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

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SUMMARY

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.* c*erevisiae 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.

Keywords: *Candida albicans* - monocarboxylate transport – lactate – *JEN1* – yeast

Abbreviations list

GFP – green fluorescence protein

GPD – glyceraldehyde 3-phosphate dehydrogenase

MFS – major facilitator superfamily

MOPS – 3-morpholinopropanesulfonic acid

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* 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.

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.

“Insert figure 1 near here”

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.

"Insert figure 2 near here"

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).

"Insert figure 3 near here"

_Ca_Cat8p 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.

_Ca_Jen1-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).

"Insert figure 4 near here"

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.

"Insert figure 5 near here"

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).

"Insert table 4 near here"

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.

EXPERIMENTAL PROCEDURES

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.

"Insert table 1 near here"

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.

"Insert table 2 near here"

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).

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.

"Insert table 3 near here"

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 pIJ_2 as a probe for *CaJEN1*. Densiometer scanning was performed using the Integrated Density Analysis program from the EagleSight® Software, version 3.2 (Stratagene, CA).

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TABLES

Table 1 Yeast strains used in this work.

Strain	Relevant genotype	Source or Reference
C. albicans		
RM1000	*ura3::imm434/ura3::imm434 his1::hisG/his1::hisG*	Negredo *et al*., 1997
CPK1	*ura3::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 jen1Δ*	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/

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

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

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

FIGURE LEGENDS

Figure 1

Transport of lactate in *Candida 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.

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).

Figure 3

CaJEN1 expression in *Candida 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.

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.

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.

FIGURES

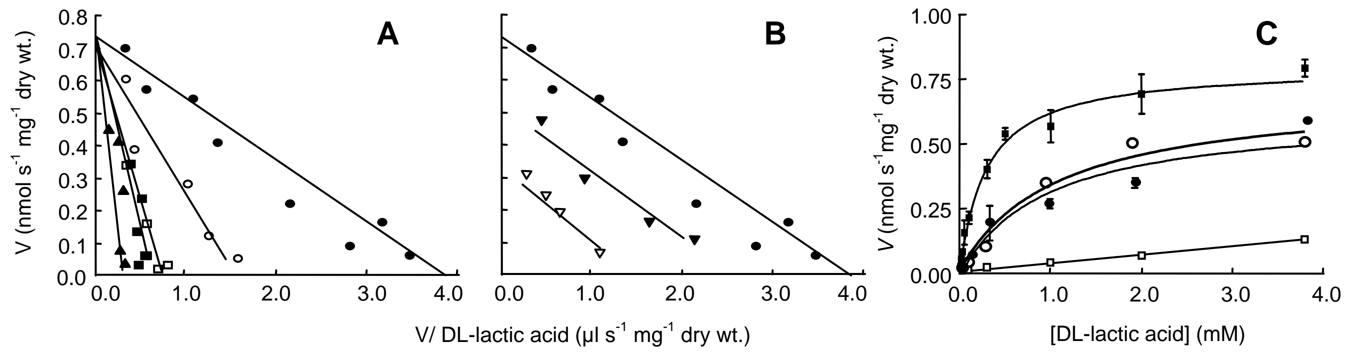
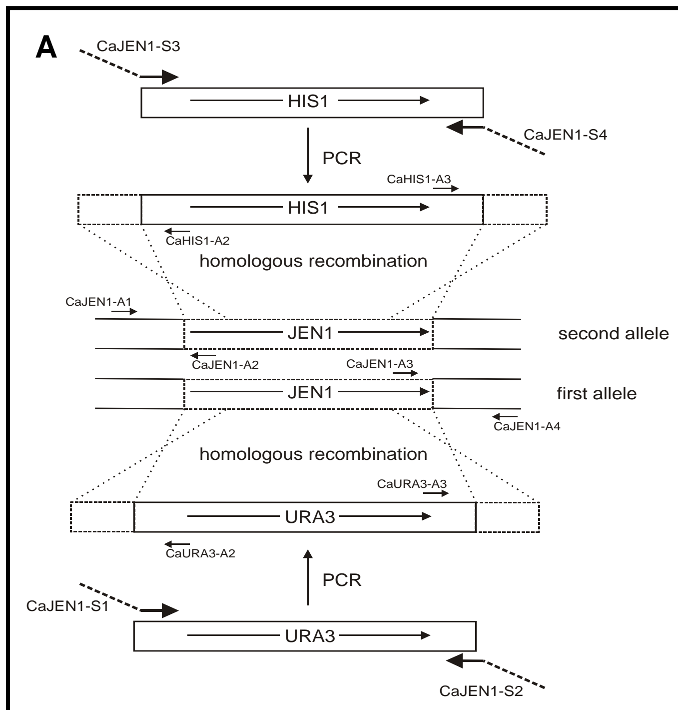


Figure 1



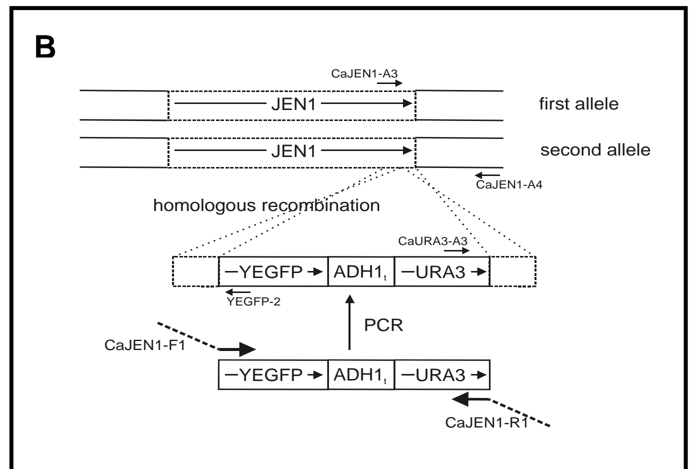
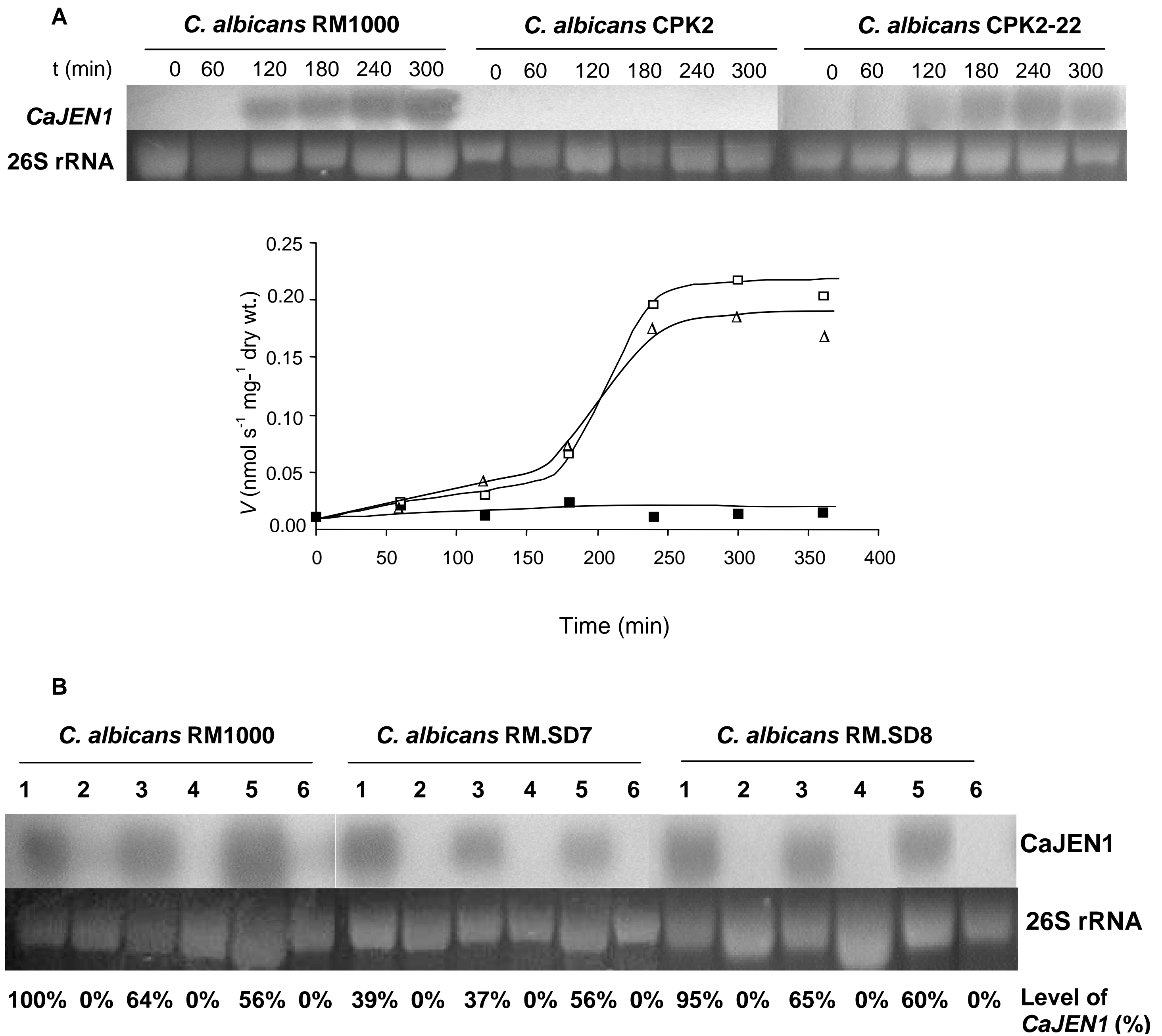


Figure 2



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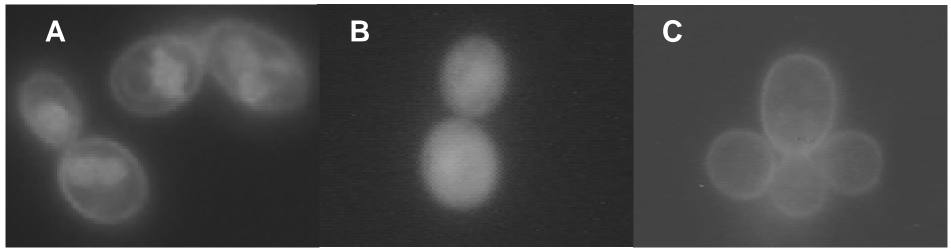

Figure 4

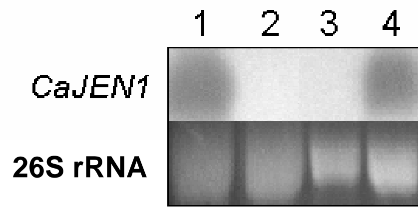

Figure 5