

# The regulatory subunit of protein kinase A promotes hyphal growth and plays an essential role in *Yarrowia lipolytica*

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*Yarrowia lipolytica*.

## Abstract

The gene encoding the regulatory subunit (*RKA1*) of the cAMP-dependent protein kinase (PKA) of *Yarrowia lipolytica* was isolated to analyze the role of the PKA pathway in the dimorphic transition of the fungus. The gene encoded a protein of 397 amino acids that exhibits significant homology to fungal PKA regulatory subunits. Attempts to disrupt the gene by double homologous recombination, or the Pop-in Pop-out technique, were unsuccessful. The gene could be mutated only in merodiploids constructed with an autonomous replicating plasmid. Loss of the plasmid occurred with growth under nonselective conditions in the whole population of merodiploids carrying the mutation in the plasmid, but in merodiploids with the mutation at the chromosome, a resistant population prevailed. These data suggest that *RKA1* is essential in *Y. lipolytica*. cAMP addition inhibited the dimorphic transition of the parental strain, but merodiploids carrying several copies of *RKA1* were more resistant to cAMP. These results, and the observation that *RKA1* was upregulated in mycelial cells, indicate that an active PKA pathway promotes yeast-like growth and opposes mycelial development. This behavior is in contrast to that of *Candida albicans*, where the PKA pathway favors hyphal growth.

## Introduction

*Yarrowia lipolytica* is a dimorphic fungus that has received increasing attention because of its biotechnological applications, e.g. degradation of hydrocarbons, oils and fatty acids, organic acid accumulation, and secretion of heterologous proteins (Barth & Gaillardin, 1996; Fickers *et al.*, 2005). This yeast is also an interesting model for the analysis of fungal dimorphism (Rodríguez & Domínguez, 1984; Guevara-Olvera *et al.*, 1993; Domínguez *et al.*, 2000).

This organism grows under natural conditions as a mixture of budding yeast and mycelium, but in the laboratory, different effectors favor the preferential development of either morphology. These include pH of the medium, carbon and nitrogen sources, and some specific factors such as citrate or serum (Rodríguez & Domínguez, 1984; Kim *et al.*, 2000; Ruiz-Herrera & Sentandreu, 2002; Szabo & Stofaníková, 2002).

It is known that environmental stimuli relay information that affects differentiation processes of the cell via signaling pathways. In most dimorphic fungi, mitogen-activated protein kinases (MAPK) and the cAMP-dependent protein kinase A (PKA) pathways are involved in the regulation of

the dimorphic transition (reviewed by Lengeler *et al.*, 2000; Lee *et al.*, 2003). Recently, we reported that in *Y. lipolytica*, a Ste11-dependent MAPK pathway plays a central role in the yeast-to-mycelium transition (Cervantes-Chávez & Ruiz Herrera, 2006). We have now analyzed the possible role of the PKA pathway in the phenomenon, taking into consideration that the second messenger cAMP is an important mediator of environmental stimuli. In eukaryotes, the cAMP-activated PKA is a ubiquitous signaling molecule and the main target of cAMP (reviewed by Johnson *et al.*, 2001; Taylor *et al.*, 2004, 2005). When inactive, the PKA heterotetramer is composed of two catalytic subunits attached to a dimer of regulatory PKA (rPKA) subunits (Taylor *et al.*, 2004, 2005). When the cAMP concentration is increased either by adenylate cyclase activation or by entry of exogenous nucleotide into the cell, two molecules of this compound bind to each rPKA subunit, releasing the catalytic subunits from the heterotetramer; these are now able to phosphorylate their target proteins on serine or threonine residues (Johnson *et al.*, 2001; Taylor *et al.*, 2004, 2005).

In the present study, we isolated the *RKA1* gene encoding the rPKA from *Y. lipolytica*. This gene was targeted on the basis of the previous observation that addition of cAMP

inhibited dimorphism of *Y. lipolytica* (Ruiz-Herrera & Sentandreu, 2002). *RKA1* appears to be an essential gene, and activation of the cAMP pathway triggers yeast growth in this fungus.

## Materials and methods

### Strains and culture conditions

The *Y. lipolytica* strains used in this work and their relevant characteristics are shown in Table 1. *Escherichia coli* strain Top10 was used routinely for plasmid propagation.

*Yarrowia lipolytica* strains were maintained at  $-70^{\circ}\text{C}$  in 50% (v/v) glycerol. When required, they were transferred to liquid or solid YPD medium (1% yeast extract, 2% peptone, 2% glucose, plus 2% agar when required) or YNB medium [0.67% YNB without amino acids/ammonium sulfate (Difco), 1% glucose, 0.5% ammonium sulfate, supplemented with the necessary requirements]. Carbon sources (glucose, galactose, glycerol or potassium acetate) were added at a final concentration of 2%. *Yarrowia lipolytica* was incubated routinely at  $28^{\circ}\text{C}$  for variable periods of time. Whenever required, YNB medium was supplemented with uracil ( $22.4\text{ mg L}^{-1}$ ) or leucine ( $262\text{ mg L}^{-1}$ ). Selection against the *URA3* gene marker was conducted on YNB plates supplemented with  $1\text{ g L}^{-1}$  5-fluoroorotic acid (Sigma-Aldrich). When necessary, cAMP sodium salt (Sigma-Aldrich) was dissolved in water, sterilized by filtration, and added to the sterile media at different concentrations. Growth rate was monitored by measuring the OD of the cultures at 600 nm in a Pharmacia LKB ultrospec III. *Escherichia coli* was grown at  $37^{\circ}\text{C}$  in Luria–Bertani medium (1% tryptone, 0.5% yeast extract and 0.5% sodium chloride) with ampicillin ( $100\text{ }\mu\text{g mL}^{-1}$ ) or kanamycin ( $50\text{ }\mu\text{g mL}^{-1}$ ) for plasmid selection.

### Genetic transformation

Genetic transformation of *Y. lipolytica* was carried out by the lithium acetate method (Barth & Gaillardin, 1996).

**Table 1.** Strains of *Yarrowia lipolytica* used in this work

Name	Relevant genotype	Reference
P01A	MatA, <i>leu 2-270</i> , <i>ura 3-52</i>	INRA*
Ar1	MatA, <i>ura 3-52</i> , pMD2	This work
Ar20	MatA, <i>ura 3-52</i> , pMD2	This work
MDR14	MatA, <i>RKA1</i> , pMD2 $\Delta$ <i>rka1</i>	This work
MDR39	MatA, <i>RKA1</i> , pMD2 $\Delta$ <i>rka1</i>	This work
MDR41	MatA, <i>RKA1</i> , pMD2 $\Delta$ <i>rka1</i>	This work
MDR48	MatA, $\Delta$ <i>rka1</i> , pMD2	This work
MDR49	MatA, <i>RKA1</i> , pMD2, pMD2 $\Delta$ <i>rka1</i>	This work
MDR95	MatA, <i>RKA1</i> , pMD2, pMD2 $\Delta$ <i>rka1</i>	This work
MDR48A–MDR48L	MatA, $\Delta$ <i>rka1</i> , pMD2	This work

\*Kindly made available by C. Gaillardin.

*Escherichia coli* transformation was performed by standard procedures (Sambrook & Russell, 1999).

### Induction of the dimorphic transition

Dimorphic transition of *Y. lipolytica* was induced basically as reported previously by Ruiz-Herrera & Sentandreu (2002). The media composition is described for each experiment. 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.

### Merodiploid construction

The parental *Y. lipolytica* strain P01A (*leu2*, *ura3*, see Table 1) was transformed with the autoreplicative plasmid pMD2 (see below) harboring the wild-type *RKA1* gene and the *LEU2* gene marker. Merodiploid strains were recovered on plates of YNB plus uracil. Afterwards, two merodiploids (Ar1 and Ar20) were selected for mutagenesis by transformation with the disruption cassette (DCD, see below), and mutation was confirmed by PCR and Southern blot.

### Plasmid curing

Elimination of pMD2 plasmid from the mutagenized merodiploid was carried out by subculturing each of the strains at  $28^{\circ}\text{C}$  for 48 h on plates of both solid YPD and YNB media without supplements. These media are, respectively, non-selective or selective for both auxotrophic marker genes (*LEU2* and *URA3*).

The procedure involved the selection of 12 colonies from each mutagenized merodiploid, which were streaked on plates of selective and nonselective media, and incubated at  $28^{\circ}\text{C}$ . After 48 h, a loopful of cells was taken from YPD plates and transferred to fresh plates. Subculture steps were conducted 25 times, or until strains failed to grow on selective medium. Plasmid elimination experiments were also conducted in media with osmotic stabilizer (0.5 or 1 M sorbitol) and with different carbon sources: glucose, galactose, glycerol, or potassium acetate.

### Techniques for nucleic acid manipulation

Genomic DNA from *Y. lipolytica* was isolated by the glass bead lysis method, as described by Hoffman & Winston (1987). Isolation of yeast plasmids for *E. coli* transformation was conducted as described by Barth & Gaillardin (1996). Plasmid DNA isolation from *E. coli* was performed by standard procedures (Sambrook & Russell, 1999). DNA enzymatic reactions such as digestion, ligation and vector dephosphorylation 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). Total RNA was isolated according to Jones *et al.* (1985). Southern and Northern blots were performed by standard techniques (Sambrook & Russell, 1999). DNA probes were labeled with [<sup>32</sup>P]dCTP, using the random primer labeling system (Amersham Biosciences). Hybridization of rRNA with a 5.8-kb fragment of the human 28S rRNA gene was used as a loading control.

### PCR conditions

Routine PCR reactions were conducted using Taq DNA polymerase (Invitrogen), following the 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 a primer-specific temperature for 60 s (Table 2), and polymerization at 72 °C (1 min kb<sup>-1</sup> of DNA target length). When required, the expanded high-fidelity PCR system (Boehringer Mannheim) was used according to the manufacturer's recommendations. An extension period of 7 min at 72 °C was programmed for those PCR products that were cloned into pCR2.1 TOPO (Invitrogen).

### DNA sequencing

DNA sequencing was performed with an ABI PRISM 377 DNA automated sequencer (Perkin Elmer). Double-stranded DNA was used as a template. Universal primers, reverse primers and some specific primers were used (Table 2). The sequence of *RKA1* was submitted to EMBL under the accession number AJ579715.2.

### Plasmid construction

Plasmid pRKA1 contained an *RKA1* gene fragment obtained by nested-PCR amplification using degenerate primers 2572, 2573 and 2574 (Table 2), and genomic DNA from

strain P01A. Plasmids p521 and p55 isolated from a sub-genomic library constructed into pBKS<sup>+</sup> with KpnI harbor a *c.* 3.8-kb truncated *RKA1* gene. Plasmid pRKA2 is a pCR2.1 TOPO harboring the full *RKA1* gene amplified in a PCR reaction with primers 1009 and 1008 (Table 2) using genomic DNA from strain P01A.

Plasmid pDC4 harbors an *RKA1* disruption cassette by double crossover. It was constructed as follows. The *RKA1* 3'-flanking fragment (*c.* 0.7 kb) was PCR-amplified from plasmid pRKA2 using primers 1010 (containing a BamHI sequence) and 1008 (Table 2), and cloned into pCR2.1 TOPO, yielding plasmid pRKAT1. The 3'-flanking region was then subcloned as a BamHI–EcoRI fragment into pCR2.1 TOPO, giving rise to plasmid pDC1. Next, it was subcloned as a BamHI–NotI fragment into the pBKS<sup>+</sup> vector, giving plasmid pDC2. The *RKA1* 5'-flanking region (*c.* 0.8 kb) was PCR-amplified using primers 1009 and 1011 (with a BamHI sequence; Table 2) and cloned into pCR2.1 TOPO, yielding plasmid pRKAP1. Next, it was digested with KpnI and XhoI, and subcloned into these sites of pDC2, yielding plasmid pDC3. Finally, the *Y. lipolytica* *URA3* gene was subcloned from plasmid pINA444 (Barth & Gaillardin, 1996) as a *c.* 1.7-kb HinCII fragment into plasmid pDC3, thus creating the pDC4 plasmid.

Plasmid pDPP2, used for attempting the disruption of the wild-type *RKA1* gene by the Pop-in Pop-out technique (Böeke *et al.*, 1987), was constructed as follows. The *RKA1* 5'-flanking region was PCR-amplified with primers 2822 and 1011 (with a BamHI sequence; Table 2), using genomic DNA from the P01A strain. The *c.* 1.2-kb PCR product was cloned into pCR2.1 TOPO, resulting in plasmid pDPP1. Next, the 5'-flanking region was digested from this plasmid with EcoRI and BamHI, whereas the 3'-flanking region was digested from plasmid pRKAT1 with the same enzymes. Digested fragments were ligated at the BamHI site, and the resulting *c.* 1.87-kb EcoRI fragment was introduced into the EcoRI site of pUCURA3 (pUC18 vector, which harbors the *Y. lipolytica* *URA3* gene) to generate plasmid pDPP2.

Plasmid pMD2, used to construct the *RKA1* merodiploid strains, was prepared as follows. A BamHI–SacI (about 3.1 kb) fragment containing the 5'-region of the *RKA1* gene was rescued from plasmid p521. The 3'-end of the gene was excised from plasmid pRKA2 as a SacI–BamHI fragment (*c.* 1.1 kb). Both fragments were ligated at the SacI site, and the resulting BamHI fragment (about 4.2 kb) was subcloned into the BamHI site of pCR2.1 TOPO to produce plasmid pMD1. Next, the *RKA1* gene was recovered by digestion with BamHI and cloned at this site into the autoreplicative vector pINA240 (which harbors the *LEU2* gene; Barth & Gaillardin, 1996) to generate plasmid pMD2. All constructions used in this work were verified by their restriction patterns and sequencing.

**Table 2.** Primers used in this work

Name	Sense	Sequence 5'–3'	<i>T<sub>m</sub></i> (°C)
2572	Forward	GGIGAITWYTTYTAYRTIGTIGAG	46
1009	Forward	CTCTCTCAGGTCCAGGGTTAGGGTTGC	70
2822	Forward	CTCGCTCTCCAACTATGTCCATCGTC	71
1010	Forward	GCGCGGATCCAAGGAAAATGACCCAC	70
1107	Forward	GGATGGCCCCGAGGTTGTCAAGCAG	68
2761	Forward	ACCCTAACCTGTTC	50
2573	Reverse	DGCIARYTCICCRARWAITYDCC	46
2574	Reverse	RGCIGCISYIGNCGGTRTRCAT	46
1008	Reverse	GTTACCCCAAGACATTCCTTACCCAC	70
1011	Reverse	CGCGGGATCCAGAATAGCTTGCGTCCAG	70
2821	Reverse	TTCTCGTTTGGCCAGCCGTTTCGTTG	71
2763	Reverse	GCCATGAGATAAACGTC	55
1109	Reverse	GTAGGAGGGCATTITGGTGGTGAAGAG	70

## Results

### Isolation of *RKA1*, the gene encoding the PKA regulatory subunit of *Y. lipolytica*

Degenerate primers were designed from the sequences of fungal genes encoding rPKA. To this end, a search for rPKA proteins in databases was conducted. Alignment from *Blumeria graminis*, *Neurospora crassa*, *Cryptococcus neoformans*, *Trichoderma atroviridae* and *Ustilago maydis* rPKA proteins was done using the MEGALIGN program. Three degenerate primers, 2572, 2573 and 2574 (Table 2), were designed according to the regions where the proteins showed the most conserved sequences. When primers 2572 and 2573 (Table 2) were used for a PCR reaction, the products ran as a smear in gel electrophoresis. Therefore, the reaction products were used for a nested-PCR reaction with primers 2572 and 2574 (Table 2), amplifying a fragment about 144 bp in length (data not shown). Sequence analysis of this revealed an ORF of 48 amino acid residues, which shared high similarity with the rPKA proteins from *Saccharomyces cerevisiae* (68%), *Kluyveromyces lactis* (65%), *Candida glabrata* (65%), *Ca. albicans* (53%), *Aspergillus nidulans* (47%), *Asp. fumigatus* (47%) and *B. graminis* (45%). This similarity suggested that it corresponded to a fragment of the *RKA1* gene from *Y. lipolytica*.

In order to clone the full-length gene, we proceeded to construct a subgenomic library. Southern hybridization revealed KpnI as the most suitable restriction enzyme with which to construct this subgenomic library, based on the fact that it gave only one hybridization signal of about 3.8 kb in size. Taking into account that fungal genes encode rPKA proteins about 400 amino acids in length (*N. crassa*, 385 amino acids; *Asp. nidulans*, 412 amino acids; *K. lactis*, 466 amino acids, *Colletotricum lagenarium*, 391 amino acids), we assumed that the full gene might be contained in this fragment. Accordingly, genomic DNA was digested with KpnI, and after electrophoresis, fragments of c. 3.8 kb were recovered from the agarose gel and ligated into pBKS<sup>+</sup> vector. In total, 3500 colonies were screened by hybridization with the PCR fragment as a probe (144 bp), labeled with [<sup>32</sup>P]dCTP (see 'Materials and methods'). Plasmid DNA was recovered from the four positive colonies identified (numbers 5, 55, 68 and 521). Restriction analysis revealed a KpnI fragment of the expected size (3.8 kb). Because all of them showed the same restriction pattern, plasmids p55 and p521 from colonies 55 and 521 respectively were selected for further analysis. Two PstI fragments (603 and 2294 bp) and one ClaI–KpnI fragment (2097 bp in length) were subcloned. All of them were sequenced with universal and reverse primers. Sequence-specific primers 2761 and 2763 were designed to rule out ambiguities in an internal portion of the ClaI–KpnI fragment. Sequence analysis

revealed a truncated *RKA1* gene lacking the 3'-end of the gene.

Subsequently, the whole gene was cloned using information from the ongoing *Y. lipolytica* genome sequencing project (<http://cbi.labri.fr/Genolevures/>). To this end, a BLAST search was conducted using as a query the previously cloned truncated ORF. A match YALIOFO4422G located in chromosome F was found. Primers 1009 and 1008 were designed over this sequence. In a PCR reaction with genomic DNA from strain P01A, we amplified and cloned a PCR product of about 2.5 kb. The sequence revealed an ORF of 1191 bp with no canonical introns, which encodes a protein composed of 397 amino acids with a theoretical molecular mass of 42.4 kDa. In the 5'-regulatory sequence, a putative TATA box was located at –385 bp. In the 3'-regulatory region, neither a polyA signal nor the tripartite termination TAG...TA(T)GT...TTT was found.

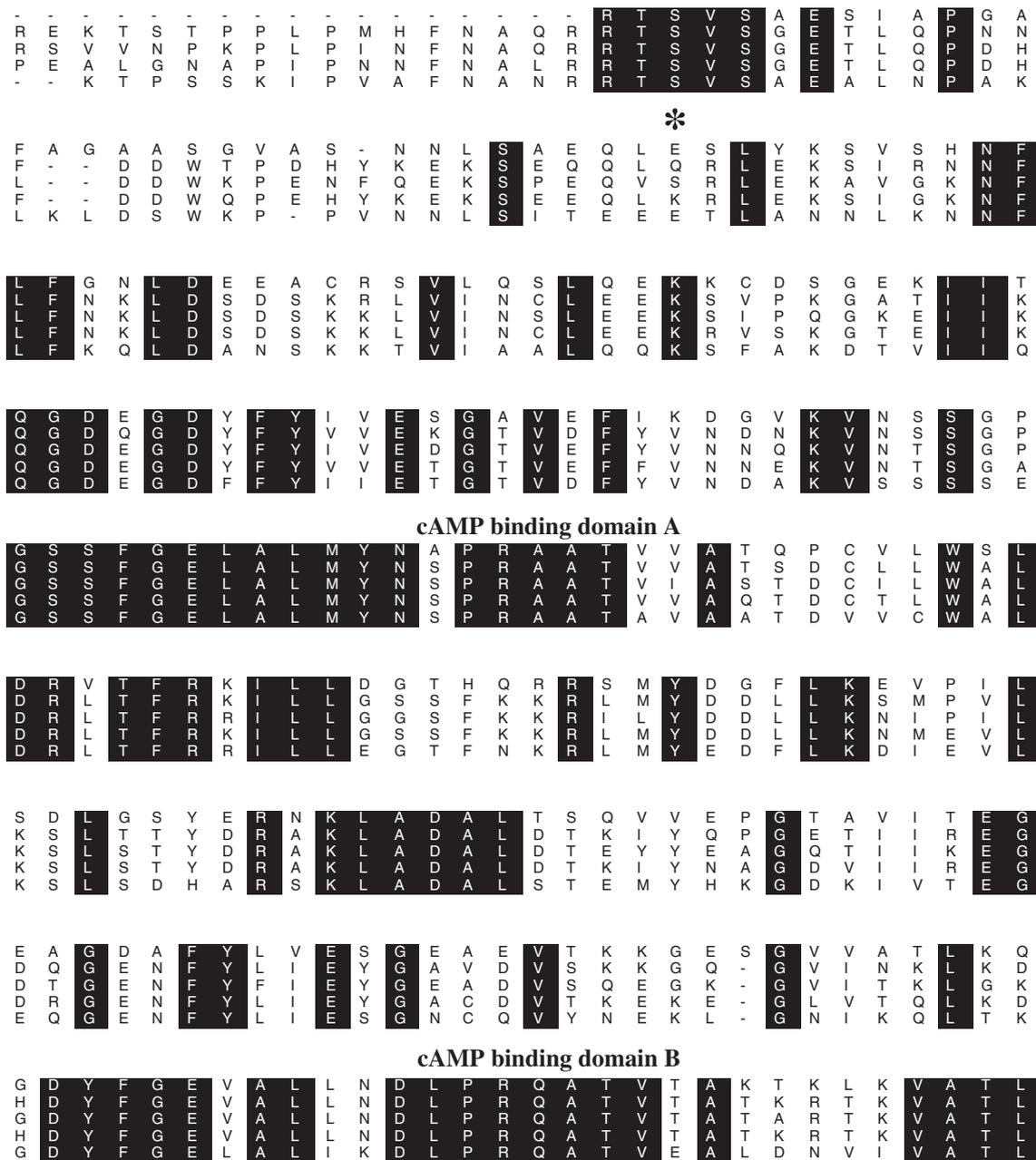
According to Southern hybridization data, *RKA1* is a single-copy gene. Later, this result was corroborated when all of the sequencing project data for the *Y. lipolytica* genome became accessible (<http://cbi.labri.fr/Genolevures/>). The *RKA1* gene shared high similarity with the rPKA proteins from *Ashbya gossypii* (59%), *Sa. cerevisiae* (56%), *K. lactis* (57%), *Ca. glabrata* (49%) and *Ca. albicans* (49%) (Fig. 1).

Comparison of the protein encoded by *Y. lipolytica* *RKA1* in databases (see also Fig. 1) indicates that it has the relevant characteristics of fungal rPKA proteins according to Canaves & Taylor (2002). It has the autoinhibition domain, the serine residue that is susceptible to phosphorylation at position 125 within the consensus RRTSVSAES, and the cAMP-binding domains A and B located at the C-terminus (Fig. 1). The A domain is well conserved except for an A residue instead of the common S residue, whereas the B domain shows some degree of divergence from the consensus. As occurs in *Sa. cerevisiae* (AY558087.1), *K. lactis* (Q6PCK7), *Ca. glabrata* (CR380955.1) and *Ca. albicans* (AF317472.1), the B domain contains a Q residue (Fig. 1) instead of A. The importance of this amino acid for the determination of nucleotide specificity (cAMP or cGMP) has been demonstrated (Canaves & Taylor, 2002).

### Attempts to disrupt the *RKA1* gene

#### By double crossover

Strain P01A was transformed with the disruption cassette harbored by plasmid pDC4, previously amplified by PCR using primers 1008 and 1009 (Table 2). Several *Ura*<sup>+</sup> transformants were recovered on plates of leucine-containing YNB media. PCR was used to identify gene disruption mutants using primers 2822 and 1008 (Table 2). A fragment



Yl  
Sc  
Kl  
Cg  
Ca

**Fig. 1.** Sequence alignment of the most conserved region of the predicted product of the *Yarrowia lipolytica* *RKA1* gene (Yl) with the homologous proteins from *Saccharomyces cerevisiae* (Sc; AY558087.1), *Kluyveromyces lactis* (Kl; Q6PCK7), *Candida glabrata* (Cg; CR380955.1) and *Candida albicans* (Ca; AF317472.1). Dashes indicate gaps in the alignment, and identical residues are shown in boxes. The serine residue susceptible to phosphorylation is indicated by an asterisk. The A and B cAMP-binding domains located at the C-terminus are shown. The Q residue in the B cAMP-binding domain involved in the specificity of the binding nucleotide is indicated by a dot.

c. 3.5 kb should be amplified in null mutant strains ( $\Delta rka1$ ), whereas a fragment c. 3.0 kb was expected for the wild-type gene. Three independent transformation events were carried out, and 400 transformants were analyzed by PCR. In all

cases, only the band corresponding to the wild-type gene was amplified (data not shown). These results suggested that all *Ura*<sup>+</sup> transformants were obtained by nonhomologous integration of the disruption cassette.

### By the Pop-in Pop-out technique

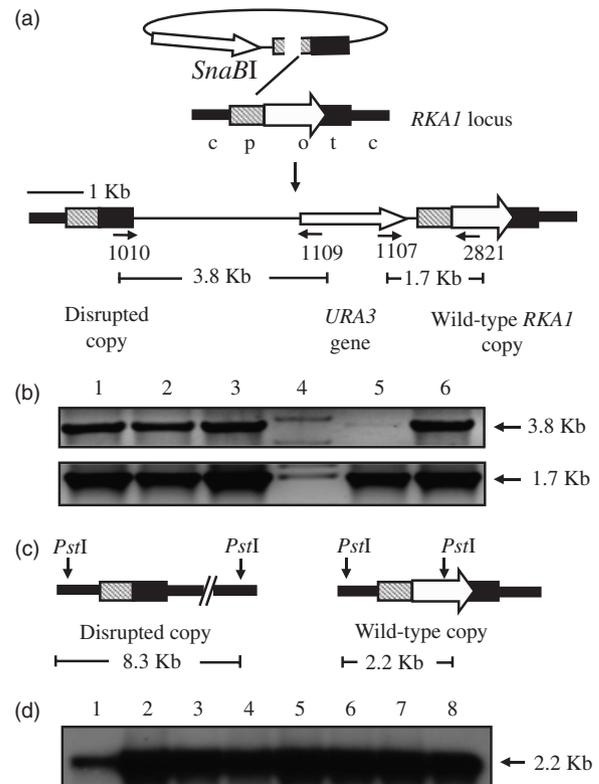
The negative results obtained after several attempts to disrupt the *RKA1* gene by double crossover prompted us to use the Pop-in Pop-out technique as an alternative strategy (Böeke *et al.*, 1987). To this aim, the P01A strain was transformed using plasmid pDPP2 previously linearized with *Sna*BI (see 'Materials and methods'). Several *Ura*<sup>+</sup> transformants were recovered, and homologous integration of the pDPP2 plasmid was confirmed by PCR using primers pairs 1010/1109 and 1107/2821 (Table 2), which should amplify bands of about 3.8 or 1.7 kb respectively (Fig. 2a). Several transformants with the correct amplification pattern were identified (Fig. 2b). Two strains were selected to conduct a counterselection against the *URA3* gene on YNB/5-fluoroorotic acid media (see 'Materials and methods'). Several fluoroorotic acid-resistant strains were recovered, and the *RKA1* gene copy (wild-type or disrupted) harbored by them was identified by PCR. With the use of primers 1008 and 1009 (Table 2), amplification of bands *c.* 1.3 or 2.5 kb would reveal the disrupted or wild-type genes respectively. Even though the transformation event and fluoroorotic acid selection were repeated, and more than 300 colonies were analyzed by PCR, we only identified cells containing the wild-type *RKA1* gene (data not shown). Additionally, several putative transformants were checked by Southern hybridization, and only the signal corresponding to the wild-type gene was obtained (Fig. 2c and d).

The same negative results were obtained when fluoroorotic acid selection was conducted in media supplemented with 0.5 or 1 M sorbitol as an osmotic stabilizer (not shown). When fluoroorotic acid plates were incubated for a longer time (7 days), we observed the appearance of smaller colonies, but all of them proved to bear the wild-type gene when they were analyzed by PCR (data not shown).

Considering the negative results when we attempted to obtain a  $\Delta rka1$  mutant by double homologous recombination, and taking into consideration that, with use of the Pop-in Pop-out technique, an average of *c.* 50% of the fluoroorotic acid-resistant population should correspond to mutants, we entertained the possibility that the function of *RKA1* is essential in *Y. lipolytica*.

### Analysis of the essential functionality of the *RKA1* gene in *Y. lipolytica*

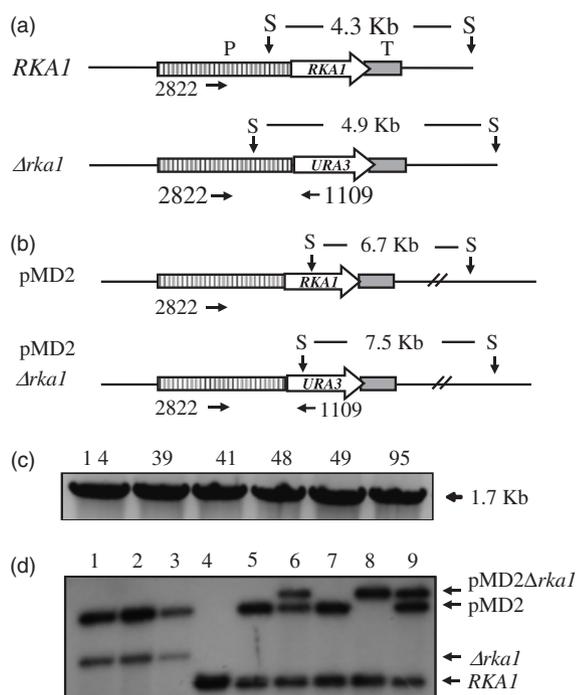
To test our hypothesis about the essential role of this gene, *RKA1* merodiploid strains were constructed (see 'Materials and methods'). Merodiploid strains Ar1 and Ar20 were selected to try to disrupt the *RKA1* gene. After they were transformed using the disruption cassette by double crossover (see 'Materials and methods'), we recovered 139 transformants on YNB medium, demonstrating that they contained both genetic markers (*LEU2* and *URA3*). In order



**Fig. 2.** *RKA1* gene disruption by the Pop-in Pop-out procedure. (a) Schematic representation of strain P01A transformation with plasmid pDPP2 previously linearized with *Sna*BI. c, chromosome; p, 5'-flanking region; o, *RKA1* ORF; t, 3'-flanking region. (b) Homologous integration (Pop-in) demonstrated by the presence of two amplified fragments by PCR in *Ura*<sup>+</sup> transformants (lanes 1–3, 5 and 6). Lane 4: size markers ( $\lambda$  PstI). (c). Schematic representation of the two expected *RKA1* alleles after the Pop-out event. (d) Southern hybridization from some *Ura*<sup>+</sup> transformants showing only the presence of the wild-type fragment (lanes 1–7). Lane 8: parental strain. Genomic DNA was digested with *Pst*I and hybridized with the 3'-flanking region sequence of the *RKA1* gene.

to identify those transformants in which the disruption cassette had been integrated at the homologous locus, they were PCR-tested using primers 2822 and 1109 (Table 2). The expected band around 1.7 kb was successfully amplified in only six transformants named MDRs (numbers 14, 39, 41, 48, 49 and 95; Fig. 3c).

Taking into account that chromosomal and extrachromosomal *RKA1* genes are alike, it was expected that the disruption cassette could be targeted to either of them. Therefore, the presence of either copy (wild type or disrupted) of the *RKA1* gene was analyzed by Southern hybridization. To this end, DNA was isolated from these strains, digested with *Sac*I, and probed with the 3'-flanking region of the gene. Hybridization signals around 4.3 kb (*RKA1*) or 4.9 kb ( $\Delta rka1$ ) for the chromosomal gene should be obtained, whereas for the extrachromosomal gene,



**Fig. 3.** Scheme of *RKA1* gene disruption by double crossover in merodiploid strains. (a) Schematic representation of the disruption cassette integration in the chromosome. *RKA1*, wild-type chromosome gene;  $\Delta rka1$ , mutagenized chromosome gene. (b) Schematic representation of the disruption cassette integration in the plasmids; pMD2, scheme of wild-type plasmid; pMD2  $\Delta rka1$ , scheme of mutagenized plasmid. P and T, 5'- and 3'-flanking regions, respectively; 2822 and 1109, primers used to confirm homologous integration. (c) Confirmation of homologous integration of disruption cassette in the indicated strains. (d) Determination by Southern blot of the site of integration (chromosome or plasmid) of the disruption cassette. Lanes 1–3: strains in which integration occurred in the chromosome. Lane 4: parental P01A strain. Lanes 5 and 7: control merodiploids (Ar20 and Ar1 respectively). Lanes 6 and 9: strains carrying both wild-type and disrupted plasmids. Lane 8: strain carrying only mutated plasmids. DNA was digested with *Sac*I (S); the 3'-flanking region of the *RKA1* gene was used as a probe.

signals around 6.7 kb pMD2 (*RKA1*) or 7.5 kb pMD2 $\Delta rka1$  were expected (Fig. 3a and b). The hybridization data obtained identified the MDR48 strain as the only one whose chromosome *RKA1* gene had been disrupted (Fig. 3d, lanes 1–3).

Resident plasmids of the MDR strains were recovered by transformation into *E. coli*, and their identity was determined by restriction analysis with *Sac*I. The restriction pattern revealed that plasmids obtained from MDR strains 14, 39 and 41 harbored the mutagenized  $\Delta rka1$  allele (pMD2 $\Delta rka1$ ); in contrast, all plasmids from strain MDR48 were wild type (pMD2). MDR strains 49 and 95 contained both wild-type and mutagenized plasmids (data not shown). These results agreed with the hybridization pattern obtained (representative data are shown in Fig. 3d).

Next, colonies from MDR strains were subjected to a plasmid curing process. Theoretically, if *RKA1* were an essential gene, the strain with the chromosomal gene disrupted by the *URA3* gene should survive only if it conserved the wild-type gene present in plasmid pMD2 (*LEU2* gene marker). To investigate this possibility, we recovered 12 isolated colonies from each MDR strain, including the merodiploid Ar20 as a control (see 'Materials and methods'). In the course of subculturing in complete medium, colonies from MDR strains 14, 39, 41, 49 and 95, as well as the control Ar20, became unable to grow on selective YNB medium, revealing in this way the elimination of either plasmid pMD2 or pMD2 $\Delta rka1$ . The rate of plasmid elimination was specific for each strain tested, the average being the sixth subculture, at which none of the original 12 colonies (see above) survived on the selective medium. The exceptions were those colonies coming from strain MDR48 (MDR48A–MDR48L), which did not lose the capacity to grow on selective YNB medium (without requirements) even after 25 subculture steps, indicating that they still possessed the pMD2 plasmid.

Attempts to cure the plasmids in clones from the MDR48 strain were also made in media supplemented with 1 M sorbitol and different carbon sources (glucose, galactose, potassium acetate or glycerol). Even after 25 subculture steps (10 in glucose media and 15 in the other carbon sources), the original 12 clones derived from the MDR48 strain did not lose the pMD2 plasmid, as evidenced by their ability to grow on selective YNB medium. These and the previous results provide evidence that the *RKA1* gene is essential in *Y. lipolytica*.

### Expression of the *RKA1* gene during the dimorphic transition

To determine whether this gene was regulated at the transcriptional level during the yeast-to-mycelium transition, we grew the parental P01A and merodiploid Ar20 strains under inducing conditions (YNB plus citrate, pH 7) or noninducing conditions (YNB plus citrate, pH 3). As expected, both strains grew in a yeast-like way under noninducing conditions, and as mycelia under inducing conditions (Fig. 4). Total RNA was isolated and analyzed by Northern blot. A modest increase in *RKA1* expression (1.1- and 1.3-fold at 8 and 12 h, respectively) was observed in samples from mycelial cells (pH 7; Fig. 5, lanes 1 and 2), as compared with samples from cells growing with yeast morphology (pH 3; Fig. 5, lanes 3 and 4). Nevertheless, a greater difference in mRNA levels (3.0- and 2.7-fold at 8 and 12 h, respectively) between these two conditions (mycelium or yeast) was observed in samples from the merodiploid strain (compare lanes 5 and 6 with lanes 7 and 8 in Fig. 5). This is probably because the replicative plasmid pMD2

carrying the *RKA1* gene is present in several copies in the cell, giving rise to gene overexpression.

### Influence of cAMP on cell morphology in *Y. lipolytica* wild-type and merodiploid strains

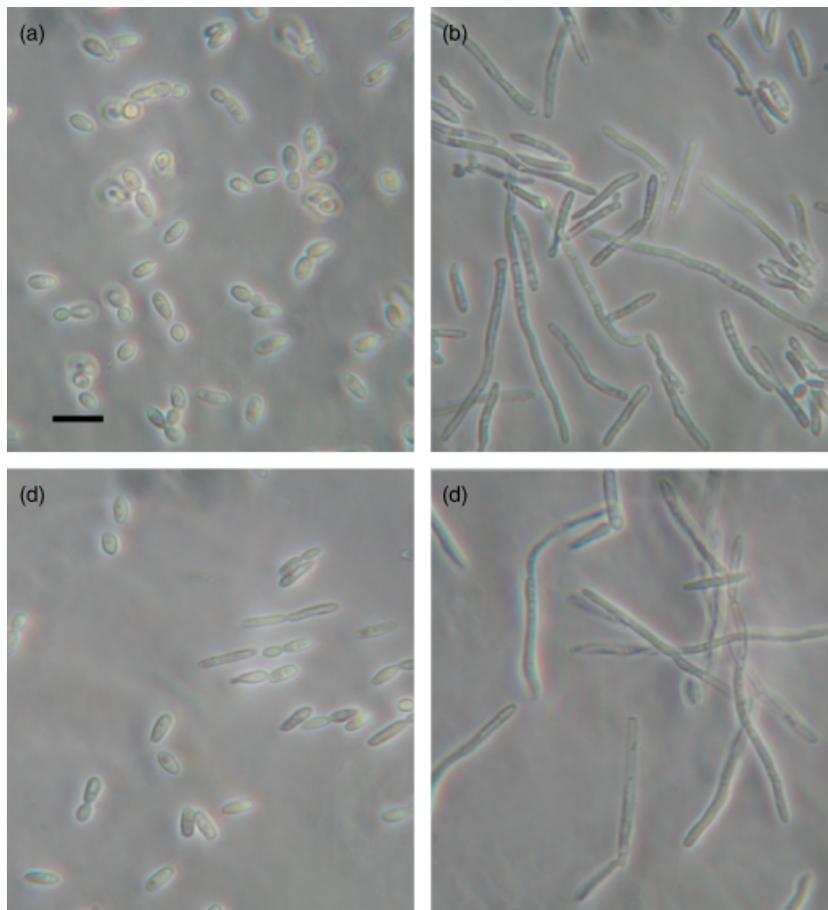
It was previously reported that the dimorphic transition of the *Y. lipolytica* wild-type strain W29 was inhibited by addition of cAMP to the induction media (Ruiz-Herrera & Sentandreu, 2002). Accordingly, we scored the effect of exogenous cAMP on the dimorphic transition for both the parental strain and two merodiploid strains. In the control cultures without cAMP addition, cells grew with the typical mycelial morphology, both in citrate–glucose and in citrate–glucose plus serum media of neutral pH. However, when cAMP was added to either medium, the higher the nucleotide concentration, the lower were the percentages of mycelial cells observed. When the behavior of the Ar1 and Ar20 strains was analyzed, we observed a refractory response to cAMP. For example, 50% of the population was still able to form mycelial cells at 25 mM cAMP, a concentration at which only 2% of the cells from the parental strain P01A were able to grow in the hyphal form (Fig. 6). Growth curves indicated that at the concentrations used, cAMP had no

influence on the growth rate of the *Y. lipolytica* strains (data not shown).

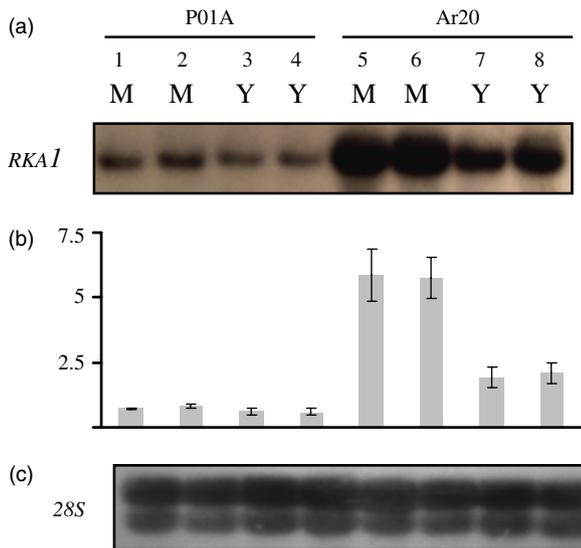
Expression of *RKA1* was measured by Northern blot analysis in this experiment, in the absence or presence of 20 mM cAMP. We observed that cAMP addition brought about an inhibition in the expression of the gene in the parental strain (4.6-fold; Fig. 7). No inhibition was observed in the Ar20 strain, probably because the overexpression of *RKA1* saturates the system.

### Discussion

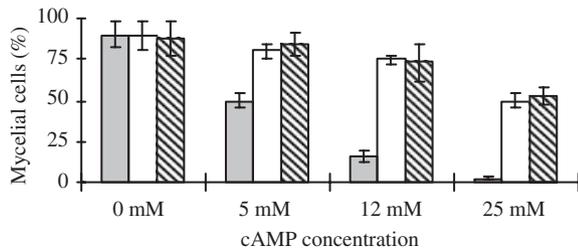
It is widely known that cAMP regulates different physiological processes in fungi including growth, metabolism, cell cycle progression, aging, stress resistance, and cell differentiation. Particularly in human pathogenic fungi such as *Ca. albicans*, *Asp. fumigatus* and *Cr. neoformans* (Hull & Heitman, 2002; Dhillon *et al.*, 2003; Liebmann *et al.*, 2004), and also in plant fungal pathogens such as *Magnaporthe grisea* and *U. maydis*, morphologic changes as well as developmental aspects related to pathogenicity are regulated by the cAMP-dependent PKA signal transduction pathway (Lee *et al.*, 2003).



**Fig. 4.** Photographs of *Yarrowia lipolytica* strains grown at 28 °C in YNB medium containing 0.5%  $(\text{NH}_4)_2\text{SO}_4$ , 1% glucose and requirements for 12 h. (a) P01A strain grown in medium containing citrate buffer, pH 3. (b) P01A strain grown in medium containing citrate buffer, pH 7. (c) Ar20 strain grown in medium containing citrate buffer, pH 3. (d) Ar20 strain grown in medium containing citrate buffer, pH 7. Magnification bar: 10  $\mu\text{m}$  for all photographs.



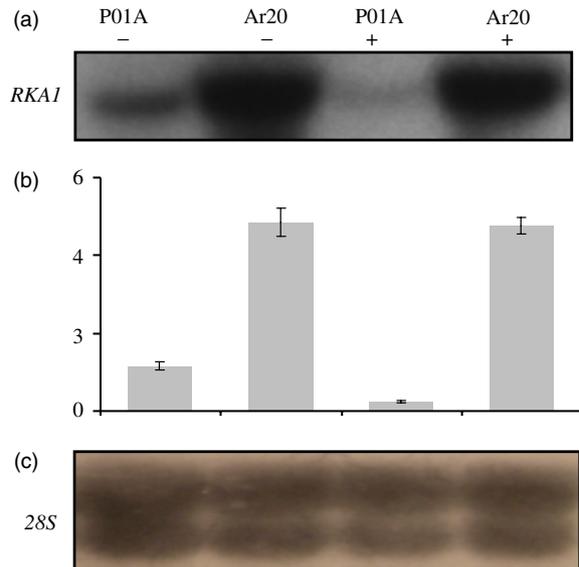
**Fig. 5.** Determination of *RKA1* transcript levels in the yeast and mycelial forms. Strains were grown as described for Fig. 4. Lanes 1, 2, 5 and 6: medium containing citrate buffer, pH 7. Lanes 3, 4, 7 and 8: medium containing citrate buffer, pH 3. Lanes 1, 3, 5 and 7: 8-h incubation. Lanes 2, 4, 6 and 8: 12-h incubation. (a) Northern analysis of the *RKA1* transcript. The 348-bp PstI–HindIII fragment corresponding to the *RKA1* ORF was used as a probe. (b) Densitometric analysis of the autoradiographic data of (a). The y-axis scale is shown in densitometric units. (c) Hybridization of the 28S gene used as a control. M, mycelium; Y, yeast.



**Fig. 6.** Effect of exogenous cAMP during the yeast-to-mycelium transition in parental and merodiploid strains. Parental strain P01A (gray bars) and merodiploid strains Ar1 and Ar20 (white and striped bars respectively) were grown as in Fig. 4 with citrate buffer (pH 7). The nucleotide was added at the indicated concentrations at the time of inoculation. Cell morphology was scored after 16 h of incubation. Results are averages of three experiments, each with duplicate samples.

In most organisms, PKA activity is regulated by the levels of cAMP, which affect binding of the regulatory and catalytic subunits (reviewed by Johnson *et al.*, 2001). Also important for PKA activity in higher eukaryotic organisms are PKA anchor proteins (AKAPs) that target the PKA holoenzyme to specific subcellular locations (Colledge & Scott, 1999).

In this study, we have analyzed the possible role of the PKA pathway in the dimorphic transition of *Y. lipolytica*. To this end, we isolated the gene encoding the PKA regulatory subunit, with the objective of isolating null mutants by



**Fig. 7.** Effect of cAMP on *RKA1* expression. Strains were incubated for 12 h as in Fig. 4 with citrate buffer (pH 7) with (+) or without (-) 20 mM cAMP. (a)–(c) as described for Fig. 5.

reverse genetics, and analyzing their phenotype. We found that the corresponding gene (*RKA1*) is present in a single copy according to Southern hybridization data and by *in silico* analysis conducted in the *Y. lipolytica* database. This agrees with data from *Sa. cerevisiae*, *U. maydis*, *N. crassa*, *M. grisea*, *Cr. neoformans*, *Ca. albicans* and *Schizosaccharomyces pombe* (Toda *et al.*, 1987; Gold *et al.*, 1994; Bruno *et al.*, 1996; Adachi & Hamer, 1998; D'Souza *et al.*, 2001; Cassola *et al.*, 2004; Stiefel *et al.*, 2004). Genes encoding the PKA regulatory subunit have been cloned from several fungi, but the corresponding mutants have been isolated from only a few of them. In *M. grisea* as well as in *Sc. pombe*, mutants in the regulatory subunit gene were obtained as suppressors of the adenylate cyclase mutant phenotype (*sum-99* and *cgs1-1*, *cgs1-10*, respectively). Suppressor *sum-99* from *M. grisea* harbors a point mutation in one cAMP-binding domain; therefore, its PKA activity was still partially enhanced by cAMP addition, suggesting that the *SUM1* gene was not completely inactivated (Adachi & Hamer, 1998). In *Sc. pombe*, suppressor *cgs1-10* conserves the wild-type coding sequence. Even though the identity of the mutation for the *cgs1-1* allele is not known, it resulted in a great loss of *cgs1*<sup>+</sup> function, and as a consequence of this, mutants showed a high PKA activity (Stiefel *et al.*, 2004). In contrast, deletion in the regulatory subunit gene (*ubc1*) in *U. maydis* was achieved both as a suppressor of an adenylate cyclase (*uac1*) mutant, and as an individual *ubc1* mutant (Gold *et al.*, 1994). Mutants in the PKA regulatory gene in *N. crassa* *mcb1* (conditional) and in *Asp. niger* 9ΔR (null mutant) resulted in loss of its growth polarity pattern. In

*N. crassa*, this kind of growth persists until cells rupture (Bruno *et al.*, 1996; Staudohar *et al.*, 2002). This phenotype was reverted by deletion of the gene encoding the catalytic subunit (Banno *et al.*, 2005).

In the present study, all of our attempts to disrupt the *RKA1* gene from *Y. lipolytica* in wild-type cells by double crossover, as well as by the Pop-in Pop-out procedure, were unsuccessful. All transformants obtained by the double crossover approach resulted from ectopic integration of the disruption cassette. This result was unexpected, given that, in *Y. lipolytica*, homolog recombination is easily conducted, even using flanking homolog fragments shorter than the ones we used (Barth & Gaillardin, 1996). Using the Pop-in Pop-out technique, which theoretically gives rise to an equal proportion of mutant and wild-type strains (Böeke *et al.*, 1987), we only recovered strains carrying the wild-type gene.

*Saccharomyces cerevisiae* and *Ca. albicans* mutants with upregulated PKA pathways have shown severe cell wall construction defects, as well as high sensitivity to stress conditions (Jones *et al.*, 2003; Jung & Stateva 2003; Jung *et al.*, 2005). We thus entertained the possibility that our failure to obtain  $\Delta rka1$  mutants might have been due to alterations in the cell wall rigidity, and prompted the use of hypertonic conditions to isolate the putative mutants. Nevertheless, even though several 5-fluoroorotic acid screens were conducted in a medium supplemented with an osmotic stabilizer (sorbitol), not a single  $\Delta rka1$  mutant was isolated.

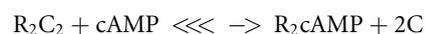
We also considered that a possible failure to obtain  $\Delta rka1$  mutants was related to the carbon source used during mutant selection, as *Sa. cerevisiae* *bcy1* mutants are not viable in nonfermentable carbon sources (Toda *et al.*, 1987), and the rate of growth of *Asp. fumigatus* was affected by cAMP addition only in the presence of nonfermentable carbon sources (Oliver *et al.*, 2001). Nevertheless, this possibility was dismissed when negative results were obtained in media supplemented with sorbitol as osmotic support, using fermentable or nonfermentable carbon sources. Collectively, these data are consistent with the results that would be expected if *RKA1* was an essential gene in *Y. lipolytica*. The essential nature of *RKA1* is probably due to an unregulated PKA activity that unleashes deleterious effects in the fungus.

In confirmation of the above results, mutants defective in *RKA1* could only be obtained by transformation of merodiploids derived from the parental strain that carried an autonomous replicating plasmid (pMD2) bearing the wild-type *RKA1* gene. Those merodiploids carrying the *RKA1* deleted gene at the chromosome or the plasmid were viable, but as expected, the population of the strains carrying the mutation in the plasmid that lost it during the transfers prevailed in the nonselective medium, because of their selective advantage. On the other hand, only the population

that maintained the plasmid carrying the wild-type *RKA1* gene survived in the selective medium.

Until now, only in *Ca. albicans* has it been reported that deletion of the gene encoding the regulatory subunit gene (*BCY1*) is lethal (Cassola *et al.*, 2004). In *Asp. niger* nine  $\Delta R$  mutants, PKA activity did not show any change with respect to the wild-type strain, suggesting the possible existence of another gene carrying out similar functions (Staudohar *et al.*, 2002). Recently, Zhao *et al.* (2006) reported that an *Asp. fumigatus* mutant  $\Delta pkaR$  showed reduced growth, as well as germination defects; these mutants were more sensitive to oxidative stress damage.

In *Ca. albicans*, both the PKA and MAPK pathways act together to regulate the dimorphic transition (Dhillon *et al.*, 2003). Although in *Y. lipolytica* and *Ca. albicans* the yeast-to-mycelium transition has some inducers in common (neutral pH and serum), it is clear that the PKA pathway exerts opposite roles on dimorphism in both ascomycetes, as evidenced by the observation that the yeast-to-mycelium transition in *Y. lipolytica* is inhibited by exogenous cAMP (Ruiz-Herrera & Sentandreu, 2002; this study). It is known that cAMP binds to the regulatory subunit, giving rise to the activation of the catalytic subunit. Moreover, according to our results, cAMP inhibits expression of *RKA1*, thus leading to a further activation of the catalytic subunit. In contrast, merodiploid strains containing several copies of the *RKA1* gene were more resistant to the effect of exogenous cAMP than was the wild-type strain. This phenomenon is probably due to the fact that the levels of the *RKA1* transcript were not affected by the concentration of cAMP used, and therefore the higher amounts of rPKA produced altered the equilibrium in the dissociation reaction towards the undissociated (inactive) form of the PKA holoenzyme according to the equation:



It is important to stress that our previous results (Cervantes-Chávez & Ruiz Herrera, 2006) demonstrated that the yeast-to-mycelium transition in *Y. lipolytica* requires a functional Ste11-dependent MAPK pathway. Accordingly, it is evident that the PKA and MAPK pathways involved in the yeast-to-mycelium transition in this fungus are antagonistic. This behavior does not agree with that of *Ca. albicans*, in which both pathways are required for mycelial growth (Dhillon *et al.*, 2003).

Even though the inducers of the yeast-to-mycelium transition are different in *Y. lipolytica* and *U. maydis* (Ruiz-Herrera *et al.*, 1995), the roles of both the MAPK (Cervantes-Chávez & Ruiz Herrera, 2006) and PKA (this study) pathways in dimorphism are similar. The first is essential for mycelium formation, whereas the second is involved in yeast-like growth. In this sense, it is worth recalling that a PKA pathway switched off by deletion in the adenylate

cyclase (*uac1*) or in the catalytic subunit (*adr1*) genes resulted in a constitutive filamentous phenotype in *U. maydis* (Gold *et al.*, 1994; Dürrenberger *et al.*, 1998), whereas exogenous cAMP addition, or mutation in the gene encoding the regulatory subunit gene (*ubc1*), restored budding growth in *uac1* mutants (Gold *et al.*, 1994). Additionally, the filamentation response of *U. maydis* to acidic pH, as in *Y. lipolytica*, requires a functional pheromone-responsive MAPK pathway (Martinez-Espinoza *et al.*, 2004). In contrast, *cac1* (adenylate cyclase) mutants in the basidiomycete *Cr. neoformans* maintain the yeast morphology, like the wild-type cells (Alspaugh *et al.*, 2002).

All these data constitute evidence that the organization of the transduction pathways that transfer the different signals affecting morphogenesis in fungi is an extremely plastic phenomenon.

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