-malic acid (D) at different concentration by *D. hansenii* CBS767 at pH 5.0, 30ºC. Cells were cultivated in YNB pyruvic acid, acetic acid, succinic acid and malic acid until mid-exponential growth phase to access the transport of lactate or acetate, succinate, and malate, respectively.

Figure 2. Expression profiles of *DH17*, *DH18*, *DH24* and *DH27* genes of *D. hansenii* CBS767 strain in the presence of glucose (Glu), glycerol (Gly), lactic acid (Lact), succinic acid (Suc) and citric acid (Cit). RT-PCR was performed from cells grown in the presence of the mentioned carbon sources and collected at mid-exponential phase. Actin was used as an endogenous control for all conditions tested.

Figure 3. Epifluorescent and contrast phase microscopy of a GFP-tagged version of Dh17, Dh18, Dh24, and D27 expressed in *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ. In all cases, *D. hansenii* Jen1 homologues labelled the plasma membrane.

Figure 4. Growth phenotypes of *D. hansenii CBS767* and *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ strains heterologously expressing *DH17*, *DH18*, *DH24* and *DH27* on media containing glucose (2%), acetic acid (0.5 %), lactic acid (0.5%), pyruvic acid (0.5 %), malic acid (1 %), succinic acid (1%) and citric acid (1 %) as sole carbon and energy source. All *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ strains are isogenic expressing from a low copy plasmid. The negative control is a strain carrying the corresponding empty vector. Cells were serially diluted; 3 µl drops of each dilution were spotted onto the plates and grown during 5 days at 18ºC.

Figure 5. Uptake velocity of radiolabelled lactic acid (A), acetic acid (B), succinic acid (C) and malic acid (D), at 1mM final concentration, by *D. hansenii CBS767* at pH 5.0 and 26ºC, and *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ heterologously expressing *DH17*, *DH18*, *DH24* and *DH27* and its mutants, at pH 5.0 and 30ºC. The *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ-p146GPD was used as a control. Cells

were cultivated in glucose until mid-exponential growth phase, washed and transferred during 4 h to YNB supplemented with the carboxylic acid which would be accessed for the uptake.

Figure 6. Initial uptake rates of radiolabelled, ^{14}C-acetic acid, ^{14}C-succinic acid, and ^{14}C-malic acid at different concentration by *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ heterologously expressing *DH17*(A), *DH18* (B) or *DH24* (C), and *DH27* (D), at pH 5.0, 30ºC. Cells were cultivated in glucose until mid-exponential growth phase, washed and transferred during 4 h to YNB supplement with the carboxylic acid, which would be accessed for uptake.

Chapter IV

Figure 1. Lactate uptake in the presence of sugar acids in *S. cerevisiae jen1*Δ a*dy2*Δ expressing the ScJen1-S271Q. A) Relative capacity (%) of ^{14}C-lactic acid uptake (60 μM), at pH 5.0, 26ºC, in the absence or presence of non-labelled sugar acids (10 mM). Lactic acid was used as positive control and succinic acid as a negative control. B) Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-lactic acid as a function of the acid concentration at pH 5.0, 26ºC, in the absence (●) and in the presence of saccharic acid 20 mM (■) and 30 mM (▲). Inset: the apparent K_m of labelled lactic acid uptake plotted against the saccharic acid concentration. The estimated K_i is 21.8 mM for total saccharic acid. The data shown are mean values of at least three independent experiments and the error bars represent the standard deviation. *Significant differences ($p < 0.05$).

Figure 2. Molecular docking of ScJen1 and ScJen1-S271Q with saccharate (orange) and lactate (green). A) The 3D transversal view of ScJen1 and ScJen1-S271Q protein surface with the identification of the lactate and saccharate binding sites (1, 2, 3, 4) obtained in the docking analysis. The 3D ScJen1 and ScJen1-S271Q structural models used in this study were obtained from the crystal structure of PiPT transporter (PDB 4J05). B) 2D view of the molecular structure of the interaction between the ligands (saccharate and lactate) and the proteins (ScJen1 and ScJen1-S271Q). 1, 2, 3 and 4 are relative to the binding sites identified. The arrows indicate hydrogen bond interaction between the amino acid residues and functional groups or atoms in the molecular structure of the ligand.

Figure 3. Relative capacity (%) of lactate and succinate uptake in *C. albicans*, *D. hansenii, K. lactis and Y. lipolytica* measured in the absence and presence of non-labelled mucic, saccharic, xylonic, xylaric and gluconic acid (10 mM), pH 5.0 and 26ºC. The data shown are mean values of at least three independent experiments and the error bars represent the standard deviation. *Significant differences ($p \leq 0.05$).

Figure 4. Relative capacity (%) of *S. cerevisiae jen1*Δ *ady2*Δ cells expressing *C. albicans* homologues (CaJen1 and CaJen2) to transport lactate and succinate in the absence and presence of mucic,

saccharic, xylonic, xylaric and gluconic acid. A) Uptake of ^{14}C-lactic acid (60 μM) in cells transformed with the pCaJen1 plasmid. B) Uptake of ^{14}C-succinic acid (20 μM) in cells transformed with pCaJen2. The data shown are mean values of at least two independent experiments and the error bars represent the standard deviation. *Significant differences ($p \leq 0.05$).

Figure 5. Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-succinic acid as a function of the acid concentration at pH 5.0, 26ºC in *S. cerevisiae jen1Δ ady2*Δ pCaJen2 in the presence of:

A) no inhibitor (●), xylaric acid 20 mM (■) and xylaric acid 30 mM (▲);

B) no inhibitor (●), gluconic acid 20 mM (■) and gluconic acid 30 mM (▲);

C) no inhibitor (●), mucic acid 20 mM (■).

Insets: the apparent K_m of labelled succinic acid uptake was plotted against the concentration of xylaric (A) and gluconic (B) acid. The estimated K_i is 20.2 mM for total xylaric acid and 25.9 mM for total gluconic acid. For mucic acid, it was not possible to estimate the K_i value.

Figure 6. Molecular docking of CaJen2 with malate, succinate, gluconate, mucate, and xylarate. A) Transversal view of CaJen2 protein surface with the identification of the binding sites obtained in docking analysis. Ligand molecules are represented in their stick molecular structure coloured blue (succinate), red (malate), yellow (gluconate), purple (mucate) and green (xylarate). B) 2D view of the interactions between ligands (malate, succinate, gluconate, mucate, and xylarate) and CaJen2 residues. The 3D CaJen2 structural model used in this study was modulated from the crystal structure of the GLUT3 transporter (PDB 4ZW9).

Figure 7. Relative capacity (%) of *S. cerevisiae jen1*Δ *ady2*Δ cells expressing *K. lactis* homologues (KlJen1 and KlJen2) to transport lactate and succinate in the absence and presence of mucic, saccharic, xylonic, xylaric and gluconic acid. A) ^{14}C-lactic acid uptake (60 μM) in cells transformed with pKlJen1. B) ^{14}C-succinic acid (20 μM) uptake in cells transformed with pKlJen2. The data shown are mean values of at least two independent experiments and the error bars represent the standard deviation. *Significant difference ($p \leq 0.05$).

Figure 8. Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-succinic acid as a function of the acid concentration at pH 5.0, 26ºC in *S. cerevisiae jen1*Δ *ady2*Δ pKlJen2 cells in the presence of A) no inhibitor (●), gluconic acid 20 mM (■) and gluconic acid 30 mM (▲); B) no inhibitor (●), saccharic acid 20 mM (■) and saccharic acid 30 mM (▲). Insets: the apparent K_m of labelled succinic acid uptake against (A) gluconic and (B) saccharic acid concentration. The estimated K_i is K_i is 22.7 mM and 24.8 mM for total gluconic and saccharic acid, respectively. The data shown are mean values of at least three independent experiments and the error bars represent the standard deviation.

Figure 9. Molecular docking of KlJen2 with malate, succinate, gluconate, and saccharate. A) 3D transversal view of KlJen2 protein surface with the identification of the ligands binding sites: succinate (blue), malate (red), gluconate (yellow) and saccharate (orange). B) 2D view of interactions between ligands (malate, succinate, gluconate, and saccharate) and KlJen2 residues. The 3D KlJen2 structural model used in this study was obtained from the crystal structure of PiPT transporter (PDB 4J05).

Chapter V

Figure 1. Conserved domain 379NXX[S/T]HX[S/T]QDXXXT391 of the Jen1 group transporters. ScJen1 - *Saccharomyces cerevisiae*; CaJen1 - *Candida albicans*; KlJen1 - *Kluyveromyces lactis*; Dh17 - *Debaryomyces hansenii*; Cn04, Cn36 - *C. neoformans.* Multiple sequence alignment was performed using T-Coffee (http:// ebi.ac.uk/Tools/msa/tcoffee)

Figure 2. The phylogenetic tree presenting the evolutionary relationships of different carboxylic acids transporters and potential 3BP transporters in *C. neoformans* cells. ScJen1 - *Saccharomyces cerevisiae*; CaJen1, CaJen2 - *Candida albicans*; KlJen1, KlJen2 - *Kluyveromyces lactis*; Dh17 - *Debaryomyces hansenii*; Cn04, Cn36 - *C. neoformans*. Evolutionary analyses were carried out in MEGA7.

Figure 3. Impact of 3BP on the expression level of genes encoding potential 3BP transporters and ABC pump (Δ*CNAG_06348*) in *C. neoformans* cells

Figure 4. The uptake kinetics of ^{14}C-3BP in the different strains of *C. neoformans. C. neoformans* H99 - parental strain, Δ*CNAG_04704* - *C. neoformans* with a deletion in genes encoding Cn04 protein, Δ*CNAG_06536* - Cn36, Δ*CNAG_06348* - ATP-binding cassette transporters. Substrate concentration range: 0-2 mM.

Figure 5. A) The uptake kinetics of ^{14}C-3BP in *S. cerevisiae* strains Δ*jen1* Δady2 transformed by empty plasmid (*S. cerevisiae jen1*Δ *ady2*Δ p416GPD) and by plasmid contains *CNAG_04704* gene (*S. cerevisiae jen1*Δ *ady2*Δ p416-*CNAG_04704*). Substrate concentration range: 0-2 mM. B) Initial uptake rates in *S. cerevisiae jen1*Δ *ady2*Δ p416-*CNAG_04704* of radiolabeled 3BP in the presence or absence of different concentrations of energetic inhibitors

Figure 6. Epifluorescent and contrast phase microscopy of GFP-tagged version of Cn04 expressed in *S. cerevisiae* Δ*jen1* Δ*ady2* labelled the plasma membrane

Chapter VI

Figure 1 – The functional role of *yaaH* and *actP* genes as acetate transporters in *E. coli*. The graphic represents the percentage of 0.5 mM [1-^{14}C]-acetic acid uptake, at pH 6.0, considering 100% the velocity of transport found for the wild-type *E. coli* MG1693 strain. The cells were collected at the mid-exponential growth phase in minimal medium with glucose 1 % (w/v), containing the required supplements for growth as indicated in materials and methods. The strain Δ*yaaH* Δ*actP* was transformed with the plasmids pSVA9 and pSVA10, expressing *yaaH* from a high or low copy number plasmid, respectively (Table 2). Each data point represents the mean ± SD of 3 independent experiments (n=9).

Figure 2 – Energetics of the YaaH transporter. Effect of pH and of CCCP, valinomycin and monensin on the uptake of [1-^{14}C]-acetic acid 0.5 mM, in cells of *E. coli* MG1693 Δ*yaaH* Δ*actP* transformed with pSVA9 or pUC18 plasmids, grown as indicated in Figure 1 legend. A) Transport activity was determined in cells buffered in potassium phosphate at the pH values indicated. B) Cells were pre-incubated with the compounds mentioned, at the concentration indicated, pH 6.0, for 1 min before adding the radiolabeled substrate. Each data point represents the mean ± SD of 3 independent experiments (n=6).

Figure 3 – Acetic acid kinetics of the YaaH transporter. Eadie-Hofstee plots of initial uptake rates of [1-^{14}C]-acetic acid, pH 6.0, in glucose-grown cells, as indicated in Figure 1 legend. *E. coli* strains used: ●, Δ*yaaH* Δ*actP* transformed with the pSVA9 plasmid, expressing the *yaaH* transporter; ▲, Δ*actP* transformed with pUC18 multicopy plasmid; ○, Δ*yaaH* Δ*actP* transformed with the pUC18 multicopy plasmid. Inset:

Kinetics of [1-^{14}C]-acetic acid uptake at pH 6.0, as a function of the acid concentration. Each data point represents the mean ± SD of 3 independent experiments (n=9).

Figure 4 – Acetate uptake profiles of cells grown on acetic acid. Growth profiles of *E. coli* MG1693 grown on minimal medium with acetic acid 1.67 mM (A), 8.3 mM (B), 16.6 mM (C), 33.3 mM (D), 50 mM (E), 66.7 mM (F) and 83.3 mM (G), pH 6.0. Symbols: ■, OD_{600}; ▲ glucose (g/L);▼, acetic acid (g/L); bars, transport activity measured with [^{14}C]-acetic acid, 0.5 mM, pH 6.0.

Figure 5 – The physiological role of YaaH and ActP on acetate assimilation. Growth profiles of *E. coli* MG1693 (●) and isogenic Δ*actP* (▲), Δ*yaaH* (□) and Δ*yaaH* Δ*actP* (▼) mutants in minimal medium with acetic acid 1.67 mM (A), 8.3 mM (B), 16.6 mM (C), 33.3 mM (D), 50 mM (E), 66.7 mM (F) and 83.3 mM (G), pH 6.0. H) Relative fold expression of *yaaH* and *actP* genes evaluated by RT-PCR in wild-type cells collected at 6, 12 and 24 hours of growth, normalized to *16S* with standard

deviations of ΔC_T values propagated for each fold change value, as described in the experimental procedures. Each data point represents the mean ± SD of 3 independent experiments (n=6).

Figure 6 – Acetate uptake profiles of cells grown on glucose. Growth profiles of *E. coli* MG1693 grown on minimal medium with glucose 0.2 %, w/v (A) and 0.4 %, w/v (B). Symbols: ■, OD_{600}; ▲, glucose (g/L); ▼, acetic acid (g/L); bars, transport activity measured with [^{14}C]-acetic acid, 0.5 mM, pH 6.0. C) Relative fold expression of *yaaH* and *actP* genes evaluated by RT-PCR in wild-type cells collected at 6, 12 and 24 hours of growth, normalized to *16S* with standard deviations of ΔC_T values propagated for each fold change value, as described in the experimental procedures. Each data point represents the mean ± SD of 3 independent experiments (n=6).

Figure 7 – Distinct physiological roles of the two *E. coli* acetate transporters. Growth profiles of *E. coli* MG1693 (A), and isogenic Δ*actP* (B), Δ*yaaH* (C) and Δ*yaaH* Δ*actP* (D) mutants in minimal medium with glucose 1% (w/v) and the required supplements, as indicated in materials and methods. Symbols: ■, OD_{600}; ▲, glucose (g/L); ▼, acetic acid (g/L); bars, transport activity measured with [^{14}C]-acetic acid, 0.5 mM, pH 6.0. E) Time course analysis for acetic acid uptake of the wild-type strain (dark bars, data from A) compared to the sum of the activities of the strains Δ*actP* (light grey bars, data from B) and Δ*yaaH* (dark grey bars, data from C). F) Relative fold expression of *yaaH* and *actP* genes evaluated by RT-PCR normalized to *16S* with standard deviations of ΔC_T values propagated for each fold change value, as described in the experimental procedures. Each data point represents the mean ± SD of 3 independent experiments (n=6).

Figure 8 – Substrate specificity of the YaaH transporter. Eadie-Hofstee plots of initial uptake rates of [1-^{14}C]-acetic acid, pH 6.0 in the absence (○) and in the presence (■, ▲) of non-labelled acids, at the concentration indicated in each plot. The cells were grown and collected as described in Figure 1 legend. Each data point represents the mean ± SD (n=6).

Figure 9 – Succinic acid kinetics of the YaaH transporter. Initial uptake rates of [1,4-^{14}C]-succinic acid, pH 6.0 as a function of the acid concentration in *E. coli* Δ*yaaH* Δ*actP* cells transformed with the plasmid pSVA9 (■). As a control, the same strain was transformed with the pUC18 plasmid (□). The cells were grown and collected as described in Figure 1 legend. Each data point represents the mean ± SD (n=6).

Figure 10 – Site-directed mutagenesis of the *yaaH* gene. (A) Predicted transmembrane segments V and VI of YaaH (http://www.cbs.dtu.dk/services/TMHMM/) aligned with Ady2 using ClustalW2. The conserved residues are marked with a star (*) and the residues mutated in this work are highlighted in black boxes. (B) Initial uptake rates of D,L-[U-^{14}C]-lactic acid, pH 5.0 as a function of

the acid concentration. Strains of *E. coli* Δ*yaaH* Δ*actP* transformed with plasmids pSVA9 (●), pA164G (▲) or pL131V (▼). As a control, the same strain transformed with the pUC18 plasmid was used (○). (C) Eadie-Hofstee plot of the initial uptake rates of D,L-[U-^{14}C]-lactic acid, pH 5.0 in the absence (●) and in the presence (▲, △,■,□,) of non-labelled acetic and succinic acid, at the concentration indicated in each plot in cells transformed with pA164G (D) Eadie-Hofstee plot of initial uptake rates of [^{14}C]-lactic acid, pH 5.0 in the absence (●) and in the presence (▲, △,■,□,) of non-labelled acetic and succinic acid, at the concentration indicated in each plot in cells transformed with pL131V. Cells were grown and collected as described in Figure 1 legend. Each data point represents the mean ± SD of 3 independent experiments (n=6).

Chapter VII

Figure 1. Maximum likelihood phylogenetic tree of AceTr family (TCDB 2.A.96) present in eukaryotic and prokaryotic genomes. Branch lengths are proportional to sequence divergence. Major taxonomic groups are indicated in different shades of grey. Homologues relevant for the discussion through the manuscript are highlighted. Groups indicated as E1, E2, P1, P2, P3, and P4 were created to facilitate the following of the tree description in the main text and are not meant to provide any type of classification.

Figure 2. Acetate uptake and transport energetics of *E. coli 3*Δ heterologously expressing pAceP: A) Initial uptake rates of radiolabelled ^{14}C-acetic acid at different concentration by *E. coli 3*Δ heterologously expressing pAceP or pUC18, at pH 6.0, 30◦C; b) Transport energetics: effect of CCCP, valinomycin, and monensin on the uptake of 0.1 mM ^{14}C-acetic acid in cells of *E. coli 3*Δ pAceP; C) transport specificity: the uptake of 0.1 mM ^{14}C-acetic acid in in *E. coli* 3Δ heterologously expressing pAceP or pUC18 measured in the absence and presence of non-labelled formic, pyruvic, lactic, succinic and malic acid (10 mM), pH 6.0 and 30◦C. Cells were pre-incubated with the compounds mentioned, at the concentration indicated and pH 6.0 for 1 min before adding the radiolabelled substrate. Each data point represents the mean ± S.D. for three independent experiments (n =9).

Figure 3. A) Initial uptake rates of radiolabeled ^{14}C-acetic acid at different concentrations by *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ cells heterologously expressing pGpr1, pAdy2, pFun34 and p416GPD as a control at pH 6.0, 30°C and respective kinetic parameters. Cells were cultivated in glucose until exponential growth phase, washed and transferred to YNB supplemented with acetic acid (0.5%) during 6 hours. Each data point represents the mean ± S.D. for three independent

experiments (n =9). B) Epifluorescence and contrast phase microscopy of a GFP-tagged version of *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ cells expressing *GPR1*, before and during 6h of exposure of cells to 0.5% acetic acid, at pH6, 30°C. Scale bars = 7.5 μm.

Figure 4. A) The percentage of 1 mM ^{14}C-acetic acid uptake, at pH 6.0, considering the velocity of transport found for the *S. cerevisiae ady2*Δ *jen1*Δ strains expressing pAdy2 as 100%. Cells were cultivated in glucose until mid-exponential growth phase, washed and transferred during 6 h to YNB supplement with the acetic acid (0.5%) and solid YNB acetic acid (0.5 %) for growth tests shown in the images above each bar of Ady2 alleles and in p416GPD. B) The percentage of 0.5 mM ^{14}C-acetic acid uptake, at pH 6.0, considering the velocity of transport found for the *E. coli 3*Δ strains expressing pSatP as 100%. Cells were collected at mid-exponential growth phase from minimal medium with glucose 1% (w/v). Each data point represents the mean ± S.D. for three independent experiments (n =9).

Figure 5. Epifluorescence microscopy localization of Ady2::GFP and mutant alleles expressed in *S. cerevisiae ady2*Δ *jen1*Δ. Cells were collected at mid-exponential growth phase from YNB glucose 1% (w/v), washed and transferred during 6 h to YNB, supplement with the acetic acid (0.5%), and observed by epifluorescence microscopy.

Figure 6. Molecular docking of SatP 3D model, based on SatP_Ck structure, with the substrates acetate and succinate. Transversal (A) and cytoplasmic (B) view of SatP 3D model with the identification of the NPAPLGLM motif colored in black side chains. C) Zoomed transversal view of the binding sites S1 and S2 for acetate and succinate, with the putative binding residues of the NPAPLGL motif colored in red side chains (N8, A10, G13, L14).

Figure 7. A) The percentage of 0.5 mM ^{14}C-acetic acid uptake, at pH 6.0, considering the velocity of transport found for the *E. coli 3*Δ strains expressing pSatP as 100%. Cells were collected at mid-exponential growth phase from minimal medium with glucose 1% (w/v). Each data point represents the mean ± S.D. for three independent experiments (n =9). B) Transversal and cytoplasmic view of SatP 3D model obtained with the identification of the R122 and Q125 in blue side chains and the identification of the NPAPLGM residues in black side chains. Dash red line is indicative of hydrogen bonds.

List of tables

Chapter III

Chapter IV

Chapter V

Chapter VI

Chapter VII

List of abbreviations

3BP - 3-bromopyruvate

3HP - 3-Hidroxylpropionic

AAB - acetic acid bacteria

ABC - ATP-binding cassette

AceTr - Acetate Uptake Transporter

ack acetate kinase

acs - acetylCoA synthetase

ANOVA - one-way analysis of variance

ART - arrestin-related trafficking adaptors

CCCP - carbonyl cyanide m-chlorophenyl hydrazine

C_T - threshold cycle

Dh - *Debaryomyces hansenii*

Ec – *Escherichia coli*

DSB - double strand breaks

DNA - Deoxyribonucleic acid

GFP - green fluorescent protein

H^+ - proton

JTT - Jones-Taylor-Thornton

K_d - diffusion constant

K_i - inhibition constant

K_m – affinity constant

k_{mobs} – inhibitor's k_m

LAB - lactic acid bacteria

LB - Luria-Bertani

LOMETS - Local Meta-Threading-Server

MCT - monocarboxylate transporter

MFS - Major Facilitator Superfamily

MIC - minimal inhibitory concentration

MIP - Major Intrinsic Protein

MM - Minimal Media

MVBs - multivesicular bodies

NAD - adenine dinucleotide

NBD - nuclear binding domains

PDR - Pleiotropic Drug Resistance

PDR - pleiotropic drug resistance

pHi - intracellular pH

pta - acetyl-CoA phosphotransferase

RMSD - *root-mean-square deviation*

ROS – reactive oxygen species

Sc - *Saccharomyces cerevisiae*

SGD - Saccharomyces Genome Database

SIP - small and basic intrinsic proteins

SSS - Solute:Sodium Symporter

TCA - tricarboxylic acid

TCDB - Transport Classification Database

TMS - transmembrane segments

WARE - weak-acid response element

WGD - Whole Genome Duplication

XIP -intrinsic proteins

YAC - yeast artificial vectors

YNB - yeast nitrogen base

ZΔpH - electrochemical potential

Thesis outline and aims

Thesis outline

As the demand for sustainable energy increases, production of platform chemicals from renewable feedstocks using microbial fermentation has become more attractive as a replacement for petroleum-based fuels and chemicals. Organic acids can be used as building blocks of a wide range of industrial chemicals. Their presence goes from ancient to modern foods and beverages, as well as drugs, detergents, cosmetics, polymers, plastics, resins, and many other biochemical or chemical products.
Biosynthesis of organic acids in high and sustainable yields requires the engineering of not only the microorganisms enzymatic and metabolic machinery, but also of plasma membrane transporters able to export the acids to the extracellular space to improve the host's tolerance and decreasing downstream processing costs. Only a deep study in organic acids transporters will allow significant progress for future applications on the increased microbial production of these molecules based on exploitation and engineering of membrane transporters.
This PhD project aimed at solving the structural and functional features of carboxylate transporters and exploit this knowledge towards the engineering of biotechnological relevant industrial microorganisms. The work plan had the following goals:

1. To uncover and characterize new organic acid transporters in prokaryotes and eukaryotes.

2. To screen and engineer carboxylate transporters to increase commercial valuable organic acids bioproduction: monocarboxylic and dicarboxylic acids, and sugar acids such as xylonic, gluconic, mucic, saccharic and xylaric acids.

3. To accomplish a structural-functional analysis of the Acetate transporter AceTr family. Rational site-directed mutagenesis of targeted residues of the yeast (Ady2) and in *E. coli* (SatP) transporters were performed aiming to unravel the role of critical amino acid residues crucial for function, activity, and substrate specificity.

This thesis is organized throughout 8 chapters:

a) Chapter I, subdivided into sub-chapters, corresponds to an introduction to the *state of the art* in the research field explored in this thesis. It regards the economic and environmental importance of microbial production of essential industrial building blocks, namely organic acids, as an alternative to the petroleum-based industry. In addition, it also addresses the application and engineering of membrane transporters to boost cell factories productivity and sustainability.
b) Chapter II relies on a published book chapter entitled **"Carboxylic Acids Plasma Membrane Transporters in *Saccharomyces cerevisiae*"**, which describes Jen1 and AceTr family and highlights the mechanisms involving carboxylic acid transporters and yeast cell response to environmental stimulus.
c) Chapter III is based on the published manuscript **"The *Debaryomyces hansenii* carboxylate transporters Jen1 homologues are functional in *Saccharomyces cerevisiae*",** it is reported the functional characterization of the four Jen1 homologues from *Debaryomyces hansenii* by heterologous expression in *S. cerevisiae*. The purpose of this study was to uncover the functional role of the *D. hansenii JEN1* homologues. Therefore, the four genes

DEHA2D18920p, *DEHA2E24024p*, *DEHA2F17402p,* and *DEHA2F27126p* were heterologously expressed in an *S. cerevisiae jen1*Δ *ady2*Δ strain.

d) Chapter IV addresses a first authorship published work **"Yeast as a tool to express sugar acid transporters with biotechnological interest"**. In this study, we used the model organism *S. cerevisiae* expressing distinct Jen1 homologues to assess their ability to transport specialty sugar acids, such as gluconic, mucic, saccharic, xylonic and xylaric acid envisaging it's the application of these transporters in microbial cell factories.

e) Chapter V relies on a co-authorship submitted work entitled **"The *Cryptococcus neoformans* monocarboxylate transporter Cn04 is responsible for increased 3-bromopyruvate sensitivity"**. In this study, we conducted several studies to identify the membrane protein responsible for 3BP transport in pathogen *C. neoformans*. The 3BP uptake and sensitivity were analyzed in three *C. neoformans* mutants with deletions of genes encoding putative carboxylates transporters. Moreover, in this article, we also describe the heterologous expression of functionally characterized carboxylate transporters from *C. neoformans* in the yeast *S. cerevisiae jen1*Δ *ady2*Δ.

f) Chapter VI is based on the published work **"SATP (YaaH), a succinate-acetate transporter protein in *Escherichia coli*"**. It accesses the role of the AceTr member, YaaH (SatP), in the transport of organic acids, namely acetic and succinic acid.

g) Chapter VII relies on a first authorship published work **"The Acetate Uptake Transporter family motif "NPAPLGL(M/S)" is essential for substrate uptake"**. It addresses a phylogenetic analysis of AceTr family and further characterization of two of its members. We heterologously expressed the *GPR1* from *Y. lipolytica* in *S. cerevisiae* and *aceP* from *M. acetivorans* in *E. coli*. We also analyzed the role of each residue of the conserved motif NPAPLGL(M/F) in substrate transport of both membrane proteins SatP and Ady2. Complementary molecular docking analyzes were used to uncover putative binding sites in the vicinity of this conserved motif.

h) Chapter VIII regards a general discussion of the results and advances achieved throughout this thesis is described. Moreover, future perspectives and new challenges are also discussed.

All the experimental work conducted in this work was performed at the Molecular and Environmental Biology Centre (CBMA), Department of Biology, from the University of Minho under the supervision of Professor Margarida Casal, Professor Sandra Paiva, and Professor George Diallinas. We have established a close collaboration with Merja Penttilla from VTT Technical Research Center, which coordinates a team responsible for the development of microbial cell factories to produce sugar acids. Also, we also established a close collaboration with Professor Stanislaw Ułaszewski and the PhD student Katarzyna Niedźwiecka from the Institute of Genetics and Microbiology, University of Wroclaw (Poland), which were involved in the study of the functional characterization of the 3BP transporter from *C. neoformans*.

The present work resulted in the publication of four articles, submission of another two scientific manuscripts, preparation of another two manuscripts and a provisional patent application (PPA), as detailed below:

a) Published and submitted articles:

Casal, M., Queiros, O., Talaia, G., **Ribas, D**., and Paiva, S. (2016). Carboxylic Acids Plasma Membrane Transporters in Saccharomyces cerevisiae. In Advances in experimental medicine and biology, pp. 229-251.

Ribas, D., Sá-Pessoa J, Soares-Silva I, Paiva S, Nygård Y, Ruohonen L, Penttilä M, and M., C. (2017). Yeast as a tool to express sugar acid transporters with biotechnological interest. FEMS yeast research 17.

Sá-Pessoa, J., Paiva, S., **Ribas, D.**, Silva, I.J., Viegas, S.C., Arraiano, C.M., and Casal, M. (2013). SATP (YaaH), a succinate-acetate transporter protein in Escherichia coli. The Biochemical journal 454, 585-595.

Soares-Silva, I., **Ribas, D.**, Foskolou, I.P., Barata, B., Bessa, D., Paiva, S., Queiros, O., and Casal, M. (2015). The Debaryomyces hansenii carboxylate transporters Jen1 homologues are functional in Saccharomyces cerevisiae. FEMS yeast research 15, fov094.

b) Submitted articles:

Ribas D., Soares-Silva, I., Vieira, D., Sousa-Silva, Sá-Pessoa, J., M., Azevedo-Silva, J., Paiva, S., Viegas, S., Arraiano, Diallinas, G., S., Paiva, S, Soares, P., Casal, M., (2018). The Acetate Uptake Transporter family motif "NPAPLGL(M/S)" is essential for substrate uptake and binding. *Accepted in Fungal Genetics and Biology*

Niedźwiecka, K., **Ribas, D.**, Casal, M., Ułaszewski, S. (2018). The *Cryptococcus neoformans* monocarboxylate transporter Cn04 is responsible for increased 3-bromopyruvate sensitivity**.** *Submitted in* FEMS yeast research

c) In preparation for submission

Ribas, D., Soares-Silva, I., Casal, M. (2018). Membrane transporters: the key to boost microbial production of organic acids. *In preparation.*

Talaia, G., **Ribas, D**., Casal, M., Diallinas, G., Paiva, S. (2018). The cytosolic domains of Jen1 are critical for subcellular localization, function and turnover. *In preparation*

d) International patent application:

Reference code: P565.7

Chapter 1

Introduction

Adapted from:

Ribas, D., Soares-Silva, I., Casal, M. (2018). Membrane transporters: the key to boost microbial production of organic acids. *In preparation*

Chapter I

1. The bioeconomy as a driving force for research and innovation

Presently, the overexploitation of natural resources, their quality decrease and the loss of biodiversity, represent an economic burden in most developed countries. With the increasing dimensions of the global economy and population levels, the world will face a period of greater demand for natural resources and energy, dangerously threatening the balance and sustainability of the ecosystems [1]. Thus, a new economic vision for the exploitation of earth resources has been proposed: the "*Bioeconomy*". This is a social and economic approach, where the primary production is either achieved through available biomass, such as agriculture, forestry, and fishing, or through the proper use of waste streams to produce food, feed, fuels, energy, and other materials (Figure 1) [2-4].

As per the definition, bioeconomy is an interdisciplinary field, which interlinks biological innovations with that of the economic activity, with broad applicability in different sectors, such [5, 6]:

- Industrial applications to limit environmental impacts, including synthetic polymers, bio-plastic material, enzymes, microbial biosynthesis, biofuel developments, replacement of chemical fertilizers with natural biological nitrogen fixation and other environmental applications, like bioremediation, biosensors;
- Veterinary applications to improve plant and animal breed through cross-breeding, health, sanitation;
- Human healthcare, such as pharmacogenetics, and biopharmaceuticals.

The European Commission and the US were pioneers in the development of bioeconomy strategies. These envisage future biobased industries producing commodities, such as fuels, chemicals, plastics, textiles, using biomass as the feedstock instead of fossil resources [7-10]. This bioeconomy vision is spreading and developing nations, e.g., India, Russia, Brazil, and China, that in the past relied on exporting their natural resources, are now creating a bioeconomy based on the production of higher value products. However, in these countries, the bioeconomy strategy is only partial, since policies that suggest a developing bioeconomy are favoured but without having a full dedicated bioeconomy strategy [4].

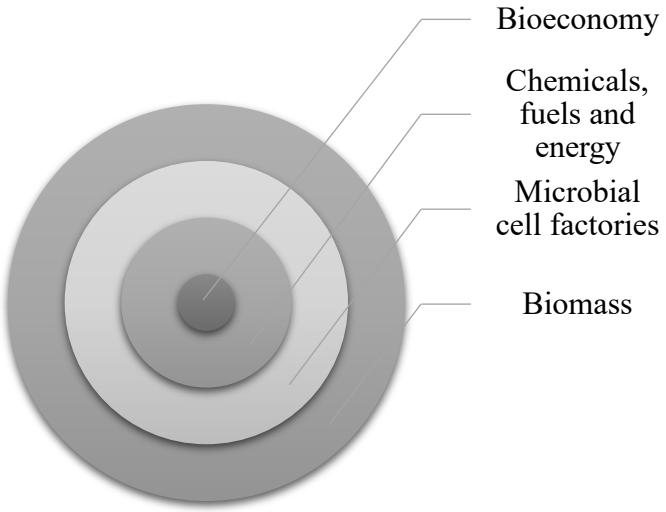

Figure 1. Bioconversion of biomass into chemicals (food, feed, platform chemicals), fuels and energy by microbes raises the foundations for bioeconomy establishment. (Adapted from Philp, 2015 [4])

1.1 The scope of bioeconomy

The global demand for natural resources, namely water, and energy is estimated to grow, between 30% to 40% in the next twenty years [6, 11]. Accordingly, overall demand for food and animal feed was estimated to grow 60%, whereas the area of arable farmland per capita may decrease at the rate of 1.5% per year [12].

Due to the overexploitation of natural resources and the potentially increasing demands for agriculture products, fishing stocks and forestry resources, developing countries are predicted to face unprecedented environmental, social and economic pressure: growing demands for biological resources, due to interdependencies on global trade and competition [13]; rising pandemics episodes due to increased mobility around the world and precarious food distribution [14]; increased environmental pollution and extreme weather conditions due to climatic changes [15].

Thus, based on the current industry trends and economic growth regarding technology as well as demography, it is imperative to connect the growing economy with renewable resources for better sustainability [16]. In this regard, biosciences are the basis of many technological developments, with a potential to solve some of the most significant challenges globally faced. In fact, biosciences are helping to understand the severity of these challenges and need for new solutions for the above-mentioned issues [14]. The research community remains fragmented, with American and European regional clusters most active and tightly interconnected [6]. The aim is to acquire significant benefits in diverse segments to reduce the loss of natural resources and therefore conserve the environment. Some strategies could include, an increased agricultural income and production, building new values

chains, supplying the increasing energy demands through advances in the biofuel production, creation of competitive infrastructure, replacement of oil-based chemicals and materials. Soon, biorefineries are also expected to increase the production of chemical platforms from renewable biomass resources [5, 6, 17].

1.2 The European bioeconomy policies

Although conceptualization of bioeconomy occurred in the 90's [18], European countries started formulating different bioeconomy policies and strategies in the mid of the 20th century. The foundation of bioeconomy was laid by the European Commission in 1993, through the need for non-physical, knowledge-based investments as well as advanced biotechnical approaches. The European commission's proposal was directed towards the establishment of smarter and greener bioeconomy for sustainable economic growth of countries. Currently, the European bioeconomy has a market size of over 2 trillion euros with the provision of 22 million jobs across several industry sectors, contributing to 9% of the total European labour force [19].

In Europe, waste production per person is approximately six tons per year. This is a significant waste stream lost, that could be used as a secondary raw material. By the end of the year 2010, only 36% of the total generated waste was recycled, and 64% of the landfilled used as biomass, for economic sustainability [9]. These figures highlight the current European economy and opportunities to formulate better integrative policies to expand the output of the bio-based products.

European Bioeconomy has recently supported a lead market initiative that was particularly active in 2007-2011, and it has encouraged bio-based product commercialization as one of the six principal sectors, which will be supported by new products and services. In this regard, various funding mechanisms are being intended, like Horizon 2020, to structure European framework during the year 2014-2020 for research and innovations in different industry segments. European government is supporting the theme of the building of Knowledge-based Bio-Economy and funding the same for their relevant, innovative applications in food, agricultural, fisheries, and biotechnology, with an intention to support research programs and better availability of funding [9].

In 2011, the European Commission [9] conducted a public consultation on bioeconomy, which eventually got a massive response from various organizations and even individuals across different member states of the European Union. As a result, combined strategies along with the detailed action plan was introduced, entitled "Innovating for sustainable growth: A bioeconomy for Europe" [9]. The preliminary aim of these strategies was to improve bioeconomy based on knowledge, encourage biotechnical advancements and innovations, as well as to enhance the productivity of natural resources in a more sustainable manner [20]. The strategical actions are segmented around three main

pillars, such as investments in research, skills, and innovations along with market enhancement and competitiveness. Interestingly, various member states of the European Union have started different strategic action plans to promote dynamic policy background for the development of sustainable bioeconomy in Europe [20].

Various barriers were pinpointed by experts in the successful implementation of natural resources for the improvement of bioeconomy. One of the primary issues is the management of value chain, right from initial extraction, logistics and trading towards the final production of refined bio-resources into multiple economically valuable products, as well as its recycling capacity. Technical challenges need to be tackled as well: like finding the best bio-resource and its availability, applications of different bio-based feedstocks and process validation, marketing problems, production, and availability of valuable by-products [4, 6].

2. Microbial bio-based production

Humankind uses microbial fermentation since ancient times, mostly for food and feed applications to extend the lifetime, quality, and flavour of these products. Most of these properties are achieved by the microbial production of organic acids, alcohols, amino acids, and vitamins. The first report of microbial fermentation at industrial scale is from 1923, with the commercialization of citric acid produced by the fungi *Aspergillus nidulans*. Later the introduction of penicillin followed by other antibiotics highlighted the role of microbial fermentation at the industrial scale. This was further supported by the production of enzymes in the 1970s through the revolution of DNA recombinant technology along with genetic engineering in the next years (see reviews [21, 22]).

Organic acids are currently an essential group of platform chemicals produced by microbes. However, the chemical synthesis of these compounds from petroleum derivatives is the major source of production to fulfil the world demands for these acids. Most of the organic acids produced industrially have as destination the food industry. Nevertheless, their potential as building blocks for the synthesis of several chemicals, drugs, and biopolymers, along with the increasing concerns related to greenhouse gas emission and limited availability of natural resources, constitute a promising future for the bioproduction of this class of compounds [23, 24] Currently several organic acids are industrially generated *via* microbes, such as succinic, lactic, citric, gluconic and acetic acid [23].

2.1 Industrial applications of organic acids

Among the top twelve value-added platform chemicals, most of them are organic acids, namely succinic, fumaric, malic, furan dicarboxylic, 3-Hidroxylpropionic (3HP), glucaric, levulinic and itanoic acid [25].

Organic acids and their derivatives are well known for their extensive industrial applications. The market of organic acids envisages a broad range of applications like plastic, tanning, textiles, colours, lubricants, metals, food, and beverage industries. In the petrochemical industry, organic acids are used in the manufacture of intermediate and end-use petrochemical products, such as biogas, alcohol, paper and oil [23]. They are also additives in the manufacturing of perfumes, insecticides, pesticides, lubricants, household cleaners, leather and adhesives.

Carboxylic derivatives of organic acids, designated as carboxylic acids are critical in the food and beverage industries, as well as in the pharmaceutical industry [26]. Lactic and acetic acids served for centuries as food preservatives, namely in fermented foods and beverages, such as bread, cheese, yoghurt, beer, and wine. Some of the most common carboxylic acids are used in drug manufacturing, namely adipic acids, azelaic acids, fumaric acid, maleic acid, oxalic acid and succinic acid [26]. In addition to being used as pharmaceuticals, acids such as succinic acid and maleic acid are also being used as raw materials in dye and lacquer, plastic, textile and metal industries [27]. Furthermore, organic acids such as citric acid, succinic acid, gluconic acid, malic acid are commonly used in the industrial production of large-scale biodegradable polymers [26]. Figure 2 shows the predicted evolution of organic acid production between 2015-2024 according to the industrial sector and/or application as previously mentioned.

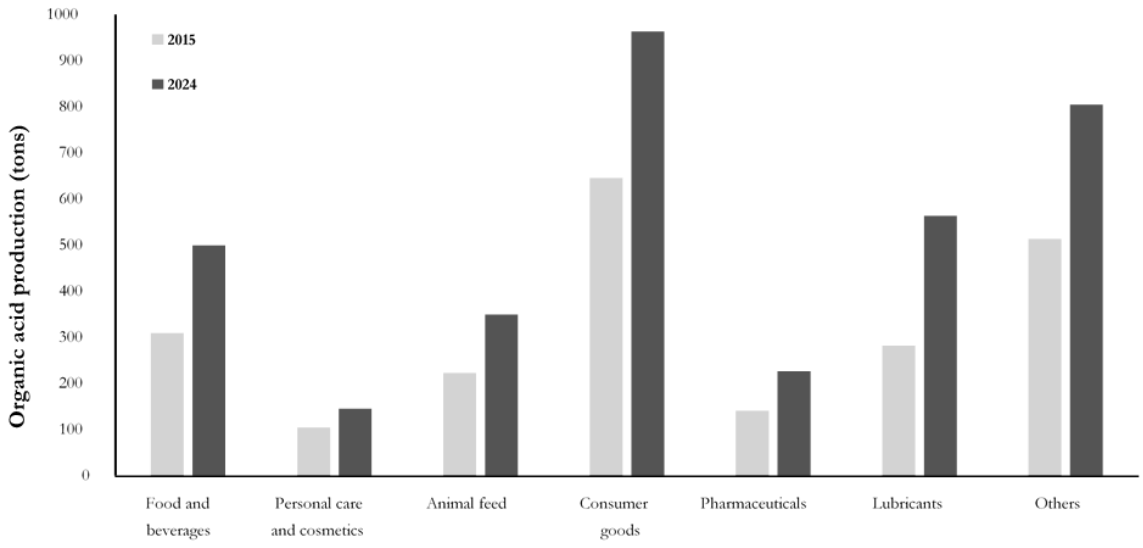

Figure 2. Annual production of organic acids according to market sector/application between 2015 and 2024. (Adapted from Yashwant, 2017 [28]).

2.2 The global market for organic acids

The global organic acid market was valued at 20 billion euros in 2016. The forecasts predict an annual growth of 8.3%, which should reach 35 billion euros by the year of 2023, with an impact in a broad range of industrial sectors (Figure 2). The most significant contributions to this growth are the rising market and the demands for bio-based organic acids, the use of renewable sources and the growing demand from developing countries. The speculation upon raw material prices and availability, as well as upon organic acids prices can create strains this market [28].

The Asia Pacific region dominates the global organic acid market, followed by North America and Europe. The Asia Pacific region will experience the fastest growth in this market between 2017-2022. The reasons for this economic performance are the growing economy and industrialization in countries of this region, and the settlement of new industries, such as food, beverage, pharmaceutical, and animal feed industries [28, 29].

Regarding the European market for organic acids, it stands for 1.7 billion euros, and it is estimated to grow annually around 5.91 % reaching a value of 2.9 billion euros in 2026. The increasing population, high purchasing power and growing attention towards health properties of natural products should support this growth. Moreover, new administration policies and government legislation for the use of chemicals as preservatives in food, feed, and agriculture will increase the demand for organic acids [29].

The market size by product is segmented into acetic acid, propionic acid, formic acid, valeric acid, isovaleric acid, butyric acid, isobutyric acid, caproic acid, citric acid, stearic acid, and other carboxylic acids. Acetic acid is the organic acid with bigger market size due to its application in food industry, either for its use in vinegar but also as preservative and flavour enhancer in several food products. On the other hand, stearic acid is predicted to have one of the most significant market size growth until 2024, due to its potential for the production of cosmetics, soap and detergent industries [29].

A quarter of the global production of organic acids is accomplished by six companies, namely BASF SE, Celanese Corporation, LyondellBasell Industries, Jiangsu Sopo Group, Eastman Chemical Company, and Dow Chemical Company [29].

3. Microbial production of organic acids

The first reports of microbial production of organic acids date back from 1823 for acetic acid and 1893 for citric acid. However, it was only six years after the isolation of an *A. nidulans* strain capable of producing significant amounts of citric, in 1923, that citric acid produced by fermentative process was added to the market by the Pfizer and Co Inc company. Since then, and due to the range of

applications of this class of compounds, several other organic acids started to be produced by microbes and are now commercialized [21, 27].

The industry of microbial production is in continuous development to increase cell factory productivity, yields and range of products. The development of recombinant DNA technology along with synthetic biology contributed to this evolution, allowing a rational engineer of organic acid producing microbes. Meanwhile, beyond the classical industrial microbes, such as *E. coli, S. cerevisiae* and *Corynebacterium glutamicum*, other microorganisms were isolated from natural sources and found to display a high capacity to generate organic acids [30-32]. This is the case of the filamentous fungi *A. niger*, which is exploited mostly for the large-scale production of citric acid since the beginning of the 20th century. Another example is lactic acid bacteria (LAB) used to produce lactic acid mainly in the food industry [26]. Moreover, endophytic fungi that are found to be associated symbiotically with internal plant tissues, are being identified as potential sources of several organic acids for industrial applications [33-35].

One of the most common processes exploited for the microbial production of organic acids is submerged fermentation, in which microorganisms grow in a liquid broth [36]. However, recent investigations found solid-state fermentation to be more efficient for several microorganisms, like filamentous fungi, by simulating their natural habitat [37, 38], which is particularly advantageous for the fermentation of agro-industrial wastes difficult to solubilize [39, 40].

Other derivatives of organic acids are the sugar acids, which are currently generating considerable interest due to their potential as platform chemicals and precursors in the manufacture of biomass-derived plastics and pharmaceutical drugs [41].

We will further detail the microbial production of acetic, lactic, 3HP, fumaric, succinic, xylonic, gluconic, saccharic and mucic acids, considering the industrial relevance of these compounds and the scope of this thesis dissertation.

3.1 Acetic acid

Acetic acid, a monocarboxylic acid also known as ethanoic acid (Figure 3), is the most demanded organic acid in the world and it is used as a substrate for many industrial processes. Applications of acetic acid range from food additives and preservatives to anti-fungal creams [42]. It also has application in the manufacture of dyes and perfumes, distinct types of fibres, textiles, wood glues and pesticides, and blood testing components.

The acetic acid bacteria (AAB), due to their natural ability to produce significant amounts of acetic acid, are the main organisms used in industry. Many of these AAB are from the genera *Clostridium* and *Acetobacterium* and so far, over one hundred species of these acetogens bacteria have been

identified. Some of the most commonly studied are *Clostridium thermoaceticum*, *Acetobacterium woodii*, and *Clostridium ljungdahlii* [43]. At the industrial scale, acetic acid is mainly produced by submerged fermentation, which refers to an aerobic process in which the ethanol in beverages such as spirits, wine or cider is oxidized to acetic acid by AAB [44]. The genetic engineering of the acetate producer *Acetobacter aceti spp. Xylinum,* involving the cloning of the aldehyde dehydrogenase gene from *Acetobacter polyoxogenes,* increased the rate of acetic acid production by over from 1.8 to 4 g /L/h and the titer from 68 to 97 g/L [45].

The Wacker company started its 500 tonnes/year bio-based acetic acid pilot plant in Burghausen, Germany, in 2009. In this bioprocess, the straw is used as feedstock, but it other sugar-based biomass raw materials can be used as well [23].

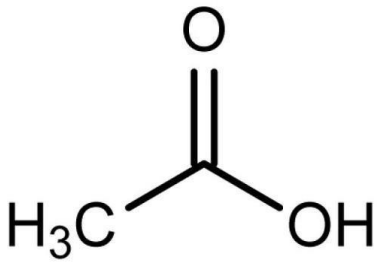

Figure 3. Acetic acid

3.2 Lactic acid

The microbial production of lactic acid or Hydroxypropanoic, a monocarboxylic acid (Figure 4), has been used for decades envisaging its application in the food, cosmetic, pharmaceutical and textile industries. Nevertheless, most of the lactic acid currently produced is converted in polylactic acid, widely used to produce bioplastics. LAB are producers of lactic acid and traditionally exploited for the food industry. However, regarding the biosynthesis of pure lactic acid, these microbes do not allow an economically viable process, due to the nutritional and neutral pH conditions requirements [26, 46]. Other microorganisms, such as *Rhizopus oryzae,* produce naturally lactic acid reaching a titer of 231 g/L of lactic acid [47]. Similarly to the LAB, the culture conditions are a bottleneck for the economic sustainability of this bioprocess. For that reason, the robustness of the yeast *S. cerevisiae*, namely tolerance to low pH and low nutritional requirements, makes it suitable for the production of lactic acid. Since *S. cerevisiae* is not a natural producer of lactic acid, metabolic engineering tools were used to create a metabolic pathway to produce lactic acid and increase the productivity and yields of this microbe [48]. In 2008, a *S. cerevisiae* strain was engineered by Cargill company to produce lactic acid for commercial purposes. This strain has a deletion in the pyruvate

decarboxylase1 gene and heterologously expresses the L-lactate dehydrogenase gene from *Lactobacillus casei*. Along with other laboratory selection and mutagenesis tools, it reached a titer of 135g/l of lactic acid at pH 3.0. Thus 90% of these titers are in the free acid form, which avoids the use of pH buffer chemicals reducing significantly downstream costs [46].

Several companies commercialized bio-based lactic acid, such as ADM, Cargill, BASF, PURAC and BioAmber [23].

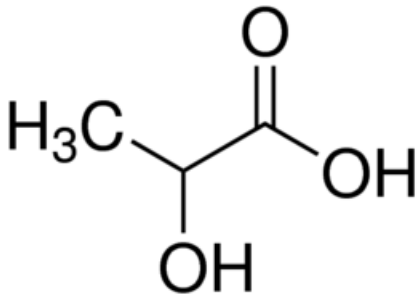

Figure 4. Lactic acid

3.3 3-Hydroxypropionic acid

The monocarboxylic acid 3-Hydroxypropionic acid (3HP) (Figure 5) is a building block that can be processed in 3-carbon intermediates as an alternative to the petrochemical industry. The 3HP acid is converted to acrylic acid, acrylamide, acrylonitrile, ethyl 3HP, 1,3-propanediol and malonic acid [23, 24].

For the production of 3HP from glycerol, two bacterial species are used, *E. coli* and *Klebsiella pneumonia*. The recurrent costs related to nutrient requirements, namely vitamin B12 have impelled industries and research laboratories to look for another microorganism, like *S. cerevisiae* [49-51], for the production of this acid. Indeed, the production of 3HP from sugars by engineered *S. cerevisiae* is possible by heterologous expression of malonyl-CoA reductase derived from *Chloroflexus aurantiacus*, similarly to what happens in *E. coli.* This approach combined with metabolic flux engineering of 3HP through the overexpression of *PDC*, *ALD*, *ACS* and *ACC* genes resulted in a 3HP titer of 9.5 g/L with a yield of 0.09 g/g glucose [52]. However, in *E. coli,* the higher production titer reported for 3HP is 48.4 g/L with a return of 0.53 g/g glucose [53]. Despite the significant differences between these two producers, the possibility of low pH fermentation is a promising advantage for production in yeast [24].

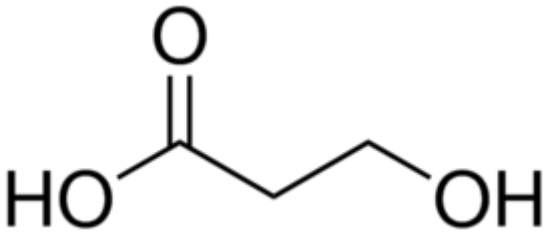

Figure 5. 3-Hydroxypropioninc acid

3.4 Succinic acid

Currently, large-scale production of commercially available succinic acid, a dicarboxylic acid (Figure 6), is mainly achieved through the chemical synthesis of petroleum-derived maleic anhydride. This limits the exploitation of this product due to its high production cost [26]. Recently, it has been postulated that succinic acid can be produced more cost-effectively, by microbial fermentation using renewable carbon sources, such as sugar cane, molasses, glycerol and others [24]

Production of succinic acid through a process of fermentation is being achieved from a wide variety of bacteria, like *Anaerobiospirillum succiniciproducens* [54], *Actinobacillus succinogenes* [55], *Mannheimia succiniciproducers* [56] and *E. coli* [57].

Biotechnology companies such as DSM/Roquette (joint Venture Reverdia) have been able to engineer the yeast *S. cerevisiae* to generate succinic acid in a titer of 100g/L at low pH conditions, which as aforementioned as a significant impact in the costs of downstream processes and nutrient requirements [58]. Besides Roquette company, also Reverdia, Myriant, BASF/PURAC and BioAmber companies biosynthesise succinic acid at industrial scale [23].

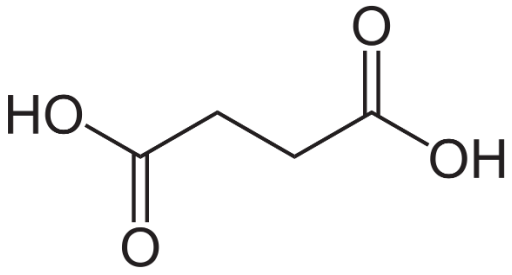

Figure 6. Succinic acid

3.5 Fumaric Acid

The dicarboxylic acid fumaric acid (Figure 7) is one of the most versatile organic acids. It has several applications in petrochemical industry as a building block to produce synthetic resins, coating

compound, and synthetic polymers. In the food industry, fumaric acid serves as na acidulant agent in bread, wine, dairy products, beverages, and processed meat. In the pharmaceutical segment, fumaric acid is a co-component in various drugs and drug-based byproducts against skin diseases. It is indeed a primary component of many skin care products, hair products, and bath products [59].

Microbial production of this organic acid relies on the *R. oryzae* fungi, taking advantage of its ability to use renewable feedstocks (Xu et al. 2012). Recently, a new approach of simultaneous saccharification and fermentation of cornstarch using an engineered strain of *R. oryzae* allowed a 1.28 fold increment in fumaric acid production, corresponding to 44.10 g/L of fumaric acid [60].

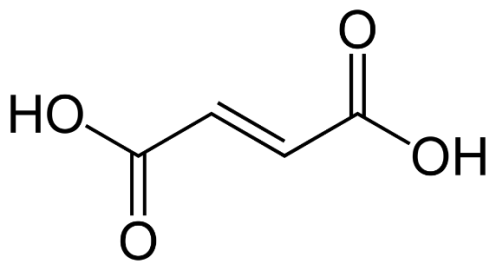

Figure 7. Fumaric acid

3.6 Xylonic acid

Xylonic acid is a five-carbon sugar monocarboxylic acid (Figure 8), occurring naturally in different food products, such as bread, milk and milk products, cheese products. It can be an excellent substitute for gluconic acid and used as cement retardant, production of polyamides and other polyesters as well, as bioplastics [23, 41]

In 1898, the fermentative production of xylonic acid was reported for the first time. Since then, several different bacteria were described as xylonate producers such as *Pseudomonas sp.*, *Acetobacter sp.*, *Aerobacter sp.*, *Gluconobacter sp.* and *Erwinia sp.*, as well as other fungi species, particularly *A. niger*. High yields of D-xylonate from D-xylose make *Gluconobacter oxydans* an attractive choice, even more considering its ability to synthesize D-xylonate directly from plant biomass hydrolysates. Xylonate has been produced by genetically modified *E. coli, S. cerevisiae* and *Kluyveromyces lactis* engineered strains. Expression of NAD(+)-dependent D-xylose dehydrogenase of *Caulobacter crescentus* in either *E. coli* or in a robust, hydrolysate-tolerant, industrial *Saccharomyces cerevisiae* strain has resulted in D-xylonate titres, comparable to those seen with *G. oxydans*, at a volumetric rate approximately 30% of that observed with *G. oxydans* [41, 61, 62].

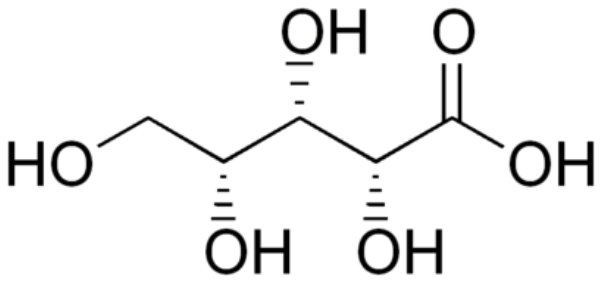

Figure 8. Xylonic acid

3.7 Gluconic Acid

In recent years we have witnessed the commercial acceptance of the monocarboxylic gluconic acid (Figure 8) as a low-cost fermentation-derived product, especially in the agro and food-industry [63]. In the food industry, it acts as a flavouring and leavening agent, as a fat absorption intervenient and mineral supplement. In the textile industry, gluconic acid is used to prevent polyamide and polyester desizing and iron deposition. Due to its chelating properties, it has several applications in metallurgy industry, concrete resistance, and mouthwashes products. The zinc gluconate salt is used in common cold treatment, wound healing and in several diseases caused by zinc deficiencies, such as mental lethargies, skin changes, delayed sexual maturation and immunodeficiency [63].

The microbial production of gluconic acid can be achieved through low-cost carbohydrate sources, like sugarcane molasses, grape must [64]. Several microbes like bacteria from the genus *Pseudomonas*, *Acetobacter* as well as *Gluconobacter* were proven to be useful in the production of gluconic acid. However, the fungi *A. niger* is found to be the most efficient producer microbe [63]. In fact, this filamentous fungus generates gluconic acid at an industrial scale in fed-batch fermentation conditions, at buffered pH around 6.0 – 6.5 and 34º C, reaching a titer of 360 g/L [65].

Bio-based gluconic acid is currently being commercialized by Roquette and Purac [23].

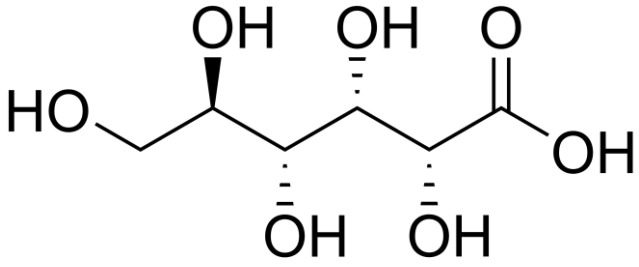

Figure 9. Gluconic acid

3.8 Saccharic acid

Saccharic acid, also known as glucaric acid, is a dicarboxylic acid (Figure 10) found in nature. Since 1888, saccharic acid is produced by chemical synthesis by nitric acid oxidation of D-glucose [66].

This sugar acid was found to promote cholesterol reduction and anticancer activity. It is also a chemical platform used for biopolymer production, such as nylon, polyesters, and biodegradable fibres [66-68].

Despite the inexistence of microbial production reports at industrial scale, an engineered *E. coli* strain was able to synthesize 1g/L of saccharic acid from glucose. A metabolic pathway was created in this strain inserting *INO1* gene from *S. cerevisiae* coding for myo-Inositol-1-phosphate synthase expression; MIOX gene from mice coding for myo-inositol oxygenase; and uronate dehydrogenase *udh* gene from *Pseudomonas syringae* [67]. After increasing MIOX activity and myo-inositol transport, the production of glucaric acid was significantly increased to 4.85 g/L [69, 70]. Also, the coexpression of MIOX and *udh* genes in the host *Pichia pastoris,* allowed to obtain a titer of 6.61 g/L of saccharic acid [71].

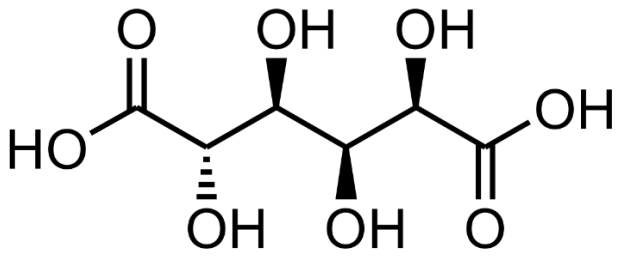

Figure 10. Saccharic acid

3.9 Mucic acid

Mucic acid or galactaric acid is a dicarboxylic acid (Figure 11) commercially produced from D-galactose oxidation or electrolytic oxidation of D-galacturonate. Mucic acid is used as a chelator and leavening agent, in skin care products and to produce biopolymers [72, 73]. There are no reports of commercial mucic acid microbial production [73]. Nevertheless, metabolic engineering of *Hypocrea jecorina* and *A. niger* by deletion of D-galacturonate reductase *gar1* and *gaaA* gene, respectively, along with heterologous expression of D-galacturonic acid dehydrogenase *udh* gene from *Agrobacterium tumefaciens*, allowed the bioproduction of mucic acid. In the engineered *H. jecorina* a titer of 5.9 g/L of mucic acid was obtained against 1 g/L mucic acid from *A. niger* strain [72]. Mucic acid can also be produced biotechnologically, using either genetically modified *Trichoderma reesei* or *E. coli* at 10 g/L and 20g/L, respectively [74, 75]. Similarly to the previous studies, these strains heterologously express the *udh* gene under disruption of the native D-galacturonate metabolic pathway.

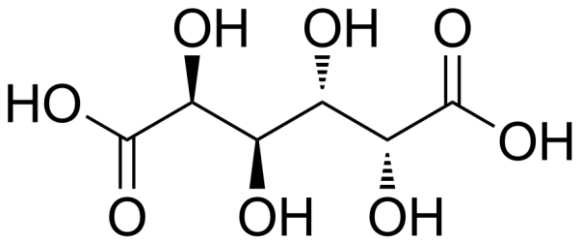

Figure 11. Mucic acid

3.10 Microbial production of organic acids from renewable resources

Although the industry of microbial fermentation has shown to be a reliable, cost-competitive, feasible, eco-friendly and sustainable alternative to petrochemical industry for the production of some organic acids, it needs to be in continuous development to overcome the current and future global economic and environmental constraints [76]. The costs of the upstream processing associated with fermentation media are estimated to account for 60% of the operating expenses, which represents an economic burden, challenging to handle by biotechnology companies [23]. Moreover, rising environmental concerns and the depletion of fossil-carbon resources, have led to the use of inexpensive feedstocks by the biotech industry, opening the path for efficient and sustainable bio-production of organic acids from non-food renewable resources such as lignocellulose, food processing by-products and agro-industrial wastes [23, 77].

In a scenario where feedstock cost increase and sugar prices are volatile, the industry of microbial production has focused its research pipeline in the exploitation of renewable feedstocks: agricultural residues such as cereal straws, corn stover and wheat straw, forest residues like hardwoods, and industrial wastes or by-products such as glycerol and cheese whey [23].

Due to its low cost, availability and carbon content, lignocellulose is one of the best renewable feedstock for production of organic acids. Chemical and enzymatic pretreatment of cellulose and hemicellulose result in considerable amounts of sugars, which can be further assimilated by microbial cell factories for the further production of specialty organic acids, such as fumaric, propionic, butyric, gluconic and succinic acids. The most used lignocellulose feedstocks for microbial production processes are sweet sorghum, non-edible crops like Jerusalem artichoke, sugarcane bagasse and industrial fibrous waste from juice extraction [23, 77] *E. coli* [78] and *A. succinogens* [79] were engineered to produce succinic acid from lignocellulosic feedstocks, such as corn stover and sugarcane bagasse hydrolysate, respectively.

The agro-industrial industry generates every year unmeasured amounts of wastes with high potential for microbial fermentation processes, due to their low-cost and high carbon and sugar content. Most of these agro-industrial renewable feedstocks come from food processing plants, such as corncob

molasses and sugarcane molasses [23, 77]. Sugarcane molasses are recycled for the bioproduction of succinic acid, as well as of butyric acid by *Clostridium tyrobutyricum [80]*. Corncob molasses, rich in xylitol, were tested for the biosynthesis of propionic acid by *Propionibacterium acidipropioni* [81]. Nowadays, glycerol, a by-product from the biodiesel industry, exceeded its industrial demand [23]. This surplus has led the biotech industry to look for bioprocesses to convert glycerol into platform-chemicals such as organic acids [82]. However, crude glycerol has a significant amount of impurities (methanol, salts, free fatty acids, and free methyl esters) that can affect the productivity of cell factories. Nevertheless, when compared to glucose, higher organic acid yield and lower by-product formation were found for the production of propionic acid by *Propionibacterium freudenreichii.* On the other hand, glycerol can trigger cellular redox imbalance diminishing cell growth and cell biosynthesis performance under anaerobic conditions[83]. Regardless of these constraints, glycerol was found to be a reasonable precursor for the bioproduction of glyceric, α-ketoglutaric, propionic and 3HP acid [83-86].

Cheese whey is the principal co-product of the dairy industry. The Europe region stands alone for a quarter of the total waste generated. Around 60% of cheese whey enters the composition of whey protein products and dietary supplements, but the remaining waste, rich in lactose, represents an environmental risk and recycling technologies are far from being economically sustainable. [23, 87]. Regarding microbial fermentation, cheese whey was proven to be an excellent feedstock to produce lactic, citric, lactobionic, acrylic and succinic acid [23]. The use of cheese whey as a carbon source avoids pretreatment and saccharification methods, although it is necessary that cell factories have metabolic pathways for the degradation of lactose, which is not so common for many industrial microbes [27, 88-90].

3.11 *Saccharomyces cerevisiae* as a cell factory for organic acid production

The yeast *S. cerevisiae* is one of the preferred cell factories for bioproduction of organic acids, due to its ability to grow in low pH conditions associated with low nutrient requirements, robustness, and easy genetic manipulation [23, 91]. When compared with *E. coli*, one of the preferable hosts for bio-based production, *S. cerevisiae* is quite appealing due to the higher tolerance to low pH conditions, the lesser requirement of maintenance for fermentation process and less vulnerability to contamination, namely phage infection. On the other hand, the higher product tolerance is the key factor towards better performance [92].

Due to *S. cerevisiae's* ability to grow at low pH, this yeast species avoids the use of buffering agents. Thus, at the end of the fermentation period (the organic acid is in the undissociated form), there is no generation of salts in downstream processing [23, 24, 91-93]. Moreover, this yeast is by far one of

the best-characterized model organisms, where a detailed depiction of its metabolic pathways is available. The baker yeast was the first eukaryotic organism to have its entire genome sequenced, and several research tools are available to study *S. cerevisiae* [23, 26, 94]. Its efficient recombination machinery fixes various types of DNA damage, including double strand breaks (DSB). Due to the DSB repairing machinery, *S. cerevisiae* has a unique ability to allow genetic transformation by homologous recombination [95]. Also, a variety of selectable markers are available, both complementing auxotrophies markers or antibiotic resistant gene markers [96]. A plethora of vectors are available for *S. cerevisiae*: the 2 µ episomal vectors (high copy number) dedicated for protein expression; centromeric vectors characterized by low copy number and high stability ideal for complementation studies or analysis of mutant alleles; integrative vectors devoid of replicating origin and yeast artificial vectors (YAC) allowing autonomous and stable replication fine for the expression of entire metabolic pathways (see review Sandström et al, 2014 [93]).

Moreover, a vast amount of information gathered during decades was consolidated in the Saccharomyces Genome Database (SGD, http://www.yeastgenome.org), which contains manually curated information about every gene and pathway, the SPELL database of microarray expression studies, and Yeast GFP Fusion Localization database.

For a successful *S. cerevisiae* strain development strategy, not only strain robustness but also heterologous pathway expression via the removal of competitive pathways and the maintenance of redox and energy balance must be taken into consideration. There are certain disadvantages as well that should be taken into consideration, while choosing *S. cerevisiae* as a platform, such as its inability to use xylose, which is one of the most abundant sugars generated from hemicellulosic hydrolysates and inhibition of pentose metabolism triggered by weak acids, especially at low pH conditions [23, 97].

Up to now, *S. cerevisiae* was successfully tested for the production of several organic acids such as fumaric, glycolic, itanoic, lactic, malic, muconic, pyruvic, succinic, 3-HP and xylonic acid (see review Sandström et al, 2014 [93]). Many known biotech companies are using engineered strains of *S. cerevisiae* to produce organic acids, such as lactic, succinic acid and malic acid, on a large scale [24, 41, 77].

4. Membrane transporters as tools for the development of cell factories

Microbial production of organic acids comprises several transport processes, such as substrate uptake, transport between organelles and product export [26, 98, 99]. These processes, mostly ruled by membrane proteins, are critical for the biosynthesis of organic acids and their purification from the culture broth [26].

With few exceptions, neither substrates nor products of cell metabolism can cross the plasma membrane freely. Instead, these molecules require the activity of membrane transporter proteins to cross the cell membrane outwards or inwards. Thus, the productivity of microbial cell factories is significantly affected by the influx of substrates and efflux of products and by-products.

Nowadays, most industrial microorganisms are metabolically engineered to produce specific products and/or to metabolize specific substrates. For decades, the engineering of the flux processes, transport mechanisms and transport energetics of these compounds were underestimated, and special attention was given to the engineering of metabolic pathways. But recently, the scientific community and biotech companies started to focus their efforts in transporters engineering envisaging the development and improvement of microbial cell factories [26, 98, 99].

The microbial fermentation industry faces two major bottlenecks in the production line: the first related with product accumulation and toxicity insde the cell and low product titers in the extracellular medium; the second associated with cell factory capacity to assimilate and uptake carbon and energy sources for product biosynthesis. These two blockages in microbial fermentation can be overcome by transport activity of endogenous or exogenous membrane transporters, importers and/or exporters, as well as their genetic manipulation regarding expression levels and generation of mutant alleles with increased transport capacity [98-100].

4.1 The role of membrane transporters in the uptake of renewable substrates

The use of low-cost renewable feedstocks in microbial production processes is an increasingly used approach to turn bioproduction of chemical platforms competitive with petrochemical derivatives. However, the substrates, the carbon and energy sources made available in these renewable feedstocks are in most cases hardly assimilated and metabolized by cell factories. This bottleneck is associated with the inexistence of membrane proteins or extracellular enzymes able to respectively uptake or convert substrates in assimilated forms. Therefore, besides the exploitation of membrane proteins to increase product efflux, membrane proteins are also engineered in microbial cell factories to increase substrate influx to increase production yields [77, 98, 101].

Among the renewable feedstocks available for microbial fermentation industry, cheese whey and lignocellulosic biomass are among the most used. Whereas lactose is the most abundant sugar in cheese whey, xylose along with arabinose are the most abundant in lignocellulosic hydrolysates [23, 77]. Since *S. cerevisiae* is considered as one of the most promising microorganisms to produce chemical platforms, including organic acids and ethanol [91], several experiments regarding the functional expression of lactose, xylose, and arabinose transporters in *S. cerevisiae* were reported:

- The expression of *K. lactis* lactose permease encoding gene, *LAC12*, in an engineering *S. cerevisiae* flocculent strain allowed lactose consumption. In this work, a recombinant *S. cerevisiae* flocculent strain heterologously expressing the b-galactosidase *LAC4* gene and lactose permease *LAC12* gene of *K. lactis* was used for ethanol production from lactose in a continuous culture operation. This approach resulted in a ethanol production yield of 0.51 g/g of lactose. In continuous fermentation conditions, this engineered *S. cerevisiae* strain reached an ethanol titer of 11 g/L/h, which represents a seven-fold raise compared with conventional systems [102].

- The functional heterologous expression of xylose transporters in *S. cerevisiae* for the conversion of this lignocellulosic sugar in building blocks was reported several times in the literature. More than 80 heterologous xylose transporters or putative xylose transporters encoding genes have already been expressed in *S. cerevisiae* [91]: *SUT1*, *SUT2*, *XUT1*, *XUT3* (Xyp33), *XUT4*, Xyp29 (*STL12*), SUT3 (Xyp37) from *S. stipitis*, and *GXF1* from *Candida intermedia*, At5g59250 from *A. thaliana*, An29-2 and An25 from *Neurospora crassa*, and xtrD from *A. nidulans*, MgT05196 from *Meyerozyma guilliermondii* and Xylh from *D. hansenii*. These have been shown to enable the HXT-null *S. cerevisiae* strains to transport xylose, although most of them display a preference for glucose over xylose. Nevertheless, more than 80 percent of these putative transporters or annotated sugar transporters were not functional in *S. cerevisiae*, probably due to misfolding or improper localization [91].

- Along with xylose, arabinose is the second most abundant sugar present in lignocellulosic hydrolysates. Due to the lack of proper arabinose uptake in *S. cerevisiae*, two characterized L-arabinose transporters, LAT-1 from *N. crassa* and MtLAT-1 from *Myceliophthora thermophila* were expressed in this yeast. The expression of both transporters in an engineered strain of *S. cerevisiae* containing L-arabinose metabolic pathway display much faster L-arabinose utilization, greater biomass accumulation, and higher ethanol production than the control strain [103].

4.2 The role of membrane transporters in product efflux

One of the first reports of membrane transporter exploitation to increase product export and extracellular accumulation dates to 1960s. At this time, the bacteria *C. glutamicum* was the microbe used to produce glutamate. In this regard, several experimental procedures and alterations in fermentation conditions increased the productivity and the efflux of glutamate. Later, this increment

in glutamate efflux was associated with mechanic tension, which triggered the activation of MscS glutamate efflux pump. After this, several pumps similar to MscS were described as playing a critical role in the product efflux in the microbial biosynthesis of other amino acids, namely lysine, isoleucine, threonine, methionine, and others. [98, 101]

Regarding the microbial production of organic acids, several reports pointed to the effectiveness and contribution of membrane transporters for product efflux, mostly in the last decade:

- The malic acid titer produced by an engineered *S. cerevisiae* strain was increased up to tenfold through the expression of the *S. pombe* malate transporter SpMae1. In this study an engineered glucose-tolerant, C_2-independent pyruvate decarboxylase-negative *S. cerevisiae* strain served as a platform to evaluate the effect of three genetic modifications, namely overexpression of the native pyruvate carboxylase encoded by *PYC2*, overexpression of an allele of the malate dehydrogenase *MDH3* gene, and functional expression of the *S. pombe* malate transporter gene Sp*MAE1*. These modifications *per se* improved malate production, although the combination of all genetic modifications reached the highest titer of malate, approximately 59g/L. Nevertheless, the engineered strains still produced significant amounts of pyruvate, indicating that the metabolic pathway for malate synthesis could be improved. [104].

- Overexpression of the *S. cerevisiae* mitochondrial succinate-fumarate transporter *SFC1* gene enhanced fumarate export and production by 47.6 % in this yeast [105]. Based on genome-scale metabolic *in silico* model, an engineered *S. cerevisiae* strain wa created to produce fumaric acid. In a first step, deletion *FUM1* gene, coding for fumarase, led to a yield of 610 mg/ L of fumarate without any noticeable change in growth in fed-batch culture. Since flux balance analysis showed pyruvate carboxylase as one of the factors limiting higher fumarate production, the *RoPYC* gene was cloned in the previously engineered strain. As a result, the strain reached a titer of 1134 mg/ L of fumarate. Also, the overexpression *SFC1* gene encoding a succinate-fumarate transporter in the S. *cerevisiae* manipulated strain resulted in the production of 1675 mg/L of fumarate in batch culture [105].

- In a *S. cerevisiae* strain engineered for lactate production, constitutive expression of *JEN1* and *ADY2* carboxylate transporters encoding genes resulted in a higher accumulation of lactate in the extracellular space. In this work, the lactate-dehydrogenase *LDH* gene from *L. casei* was expressed in *S. cerevisiae* wild-type strain and in the isogenic mutants *jen1*Δ,

*ady2*Δ and *jen1*Δ *ady2*Δ to allow lactate production. All the strains expressing *LDH* were able to produce higher titers of lactic acid compared with the wild-type strain and the isogenic mutant strains. Moreover, the constitutive expression of *JEN1* or *ADY2* genes along with *LDH* resulted in the higher external accumulation of lactic acid in the presence of glucose. However, upon glucose consumption, lactate consumption was also more pronounced in these strains [106].

- Expression of two previously identified *Aspergillus terreus* genes encoding putative organic acid transporters (*mttA*, *mfsA*) increased itaconic acid production in an *A. niger* cis-aconitate decarboxylase expressing strain. In previous experiments, the heterologous expression of cis-aconitate decarboxylase gene (*cadA*) from *A. terreus* allowed the production of itanoic acid by *A. niger* [107, 108]. Using this producer strain, two *A. terreus* putative organic acid transporters encoding genes, *mttA,* and *mfsA* were singly expressed or co-expressed. The resultant strains expressing *mtta* and/or *mfsA* displayed an increased itaconic acid production when compared with *A. niger* strain expressing only cis-aconitate decarboxylase. Interestingly, the production did not increase further when both transporters were co-expressed. However, oxalic acid was accumulated as a by-product in the culture of mfsA transformants, which led to further manipulation of the producing strain to avoid the accumulation of these compound and increase itanoic acid production [109].

- Modulating the expression of *E. coli* transporter genes *dcuB* and *dcuC* in combination led to a 34% increase of succinic acid titer in engineered *E. coli* strain. In this work, four *E. coli* Dcu C4-dicarboxylates transporters were exploited for their capacity on succinate export. Whereas deletion of *dcuA* and *dcuD* did not affect the export of this organic acid, *dcuB* and *dcuC* deletion led to 15% and 11 % decrease of succinate extracellular titer, respectively. Deletion of both *dcuA* and *dcuD* genes resulted in 90 % decrease of succinate titer. As result, a ribosome binding site library was investigated to modulated and increase the co-expression of *dcuA* and *dcuD*, which led to 34 % increase of succinate titer produce by *E. coli* [110].

- The overexpression of the *C. glutamicum* succinate exporter, SucE, increased succinate yield in na engineered strain. A dual-route for anaerobic succinate production was engineered in *C. glutamicum*, which involved the reconstruction of the glyoxylate pathway by overexpressing isocitrate lyase, malate synthase, and citrate synthase. This succinate producer strain reached a succinate yield of 1.34 mol/mol of glucose. Additionally, the overexpression of the endogenous succinate exporter, SucE, increased succinate yield to 1.43

mol/mol of glucose. In anaerobic fed-batch fermentation, the *C. glutamicum* succinate producer strain overexpressing SucE transporter led to a titer of 109g/L succinate [111]

- Employing the two-dicarboxylic acid transporter Sfc1 and SpMae1 led to an improvement of fumarate production in *C. glabrata*. This work endorsed the metabolic engineering of the tricarboxylic acid (TCA) cycle in *C. glabrata* to construct the oxidative pathway for fumarate production. Thus, a set of genetic modifications to manipulate the oxidative pathway was applied in α-ketoglutarate dehydrogenase complex, succinyl-CoA synthetase, and succinate dehydrogenase. As a result, the *C. glabrata* producer strain reached a fumarate titer of 8.24 g/L. The overexpression of argininosuccinate lyase gene led to a fumarate increase up to 9.96 g/L. This result was associated with accumulation of argininosuccinic acid detected intracellularly in the earlier engineered strain. The expression of two dicarboxylic acids transporters, Sfc1 and SpMae1, allowed an improvement of fumarate production to a titer of 15.76 g/L fumarate. [112].

- The overexpression of the endogenous genes *dcuB* and *dcuC* from *E. coli* increased fumaric acid yield by 48.5% and 53.1%, respectively. In a similar approach to the one mentioned earlier [110], a set of C4-dicarboxylate transporters from different organisms sources were cloned in an *E. coli* fumaric acid producing strain to evaluate their impact on the production of this organic acid. However, the overexpression of endogenous C4-dicarboxylate transporters DcuB and DcuC displayed a higher impact in the production of fumaric acid, leading to an increase of fumaric acid yield by 48.5% and 53.1%, respectively. In fed-batch fermentation culture, the fumaric acid producer strain overexpressing *dcuB* gene produced 9.42 g/L of fumaric acid after 50 hours [113].

- The overexpression of two endogenous malate transporter genes in the natural malic acid producer *Ustilago trichophora* RK089 improved the production yields by 54%. In this study, the overexpression of pyruvate carboxylase, two malate dehydrogenases (*mdh1*, *mdh2*), and two malate transporters (*ssu1, ssu2*) where carried in a laboratory-evolved *U. trichophora* strain. This strain overexpressing of *mdh1*, *mdh2*, *ssu1*, and *ssu2* reached an extracellular matale titer of 120 g/L [114].

4.3 Engineering membrane transporters

To find a membrane transporter with specificity for a certain product or substrate might not be enough to achieve the levels of productivity needed to turn a bioproduction process cost-effective and

sustainable. Frequently, membrane proteins display substrate promiscuity, or altered transport affinity and capacity when expressed heterologously. However, engineering membrane proteins to reduce these containments is also possible and often used to tune and boost the transport activity of membrane proteins towards a specific compound. This approach is frequently achieved by direct evolution experiments, either by mutagenesis or recombination involving methods of synthetic biology [91, 98, 101].

Whereas it is common to look for exogenous transporters to be cloned into producer strains, sometimes the endogenous transportome of the producer strain could display a fine pool for selecting the proper transporters for further engineering. *S. cerevisiae* genome encodes 85 members of the Major Facilitator Superfamily of multidrug efflux pumps and at least 25 members of the ABC multidrug transporter family, all promising targets to be used in product efflux [98]. On the other hand, it encodes twenty transporter proteins belonging to the Hexose Transporter Family with specificity for several sugars and thus with the potential to be exploited in yeast cell factories for the uptake of renewable sugars from agro-industrial wastes [91, 115].

As noteworthy, the *S. cerevisiae* Gal2p membrane protein is a known galactose permease and it has been reported that it also has specificity for L-arabinose, but with low transport capacity [116]. Homologous modelling and L-arabinose docking studies identified nine residues of Gal2p interacting with L-arabinose. Most of the tested mutations affected L-arabinose transport capacity, and some (F85S, F85G, F85C, and F85T) increased significantly L-arabinose transport. In fact, the F85S mutation improved xylose transport and allowed an increment of 40% in *S. cerevisiae* growth rate [117]. Another similar study using a growth-based screening platform allowed the identification of two residues in *S. cerevisiae* hexose transporters Hxt7 and Gal2 that can be mutated to yield glucose-insensitive xylose transporters. [118]. Also, the substrate specificity of the yeast Jen1 lactate transporter was also altered in previous works, which allowed the permease to transport dicarboxylic acids, such as succinic acid [119, 120]. This dissertation will further detail these works in Chapter 2.

5. Future perspectives for transport engineering

The impact of membrane transporters in cell factory productivity is still slight, at the industrial scale. Nevertheless, the research on the effects of the transport engineering in producer strains underlines the benefits of this approach, especially in the yeast *S. cerevisiae* as it was described previously and summarized in Figure 11.

The functional and structural characterization of membrane proteins is still a slow process and more tools are required [98, 99]. Nevertheless, major advances in the future are expected, as optimized directed evolution techniques will guide the selection and engineering of membrane transporters [98].

Also, new roles of membrane transporters in productivity of cell factories, rather than the efflux and influx of molecules, start to be uncovered, namely at the level of strain tolerance to by-products. A recent study reported the impact on ethanol production through the deletion of the *S. cerevisiae* acetate transporter coding gene *ADY2* [121]. An increment of 14.7% in ethanol production was registered for a yeast strain lacking *ADY2* gene, in which acetate uptake from the extracellular space was impaired and consequently reduced the intracellular toxicity of acetate and increased the tolerance towards this organic acid [121].

The versatility and plasticity of membrane transporters hold a promising future in the biotechnology industry, as well as in the contribution for the implementation of further bioproduction of platform chemicals at the industrial scale.

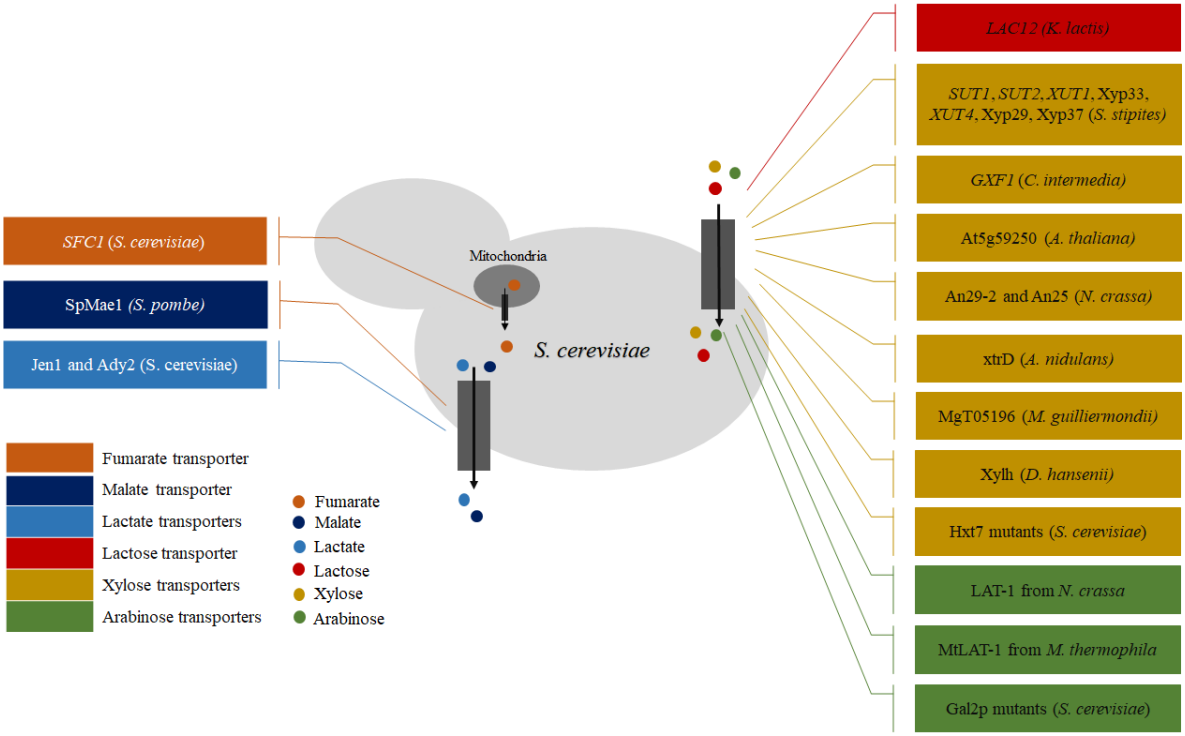

Figure 12 – The expression of endogenous or exogenous encoding membrane transporter genes in engineered strains of *S. cerevisiae* allowed the uptake of renewable substrates (lactose, xylose or arabinose) as well as the export and extracellular accumulation of specialty organic acids (fumarate, malate or lactate) as it was reviewed earlier in this chapter. The red, yellow, green, orange, dark blue and light blue colours are used to identify lactose, xylose, arabinose, fumarate, malate and lactate transporters/transporters encoding genes and the correspondents' substrates, respectively. The coloured circles represent the substrates. The black arrows indicate the direction of the substrate, either into the cytoplasm or into the extracellular space.

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Chapter I

Chapter II

Carboxylic acids plasma membrane transporters in *Saccharomyces cerevisiae*

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Personal contribution: I was involved in the writing of the introduction and the sections describing: Jen1 as a member of MFS family; Jen1 structure; and Ady2 as a member of AceTr family.

Chapter II

Carboxylic acids plasma membrane transporters in *Saccharomyces cerevisiae*

Abstract

This chapter covers the functionally characterized plasma membrane carboxylic acids transporters Jen1, Ady2, Fps1 and Pdr12 in the yeast *Saccharomyces cerevisiae,* addressing also their homologues in other microorganisms, as filamentous fungi and bacteria. Carboxylic acids can either be transported into the cells, to be used as nutrients or extruded in response to acid stress conditions. The secondary active transporters Jen1 and Ady2 can mediate the uptake of the anionic form of these substrates by a H^+-symport mechanism. The undissociated form of carboxylic acids is lipid-soluble, crossing the plasma membrane by simple diffusion. Furthermore, acetic acid can also be transported by facilitated diffusion *via* Fps1 channel. At the cytoplasmic physiological pH, the anionic form of the acid prevails and it can be exported by the Pdr12 pump. This review will highlight the mechanisms involving carboxylic acids transporters, and the way they operate according to the yeast cell response to environmental changes, as carbon source availability, extracellular pH, and acid stress conditions.

Chapter II

1. Introduction

Carboxylic acids are ubiquitous organic compounds that can be used by the cell machinery as natural substrates or found as final products or by-products of fermentative processes. As weak acids, they can partially dissociate according to their pK_a and to the pH of the aqueous solution, following the Henderson– Hasselbalch equation [$pH = pK_a + \log(A^-/HA)$]. One of the most important factors influencing carboxylic acids transport across biological membranes is the environmental pH. When the pH is below the pK_a of the acid, the protonated undissociated form predominates and it is able to cross biological membranes by passive diffusion. However, when the pH is above pK_a of the acid, the charged anionic form predominates, requiring a transporter to cross the biological membrane [1].

The first evidence for a carboxylic acid transporter in yeasts was described in *Schizosaccharomyces pombe*, associated with the uptake of malic acid [2]. The gene encoding this activity (*MAE1*) was also the first carboxylate permease gene identified in yeasts [3]. According to the Transport Classification Database (TCDB, www.tcdb.org), Mae1 displays ten predicted transmembrane segments (TMS), belonging to the Tellurite resistance/Dicarboxylate Transporter family (TC 2.A.16). Evidence for the existence of a transporter mediating the uptake of lactic acid was found for the first time in the yeasts *Candida utilis* [4] and *Saccharomyces cerevisiae* [5] and the associated gene was named *JEN1* [6]. This transporter is a member of the Major Facilitator Superfamily (MFS) belonging to the lactate/pyruvate:H^+ sub-family (TC 2.A.1.12.2). Some years later, the Ady2 acetate-propionate-formate transporter was described in *S. cerevisiae*. This transporter is expressed in the presence of non-fermentable carbon sources and subjected to glucose repression [7]. Ady2 displays six predicted TMS and belongs to the Acetate Uptake Transporter (AceTr) family (TC2.A.96.1.4). Additional roles have been attributed to this protein, namely in ammonia export [8] and *ascus* formation [9].

Jen1 and Ady2, involved in the uptake of the anionic form of monocarboxylic acids are so far the only secondary active transporters assigned in *S. cerevisiae*. At lower pH values, when the undissociated form of the acid prevails, carboxylic acids can also cross the plasma membrane by simple diffusion. However, it has been found that the aquaglyceroporin Fps1, a channel primarily involved in osmoadaptation, can mediate the entry of undissociated acetic acid by facilitated diffusion [10]. Both simple and facilitated diffusion correspond to energy independent transport mechanisms. Once in the cytosol, at neutral pH values, the acids dissociate, releasing toxic counter-anions and protons, inducing an intracellular acidification that affects cell homeostasis. However, *S. cerevisiae* can still grow in the presence of high concentration of weak carboxylic acids at low pH values, though presenting increased lag phase duration and lower biomass yields [11]. Pdr12, an ATP-binding cassette (ABC) transporter, member of the Pleiotropic Drug Resistance (PDR) family, was

demonstrated to be essential to the acquisition of tolerance to weak acid stress, being involved in the extrusion of the carboxylate anions and participating in cellular detoxification [12].

The importance of carboxylic acids transporters in yeast relies thus on two fundamental processes, the uptake of the acids to be used as nutrients and their extrusion in response to acid stress conditions. The present review will focus on the role of the currently known plasma membrane carboxylic acids transporters in *S. cerevisiae* Jen1, Ady2, Fps1, and Pdr12, addressing also their homologues found in other microorganisms, as filamentous fungi and bacteria.

2. The carboxylic acids transporter Jen1, a member of the Major Facilitator Superfamily

Jen1 was the first monocarboxylic acids transporter described in fungi [6]. Besides its role in the uptake of lactate, pyruvate, acetate and propionate [6], it also transports the micronutrient selenite [13] and the antitumor compound 3-bromopyruvate [14]. The transport of the substrate is bidirectional, being Jen1 also involved in the acids efflux [15, 16]. The kinetic parameters estimated for this transporter, concerning lactate uptake in *S. cerevisiae* W303-1A lactic acid-grown cells are V_{max} of 0.40 nmol of lactic acid s^{1} mg of dry $weight^{1}$ and K_m of 0.69 mM lactic acid [6]. When overexpressed in the host *Pichia pastoris* a fivefold increase in V_{max} was achieved ($V_{max} = 2.15 \pm 0.14$ nmol of lactic acid s^{1} mg of dry $weight^{1}$), whereas the K_m was of the same order of magnitude ($K_m = 0.54 \pm 0.08$ mM) [17]. The reconstitution of Jen1 in *P. pastoris* vesicles demonstrated that the proton motive force is necessary for transport, confirming the proton-symport mechanism. Kinetic properties of the reconstituted transporter were found to be similar to the ones reported for *S. cerevisiae* intact cells [17]. This evidence clearly demonstrated Jen1 as a fully functional lactate permease.

In *Kluyveromyces lactis* two Jen1 homologues were identified, one encoding a monocarboxylate transporter (KlJen1), for lactate and pyruvate, and the other a dicarboxylate transporter (KlJen2) for malate and succinate [18, 19]. A similar situation was found in *Candida albicans* where CaJen1 transports monocarboxylic acids, such as lactate, whereas CaJen2 transports the dicarboxylic acids succinate and malate. The phylogenetic analysis of these Jen1 homologues suggested the existence of two functional clusters, Jen1 and Jen2, comprising the functionally characterized monocarboxylate and dicarboxylate transporters, respectively [1]. The *Yarrowia lipolytica* genome encodes six Jen1 homologues. The YlJen1 encoding genes have their expression increased in the presence of different carboxylic acids. Recently they were associated with mono-, di- and tricarboxylic acids transport, such as lactate, pyruvate, fumarate, malate, succinate, and citrate, suggesting the ability to transport these acids [20, 21].

Using the information that resulted from the sequencing of various yeast and fungi genomes it has been possible to trace the evolution of the Jen1 family members [1, 20, 22, 23]. Since YlJen homologues represent a separated cluster, they were designated to their own subfamily called Jen3 [20]. YlJen1 and YlJen5 were considered ancestors of Jen1, therefore also known as preJen1 proteins. These proteins are considered an evolutionary intermediate between Jen2 (the ancestral homologue) and Jen1 members [20, 22]. Jen1 transporters differentiated from preJen1 in *K. lactis* and they are present as a single copy in *Saccharomyces* species. Post-WGD (Whole Genome Duplication) species (such as *Saccharomyces* genus) in general lack Jen2, indicating that this precursor was evolutionary lost prior to the WGD [22].

2.1 Expression and Regulation

In *S. cerevisiae* the regulation of Jen1 expression has also been studied over the past years at the transcriptional, post-transcriptional and post-translational levels. In lactic acid, pyruvic acid, acetic acid or glycerol-grown cells *JEN1* is highly expressed, whereas in glucose, formic and propionic acid-grown cells it is undetectable [6]. *JEN1* glucose repression involves the transcription factors Mig1 and Mig2 [24]. When glucose is withdrawn, Hap2/3/4/5 complex, Adr1, and Cat8 transcription factors act together (Fig. 1), upregulating numerous genes involved in the utilization of non-fermentable carbon sources, including *JEN1* and *ADY2* [24-27]. The kinase Snf1, a AMPK homologue, is also involved in the release of glucose repression of both genes. It phosphorylates Mig1 resulting in its translocation from the nucleus, activating Cat8 and Adr1 [26, 28, 29]. It was also demonstrated that *SNF1* deletion inhibits H3 acetylation at the *ADY2* promoter, impairing chromatin remodelling and *ADY2* transcription, in a process independent of Adr1 and Cat8 [29].

The influence of the carbon source in *JEN1* expression has also been highlighted at the post-transcriptional level, particularly in mRNA turnover [30]. The transcriptional map of yeast genome revealed two *JEN1* transcripts with 2200 and 1900 nucleotides [31]. It has been found that, in *S. cerevisiae* W303-1A lactic-grown cells, the long-mRNA decay is triggered by a pulse of glucose. This was in contrast to what was observed for CEN.PK2-1C.

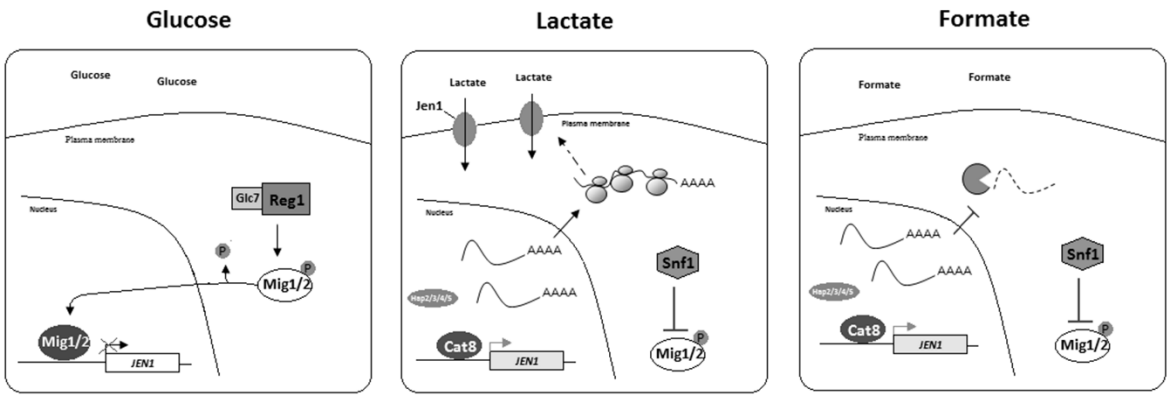

Figure 1. Post-transcriptional regulation of *JEN1*. In **glucose**, the transcription of *JEN1* is repressed by Mig1/2 transcription factors. In the cytoplasm, Mig1/2 are activated by dephosphorylation through a Reg1/Glc7 phosphatase and, then, imported into the nucleus [32]. In **lactate**, as sole carbon source, the transcriptional factors Cat8, Adr1, and Hap2/3/4/5 are able to activate *JEN1* expression. The inactivation of Mig1/2 by phosphorylation, through Snf1 protein kinase, leads to their export to the cytoplasm. Therefore, in these conditions, the formation and maturation of mRNA is unaffected which ultimately leads to the expression of the lactate transporter at the plasma membrane. Cat8, Adr1, and Hap2/3/4/5 are transcriptional activators associated to non-fermentable carbon sources growth [25, 33, 34], as lactate. Interestingly, *CAT8* is repressed at the DNA level by Mig1/2 proteins [35]. In **formate**, as sole carbon source, the transcription of *JEN1* is also active, however, the early synthesized mRNA is targeted for degradation, consequently, no Jen1 protein is produced. Dhh1 RNA helicase, Pat1, and Lsm are involved in *JEN1* mRNA degradation, in this condition cells, where the long-mRNA retained a half-life time of 17–20 min, independently of the addition of glucose to the culture medium. However, when CEN.PK2-1C cells were grown on ethanol, the *JEN1* long-mRNA half-life time was reduced from 26 to 7 min. upon the pulse of glucose. Mapping of the *JEN1* 5' and 3' UTR transcripts revealed multiple transcription start sites located at positions -51 (for the long transcript, translated), and C391 or C972 (for the short transcripts, not translated). It was demonstrated that when *JEN1*(C391) small transcript is present, it works as a glucose sensor, promoting *JEN1*(-51) protein-coding mRNA rapid decay [36].

The Dhh1 RNA helicase and the Pat1-Lsm decapping enhancers were found to play a crucial role in the mechanisms underlying the regulation of *JEN1* 5'3' mRNA decay pathway [37]. Dhh1 together with Pat1-Lsm complex controls the translation initiation, by targeting mRNAs to the P-bodies, contributing to the recruitment of the mRNA decapping machinery (reviewed by [38]). When cells were incubated in formic acid *JEN1* mRNA accumulates in *dhh1*, *pat1* or *lsm* mutants, contrarily to what was found in the wild-type strain [37]. In this condition, it was found that *JEN1* mRNA, although transcriptionally active, was targeted for degradation *via* Dhh1 and Pat1-Lsm complex (Fig. 1). Microarray analysis revealed that this mechanism of regulation is also shared by other genes involved in non-fermentable carbon source metabolism, as *ADY2*, *CAT8,* and *HAP4*. Besides its general effect in the global cytoplasmic mRNA decay, Dhh1 has additional roles in the post-transcriptional or transcriptional regulation of several genes in response to environmental stimuli [37].

Another level of Jen1 regulation involves protein trafficking and turnover. The addition of a pulse of glucose to lactic acid-grown cells very rapidly triggered the loss of Jen1 activity and endocytosis, followed by vacuolar degradation [39]. The HECT E3 ubiquitin ligase Rsp5, the unique member of the Nedd4 family in yeast, modifies Jen1 at the cell surface by polyubiquitylation [40]. Jen1 has been reported as one of the first examples where endocytic internalization and sorting at multivesicular bodies (MVBs) require ubiquitin-K63 linked chain(s). It has also been demonstrated that the yeast Rod1, a protein of the ART family (arrestin-related trafficking adaptors), is essential for glucose-induced Jen1 ubiquitylation and endocytosis. In lactic acid-grown cells, Snf1 protein kinase inactivates Rod1 by phosphorylation. Upon a pulse of glucose, the PP1 phosphatase Glc7/Reg1 activates Rod1, which also dephosphorylates Snf1 inactivating it [41] (Fig. 2). Therefore, Rod1 serves as a relay between glucose signalling and endocytosis.

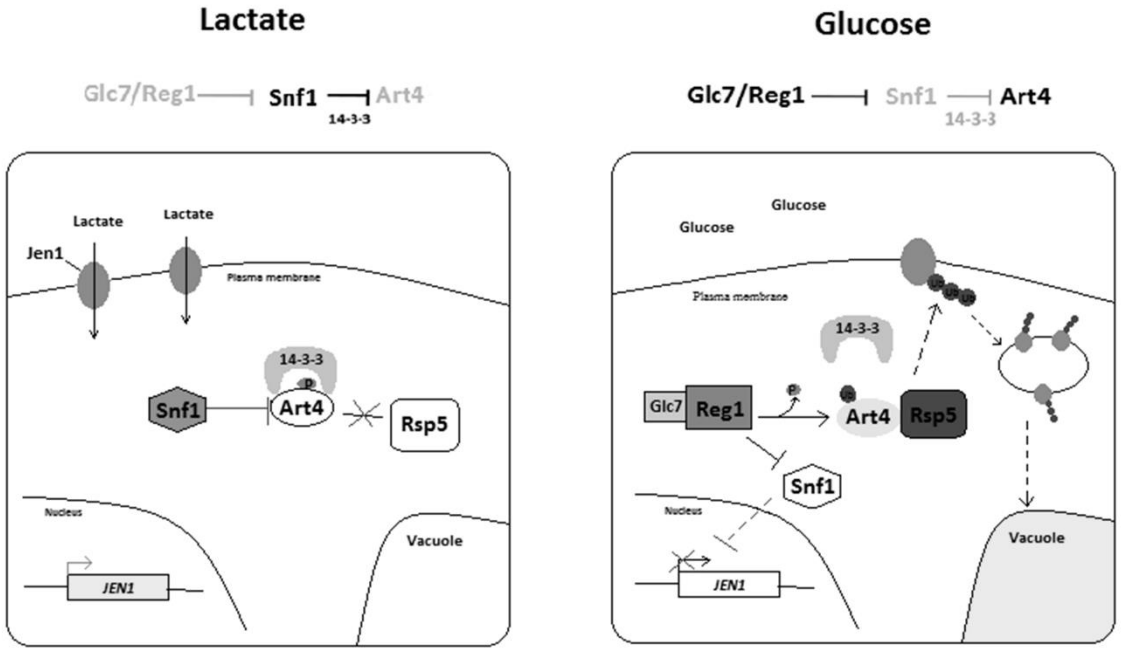

Figure 2. Jen1 inactivation by glucose. In **lactate**, as only carbon source, Jen1 is expressed at the plasma membrane. In this condition, Rsp5 ubiquitin ligase is unable to target Jen1 for degradation *via* the arrestin-like protein Rod1. Rod1 phosphorylation is dependent on Snf1 protein kinase. Phosphorylated Rod1 interacts with 14-3-3 proteins, becoming inaccessible to Rsp5, thus impairing Jen1 degradation. Upon a pulse of **glucose**, Jen1 is rapidly removed from the plasma membrane. Reg1/Glc7 phosphatase is involved in Rod1 activation and Snf1 inactivation, through dephosphorylation. As a consequence, Rod1 is released from a phospho-dependent interaction with 14-3-3 proteins and ubiquitylated by Rsp5. Finally, ubiquitylated-Rod1-Rsp5 complex ubiquitylates Jen1 resulting in its endocytosis and vacuolar degradation.

2.2 Jen1 structure

Jen1 shares the common topology of the MFS members (Fig. 3), known as MFS fold, which comprises 12 TMS organized in 6+6 folded domains in the N- and C-termini, separated by a central cytoplasmic loop [42]. Currently, several members of this superfamily, covering six subfamilies, have their structure solved, namely, the lactose/H^+ symporter LacY [43], the glycerol-3-phosphate/Pi antiporter GlpT [44], the multidrug/H^+ antiporter EmrD [45], the L-fucose/H^+ symporter FucP [46], the oligopeptide/H^+ symporters PepTSo and PepTSt [47], and the D-xylose/H^+ symporter XylE [48]. Jen1 structural-functional relationships have been elucidated by a rational mutational analysis of conserved amino acid residues. The conserved sequence ^{379}**N**XX[S/T]**H**X[S/T]**QD**^{387}, located towards the periplasmic side of putative transmembrane segment seven (7-TMS), is part of the substrate translocation pathway [49]. The residue N379 was found to be irreplaceable, and crucial for protein activity. Residues H383 and D387 affect both the transport capacity and the specificity. On the other hand, Q386N substitution reduces the binding affinity for all Jen1 substrates, while Q386A increases the affinity for pyruvate [49]. In addition, the conserved residues F270 (5-TMS) and Q498 (11-TMS) are essential for the substrate specificity, as they are involved in the distinction between mono- and dicarboxylates. The residues N501 (11-TMS) and R188 (2-TMS) are important players in the protein function as they are irreplaceable for Jen1 activity [50] (Fig. 3).

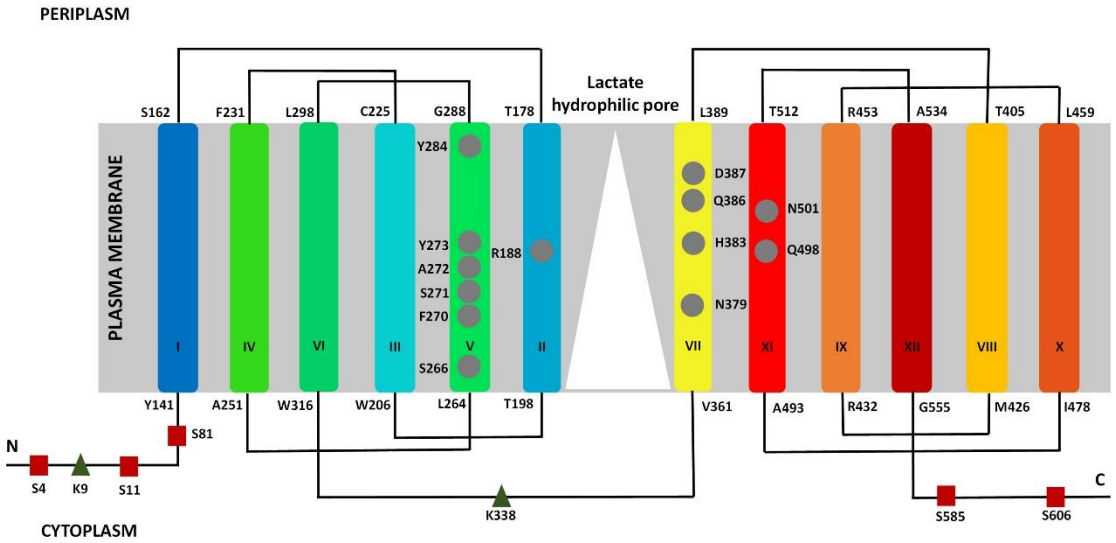

Figure 3. ScJen1 overall topology. The predicted transmembrane segments are coloured in rainbow spectrum, from the I TMS to XII TMS. Residues involved in substrate binding, specificity and translocation are shown as circles: II TMS (R188), V TMS (S266, F270, S271, A272, Y273, Y284) VII TMS (N379, H383, Q386, D387) and XI TMS (Q498, N501). Residues marked with squares
(S4, S11, S81, S585, S606) are predicted targets for phosphorylation. Ubiquitylated residues (K9, K338) are shown as triangles. The hydrophilic lactate pore is designated by a white triangle between the II TMS and VII

TMS, pointing the transport of lactate molecule from periplasm to cytoplasm. The N-terminus and C-terminus of the protein are annotated as N and C, respectively.

The predicted structural similarity of Jen1 with the GlpT permease revealed that all the polar residues above mentioned, namely R188 (2-TMS), S266, F270, S271, A272, Y273 (5-TMS), N379, H383, D387 (7-TMS) and Q498, N501 (11-TMS), are perfectly aligned in an imaginary axis that lies parallel to the protein pore (Fig. 4). Furthermore, docking calculations revealed a 'trajectory-like' substrate displacement along the Jen1 pore, where R188 plays a major dynamic role mediating the orderly relocation of the substrate by subsequent H^+-bond interactions involving also residues H383, N501 and Q498 [50]. The association between structural-functional studies and prediction models provided extremely valid information on the structural properties and features of Jen1. The conserved amino acids residues, in particular, the polar and charged present in TMS, were found essential for binding and translocation of both the substrate (the anionic form of the acid) and the co-substrate (the proton) [50].

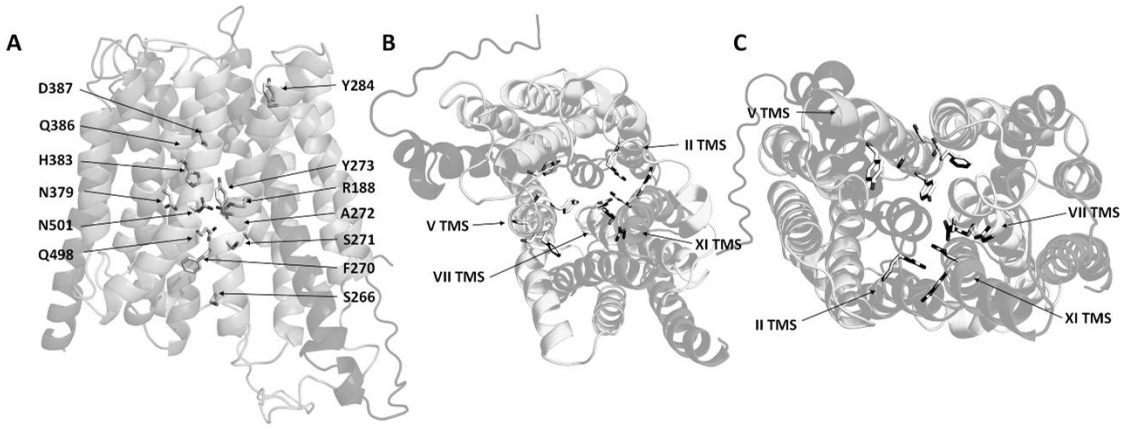

Figure 4. Jen1 predicted 3D structural model: (**A**) transversal, (**B**) cytoplasmic and (**C**) periplasmic view. Residues critical for substrate binding, specificity and trajectory are shown as stick molecular structures and identified by black arrows: II TMS (R188), V TMS (S266, F270, S271, A272, Y273, Y284) VII TMS (N379, H383, Q386, D387) and XI TMS (Q498, N501). The Jen1 model was obtained with Modeller software (Version 9.14, Ben Web, CA, USA) based on GlpT transporter X-ray structure. The images were obtained with the PyMOL Molecular Graphics System (Version 1.5.0.4 Schrödinger, LLC).

3. The carboxylic acid transporter Ady2, a member of the AceTr Family

Members of the Acetate Uptake Transporter (AceTr) Family (TC 2.A.96.1.4) are found in archaea, eubacteria as well as in simple or complex eukaryotes. They share several conserved motifs namely

the amino acid residues sequences NP(A/V/G)P(L/F/V)GL and (Y/F)G(X)FW [51]. The NPA sequence found in the first motif is typical of the Major Intrinsic Protein (MIP) family, also known as aquaporins. The AceTr members have a similar size and contain five to six predicted TMS, characteristic of the MIP family. However, while the characteristic NPA motif is present twice in MIP proteins, between the second and the third, and between the fifth and sixth TMS [52], in AceTr proteins it is present only once in the N-terminal part at the first predicted TMS [51].

Gpr1 (TC 2.A.96.1.2) from *Yarrowia lipolytica* was the first AceTr family member identified in yeasts [53], shown to be involved in acetic acid sensitivity, cell and colony morphology, yeast-to-hyphae transition and cell life span [54]. *GPR1* transcription is induced or derepressed in acetic acid or ethanol supplemented medium, compared to glucose-grown cells. Its localization at the plasma membrane is supported by subcellular fractionation studies and by fluorescence microscopy analysis [51].

The *S. cerevisiae ADY2* (TC 2.A.96.1.4), along with *ATO3* (TC 2.A.96.1.5) and *FUN34* (TC 2.A.96.1.5) genes are homologous of *Y. lipolytica GPR1* gene [54]. *ADY2* was first characterized as a gene required for proper *ascus* formation on a sporulation medium in which acetate is the main carbon source [9]. Other reports associate *ADY2* and the two homologue genes *ATO3* and *FUN34* with ammonium export [8]. Microarray analysis suggested the involvement of *ADY2* in acetate utilization and its subsequent disruption abolished active acetate transport [7]. Despite this evidence, the assignment of Ady2 as a carboxylate transporter held some controversy due to its multifunctional activity. However, the identification of Ady2 homologues as acetate transporters, namely AcpA (TC 2.A.96.1.3) from *Aspergillus nidulans* [55] and SatP (TC 2.A.96.1.1) from *E. coli* [56], strongly supported this assignment for *S. cerevisiae* Ady2.

AcpA is a transporter essential for the uptake and use of acetate as sole carbon source in *A. nidulans* [55], with high-affinity and high-capacity for acetate, and low capacity for other monocarboxylates. The AcpA transporter has an unusual expression profile showing activity in resting *A. nidulans* conidiospores, consistent with a role in spore maintenance or homeostasis [57]. The early activity of AcpA in resting conidiospores might be justified since transporters are efficient scavengers that can supply metabolites avoiding the cost of biosynthesis. In addition, the non-repressibility of AcpA by a primary carbon source such as glucose shows that it serves cellular needs for acetate accumulation other than acting as a carbon source supplier [57]. The AcpB *A. nidulans* was found to be responsible for residual acetate transport in mycelia and no function was found for AcpC. These results are in agreement with the lack of detectable transcription and minimal evolutionary conservation in fungi [57]. The AlcS (TC 2.A.96.2.1), another *A. nidulans* plasma membrane protein of the AceTr family,

is induced by ethanol and repressed by glucose [58], however, it is not involved in the transport of carboxylic acids [59].

The SatP protein from *E. coli*, previously known as YaaH, is a succinate acetate/H^+ symporter, differing from the other family members. The ability to transport both a monocarboxylic acid and a dicarboxylic acid was reported for the first time in this family. Before this, other AceTr family members were associated exclusively to the transport of monocarboxylates [56]. From a physiological point of view, the SatP transporter was found to play an important role for *E. coli* cells prior to the acetate switch momentum. When cells are grown aerobically in glucose, along with sugar consumption, the excretion of acetate occurs. However, when glucose is depleted, cells switch the metabolism to use acetate as carbon source. SatP is highly expressed and active during the exponential phase of growth, prior to the acetate switch, most likely being involved in acetate efflux [56]. Besides SatP, *E. coli* cells express another acetate transporter, the ActP, which is transcribed along with acetyl-CoA synthetase. This transporter belongs to the Sodium: Solute Symporter family and is highly specific for short-chain aliphatic monocarboxylates, namely acetate, glycolate and propionate [60]. When *E. coli* cells are grown aerobically in glucose, ActP is mostly expressed and active after the acetate switch phase, which suggests its involvement in acetate uptake. SatP contributes to the acetate–succinate intracellular balance, although it is not crucial for cells to grow on glucose or acetic acid [56].

In the archaea *Methanosarcina acetivorans*, the *MA4008* gene was found by quantitative transcription analysis of acetic acid *versus* methanol-grown cells, with 125-fold induction [61]. This high level of expression is only achieved in the presence of acetate, as the only carbon source. Methanol plus acetic acid-grown cells display lower levels of expression than cells grown in sole methanol, which is the preferred carbon source of this species. In addition, the expression of *MA4008* was similar to acetate kinase (*ack*) and acetyl-CoA phosphotransferase (*pta*) encoding genes which are required for acetate utilization in bacteria. This indicates that the *MA4008* gene is expressed only in the absence of a rich carbon source, suggesting its putative role in acetate uptake [61].

A phylogenetic tree of the AceTr family members functionally analyzed is presented in Fig. 5. The *S. cerevisiae* and *Y. lipolytica* members form a distinct clade with similar evolutionary distance from the *A. nidulans* AcpA and AcpB clades. The AcpA clade includes members from 21 species of *Aspergillus* genus with known genomes and most ascomycetes. A second clade, which includes AcpB, close to the AcpA proteins, is also partially conserved in *Aspergillus* sp. or other dikarya. The AcpC *A. nidulans* clade includes few sequences and is significantly more distant from AcpA, AcpB or the yeast clades [57]. The AceTr bacteria homologues are the most distant members from the fungi species, possibly due to evolutionary distance factors, such as substrate specificity. The SatP

transporter accepts both acetate and succinate [56] in contrast to known fungi homologues, which do not.

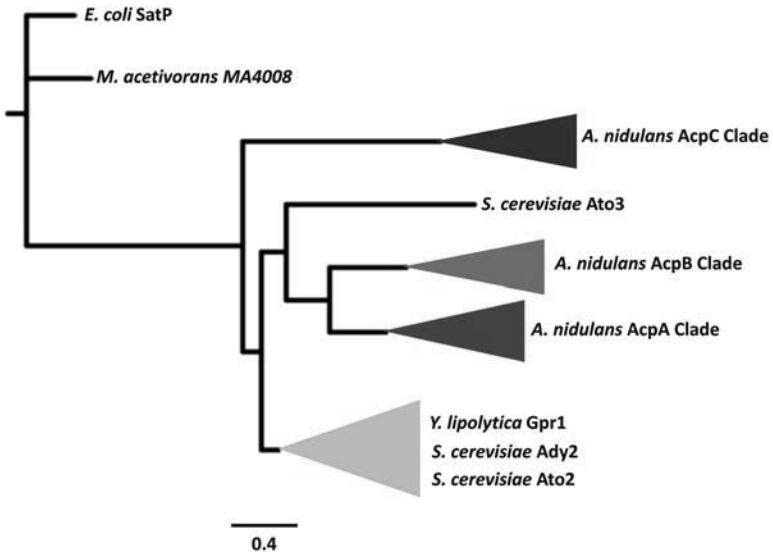

Figure 5. Phylogenetic tree of AceTr family. A phylogenetic tree was constructed from alignments of bacteria and fungi AceTr homologous protein sequences, using the Nearest-NeighbourJoining algorithm. NCBI Protein accession numbers are shown next to the species name. Microbial species shown in the tree and protein clades are as follows: *Aspergillus sp.* (AcpA, AcpB, and AcpC clades); *Saccharomyces cerevisiae; Yarrowia lipolytica; Escherichia coli; Metanosarcina acetirovans*. The SatP transporter from *E. coli* was used as an outgroup. The scale bar indicates the number of amino acid substitution per site.

The Ady2 transporter has potential biotechnological applications for the efflux of carboxylic acids, such as in lactate secreting *S. cerevisiae* cell factories [16]. In *S. cerevisiae* strains, engineered to produce lactate from glucose, the overexpression of Ady2 promotes the increase of lactic acid accumulation in the extracellular medium. Upon glucose exhaustion, a switch in the overall cell metabolism occurs, and the extracellular acid is again transported inside the cell *via* Ady2 [16]. In order to uncover novel lactate transporters in *S. cerevisiae,* a laboratory evolution approach was carried out in a *jen1* deleted strain. As a result, two independent evolved mutants associated with a gain of function for lactate uptake were selected. The whole-genome resequencing identified two single-nucleotide changes both located in *ADY2*, the L219V and A252G [62]. The two previous amino acid residues were equivalently mutated in the bacteria homologue SatP, specifically in L131 and A164. Both mutations enhanced the lactate uptake [56]. Overall, these results demonstrate the relevance of carboxylate transporters as modulators of the production and secretion of carboxylic acids by microbial cells. The molecular engineering of these transporters can improve microbial cell factories for organic acids bio-production.

4. Role of plasma membrane transporters in carboxylic acid stress resistance

Short-chain carboxylic acids are important biotechnological compounds with large application in the so-called Bio-based economy for obtaining building-block chemicals from sustainable biomass [63]. Additionally, in food and beverages industries, short-chain monocarboxylic acids like acetic, sorbic, benzoic or propionic acids are widely used as preservatives, in order to prevent microbial spoilage [64]. It has been observed that some yeasts are acid resistant and able to proliferate in acidic environments [12, 65]. These properties are desirable for industrial carboxylic acids production, avoiding the addition of neutralizing agents to the medium and expensive downstream processes to regenerate undissociated carboxylic acids, as it happens when less tolerant bacteria are used [66]. However, concerning the use of carboxylic acids as food and beverage preservatives, the capacity to resist to an acidic environment can lead to the development of contaminant yeasts and food spoilage [12], requiring higher concentrations of the acids with deleterious effects in human health. In this way, a deep understanding of the mechanisms underlying acid resistance and stress response is fundamental to control these biotechnological processes.

The maintenance of the intracellular pH (pHi) and the development of a stress response are crucial for yeast cell survival in an acidic environment. Intracellular acidification can affect the redox homeostasis, enzymatic activities (consequently, altering metabolic pathways and energy yield) or nutrient transport across the cell [1, 67]. On the other hand, the accumulation of the carboxylic acid counter-ion (RCOO) induces toxic effects such as high internal turgor pressure, oxidative stress and lipid peroxidation [68, 69]. Furthermore, organic acids can cause changes in membrane and cell wall structure and composition [70, 71]. Carboxylic acids stress response in yeast includes (i) a general and pleiotropic stress response that affects the expression of several genes under the control of Msn2 and Msn4 transcription factors [72, 73] and (ii) a specific response that involves the efflux of the organic anions by the pump Pdr12 and the efflux of protons by the pump Pma1 [12, 74, 75]. The rate of entrance of the undissociated form of carboxylic acids into the cell (by simple diffusion or *via* Fps1) and the efflux of the organic anions (*via* Pdr12) are key events in yeast adaptation to the acidic environment (Fig. 6).

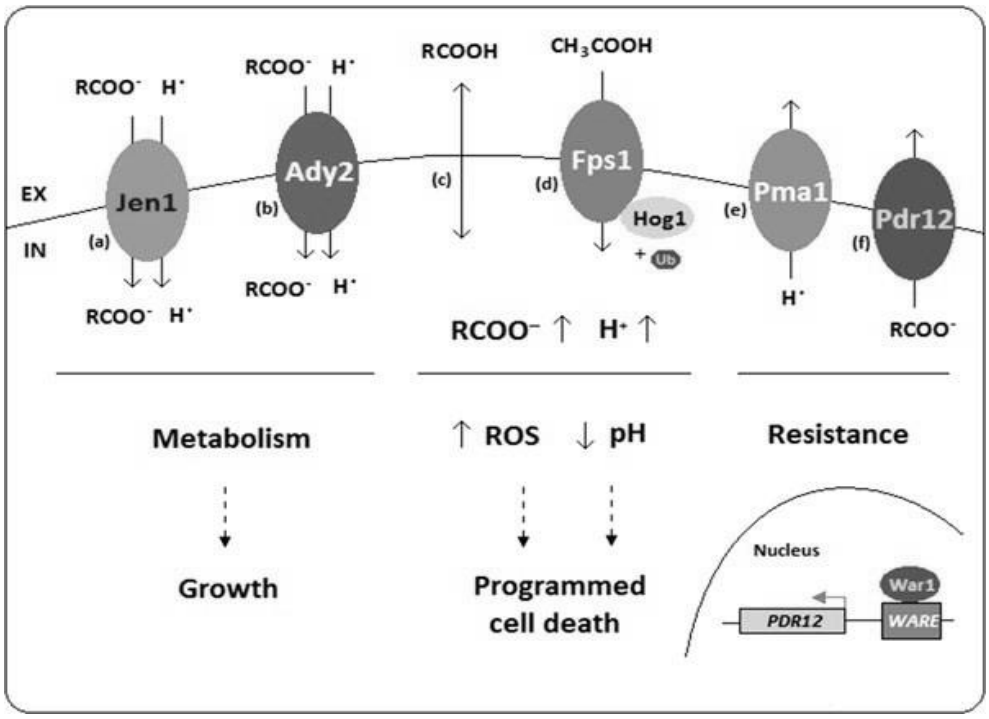

Figure 6. Mechanisms of carboxylic acids transport in*S. cerevisiae*. Jen1 and Ady2 are able to mediate the transport of carboxylates ($RCOO^-$, (**a**) lactate, pyruvate, acetate or propionate; (**b**) acetate, propionate, formate or lactate, respectively) into the cell, where they are used as carbon and energy sources. When the undissociated form of the acid (RCOOH) prevails, it enters by simple diffusion (**c**) and in the case of acetic acid (CH_3COOH) also through the Fps1 channel (**d**). The accumulation of protons (H^+) and anions (RCOO) may cause severe acidic conditions (low pH and high levels of ROS), which eventually lead to programmed cell death. Acid stress triggers the efflux of H^+ by Pma1 (**e**) and of carboxylates by Pdr12 (**f**) sorbate, propionate, benzoate or levulinate). To increase the expression of Pdr12, cell activates the War1 transcription factor which induces the transcription of *PDR12*, through *WARE* cis-acting response element. Furthermore, the ubiquitylation and degradation of Fps1, promoted by Hog1 MAPK pathway, serves as a mechanism to avoid acetic acid facilitated diffusion.

4.1 The pleiotropic drug resistance Pdr12 pump

Pdr12 is a member of a large ATP-binding cassette (ABC) transporters PDR subfamily. Contrarily to other members of this subfamily that have a broad range of substrates, Pdr12 is specifically involved in the efflux of carboxylic acids [76].

The PDR subfamily is characterized by the presence of nuclear binding domains (NBD), that contain three conserved motifs: Walker A, Walker B, and ABC signature or C-loop, alternating with TMS containing six transmembrane segments, in the following pattern NBD-TMS-NBD-TMS, a reverse

topology of the classic ABC transporters [76-78]. The two NBD work in tandem forming the ATP binding site, while TMS bind the substrate and translocate it across the plasma membrane[79].

In *S. cerevisiae* the PDR subfamily comprises the following members: Pdr5, Pdr10, Pdr15, Snq2, Pdr12, Aus1, Pdr11, Pdr18, Adp1 and YOL075c [80, 81]. An extensive phylogenetic analysis of 349 PDR proteins from 55 fungal species identified nine clusters (A, B, C, D, E, F, G, H1, H2), being Pdr12-like pumps associated with cluster D, which also includes Snq2. This cluster, as well as the clusters A (Pdr5-like) and E (Aus1/Pdr11-like), only contains Saccharomycotina members [78].

Another phylogenetic study, based on Génolevures database comprising nine hemiascomycetous species, identified a family (GL3C0025) containing all PDR proteins of *S. cerevisiae*, with 62 members clustered in 5 groups (A to E). Cluster A grouped Pdr12 together with eight other members from *Candida glabrata*, *Zygosaccharomyces rouxii*, *Saccharomyces kluyvery*, *K. lactis,* and *Y. lipolytica*. In this analysis, the close homologue Snq2 was represented in cluster B [76]. Although the function of Pdr12 and Snq2 diverge (short chain carboxylic acids efflux and antifungal azoles or other hydrophobic compounds extrusion, respectively), they share high sequence identity (46 %), what may suggest a common ancestral to both clusters [76].

Pdr12 is located at the plasma membrane and it is essential to yeast adaptation to weak acid stress [12]. Its correct expression, trafficking and/or functionality is controlled by Pdr10, another member of ABC subfamily that shares 36 % identity with Pdr12. Specifically, Pdr10 controls the local environment of Pdr12 in the membrane and its partition in lipid rafts [82].

Pdr12 is specifically involved in the efflux of propionic, sorbic and benzoic acids, increasing cells resistance to these acids [12, 74]. Carboxylic acids with aliphatic chain lengths higher than C7 (thus, with a high degree of hydrophobicity), or highly hydrophilic (lactic, acetic and formic) acids, are not exported by Pdr12 [74].In accordance to this, it has been observed that the presence of propionic, sorbic or benzoic acids, as well as acidic pH, strongly induces *PDR12* expression, differently to what is observed with acetic and formic acids [70, 83].

In contrast with a wild-type strain, *pdr12* mutant cells present higher sensitivity to sorbic, benzoic, propionic and levulinic acids, as well as to other carboxylic acids ranging from C1 to C7 [12, 74, 84]. The opposite is observed when overexpressing *PDR12* [84]. The influence of Pdr12 in carboxylic acid stress response depends on the degree of the acid hydrophobicity, as *PDR12* expression decreases the tolerance to the hydrophilic formic, acetic, lactic and glycolic acids, in contrast to what happens for the moderately hydrophobic sorbic, propionic and levulinic acids [84].

The release of H^+ and of the charged anion ($RCOO^-$) due to the acid dissociation alters the plasma membrane electrochemical potential ($Z\Delta pH$). Pma1 is able to mediate the efflux of the protons, neutralizing the cytoplasm acidification. But there is still a charge imbalance. Thus, the extrusion of

the anionic form of the acid by Pdr12 avoids its toxic accumulation and restores the charge balance at expenses of intracellular ATP [12, 74].

The carboxylic acid stress response is regulated by different pathways, namely through the transcription factors Msn2 and Msn4, Haa1, Rim1 and War1 (reviewed by Mira et al., 2010 [69]). *PDR12* promoter contains a weak-acid response element (*WARE*), recognized by the zinc-fingerproteinWar1, exclusively involved in *PDR12* expression regulation [85]. Deletion of *WAR1* leads to a failure of Pdr12 accumulation at the plasma membrane and to a decreased resistance to the moderately hydrophobic carboxylic acids. This phenotype is restored by the constitutive expression of *PDR12* in the *war1* null mutant [85, 86]. War1 is activated by carboxylic acids, putatively through the modification of the phosphorylation status of two serine residues at the positions 923 and 930 [87]. It has been observed that the carboxylic acid stress induces a conformational change of War1, enhancing its association to *PDR12* promoter [88]. In fact, gel electrophoresis assays demonstrated a mobility shift of War1 in carboxylic acid-stressed cells, due to changes in its phosphorylation status. However, a War1 kinase has not been identified so far [85]. War1 is conserved among several yeast species and an orthologue found in *C. albicans* was shown to mediate sorbic acid tolerance, like in *S. cerevisiae* [89].

In contrast to what happens with War1, Pdr12 is negatively regulated by Cmk1, a calmodulin protein kinase [74, 84]. Like the strain overexpressing *PDR12*, the deletion of *CMK1* increases the resistance to levulinic and propionic acids and the sensitivity to formic, acetic, glycolic and lactic acids. However, the role of Cmk1 is not limited to Pdr12 negative regulation, as improved tolerance to the acetic and formic acids observed in *pdr12* mutant strain was further enhanced by *CMK1* deletion, indicating an additional role of Cmk1 in the carboxylic acids resistance capacity [84].

Phylogenetic studies indicate the existence of Pdr12 homologues in other yeast species. In *C. glabrata* and *C. albicans*, like in *S. cerevisiae*, an increased resistance to sorbic and benzoic acids is accompanied by an increased expression of Pdr12, mediated by the activation of War1 [90, 91]. Furthermore, in *C. glabrata* Hog1 is required for full activation of *PDR12* [92]. In *K. lactis*, Pdr12 is associated with the efflux of the 4-methylthiooxobutyric acid, a fusel acid derived from methionine [93], a feature well described in *S. cerevisiae* [94], suggesting an analogue function for Pdr12 proteins in these two species. Although *PDR12* orthologues have been annotated in several yeast genomes, a functional role has only been studied in detail in *S. cerevisiae.*

4.2 The Aquaglyceroporin Fps1

The yeast aquaglyceroporin Fps1 is able to mediate the uptake of the undissociated form of acetic acid into the cell [10]. *S. cerevisiae* genome encodes another member of aquaglyceroporins, Yfl045c,

whose function is still unclarified [95]. Both Fps1 and Yfl045c belong to the MIP family, a group of integral membrane proteins present in all organisms. These transporters form channels or pores through biological membranes, mediating the transport of small uncharged molecules, such as water or glycerol [96, 97]. Fps1, in contrast to what is described for other aquaglyceroporins, does not act as a monomer, but most probably as a homotetramer, as described for aquaporins [98].

MIP channels have two symmetrical halves and are characterized by the presence of 6 TMS and two important loops, B, and E, containing the highly conserved NPA motif. However, in Fps1-like proteins, these loops are less conserved presenting the NPX and NXA motifs [99]. The loops B and E are located between second and third TMS and fifth and sixth TMS respectively, consisting in half-helices entering the membrane in opposite sites, forming a channel pore [100].

More than 400 MIPs have been found in fungal available genomes, phylogenetically classified according to protein similarity and substrate selectivity [101]. According to this classification, besides the traditional and dominant clusters of aquaporins (water channels) and aquaglyceroporins (water and small uncharged molecules channels), two new clusters have been identified, XIP (xintrinsic proteins) and SIP (small and basic intrinsic proteins) groups, which are also present in plants. Aquaglyceroporins cluster contains at least seven subfamilies in fungi, including Fps1-like subfamily which is found invariably in yeast Ascomycota [101].

Fps1 was firstly described as being involved in glycerol efflux in response to osmotic stress [102]. However, Fps1 was shown to be also involved in the influx of arsenite, antimonite and the undissociated form of acetic acid [10, 103]. Regulation of Fps1 expression is dependent on the Hog1 MAPK pathway [97]. Hog1 is activated by hyperosmotic stress, binding in these conditions to the N-terminal of Fps1, leading to the channel closure and preventing glycerol outflow [104]. In hypoosmotic conditions, water enters the cell causing turgor pressure, inducing the opening of the channel and glycerol efflux through Fps1 [97]. Acetic acid stress activates Hog1 MAPK pathway leading to Fps1 decay. Hog1 promotes Fps1 phosphorylation, ubiquitylation, endocytosis and degradation in the vacuole, impairing acetic acid uptake and therefore, rendering the cells more resistant to the acidic stress [10, 91] (Fig. 6).

FPS1 orthologues have been found in *Zygosaccharomyces rouxii*, *K. lactis*, *Kluyveromyces marxianus* and *Pichia angusta* genomes but not in *C. albicans*, *Candida tropicalis*, *D. hansenii* and *Pichia sorbitophila*. The heterologous expression of *FPS1* from the first three above mentioned yeast species in a *S. cerevisiae fps1* mutant complements its phenotype regarding the capacity to recover from a hypoosmotic shock, indicating their conserved role in glycerol efflux [105]. In *C. glabrata* two orthologues were found: CgFps1 and CgFps2, both regulated by the Hog1 pathway. Like in *S. cerevisiae*, they are associated with glycerol efflux and tolerance to osmotic stress where CgFps1 has

a more important role than CgFps2 [106]. Only *CaFPS1* was able to complement the *fps1* mutant phenotype of *S. cerevisiae*, reflecting the higher degree of homology between ScFps1 and CaFps1 [106]. Despite the similarity of functions of *S. cerevisiae FPS1* yeast orthologues concerning glycerol transport and osmotic stress response, no role concerning carboxylic acid stress, and more precisely, concerning the response to acetic acid stress, has yet been attributed to them.

5. Discussion and final remarks

Carboxylic acids are intermediates of central metabolic pathways like glycolysis and tricarboxylic acid cycle (TCA), thus playing a key role in the metabolism of all cells. The study of the metabolism of carboxylic acids in yeasts is also important from a biotechnological point of view, particularly regarding the production and preservation of foods and beverages, but also in the pharmaceutical and biomedical industries.

S. cerevisiae can use a wide range of nutrients as carbon and energy sources, with a preference for carbohydrates, in particular, glucose or other related rapidly fermentable sugars, like fructose and mannose (review by Conrad et al., 2014 [107]). The transition from a fermentative to an oxidative metabolism requires sugar depletion and is associated with a metabolic reprogramming, namely of TCA, oxidative phosphorylation, glyoxylate cycle and gluconeogenesis, and membrane transporters [1, 108, 109]. In this chapter, we reviewed the role of carboxylic acid transporters in the capacity of the yeast cell to adapt and respond to environmental changes (Fig. 6). On one hand, Jen1 and Ady2, expressed only in the absence of glucose, are involved in the metabolic use of monocarboxylic acids. On the other hand, Fps1 and Pdr12 are related to acid stress responses. The entrance of the undissociated acid in the cell, either by simple diffusion or *via* Fps1, in the case of acetic acid, imposes a severe stress: the anion accumulation is toxic and the released protons acidify the intracellular pH. Pumps specific for protons (Pma1) and for monocarboxylate anions (Pdr12) are crucial for the cells to recover and essential to maintain an equilibrated intracellular environment and cellular homeostasis.

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Chapter III

The *Debaryomyces hansenii* carboxylate transporters Jen1 homologues are functional in *Saccharomyces cerevisiae*

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Chapter III

The *Debaryomyces hansenii* carboxylate transporters Jen1 homologues are functional in *Saccharomyces cerevisiae*

Abstract

We have functionally characterized the four *Saccharomyces cerevisiae* (Sc) Jen1 homologues of *Debaryomyces hansenii (*Dh*)* by heterologous expression in *S. cerevisiae*. *D. hansenii* cells display mediated transport for the uptake of lactate, acetate, succinate and malate. DhJen genes expression was detected by RT-PCR in all carbon sources assayed, namely lactate, succinate, citrate, glycerol and glucose. The heterologous expression in the *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ strain demonstrated that the *D. hansenii JEN* genes encode four carboxylate transporters. *DH27* gene encodes an acetate transporter (K_m 0.94 ± 0.17 mM; V_{max} 0.43 ± 0.03 nmol s^{-1} mg^{-1}), *DH17* encodes a malate transporter (K_m 0.27 ± 0.04 mM; V_{max} 0.11 ± 0.01 nmol s^{-1} mg^{-1}) and both *DH18* and *DH24* encode succinate transporters with the following kinetic parameters respectively, K_m 0.31 ± 0.06 mM; V_{max} 0.83 ± 0.04 nmol s^{-1} $mg^{-1}$and K_m 0.16 ± 0.02 mM; V_{max} 0.19 ± 0.02 nmol s^{-1} mg^{-1}. Surprisingly no lactate transporter was found, although *D. hansenii* presents a mediated transport for this acid. This work advanced the current knowledge on yeast carboxylate transporters by characterizing four new plasma membrane transporters in *D. hansenii*.

Chapter III

Introduction

Short-chain carboxylic acids have a central role in cell metabolism and are used by many organisms as sole carbon and energy sources. In this context, the study of carboxylate transporters is of great significance since the uptake of these nutrients across cellular membranes is essential for the cell metabolism. In recent years these transporters have also been a focus of attention due to their important role in the production of organic acids with biotechnological interest by microorganisms. It is known that these transporters are involved in the export of acids, decreasing their cell toxicity and improving the productivity [1, 2].

In *Saccharomyces cerevisiae,* acetate and lactate can be efficiently utilised as sole carbon and energy sources, requiring mediated transport systems to cross the plasma membrane. In this yeast species, activity for at least two monocarboxylate proton symporters have been found [3, 4]: one is encoded by *JEN1* [5], and the second by *ADY2* [6]. Jen1p is capable of binding and transporting lactate, pyruvate, acetate, and propionate [5]. As for the Ady2p permease, it is involved in the uptake of acetate, propionate, and formate [6]. More recently, Jen1 gene was also characterized as a selenite transporter [7].

With the increasing number of sequenced genomes, several *JEN1* homolog genes have been identified by homology search in other fungi. Until now, few are functionally characterized, namely, in the yeasts *Candida albicans, Kluyveromyces lactis*, *Yarrowia lipolytica* and also in filamentous fungi, *Metharizyum anisopliae, Beuvaria bassiana* and *Aspergillus nidullans,* although in these last three species, the involvement of these genes in the carboxylate uptake was not determined [8-12]. In *K. lactis* two *JEN1* homolog genes were identified, one encoding a monocarboxylate transporter (*JEN1*) that transports lactate and pyruvate, and the other a dicarboxylate transporter (*JEN2*) that transports malate and succinate [10, 13]. A similar situation is described in *C. albicans* where Jen1 and Jen2 transporters also have different specificities: Jen1 transports short-chain monocarboxylic acids such as lactate, whereas Jen2 transports short-chain dicarboxylic acids such as succinate and malate [11, 14].

Two recent publications describe the characterization of six *JEN1* homologous genes of *Yarrowia lipolytica*. Dulermo and co-workers propose that these genes form a new Jen3 subfamily of transporters for fumarate, malate, and succinate, not having the ability to transport monocarboxylic acids [15]. In the second work, using a different methodology, the authors find different substrate specificities for the same genes, including mono, di, and tricarboxylic acids [16]. However, both works fail to measure substrate uptake kinetics, and thus further studies are still necessary to fully characterize this group of genes.

In the yeast *Debaryomyces hansenii*, four *ScJEN1* genes homologues are described in Genolevures (http://genolevures.org/). According to this comparative genomics project, *D. hansenii* seems to have the highest coding capacity among the 18 species of hemiascomycete yeasts analysed and is also the yeast with the most redundant genome, with an overall redundancy of 49.2%. *D. hansenii* is well known for being a highly osmotolerant and halotolerant yeast that normally inhabits hyper-saline environments such as seawater and concentrated brines [17]. Although this yeast is capable of growing in mono-, di and tricarboxylic acids as the only carbon and energy source the carboxylic acid transporters have not been characterized yet. Thus the purpose of this study was to uncover the functional role of *D. hansenii JEN1* homologues. Therefore, the four genes *DEHA2D18920p*, *DEHA2E24024p*, *DEHA2F17402p*, *DEHA2F27126p* were heterologously expressed, under the control of a *GPD* constitutive promoter, in a *S. cerevisiae jen1*Δ *ady2*Δ strain, that presents no activity for plasma membrane carboxylate permeases.

Materials and methods

Yeast strains, plasmids and growth conditions

The yeast strains and the plasmids used in this work are listed respectively in Tables 1 and 2. The *S. cerevisiae* strain W303-1A *jen1*Δ *ady2*Δ, lacking monocarboxylate uptake capacity, was used to express *ScJEN1* homologues. The cultures were maintained on slants of yeast extract (1%, w/v), peptone (1%, w/v), glucose (2%, w/v) and agar (2%, w/v) or minimal media with the required supplements for growth of the strains with auxotrophies. Yeast cells were grown in yeast nitrogen base (Difco), 0.67%, w/v (YNB medium), supplemented with adequate requirements for prototrophic growth or in yeast extract (1%, w/v), peptone (1%, w/v) (YP medium). Carbon sources were glucose (2%, w/v), acetic acid (0.5%, w/v, pH 5.0), lactic acid (0.5%, v/v, pH 5.0), pyruvic acid (0.5%, w/v, pH 5.0), succinic acid (1%, w/v, pH 5.0), malic acid (1% w/v, pH 5.0). Growth was carried out at 30 ºC, both in solid or liquid media. Cultures were always harvested during the exponential phase of growth. YNB glucose-containing media was used for growth of yeast cells under repression conditions. For derepression conditions, glucose-grown cells were centrifuged, washed twice in ice-cold deionised water and cultivated into fresh YNB medium supplemented with the suitable carbon source.

For drop tests, cells were grown on YNB Glu $^{-}$Ura media, until mid-exponential phase and diluted to an OD_{640nm} of 0.1. A set of three 1:10 serial dilutions were performed and 3 µl of each suspension was inoculated in the desired medium, using YNB Glu $^{-}$Ura as a control. Cells were incubated at 18

ºC for 5 days. At 18 ºC carboxylic acid uptake by diffusion is drastically reduced so that growth on carboxylic acid as sole carbon source is directly dependent on a functional transporter [18].

Table 1. Yeast strains used in this work.

Strain	Relevant genotype	Source or Reference
***D. hansenii* CBS767**	Wild-type	
S. cerevisiae* W303-1A *ady2Δjen1Δ	a *ade2 leu2 his3 trp1 ura3*	Soares-Silva et al 2007 [18]
***S. cerevisiae jen1Δady2Δ* p416GPD**	a *ade2 leu2 his3 trp1*	Soares-Silva et al 2007 [18]
***S. cerevisiae jen1Δady2Δ* pDS-I**	a *ade2 leu2 his3 trp1*	Soares-Silva et al 2007 [18]
***S. cerevisiae jen1Δady2Δ* p416-*Dh*17**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-*Dh*18**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-*Dh*24**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-*Dh*27**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-*Dh*17-L501S**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-*Dh*17-GFP**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-*Dh*18-GFP**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-*Dh*24-GFP**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-*Dh*27-GFP**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-Dh27-H292N**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-Dh27-A299Q**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-Dh27-H292N /A299Q**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-Dh27-H292N-GFP**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-Dh27-A299Q-GFP**	a *ade2 leu2 his3 trp1*	(this work)
***S. cerevisiae jen1Δady2Δ* p416-Dh27-H292N/A299Q-GFP**	a *ade2 leu2 his3 trp1*	(this work)

Table 2. Plasmids used in this work.

Plasmids	Characteristics	Source or reference
p416GPD	glyceraldehyde-3-phosphate dehydrogenase promoter	Mumberg, et al 1995 [19]
pDS-I	Sc JEN1 clone under the control of GPD promoter	Soares-Silva et al 2003 [20]
p416-*Dh*17	DEHA2F17402 clone under the control of GPD promoter	this work
p416-*Dh*18	DEHA2D18920 clone under the control of GPD promoter	this work
p416-*Dh*24	DEHA2E24024 clone under the control of GPD promoter	this work
p416-*Dh27*	DEHA2F27126 clone under the control of GPD promoter	this work
p416-*Dh*17-GFP	DEHA2F17402 clone with the GFP gene	this work
p416-*Dh*18-GFP	DEHA2D18920 clone with the GFP gene	this work
p416-*Dh*24-GFP	DEHA2E24024 clone with the GFP gene	this work
p416-*Dh*27-GFP	DEHA2F27126 clone with the GFP gene	this work
pFA6a-GFP6s+KanMx6A	Contains the GFP gene	Wach et al., 1997 [21]
p416-*Dh*17-S501	DEHA2F17402 clone with L501S mutation	this work
p416-Dh27-H292N	DEHA2F27126 clone with H292N mutation	this work
p416-Dh27-A299Q	DEHA2F27126 clone with A299Q mutation	this work
p416-Dh27-H292N/A299Q	DEHA2F27126 clone with H292N/A299Q mutation	this work
p416-Dh27-H292N-GFP	DEHA2F27126 clone with H292 mutation and GFP gene	this work
p416-Dh27-A299Q-GFP	DEHA2F27126 clone with A299Q mutation and GFP gene	this work
p416-Dh27-H292N/A299Q-GFP	DEHA2F27126 clone with H292N/A299Q mutation and GFP gene	this work

Transport assays

Cells incubated under derepression conditions were harvested by centrifugation, washed twice with ice-cold deionized water and resuspended in ice-cold deionized water to a final concentration of about 15-30 mg dry wt. ml $^{-1}$.30 µl of yeast cell suspension were mixed in microtubes with 60 µl of 0.1 M potassium phosphate buffer, pH 5.0. After 2 min of incubation, the reaction was started by the addition of 10 ml of an aqueous solution of the labelled acid at different concentrations and pH 5.0 and stopped by the addition of cold 120 mM non-labelled acid, pH 5.0. A first evaluation of the transport capacity of the different strains was performed with 1mM final concentration of labelled substrates, while transport kinetics characterization assays were accessed in a range from 0.05 to 4 mM final concentrations of labelled substrates. The reaction mixtures were centrifuged for 3 min at 13200 rpm, the pellet was resuspended by vortex in 1 ml of deionized cold water and centrifuged again for 3 min at 13200 rpm. The pellet was finally resuspended in 1 ml of scintillation liquid (Opti-Phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). Radioactivity was measured in a Packard Tri-Carb 2200CA liquid scintillation spectrophotometer with disintegrations per minute correction. The following radioactive labelled substrates were utilised: [1-^{14}C] acetic acid, sodium salt (GE

Healthcare, London, UK); D,L-[U-^{14}C] lactic acid, sodium salt (Perkin Helmer, Massachusetts, USA); [2,3-^{14}C] succinic acid, (Moravek Biochemicals, California, USA); L-[2,3-^{14}C] malic acid (Amersham, New Jersey, USA). Non-specific ^{14}C adsorption to the cells, as well as the diffusion component, was determined by adding labelled acid after ice-cold water. The values estimated represent less than 5% of the total incorporated radioactivity. The transport kinetics best fitting the experimental initial uptake rates and the kinetic parameters were determined by a computer-assisted non-linear regression analysis (GraphPad Software, San Diego, CA, USA). The data shown are mean values of at least three independent experiments, with three replicas of each one.

Cloning strategies of DhJen1 homologs

Different strategies were used to clone the DhJen homologues.

Sequence data for *D. hansenii* were obtained from the Genolevures at http://genolevures.org/ [22]. Using the BLASTp program four ORFs were identified revealing homology to the *JEN1* of *S. cerevisiae*, *DEHA2D18920p*, *DEHA2E24024p*, *DEHA2F17402p*, *DEHA2F27126p*, hereinafter referred to as *DH18*, *DH24*, *DH17* and *DH27* respectively. Genes were amplified from the strain *D. hansenii* CBS767 using the primers listed in Table 3. The chromosomal DNA of *D. hansenii* was obtained as described by Charles S. Hoffman [23].

The genes *DH18* and *DH27* were cloned in the plasmid pGEM®-T Easy vector (Promega, Madison, USA), by amplification with the ACCUZYME™ DNA Polymerase (Bioline, London, UK) with the primers listed in Table 3 using chromosomal DNA. DNA cloning and manipulation were performed according to standard protocols [24]. The *Bam*HI *Hind*III (Fermentas, Vilnius, Lithuania) fragment of these plasmids, containing the *DH18* and *DH27* genes were then cloned in the centromeric vector p416GPD under the control of the Glyceraldehyde-3-phosphate dehydrogenase constitutive promoter) [25].

The gene *DH24* was cloned by gap repair directly in the vector p416GPD as described previously [26], using the primers listed in Table 3, as the strategy used for the genes *DH18* and *DH27* did not result in *E. coli* transformants for *DH24* gene, probably due to the toxicity of this gene in *Escherichia coli*.

The *DH17* gene was chemically synthesized with an optimal codon usage for expression in *S. cerevisiae*, since no expression was obtained with the original gene cloned in the p416GPD vector (data not shown). The synthetic *DH17* gene was codon optimized by OptimumGene software tool (GenScript, Piscataway, NJ, USA Inc.) and ordered to GenScript (GenScript, Piscataway, NJ, USA Inc.). The synthetic version of *DH17* with *Xba*I and *Hind*III (Fermentas, Vilnius, Lithuania) restriction sites was cloned in the p416GPD vector [25].

For GFP fusion proteins, GAP repair technique was performed as described previously [18], using the primers listed in Table 3.

Table 3. Oligonucleotides used for cloning, expression, site-directed mutagenesis, and GFP tagging.

Gene Cloning	
DEHA2F17402_For	CGGGATCCTCTTTGCCGCAAAGTGAGTT
DEHA2F17402_Rev	CCCAAGCTTTCGCTTACTTCCTAATTGGGA
DEHA2D18920_For	CGGGATCCGTGAAGATATGGAACGATCCA
DEHA2D18920_Rev	CCCAAGCTTGATTGAATGCCCACTTGACA
DEHA2F27126_For	CGGGATCCTCTATCCCAAGGCAAAATGA
DEHA2F27126_Rev	CCCAAGCTTTCTGTAGCTTGACGTGAGAAA
Site-directed mutagenesis	
Dh27-H292N_fwd	GTTTTATTTGCGGCGGGAATTAATTTTACGTCCCATGGATC
Dh27-H292N_rev	GATCCATGGGACGTAAAATTAATTCCCGCCGCAAATAAAAC
Dh27-A299Q_fwd	ACGTCCCATGGATCACAAGACTTGTATCCAACTTTC
Dh27-A299Q_rev	GAAAGTTGGATACAAGTCTTGTGATCCATGGGACGT
RT-PCR	
RT_Dh17fwd	TGCTTGTTGTCTTCTTTTCGTC
RT_Dh17rev	AATAAATGCACCGCCCATTA
RT_Dh18fwd	GGGGCTTTCATTTTTGGATA
RT_Dh18rev	TCTTCTGCCAAGAAAGTTTGAA
RT_DH24FWD	ATGGGTGGTATGTTCGGAAA
RT_DH24REV	CAAACCACCTGTGATTGCAC
RT_Dh27fwd	TTTACGTCCCATGGATCAGC
RT_Dh27rev	CCCTGGCTTATTGAGAGGAA
RT_DhACT1fwd	GATTATGAAGTGTGATGTC
RT_DhACT1rev	TTAGAAACACTTATGATGAAC
GFP-fusion	
Dh17-GFP	TCTCCCAACAAAAGTTTGAAGTTTCTCATAAAGACAGTAAAGGAGAAGAACTTTTC
Dh18-GFP	GAAAGTAGAAAATCGTATTCAATAGATAAAATTGAGATAGCTAAAAGTAAAGGAGAAG AACTTTTC
Dh24-GFP	AGAATAACGAAAAGAAGCCAGCAATTATTCACGTAGAAAGTAAAGGAGAAGAACTTTT CACT
Dh27-GFP	GATGTTCTGGTAATTGAAGAAGAGACTATGATTATTCAAAAGCAAAGTAAAGGAGAAG AACTTTTC
GFPrev	GTGAATGTAAGCGTGACATAACTAATTACATGATATCGACAAAGGAAAAGGGGCCTGTT AAACAGATCTATATTACCCTG

Heterologous expression

The heterologous expression of *DH17*, *DH18*, *DH24* and *DH27* was performed in the *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ strain which, under the conditions tested, is unable to use and to transport actively carboxylic acids [18].

Construction of mutations

Gene mutations were constructed in plasmids p416Dh27 and p416Dh27-GFP with oligonucleotide-directed mutagenesis as previously described [18]. The oligonucleotides used are listed in Table 1. Mutations were confirmed by sequencing. The mutant alleles were introduced in a *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ strain, and transformants were selected on complementation of uracil auxotrophy. As a control, the strain was also transformed with the original vector p416GPD.

Epifluorescent microscopy

Samples for fluorescence microscopy were grown in YNB glucose, at 30 °C until mid-exponential growth phase and then shifted under derepressed conditions for expression (YNB acetate 0.5% (v/v); YNB succinate 1% (v/v); YNB malate 1% (v/v pH 5.0) for 4 h [18]. Samples were at this point immobilized on coverslips using one volume of low-melting agarose and then directly observed on a Leica DM5000B epifluorescent microscope with appropriate filters. The resulting images were acquired with a Leica DFC 350FX R2 digital camera using the LAS AF V1.4.1 software.

RNA analysis

Gene expression analysis in *D. hansenii* was performed by Reverse Transcriptase PCR. Cells were grown at 30 ºC in YNB media supplemented with the appropriate carbon source (glucose 2% w/v; glycerol 2% w/v; lactic acid 0.5% w/v, pH 5.0; malic acid 1% w/v, pH 5.0 or citric acid 1% w/v, pH 5.0) with the required supplements for growth of the strains with auxotrophies. At mid-exponential phase, cells were harvested and total RNA was isolated using the SV total RNA Isolation System (Promega), according to manufacturer's instructions. RT-PCR reactions were performed with primers presented in Table 3, based on the sequence of *D. hanseni JEN1* homologues, and as a reference the sequence of *DEHA2D05412* (Actin) [27] using the following conditions: 95 °C 5 min (95 °C 30 sec, 50 °C 30 sec, 72 °C 60 sec) 30 cycles 72 °C 5 min and 4 °C hold.

Percentage of shared amino acids between Jen homologues

The amino acid sequences of Jen1 homologues proteins functionally characterized at the moment were obtained in NCBI database. The Jen1 homologues selected belong to *C. albicans*, *D. hansenii*, *K. lactis*, *S. cerevisiae,* and *Y. lipolytica* species. Multiple sequence alignment was performed with M-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee; [28]) and curation of the alignment with trimAI online version [29]. The percentage of shared aligned amino acids between each Jen1 homologue selected was calculated using the LALIGN tool from Swiss Institute of Bioinformatics (http://embnet.vital-it.ch/software/LALIGN_form.html).

Sequence alignment

Gene sequences for ScJen1 homologues were obtained at NCBI. Reference sequences ScJen1 NP_012705.1, KlJen1 XP_454682.1, KlJen2 XP_455537.1, CaJen1 XP_716108.1, CaJen2 XP_717031.1, Dh17 XP_461118.2, Dh18 XP_459308.2, Dh24 XP_002770539.1, Dh27 XP_461518.2; protein multiple sequence alignment was performed with ClustalW2 [30].

RESULTS

Debaryomyces hansenii characterization

The yeast *D. hansenii* is able to grow in mono-, di- and tri-carboxylic acids as sole carbon and energy source [31]. This yeast has an increased growth on these carbon sources when compared to other yeasts such as *S. cerevisiae* that presents very poor growth on di- and tri-carboxylic acid-containing medium (data not shown). To characterize the *D. hansenii* transport of carboxylic acids, cells were grown on these substrates and the uptake of labelled substrates was measured. *D. hansenii* cells grown on pyruvic or acetic acids were used to measure the initial uptake rates of labelled lactic and acetic acids, respectively. The application of a computer-assisted non-linear regression analysis to the experimental data, agreed with the presence of a mediated transport system for labelled DL-lactic acid (K_m 0.93 ± 0.30 mM; V_{max} 0.55 ± 0.06 nmol s^{-1} mg^{-1}) and acetic acid (K_m 0.75 ± 0.14 mM; V_{max} 0.39 ± 0.02 nmol s^{-1} mg^{-1}) (Figure 1 A and B).

Initial uptakes rates of labelled succinic and malic acids were measured for *D. hansenii* succinic or malic acids grown-cells. In both cases, a Michaelis–Menten kinetics was found, indicating the presence of a mediated transport system (Figure 1 C and D). In succinic acid grown-cells, the estimated kinetic parameters were: K_m = 0.39 ± 0.08 mM succinic acid and V_{max} = 0.26 ± 0.02 nmol succinic acid s^{-1} mg^{-1} dry wt. As for malic acid-grown cells the following parameters were found K_m = 0.61 ± 0.10 mM malic acid and V_{max} = 0.28 ± 0.02 nmol malic acid s^{-1} mg^{-1} dry wt.. Additionally,

citric acid-grown cells were tested but no mediated transport system was detected for this acid (data not shown).

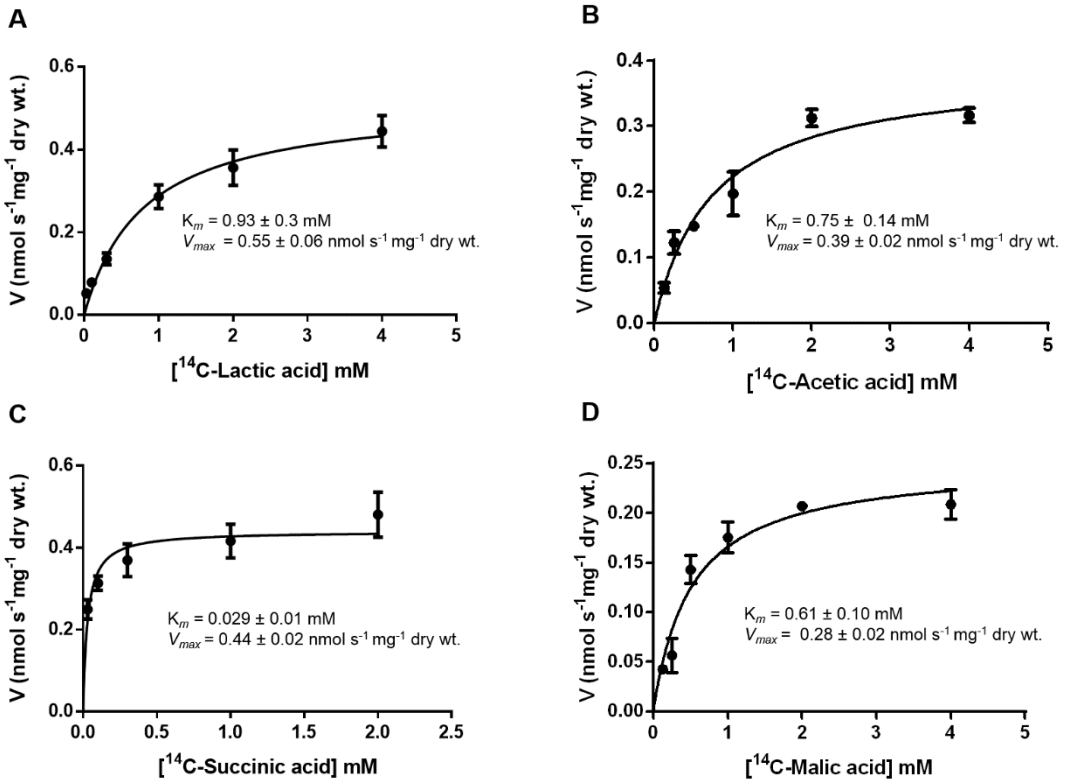

Figure 1. Initial uptake rates of radiolabelled, ^{14}C-lactic acid (A), ^{14}C-acetic acid (B), ^{14}C-succinic acid (C) and ^{14}C-malic acid (D) at different concentration by *D. hansenii* CBS767 at pH 5.0, 30ºC. Cells were cultivated in YNB pyruvic acid, acetic acid, succinic acid and malic acid until mid-exponential growth phase to access the transport of lactate or acetate, succinate, and malate, respectively.

Common BLASTp analysis unveiled four ScJen1 homologues in *D. hansenii*: *DH17*, *DH18*, *DH24*, and *DH27*. The Dh protein most similar to ScJEN1 is Dh17 (44% similarity) although the similarity doesn't differ much between them, ranging from 37 to 44% (Table 4). Two of the Dh proteins, Dh17 and Dh27, show much more similarity (64%) between them than any other homologues, whereas a similarity pattern between Dh17, Dh18, and Dh24 with YlJen protein group is noticeable. Moreover, Dh18 is more than 50% similar with KlJen2 protein and Dh24 displays similarities with KlJen2 and CaJen2.

Table 4. Percentage of shared amino acids between Jen1 characterized homologs of *D. hansenii, S. cerevisiae, C. albicans, and K. lactis* and *Y. lipolytica.*

	Dh17	Dh18	Dh24	Dh27	ScJen1	CaJen1	KlJen1	CaJen2	KlJen2	YlJen1	YlJen2	YlJen3	YlJen4	YlJen5	YlJen6
Dh17	100														
Dh18	46	100													
Dh24	48	54	100												
Dh27	64	43	47	100											
ScJen1	44	37	40	42	100										
CaJen1	55	41	43	54	41	100									
KlJen1	43	37	39	42	65	38	100								
CaJen2	45	49	57	43	38	44	40	100							
KlJen2	45	54	58	42	37	41	36	49	100						
YlJen1	52	62	56	49	40	47	40	50	53	100					
YlJen2	49	56	55	47	41	44	39	47	49	76	100				
YlJen3	50	61	54	47	42	46	39	49	51	78	70	100			
YlJen4	50	58	54	47	43	44	39	50	50	75	64	74	100		
YlJen5	45	51	55	43	37	41	36	44	45	63	54	60	61	100	
YlJen6	50	60	55	49	39	47	39	48	51	71	64	72	68	60	100

Expression of *JEN1* homologues in *D. hansenii* was analysed by RT-PCR. The transcripts of Dh*Jen* homologues were found in all tested carbon sources, namely glucose, glycerol, lactic acid, succinic acid and citric acid (Figure 2). However the four genes presented different expression patterns in the different culture media: a high expression level was found for *DH17* in glycerol and citrate and no expression in succinate, *DH18* had no traceable expression in glycerol, *DH24* was highly expressed in glycerol, and *DH27* displayed low expression levels in lactate.

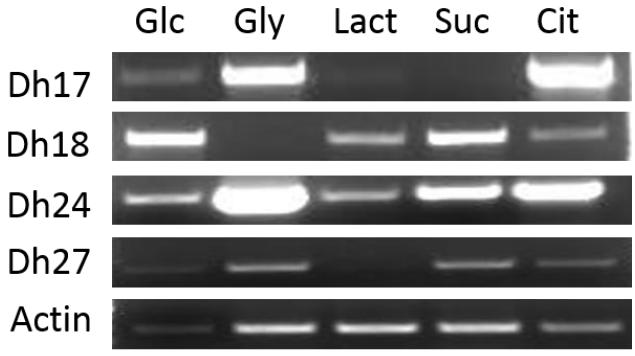

Figure 2. Expression profiles of *DH17*, *DH18*, *DH24* and *DH27* genes of *D. hansenii* CBS767 strain in the presence of glucose (Glu), glycerol (Gly), lactic acid (Lact), succinic acid (Suc) and citric acid (Cit). RT-PCR was performed from cells grown in the presence of the mentioned carbon sources and collected at mid-exponential phase. Actin was used as an endogenous control for all conditions tested.

Functional expression of the *D. hansenii JEN1* gene homologs in *Saccharomyces cerevisiae*

For the evaluation of *D. hansenii* gene function, the strain *S. cerevisiae jen1*Δ *ady2*Δ was used for heterologous expression. This *S. cerevisiae* strain lacks any activity for plasma membrane carboxylate transporter described so far in the literature (Casal et al. 1999, Paiva et al. 2004), and has been successfully utilized before, to characterize the *JEN1* homologues in *K. lactis* [13], *C. albicans* [11, 14] and *Plasmodium falciparum* [32].

Fluorescence microscopy analysis of *S. cerevisiae W303-1A jen1*Δ *ady2*Δ cells expressing *D. hansenii* Jen1 homologues tagged with GFP (Dh17-GFP; Dh18-GFP; DH24-GF; DH27-GFP) as a reporter gene, revealed that the fusion proteins were localized at the plasma membrane (Figure 3).

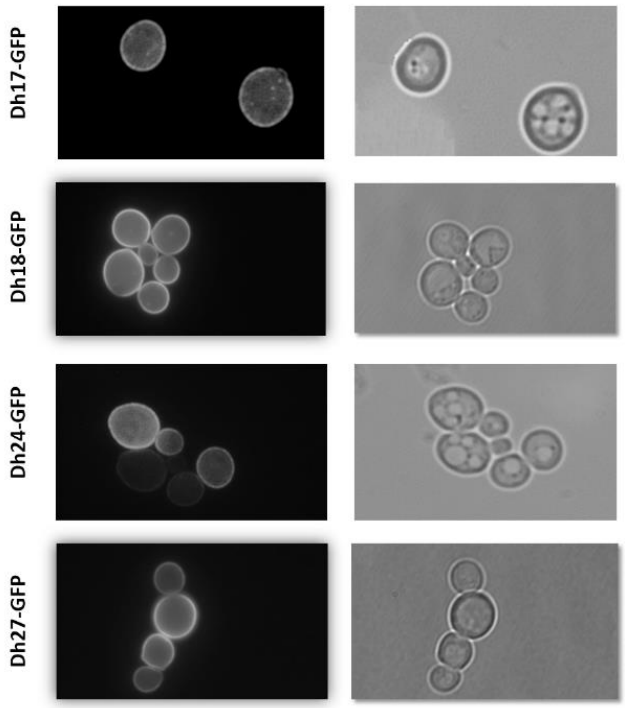

Figure 3. Epifluorescent and contrast phase microscopy of a GFP-tagged version of Dh17, Dh18, Dh24, and D27 expressed in *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ. In all cases, *D. hansenii* Jen1 homologues labelled the plasma membrane.

The growth of control strains and *S. cerevisiae* strains expressing *D. hansenii JEN1* gene homologues were tested in media containing monocarboxylic, dicarboxylic or tricarboxylic acids as sole carbon and energy sources. As seen in Figure 4, a *S. cerevisiae* strain expressing the *DH17* gene was able to grow in malic acid, the strains expressing the genes *DH18* and *DH24* grew in medium with succinic acid and the strain Dh27 in acetic acid. None of the tested strains were able to grow in lactic and

pyruvic acids, or in citric acid as sole carbon and energy sources. These results reinforce the role of these proteins as full functional transporters in *S. cerevisiae*.

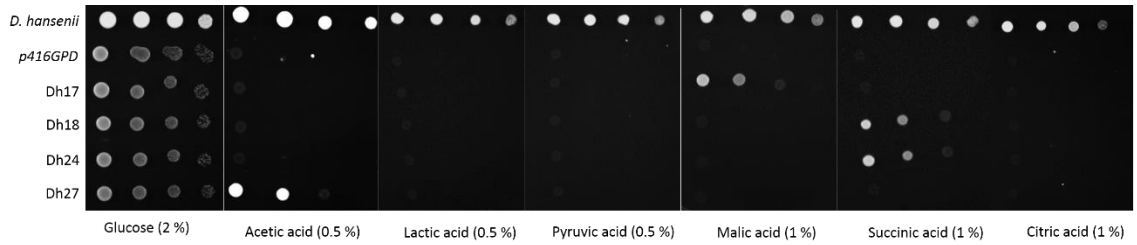

Figure 4. Growth phenotypes of *D. hansenii CBS767* and *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ strains heterologously expressing *DH17*, *DH18*, *DH24* and *DH27* on media containing glucose (2%), acetic acid (0.5 %), lactic acid (0.5%), pyruvic acid (0.5 %), malic acid (1 %), succinic acid (1%) and citric acid (1 %) as sole carbon and energy source. All *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ strains are isogenic expressing from a low copy plasmid. The negative control is a strain carrying the corresponding empty vector. Cells were serially diluted; 3 µl drops of each dilution were spotted onto the plates and grown during 5 days at 18ºC.

Mutation of Dh27 transmembrane segment 7

Since none of the *D. hansenii* genes studied here encoded a lactate transporter we set to analyse these protein sequences to see if the conserved domain localized in TMS7 N379XX[S/T]H383X[S/T]Q386D387XXXT391 [18], and considered as a 'signature' motif of this group of transporters, was also conserved in these proteins. By doing a multiple alignments of the DhJen proteins and the already functionally characterized Jen1 proteins (data not shown) we identified two substitutions in this domain (N379H and Q386A) in the Dh27 protein.

To test if the absence of lactate transport activity could be due to the absence of this conserved motif, we created a Dh27 mutant protein with the signature motif of this transporter family. Two single (H292N and A299Q) and one double mutant (H292N/A299Q) were obtained by site-direct mutagenesis. None of these mutants were able to grow in the medium containing lactic acid, as sole carbon and energy source, suggesting that these mutations were not responsible for the absence of lactic acid transport in the Dh27 gene (Figure 5).

Since the *D. hansenii* is a yeast from the CTG clade, that uses a non-standard genetic code, we analysed the presence of CTG codons in Dh27 gene. We found a sole CTG codon that encodes Ser494 in *D. hansenii* while in *S. cerevisiae* it codes for a leucine. This serine is located at the end of the protein that is 506 amino acids long. The alignment of several Jen1p homologues showed that this serine is not conserved. Thus, the substitution S494L when the DH27 protein is expressed in *S. cerevisiae* is unlikely to be responsible for the absence of lactate uptake activity, especially since

previous studies have reported that amino acids responsible for substrate specificity are located within transmembrane segments [18, 33].

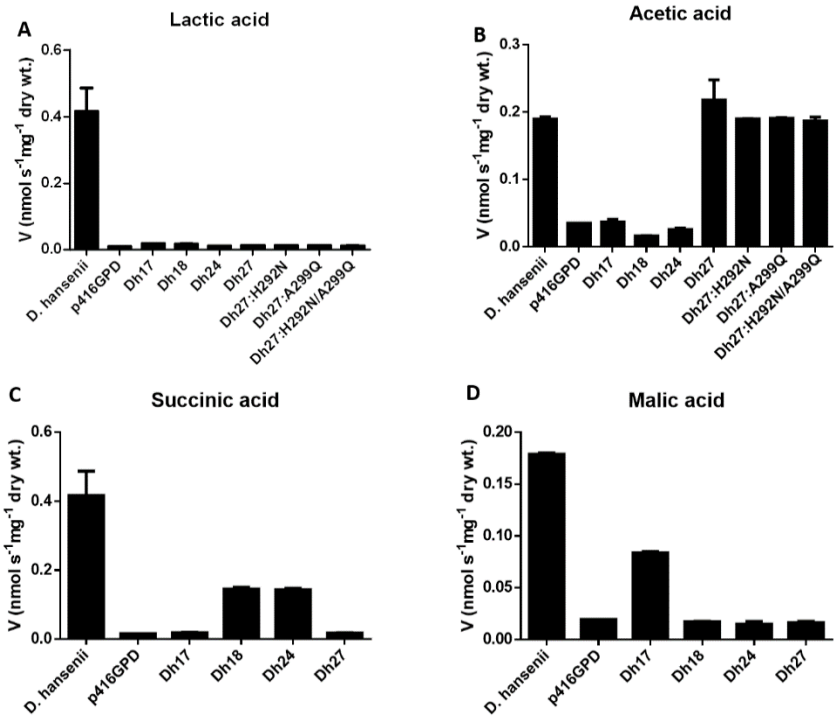

Figure 5. Uptake velocity of radiolabelled lactic acid (A), acetic acid (B), succinic acid (C) and malic acid (D), at 1mM final concentration, by *D. hansenii CBS767* at pH 5.0 and 26ºC, and *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ heterologously expressing *DH17*, *DH18*, *DH24* and *DH27* and its mutants, at pH 5.0 and 30ºC. The *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ-p146GPD was used as a control. Cells were cultivated in glucose until mid-exponential growth phase, washed and transferred during 4 h to YNB supplemented with the carboxylic acid which would be accessed for the uptake.

Functional analysis of Jen1 transporters

The carboxylate uptake displayed by *S. cerevisiae* strains expressing DhJen transporters confirmed the data observed in the growth tests (Figure 5). *DH27 gene* presented an increased ability to transport acetate, *DH17* to transport malate and both *DH24* and *DH18* to transport succinate. Again, we did not observe an increased lactate uptake capacity in any of the tested strains.

Based on these results, kinetic parameters were determined for acetic, succinic and malic acids uptake (pH 5.0) in the strains where an increased transport capacity was observed (Figure 6). The expression of *DH17* gene allowed the cells to transport labelled malic acid by a mediated mechanism (K_m 0.27

± 0.04 mM; V_{max} 0.11 ± 0.01 nmol s^{-1} mg^{-1}). Whereas *DH18* (K_m 0.31 ± 0.06 mM; V_{max} 0.83 ± 0.04 nmol s^{-1} mg^{-1}) and *DH24* (K_m 0.16 ± 0.02 mM; V_{max} 0.19 ± 0.02 nmol s^{-1} mg^{-1}) enabled the uptake of succinic acid by active transport. At last, *DH27* expression in *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ re-establishes the acetate uptake in this strain with the following kinetic parameters: K_m 0.94 ± 0.17 mM; V_{max} 0.43 ± 0.03 nmol s^{-1} mg^{-1}. In addition, the mutations in the *DH27* TMS 7 conserved domain did not affect the acetate transport properties in *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ cells.

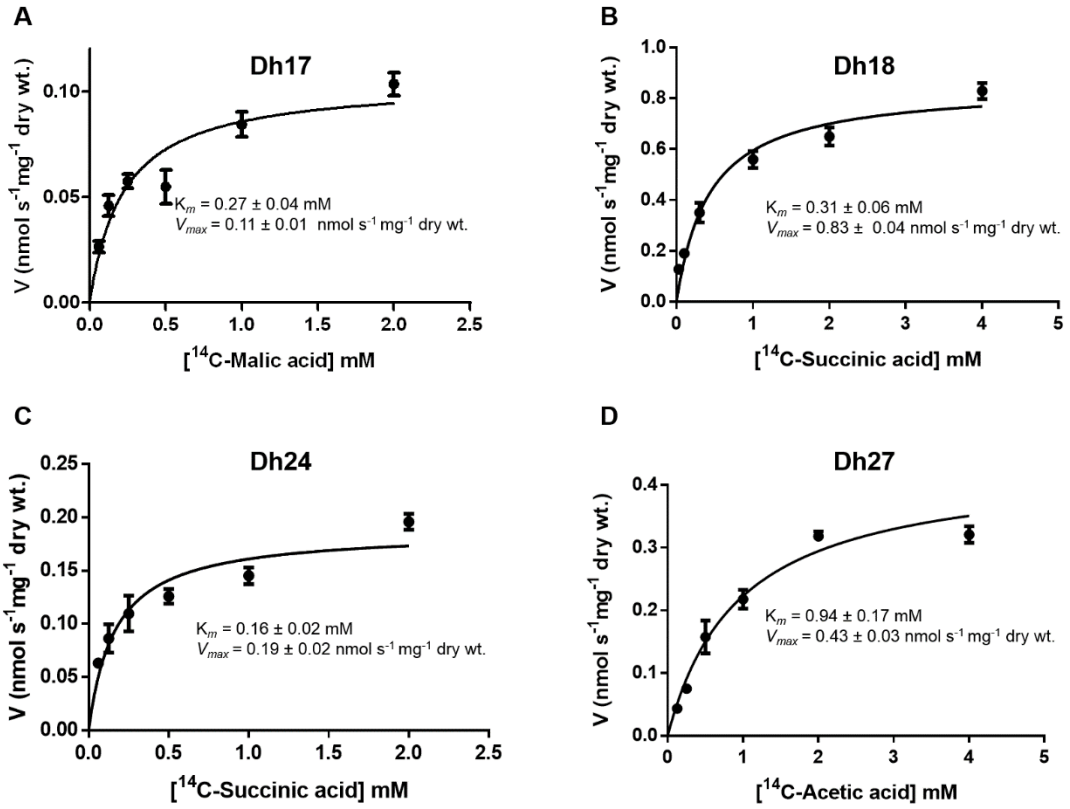

Figure 6. Initial uptake rates of radiolabelled, ^{14}C-acetic acid, ^{14}C-succinic acid, and ^{14}C-malic acid at different concentration by *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ heterologously expressing *DH17*(A), *DH18* (B) or *DH24* (C), and *DH27* (D), at pH 5.0, 30ºC. Cells were cultivated in glucose until mid-exponential growth phase, washed and transferred during 4 h to YNB supplement with the carboxylic acid, which would be accessed for uptake.

Discussion

In this work, we functionally characterized the ScJen1 homologues of *D. hansenii* by heterologous expression in a *S. cerevisiae* strain with no carboxylic acid transporters. Four *ScJen1* homologous genes were identified in *D. hansenii*. In a first attempt to characterize these genes, a phylogenetic study was carried out demonstrating that two of the genes belonged to the Jen1 cluster (Dh17 and 27)

and the other two to the Jen2 cluster (Dh18 and Dh24) [34]. An initial characterization of the *D. hansenii* carboxylate uptake system revealed the existence of mediated transport systems for the uptake of monocarboxylates, lactate and acetate, and dicarboxylates, succinate, and malate. The kinetic parameters obtained were similar to the ones found by our group in other yeasts [3, 4, 11, 13, 14].

The expression of the DhJen genes was detected in all the carbon sources tested (glucose, glycerol, lactic acid, succinic acid and citric acid), although different expression levels were found. Contrarily to what is found in *S. cerevisiae*, all four DhJen genes are not subjected to glucose repression. However, this behaviour is similar to what is found for the ScJen1 homologues in *Yarrowia lipolytica*, where expression in glucose was also observed in three of the six genes [15], and two of them were not expressed in any of the carbon sources tested.

In this work, we demonstrate that the four *D. hansenii* ScJen1 homologues are functional carboxylate transporters in *S. cerevisiae jen1*Δ *ady2*Δ strain, an expression system that has been widely used by our group [18, 33] and others [32]. This approach revealed that *D. hansenii* JEN genes encode four carboxylate transporters: an acetate, a malate and two succinate transporters, these last two with different kinetic parameters. Surprisingly, no lactate transporter was found in *D. hansenii* Jen1 homologues, although this yeast presents a mediated transport system for this acid. As previously mentioned, in other yeasts, lactate transporters are encoded by Jen1 homologs [5, 10, 11, 13-15]. An acetate transporter was the sole monocarboxylate transporter here identified with the particularity of not having the signature motif of the lactate/pyruvate:H^+ symporter subfamily, widely conserved in Jen homologues [18]. Since in *S. cerevisiae* the *ADY2* gene is also capable of mediating the uptake of lactate [1], it is possible that in *D. hansenii* Jen genes have lost the ability to transport lactate and the lactate transporter is encoded by an *ADY2* homolog. Note that in this yeast there are three *ADY2* homologues (DEHA2C07810g, DEHA2E24222g, and DEHA2G03564g). Additionally, no citrate transporter was found although these genes were expressed in citric acid-grown cells.

The existence of two succinate transporters is also noteworthy. The expression of both transporters was detected in different several carbon sources and also on succinate. Despite this, the kinetic parameters measured in *D. hansenii* succinate-grown cells are consistent with the activity of only one transporter. To understand the activity of these transporters we tested the kinetic parameters of succinate uptake in *D. hansenii* cells grown in glucose, lactic acid and citric acid (data not shown). In none of the situations, the kinetic parameters obtained are in agreement with the activity of two transport systems. Additionally, the measured kinetic parameters for succinate were close to the ones presented by the *DH18* expressed in *S. cerevisiae* presenting a K_m near 0.31. The kinetic parameters were the following: glucose-grown cells K_m 0.36 ± 0.09 mM and V_{max} 0.18 ± 0.01 nmol s^{-1} mg^{-1} ,

lactic acid-grown cells K_m 0.33 ± 0.06 mM and V_{max} 0.14 ± 0.01 nmol s^{-1} mg^{-1}, and citric acid-grown cells K_m 0.37 ± 0.12 mM and V_{max} 0.21 ± 0.02 nmol s^{-1} mg^{-1}. These results suggest that the activity of the *DH18* transporter is the one detected under the experimental conditions tested. This could be explained by the existence of different expression patterns since *DH18* has a higher transport capacity but lower affinity than *DH24*. A recent phylogenetic analysis, by Dulermo and co-workers [15], showed that these two genes are in two different clusters: *DH24* belongs to Jen2 cluster and *DH18* in Jen3 cluster, which is a new phylogenetic group described by the authors that contain all the *Y. lipolytica* genes. Possibly the regulation of these two genes is the evolutionary justification for the existence of two succinate transporters, however further studies are needed to fully characterize the mode of regulation of these transporters. Interestingly, *DH17* gene that was assigned to the Jen1 cluster in both studies [15, 34] is, in fact, a dicarboxylate transporter, and not a monocarboxylate transporter.

Additional studies will be required to identify *D. hansenii* lactate permease encoding gene as it remains unidentified. This work, however, extended the current knowledge on the yeast carboxylate transporters by characterizing four new plasma membrane transporters in *D. hansenii*. These transporters differ from previously identified carboxylate transporters in their substrate profile, since they seem to be highly specific for the uptake of acetate, malate, and succinate. DhJen transporters can have biotechnological interest with impact in the microbial production of organic acids. *D. hansenii* is a high osmotolerant and halotolerant microorganism, which has a wide range of applications in biotechnology, being used in food industry, bioremediation or alternative energy production, among others [35]. One growing field in biotechnology is the production of short chain carboxylic acids. These acids can be used in food, cosmetic or pharmaceutical industry, in the production of biopolymers and other desirable compounds, as building–block chemicals and can even be substitutes for petroleum-derived chemicals. The bioproduction of these compounds is gaining more importance, as the chemical methods used to produce them were proven uncompetitive since the starting materials are often worth more than the final product. The use of yeasts with the characteristics of *D. hansenii* in this biotechnological field can be very attractive, due to its capacity to grow in extreme conditions that are usually harmful to other microorganisms. One of the limiting steps for carboxylates production by yeasts is their export by the cell [36]. The identification of novel carboxylate transporters that can be, according to our previous results [1], manipulated for such efflux, could be of great importance for the use of halotolerant yeasts like *D. hansenii* as carboxylic acid producing organisms.

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Chapter IV

Yeast as a tool to express sugar acid transporters with biotechnological interest

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Chapter IV

Yeast as a tool to express sugar acid transporters with biotechnological interest

Abstract

Sugar acids can be used as platform chemicals to generate primary building blocks of industrially relevant products. Microbial production of these organic compounds at high yields requires the engineering of the enzymatic machinery and the presence of plasma membrane transporters able to export them outside the cells. In this study, several yeast carboxylic acid transporters belonging to the Jen family were screened for the transport of biotechnologically relevant sugar acids, namely gluconic, saccharic, mucic, xylaric and xylonic acid, and functionally characterized in *Saccharomyces cerevisiae*. We show that Jen permeases are capable of transporting most of these sugar acids, although with different specificities. Saccharate is a substrate of the transporters ScJen1-S271Q and KlJen2, gluconate of CaJen2 and KlJen2, and xylarate and mucate of CaJen2. A molecular docking approach of these transporters identified the residues that play a major role in the substrate binding of these sugar acids, namely R188 (ScJen1), R122 (CaJen2) and R127 (KlJen2), all equivalent residues (TMS II). The identification of Jen members as sugar acid transporters can contribute to engineering efficient microbial cell factories with increased sugar acid production, as the ScJen1 is able to promote substrate efflux.

Introduction

About 50% of the most demanded biologically synthetized platform chemicals are organic acids, including sugar acids, such as gluconic, mucic, saccharic and xylonic acid [1, 2]. The range of applications of these acids is very extended, embracing the food, pharmaceutical and chemical industry [1].

The microbial production of carboxylic acids is an environmentally sound alternative to oil-based chemicals [3]. *Escherichia coli* and *Saccharomyces cerevisiae* have been suggested as promising cell factories to produce carboxylic acids, such as lactic, malic, xylonic and succinic acids (see review [4]). Significant titers were obtained with these microbes [5, 6], although a major limiting factor to overcome during the fermentation process is the product toxicity [7-9]. Other organisms such as lactic-acid bacteria have been used for industrial purposes, although they have some well-known drawbacks: they have complex nutritional requirements due to their reduced ability to synthesize B-type vitamins and amino acids; they require costly downstream processing approaches and many bacterial species are unable to grow at low pH [10]. The utilization of acid-tolerant microorganisms, such as the yeast *S. cerevisiae* with a higher predisposition for growing at lower pH and enhanced resistance mechanisms to weak carboxylic acid stress, would reduce the cost for pH titrants [11]. The toxic effect of an acid is intrinsically related to both the anion and proton accumulation in the cytoplasm, which affect the plasma membrane integrity [12-14]. The chemical state of the acid results from the intracellular pH. When the extracellular pH is below the pK_a of the acid, the undissociated/uncharged form of the molecule predominates, crossing the biological membranes by passive diffusion, depending on its lipid solubility and cell membrane permeability. However, when the pH is above the pK_a of the carboxylic acid, the dissociated or anion form predominates, which implies a mediated transport mechanism to accomplish the uptake/export of the molecule (reviewed by [15]). In order to cope with high levels of carboxylic acid production, cell engineering approaches are required to introduce an efficient mechanism to transport the acid out of the cell, avoiding the intracellular accumulation of acid anions, and ultimately increasing the extracellular acid titers [5].

The Jen family, which belongs to the Major Facilitator Superfamily, is associated with plasma membrane transport of carboxylic acids in fungi. The first member of this family to be described was ScJen1 from *S. cerevisiae*, a lactate proton symporter with a K_m of 0.29 mM and a V_{max} of 0.4 nmol^{-1} s^{-1} mg^{-1} dry wt [16, 17]. Besides lactate, ScJen1 also transports acetate, propionate, and pyruvate (reviewed by [18]. The ScJen1 has 12 predicted transmembrane segments (TMS). Through rational mutagenesis of conserved amino acid residues, ScJen1 structural-functional properties have been elucidated. The conserved motif ^{379}NXX[S/T]HX[S/T]QD^{387}, located towards the periplasmic side of the predicted TMS VII, is involved in the substrate translocation pathway [19]. The N379 residue of

this domain along with the N501 (TMS XI) and R188 (TMS II) residues were reported to be irreplaceable for the ScJen1 activity [19, 20]. Both H383 and D387 residues affect the specificity and transport capacity of the symporter. Additionally, the Q386 residue is involved in substrate affinity (Soares-Silva *et al.* 2007) and F270 (TMS V) and Q498 (TMS XI) are critical for substrate specificity, in particular for the differentiation between mono- and dicarboxylates[20].

Other Jen homologues from *Candida albicans* [21, 22], *Kluyveromyces lactis* [23, 24], *Debaryomyces hansenni* [25], *Yarrowia lipolytica* [26, 27] and *Aspergillus nidulans* [28] were also reported to transport mono-, di- and/or tricarboxylates (reviewed in [15, 18]. Ady2 is another carboxylate transporter from *S. cerevisiae* belonging to the acetate transporter family (AceTr) [29, 30]. Pacheco and colleagues (2012) in an attempt to create an improved *S. cerevisiae* strain for the production of lactate, overexpressed the ScJen1 and Ady2 transporters in a strain expressing the l-LDH gene from *Lactobacillus casei*, demonstrating their role as efflux permeases by successfully increasing the extracellular titration of lactate [31]. The reversibility of ScJen1 transport had already been demonstrated in a previous work where the permease activity was reconstituted in membrane vesicles [32]. Depending on the substrate and proton gradients, the ScJen1 was able to promote lactate uptake or efflux.

In this study, we used the model organism *S. cerevisiae jen1*Δ *ady2*Δ [19] expressing distinct Jen homologues to assess their ability to transport gluconic, mucic, saccharic, xylonic and xylaric acid, an attractive C5 diacid despite not having biotechnological production. The identification of sugar acid transporters belonging to Jen family can be a valuable tool for the improvement of engineered yeasts strains tuned for the production of these acids.

Materials and methods

Yeasts species, strains, plasmids and growth conditions

The yeast strains and plasmids used in this work are listed in tables 1 and 2, respectively. The yeast cultures were maintained on slants of yeast extract (1%, w/v), peptone (1%, w/v), glucose (2%, w/v) and agar (2%, w/v) or in yeast nitrogen base (YNB medium, Difco), 0.67%, w/v, supplemented with the adequate requirements for prototrophic growth. Yeast cells were grown in YNB medium. Carbon sources were glucose (2%, w/v), lactic (0.5%, v/v, pH 5.0) or succinic acid (1%, w/v, pH 5.0). Growth in liquid media was performed in Erlenmeyer flasks, with a quintuple capacity of the volume of culture medium utilized. Cultures were always harvested during the exponential phase of growth. Cultures were incubated at proper temperature (as given in tables and legends) in an orbital incubator at 200 rpm. Growth evaluation in liquid medium was performed by OD measurement at 640 nm. For

derepression conditions, glucose-grown cells were centrifuged, washed twice with sterile deionized water and cultivated in fresh YNB medium supplemented with lactic acid or succinic acid for 4 h.

Table 1. Yeast species and strains used in this work.

Strain	Genotype	Source or reference
***S. cerevisiae* W303-1A**	MATα *ade2 leu2 his3 trp1 ura3*	(Thomas and Rothstein, 1989) [33]
S. cerevisiae jen1Δ ady2Δ	W303-1A; *JEN1::KanMX4 ADY2::HphMX4*	(Soares-Silva *et al.* 2007) [19]
***S. cerevisiae jen1Δ ady2Δ* p416GPD**	*jen1*Δ *ady2*Δ transformed with p416GPD	(Soares-Silva *et al*. 2007) [19]
***S. cerevisiae jen1Δ ady2Δ* pScJen1**	*jen1*Δ *ady2*Δ transformed with pScJen1	(Soares-Silva *et al.* 2003) [32]
***S. cerevisiae jen1Δ ady2Δ* pScJen1-S271Q**	*jen1*Δ *ady2*Δ transformed with pScJen1-S271Q	(Soares-Silva *et al*. 2011) [20]
***S. cerevisiae jen1Δ ady2Δ* pScJen1-A272G**	*jen1*Δ *ady2*Δ transformed with pScJen1-A272G	(Soares-Silva *et al*. 2011) [20]
***S. cerevisiae jen1Δ ady2Δ* pScJen1-F270G**	*jen1*Δ *ady2*Δ transformed with pScJen1-F270G	(Soares-Silva *et al*. 2011) [20]
***S. cerevisiae jen1Δ ady2Δ* pScJen1-F270A**	*jen1*Δ *ady2*Δ transformed with pScJen1-F270A	(Soares-Silva *et al*. 2011) [20]
***S. cerevisiae jen1Δ ady2Δ* pScJen1-F270Q/S271Q**	*jen1*Δ *ady2*Δ transformed with pScJen1-F270Q/S271Q	(Soares-Silva *et al*. 2011) [20]
***S. cerevisiae jen1Δ ady2Δ* pScJen1-Q498A**	*jen1*Δ *ady2*Δ transformed with pScJen1-Q498A	(Soares-Silva *et al*. 2011) [20]
***S. cerevisiae jen1Δ ady2Δ* pScJen1-Y284Q**	*jen1*Δ *ady2*Δ transformed with pScJen1-Y284Q	(Soares-Silva *et al*. 2011) [20]
***S. cerevisiae jen1Δ ady2Δ* pScJen1-Y284A**	*jen1*Δ *ady2*Δ transformed with pScJen1-Y284A	(Soares-Silva *et al*. 2011) [20]
***S. cerevisiae jen1Δ ady2Δ* pScJen1-Q386A**	*jen1*Δ *ady2*Δ transformed with pScJen1-Q386A	(Soares-Silva *et al*. 2007) [19]
***S. cerevisiae jen1Δ ady2Δ jen1Δ ady2Δ* pCaJen1**	*jen1*Δ *ady2*Δ transformed with pCaJen1	(Soares-Silva *et al.* 2004) [21]
***S. cerevisiae jen1Δ ady2Δ jen1Δ ady2Δ* pCaJen2**	*jen1*Δ *ady2*Δ transformed with pCaJen2	(Vieira *et al*. 2010) [22]
***S. cerevisiae jen1Δ ady2Δ jen1Δ ady2Δ* pKlJen1**	*jen1*Δ *ady2*Δ transformed with pKlJen1	(Lodi *et al*. 2004) [23]
***S. cerevisiae jen1Δ ady2Δ jen1Δ ady2Δ* pKlJen2**	*jen1*Δ *ady2*Δ transformed with pKlJen2	(Lodi *et al*. 2004) [23]
***Candida albicans* RM1000**	*ura3::imm434/ura3::imm434, his1::hisG/his1::hisG*	(Negredo *et al*. 1997) [34]
Kluyveromyces lactis	Type strain CBS 2359	CBS 2359
Yarrowia lipolytica	Type strain ISA 1718	ISA 1718
Debaryomyces hansenii	Type strain CBS 767	CBS 767

Table 2. Plasmids used in this work.

Plasmids	Characteristics	Source/reference
p416GPD	Glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter	(Mumber et al. 2995) [35]
pScJen1	*ScJen1* cloned in p416 under the control of GPD promoter	(Soares-Silva et al. 2003) [32]
pScJen1-S271Q	*ScJen1*-S271Q cloned in p416 under the control of GPD promoter	(Soares-Silva *et al*. 2011) [20]
pScJen1-A272G	*ScJen1*-A271G cloned in p416 under the control of GPD promoter	(Soares-Silva *et al*. 2011) [20]
pScJen1-F270G	*ScJen1*-F270G cloned in p416 under the control of GPD promoter	(Soares-Silva *et al*. 2011) [20]
pScJen1-F270A	*ScJen1*-F270A cloned in p416 under the control of GPD promoter	(Soares-Silva *et al*. 2011) [20]
pScJen1-F270Q/S271Q	*ScJen1*-F270Q/S271Q cloned in p416 under the control of GPD promoter	(Soares-Silva *et al*. 2011) [20]
pScJen1-Q498A	*ScJen1*-Q498A cloned in p416 under the control of GPD promoter	(Soares-Silva *et al*. 2011) [20]
pScJen1-Y284Q	*ScJen1*-Y284Q cloned in p416 under the control of GPD promoter	(Soares-Silva *et al*. 2011) [20]
pScJen1-Y284A	*ScJen1*-Y284A cloned in p416 under the control of GPD promoter	(Soares-Silva *et al*. 2011) [20]
pScJen1-Q386A	*ScJen1*-Q386A cloned in p416 under the control of GPD promoter	(Soares-Silva *et al*. 2007) [19]
pCaJen1	*CaJen1* clone in p416 under the control of GPD promoter	(Soares-Silva et al. 2004) [21]
pCaJen2	*CaJen2* cloned in p416 under the control of GPD promoter	(Vieira et al. 2010) [22]
pKlJen1	*KlJen1* cloned in p416 under the control of GPD promoter	(Queirós et al. 2007) [24]
pKlJen2	*KlJen2* cloned in p416 under the control of GPD promoter	(Queirós et al. 2007) [24]

Transport assays

For uptake measurements, cells were harvested during exponential phase by centrifugation (5000 rpm, 2 minutes). The samples were washed twice with ice-cold deionized water and resuspended in ice-cold deionized water to a final concentration of about 25–35 mg dry weight/mL. The reaction mixtures were prepared in 1.5 mL microtubes tubes containing 60 µL of KH_2PO_4 (0.1 M, pH 5.0), and 30 µL of the yeast cell suspension. After 2 minutes of incubation at 26ºC, the reaction was started by the addition of 10 µL of a solution of the radiolabelled substrate, at the desired pH and concentration, rapidly mixed by vortexing, and incubated at 26ºC. After one minute, 100 µL of non-labelled substrate at 100 mM was added, quickly mixed by vortexing and chilled on ice, to stop the reaction. The reaction solutions were centrifuged for 5 minutes at 13200 rpm. The supernatant was carefully rejected, the pellet was resuspended in 1 mL of deionized cold water and centrifuged for 5 minutes at 13200 rpm. The resulting pellet was resuspended in 1 mL of scintillation liquid (Opti-Phase HiSafe II; LKB FSA Laboratory Supplies). Non-specific ^{14}C adsorption to the cells, as well as the diffusion component, was determined by adding a 1000-fold concentrated mixture of labelled and unlabelled acid. The values estimated represent less than 5 % of the total incorporated radioactivity. The inhibition assays were carried out by adding simultaneously the labelled and non-labelled substrates. The inhibition constant (K_i) was deduced by the effect of different concentrations of the non-labelled inhibitor in uptake velocities. The K_i values of competitive transport and the capacity to transport the inhibitory are an indirect indication for transport of the inhibitory molecule through the

carrier.

The following radioactive labelled substrates were utilized, D, L-[^{14}C]-lactic acid (Perkin Elmer) and [^{14}C]-succinic acid (Moravek Biochemicals) with a specific activity of 2000 dpm. Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer, with dpm correction. The transport kinetics best fitting the experimental initial uptake rates, as well as statistical analysis (One-Way ANOVA) were determined by a computer-assisted non-linear regression analysis using GraphPad Prism version 6.0 for Windows. The data shown are mean values of at least three independent experiments, with three replicas each.

Three-dimensional structural modelling and molecular docking

To obtain predicted Jen transporter 3D structures, the amino residues sequences were threaded through PDB library using LOMETS (Local Meta-Threading-Server) [36]. LOMETS (on-line web service for protein structure prediction) was used to generate a 3D model by collecting high-scoring target-to-template alignments from 10 locally-installed threading programs (FFAS-3D, HHsearch, MUSTER, Neff-PPAS, pGenTHREADER, PPAS, PRC, PROSPECT2, SP3, and SPARKS-X). The threading folds were considered based on the “Confidence Score”. The "Confidence Score" indicates the confidence of the predicted template which is based on a scoring function that takes into account the Z-score of the template, the confidence of the particular server and the sequence identity between the query and the template [36]. The PipT phosphate transporter (PDB code 4J05A) was the top-ranked template threading identified in LOMETS for ScJen1 and KlJen2, and the GLUT3 glucose transporter (PDB code 4zw9A) was the top-ranked template threading identified in LOMETS for CaJen2.

Molecular docking simulations were performed using AutoDockvina with a Lamarckian genetic algorithm as a scoring function [37]. Chimera [38], a graphical user interface for AutoDock Vina, was used to perform virtual screening. The binding affinities and RMSD (*root-mean-square deviation)* scores for each ligand with nine different poses were determined. Primarily proteins and ligand molecules were prepared and optimized. These results were analysed with Chimera and the 2D and 3D ligand interaction images were rendered in Free Maestro version (Schrödinger Release 2016-1: Maestro, version 10.5, Schrödinger, LLC, New York, NY, 2016). The structure of all carboxylic acids was obtained from the Zinc database [39]. The docking studies were performed with the dissociated form of each carboxylic acid.

Results

Screening ScJen1 alleles able to transport gluconic, mucic, saccharic, xylaric and xylonic acids

The transport of radiolabelled lactic acid was assessed in the presence of gluconic, mucic, saccharic, xylaric and xylonic acid (10 mM) in *S. cerevisiae jen1*Δ *ady2*Δ pScJen1. The presence of non-labelled acids allows the identification of potential inhibitors of lactic acid uptake, being indicative of an interaction with the substrate binding site. Since no inhibition of lactate uptake was detected for the wild-type ScJen1 (data not shown), a collection of ScJen1 transporter alleles was tested. The alleles that possess altered kinetic parameters for carboxylic acid uptake were selected, namely A272G, Y284Q, Y284A, Q386A, Q498A, S271Q, F270G, F270A and F270Q/S271Q (Soares-Silva et. 2007; Soares-Silva *et al.* 2011). The ScJen1-S271Q was the only allele that presented a phenotype, where the uptake of labelled lactate was inhibited by 50% in the presence of non-labelled saccharic acid (Fig. 1A). A further characterization of the transport capacity of saccharate by the S271Q mutant was done by performing inhibitions of lactate uptake in the presence of this substrate at different concentrations. The Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-lactic acid uptake in the absence and presence of saccharic acid (20 and 30 mM) revealed a competitive inhibition profile for the S271Q mutant (Fig. 1B). These results suggest that saccharic acid is a substrate of the ScJen1S271Q transporter. The estimated K_i value of saccharic acid was 21.8 mM (Fig. 1B, inset).

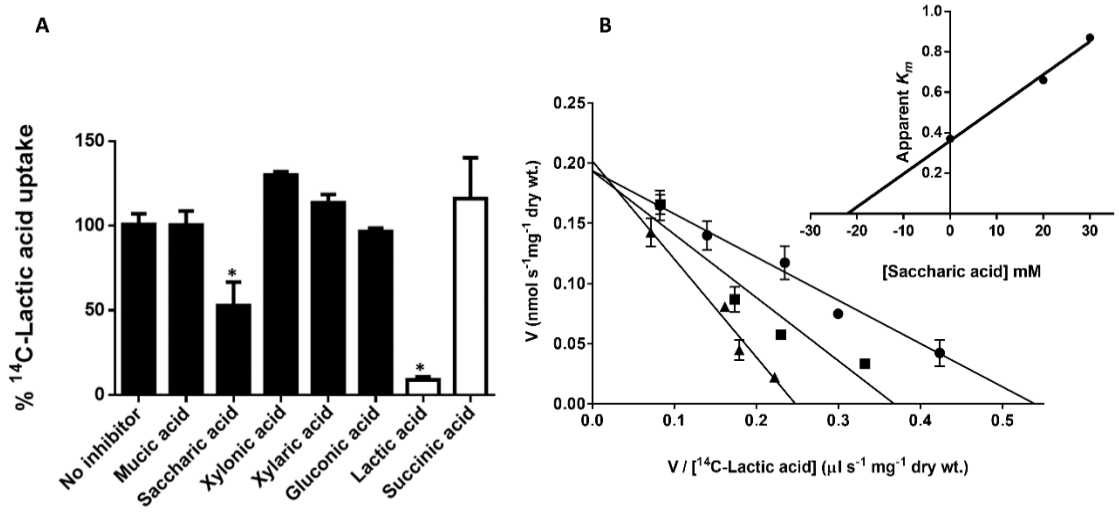

Figure 1. Lactate uptake in the presence of sugar acids in *S. cerevisiae jen1*Δ a*dy2*Δ expressing the ScJen1-S271Q. A) Relative capacity (%) of ^{14}C-lactic acid uptake (60 µM), at pH 5.0, 26ºC, in the absence or presence of non-labelled sugar acids (10 mM). Lactic acid was used as positive control and succinic acid as a negative control. B) Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-lactic acid as a function of the acid concentration at pH 5.0, 26ºC, in the absence (●) and in the presence of saccharic acid 20 mM (■) and 30 mM (▲). Inset: the apparent K_m of labelled lactic acid uptake plotted against the saccharic acid concentration. The estimated K_i is 21.8 mM for total saccharic acid. The data shown are mean values of at least three independent experiments and the error bars represent the standard deviation. *Significant differences ($p < 0.05$).

Studies of modelling by docking of lactate and saccharate were performed for both ScJen1 and ScJen1-S271Q alleles (Fig. 2). The native ScJen1 structure displays two putative binding sites for lactate (Fig. 2A): one located close to the periplasmic side of the membrane; the other in the central pore closer to the cytoplasm. The docking of saccharate revealed a sole binding site in the ScJen1 structure, close to the periplasmic side of the membrane.

The predicted model of ScJen1-S271Q revealed the existence of two putative binding sites for both saccharate and lactate, suggesting that in this allele the two acids share the same binding sites (Fig. 2A). Taking into account the position of each ligand, as well as their structural overlap, it is noteworthy that the binding sites identified in Scjen1 and ScJen1-S271Q are occupying the same spatial position in the structure of the protein. A close view of the interactions between ligands and protein residues confirmed the previous observation (Fig. 2B). In the ScJen1 first binding site, saccharate interacts with the T382 and Q386 residues and lactate with the Q386 residue by hydrogen bonds. Moreover, in ScJen1 the second binding site exclusive for lactate displayed a hydrogen bond

with the Y497 residue and a salt bridge with the R188 residue. Similarly, in the Scjen1-S271Q first binding site, saccharate interacts with the T382 residue and lactate with the Q386 residue. However, in the second binding site, saccharate interacts by a salt bridge with the R188 residue, which is also involved in lactate binding, as well as with the Y273, Y246 and N379 residues by hydrogens bonds. The residue Y497 is not involved in saccharate binding but is important for lactate binding.

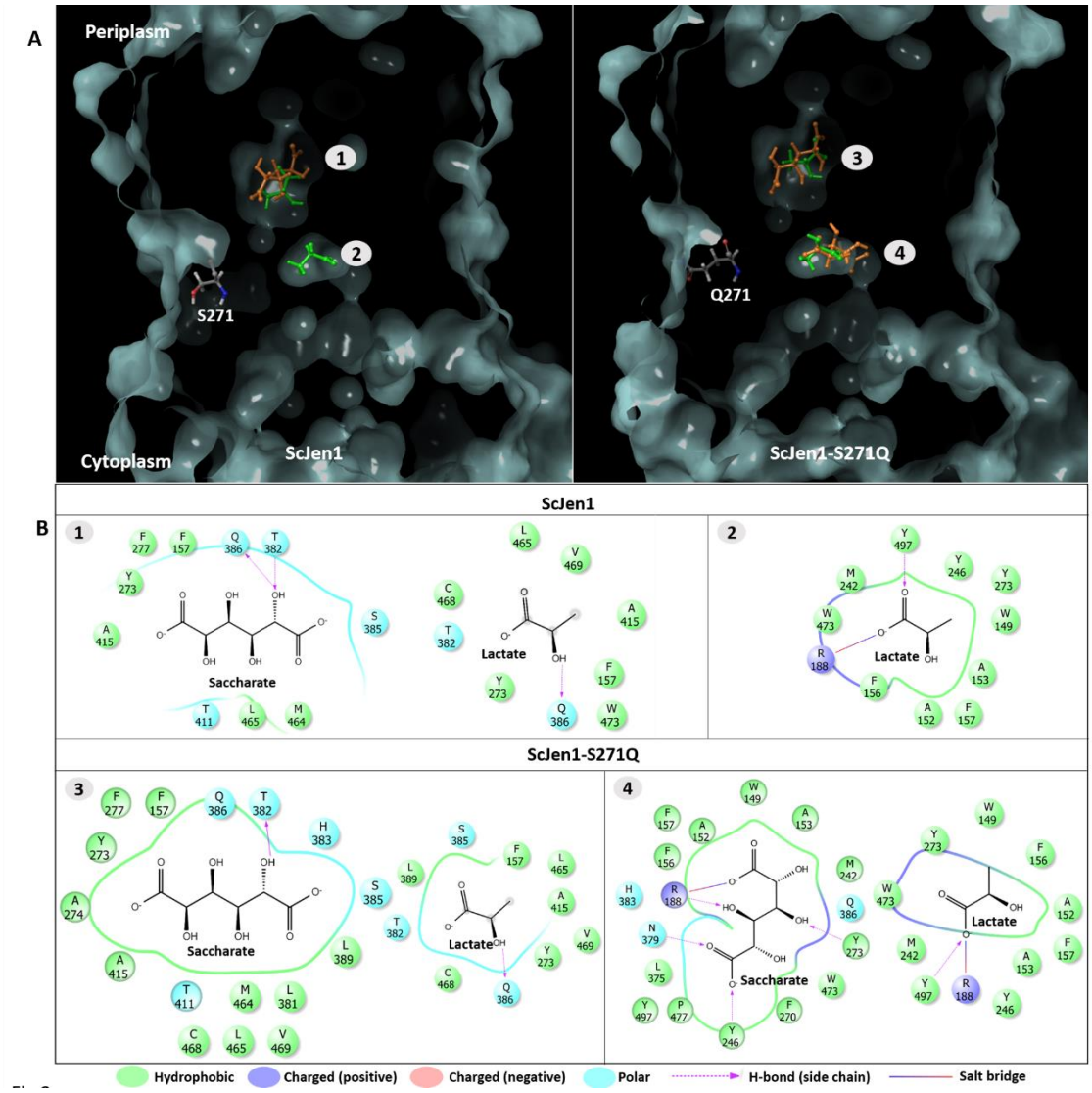

Figure 2. Molecular docking of ScJen1 and ScJen1-S271Q with saccharate (orange) and lactate (green). A) The 3D transversal view of ScJen1 and ScJen1-S271Q protein surface with the identification of the lactate and saccharate binding sites (1, 2, 3, 4) obtained in the docking analysis. The 3D ScJen1 and ScJen1-S271Q structural models used in this study were obtained from the crystal structure of PiPT transporter (PDB 4J05). B) 2D view of the molecular structure of the interaction between the ligands (saccharate and lactate) and the proteins (ScJen1 and ScJen1-S271Q). 1, 2, 3 and 4 are relative to the binding sites identified. The arrows

indicate hydrogen bond interaction between the amino acid residues and functional groups or atoms in the molecular structure of the ligand.

Assessing non-saccharomyces yeast species ability to transport gluconic, mucic, saccharic, xylaric and xylonic acid

Homologous proteins of Jen family, able to transport mono- (Jen1) and dicarboxylic acids (Jen2) were found in the yeasts *Candida albicans* [21, 22], *Kluyveromyces lactis* [23, 24], *Debaryomyces hansenni* [25] and *Yarrowia lipolytica* [26, 27]. The uptake of labelled lactic and succinic acid was measured in the above-mentioned yeasts species, in the presence of gluconic, mucic, saccharic, xylaric and xylonic acid (Fig. 3). In all these species the uptake of labelled lactic acid was not inhibited by the presence of any of the acids tested. Similar results were found for the uptake of labelled succinic acid in *D. hansenii* and *Y. lipolytica*. However, in *C. albicans* and *K. lactis* the uptake of labelled succinic acid was inhibited in the presence of all the above-mentioned acids. In addition, in *D. hansenii cells* lactate and succinate uptake was higher in the presence of xylonic and xylaric acid, as well as mucic, saccharic and xylonic acid, respectively. Whereas in *K. lactis,* the lactate uptake increased in the presence of mucic and xylonic acid.

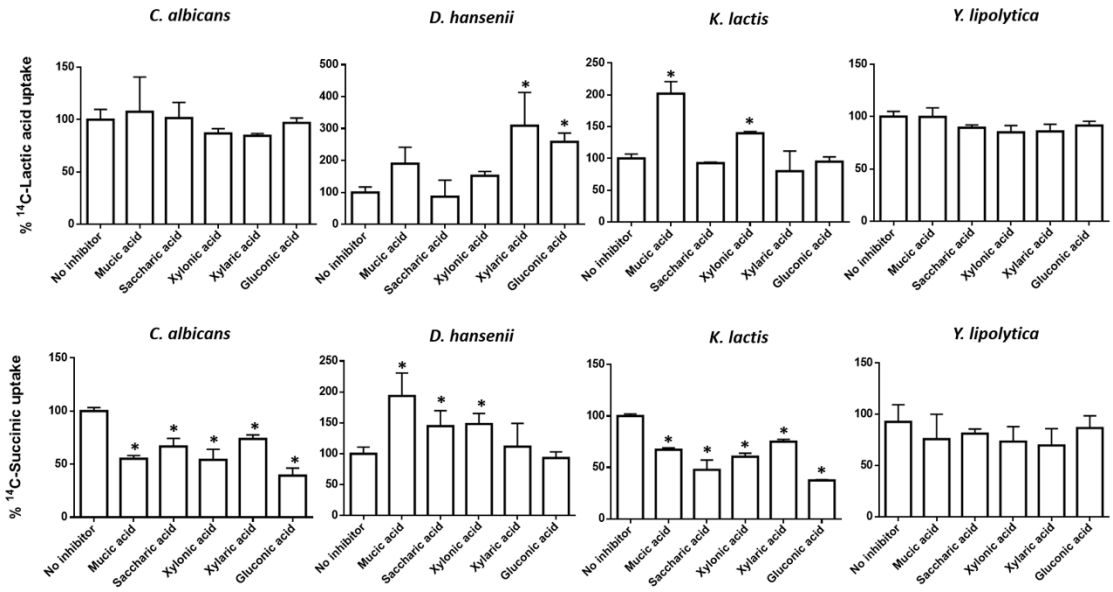

Figure 3. Relative capacity (%) of lactate and succinate uptake in *C. albicans*, *D. hansenii, K. lactis and Y. lipolytica* measured in the absence and presence of non-labelled mucic, saccharic, xylonic, xylaric and gluconic acid (10 mM), pH 5.0 and 26ºC. The data shown are mean values of at least three independent experiments and the error bars represent the standard deviation. *Significant differences ($p \leq 0.05$).

Specificity profiles of CaJen1 and CaJen2 for gluconic, mucic, saccharic, xylaric and xylonic acid

*S. cerevisiae jen1*Δ *ady2*Δ strains expressing the monocarboxylate CaJen1 or the dicarboxylate CaJen2 homologues were used to evaluate the inhibition profile of gluconic, mucic, saccharic, xylaric and xylonic acid (10 mM). None of these acids inhibited the uptake of ^{14}C-lactic acid uptake in cells expressing CaJen1 (Fig. 4A). In cells expressing CaJen2, succinate uptake was inhibited by 50%, 40% and 80% by mucic, xylaric and gluconic acid, respectively (Fig. 4B).

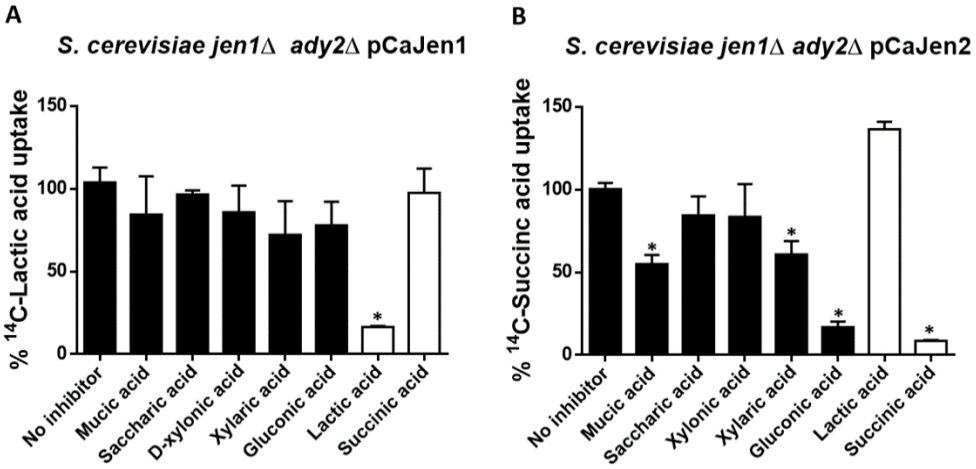

Figure 4. Relative capacity (%) of *S. cerevisiae jen1*Δ *ady2*Δ cells expressing *C. albicans* homologues (CaJen1 and CaJen2) to transport lactate and succinate in the absence and presence of mucic, saccharic, xylonic, xylaric and gluconic acid. **A**) Uptake of ^{14}C-lactic acid (60 μM) in cells transformed with the pCaJen1 plasmid. **B)** Uptake of ^{14}C-succinic acid (20 μM) in cells transformed with pCaJen2. The data shown are mean values of at least two independent experiments and the error bars represent the standard deviation. *Significant differences ($p \leq 0.05$).

Eadie-Hofstee plots of the initial uptake rates of labelled succinic acid in the presence of mucic, xylaric and gluconic acid revealed a competitive inhibition profile for these acids (Fig. 5). The inhibition constants for xylaric acid (K_i 20.2 mM) and gluconic acid (K_i 25.9 mM) were estimated (Fig. 5, insets). Mucic acid acted as an inhibitor when using the highest concentration possible to use in solution (20 mM), impairing the estimation of the K_i for this acid (Fig. 5C).

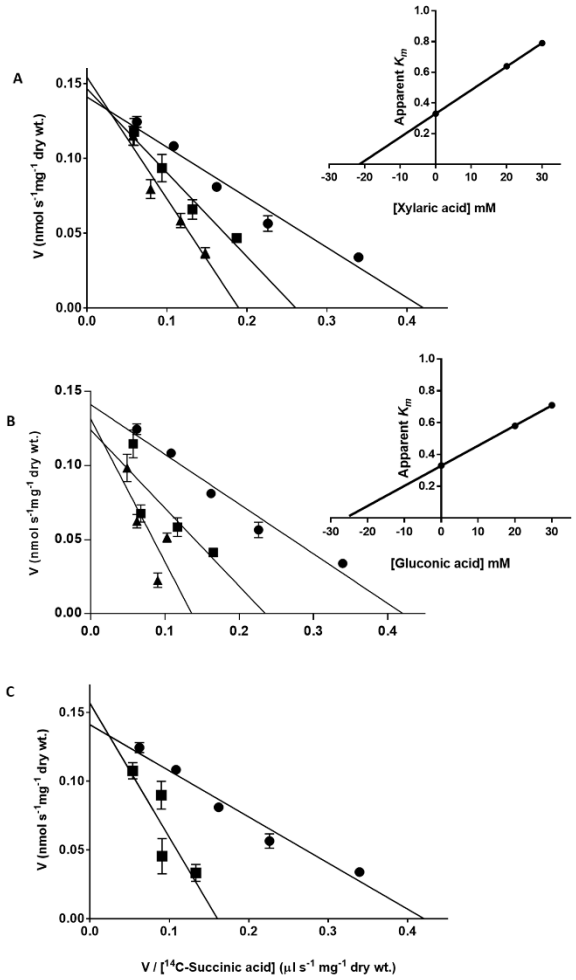

Figure 5. Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-succinic acid as a function of the acid concentration at pH 5.0, 26ºC in *S. cerevisiae jen1Δ ady2*Δ pCaJen2 in the presence of:

A) no inhibitor (●), xylaric acid 20 mM (■) and xylaric acid 30 mM (▲);

B) no inhibitor (●), gluconic acid 20 mM (■) and gluconic acid 30 mM (▲);

C) no inhibitor (●), mucic acid 20 mM (■).

Insets: the apparent K_m of labelled succinic acid uptake was plotted against the concentration of xylaric (A) and gluconic (B) acid. The estimated K_i is 20.2 mM for total xylaric acid and 25.9 mM for total gluconic acid. For mucic acid, it was not possible to estimate the K_i value.

Molecular docking of CaJen2 with malate, succinate, gluconate, mucate or xylarate unveiled one single putative binding site common to all these ligands (Fig. 6A). Moreover, the R122 residue interacts with all the substrates analysed, either by hydrogen bonds or by salt bridges (Fig. 6B). Interestingly, mucate interacted with the same residues (W83, R122, and Q204) as malate and

succinate, whereas gluconate interacted with Q204 like the aforementioned ligands and additionally with Y417. Xylarate only interacted directly with R122.

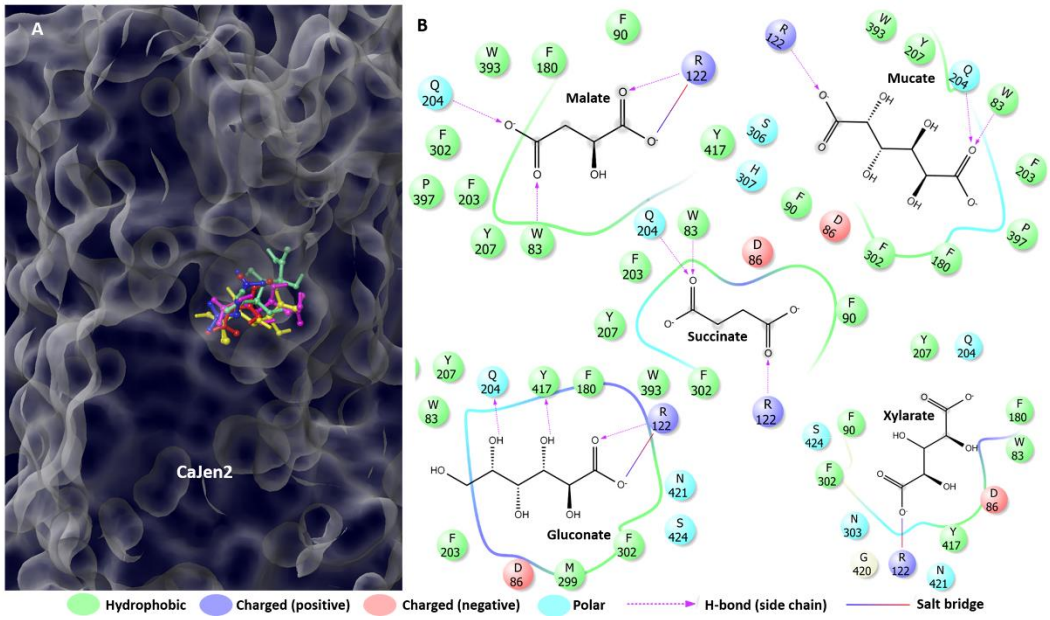

Figure 6. Molecular docking of CaJen2 with malate, succinate, gluconate, mucate, and xylarate. A) Transversal view of CaJen2 protein surface with the identification of the binding sites obtained in docking analysis. Ligand molecules are represented in their stick molecular structure coloured blue (succinate), red (malate), yellow (gluconate), purple (mucate) and green (xylarate). B) 2D view of the interactions between ligands (malate, succinate, gluconate, mucate, and xylarate) and CaJen2 residues. The 3D CaJen2 structural model used in this study was modulated from the crystal structure of the GLUT3 transporter (PDB 4ZW9).

Specificity profiles of KlJen1 and KLJen2 for gluconic, mucic, saccharic, xylaric and xylonic acid

*S. cerevisiae jen1*Δ *ady2*Δ cells expressing the *K. lactis* monocarboxylic KlJen1 and dicarboxylic KlJen2 transporters [23, 24] were used to assess their specificity for gluconic, mucic, saccharic, xylaric and xylonic acids. The uptake of ^{14}C-lactic acid in cells expressing the *KlJen1* was not inhibited by any of the carboxylic acids mentioned above (Fig. 7A). Nonetheless, both saccharic and gluconic acid (10 mM) significantly decreased the uptake of ^{14}C-succinic acid in cells expressing *KlJen2* (Fig. 7B).

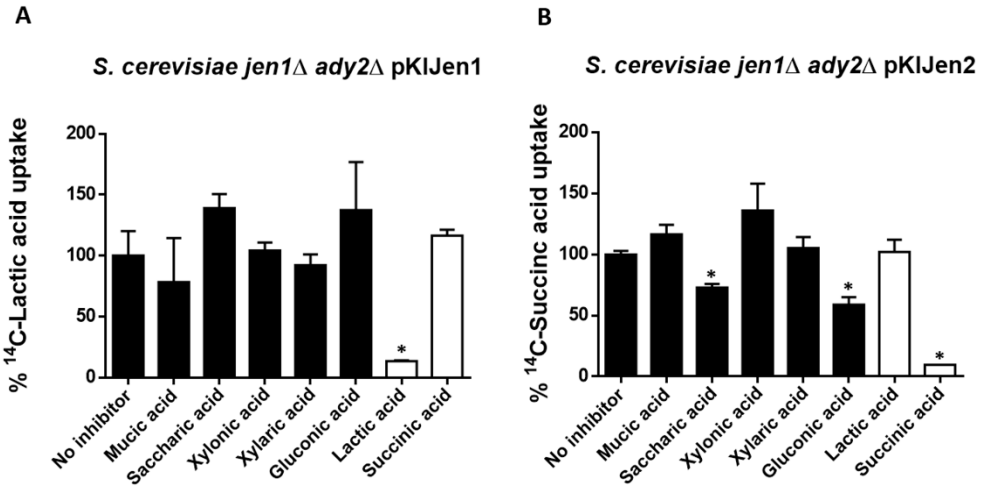

Figure 7. Relative capacity (%) of *S. cerevisiae jen1*Δ *ady2*Δ cells expressing *K. lactis* homologues (KlJen1 and KlJen2) to transport lactate and succinate in the absence and presence of mucic, saccharic, xylonic, xylaric and gluconic acid. **A)** ^{14}C-lactic acid uptake (60 μM) in cells transformed with pKlJen1. **B)** ^{14}C-succinic acid (20 μM) uptake in cells transformed with pKlJen2. The data shown are mean values of at least two independent experiments and the error bars represent the standard deviation. *Significant difference ($p \leq 0.05$).

Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-succinic acid uptake in the presence of different concentrations of saccharic and gluconic acids displayed a competitive inhibition profile (Fig. 8). The K_i value of gluconic acid was 22.7 mM (Fig. 8A, inset) and the K_i of saccharic acid was 24.8 mM (Fig. 8B, inset).

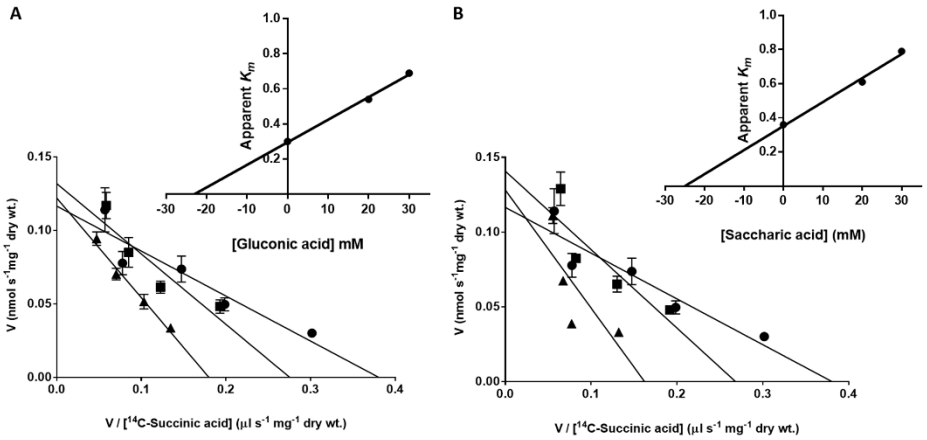

Figure 8. Eadie-Hoffstee plots of the initial uptake rates of ^{14}C-succinic acid as a function of the acid concentration at pH 5.0, 26ºC in *S. cerevisiae jen1*Δ *ady2*Δ pKlJen2 cells in the presence of A) no inhibitor (●), gluconic acid 20 mM (■) and gluconic acid 30 mM (▲); B) no inhibitor (●), saccharic acid 20 mM (■) and saccharic acid 30 mM (▲). Insets: the apparent K_m of labelled succinic acid uptake against (A) gluconic and (B) saccharic acid concentration. The estimated K_i is K_i is 22.7 mM and 24.8 mM for total gluconic and saccharic acid, respectively. The data shown are mean values of at least three independent experiments and the error bars represent the standard deviation.

The docking of malate, succinate, gluconate, or saccharate in KlJen2 allowed to uncover a single putative binding site common to all the ligands analysed (Fig. 9A). The R127 residue is involved in direct interactions with all the carboxylic acids tested, predominantly by salt bridges (Fig. 9B). This residue was the only residue interacting with malate, succinate, and gluconate. Saccharate additionally interacted with H308.

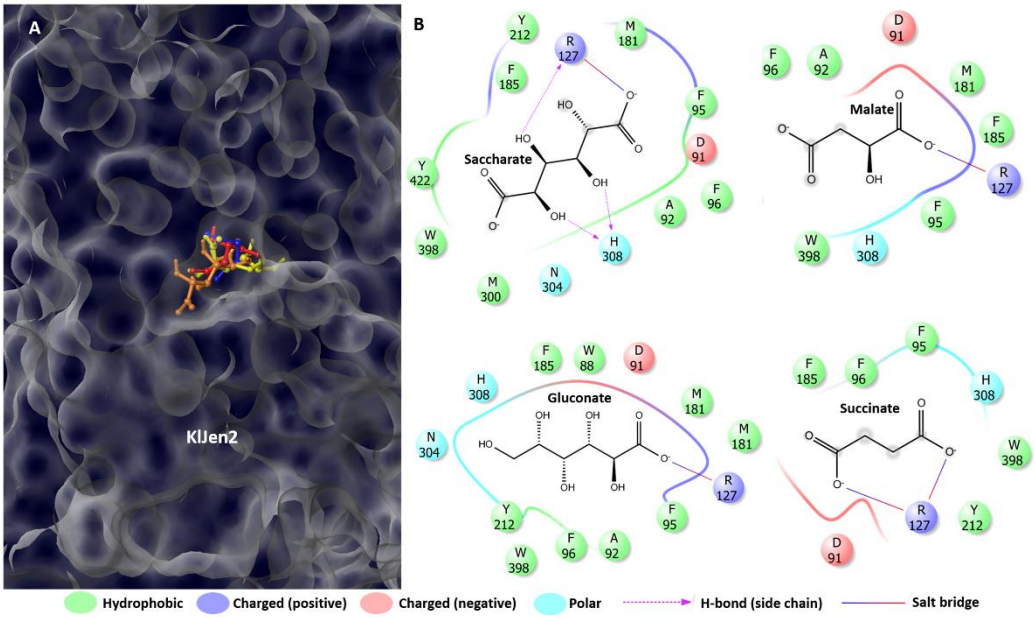

Figure 9. Molecular docking of KlJen2 with malate, succinate, gluconate, and saccharate. A) 3D transversal view of KlJen2 protein surface with the identification of the ligands binding sites: succinate (blue), malate (red), gluconate (yellow) and saccharate (orange). B) 2D view of interactions between ligands (malate, succinate, gluconate, and saccharate) and KlJen2 residues. The 3D KlJen2 structural model used in this study was obtained from the crystal structure of PiPT transporter (PDB 4J05).

Discussion

This study focused on the identification of yeast carboxylate transporters belonging to Jen family with specificity for sugar acids with biotechnological interest. Ultimately these transporters could be used as a tool to improve production titers in engineered strains of *S. cerevisiae* tuned to produce sugar acids. Our results show that saccharate is a substrate of the transporters ScJen1-S271Q and KlJen2, gluconate of CaJen2 and KlJen2, and xylarate and mucate of CaJen2.

The expression system used in this work has been successfully utilized before for the characterization of the Jen homologues of *K. lactis* [24], *C. albicans* [21, 22], *D. hansenii* [25] and *Plasmodium falciparum* [40]. The *S. cerevisiae jen1*Δ *ady2*Δ strain [20], which presents no activity for the uptake of carboxylates, was used to express the Jen permeases from C. *albicans,* and *K. lactis* as well as the ScJen1 mutant alleles.

Herein, we describe that S271Q allele shows a K_i value of 21.8 mM for saccharate. The S271Q allele was previously associated with loss of function for selenite transport, but not for lactate, uncovering the role of this residue in the ScJen1 transporter specificity. The gain of function of ScJen1 enabling the transport of dicarboxylic acids in mutant alleles of ScJen1 was already reported [20]. The F270G,

F270G/S271Q, and Q498A mutants are able to transport the dicarboxylates succinic and malic acid with a K_i of 1.9 mM and 3.4 mM (F270G), 1.9 mM and 28.1 mM (F270G/S271Q) and 29.8 mM and 33.5 mM (Q498A), respectively [20]. Molecular docking analyses in the present study showed that S271Q mutation plays a role in saccharate transport, although it never interacts directly with this substrate. This phenotype could be due to the localization of S271 residue in the vicinity of F270, a critical residue in substrate specificity that has a significant impact on ScJen1 transport characteristics. While ScJen1 allele only displays one putative binding site common to saccharate and lactate in the upper part of the pore, the ScJen1-S271Q mutant has an extra putative binding site for saccharate shared with lactate, right in the middle of the pore. This last binding site comprises interactions between saccharate and the protein, involving two of the most important residues of ScJen1 functionality, the R188 and N379 residues. Previous docking analyses have reported that the R188 residue plays a major dynamic role mediating the orderly relocation of the substrate [20]. This interaction between saccharate and both the R188 and N379 residues, observed exclusively in the S271Q mutant, could explain the reason why lactate uptake was reduced in the presence of saccharate, i.e. saccharate is competing with lactate for the transporter binding site.

It was previously reported that Jen1 family has members in several yeasts, including *C. albicans*, *D. hansenii*, *K. lactis,* and *Y. lipolytica*. In order to identify the possible involvement of Jen transporters in the transport of sugar acids, the uptake of lactate and succinate was tested in these yeasts in the presence of sugar acids. However, only the uptake of radiolabelled succinic acid was inhibited by gluconic, mucic, saccharic, xylaric or xylonic acid in cells of *C. albicans and K. lactis*. Interestingly the uptake of lactate and succinate was higher in *D. hansenii* in the presence of some of the sugar acids tested. Although to a lesser extent, a similar profile was observed in *K. lactis* cells. It is likely that in these yeast species, the presence of these acids triggers a stimulation of lactate and succinate uptake by unknown mechanisms.

The inhibitory effect of sugar acids in the radiolabelled succinic acid uptake was only detected in *S. cerevisiae jen1*Δ *ady2*Δ transformed with the dicarboxylate transporters, CaJen2 and KlJen2. Molecular docking experiments with carboxylic acids shown to inhibit the transport of succinate were carried out. Malate was also included in the docking tests since it is a known substrate for CaJen2 and KlJen2 [21-24]. In both CaJen2 and KlJen2 docking experiments, only one putative binding site common to all carboxylic acids tested was found. In CaJen2, mucate presented a similar binding pattern as the known transported substrates (i.e. malate and succinate, interacting with the same residues (W83, R122, and Q204), whereas gluconate interacted with Q204, R122, and Y417 and xylarate bound exclusively to R122. Interestingly, the Q204 residue is the equivalent of the F270 residue from ScJen1, which was described as critical for substrate specificity [20]. Actually, the

F270Q mutation in ScJen1 increased the range of substrate spectrum and it is likely to be one of the reasons for CaJen2 transporting such a number of carboxylic acids and particularly dicarboxylic acids. On the other hand, in KlJen2 docking tests, gluconate only interacted with the R127 residue in the same way as malate and succinate, whereas saccharate, besides R127 interaction, also interacted with H308. The H308 residue is the equivalent of H383 residue in ScJen1, which was described to be involved in substrate specificity and transport capacity [20]. Similarly to ScJen1, there is a critical residue that seems to play an important role in substrate binding in CaJen2 and KlJen2. This residue is R122 (CaJen2) and R127 (KlJen2), respectively. These residues are the equivalent residues of R188 from ScJen1, known to be irreplaceable for transport activity [20].

Herein we found strong evidence that saccharate is a substrate of the transporters ScJen1-S271Q and KlJen2, gluconate of CaJen2 and KlJen2, and xylarate and mucate of CaJen2. Considering the nature of sugar acids, it is likely that the transport of these molecules is by an active mechanism, similarly to mono- and dicarboxylates [16, 21, 24]. Moreover, we did not find any Jen transporter with specificity for xylonate although succinate transport in the yeasts *C. albicans* and *K. lactis* was inhibited by this acid, suggesting the presence of an alternative transport system for succinate that binds xylonate. According to the transport classification database (TCDB; http://www.tcdb.org) there are few characterized eukaryotic transporters with specificity for the sugar acids here tested. Two gluconate transporters are identified, the Ght3 gluconate:H^+ symporter from *Schizosaccharomyces pombe* and the CIC-5 of *Sus scrofa,* a Cl^-:H^+ antiporter with substrate preference for acetate and gluconate [41, 42]. The *Homo sapiens* CIC5 homologue (CIC-5) is associated with Dent's disease [43]; the SLC26A7 of *H. sapiens* is a chloride/sulfate/oxalate permease or channel, with specificity for saccharate [44]; there are no eukaryotic transporters known for xylonate, xylarate, and mucate, and only one prokaryotic putative mucate:H^+ symporter is reported, the GarP of *E. coli* [45].

The results of this study have high biotechnological potential for the microbial production of sugar acids with added industrial value. The transporter proteins here identified could be (over)expressed in industrial production organisms, where acid accumulation and tolerance is a bottleneck. The ScJen1 permease and other Jen1 homologues transport their substrates by a proton symport mechanism, and similarly to other permeases it is possible to reverse the uptake activity if the substrate gradient and/or proton motive force favours this mechanism [16, 21, 24, 32]. In reconstituted heterologous *P. pastoris* membrane vesicles, it was demonstrated that ScJen1 is able to export lactic acid after a proton gradient change or the addition of another substrate of the permease, like pyruvate, at high concentrations [32]. Considering the role of ScJen1 in lactate efflux [31], it is expectable that the expression of Jen permeases in sugar acid producing strains will increase the flux towards extracellular product accumulation. Mojzita *et al.* (2010) suggested that export of mucate may be

limiting in *Hypocrea jecorina* [46]. Kell *et al*. (2015) argued that transporters are largely undervalued proteins that could greatly contribute to the control of biotechnological fluxes. In addition to gain of functions obtained via random mutagenizes, several studies already demonstrate rational design approaches for improved transporters: beyond the studies of Soares-Silva and colleagues (2008 and 2011) previously pointed here and showing the effect of site-directed mutagenesis in the transport affinity and specificity of ScJen1; MdfA of *E. coli* has been converted from a mono into a divalent cationic drug transporter by inserting an additional acidic residue into the putative recognition pocket [47]; there are also various both rational and site-directed mutagenesis attempts on engineering xylose transport and thus improving xylose fermentation in yeast [48, 49].

Increased knowledge on transporters and their specificity is needed for predictive and quantitative structure-activity relationship models, ultimately leading to design of transporters [50]. The docking studies of the acids performed in this study provide leads on how to further engineer these transporters or their homologues for altered affinity and specificity. In conclusion, this work provides novel understanding on sugar acids transport and is a step forward in developing microbial production of sugar acids with added industrial value.

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Chapter V

The *Cryptococcus neoformans* monocarboxylate transporter Cn04 is responsible for increased 3-bromopyruvate sensitivity

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Personal contribution: I was a co-author of this work. I collaborate in the experimental design of this work, as well as in the determination and evaluation of 3BP uptake assays in *C. neoformans* and *S. cerevisiae* cells, and manuscript writing

Chapter V

The *Cryptococcus neoformans* monocarboxylate transporter Cn04 is responsible for increased 3-bromopyruvate sensitivity

Abstract

In the last decades, 3-bromopyruvate (3BP) has been intensively studied as a promising anticancer and antimicrobial agent. Due to the mechanisms of action of 3BP at the intracellular level, especially in the glycolytic pathway, the transport of this drug inside the cell is a critical step for the toxicity and sensitivity in both, cancer and pathogenic organisms.

The *Cryptococcus neoformans* pathogen is responsible for cryptococcosis in humans, which in most of the cases could be lethal in immunocompromised patients. This pathogenic yeast-like fungus is one of the most sensitive species of microorganisms toward 3BP action. Its cells exhibit the highest uptake rate of this compound among all tested fungal strains.

We functionally characterized the Cn04 protein, a Jen1 homologue of *Cryptococcus neoformans*, and its role in the phenotypic sensitivity to this compound. Gene expression analysis showed that *CNAG_04704* gene, which codes for Cn04, is kept at basal levels when *C. neoformans* cells are treated with 3BP. The deletion of the *CNAG_04704* was found to impair the mediated transport of 3BP and decrease the 3BP sensitivity of *C. neoformans* cells. Further heterologous expression of the Cn04 transporter in the yeast *S. cerevisiae jen1*Δ *ady2*Δ strain restored both, the mediated transport of 3BP and the sensitivity of cells to this carboxylic acid. We also identified a 3BP proton symporter mechanism for Cn04 with a K_m of 0.45 mM for 3BP.

The identification of 3BP transporters in cells of pathogens is of great importance for both understanding the mechanisms of action of this molecule and to anticipate the application of this compound as an antimicrobial drug as well as to identify the best targets to boost 3BP efficacy.

Chapter V

Introduction

3-bromopyruvate (3BP) is a promising anticancer and antimicrobial drug created by the bromination of pyruvate[1-4]. Its properties are mainly due to the inhibitory activity of pivotal glycolytic enzymes which leads to ATP depletion and cell death [5-8]. Moreover, 3BP increases the generation of free radicals and decreases the concentration of the reduced form of glutathione in different cancer and microbial strains[9-16]. 3BP is not a substrate of any drug-efflux transporters belonging to the ATP-binding cassette (ABC) superfamily, which is responsible for the phenotype of resistance to many anticancer and antifungal drugs [17]. In the case of *Saccharomyces cerevisiae* cells, 3BP uptake takes place mainly via the lactate/pyruvate-proton symporter Jen1, which is glucose-repressible [17], whereas in the case of mammalian cells the monocarboxylate transporters (MCTs) are responsible for 3BP uptake [18]. Due to the 'Warburg effect', members of this family of permeases are overexpressed in tumour cells [19]. It is worth noting that the acidification of the extracellular environment of cancer, caused by the removal of excess lactate from tumour cells, increases the 3BP stability [20]. Furthermore, the 3BP uptake by MCT1 transporter is higher when the extracellular milieu has lower than physiological pH[21]. This favours the selective action of 3BP targeted on cancer cells [19]. All proteins identified so far as 3BP transporters belong to the major facilitator superfamily (MFS) [17, 22]. In addition to protein systems, it has also been demonstrated that a small amount of 3BP enters cells by simple diffusion [23].

Among all investigated microbial species, the most sensitive to 3BP was *Cryptococcus neoformans* [4, 14, 24]. *C. neoformans* and *Cryptococcus gattii* are the only two significant human pathogens among 37 described species belonging to the genus *Cryptococcus* [25]. A serious disease caused by their infection is called cryptococcosis. The frequency of its diagnosis is growing year by year. This is related to an increase in the number of patients with immunosuppression, who are most vulnerable to *Cryptococcus* spp. infection [26]. Each year, fungi cause about two million invasive human infections, and nearly half of them are caused by *C. neoformans*. The mortality rate of infections due to this pathogen is up to 60% [27]. Studies have shown two main reasons for high sensitivity of *C. neoformans* to 3BP. One of them is the naturally low level of the reduced form of glutathione, which is a main cellular antioxidant. In addition, it was observed that *C. neoformans* cells exhibit the highest uptake velocities of the compound compared to the other tested strains of microorganisms[4, 14, 24]. The 3BP transport into cells is consistent with a Michaelis-Menten kinetics, which indicates the presence of a permease mediating the transport of this compound [4].

The genome of *C. neoformans* var. *grubii* has a size of about 19 Mb and consists of 14 chromosomes. So far, 6572 genes encoding the proteins have been identified. Notwithstanding, the function of most of them remains unknown [25]. In this study, we conducted a number of studies to identify the

membrane protein responsible for 3BP transport in *C. neoformans* cells. Due to the 3BP chemical structure, we assumed that the uptake of this drug would also take place via the lactate and/or pyruvate transporters as well. Consequently, 3BP uptake and sensitivity were analyzed in three *C. neoformans* mutants with deletions of genes encoding potential carboxylates transporters. Moreover, in this paper, we also describe the heterologous expression of functionally characterized carboxylate transporters from *C. neoformans* in the yeast *S. cerevisiae jen1*Δ *ady2*Δ strain with a deletion of genes encoding carboxylate transporters.

Materials and Methods

Fungal strains and growth conditions

Genotypes and references of fungal strains used and obtained in this study are listed in Table 1. All fungal strains were cultured at 28°C on full YPD medium (2% w/v peptone, 1% w/v yeast extract, pH=5.5) and on synthetic minimal SD medium (0.67% w/v of yeast nitrogen base without amino acids, pH=5.5). Media contained 2% of glucose, sucrose or DL-lactate as a carbon source. Microbiological media were solidified by agar (2% w/v) if needed. In the case of *S. cerevisiae* strains, the media were supplemented with suitable amino acids at the concentration 10 μg/ml.

Table 1. Fungal strains

Strain	Genotype	Reference
***C. neoformans var. grubii* H99**	Wild-type	(Sudarshan *et al.* 1999) [28]
C. neoformans var. grubii* H99 *ΔCNAG_04704	H99 *ΔCNAG_04704*	Madhani Lab
C. neoformans var. grubii* H99 *ΔCNAG_06536	H99 *ΔCNAG_06536*	Madhani Lab
C. neoformans var. grubii* H99 *ΔCNAG_06348	H99 *ΔCNAG_06348*	Madhani Lab
***Saccharomyces cerevisiae* W303-1A**	*MATa ade2 leu2 his3 trp1 ura3*	(Thomas and Rothstein 1989) [29]
S. cerevisiae* W303-1A *jen1Δ ady2Δ	*MATa ade2 leu2 his3 trp1 ura3*	(Soares-Silva *et al.* 2007) [30]
***S. cerevisiae jen1Δ ady2Δ* p416GPD**	*MATa ade2 leu2 his3 trp1*	this work
S. cerevisiae jen1Δ ady2Δ* p416-*JEN1	*MATa ade2 leu2 his3 trp1*	this work
S. cerevisiae jen1Δ ady2Δ* p416-*CNAG_04704	*MATa ade2 leu2 his3 trp1*	this work
***S. cerevisiae jen1Δ ady2Δ* pGREG596**	*MATa ade2 leu2 his3 trp1*	this work
S. cerevisiae jen1Δ ady2Δ* pGREG596 *CNAG_04704	*MATa ade2 leu2 his3 trp1*	this work

Spot test method

The minimal inhibitory concentration (MIC) values of 3BP (Sigma-Aldrich, Germany) were determined according to a spot test method[31]. All tested strains were grown to an exponential phase and next diluted to an OD_{600nm}≈0.2. 3 μl of the suspensions were spotted in 10-fold serial dilutions

onto the minimal SD solid medium containing different concentrations of 3BP and suitable amino acids at the concentration 10 μg/ml when needed. Plates were incubated at 28°C for 48 hours (*S. cerevisiae* strains) or 72 hours (*C. neoformans* strains).

3-bromopyruvate transport assays

The [^{14}C]-labeled 3BP uptake assays in *C. neoformans* and *S. cerevisiae* cells was measured as described earlier [30]. The measurement of radioactivity was performed using a Backman LS100 scintillation counter. The results were converted to dry cell mass. All charts and calculations were made using GraphPad Prism 5.01 program.

Examination of the level of genes expression

Changes in the level of gene expression in the *C. neoformans* genome were investigated using the real-time polymerase chain reaction method, as described previously [14]. The *CNAG_00483* (actin) as housekeeping gene was used for normalization. Outcomes were obtained from three independent experiments. Primers used for real-time PCR assays are listed in Table 2. They were designed based on the sequence of genes deposited in the GenBank database. Their synthesis was performed by Genomed Company, Warsaw.

Table 2. Oligonucleotides used for real-time PCR

Primer	Sequence 5' - 3'
***CNAG_00483*_For**	TCTGGTATGTGCAAGGCTGG
***CNAG_00483*_Rev**	CGTAAGAGTCCTTCTGGCCC
***CNAG_04704*_For**	TTCTCCAACAGGGTTACGCC
***CNAG_04704*_Rev**	TTGGATTCGGGGAGACAAGC
***CNAG_06536*_For**	TGCGTTGTCGATGATTGGGA
***CNAG_06536*_Rev**	GATATGCTACGCCACCGACA
***CNAG_06348*_For**	AGAAGGATGCTCTTGCCCAC
***CNAG_06348*_Rev**	CAGAGGAAGCGGCGATAGAG

Cloning strategy and heterologous expression

The gene *CNAG_04704* was cloned by gap repair directly in the vector p416GPD as described previously [32]. The GFP fusion was also performed by gap repair. Oligonucleotides and plasmids used for gap repair are listed in Table 3 and 4. The heterologous expression of CNAG_04704 was performed in the *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ strain which, under the conditions tested, is unable to use and to transport actively carboxylic acids [30].

Table 3. Oligonucleotides used for genes transformation

Primer	Sequence 5' - 3'
***CNAG_04704*_GAP_For**	GTTTTAAAACACCAGAACTTAGTTTCGACGGATTCTAGCCTTGCTTTCCC CACCTATG
***CNAG_04704*_GAP_Rev**	GATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGCTCTTGAA ACATCAGCATTC
***JEN1*_GAP_For**	TAGTTTTAAAACACCAGAACTTAGTTTCGACGGATTCTAGATGTCGTCG TCAATTACAGAT
***JEN1*_GAP_Rev**	ATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGTTAAACGGT CTCAATATGCT
***CNAG_04704*_GFP_For**	CTTTAACGTCAAGGAGAAAAAACCCCGGATTCTAGAGCGGCCATGTCTA CCTCAAACCTTTC
***CNAG_04704*_GFP_Rev**	GTTTGCGTACTCACTGTACACCCCATACTAGTGCGGCCAGCCATTTCCTT GTGCTGAAC

Table 4. Plasmids used

Name	Features	Reference
p416-GPD	Vector with *URA3* and Amp^R markers, with constitutive promoter for expression: glyceraldehyde-3-phosphate dehydrogenase	Mumberg et al. (1995) [39]
p416-Cn04	*CNAG_04704* clone under the control of GPD promoter	this work
p416-Cn04-GFP	*CNAG_04704* fused with GFP tag	this work

Epifluorescence microscopic observations

The microscopic observation was preceded by the procedure described earlier [30]. Cells were grown until mid-exponential phase in SD medium glucose 2% and then collected for microscopy analysis. Samples were observed on a Leica DM5000B epifluorescent microscope with suitable filters. The images were obtained with a Leica DFC 350FX R2 digital camera using the LAS AF V1.4.1 software.

Sequence alignment and phylogenetic analysis

Protein sequences were obtained at NCBI and the protein multiple sequence alignment and phylogenetic tree were obtained using the web server T-coffee. (T-Coffee: A novel method for fast and accurate multiple sequence alignment[33].

Statistical and *in silico* analysis

Outcomes are presented as the mean ± SD from of at least three irrespective experiments. Statistical significance was estimated by one-way analysis of variance (ANOVA) using GraphPad Prism 5.01, and with Tukey's multiple comparison tests. The minimal level of significance amounted P=0.05.

Results

C. neoformans genes encoding potential 3BP transporters

In silico analysis using an online database (NCBI) allowed the identification of two potential carboxylates transporters of *C. neoformans* var. *grubii* H99 cells, assigned in this manuscript as Cn04 (gene: *CNAG_04704*) and Cn36 (gene: *CNAG_06536*) (Table 5).

Table 5. Putative 3BP transporters analyzed in this work

Gene name	Protein name	Submitted protein name (according to uniprot.org)
CNAG_04704	Cn04	MFS transporter, SHS family, lactate transporter
CNAG_06536	Cn36	Monocarboxylic acid transporter

Multiple sequence alignment (with characterized Jen1 homologous proteins) revealed that Cn04 has exactly the same conserved residues at the functional critical motif "^{379}NXX[S/T]HX[S/T]QDXXXT^{391}" as Jen1 from *S. cerevisiae*, excluding the equivalent residue of tyrosine 382 (the number refers to Jen1) replaced by a serine residue, whereas Cn36 exhibits only one residue (N379) equal to Jen1 and all other conserved residues from the motif are different from the Jen1 motif (Fig. 1).

```
ScJen1          MKRTVQKYWLLFAYL-VVLLVGPNYLTHASQDLLPTMLRAQLGLSKDAVT
CaJen1          ILVTFKTEWLIFSYL-VLLYAGWNFTTHGSQDLYVTMITKQYHVGLDKKT
KlJen1          VKKTVSKYWLLFGYL-ILLLVGPNYLTHASQDLFPTMLRAQLRFSEDAVT
Dh17            ISSVFKTEWLMFVYL-VVLMSGYNFMSHGSQDLYPTLLVKQHNVGPDRKT
Cn04            -GNMFRTNWLRWIWA-VCLMTFFNFFSHGSQDLYPTYLKTTKGLSSSLAS
Cn36            -----KNSVALTGSLIILFVSMGNFIPTVWLPSYA----DDLKLRYLDGT
```

Figure 1. Conserved domain 379NXX[S/T]HX[S/T]QDXXXT391 of the Jen1 group transporters. ScJen1 - *Saccharomyces cerevisiae*; CaJen1 - *Candida albicans*; KlJen1 - *Kluyveromyces lactis*; Dh17 - *Debaryomyces hansenii*; Cn04, Cn36 - *C. neoformans.* Multiple sequence alignment was performed using T-Coffee (http:// ebi.ac.uk/Tools/msa/tcoffee)

Construction of a phylogenetic tree also showed that the Cn04 protein is the most closely related to the Jen1 and Jen2 groups of transporters among all analyzed cryptococcal proteins (Fig. 2).

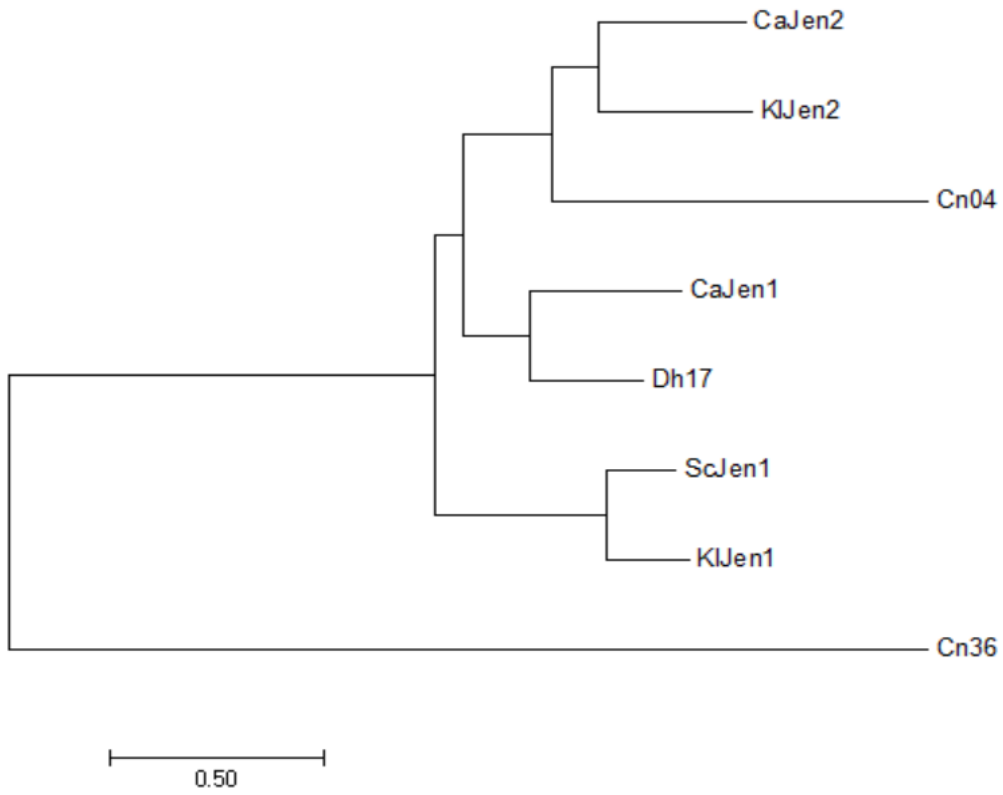

Figure 2. The phylogenetic tree presenting the evolutionary relationships of different carboxylic acids transporters and potential 3BP transporters in *C. neoformans* cells. ScJen1 - *Saccharomyces cerevisiae*; CaJen1, CaJen2 - *Candida albicans*; KlJen1, KlJen2 - *Kluyveromyces lactis*; Dh17 - *Debaryomyces hansenii*; Cn04, Cn36 - *C. neoformans*. Evolutionary analyses were carried out in MEGA7.

Further bioinformatic analysis showed that Cn04 is also characterized by a higher percentage of amino acid similarity to the yeast transporter ScJen1 (30.3%) compared to Cn36 (19.3%) (Table 6).

Table 6. The similarity (in percentage) between the amino acid sequence of analyzed proteins and short-chain carboxylic acid transporters in *Saccharomyces cerevisiae*, *Candida albicans* and *Kluyveromyces lactis* cells (http://xylian.igh.cnrs.fr/bin/align-guess.cgi)

	Cn04	Cn36	ScJen1	ScAdy2	ScQdr2	CaJen1	CaJen2	KlJen1	KlJen2
Cn04	100								
Cn36	21.4	100							
ScJen1	30.3	19.3	100						
ScAdy2	16.0	17.3	15.8	100					
ScQdr2	19.1	18.9	15.5	15.2	100				
CaJen1	31.0	19.1	37.1	15.5	19.9	100			
CaJen2	35.2	20.1	31.4	14.7	20.1	40.6	100		
KlJen1	27.7	19.4	58.3	16.1	18.9	37.2	35.4	100	
KlJen2	39.2	18.9	34.4	16.2	19.7	38.5	48.6	32.5	100

Expression analysis of *CNAG_04704 and CNAG_06536*

Analysis of selected candidate proteins for 3BP mediated transport began with gene expression studies using the real-time PCR method. Changes in the expression level of examined genes under the influence of incubation in the presence or absence of 3BP were determined. It was observed that *CNAG_06536* exhibits significant overexpression compared to the control without the compound. Only the *CNAG_04704* gene showed an expression level comparable to the control. For comparison purposes, the changes in expression level of the *CNAG_06348* gene encoding the ABC transporter were measured (Fig. 3).

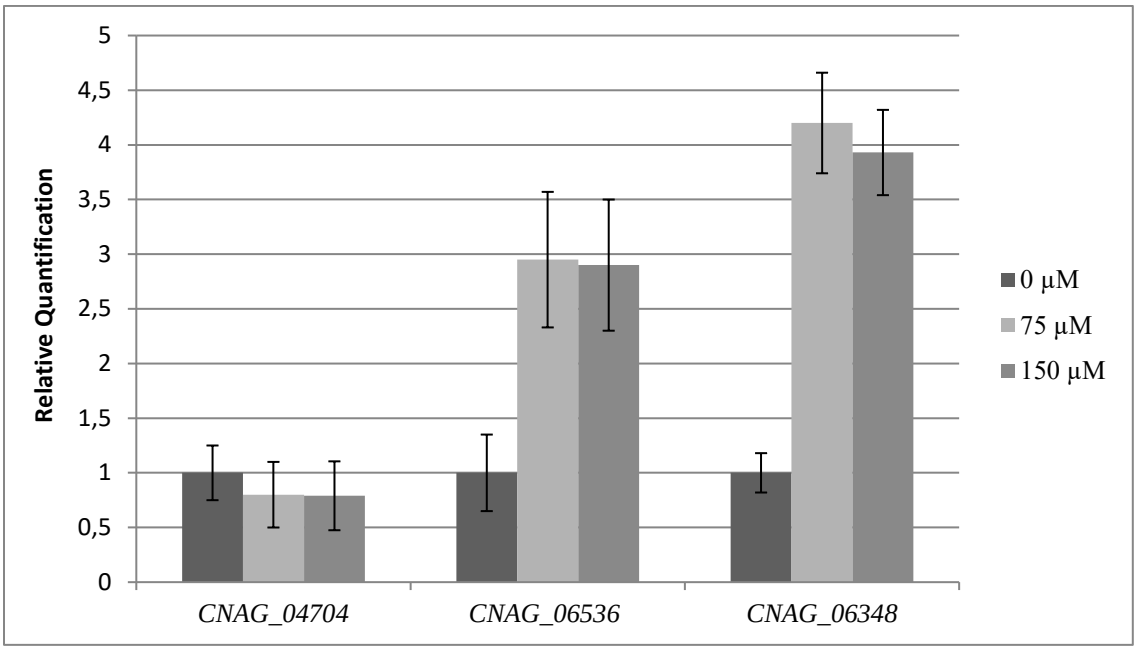

Figure 3. Impact of 3BP on the expression level of genes encoding potential 3BP transporters and ABC pump (Δ*CNAG_06348*) in *C. neoformans* cells

The role of Cn04 and Cn36 in *C. neoformans* sensitivity to 3BP

To analyze the role of *CNAG_04704* and *CNAG_06536* genes, changes in the phenotypic sensitivity to 3BP of *C. neoformans* strains with single deletions for each gene were examined. For control purposes, the MIC values of a strain without the gene encoding ATP-binding cassette transporters (*CNAG_06348*) were also determined. Tests were carried out on SD medium with sucrose and glucose as a carbon source. Differences in the carbon source did not affect outcomes of the experiment. The results show an increase of the resistance in the case of the strain with a deletion of the *CNAG_04704* gene. Compared to the wild-type strain (*C. neoformans* H99), the MIC value of this strain increased from 0.2 mM to 0.225 mM (Table 7).

Table 7. Phenotypic tests toward sensitivity to 3BP of *C. neoformans* H99 strains with deletion of genes encoding different proteins: Δ*CNAG_04704* - Cn04, Δ*CNAG_06536* - Cn36, Δ*CNAG_06538* - Cn38, Δ*CNAG_06348* - ATP-binding cassette transporter. SD medium with sucrose (**A**) and glucose (**B**) as the sole carbon source

A

C. neoformans strain	0 mM	0.175 mM	0.2 mM	0.225 mM	MIC [mM]
C. neoformans H99					0.2
Δ*CNAG_04704*					0.225
Δ*CNAG_06536*					0.2
Δ*CNAG_06348*					0.2

B

C. neoformans strain	0 mM	0.175 mM	0.2 mM	0.225 mM	MIC [mM]
C. neoformans H99					0.2
Δ*CNAG_04704*					0.225
Δ*CNAG_06536*					0.2
Δ*CNAG_06348*					0.2

It was previously demonstrated that *C. neoformans* H99 has a mediated transport of 3BP [4]. Here, we measured the uptake rates of 3BP in *C. neoformans* H99 strains single deleted in *CNAG_04704*, *CNAG_06536* or *CNAG_06348* (Fig 4.).

The kinetic parameters of each strain are shown in Table 8. Excluding the strain *C. neoformans* var. *grubii* H99 *ΔCNAG_04704*, all strains used to measure 3BP initial uptake rates displayed Michaelis-Menten kinetics for this compound, whereas the deletion of *CNAG_04704* in *C. neoformans* cells abolished the mediated transport of 3BP and the plot obtained is representative of a first order kinetics. The strains of *C. neoformans* used in the previous experiment were also grown in minimal media, with lactate as the sole carbon and energy source, although no phenotypic differences were registered between strains (data not shown).

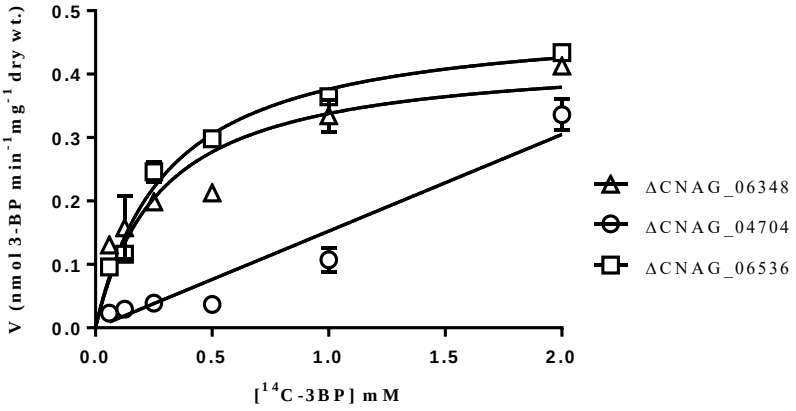

Figure 4. The uptake kinetics of ^{14}C-3BP in the different strains of *C. neoformans*. *C. neoformans* H99 - parental strain, Δ*CNAG_04704* - *C. neoformans* with a deletion in genes encoding Cn04 protein, Δ*CNAG_06536* - Cn36, Δ*CNAG_06348* - ATP-binding cassette transporters. Substrate concentration range: 0-2 mM.

Table 8 Comparison of the estimated kinetic parameters for 3BP in analyzed strains of *C. neoformans*

Strain	K_m[mM]	V_{max} [nmol $s^{-1}mg^{-1}$ dry wt.]
***C. neoformans* H99**	0.62 ± 0.23	0.65 ± 0.10
Δ*CNAG_04704*	(undefined)	(undefined)
Δ*CNAG_06536*	0.30 ± 0.03	0.49 ± 0.01
Δ*CNAG_06348*	0.28 ± 0.06	0.43 ± 0.03

Heterologous expression of Cn04 in *S. cerevisiae*

In order to functionally characterize Cn04 protein regarding its 3BP transport activity, the *C. neoformans* Jen1 homolog was heterologously expressed in the yeast *S. cerevisiae*. For this purpose, *S. cerevisiae jen1*Δ *ady2*Δ cells were used. The strain *S. cerevisiae ady2*Δ *jen1*Δ does not display mediated transport of 3BP and was used previously to characterize several carboxylate transporters [30, 34-36]. The *CNAG_04704* cryptococcal gene encoding a potential 3BP transporter was inserted in the p416GPD vector using the gap-repair method, resulting in the strain *S. cerevisiae ady2*Δ *jen1*Δ p416-*CNAG_04704* (Table 1). For comparative purposes, a *S. cerevisiae ady2*Δ *jen1*Δ strain expressing *JEN1*, which encodes the major 3BP transporter in *S. cerevisiae* cells (ScJen1), or carrying the p416GPD empty vector, was also used. The analysis of phenotypic susceptibility toward 3BP of *S. cerevisiae jen1*Δ *ady2*Δ strain and resulting transformants was carried out. We observed that similarly to the strain expressing *JEN1*, the *S. cerevisiae jen1*Δ *ady2*Δ p416-*CNAG_04704* exhibits

greater sensitivity to 3BP than the *S. cerevisiae* Δ*jen1* Δ*ady2* strain transformed with empty plasmid *S. cerevisiae jen1*Δ *ady2*Δ p416GPD (Table 9).

Table 9. Phenotypic tests toward sensitivity to 3BP of *S. cerevisiae* strains. SD medium with sucrose as the sole carbon source

3BP (mM)	strain				
	S. cerevisiae W303-1A	*S. cerevisiae* W303-1A Δ*jen1* Δ*ady2*	*S. cerevisiae* W303-1A Δ*jen1* Δ*ady2* p416GPD	*S. cerevisiae* W303-1A Δ*jen1* Δ*ady2* p416- *JEN1*	*S. cerevisiae* W303-1A Δ*jen1* Δ*ady2* p416-*CNAG_04704*
0					
1.5					
1.8					
2.0					
2.2					
2.5					
2.8					
MIC	1.8 mM	2.8 mM	2.8 mM	1.8 mM	2.2 mM

The 3BP uptake assays indicated that expression of the cryptococcal *CNAG_04704* gene in *S. cerevisiae jen1*Δ *ady2*Δ cells restores the mediated transport of 3BP, with an affinity constant of approximately 0.45 mM (Fig. 5). In addition, the study of the 3BP transport energetics displayed a proton symporter system, since the presence carbonyl cyanide m-chlorophenyl hydrazine (CCCP), which is responsible for disruption of the proton gradient, significantly inhibited the 3BP transport in yeast cells expressing *CNAG_04704*.

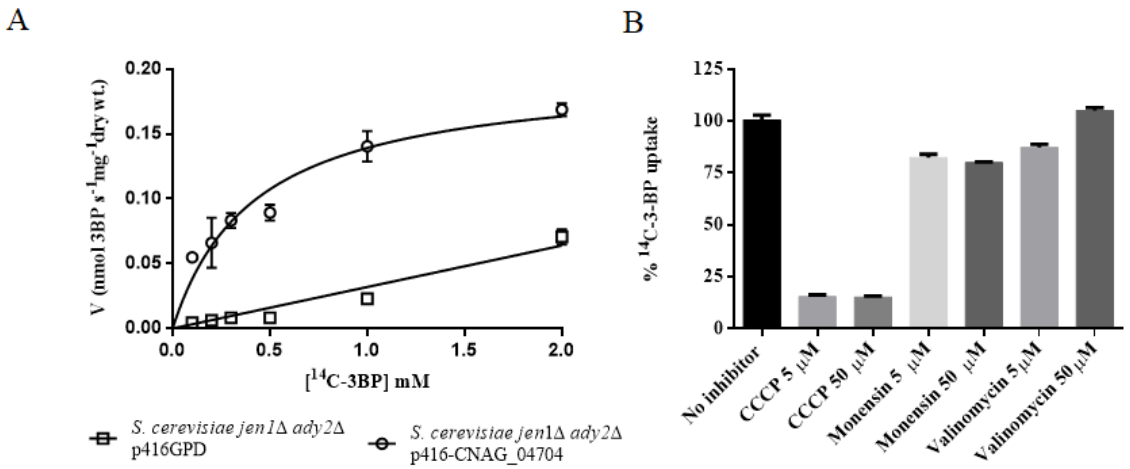

Figure 5. A) The uptake kinetics of ^{14}C-3BP in *S. cerevisiae* strains Δ*jen1* Δady2 transformed by empty plasmid (*S. cerevisiae jen1*Δ *ady2*Δ p416GPD) and by plasmid contains *CNAG_04704* gene (*S. cerevisiae jen1*Δ *ady2*Δ p416-*CNAG_04704*). Substrate concentration range: 0-2 mM. **B)** Initial uptake rates in *S. cerevisiae jen1*Δ *ady2*Δ p416-*CNAG_04704* of radiolabeled 3BP in the presence or absence of different concentrations of energetic inhibitors

To reveal the location of Cn04 expression in *S. cerevisiae*, we add a green fluorescent protein (GFP) tag by gap repair in the p416-CN04 vector at the end of *CNAG_04704* gene, resulting in the p416-CN04-GFP. This last vector was then transformed in the *S. cerevisiae jen1*Δ *ady2*Δ strain and used for fluorescence microscopy analysis. Observation using a fluorescence microscope revealed the Cn04-GFP protein labelled at the plasma membrane (Fig. 6).

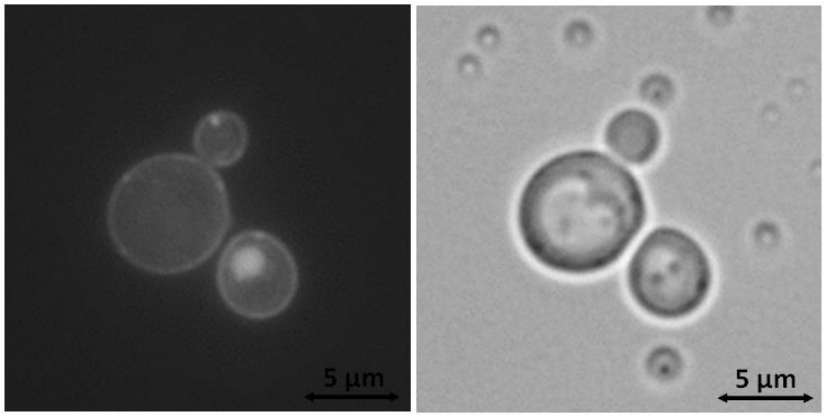

Figure 6. Epifluorescent and contrast phase microscopy of GFP-tagged version of Cn04 expressed in *S. cerevisiae* Δ*jen1* Δ*ady2* labelled the plasma membrane

Discussion

The 3-bromopyruvate is a promising drug, showing strong anticancer and antimicrobial properties. It is a small molecule, which differs from pyruvate in only a single atom. In the case of all tested fungi, the transport of the compound into cells is consistent with the enzymatic Michaelis-Menten curve [4]. This indicates the presence of a mediated transport system. The uptake velocities of 3BP were higher in the case of strains which are more sensitive to 3BP [4]. Among all tested fungal species, the most sensitive was *C. neoformans*, which also exhibits the highest rate of 3BP uptake [4]. There are known transporters of 3BP, inter alia in *S. cerevisiae* and in human cells. In both cases, they are monocarboxylic acid transporters belonging to the major facilitator superfamily (MFS) [17, 22]. Proteins responsible for the transport of 3BP in the *C. neoformans* cells remained unknown until recently. Due to the chemical structure of 3BP, it was highly probable that the transport of this compound would also take place via the pyruvate or lactate transporters.

It was demonstrated that one of two screened proteins, namely Cn04, plays a role as a 3BP transporter in *C. neoformans* cells. In addition, it is also a functional 3BP membrane transporter in the *S. cerevisiae jen1*Δ *ady2*Δ strain. Cn04, like Jen1 and MCTs, belongs to the major facilitator

superfamily of proteins (according to NCBI) [17]. Multiple sequence alignment revealed that Cn04, unlikely Cn36, exhibits high similarity with respect to the conserved motif ^{379}NXX[S/T]HX[S/T]QDXXXT^{391} (the numbers refer to Jen1), known to be critical for Jen1 transport activity, specificity and capacity. The conserved motif ^{379}NXX[S/T]HX[S/T]QDXXXT^{391} predicted to be located at the seven transmembrane segment is reported to be involved in the substrate translocation pathway of Jen1 [30]: the residue N379 is described as irreplaceable for the protein activity; the residues H383 and D387 play a role in transport capacity and specificity, whereas the Q386N substitution diminished the substrate affinity of Jen1. Interestingly, the only conserved residue for which Cn04 differs from Jen1 is the equivalent residue of tyrosine 382, replaced by a serine residue. This residue substitution is common to other Jen1 homologs, although there are no reports about its role in transport activity. Similarly to the alignment results, phylogenetic analysis confirmed that the Cn04 protein is closely related to the transporters belonging to the Jen1 and Jen2 proteins, known to transport carboxylic acids. In contrast, Cn36 proteins are isolated and in a distinct branch.

Significant differences in the expression levels of genes encoding Cn04 and Cn36 under the action of 3BP were observed. Unlike the *CNAG_04704* gene, whose expression was not increased during incubation in the presence of the compound, the *CNAG_06536* gene was significantly over-expressed. This may indicate that the gene *CNAG_04704* is characterized by constitutive expression, as it showed similar expression when 3BP was absent. Another hypothesis could be related to the capacity of Cn04 to transport 3BP, which can lead *C. neoformans* cells to maintain the expression of *CNAG_04704* at basal levels in order to reduce the toxicity of intracellular 3BP. In contrast, expression of other genes is induced by the influence of 3BP. The reaction of the *CNAG_06536* gene was more similar to the reaction of the gene encoding the multidrug efflux pump. The significant increase in expression of the *CNAG_06348* gene, which is a homolog of yeast *PDR5*, is probably a cellular reaction to the presence of the xenobiotic. Therefore, despite the weak functioning of the efflux pump due to the depletion of ATP caused by 3BP action [4, 8, 15], the cell is trying to increase its chances of getting rid of harmful substances. The previous studies carried out on *S. cerevisiae* indicate that 3BP is not a substrate of the efflux pumps involved in the pleiotropic drug resistance (PDR) network which confers resistance to many anticancer and antifungal drugs [17]. However, it should be added that complexes of the reduced form of glutathione with 3BP formed to inactivate the compound are removed from the cell just using ABC pumps [14].

Analysis of the phenotypic sensitivity of *C. neoformans* strains with deletions in different genes demonstrated that only the Δ*CNAG_04704* strain is characterized by higher resistance toward 3BP. Deletion of the *CNAG_06536* gene did not change the sensitivity of cells. Therefore, we can

assume that the protein encoded by this gene may not perform or perform only a marginal function in 3BP transport. This is confirmed by the results of uptake assays, which showed that 3BP transport in cells without Cn36 displayed a Michaelis-Menten kinetics, similarly to what was reported for *C. neoformans* wild-type strain [4], whereas the 3BP transport rate of the cells of the *ΔCNAG_04704* strain is significantly lower than the others strains tested and the mediated transport kinetics is replaced by a first order kinetics. Based on the analysis of obtained outcomes, we can also conclude that in *C. neoformans* cells, unlike in the case of *S. cerevisiae* [17], the 3BP uptake is not repressed by glucose. In addition, as described earlier, 3BP is not a substrate for efflux pumps [17]. Studies using *C. neoformans* mutants without the genes encoding the ABC transporters confirmed the above statement. The deletion of these genes did not alter the sensitivity of the strains to 3BP.

The heterologous expression of the *CNAG_04704* gene in the *S. cerevisiae jen1*Δ *ady2*Δ strain, which lacks the mediated transport of carboxylic acids, restored the capacity for the mediated transport of 3BP, and it consequently became more sensitive to this compound. This procedure allowed identification of a 3BP proton symporter mechanism with a K_m of 0.45 mM. The reported K_m of ScJen1 is approximately 2 mM of 3BP [17]. Meanwhile, Cn04 tagged with GFP showed strong expression of the tested protein, mainly in the cell membrane of *S. cerevisiae jen1*Δ *ady2*Δ. Accordingly, we can infer and define the protein encoded by the *CNAG_04704* gene as a 3BP transporter in *C. neoformans* cells.

In this study, we revealed the role of Jen1 homologs in 3BP transport and sensitivity in the pathogen *C. neoformans*. It is evident that these transporters play a critical role in the mechanisms of action of 3BP, as the first players to ensure the entrance of this drug into cells. These results highlight the role of carboxylate transporters in 3BP uptake already reported in microbial cells, as well as in mammalian cells, and open new treatment approaches in the pharmaceutical field: the assessment and identification of Jen1 homologs in other pathogen species could open new possibilities to use 3BP as an effective antimicrobial agent.

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Chapter V

Chapter VI

SATP (YaaH), a succinate–acetate transporter protein in *Escherichia coli*

Adapted from:

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Personal contribution: I collaborated in the RT-PCR experiments and data analysis, as well as in the review process and answers elaboration to referees after submission.

Note: This chapter was previously reported in the Doctoral thesis of Joana Sá-Pessoa, which is entitled "Structural-functional studies of plasma membrane carboxylate transporters in yeast" and is available in the "Repositorium" of the University of Minho.

Chapter VI

SATP (YaaH), a succinate–acetate transporter protein in *Escherichia coli*

Abstract

In this work, we describe a new carboxylic acid transporter in *Escherichia coli* encoded by the gene y*aaH*. In contrast to what had been described for other YaaH family members, the *E. coli* transporter is highly specific for acetic acid (a monocarboxylate) and for succinic acid (a dicarboxylate), presenting the following affinity constants at pH 6.0, 1.24 ± 0.13 mM acetic acid and 1.18 ± 0.10 mM succinic acid. In glucose grown-cells, the Δ*yaaH* mutant is compromised for the uptake of both labelled acetic and succinic acids. YaaH, together with ActP, previously described as an acetate transporter, affect the use of acetic acid as sole carbon and energy source. Both genes have to be deleted simultaneously to abolish acetate transport. The uptake of acetate and succinate was restored when *yaaH* was expressed in *trans* in Δ*yaaH* Δ*actP* cells. We also demonstrate the critical role of YaaH amino acid residues Leu131 and Ala164 on the enhanced ability to transport lactate. Due to its functional role in acetate and succinate uptake, we propose its assignment as SatP: the Succinate-Acetate Transporter Protein.

Introduction

The acetate switch in *E. coli* has been defined as a dynamic alteration of the cell physiology that occurs when acetate dissimilation equals its assimilation. This happens when cells are transiting from a rapid growth phase, associated to the production and excretion of acetate, to a slower growth phase, supported by the import and utilization of acetate [1]. Acetic acid is a weak carboxylic acid that can dissociate in aqueous solution, and the amount of both anionic and lipophilic form is dependent on the pH of the medium. As the pK_a of acetic acid is 4.76, at neutral pH the acetate ion accounts for more than 99% of the acid form. Thus, acetic acid producing cells must possess plasma membrane transporters to efficiently export acetate in its anion form out of the cell. Although the metabolic pathways associated with acetate dissimilation and assimilation in *E. coli* have been extensively studied (for a review see Wolfe, 2005 [1]), the identification of the plasma membrane transporters responsible for the export and/or import of acetate have not been yet fully elucidated. An acetate permease (*actP*), cotranscribed with acetylCoA synthetase (*acs*) is involved in scavenging micromolar concentrations of acetate from the extracellular medium in *E. coli* [2]. The ActP transporter belongs to the Sodium:Solute Symporter Family (http://www.tcdb.org/) and is highly specific for short-chain aliphatic monocarboxylates, namely acetate, glycolate and propionate. However, Gimenez *et al.* (2003) also claimed that besides ActP, another transporter must exist in *E. coli* since the *actP* deficient mutant strain displays low but not null rates of acetate transport. The identification of such transporter remains to be done and our working hypothesis is based on the assumption that this function should be accomplished by the YaaH protein.

The AceTr (former YaaH) family members are polytopic proteins with a predicted topology arranged in six transmembrane segments, containing a conserved motif (N-P[AV]-P-[LF]-G-L-x-[GSA]-F) located at the first putative transmembrane region of the N-terminus of the protein (http://www.tcdb.org/). Members of the AceTr family are found in archaea, eukaryotes and bacteria, with some members experimentally demonstrated as acetate transporters such as Ady2 in the yeast *Saccharomyces cerevisiae* [3] and AcpA in the filamentous fungus *Aspergillus nidulans* [4]. An *acpA* deletion in *A. nidulans* leads to reduced growth on acetate as a sole carbon source, at low concentration and in a high pH medium, which is fully restored upon reintroduction of the *acpA* gene. This gene is also required for the induction of the acetate assimilation pathways and direct measurement of acetate incorporation into germinating conidia confirmed that AcpA is fundamental for acetate uptake, especially at low substrate concentrations [4]. The *acpA* transcript level increases as the demand for acetate uptake increases and its expression is induced in the presence of several weak monocarboxylic acids such as glyoxylate, propionate, lactate, pyruvate and formate [4].

Acetic acid-grown cells of the yeast *S. cerevisiae* display activity for a monocarboxylate /proton symporter, shared by acetate, propionate and formate, dependent on the ΔpH across the plasma membrane [5], found to be associated to Ady2 expression. *S. cerevisiae ADY2* is subjected to glucose repression and its expression occurs upon a shift from glucose medium to a non-fermentable carbon source, such as acetic acid [3]. The deletion of *ADY2* results in the loss of mediated acetate uptake measured at pH 6.0, implying that this gene is essential for the acetate permease activity in *S. cerevisiae* [3]. The same protein was also hypothesized to be an ammonium exporter [6, 7] since the null mutant displays a reduction in ammonia production when cells are growing in colonies [6], and the appearance of Ady2 at the plasma membrane correlated with ammonia release [7]. However, more recently, two independent teams have found that Ady2 is also implicated in lactic acid uptake [8, 9]. It was demonstrated that the double mutant strain for Ady2 and for the lactate permease Jen1 (*ady2*Δ *jen1*Δ), but with *ADY2* gene expressed in a centromeric plasmid under the control of a strong constitutive promotor, displays a Michaelis–Menten kinetics for initial uptake rates of labelled lactic acid at pH 5.0 [8]. By using a laboratory evolution strategy of a *S. cerevisiae jen1*Δ strain, Kok *et al.* (2012) found two genomic mutants in *Ady2*, Leu219Val and Ala252Gly, with enhanced ability to grow on lactic acid. All these evidence supports a role for Ady2 as a monocarboxylate transporter located at the plasma membrane of yeast cells. Although not directly shown to be functionally implicated in acetate uptake, other YaaH family members have been linked to acetic acid adaptation, such as Gpr1 in the yeast *Yarrowia lipolytica* and MA4008 in the archaea species *Methanosarcina acetivorans*. In *Y. lipolytica GPR1* mRNA expression is enhanced by acetic acid and although its deletion did not impair growth on acetic acid as a carbon source [10], mutations in the C-terminal part of Gpr1 were found to be detrimental for acetic acid sensitivity [11]. In the methanogen archaeobacteria *M. acetivorans* by quantitative transcription analysis, the gene *MA4008* was found to be highly expressed in acetic acid-grown cells versus methanol-grown cells. The expression of this gene was similar to acetate kinase (*ack*) and acetyl-CoA phosphotransferase (*pta*), two of the enzymes required for acetate utilization, suggesting a role of *MA4008* in acetate uptake [12].

The YaaH protein of *E. coli*, responsible for naming the family to which it belongs to, has its annotation exclusively based on homology assumptions. In this work, we present for the first time experimental evidence for the functional role of YaaH as an acetate/succinate transporter proposing a new nomenclature for this protein.

Material and methods

Bacterial strains, plasmids and growth conditions

All the bacterial strains and plasmids used in this study are listed in Table 1 and 2, respectively. All strains used are isogenic with the wild-type *E. coli* K12 derivative strain MG1693. The cultures were maintained on slants of Luria-Bertani (LB). All strains were grown in Minimal Media (MM) [13] [34 mM NaH_2PO_4, 64 mM K_2HPO_4, 20 mM $(NH_4)_2SO_4$, 1 µM $FeSO_4$, 0.1 mM $MgSO_4$, and 10 µM $CaCl_2$] in shake flasks, ratio 1:10, at 37 °C and 200 r.p.m. throughout this study, unless stated otherwise. Carbon sources were glucose (1%, w/v) or acetic acid (1.67, 8.3, 16.6, 33.3, 50, 66.7 and 83.3 mM, pH 6.0) as given in figure legends. Growth medium was supplemented with thymine (50 µg/mL) and the following antibiotics when appropriate: ampicillin (100 µg/mL), chloramphenicol (50 µg/mL) and kanamycin (50 µg/mL).

For the growth experiments in acetic acid, *E. coli* strains were cultured overnight in minimal media supplemented with glucose 1 % (w/v), collected by centrifugation at 5000 *g* for 1 min, washed twice in minimal media (carbon source free) and diluted to an optical density at 600 nm of 0.05 in 150 µL of minimal media, pH 6, supplemented with 1.7, 8, 16, 33.3, 50.0, 66.7 or 83.3 mM acetic acid, into a 96-well microplate. Growth was monitored for 36 h using a Spectramax plus 384 absorbance microplate reader (Molecular Devices).

Table 1 – List of strains used in this work.

Strain	Relevant markers/genotype	Reference
MG1693	thyA715	(Arraiano et al. 1988) [14]
BBC232	MG1693 yaaH(yaaH::Cm^R)	The present study
BBC233	MG1693 actP(actP::Kan^R)	The present study
BC234	MG1693 yaaHactP(yaaH::Cm^R/actP::Kan^R)	The present study
BBC235	BBC234 transformed with pUC18	The present study
BBC236	BBC234 transformed with pSVA9	The present study
BBC237	BBC234 transformed with pWSK29	The present study
BBC238	BBC234 transformed with pSVA10	The present study
BBC239	BBC234 transformed with pL131V	The present study
BBC240	BBC234 transformed with pA164G	The present study
BBC241	**BBC233 transformed with pUC18**	**The present study**

Table 2. List of plasmids used in the present study

Plasmid	Comments	Origin/marker	Reference/source
pKD3	Template for mutants construction; carries chloramphenicol-resistance cassete	oriRγ /Amp^R	(Datsenko and Wanner 2000)[15]
pKD4	Template for mutants construction; carries kanamycin-resistance cassete	oriRγ /Amp^R	(Datsenko and Wanner 2000) [15]
pKD46	Temperature-sensitive λ-red recombinase expression plasmid	oriR101/Amp^R	(Datsenko and Wanner 2000)[15]
pUC18	High-copy plasmid, constitutive expression	pMB1/Amp^R	Fermentas
pWSK29	Low-copy plasmid, constitutive expression	pSC101/Amp^R	(Rong Fu and Kushner 1991) [16]
pSVA9	pUC18 derivative; constitutive expression of yaaH	pUC18/Amp^R	The present study
pSVA10	pWSK29 derivative; constitutive expression of yaaH	pWSK29/Amp^R	The present study
pL131V	pSVA9 with the substitution L131V in yaaH	pMB1/Amp^R	The present study
pA164G	**pSVA9 with the substitution A164G in yaaH**	**pMB1/Amp^R**	**The present study**

Construction of *E. coli* mutants

The *yaaH* (BBC232) and *actP* (BBC233) null mutants were constructed using the primer pairs dyaaH1/dyaaH2 and dactP1/dactP2, respectively, and following the λ-*red* recombinase method [15] with few modifications, as previously described [17]. The chloramphenicol-resistance cassette of plasmid pKD3 replaces nucleotides +5 to +535 of the *yaaH* gene and the kanamycin-resistance cassette of plasmid pKD4 replaces nucleotides +69 to +1596 of *actP*. The gene deletions were verified by colony PCR using the primer pair P1yaaH/P2yaaH for *yaaH* and kt/P3actP and emb282/P1actP for *actP*. All chromosomal mutations were subsequently transferred to a fresh genetic background (MG1693 strain) by P1 transduction. The same method was used for the construction of the double mutant *yaaH*/*actP* (BBC234), from the respective single mutants.

For the construction of pSVA9 plasmid expressing *yaaH*, a PCR fragment containing the entire *yaaH* coding sequence was amplified from MG1693 chromosome using the primer pair P3yaaH and P5yaaH. The resultant PCR fragment was cleaved with *Xba*I and *Hind*III and ligated into the high copy pUC18 plasmid (GenBank/EMBL accession number L09136) digested with the same enzymes. Plasmid pWSK29 (GenBank accession number AF016889-1), expressing *yaaH* gene under its own putative promoter signals (pSVA10), was constructed using the same strategy but with the primer pair P4yaah/P5yaaH. Correct clones were verified by colony PCR and sequencing. All primers were obtained from StabVida (Portugal) and are listed in Table 3.

Table 3 – List of oligos used in this work.

Oligonucleotide	Sequence 5′→3′
dyaaH1	CGAGCGGGGGGATCTCAAAACAATTAGTGGGATTCACCAATCGGCAG AACGTGTAGGCTGGAGCTGCTTC
dyaaH2	TCAGGGAAATTATTTCACCATTCATTCGATGATGATTTTTGAGGAATTAT GGGGTCCATATGAATATCCTCCTTAG
P1yaaH	ATGCCGCGCCCTGAAAACACTAC
P2yaaH	AGTGCAAGACGCGACGTTAGCGAAT
P3yaaH	GTTTTT**TCTAGA**CCATTCATTCGATGATGATTTTTGAG
P4yaaH	GTTTTTTCTAGACAGGTCTGATACCGGAAAC
P5yaaH	GTTTTTT**AAGCTT**GCAAGACGCGACGTTAGC
dactP1	GATTAATGCGCGCGGCCTTGCTCAACGCCAAAGCCGGTCTGGGAGCG GATAAACTGGTGTAGGCTGGAGCTGCTTC
dactP2	CGGCGCTTGCCGCCACACTCCCTTTCGCAGCTAACGCCGCGGATGCT ATTAGGGTCCATATGAATATCCTCCTTAG
P1actP	TCTACATCTGGCGGGCGAAC
P3ActP	AGGCATATTCTCTGCATTATC
kt	CGGCCACAGTCGATGAATCC
Emb282	ACGCTTGATCCGGCTACCTGCCC
P6yaaHmut (L131V)	CAATTCGTTTTCTTTAGC**GTG**ACCGTGCTGTTTGCC
P7yaaHmut (A164G)	GATCTGCGGTGCCAGC**GGC**ATCTATCTGGCGATG
RT_16s fwd	AAGTCGAACGGTAACAGGAAG
RT_16s rev	AGGCAGTTTCCCAGACATTAC
RT2_yaaH fwd	TTTTGCTGGTCTGCTGGAGT
RT2_yaaH rev	AGACCCAGTTTCGGCATCAG
RT2_actP fwd	TCTTTCGGCCTGTGGTTCTC
RT2_actP rev	**AGCACCACCGCAATGTGATA**

The restriction sequences in the primers for cloning procedures are shown in bold. The sequence of homology to the gene in the primers used for the generation of deletion mutants is underlined. The codon mutated is shown in bold underlined.

HPLC measurements

Samples at specific time points of growth were analysed for their concentration in organic acids namely succinic and acetic acids and in carbohydrates namely glucose. Cellular density was measured at 600 nm on a Genesys 20 spectrophotometer (Thermo Spectronic) and 1.5 mL samples were collected and centrifuged for 5 min at 16100 *g*. The supernatants were filtered through 0.22 µm syringe filters and were monitored using a Rezex™ 8 µm ROA-organic acid H+ (8 %) HPLC column (Phenomenex). 2.5 mM H_2SO_4 was used for the mobile phase, the column was maintained at 60 °C and detection was by refractive index measurement with an Elite LaChrom L-2490 RI detector (VWR

Hitachi) at 40 °C. An Elite LaChrom (VWR Hitachi) chromatography system was used with the EZChrom Elite 3.3.2 SP2 software for data collection and analysis.

Transport assays

Cells were incubated in minimal media supplemented with the respective carbon source and harvested by centrifugation at specific time points (as given in figure legends). Cells were then washed in minimal media and resuspended in a 0.1 M potassium phosphate buffer (pH 6.0) containing 0.25 mM $MgCl_2$ to a final concentration of about 5-15 mg dry weight ml^{-1}. 90 µL of bacterial cell suspension was placed in microtubes and after 2 min of incubation at 37 °C, the reaction was started by the addition of 10 µL of an aqueous solution of [1-^{14}C] acetate (s.a. 10000 dpm/nmol), sodium salt (Amersham Biosciences) at 0.5 mM concentration and pH 6.0 (unless otherwise stated), and stopped by the addition of cold 100 mM non-labelled acid, pH 6.0, after 2 min. A time course assay was carried out for acetic acid uptake and the initial uptake rate of this carboxylate was linear for 3 min (data not shown) so we chose an incubation time of 2 min for the transport kinetics determination. The reaction mixtures were centrifuged for 5 min at 16100 g, the pellet was resuspended by vortex in 1 mL of minimal media and centrifuged again for 5 min at 16100 g. The pellet was finally resuspended in 1 mL of scintillation liquid (Opti-Phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). Radioactivity was measured in a Packard Tri-Carb 2200CA liquid scintillation spectrophotometer with disintegrations per minute correction. The inhibition effect of non-labelled substrates on the initial uptake velocities of labelled acid was assayed by adding simultaneously the labelled and non-labelled substrate. Non-specific ^{14}C adsorption to the cells, as well as the diffusion component, was determined by adding a mixture of labelled acid and unlabelled acid 1000-fold concentrated. The values estimated represent less than 5-10 % of the total incorporated radioactivity. Uptake measurements were also performed using [1,4-^{14}C]-succinic acid (s.a. 4000 dpm/nmol), purchased from Moravek Biochemicals, and with D,L-[U-^{14}C]-lactic acid (s.a. 4000 dpm/nmol), purchased from Amersham Biosciences, at the desired concentration as described for acetic acid. The transport kinetics best fitting the experimental initial uptake rates and the kinetic parameters were determined by a computer-assisted non-linear regression analysis (using GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com). The data shown are mean values of at least three independent experiments, with three replicas of each.

Gene expression analysis by RT-PCR

Cells were incubated in minimal media supplemented with 1% glucose (w/v) or 1.67 mM (0.1 %, v/v) to 83.3 mM (0.5 %, v/v) acetic acid and $1x10^9$ cultured bacterial cells were harvested by centrifugation at specific time points (as given in figure legends). For total RNA isolation and purification, the kit GRS Total RNA Kit – Blood & Cultured Cells from GRISP Research Solutions© was used according to the manufacturer instructions. Purity and concentration of total RNA were evaluated by measuring the optical density at 260 and 280 nm using a NanoDrop1000 spectrophotometer.

Transcription of isolated RNA to cDNA was performed with the iScript™ cDNA Synthesis Kit (Bio-Rad). Relative quantitative RT-PCR of the cDNA samples was carried out in a CFX96 Touch™ Real-Time PCR Detection System from Bio-Rad using KAPA SYBR® FAST qPCR Master Mix with SYBR® Green I as the detection agent. The primers used to amplify the selected genes using qPCR were designed using Primer Blast [18, 19] and are listed in Table 3. Reactions were set up in a total volume of 20 µl using 2 µl of cDNA (diluted to 10^{-1}), 10 µl KAPA SYBR® FAST qPCR master mix (Kapa Biosystems), nuclease-free water and 200 nM of each gene-specific primer (Table 3) and performed in the CFX96 Touch™ machine (Bio-Rad). Amplification conditions were as follows: 95 ºC for 3 min, 40 cycles at 95 ºC for 3 s and 57.6 ºC for 30 s. Specificity of the PCR products was confirmed by analysis of the dissociation curve. The melting curve program consisted of temperatures between 65 ºC and 95 °C with a heating rate of 0.5 °C/5 s and a continuous fluorescence measurement. Additionally, the amplicons' expected size and the absence of nonspecific products were confirmed by analysis of the real-time PCR products in 1 % agarose gels (w/v) in 1 × TAE, stained with Midori Green and visualized under UV light. A negative control without template was conducted for each gene in each PCR run, and a control of DNA contamination was implemented by using the purified RNA samples as template. A positive control with the target cloned in an expression plasmid was conducted for each gene in each PCR run. qPCR reactions were performed in triplicate for each cDNA sample tested. Threshold cycle (C_T) values were calculated by using Bio-Rad CFX Manager software, and fold changes were calculated as $2^{-\Delta\Delta}CT$ with inner normalization to the 16S rRNA housekeeping gene [20]. All samples were then compared to the expression levels of the mid-exponential (3 hours) samples of growth in glucose and expressed as relative fold expression. Standard deviations of ΔC_T values for three biological triplicates were propagated to obtain standard errors for each fold change value [21].

Construction of *yaaH* mutations by site-directed mutagenesis

yaaH mutations were constructed in the plasmid pSVA9, with the oligonucleotide-directed mutagenesis technique [22]. The mutagenesis was performed using the DNA Polymerase KAPA HiFi™ (Kapa Biosystems) with proofreading activity as follows: 10 ng of the template plasmid pSVA9 were combined with 10 pmol of the forward oligonucleotides P6yaaHmut or P7yaaHmut and the respective complementary reverse oligonucleotides (Table 3) containing the desired substitution (L131V or A164G) and used in the following PCR reaction: 5 min at 95 °C followed by 25 cycles of 20 s at 98 °C, 15 s at 65 °C, 5 min at 72 °C, and a final extension step of 5 min at 72 °C. In order to destroy the parental strands, the PCR reaction was incubated with the restriction enzyme *Dpn*I (Fermentas) for 2 h at 37 °C. This mixture was then used to transform *E. coli* XL1-Blue and plasmid extraction was performed on several clones with the Gene Elute™ Plasmid Miniprep Kit (Sigma) to obtain plasmids pL131V and pA164G. Mutations were confirmed by sequencing using appropriate oligonucleotides for both DNA strands. The genes containing the desired mutations were introduced in *E. coli* Δ*yaaH* Δ*actP* cells made competent by the rubidium chloride method [23]

Results

Distinct physiological roles for *yaaH* and *actP* on acetate uptake

Single and double mutants for the genes *yaaH* and *actP* were constructed in the strain *E. coli* MG1693, as described in the Experimental Procedures. The uptake of [^{14}C]-acetic acid 0.5 mM pH 6.0, was assessed in all above mentioned strains aerobically grown on mineral media containing glucose, as sole carbon and energy source, collected in mid-exponential phase (Figure 1). A strong decrease in the transport activity of labelled acetic acid was found in the double mutant Δ*yaaH* Δ*actP*, whereas for the respective single mutants only a partial reduction in activity was detected compared with the wild-type strain (100% of activity). These observations were indicative of separate roles for YaaH and ActP in acetate transport.

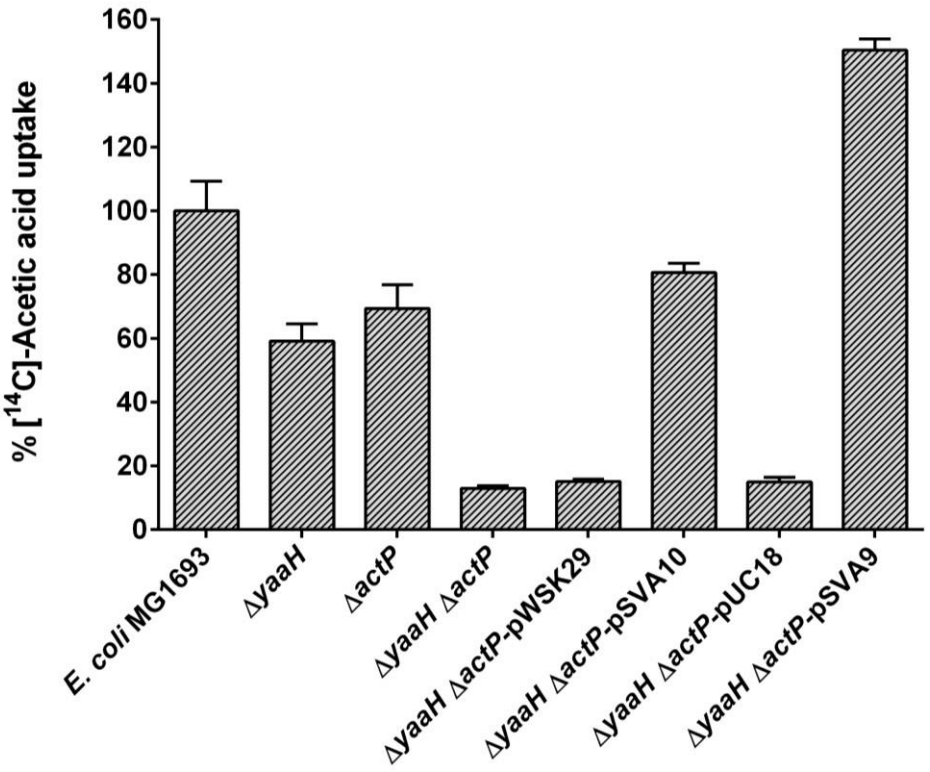

Figure 1 – The functional role of *yaaH* and *actP* genes as acetate transporters in *E. coli*. The graphic represents the percentage of 0.5 mM [1-^{14}C]-acetic acid uptake, at pH 6.0, considering 100% the velocity of transport found for the wild-type *E. coli* MG1693 strain. The cells were collected at the mid-exponential growth phase in minimal medium with glucose 1 % (w/v), containing the required supplements for growth as indicated in materials and methods. The strain Δ*yaaH* Δ*actP* was transformed with the plasmids pSVA9 and pSVA10, expressing *yaaH* from a high or low copy number plasmid, respectively (Table 2). Each data point represents the mean ± SD of 3 independent experiments (n=9).

The uptake of acetate was restored when *yaaH* was expressed in *trans* in Δ*yaaH* Δ*actP* cells either from a low or a high copy number plasmid (Figure 1). The level of activity produced from the low copy number plasmid was very similar to the one found for the single Δ*actP* mutant, while for the high copy number plasmid the uptake values exceeded, in about 50 %, the activity of the wild-type strain.

Energetics and kinetics of the YaaH transporter

The effect of pH on the acetate transport activity associated with YaaH expression was measured in *E. coli* Δ*yaaH* Δ*actP* cells transformed with the high copy plasmid, not expressing (pUC18) or

expressing *yaaH* (pSVA9). Data was taken from cells growing exponentially in mineral media with glucose as sole carbon and energy source (Figure 2

A). Under the pH range analysed, the uptake was maximum at pH 6.0 in cells harbouring pSVA9, with a decrease of activity in more acidic or more alkaline pH values while, in cells transformed with pUC18, this pH effect was not found.

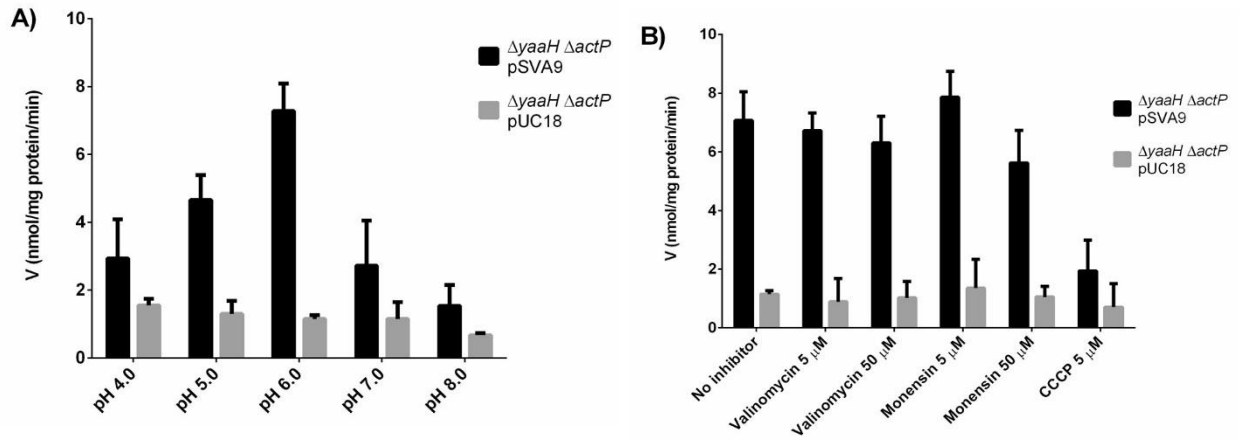

Figure 2 – Energetics of the YaaH transporter. Effect of pH and of CCCP, valinomycin and monensin on the uptake of [1-^{14}C]-acetic acid 0.5 mM, in cells of *E. coli* MG1693 Δ*yaaH* Δ*actP* transformed with pSVA9 or pUC18 plasmids, grown as indicated in Figure 1 legend. A) Transport activity was determined in cells buffered in potassium phosphate at the pH values indicated. B) Cells were pre-incubated with the compounds mentioned, at the concentration indicated, pH 6.0, for 1 min before adding the radiolabeled substrate. Each data point represents the mean ± SD of 3 independent experiments (n=6).

The protonophore CCCP that collapses the proton motive force, lowered transport to almost negligible values at pH 6.0 in cells expressing *yaaH* (Figure 2 B). The potassium ionophore valinomycin and the sodium ionophore monensin, that disrupt the membrane electrical potential Δψ, had no significant effect on acetate uptake. In *E. coli* Δ*yaaH* Δ*actP* cells transformed with pUC18, no visible inhibitory effect was observed with the protonophores and ionophores tested.

The dependence of the transporter activity on ΔpH, together with the effect of the protonophore CCCP, suggest that YaaH behaves as a secondary active acetate/proton symporter, energetically dependent on the proton motive force.

The initial uptake rates of [^{14}C]-acetic acid as a function of acetic acid concentration in *E. coli* Δ*yaaH* Δ*actP* cells transformed with the empty pUC18 plasmid were residual for acetic acid uptake (Figure 3). Cells of the Δ*actP* mutant were used to assess the kinetic parameters associated with YaaH activity revealing an apparent K_m of 1.24 ± 0.13 mM acetic acid and an apparent V_{max} of 8.72 ± 0.37 nmol acetic acid mg protein^{-1} min^{-1} (Figure 3). As expected when overexpressing *yaaH* in Δ*yaaH* Δ*actP*

cells, a higher acetate transport activity (apparent V_{max} of 14.49 ± 0.63 nmol acetic acid mg protein^{-1} min^{-1}) was found. All these findings support the involvement of YaaH protein as an acetate transporter in *E. coli*.

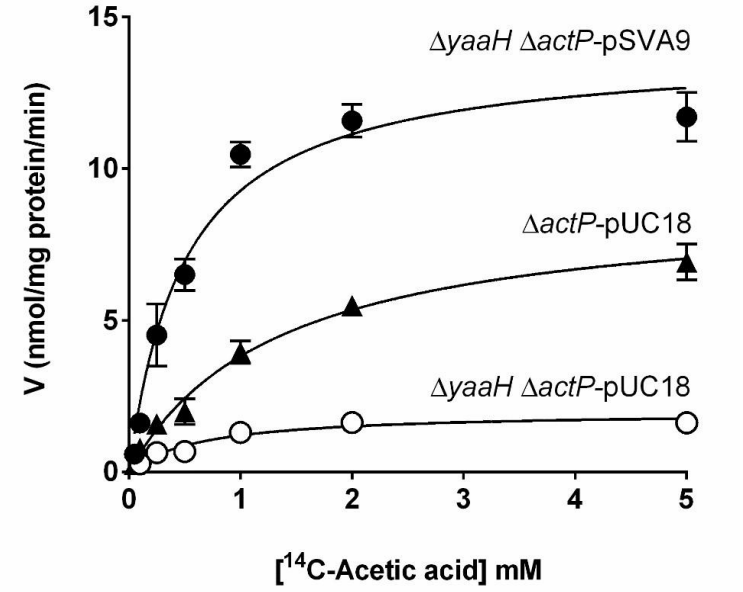

Figure 3 – Acetic acid kinetics of the YaaH transporter. Eadie-Hofstee plots of initial uptake rates of [1-^{14}C]-acetic acid, pH 6.0, in glucose-grown cells, as indicated in Figure 1 legend. *E. coli* strains used: ●, Δ*yaaH* Δ*actP* transformed with the pSVA9 plasmid, expressing the *yaaH* transporter; ▲, Δ*actP* transformed with pUC18 multicopy plasmid; ○, Δ*yaaH* Δ*actP* transformed with the pUC18 multicopy plasmid. Inset: Kinetics of [1-^{14}C]-acetic acid uptake at pH 6.0, as a function of the acid concentration. Each data point represents the mean ± SD of 3 independent experiments (n=9).

The physiological role of YaaH and ActP on acetate assimilation

Wild-type cells display ability to growth on acetic acid (pH 6.0) in a range of concentrations varying from 1.67 mM (0.01 %, v/v) to 83.3 mM (0.5 %, v/v) (Figure 4), with concomitant acetic acid consumption as well as measurable acetic acid uptake.

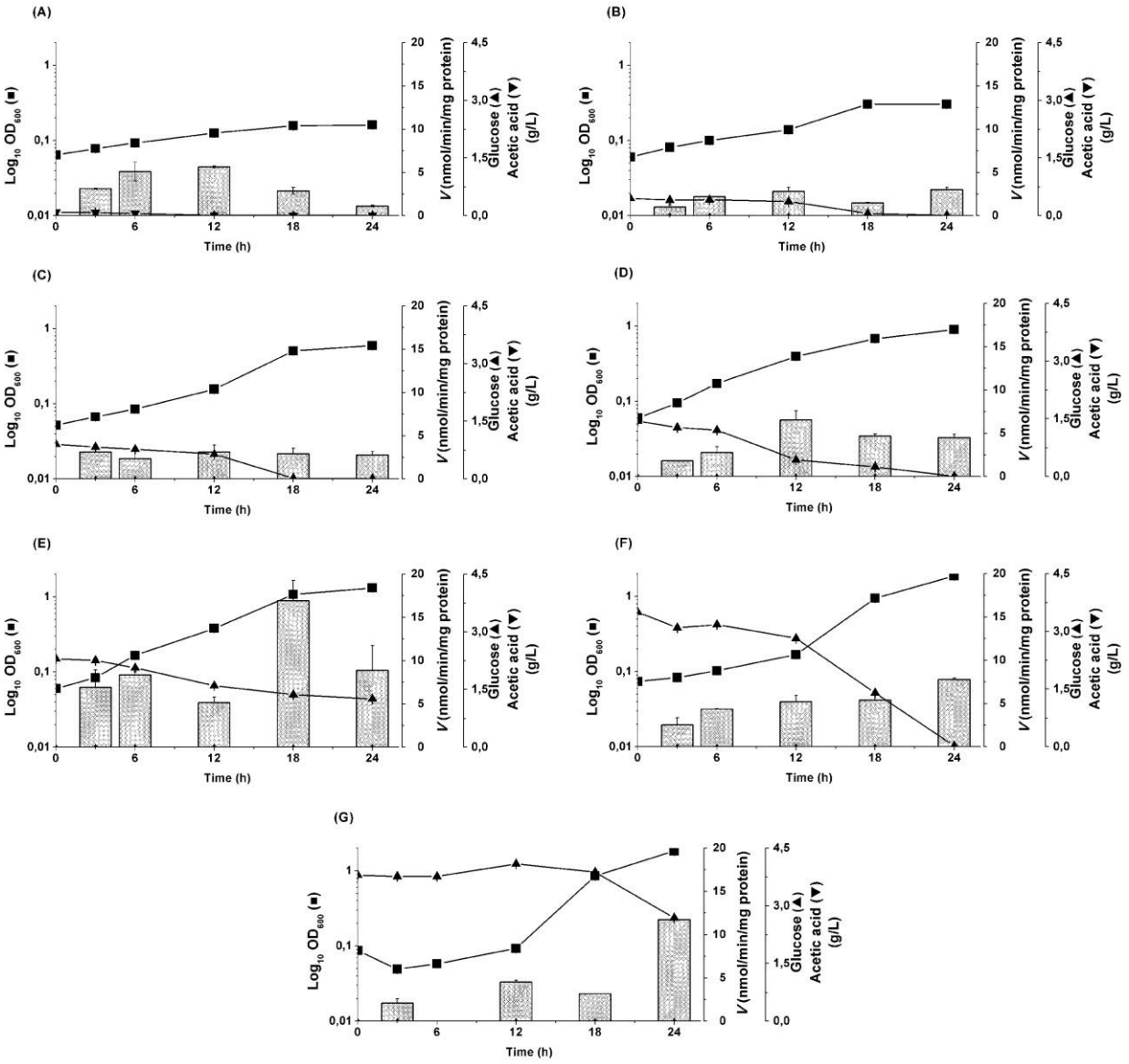

Figure 4 – Acetate uptake profiles of cells grown on acetic acid. Growth profiles of *E. coli* MG1693 grown on minimal medium with acetic acid 1.67 mM (A), 8.3 mM (B), 16.6 mM (C), 33.3 mM (D), 50 mM (E), 66.7 mM (F) and 83.3 mM (G), pH 6.0. Symbols: ■, OD_{600}; ▲ glucose (g/L);▼, acetic acid (g/L); **bars,** transport activity measured with [^{14}C]-acetic acid, 0.5 mM, pH 6.0.

Based on this evidence we wondered about the role of acetate transporters in cells' ability to grown on acetic acid. We have found that single and double mutants revealed a significant alteration in the ability to grow in acetic acid as sole carbon and energy source at 66.7 mM (0.4 %, v/v). This phenotype was even more pronounced at 83.3 mM (0.5 %, v/v), where the double and the Δ*actP* mutants display a longer lag phase (Figure 5). The mRNA expression of *yaaH* and *actP* accessed at three-time points (6, 12 and 24 h) has a similar profile (Figure 5 H), with *yaaH* presenting a lower

relative fold expression when compared to *actP*. In general, the expression of both genes increased over time independent of the acetic acid concentration used for growth with a maximum level detected at 24 h, in cultures with 66.7 mM acetic acid. Overall, these results highlight the relevance of acetate transporters on acetic acid utilization.

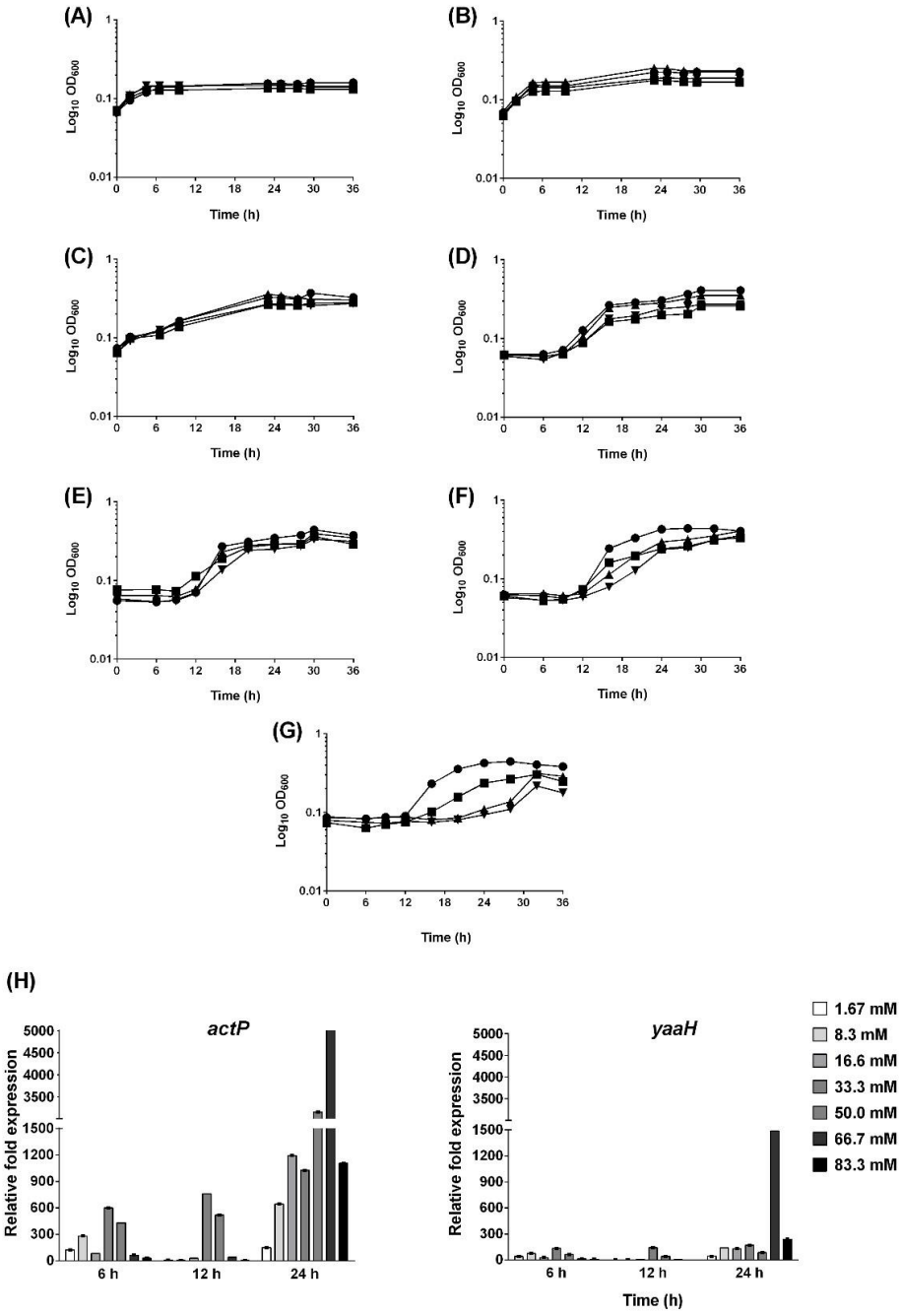

Figure 5 – The physiological role of YaaH and ActP on acetate assimilation. Growth profiles of *E. coli* MG1693 (●) and isogenic Δ*actP* (▲), Δ*yaaH* (■) and Δ*yaaH* Δ*actP* (▼) mutants in minimal medium with acetic acid 1.67 mM (A), 8.3 mM (B), 16.6 mM (C), 33.3 mM (D), 50 mM (E), 66.7 mM (F) and 83.3 mM (G), pH 6.0. H) Relative fold expression of *yaaH* and *actP* genes evaluated by RT-PCR in wild-type cells collected at 6, 12 and 24 hours of growth, normalized to *16S* with standard deviations of ΔC_T values propagated for each fold change value, as described in the experimental procedures. Each data point represents the mean ± SD of 3 independent experiments (n=6).

Distinct physiological roles for distinct acetate transporters

The observation that both YaaH and ActP act independently in the transport of acetate raised the question about what their respective distinctive physiological role might be during aerobic growth on glucose. We evaluated the expression of both *yaaH* and *actP* in cells grown on 0.2, 0.4 and 1.0 % w/v glucose in the wild-type strain as well as the relative acetic acid uptake over time. At low (0.2 %) and intermediary (0.4 %) glucose concentration *yaaH* expression was almost negligible (Figure 6) while *actP* had a peak following entry into stationary phase. Taking the expression pattern into account we can infer that at these concentrations of glucose the relative activity for acetate uptake is probably more associated with *actP*.

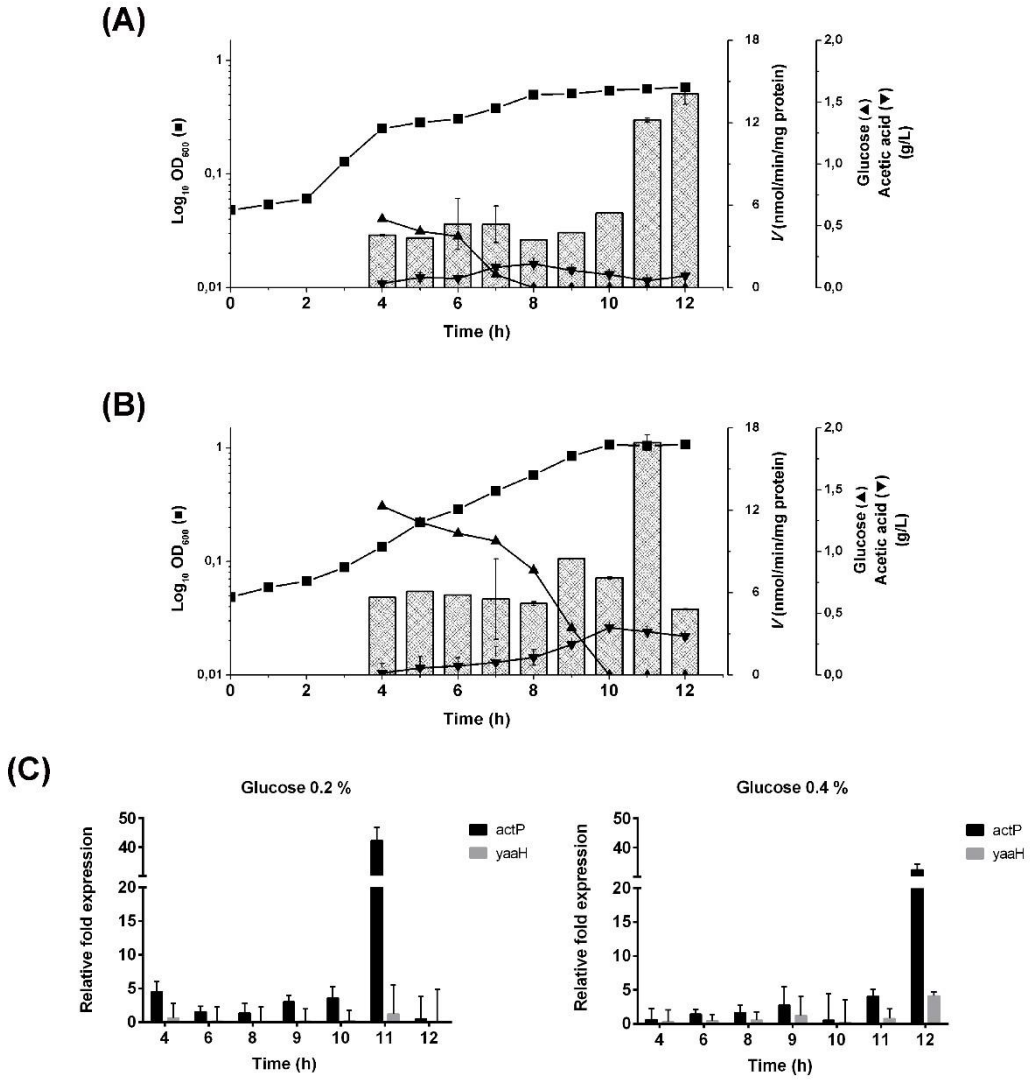

Figure 6 – Acetate uptake profiles of cells grown on glucose. Growth profiles of *E. coli* MG1693 grown on minimal medium with glucose 0.2 %, w/v (A) and 0.4 %, w/v (B). Symbols: ■, OD_{600}; ▲, glucose (g/L); ▼, acetic acid (g/L); **bars,** transport activity measured with [^{14}C]-acetic acid, 0.5 mM, pH 6.0. C) Relative fold expression of *yaaH* and *actP* genes evaluated by RT-PCR in wild-type cells collected at 6, 12 and 24 hours of growth, normalized to *16S* with standard deviations of ΔC_T values propagated for each fold change value, as described in the experimental procedures. Each data point represents the mean ± SD of 3 independent experiments (n=6).

A distinct behaviour was found for cells grown on high (1 %) glucose concentration where both *yaaH* and *actP* are expressed. To access the distinct physiological role of each transporter we compared the behaviour of the single and double mutant strains with the wild-type. In the experiment described in Figure 7, the glucose consumption profiles over time (Figures 5A-5D, ▲) were very similar in all

strains, which displayed the following specific growth rates (hr^{-1}): wild type 0.15; Δ*actP* 0.14; Δ*yaaH* 0.14; Δ*yaaH* Δ*actP* 0.12. Regarding acetate accumulation in the extracellular medium (Figures 5A-5D, ▼) the levels detected were identical in all four strains.

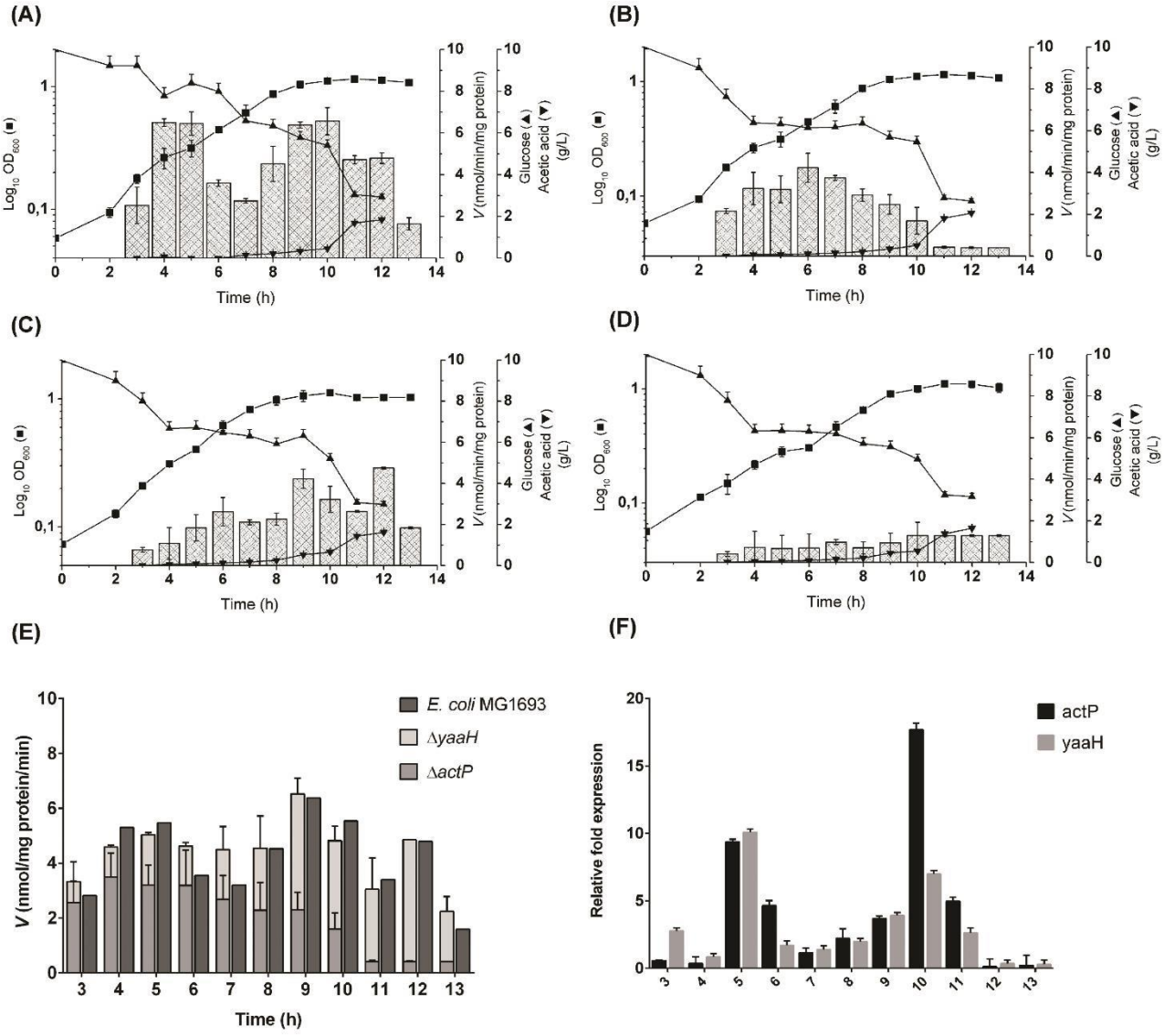

Figure 7 – Distinct physiological roles of the two *E. coli* acetate transporters. Growth profiles of *E. coli* MG1693 (A), and isogenic Δ*actP* (B), Δ*yaaH* (C) and Δ*yaaH* Δ*actP* (D) mutants in minimal medium with glucose 1% (w/v) and the required supplements, as indicated in materials and methods. Symbols: ■, OD_{600}; ▲, glucose (g/L); ▼, acetic acid (g/L); **bars,** transport activity measured with [^{14}C]-acetic acid, 0.5 mM, pH 6.0. E) Time course analysis for acetic acid uptake of the wild-type strain (dark bars, data from A) compared to the sum of the activities of the strains Δ*actP* (light grey bars, data from B) and Δ*yaaH* (dark grey bars, data from C). F) Relative fold expression of *yaaH* and *actP* genes evaluated by RT-PCR normalized to *16S* with standard deviations of ΔC_T values propagated for each fold change value, as described in the experimental procedures. Each data point represents the mean ± SD of 3 independent experiments (n=6).

The four strains achieved the same final OD_{600} after 13 h of growth (Figure 7 A-D, ■**)**, however, regarding the acetate transport activity, a distinct profile was observed for each strain (Figure 7 A-D, bars). In the wild-type strain, two peaks of activity were detected (Figure 7 A), which in fact can be correlated with the behaviour of the single mutants. The first peak, between 4-6 hours of growth, is mostly associated with YaaH activity (Figure 7 B), whereas the second peak, between 8-12 hours, correlates with the ActP permease (Figure 7 C). The double mutant displays only a residual acetate uptake activity all over the growth curve (Figure 7 D). It is worth mentioning that acetate uptake activity profile detected in wild-type correlates well with the sum of the values found for each single mutant (Figure 7 E).

In the wild-type strain, the mRNA expression profile was similar for both genes (Figure 7 F), where two main peaks are detected, one at 5 h (middle exponential growth phase) and another at 10 h (stationary growth phase). These peaks of expression correlate with the mutants' acetate uptake profiles. This behaviour suggests the existence of an unknown regulatory system at the transcriptional and/ or translational level connecting the expression of these two genes.

Overall, the differences found among the four strains lead us to postulate distinct physiological roles played by the two transporters when cells are grown in high glucose concentrations, with YaaH being more active at the exponential growth phase, while ActP is most active in the entry to stationary growth phase [2].

YaaH as a succinate transporter

The range of YaaH transporter substrates was determined in the strain *E. coli* Δ*yaaH* Δ*actP* transformed with the pSVA9 plasmid, by adding non-labelled substrates (30 mM) to the reaction mixture containing labelled acetic acid, 0.3 mM, pH 6.0. Boric, oxalic, pyruvic, lactic, malic and citric acid had no inhibitory effect on the uptake of acetate (not shown). However, an inhibitory effect, higher than 80 %, was found for formic, propionic, benzoic, salicylic and butyric acid (all monocarboxylic acids), as well as the dicarboxylate succinic acid. As can be seen in Figure 8 all these monocarboxylic acids behaved as noncompetitive inhibitors of acetic acid uptake, possibly by affecting the binding site for acetic acid. A different result was found for succinic acid which behaved as a competitive inhibitor for acetate uptake by the YaaH transporter (Figure 8), where an alteration of the slope (K_m) of the plots is observed instead of an alteration on the V_{max}.

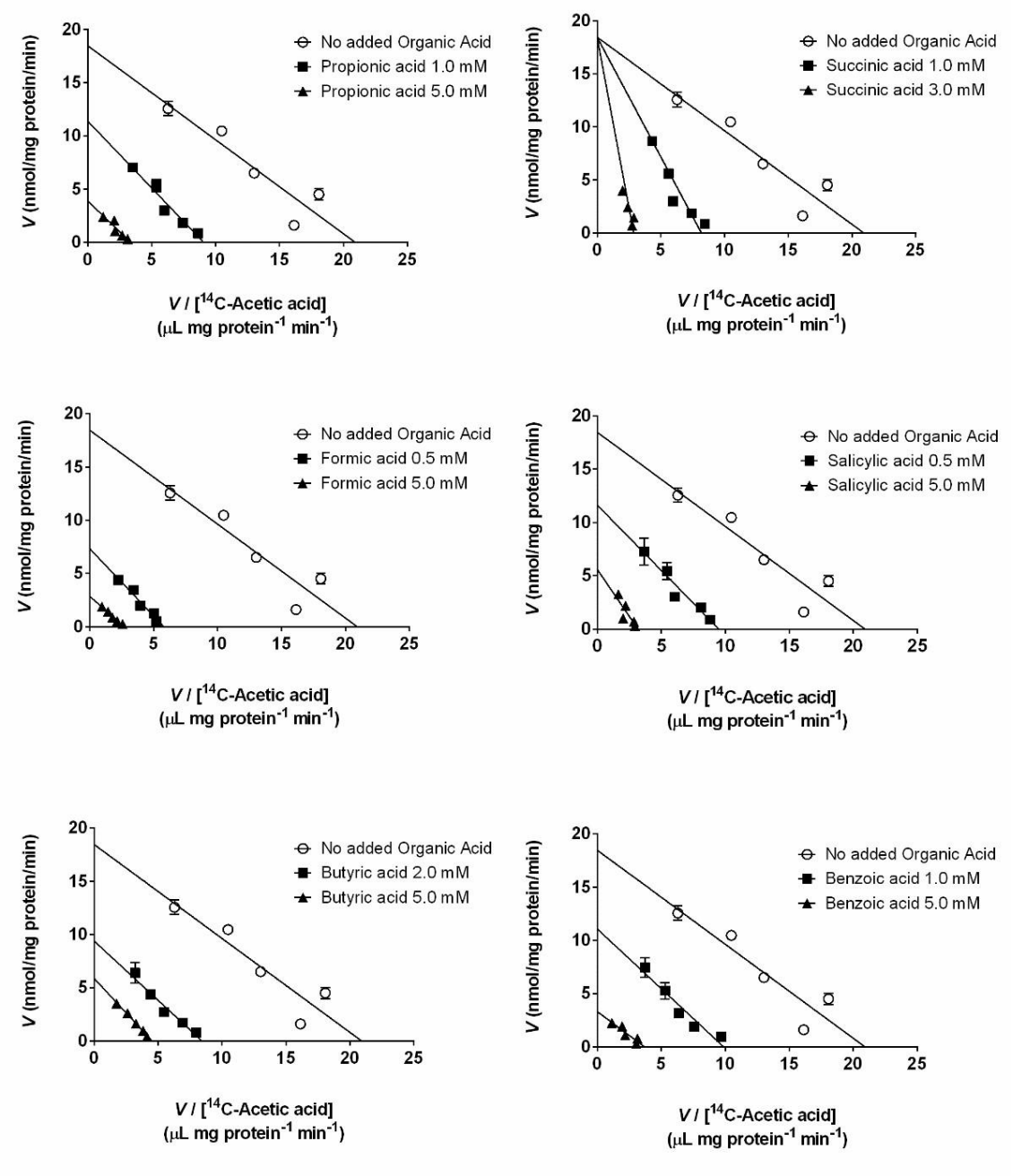

Figure 8 – Substrate specificity of the YaaH transporter. Eadie-Hofstee plots of initial uptake rates of [1-^{14}C]-acetic acid, pH 6.0 in the absence (○) and in the presence (■, ▲) of non-labelled acids, at the concentration indicated in each plot. The cells were grown and collected as described in Figure 1 legend.
Each data point represents the mean ± SD (n=6).

To confirm this observation the kinetic parameters for the initial uptake rates of [1,4-^{14}C] succinic acid were determined in cells of the double mutant transformed with pSVA9 plasmid (Figure 9),

using as control the same strain transformed with the pUC18 plasmid. In both strains labelled succinic acid exhibited a Michaelis-Menten kinetics with the following kinetic parameters: apparent K_m of 1.18 ± 0.10 mM succinic acid and apparent V_{max} values of 10.05 ± 0.34 nmol succinic acid mg protein^{-1} min^{-1} for pSVA9 transformants; apparent K_m of 2.19 ± 0.33 mM succinic acid and apparent V_{max} of 5.50 ± 0.43 nmol succinic acid mg protein^{-1} min^{-1} for pUC18 transformants. This data suggests that YaaH plays a role as a succinic acid transporter.

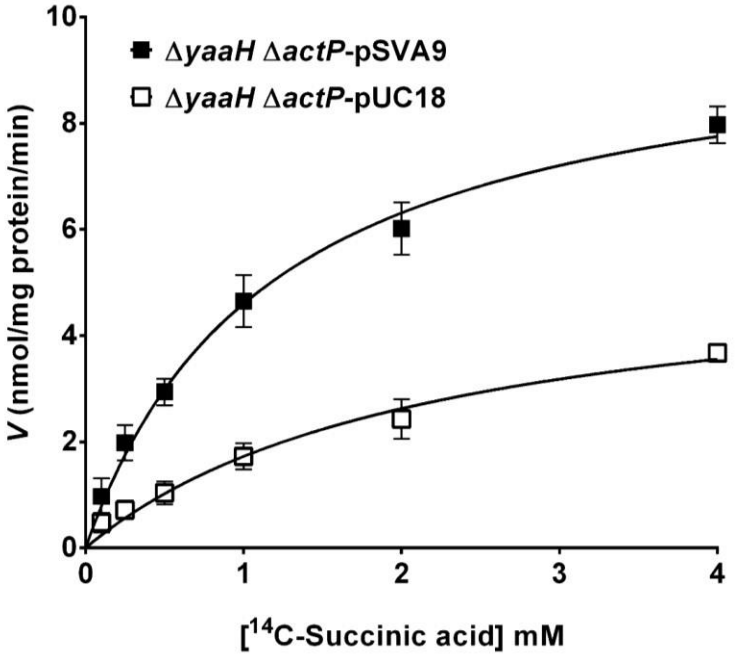

Figure 9 – Succinic acid kinetics of the YaaH transporter. Initial uptake rates of [1,4-^{14}C]-succinic acid, pH 6.0 as a function of the acid concentration in *E. coli* Δ*yaaH* Δ*actP* cells transformed with the plasmid pSVA9 (■). As a control the same strain was transformed with the pUC18 plasmid (□). The cells were grown and collected as described in Figure 1 legend. Each data point represents the mean ± SD (n=6).

To further elucidate the functional role of YaaH in succinate uptake, the concentration of succinic acid was measured in the extracellular media from experiments described in Figure 7. In all strains, succinic acid appeared as a minor sub-product during growth in glucose, but two distinct profiles were observed among the four strains studied (not shown). A sole peak of 10 ± 0.5 mg/L of succinic acid was detected in the samples collected at 10 h of growth both in the wild-type and Δ*actP* strains. However, in the strains, Δ*yaaH* and Δ*yaaH* Δ*actP* 14 ± 0.7 and 16 ± 0.8 mg/L of succinic acid were found at 9 h and 10 h of growth, respectively. The highest levels of succinic acid found in the Δ*yaaH*

deleted strains, both single and double mutant, are consistent with a decrease of succinate import due to the absence of the YaaH protein.

Two single residue mutations change the specificity in YaaH

Two single-nucleotide mutations (C755G/Leu219Val and C655G/Ala252Gly) in the yeast Ady2 promote a change in substrate specificity of this transporter allowing an efficient utilization of lactic acid [9] so we asked whether these residues could behave as in its bacteria homologue. The equivalent residues were identified based on the alignment between YaaH and Ady2 using ClustalW2 [24] (Figure 10 A) and the two mutants (Leu131Val and Ala164Gly) were obtained by site-directed mutagenesis in the pSVA9 plasmid.

The YaaH alleles were tested for the uptake of labelled D,L-[U-^{14}C]-lactic acid, pH 5.0 (Figure 10 B) and as expected, both substitutions improved the affinity and capacity of the acid uptake. The kinetic parameters estimated from the plots shown in Figure 10 B revealed an apparent K_m of 4.15 ± 0.59 mM lactic acid for the wild-type, 2.88 ± 0.45 mM lactic acid for Ala164Gly and the highest affinity detected for the Leu131Val mutant with an apparent K_m of 1.97 ± 0.25 mM lactic acid. The transport capacity in both mutants was also increased, with the following apparent V_{max} (nmol lactic acid.min^{-1}.mg protein^{-1}): 10.15 ± 0.76 for pSVA9, 11.17 ± 0.82 for Ala164Gly, 14.25 ± 0.79 for Leu131Val. In both mutants lactic acid uptake was competitively inhibited by acetic and succinic acid, showing that these acids are still accepted by the YaaH transporter (Figure 10 C). All these assays were performed on glucose-grown cells, conditions where the *E. coli* lactate transporters (*lldP* and *glcA*) are down-regulated, thus the level of lactic acid transport detected in cells transformed with the empty pUC18 plasmid (Figure 8 B, ○) is only basal.

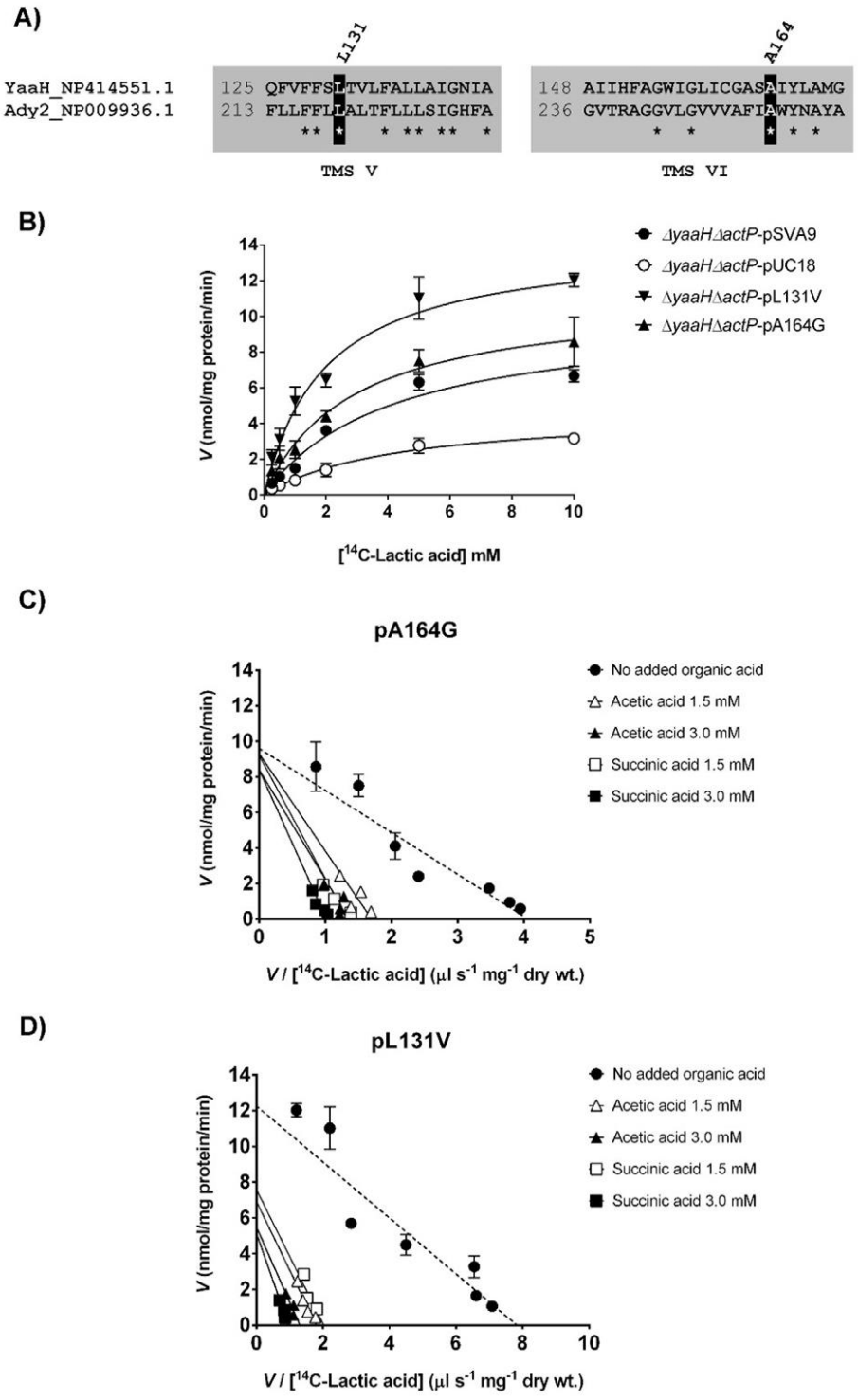

Figure 10 – Site-directed mutagenesis of the *yaaH* gene. (A) Predicted transmembrane segments V and VI of YaaH (http://www.cbs.dtu.dk/services/TMHMM/) aligned with Ady2 using ClustalW2. The conserved residues are marked with a star (*) and the residues mutated in this work are highlighted in black boxes. (B) Initial uptake rates of D,L-[U-^{14}C]-lactic acid, pH 5.0 as a function of the acid concentration. Strains of *E. coli* Δ*yaaH* Δ*actP* transformed with plasmids pSVA9 (●), pA164G (▲) or pL131V (▼). As a control, the same

strain transformed with the pUC18 plasmid was used (○). (C) Eadie-Hofstee plot of the initial uptake rates of D,L-[U-^{14}C]-lactic acid, pH 5.0 in the absence (●) and in the presence (▲, △,■,□,) of non-labelled acetic and succinic acid, at the concentration indicated in each plot in cells transformed with pA164G (D) Eadie-Hofstee plot of initial uptake rates of [^{14}C]-lactic acid, pH 5.0 in the absence (●) and in the presence (▲, △,■,□,) of non-labelled acetic and succinic acid, at the concentration indicated in each plot in cells transformed with pL131V. Cells were grown and collected as described in Figure 1 legend. Each data point represents the mean ± SD of 3 independent experiments (n=6).

Discussion

YaaH is an acetate/proton symporter

In this work we demonstrate that acetate transport in *E. coli* occurs via, at least, two distinct proteins: ActP, a member of the Solute:Sodium Symporter (SSS) Family (TCDB # 2.A.21, www.tcdb.org) and YaaH, a member of the AceTr Family (TCDB # 2.A.96), here characterized. Our data fully agrees with the previous work of Wagner *et al.* (1972) [25] reporting the presence of two separate processes for acetate transport that to our knowledge are, most probably, associated with the ActP and YaaH proteins.

The single mutants on the genes *yaaH* and *actP* revealed a partial reduction in acetate uptake when compared with the wild-type strain while the double mutant displays a significant reduction, indicative of separate roles for YaaH and ActP in acetate transport activity. Additionally, the level of acetate uptake activity in Δ*yaaH* Δ*actP* cells, expressing *yaaH* gene in *trans*, fully agrees with its role as a transporter. When *yaaH* is expressed in a low copy number plasmid, a lower apparent V_{max} was found when compared to the one observed for the wild-type since the *actP* gene is missing. However, as expected, when using the high copy number plasmid there was an increase in the acetate uptake, associated with the overexpression of the YaaH protein. A 50 % increase in activity is quite significant since overexpressed membrane proteins have to be accommodated at the plasma membrane, limiting the level of maximum activity. The basal level of acetate uptake found in the double mutant Δ*yaaH* Δ*actP* (Figure 1) can be attributed either to the existence of other transporters or to the simple diffusion of the undissociated form of the acid across the plasma membrane. It is commonly accepted that the undissociated form of the acid enters bacteria mostly by diffusion [26] while the dissociated form needs assistance from acetate carrier systems [27]. As our assays were carried out at pH 6.0, and taking into account the pK_a of acetic acid (4.74), only 5.44 % of the acid is in its lipid-soluble (uncharged) form. Since the acid is mostly in the dissociated form, passive diffusion is limited by its solubility and permeability [26, 28]. Acetate uptake in glucose-grown cells of the double mutant Δ*yaaH* Δ*actP* follows a saturable kinetics in a pH ranging from 5.0 to 7.0 (not

shown) with similar kinetic parameters found for Δ*yaaH* Δ*actP* transformed with pUC18 (Figure 3). Overall, our data suggest that the existence of other yet unidentified acetate transporter(s) in *E. coli* cannot be ruled out.

The YaaH acetate uptake activity was optimal at pH 6.0 with decreasing activity both at more acidic and alkaline pH values. This observation further strengthens our hypothesis about the minor role that passive diffusion plays on acetate influx in the conditions analysed. If the undissociated acid was permeating the membrane mostly by simple diffusion, decreasing the pH would increase the acid influx due to an increase in the relative amount of the undissociated acid, but this increase in uptake was not observed. Furthermore, the inhibition of acetate transport by the uncoupler CCCP in the Δ*yaaH* Δ*actP* transformed with pSVA9, indicates that the driving force used by YaaH is the transmembrane electrochemical potential. The fact that this uncoupler had no effect on Δ*yaaH* Δ*actP* cells carrying pUC18 demonstrates that CCCP, in the conditions and incubation times tested, had no other pleiotropic effects [29, 30].

In summary, our results suggest that YaaH behaves as a secondary active acetate/proton symporter with an apparent K_m of 1.24 ± 0.13 mM acetic acid and an apparent V_{max} of 8.72 ± 0.37 nmol acetic acid mg protein^{-1} min^{-1}, at pH 6.0, energetically dependent on the proton motive force, as many carboxylate transporters found in microorganisms [2, 31-34].

YaaH is a succinic acid transporter

In contrast to what had been described for other family members [4, 5], the YaaH transporter in *E. coli* seems to be highly specific for acetic acid (a monocarboxylic acid) and for succinic acid (a dicarboxylic acid). It had been reported that *E. coli* cells lacking the known aerobic and anaerobic C4-dicarboxylate carriers (DctA, DcuA, DcuB, DcuC, and DcuD or CitT) still display the ability to aerobically grow on succinate at pH 6.0, by expressing a different succinic acid carrier [35]. The authors mentioned that such carrier was not specific for C4dicarboxylates, displaying also low affinity for monocarboxylates, such as propionate, butyrate and acetate. A recent study in *E. coli* reported that in succinic acid aerobically grown cells, two succinate transporters DctA and DauA co-exist, with DauA as the lower affinity and lower capacity transporter. The authors demonstrated that DauA is essential for the expression and activity of DctA, with DctA as the main transporter at pH 7.0 and DauA at pH 5.0 [36]. The mutants Δ*dauA* at pH 5.0 and Δ*dctA* at pH 7.0, grown in succinate, display residual succinic acid transport activity pointing to the presence of a very low-affinity uncharacterized transporter [36]. Our finding that YaaH is able to transport succinic acid with a lower apparent K_m (1.18 ± 0.10 mM succinic acid, at pH 6.0) compared to the ones found for the above mentioned transporters, can contribute to filling this gap. Furthermore, YaaH only recognizes

succinate and does not accept other C4-dicarboxylates, a feature that is in good agreement with the literature [35].

Taking into account the role of the YaaH protein in acetic and succinic acid uptake, we propose a new nomenclature for this permease, SatP, a Succinate-Acetate Transporter Protein.

L131V and A164G change the specificity of YaaH

The yeast homologue of YaaH is Ady2, a monocarboxylate/proton symporter, shared by acetate, propionate, lactate and formate, whose expression is repressed by glucose [3, 5, 8]. By direct evolution for growth in lactic acid two mutant alleles Leu219Val and Ala252Gly where identified in Ady2 [9]. We wondered if the corresponding mutations in YaaH, Leu131Val and Ala164Gly (Figure 10 A), would lead to a gain of function for lactic acid uptake. It is known that the uptake of lactate in *E. coli* is mediated by two distinct proton symporters: the L-lactate permease (*lldP*) and the glycolate permease (*glcA*) [31]. These transporters are repressed in glucose [37] thus only a basal level of lactic acid uptake was detected in the conditions used in the present work for the control strain (Δ*yaaH* Δ*actP* transformed with pUC18). Glucose-grown cells of Δ*yaaH* Δ*actP* expressing the *yaaH* mutant alleles L131V and A164G display an enhanced affinity and capacity for lactic acid revealing an acquired ability to transport this substrate compared with the wild-type allele. Despite the distinct range of substrates of YaaH and Ady2 they share common molecular traits regarding the substrate binding site since mutations in conserved amino acid residues lead to similar physiological behaviour, as we have shown for the gain of function for lactic acid uptake.

YaaH and the acetate switch

In the present work, we addressed the physiological relevance of the existence of two acetate carriers ActP and YaaH by following their activity along aerobic growth in glucose minimal media. When *E. coli* is grown aerobically in glucose as the carbon source a rapid growth phase is observed, with the consumption of the sugar and the excretion of acetate. This is followed by a slower growth phase with a switch to the use of the excreted acetate as a source of carbon and energy [1]. The formation of acetate depends primarily on the PTA-ACKA pathway with PTA (acetyl-CoA transferase) reversibly converting acetyl-CoA and inorganic phosphate to acetyl~P and coenzyme A while ACKA (acetate kinase) reversibly converts acetyl-P and ADP to acetate and ATP (for a review see Wolfe, 2005). YaaH functions primarily during the exponential phase of growth prior to the acetate switch. When the carbon flux exceeds the capacity of the central metabolic pathways, acetate is assimilated by acetyl-CoA synthetase (*acs*), which activates acetate to acetyl-CoA with the concomitant

conversion of ATP to AMP and pyrophosphate [38, 39]. Acetyl-CoA can be used in the tricarboxylic acid cycle via the glyoxylate bypass [40]. ActP seems to be involved in this step being responsible for the reutilization of acetate, scavenging micromolar concentrations of this compound [2]. This idea was reinforced in this work by showing a more active role of this permease at the stationary phase after the acetate switch. Furthermore, previous work has shown that transcription of the *acs–yjcH–actP* operon is low in the presence of glucose excess due to low cAMP levels that regulate this operon [41] and that *acs* transcription increases with extracellular acetate formation [39]. This is in accordance with what was observed for *actP* expression that increased at the entry into stationary phase.

YaaH in acetate assimilation

We have shown that cells' ability to assimilate acetic acid is not strictly dependent on either YaaH or ActP activity. As shown in Figure 5, wild-type and deleted mutant strains were able to grow over a range of concentrations varying from 1.67 to 83.3 mM acetic acid, as sole carbon and energy source at pH 6.0. Both YaaH and ActP display a functional role at the highest concentrations, followed by an increased level of expression, with a peak for both genes at 66.7 mM acetic acid. These are evidence for the role of these transporters in acetate assimilation allowing cells to better control the uptake of the acid, in particular at very high concentrations.

Acetate-metabolizing cultures of *E. coli* are particularly relevant for the biotechnology industry since the accumulation of acetate in the extracellular medium poses an obstacle to high cell density cultivation and protein production [42, 43]. Acetate toxicity and growth inhibition can be attributed to an acidification of the cytoplasm. It has been assumed that acetate excretion into the environment is a problem due to the lipophilic nature of the undissociated form that can permeate membranes, uncoupling the transmembrane pH gradient and becoming highly toxic [26, 28, 44]. However, acetate seems to have only a small effect on the decrease of the intracellular pH and therefore the growth inhibition is possibly not only correlated to the acidification of the intracellular medium [28] but rather to a complex result of the anion balance of the cell [45]. Although not crucial for cells to grow on glucose or acetic acid, YaaH and ActP contribute to the acetate-succinate intracellular balance. Acetate transporters might be future targets for improving existing protein expression systems as *E. coli* is one of the most important microorganisms for biotechnological applications where acetate represents a major unwanted by-product.

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Chapter VII

The Acetate Uptake Transporter family motif "NPAPLGL(M/S)" is essential for substrate uptake

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Personal contribution: I collaborated in the laboratory experiments and manuscript writing.

Chapter VII

The Acetate Uptake Transporter family motif "NPAPLGL(M/S)" is essential for substrate uptake.

Abstract

Organic acids are recognized as one of the most prevalent compounds in ecosystems, thus the transport and assimilation of these molecules represent an adaptive advantage for organisms. The AceTr family members are associated with the active transport of organic acids, namely acetate and succinate. The phylogenetic analysis shows this family is dispersed in the tree of life. However, in eukaryotes, it is almost limited to microbes, though reaching a prevalence close to 100% in fungi, with an essential role in spore development. Aiming at deepening the knowledge in this family, we studied the acetate permease AceP from *Methanosarcina acetivorans*, as the first functionally characterized archaeal member of this family. Furthermore, we demonstrate that the yeast Gpr1 from *Yarrowia lipolytica* is an acetate permease, whereas the Ady2 closest homologue in *Saccharomyces cerevisiae*, Fun34, has no role in acetate uptake. In this work, we describe the functional role of the AceTr conserved motif NPAPLGL(M/S). In this work we further unveiled the role of the amino acid residues R122 and Q125 of SatP as essential for protein activity.

Chapter VII

Introduction

Organic acids are one of the most prevalent and abundant organic compounds on earth's surface and they are vital intermediates for cell metabolism [1]. The cell homeostasis of organisms is highly dependent on the transport of specific molecules across the cell membrane. When the environmental pH is below the p*Ka* of the acid, the protonated form predominates and crosses the cell membrane by passive diffusion according to its diffusion coefficient. However, when the pH is above the acid p*Ka* the anionic form of the acid predominates, requiring a transporter protein to cross the membrane [2, 3].

The AceTr family was recently reassigned as the Acetate Uptake Transporter Family (TCDB 2.A.96) due to the functional characterization of several members as acetate transporters. AceTr proteins display six transmembrane segments (TMS), sharing the conserved motif NPAPLGL(M/F) located at the beginning of the first TMS [3, 4]. Members of this family have been reported in the literature, however their function as acetate transporters has not been proven yet, namely Gpr1, Fun34 and AceP. The *Yarrowia lipolytica* Gpr1 protein, the first family member to be reported in literature, was found to be expressed in the presence of acetic acid or ethanol [4] and it was shown to be involved in acetic acid sensitivity, cell and colony morphology, yeast-to-hyphae transition and cell lifespan [4, 5]. On the other hand Ady2, the Gpr1 homologue in *Saccharomyces cerevisiae*, was found to be expressed in acetic acid grown cells, behaving as a proton-symporter for the anionic form of the acid [6, 7]. The Ady2 homozygous null mutant in diploid cells results in reduced sporulation and higher frequency of abnormal asci, forming dyads instead of tetrads [8]. *S. cerevisiae* has two other AceTr members, Fun34 (Ato2) and Ato3, which were reported to be involved in ammonium export, along with Ady2 [9]. In the filamentous fungi *Aspergillus nidulans,* four AceTr homologs were found, AcpA, AcpB, AcpC, and AlcS, [10-12]. The AcpA protein is essential for the uptake and use of acetate as a sole carbon source and contributes for spore maintenance and homeostasis [10, 12]. AcpB was found to be responsible for residual acetate transport in mycelia, whereas no function related to carboxylic acids transport was assigned for both AcpC [12] and AlcS proteins [11, 13]. SatP is the sole AceTr homolog in *Escherichia coli*. Unlike other AceTr members, it transports both mono- and dicarboxylic acids being a succinate/acetate proton symporter, which is mostly active during exponential growth phase during glucose consumption [14]. In archaea, the *aceP* gene from *Methanosarcina acetivorans* was found to be overexpressed in the presence of acetate, however, this high level of expression is only reached when acetate is used as a sole carbon source [15]. The crystal structure of the SatP homolog from *Citrobacter koseri* (SatP_Ck) recently released, revealed a complex anion channel with four acetate-binding sites aligned in a single file interrupted by three hydrophobic constrictions [16].

In this study, we carried out a deep phylogenetic analysis of AceTr family and functionally characterized new members of this family: the yeast GPR1 from *Y. lipolytica* and Fun34 from *S. cerevisiae* and the archaea aceP from *M. acetivorans*. Here we demonstrate the crucial role played by each residue of the conserved amino acid residues from the motif NPAPLGL(M/F). SatP molecular modelling studies allowed the identification of other amino acid residues essential for SatP activity.

Materials and methods

Homology search

We downloaded over 10000 proteomes from NCBI Assembly platform as individual FASTA files. A BLAST search, with a cut-off e-value 10^{-5}, was performed on this database using three queries, a bacterial, an archaeal and an eukaryotic member: Satp from the organism *E. coli*, AceP from *M. acetivorans* and Ady2 from *S. cerevisiae*. To avoid redundancies, only sequences from a single genome of a given species were considered.

Alignment and Phylogenetic reconstruction

Retrieved protein sequences were aligned with PROMALS3D [17], a multiple-alignment algorithm that incorporates secondary structure prediction. Sequences that were not aligning extensively across the conserved region of the alignment were further excluded from the phylogenetic analysis. A phylogenetic reconstruction was performed using Maximum Likelihood, more appropriate for the deeper divergences under analysis here, using MEGA6 [18] and the Jones-Taylor-Thornton (JTT) substitution model. Bootstrap was performed for 1000 repetitions. Obtained phylogenetic tree was displayed and edited in FigTree v.1.3.1. (http://tree.bio.ed.ac.uk/).

Three-dimensional modelling and molecular docking

The three-dimensional modelling analysis was performed for both SatP and Ady2, using as template the crystal structure of the SatP_Ck protein. The residue sequence of SatP and Ady2 were threaded in the Swiss-Model server [19] and the PDB file of SatP_Ck was added as a template model. Since SatP three-dimensional modelling obtained the best score for protein structure prediction, it was further considered for molecular docking analysis. Molecular docking simulations were performed as described before [20].

Strains, plasmids and growth conditions

The strains and plasmids used in this work are listed in Tables 1 and 2 respectively. The *S. cerevisiae* strain W303-1A *jen1*Δ *ady2*Δ, lacking monocarboxylate uptake capacity, was used to express the *ADY2* alleles and the *GPR1* from *Y. lipolytica*. The cultures were maintained on slants of yeast extract (1%, w/v), peptone (1%, w/v), glucose (2%, w/v) and agar (2%, w/v) or yeast nitrogen base (Difco), 0.67%, w/v (YNB medium), supplemented with adequate requirements for prototrophic growth. For drop tests, cells were grown on YNB Glu −Ura media, until mid-exponential phase and diluted to an OD 640nm of 0.1. A set of three 1:10 serial dilutions were performed and 3 µl of each suspension was inoculated in YNB acetic acid 0.5% (Oxoid agar 2%) pH 5.5, using YNB Glu −Ura as a control. Cells were incubated at 18◦C for 5 days. At 18◦C, carboxylic acid uptake by diffusion is drastically reduced, so that growth on carboxylic acid as sole carbon source is directly dependent on a functional transporter [21]. The *E. coli* strain *actP*Δ *lldp*Δ *satP*Δ (assigned in this study as *E. coli* 3Δ) was used to express the *satP* alleles and the *aceP* gene from *M. acetivorans*. Bacterial strains were grown as previously described [14].

Construction of *E. coli actP*Δ *lldP*Δ *satP*Δ

The *lldP* null-mutant was constructed using the primer pairs dlldP1/dlldP2 and following the λ-red recombinase method [22] with a few modifications, as described previously [14, 23]. The chloramphenicol-resistance cassette of plasmid pKD3 replaces nucleotides +50 to +1587 of the *lldP* gene. The gene deletion was verified by colony PCR using the primer pair P1lldP/P2lldP and the chromosomal mutation subsequently transferred to a fresh genetic background (MG1693 strain) by P1 phage transduction, to generate *E. coli* MG1693 *lldP* (Δ*lldP*::Cm^R) strain. To construct the triple *yaaH*/*actP*/ *lldP* mutant, the antibiotic resistance genes of the double *yaaH*/*actP* mutant (BBC234) (Sá-Pessoa et al., 2013) were eliminated using a helper plasmid encoding the FLP recombinase (pCP20) and following the procedures previously described [22]. The loss of the kanamycin and chloramphenicol resistance was confirmed, and the strain used as a receptor, in the P1 phage transduction of *lldP* mutation from the single *lldP* null-mutant. The chloramphenicol resistance was used for selection of the *lldP* mutation and the presence of the three, *yaaH*, *actP* and *lldP*, gene deletions were confirmed by PCR using specific primers: P1yaaH/P2yaaH for *yaaH*; P1ActP/P4actP for *actP* and P1lldP/P2lldP for *lldP* (primers listed in Table 3).

Cloning strategy of *aceP* gene

The *aceP* gene was chemically synthesized with an optimal codon usage for expression in *E. coli* since no expression was obtained with the original gene cloned into the pUC18 vector. The synthetic

aceP gene was codon optimized by the OptimumGene software tool (GenScript, Piscataway, NJ, USA Inc.). The synthetic version of *aceP* flanked by *KpnI* and *NdeI* restriction sites was cloned in the puc18 vector, originating the pAceP vector.

Heterologous expression of *GPR1*

The pGPR1 plasmid was constructed by restriction enzyme cloning and the pGPR1::GFP plasmid through GAP repair technique [24]. The GPR1 gene was amplified by PCR, from genomic DNA extracted from *Yarrowia lipolytica* PYCC 4811 [25], using the primers Gpr1_FWD and Gpr1_REV (Table 3). The amplified GPR1 was digested using *Hind*III and *Sal*I enzymes (*ThermoFisher Scientific*; USA). The final product was inserted into the vector p416GPD, previously digested with the same restriction enzymes using the same conditions. The resultant plasmid pGPR1 was cloned into *S. cerevisiae* W303-1A *jen1Δ ady2Δ* strain. To obtain the plasmid pGPR1-GFP, the GFP gene was inserted as described by Bessa et al., 2012, using the primers Gpr1-gfp_FWD and the Gpr1-gfp_REV (Table 3).

Cloning strategy for *FUN34*

The pFUN34 plasmid was constructed by restriction enzyme cloning. The *FUN34* gene was amplified by PCR, from genomic DNA extracted from *S. cerevisiae S288c*, using the primers Fun34_FWD, Fun34_REV (Table 3). The amplified versions were digested using *BamH*I and *Xho*I enzymes considering. The final products were inserted into the vector p416GPD, previously digested with the same restriction enzymes using the same conditions. The final plasmid, pFUN34 was cloned into *S. cerevisiae* W303-1A *jen1Δ ady2Δ* strain.

Construction of Ady2 and SatP mutants

Site-directed mutagenesis was performed as previously described [21]. For *GFP* fusion proteins with the *ADY2*, GAP repair technique was performed as previously described [21], using the primers listed in Table 3.

Transport assays

Measurement of transport activity in yeast and bacterial strains was performed as previously described by Soares-Silva et al. (2007) and Sá-Pessoa et al. (2013), respectively. The radiolabeled The radiolabeled substrates used were the following: [1-^{14}C] acetic acid, sodium salt (GE Healthcare, London, UK) and [2,3-^{14}C] succinic acid (Moravek Biochemicals, California, USA).

Epifluorescent microscopy

Microscopy analysis was performed as described in Soares-Silva et al. (2007) on a Leica DM5000B epifluorescent microscope with a Leica DFC 350FX R2 digital camera using the LAS AF V1.4.1 software.

Table 1. List of plasmids used in this study

Plasmid	Characteristics	Reference
pUC18	High-copy plasmid, constitutive expression	(Norrander *et al.* 1983) [26]
PSatP	pUC18 derivative; constitutive expression of *SatP*	(Sá-Pessoa et al, 2013) [14]
pSatP-N8A	pSatP with the substitution N8A in SatP	This study
pSatP-P9A	pSatP with the substitution P9A in SatP	This study
pSatP-A10T	pSatP with the substitution A10T in SatP	This study
pSatP-P11A	pSatP with the substitution P11A in SatP	This study
pSatP-L12A	pSatP with the substitution L12A in SatP	This study
pSatP-G13A	pSatP with the substitution G13A in SatP	This study
pSatP-L14A	pSatP with the substitution L14A in SatP	This study
pSatP-M15A	pSatP with the substitution M15A in SatP	This study
p416GPD	Glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter	(Mumberg et al. 1995) [27]
pDS1::GFP	p416 derivative, constitutive expression of JEN1::GFP	(Soares-Silva *et al*. 2007) [21]
pAdy2::GFP	p416 derivative, constitutive expression of ADY2::GFP	This study
pAdy2::GFP-N89A	pAdy2::GFP with substitution N89A in Ady2	This study
pAdy2::GFP-P90A	pAdy2::GFP with substitution P90A in Ady2	This study
pAdy2::GFP-A91T	pAdy2::GFP with substitution A91T in Ady2	This study
pAdy2::GFP-P92A	pAdy2::GFP with substitution P92A in Ady2	This study
pAdy2::GFP-L93A	pAdy2::GFP with substitution L93A in Ady2	This study
pAdy2::GFP-G94A	pAdy2::GFP with substitution G94A in Ady2	This study
pAdy2::GFP-L95A	pAdy2::GFP with substitution L95A in Ady2	This study
pAdy2::GFP-S92A	pAdy2::GFP with substitution S92A in Ady2	This study
PAceP	pUC18 derivative; constitutive expression of *aceP*	This study
p416::Gpr1	p416GPD with constitutive expression of *GPR1*	This study
p416::Gpr1-GFP	p416GPD with constitutive expression of *GPR1::GFP*	This study

Table 2. List of strains used in this study

Strain	Genotype	Reference
E. coli MG1693	thyA715	(Arraiano *et al*. 1988) [28]
E. coli ΔlldP	*MG1693 lldP (ΔlldP::Cm^R)*	This study
***E. coli* 3Δ**	*MG1693 yaaH actP lldP (ΔyaaH /ΔactP/ΔlldP::Cm^R)*	This study
***E. coli* 3Δ pUC18**	*E. coli* 3Δ transformed with pUC18	This study
***E. coli* 3Δ pSatP**	*E. coli* 3Δ transformed with pSatP	This study
***E. coli* 3Δ pSatP-N8A**	*E. coli* 3Δ transformed with pSatP-N8A	This study
***E. coli* 3Δ pSatP-P9A**	*E. coli* 3Δ transformed with pSatP-P9A	This study
***E. coli* 3Δ pSatP-A10T**	*E. coli* 3Δ transformed with pSatP-A10T	This study
***E. coli* 3Δ pSatP-P11A**	*E. coli* 3Δ transformed with pSatP-P11A	This study
***E. coli* 3Δ pSatP-L12A**	*E. coli* 3Δ transformed with pSatP-L12A	This study
***E. coli* 3Δ pSatP-G13A**	*E. coli* 3Δ transformed with pSatP-G13A	This study
***E. coli* 3Δ pSatP-L14A**	*E. coli* 3Δ transformed with pSatP-L14A	This study
***E. coli* 3Δ pSatP-M15A**	*E. coli* 3Δ transformed with pSatP-M15A	This study
***E. coli* 3Δ pAceP**	*E. coli* 3Δ transformed with pAceP	This study
***S. cerevisiae* W303-1A**	MATα *ade2 leu2 his3 trp1 ura3*	(Thomas and Rothstein 1989) [29]
S. cerevisiae jen1Δ ady2Δ	W303-1A; *JEN1::KanMX4 ADY2::HphMX4*	(Soares-Silva *et al*. 2007) [21]
***S. cerevisiae jen1Δ ady2Δ* p416GPD**	*jen1Δ ady2Δ* transformed with p416GPD	(Soares-Silva *et al*. 2007) [21]
***S. cerevisiae jen1Δ ady2Δ* pAdy2**	*jen1Δ ady2Δ* transformed with *p416::Ady2*	This study
***S. cerevisiae jen1Δ ady2Δ* pAdy2::GFP**	*jen1Δ ady2Δ transformed with pAdy2::GFP*	This study
***S. cerevisiae jen1Δ ady2Δ* pAdy2::GFP-N89A**	*jen1Δ ady2Δ transformed with pAdy2::GFP-N89A*	This study
***S. cerevisiae jen1Δ ady2Δ* pAdy2::GFP-P90A**	*jen1Δ ady2Δ transformed with pAdy2::GFP-P90A*	This study
***S. cerevisiae jen1Δ ady2Δ* pAdy2::GFP-A91T**	*jen1Δ ady2Δ* transformed with pAdy2::GFP-A91T	This study
***S. cerevisiae jen1Δ ady2Δ* pAdy2::GFP-P92A**	*jen1Δ ady2Δ* transformed with pAdy2::GFP-P92A	This study
***S. cerevisiae jen1Δ ady2Δ* pAdy2::GFP-L93A**	*jen1Δ ady2Δ* transformed with pAdy2::GFP-L93A	This study
***S. cerevisiae jen1Δ ady2Δ* pAdy2::GFP-G94A**	*jen1Δ ady2Δ* transformed with pAdy2::GFP-G94A	This study
***S. cerevisiae jen1Δ ady2Δ* pAdy2::GFP-L95A**	*jen1Δ ady2Δ* transformed with pAdy2::GFP-L95A	This study
***S. cerevisiae jen1Δ ady2Δ* pAdy2::GFP-S92A**	*jen1Δ ady2Δ* transformed with pAdy2::GFP-S92A	This study
***Yarrowia lipolytica* PYCC 4811**	Wild-type	Collection
***S. cerevisiae* S288c**	Wild-type	Collection
***S. cerevisiae jen1Δ ady2Δ* pGpr1**	*jen1Δ ady2Δ* transformed with *p416::Gpr1*	This study
***S. cerevisiae jen1Δ ady2Δ* pGpr1-GFP**	*jen1Δ ady2Δ* transformed with *p416::Gpr1-GFP*	This study

Table 3. Oligonucleotides used for strain construction, cloning, expression, site-directed mutagenesis and GFP tagging.

Name	Sequence
N89A_FWD	GTGCACAAATTTGCTGCTCCTGCGCCCTTAGG
N89A_REV	CCTAAGGGCGCAGGAGCAGCAAATTTGTGCAC
P90A_FWD	CACAAATTTGCTAATGCTGCGCCCTTAGGTC
P90A_REV	GACCTAAGGGCGCAGCATTAGCAAATTTGTG
A91T_FWD	CAAATTTGCTAATCCTACGCCCTTAGGTCTTTC
A91T_REV	GAAAGACCTAAGGGCGTAGGATTAGCAAATTTG
P92A_FWD	GCTAATCCTGCGGCCTTAGGTCTTTCAGCC
P92A_REV	GGCTGAAAGACCTAAGGCCGCAGGATTAGC
L93A_FWD	GCTAATCCTGCGCCCGCAGGTCTTTCAGCCTTC
L93A_REV	GAAGGCTGAAAGACCTGCGGGCGCAGGATTAGC
G94A_FWD	GCTAATCCTGCGCCCTTAGCTCTTTCAGCCTTCGCG
G94A_REV	CGCGAAGGCTGAAAGAGCTAAGGGCGCAGGATTAGC
L95A_FWD	CCTGCGCCCTTAGGTGCTTCAGCCTTCGCGTTG
L95A_REV	CAACGCGAAGGCTGAAGCACCTAAGGGCGCAGG
N8A_FWD	CAACACTAAGTTGGCTGCTCCGGCACCGCTGGGC
N8A_REV	GCCCAGCGGTGCCGGAGCAGCCAACTTAGTGTTG
P9A_FWD	CACTAAGTTGGCTAATGCGGCACCGCTGGGCCTG
P9A_REV	CAGGCCCAGCGGTGCCGCATTAGCCAACTTAGTG
A10T_FWD	CTAAGTTGGCTAATCCGACACCGCTGGGCCTGATGG
A10T_REV	CCATCAGGCCCAGCGGTGTCGGATTAGCCAACTTAG
P11A_FWD	GTTGGCTAATCCGGCAGCGCTGGGCCTGATGGGC
P11A_REV	GCCCATCAGGCCCAGCGCTGCCGGATTAGCCAAC
L12A_FWD	GCTAATCCGGCACCGGCGGGCCTGATGGGCTTC
L12A_REV	GAAGCCCATCAGGCCCGCCGGTGCCGGATTAGC
G13A_FWD	CTAATCCGGCACCGCTGGCCCTGATGGGCTTCGGC
G13A_REV	GCCGAAGCCCATCAGGGCCAGCGGTGCCGGATTAG
L14A_FWD	CCGGCACCGCTGGGCGCGATGGGCTTCGGCATG
L14A_REV	CATGCCGAAGCCCATCGCGCCCAGCGGTGCCGG
M15A_FWD	GGCACCGCTGGGCCTGGCGGGCTTCGGCATGACC
M15A_FREV	GGTCATGCCGAAGCCCGCCAGGCCCAGCGGTGCC
ady2_gfp_fwd	CCCATTACCATCTACTGAAAGGGTAATCTTTAGTAAAGGAGAAGAAC
ady2_gfp_rev	CATAACTAATTACATGACTCGAGCTATTTGTATAGTTCATCCATGCC
dlldP1	GTTAAGACATAAGCCTGAAGCGTGGTGATCACGCCCACTATACAGGTGAAGAGTGTAGGCTGGAGCTGCTTC
dlldP2	ATGAATCTCTGGCAACAAAACTACGATCCCGCCGGGAATATCTGGCTTTCGGTCCATATGAATATCCTCCTTAG
P1lldP	ACGATGTGCGTGGACTCCAG
P2lldP	ATCAATCAGCGCCCGCAC
P1yaaH	ATGCCGCGCCCTGAAAACACTAC
P2yaaH	AGTGCAAGACGCGACGTTAGCGAAT
P1actP	TCTACATCTGGCGGGCGAAC
P4actP	ACAGAGTGGTTATCGTTAATCAG
Gpr1_FWD	GGCAAGCTTATGAACACCGAAATCCCC
Gpr1_REV	GCGGTCGACTTAGTCCTTCTTGACGAA
Gpr1-gfp_FWD	TTTCCTCAAAGAGATTAAATACTGCTACTGAAAATATGAACACCGAAATCCCCGA
Gpr1-gfp_REV	ACTCGAGGTCGACGGTATCGATAAGCTTTACTATTTGTATAGTTCATCCA

Results

Phylogenetic analysis of AceTr family

A total of 818 hits were obtained for the BLAST search in the NCBI's Assembly database, using complete genomes only, with the 3 proteins of the AceTr family: Ady2, SatP, *and* AceP. Homologues were present across a wide range of organisms: most of them were detected in bacteria, archaea, and fungi. Hypothetical AceTr homologs were also detected in the genus *Leishmania,* three Viridiplantae and the Rhodophyta *Cyanidioschyzon merolae*. 12 sequences that lacked large conserved regions were excluded from a final dataset of 806 sequences. For a clear description of the tree, some sections were labelled as E1, E2, and P1 to P4.

The Phylogenetic tree (Figure 1) suggests a basal split between eukaryotic and prokaryotic organisms. Eukaryotes and prokaryotes form two monophyletic clades with an exception from each subclade. The exception in the eukaryotic clade is the protein of bacteria *Gordonia polyisoprenivorans*, which might be dubious, whereas the exception in prokaryotic clade is from the pathogenic trypanosome *Leishmania* genus, which likely represents a case of horizontal gene transfer. The prokaryotic subclade shows bacteriais and archaeal homologs intertwined within the tree. The tree splits deeply into two branches, labelled P1-3 and P4 in Figure 1. P4 contains mostly homologs present in the bacterial phylum Firmicutes, so far uncharacterized. P1-3 also contains another split between a clade containing a high percentage of homologues present in Actinobacteria (P3), a monophyletic archaea sub-branch and homologues from various other bacteria (including Phyla Firmicutes, Proteobacteria and others), and a second more frequent clade (P1-2) where bacterial homologues from phylum Proteobacteria are the most common (forming the totality of minor clade P2). The large subclade P1 also contains other bacteria, several archaea and the mentioned-above subclade of eukaryotic *Leishmania* genus. The P1 subclade contains the SatP from bacteria *E. coli* [14] and AceP (this study) from archaea *M. acetivorans*, two transporters functionally characterized as acetate transporters and two *M. acetivorans* homologs not functionally characterized (MA_0103 and MA_4393). It is important to note that while prokaryotic homologs dominate the tree, only 15% of the genomes in the database contained at least one member of the AceTr family (14% in bacteria and 31% in archaea), reflecting mostly the large numbers of sequenced prokaryotic genomes. The eukaryotic branch has a deep divergence between homologs present in fungi and other eukaryotes, *Leishmania sp., Thalassiosira pseudonana* (a marine diatom) abd *Cyanidioschyzon merolae* (red algae). The presence of this deep clade suggests a probable more ancient presence in a wider range of eukaryotes. Homologues belonging to the AceTr family were detected in 97% of the genomes analyzed in fungi. The large clade splits into two subclades. The minor (E2) holds mostly homologues present in

filamentous ascomycetes. A second one (E1) includes homologues from a wider taxonomic range of fungi, including Basidiomycota and a few filamentous Ascomycetes, namely experimentally tested homologues in *Aspergillus nidulans AcpA, AcpB, and AcpC*. In the remaining clade, most sequences were mainly detected in Saccharomycetes, including a large sub-clade formed by Saccharomycetales only, although one subclade includes the AcpA and AcpB. The large clade containing only Saccharomycetales has the three homologues of *S. cerevisiae*. Ady2 and Fun34 are very similar phylogenetically with Ato3, suggesting a duplication and evolution of the gene dating to an earlier divergence within the Saccharomycetales evolution. This duplication probably occurred before the whole genome duplication event in yeast, since the yeasts *Kluyveromyces marxianus* and *Kluyveromyces lactis* have homologs in a cluster together with both Ady2/Fun34 and Ato3 from *S. cerevisiae*. This major clade of Saccharomycetales also holds the homologues of *Yarrowia lipolytica*, including the Gpr1 protein.

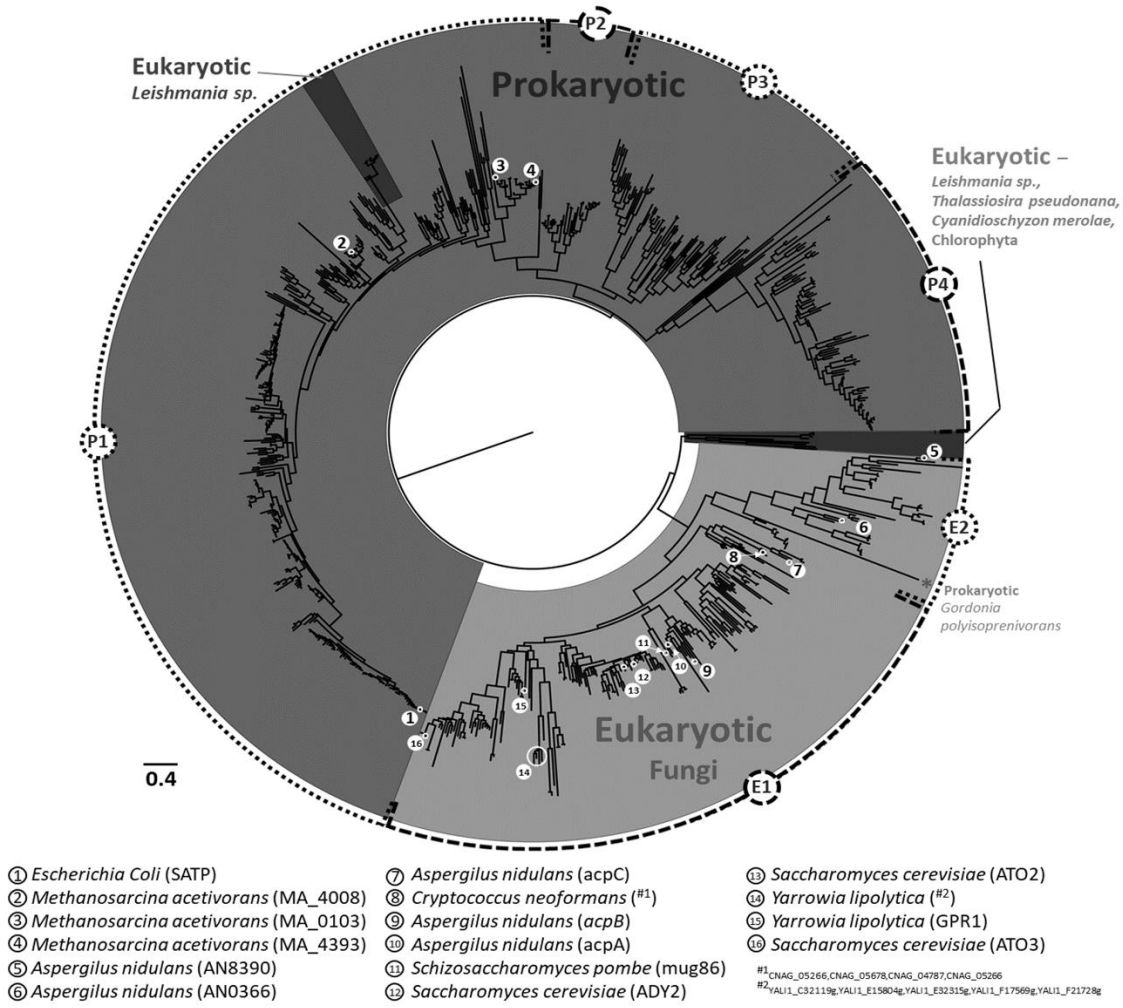


Figure 1. Maximum likelihood phylogenetic tree of AceTr family (TCDB 2.A.96) present in eukaryotic and prokaryotic genomes. Branch lengths are proportional to sequence divergence. Major taxonomic groups are indicated in different shades of grey. Homologues relevant for the discussion through the manuscript are highlighted. Groups indicated as E1, E2, P1, P2, P3, and P4 were created to facilitate the following of the tree description in the main text and are not meant to provide any type of classification.

The *aceP* gene codes for an acetate permease

Methanosarcina acetivorans uses acetate as a source of carbon and as a source of energy by breaking down acetate to produce carbon dioxide and methane. It was previously shown that *aceP* gene from *M. acetirovans* is overexpressed in the presence of acetate (Rohlin and Gunsalus, 2010). The *M. acetivorans* species has an optimal growth temperature between 35 ^{o}C and 45 ^{o}C and optimal pH between 6.5 and 7.0 [30], close to the model organism *E. coli,* which led us to attempt the heterologous

expression of *aceP* in this last organism. In addition, the tools for genetic manipulation of *M. acetivorans* are scarce, and culture conditions are difficult to achieve. The heterologous expression of *aceP* was performed in the *E. coli 3*Δ strain, deleted for three monocarboxylate transporters, ActP an acetate transporter [31], LldP lactate transporter [32], and SatP acetate/succinate [14]. An optimized version of *aceP* was created using the GeneScript codon usage adaptation algorithm (GeneScript, USA). The vector pAceP was transformed in the *E. coli 3*Δ strain, and the acetate uptake measured after growth in minimal medium with glucose as a carbon source, during the exponential growth phase. The *E. coli 3*Δ pAceP was able to recover the mediated transport of acetate, with a Michaelis-Menten kinetics, displaying a K_m of 0.49 ± 0.07 mM of acetic acid and a V_{max} of 46.4 ± 2.9 nmol min^{-1} mg^{-1} protein (Figure 2A).

The energetics of the acetate uptake in the *E. coli 3*Δ pAceP strain was further evaluated. We verified that the protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), which collapses the proton motive force, lowered acetate transport to almost negligible values at pH 6.0 in cells expressing AceP (Figure 2B). The potassium ionophore valinomycin and the sodium ionophore monensin, which disrupt the membrane electrical potential ($\Delta\psi$), had no significant effect on acetate uptake. These data suggest that acetate uptake occurs by a proton symporter mechanism, similar to other AceTr acetate transporter members yet functionally characterized: AcpA, AcpB, Ady2 and SatP. The measurement of labelled acetate uptake in the presence of non-labelled acids allowed the identification of potential inhibitors of acetic acid uptake, being indicative of an interaction of these acids with the transporter protein. None of the carboxylic acids tested inhibited the acetate uptake in *E. coli 3*Δ cells expressing *aceP* (Figure 2C). Thus, this acetate transporter is not able to bind the carboxylic acids here tested, namely the monocarboxylates lactate, pyruvate and formate, and the dicarboxylates malate and succinate.

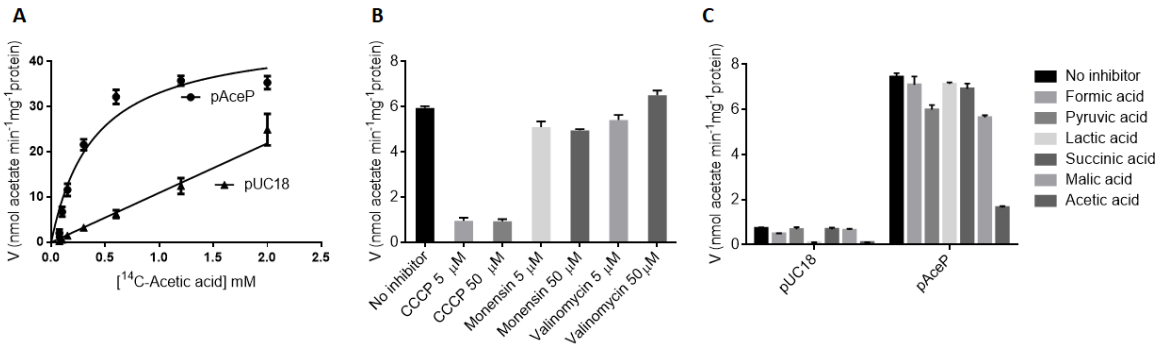


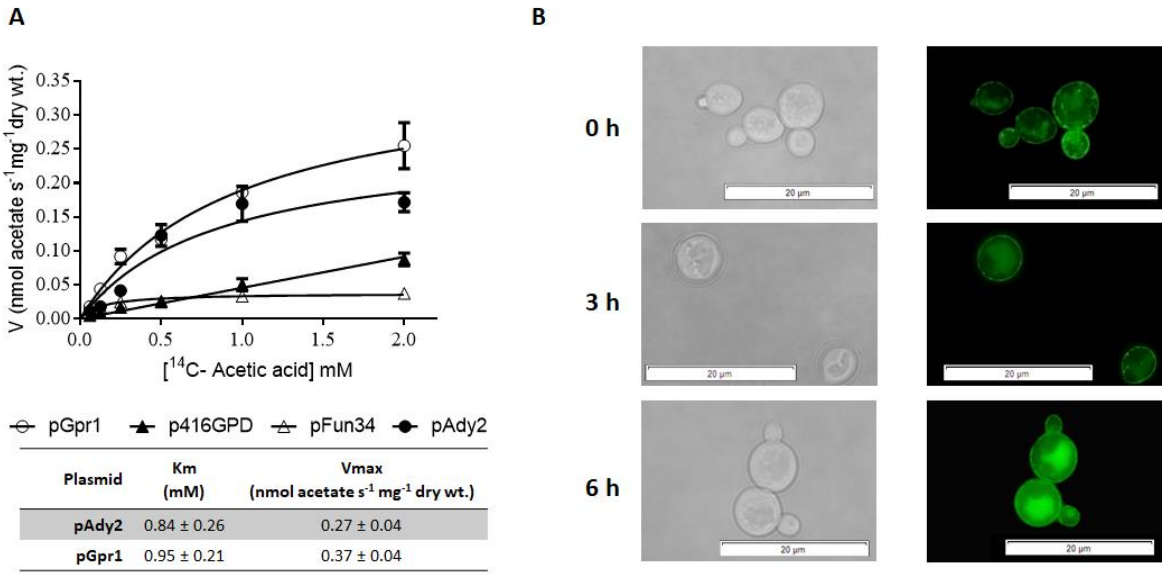
Figure 2. Acetate uptake and transport energetics of *E. coli 3*Δ heterologously expressing pAceP: A) Initial uptake rates of radiolabelled ^{14}C-acetic acid at different concentration by *E. coli 3*Δ heterologously expressing pAceP or pUC18, at pH 6.0, 30◦C; b) Transport energetics: effect of CCCP, valinomycin, and monensin on the uptake of 0.1 mM ^{14}C-acetic acid in cells of *E. coli 3*Δ pAceP; C) transport specificity: the uptake of 0.1 mM ^{14}C-acetic acid in in *E. coli* 3Δ heterologously expressing pAceP or pUC18 measured in the absence and presence of non-labelled formic, pyruvic, lactic, succinic and malic acid (10 mM), pH 6.0 and 30◦C. Cells were pre-incubated with the compounds mentioned, at the concentration indicated and pH 6.0 for 1 min before adding the radiolabelled substrate. Each data point represents the mean ± S.D. for three independent experiments (n =9).

Gpr1 mediates acetate transport in *S. cerevisiae*

The functionality of the Gpr1 membrane protein from *Y. lipolytica* was accessed through heterologous expression in the *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ, a strain, which does not have activity for plasma membrane carboxylate mediated transport under the conditions tested [20, 21, 33, 34]. Mid-exponential *S. cerevisiae* cells expressing *GPR1* grown on glucose (minimal medium YNB-Glu) were collected, washed and incubated for 6h in minimal medium containing 0.5% acetic acid (pH 6.0), as sole carbon and energy source. The expression of Gpr1 was able to restore the acetate-mediated transport in *S. cerevisiae jen1*Δ *ady2*Δ strain with a K_m of 1.13 ± 0.32 mM and V_{max} of 0.41 ± 0.06 nmol acetate s^{-1} mg^{-1} dry weight, at pH6.0 (Figure 3A). A GFP-tagged version of Gpr1p was analyzed by fluorescence microscopy revealing that the fusion protein was localized at the plasma membrane in glucose and acetic acid grown cells for at least 6 hours (Figure 3B).

Fun34 does not transport acetate

Following the methodology described in the previous section, the activity of the Fun34 protein, the closest homolog of the acetate transporter Ady2 from *Saccharomyces cerevisiae*, was accessed by constitutive expression in the *S. cerevisiae jen1*Δ *ady2*Δ strain. The results revealed that the overexpression of Fun34 does not restore the mediated acetate transport at pH 6.0 (Figure 3A).



Plasmid	Km (mM)	Vmax (nmol acetate s^{-1} mg^{-1} dry wt.)
pAdy2	0.84 ± 0.26	0.27 ± 0.04
pGpr1	0.95 ± 0.21	0.37 ± 0.04

Figure 3. A) Initial uptake rates of radiolabeled ^{14}C-acetic acid at different concentrations by *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ cells heterologously expressing pGpr1, pAdy2, pFun34 and p416GPD as a control at pH 6.0, 30°C and respective kinetic parameters. Cells were cultivated in glucose until exponential growth phase, washed and transferred to YNB supplemented with acetic acid (0.5%) during 6 hours. Each data point represents the mean ± S.D. for three independent experiments (n =9). B) Epifluorescence and contrast phase microscopy of a GFP-tagged version of *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ cells expressing *GPR1*, before and during 6h of exposure of cells to 0.5% acetic acid, at pH6, 30°C. Scale bars = 7.5 µm.

The residues of the conserved motif NPAPLGL(M/S) are crucial for protein activity

The motif ^{89}NPAPLGL(M/S)^{96} (numbers refer to Ady2 sequence) exhibit an extensive conservation among the AceTr family members (. Most of the conserved residues are polar and hydrophobic and thus have the potential to interact with charged substrates, e.g. acetate, succinate, a feature that was recently revealed by the 3D structure recently realized [16]. We performed site-directed mutagenesis both in the Ady2 and SatP conserved motif NPAPLGL(M/S) by replacing each residue by an alanine residue, except for the alanine residues 91 (Ady2) and 10 (SatP), which were both replaced by tyrosine residues. All the single mutations, from both Ady2 or SatP, decreased acetate transport (Figure 4 A, B). Accordingly, all Ady2 mutant alleles displayed poor growth in YNB acetic acid (0.5%) as sole carbon and energy source when compared with the native allele. Since SatP is known to mediate the transport of succinate, we also measured succinate uptake in the SatP mutant alleles (Figure 4B). Similar to acetate uptake, the SatP mutant alleles displayed a succinate uptake capacity significantly reduced when compared to the wild-type allele. In order to distinguish whether this is due to an

incorrect protein location or lack of transporter function, we used mutant alleles expressing an Ady2::GFP chimeric transporter. In all Ady2 mutants, proteins were localized by epifluorescence microscopy at the plasma membrane (Figure 5). These results strongly suggest that in these mutants presenting low or no transport capacity, the lack of transporter activity is not a consequence of altered protein trafficking, but rather due to a modification on the mechanism of substrate transport *per se*.

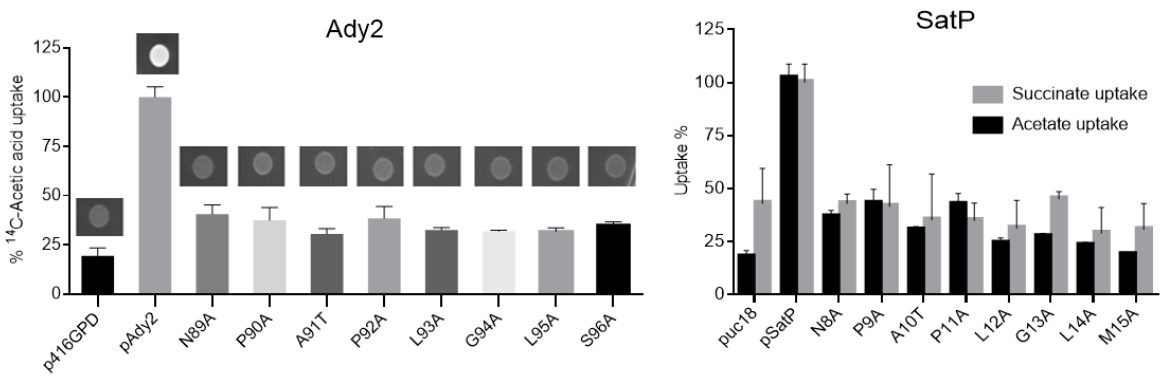


Figure 4. A) The percentage of 1 mM ^{14}C-acetic acid uptake, at pH 6.0, considering the velocity of transport found for the *S. cerevisiae ady2*Δ *jen1*Δ strains expressing pAdy2 as 100%. Cells were cultivated in glucose until mid-exponential growth phase, washed and transferred during 6 h to YNB supplement with the acetic acid (0.5%) and solid YNB acetic acid (0.5 %) for growth tests shown in the images above each bar of Ady2 alleles and in p416GPD. B) The percentage of 0.5 mM ^{14}C-acetic acid uptake, at pH 6.0, considering the velocity of transport found for the *E. coli 3*Δ strains expressing pSatP as 100%. Cells were collected at mid-exponential growth phase from minimal medium with glucose 1% (w/v). Each data point represents the mean ± S.D. for three independent experiments (n =9).

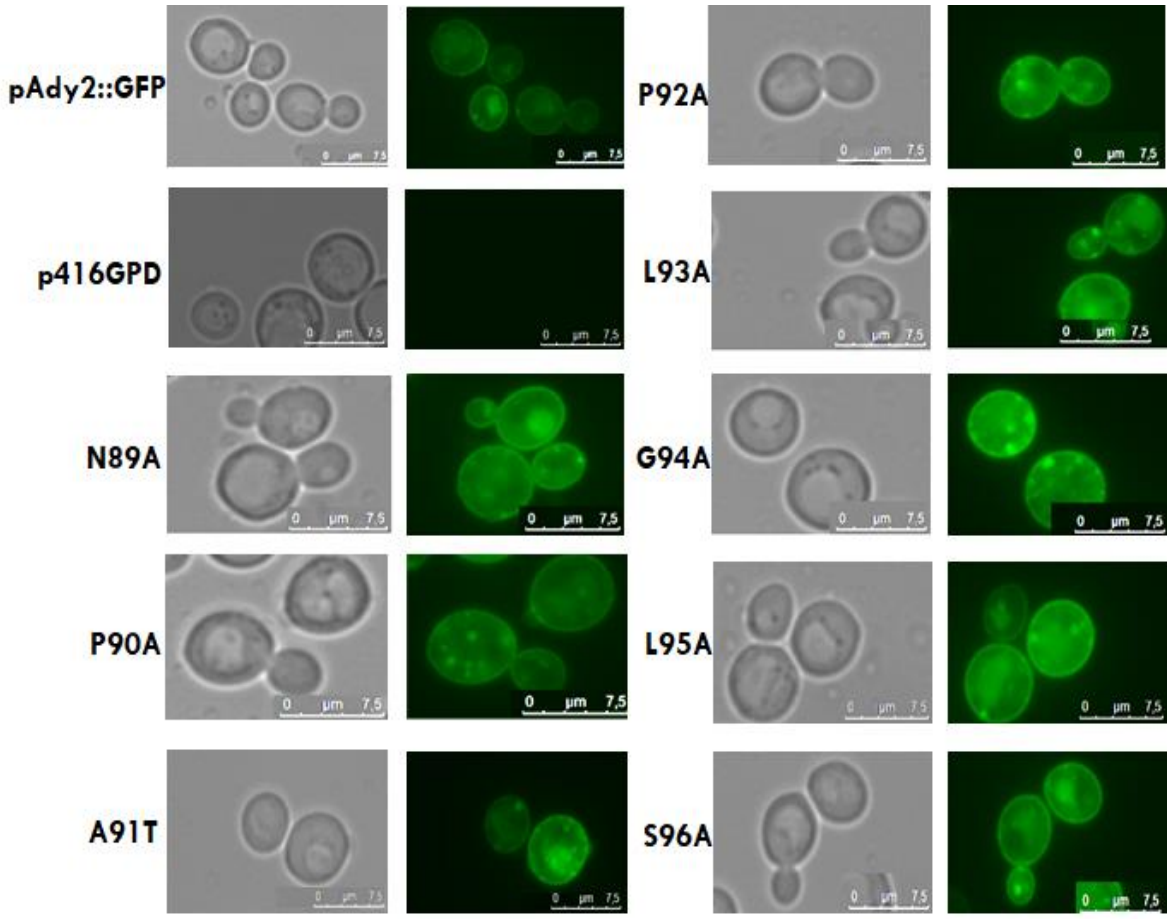


Figure 5. Epifluorescence microscopy localization of Ady2::GFP and mutant alleles expressed in *S. cerevisiae ady2*Δ *jen1*Δ. Cells were collected at mid-exponential growth phase from YNB glucose 1% (w/v), washed and transferred during 6h to YNB, supplement with the acetic acid (0.5%), and observed by epifluorescence microscopy.

SatP 3D model

Molecular docking based on a SatP 3D model obtained from the crystal structure of SatP_Ck (PDB 5YS3) uncovered the residues involved in acetate and succinate binding of the conserved motif NPAPLGLM (Figure 6A, B), located in TMS 1. The residues N8, A10, G13, were identified as putative binding residues in the binding site S1, whereas the residues G13, L14, were identified as putative binding residues in the binding site S2. The SatP model suggests that succinate uses that same binding sites (S1 and S2) as acetate (Figure 6C).

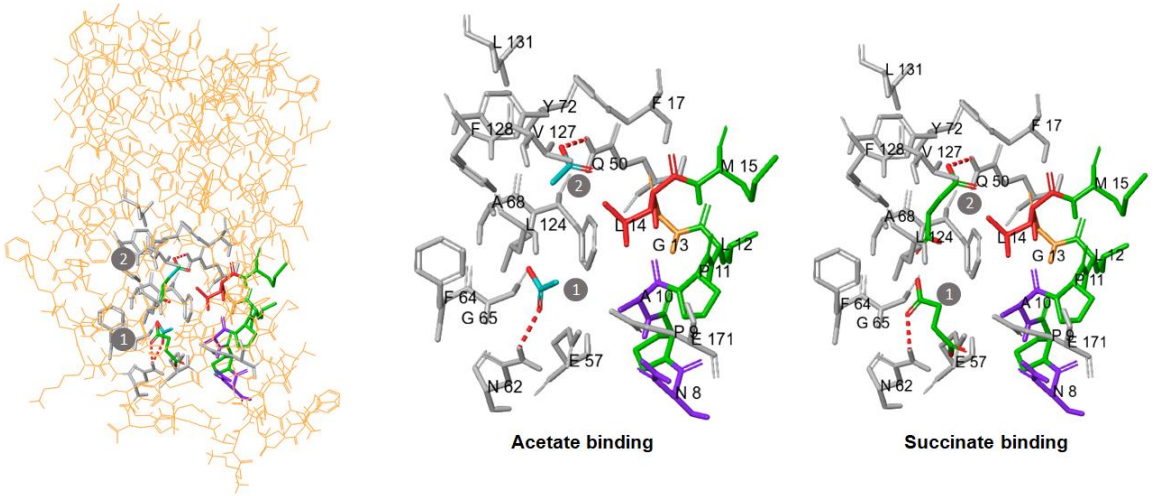


Figure 6. Molecular docking of SatP 3D model, based on SatP_Ck structure, with the substrates acetate and succinate. Transversal (A) and cytoplasmic (B) view of SatP 3D model with the identification of the NPAPLGLM motif colored in side chains. C) Zoomed transversal view of the binding sites S1 and S2 for acetate and succinate, with the putative binding residues of the NPAPLGL motif colored (N8, A10, G13, L14).

The SatP 3D model was used to identify novel residues essential for transporter's activity. A predicted hydrogen bond was found between the residues R122 and Q125 are facing the cytoplasmic side, at the beginning of the TMS 5 (Figure 7A). The replacement of the R122 by an alanine or a valine (the Ady2 equivalent residue) reduced significantly the acetate uptake (Figure 7B). The replacement of the Q125 by an alanine resulted in the abolishment, while the replacement by phenylalanine (the Ady2 equivalent residue) had no impact on acetate uptake (Figure 7B).

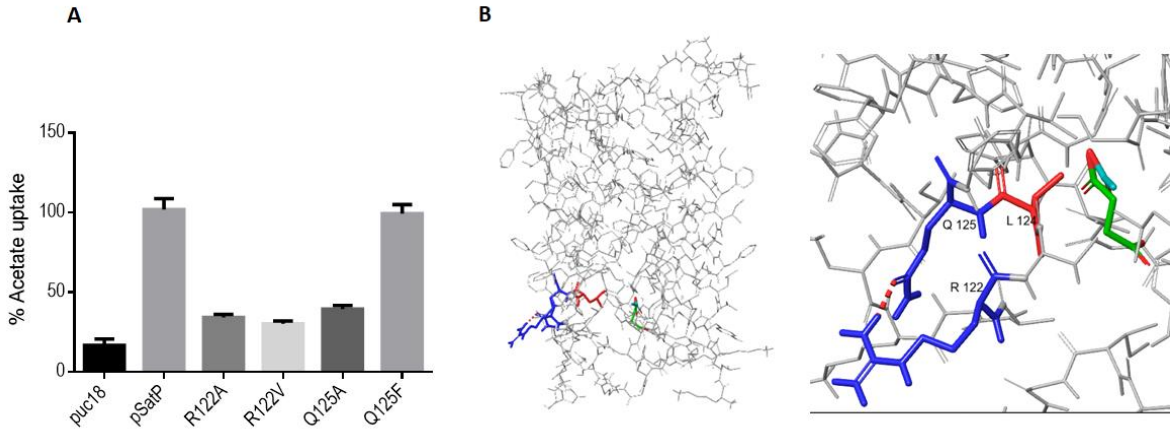


Figure 7. A) The percentage of 0.5 mM ^{14}C-acetic acid uptake, at pH 6.0, considering the velocity of transport found for the *E. coli 3*Δ strains expressing pSatP as 100%. Cells were collected at mid-exponential growth phase from minimal medium with glucose 1% (w/v). Each data point represents the mean ± S.D. for three independent experiments (n =9). B) Transversal and cytoplasmic view of SatP 3D model obtained with the identification of the R122 and Q125 in blue side chains. Dash red line is indicative of hydrogen bonds.

Discussion

The AceTr-Acetate Transporters are found in all domains of life. The phylogenetic analysis of all the homologs so far sequenced, suggests a basal split between eukaryotic and prokaryotic organisms that form two monophyletic clades. Overall, the phylogenetic tree indicates that an ancestral form of this family was present in the primordial organisms in the root of the tree of life. However, although present in a taxonomically diverse range of organisms, the AceTr homologs are limited to very specific groups of prokaryotic and eukaryotic organisms. This fact strongly appoints to their loss in most organisms during evolution. While this trend is obvious for most eukaryotic organisms outside fungi (homologs detected in only 10% of analyzed genomes), the picture is similar for prokaryotic organisms, with members of the AceTr family detected in only 15% of the genomes in the database. On a drastic shift, homologs were detected in 97% of the fungal genomes analyzed. These data suggest that these transporters play a key role in fungi. In fact, the Ady2 from *S. cerevisiae* was described as essential for proper ascus formation [8] and later AcpA from *A. nidulans* as the major acetate transporter during germination of conidiospores [12]. In addition, the Ady2 homolog of *Cryptococcus neoformans* was also associated with acetate transport and described as indispensable for survival during physiologically relevant starvation conditions [35]. According to the phylogenetic analysis, the only fungal genomes without AceTr members were the *Encephalitozoon,* atypical fungi that belong to phylum Microsporidia [36]. Based on this evidence, it is tempting to postulate that this family of transporters is critical for spore germination process in fungi, and thus greatly conserved throughout evolution.

In this work, we extended the number of AceTr members functionally characterized as acetate transporters, and for the first time to an archaea member, the *aceP* gene from *M. acetivorans*. The *Methanosarcineae* are the most metabolically diverse methanogens, that thrive in a broad range of environments, and are unique among the Archaea in forming complex multicellular structures. Species, like *M. acetivorans* display high methanogenesis metabolic activity. In nature, the methane produced is mostly derived from acetate with a strong impact on global warming [37]. Here we demonstrate that the *aceP* from *M. acetivorans* is highly specific for acetate. Additionally, we

characterized the acetate transport capacity of Gpr1 from *Y. lipolytica*, an AceTr family member, until now only described as being involved in cells sensitivity to acetic acid [4]. Gpr1p was reported to have an important role over stress cue, triggered by the exposure of cells to acetic acid, and to be involved in the repression of genes that encode glyoxylate cycle enzymes [4, 5]. Fluorescence microscopy analysis of *S. cerevisiae* cells harbouring Gpr1-GFP revealed that the fusion protein was detected at the plasma membrane, as previously reported [4, 38]. The yeast *Y. lipolytica* displays specific physiological, metabolic and genomic characteristics, which differentiates it from the model yeast *S. cerevisiae* [39]. Although the whole genome duplication event did not occur in *Y. lipolytica*, its genome size is almost the double of *S. cerevisiae* having six AceTr members. Nevertheless, Gpr1 and Ady2, two of the AceTr members of these phylogenetically distant yeast species, maintained their activity as acetate transporters over evolution. We also found that the closest homologue of Ady2 in *S. cerevisiae*, the Fun34 membrane protein (sharing 70% amino acid identity with Ady2), is not involved in acetate transport. The differences found in acetate uptake between the strain expressing the empty vector and the strain overexpressing a nonfunctional protein (Fun34) is, most probably, related with the alteration of membrane composition that affects the diffusion of the acid across the plasma membrane.

To gain insights into the structure of AceTr family members, the conserved motif NPAPLGL(M/S) of Ady2 and SatP proteins, was analyzed by site-directed mutagenesis. The substrate uptake in these two proteins was significantly reduced when each of the residues of the domain was mutated. However, these same mutant alleles did not affect plasma membrane targeting of Ady2. Accordingly the recently released SatP_Ck 3D structure, an ortholog of SatP, the conserved motif NPAPLGL(M/S) is located at the cytoplasmic vestibule of the protein, in the vicinity of the first (S1) and second (S2) acetate binding sites [16]. Our molecular docking analysis using SatP 3D model uncovered two putative binding sites accepting both acetate and succinate as substrates. Is it also noticeable that the residues N8, A10, L13 e G14 are the residues involved in the S1 and S2 binding sites, as it is highlighted in figure 6 in red side chains. The N8 residue was previously described as essential for protein activity since the N8A mutant allele lost almost all acetate conductivity [16]. The F17 and L14 residues were reported as essential players in the S2 binding site, determining the flux direction of the acetate anions and creating hydrophobic constriction sites [16]. Our results demonstrate the impact of the NPAPLGL(M/S) conserved residues in acetate uptake and reinforce their role as S1 and S2 binding sites [16]. Here, we also describe the role of SatP R122 and Q125 residues in substrate acetate uptake. According to the SatP 3D model, the charged R122 and polar Q125 form a hydrogen bond (see arrow, Figure 7A) and are located in the cytoplasmic side, in the opposite position of the S1 binding site. Most probably these residues may help to attract negatively

charged acetate/succinate anions to the cytoplasmic vestibule through electrostatic interactions as proposed by Qiu and colleagues (2018). The proposed structure for the SatP_CK protein discloses a hexamer structure, acting as a channel [16]. The monomers of the hexamer are reported to function independently and to transport different anions, such as acetate. The substrate transport is dependent on four binding sites for acetate and regulated by a gated mechanism in the interior of the pore of each monomer. The SatP_CK homologues Ady2, SatP, AceP and Gpr1 have been experimentally reported by our group to function as proton symporters, with the acetate uptake fitting to a Michaelis-Menten kinetics. In addition, proton flux studies (Ady2) and transport energetic assays (SatP, AceP) support their assignment as permeases dependent on proton-motif force [6, 7, 14]. Both the conclusion based on our data and that of Qiu and collegues (2018) are well supported. Although both studies seem contradictory the differences might arise from differences in experimental methodologies. We think that to fully determine whether members of the AceTr family act as channels or permeates further studies are required.

Acetate is a ubiquitous compound used by cells in distinct metabolic pathways [1]. In this work, we demonstrated that the AceTr family is widespread in bacteria, archaea, fungi and nematodes, with a prevalence close to 100% in fungi organisms. Acetate transporters of this family have been reported to play a role in normal sporulation of fungi organisms, development of asci (dyads) [8], survival during physiologically relevant starvation conditions [35], cell and colony morphology, yeast-to-hyphae transition and cell lifespan [4, 5], which explains the wide distribution of AceTr family in the tree of life and specially the elevated prevalence found in fungi organisms. In addition, we disclosed the role of eleven amino acid residues in the substrate transport activity of AceTr transporters, namely the residues of the conserved motif NPAPLGL(M/S), which we propose to be considered the signature motif of AceTr family.

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Chapter VIII

General discussion and future perspectives

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General Discussion

The microbial production of organic acids has become a fast-moving research field due to the versatility of these compounds as platform chemicals. In the last decades, the panoply of biosynthesized organic acids has increased significantly [1, 2]. However, most of these molecules are reported to be toxic to cell factories, as they might cause membrane disruption and perturb metabolism by cytosol acidification and anion accumulation [3-6]. The overexpression of membrane transporters to export organic acids has been suggested to improve the organism tolerance and the extracellular titers [7, 8].

A similar genetic engineering approach has also been exploited to allow the consumption or enhances the assimilation of renewable substrates in cell factories [9, 10]. *S. cerevisiae* is considered one of the most appealing hosts for the production of these platform chemicals, thanks to its tolerance to low pH. This reduces the need to maintain a neutral pH, via alkali addition, and therefore decreases the risk of contamination [3, 8].

Approximately 20-30 % of the genome in an average organism is thought to encode integral membrane proteins, and half of them are predicted to be membrane transporters [11, 12]. In yeasts, the transport of mono- and dicarboxylic acids is mainly carried out by a well-known group of plasma membrane proteins, belonging to the Jen1 family [13, 14], firstly described in *S. cerevisiae* [15]. The ScJen1p is a member of the Major Facilitator Superfamily, contains 12 TMS, localizes at the plasma membrane, and it is regulated through a complex pathway of different expression stages [13, 16, 17]. Besides its role as lactate; pyruvate; acetate; propionate/H^+ symporter, ScJen1p is also involved in selenite and 3BP drug transport [18, 19]. Some species, like *C. albicans* and *K. lactis* in addition to Jen1 monocarboxylate transporter, also possess a Jen2 protein, upregulated when exposed to dicarboxylic acids, namely malate, and succinate [13, 14]. Jen1 family members share a conserved motif at the seventh TMS, NXX(S/T)HX(S/T)QDXXXT, which is involved in transport activity and substrate affinity [20, 21]. Another family associated with the transport of organic acids in microorganisms is the AceTr family, which is found in archaea, bacteria, and eukaryotes. AceTr members share conserved motifs, namely the amino acid residues sequences NP(A/V/G)P(L/F/V)GL [22]. The *ADY2* along with *ATO3* and *FUN34* genes of *S. cerevisiae* are members of the AceTr Family [23]. *ADY2* was first characterized as a gene required for proper ascus formation on a sporulation

medium in which acetate was the main carbon source [24]. Transport and microarray analyses of a Δ*ady2* mutant strain unravelled its role as an active acetate transporter [25]. Other reports associate *ADY2* and the two homologue genes *ATO3* and *FUN34* with ammonium export [26].

In this work, we focused on the functional and structural characterization of Jen1 and AceTr family members:

- **Expression of DhJen1 homologues in *S. cerevisiae jen1*Δ *ady2*Δ restores carboxylate transport.**

In chapter 3, we address the functional characterization of the ScJen1 homologues of *D. hansenii* by heterologous expression in a *S. cerevisiae jen1*Δ *ady2*Δ strain with no carboxylate transport. Four *ScJen1* homologues *D. hansenii* and showed that these proteins are functional carboxylate transporters in *S. cerevisiae jen1*Δ *ady2*Δ strain: an acetate (Dh27), a malate (Dh17) and two succinate transporters (Dh18; Dh24). These transporters differ from previously identified carboxylate transporters in their range of substrate specificity since they display high specificity for the uptake of acetate, malate and succinate. Whereas, it was reported that the ScJen1 has specificity for lactate, acetate, pyruvate, and propionate [15].

Although *D. hansenni* presents a mediated transport system for lactate, we did not find any functional lactate transporter in this study. Additional studies will be required to identify *D. hansenii* lactate permease encoding gene as it remains unidentified. Other carboxylate transporter family members, such as AceTr and MCT families, should be screened to find the protein(s) involved in lactate transport in this yeast.

- **The Jen1 homologues were found to have specificity for sugar acids**

Our work focused on the identification of yeast carboxylate transporters belonging to Jen family with specificity for sugar acids with biotechnological interest.

We found robust evidence that saccharate is a substrate for ScJen1-S271Q and KlJen2 transporters, gluconate for CaJen2 and KlJen2, and xylarate and mucate of CaJen2. This work envisaged the application of these transporters, as exporters of these compounds, while it did not evaluate directly the export activity of these proteins. Although considering the chemical structure of sugar acids, it is likely that the transport of these molecules is driven by an active mechanism, similarly to mono- and dicarboxylates [15, 27, 28]. Since the ScJen1 permease and other Jen1 homologues transport their substrates by a proton symport mechanism, and similarly to other permeases, it is possible to reverse

the uptake activity if the substrate gradient and/or proton motive force favours this mechanism [15, 27-29].

The transporter proteins identified in this study as sugar acid transporters could be (over)expressed in industrial production organisms, particularly in *S. cerevisiae* strains, in which acid accumulation and tolerance is a bottleneck to produce these acids.

However, succinate transport in *C. albicans* and *K. lactis* was inhibited in the presence of xylonate, we did not find any Jen transporter with specificity for xylonate, suggesting the presence of an alternative transport system for succinate that binds xylonate.

- **The Cn04 membrane protein is responsible for enhanced 3BP sensitivity in *C. neoformans* pathogen**

In this study, we uncovered the role of a Jen1 homologue in 3BP transport and sensitivity in the pathogen *C. neoformans*. As shown in chapter 5 we showed that one of two screened proteins, namely Cn04, plays a role as a 3BP transporter in *C. neoformans* cells. Also, it is also a functional 3BP membrane transporter in the *S. cerevisiae jen1*Δ *ady2*Δ strain. Like Jen1 and MCTs [19, 30], Cn04 play a critical role in the mechanisms of action of 3BP, as the first player to ensure the entrance of this drug into the cells. These results highlight the role of carboxylate transporters in 3BP uptake already reported in microbial cells, as well as in mammalian cells, and open new treatment approaches in the pharmaceutical field: the assessment and identification of Jen1 homologs in other pathogen species could open new possibilities to use 3BP as an active antimicrobial agent.

- **The *E. coli* AceTr member transports both succinate and acetate**

In chapter 6, we reported that YaaH behaves as a secondary active acetate/proton symporter and considering the role of the YaaH protein in acetic and succinic acid uptake, we proposed new nomenclature for this permease, SatP, a Succinate-Acetate Transporter Protein.

In this work, we proved that acetate transport in *E. coli* occurs driven by two proteins, ActP and SatP. Whereas SatP is active during the exponential phase of growth, before the acetate switch, ActP is active during the stationary phase.

Despite their distinct range of substrates of SatP and Ady2 share common molecular traits on the substrate binding site. Mutations in conserved amino acid residues lead to similar transport-related phenotypes, as we have shown for the gain of function for lactic acid uptake.

- **The *aceP* and *GPR1* genes encode for acetate permeases and phylogenetic analysis of AceTr family uncovers an ubiquitous occurrence in fungi organisms. The NPAPLG (M/S) is the signature motif of AceTr family.**

In Chapter 7, we extended the number of AceTr members functionally characterized as acetate transporters, and for the first time to an archeal member, the *aceP* gene from *M. acetivorans*. Additionally, we characterized the acetate transport capacity of Gpr1 from *Y. lipolytica*, an AceTr family member.

Moreover, we report the motif NPAPLGL(M/S) as a 'signature' motif of this group of transporters, since substitutions in the residues of this domain abolished the transport activity and resulted in poor growth in acetate, as sole carbon and energy source, although membrane targeting and stability was not affected by Ady2 mutant alleles.

According to this study, the members of AceTr family were lost during the evolution of both eukaryotic and prokaryotic organisms, excluding few exceptions. It is biologically reasonable to consider that evolutionary pressures led to alternate physiological processes to evolve from a catalogue of alternatives, present in ancestral organisms (especially in prokaryotic organisms characterized by their evolutionary plasticity). Nevertheless, we must consider the presence of AceTr family members in almost all analysed fungi organisms, which suggests the existence of a specific and essential role in fungal evolution, possibly in sexual reproduction through sporulation.

Final remarks and future perspectives

Currently, the knowledge on carboxylate transporters is of significant importance taking into account that the substrates of these proteins are among the most highly valued compounds. The present work underlined the wide range of applications of carboxylate transporters in the biotechnology industry, increased the list of microbial carboxylate transporters characterized and gave new structural and phylogenetic insights on the AceTr family.

This work reported the functional characterization of several carboxylate transporters belonging to Jen1 and AceTr family: the four *D. hansenni* Jen1 homologues (Chapter 3)*;* the Cn04 3BP transporter from *C. neoformans* (Chapter 5)*;* the SatP transporter from *E. coli* (Chapter 6); the AceP membrane protein from *M. acetirovans* (Chapter 7)*;* the Gpr1 acetate permease from *Y. lipolytica* (Chapter 7). Moreover, new substrates were reported - sugar acids - for other previously characterized Jen1 homologues, namely ScJen1-S271Q, KlJen2, CaJen2 (Chapter 4).

Future work should focus on the exploitation of the above-mentioned carboxylate transporters in *S. cerevisiae* engineered strains producers of organic acids and sugar acids. This work would evaluate

the effect on the extracellular titers of organic acids upon expression of these carboxylate transporters, to investigate its impact on the productivity and yields of cell factories

Moreover, the information on the identification of new AceTr members and its characterization as acetate transporters should be used to create new cell factories strains with increased tolerance to acetic acid. Extracellular acetic acid accumulation and toxicity during fermentation are reported as one of the major bottlenecks in cell factories productivity [31]. In *S. cerevisiae* it was already demonstrated that *ADY2* deletion improved acetic acid tolerance and resulted in increased ethanol production [32]. We propose deletions of SatP and ActP genes in expression strains of *E. coli*, as well other AceTr members of other industrial microbes to overcome acetic acid toxicity during fermentation.

We also propose for future work to assess the effect of the antimicrobial 3BP drug in other pathogen yeasts expressing Jen1 homologues since this family of transporters has displayed specificity for this compound [19]. This work could potentially suggest the use of 3BP as an alternative to classic antifungals to treat yeast related human infections.

Meanwhile the functional and structural studies of AceTr family members were complemented with a new set of mutations in several conserved residues of Ady2 and SatP (Supplementary Table S1) and currently, the impact of these mutations in acetate growth and transport is under investigation. The recently released structure of an AceTr member, SatP_Ck [33], will provide support to identify further essential residues of AceTr members. as it will help in the validation of the results of the present study presented in chapter VIII, describing the essential role in substrate uptake of the conserved residues NPAPLGL(M/S).

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Supplementary information.

Table S1 – List of mutations in Ady2 and SatP under investigation

Ady2 mutants				SatP mutants
A88V	T146A	D178A	H230A	A33T
F98A	F147A	L191A	R239A	D35P
G129A	T150A	L191I	G242A	K59A
G130A	L152A	W194A	Y254A	D92A
Q133A	C153A	F197A	Y254W	D92E
A136G	S154A	T198A	V260A	Q97A
G137A	Y155A	M211A	T262A	Q97N
E140A	F158A	F212A	Y267A	Q125A
A142K	F158I	F216A	R271A	Q125F
L143K	W159A	F223A		R122V
E144A	W159Y	L226A		R122A
N145A	E177A	L226I		F177N