Fungal Genetics and Biology 46 (2009) 390–399

Contents lists available at ScienceDirect

Fungal Genetics and Biology

journal homepage: www.elsevier.com/locate/yfgbi

Regulatory role of the PKA pathway in dimorphism and mating in *Yarrowia lipolytica*

José A. Cervantes-Chávez [a], Florencia Kronberg [b], Susana Passeron [b], José Ruiz-Herrera [a,*]

[a] Departamento de Ingeniería Genética, Unidad Irapuato, Centro de Investigación y de Estudios Avanzados del IPN. km. 9.6 Libramiento Norte, Carretera Irapuato-León, 36500 Irapuato, Gto. México

[b] Cátedra de Microbiología, Facultad de Agronomía, Universidad de Buenos Aires, IBYF-CONICET. Avda. San Martin 4453, C1417DSE, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 5 November 2008
Accepted 10 February 2009
Available online 26 February 2009

Keywords:
Yarrowia lipolytica
Fungal dimorphism
PKA pathway
Pka catalytic subunit

ABSTRACT

Previous studies on the dimorphic transition of *Yarrowia lipolytica* suggested opposite roles for MAPK and PKA pathways in this phenomenon. To obtain conclusive evidences for these opposite roles we isolated and disrupted the unique gene encoding the Pka catalytic subunit (*TPK1*). *TPK1* was regulated only at the post-transcriptional level, with Pka activity increasing during yeast-like growth. *tpk1* null mutants were viable and without growth defects, but more sensitive to different stress conditions. Δ*tpk1* mutants were mating-deficient, and grew constitutively in the mycelial form, whereas Δ*ste11* (Mapkkk-less)/Δ*tpk1* double mutants grew in the yeast form, indicating that this is the default growth pattern of the fungus. Our data confirm that MAPK and PKA pathways operate in opposition during the dimorphic behavior of *Y. lipolytica*, but synergic in mating. These data stress the idea that in different fungi both signal transduction systems may operate distinctly or even be antagonistic or synergic in the coordination of cell responses to different stimuli.

1. Introduction

Yarrowia lipolytica is a non-conventional yeast which has received much attention due to its possible biotechnological applications. These include its capacity to degrade fatty acids and hydrocarbons, to express heterologous proteins, and to produce organic acids and compounds for the flavor industry (Barth and Gaillardin, 1996; Waché et al., 2003; Madzak et al., 2004; Fickers et al., 2005). *Y. lipolytica* has also been useful as a cell differentiation model system because its dimorphic properties. Accordingly, it is possible to obtain almost homogenous populations of yeast or mycelium by manipulation of the growth conditions. These include the carbon source, specific compounds, such as citrate or serum, as well as the medium pH (Rodríguez and Domínguez, 1984; Guevara-Olvera et al., 1993; Domínguez et al., 2000; Ruiz-Herrera and Sentandreu, 2002; Szabo and Stofaníková, 2002). The phenomenon of dimorphism is particularly important since in a number of fungi pathogenic for humans or plants, their dimorphic capacity is directly related to their virulence (D'Souza et al., 2001; Nemecek et al., 2006; Biswas et al., 2007; Klein and Tebbets, 2007; Nadal et al., 2008). In this sense it is interesting to note that the conditions that regulate dimorphism in *Y. lipolytica* are similar to those from *Candida albicans*, probably the most important human pathogenic fungus.

Evidence exists that external signals that influence the fungal dimorphic response, are basically sensed through the operation of two signaling transduction mechanism: the mitogen activated protein kinase (MAPK), and the cyclic-AMP (cAMP) dependent protein kinase (PKA) pathways (Lengeler et al., 2000; Lee et al., 2003; Martínez-Espinoza et al., 2004).

We have described that cAMP addition inhibited the yeast-to-mycelium transition of *Y. lipolytica* (Ruiz-Herrera and Sentandreu, 2002), contrary to what occurs in *C. albicans* (Sonneborn et al., 2000; Cloutier et al., 2003; Souto et al., 2006). We also reported that *Y. lipolytica* carrying mutations in the gene encoding the MAPKKK (mitogen activated kinase kinase kinase) Ste11 grow constitutively in the yeast-like form (Cervantes-Chávez and Ruiz-Herrera, 2006). In a further report we described that merodiploid strains carrying several copies of the *RKA1* gene that encodes the Pka regulatory subunit (rPka), showed refractory response to the negative effect exerted by cAMP on the dimorphic transition in a dose dependent manner and that *RKA1* was upregulated at the transcriptional level under conditions that promoted the dimorphic transition (Cervantes-Chávez and Ruiz-Herrera, 2007). All together these results suggested that in *Y. lipolytica*, MAPK and PKA pathways are oppositely involved in dimorphism: while a MAPK signaling pathway is necessary for mycelial growth, a functional PKA pathway is required for growth in the yeast-like form.

In the present study we analyzed the validity of this hypothesis, and studied in deeper detail the mechanism of regulation of the dimorphic transition by the PKA and MAPK pathways in *Y. lipolytica*. Accordingly, we proceeded to isolate and disrupt the gene encoding the catalytic subunit of Pka (cPka), and to analyze the phenotypic characteristics of the corresponding mutants.

* Corresponding author. Fax: +52 462 623 9649.
E-mail address: jruiz@ira.cinvestav.mx (J. Ruiz-Herrera).

1087-1845/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2009.02.005

2. Materials and methods

2.1. Strains and culture conditions

The *Y. lipolytica* strains used in this work and their relevant genotypes are shown in Table 1. *Escherichia coli* Top10 strain was used routinely for plasmid propagation. *Y. lipolytica* strains were maintained at −70 °C in 50% (v/v) glycerol. When required they were transferred to liquid or solid (2% agar) YPD medium (1% yeast extract, 2% peptone and 2% glucose) or YNB medium [0.67% YNB without amino acids/ammonium sulfate (Difco), 1% glucose, 0.5% ammonium sulfate, supplemented with the necessary requirements], and incubated routinely at 28 °C for variable periods of time. Whenever required, YNB medium was supplemented with uracil (22.4 mg/l) or leucine (262 mg/l). Selection against the *URA3* genetic marker was conducted on YNB plates supplemented with 0.1% FOA (5-fluoroorotic acid; Sigma–Aldrich, St. Louis, MO). Where indicated, cAMP sodium salt (Sigma–Aldrich, St. Louis, MO) or myristoylated PKI (14–24) amide (Myr-PKI, Biomol Research Lab, Burlington, ON) were dissolved in water, sterilized by filtration and added to the sterile media at concentrations indicated in each experiment. Growth rate was measured by the Optical Density (OD) of the cultures at 600 nm in a Pharmacia LKB Ultrospec III. *E. coli* was grown at 37 °C in Luria-Bertani medium (1% tryptone, 0.5% yeast extract and 0.5% sodium chloride) with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) for plasmid selection.

2.2. Preparation of crude extracts and assay of Pka activity

Cells ($1–2 \times 10^{-7}$) were suspended in 300 μl 10 mM sodium phosphate buffer, pH 6.8 containing 1 mM EGTA, 1 mM EDTA, 10 mM β-mercaptoethanol and protease inhibitors [one tablet 'complete mini' protease mix (Roche, USA) per 10 ml lysis buffer], and lysed by disruption with glass beads. The suspension was spun down in a microcentrifuge at maximum speed for 30 min and the supernatant was used immediately for enzymatic assays. All operations were performed at 4 °C. Pka assays were performed as previously described (Zelada et al., 1998). Briefly, phosphotransferase activity was measured in a final volume of 60 μl containing 20 mM Tris·HCl buffer, pH 7.5, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 0.1 mM kemptide (Sigma–Aldrich, St. Louis, MO), 0.1 mM [γ-^{32}P]ATP (0.1–0.5 Ci/mmol, New England Nuclear, Waltham Massachusetts) and 10 μM cAMP when indicated. After incubation for 10 min at 30 °C, 50 μl aliquots were spotted on squares of phosphocellulose paper (P-81, Whatman, Chandler, AZ) and dropped into 75 mM phosphoric acid for washing. Radioactivity was measured by liquid scintillation. In all assays the amount of extract was adjusted in order to minimize endogenous kinase activity and the reactions were carried out under conditions of linearity respect to the amount of extract and the time of incubation. Pka specific activity was expressed as pmoles ^{32}P incorporated to kemptide mg^{-1} protein min^{-1}.

Table 1
Yarrowia lipolytica strains used in this work.

Strain	Relevant genotype	Source
P01A	MatA, *ura 3-52, leu 2-270*	INRA*
E151	MatB, *his1, leu 2-270*	INRA
E121	MatA, *lys 11-23*	INRA
E151A	MatB, *his1* + pINA240	Cervantes-Chávez and Ruiz-Herrera (2006)
AC1	MatA, *ura 3-52, leu2-270, Δste11*	Cervantes-Chávez and Ruiz-Herrera (2006)
AC11	MatA, *ura 3-52, leu 2-270, Δtpk1*	This work
AC12	MatA, *ura 3-52, leu 2-270, Δtpk1*	This work
AC33	Mata, *ura 3-52, leu 2-270, Δtpk1, Δste11*	This work
AC41	MatA, *ura 3-52, leu 2-270, Δtpk1, Δste11*	This work
AC03	MatA, *leu2-270, TPK1*	This work
AC29	MatA, *leu2-270, TPK1*	This work
AC31	MatA, *leu2-270, TPK1*	This work

* Institut National de la Recherche Agronomique.

2.3. Protein determination

Protein was quantified by the method of Bradford (1976) using bovine serum albumin as standard.

2.4. Genetic transformation and mutant isolation

Genetic transformation of *Y. lipolytica* was carried out by the lithium acetate method (Barth and Gaillardin, 1996). *E. coli* transformation was performed by standard procedures (Sambrook and Russell, 1999). *Y. lipolytica* mutants were obtained according to the "Pop-in Pop-out" methodology described by Boeke et al. (1987).

2.5. Induction of the yeast-to-mycelium transition

Dimorphic transition of *Y. lipolytica* was basically assayed as previously described (Ruiz-Herrera and Sentandreu, 2002). Cells were observed with a Leica DMRE microscope. At least 500 cells were scored to determine the ratio of yeast and mycelial cells. For each assay, three experiments with duplicate samples were performed.

2.6. Mating assays

Mating assays were conducted as previously reported (Cervantes-Chávez and Ruiz-Herrera, 2006). For each assay, three experiments with duplicate samples were performed.

2.7. Stress assays

Different stress conditions on *Y. lipolytica* were tested on solid YPD or YNB media. Cells were grown in YPD liquid medium at 28 °C until the stationary phase was reached, collected and washed with sterile distilled water by centrifugation. Cell suspensions were adjusted to contain 1×10^8 cells ml^{-1} (counted with a Neubauer chamber), tenfold serial dilutions were prepared and 10 μl were spotted on agar plates containing different media and additions, or after cells were treated with specific reagents as indicated for each experiment. Plates were incubated at 28 °C for 48 h or at 36 °C for 72 h, and photographed using a Cannon camera model Eorebeld.

To analyze the utilization of different carbon sources, cell dilutions prepared as above were spotted on plates of complete medium (1% yeast extract and 2% peptone) or YNB, supplemented with 2% carbon source (glucose, glycerol, sodium acetate, ethanol or galactose), and incubated and photographed as above.

2.8. Techniques for nucleic acids manipulations

Genomic DNA from *Y. lipolytica* was isolated by the glass bead lysis method as described by Hoffman and Winston (1987). Total RNA was isolated according to Jones et al. (1985). Southern and Northern blots were performed by standard techniques (Sambrook and Russell, 1999) using 10 or 30 μg of nucleic acid samples, respectively. DNA probes were labeled using the random primer labeling system and [^{32}P] α dCTP (Amersham Biosciences, Buckinghamshire, UK). Ribosomal RNA stained with ethidium bromide was used as loading control.

Plasmid DNA isolation from *E. coli* was performed by standard procedures (Sambrook and Russell, 1999). DNA enzymatic reactions such as digestion, ligation and vector dephosporylation were performed as recommended by the manufacturers of the reagents used (Invitrogen, New England Biolabs). DNA for sequencing, ligation and random primer labeling reactions was purified using the QIAquick Gel extraction Kit (QIagen, Valencia, CA).

2.9. Polymerase chain reaction (PCR) conditions

Routine PCR reactions were conducted using Taq DNA polymerase (Invitrogen, Carlsbad, CA) and the following general program: an initial cycle of 94 °C for 5 min; amplification (30–35 cycles) at 94 °C for 30 s followed by annealing at primer-specific temperature for 60 s (see Table 2), and polymerization at 72 °C (1 min kb^{-1} of DNA target length). When required, the expanded high fidelity PCR system (Boehringer, Mannheim) was used according to the manufacturer's instructions. An extension period of 7 min at 72 °C was programmed for those PCR products that were cloned into pCR2.1 or pCR4 TOPO (Invitrogen).

2.10. DNA sequencing

DNA sequencing was performed with an ABI PRISM 377 DNA automated sequencer (Perkin Elmer). Double stranded DNA was used as template. Universal, reverse and some specific primers were used (see Table 2). The sequence of the *TPK1* gene was submitted to EMBL with accession No. FM865406.

2.11. Plasmid construction

pACa harbors a 3.1 kb fragment amplified by PCR with primers AC106 and AC107 (Table 2) containing the full *TPK1* ORF (993 bp) plus 889 bp from 5′UTR, and 1283 bp corresponding to the 3′UTR, cloned into pCR2.1 vector. Plasmid pACc used to disrupt the wild type *TPK1* gene according to the Pop-in Pop-out procedure was constructed as follows: 95% of the ORF including 98 bp belonging to the 3′non coding region were eliminated as a *Hinc*II fragment (1038 bp) from plasmid pACa. The rest of the plasmid was self-ligated, rendering attached promoter and terminator sequences (P/T) giving rise to plasmid pACb. Next, the P/T sequence (*ca* 2 kb) was recovered as an *EcoR*I-*Xba*I fragment from plasmid pACb and subcloned into the same restriction sites of pURA3 plasmid (pUC19 harboring the *URA3* gene from *Y. lipolytica*).

Plasmid pACe was constructed to complement the Δ*tpk1* mutation. Firstly, the *TPK1* gene was amplified as indicated above and cloned into pCR4 vector giving rise to plasmid pACd. This plasmid was opened at the *BamH*I site located in the 3′UTR, leaving in this way a terminator of 431 bp. Next, *URA3* gene (selection marker) was recovered as a *BamH*I fragment from plasmid pURA3-B (*URA3* gene flanked by *BamH*I restriction sites into pCR2.1 vector) and subcloned into plasmid pACd producing in this way plasmid pACe which harbors the *TPK1* wild type gene containing *ca* 1 kb of promoter sequence and 0.4 kb of the terminator followed by the *URA3* gene and 0.7 kb of the 3′non coding sequence. Finally, the complementation cassette (4.8 kb) was recovered by PCR using primers AC106 and AC107.

All constructions were checked by digestion pattern and by PCR as well as by sequencing.

3. Results

3.1. Influence of activators and inhibitors of the PKA pathway on the yeast-to-mycelium transition

We have previously shown that exogenous addition of cAMP inhibits the *in vitro* yeast-to-mycelium dimorphic transition of *Y. lipolytica* (Ruiz-Herrera and Sentandreu, 2002; Cervantes-Chávez and Ruiz-Herrera, 2007). Here we confirmed these results, and demonstrated that this effect involved the PKA pathway. When cells from the P01A strain growing under conditions that induce mycelial morphology were treated with 20 mM cAMP, the proportion of mycelial cells decreased. This inhibitory effect of cAMP was reverted by the specific Pka inhibitor Myr-PKI. After its addition, the proportion of mycelial cells was similar to that observed without cAMP treatment (Table 3).

3.2. Isolation of the TPK1 gene

Further experiments involved the isolation and disruption of the gene encoding the Pka catalytic subunit. An *in silico* analysis was conducted on the genome of *Y. lipolytica* (http://www.genolevures.edu) using as queries the protein sequences from several fungal Pka catalytic subunits. Only the sequence YALI0C08305g matched with these proteins, sharing 85% homology with *C. albicans* Tpk2p, and 83%, 75% and 72% with *Saccharomyces cerevisiae* Tpk2p, Tpk3p and Tpk1p, respectively. Primers AC106 and AC107 (Table 2) were designed over this sequence to clone the corresponding complete gene from *Y. lipolytica*. A 3.1 kb PCR product was amplified using genomic DNA from P01A strain as template and cloned into the pCR2.1 vector. This sequence encoded a putative 38067 Mr protein comprising 330 amino acids, showing a high degree of homology to fungal Pkas (data not shown). The *Y. lipolytica* Pka protein is of a similar size to most fungal catalytic subunits, but lacks *ca* 100 aa residues corresponding to the *N*-terminus in comparison to Pka subunits from *S. cerevisiae* and *C. albicans*, wich are larger.

In most eukaryotes including some fungi, catalytic subunits of Pka proteins are encoded by more than one gene. Therefore a Southern hybridization was conducted using genomic DNA from *Y. lipolytica* and probed with a fragment spanning most of the *Y. lipolytica* Pka coding region. We found that the size of the single fragment obtained corresponded to that expected from the cloned coding sequence. The same hybridization pattern was obtained no matter whether we used high or low conditions of hybridization

Table 2
Primer used in this work.

Name	Sense	Sequence 5′ → 3′	Tm (°c)
AC106	F*	GTTCGTCATACCAGCCGAGTCCCGCC	68
AC107	R**	TCTAGACGCTCATTGAACACCCGGACC	68
AC114	F	CCCGGAGGAGGAGCTGGACTATGGAAT	68
1109	r	GTAGGAGGGCATTTTGGTGGTGAAGAG	70
AC141	R	GAGGGGTGGAGACTAGTTTCTATGAGAC	67
M13F	F	GTAAAACGACGGCCAG	55
M13R	R	CAGGAAACAGCTATGAC	55

* F, forward.
** R, reverse.

Table 3
Modulation of the PKA pathway and its effect on *Y. lipolytica* morphology.

Additions	Mycelial cells (%)
None	77 ± 5
cAMP (20 mM)	30 ± 2
cAMP (20 mM) + Myr-PKI (10 μM)	70 ± 4.7

P01A cells were grown under mycelial inducing conditions in YNB medium as described in Material and methods. Data are expressed as proportion of mycelial cells. Values are means ± standard deviation (SD) from three independent experiments performed in duplicate.

stringency (data not shown). Altogether these results, as well as *in silico* analysis, and Pka activity measurements (see below), indicate that *Y. lipolytica* possesses a single gene encoding the Pka catalytic subunit.

3.3. Analysis of regulation of TPK1 at the transcriptional level

We analyzed by Northern blot whether *Y. lipolytica TPK1* was under transcriptional regulation during the yeast-to-mycelium switch. Only very small changes in gene expression were observed during the process. Under conditions that induce mycelial growth, the highest expression level was observed 4 h after the morphogenetic stimulus, and the lowest at 24 h (Fig. 1 panel A). Under non-inducing conditions at pH 3, almost a constitutive expression pattern was observed (Fig. 1 panel A). As expected, no transcript was detected in Δ*tpk1* mutants as compared to parental P01A and complemented strains (see Fig. 1 panel C, and below).

It has been described that of *S. cerevisiae* mutants in *BCY1* encoding a Pka regulatory subunit grow well in glucose only (Toda et al., 1987). Since our preliminary results suggested opposite roles for the PKA pathway in *S. cerevisiae* and *Y. lipolytica*, transcript levels of *Y. lipolytica TPK1* were measured in cells grown with different carbon sources: glucose, glycerol, sodium acetate, or peptone. No significant differences in growth or transcript levels were observed among the different cultures, but in all carbon sources tested, a small increase in the amount of transcript was detected during the stationary phase (24 h; see Fig. 1 panel B).

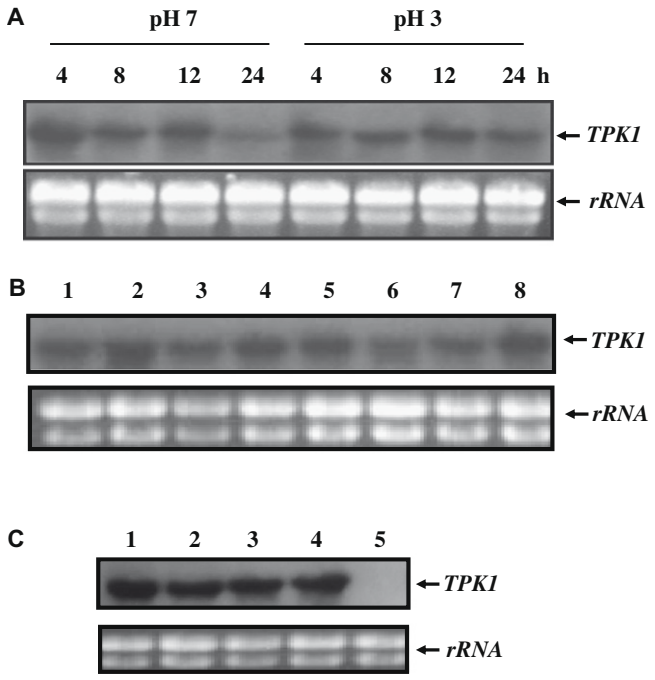

Fig. 1. *TPK1* transcript analysis. Panel A) Northern analysis of RNA isolated from P01A strain grown for the indicated periods of time in YNB medium containing 0.5% $(NH_4)_2SO_4$, 1% glucose, and 100 mM citrate buffer pH 3 or 7. Panel B) Northern analysis of RNA isolated from P01A strain grown in YNB medium pH 7 supplemented with 2% of the following carbon sources: Lanes 1 and 2, glucose; lanes 3 and 4, glycerol; lanes 5 and 6, sodium acetate; lanes 7 and 8, peptone. Lanes 1, 3, 5 and 7, 12 h incubation. Lanes 2, 4, 6 and 8, 24 h incubation. Panel C) Northern analysis of RNA isolated from parental, mutant, and complemented strains grown at pH 7 as in panel A for 12 h. Lane 1, P01A; lanes 2–4 AC03, AC29 and AC31 complemented strains, respectively; lane 5, Δ*tpk1* strain. As probe, a *ca* 1 kb *Hin*cII fragment belonging to the ORF fragment obtained from plasmid pACa was used. rRNAs are shown as loading control.

3.4. Disruption of TPK1 gene by the Pop-in Pop-out technique

The procedure of Boeke et al. (1987) for wild type gene replacement was followed. We included in the analysis the parental P01A strain, and a Δ*ste11* mutant (AC1) lacking the MAPKKK Ste11, and therefore defective in the MAPK pathway (Cervantes-Chávez and Ruiz-Herrera, 2006). This mutant was included to analyze the phenotype of a double mutant defective in both the PKA and the MAPK signal transduction pathways. Cells were transformed by the lithium acetate procedure using plasmid pACc previously linearized with *Bgl*II. Several Ura+ transformants were recovered, and the single homologous recombination step was verified by PCR producing a band *ca* 1.8 kb in size using primers AC114 and 1109 (data not shown). Two transformants for either transformed genotype were chosen to carry out the Pop-out step. FOA resistant strains (*ura*−) were recovered on YNB plates containing uracil, leucine and FOA. Some of them were screened for the presence of the *TPK1* or Δ*tpk1* alleles by PCR using primers AC106 and AC107, which would amplify a band around 3.1 kb for the wild type gene, or a 2.1 kb band for the mutant allele (data not shown). Δ*tpk1* deletion was confirmed by Southern hybridization: genomic DNA was digested with *Xho*I and hybridized with the promoter region of the gene. A 6.9 kb signal revealed the presence of the mutant allele, whereas a 4.7 kb signal identified the wild type one (results not shown). Δ*tpk1* mutants (AC11 and AC12) and double Δ*tpk1*/Δ*ste11* mutants (AC33 and AC41) were selected for further analysis.

3.5. Genetic complementation of Δtpk1 mutants

Re-introduction of the *TKP1* gene into its original genomic context was achieved by transforming Δ*tpk1* mutants with the cassette obtained by PCR from plasmid pACe, which was amplified using primers AC106 and AC107. Uracil prototrophic transformants were recovered on YNB plates supplemented with leucine. Homologous integration was analyzed by PCR using primers 1109 and AC141; the presence of an amplification band *ca* 1.8 kb revealed the homologous integration (data not shown). Integration into the corresponding *TPK1* locus was confirmed by Southern blot, using as a probe almost the entire gene. The presence of hybridization signals around 3.2 and 4.7 kb (*TPK1*); 6.9 kb (Δ*tpk1*); 2.3 and 4.7 kb (*TPK1* complemented strain) confirmed the respective alleles. In some complemented strains the correct transcription of *TPK1* was verified by Northern blot (Fig. 1 panel C).

3.6. Determination of Pka activity

Various aspects of the regulation of Pka activity were investigated and these are detailed below:

(a) Activity of Pka could be modulated by exogenous cAMP or Myr-PKI addition. Protein extracts from the P01A strain were prepared and phosphorylation of kemptide substrate was used to determine Pka activity. Our data indicate that activity detected was strictly dependent on cAMP and sensitive to the specific inhibitor Myr-PKI. A very low level of Pka activity was detected under normal conditions without cAMP (41 ± 5 pmoles mg^{-1} min^{-1}), whereas a sharp increase in activity was observed after addition of 10 μM cAMP (1780 ± 25 pmole mg^{-1} min^{-1}). On the other hand, addition of the Myr-PKI (10 μM), in the presence of cAMP, resulted in an eightfold drop in Pka activity (209 ± 9 pmoles mg^{-1} min^{-1}). These findings confirmed that the enzymatic activity observed in *Y. lipolytica* is specific for Pka.

(b) All detected Pka activity in *Y. lipolytica* is represented by the *TPK1* gene product. Extremely low levels of Pka activity were detected in Δ*tpk1* mutant strains, assayed either in logarith-

mic or in stationary phase (Fig. 2 panel A). These results reveal that all the Pka activity quantified in this fungus was due to the enzyme encoded by the *TPK1* gene, given that in a complemented strain (AC31), activity was recovered to wild type values (Fig. 2 panel A). Interestingly, in P01A or complemented strains, a two fold higher activity was detected at the stationary phase as compared to the logarithmic phase (Fig. 2 panel A).

(c) Pka activity during the yeast-to-mycelium transition. Analysis of the levels of enzyme activity at different times during yeast or mycelial growth, revealed a peculiar kinetics. At pH 7 (conditions that favor mycelial growth), activity reached a maximum at 8 h of incubation, then decreased to almost undetectable levels at 12 h (Fig. 2 panel B). In contrast, measurements of Pka activity under conditions favoring yeast growth (pH3), revealed very low activity during the first 4 h, but subsequently the activity showed a sharp increase to peak at 8 h, remaining almost constant until 24 h of incubation (Fig. 2 panel B).

3.7. Phenotypic analysis of Δtpk1 mutants

Phenotypic analysis of Δ*tpk1* mutants involved different assays. The results obtained are described below:

(a) Analysis of growth. No differences in growth rate of P01A, AC1, AC11, AC12, AC33 and AC41 strains were observed in liquid YNB or YPD media adjusted to pH 3 or pH 7 with 100 mM citrate buffer (data not shown). Also no difference in growth of parental, complemented or Δ*tpk1* mutant strains was observed when incubated at 28 °C on plates of solid complete (1% yeast extract and 2% peptone) or minimal (YNB) media supplemented with 2% of the carbon source (glucose, sodium acetate, glycerol, ethanol or galactose), but when temperature of incubation was raised to 36 °C, Δ*tpk1* mutants showed a small decrease in growth when incubated with sodium acetate, glycerol or ethanol. On the other hand, when galactose was used as carbon source, Δ*tpk1* mutants hardly grew at 36 °C (data not shown).

(b) Response to ionic or osmotic stress. Parental, Δ*tpk1* mutants and complemented strains were subjected to osmotic stress using different agents. We did not observe differences in the growth of parental and mutant strains incubated at 28 °C or 36 °C on YNB or YPD solid media made hypertonic with 1.5 M sorbitol, 1.5 M NaCl, or 1.5 M KCl (data not shown).

(c) Response to heat shock, acid, oxidative or saline stress. Δ*tpk1* mutants proved to be more susceptible to acid (300 mM acetic acid) or oxidative (100 mM H_2O_2) stress as compared with the parental or complemented strains. It is worth mentioning that a stronger deleterious effect was observed when plates were incubated at 36 °C (see representative results in Fig. 3 panels B and D), in contrast to plates kept at 28 °C (Fig. 3 panels A and C). Similar behavior was observed when cells were heat shocked at 46 °C for 35 min: mutants were more sensitive than the P01A strain when further incubated at 36 °C, in contrast to plates conserved at 28 °C (Fig. 4 panels A and B). Interestingly, resistance to the toxic effect exerted by LiCl was observed in Δ*tpk1* or Δ*tpk1*/Δ*ste11* mutants, given that they were able to grow at concentrations at which the parental or complemented strains showed minimal growth. Once again, sensitivity was more evident in plates incubated at 36 °C in contrast to plates incubated at 28 °C (Fig. 4 panels C with D).

(d) Effect of agents that perturb cell wall and membrane integrity. Using the same assay procedure employed for the other agents, we assayed non-lethal concentrations of congo red, SDS or caffeine that did not affect growth of P01A and complemented strains. Growth of Δ*tpk1* mutants was strongly diminished when plates supplemented with congo red were incubated at 36 °C, whereas at 28 °C, growth differences were not significant (Fig. 5 panels A and B). Addition of SDS produced a more severe effect, given that even at 28 °C Δ*tpk1* strains showed difficulties in growth, and at 36 °C they were unable to grow at all (Fig. 5 panels C and D). On the other hand, caffeine (10 mM) had no effect on growth of the mutant strains (not shown).

(e) Dimorphic transition. We found that Δ*tpk1* mutants grew constitutively and almost uniformly in the hyphal morphology under conditions that do not support the yeast-to-mycelium dimorphic transition of the parental P01A strain (YNB buffered to pH3 using 100 mM citrate, Tris or phosphate; see Table 4 and Fig. 6). Interestingly, we observed that the phenotype of Δ*tpk1*/Δ*ste11* double mutants (AC33 or AC41) was indistinguishable from that of Δ*ste11* single mutants (Table 4 and Fig. 6), that grow constitutively in the yeast form (Cervantes-Chávez and Ruiz-Herrera, 2006). All experiments described above were also conducted in the presence of 20 mM cAMP and showed similar results to experiments in the absence of cAMP (data not shown). These data confirmed that *Y. lipolytica* has no other Pka catalytic subunit that could be activated by cAMP to replace Tpk1 functions.

(f) Mating capacity. We tested crosses of single Δ*tpk1* (AC11 and AC12) or double Δ*tpk1*/Δ*ste11* (AC33 and AC41) mutants with a sexually compatible wild type strain (E151 or 151A). As controls we used the parental (P01A), a comple-

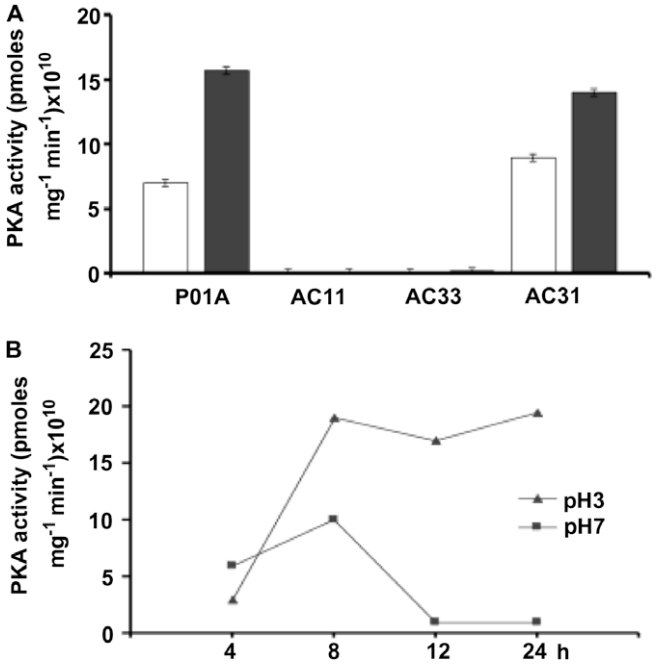

Fig. 2. Determination of Pka activity. Panel A) Pka activity was compared in protein extracts from P01A (parental), AC11 (Δ*tpk1*); AC33 (Δ*tpk1*/Δ*ste11*) and complemented AC31 (*TPK1*) strains. Cells were grown in liquid YPD medium, and collected when they reached logarithmic (white bars) or stationary phase (black bars). Values are means ± standard deviation (SD) from three independent experiments performed in duplicate. Panel B) Pka activity analyzed from P01A strain growing in YNB medium with citrate buffer pH 7 for mycelium growth, or at pH 3 for yeast growth. Samples were withdrawn after 4, 8, 12 or 24 h incubation. Activity was assayed in the presence of 10 μM cAMP. Data shown are representative of three independent experiments.

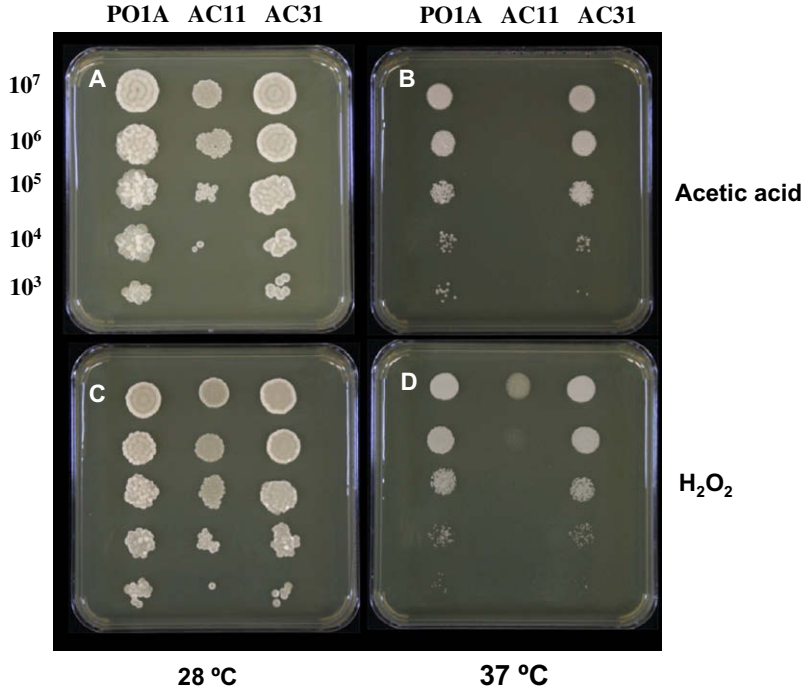

Fig. 3. Response of *Y. lipolytica* strains to acid or oxidative stress. Parental P01A, AC11 (Δ*tpk1*) and complemented AC31 (*TPK1*) strains were grown on YPD liquid medium at 28 °C until stationary phase, and cell numbers were determined using a Neubauer chamber. 10^8 cells ml^{-1} aliquots were treated with 300 mM acetic acid for 1.5 h (panels A and B) or 100 mM H_2O_2 for 4 h (panels C and D) at 28 °C with shaking. Then, serial decimal dilutions were prepared and 10 μl of each dilution was spotted on YPD solid media. Plates were incubated at 28 °C for 48 h (panels A and C) or at 36 °C for 72 h (panels B and D).

mented (AC31) and a Δ*ste11* (AC1) strains. Mating reactions were spread over plates of media for diploid selection and incubated at 28 °C. In the P01A × E151 cross, diploid colonies grew after 3 days of incubation, whereas, as expected, no diploids were obtained for the cross between AC1 and E151 as previously reported (Cervantes-Chávez and Ruiz-Herrera, 2006). It was observed that mating efficiency of Δ*tpk1* mutants was severely decreased, since only a few dip-

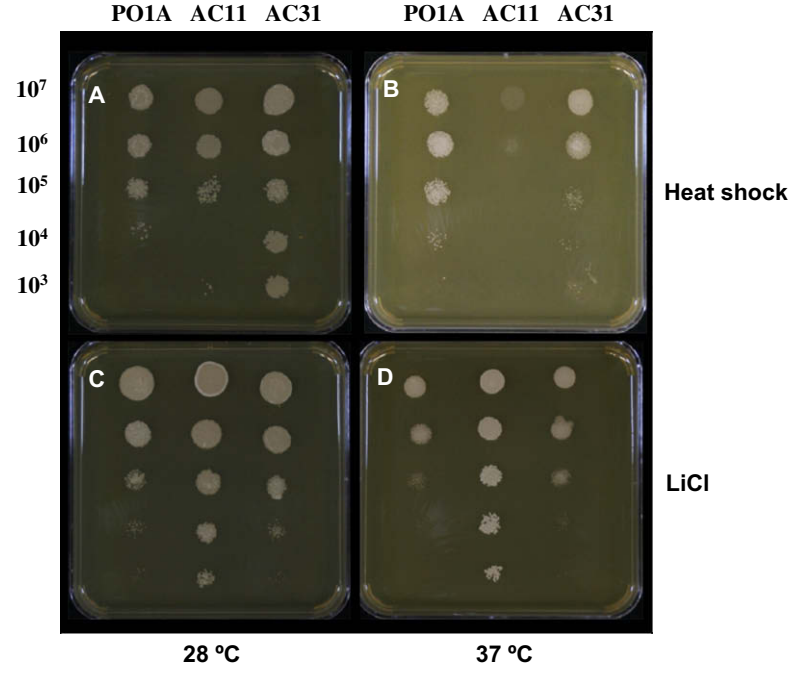

Fig. 4. Response of *Y. lipolytica* strains to heat shock or LiCl. Parental P01A, AC11 (Δ*tpk1*) and complemented AC31 (*TPK1*) strains were grown as in Fig. 3. Panels A and B) An aliquot of 1×10^8 cells ml^{-1} was heated at 46 °C for 35 min, serial decimal dilutions were prepared as in Fig. 3, and 10 μl of each dilution was spotted on YPD plates. Panels C and D) The same strains without any treatment were diluted as above and spotted on YPD plates supplemented with 350 mM LiCl. Plates were incubated at 28 °C for 48 h (panels A and C) or at 36 °C for 72 h (panels B and D).

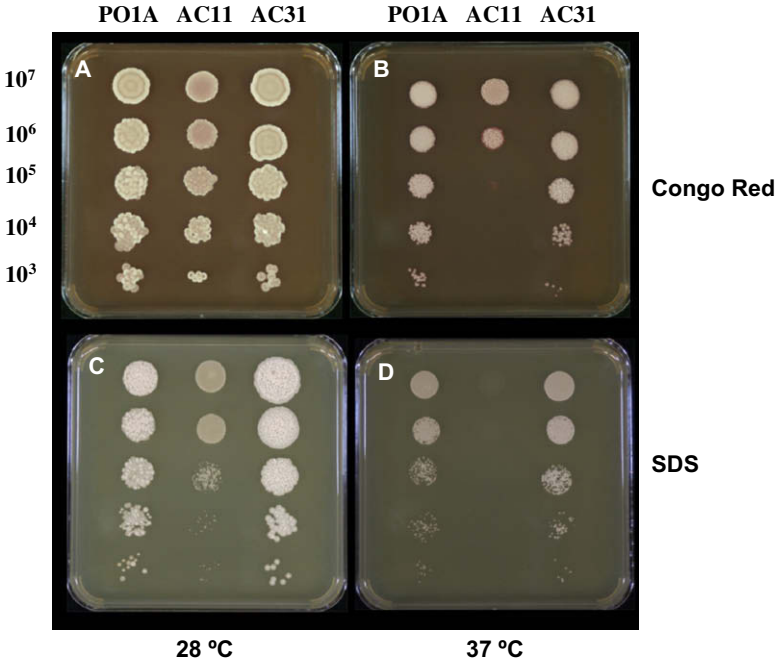

Fig. 5. Cell surface stability of *Y. lipolytica* strains. Parental P01A, AC11 (Δ*tpk1*) and complemented AC31 (*TPK1*) strains were grown, diluted and spotted as in Fig. 3 on YPD plates supplemented with 13 mg/ml congo red (panels A and B) or with 0.017% SDS (panels C and D). Plates were incubated at 28 °C for 48 h (panels A and C) or at 36 °C for 72 h (panels B and D).

loids were obtained on selective media, in contrast to the results obtained with the complemented strain (AC31), which exhibited a mating capacity similar to that of the P01A strain (Table 5). Double mutants AC33 and AC41 were sterile (Table 5).

4. Discussion

Microorganisms exhibit the ability to adapt to a wide variety of environments, a feat that is achieved by activation of several signaling cascades. In this sense, fungi are not the exception (Lengeler et al., 2000). Fungal dimorphism is a cell differentiation process resulting from adaptation of the organisms to the prevailing environmental conditions, and several inducers of *in vitro* dimorphic growth in different fungi have been identified. With some exceptions, these signals are transduced and internalized by means of specific MAPK or PKA pathways (Mösch et al., 1999; Lengeler et al., 2000; Xu, 2000).

In this work we confirmed that cAMP inhibited mycelial growth of *Y. lipolytica*, and demonstrated that this effect was reversed by Myr-PKI, contrasting with the total inhibition of germ tube formation by Myr-PKI in *C. albicans* (Cloutier et al., 2003).

Southern and *in silico* analyses, as well as Pka activity measurements showed that *Y. lipolytica* has only one gene encoding the Pka catalytic subunit (denominated *TPK1*). A single gene was also found in *Schizosaccharomyces pombe* (Maeda et al., 1994) and *Aspergillus niger* (Bencina et al., 1997), while two genes are present in *Ustilago maydis* (Dürrenberger et al., 1998), *C. albicans* (Sonneborn et al., 2000; Bockmühl et al., 2001), *Aspergillus fumigatus* (Liebmann et al., 2004); *Cryptococcus neoformans* (Hicks et al., 2004), *A. nidulans* (Shimizu and Keller, 2001; Ni et al., 2005), and three genes exist in *S. cerevisiae* (Toda et al., 1987). In spite of gene multiplicity in these fungi, generally only one is responsible for most of the Pka activity.

The observation that viability of *Y. lipolytica* single (Δ*tpk1*) or double (Δ*tpk1*/Δ*ste11*) mutants was not compromised was unexpected, since in most cases mutation of these genes is lethal e.g. *S. cerevisiae* triple (Δ*tpk1*, Δ*tpk2* and Δ*tpk3*) or *A. nidulans* double (Δ*pkaA* and Δ*pkaB*) mutations (Toda et al., 1987; Shimizu and Keller, 2001; Ni et al., 2005). Only *U. maydis* mutants defective in the two genes encoding catalytic Pka subunits (*adr1* and *uka1*) have been reported to be viable. It also contrasts with our previous observation that mutation of the regulatory subunit of Pka is lethal in *Y. lipolytica* (Cervantes-Chávez and Ruiz-Herrera, 2007). These data disclose the odd situation that whereas the effects of a constitutively active Pka are unbearable for cell viability, its lack of activity has almost no effect, at least under controlled *in vitro* conditions. Also in contrast with other fungi growth rate of *Y. lipolytica* Δ*tpk1* mutants showed no differences with their parental strain. Mutants impaired in the PKA pathway showed reduced growth: *S. pombe pka* (Maeda et al., 1994), *Colletotricum trifolli pkaC* (Yang and Dickman, 1999), *A. nidulans pkaA* (Shimizu and Keller, 2001), *A. niger pka* (Staudohar et al., 2002); *Botrytis cinerea* or *Sclerotinia sclerotiorum* defective in the gene encoding adenylyl cyclase (Klimpel et al., 2002; Jurick and Rollins, 2007), *Colletotrichum lagenarium cpk1* (Yamauchi et al., 2004), and *A. fumigatus pkaC1*

Table 4
Cell morphology of different strains grown under non-inducing yeast-to-mycelium conditions.

Growth medium	Percent of mycelial cells				
	P01A	AC11	AC31	AC1	AC33
YNB-Glc citrate pH3	3	82	5	1	0
YNB-Glc tris pH3	0	73	4	0	0
YNB-Glc phosphate pH3	0	75	0	0	0

Cells were incubated with shaking at 28 °C for 14 h in media containing glucose (glc) and the indicated buffer (100 mM). P01A, parental strain; AC11, Δ*tpk1*; AC31, *tpk1* (complemented); AC1, Δ*ste11*, and AC33, Δ*tpk1*, Δ*ste11*. Results are the average of three experiments with duplicate samples each. SD did not exceed a value of 3.

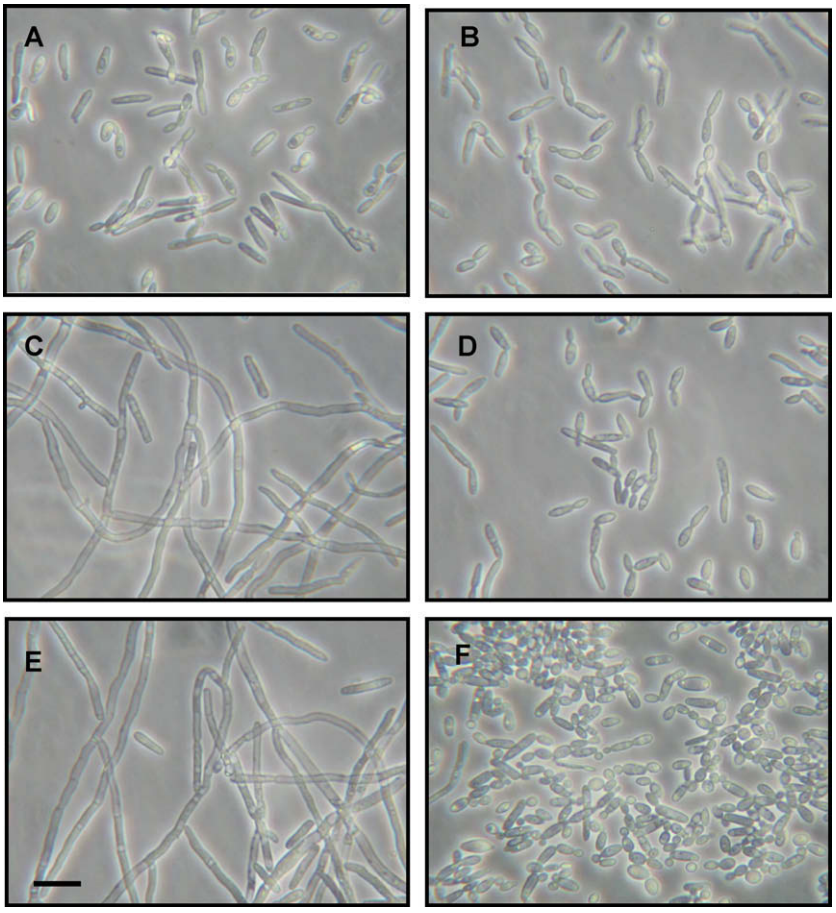

Fig. 6. Cell morphology of *Y. lipolytica* strains grown under yeast growth-inducing conditions. 3×10^6 cells ml^{-1} were inoculated on YNB medium plus requirements buffered with 100 mM citrate pH 3. Cultures were incubated with shaking at 28 °C. Samples were withdrawn after 14 h and the morphology was scored. Panel A, P01A; panel B, AC1 (Δ*ste11*); panel C, AC11 (Δ*tpk1*); panel D, AC33 (Δ*tpk1*/Δ*ste11*); panel E, AC12 (Δ*tpk1*) and panel F, AC31 (*TPK1* complemented strain). Magnification bar, 10 μm.

Table 5
Mating efficiency of wild type, mutants and complemented strains.

Cross	Mating efficiency[a]
E151 × P01A	$7.1 \times 10^{-4} \pm 0.8 \times 10^{-4}$
E151 × AC1	0
E151 × AC11	$1.7 \times 10^{-6} \pm 0.3 \times 10^{-6}$
E151 × AC12	$1.2 \times 10^{-6} \pm 0.3 \times 10^{-6}$
E151 × AC33	0
E151 × AC41	0
E151A × AC31	$6.2 \times 10^{-4} \pm 1 \times 10^{-4}$

[a] Mean of diploids per total cell numbers spread on selective media in triplicate experiments ± standard deviations of the mean. P01A, parental strain (*MATA*); E151, *MATB* strain; E151A, *MATB* strain transformed with pINA240 plasmid; AC1, Δ*ste11*; AC11 and AC12, Δ*tpk1* mutants; AC33 and AC41, Δ*ste11*/Δ*tpk1* double mutants; AC31, *TPK1* complemented strain.

(Liebmann et al., 2004). Interestingly *Y. lipolytica* Δ*tpk1* mutants showed growth problems when galactose was used as carbon source at 36 °C. This result suggests a role for Pka in its metabolism, although its level of action remains unknown. No antecedent for a similar phenomenon could be found in the literature.

TPK1 was not regulated at the transcriptional level, showing a practically constitutive expression pattern during conditions that induce the morphogenetic switch. These results contrast with the transcriptional up-regulation of the gene encoding Pka regulatory subunit Rka1 in mycelial cells (Cervantes-Chávez and Ruiz-Herrera, 2007), and suggest that transcriptional regulation of the PKA pathway may rest on the regulatory subunit. These findings differ from other fungi, whose genes encoding the Pka catalytic subunit display transcriptional regulation during some cell differentiation programs, such as conidiation in *C. trifolli* (Yang and Dickman, 1999) or spore germination in *Blastocladiella emersonii* (Marques et al., 1992; de Oliveira et al., 1994). In *Mucor circinelloides*, *pkaC* gene was expressed only in the yeast form (Wolff et al., 2002). *TPK1*, but not *TPK2* from *C. albicans* was subjected to transcriptional regulation during the dimorphic switch (Souto et al., 2006). In *A. nidulans* the *pkaB* gene was transcriptionaly regulated during sexual and asexual development, whereas expression of *pkaA* was constitutive (Ni et al., 2005; Shimizu and Keller, 2001). Additionally, variations in mRNA levels of *TPK1* and *TPK2* in *C. albicans* as well as of *pkaB* in *A. nidulans* were observed during vegetative growth (Souto et al., 2006; Ni et al., 2005).

Lack of transcriptional regulation of the *TPK1* gene in *Y. lipolytica* suggested a possible regulation mechanism at the translational or post-translational levels. Indeed, we found variations of Tpk1 activity in the course of the dimorphic transition consistent with our previous observations for cAMP (Ruiz-Herrera and Sentandreu, 2002; Cervantes-Chávez and Ruiz-Herrera, 2007).

Activation of the PKA pathway by addition of cAMP in *C. albicans* and *S. cerevisiae* leads to the formation of hyphae or pseudohyphae, respectively (Sabie and Gadd, 1992; Lorenz and Heitman, 1997). Similarly, addition of dbcAMP, the permeable derivative of cAMP, to the human pathogen *Paracoccidiodes brasiliensis* interferes with its transition from the mycelium to the yeast form (Chen et al., 2007). These data indicate that mycelial or pseudomycelial growth in these fungi is associated with a functional PKA pathway.

In contrast, in *U. maydis* a functional Pka is required for yeast but not for mycelial growth (Gold et al., 1994; Martínez-Espinoza et al., 2004). Our previous results (Cervantes-Chávez and Ruiz-Herrera, 2007) suggested that the behavior of *Y. lipolytica* was similar to *U. maydis*, and contrary to *C. albicans*, although it is important to recall that mycelial growth in *U. maydis* occurs at acidic pH, contrary to *Y. lipolytica* where it is induced by neutral pH (Ruiz-Herrera and Sentandreu, 2002). These observations are now supported by the results here reported; Δ*tpk1* mutants grew constitutively in the hyphal form. Previously we reported that *Y. lipolytica* Δ*ste11* mutant deficient in a Map kinase kinase kinase (MAPKKK) grew constitutively in the yeast-like form (Cervantes-Chávez and Ruiz-Herrera, 2006). Since deletion of *TPK1* gene in this genetic background did not reverse the yeast-like growth of the double mutants, it may be suggested that the default status (in the absence of external stimuli) of the *Y. lipolytica* pattern of growth is the yeast-like one. These data also confirm the opposite roles of the PKA and MAPK pathways in *Y. lipolytica* dimorphism, in contrast to *C. albicans* where both pathways act together to regulate the dimorphic transition (Biswas et al., 2007).

The finding that deletion of *Y. lipolytica TPK1* resulted in lower mating efficiency and that a Δ*tpk1*/Δ*ste11* double mutant was completely unable to mate are of interest as they suggest synergy between the MAPK and PKA pathways, in contrast to their opposing roles during filamentation This result agrees with *C. neoformans* varieties where PKA mutation dramatically reduced mating efficiency (D'Souza et al., 2001; Hicks et al., 2004).

C. albicans tpk1 or *tpk2* mutants were found to be very sensitive to 0.4 M NaCl (Bockmühl et al., 2001), but we found that *Y. lipolytica* Δ*tpk1* or Δ*tpk1*/Δ*ste11* were not affected by 1.5 M NaCl, KCl or sorbitol. On the other hand, these mutants were more resistant to Li^+ ions than the parental or complemented strains when incubated at 36 °C. This result contrasts with those found for *C. neoformans pka1Δ* mutants, which were more sensitive to LiCl than the parental strain (Hu et al., 2007). It may be suggested that the *Y. lipolytica* behavior might be related to a negative control by Pka of a membrane ion-activated ATPase similar to the *ENA* system, as occurs in *S. cerevisiae* (Ruiz and Ariño, 2007).

Alterations in stress tolerance to different insults are variable in mutants affected in the genes encoding Pka in different fungi. *S. cerevisiae*, *C. albicans* and *C. neoformans* mutants with higher levels of Pka activity than their respective parental strains are more sensitive to heat shock and oxidative or acid stress (Toda et al., 1987; Jung and Stateva, 2003; Phillips et al., 2006; Wilson et al., 2007; Hu et al., 2007), than those harboring non-functional Pka (Versele et al., 2004; Harcus et al., 2004; Phillips et al., 2006). Contrary to this, *A. nidulans* Δ*pkaA* mutants were more sensitive to oxidative (H_20_2) stress, but Δ*pkaB* mutants were resistant (Ni et al., 2005), and both mutants were tolerant to heat shock (Ni et al., 2005), similarly to *S. cerevisiae*, *C. albicans* or *C. neoformans*. Interestingly, we observed that *Y. lipolytica* Δ*tpk1* or Δ*tpk1*/Δ*ste11* mutants were more sensitive than the parental or complemented strains to acid (acetic), oxidative (H_2O_2) or heat shock treatments. In *C. neoformans* resistance to heat shock was associated with up-regulation of genes encoding heat shock proteins (Hu et al., 2007). It may be, therefore suggested that regulation by Pka of the genes involved in stress response in all these fungi, *Y. lipolytica* included, may be either positive or negative.

Substances such as SDS, congo red, calcofluor white or caffeine are agents widely used to reveal a weak or perturbed cell surface. Mutants of *S. cerevisiae* and *C. albicans* with a constitutively activated PKA pathway by deletion of the phosphodiesterase gene (*pde2*) are very sensitive to these agents (Jones et al., 2003; Jung et al., 2005; Wilson et al., 2007; Hu et al., 2007), and *C. albicans* mutants with a constitutively shut down PKA pathway by deletion of adenylyl cyclase (*cdc35*) or *ras1* genes, show increased resistance against calcofluor white (Harcus et al., 2004). In contrast to these results, Δ*tpk1 Y. lipolytica* mutants were very sensitive to congo red and SDS, but not to caffeine.

All these data provide further evidence that the functions regulated by Tpk1 in *Y. lipolytica* oppose those observed in other fungi, as genes required for cell surface integrity are negatively regulated in *S. cerevisiae* and *C. albicans*, and positively regulated in *Y. lipolytica*. The observation that the effects of these stresses in *Y. lipolytica* were greater at higher temperatures (36 °C) suggests that heat stress has an additive effect.

Summarizing our results in the light of other system, it is evident that PKA and MAPK pathways may operate differently depending on the fungal species, in some systems even acting either cooperatively or in opposition during the control of several physiological responses. It is likely that the final outcome of their operation depends on their effect on the down-stream receptors receiving the different signals carried by the two systems.

Acknowledgments

This work was partially supported by CONACYT México, CONCYTEG, Guanajuato, México, and CONYCET, Argentina. JACCH postdoctoral fellowship was supported by CONACYT. We are grateful to Claude Gaillardin for providing several plasmids and *Y. lipolytica* strains, and Alicia Mireles and Yesenia Ruiz for skilled technical support, as well as to Mr. Antonio Cisneros for photographs.

References

Barth, G., Gaillardin, C., 1996. *Yarrowia lipolytica*. In: Wolf, K. (Ed.), Nonconventional Yeasts in Biotechnology. Springer, Berlin, pp. 313–388.

Bencina, M., Panneman, H., Ruijter, G.J.G., Legisa, M., Visser, J., 1997. Characterization and overexpression of the *Aspergillus niger* gene encoding the cAMP-dependent protein kinase catalytic subunit. Microbiology 143, 1211–1220.

Biswas, S., Van Dijck, P., Datta, A., 2007. Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. Microbiol. Mol. Biol. Rev. 71, 348–376.

Bockmühl, D.P., Krishnamurthy, S., Gerads, M., Sonneborn, A., Ernst, J.F., 2001. Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of *Candida albicans*. Mol. Microbiol. 42, 1243–1257.

Boeke, J., Trueheart, D.J., Natsoulis, G., Fink, G.R., 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. 154, 164–174.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochem. 72, 248–259.

Cervantes-Chávez, J.A., Ruiz-Herrera, J., 2006. *STE11* disruption reveals the central role of a MAPK pathway in dimorphism and mating in *Yarrowia lipolytica*. FEMS Yeast Res. 6, 801–815.

Cervantes-Chávez, J.A., Ruiz-Herrera, J., 2007. The regulatory subunit of protein kinase A promotes hyphal growth and plays an essential role in *Yarrowia lipolytica*. FEMS Yeast Res. 7, 929–940.

Chen, D., Janganan, T.K., Chen, G., Marques, E.R., Kress, M.R., Goldman, G.H., Walmsley, A.R., Borges-Walmsley, M.I., 2007. The cAMP pathway is important for controlling the morphological switch to the pathogenic yeast form of *Paracoccidioides brasiliensis*. Mol. Microbiol. 65, 761–779.

Cloutier, M., Castilla, R., Bolduc, N., Zelada, A., Martineau, P., Bouillon, M., Magee, B.B., Passeron, S., Giasson, L., Cantore, M.L., 2003. The two isoforms of the cAMP-dependent protein kinase catalytic subunit are involved in the control of dimorphism in the human fungal pathogen *Candida albicans*. Fungal Genet. Biol. 38, 122–141.

de Oliveira, J.C., Borgues, A.C., Marques, Mdo. V., Gomes, S.L., 1994. Cloning and characterization of the gene for the catalytic subunit of cAMP dependent protein kinase in *Blastocadiella emersonii*. Eur. J. Biochem. 15, 555–562.

Domínguez, A., Fermiñán, E., Gaillardin, C., 2000. *Yarrowia lipolytica*: an organism amenable to genetic manipulation as a model for analyzing dimorphism in fungi. In: Ernst, J.F., Schmidt, A. (Eds.), Dimorphism in Human Pathogenic and Apathogenic Yeast. Karger, Basel, pp. 151–172.

D'Souza, C.A., Alspaugh, J.A., Yue, C., Harashima, T., Cox, G.M., Perfect, J.R., Heitman, J., 2001. Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. Mol. Cell. Biol. 21, 3179–3191.

Dürrenberger, F., Wong, K., Kronstad, J.W., 1998. Identification of a cAMP-dependent protein kinase catalytic subunit required for virulence and morphogenesis in *Ustilago maydis*. Proc. Natl. Acad. Sci. USA 95, 5684–5689.

Fickers, P., Benetti, P.H., Waché, Y., Marty, A., Mauersberger, S., Smit, M.S., Nicaud, J.M., 2005. Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica* and its potential applications. FEMS Yeast Res. 5, 527–543.

Gold, S., Duncan, G., Barret, K., Kronstad, J., 1994. cAMP regulates morphogenesis in the fungal pathogen *Ustilago maydis*. Genes Dev. 8, 2805–2816.

Guevara-Olvera, L., Calvo-Mendez, C., Ruiz-Herrera, J., 1993. The role of polyamine metabolism in dimorphism of *Yarrowia lipolytica*. J. Gen. Microbiol. 193, 485–493.

Harcus, D., Nantel, A., Marcil, A., Rigby, T., Whiteway, M., 2004. Transcription profiling of cyclic AMP signaling in *Candida albicans*. Mol. Microbiol. 15, 4490–4499.

Hicks, J.K., D'Souza, C.A., Cox, G.M., 2004. Cyclic AMP-dependent protein kinase catalytic subunits have divergent roles in virulence factor production in two varieties of the fungal pathogen *Cryptococcus neoformans*. Eukaryot. Cell 3, 14–26.

Hoffman, C.S., Winston, F., 1987. A ten minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57, 267–272.

Hu, G., Steen, B.R., Lian, T., Sham, A.P., Tam, N., Tangem, K.L., Kronstad, J.W., 2007. Transcriptional regulation by protein kinase A in *Cryptococcus neoformans*. PLoS Pathogens 3, e42.

Jones, J.D.G., Dunsmuir, P., Bedbrook, J., 1985. High level expression of introduced chimeric genes in regenerated transformed plants. EMBO J. 4, 2411–2418.

Jones, D.L., Petty, J., Hoyle, D.C., Hayes, A., Ragni, E., Popolo, L., Oliver, S.G., Stateva, L.I., 2003. Transcriptome profiling of a *Saccharomyces cerevisiae* mutant with constitutively activated Ras/cAMP pathway. Physiol. Genomics 16, 107–118.

Jung, W.H., Stateva, L.I., 2003. The cAMP phosphodiesterase encoded by *CaPDE2* is required for hyphal development in *Candida albicans*. Microbiology 149, 2961–2976.

Jung, W.H., Warn, P., Ragni, E., Popolo, L., Nunn, C.D., Turner, M.P., Stateva, L., 2005. Deletion of *PDE2*, the gene encoding the high-affinity cAMP phosphodiesterase, results in changes of the cell wall and membrane in *Candida albicans*. Yeast 22, 285–294.

Jurick II, W.M., Rollins, J.A., 2007. Deletion of the adenylate cyclase (*sac*1) gene affects multiple developmental pathways and pathogenicity in *Sclerotinia sclerotiorum*. Fungal Genet. Biol. 44, 521–530.

Klein, B.S., Tebbets, B., 2007. Dimorphism and virulence in fungi. Curr. Opin. Microbiol. 10, 314–319.

Klimpel, A., Gronover, C.S., Williamson, B., Stewart, J.A., Tudzynski, B., 2002. The adenylate cyclase (BAC) in *Botrytis cinerea* is required for full pathogenicity. Mol. Plant Pathol. 3, 439–450.

Lee, N., D'Souza, C.A., Kronstad, J.W., 2003. Of smuts, blasts, mildews, and blights: cAMP signaling in phytopathogenic fungi. Annu. Rev. Phytopathol. 41, 399–427.

Lengeler, K.B., Davidson, R.C., D'Souza, C., Harashima, T., Shen, W.C., Wang, P., Pan, X., Waugh, M., Heitman, J., 2000. Signal transduction cascades regulating fungal development and virulence. Microbiol. Mol. Biol. Rev. 64, 746–785.

Liebmann, B., Müller, M., Braun, A., Brakhage, A.A., 2004. The cyclic AMP-dependent protein kinase A networks regulates development and virulence in *Aspergillus fumigatus*. Infect. Immun. 72, 5193–5203.

Lorenz, M.C., Heitman, J., 1997. Yeast pseudohyphal growth is regulated by gpa2, a G protein alpha homolog. EMBO J. 16, 7008–7018.

Madzak, C., Gaillardin, C., Beckerich, J.M., 2004. Heterologous protein expression and secretion in the non-conventional yeast *Yarrowia lipolytica*: a review. J. Biotechnol. 109, 63–81.

Maeda, T., Watanabe, Y., Kunitomo, H., Yamamoto, M., 1994. Cloning of the pka1 gene encoding the catalytic subunit of the cAMP-dependent protein kinase in *Schizosaccharomyces pombe*. J. Biol. Chem. 269, 9632–9637.

Marques, Mdo.V., Borges, A.C., de Oliveira, J.C., Gomes, S.I., 1992. Coordinate pretranslational control of cAMP-dependent protein kinase subunit expression during development in the water mold *Blastocladiella emersonii*. Dev. Biol. 149, 432–439.

Martínez-Espinoza, A.D., Ruiz-Herrera, J., León-Ramírez, C.G., Gold, S.E., 2004. MAP Kinase and cAMP signaling pathways modulate the pH-induced yeast-to-mycelium dimorphic transition in the corn smut fungus *Ustilago maydis*. Curr. Microbiol. 49, 274–281.

Mösch, H.U., Kübler, E., Krappmann, S., Fink, G.R., Braus, G.H., 1999. Crosstalk between the Ras2p-controlled mitogen-activated protein kinase and cAMP pathways during invasive growth of *Saccharomyces cerevisiae*. Mol. Biol. Cell 10, 1325–1335.

Nadal, M., García-Pedrajas, M.D., Gold, S.E., 2008. Dimorphism in fungal plant pathogens. FEMS Microbiol. Lett. 284, 127–134.

Nemecek, J.C., Wüthrich, M., Klein, B.S., 2006. Global control of dimorphism and virulence in fungi. Science 312, 553–558.

Ni, M., Rierson, S., Seo, J.A., Yu, J.H., 2005. The *pkaB* gene encoding the secondary protein kinase A catalytic subunit has a synthetic lethal interaction with *pkaA* and plays overlapping and opposite roles in *Aspergillus nidulans*. Eukaryot. Cell 4, 1465–1476.

Phillips, A.J., Crowe, J.D., Ramsdale, M., 2006. Ras pathway signaling accelerates programmed cell death in the pathogenic fungus *Candida albicans*. Proc. Natl. Acad. Sci. USA 103, 727–731.

Rodríguez, C., Domínguez, A., 1984. The growth characteristics of *Saccharomycopsis lipolytica*: morphology and induction of mycelial formation. Can. J. Microbiol. 30, 605–612.

Ruiz, A., Ariño, J., 2007. Function and regulation of the *Saccharomyces cerevisiae* ENA sodium ATPase system. Eukaryot. Cell 6, 2175–2183.

Ruiz-Herrera, J., Sentandreu, R., 2002. Different effectors of dimorphism in *Yarrowia lipolytica*. Arch. Microbiol. 178, 477–483.

Sabie, F.T., Gadd, G.M., 1992. Effect of nucleosides and nucleotides and the relationship between cellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and germ tube formation in *Candida albicans*. Mycopathologia 119, 147–156.

Sambrook, J., Russell, D.W., 1999. Molecular Cloning: A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shimizu, K., Keller, N.P., 2001. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. Genetics 157, 591–600.

Sonneborn, A., Bockmühl, D.P., Gerads, M., Kurpanek, K., Sanglard, D., Ernst, J.F., 2000. Protein kinase A encoded by *TPK*2 regulates dimorphism of *Candida albicans*. Mol. Microbiol. 35, 386–396.

Souto, G., Giacometti, R., Silberstein, S., Giasson, L., Cantore, M.L., Passeron, S., 2006. Expression of *TPK*1 and *TPK*2 genes encoding Pka catalytic subunits during growth and morphogenesis in *Candida albicans*. Yeast 23, 591–603.

Staudohar, M., Bencina, M., van de Vondervoort, P.J.I., Panneman, H., Legisa, M., Visser, J., Ruijter, G.J.G., 2002. Cyclic AMP-dependent protein kinase is involved in morphogenesis of *Aspergillus niger*. Microbiology 148, 2635–2645.

Szabo, R., Stofaníková, V., 2002. Presence of organic sources of nitrogen is critical for filament formation and pH–dependent morphogenesis in *Yarrowia lipolytica*. FEMS Microbiol. Lett. 206, 45–50.

Toda, T., Cameron, S., Sass, P., Zoller, M., Wigler, M., 1987. Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. Cell 50, 277–287.

Versele, M., Thevelein, J.M., Van Dijck, P., 2004. The high general stress resistance of the *Saccharomyces cerevisiae fil1* adenylate cyclase mutant (Cyr $I^{Lys1682}$) is only partially dependent on trehalose, Hsp104 and overexpression of Msn2/4-regulated genes. Yeast 21, 75–86.

Waché, Y., Aguedo, M., Nicaud, J.M., Belin, J.M., 2003. Catabolism of hydroxyacids and biotechnological production of lactones by *Yarrowia lipolytica*. Appl. Microbiol. Biotechnol. 61, 393–404.

Wilson, D., Tutulan-Cunita, A., Jung, W., Hauser, N.C., Hernandez, R., Williamson, T., Piekarska, K., Rupp, S., Young, T., Stateva, L., 2007. Deletion of the high-affinity cAMP phosphodiesterase encoded by *PDE2* affects stress responses and virulence in *Candida albicans*. Mol. Microbiol. 65, 841–856.

Wolff, A.M., Appel, K.F., Peterssen, J.B., Poulsen, U., Arnau, J., 2002. Identification and analysis of genes involved in the control of dimorphism in *Mucor circinelloides* (syn. *racemosus*). FEMS Yeast Res. 2, 203–213.

Xu, J.R., 2000. Map Kinases in fungal pathogens. Fungal Genet. Biol. 31, 137–152.

Yamauchi, J., Takayanagi, N., Komeda, K., Takano, Y., Okuno, T., 2004. cAMP-PKA signaling regulates multiple steps of fungal infection cooperatively with Cmk1 MAP kinase in *Colletotrichum lagenarium*. Mol. Plant-Microbe Interact. 17, 1355–1365.

Yang, Z., Dickman, M.B., 1999. *Colletotrichum trifolii* mutants disrupted in the catalytic subunit of cAMP-dependent protein kinase are nonpathogenic. Mol. Plant-Microbe Interact. 12, 430–439.

Zelada, A., Passeron, S., Lopes-Gomes, S., Cantore, M.L., 1998. Isolation and characterisation of cAMP-dependent protein kinase from *Candida albicans*. Purification of the regulatory and catalytic subunits. Eur. J. Biochem. 252, 245–252.