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

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SYNOPSIS

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 $V_{\text{max}}$ 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 $V_{\text{max}}$ (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 [1]. 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 [2]. At concentrations occurring in must fermentation acetic acid induces cell death [3,4] and inhibits metabolic fermentation/respiration activities [5].

The ABC transporters Yor1p [6] and Pdr12p [7,8] 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 AZRI is required for adaptation to acetic acid and to low molecular-weight organic acids [9]. 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 [2, 10, 11]. 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 [12]. JEN1 is the only S. cerevisiae member of the Sialate-Proton Symporters subfamily (TC#2.A.1.12) belonging to the Major Facilitator Superfamily [13]. 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 [13].
Jen1p is rapidly and irreversibly inactivated upon the addition of glucose to induced cells [14]. 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 [15]. 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 rpm and P. pastoris cultures were grown at 30°C, 250 rpm. The media were supplemented with zeocin (25 - 100 µg/ml) and ampicilin (100 µg/ml) whenever necessary. The E. coli XL1-Blue strain was used for plasmid propagation and
amplification according to [16]. 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
[16]. The yeast shuttle vectors p416GPD (CEN6/ARS4) and p426GPD (2µ) were
kindly provided by Dr. Dominik Mumberg [17]. The gene JEN1 was amplified from S.
cerevisiae W303-1A genomic DNA by PCR using primers J7
(CCGGAATTCGTTACATAGAAGCGAACACG) and J8
(CGCGGATCCAGTTTCAAAAGTTTTTCCTCAAAG), (MWG Biotech) and
Platinum Taq high fidelity DNA polymerase (Gibco Cat. No. 11304-011). The primers
introduced a BamHI (J8) and an EcoRI (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).

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
(GCTCTAGAACGCTCTCAATATGCTCC). JEN1 was also cloned in the replicative
expression vector pZPARS [18]. For this purpose, JEN1 was amplified by PCR from the
S. cerevisiae W303-1A genome, using the primers JP-SalI
(ACGCGTCGACGTCGTCGTCAATTACAGATGAGAAAATATCTGG) and JP-NotI
(ATAAGAATGCGGCCGCATTAAACGGTCTCTCAATATGCTCCTCATATGTC). 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
(TGACATGCGAGAATTTGGAAC) and JR (GGAATTTTCATGGCACAACA), based
on the sequence of JEN1, and PF (AAAGATATGGATCATCACTCATGGAAGAA) 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 *Sall* (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. [19] 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 (GATACCCCGACACCAAGAC). Considering that all *P. pastoris* KM71H transformants have a Mut<sup>s</sup> phenotype, X-33 integration transformants were tested for their Mut<sup>+</sup> or Mut<sup>s</sup> phenotype in MM medium. All the transformant strains analysed presented a Mut<sup>+</sup> 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<sub>600</sub> = 2.0 – 6.0. The cells were harvested and resuspended in 200 ml MM medium to an OD<sub>600</sub> 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. [20]. 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 [12]. 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.* [21]. 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 *et al.* [22].

**SDS-polyacrylamide gel electrophoresis, and Western-blotting**

Protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Hybond™-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 [15]. The substrates were DL-[14C]lactic acid (sodium salt, Amersham Pharmacia Biotech) (4000 dpm/nmol), pH 5.0 and [14C]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 [23] 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 26 ºC.
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 promoter (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 [12, 14] 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 [14]. 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.

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* [2, 12]. 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*.

**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. 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.

**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 (pZ-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.

**Expression analysis of _JEN1_ in _Pichia pastoris_**

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 e-myc tags (Figure 5).
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 $k_d = 0.040 \pm 0.002 \, \mu l \, s^{-1} \, mg^{-1}$ 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.

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 [10].

DISCUSSION

This is the first report of fully functional reconstitution of a *S. cerevisiae* permease in the heterologous host *Pichia pastoris*. We therefore will comment on some features of the cloning system used. The *Pichia 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 [24, 25, 26]. By slot-blot hybridisation we verified that the clone KM71H (pB-JEN1)-1 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 [27]. Fortunately, this phenomenon was not observed for JEN1 expression in the KM71H (pB-JEN1)-1 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 $K_m = 0.54 \pm 0.08$ mM lactic acid and $V_{\text{max}} = 2.15 \pm 0.14$ nmol lactic acid s$^{-1}$ mg$^{-1}$ dry weight while in S. cerevisiae W303-1A the $V_{\text{max}}$ 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 [12]. 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 $V_{\text{max}}$ using the strong GPD promoter while the use of a multicopy vector inhibited growth. Even though it appears that, as in S. cerevisiae [28], 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 [29, 30], 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.* [30] 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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**Abbreviations used**

- FCCP - carbonyl cyanide *p*-(trifluoromathoxy)phenylhydrazone
- GPD - glyceraldehyde 3-phosphate dehydrogenase
- AOX – alcohol dehydrogenase
- HPLC - high performance liquid chromatography
- TMPD - *NNN'N'*-tetramethyl-*p*-phenylenediamine
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### Table 1. Yeast strains

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<td>KM71H (pB-JEN1)-II</td>
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<td>BLC555</td>
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<td>BLC556</td>
<td>KM71H (pB-JEN1)-IV</td>
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**Table 2.** Kinetic parameters for the transport of monocarboxylic acids in *S. cerevisiae jen1Δ* 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).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Culture media</th>
<th>Kinetic parameters</th>
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<tr>
<td></td>
<td></td>
<td>Acetic acid</td>
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<tr>
<td></td>
<td></td>
<td>$K_m$ (mM)</td>
<td>$V_{max}$ (nmol s$^{-1}$ mg dry wt$^{-1}$)</td>
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<tr>
<td></td>
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<td>Lactic acid</td>
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<tr>
<td></td>
<td></td>
<td>$K_m$ (mM)</td>
<td>$V_{max}$ (nmol s$^{-1}$ mg dry wt$^{-1}$)</td>
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</tr>
<tr>
<td>p416GPD</td>
<td>G</td>
<td>No activity</td>
<td>No activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>No activity</td>
<td>No activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>1.50±0.50</td>
<td>2.69±0.39</td>
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</tr>
<tr>
<td>pDS1</td>
<td>G</td>
<td>No activity</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>No activity</td>
<td>No activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
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<td>1.04±0.26</td>
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<td>1.57±0.62</td>
<td>3.07±0.53</td>
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<tr>
<td>p426GPD</td>
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<td>No activity</td>
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<tr>
<td></td>
<td>GA</td>
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<td></td>
<td>GA</td>
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<td>No activity</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>1.06±0.47</td>
<td>2.86±0.48</td>
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Not determined
Table 3. Specific growth rate (h\(^{-1}\)) of *S. cerevisiae jen1Δ* 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.

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Specific growth rate (h(^{-1})) of the transformants</th>
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<tr>
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<td>Glucose (% w/v)</td>
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<td>0.00</td>
</tr>
<tr>
<td>2.0</td>
<td>0.25</td>
</tr>
<tr>
<td>0.0</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Figure 1
Detection of JEN1 expression by RT-PCR in cells of Saccharomyces cerevisiae jen1Δ 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).

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.

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

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.
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.

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 °C, 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.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5
Figure 6
Figure 7.