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

Isabel SOARES-SILVA, Dorit SCHULLER, Raquel P. ANDRADE, Fátima BALTAZAR, Fernanda CÁSSIO and Margarida CASAL[1]

Departamento de Biologia, Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

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 of dry $weight^{-1}$. 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 on either glucose and/or acetic acid. The highest V_{max} (0.84 nmol · s^{-1} · mg of dry $weight^{-1}$) 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 with 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 the membrane-vesicle levels.

Key words: functional reconstitution, heterologous expression, *JEN1*, lactate transporter, *Pichia pastoris*, yeast.

INTRODUCTION

Lipophilic weak carboxylic acids are used as food preservatives. The undissociated acid accumulates into microbial cells by simple diffusion until equilibrium of concentrations 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 pK_a 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 metabolize 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 *AZR1* is required for adaptation to acetic acid and to low-molecular-mass 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, whereas 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 is dependent on the expression of *JEN1* [12]. *JEN1* is the only *S. cerevisiae* member of the sialate–proton symporters subfamily [TC (Transport Commission) no. 2.A.1.12; http://tcdb.ucsd.edu/tcdb/background.php] 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–green fluorescent protein fusion protein in a set of strains with mutations in specific steps of the secretory and endocytic pathways [15]. However, none of the above data discriminate the possibilities of whether Jen1p has regulatory (or sensor) or transport function.

The purpose of the present study was 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 medium contained Difco yeast nitrogen base

Abbreviations used: FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; GPD, glyceraldehyde-3-phosphate dehydrogenase; AOX, alcohol dehydrogenase; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; RT-PCR, reverse transcriptase PCR; YNB, yeast nitrogen base.

[1] To whom correspondence should be addressed (e-mail mcasal@bio.uminho.pt).

Table 1 Yeast strains

Strain	Relevant genotype	Source or reference
S. cerevisiae		
W303-1A	*a ade2 leu2 his3 trp1 ura3*	[31]
BLC203	W303-1A *jen1*Δ::*HIS3*	[12]
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

(YNB) adjusted to the indicated pH with HCl or NaOH and supplemented with the adequate requirements for prototrophic growth. For growth of *S. cerevisiae*, YNB media were supplemented with different concentrations of glucose and/or acetic acid, as indicated in the text. For growth of *P. 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) and sorbitol (1.0 M); MGY medium, YNB (1.34 %, w/v), biotin (4.0×10^{-5} %, w/v) and glycerol (1.0 %, w/v); MM medium, YNB (1.34 %, w/v), biotin (4.0×10^{-5} %, w/v) and methanol (0.5 %, v/v); MD medium, YNB (1.34 %, w/v), biotin (4.0×10^{-5} %, w/v) and glucose (2.0 %, w/v). Agar (2.0 %, w/v) was added for solid media. Liquid *S. cerevisiae* cultures were grown at 28 °C and 180 rev./min and *P. pastoris* cultures were grown at 30 °C and 250 rev./min. 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 (catalogue no. 51270), maintained at 50 °C. The mobile phase was H_2SO_4 (0.025 M, 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*/*ARSH4*) and p426GPD (2μ) were kindly provided by Dr Dominik Mumberg (Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Germany) [17]. The gene *JEN1* was amplified from *S. cerevisiae* W303-1A genomic DNA by PCR using primers J7 (5′-CCGGAATTCGTTACATAGAGAAGCGAACACG-3′) and J8 (5′-CGCGGATCCAGTTTCAAAAGTTTTTCCTCAAAG-3′; MWG Biotech) and Platinum *Taq* high-fidelity DNA polymerase (Gibco, catalogue no. 11304-011). The primers introduced a *Bam*HI (J8) and an *Eco*RI (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 (5′-CCGGAATTCGAAAATATGTCGTCGTCAATTACAG-3′) and JB-XbaI (5′-GCTCTAGAACGGTCTCAATATGCTCC-3′). *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 (5′-ACGCGTCGACGTCGTCGTCAATTACAGATGAGAAAATATCTGG-3′) and JP-NotI (5′-ATAAGAATGCGGCCGCATTAAACGGTCTCAATATGCTCCTCATATGTC-3′). 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 (reverse transcriptase PCR) reactions were performed with primers JF (5′-TGACATGGCAGAATTGGAAC-3′) and JR (5′-GGAATTTCTCATGGCCAACA-3′), based on the sequence of *JEN1*, and PF (5′-AAAGATATGGTCATCATCAGAAGAA-3′) and PR (5′-CAAACTTGGATGCTTGGTAGACA-3′) as a reference based on the sequence of *PDA*. The Ready To-Go™ RT-PCR Beads from Amersham Biosciences (catalogue no. 27-9556-01) were used, in combination with 300 ng of DNase-treated RNA and oligo(T)$_{12-18}$ as first-strand primer.

Selection of *P. 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. [19] and in accordance to the guidelines provided by the EasySelect™ *Pichia* Expression Kit Instruction Manual (Invitrogen) using the 3′AOX1 (5′-GCAAATGGCATTCTGACATCC-3′) and 5′AOX1 (5′-GACTGGTTCCAATTGACAAGC-3′) primers (where AOX is alcohol dehydrogenase). Another PCR reaction was performed to amplify of an internal fragment of the *S. cerevisiae* gene using specific primers Jinv-1 (5′-GAAAGTGGCCGTACATTAC-3′) and JC (5′-GATACCCCAGACACCAAAGAC-3′). 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.

P. pastoris growth conditions for recombinant protein expression

Cells previously grown in solid MD medium for 48 h were inoculated in 100 ml of MGY medium in a 1 litre flask and grown in a shaking incubator until the culture reached $D_{600} = 2.0$–6.0. The cells were harvested and resuspended in 200 ml of MM medium to a D_{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 h to maintain induction. Cell-suspension samples of 15 ml were collected over time and analysed for recombinant protein expression.

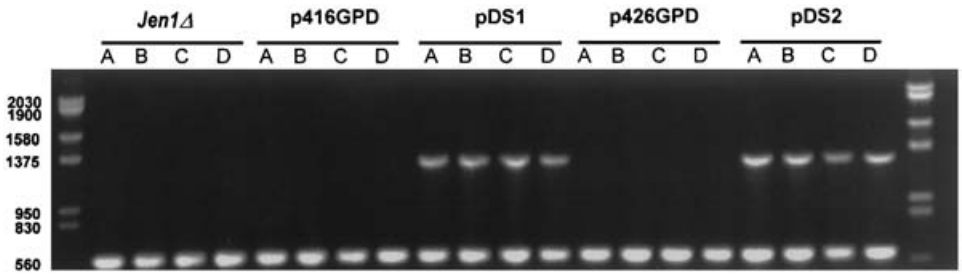

Figure 1 Detection of *JEN1* expression by RT-PCR in cells of *S. cerevisiae jen1*Δ not transformed or transformed with the referred plasmids

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, and acetic acid, 0.25 %, v/v, pH 4.0); D (YNB; acetic acid, 0.25 %, v/v, pH 4.0).

Hybridization analysis

Samples of 10 μg of genomic DNA from each *P. pastoris* transformant strain were blotted on to a positively charged nylon membrane (GeneScreenPlus Hybridization Transfer Membrane; NEN™ Life Sciences Products, Boston, MA, U.S.A.) using a PR648 slot-blot filtration manifold (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). 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 on to a positively charged nylon membrane and the membrane was hybridized for *JEN1* [12]. Densitometer scanning was performed using the Integrated Density Analysis program from EagleSight® software, version 3.2 (Stratagene, La Jolla, CA, U.S.A.).

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 of protein/ml) and stored in liquid nitrogen. Total yeast extracts were obtained by the method of Volland et al. [22].

SDS/PAGE and Western blotting

Protein samples were separated by SDS/PAGE (10 % gels) and transferred to a PVDF membrane (Hybond™-P, Amersham Biosciences). The proteins were probed with chicken polyclonal antibody raised against a 13-amino-acid peptide of the N-terminal region (EVYNPDHEKLYHN) of Jen1p. Primary antibody was detected with a horseradish-peroxidase-conjugated anti-chicken IgG secondary antibody (Sigma) detected by enhanced chemiluminescence (ECL®; Amersham Biosciences).

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 of dry weight per ml. Uptake rates of labelled monocarboxylic acids were estimated as described previously [15]. The substrates were D,L-[^{14}C]lactic acid (sodium salt, Amersham Biosciences; 4000 d.p.m./nmol), pH 5.0, and [^{14}C]acetic acid (sodium salt; Amersham Biosciences; 3000 d.p.m./nmol), pH 5.0. A computer-assisted non-linear regression analysis program (GraphPad Software, San Diego, CA, U.S.A.) 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 mean 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 D,L-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 (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine). Aliquots of 15 μl were withdrawn at appropriate intervals and diluted with 2.0 ml of ice-cold 100 mM LiCl. The mixtures were filtered through nitrocellulose filters (pore size. 0.45 μm; Macherey-Nagel) and washed with 2.0 ml of 100 mM LiCl. The filters were introduced into vials and radioactivity was measured as described above. Experiments were carried out at 26 °C.

RESULTS

Constitutive expression of *JEN1* in *S. cerevisiae*

The *S. cerevisiae* strain carrying a genomic deleted *jen1*Δ allele was transformed with either of the plasmid p416GPD and p426GPD (native plasmids) and with the corresponding plasmid containing a copy of *JEN1* under the control of the GPD (glyceraldehyde-3-phosphate dehydrogenase) constitutive promotor (pDS1 and pDS2 respectively; Table 1). *JEN1* transcription was analysed 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 was previously shown [12,14] in the *S. cerevisiae* strain 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 min 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.

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). Units for V_{max} are nmol · s^{-1} · mg of dry weight^{-1}.

		Kinetic parameter			
		Acetic acid		Lactic acid	
Plasmid	Culture medium	K_m (mM)	V_{max}	K_m (mM)	V_{max}
p416GPD	G	No activity	No activity	No activity	No activity
	GA	No activity	No activity	No activity	No activity
	A	1.50 ± 0.50	2.69 ± 0.39	No activity	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	No activity	No activity
	GA	No activity	No activity	No activity	No activity
	A	1.67 ± 0.31	1.39 ± 0.68	No activity	No activity
pDS2	G	No activity	No activity	No activity	No activity
	GA	No activity	No activity	No activity	No activity
	A	1.06 ± 0.47	2.86 ± 0.48	Not determined	Not determined

Transport of monocarboxylic acids in *S. 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, whereas when cloned in the multicopy plasmid, the uptake of labelled lactic acid obeys a simple diffusion mechanism. In cells carrying no functional copy of *JEN1*, the transport of labelled lactic acid also obeyed 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 (sorbitol, 100 mM) sugar 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 (in either the presence or absence of acetic acid) activity of 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 *S. cerevisiae*

In order to determine whether the constitutive expression of *JEN1* in the presence of glucose is associated with the altered consumption of acetic acid, the supernatants of cultures grown in YNB containing glucose and acetic acid were analysed by HPLC. As can be observed in Figure 2, the consumption of both substrates

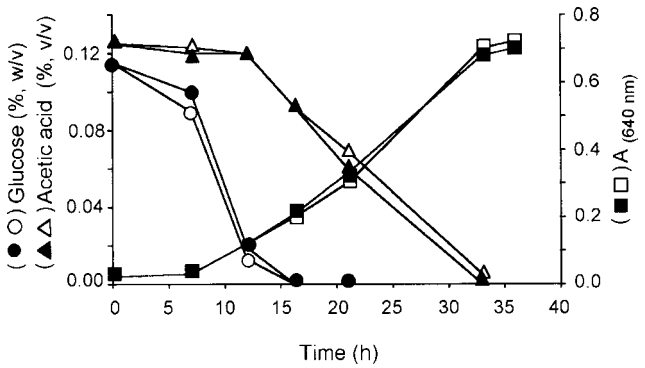

Figure 2 Growth of *S. cerevisiae* L19 (open symbols) and L23 (closed symbols) strains in YNB medium containing glucose (0.1 %, w/v) and acetic acid (0.125 %, v/v), pH 4.0

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 medium			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	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	0.25	6.0	0.16 ± 0.01	0.13 ± 0.01

was identical in a mixed-substrate medium, containing glucose and acetic acid, at pH 4.0. In cells either expressing *JEN1* or not, 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 under all the conditions tested, at either pH 4.0 or 6.0. In the media containing glucose and acetic acid, the growth rate decreased and the exponential 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.PK113-13D), and no differences in growth were detected between the strains.

Lactate permease activity in *P. 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 incubated further 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 (results not shown). Additionally, the *P. pastoris*

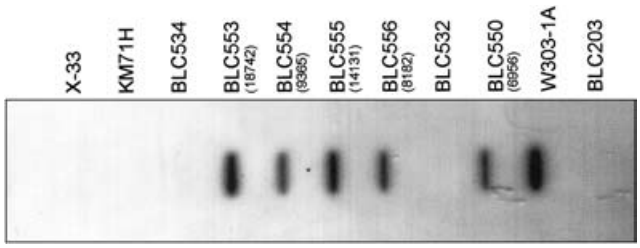

Figure 3 Slot-blot analysis of 10 μg of genomic DNA from *P. pastoris* transformants, using an *JEN1*-specific probe

Numbers in parentheses refer to the measured spot densities.

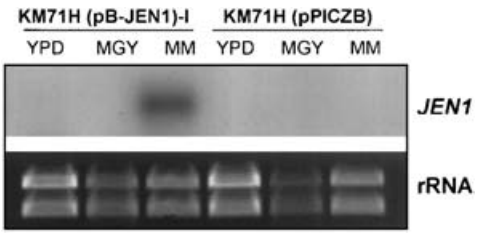

Figure 4 Northern blot analysis of *JEN1* expression in *P. pastoris* KM71H recombinant strains

Samples were taken from cells grown in YPD or MGY and from cells transferred to MM medium for 24 h.

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 (results not shown). In the strains containing the empty vectors (Table 1), no measurable permease activity could be found during the induction time-course tested (results 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 hybridized as expected with the parental *S. cerevisiae* W303-1A genomic DNA control, and failed to produce any signal in all negative controls, whereas 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 *P. 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* KM71H (pB-JEN1)-I membranes is larger than the one detected in *S. cerevisiae*, which is expected

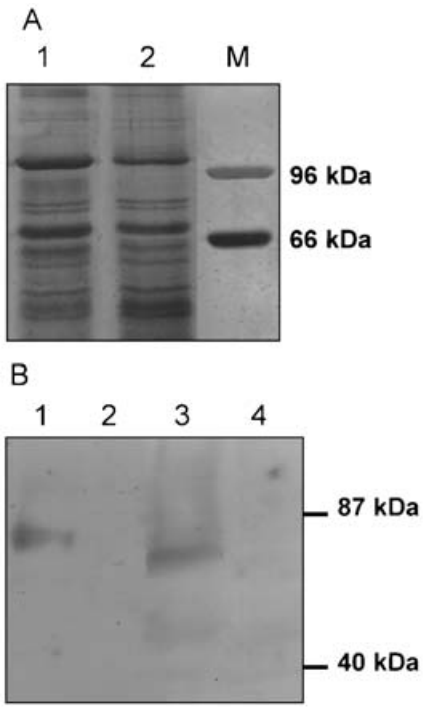

Figure 5 Expression analysis of Jen1p

(**A**) Silver-stained SDS/PAGE of plasma membrane proteins prepared from *P. pastoris* 24 h-methanol-induced cells. Lane 1, *P. pastoris* KM71H (pB-JEN1)-I; lane 2, *P. pastoris* KM71H pPICZB; lane M, SDS/PAGE Standard (Bio-Rad). (**B**) Western blot using a specific Jen1p antibody. Lane 1, plasma membrane proteins from *P. pastoris* KM71H (pB-JEN1)-I 24 h-methanol-induced cells; lane 2, plasma membrane proteins from *P. pastoris* KM71H pPICZB 24 h-methanol-induced cells; lane 3, crude extracts of *S. cerevisiae* W303-1A lactic acid-grown cells; lane 4, crude extracts of *S. cerevisiae* W303-1A glucose-grown cells.

as Jen1p expressed in *P. pastoris* has the hexahistidine and c-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 $k_d = 0.040 \pm 0.002\ \mu l \cdot s^{-1} \cdot mg$ of dry weight^{-1}. 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 D,L-lactic acid and $V_{max} = 2.15 \pm 0.14$ nmol of lactic acid $\cdot s^{-1} \cdot$ mg of dry weight^{-1}.

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 bovine heart cytochrome *c* 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

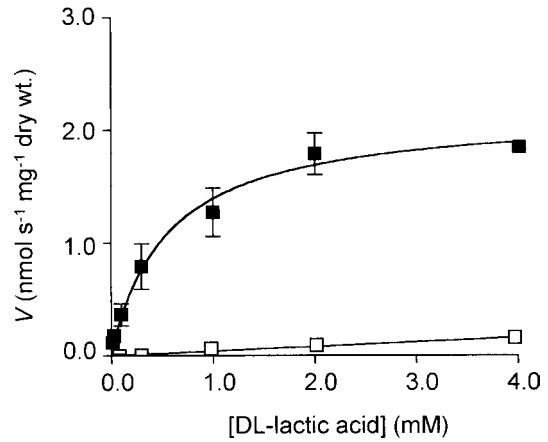

Figure 6 Initial uptake rates of labelled lactic acid (pH 5.0) measured in *P. pastoris* (pZB-JEN1)-I (■) and pPICZB (□) transformants after 24 h of induction in methanol-containing medium

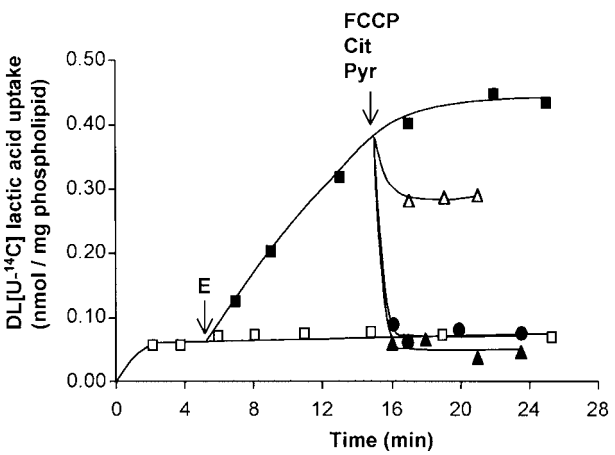

Figure 7 Uptake of radiolabelled lactic acid in hybrid vesicles prepared from *P. pastoris* KM71H (pZB-JEN1)-I methanol-induced cells

The assays were performed with 158 μM radiolabelled 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 (Pyr; ●) and 80 mM citric acid (Cit; △) were added.

contribution of the protonmotive 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 [carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone]. 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 *P. pastoris*. We will therefore 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 [24–26]. By slot-blot hybridization we verified that the clone KM71H (pB-JEN1)-I presenting the highest levels of lactic acid transport also exhibited increased the *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)-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 $K_m = 0.54 \pm 0.08$ mM lactic acid and $V_{max} = 2.15 \pm 0.14$ nmol of lactic acid $\cdot s^{-1} \cdot$ mg of dry weight^{-1} while in *S. cerevisiae* W303-1A the V_{max} was previously estimated to be 0.40 nmol of lactic acid $\cdot s^{-1} \cdot$ mg of dry weight^{-1} 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 with wild-type *S. cerevisiae*. 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* [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)-I with proteoliposomes. The properties of the reconstituted lactate uptake agreed with those of the permease evaluated in *S. cerevisiae* cells. The involvement of the protonmotive force was directly demonstrated in reconstituted *P. pastoris* vesicles by instant release of lactate upon addition of protonophore thus confirming 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 the 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 five members of the monocarboxylate porter subfamily nor the *YHL008c* gene were involved in monocarboxylate transport under all tested conditions.

We are especially grateful to Professor André Goffeau for his encouragement throughout the work and critical analysis of the manuscript. The pZPARS vector was kindly provided by Dr D. Cahill. This study was supported by the Portuguese grant POCTI/1999/BME/36625 (Eixo 2, Medida 2.3, QCAIII-FEDER). I.S.-S., R.P.A. and F.B. received fellowships from the Portuguese Government (grant nos. SFRH/BD/4699/2001, BD/15737/98 and SFRH/BPD/7170/2001 respectively).

REFERENCES

1 Rottenberg, H. (1979) The measurement of membrane potential and ΔpH in cells, organelles, and vesicles. Methods Enzymol. **55**, 547–569
2 Casal, M., Cardoso, H. and Leão, C. (1996) Mechanisms regulating the transport of acetic acid in *Saccharomyces cerevisiae*. Microbiology **142**, 1385–1390
3 Pinto, I., Cardoso, H., Leão, C. and van Uden, N. (1987) High enthalpy and low enthalpy death in *Saccharomyces cerevisiae* induced by acetic acid. Biotechnol. Bioeng. **33**, 1350–1352
4 Ludovico, P., Sousa, M. J., Silva, M. T., Leão, C. and Côrte-Real, M. (2001) *Saccharomyces cerevisiae* commits to a programmed cell death process in response to acetic acid. Microbiology **147**, 2409–2415
5 Pampulha, M. E. and Loureiro-Dias, M. C. (2000) Energetics of the effect of acetic acid on growth of *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. **184**, 69–72
6 Cui, Z., Hirata, D., Tsuchiya, E., Osada, H. and Miyakawa, T. (1996) The multidrug resistance-associated protein (MRP) subfamily (Yrs1/Yor1) of *Saccharomyces cerevisiae* is important for the tolerance to a broad range of organic anions. J. Biol. Chem. **271**, 14712–14716
7 Piper, P., Mahe, Y., Thompson, S., Pandjaitan, R., Holyoak, C., Egner, R., Muhlbauer, M., Coote, P. and Kuchler, K. (1998) The pdr12 ABC transporter is required for the development of weak organic acid resistance in yeast. EMBO J. **17**, 4257–4265
8 Holyoak, C. D., Bracey, D., Piper, P. W., Kuchler, K. and Coote, P. J. (1999) The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. J. Bacteriol. **181**, 4644–4652
9 Tenreiro, S., Rosa, P. C., Viegas, C. A. and Sá-Correia, I. (2000) Expression of the *AZR1* gene (ORF *YGR224w*), encoding a plasma membrane transporter of the major facilitator superfamily, is required for adaptation to acetic acid and resistance to azoles in *Saccharomyces cerevisiae*. Yeast **16**, 1469–1481
10 Cássio, F., Leão, C. and van Uden, N. (1987) Transport of lactate and other short-chain monocarboxylates in the yeast *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **53**, 509–513
11 Casal, M., Blazquez, M. A., Gamo, F. J., Gancedo, C. and Leão, C. (1995) Lack of lactate–proton symport activity in *pck1* mutants of *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. **128**, 279–282
12 Casal, M., Paiva, S., Andrade, R. P., Gancedo, C. and Leão, C. (1999) The lactate–proton symport of *Saccharomyces cerevisiae* is encoded by *JEN1*. J. Bacteriol. **181**, 2620–2623
13 De Hertogh, B., Carvajal, E., Talla, E., Dujon, B., Baret, P. and Goffeau, A. (2002) Phylogenetic classification of transporters and other membrane proteins from *Saccharomyces cerevisiae*. Funct. Integr. Genomics **2**, 154–170
14 Andrade, R. P. and Casal, M. (2001) Expression of the lactate permease gene *JEN1* from the yeast *Saccharomyces cerevisiae*. Fungal Genet. Biol. **32**, 105–111
15 Paiva, S., Kruckeberg, A. L. and Casal, M. (2002) Utilization of green fluorescent protein as a marker for studying the expression and turnover of the monocarboxylate permease Jen1p of *Saccharomyces cerevisiae*. Biochem. J. **363**, 737–744
16 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd edn, Cold Spring Harbor Press, Cold Spring Harbor, NY
17 Mumberg, D., Muller, R. and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. Gene **156**, 119–122
18 Lueking, A., Holz, C., Gotthold, C., Lehrach, H. and Cahill, D. (2000) A system for dual protein expression in *Pichia pastoris* and *Escherichia coli*. Protein Expression Purif. **20**, 372–378
19 Linder, S., Schliwa, M. and Kube-Granderath, E. (1996) Direct PCR screening of *Pichia pastoris* clones. Biotechniques **20**, 980–982
20 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Siedman, J. G. and Struhl, K. (1996) Current Protocols in Molecular Biology, John Wiley & Sons, NY
21 Van Leeuwen, C. C., Postma, E., Van den Broek, P. J. and Van Steveninck, J. (1991) Proton-motive force-driven D-galactose transport in plasma membrane vesicles from the yeast *Kluyveromyces marxianus*. J. Biol. Chem. **266**, 12146–12151
22 Volland, C., Urban-Grimal, D., Geraud, G. and Haguenauer-Tsapis, R. (1994) Endocytosis and degradation of the yeast uracil permease under adverse conditions. J. Biol. Chem. **269**, 9833–9841
23 Gerós, H., Cássio, F. and Leão, C. (1996) Reconstitution of lactate proton symport activity in plasma membrane vesicles from the yeast *Candida utilis*. Yeast **12**, 1263–1272
24 Cregg, J. M., Vedvick, T. S. and Raschke, W. C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. Biotechnology (NY) **11**, 905–910
25 McGrew, J. T., Leiske, D., Dell, B., Klinke, R., Krasts, D., Wee, S. F., Abbott, N., Armitage, R. and Harrington, K. (1997) Expression of trimeric CD40 ligand in *Pichia pastoris*: use of a rapid method to detect high-level expressing transformants. Gene **187**, 193–200
26 Cai, J., Daoud, R., Georges, E. and Gros, P. (2001) Functional expression of multidrug resistance protein 1 in *Pichia pastoris*. Biochemistry **40**, 8307–8316
27 Romanos, M. A., Scorer, C. A. and Clare, J. J. (1995) Expression of cloned genes in yeast. In DNA Cloning 2: Expression Systems, A Practical Approach Series (Glover, D. and Hames, D., eds.), pp. 123–167, Oxford University Press, Oxford
28 De Kerchove d'Exaerde, A., Supply, P. and Goffeau, A. (1996) Review: subcellular traffic of the plasma membrane H^+-ATPase in *Saccharomyces cerevisiae*. Yeast **12**, 907–916
29 Halestrap, A. P. and Price, N. T. (1999) The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochem. J. **343**, 281–299
30 Makuc, J., Paiva, S., Schauen, M., Kramer, R., Andre, B., Casal, M., Leão, C. and Boles, E. (2001) The putative monocarboxylate permeases of the yeast *Saccharomyces cerevisiae* do not transport monocarboxylic acids across the plasma membrane. Yeast **18**, 1131–1143
31 Thomas, B. J. and Rothstein, R. (1989) Elevated recombination rates in transcriptionally active DNA. Cell **56**, 619–630

Received 4 August 2003; accepted 8 September 2003
Published as BJ Immediate Publication 8 September 2003, DOI 10.1042/BJ20031180