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Characterization of monocarboxylate permeases in yeast - functional and structural analysis

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Declaration

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(Isabel João Soares da Silva)

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Resumo

Os ácidos carboxílicos de cadeia curta são compostos importantes produzidos de forma natural no decorrer de numerosos processos metabólicos, para além de poderem ser utilizados como única fonte de carbono e energia por diversos seres vivos, incluindo leveduras. É neste contexto que o estudo das permeases de monocarboxilatos se reveste de especial importância, dado que o transporte destes nutrientes através da membrana é um factor essencial no metabolismo da grande maioria das células. Com o trabalho descrito na presente tese procurou-se desenvolver o conhecimento actual sobre permeases de monocarboxilatos de leveduras, através de um aprofundamento do estudo da permease de lactato/piruvato de *Saccharomyces cerevisiae* codificada pelo gene *JEN1*.

A primeira etapa deste trabalho consistiu na expressão heteróloga do gene *JEN1* na levedura *Pichia pastoris.* Este gene foi clonado num vector do tipo integrativo (pPICZB) assim como num vector replicativo vector (pZPARS), e posteriormente expresso em duas estirpes diferentes, Mut^+ e Mut^S. A confirmação da expressão do gene *JEN1* foi efectuada em células induzidas 24 horas em metanol tanto ao nível do mRNA como ao nível proteico. A actividade máxima da permease obtida em células de *P. pastoris* apresentou um V_{max} de 2,1 nmol s^{-1} mg^{-1} peso seco. Este valor representou um aumento de cinco vezes comparativamente com os parâmetros descritos na literatura para células da estirpe selvagem crescidas em lactato. Numa segunda fase, a actividade da permease foi reconstituída em vesículas híbridas, obtidas por fusão de membranas extraídas a partir de células de *P. pastoris* induzidas em metanol e lipossomas de *Escherichia coli* contendo citocromo *c* oxidase, como proteína geradora de uma força proto-motriz. A actividade da proteína reconstituída apresentou propriedades semelhantes à proteína selvagem de *S. cerevisiae* no que diz respeito ao simporte de protões como mecanismo acoplado ao transporte de lactato e também aos inibidores testados. Com este trabalho foi possível demonstrar a funcionalidade da proteína Jen1 de *S. cerevisiae* como sendo um transportador de lactato.

A caracterização molecular de outros genes codificantes de permeases de monocarboxilatos levou-nos ao estudo do transporte de ácidos monocarboxílicos na levedura *Candida albicans.* A presença de uma permease de monocarboxilatos foi identificada em células da estirpe *C. albicans* RM1000 crescidas em lactato. Estudos de inibição demonstraram que o transporte de lactato é inibido competitivamente por piruvato e propionato, mas não por acetato que se revelou ser um inibidor não competitivo. Uma pesquisa de homólogos do gene *ScJEN1* em *C. albicans* revelou a existência de uma ORF com 61% de similaridade que foi designada por *CaJEN1*. Deleções de ambos os alelos de *C. albicans* originaram uma ausência de detecção do *CaJEN1* mRNA e da actividade da permease. Em células da estirpe selvagem *C. albicans* RM1000 crescidas em glucose não se verificou expressão do *CaJEN1* assim como não foi possível detectar actividade da permease. A expressão heteróloga do gene *CaJEN1* foi efectuada na estirpe *S. cerevisiae jen1*Δ, mas não foi recuperado o transporte de ácido láctico. A levedura *C. albicans* possui uma alteração no código genético o que faz com que a proteína CaJen1 ao ser expressa em *S. cerevisiae* possua uma mutação S217L. Foi aplicada a técnica de mutagénese dirigida de forma a restabelecer a sequência proteica original, sendo desta forma recuperada actividade da permease em *S. cerevisiae.* Com este estudo confirmou-se que o gene *CaJEN1* codifica um transportador de monocarboxilatos em *C. albicans*. Estudos adicionais com uma estirpe deletada no gene *CaCAT8* demonstraram que a expressão deste gene influencia a expressão do *CaJEN1,* fenómeno idêntico ao que se verifica com os correspondentes genes homólogos de *S. cerevisisae.*

Na última parte do trabalho efectuou-se uma pesquisa de domínios da ScJen1p envolvidos no reconhecimento/transporte do substrato. Foi efectuada uma análise por mutagénese da sequência ^{379}NXX[S/T]HX[S/T]QDXXXT^{391} em cinco dos aminoácidos conservados. A substituição dos resíduos N379, H383 ou D387, mesmo que por aminoácidos muito similares, provocou uma acentuada redução do transporte de lactato e piruvato mas manteve transporte de acetato mensurável. Ensaios de inibição de transporte de acetato demonstraram que estes mutantes têm a capacidade de ligar mas não de transportar lactato e piruvato. Ensaios de transporte com o duplo mutante H383D/D387H demonstraram a existência de interacções entre estes dois aminoácidos, dado que este exibe uma perda de função para o transporte de lactato e piruvato mas apresenta parâmetros cinéticos idênticos ao Jen1p para o transporte de acetato. As

mutações nos resíduos N379, H383 e D387 afectam simultaneamente a capacidade de transporte e a especificidade da Jen1p. Quanto aos aminoácidos Q386 eT391 o seu efeito parece ser mais ao nível da afinidade de ligação ao substrato do que na capacidade de transporte. Na sua globalidade estes resultados sugerem que a sequência aqui estudada intervém na via de translocação do substrato da permease de lactato/piruvato de *S. cerevisiae*.

Abstract

Short chain carboxylic acids are important compounds that result from normal cell metabolism and that can be used as sole carbon and energy source by different organisms. In this context the study of monocarboxylate permeases is of great significance since the uptake of these nutrients across cellular membranes is essential for the metabolism of most cells. With the work presented in this thesis we seek to increase the current knowledge on yeast monocarboxylate permeases by studying the *Saccharomyces cerevisiae* lactate/pyruvate proton symporter, encoded by the *JEN1* gene.

The first step of this work was the heterologous expression of the *JEN1* gene in *Pichia pastoris. JEN1* was cloned in an integrative vector (pPICZB) and a replicative vector (pZPARS), and expressed in two different strains, Mut^+ and Mut^S. *JEN1* expression was confirmed in 24 hour methanol-induced cells both at mRNA and protein level. Maximum lactate permease activity was obtained in *P. pastoris* cells with a V_{max} of 2.1 nmol s^{-1} mg^{-1} dry weight, representing a 5 fold increase in the permease activity when compared to the wild-type lactate-grown cells. In the second part of this work the lactate permease activity was reconstituted in hybrid vesicles, obtained by fusion of plasma membranes from *P. pastoris* methanol-induced cells with *Escherichia coli* liposomes containing cytochrome *c* oxidase, as proton-motive force. The reconstituted lactate uptake activity presented similar properties with those of the permease evaluated in *S. cerevisiae* in what concerns the proton symporter mechanism and the inhibitors tested. This work demonstrated that *S. cerevisiae* Jen1p is a fully functional lactate transporter.

The molecular identification of other genes encoding monocarboxylate permeases led us to study the uptake of monocarboxylates in the yeast *Candida albicans.* A lactate permease was biochemically identified in *C. albicans* RM1000 lactate-grown cells. Inhibition assays demonstrated that lactate uptake was competitively inhibited by pyruvic and propionic acids but not by acetic acid, that behaved as a non-competitive

substrate. A *ScJEN1* homologue search within the *C. albicans* genome revealed the existence of an ORF possessing 61% similarity, that was named *CaJEN1*. Deletions of both *CaJEN1* alleles resulted in absence of mRNA detection and in the lack of measurable lactate uptake. In the presence of glucose no *CaJEN1* expression and lactate permease activity were detected. Heterologous expression of *CaJEN1* was performed in *S. cerevisiae jen1*Δ strain, but no activity for the permease was achieved with the native *CaJEN1* gene. Due to a difference in the *C. albicans* genetic code, site directed mutagenesis was performed to re-establish the CaJen1p codon 217 as a serine when expressed in *S. cerevisiae*, and permease activity was recovered. This was the confirmation that the *CaJEN1* gene codes for a monocarboxylate transporter. Additionally, studies with a *CaCAT8* deletion strain demonstrated that the *CaJEN1* transcription level is influenced by the expression of this gene, in a similar way to what happens to the corresponding *S. cerevisiae* homologs.

The last part of this work was dedicated to the search for ScJen1p domains involved in substrate recognition/transport. This was performed using a mutational analysis of the conserved sequence ^{379}NXX[S/T]HX[S/T]QDXXXT^{391}. The study focused on five amino acids, charged or polar, and highly conserved. Substitution of amino acid residues N379, H383 or D387, even with very similar amino acids, resulted in a very dramatic reduction of lactate and pyruvate uptake, but conserved measurable acetate transport. Acetate transport inhibition assays showed that these mutants conserve the ability to bind but not transport lactate and pyruvate. The double mutant H383D/D387H, demonstrated the existence of an interaction between these two aminoacids, since it behaves as a total loss-of-function allele for lactate and pyruvate uptake, but fully restores the kinetic parameters of Jen1p for acetate transport. Thus, residues N379, H383 or D387 affect both the transport capacity and the specificity of Jen1p. The Q386 and T391 residues seem to contribute more for substrate binding affinity than transport capacity. Overall the results obtained suggest that the conserved sequence here studied is part of the substrate translocation pathway in the *S. cerevisiae* proton symporter monocarboxylate permease.

Abbreviations

Main abbreviations

5-FOA	5-fluoroorotic acid
$A_{\#\#\#}$	absorbance at ### nm
a.a.	aminoacid
ABC	ATP binding cassette
BLAST	Basic Local Alignment Research Tool
bp	base pairs
ºC	degrees Celsius
Ci	Curie
c.p.m.	counts per minute
Da	Dalton
d.p.m.	disintegrations per minute
Dry wt	dry weight
EC	enzyme commission
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tags
EtBr	ethidium bromide
FCCP	carbonylcyanide p-trifluoromethoxyphenylhydrazone
g	gram
g	gravity acceleration
GFP	green fluorescence protein
h	hour
HPLC	high performance liquid chromatography
IUBMB	international union of biochemistry and molecular biology
K_d	difusion constant
K_i	inhibition constant
K_m	Michaelis-Menten constant
l	Liter
M	Molar
MCF	mitochondrial carrier family
Mch	monocarboxylate homolog
MCS	multiple cloning site
MCT	monocarboxylate transporters
MFS	major facilitator superfamily

min	minute
MOPS	3-(N-morpholino)propane-sulfonic acid
NCBI	National Center for Biotechnology Information
$OD_{###}$	optical density at ### nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDR	pleiotropic drug resistance
Pi	inorganic phosophate
PMF	proton motive force
RNase	ribonuclease
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SGD	*Saccharomyces* genome database
SMF	sodium motive force
TC	transport classification system
TCA	tricarboxylic acid cycle
TCBD	transport classification database
T_m	melting temperature
TMS	transmembrane segment
U	Unit of enzymatic activity
u.p.	ultra pure
UV	ultra-violet light
V	Volt
v; vol.	volume
V_{max}	maximum velocity
w	weight
wt	wild type
YNB	Yeast Nitrogen Base
YTPdb	yeast transport protein database

Nucleic acids

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
A	Adenine
T	Thymine
C	Cytosine
G	Guanine
dATP	2’-deoxyadenosine-5´-triphosphate
dTTP	2’-deoxycitidine-5´-triphosphate
dCTP	2’-deoxyguanosine-5´-triphosphate
dGTP	2’-deoxythymidine-5´-triphosphate
dNTP	2’-deoxynucleotide-5´-triphosphate

Amino Acids

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

Chapter 1

General Introduction

Chapter 1

General Introduction

Nutrient transport across the plasma membrane

The maintenance of an equilibrated intracellular environment is of crucial importance to cell homeostasis being this equilibrium strongly dependent on the activity of several transport systems located mainly at the plasma membrane level. Simple diffusion also contributes, to a lesser extent, to the transport of molecules that cross biological membranes. In this mechanism there are no membrane proteins or transporters involved, it is the simplest process of transport, also designated by non mediated, and it is most relevant for lipophilic substrates where it can account for 10% of the total flux (Jarvis, 2000). In contrast, the transport involving proteins is designated as mediated. Transport systems intervene in several processes, being the most important the entrance of essential nutrients to the cytoplasm and from there to other organelles, enabling the metabolism of exogenous metabolites and molecules. They are also involved in the excretion of metabolic end products, toxic substances, as well as in cell defence against extracellular substances. Additionally,

transport systems also permit nucleic acids transfer between organisms, allowing genetic transfer and thus enabling species diversification (for a review see Saier, 2000).

Permeases are proteins or protein complexes that assist the movement of substances across cellular membranes. Although they can catalyze a chemical or electron transfer reaction that impels the substance movement they are not permanently altered in the process. In what concerns the energetics of a transport system this can be classified as passive or active transport. The first type is energy independent, the substances cross membranes according to their gradient and additionally, in the case of charged molecules, to the electric potential generated across the membrane. It includes simple diffusion, facilitated diffusion and diffusion trough channels. In contrast, a transport is considered active when the solute is transported against its chemical and/or electrical gradient. Active transport can be divided in two types according to the type of energy used. Primary active transport uses a primary source of energy, like a chemical reaction, light absorption or electron force. When a secondary source of energy such as an ion electrochemical gradient is used, the transport is named secondary active transport. The electrochemical gradient is generated at the expense of a primary active transport and the most common electrochemical gradients are the Proton Motive Force (PMF) and the Sodium Motive Force (SMF) (Harold, 1986). Active transporters can be divided in three groups: uniporters that transport only one molecular species; symporters, that transport two or more substrates in the same direction; antiporters, that catalyze the exchange of one or more substances for another (Figure 1).

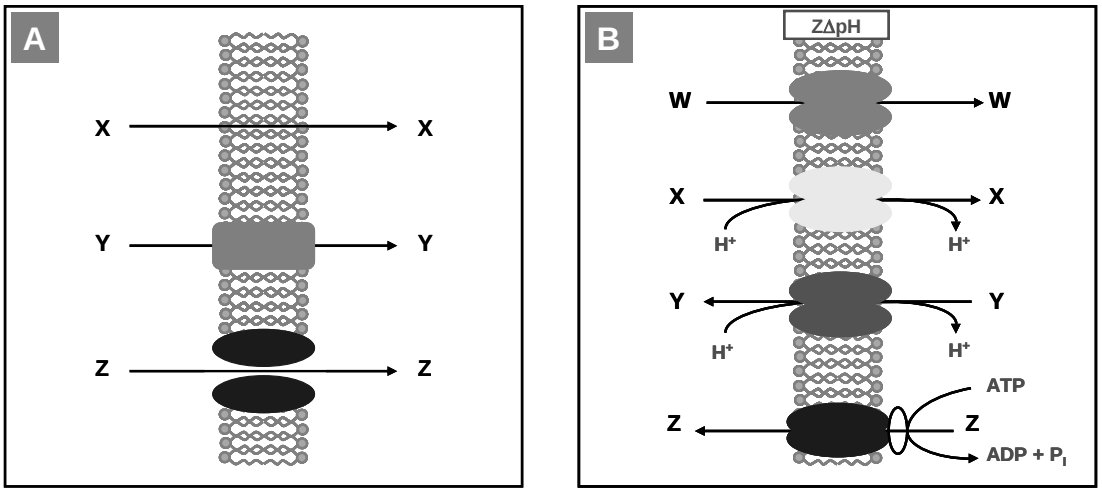

Figure 1 – Schematic representation of different types of transporter mechanisms. A) Passive transport: (from top to bottom) passive diffusion, facilitated diffusion and diffusion trough channels; B) Secondary active transport: (from top to bottom) uniporter, symporter, antiporter and primary active transport.

With the growing number of fully sequenced genomes, researchers all over the world realized that membrane proteins have a crucial role in metabolic context, since they account for roughly one third of the total proteins encoded in genomes (Loll, 2003). For instance, in *Saccharomyces cerevisiae*, the first eukaryote organism to have its genome fully sequenced, it is estimated that 30% of the proteome are membrane proteins, from which 6% is responsible for the transport of small molecules trough the plasma membrane (Paulsen *et al.*, 1998; Van Belle and André, 2001). The characterization of all yeast transport proteins or the entire yeast "transportome" is of great importance to the understanding of the role of transport systems in the eukaryotic metabolism.

The Transporter Classification system

With the increasing number of functionally characterised transport systems and the breakthrough of gene sequencing technologies revealing the primary structures of transmembrane proteins, the importance of elaborating a classification

system for these proteins became crucial. Most of the hydrophobicity analysis performed in transporters revealed the strikingly hydrophobic nature of different types of integral membrane transporters (Saier, 2000). Current approaches involving multidisciplinary teams are slowly obtaining information about the three dimensional structure of transporters, and recent advances have determined the high resolution structure of one of the most extensively studied transporter, the lactose permease from *Escherichia coli*, *LacY* (Abramson *et al.*, 2003).

It is recognized that highly homologous proteins will generally have a very similar three dimensional structure. This is in accordance with the fact that the degree of similarity of a protein's tertiary structure correlates with the degree of similarity of the primary structure. For this reason, Saier and co-workers have developed a classification system for transmembrane solute transporters, named Transport Classification system (TC - http://www-biology.ucsd.edu/~msaier/transport/). This system takes into account previous work devoted to the phylogenetic characterization of permease families (Saier *et al.*, 1998) and it is based on the classification system developed for enzymes by the Enzyme Commission (EC). In contrast to the EC, based solely on the function, the TC is based both on functional and phylogeny criteria and was designed to provide a classification system for all transmembrane transport proteins found in living organisms. The TC system was adopted by the International Union of Biochemistry and Molecular Biology (IUBMB) as the internationally acclaimed system for the classification of transporters (Busch *et al.*, 2004).

The Transporter Classification Database (TCDB - http://www.tcdb.org/) is a web database where the transporter classification (TC) system data is available. It contains sequence, classification, structural, functional and evolutionary information about transport systems from a variety of living organisms. The TCBD contains information compiled from 10000 references, referent to approximately 3000 representative transporters and putative transporters, classified into 400 families, presenting statistical analyses of these families and their constituent proteins. Additionally, it identifies transporter types for size and topological differences and analyzes the families for the numbers and organism sources of their constituent members (Saier *et al.*, 2006).

The TC system uses a four digit classification system for each protein. The first digit classifies permeases according to type of transport and energy coupling

mechanism, being each category assigned with a one-component TC number (W). The secondary level of classification corresponds to the phylogenetic family (or superfamily) to which a particular permease is assigned, and each family is assigned a two-component TC number (W.X). The third level of classification refers to the phylogenetic cluster within a family (or the family within a superfamily) to which the permease belongs, and each cluster receives a three-component TC number (W.X.Y). Finally, the last level of categorization is based on substrate specificity and polarity of transport, and each protein is assigned a four component TC number (W.X.Y.Z) (Saier, 2000).

Several families were grouped in large Superfamilies like the Voltage-gated Ion Channel superfamily VIC (TC 1.A.1), the Major Facilitator Superfamily MFS (TC 2.A.1) and the ATP-binding cassette (ABC) superfamily (TC 3.A.1) (Saier, 2000). These two last superfamilies occur ubiquitously in all living organisms, and account for almost half of the solute transporters encoded within the genome of microorganisms, being also prevalent in higher organisms (Saier *et al.*, 1998). The permeases belonging to the ABC superfamily are mostly multicomponent primary active transporters, capable of transporting both small molecules and macromolecules at the expense of ATP hydrolysis. The MFS transporters are single-polypeptide secondary carriers that transport small solutes in response to chemiosmotic ion gradients. This family was initially believed to function primarily in the uptake of sugars, but further studies revealed the existence of other kinds of transporters, such as drug efflux systems that were further subdivided in two different families based on topological characteristics (Goffeau *et al.*, 1997).

In 1998, and due to an increasing degree of diversity within the MFS, Saier and co-workers (Pao *et al.*, 1998) systematically identified the sequenced proteins that belonged to the MFS Superfamily. This study pointed out that typically the members of the MFS presented a very similar topology: two sets of 6 Transmembrane Segments (TMS) connected by a cytoplasmatic loop. Seventeen families were initially proposed in this study, but the MFS has been enlarged to 37 distinct families by the TC system in 2002. Presently, the Transport Classification Database consideres 49 families within the Major Facilitator Superfamily.

Transport and metabolism of carboxylic acids in *Saccharomyces cerevisiae* – physiological aspects

Short chain organic acids are weak acids that are partially dissociated in an aqueous system, being the degree of dissociation dependent on acid pKa and the pH of the medium. Previous studies performed in different biological systems have demonstrated that the transport of weak carboxylic acids can occur mainly by two different manners: the undissociated form by simple diffusion, and the anionic form by a mediated transport system. At low pH the undissociated form is favoured and, being lipid soluble, it crosses the plasma membrane by simple diffusion. Once inside the cell the acids dissociate given the neutral intracellular pH. The acid anion will tend to accumulate intracellularly to very high levels as, being charged, it cannot readily diffuse out of the cell. Consequent proton release results in a decrease of the intracellular pH that can cause alterations in several metabolic pathways (Krebs *et al.*, 1983) (Figure 2). Additionally, it can also influence free radical production, leading to the severe oxidative stress, which is a major component of weak organic acid stress in aerobic *S. cerevisiae* (Piper *et al.*, 2001).

The yeast *S. cerevisiae* is able to use several carbon substrates as a source of cellular biomass and metabolic energy. Monossacharides such as glucose and fructose are among the preferred substrates, but yeast cells are also able to use nonfermentable carbon sources like glycerol, ethanol and short chain weak carboxylic acids, namely lactate and acetate (Barnett and Kornberg, 1960; Barnett, 1976). At the industrial level these acids play a very important role as they may constitute final products or subproducts of fermentative processes (Radler, 1993). Another important aspect concerning this group of compounds is the use of carboxylic acids such as benzoic, sorbic, acetic and propionic acids as food and beverage preservatives, avoiding the growth of contamination microorganisms (Holyoak *et al.*, 1999).

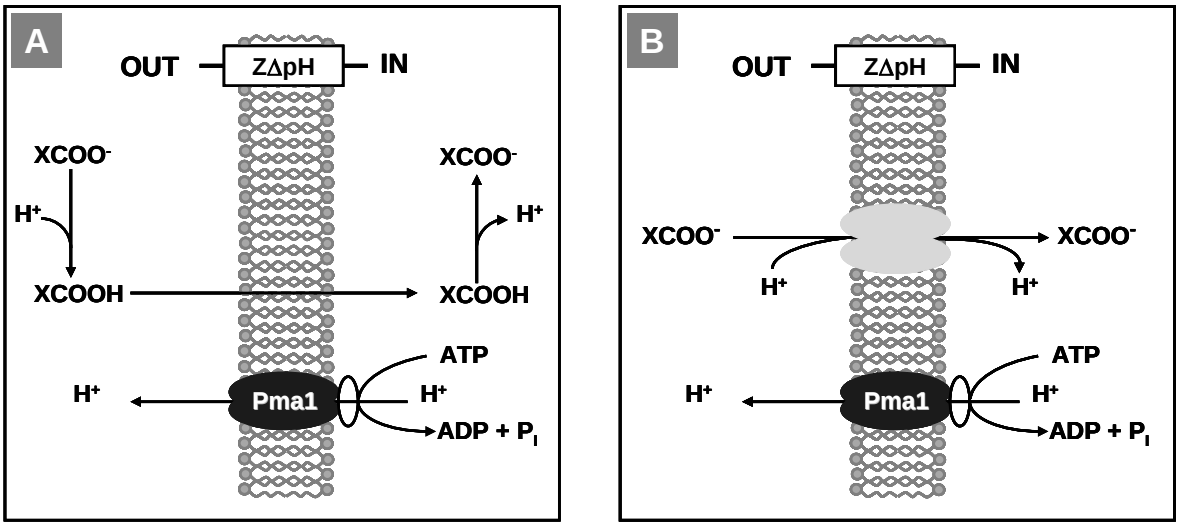

Figure 2 - Schematic model for the transport of carboxylic acids in yeast cell. **A**) A protonated, uncharged form of the acid (XCOOH) is shown as freely permeable to the cell membrane and readily entering by diffusion. The concentration of the undissociated form of the acid inside and outside should be the same, regulated by the intra and extracellular pH and the acid dissociation constant. A higher pH on the cytosolic side of the membrane will cause a substantial fraction of this acid to dissociate to the anion ($XCOO^-$), a form which is relatively membrane-impermeant and that therefore will accumulate inside the cell. **B**) The dissociated acid ($XCOO^-$) requires the presence of a transporter protein to cross the plasma membrane. In the majority of weak acid transporter systems a proton symporter mechanism occurs (see text). The electrochemical potential difference across the plasma membrane ($Z\Delta pH$) is maintained largely through plasma membrane H^+-ATPase (Pma1)-catalysed proton extrusion (adapted from Piper *et al.*, 2001).

The utilization of carboxylic acids as sole carbon and energy source depends on the transport of the nutrient trough the plasma membrane and the existence of adequate metabolic processes for degradation. The use of nonfermentable substrates requires aerobic conditions. Under these conditions the oxidative metabolism of mitochondria produces ATP and also the gluconeogenesis regenerates sugar phosphates for biosynthetic purposes. Additionally, biosynthetic intermediates must be generated trough anaplerotic pathways such as the glyoxylate cycle and pyruvate carboxylate (Gancedo and Serrano, 1989; Gancedo, 1998).

The first step of nonfermentable carbon sources utilization is the transport of the molecules inside the cell. Some substances require the presence of a transporter

and others diffuse freely across the plasma membrane. This is the case of ethanol present in the extracellular medium produced after fermentative growth in glucose media, being consumed after glucose depletion during a second respiratory growth phase (Beck and von Meyenburg, 1968). Once inside the cell it is metabolised by oxidation and further converted to acetate and subsequently to acetyl-CoA (Figure 3).

Acetate and lactate are monocarboxylates that can be efficiently utilised by *S. cerevisiae* as sole carbon and energy sources, requiring mediated transport systems to cross plasma membrane (Cássio *et al.*, 1987; Casal *et al.*, 1996). Several pathways must be active in order for them to be metabolised, namely the tricarboxylic acid cycle (TCA), gluconeogenesis pathway and glyoxylate cycle. Acetate is converted to acetil-CoA, by the acetyl-CoA-synthase and this way it can be used to generate intermediates of the TCA. The glyoxylate bypass is very important when cells are growing in acetate since this pathway provides 4 and 3 carbon compounds necessary for biosynthesis (Figure 3). As for lactate it is first oxidized to pyruvate by the L-lactate: cytocrome c oxidoreductase, and then it is decarboxylated to acetil-CoA by the pyruvate dehydrogenase complex.

Glycerol, another nonfermentable carbon source, is actively transported inside the cell trough the Stl1p transporter (Ferreira *et al.*, 2005), and is directed to the glycolytic pathway at the level of dihydroxyacetone phosphate (DHAP), that can be converted to pyruvate or to glucose-6-phosphate trough the gluconeogenesis.

The pattern of gene expression is very dependent on the utilized carbon source. These changes allow cells to adapt to the most convenient substrate available. Since glucose is one of *S. cerevisiae*'s preferred carbon sources, its presence controls the pattern of gene expression and the phenomena designated as yeast catabolite repression is observed. In the presence of glucose, genes encoding crucial enzymes of the gluconeogenesis and glyoxylate cycle are repressed, as well as genes from the Krebs cycle and respiratory chain (Gancedo, 1998).

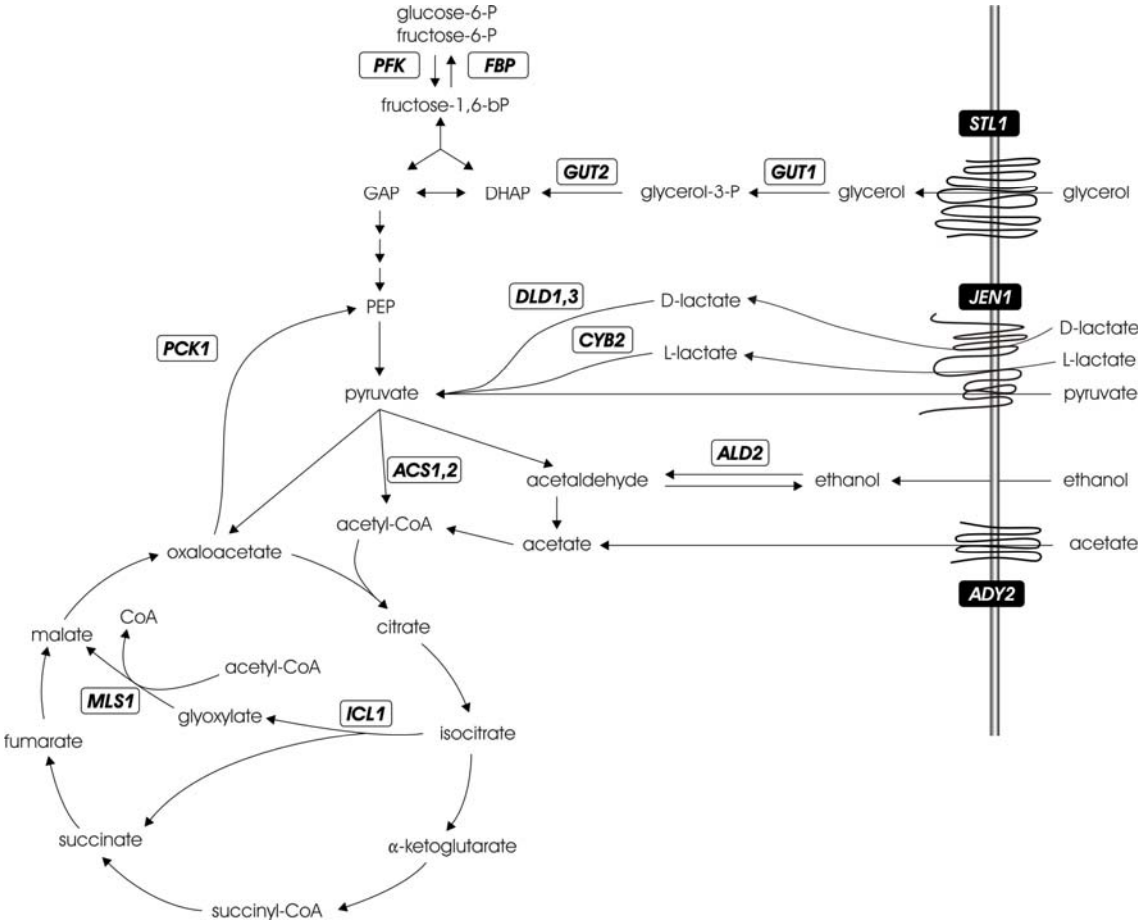

Figure 3 – Schematic representation of the oxidative and gluconeogenic metabolism in *S. cerevisiae*. Some genes involved in the utilisation of non fermentable carbon source are indicated in the figure: STL1- Glycerol proton symporter; JEN1- Lactate transporter; ADY2 - Acetate transporter; GUT1 - Glycerol kinase; GUT2- Mitochondrial glycerol-3-phosphate dehydrogenase; FBP1 - Fructose-1,6-bisphosphatase; PFK1,2- phosphofructokinase; PCK1 - Phosphoenolpyruvate carboxykinase; DLD1,3 - D-lactate dehydrogenase; CYB2 - L-lactate cytochrome-c oxidoreductase; ACS1/ACS2 - Acetyl-coA synthetase; ICL1-Isocitrate lyase; MLS1- malate synthase.

In a medium where glucose is the carbon source fermentation occurs, producing ethanol and enabling a rapid growth. When glucose is consumed, *S. cerevisiae* cells start using ethanol as a carbon source for aerobic growth. A diauxic growth curve is observed, being the nonfermentable carbon source consumed only upon exhaustion of glucose. A metabolic reprogramming occurs during the diauxic shift and the expression patterns of many genes change. mRNA levels for

approximately 700 genes are induced, and the mRNA levels for approximately 1000 genes decline (De Risi *et al.*, 1997). Within the highly induced genes are the ones coding for the enzymes aldehyde dehydrogenase and acetyl–coenzyme A(CoA) synthase which function together to convert the products of alcohol dehydrogenase into acetyl-CoA, which in turn is used to fuel the tricarboxylic acid (TCA) cycle and the glyoxylate cycle. On the other hand the transcription of the pyruvate decarboxylase gene is decreased and the pyruvate carboxylase induced, redirecting pyruvate to oxalacetate, where it can serve to supply the TCA cycle and gluconeogenesis.

Several regulators intervene in the glucose repression phenomena but we will focus only in the main aspects (for a review consult Gancedo, 1998; Carlson, 1999). Glucose repression acts at various levels, e.g. mRNA level, translation rate and protein degradation rate, but the main effect occurs at the transcriptional level. The best characterized transcription factor involved in glucose repression at gene level is the Mig1p, being able to bind to the promoters of a variety of genes and stop its transcription (Ostling *et al.*, 1996). The Mig1 is regulated by Snf1, a protein kinase that is inactive in the presence of glucose and becomes active when glucose is limiting (Wilson *et al.*, 1996). It has a crucial role in the control of gene expression since it is required for the derepression of most genes repressed by glucose. A *snf1* mutant is unable to grow on any nonfermentable carbon source, however conserves an apparent normal growth in the presence of glucose. When Snif1p is active the inactivation of the Mig1 occurs and consequently a shift in the localization of the protein from the nucleus to the cytoplasm (De Vit *et al.*, 1997) (Figure 4).

The Cat8 is a protein necessary for the derepression of several genes involved in the utilization of nonfermentable carbon sources such as the gluconeogenic enzymes fructose-1,6-biphosphatase, phosphoenolpyruvate carboxykinase, and isocitrate lyase (Hedges *et al.*, 1995). Additionally, it is highly involved in the reprogramming of carbon metabolism during the diauxic shift in *S. cerevisiae* (Haurie *et al.*, 2001). It is strongly repressed by glucose and is regulated by the Hap2/3/4/5 complex which binds the promoter of *CAT8*. This complex is responsible for the transcription activation of a large number of genes when yeast grows on a nonfermentable carbon (Rández-Gil *et al.*, 1997). The Snf1 protein kinase also regulates the Cat8p activity, undergoing phosphorylation under

derepressing conditions. When glucose is present the dephosphorylation of Cat8 occurs (Rández-Gil *et al.*, 1997).

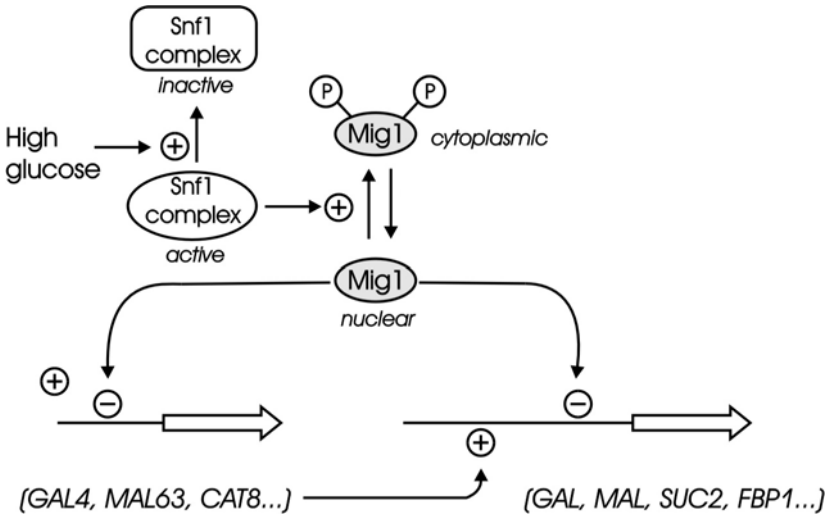

Figure 4 - Schematic view of the Mig1 regulation. Mig1 is found in the nucleus in the presence of glucose, repressing genes encoding activators such as *GAL4* and *MAL63* and genes involved in the metabolism of alternative carbon sources. When glucose is removed Mig1 is phosphorilated in a Snf1 dependent way and is translocated to the cytoplasm (adapted from Gancedo, 1998).

Given the importance of carboxylic acids in yeast metabolism, physiological studies were undertaken aiming at the characterization of the type of mechanism involved in the uptake of these acids in several yeasts. One of the first studies to report the existence of a mediated transport system for a carboxylic acid was performed in *Kluyveromyces lactis* for L-malic acid (Zmijewski and Macquillan, 1975). Two years latter a mediated transport system for the uptake of pyruvate and lactate was described in *S. cerevisiae* mitochondria (Briquet, 1977). A transport system for these acids was reported also in the plasma membrane of the yeasts *Candida utilis*, *S. cerevisiae* and latter in *Torulaspora delbrueckii* in cells growing in lactic acid (Leão and van Uden, 1986; Cássio *et al.*, 1987; Casal and Leão, 1995). All the described plasma membrane transporters are proton symporters with a very

similar range of substrates, all accepting lactate, pyruvate, acetate and propionate. In *Kluyveromyces marxianus* the monocarboxylate transporter works trough a different mechanism since no proton movements are associated with lactate uptake, suggesting that the transporter is a uniporter (Fonseca *et al.*, 1991). Although this transport mechanism is different, the range of substrates is very similar with the exception of glycolic acid that is also a substrate of the permease. However this substrate was not tested in the former cases.

Besides the lactate/pyruvate proton symporter, when cells are grown in acetate or ethanol another monocarboxylate permease with a different pattern of regulation and specificity was reported for *S. cerevisiae*. The protein is also proton symporter that transports, acetate, propionate and formate (Casal *et al.*, 1996). Similar permeases were identified in *Zygosaccharomyces bailii* (Gerós *et al.*, 1996) and *Dekkera anomala* (Gerós *et al.*, 1996; Sousa *et al.*, 1996), but in *D. anomala* the permease is also able to transport sorbate.

In *Z. bailii*, activity for a transporter specific for acetate is present in glucose-grown cells (Sousa *et al.*, 1996), however all the other plasma membrane monocarboxylate transporters referred so far are repressed by glucose. In yeasts this mechanism is well described for proteins whose metabolical role is not closely related to glucose, such as proteins involved in mitochondrial biogenesis and oxidative phosphorilation, gluconeogenesis as well as those involved in the transport and metabolism of nonfermentable carbon sources. In *S. cerevisiae*, the genes that are being activated are mostly genes involved in glycolysis and fermentation (De Risi *et al.*, 1997).

A different group of transporters is involved in the uptake of dicarboxylates. As it was already referred, the first member to be identified was the malic acid permease in *K. lactis* (Zmijewski and Macquillan, 1975), with substrate specificity for malate, succinate, fumarate and α-cetoglutarate. Additional dicarboxylate transporters have since then been identified in several yeasts, namely *Candida sphaerica* (Côrte-Real *et al.*, 1989) *Hansenula anomala* (Côrte-Real and Leão, 1990), *C. utilis* (Cássio and Leão, 1993), and *K. marxianus* (Queirós *et al.*, 1998). In all these yeasts the dissociated form is transported across the plasma membrane trough a proton symport mechanism, which is induced by the substrate and subjected to glucose repression. In *Schizosaccharomyces pombe* an identical permease is present that it is not glucose repressed (Osothsilp and Subden, 1986; Sousa *et al.*,

1992). Instead, it requires the presence of an assimilable carbon source, like glucose or glycerol, to transport malate. This is related with the fact that this yeast species is not capable of growing on malate as the sole carbon and energy source. Another important aspect is that the *S. pombe* malic enzyme encoded by *Mae2,* responsible for promoting the oxidative decarboxylation of malate to pyruvate using $NAD(P)^+$ (Boles *et al.*, 1998), is only induced in high levels of glucose or under anaerobic conditions (Viljoen *et al.*, 1999). Cells cultivated in a medium containing malic acid are unable to accomplish gluconeogenesis since the malic enzyme is not present to convert malate to pyruvate, and so cell growth is impaired. *Z. bailii* also requires the presence of an assimilable carbon source to express the dicarboxylate transporter. In this case, dicarboxylates are transported by a facilitated diffusion mechanism that is induced by glucose and repressed by fructose (Baranowski and Radler, 1984).

Few tricarboxylate transporters have been characterized so far in yeast. A low and a high affinity transport system was identified in the species *C. utilis* (Cássio and Leão, 1991). The high affinity transporter is specific for citrate and isocitrate. A system with low affinity for a range of substrates including, mono, di and tricarboxylates, as well as glycine and glutamate, was also identified in this specie. The uptake mechanisms are also different since the high affinity transporter works trough a facilitated diffusion mechanism and the low affinity trough a proton symporter, but both are glucose repressed (Cássio and Leão, 1991; Cássio and Leão, 1993). In *S. cerevisae* isolated mitochondria evidence for a citrate transporter was also found (Perkins *et al.*, 1973), being the transporter shared by citrate, isocitrate and malate (Evans *et al.*, 1983).

Genes coding for carboxylate transporters

Despite the vast physiological knowledge on yeast carboxylate transporters, genes coding for transporters were unknown for a long time. The first carboxylate transporter gene to be identified belonged to the mammalian monocarboxylate transporters. Extensive work was performed with this group of proteins due to the importance of lactate and pyruvate uptake/extrusion in mammalian metabolism.

In the present subchapter we will focus on the molecular characterization of carboxylate transporters.

The Monocarboxylate Porter family (MCP)

Carboxylic acids play a fundamental role in the metabolism of most cells, being their uptake and excretion crucial processes in the regulation of the energy metabolism as well as the acid-base equilibrium in many cells and tissues (Garcia *et al.*, 1994). In mammals, some tissues like red blood cells and tumour cells depend on the production of lactic acid from glucose to obtain most of the ATP needed under normal physiological conditions. Other tissues are also dependent on this pathway under hypoxia or ischemia (Halestrap and Price, 1999). If high rates of glycolysis are occurring in the cell, high lactate concentrations are being reached inside them, and thus the need to remove it from the cell to prevent the decrease of intracellular pH and keep up with the velocity of lactic acid production. On the other hand, lactic acid is rapidly imported into the cells in tissues where it may become a major respiratory fuel or a major gluconeogenic substrate, like in the liver during the Cori cycle. Other monocarboxylates that have important roles in the metabolic context include pyruvate, acetate and ketone bodies (Poole and Halestrap, 1993).

Originally, it was thought that the diffusion of the non dissociated weak carboxylic acids was responsible for the crossing of these nutrients trough the plasma membrane. In mammals the uptake of lactate was extensively characterized in erythrocytes, and it was demonstrated that the uptake of this acid was specifically inhibited by α-cyano-4-hydroxycinnamate and organomercurials, thus proving the existence of a mediated transport system for a monocarboxylate. In 1982, Jennings and Adam-Lackey evidenced that the transport of lactate obeyed a Michaelis-Menten kinetics and was associated with the presence of a membrane protein present in rabbit erythrocytes. The unequivocal identification of a transporter was achieved by Poole and Halestrap in 1992, after solubilization of the protein and reconstitution of the transport activity in proteoliposomes. The protein was further characterized and named monocarboxylate transporter 1 (MCT1), being found that it had an N-terminal identical to a putative transporter previously cloned from Chinese-hamster ovary cells (Kim *et al.*, 1992). The next transporter to be cloned was isolated from

hamster liver, and named MCT2 (Garcia *et al.*, 1995). It presented 60% identity with MCT1 and was characterized by expression in insect cells. It was due to the study of the X-inactivating sequence of the X chromosome that another gene with a 27% identity to the human MCT1 was identified being designated as XPTC (Lafreniere *et al.*, 1994). MCT3 was identified in chicken retinal pigment epithelium and its function was confirmed by heterologous expression in a thyroid epithelial cell line (Yoon *et al.*, 1997). By performing a search against the four known MCT sequences in the Expression Sequence Tags (EST) database, four human MCT-related sequences were identified and cloned from a human cDNA library (Price *et al.*, 1998) which were named MCT3 to MCT6. In this work the authors found additional potential MCT homologs by searching databases containing protein sequences from other organisms, namely *S. cerevisiae*, *Caenorhabditis elegans* and *Sulfolobus solfataricus*. With the identification of non-mammalian MCT homologues a new family of transporters was proposed containing both eukaryote and prokaryote members (Price *et al.*, 1998).

All the identified members had a predicted topology of 10 to 12 transmembrane domains (TMD) with the C- and N-terminus located in the cytoplasm. Another characteristic of this family was the existence of a loop between the TMD 6 and 7. Topological studies were performed for MCT1, revealing that the predicted topology of 12 TMD was correct in what concerns the localization of the C and N terminus, as well as of the loop between the 6^{th} and 7^{th} TMD (Poole *et al.*, 1996).

The MCT9 was also identified in a human EST database (Halestrap and Price, 1999). Kim and co-workers reported the existence of an aromatic amino acid transporter *TAT1* belonging to the MCT family. The Tat1p transports amino acids, and not lactate or pyruvate, trough a facilitated diffusion mechanism, and so this transporter differs from the other MCTs both in substrate range and transport mechanism, since the uptake is not driven trough a H^+ gradient like the remaining MCT members (Kim *et al.*, 2001).

More recently, four new genes that belong to the MCT family, MCT11, MCT12, MCT13 and MCT14 were discovered trough homology search in databases by Halestrap and Meredith (Halestrap and Meredith, 2004). In this study the authors also report unpublished results concerning the Mct8p specificity. This gene is the closest to the *TAT1*, and it was found to transport thyroid hormones T4 and T3, but

doesn't transport aromatic amino acids, nor lactate, appearing to have a completely different specificity. Presently, the MCT family comprises 14 members, and since the beginning of the studies with the monocarboxylates transporters many changes in the nomenclature have occurred. In Table 1 we can find the actual name of the human MCT as well as the corresponding human gene name, and known substrates for each permease.

Table 1 – Human genes belonging to the Monocarboxylate Porter Family (adapted from Halestrap and Meredith, 2004) The Human Genome Organization (HUGO) Nomenclature Committee Database includes more than 40 transporter families of the SLC (Solute Carrier) gene series. The SLC families represent a major portion of the transporter-related genes and additional SLC transporters are constantly being identified (Hediger *et al.*, 2004).

Human Gene Name	Protein Name	Aliases	Predominant Substrates	Transport type /coupling ions
SLC16A1	MCT1		Lactate, pyruvate, ketone bodies	Co transporter/ H^+ or exchanger/ monocarboxylates
SLC16A2	MCT8	XPCT (was MCT7)	T3, T4	Facilitated transporter
SLC16A3	MCT4	was MCT3		Co transporter/ H^+
SLC16A4	MCT5	was MCT4		
SLC16A5	MCT6	was MCT5		
SLC16A6	MCT7	was MCT6		
SLC16A7	MCT2		Lactate, pyruvate, ketone bodies	Co transporter/ H^+
SLC16A8	MCT3	REMP	Lactate	Co transporter/ H^+
SLC16A9	MCT9			
SLC16A10	TAT1		Aromatic a.a.	
SLC16A11	MCT11			
SLC16A12	MCT12			
SLC16A13	MCT13			
SLC16A14	MCT14			

A cladogram illustrating the phylogentic relationships existent between the different human MCT members and the *S. cerevisiae* monocarboxylate homologs (Mch) is presented in Figure 5. The MCT and the Mch form two different clades. Within the MCT cluster the four genes (SLC16A1, SLC16A3, SLC16A7, SLC16A8) that are known to be proton co-transporters are in two adjacent sub-clades, and the two genes presenting a non-monocarboxylate substrate specificity, MCT8 (SLC16A2) and TAT1 (SLC16A10), are together in the more distant sub-clade. As for the *S. cerevisiae* Mch they form a different clade being the Mch1 more unrelated to the other members.

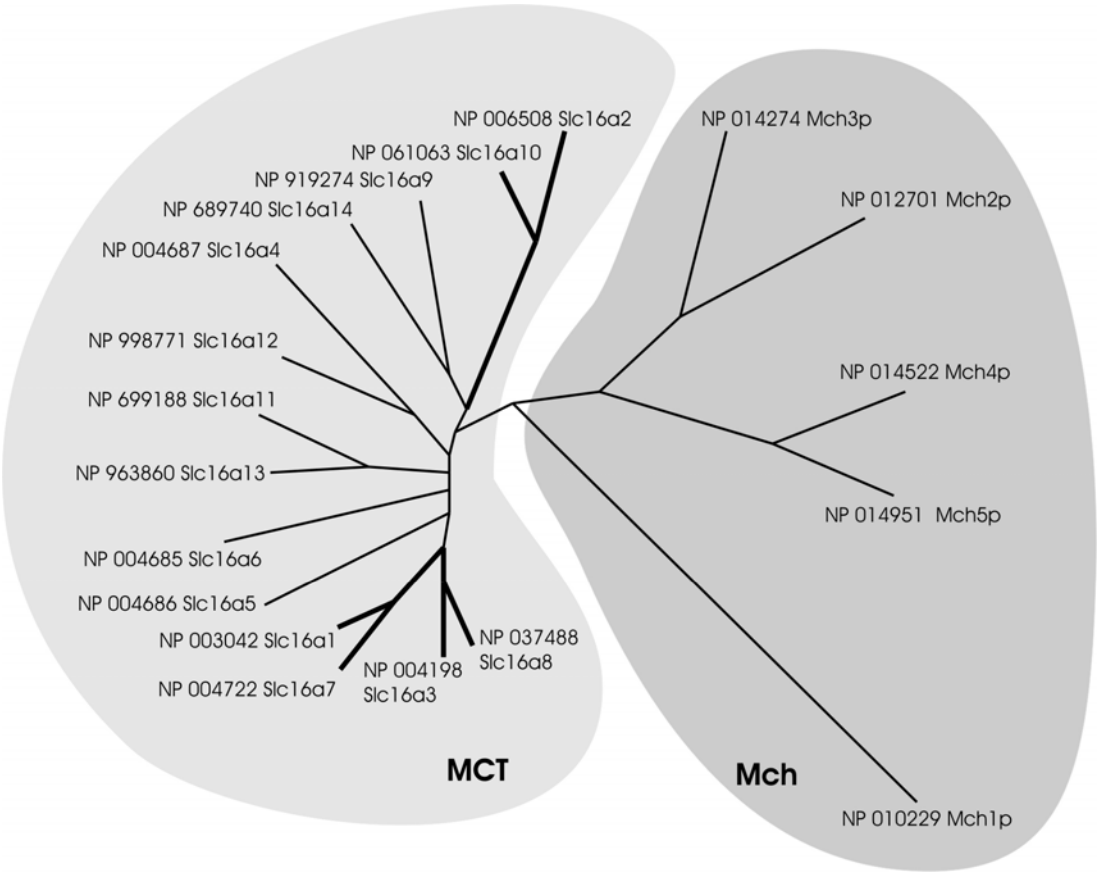

Figure 5 - Unrooted tree representing the phylogenetic relationship between the MCTs and the Mch. The corresponding aminoacid sequences were analyzed using ClustalW for sequence alignment and PHYLIP for tree plotting (http://evolution.genetics.washington.edu/phylip.html). GenBank Accession numbers are indicated for all sequences.

The MCTs have been included in the Major Facilitator Superfamily, and according to the TC system they belong to the Monocarboxylate Porter (MCP) family, (TC 2.A.1.13). In 1998 this family included only 13 members, all from eukaryotes. Besides the mammalian members, three belonged to *C. elegans* and four to *S. cerevisiae*. In phylogenetic analysis the mammalian members cluster together as well as the *C. elegans* members that were clustered in a second group. As for the *S. cerevisiae* members they were divided in two different clusters (Pao *et al.*, 1998). In 1999, Halestrap and Price reviewed the monocarboxylate transporter sequences, dividing the known monocarboxylate transporters into two different groups, those related with MCT1 and those unrelated with MCT1. In the MCT1 related genes, besides the mammalian members, genes from *Drosophila melanogaster* and bacterial species like *E. coli* and *Bacillus subtilus* were also found.

Recently, the first *Aspergillus nidulans* MCT homologue *AmcA* was described. It has a similar topology to the one presented by the members of the MCP family, and 31% of identity to Mch5 and 29% to the Mch4 from *S. cerevisiae*. The expression of *AmcA* is increased in medium containing acetate or pyruvate as single carbon source (Semighini *et al.*, 2004). Two other *A. nidulans* genes that present a high level of identity to the *AmcA*, *AmcB* and *AmcC* were also identified in this work, and hence three *A. nidulans* genes seem to belong to the MCP family.

In mammals the MCT genes present different tissue distribution and this fact could explain the need for several homologous genes in mammals. Also, another possible explanation is that some MCT members have different specificities besides the transport of monocarboxylates (Halestrap and Meredith, 2004). In *S. cerevisiae*, the existence of different monocarboxylate transporter homologues can only be explained by differences in substrate specificities or affinities.

The yeast monocarboxylate permease homologs (MCH family)

A first attempt to classify all the putative permeases and other membrane proteins from *S. cerevisiae* belonging to the MFS was made by Goffeau and his co-workers after the complete genome sequencing of the yeast *S. cerevisiae* (Nelissen *et al.*, 1997). 186 proteins were identified as possible members of the MFS, divided in 23 families. One of the families was designated as Monocarboxylate Permease

Homologues (Mch) and included four members, YOR306c, YOL119c, YKL221W and YNL125c. Another analysis was latter published in the Yeast Transport Protein database (YTPdb – http://alize.ulb.ac.be/YTPdb) by Bruno André, that showed another gene, *YDL054c,* with big similarities with the remaining MCH genes. This was also reported in a transporter and membrane protein classification study that revealed the existence of 5 *S. cerevisiae* members of the MCP family (De Hertogh *et al.*, 2002).

S. cerevisiae presents activity for two monocarboxylate-proton symporter permeases: one is shared by lactate, pyruvate, acetate and propionate (Cássio *et al.*, 1987) and another by acetate, propionate and formate (Casal *et al.*, 1996). The gene encoding the lactate/pyruvate permease was found by complementation of a strain deficient in lactate transport. The identified gene is the *JEN1* gene that has no homology with the mammalian monocarboxylate transporters (MCT) or the lactate permease of *E. coli.* Instead it is classified as belonging to the Sialate Porter family T.C. - 2.A.1.12 together with the *NanT* sialic acid transporter from *E. coli* (Saier, 2000).

In order to characterize the *S. cerevisiae* Mch family members, a functional analysis of the five Mch genes was carried out (Makuc *et al.*, 2001). Additionally in this study it was included the *JEN1* gene, as well as the gene *YHL008c* which was proposed to code for an acetate-proton symporter since it resembles the bacterial formate-nitrite transporters (Paulsen *et al.*, 1998). It was demonstrated that the simultaneous deletion of the five Mch genes did not affect the monocarboxylate uptakes rates. The deletion of the gene *YHL008c*, proposed to be an acetate transporter, did not affect the uptake of acetate. In fact, the only gene that affected monocarboxylate uptake was the *JEN1* gene. This result reinforced the idea that *JEN1* gene encodes a monocarboxylate permease even though it is not an homolog of the mammalian MCT. It was also demonstrated that the 5 Mch members were not involved in the secretion of monocarboxylates, as happens with some of the mammalian members. None of the Mch genes were repressed by glucose nor required the presence of lactate to be induced similarly to the monocarboxylate transport systems of *S. cerevisiae* (Cássio *et al.*, 1987; Casal *et al.*, 1996). The Mch expression pattern in medium containing different carbon sources (lactate, ethanol or pyruvate) revealed that the gene with the highest expression level was Mch5, followed by Mch4, Mch3 and Mch1. The transcription of Mch2 was hardly detected.

Therefore, the expression level of all the genes in the different carbon sources was very similar. Mch-GFP fusions were constructed to identify the localization of all the Mch proteins, but only enabled the determination of the Mch4 localization at the vacuole membrane. Mch3 location was dubious but a western-blotting of a HA-tagged version of Mch3 confirmed the location in the mitochondria (Makuc *et al.*, 2001), thus none of them are located at the plasma membrane level like the MCT members.

It was only recently that the true function of a Mch was experimentally determined (Reihl and Stolz, 2005). The authors demonstrated that Mch5 (YOR306c) is a high affinity transporter of riboflavin (vitamin B_2). The uptake is not inhibited by protonophores and does not require metabolic energy working by a facilitated diffusion mechanism, enabling riboflavin uptake only if the cytoplasmic concentrations are lower than the outside concentrations. Heterologous expression in *S. pombe*, which does not have an Mch5 homolog nor presents activity for a riboflavin transporter, confirmed that the protein functions as a riboflavin transporter. The other Mch proteins were tested to see if they shared the same function as the Mch5, but it was demonstrated that the *MCH5* performs a unique function within the yeast Mch gene family. A sucrose density gradient centrifugation revealed the Mch5 distribution pattern as being identical to the one of Pma1, a plasma membrane ATPase, confirming its localization at the plasma membrane (Reihl and Stolz, 2005).

The yeast monocarboxylates homologs do not transport the same substrates as their mammalian equivalents. Also, the nature of the substrates seems to be very different since the mammalian MCT transports negatively charged molecules, like lactate, pyruvate, and also the thyroid hormones, and the Mch5 transports a neutral substrate. They also differ in the fact that the mammalian proteins are proton symporters and the Mch5p is a facilitator. Although they have a common ancestral it seems that the Mch and the MCT diverged in what concerns specificity and energetics. In fact, in the phylogenetic tree presented in Figure 5 this divergence is patent since the two groups of transporters belong to different clades.

The dicarboxylate permease from Schizosaccharomyces pombe

The first report of the functional analysis of a gene encoding a dicarboxylate permease in yeast was published in 1995. In this work it was demonstrated that the *MAE1* gene from *S. pombe* encodes the permease for malate and other C4 dicarboxylic acids. This gene belongs to the Telurite-Resistance Dicarboxylate Transporter Family (T.C.-2.A.16.2.1). The methodology for identifying the gene was the complementation of a mutant phenotype, in this case the absence of a mediated transport system for malate (Grobler *et al.*, 1995). The gene *MAE1* was cloned from a *S. pombe* genomic library and the permease possesses 438 amino acids and ten putative transmembrane domains. Expression analysis by Northern-blot confirmed that the *MAE1* gene was not subject to catabolite repression and transport assays identified L-malate, succinate and malonate as substrates of the permease.

The lactate/pyruvate permease of Saccharomyces cerevisiae

The first gene encoding a fungal monocarboxylate permease was identified in *S. cerevisiae*. The complementation of a mutant unable to transport lactate revealed that the *JEN1* gene was the gene encoding the lactate/pyruvate permease of *S. cerevisiae* (Casal *et al.*, 1999). The real function of this gene was questioned for some time, since some authors postulated that this gene could be a regulator of the lactate/pyruvate permease and not the permease itself (Halestrap and Price, 1999; Makuc *et al.*, 2001). This question was based on the fact that the *JEN1* gene has no homology with monocarboxylate permeases described in mammals nor to the lactate permease from *E. coli, LctP* (T.C. - 2.A.14.1.1) and is an homolog to the sialic acid transporter *NanT* (T.C. – 2.A.13.1.1) from *E. coli*. Sialic acids are monocarboxylated monosaccharides, normally present in mammal sugar side chains of glycoproteins, glycolipids, and glycosaminoglycans whose carboxylic group is deprotonated at physiological pH (Vimr *et al.*, 2004). Several studies were carried out in order to better characterize the Jen1p. *In silico* topological studies demonstrated that the Jen1p had 12 putative transmembrane domains and a JEN1-GFP fusion demonstrated that, under induction conditions, the protein was localized at the plasma membrane (Paiva *et al.*, 2002).

Glucose repression acts at different cellular levels in the *JEN1* regulation. In the presence of glucose there is no detection of *JEN1* gene mRNA (Casal *et al.*, 1999), since the transcription factors Mig1p and Mig2p involved in glucose repression are down-regulating the *JEN1* gene (Bojunga and Entian, 1999). Glucose regulation also acts at the post-transcriptional level. Addition of a pulse of glucose to cells expressing the *JEN1* gene promotes an increase in mRNA degradation, resulting in a decrease of the *JEN1*-mRNA half-life from 15 minutes to 3 to 4 minutes (Andrade and Casal, 2001; Andrade *et al.*, 2005). Recently, a mechanism for the glucose accelerated *JEN1* mRNA decay was proposed. A second transcript of the *JEN1* was mapped to the position +391, and the presence of this transcript was correlated with the observation of glucose-triggered mRNA degradation. In a strain overexpressing the +391 *JEN1* transcript, the JEN1-mRNA half life always diminished, even in the absence of glucose. The authors propose that the alternative *JEN1* transcript works as a glucose sensor that, in the presence of even small amounts of glucose, accelerates the *JEN1* mRNA decay (Andrade *et al.*, 2005). In addition to the Mig1p and Mig2p, other proteins regulate the transcriptional level of the *JEN1* gene. The Cat8p is necessary for a full derepression during a shift from fermentative to respiratory growth (Bojunga and Entian, 1999) and the Hap2/3/4/5 complex is necessary for a derepression of the *JEN1* gene in medium containing lactate (Lodi *et al.*, 2002). The protein kinase Snf1p, that plays an essential role in the release of glucose-repressible genes from glucose repression (Carlson, 1999) is also involved in the control of *JEN1* transcription (Lodi *et al.*, 2002).

Regulation of this system occurs also at the protein level. Addition of glucose to cells expressing a Jen1-GFP fusion at the plasma membrane promotes the rapid internalization of the protein (Paiva *et al.*, 2002), dependent on the End3 endocytosis pathway, and it is targeted for degradation in the vacuole. Experiments performed in a *doa4*Δ mutant strain showed that ubiquitination is associated with the turnover of the permease. Additionally, the targeting of the Jen1-GFP fusion to the plasma membrane occurs in a Sec6-dependent process (Paiva *et al.*, 2002).

The doubts concerning the real function of the Jen1p were dissipated with the heterologous expression of the Jen1p in *Pichia pastoris.* This work is described in chapter 2 of the present thesis. The reconstitution of the lactate permease activity in hybrid vesicles of fused *P. pastoris* membranes expressing the Jen1p with *E. coli*

lipossomes, demonstrated that the Jen1p is a fully functional lactate transporter and not only a regulator of its expression/activity.

JEN1 homologous genes have been described in the literature for several species. The *Candida albicans* homolog is described in chapter 3 of this thesis, and other homologs were also described in *Metarhizium anisopliae* and *Kluyveromyces lactis* (Fang *et al.*, 2003; Lodi *et al.*, 2004). In *K. lactis* two *JEN1* homologs were described, the *KlJEN1* a monocarboxylate transporter and *KlJEN2*, a dicarboxylate transporter. The KlJen1p is required for lactate uptake and the KlJen2p is required for succinate and malate transport (Lodi *et al.*, 2004; Pereira, 2006). The uptake of succinate is inhibited by fumarate thus indicating that the KlJen2p is also capable of binding this substrate. The heterologous expression of *KlJEN1* in a *S. cerevisiae jen1*Δ strain restored the capacity of the strain to transport labelled lactic acid. As for the *KlJEN2* expression it enabled *S. cerevisiae* to uptake succinic and malic acids by a mediated mechanism (Pereira, 2006). An alignment of the ScJen1 and known homologs from *K. lactis*, *C. albicans*, *M. anisopliae, Neurospora crassa* and *Beauveria bassiana* is presented in Figure 6. The corresponding cladogram is also presented. It is possible to identify two subclades, one formed between the yeast *JEN1* homologs and the other by the filamentous fungi homologs. In the middle of the two subclades is the *KlJEN2* that has different substrate specificity from the yeast *JEN1* homologs.

```
BbJEN1          ---------------------------------MATPSSHPVANEPADDLAQGII----- 22
MaJEN1          ---------------------------------------MSPRENEPADALAKGII----- 17
NcJEN1          ---------------------------------------MESTHEPADPVAKGIL----- 16
KlJEN2          ----------------------------MAAESIVSRDESIASLEKAEGRITYLK----- 27
ScJEN1          MSSSITDEKISGEQQQPAGRKLYYNTSTFAEPPLVDGEGNPINYEPEVYNPDHEKLYHNP 60
KlJEN1          ----MNNNNITPESD--SMKSNDNDQTNDYMPDVADFDHTQTNTNEIARAISHPGSVLSR 54
CaJEN1          ------------------------------MEEKARADHISITNSNELNYDDYSNSYKN- 29
                                                          .

BbJEN1          ---------------------------------PTARQSLRDLFVWKQRVVVSNAYGETR 49
MaJEN1          ---------------------------------PTAKQSFKDLFIWRQRVVLSNEYGETR 44
NcJEN1          ---------------------------------PTARQSWKDLFIWKQRVVVTNVYGETA 43
KlJEN2          ---------------------------------PQSRITWSDAXKYLATRIPTLFPTKAS 54
ScJEN1          SLPAQSIQDTRDDELLERVYSQDQGVEYEEDEEDKPNLSAASIKSYALTRFTSLLHI--- 117
KlJEN1          VASYVSRKDRYVDENGNEVWQDDE-VSILMEEDETPDFTWKNIRHYAITRFTTLTEL--- 110
CaJEN1          -------QPELDDELFN---------------EKPDLSLSSISRYFATRLTTLLDLPLY 66
                                               .  :  .   :    . :

BbJEN1          CEWQQPDKFKNPIALFAQLTPKAWLFFIVGWLAWVADAFDFHALSIQTVKLAKYYGRTKA 109
MaJEN1          CEWRDPDRFVNPISLMAQLSAKNWLFFLVGFLSWTADAFDFHALSIQTKKLAVYYGRSKT 104
NcJEN1          TEWAKPVPLKNPISLLAQLSGRDWICFLVGFCAWSADAFDFHALSIQQVKLAAYYGVSKT 103
KlJEN2          IREARKEYPINPFPALRSMNWLQTQYFIVGFLAWTWDALDFFAVSLNMTNLAKDLDRPVK 114
ScJEN1          --HEFSWENVNPIPELRKMTWQNWNYFFMGYFAWLSAAWAFFCVSVSVAPLAELYDRPTK 175
KlJEN1          --HRVSMENIDPIPELRKMTLHNWNYY--------------------------LDRX-- 139
CaJEN1          HTHKKWYEVINPIPGLKSMSKSDWNFYCLGFFAWALDAMDFFCVSVAAPEIANTLNISVT 126
                          :*:. : .:.      :                            .
```

```
BbJEN1          DITTAITLTLLLRSVGAAVFGLAGDKWGRKWPMVFNMLVLGLLQIATIYSTKFSHFLAVR 169
MaJEN1          DITTAITLTLLLRSIGAAFFGLAGDKWGRKWPMVVNMLVLGLLQIATIYSQTFQQFLAVR 164
NcJEN1          SVSTAITLTLLLRSIGAAAFGLAGDRWGRKWPMVVNMIVLGILQIATIYSSTYSQFLGVR 163
KlJEN2          DISHAITLVLLLRVIGALIFGYLGDRYGRKYSFVLTMALIIVIQIGTGFVNSFSAFLGCR 174
ScJEN1          DITWGLGLVLFVRSAGAVIFGLWTDKSSRKWPYITCLFLFVIAQLCTPWCDTYEKFLGVR 235
KlJEN1          ---------LFX----------XNGLTSLVWG------LFLICQLCTPWAKTYTQFLGVR 174
CaJEN1          DVTWGVTLVLMLRSVGAVIFGIASDYFGRKWTYISIVTLFVVVEVGTGFVQTYQQFLGVR 186
                         *:             .  .  :       :: : :: * :  .:  **. *

BbJEN1          SLFGLFMGGVYG-------------------NAIAMALEQCPSNARGLMSGILQQGYSFG 210
MaJEN1          SLFGLFMGGVYG-------------------NAIAMALEQCPSNARGLMSGILQQGYSFG 205
NcJEN1          ALFGLFMGGVYG-------------------NAIAMALENSPVDARGLMSGILQQGYAFG 204
KlJEN2          AIFGIIMGSVFGSAFLGCRAIFXIIMGSVFGVASXTALENAPNKAKSILSGIFQEGYAFG 234
ScJEN1          WITGIAMGGIYG-------------------CASATAIEDAPVKARSFLSGLFFSAYAMG 276
KlJEN1          WISGIAMGGIYA-------------------CASATAIEDAPVKARSFLSGLFFTAYAMG 215
CaJEN1          AIFGILMGAMYP-------------------IAMVTALEGQPIAARSVLSGLFLPGYCFG 227
                 : *: **.::                     *   *:*  *  *:..:**::  .*.:*

BbJEN1          -YVLAACANLGVG---GAVDSWKIVFWIGAGLSIAVGLIRMLFPESQQFIEAKKTG---- 262
MaJEN1          -YVLAACANLGVG---GGTDTWKIVFWIAAGFSIAVGLVRILFPESQQFLEAKKNG---- 257
NcJEN1          -YVCAACANLGVG---GDTDSWKTVFWIAAGLSIGVGIIRCFFPESKQFLEARKEG---- 256
KlJEN2          XLLGVVFQRAIVD---NSPHGWRAIFWFSAGPPVLFIAWRLMLPESQHYVERVRLE---- 287
ScJEN1          -FIFAIIFYRAFG-YFR-DDGWKILFWFSIFLPILLIFWRLLWPETKYFTKVLKAR---- 329
KlJEN1          -FIFAIIFYRAFL-NVNGENYWKVQFWFSIWLPAVLILWRLVWPETKYFTKVLKAR---- 269
CaJEN1          -YIMAMVWYRAFAGTYKEGEGWRSLIWFSGGLSLILIVWRLFTPESPDYIKMKIKKEKFN 286
                  : .      .       . *:  :*:.   .  .   * . **:  : :

BbJEN1          ---------------KKASS-PGAFWRETKVMLAKEWKMCVYCIFLMTWFNFYSHTSQDS 306
MaJEN1          ---------------KKAAS-PGAFWHDTVKMLKQEWKMRVYCIILMTWFNYYSHTSQDS 301
NcJEN1          ---------------KAHAN-PSQFWRETKVMLRQEWKMCVYCCILMTWFNCN-HTSQDN 299
KlJEN2          ---------------KLENDGKSQFWKNAKLACSQYWLSMIYLVLLMAGFNFSSHGSQDL 332
ScJEN1          KLILSDAVKANGGEPLPKANFKQKMVS-MKRTVQKYWLLFAYLVVLLVGPNYLTHASQDL 388
KlJEN1          QLMRDDAIAKNGGQPLPKLSFKQKFAN-VKKTVSKYWLLFGYLILLLVGPNYLTHASQDL 328
CaJEN1          QQQRLKEQEQNGGVAVKEKKFWQKIDKSILVTFKTEWLIFSYLVLLYAGWNFTTHGSQDL 346
                               .    :           *    *  .* .  *   * ***

BbJEN1          YTTFMLTEKELSNKGASRASIMMKVGACVGGTIIGYASQFLGRRRTIIGSALMSAMLIPA 366
MaJEN1          YTTFMLTQKGLDNAGASRASILMKAGACVGGTILGYMSQFIGRRRTIISSCLMSAVLIPA 361
NcJEN1          YTTFVLRAKEMDNSAASRASIIMKAGACVGGTIIGYLSQYFGRRRTIIVSSLISGCMIPA 359
KlJEN2          FPTMLTSQYQFSADASTXTNSVANLGAIAGGIIVAHASSFFGRRFSIIVCCIGGGAMLYP 392
ScJEN1          LPTMLRAQLGLSKDAVTVIVVVTNIGAICGGMIFGQFMEVTGRRLGLLIACTMGGCFTYP 448
KlJEN1          FPTMLRAQLRFSEDAVTVAIVVVCLGSIAGGMFFGQLMEITGRRVGLLLALIMAGCFTYP 388
CaJEN1          YVTMITKQYHVGLDKKTVIIVVSNIGGIIGGIIMGQASELLGRRLTVVISIVCAGAFLYP 406
                  *::     ..    :    :   *.  ** :..   .  ***  :: .   .. :  .

BbJEN1          WILPRGEASLSASGFFMQFFVQGAWGVIPIHLNELSPAAYRSTFPGVTYQIGNMISSPSA 426
MaJEN1          WILPTGERALSATGFFMQFFVQGAWGVIPIHLNELSPVAFRSTFPGVTYQLGNMISSPSA 421
NcJEN1          WILPNSERALSATGFFMQFFVQGAWGVIPIHLNELAPPAFRSSFPGITYQVGNMVSSPSA 419
KlJEN2          WGFVANKSGINASVFFLQFFVQGAWGIVPIHLTELAPTEFRALITGVAYQLGNMISSASS 452
ScJEN1          AFMLRSEKAILGAGFMLYFCVFGVWGILPIHLAELAPADARALVAGLSYQLGNLASAAAS 508
KlJEN1          AFMLKTSSAVLGAGFMLWFSILGVWGVLPIHLSELSPPEARALVSGLAYQLGNLASAASV 448
CaJEN1          SFFN--PDRNWPAYIFLNAFVFGSFSVGPAYLLELVNSTHRTLLSGVAYQLGNLVSSASA 464
                  :         : :::   : * :.: * :* **     *: ..*::**:**: *:.:

BbJEN1          QIVNAIAEKTFIT--LKSGHRVEAYGPVMGVATAIIALGIIFSTMVGPEKRGRAFENAVA 484
MaJEN1          QIVNAIAEKMFVT--TPDGRRAEAYGPVMGIATAIIALGIVFTTMFGPERRGRDFENKVA 479
NcJEN1          QIVNAVSEKIHIV--SHTGKLVEAYGPTMGIATAIIVMGIVVTTAFGPEKRGREFEKALP 477
KlJEN2          TIEASIGERFPLE--GRED--AYDYGKVMCIFMGCVFAYLLIVTVLGPENKGGELRLSTT 508
ScJEN1          TIETQLADRYPLERDASGAVIKEDYAKVMAILTGSVFIFTFACVFVGHEKFHRDLSSPVM 568
KlJEN1          VIENDLADLYPIEWNSAVKVTNKDYSKVMAILTGSSVIFTFVLVFVGHEKFHRDLSSPHL 508
CaJEN1          TIEAKIGERFPLKDQPG----MFDYGKVMCIFCGAIFAYM-------------------- 500
                 *   :.:   :            *. .* :  .

BbJEN1          G----------------VETASSEKTSADEEDVEK-----------------------A 504
MaJEN1          G----------------M------------------------------------------ 481
NcJEN1          AGMNLQKQHGKQVDDLEMETGHMEKVTSELDDEKREGRAVQIFIIFLGPERFHRDLQIHD 537
KlJEN2          G----------------TEQDDEESQNNNIIRRNCRG---------------------W 530
ScJEN1          K-------------------KYINQVEEYEADGLSISDIVEQKTECASVKMIDSNVSKTY 609
KlJEN1          K-------------------SYIERVDQTEEVAAMTG---------STANSISSKPSDDQ 540
CaJEN1          ------------------------------------------------------------

BbJEN1          TVELSEVK-------------- 512
MaJEN1          ---------------------
NcJEN1          TDEIEELQQDSSSNNESVKERV 559
KlJEN2          TSFGSKFQARNST--------- 543
ScJEN1          EEHIETV--------------- 616
KlJEN1          LEKVSV---------------- 546
CaJEN1          ---------------------
```

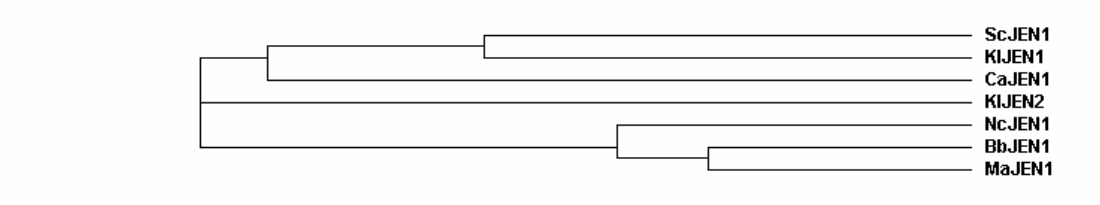

Figure 6 – Multiple sequence alignment and corresponding cladogram of known ScJen1 homologs, performed with the ClustalW software (www.ebi.ac.uk/clustalw).

The acetate permease from Saccharomyces cerevisiae

Despite the vast knowledge on acetate metabolism, the genes coding for acetate permeases were only recently discovered. In *E. coli* the acetate permease was identified and named *ActP*, it belongs to the Solute:Sodium Symporter (T.C. - 2.A.21.7.2). Resembling the lactate permease *LctP*, it is located in an operon, together with the *Acs* gene encoding an Acetyl-CoA synthetase (EC 6.2.1.1), and the gene *yjcH* of unknown function (Gimenez *et al.*, 2003). It has a predicted structure of 14 transmembrane domains and is highly specific for short-chain aliphatic monocarboxylates. Besides acetate it transports glycolate, and is greatly inhibited by propionate, and to a lesser degree by L-lactate, oxalate and butyrate.

Several attempts were made towards the identification of the gene encoding the acetate permease first characterized in *S. cerevisiae* in 1996 by Casal and co-workers (Paiva *et al.*, 1999; Paiva, 2002).

With the advent of the sequencing genome projects, new global analysis technologies have been developed, such as the Microarrays technique. In *S. cerevisiae*, a comparison between the gene expression profile of glucose grown cells and cells shifted to a medium containing acetate as the sole carbon and energy source evidenced out the global adaptation strategy of cells growing on glucose and then shifted to a poor carbon source. In addition to general responses like the derepression of gluconeogenic genes and the induction of the glyoxylate and TCA cycles, previously unreported responses were descrived that include the activation of the translation machinery, rRNA maturation and mitochondria biogenesis. In what concerns transporters, many carriers were shown to be upregulated, including the *JEN1* gene, but the Mch genes were downregulated.

Of all the genes strongly activated, only three were putative membrane proteins and belonged to the same YaaH family (T.C. - 9.B.33), the YCR010c/*ADY2*, YNR002c/*FUN34* and YDR384c, all homologous to the *GPR1* gene of *Yarrowia lipolytica*. The deletion of the *GPR1* was reported to cause a defect in acetic acid utilization (Tzschoppe *et al.*, 1999). Deletion studies demonstrated that the *ADY2* was the only gene involved in the acetate uptake and so the gene encoding the acetate transporter. Microarrays analysis of the *S. cerevisisae ady2*Δ strain demonstrated that this gene is not involved in a global transcriptional response to the presence of acetate, thus indicating that this gene is directly involved in acetate transport. *ADY2* gene was first described as being involved in other cellular processes, namely proper *ascus* formation on sporulation medium, containing acetate as the main carbon source (Rabitsch *et al.*, 2001). It was reported that it was involved in ammonia production in *S. cerevisiae* during yeast colony development, together with the two other genes belonging to the same family *YNR002c* and *YDR384c* (Palkova *et al.*, 2002). The authors verified that these genes are strongly induced in the alkali phase of colony growth, when ammonia production is increased. Additionally, the corresponding deleted strains present a lower ammonium export. Based on this fact the authors proposed that the proteins are ammonium/H^+ antiporters, extruding ammonium from the cell and importing protons (Palkova *et al.*, 2002).

Although the true function of the Ady2p remains to be determined, we can find in the literature several similar expression patterns between *ADY2* and *JEN1*. In what concerns the regulation of gene expression, vaious proteins have been described as regulation factors intervening at the transcription level of these genes. The Cat8p is a known regulator of the expression of genes connected with the nonfermentable carbon sources metabolism (Hedges *et al.*, 1995). It was first described as being involved in the derepression of the gluconeogenic genes *FBP1*, *PCK1*, *ACR1*, *ICL1* and *MLS1* (Hedges *et al.*, 1995) and later the *JEN1* and the *IDP2* (Bojunga and Entian, 1999). During the diauxic shift the Cat8p regulator is highly expressed, and a comparison between the wild-type strain and a *Cat8* mutant revealed that the expression of several genes is dependent of the expression of this gene (Haurie *et al.*, 2001), among them are the *ADY2* and the *JEN1*. These genes present a similar expression profile in the wild-type strain, and absence of expression in the *Cat8* mutant during the diauxic shift. In what concerns the *ADY2*

homologous genes, the Cat8p has an opposite effect in the *YNR002c* expression, being more expressed in the absence of this regulator, and as for the *YDR384c*, no Cat8p regulatory effect was described (Haurie *et al.*, 2001).

Adr1p is another regulator of a large number of genes involved in the metabolism of nonfermentable carbon sources that acts cooperatively with the Cat8p (Young *et al.*, 2003). Expression of the gene *ADY2* is Adr1 dependent, and this regulator was described to bind to the promoter of the gene. The *JEN1* promoter is also bound by Adr1p but it is unclear if the expression is Adr1p dependent since the significance value for *ADR1*-dependent expression was just below the cutoff (Young *et al.*, 2003). For both genes, the binding of Cat8p to the corresponding promoter is independent of the presence of Adr1p (Tachibana *et al.*, 2005). Additionally, both require the presence of the Snf1 protein kinase to be fully expressed, since the *SNF1*/*snf1* expression ratio is around 30.

Recently, the Sok2p was described as a transcriptional repressor involved in starvation-induced metabolic changes, as well as in sporulation and pseudohyphal growth regulation (Shenhar and Kassir, 2001). An expression study of the *sok2* mutant performed by Palkova and co-workers revealed the involvement of this regulator in the adaptation of colonies to long term survival (Vachova *et al.*, 2004). This study shows that the genes *JEN1*, *ADY2* and also its homologs *YNR002c* and *YDR384c* are all down regulated in the *sok2* mutants when compared to the wild-type colonies. Additionally, the *ADY2* and *JEN1* expression pattern in colonies during different development stages is very similar.

Other global expression studies were published where a similar expression profile for the *ADY2* and *JEN1* is observed. These include the evaluation of the transcriptional response of *S. cerevisiae* grown under limiting conditions for carbon, nitrogen, phosphorous or sulphur and the comparison of gene expression of yeast grown in oleate versus glucose (Kal *et al.*, 1999; Boer *et al.*, 2003). In the first case both genes are specifically highly expressed under carbon limitation. As for the second case, both genes present a high induction level in oleate-grown cells when compared to glucose.

Overall, these results evidence a resemblance between regulation patterns and expression profiles of the *ADY2* and *JEN1* genes. This fact could be sustained by the existence of a functional proximity between the two proteins, which would be best supported by *ADY2* as an acetate transporter rather than an ammonia exporter.

Nevertheless, the true function of the *ADY2* gene remains to be confirmed. Additional studies such as expression in a heterologous system or the characterization of the transport activity in vesicles will enable to define the functions of this gene.

The mitochondrial carboxylate transporters

Mitochondria intervene in several metabolic pathways, having a major role in the metabolic context. Inside the mitochondria, different energy generating pathways can be found such as the citric acid cycle or the fatty acid β-oxidation, and the coupling of NADH production to the oxidative phosphorylation. Mitochondria are also able to replicate their own DNA, and to transcribe and translate the mitochondrial mRNA. In order to carry out these pathways, an exchange of solutes has to occur between the mitochondria and the cytoplasm and other components. Several mitochondrial transporters were identified trough physiological studies in isolated mitochondria of a number of organisms. With the complete sequencing of the yeast mitochondrial genome it was demonstrated that it encodes mainly genes connected with mRNA translation, such as tRNAs and ribosomal RNAs (Foury *et al.*, 1998). There is no mitochondrial encoded transporter known so far.

Based on the first mitochondrial carrier sequences obtained, researchers noticed that they form a family of proteins with a related sequence and structure (for a review see Kunji, 2004). In 1996, with the sequencing of the entire yeast genome a search for mitochondrial carriers was carried out. The search was based on homology criteria and it was possible to identify the mitochondrial carrier family (MCF) (TC-2.A.29). This family is presently composed by 35 yeast members, from which 18 have been functionally characterized (Hildyard and Halestrap, 2003). These proteins possess a tripartite structure of three tandem repeated homologous sequences with around 100 amino acids each, that form two transmembrane helices connected by a loop. The mitochondrial carriers have a different range of substrates connected mostly with mitochondrial metabolism such as the Krebs cycle intermediates. Seven carboxylate transporters were so far identified (Table 2).

Table 2 – Carboxylate mitochondrial carriers in yeast.

Gene name	SGD ID	Carrier substrates	Reference
CTP1	YBR291c	Citrate	(Kaplan *et al.*, 1995)
SFC1	YJR095w	Succinate/fumarate	(Palmieri *et al.*, 1997)
OAC1	YKL120w	Oxaloacetate	(Palmieri *et al.*, 1999)
DIC1	YLR348c	Dicarboxylate	(Palmieri *et al.*, 2000)
ODC2	YOR222w	Oxidicarboxylate	(Palmieri *et al.*, 2001)
ODC1	YPL134c	Oxidicarboxylate	(Palmieri *et al.*, 2001)
	YIL006w	Pyruvate	(Hildyard and Halestrap, 2003)

One of the first and most studied mitochondrial transporters was the pyruvate carrier, whose existence was first demonstrated with the specific inhibition of pyruvate uptake in rat liver mitochondria by α-cyano-4-hydroxycinnamate (Halestrap, 1976). In yeast, the mechanism of mitochondrial pyruvate transport was studied in isolated mitochondria and the existence of a proton symporter mediated transport system for the uptake of pyruvate and also lactate was demonstrated by Briquet in 1977. At that time it was suggested that the pyruvate carrier was not encoded in the mitochondrial genome. Hildyard and Halestrap (2003) tested all the genes belongings to the mitochondrial carrier family of unknown function and demonstrated that only the strain deleted in the *YIL006w* gene presented no mitochondrial mediated uptake of pyruvate. Additionally, the pyruvate uptake in the *YIL006w* mutant strain was not sensible to the α-cyano-4-hydroxycinnamate inhibitor, thus confirming that the mitochondrial pyruvate transporter is encoded by the *YIL006w* gene.

Carboxylate efflux pumps

The use of lipophilic weak acids as food preservatives is very widespread. These operate at low pH, where the undissociated form of the acid is favoured. The undissociated form can cross the plasma membrane, and once inside it dissociates resulting in the formation of protons and anions that cannot cross the plasma membrane, as previously described. This has a deleterious effect on cell metabolism

and several ways of action have been proposed like membrane disruption, inhibition of essential metabolic reactions, decrease in ATP yield, alterations on the intracellular pH homeostasis and accumulation of toxic anions (Krebs *et al.*, 1983; Eklund, 1985; Pampulha and Loureiro-Dias, 1990; Pampulha and Loureiro-Dias, 2000) depending on the acid, pH and concentration. In yeast, these effects characteristically cause an extended lag phase and cell stasis, rather than cell death (Piper *et al.*, 1998), although it has been demonstrated that acetic acid is able to induce apoptosis in *S. cerevisiae* and in *Z. bailii*, when present in concentrations between 20 mM and 80 mM (Ludovico *et al.*, 2001; Ludovico *et al.*, 2003). Despite the toxic effect of weak acids, some yeasts and molds are able to adjust and grow in the presence of maximum allowed levels of these preservatives. To overcome the inhibitory effect of weak acids, yeasts shift ATP spending from anabolic processes to the maintenance of cell homeostasis, resulting in a decreased biomass yield. Another proposed consequence is the efflux of the anion.

Special attention has been paid to the resistance mechanisms caused by acid stress. The study of benzoic acid tolerance in *Z. bailii* presented some results that supported the extrusion of the acid (Warth, 1977). In *S. cerevisiae* it was found that the intracellular concentration of this acid was lower than that which would be expected if equilibrium due to diffusion across the plasma membrane was established (Verduyn *et al.*, 1992), indicating that the acid was being extruded from the cell. Confirmation was obtained trough the demonstration that benzoic acid was extruded from *S. cerevisiae* by an energy-dependent mechanism. The extrusion happened only in induced cells like benzoic-grown cells and after a pulse of glucose or acids, e.g., octanoic, sorbic, hexanoic, salicylic, butyric and propionic (Henriques *et al.*, 1997).

In 1997, Piper and co-workers studied the effect of *S. cerevisiae* exposure to sorbic acid at the plasma membrane protein level. They observed strong induction of two membrane proteins identified trough microsequencing, the Hsp30 and the Pdr12 (Piper *et al.*, 1997; Piper *et al.*, 1998). Hsp30 is a protein involved in the stress response pathway to weak acid exposure that is also induced by heat and ethanol shock. The *Hsp30*Δ strain presents reduced biomass yields as well as a longer lag phase when exposed to sorbate (Piper *et al.*, 1997; Piper *et al.*, 1998). The Pdr12p is an ATP binding cassette transporter belonging to the Pleiotropic Drug Resistance (PDR) Subfamily (TC – 3.A.1.205.3). It was demonstrated that Pdr12p is required

for the adaptation of yeast to weak acid stress, given that the mutant strain is sensitive at low pH to sorbic, benzoic and propionic acids as well as a range of carboxylic acids ranging from C1 to C7 (Holyoak *et al.*, 1999). Another important feature of the deleted *pdr12* strain is the absence of active benzoate efflux (Piper *et al.*, 1998). Pdr12p seems to be responsible for the active efflux of the acid, diminishing its toxic effect. The study of fluorescein efflux, a water soluble monocarboxylate, in wild-type and *pdr12*Δ strain revealed that the Pdr12p was also responsible for the extrusion of this molecule from the cell (Holyoak *et al.*, 1999). The inhibition of glucose mediated extrusion of fluorescein by vanadate and the accumulation of ATP in the *pdr12* mutant strain demonstrated that the extrusion mechanism is ATP dependent. Additionally, inhibition studies demonstrated that the extrusion of fluorescein was inhibited by sorbic and benzoic acids at low pH, indicating that these acids are extruded from the cell by the same protein.

In order to maintain the resistance to weak acids the cells have to diminish the entrance of the acid's undissociated form, otherwise the acid would re-enter the cell and dissociate with the anion being pumped out of the cell at the cost of ATP consumption, re-starting the same cycle would result in a large energy spent as well as in a large proton influx. In cells adapted to benzoic acid, the diffusion coefficient of organic weak acids across the plasma membrane is lower, thus reducing the entrance of weak acids into the cell (Loureiro-Dias, 1998).

Another gene belonging to the ABC transporters that is involved in weak acid resistance is the *YRS1* gene also known as *YOR1* (TC-3.A.1.208.3.). The disruptant strain exhibited increased sensitivity to the carboxylic acids such as acetic, propionic, and benzoic acids (Cui *et al.*, 1996; Decottignies *et al.*, 1998).

The gene *AZR1* was also reported to be involved in the resistance to acetic acid as well as to azoles and to a lesser extent to propionic acid (Tenreiro *et al.*, 2000). *AZR1* is a member of the Drug:H^+ Antiporter-2 Subfamily of the Major Facilitator Superfamily (TC-2.A.1.3). The Azr1p was shown to be involved in the reduction of the acetic acid-induced latency phase, but was not related with the active efflux of acetate as the Pdr12p.

The same group identified another gene of the MFS involved in multidrug resistance (Tenreiro *et al.*, 2002), the *AQR1* gene (TC-2.A.1.2). This protein confers resistance to a broader range of substrates, including short chain carboxylic acids (C_2-C_6, e.g. acetic, propionic and butyric acids), ketaconazole, cationic dyes and

quinoline containing drugs. The *AQR1* gene was later described as being implicated in the excretion of excess amino acids possibly trough a mechanism of vesicle formation followed by a release to the extracellular space by exocytosis (Velasco *et al.*, 2004). The authors postulate that the transport of monocarboxylates and other substrates besides amino acids by Aqr1 may correspond to an alternative to its natural function the mediated excretion of amino acids, due to the existence of similarities in the structure of the different compounds.

Recently, Piper and co-workers have demonstrated that the acetate sensitivity phenotype reported to yeast mutants lacking the transporters Pdr12p and Azr1p is an artefact that results from the use of *trp1* mutation in the strains like the W303-1A (Bauer *et al.*, 2003). The effects of acetate sensitivity are only seen in auxotrophic mutants that need to assimilate aromatic amino acids from the culture medium. The presence of weak acids inhibits the uptake of these amino acids, and this phenotype is overcome by supplementing the medium with higher levels of amino acids, or by overexpressing the Tat2p high affinity tryptophan permease. It remains to be studied if in other transporters, such as the Yrs1p and the Aqr1p, the acetate resistance is also an artefact due to the use of *trp1* mutants and if the artefact is exclusive for acetate or affects other carboxylic acids.

Permease structure

Membrane proteins, such as channels, transporters, and receptors, are important components of several fundamental biological processes. Additionally they play an important role in biomedical and biotechnological fields since the majority of drug targets are integral membrane proteins.

Within membrane proteins, transporters represent a very important role. The Major Facilitator Superfamily represents the largest group of secondary active transporters, comprising 25% of all transporters (Saier, 2000). As previously reported, the members of the MFS are ubiquitous in living organisms, and they have very broad substrate specificity such as ions, sugars, sugar–phosphates, drugs, neurotransmitters, nucleosides, amino acids, peptides and other hydrophilic solutes.

One of the best studied members of the MFS is the lactose permease from *E. coli* (LacY). Several studies of site-directed biochemical and biophysical techniques were undertaken allowing the construction of a *LacY* structural model. Additionally, extensive mutational analysis led to a proposed mechanism for energy coupling between sugar and H^+ transport, and the discovery of substrate binding site (Venkatesan and Kaback, 1998).

The first membrane protein structure to be determined was the photosynthetic reaction centre (PSRC) from *Rhodopseudomonas viridis* in 1985 (Deisenhofer *et al.*, 1985). Only recently the crystallization and three dimensional structure of a MFS permease was obtained. In 2002 a cryo-electron microscopy map at a 6.5-Å resolution of the oxalate-formate antiporter (OxlT) was achieved, enabling the first glance of an MFS protein structure (Hirai *et al.*, 2002). In the following year the X-ray crystallographic structures of two proteins were published simultaneously: the lactose/proton symporter (LacY) and the Pi/glycerol-3-phosphate antiporter (GlpT) at a resolution of 3.5 Å and 3.3 Å, respectively (Abramson *et al.*, 2003; Huang *et al.*, 2003). In all the determined structures a common topology was identified, 12 transmembrane helices forming two distinct domains named N and C-terminal. This fact suggests that members of this superfamily have evolved conserving secondary and tertiary structure elements although the substrate transport mechanism is different since the LacY is a symporter and both GlpT and OxlT are antiporters.

LacY and GlpT structure determination was obtained in different manners. A LacY C154G mutant that presented an increased substrate affinity but low level of transport and enhanced thermostability was used to obtain crystals in the presence of the high-affinity lactose homologue β-D-galactopyranosyl-1-thio-β-D-galactopyranoside (Smirnova and Kaback, 2003). As for the GlpT, a screening within protein truncated versions enabled the identification of a more stable protein that was then purified and used to obtain crystals (Lemieux *et al.*, 2003). Both structures reveal 12 transmembrane helices that have a trapezoid-like shape, with an internal large cavity that is opened to the cytoplasm and closed to the periplasm (Figure 7). The transporters are divided in two similar domains, N and C-terminal, formed by 6 helix groups connected by an extended loop between helix 6 and 7. The hydrophilic cavity is located between the two domains and is formed from H-I, H-II, H-IV and H-V of the N-terminal domain and H-VII, H-VIII, H-X and H-XI of the

C-terminal domain. The remaining helices are in contact with the lipid bilayer, not contacting with the cavity (Abramson *et al.*, 2004)

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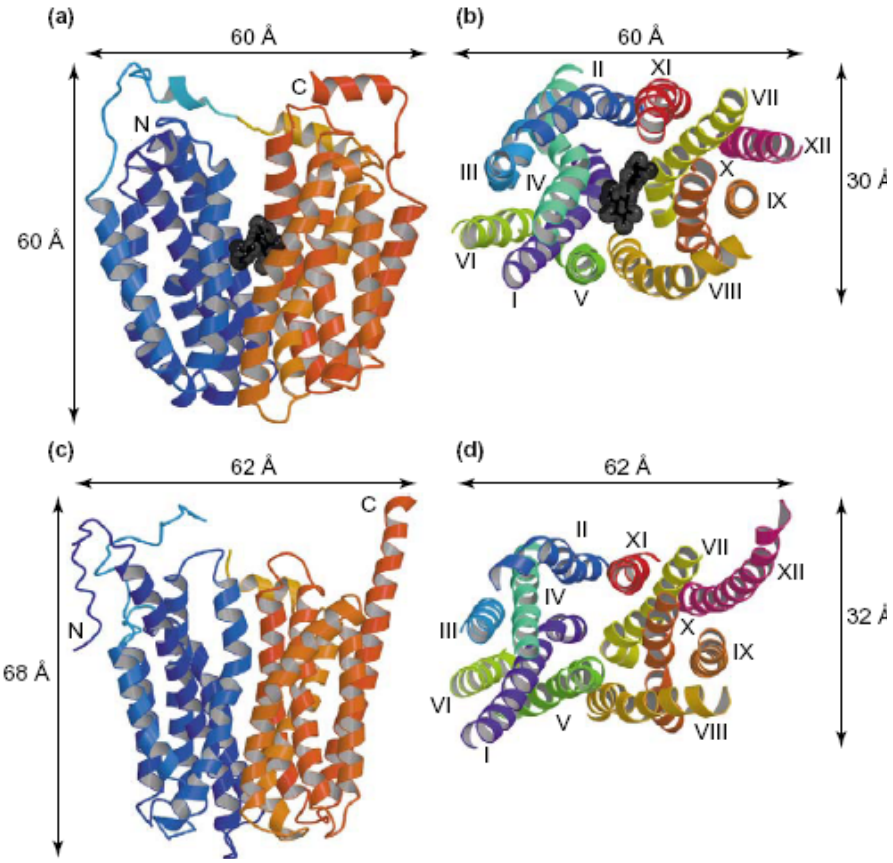

Figure 7 - Structure of LacY and GlpT. (a) Representation of LacY viewed parallel to the membrane. The N- and C-terminal domains are coloured blue and red, respectively; LacY substrate is represented in black. (b) Representation of LacY viewed along the membrane from the cytoplasmic side. (c) Representation of GlpT viewed parallel to the membrane. The colour scheme is as in (a). (d) Representation of GlpT viewed along the membrane from the cytoplasmic side. (b) (adapted from Abramson *et al.*, 2004).

The substrate binding site is located within the internal cavity, that is better characterized in the LacY than in GlpT due to the fact that the protein crystal structure was achieved in the presence of the substrate, and as it was already referred due to the extensive work performed on the permease by Kaback and his co-workers. In both proteins the substrate binding site is formed by the helices H-I, H-IV and H-V of the N-terminal domain, and H-VII and H-XI of the C-terminal

domain. In the LacYp the sugar binds at a similar distance of the cytoplasm and periplasm. On the contrary, in the GlpT the two arginines residues Arg45(H-I) Arg269(H-269) that form the binding site are closer to the periplasm than the binding site residues of LacY. Several LacY residues are involved in substrate binding, namely Trp151 (H-V), Met23 (H-I), Arg144 (H-V), Glu126 (HIV), Glu269 (H-VIII), of the N-terminal domain and Lys358 (H-XI), Asp237 (H-VII) of the C-terminal (Abramson *et al.*, 2003) (Figure 8). The N-terminal substrate binding residues seem to be more connected with substrate specificity and the C terminal with substrate affinity (Kaback, 2005). As for the residues involved in proton coupling and translocation they are located in the C-terminal domain: Tyr236 (H-VII) Asp240 (H-VII), Lys319 (H-X), His322 (H-X), Glu325 (H-X) and Arg302 (H-IX).

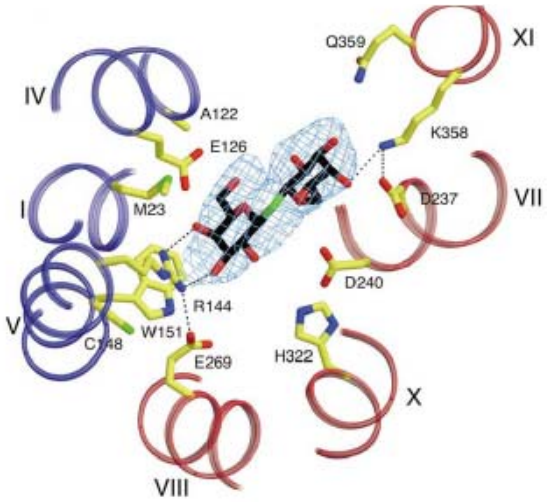

Figure 8 – Detailed structure of *LacY* substrate binding site. Representation of the helices involved in substrate binding and amino acids involved in substrate or proton ligation (adapted from Abramson *et al.*, 2003).

Recent work on the LacY permease substrate binding site demonstrated the absence of binding site when the protein ligand is not present. The substrate is the responsible for the formation of the sugar binding-site which is interrelated with the deprotonation of the residue Glu269 (H-VIII) (Mirza *et al.*, 2006).

Another important question that was further elucidated with the publication of the MFS-member structures was the mechanism of substrate translocation across the plasma membrane. Two models were proposed based on the three dimensional structure as well as in previous biochemical data (Figure 9).

For both permeases an alternating access model of transport is proposed with a unique substrate-binding site that is accessible from only one side of the membrane at a time. The mechanism for the lactose/proton symporter can be described in six steps, starting from the outward-facing conformation: protonation of LacY, binding of substrate, conformational change to the inward-facing conformation, release of substrate, release of proton, and return to the outward-facing conformation.

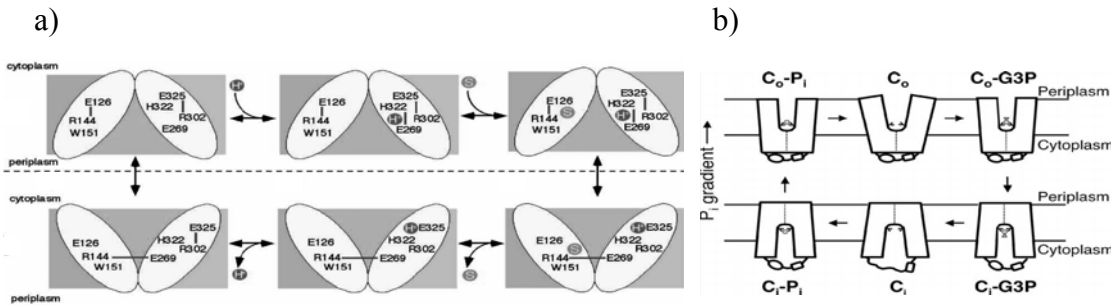

Figure 9 – Proposed models for substrate translocation of a) lactose/proton symporter (LacY) and the b) Pi/glycerol-3-phosphate antiporter (GlpT) (Lemieux *et al.*, 2004; Kaback, 2005).

As for the glycerol-3-phosphate/Pi antiporter, the overall model is quite similar to the lactose permease: binding of substrate to protein in the outward-facing conformation, conformational change to the inward-facing conformation, higher concentration of Pi in the cytoplasm results in release of substrate and binding of Pi, conformational change to the outward-facing conformation, due to higher permease affinity to glycerol-3-phosphate, release of Pi and binding of substrate.

Since the publication of the MFS transporter structures, our knowledge of structural data has significantly increased and several structural models are now proposed based on the LacY and GlpT three dimensional structures. A model for the phosphate/proton symporter transporter Pho84 of *S. cerevisiae* and the osmoprotectant/proton symporter ProP from *E. coli* was elaborated based on the GlpT structure (Lagerstedt *et al.*, 2004; Wood *et al.*, 2005). Also structural models

for non MFS members like the melibiose transporter from *E. coli* (MelB) belonging to the glycoside-pentoside-hexuronide:cation symporter family (TC-2.A.2.1) are being modulated based on a comparison with the MFS resolved protein structure, although previous work on two dimensional crystallization followed by cryoelectron microscopy had already a projected structure with 8 Å resolution (Hacksell *et al.*, 2002; Purhonen *et al.*, 2005).

The number of determined integral membrane protein structures is growing although it is rather small when compared to other kinds of proteins. In the website 'Membrane Proteins of Known Structure' (http://www.mpibp-frankfurt.mpg.de/michel/public/memprotstruct.html), 56 different membrane protein structures are listed, and according to another website 'Membrane Proteins of Known 3D Structure' (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html), there are currently 97 unique membrane protein structures. This number is continuously increasing and reflects the efforts of several multidisciplinary teams in optimizing protein production, protein purification, and crystal growth that ultimately enables protein structure determination.

Outline of the present thesis

Carboxylic acids are important compounds in the yeast cell functionality, since they can be used as carbon and energy sources and are subproducts of yeast metabolism. In light of the importance of this group of compounds, intensive physiological studies have contributed significantly to the understanding of the metabolism and transport of these nutrients across the plasma membrane in yeasts.

The aim of this thesis was to increase the current knowledge on yeast monocarboxylate permeases, especially on the Lactate/Pyruvate Proton Symporter.

In the general introduction a bibliographic revision on yeast carboxylate transporters is presented. The physiological characterization of many carboxylate transporter systems has been done for several years now, resulting in a vast knowledge on yeast carboxylate uptake systems in several yeast species. Nevertheless, the molecular characterization of these systems evolved very recently, and the identification of genes encoding carboxylate transporters was only achieved latter on. Several groups of transporters are focused, the plasma membrane carboxylate transporters, including the mammalian members, were the first to be described, followed by the mitochondrial membrane carboxylate transporters and the efflux pumps. Finally, a revision on the current knowledge of permease structure is presented. This field was greatly enriched with the recent achievement on the crystallization and structure determination of two transporters belonging to the Major Facilitator Superfamily. This major breakthrough enlightened the field of permease structure determination and made way for new structure predictions based on previously determined structures.

In chapter two the work concerning the heterologous expression in *Pichia pastoris* is presented. With this study the doubts concerning the true function of the Jen1p permease are dissipated, since besides achieving the uptake of lactate in *P. pastoris* strains expressing the *JEN1* gene, the activity for a mediated lactate uptake system was recovered in hybrid vesicles. This fact ultimately confirmed that Jen1p was the lactate/pyruvate permease, and not a mere regulator of its expression as it was previously proposed (Halestrap and Price, 1999; Makuc *et al.*, 2001).

After the sequencing of the *S. cerevisiae* genome several other projects were started, aiming at the sequencing of several yeast genomes. This also happened with *C. albicans*, a well known pathogenic yeast that represents a major pharmaceutical interest since the number of infected patients has dramatically increased in the past few years. Using a comparative genomic strategy we aimed at the identification of a monocarboxylate permease. In chapter three the characterization of the lactate uptake mechanism of *C. albicans* is described as well as the cloning of the lactate permease. Additionally, the effect of the CaCat8p, an homolog of the known *S. cerevisiae* Cat8p regulator, on the lactate permease expression was also evaluated.

Chapter four describes the results obtained for a structural/functional study of the Jen1p. A conserved domain of the permease was identified by performing an alignment with Jen1p homologs and homologous sequences deposited in databases. A mutational analysis of five amino acids composing the conserved sequence highlighted the importance of this domain, and the possible contribution of each amino acid for protein function.

In chapter five, a general discussion of the described work is presented. The main outcomes of the studies here detailed are described, as well as some perspectives for new research possibilities.

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Chapter 2

Functional expression of the lactate permease Jen1p of *Saccharomyces cerevisiae* in *Pichia pastoris*

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Adapted from:

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Abstract

In *Saccharomyces cerevisiae* the activity for the lactate proton-symporter is dependent on *JEN1* gene expression. *Pichia pastoris* was transformed with an integrative plasmid containing the *JEN1* gene. After 24 h of methanol induction, Northern and Western-blotting analyses indicated the expression of *JEN1* in the transformants. Lactate permease activity was obtained in *P. pastoris* cells with a V_{max} of 2.1 nmol s^{-1} mg^{-1} dry weight. Reconstitution of the lactate permease activity was achieved by fusing plasma membranes of *P. pastoris* methanol-induced cells with *Escherichia coli* liposomes containing cytochrome *c* oxidase, as proton-motive force. These assays in reconstituted heterologous *P. pastoris* membrane vesicles demonstrate that *S. cerevisiae* Jen1p is a functional lactate transporter. Moreover a *S. cerevisiae* strain deleted in the *JEN1* gene was transformed with a centromeric plasmid containing *JEN1* under the control of the glyceraldehyde 3-phosphate

dehydrogenase constitutive promotor. Constitutive *JEN1* expression and lactic acid uptake were observed in cells grown either on glucose and/or acetic acid. The highest V_{max} (0.84 nmol s^{-1} mg^{-1} dry weight) was obtained in acetic acid-grown cells. Thus overexpression of the *S. cerevisiae JEN1* gene in both *S. cerevisiae* and *P. pastoris* cells resulted in increased activity of lactate transport when compared to the data previously reported in lactic acid-grown cells of native *S. cerevisiae* strains. *Jen1p* is the only *S. cerevisiae* secondary porter characterized so far by heterologous expression in *P. pastoris* at both the cell and membrane vesicle levels.

Introduction

Lipophilic weak carboxylic acids are used as food preservatives. The undissociated acid accumulates into microbial cells by simple diffusion until equilibrium of concentrations in both sides of the plasma membrane (Rottemberg, 1979). The acid dissociates in the cytoplasm resulting in toxic accumulation of protons and anions. This phenomenon depends on the microbial strain, the external pH, the external concentration of the acid molecule and its physical characteristics such as pKa and lipid-buffer partition value.

Monocarboxylic acids are normal end products of the alcoholic fermentation carried out by *Saccharomyces cerevisiae*. Lactic acid, pyruvic acid or acetic acid can be used as sole carbon and energy sources by *S. cerevisiae.* However glucose-grown cells are not able to metabolise these acids (Casal *et al.*, 1996). At concentrations occurring in must fermentation acetic acid induces cell death (Pinto *et al.*, 1987; Ludovico *et al.*, 2001) and inhibits metabolic fermentation/respiration activities (Pampulha and Loureiro-Dias, 2000).

The ABC transporters Yor1p (Cui *et al.*, 1996) and Pdr12p (Piper *et al.*, 1998; Holyoak *et al.*, 1999) have been reported to contribute to tolerance to monocarboxylic acids in *S. cerevisiae*, possibly by directly extruding the toxic anion through the plasma membrane. Expression of the major facilitator *AZR1* is required for adaptation to acetic acid and to low molecular-weight organic acids (Tenreiro *et al.*, 2000). Two monocarboxylate-proton symporters have been described in *S. cerevisiae*: one is shared by acetate, propionate and formate, while the other

transports lactate, pyruvate, acetate and propionate (Cássio *et al.*, 1987; Casal *et al.*, 1995; Casal *et al.*, 1996). The first system is constitutively expressed in cells growing on non-fermentable carbon sources while the lactate transporter is specifically induced by lactate. Both systems are totally repressed by glucose.

In *S. cerevisiae* it was demonstrated that the activity for the lactate proton-symporter in dependent on the expression of *JEN1* (Casal *et al.*, 1999). *JEN1* is the only *S. cerevisiae* member of the Sialate-Proton Symporters subfamily (TC#2.A.1.12) belonging to the Major Facilitator Superfamily (De Hertogh *et al.*, 2002). However members of other phylogenic subfamilies can be expected to transport monocarboxylic acids such as the five MCP Monocarboxylate Porters, the FNT Acetate:H^+ Symporter YHL008c or even the *SSU1* Putative Transporter of Unknown Mechanism (De Hertogh *et al.*, 2002).

Jen1p is rapidly and irreversibly inactivated upon the addition of glucose to induced cells (Andrade and Casal, 2001). Some of the factors involved in proper localization and turnover of the Jen1 protein were revealed by expression of the JEN1-GFP fusion in a set of strains with mutations in specific steps of the secretory and endocytic pathways (Paiva *et al.*, 2002). However none of the above data discriminates the possibilities whether Jen1p has regulatory (or sensor) or transport function.

The purpose of our work is to demonstrate non-ambiguously that Jen1p is a monocarboxylate proton symporter. Therefore the *JEN1* gene was cloned in *Pichia pastoris* to produce significant amounts of active protein allowing heterologous reconstitution of lactate transport activity in membrane vesicles. The *JEN1* gene was also overexpressed in *S. cerevisiae* (at a lower efficiency however) to characterize the kinetic properties of Jen1p at the cell level.

Materials and Methods

Strains and growth conditions

Yeast strains are described in Table 1. Cultures were maintained on YPD. Minimal media contained Difco yeast nitrogen base (YNB) adjusted to the indicated

pH with HCl or NaOH and supplemented with the adequate requirements for prototrophic growth. For growth of *Saccharomyces cerevisiae,* YNB media were supplemented with different concentrations of glucose and/ or acetic acid, as indicated in the text. For growth of *Pichia pastoris*, specific media were utilized as follows: YPDS medium - glucose (2.0%, w/v), yeast extract (1.0%, w/v), peptone (1.0%, w/v), sorbitol (1.0 M); MGY medium - YNB (1.34%, w/v), biotin (4.0 x 10^{-5}%, w/v), glycerol (1.0%, w/v); MM medium - YNB (1.34%, w/v), biotin (4.0 x 10^{-5}%, w/v), methanol (0.5%, v/v); MD medium - YNB (1.34%, w/v), biotin (4.0 x 10^{-5}%, w/v), glucose (2.0%, w/v). Agar (2.0%, w/v) was added for solid media. Liquid *S. cerevisiae* cultures were grown at 28°C, 180 r.p.m. and *P. pastoris* cultures were grown at 30ºC, 250 rpm. The media were supplemented with zeocin (25 - 100 μg/ml) and ampicilin (100 μg/ml) whenever necessary. The *Escherichia coli* XL1-Blue strain was used for plasmid propagation and amplification according to (Sambrook *et al.*, 1989). Consumption of glucose and acetic acid was determined using a HPLC system (Gilson), equipped with a Merck Polyspher OA KC Column (Cat. no. 51270), maintained at 50°C. The mobile phase was sulfuric acid (0.05 N, in ultra-pure water), and the flow rate was 0.5 ml min^{-1}.

DNA manipulation and cloning techniques

DNA cloning and manipulation were performed according to standard protocols (Sambrook *et al.*, 1989). The yeast shuttle vectors p416GPD (*CEN6/ARSH4*) and p426GPD (*2μ*) were kindly provided by Dr. Dominik Mumberg (Mumberg *et al.*, 1995). The gene *JEN1* was amplified from *S. cerevisiae* W303-1A genomic DNA by PCR, using primers J7 (CCGGAATTCGTTACATAGAGAAGCGAACACG) and J8 (CGCGGATCCAGTTTCAAAAGTTTTTCCTCAAAG), (MWG Biotech) and Platinum Taq high fidelity DNA polymerase (Gibco Cat. No. 11304-011). The primers introduced a *BamH* I (J8) and an *EcoR* I (J7) restriction site at the 5' and 3' ends of *JEN1*, respectively. The amplified fragment was digested with both enzymes and cloned into the cloning array of the plasmids p416GPD and p426GPD, originating plasmids pDS1 and pDS2, respectively, which were used to transform *S. cerevisiae* (Table 1).

Table 1 - Yeast strains.

Strain	Relevant genotype	Source or Reference
S. cerevisiae		
W303-1A	a ade2 leu2 his3 trp1 ura3	Thomas and Rothstein, 1989
BLC203	W303-1A *jen1*Δ::*HIS3*	Casal *et al*., 1999
L19	W303-1A *jen1*Δ::*HIS* (p416GPD)	This work
L23	W303-1A *jen1*Δ::*HIS* (pDS1)	This work
L79	W303-1A *jen1*Δ::*HIS* (p426GPD)	This work
L81	W303-1A *jen1*Δ::*HIS* (pDS2)	This work
P. pastoris		
X-33	wild-type	Invitrogen
BLC536	X-33 (pZPARS)	This work
BLC537	X-33 (pZ-JEN1)	This work
BLC532	X-33 (pPICZB)	This work
BLC549	X-33 (pB-JEN1)-I	This work
BLC550	X-33 (pB-JEN1)-II	This work
BLC551	X-33 (pB-JEN1)-III	This work
BLC552	X-33 (pB-JEN1)-IV	This work
KM71H	*arg4 aox1*Δ :: *SARG4*	Invitrogen
BLC538	KM71H (pZPARS)	This work
BLC539	KM71H (pZ-JEN1)	This work
BLC534	KM71H (pPICZB)	This work
BLC553	KM71H (pB-JEN1)-I	This work
BLC554	KM71H (pB-JEN1)-II	This work
BLC555	KM71H (pB-JEN1)-III	This work
BLC556	KM71H (pB-JEN1)-IV	This work

The *P. pastoris* pPICZB plasmid was purchased from Invitrogen. *JEN1* was amplified by PCR from the *S. cerevisiae* W303-1A genome, using the primers JB-EcoRI (CCGGAATTCGAAAATATGTCGTCGTCAATTACAG) and JB-XbaI (GCTCTAGAACGGTCTCAATATGCTCC). *JEN1* was also cloned in the replicative expression vector pZPARS (Lueking *et al.*, 2000). For this purpose, *JEN1* was amplified by PCR from the *S. cerevisiae* W303-1A genome, using the primers JP-SalI (ACGCGTCGACGTCGTCGTCAATTACAGATGAGAAAATATCTGG) and

JP-NotI (ATAAGAATGCGGCCGCATTAAACGGTCTCAATATGCTCCTCATATGTC). The PCR products were digested and cloned in the expression vector using standard procedures, originating plasmids pB-JEN1 and pZ-JEN1, respectively. The four plasmids were used to transform *P. pastoris*, both X-33 and KM71H strains (Table 1).

RT-PCR reactions were performed with primers JF (TGACATGGCAGAATTGGAAC) and JR (GGAATTTCTCATGGCCAACA), based on the sequence of *JEN1*, and PF (AAAGATATGGTCATCATCAGAAGAA) and PR (CAAACTTGGATGCTTGGTAGACA) as a reference based on the sequence of *PDA*. The Ready To-Go™ RT-PCR Beads from Amersham (Cat. Nº 27-9556-01) were used, in combination with 300 ng DNAse treated RNA and $pd(T)_{12-18}$ as first strand primer.

Selection of Pichia pastoris *recombinant strains*

The integrative vectors were digested with the restriction enzyme *Sal*I (Roche), for integration in the *AOX1 locus*. *P. pastoris* X-33 and KM71H cells were transformed by electroporation, the transformants were selected in YPDS medium supplemented with zeocin and each transformant was purified to ensure pure clonal isolates. A direct PCR screening of the *P. pastoris* clones was performed as described by Linder *et al.* (Linder *et al.*, 1996) and in accordance to the guidelines provided by the EasySelect™ *Pichia* Expression Kit Instruction Manual (Invitrogen) using the 3'AOX1 (GCAAATGGCATTCTGACATCC) and 5'AOX1 (GACTGGTTCCAATTGACAAGC) primers. Another PCR reaction was performed by amplification of an internal fragment of the *S. cerevisiae* gene using specific primers Jinv-1 (GAAAGTGGCCGTACATTAC) and JC (GATACCCCAGACACCAAAGAC). Considering that all *P. pastoris* KM71H transformants have a Mut^s phenotype, X-33 integration transformants were tested for their Mut^+ or Mut^s phenotype in MM medium. All the transformant strains analysed presented a Mut^+ phenotype.

Pichia pastoris *growth conditions for recombinant protein expression*

Cells previously grown in solid MD medium for 48 h were inoculated in 100 ml MGY medium in a 1 L flask and grown in a shaking incubator until the culture reached OD_{600} = 2.0 – 6.0. The cells were harvested and resuspended in 200 ml MM medium to an OD_{600} of 0.5 in a 2 L flask. Methanol (100%, v/v) was added to the culture to a final concentration of 0.5% (v/v) every 24 hours to maintain induction. Cell suspension samples of 15 ml were collected along time and analysed for recombinant protein expression.

Hybridization analysis

Samples of 10 μg of genomic DNA from each *P. pastoris* transformant strain were blotted onto a positively charged nylon membrane (GeneScreenPlus Hybridization Transfer Membrane, NEN™ Life Sciences Products, Inc., Boston) using a PR648 Slot blot filtration manifold (Hoefer Scientific Instruments, San Francisco, CA). Genomic DNA preparation and slot-blot experimentation were performed according to Ausubel *et al*. (Ausubel *et al.*, 1996). Total cellular mRNA was prepared from yeast cells, 20 μg were electrophoresed on 1.5% (w/v) agarose MOPS-formaldehyde gels, blotted onto a positively charged nylon membrane and the membrane was hybridised for *JEN1* (Casal *et al.*, 1999). Densitometer scanning was performed using the Integrated Density Analysis program from the EagleSight® Software, version 3.2 (Stratagene, CA).

Preparation of plasma membranes and yeast cell extracts

Plasma membranes were prepared from *P. pastoris* as described by Van Leeuwen *et al.* (Van Leeuwen *et al.*, 1991). The purified membranes were resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 (about 3 mg protein/ml) and stored in liquid nitrogen. Total yeast extracts were obtained by the method of Volland and co-workers (Volland *et al.*, 1994).

SDS-polyacrylamide gel electrophoresis, and Western-blotting

Protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Hybond^{TM}-P, Amersham Pharmacia Biotech). The proteins were probed with chicken polyclonal antibody, raised against a 13 aminoacid peptide of the N-terminal region (EVYNPDHEKLYHN) of Jen1p. Primary antibody was detected with a horseradish peroxidase-conjugated anti-chicken immunoglobulin G secondary antibody (Sigma) detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Measurement of transport activity

Cells were harvested, washed twice with ice-cold deionized water and resuspended to a final concentration of about 25-35 mg dry weight per ml. Uptake rates of labelled monocarboxylic acids were estimated as described previously (Paiva *et al.*, 2002). The substrates were DL-[^{14}C]lactic acid (sodium salt, Amersham Pharmacia Biotech) (4000 dpm/nmol), pH 5.0 and [^{14}C]acetic acid (sodium salt, Amersham Pharmacia Biotech) (3000 dpm/nmol), pH 5.0. A computer-assisted non-linear regression analysis program (GraphPAD software, San Diego CA, USA) was used to determine the best fitting transport kinetics to the experimental data and to estimate the kinetic parameters. All the experiments were performed in triplicate, and the data represent average values.

Preparation of hybrid plasma membrane vesicles and measurement of labelled lactic acid accumulation

Fusion of proteoliposomes containing cytochrome oxidase with plasma membrane vesicles was performed by the freeze-thaw-sonication procedure as described previously (Gerós *et al.*, 1996) using a 1:20 protein/phospholipid ratio. At time zero, radioactively labelled DL-lactic acid (158 μM) was added to 200 μl of hybrid vesicles and energization was started by addition of 15 mM ascorbate, 0.015 mM cytochrome *c* and 0.15 mM TMPD. Aliquots of 15 μl were withdrawn at

appropriate intervals and diluted with 2.0 ml ice cold 100 mM LiCl. The mixtures were filtered on nitrocellulose filters (pore size 0.45 μm, Macherey-Nagel) and washed with 2.0 ml 100 mM LiCl. The filters were introduced into vials and radioactivity was measured as described above. Experiments were carried out at 26ºC.

Results

Constitutive expression of JEN1 *in* Saccharomyces cerevisiae

The *Saccharomyces cerevisiae* strain carrying a genomic deleted *jen1Δ* allele was transformed with either one of the plasmids p416GPD and p426GPD (native plasmids) and with the corresponding plasmids containing a copy of *JEN1* under the control of the glyceraldehyde 3-phosphate dehydrogenase (GPD) constitutive promotor (pDS1 and pDS2, respectively, Table 1). *JEN1* transcription was analyzed by RT-PCR. Figure 1 shows the detection of *JEN1* transcripts, prepared from exponentially growing cells in different single and mixed substrate culture media. As expected, no mRNA signal was found in strains carrying the native plasmids. In contrast and independently of the carbon source, *JEN1* expression was detected in the strains transformed with the plasmids bearing *JEN1*.

As it was previously shown (Casal *et al.*, 1999; Andrade *et al.*, 2005) in the strain *S. cerevisiae* W303-1A, *JEN1* expression is absent in glucose when under the control of its own promoter. Additionally, in lactic acid-grown cells a rapid decline of *JEN1* mRNA is observed upon the addition of glucose, the transcripts being completely absent 10 minutes after the pulse of glucose (Andrade *et al.*, 2005). These results show that despite the mechanisms of repression and degradation previously reported, the cloning under a strong promoter allows constitutive expression of the *JEN1* gene whether the carbon source is glucose, acetic acid or a mixture of both.

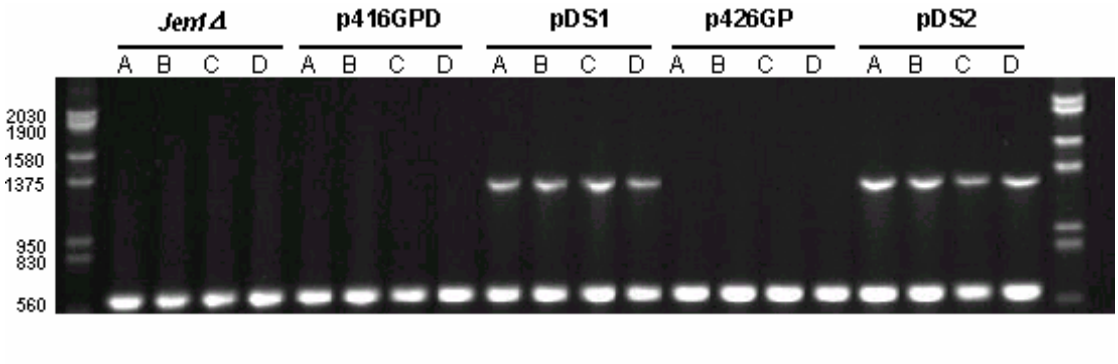

Figure 1 - Detection of *JEN1* expression by RT-PCR in cells of *S. cerevisiae jen1*Δ not transformed or transformed with the referred plasmids. Cells were collected during the first exponential growth phase. Letters indicate different culture media: A (YNB, glucose, 2.0%, w/v, pH 4,0); B (YNB, glucose, 2.0%, w/v, acetic acid, 0.25%, v/v, pH 4.0); C (YNB, glucose, 0.1%, w/v, acetic acid 0.25%, v/v, pH 4.0); D (YNB, acetic acid, 0.25%, v/v, pH 4.0).

Transport of monocarboxylic acids in Saccharomyces cerevisiae *cells expressing* JEN1 *constitutively*

Initial uptake rates of labelled lactic acid were measured at pH 5.0 in exponentially growing cells cultivated in minimal medium at pH 4.0, containing glucose (2.0%, w/v), acetic acid (0.25%, v/v) or a mixture of both glucose and acetic acid in the concentrations indicated (Table 2). Under the growth conditions used, the *JEN1* gene cloned in the centromeric plasmid induces permease activity, while when cloned in the multicopy plasmid the uptake of labelled lactic acid obeys to a simple diffusion mechanism. In cells carrying no functional copy of *JEN1* the transport of labelled lactic acid also obeyed to a simple diffusion mechanism.

Measurements of lactic acid uptake were performed in glucose grown-cells of transformed *S. cerevisiae* strains, incorporating a metabolizable (glucose, 100 mM) or a non-metabolizable sugar (sorbitol, 100 mM) in the assay buffer. The kinetic parameters obtained were of the same order of magnitude as the ones described in Table 2 in the absence of the sugar. These results indicate that the medium composition and osmotic strength do not affect the mechanism of monocarboxylic acid transport.

Transport of labelled acetic acid at pH 5.0 was also evaluated (Table 2). In acetic acid-grown cells, acetate uptake was observed in the four strains studied. This result confirms that besides *JEN1* another monocarboxylate permease is present in acetic acid-grown cells of *S. cerevisiae* (Casal *et al.*, 1996; Casal *et al.*, 1999). In glucose-grown cells (either in the presence or in the absence of acetic acid) activity for the acetate permease was only found in cells expressing *JEN1* from the centromeric plasmid. This indicates that above a certain level *JEN1* mRNA is not functionally expressed in *S. cerevisiae.*

Table 2 - Kinetic parameters for the transport of monocarboxylic acids in *S. cerevisiae jen1*Δ transformed with the indicated plasmids. Transport analyses were performed in cells exponentially growing in the following culture media: G (YNB, glucose, 2.0% w/v, pH 4.0); GA (YNB, glucose, 2.0% w/v and acetic acid, 0.25%, v/v, pH 4.0); A (YNB, acetic acid, 0.25%, v/v, pH 4.0).

Plasmid	Culture media	Kinetic parameters			
		Acetic acid		Lactic acid	
		K_m (mM)	V_{max} (nmol s^{-1} mg dry wt^{-1})	K_m (mM)	V_{max} (nmol s^{-1} mg dry wt^{-1})
p416GPD	G	No activity		No activity	
	GA	No activity		No activity	
	A	1.50±0.50	2.69±0.39	No activity	
pDS1	G	0.68±0.17	0.44±0.04	0.60±0.23	0.50±0.07
	GA	2.71±1.05	1.04±0.26	0.35±0.22	0.63±0.11
	A	1.57±0.62	3.07±0.53	0.64±0.28	0.83±0.11
p426GPD	G	No activity		No activity	
	GA	No activity		No activity	
	A	1.67±0.31	1.39±0.68	No activity	
pDS2	G	No activity		No activity	
	GA	No activity		No activity	
	A	1.06±0.47	2.86±0.48	Not determined	

Consumption of acetic acid in the presence of glucose in Saccharomyces cerevisiae

In order to determine whether the constitutive expression of *JEN1* in the presence of glucose is associated to an altered consumption of acetic acid, the supernatants of cultures grown in YNB containing glucose and acetic acid were analyzed by HPLC. As can be observed from Figure 2, the consumption of both substrates was identical in a mixed substrate medium containing glucose and acetic acid, at pH 4.0. In cells either expressing or not *JEN1,* the consumption of acetic acid was initiated only after glucose exhaustion.

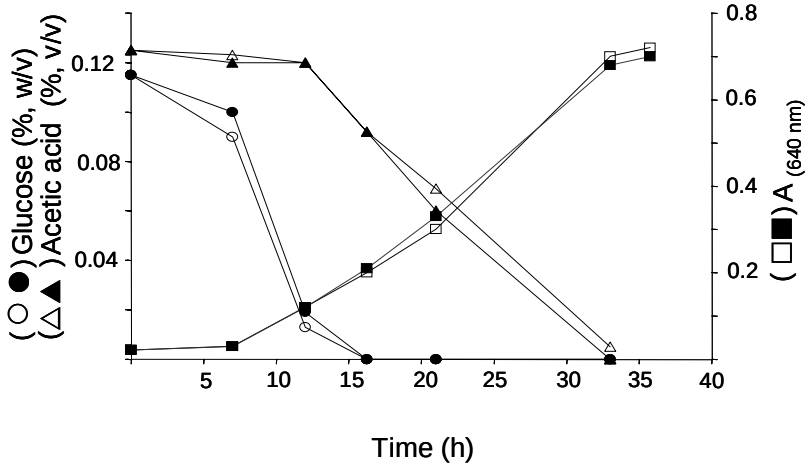

Figure 2 - Growth of *S. cerevisiae* L19 (open symbols) and L23 (closed symbols) strains in YNB medium containing glucose (0.1%, w/v) and acetic acid (0.125%, v/v), pH 4.0.

As shown in Table 3, glucose and/or acetic acid grown cells had very similar growth rates in all the conditions tested, either at pH 4.0 or 6.0. In the media containing glucose and acetic acid the growth rate decreased and the log phase increased (results not shown) with increasing amounts of acetic acid. The plasmids p416GPD and pDS1 were used to transform another *S. cerevisiae* genetic background (CEN.P113-13D), and no differences in growth were detected between the strains.

Table 3 - Specific growth rate (h^{-1}) of *S. cerevisiae jen1*Δ transformed with the plasmids p416GPD or pDS1. Cells were cultivated in YNB medium containing the indicated carbon sources, at the initial pH indicated. Data represent results of three independent experiments.

Culture media			Specific growth rate (h^{-1}) of the transformants	
Glucose (%, w/v)	Acetic acid (%, v/v)	pH	p416GPD	pDS1
2.0	0.00	4.0	0.22 ± 0.03	0.23 ± 0.02
2.0	0.25	4.0	0.22 ± 0.03	0.23 ± 0.02
2.0	0.30	4.0	0.21 ± 0.03	0.21 ± 0.03
2.0	0.40	4.0	0.18 ± 0.01	0.16 ± 0.02
2.0	0.50	4.0	0.05 ± 0.005	0.05 ± 0.004
2.0	0.60	4.0	0.05 ± 0.005	0.05 ± 0.006
2.0	0.70	4.0	No growth	No growth
0.0	0.25	4.0	0.15 ± 0.01	0.12 ± 0.02
2.0	0.00	6.0	0.30 ± 0.02	0.26 ± 0.02
2.0	0.25	6.0	0.23 ± 0.03	0.25 ± 0.02
0.0	0.25	6.0	0.16 ± 0.01	0.13 ± 0.01

Lactate permease activity in Pichia pastoris *transformants*

Jen1p permease activity was evaluated by measuring the initial uptake rates of radioactive lactic acid in different *P. pastoris* transformants. Cells were grown in mineral medium with glycerol (MGY), and further incubated for 72 h in methanol medium (MM). After 24 h induction with methanol all *P. pastoris* transformants containing the *JEN1* gene presented measurable lactic acid uptake, although with different velocities. After 48 or 72 h of induction, lactate uptake was greatly decreased. The integrative vectors generated higher uptake rates than the replicative

vectors (not shown). Additionally, the *P. pastoris* KM71H (pB-JEN1) transformant strains analysed exhibited higher lactate uptake activity than the X-33 (pB-JEN1) recombinant strains. The transformant that displayed the highest level of lactate uptake in all the tested conditions was KM71H (pB-JEN1)-I (strain BLC553, Table 1). In glycerol-containing medium (0 h induction), no measurable lactate uptake activity could be found for any of the assayed strains (not shown). In the strains containing the empty vectors (Table 1), no measurable permease activity could be found during the induction time-course tested (not shown).

Slot-blot analysis of integrated JEN1

Semi-quantitative DNA slot-blot analysis was carried out using genomic DNA isolated from the recombinant strains (Figure 3). The *S. cerevisiae JEN1* probe hybridised as expected with the parental *S. cerevisiae* W303-1A genomic DNA control, and failed to produce any signal in all negative controls, while signals were obtained with recombinant *P. pastoris* strains. The transformant BLC553 presented the highest number of genomic *JEN1* insertions, in agreement with the observation of the highest permease activity level in this transformant.

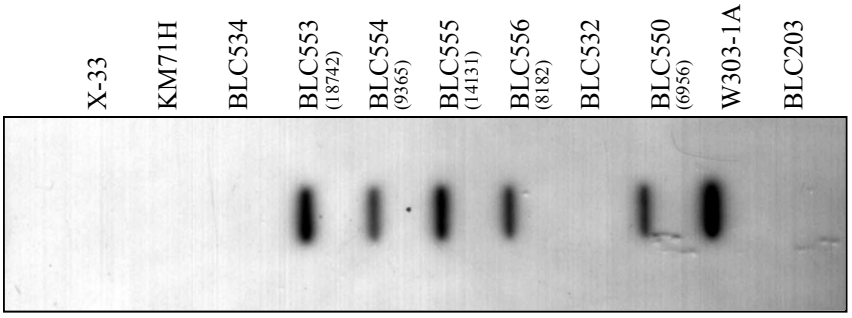

Figure 3 - Slot-blot analysis of 10 μg of genomic DNA from *P. pastoris* transformants, using a *JEN1*-specific probe. Numbers in brackets refer to the measured spot density.

No *JEN1* transcripts were detected in *P. pastoris* cells grown in media containing glucose or glycerol. However a 2.2 kb mRNA was detected in the KM71H (pB-JEN1)-I transformant induced with methanol (Figure 4).

Jen1p was detected in membrane preparations of the *P. pastoris* KM71H (pB-JEN1)-I transformant, as well as in *S. cerevisiae* lactic acid-grown cells. The size of the protein expressed in *P. pastoris* KM17H (pB-JEN1)-I membranes is larger than the one detected in *S. cerevisiae*, which is expected as Jen1p expressed in *P. pastoris* has the hexahistidine and c-myc tags (Figure 5).

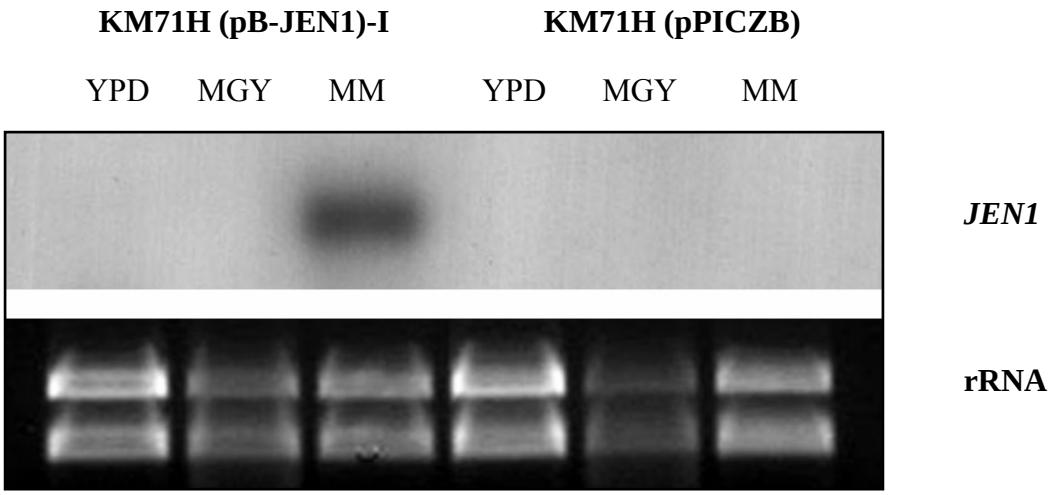

Figure 4 - Northern-blot analysis of *JEN1* expression in *P. pastoris* KM71H recombinant strains. Samples were taken from cells grown in YPD or in MGY and from cells transferred to MM medium for 24 h.

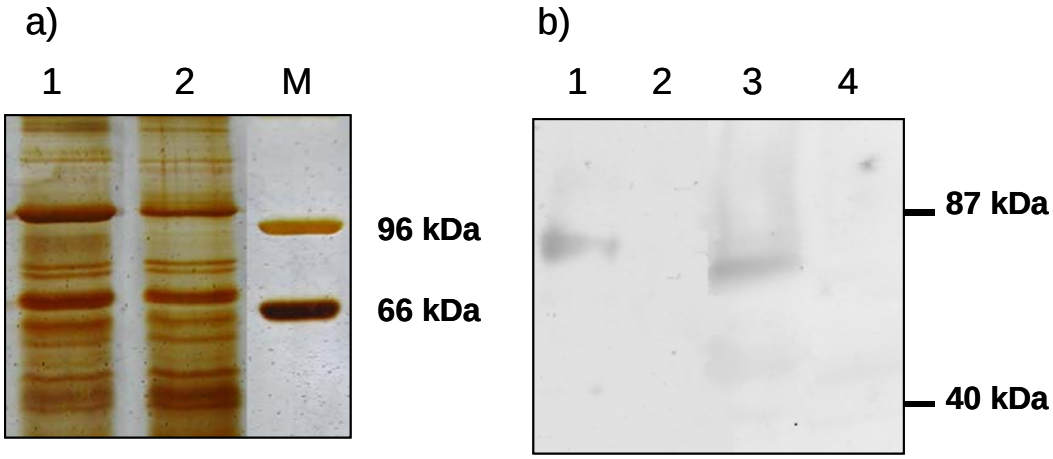

Figure 5 - Analysis of the expression of Jen1p by (a) Silver staining and (b) Western blot, using specific Jen1p antibodies, after SDS-PAGE of plasma membrane extracts from *P. pastoris* 24h-methanol induced cells. Lane 1: *P. pastoris* KM71H (pB-JEN1)-I; lane 2: *P. pastoris* KM71H pPICZB. Crude extracts of *S. cerevisiae* were used to evaluate Jen1p antibody specificity. Lane 3: W303-1A lactic acid-grown cells; lane 4: W303-1A glucose-grown cells.

Characterization of the Jen1 permease kinetic parameters

The kinetic parameters of monocarboxylate transport system were determined in the *P. pastoris* recombinant KM71H (pB-JEN1)-I recombinant strain after 24 h of induction in methanol-containing medium, measuring the initial uptake rates as a function of the labelled lactic acid concentration. *P. pastoris* KM71H transformed with the empty vector was used as a control, where labelled lactic acid was transported solely by simple diffusion (Figure 6), with $k_d = 0.040 \pm 0.002$ µl s^{-1} mg^{-1} dry weight. The presence of a mediated transport system was indicated by Michaelis-Menten saturation kinetics in the recombinant strain KM71H (pB-JEN1)-I, with $K_m = 0.54 \pm 0.08$ mM DL-lactic acid and $V_{max} = 2.15 \pm 0.14$ nmol lactic acid s^{-1} mg^{-1} dry weight.

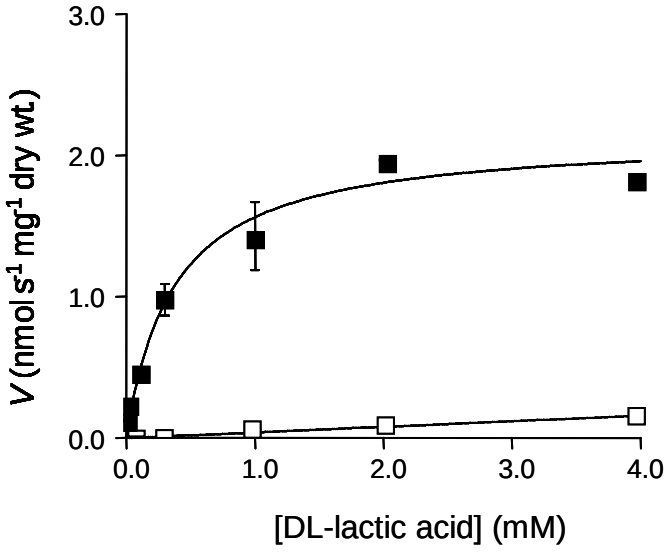

Figure 6 - Initial uptake rates of labelled lactic acid (pH 5.0) measured in P. pastoris pZB-JEN1)-I (closed symbols) and pPICZB (open symbols) transformants after 24h of induction in methanol-containing medium.

Lactic acid transport in hybrid vesicles

Plasma membranes from methanol-induced *P. pastoris* KM71H (pB-JEN1)-I recombinant were fused with *E. coli* liposomes containing beef heart cytochrome oxidase. In control vesicles obtained with *P. pastoris* transformed with the empty vector, the uptake of labelled lactic acid was negligible, before and after energization. In contrast, hybrid vesicles prepared from cells containing *JEN1* accumulated a 6-fold higher lactate concentration inside than outside at pH 6.2. (Figure 7). The essential contribution of the proton-motive force to the accumulation of lactic acid was shown by the rapid efflux of the accumulated labelled lactic acid upon addition of 10 μM FCCP. The specificity of transport was investigated by the addition of either unlabelled pyruvic acid (80 mM) or citric acid (80 mM) to the hybrid vesicles after accumulation of the labelled lactic acid. Only pyruvic acid was

able to promote the efflux of labelled lactic acid (Figure 7). Such specificity is fully consistent with the results obtained in *S. cerevisiae* whole cells (Cássio *et al.*, 1987).

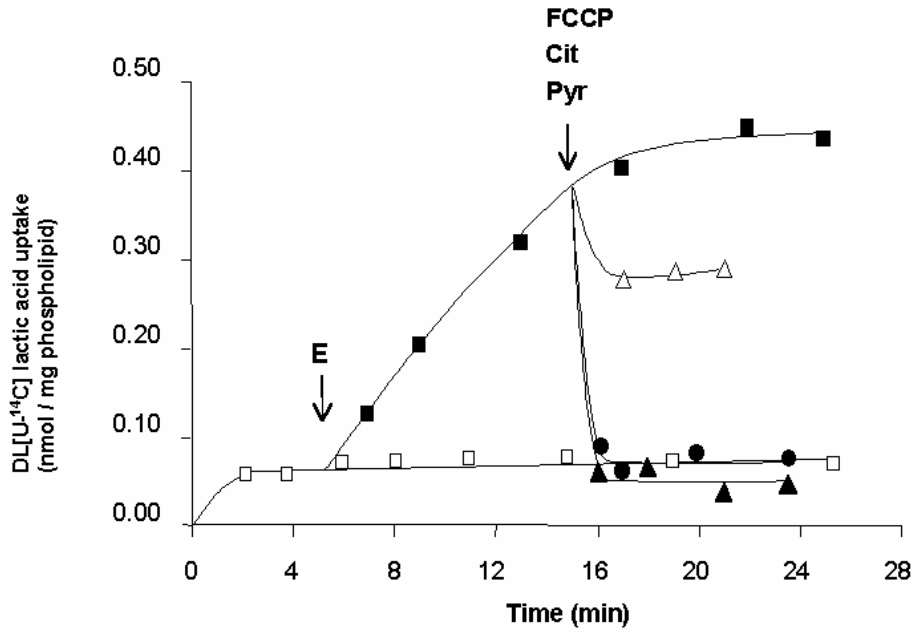

Figure 7 - Uptake of labelled lactic acid in hybrid vesicles prepared from *P. pastoris* KM71H (pZB-JEN1)-I methanol induced cells. The assays were performed with 158 μM labelled lactic acid, at pH 6.2 and 26 ºC, either with (■) or without energization (□). For energization of the system (E) 15 mM ascorbate, 0.015 mM cytochrome *c* and 0.15 mM TMPD were added to the reaction mixture. At the time indicated by the arrow, 10 μM FCCP (▲), 80 mM pyruvic acid (●) and 80 mM citric acid (△) were added.

Discussion

This is the first report of fully functional reconstitution of a *S. cerevisiae* permease in the heterologous host *P. pastoris*. We therefore will comment on some features of the cloning system used. The *P. pastoris* recombinant strains containing the *JEN1* gene were grown in glycerol and transferred to methanol-containing medium for gene induction by the strong *AOX1* promoter. All recombinant strains

presented a maximum initial rate of lactic acid uptake after 24 h of induction. After 48 or 72 h of induction, permease activity was greatly decreased indicating that continuous overproduction of the membrane protein was deleterious. Independently isolated *P. pastoris* strains transformed with the same expression vector displayed significant differences in the levels of Jen1p activity. Such clonal variation is often observed even within collections of transformants harbouring the same number of expression cassettes (Cregg *et al.*, 1993; McGrew *et al.*, 1997; Cai *et al.*, 2001). By slot-blot hybridisation we verified that the clone KM71H (pB-JEN1)-I presenting the highest levels of lactic acid transport also exhibited increased *JEN1* copy number integrated in the genome. Premature termination of transcription has been described for a number of foreign genes expressed in yeast (Romanos *et al.*, 1995). Fortunately, this phenomenon was not observed for *JEN1* expression in the KM71H (pB-JEN1)-I transformant. Northern-blot analysis in different culture media shows a transcript of similar size to that found for *S. cerevisiae*, corresponding to *JEN1* mRNA (2.2 kb). In the best *P. pastoris* transformant, the kinetic parameters for lactate uptake were found to be K_m = 0.54 ± 0.08 mM lactic acid and V_{max} = 2.15 ± 0.14 nmol lactic acid s^{-1} mg^{-1} dry weight while in *S. cerevisiae* W303-1A the V_{max} was previously estimated to be 0.40 nmol lactic acid s^{-1} mg^{-1} dry weight and the K_m = 0.69 mM lactic acid (Casal *et al.*, 1999). These results represent a 5-fold enrichment of Jen1p in *P. pastoris* transformant cells compared to the *S. cerevisiae* wild type. In contrast, the best constitutive heterologous monocarboxylate overexpression of *JEN1* in the homologous host *S. cerevisiae* had produced only a 2-fold increase in Jen1p V_{max} using the strong GPD promoter, while the use of a multicopy vector inhibited growth. Even though it appears that, as in *S. cerevisiae* (dExaerde *et al.*, 1996), excessive overexpression of membrane proteins is detrimental to *P. pastoris* cell growth, the amount of heterologous proteins produced in *P. pastoris* was much higher than in *S. cerevisiae* and sufficient for allowing the measurement of lactate transport in reconstituted membrane vesicles. The reconstitution of the activity of lactate permease of *S. cerevisiae* was achieved in hybrid vesicles obtained by fusing plasma membranes from *P. pastoris* KM71H pB-JEN1 with proteoliposomes. The properties of the reconstituted lactate uptake agreed with those of the permease evaluated in *S. cerevisiae* cells. The involvement of the proton motive force was directly demonstrated in reconstituted *P. pastoris* vesicles by instant release of lactate upon addition of protonophore confirming thus

the proton-symport mechanism previously shown in *S. cerevisiae* intact cells. Such *in vitro* measurement of Jen1p-dependent lactate uptake obtained in heterologous membrane vesicles is crucial. Indeed it is the only measurement that provides non-ambiguous demonstration of Jen1p being a lactate permease. While it is conceivable that in *S. cerevisiae* cells the loss of lactate uptake in *JEN1* deletants and its gain in constitutive overexpression conditions could result from indirect perturbation of regulatory or sensing factors, as has been postulated in literature (Halestrap and Price, 1999; Makuc *et al.*, 2001), such regulatory mechanism is very unlikely to occur in heterologously reconstituted membrane vesicles from *P. pastoris.* Indeed, when not transformed, this species does not contain lactate permease activity in the induction conditions used. In conclusion *JEN1* is a fully functional lactate permease. It is the only functional monocarboxylate transporter gene identified so far in the *S. cerevisiae* genome as Makuc *et al.* (Makuc *et al.*, 2001) have showed that neither the 5 members of the Monocarboxylate Porter subfamily nor the *YHL008c* gene were involved in monocarboxylate transport under all tested conditions.

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Chapter 3

The disruption of *JEN1* from *Candida albicans* impairs the transport of lactate

Chapter 3

The disruption of *JEN1* from *Candida albicans* impairs the transport of lactate

Adapted from:

Soares-Silva, I., Paiva, S., Kötter, P., Entian, K.-D., Casal M. **2004**.The disruption of *JEN1* from *Candida albicans* impairs the transport of lactate. Molecular Membrane Biology 21, 403-411

Abstract

A lactate permease was biochemically identified in *Candida albicans* RM1000 presenting the following kinetic parameters at pH 5.0: K_m 0.33 ± 0.09 mM and V_{max} 0.85± 0.06 nmol s^{-1} mg dry wt^{-1}. Lactate uptake was competitively inhibited by pyruvic and propionic acids; acetic acid behaved as a non-competitive substrate. An ORF homologous to *Saccharomyces cerevisiae* gene *JEN1* was identified (*CaJEN1*). Deletions of both *CaJEN1* alleles of *C. albicans* (resulting strain CPK2) resulted in the loss of all measurable lactate permease activity. No *CaJEN1* mRNA was detectable in glucose-grown cells neither activity for the lactate transporter. In a medium containing lactic acid, *CaJEN1* mRNA was detected in the RM1000 strain, and no expression was found in cells of CPK2 strain. In a strain deleted in the *CaCAT8* genes the expression of *CaJEN1* was significantly reduced, suggesting the role of this gene as an activator for *CaJEN1* expression. Both in *C. albicans* and in

S. cerevisiae cells *CaJEN1-GFP* fusion was expressed and targeted to the plasma membrane. The native *CaJEN1* was not functional in a *S. cerevisiae jen1*Δ strain. Changing ser^{217}-CTG codon (encoding leucine in *S. cerevisiae)* to a TCC codon restored the permease activity in *S. cerevisiae*, proving that the *CaJEN1* gene codes for a monocarboxylate transporter.

Introduction

The yeast *Candida albicans* is an opportunistic human fungal pathogen. In immunocompromised hosts and other susceptible individuals it can cause life-threatening systemic infections and a variety of other pathologies such as vaginitis, nosocomial bloodstream, deep tissue and oral mucosal infections (Odds, 1988). Over the past years a lot of research has been carried out to characterize this organism, both at the physiological and at the molecular level and also to elucidate its pathogenic determinants. *C. albicans* has become a model pathogenic fungus to study dimorphism, to analyse virulence, and to analyse new fungal targets (De Backer *et al*., 2000). It is an asexual diploid that exists in at least three morphogenic forms, yeast, pseudohyphae and hyphae. Strong evidence suggests that the filamentous form is implicated in the invasiveness of the organism (Odds *et al*., 1988). External pH seems to be among the factors that regulate the reversible transition between the two forms (Odds, 1985). Previous works suggest that the control of internal pH which is directly or indirectly associated with the regulation of dimorphism depends on the activity of transport systems mediating the fluxes of protons across the plasma membrane (Kaur *et al*., 1994; Stewart *et al*., 1988; Stewart *et al*., 1989). The study of carboxylate permeases is therefore of major significance to improve our current knowledge on pH-stasis and pathogenicity in *C. albicans*.

Transport to the interior of the cell is the first step in the utilization of short-chain carboxylic acids. Short-chain carboxylic acids are weak acids and they dissociate in the solution according to their dissociation constant(s), pKa(s) value(s), and to the pH of the medium. The uptake of the undissociated form of carboxylic acids through the lipid bilayer membranes can occur by a simple diffusion

mechanism. The uptake of the anionic form(s) of the acid(s), requires the presence of a mediated mechanism that catalyses their uptake to the interior of the cell. In the yeast *Saccharomyces cerevisiae* activity for at least two monocarboxylate-proton symporters was found, with differences in their mechanisms of regulation and specificity. A lactate-pyruvate-acetate-propionate transporter is induced in lactic or pyruvic acid-grown cells (Cássio *et al.*, 1987) and it is encoded by the gene *JEN1* (Casal *et al.*, 1999) and its expression depends on gene activator *CAT8* (Bojunga and Entian, 1999). *JEN1* is the first identified gene to be involved in monocarboxylate transport across plasma membranes of fungi. Biochemically, another transport system, which accepts acetate, propionate or formate was identified in cells grown in non-fermentable carbon sources (Casal *et al*., 1996). Recently, the gene *YCR010c* (*ADY2*) was identified as a new key element for this transport system (Paiva *et al*., 2004). The assembly of the diploid genome of *C. albicans* strain SC5314 became available after shotgun sequencing (Stanford Genome Technology Centre, http://www-sequence.stanford.edu/group/candida/, released May, 2002). In this work, using *JEN1* of *S. cerevisiae* as a model, we searched for genes coding for potential monocarboxylate transporters in *C. albicans*. This study reports the isolation and molecular characterization of *CaJEN1*, the first monocarboxylic acids permease gene in this species and its proof of function.

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 cultures were maintained on slants of yeast extract (1%, w/v), peptone (1%, w/v), glucose (2%, w/v) and agar (2%, w/v). Yeast cells were grown in Difco yeast nitrogen base, 6.7%, 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), lactic acid (0.5%, v/v, pH 5.0), acetic acid (0.5%, v/v, pH 6.0), pyruvic acid (0.5%, w/v, pH 5.0), propionic

acid (0.5%, v/v, pH 5.0), formic acid (0.5%, v/v, pH 5.0), glycerol (1%, w/v) and ethanol 1% (w/v). Solid media were prepared adding agar (2%, w/v) to the respective liquid media. Growth was carried out at 30 ºC, both in solid or liquid media.

Table 1 - Yeast strains used in the present work.

Strain	Relevant genotype	Source of Reference
C. albicans		
RM1000	*ura3::imm434/ura3::imm434 his1::hisG/his1::hisG*	Negredo *et al*., 1997
CPK1	*ra3::imm434/ura3::imm434his1::hisG/his1::hisG JEN1/jen1::URA3*	This work
CPK2	*ura3::imm434/ura3::imm434 his1::hisG/his1::G jen1::HIS1/jen1::URA3*	This work
CPK2-22	*ura3::imm434/ura3::imm434 his1::hisG/his1::G jen1::HIS1/JEN1 (after reintegration)*	This work
CPK20-5	(*JEN1/JEN1::YEGFP-URA3*)	This work
RM.SD2	*ura3::imm434/ura3::imm434his1::hisG/his1::hisG CAT8/cat8::URA3*	This work
RM.SD7	*ura3::imm434/ura3::imm434his1::hisG/his1::hisG cat8::URA3/cat8::HIS1*	This work
RM.SD8	*ura3::imm434/ura3::imm434his1::hisG/his1::hisG cat8::HIS1/CAT8 (after reintegration)*	This work
S. cerevisiae		
Ace720	CEN.PK 113-13D; *MATa ura3-52*	EUROSCARF*
Ace721	CEN.PK 113-13D; *MATa ura3-52 jen1*Δ	Makuc *et al*., 2001
Ace723	Ace 721 transformed with the plasmid pIJ2	This work
BLC600	Ace 721 transformed with the plasmid pUG35-CaJEN1	This work
BLC601	Ace721 transformed with the plasmid pUG35	This work
BLC602	Ace721 transformed with the plasmid p416-CaJEN1m	This work

* http://www.uni-frankfurt.de/fb15/mikro/euroscarf/

Repression and derepression conditions

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 a carbon source.

Transport assays

Cells incubated under derepression conditions were harvested by centrifugation, washed twice in ice-cold deionised water and ressuspended in ice-cold deionised water to a final concentration of about 25-40 mg dry wt. ml^{-1}. 10 µl of yeast cell suspension were mixed in 10 ml conical tubes with 30 µl of 0.1 M potassium phosphate buffer, pH 5.0. After 2 minutes of incubation at 30º C in a water bath, the reaction was started by the addition of 10 µl of an aqueous solution of the labelled acid at the desired concentration and pH value, and stopped by dilution with 5 ml of ice-cold water. The reaction mixtures were filtered immediately through Whatman GF/C membranes, the filters washed with 10-ml of ice-cold water and transferred to the scintillation fluid (Opti-phase HiSafe II; LKB FSA Laboratory Supplies, Loughborough, UK). Radioactivity was measured in a Packard Tri-Carb 2200 CA liquid scintillation spectrophotometer with disintegrations per minute correction. The effect of non-labelled substrates on the initial uptake velocities of labelled acid was assayed by adding simultaneously the labelled and non-labelled substrate. The following radioactive labelled substrate was utilised, D,L-[U-^{14}C]lactic acid, sodium salt (Amersham Pharmacia Biotech). Non-specific ^{14}C adsorption to the filters and to the cells 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).

Table 2 - Plasmids used in this work.

Plasmids	Source of reference
pCUB6	Fonzi *et al*., 1993
pGFP-URA3	Gerami-Nejal *et al*., 2001
p416GPD	Mumberg *et al*., 1995
pIJ2 (*CaJEN1* in p416GPD)	This study
pUG35	U. Güldener and J. H. Hegemann (unpublished,*)
pUG35-CaJEN1	This study
p416-CaJEN1-m	This study

* http://mips.gsf.de/proj/yeast/info/tools/hegemann/gfp.html

Cloning of the CaJEN1 *gene and site-directed mutagenesis*

Sequence data for *C. albicans* was obtained from the Stanford DNA Sequencing and Technology Centre Website at http://www-sequence.stanford.edu/group/candida. Sequencing of *C. albicans* was accomplished with the support of the NIDR and the Burroughs Wellcome Fund. Using the BLAST program an ORF was identified revealing homology to the *JEN1* of *S. cerevisiae*. This gene was named *CaJEN1* and it was cloned in the plasmid pGEM®-T Easy vector (PROMEGA), by amplification with the Pfu Turbo DNA polymerase (Stratagene) of *CaJEN1* ORF with the primers Calb1 and Calb2 using genomic DNA isolated from *C. albicans* RM1000. DNA cloning and manipulation were performed according to standard protocols (Sambrook *et al*., 1989). The *BamH* I-*Sal* I fragment of this plasmid, containing the *CaJEN1* ORF was then cloned in the p416GPD vector (Mumberg *et al*., 1995). The *C. albicans* genomic DNA contained in the plasmid pIJ2 was confirmed by sequencing. For site-specific mutagenesis the methodology described by Ansaldi *et al.* (1996) was used with *Ca*JEN1 cloned into plasmid p416GPD. The recovered plasmids were systematically checked by sequencing. The primers used for site-directed mutagenesis are indicated in Table 3.

Disruption and reintegration of the CaJEN1 and CaCAT8 genes

All genetic modifications of the *C. albicans* genome have been done by homologous recombination using PCR products with short-flanking homologies to the corresponding gene, which has to be deleted/modified (Wach *et al*., 1994). A *CaURA3* gene deletion cassette was amplified by PCR using primers (Table 3) homologous to both the *CaURA3* gene and *CaJEN1* (CaJEN1-S1/CaJEN1-S2) as well as *CaCAT*8 (CaCAT8-S1/CaCAT8-S2) and pCUB6 as template. After transformation, with the deletion cassette into *C. albicans* RM1000, correct gene replacement in uridine prototrophic colonies was verified by diagnostic PCR. In the resulting strain CPK1 (RM.SD2) the second *CaJEN1(CaCAT8)* allele was deleted using *CaHIS1* gene as selection marker. The *CaHIS1* gene deletion cassette was amplified using primers homologous to both the *CaHIS1* gene and *CaJEN1* (CaJEN1-S3/CaJEN1-S4) as well as *CaCAT*8 (CaCAT8-S3/CaCAT8-S4) and pCS-CaHIS1 (unpublished work, pRS426+CaHIS1). The resulting histidine prototrophic strain CPK2 (RM.SD7) was verified by diagnostic PCR.

To reintroduce a functional *CaJEN1* (*CaCAT8*) gene into CPK2 (RM.SD7) the WT copies of *CaJEN1* (*CaCAT8*) were amplified by PCR on chromosomal DNA using primers CaJEN1-A1/CaJEN1-A4 (CaCAT8-A1/CaCAT8-A4) and after transformation appropriate clones were then selected on plates containing 5-fluoro-orotic acid (5-FOA) (Boeke *et al*., 1984). The resulting transformants were first verified by diagnostic PCR.

Additionally the deletion of the coding region of *CaJEN1 (CaCAT8)* was verified by Southern blot analysis (not shown) on genomic DNA. Genomic DNA was prepared as described by Hoffman and Winston (1987).

CaJEN1-GFP chimeric DNA fragment construction and transformation

To generate fluorescent protein tags at the 3'-end of *CaJEN1* by PCR, plasmid pGFP-URA3 was used as template and primers CaJEN1-F1/CaJEN1-R1 (Table 3). PCR products were used to transform the strain *C. albicans* RM1000 and transformants were screened for correct integration of the fluorescent protein tag

cassette by diagnostic PCR using primer combinations CaJEN1-A3/YEGFP-2 and CaURA3-A3/CaJEN1-A4.

Plasmid pUG35-CaJEN1 was constructed by amplification of the *CaJEN1* ORF from the *C. albicans* RM1000 genomic DNA with the Accuzyme DNA Polymerase (Bioline), using the primers CaJEN1-pUG1 and CaJEN1-pUG2 (Table 3). The resulting PCR fragment was then digested with *Hind* III and *Bam*H I and ligated into pUG35 (U. Güldener and J. H. Hegemann, unpublished). *S. cerevisiae* transformant strains carrying pUG35 or pUG35-CaJEN1 plasmids were selected for further analysis (strains BLC600 and BLC601, respectively).

Microscopy

C. albicans living cells were examined with a Leitz Aristoplan epifluorescence microscope with filter cube 1001 HQ-FITC for GFP excitation. For the capture of the images, an Apogee charge-coupled device camera was used, and the micrographs were processed for display using Image Pro Plus software. *S. cerevisiae* living cells images were registered by using a Leitz Laborlux S Microscopic with accessory apparatus for fluorescence (Ploemopak Filter 12) connected to a Sony Progressive 3 CCD. The images were processed using Axio Vision 3.0 software.

RNA analysis

Total RNA was prepared from yeast cells, electrophoresed on 1.5% (w/v) agarose MOPS-formaldehyde gels (Newman, 1994) and blotted to nylon membranes by vacuum transfer. Hybridisation was carried out using a fragment of 966 bp *Cla* I – *Bam*H I from pIJ2 as a probe for *CaJEN1*. Densiometer scanning was performed using the Integrated Density Analysis program from the EagleSight® Software, version 3.2 (Stratagene, CA).

Table 3 - Oligonucleotides used for cloning, gene deletion, verification by PCR and GFP tagging. The sequences complementary to the sequences of pCUB6 (S1/S2), pCS-CaHIS1(S3/S4) and pGFP-URA3(F1/R1) are in bold letters.

Name	**Sequence**
Calb1	AGG ATC CAC TCC ATA TCA CAC CTA CAC AC
Calb2	CCC AAT GTG AAT AGC ATA TGC GGC
CaJEN1-S1	CAG ACC TTT CAT TAT CTT CAA TCT CTA GAT ATT TTG CCA CTA GAT TAA CCA CAT TAT TAG CTC ATG TTT GAC AGC TTA TC
CaJEN1-S2	TGC CCC ACA AAA AAT ACA CAT GAC TTT ACC ATA ATC AAA CAT TCC TGG TTG GTC TTT AAG CGG TCG GAC AGT GCT CCG AG
CaJEN1-S3	CAG ACC TTT CAT TAT CTT CAA TCT CTA GAT ATT TTG CCA CTA GAT TAA CCA CAT TAT TAG TCA GTG GTT GAT GAA GCG ACC
CaJEN1-S4	TGC CCC ACA AAA AAT ACA CAT GAC TTT ACC ATA ATC AAA CAT TCC TGG TTG GTC TTT AAG CTC CAA AGG CAA CAG CCT CG
CaCAT8-S1	GTC GAT AAA GTT AGA ACA ACA GCA GT CAA GTG TAT CAC AGG AGA ACA ATT TAC CAC CTA CAC TCA TGT TTG ACA GCT TAT C
CaCAT8-S2	GAC TAT CAT TAT GTG ATA TGT AAT CAT CAT GGT TTG TGG TCT TGC GTT CGT TTA TAG CAT CGG TCG GAC AGT GCT CCG AG
CaCAT8-S3	GAT GAA GAA GAT AAT GCA AGT TTA TTG AGT ATT GAA GAC TAT AAT TCT AGA CAC CGA GAC TCA GTG GTT GAT GAA GCG AC
CaCAT8-S4	TGT TTA CCA TAT CTG TAT TGC TAG TTG GCG TTT TCA ATT TCG AAG CTG CGA AAT TGT TAG CTC AAG CAA ACA TTC AAT TG
CaCAT8-A1	CAG CAT GTG ATT GGC GCA GAG
CaCAT8-A2	GCC TGC TTA GAA TCG TAT CC
CaCAT8-A3	TCT AAG TGT GAT GCT GTA CAG
CaCAT8-A4	TCA ACT AAA CAA GGA AGC AAG
CaJEN1-A1	CTA GTT TCA CCC ACA AGA ACA C
CaJEN1-A2B	TCT CTG GTG CAG CCA CCG
CaJEN1-A3B	GAG TTC AGC TAG TGC CAC C
CaJEN1-A4	GAA AAT TGG AGA GAG TTG GTG G
CaHIS1-A2	CAC CAC TCA ATA AGT TAC AGC
CaHIS1-A3	GAC GAA GAG GAC TGG GTT G
CaURA3-A2	AGG CAT GAG TTT CTG CTC TC
CaURA3-A3	TTG GCT TAT TAT GAC ACC TG
CaJEN1-F1	GAA ATC GAA GAG TTA CAA CAA GAT AGC TCT AGT AAT AAC GAG AGT GTT AAA GAA AGA GTT GGT GGT GGT TCT AAA GGT GAA GAA TTA TTC
CaJEN1-R1	AAA GAA ATT GAA CTA TCA GGA ACA CTT TAT TTC ACC TAA ATA TAA ATA ATC CGT TTA TTC TCT AGA AGG ACC ACC TTT GAT TG
CaJEN1-pUG1	CGG GAT CCA CTC CAT ATC ACA CCT ACA CAC
yEGFP-2	TCA CCT TCA CCG GAG ACA G
CaJEN1-pUG2	CCC AAG CTT AAC TCT TTC TTT AAC ACT CTC
CaJEN1mut1	CTT GCG GCA ATT GGT TGA CCT TCT AAT GCT GTA ACC ATG G
CaJEN1mut2*	GAA GGT CAA CCA ATT GCC GCA AGA TCA GTG TTG TCC GGA TTG TTT TTA CC

Results

Transport of monocarboxylic acids by lactic acid-grown cells of C. albicans

C. albicans RM1000 was able to grow in YNB medium with DL-lactic acid, pyruvic acid, acetic acid or glycerol, as sole carbon and energy source, with initial pH of 5.0, 30º C. Cells grown on DL-lactic acid (0.5%, v/v, pH 5.0) were used to measure the initial uptake rates of labelled DL-lactic acid over a concentration range from 0.016 to 3.8 mM. The uptake mechanism at pH 5.0 revealed a characteristic Michaelis-Menten kinetic. The application of a computer-assisted non-linear regression analysis to the experimental data (acid concentration 0.016 – 3.8 mM), agreed with the presence of only one mediated transport system for labelled DL-lactic acid, without contamination of a possible simple diffusion component of the undissociated acid. The values estimated for K_m and V_{max}, at pH 5.0, were respectively 0.33 ± 0.09 mM and 0.85 ± 0.06 nmol s^{-1} mg^{-1} dry wt (Figure 1). Addition of DL-lactic acid (pH 5.0) to lactic-acid grown cells induced a transient extracellular alkalisation, which is indicative for a proton uptake. To study the specificity of the permease the initial uptake rates of labelled DL-lactic acid were determined either in the presence of non-labelled enantiomers of lactic acid or other monocarboxylic acids. Both enantiomers, L- and D-lactic acid were transported by the permease, since each of them could competitively inhibit the uptake of the labelled DL-lactic acid (Figure 1A). Formic acid (from 1 to 10 mM) revealed no inhibitory effect on the acid uptake (results not shown). Additionally, pyruvic and propionic acids were competitive inhibitors of lactate transport at pH 5.0 (Figure 1A), which shows that these acids were transported by the same system as lactic acid. In contrast, acetic acid inhibited non-competitively the uptake of labelled DL-lactic acid, pH 5.0 (Figure 1B), pointing to the presence of another transport system of acetic acid. This observation is in agreement with data known for other yeast species where two monocarboxylate transporters, with distinct specificities and regulatory mechanisms can be found. In *S. cerevisiae* (Casal *et al*., 1996) and *Torulaspora delbrueckii* (Casal *et al*., 1995), besides a general monocarboxylate

transporter induced by lactic acid, activity for another acetate transporter is also found.

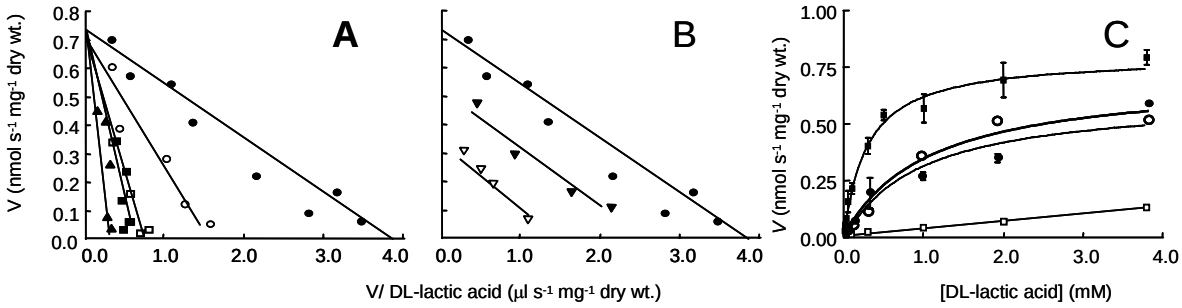

Figure 1 - Transport of lactate in *C. albicans*. A and B) Eadie-Hoffstee plots of the initial uptake rates of labelled lactic acid, at pH 5.0, by YNB-lactic acid-grown cells of *C. albicans* RM1000. (●), absence of other non-labelled substrate; (○), presence of 0.5 mM of propionic acid; (■), presence of 2 mM of D-lactic acid; (□), presence of 2 mM of L-lactic acid; (Δ), presence of 1 mM of pyruvic acid; (▼), presence of 2 mM of acetic acid; (∇), presence of 4 mM of acetic acid. C) Initial uptake rates of labelled lactic acid, pH 5.0, by YNB-lactic acid-grown cells of *C. albicans* RM1000 strains (■), CPK2 (□), CPK2-22 (○) and *S. cerevisiae* transformed with p416-CaJen1-m (●), as a function of the acid concentration.

Isolation and functional analysis of the CaJEN1 *gene*

Sequence data for *C. albicans* were obtained from the Stanford Genome Technology Centre website at http://www-sequence.stanford.edu/group/candida. Using the BLASTN program an ORF was identified revealing homology to the *JEN1* of *S. cerevisiae*. This gene was named *CaJEN1* and it encodes a protein homologous to the Jen1 protein from *S. cerevisiae*. *Ca*Jen1 protein has 12 predicted transmembrane domains and 41% of identity and 61% similarity with *S. cerevisiae* Jen1p.

To investigate the physiological function of the *CaJEN1* gene both chromosomal copies of the gene were deleted (see Materials and Methods and Figure 2), resulting in strain *C. albicans* CPK2 (see also Table 1). The uptake of

labelled DL-lactic acid, at pH 5.0, was measured in YNB-lactic acid-grown cells of *C. albicans* CPK2. The application of a computer-assisted non-linear regression analysis to the experimental data (acid concentration 0.016 – 3.8 mM) confirmed the absence of a mediated transport system in these cells, indicating that the deletion of *CaJEN1* abolished the mediated transport of lactate in *C. albicans* (Figure 1C). The strain CPK2-22, obtained by reintegration of the *CaJEN1* in the *CaJEN1* locus of the CPK2 strain, recovered the activity of the lactate carrier confirming that the permease activity is correlated with the presence of the *CaJEN1* gene (not shown).

The phenotype of *C. albicans* CPK2 strain was evaluated regarding its ability to grow on YP and YNB solid media containing glucose (2%, w/v), acetic acid (0.5%, v/v, pH 6.0), glycerol (2%, w/v), DL-lactic acid (0.5%, v/v, pH 5.0) or pyruvic acid (0.5%, v/v, pH 5.0). The cells were able to grow in all these substrates as the only carbon and energy source (not shown). Therefore, although *C. albicans* CPK2 was not able to transport lactic acid by a mediated mechanism, its ability to grow on lactic acid, as the only carbon and energy source, was not affected, when compared to the wild-type strain.

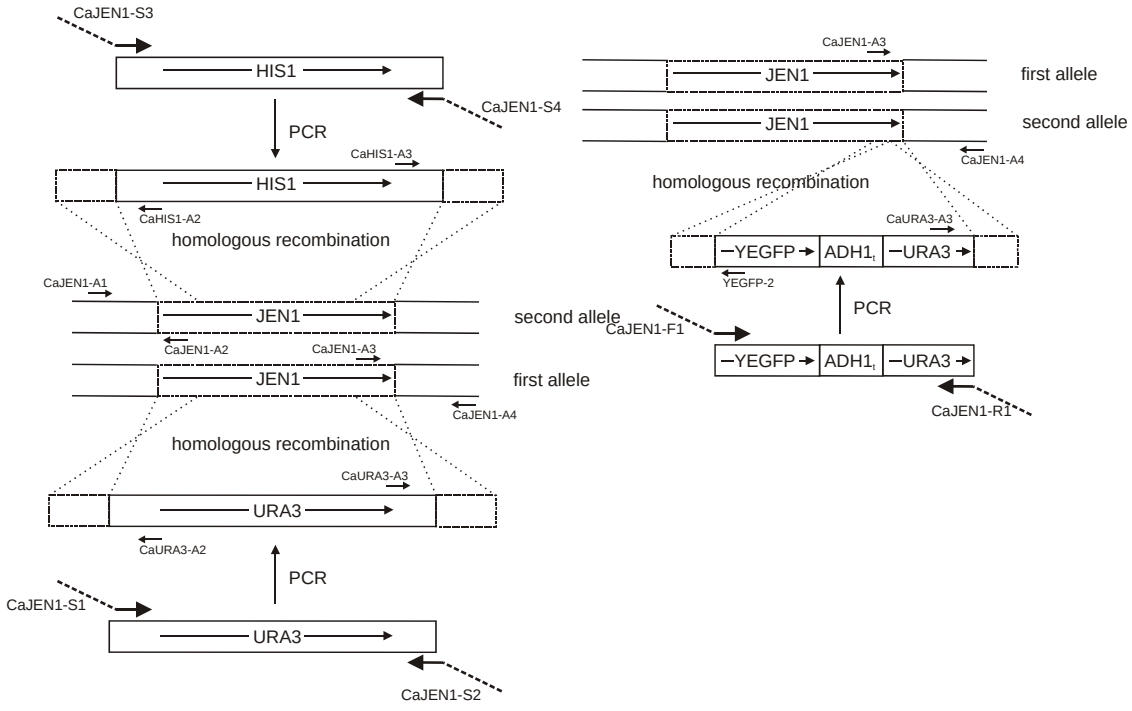

Figure 2 - Strategies for the A) disruption of *CaJEN1* (see text for explanation) and B) the construction of the *CaJEN1-GFP* fusion (see text for explanation).

No permease activity for labelled DL-lactic acid and no Ca*JEN1* expression were detected in glucose-grown cells of wild-type strain *C. albicans* RM1000, similarly to what had already been published for *JEN1* in *S. cerevisiae* (Casal *et al*., 1999, Bojunga and Entian, 1999). To follow the time course of *CaJEN1* expression glucose–grown cells were washed, transferred to YNB-lactic acid medium, and the expression of *CaJEN1* was followed by Northern-blot analysis. In cells of wild-type strain *C. albicans* RM1000, *CaJEN1* mRNA became detectable 2 h after transfer to derepressing conditions. A steady-state of *CaJEN1* mRNA expression was achieved between 2 and 5 hours (Figure 3A), with a maximum lactate carrier activity observed between 4 and 5 hours (Figure 3A). In cells of the *C. albicans* CPK2 deletion strain no *CaJEN1* mRNA was detected, even after 5 hours of derepression (Figure 3A) and no permease activity for the CPK2 strain was found (Figure 3A). In the strain CPK2-22, the *CaJEN1* mRNA was present after 2 hour incubation in lactic acid containing medium. The highest permease activity was also achieved after 4 hours of induction (Figure 3A).

CaCat8p regulatory role on CaJEN1 expression

In order to determine which carbon sources act as inducers of the *C. albicans* monocarboxylate transporter, cells of the strain RM1000 were grown in repression conditions and then washed and incubated for 4 hours in YNB medium containing different carbon sources. Total RNA was analyzed by Northern-blot (Figure 3B). *CaJEN1* expression was detected in YNB media containing DL-lactic acid, pyruvic acid or glycerol. Maximum expression was achieved with DL-lactic acid, whereas on pyruvic acid and glycerol *CaJEN1* expression was lower. In the remaining carbon sources, acetic acid, ethanol and propionic acid, no *CaJEN1* mRNA was visualized.

In the present work we studied the influence of the *C. albicans CaCAT8* on the expression of *CaJEN1*. *CaJEN1* transcripts were followed in cells of *Cacat8* deletion mutant RM.SD7 in media containing different carbon sources after 4 h of incubation. Comparing the results obtained with the isogenic wild type (Figure 3B) we found that in lactic acid and glycerol-induced cells *CaJEN1* mRNA levels were significantly lower in the *Cacat8* mutant strain, although no differences were found for pyruvic acid. *CaJEN1* transcription was controlled by the nature of the carbon

source and the regulator CaCat8p plays a significant role in the process of induction as for *ScJEN1* (Bojunga and Entian, 1999). The strain RM.SD8 presented similar levels of *CaJEN1* expression when compared to the wild type strain (Figure 3B). These observations suggest that different signalling pathways are involved in the induction of *CaJEN1* by different substrates.

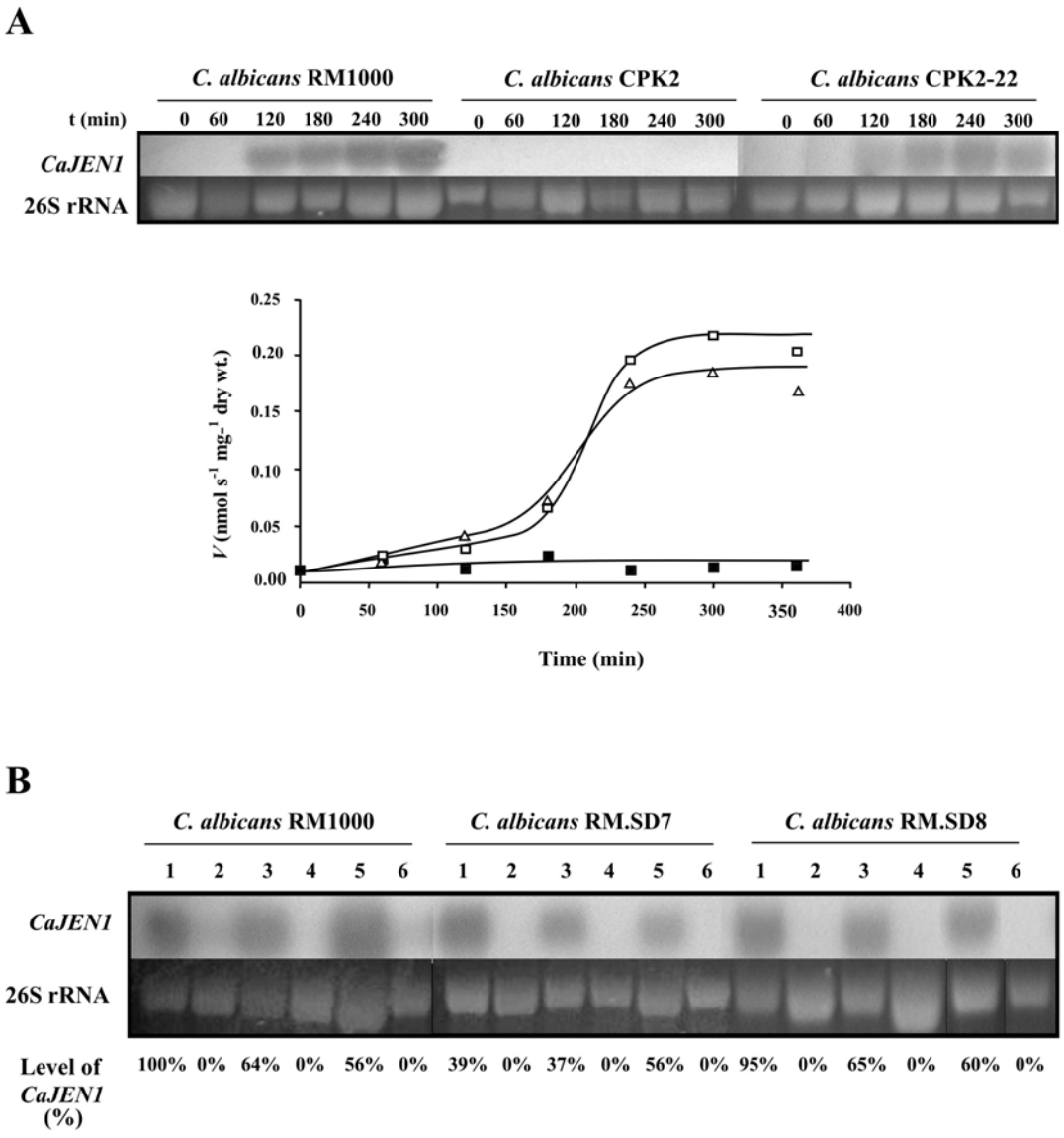

Figure 3 - *CaJEN1* expression in *C. albicans*. **A**) Exponentially growing cells of C. albicans strains RM1000, CPK2 and CPK2-22 were cultivated on YNB-glucose, washed twice with deionised water and transferred to YNB medium containing lactic acid. In distinct cell samples collected over time, analyses were performed to measure the transcriptional level of

the *CaJEN1* gene by Northern-blot (upper figure), and the lactate permease activity (lower figure). Symbols: strain RM1000 (□) CPK2 (■) and CPK2-22 (Δ) using 1.0 mM of labelled lactic acid, pH 5.0. **B**) Effect of the carbon source and *cat8* mutation in the *CaJEN1* mRNA level. Cells of *C. albicans* strains RM1000, RM.SD7 and RM.SD8 were grown on YNB medium supplemented with 2% (w/v) glucose, washed twice with deionised water and incubated for 4 h in YNB medium containing the indicated carbon sources (1) lactic acid 0.5% (v/v), pH 5.0, (2) acetic acid 0.5% (v/v), pH 6.0 (3) glycerol 1% (w/v), (4) ethanol 1% (v/v), (5) pyruvic acid 0.5% (w/v), pH 5.0, and (6) propionic acid 0.5% (v/v), pH 5.0. Each lane contains 20 μg of total RNA, the 26s rRNA is used as a control of charge. Considering the RM1000 strain grown in lactic acid as 100%, the relative percentage of *CaJEN1* expression measured by Northern blot, indicated in the figure, corresponds to average of two independent experiments.

CaJen1-GFP localization in the plasma membrane

To further support the permease function of *Ca*Jen1p, the subcellular localization of a *Ca*Jen1-GFP protein was monitored in *C. albicans* CPK20-5 living cells by fluorescence microscopy (Figure 4A). Cells pre-grown in YNB-glucose (2%, w/v) medium were analysed for the localization of Jen1-GFPp 4 h after transfer to YNB medium containing lactic acid (0.5%, v/v, pH 5.0) as sole carbon and energy source. *Ca*Jen1-GFPp was expressed and targeted to the plasma membrane (Figure 4A), even though fluorescence is also visualized in structures inside the cell.

Furthermore, the *Ca*Jen1-GFPp was also heterologously expressed, under the control of *MET25* promoter, in *S. cerevisiae* strain CEN.PK 113-13D *jen1*Δ (Table 1). Cells growing in the medium YNB-glucose with methionine (repression conditions) were collected, washed and transferred to YNB-glucose without methionine (induction conditions). In *S. cerevisiae* cells containing the *CaJEN1-GFP* construct, (plasmid pUG35 *CaJEN1*, Table 2) the fluorescence was clearly localized at the plasma membrane, whereas cells transformed with the original plasmid pUG35, Table 2, exhibited fluorescence in the cytoplasm (Figure 4B).

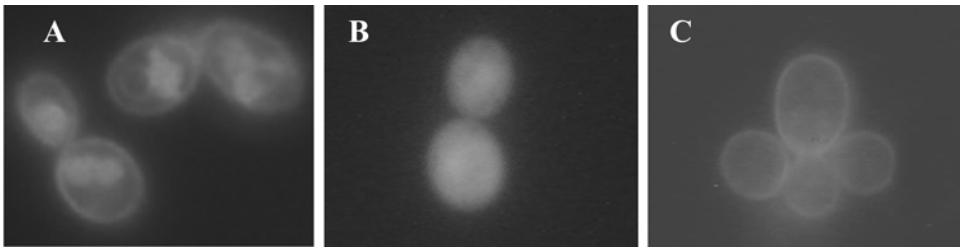

Figure 4 - Localization of CaJen1-GFP fluorescence in living cells by epifluorescence microscopy. Equal volumes of cells were resuspended in low-melt agarose and observed. **A)** *C. albicans* CPK20-5 cells growing exponentially on glucose were harvested by centrifugation, washed twice with deionised water and incubated in YNB-lactic acid. Photos were taken after 4 hours of induction. **B)** *S. cerevisiae* CEN.PK 113-13D *jen1*Δ transformed with the plasmid pUG35. The cells were grown in YNB glucose and then washed and incubated in induction conditions, YNB glucose without methionine for five hours. **C)** *S. cerevisiae* CEN.PK 113-13D *jen1*Δ transformed with the plasmid pUG35-CaJEN1. The cells were grown in YNB glucose and then washed and incubated in induction conditions, YNB glucose without methionine for five hours.

Expression and functionality of CaJEN1 *in* S. cerevisiae

For further proof of *Ca*Jen1p functioning as a lactate permease, its transport activity was followed in cells of the strain *S. cerevisiae* CEN.PK 113-13D *jen1*Δ complemented with the *CaJEN1* gene under the control of the constitutive promoter GPD (plasmid pIJ2, Table 2 and Materials and Methods), but unexpectedly the cells did not restore the activity for the lactate permease (results not shown). However, Northern-blot analysis revealed a strong *CaJEN1* mRNA signal (Figure 5) proving its expression. These results together with the correct localization of the CaJen1-GFP fusion protein indicated that *CaJEN1* was transcribed and translated but did not result in a functional protein.

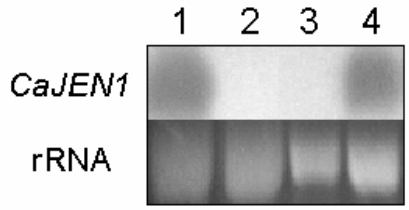

Figure 5 - Expression of *CaJEN1* in distinct yeast strains. Lactic acid grown cells of *C. albicans* RM1000 (lane 1); glucose grown cells of *C. albicans* RM1000 (lane 2); *S. cerevisiae* CEN.PK *jen1*Δ (lane 3); *S. cerevisiae* CEN.PK *jen1*Δ transformed with the plasmid pIJ2 (lane 4). 20 µg of RNA were applied at each lane. The lower row corresponds to the 26S rRNA used as a control for the charge of RNA.

A single CTG codon is found in *CaJEN1* sequence, which codes for a serine[217] in *C. albicans* while in *S. cerevisiae* it codes for a leucine. The alignment of several Jen1p analogous showed serine[217] to be strongly conserved (Table 4). Site directed mutagenesis was performed in pIJ2 substituting the CTG from *CaJEN1* for a TCC codon, which codes for serine in *S. cerevisiae.* This modification resulted in complete recovery of the lactate permease activity in *S. cerevisiae jen1*Δ strain transformed with this plasmid (Figure 1C).

Table 4 - Jen1p alignment with putative homologs available in databases.

Organism	Aminoacid sequence	Accession
Beauveria bassiana	^{287}ALEQCPSNARGLMSGILQQGYSFGY^{211}	AY187631
Metarhizium anisopliae	^{182}ALEQCPSNARGLMSGILQQGYSFGY^{206}	AY125927
Neurospora crassa	^{183}ALENSPVDARGLMSGILQQGYAFGY^{207}	AL353819
Thermoplasma acidophilum	^{120}AMESLPAKARGWVSGLIQGGYPTGY^{144}	AL445064
Kluyveromyces lactis	^{211}ALENAPNKAKSILSGIFQEGYAFGX^{235}	AL428866
Kluyveromyces lactis	^{192}AIEDAPVKARSFLSGLFFTAYAMGF^{216}	AL426631
Saccharomyces cerevisiae	^{253}AIEDAPVKARSFLSGLFFSAYAMGF^{277}	U24155
Candida albicans*	^{204}ALEGQPIAARSVLSGLFLPGYCFGY^{228}	19-2514**
Identity	A-E--P--A----SG-----Y--G-	
Consensus	ALE3APVKAR2LLSG2FQQGYAFGY	

* S – is encoded by a CTG codon, which encodes for a Serine in *C. albicans* and a Leucine in *S. cerevisiae* (Santos *et al.*, 1993)

** contig number obtained from the last assembly of the *C. albicans* genome in the Stanford Genome Technology Center

Discussion

In the present work we have identified genetically and biochemically the *CaJEN1* gene of *C. albicans*, as the first monocarboxylate transporter of this organism. The uptake of labelled DL-lactic acid, at pH 5.0 in cells grown in lactic acid followed a Michaelis-Menten kinetic and D-lactic acid, L-lactic acid, pyruvic acid as well as propionic acid were competitive inhibitors of DL-lactic acid transport system. This suggested that these acids, if transported at all, are accepted by the same carrier. However, acetic acid behaved as a non-competitive inhibitor, suggesting an independent uptake system, possibly using common co-substrates (protons). This permease behaved distinctly from Jen1p of *S. cerevisiae,* which is shared by L- and D-lactate, acetate, propionate and pyruvate (Cássio *et al.*, 1987). The observed proton movements during the initial uptake of the acid indicate a monocarboxylate-proton symport. As previously reported (Cássio *et al*., 1993 and references therein) proton flux is not only associated to the transport but also to the re-establishment of the extracellular acid-base equilibrium, which is disturbed by the transport of the acid. Studies regarding the estimation of the theoretical proton fluxes exclusively associated to the initial uptake rates of the acid and the respective proton negative charges stoichiometries, as well as correlation with the accumulation capacity of the transport system at different extracellular pH values are under development.

In a first approach, the association between the activity for the monocarboxylate transporter of *C. albicans* and the gene *CaJEN1* gene encoding for a putative permease, homologous to the Jen1 protein from *S. cerevisiae* was established by the analyses of the strain deleted in both copies of this gene. The uptake of labelled DL-lactic acid, at pH 5.0, was measured in lactic acid-grown cells of *C. albicans* CPK2 strain, the kinetics of the transport agreed with the presence of a simple diffusion of the acid, indicating that the deletion of both genomic copies of *CaJEN1* in *C. albicans* abolished the mediated transport of lactate.

Glucose grown-cells of *C. albicans* did not show activity for the lactate permease, nor *CaJEN1* gene expression. Northern-blot analyses revealed that *CaJEN1* gene expression displayed specificity with respect to the inducer, which appears to be associated with DL-lactic acid, pyruvic acid and glycerol. Cells

cultivated in ethanol, acetic acid and propionic acid showed no expression for the gene. Thus, not all the substrates of the permease are inducers of *CaJEN1* transcription. Evidences for equivalent data have been also reported for *JEN1* of *S. cerevisiae* (Andrade and Casal, 2001).

Green fluorescent protein (GFP) was used as *in vivo* reporter protein fused with the C-terminus of the *CaJEN1* gene. In *C. albicans* cells, the fusion protein *Ca*Jen1-GFP was expressed and tagged to the plasma membrane under induction conditions. In *S. cerevisiae* the fusion protein *Ca*Jen1-GFP was also tagged to the plasma membrane. Although the gene could be transcribed, *CaJEN1* cloned in the plasmid p416GPD was not functional in this organism. In the same experimental conditions, the lactate permease activity in *S. cerevisiae (jen1*Δ) strain, *ScJEN1* cloned in this plasmid restored the lactate permease activity (Soares-Silva *et al*., 2003). The CTG codon at amino acid position 217, which encodes serine in *C. albicans* and leucine in *S. cerevisiae* (Santos *et al*., 1993), was responsible for the non-functional expression of *CaJEN1* in *S. cerevisiae*. Changing the CTG codon to a TCC codon (encoding serine) revealed a functional active *Ca*Jen1 protein in *S. cerevisiae* and showed the importance of Ser217 located at the beginning of the fifth predicted transmembrane helix of Jen1p.

Cat8p is a well-known activator in *S. cerevisiae*, responsible for the derepression of a wide variety of genes for gluconeogenesis and glyoxylate enzymes (Hedges *et al*. 1995, Proft *et al*., 1995, Gancedo, 1998), as well as for the full derepression of *JEN1* (Bojunga and Entian, 1999). The level of *CaJEN1* expression was evaluated in cells of *C. albicans cat8* deleted strain and the values of expression were compared with the respective wild-type isogenic strain. Here we report that *CaJEN1* transcription is under the dependence of an external inducer, and *CaCAT8*, played a significant role in the process of induction. Live *S. cerevisiae* or *C. albicans* cells isolated from phagolysosome are upregulated for the principal enzymes of the glyoxylate cycle (isocitrate liase and malate synthase) and gluconeogenesis (frutose-1,6-bisphosphatase) (Lorenz and Fink, 2001). The most likely explanation for the induction of these enzymes in yeasts upon phagocytosis is that activation of these pathways represent the response of the cells to nutrient starvation when they colonize a mammal cell (Lorenz and Fink, 2002). As demonstrated by several studies, the presence and induction of the glyoxylate cycle, while required for full virulence is not a virulence factor per se. Whether yeast monocarboxylate

transporters also play a role during nutrient deprivation once inside the macrophages remains to be elucidated.

A recent study revealed that fluconazole has fungicidal activity for *Candida* species under *in vitro* conditions that mimic the vaginal microenvironment (Moosa *et al*., 2004). Short-chained carboxylic acids were among the substrates that had a synergistic, fungicidal effect with fluconazole. The characterization of CaJen1p, the first monocarboxylate transporter found in *C. albicans*, will certainly contribute to elucidating those interactions, which could be explored in the development of new antifungal drug targets.

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Chapter 4

The conserved sequence NXX[S/T]HX[S/T]QDXXXT of the Lactate/Pyruvate:H^+ Symporter Subfamily, is critical for Jen1p kinetics and specificity

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Adapted from:

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Abstract

In *Saccharomyces cerevisiae* Jen1p is a lactate/proton symporter belonging to the Lactate/Pyruvate:H^+ Symporter Subfamily (TC#2.A.1.12.2) of the Major Facilitator Superfamily. We investigated structure-function relationships of Jen1p using a rational mutational analysis based on the identification of conserved amino acid residues. In particular, we studied the conserved sequence ^{379}**N**XX[S/T]**H**X[S/T]**QD**XXX**T**^{391}, overlapping transmembrane helix 7. Substitution of amino acid residues N379, H383 or D387, even with very similar amino acids, resulted in a very dramatic reduction of lactate and pyruvate uptake, but conserved measurable acetate transport. Acetate transport inhibition assays showed that these mutants conserve the ability to bind, but do not transport, lactate and pyruvate. More interestingly, the double mutation H383D/D387H, while behaving as a total loss-of-function allele for lactate and pyruvate uptake, can fully

restore the kinetic parameters of Jen1p for acetate transport. Thus, residues N379, H383 or D387 affect both the transport capacity and the specificity of Jen1p. Substitutions of Q386 and T391 resulted in no or moderate changes in Jen1p transport capacities for lactate, pyruvate and acetate. On the other hand, Q386N reduces the binding affinities for all Jen1p substrates, while Q386A increases the affinity specifically for pyruvate. We also tested Jen1p specificity for a range of monocarboxylates. Several of the mutants studied showed altered inhibition constants for these acids. These results suggest that the conserved motif analyzed is part of the substrate translocation pathway in the Lactate/Pyruvate:H^+ Symporter Subfamily.

Introduction

The study of monocarboxylates transporters (MCT) is of great importance, since uptake of these nutrients across the plasma membrane plays a crucial role in the metabolism of most cells and the acid–base equilibrium status in many tissues (Garcia *et al.* 1994). The family of proton-linked monocarboxylate transporters includes mainly mammalian members (Halestrap and Price, 1999). In *S. cerevisiae*, activities for at least two monocarboxylate-proton symporters have been found: one is encoded by the *JEN1* gene (Casal *et al.* 1999) and the second by the gene *ADY2* (Paiva *et al.* 2004). *JEN1* encodes the only *S. cerevisiae* member of the Sialate:H^+ Symporter (SHS) Family (2.A.1.12.), belonging to the Major Facilitator Superfamily. The only other member of known function in this family is the *Escherichia coli NanT* gene (Vimr and Troy, 1985). Jen1p is capable of binding and transporting lactate, pyruvate, acetate and propionate (Casal *et al.* 1999). The Ady2p permease, which is involved in the uptake of acetate, propionate and formate, belongs to an evolutionary distinct family (YaaH ; 9.B.33.)

Our group has carried out considerable research in the attempt to characterize Jen1p. We have previously reported work on regulation of expression, protein turn-over, mRNA decay, heterologous expression in *Pichia pastoris* and recovery of permease activity in hybrid vesicles, which was the final proof that the Jen1p was

indeed the lactate transporter, and not a regulator of lactate transport (Andrade and Casal, 2001, Paiva *et al.* 2004, Andrade *et al.* 2005, Soares-Silva *et al.* 2003). It had already been known that in the presence of glucose there was no activity for the lactate permease in *S. cerevisiae* (Casal *et al.* 1999) However, recent work showed that glucose regulates the mRNA decay of the *JEN1* gene, as well as the protein localization in the plasma membrane, thus being a major regulation factor both at gene expression level and protein turn-over (Andrade *et al.* 2005, Paiva, 2002).

JEN1 homologues have recently been described in *Candida albicans* (Soares-Silva *et al.* 2004) and in *Kluyveromyces lactis*. In *K. lactis*., there are two carboxylic permeases with different specificities, *KlJEN1* a monocarboxylate permease that transports lactate and pyruvate, and *KlJEN2* a dicarboxylate permease that transports malate and succinate (Lodi *et al.* 2004). The *C. albicans* homologue proved to be also a lactate/proton symporter, and inhibition studies indicated that *CaJEN1* shares similar specificity as the *ScJEN1*, being inhibited by D and L-lactate, pyruvate and propionate. During the expression of the *CaJEN1* in *S. cerevisiae* it was found that a serine located in the fifth transmembrane domain is essential for the uptake of lactate (Soares-Silva *et al.* 2004).

In this work we investigate structure-function relationships in *Sc*Jen1p by using a mutational analysis of absolutely conserved residues located in a consensus motif of the Lactate/Pyruvate:H^+ symporter subfamily. Our results show that several of the residues affect Jen1p function and specificity.

Materials and Methods

Yeast strains, plasmids and growth conditions

The yeast strain used in this work to express the *JEN1* mutants was the *Saccharomyces cerevisiae* W303-1A *jen1*Δ *ady2*Δ (Paiva *et al.* 2004) that presents no uptake for monocarboxylates. 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), 6.7%, 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), or lactate (0.5%, v/v, pH 5.0). Solid media were prepared adding agar (2%, w/v) to the respective liquid media. Growth was carried out at 30 ºC, both in solid or liquid media.

Repression and derepression conditions

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 lactate for 4 hours.

Construction of the mutant strains

Plasmid isolation from *Escherichia coli* strains and DNA manipulations were performed as described by Sambrook *et al.* 1989. *JEN1* mutations were constructed in the plasmid pDS-1, with the oligonucleotide-directed mutagenesis technique. The mutagenesis was performed using the DNA Polymerase ACCUZYME (Bioline) with proofreading activity as follows: as a template, 20 ng of the plasmid pDS-1 were utilised, and the complementary oligonucleotides (20 pmol) listed in the Table 1 containing the desired substitution were used in the following PCR reaction: 30 s at 95 ºC followed by 18 cycles of 30 s at 95 ºC, 60s at the oligonucleotide T*m*, 8 min at 68 ºC, and a final extension step of 10 min at 68 ºC. In order to destroy the parental strands the PCR reaction was incubated with the restriction enzyme DpnI (NEB) for two hours at 37 ºC. This mixture was then used to transform *E. coli* and plasmid extraction was performed on several clones with the Gene Elute™ Plasmid miniprep Kit (SIGMA). Mutations were confirmed by sequencing using appropriate oligonucleotides for both DNA strands. The genes containing the desired mutations were introduced in the *S. cerevisiae jen1*Δ *ady2*Δ strain by the High Efficiency Transformation Method (Gietz and Woods, 2002) and the transformant selection

was based on complementation of a uracil auxotrophy. As a control the strain was also transformed with the original vector p416GPD.

Table 1 - Sequence of the forward primers used in the *JEN1* gene mutagenesis.

Mutation	Wt codon	Mutant codon	Primers
N379Q	aat	**ca**a	5'-gttttattggtgggtcca**caa**tacttgactcatgcttc-3'
H383K	cat	**a**a**a**	5'-ccaaattacttgact**aaa**gcttctcaagacttg-3'
Q386N	caa	**a**a**t**	5'-cttgactcatgcttct**aat**gacttgttgccaacc-3'
D387E	gac	ga**a**	5'-ctcatgcttctcaa**gaa**ttgttgccaaccatg-3'
T391S	acc	**t**cc	5'-caagacttgttgcca**tcc**atgctgcgtgccc-3'
N379A	aat	**gc**t	5'-gttttattggtgggtcca**gct**tacttgactcatgcttc-3'
H383A	cat	**gc**t	5'-ccaaattacttgact**gct**gcttctcaagacttg-3'
Q386A	caa	**gc**a	5'-cttgactcatgcttct**gca**gacttgttgccaacc-3'
D387A	gac	g**c**c	5'-ctcatgcttctcaa**gcc**ttgttgccaaccatg-3'
T391A	acc	**g**cc	5'-caagacttgtt**gcc**atgctgcgtgccc-3'
H383D	cat	**g**at	5'- ccaaattacttgact**gat**gcttctcaagacttg-3'
D387H	gac	**ca**t	5'-ctcatgcttctcaa**cat**ttgttgccaaccatg-3'

Transport assays

Cells incubated under derepression conditions were harvested by centrifugation, washed twice in ice-cold deionised water and resuspended in ice-cold deionised 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 minutes of incubation at 26º C, the reaction was started by the addition of 10 µl of an aqueous solution of the labelled acid at 60µM concentration and pH 5.0, and stopped by the addition of cold 120mM non-labelled acid, pH 5.0. The reaction mixtures were centrifuged for 3 minutes at 13200 rpm, the pellet was resuspended by vortex in 1 ml of deionised 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, United Kingdom). Radioactivity was measured in a Packard Tri-Carb 2200 CA 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. The inhibition constant (K_i) was determined by measuring the uptake rates of labelled acid in the presence of the non-labelled inhibitor at the desired concentration. The following radioactive labelled substrates were utilised, D,L-[U-^{14}C] lactate (S.a. 13000 dpm/nmol), sodium salt, [1-^{14}C] acetate (S.a. 13000 dpm/nmol), sodium salt and [1-^{14}C] pyruvate (S.a. 6000 dpm/nmol), sodium salt, all purchased from Amersham Biosciences. Non-specific ^{14}C adsorption to the cells, as well as, the diffusion component, were determined by adding a mixture of labelled acid and unlabelled acid 1000-fold concentrated. 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, as well as the inhibition constant were determined by a computer-assisted non-linear regression analysis (GraphPAD Software version 4.00, San Diego, CA, USA).

Construction of the pDS-1-GFP plasmid

The JEN1-GFP fusion was constructed in the plasmid pDS-1 (Soares-Silva *et al.* 2003), that contains the *JEN1* gene cloned under the control of the Glyceraldehyde-3-Phosphate Dehydrogenase promoter, with the gap repair technique (Ansaldi *et al.* 1996). The fragment corresponding to the GFP was amplified from the pFA6a-GFPS65T-KanMX6A plasmid with the following primers:

S1GFP-5'-gattcgaacgtctcaaagacaatagaggagcatattgagaccgttagtaaaggagaagaacttttc-3'

GFPrev-5'-gtgaatgtaagcgtgacataactaattacatgatatcgacaaaggaaaaggggcctgttaaacagatctatattaccctg-3'.

The GFP fragment was amplified using the DNA Polymerase ACCUZYME (Bioline) with proofreading activity as follows: as a template 200 ng of the plasmid pDS-1 were utilised and the complementary oligonucleotides (20 pmol) S1GFP and GFPrev were used in the following PCR reaction: 3 min at 95 ºC followed by 35

cycles of 1 min at 95 ºC, 1 min at 60 ºC, 1 min at 72 ºC, and a final extension step of 10 min at 72 ºC. The resulting PCR fragment was purified from an agarose gel with the QIAquick Gel Extraction Kit (Qiagen). The plasmid pDS-1 was digested with the EcoR I restriction enzyme (Roche) according to the manufacturer instructions, and the enzyme was inactivated at 65 ºC during 15 min. The *S. cerevisiae* W303-1A *jen1*Δ *ady2*Δ strain was transformed by the he High Efficiency Transformation Method (Gietz and Woods, 2002) with a mixture of 15 µl purified PCR fragment and 15 µl of digested pDS-1 plasmid. The transformant selection was based on complementation of a uracil auxotrophy and further visualization of GFP fluorescence under an epifluorescence microscope.

Results

A motif highly conserved in the Lactate/Pyruvate:H^+ Symporter Subfamily

Several *JEN1* homologues have been described in the literature: the *CaJEN1* from *C. albicans*, *KlJEN1* and *KlJEN2* from *K. lactis, JEN1* from *Metharizyum anisopliae* and *JEN1* from *Beuvaria bassiana* (Fang *et al.* 2003, Soares-Silva *et al.* 2004, Lodi *et al.* 2004). Functional studies have been carried out with *JEN1* from *S. cerevisiae, C. albicans* and *K. lactis,* which led to the functional characterization of *JEN1* as a monocarboxylate permease. In the case of *K. lactis,* a second homologous gene, *KlJEN2,* was recently described as a dicarboxylate permease (Lodi *et al.* 2004). A multiple alignment of several *JEN1* homologues described in literature, as well as, with homologous sequences present in databases was performed (Figure 1). In this alignment the *NanT* gene from *E. coli*, which encodes a sialate transporter was also included since the *JEN1* gene and the *NanT* are included in the same Sialate:H^+ Symporter (SHS) Family (TC#2.A.1.12) (Transporter Classification Database – TCDB – www.tcdb.org).

Various conserved regions were identified, but the conserved domain N^{379}XX[S/T]HX[S/T]QDXXXT^{391} (X represents hydrophobic amino acids and numbering refers to *Sc*JEN1p) located between the seventh and eighth predicted α-

helices had, by far, the most impressive conservation. In this domain the conserved amino acids are all polar or charged, and so have the potential to interact with the charged proton or dissociated monocarboxylates. *In silico* analysis of this motif (http://biophysics.biol.uoa.gr/SecStr/) showed that it should form a α-helix possibly followed by a short loop (results not shown).

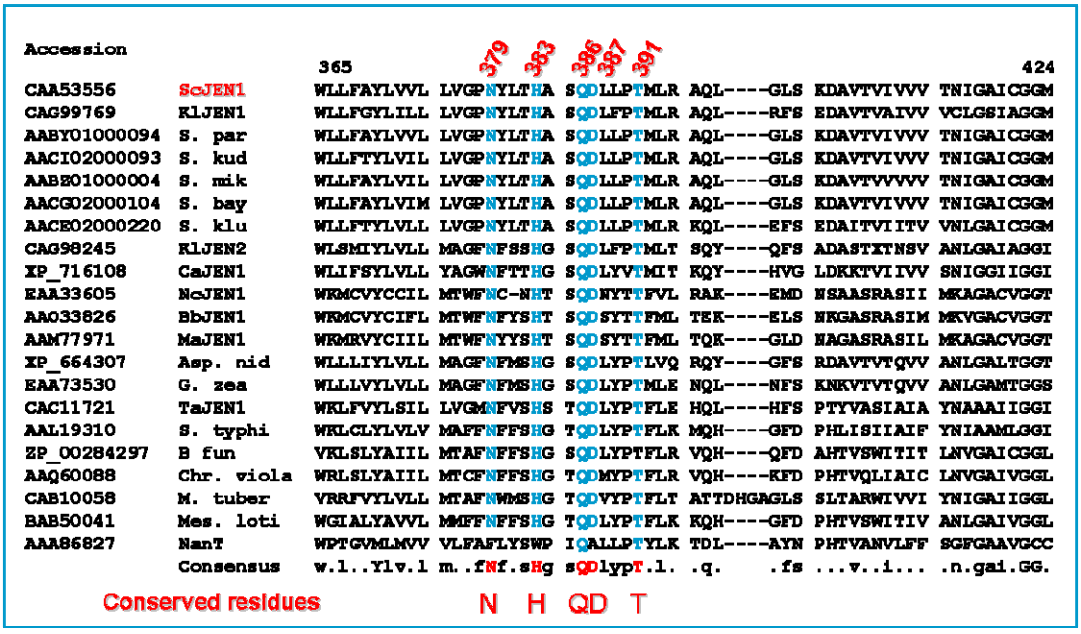

Figure 1 - Identification of a Jen1p conserved domain by multiple sequence alignment of Jen1p homologues available in the databases and the *E. coli* sialate transporter (*NanT*). The multiple sequence alignment was performed with the Multalin (INRA) bioinformatics tool. (http://prodes.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1988).

A schematic representation of ScJen1p is shown in Figure 2, the highlighted amino acids correspond to the conserved domain studied in this work.

Design and construction of Jen1p mutations

The conserved amino acids N379, H383, Q386, D387 and T391 were selected to be mutated. Two sets of mutations were done following two criteria: substitutions by isofunctional amino acids, (N379Q, H383K, Q386N, D387E and T391S) and substitutions by alanines (N379A, H383A, Q386A, D387A and T391A). The amino

acids H383 and D387 present in this domain are oppositely charged, and it is possible that an interaction is established between them that might contribute in Jen1p protein structure. In order to evaluate this hypothesis the double mutant H383D/D387H was constructed, as well as the corresponding single mutants H383D and D387H. The design of all the mutations took into account the *S. cerevisiae* codon usage. All the mutations were performed in the plasmid pDS-1 that contains the *JEN1* sequence under the control of the *GPD* promoter. This plasmid was previously used to constitutively express the *JEN1* gene in *S. cerevisiae* (Soares-Silva *et al.* 2003). The presence of the mutations was confirmed by sequencing, as described in the Materials and Methods section. The plasmids were introduced in the strain *S. cerevisiae jen1*Δ *ady2*Δ, which lacks any activity for both *S. cerevisiae* plasma membrane monocarboxylate permeases described so far in the literature (Casal *et al.* 1999, Paiva *et al.* 2004).

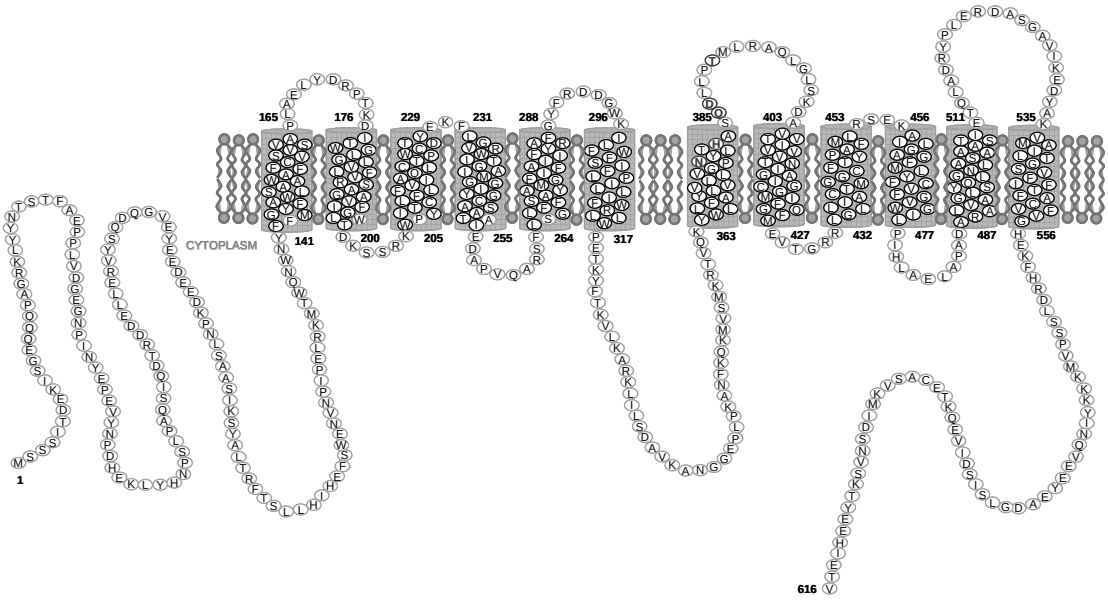

Figure 2 - Predicted topology and primary sequence of the Jen1p. The Jen1p protein belongs to the Major Facilitator Superfamily (MFS) and has a predicted topology of 6+6 transmembrane domains which is a characteristic of this group of proteins (Marger and Saier, 1993). This topology was based on the predictions obtained with the program THMHH (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (Krogh *et al.* 2001). The amino acids presented in bold were the ones further investigated in this study.

Conditions for measurement of initial uptake rates of monocarboxylates

A time assay was carried out for pyruvate, acetate or lactate uptake. As can be seen in Figure 3, for all the substrates tested, the initial uptake rate of labelled monocarboxylates was linear for at least 60 seconds. This was the incubation time chosen for the transport kinetics determination performed in this work. Growth conditions were also optimized, since the conditions previously described to express *JEN1* gene were not very efficient, due to the presence of glucose in the growth medium (see references in introduction). In the presence of glucose, the transport capacity obtained for monocarboxylates is very low and shouldn't enable the differentiation of mutants, if they were slightly affected in transport kinetics. In this work, exponentially grown cells on glucose were shifted to minimal medium containing lactic acid (0.5% w/v, pH 5.0) as a sole carbon and energy source. This resulted in a 10-fold increase in the transport capacity for monocarboxylates (results not shown). The negative effect of glucose on Jen1p was further confirmed in a strain expressing a JEN1-GFP fusion from plasmid pDS-1-GFP. After derepression in lactic acid (3 to 5 hours), it was possible to clearly visualize the protein in the plasma membrane. In contrast, on glucose most of the Jen1p was localized inside the cell (Figure 4).

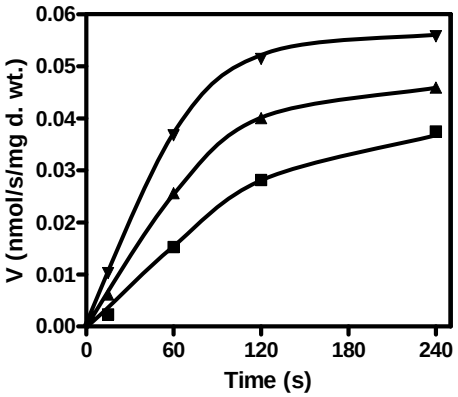

Figure 3 - Evaluation of the uptake rate of labelled lactate (▲), pyruvate (■) and acetate (▼) (60 μM final concentration), in the strain *S. cerevisiae* W303-1A pDS-1. Cells were cultivated in glucose until mid-exponential growth phase, washed and transferred to YNB lactic acid for four hours.

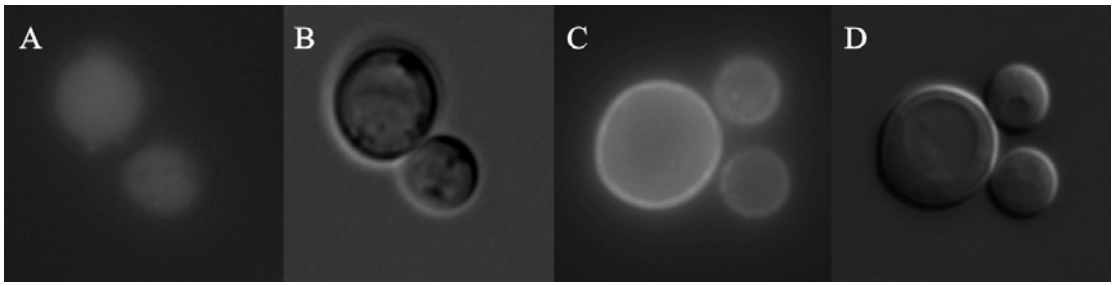

Figure 4 - Localization of Jen1-GFP fluorescence in living cells by epifluorescence microscopy. Cells of the strain *S. cerevisiae jen1*Δ *ady2*Δ pDS-1-GFP grown in YNB Glucose 2% (w/v) and collected in the mid-exponential growth phase and then observed under an epifluorescence microscope a) and also bright field b). Cells were also derepressed in YNB lactate 0.5% (v/v) pH 5.0 for four hours and observed under a epifluorescence microscope c) and also bright field d).

Lactate uptake is severely affected in several of the mutants studied

The different mutants were tested for the initial uptake rates of labelled lactate, at pH 5.0, as mentioned in Material and Methods. In Figure 5 we can observe that the substitution of amino acids N379, H383 and D387, even with very similar amino acids, resulted in a very dramatic reduction of lactate uptake. In fact, only conserved substitutions show practically measurable transport activities (N379Q, D387E). Other replacements result in total loss of transport activity. Substitution Q386A was the only that led to a slightly increased uptake rate of lactate when compared to the wild-type, while substitution by an isofunctional residue (N) decreased the uptake rate to about 50%. Regarding both T391 substitutions, Jen1p transport capacity was slightly decreased (75% of the wild-type). The double mutant H383D/D387H, as well as, the corresponding single mutants, presented practically no uptake rate.

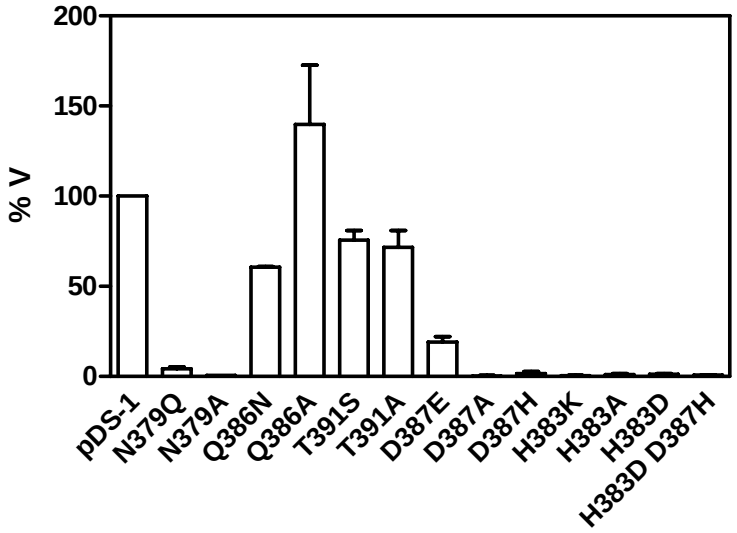

Figure 5 - Relative transport rate of labelled lactate by the Jen1p mutants. The graphic represents the percentage of the initial uptake rate of the mutants considering that the initial uptake rate of the wild-type gene (pDS-1) was 100%.

Pyruvate and acetate uptake is differentially affected in Jen1p mutants

Jen1p permease was also tested for pyruvate and acetate transport (Table 2). The results obtained for the pyruvate uptake exhibited a very similar pattern to the lactate uptake for all the mutants studied. According to the results, all substitutions in H383, N379 and in D387 resulted in total or dramatically reduced Jen1p function. On the other hand substitutions in Q386 and T391 resulted in none or moderate changes in Jen1p transport capacities. Jen1p-mediated acetate transport capacity had a more complex profile. Several substitutions considered to inactivate or dramatically reduce lactate or pyruvate transport, led to measurable acetate transport (N379Q, D387A, D387H, H383D, H383A). On the other hand, mutation Q386A, which allowed maximal lactate or pyruvate transport capacity, led to significantly reduced acetate transport (26%). Substitutions in T391 had the same minor negative effect on acetate transport, as for lactate or pyruvate transport. Most interestingly, while the double substitution H383D/D387H is considered as a total loss of-function allele for lactate or pyruvate transport, it completely restores acetate transport, which

was however compromised in the corresponding single mutants. In other words, residues N379, Q386 and especially D387 and H383 are critical for Jen1p substrate specificity.

Table 2 - Relative transport capacity (%) of lactate, pyruvate and acetate of the Jen1p mutants, compared to the wild-type (pDS-1).

	Lactate	Pyruvate	Acetate
p416GPD	< 1 %	< 1%	0 %
pDS-1	100 %	100 %	100 %
N379Q	4 %	1 %	14 %
N379A	< 1 %	2 %	< 1 %
Q386N	61 %	46 %	107 %
Q386A	140 %	117 %	26 %
T391S	76 %	60 %	60 %
T391A	72 %	44 %	47 %
D387E	19 %	5 %	36 %
D387A	< 1 %	6 %	18 %
D387H	2 %	< 1 %	7 %
H383K	< 1%	< 1 %	2 %
H383A	1 %	3 %	41 %
H383D	1%	< 1%	18 %
H383D D387H	< 1%	1%	90 %

Jen1p mutants show altered specificity profiles

In the mutants with a sufficient uptake rate (>10% of the wild-type) the kinetic parameter for substrate affinity (K_m) was determined for the three principal Jen1p substrates, lactate, pyruvate and acetate (Material and Methods). The results are presented in Table 3. The mutant Q386N has a decreased affinity for all tested substrates but this was much more significant for lactate binding (a 10-fold reduction) than for pyruvate or acetate (3-4 fold). Given that the activities are

measured at a concentration 10-fold lower than the wild-type K_m this reduction in the binding affinities for pyruvate and lactate should account for the moderate reduction in the transport capacities measured in Table 3.

Table 3 - K_m values (mM) of Jen1p mutants for the uptake of lactate, acetate, and pyruvate.

Allele	K_m Lactate (mM)	K_m Pyruvate (mM)	K_m Acetate (mM)
pDS-1	0.2	0.7	4.8
Q386N	2.7	3.0	13.7
Q386A	0.2	0.3	n.d.
T391S	0.5	4.4	12,0
T391A	0.5	n.d.	n.d.
D387E	n.d.	n.m.	11.5
H383D D387H	n.m.	n.m.	4.5

n.d. –not determined; n.m. – not measurable

Alanine substitution (Q386A) resulted in a minor increase (~3-fold) in the K_m for pyruvate. The results suggest that Q386 affects substrate binding but not transport capacity *per se*. Mutation in T391 had a minor negative effect (2-6 fold) in the binding affinity for all substrates. This reduction in K_m might account for a minor reduction in transport capacity of T391 mutations. Thus, T391 seems to have only minor importance to substrate binding. Among D387 substitutions, only the mutant with the isofunctional change D387E has sufficient transport capacity for performing kinetics. D387E was found to have a minor negative effect (2-3 fold) on both the binding and transport of acetate (Tables 2 and 3). The double mutant H383D/D387H showed an affinity for acetate nearly identical to the wild-type allele. As shown in Table 2, the same mutant restores transport capacity for acetate. These results further confirm that the double mutant fully restores Jen1p function, specifically for acetate binding and transport. Additional monocarboxylates were tested for their capacity to inhibit Jen1p-mediated uptake of labelled lactate (Q386 and T391 mutations) or acetate (H383D/D387H). Butyrate, benzoate, propionate and salicylate, were tested

at 1000-fold excess, for competition of the uptake of lactate or acetate. All tested substances inhibited the uptake of lactate or acetate by > 80% (Figure 6).

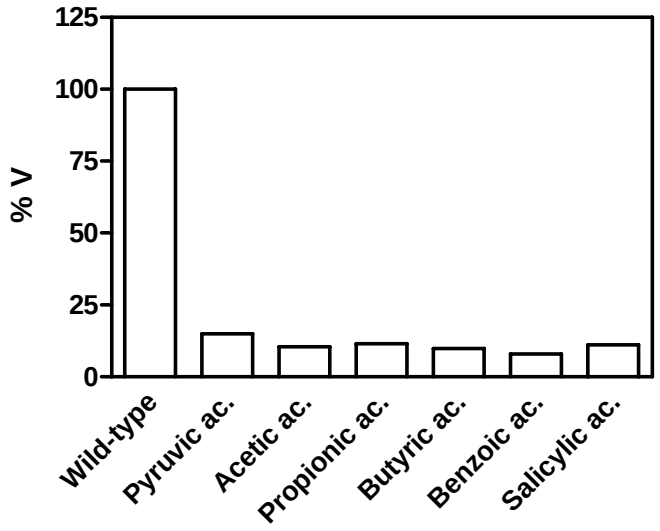

Figure 6 - Relative transport rate of labelled lactate in the presence of unlabelled inhibitors 1000-fold concentrated. The graphic represents the percentage of the initial uptake rate of the mutants considering that the initial uptake rate the wild-type gene (pDS-1) in the absence of an inhibitor was 100%.

To further characterize wild-type Jen1p and kinetically measurable mutants, we estimated the K_i values for these monocarboxylates (Table 4). Our results showed that wild-type Jen1p binds all tested monocarboxylates, at concentration ranging from 0.2 to 7.2 mM, with an affinity order: lactate > pyruvate, benzoate > propionate > acetate, butyrate > salicylate. Most mutants tested alter the binding affinities of monocarboxylates in non-hierarchical pattern. In particular, the conserved change Q386N results in an identical affinity (~2.2-2.9 mM) for all substrates, with the exception of acetate. In other words, Q386N seems to have lost the ability to discriminate several monocarboxylates. On the other hand, Q386A has also lost the ability to discriminate benzoate, propionate, butyrate and salicylate, but unlike Q386N, it can still bind lactate and pyruvate with much higher affinities. T391A can affect substrate binding both positively (salicylate, butyrate) or negatively (lactate, propionate, benzoate), while T391S has a more subtle effect.

Table 4 - K_i values (mM) of Jen1p mutants for propionate, benzoate, salicylate and butyrate.

	K_i values (mM)				
Inhibitor	wild-type	Q386N	Q386A	T391S	T391A
Propionate	1.6	2.8	4.4	2.7	2.6
Benzoate	0.7	2.2	2.3	2.5	1.9
Salicylate	7.2	2.2	3.9	4.9	2.1
Butyrate	4.8	2.5	4.3	3.6	1.8

Discussion

The Jen1p $N^{379}XX[S/T]H^{383}X[S/T]Q^{386}D^{387}XXXT^{391}$ motif that was identified and studied in this work is by far the most conserved sequence in the Lactate/Pyruvate:H^+ Symporter subfamily, and could thus be considered as a "signature" motif of this group of transporters. *In silico* analysis of the secondary structure of the sequence showed that it probably forms an α-helix overlapping with the end of TMS7 and the beginning of the following hydrophilic segment. In other words, this motif might be close to the extracellular environment. According to the topological model proposed in Figure 2, residue N379 should be buried within TMS7, H383 is at the very end of TMS7, while Q386, D387 and T391 are parts of the loop segment. We also analysed the "signature" motif sequence with a sensitive protein homology detection and structure prediction by Hhpred (http://protevo.eb.tuebingen.mpg.de/toolkit/index.php?view=hhpred). With this algorithm we built a multiple alignment of the Jen1p with a set of templates selected from the search results, as well as 3D structural models that are calculated by the MODELLER software from these alignments (results not shown). This alignment confirmed that Jen1p belongs to the MFS showing very high probability of a common topology. Comparing the topology of Jen1p with that of the LacY permease we observed that the residue N379 of Jen1p could correspond to residue

D240 in the TMS7 of LacY (Figure 7). In the solved structure, D240 was shown to interact with LacY substrates (Abramson *et al.* 2003). Based on our kinetic analysis and the fact that 379 is an irreplaceable residue, we propose that N379 also binds Jen1p substrates. It is possible that, as in the case of LacY and GlpT, the two MFS proteins with solved structures (Abramson *et al.* 2003, Huang *et al.* 2003), TMS7 of Jen1p is part of the substrate translocation pathway.

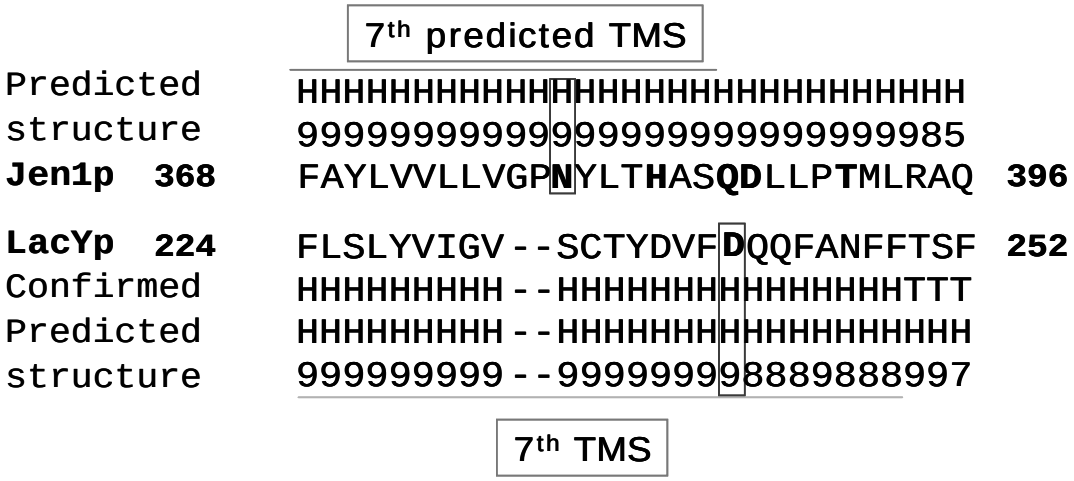

Figure 7 - Structure prediction of the conserved Jen1p sequence with the HhPred program by comparison with the resolved structure of the lactose permease from *E. coli.* In the figure the aminoacids N379 from the Jen1p and the LacY D240 are highlighted since a correspondence between the function of these two aminoacids is proposed.

An *in silico* analysis using SecStr, a program which combines six different secondary structure prediction methods (http://biophysics.biol.uoa.gr/SecStr/), was performed for the mutant versions of the $N^{379}XX[S/T]H^{383}X[S/T]Q^{386}D^{387}XXXT^{391}$ motif. This analysis (not shown) showed that Q386A, and to lesser degree T391A and D387A, affect the putative local architecture of this region. In particular, the presence of Ala residues significantly increases the probability for α-helix formation. Substitutions of Q386 and T391 resulted in no or moderate changes in Jen1p transport capacity but affect binding affinities for all Jen1p substrates. Based on these observations we could propose that these residues indirectly affect substrate

binding by determining the local architecture and flexibility of a segment close to the binding site.

The role of H383 and D387 is more complex and seems to be critical, determining the specificity of Jen1p. Single mutants lose the ability to transport lactate and pyruvate, the principal Jen1p substrates, but most conserve significant acetate uptake. Moreover, while the double mutant H383/D387 lacks any lactate and pyruvate transport capacity it has wild-type kinetics for acetate transport. This is highly suggestive of a charged interaction between these two residues. This interaction, independently of the position of the positive and negative charges, is necessary and sufficient for acetate binding and transport. In contrast, for lactate or pyruvate binding and transport, the individual positions of the charged residues are critical. This shows that acetate binds using different interactions from those involved in lactate or pyruvate binding. This is not uncommon for enzymes, as well as, for transporters (Balendiran *et al.* 1999; Koukaki *et al.* 2005).

Despite the fact that none of the mutants studied was analysed in respect to Jen1p protein stability and topology, our conclusions are not affected by this apparent drawback, since they are based on data from positive mutations affecting affinities and specificities. In the case of mutations, mostly those concerning residues N379 and D387, which dramatically reduce the capacity of Jen1p-mediated transport, we cannot formally distinguish, in this work, whether these affect topology or V_{max} *per se*. However, even in the cases of negative mutation, such as N379Q and D387E, we consider unlikely the possibility that such highly conserved mutations would affect protein stability or topology. We rather predict that these mutations will eventually prove to affect transport catalysis.

We also tested Jen1p specificity for a range of monocarboxylates. Jen1p can bind most monocarboxylates tested (lactate, pyruvate, acetate, benzoate, propionate, butyrate and salicylate). Several of the mutants studied showed non-hierarchical changes in inhibition constants for these acids. These results further suggest that the conserved motif analyzed is critical for substrate binding and translocation. Interestingly, one recently characterized member of the Lactate/Pyruvate:H^+ Symporter Subfamily, KlJen2p, is specific for dicarboxylates. This transporter conserves the residues studied herein, but is the only one, which has a polar residue (Ser), instead of a hydrophobic residue, at position 381 (numbering of *Sc*Jen1p).

Such changes, but also residues other than the ones studied here could contribute to the ability to bind dicarboxylates rather than monocarboxylates

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Chapter 5

Final considerations and future prospects

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Since the sequencing of the entire *Saccharomyces cerevisiae* genome in 1996 (Goffeau *et al.*, 1996) this yeast has become a fundamental tool for the understanding of the eukaryotic metabolism as well as basic phenomena difficult to asses in more complex organisms. Over the past years an increasing knowledge over several multiple transport systems involved in the uptake, compartmentalization and mobilisation of several specific compounds made possible to have a more complete view on the cell transport network. The purpose of the work presented in this thesis was to increase the present insight on yeast monocarboxylate transport.

The identification of the *JEN1* gene, the first gene encoding a monocarboxylate transporter to be discovered, open a new line of work in a lab where physiological studies were the main focus of research (Casal *et al.*, 1999). The discovery of the *JEN1* was surprisingly since this gene does not belong to the yeast monocarboxylate homologues as it was expected. In this context, several questions were raised in what concerned the real function of Jen1p. Physiological studies on a *S. cerevisiae jen1*Δ proved that *JEN1* expression was connected to the presence of a mediated transport mechanism for lactate and pyruvate, but did not rule out the possibility that the Jen1p

was a regulator of the lactate permease and not the permease itself. Several studies were subsequently undertaken using various approaches. mRNA expression studies demonstrated a correlation between *JEN1* transcription profiles and the lactate permease activity (Andrade and Casal, 2001). Additionally, the *JEN1* gene was fused with the reporter protein GFP (Green Fluorescence Protein) and intracellular localization studies demonstrated that the protein was expressed in lactate grown cells and located at the plasma membrane level (Paiva *et al.*, 2002). These results further supported the *JEN1* gene as encoding the lactate proton symporter of *S. cerevisiae*.

The studies presented in chapter two contributed to the dissipation of doubts concerning the function of the Jen1p. In this work the gene *JEN1* was heterologously expressed in *Pichia pastoris,* being this the first time a permease was successfully expressed in this yeast. The *JEN1* gene was expressed under the control of the methanol induced promoter of the alcohol oxidase gene, *AOX1*. After 24 hour growth in methanol maximum permease activity was reached with a V_{max} of 2.1 nmol s^{-1} mg^{-1} dry weight. This represents a five fold increase in the activity of lactate transport when compared to the data obtained for lactic acid-grown cells of the *S. cerevisiae* W303-1A strain. Additionally, the reconstitution of the lactate permease activity in hybrid vesicles of membranes of methanol-induced cells and *Escherichia coli* liposomes containing cytocrome oxidase, demonstrated that the Jen1p is a functional lactate transporter.

Attention on the yeast *Candida albicans,* one of the most commonly encountered human pathogens, increased in the last decade due to the growing number of individuals with impaired immunity, where it can become a life-threatening systemic infection agent. Due to its importance in the human health field the sequencing of the *C. albicans* genome begun in 1996, and in 2002 the Stanford Genome Technology Center (http://www-sequence.stanford.edu/group/candida) released the assembly of *Candida's* diploid genome. The availability of genome sequence data, the significance of *C. albicans,* and the need to study transport systems of other yeast beside *S. cerevisiae* led us to explore the monocarboxylate systems of this yeast. The existence of a mediated transport system for lactate uptake was physiologically evidenced in lactic acid grown-cells. The kinetic parameters obtained for labelled lactate uptake at pH 5.0 were K_m 0.33 mM and V_{max} 0.85 nmol s^{-1} mg dry wt^{-1}. From inhibition studies permease substrates were identified as being pyruvate and propionate. Aiming at the identification of the gene encoding the *C. albicans* monocarboxylate permease an homology search was performed against the *S. cerevisiae JEN1* gene. An homologous

ORF was found and named *CaJEN1*, having the the CaJen1p 12 putative transmembrane domains and 61% similarity to the *ScJen1p*. The deletion of both *CaJEN1* alleles resulted in the loss of measurable lactate permease activity. *CaJEN1* expression studies demonstrated that it is dependent on the carbon source, being absent in a medium containing glucose, acetate, propionate and ethanol and induced in a lactic acid, pyruvate or glycerol-containing medium. Expression levels of *CaJEN1* were evaluated in a strain deleted in the CaCat8 regulator, and it was demonstrated that *CaJEN1* expression was reduced, suggesting that Cat8p has a similar role to ScCat8p in activating genes involved in the utilization of nonfermentable carbon sources. The heterologous expression of *CaJEN1* was carried out in a *S. cerevisiae jen1Δ* strain. The native *CaJEN1* was not functional in this strain and the activity for the lactate permease was only recovered after a site direct mutagenesis strategy was applied. When expressing the *CaJEN1* gene in *S. cerevisiae* the CaJen1p presents a single amino acid change due to the existence of a difference in the genetic code of *S. cerevisiae* and *C. albicans* (Santos *et al.*, 1993). The change of one serine residue of the CaJen1p led to the impairment of permease activity. Alignment studies between several *CaJEN1* homologs demonstrated that this residue is part of a highly conserved domain and must have an important role in the structural/functional context. Once restored the native CaJen1 protein sequence, the permease activity was obtained in *S. cerevisiae*, evidencing that the CaJen1p is a monocarboxylate transporter.

Strucutural/funtional studies are very important to the identification of different protein domains. In what concerns the field of permease one of the best studied proteins is the Lactose permease from *E. coli* (LacY). Recently the three dimensional structure of this protein was determined (Abramson *et al.*, 2003) however this work was preceded by several site directed mutagenesis studies where identification of residues involved in substrate recognition and proton translocation was performed. This work lined the foundations for understanding permease functioning through three dimensional structure studies.

Concerning the yeast monocarboxylate permeases, little is known on the structural field. The identification of a residue that is crucial for CaJen1p functioning and recent developments in the permease structural field attracted our interest. The identification of conserved domains within the protein sequence of different homologs was the first step. Several domains were found, but the domain $N^{379}XX[S/T]H^{383}X[S/T]Q^{386}D^{387}XXXT^{391}$ was chosen to pursue this study since the

residues were all polar or charged and had the potential to interact with the charged substrate or the proton. Five residues were mutated and permease activity was evaluated in a strain presenting no activity for monocarboxylate permease. Three residues N379, H383 or D387 demonstrated to have a more important role for monocarboxylate uptake, since their substitution even for very similar amino acids resulted in a lack of activity.

The existence of a possible interaction between the charged amino acids H383 and D387 that contributed to protein structure was evaluated by the construction of a double mutant. This hypothesis was confirmed trough the recovery of Jen1p capacity to transport acetate, but not lactate or pyruvate in the double mutant. These residues seem to be critical for Jen1p substrate specificity. As for the remaining conserved residues Q386 and T391, they might be critical for determining substrate specificity and/or affinity. The results obtained evidence the importance of this domain for the overall functioning of the permease, and more important for substrate recognition. We therefore postulate that this domain can be a part of the Jen1p substrate translocation pathway.

Future prospects

In the scope of this thesis the studies performed on the *S. cerevisiae* lactate/pyruvate proton symporter deepened the knowledge on this monocarboxylate permease. In spite of the achievements so far accomplished there are still many fields to be explored.

One of the very exciting areas of research that has greatly been developed is the purification and crystallization of membrane proteins (Wiener, 2004). Over the years a lot of work was invested in obtaining crystals from membrane proteins. The first step to overcome was protein purification in sufficient quantities to allow the formation of a crystal. The second obstacle to defeat was the set up of ideal conditions for membrane protein crystal formation. This was particularly difficult due to the hydrophobic nature of these proteins that makes them largely unstable for crystal formation. The recent developments on the purification and protein crystallization of membrane proteins such as the Lactose permease- LacY and the Glycerol-3-phosphate transporter- GlpT

(Abramson *et al.*, 2003; Lemieux *et al.*, 2003) gave hope to a series of researchers long pursuing their quest.

During the work presented in this thesis the *S. cerevisiae* Jen1p permease was heterologously overexpressed in the *P. pastoris* system. Protein purification studies can be attempted aiming at the isolation of enough purified protein for further crystallization studies. If the *P. pastoris* expression system reveals itself insufficient for Jen1p protein production other overexpression systems may be tested.

Additionally, modelling studies can be performed based on the three-dimensional structures of LacY and GlpT and the results presently obtained, that point the NXX[S/T]HX[S/T]QDXXXT domain as being part of the substrate translocation pathway.

During the present thesis two homologs of the *S. cerevisiae JEN1* gene were described in *K. lactis* (Lodi *et al.*, 2004; Pereira, 2006). These highly similar genes have different specificities: the *KlJEN1* encodes a monocarboxylate permease and the *KlJEN2* a dicarboxylate transporter. A site directed mutagenesis study based on protein sequence comparison of these two genes as well as the *JEN1* gene from *S. cerevisiae* and *C. albicans* will enable the identification of additional residues/domains responsible for substrate recognition.

The understanding of yeast lactate permease has greatly improved in the past years, however knowledge on the other *S. cerevisiae* plasma membrane monocarboxylate permease has lagged behind since the identification of the gene encoding the *S. cerevisiae* acetate permease was only recently reported (Paiva *et al.*, 2004). This gene, *ADY2,* was also described as being involved in ammonia transport in *S. cerevisiae* colonies (Palkova *et al.*, 2002) and some doubts concerning the true function of this gene were raised, with the real function of the Ady2p remaining to be confirmed. In order to functionally study Ady2p the heterologous expression of this gene is suggested. Another possible approach is the study of homologous genes which will enable the understanding of the true nature of this gene. In this context the study of the archea *Methanosarcina acetivorans*, which is specialized on growing in acetate and other one carbon compounds as growth substrates and has as two genes homologous to the *ADY2* from *S. cerevisiae* (MA4008 and MA0103) is of great interest. The two *ADY2* homologs genes can be cloned and expressed in a *S. cerevisiae ady2*Δ strain. Ultimately protein purification and reconstitution of protein activity in artificial vesicles will allow the characterization of the permease substrates. This strategy can be

performed in parallel for the *ADY2* gene in order to verify protein substrates, in a similar way to what was perfomed during this thesis for the *JEN1* gene .

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