

Disruption in *Candida albicans* of the *TPS2* gene encoding trehalose-6-phosphate phosphatase affects cell integrity and decreases infectivity

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The gene *CaTPS2* encoding trehalose-6-phosphate (T6P) phosphatase from *Candida albicans* has been cloned and disrupted in this organism. The *Catps2/Catps2* mutant did not accumulate trehalose but accumulated high levels of T6P. Disruption of the two copies of the *CaTPS2* gene did not abolish growth even at 42 °C, but decreased the growth rate. In the stationary phase, the *Catps2/Catps2* mutant aggregated, more than 50% of its cells became permeable to propidium iodide and a large amount of protein was found in the culture medium. Aggregation occurred only at pH values higher than 7 and was avoided by osmoprotectants; it was never observed during the exponential phase of growth. The mutant formed colonies with a smooth border on Spider medium. Mice inoculated with 1.5×10^6 c.f.u. of wild-type cells died after 8 days, while 80% of those inoculated with the same number of c.f.u. of the *Catps2/Catps2* mutant survived for at least 1 month. Reintroduction of the wild-type *CaTPS2* gene in the *Catps2/Catps2* mutant abolished the phenotypes described. It is hypothesized that the accumulation of T6P interferes with the assembly of a normal cell wall.

Keywords: cell wall, aggregation, stress, antifungals

INTRODUCTION

Infections by opportunistic pathogens are of increasing clinical importance, particularly as the population of immunocompromised patients increases. Systemic infections by the yeast *Candida albicans* rank among the leading causes of death by microbial infections (de Pauw & Meunier, 1999; Edwards, 1991; Verduyn Lunel *et al.*, 1999). Effective drugs are available for the treatment of candidiasis (Herbrecht *et al.*, 1999; Martin, 1999), but the increasing prevalence of resistant strains indicates an urgent need for new antifungal agents (Monk & Perlin, 1994). Since the infective agent and the host are eukaryotic organisms sharing many pathways, specific targets should be looked for among those activities

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Abbreviation: T6P, trehalose 6-phosphate.

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exclusive to the micro-organism. In this context, the pathway of synthesis of trehalose appears to be a promising candidate, since this disaccharide plays important roles in many micro-organisms (François *et al.*, 1997; Van Laere, 1989) and has not been found in mammals. Trehalose biosynthesis implicates the formation of trehalose 6-phosphate (T6P) catalysed by T6P synthase (EC 2.4.1.15) followed by hydrolysis of T6P by T6P phosphatase (EC 3.1.3.12) (Cabib & Leloir, 1958). Disruption of the gene encoding T6P phosphatase, named *TPS2* in *Saccharomyces cerevisiae* or *orlA* in *Aspergillus nidulans*, makes these organisms thermo-sensitive (Borgia *et al.*, 1996; De Virgilio *et al.*, 1993). We reasoned that if disruption of the corresponding gene in *C. albicans* produced a similar phenotype, inhibitors of the T6P phosphatase activity could be interesting drugs for the treatment of candidiasis in mammals. Following this idea, we have cloned the *TPS2* gene from *C. albicans* and studied the consequences of its disruption. We report in this article that disruption of *CaTPS2* leads to aggregation of the cells in stationary phase and greatly decreases infectivity of *C. albicans* in mice.

METHODS

Strains, growth conditions and molecular biology procedures. The strains used in this work are shown in Table 1. The yeasts were grown with shaking at 30, 37 or 42 °C in 1% yeast extract, 2% peptone (YP) or in a synthetic medium (YNB, Difco yeast nitrogen base) with the adequate auxotrophic requirements and either glucose, glycerol or ethanol at 2%. Hyphae formation was induced as described by Zaragoza *et al.* (1998). Filamentation was examined on Spider medium (Liu *et al.*, 1994) in plates of 9 cm diameter with 20 ml medium and 50–100 colonies per plate. When needed, media were buffered with 0.2 M MES, pH 5, or 0.2 M HEPES, pH 7.5.

Sac. cerevisiae was transformed as described by Gietz *et al.* (1992) and *C. albicans* either by electroporation (Kohler *et al.*, 1997) or using protoplasts (Kurtz *et al.*, 1986). Recombinant DNA manipulations were done as described by Sambrook *et al.* (1989) and DNA probes were labelled using the Pharmacia oligolabelling kit. Genomic DNA was obtained from cells broken with glass beads as described by Hoffman & Winston (1987).

DNA sequencing and sequence analyses. Sequences were obtained from double-stranded plasmid DNA by the use of the cycle sequencing method and the ABI PRISM 310 capillary sequencer (Perkin Elmer Applied Biosystems). Sequences were analysed by using standard programs.

Isolation of the *C. albicans* TPS2 gene. The temperature-sensitive *Sac. cerevisiae* *tps2* strain YSH 6.106-8C was transformed with a *C. albicans* genomic DNA library in YEp322 (a gift of C. Nombela and J. Pla, Madrid, Spain). About 10⁶ transformants were plated on glucose synthetic medium and incubated at 38.6 °C. Plasmids isolated from positive colonies were tested by PCR using two degenerate oligonucleotides: 5'-GATTACGATGG[CT][AC][CT][CT]T-[AGT]AC[CT]CC[AG]AT[CT]GT-3' and 5'-GGACGAAC-TTC[AG]A[GT][AG]TT[ACGT]GC[CT]TT[ACGT]CC-3', corresponding to highly conserved sequences from known

genes encoding T6P phosphatases. A PCR reaction using these primers and DNA from the genomic library mentioned above yielded a fragment of 0.45 kb with sequence similarity to T6P-phosphatase-encoding genes; the same fragment was obtained with DNA of the isolated plasmids. Plasmid YEp352-CaTPS2-1 carried a DNA segment of 3822 bp containing the entire *CaTPS2* gene, including 700 bp upstream of the putative initiation codon and 380 bp after the stop codon. The sequence of this fragment is available at the EMBL database under accession no. AJ242990.

Chromosomal disruption of the *CaTPS2* gene. The *CaTPS2* gene was disrupted with *CaURA3* and *CaHIS1* using two disruption cassettes (Fig. 1a). The one with *CaURA3* was constructed as follows: a 4 kb *Bam*HI–*Bgl*III fragment from pCUB6K1 (C. San-José, J. Pla & C. Nombela, unpublished), a derivative of pCUB6 (Fonzi & Irwin, 1993) containing the *hisG-CaURA3-hisG* blaster cassette (Fonzi & Irwin, 1993), was inserted into the unique *Bam*HI site from plasmid YEp352-TPS2-1 to give pOZ11-3. To construct the disruption cassette with *CaHIS1*, a 1.5 kb *Bam*HI–*Bam*HI fragment from p34HHIS1 (given by J. Pla) was ligated into the unique *Bam*HI site from plasmid YEp352-TPS2-1 to produce pOZ11-4. To interrupt the corresponding genomic loci in *C. albicans* the plasmids were digested with *Hind*III and the digestion products introduced into the yeast by electroporation. Introduction of the digestion products of pOZ11-3 into *C. albicans* strain RM1000 produced strain LOZ184, introduction of those of plasmid pOZ11-4 into strain LOZ184 gave strain LOZ200 and introduction of the same products into strain RM1000 provided strain LOZ183. Correct insertion of the disruption cassette was checked by PCR and Southern analysis (Fig. 1b). *C. albicans* LOZ250 was obtained by pop-out of the *URA3* gene from strain LOZ200 after selection on YNB glucose plates with 20 µg uridine ml⁻¹ and 0.5 mg 5-fluoroorotic acid ml⁻¹.

Reintroduction of the *CaTPS2* gene into *C. albicans*. To reintroduce the *CaTPS2* gene into a *C. albicans* *tps2/tps2*

Table 1. Yeast strains used in this work

Construction of *C. albicans* strains LOZ183, LOZ184, LOZ200, LOZ250 and LOZ252 is described in Methods.

Strain	Genotype	Reference
<i>Sac. cerevisiae</i> YSH 6.106-8C	MATa <i>ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 tps2::LEU2</i>	Reinders <i>et al.</i> (1997)
<i>Sac. cerevisiae</i> CJM291	MATa <i>leu2-1 ura3-52 lys2 hxx1::LEU2 hxx2::LEU2 glk1::LEU2/pDB20-YlcHXX1</i>	Petit & Gancedo (1999)
<i>C. albicans</i> SC5314	<i>URA3 HIS1 TPS2</i> <i>URA3 HIS1 TPS2</i>	Gillum <i>et al.</i> (1984)
<i>C. albicans</i> RM1000	<i>ura3::imm434 his1::hisG TPS2</i> <i>ura3::imm434 his1::hisG TPS2</i>	Negredo <i>et al.</i> (1997)
<i>C. albicans</i> LOZ183	<i>ura3::imm434 his1::hisG tps2::HIS1</i> <i>ura3::imm434 his1::hisG TPS2</i>	This work
<i>C. albicans</i> LOZ184	<i>ura3::imm434 his1::hisG tps2::hisG-CaURA3-hisG</i> <i>ura3::imm434 his1::hisG TPS2</i>	This work
<i>C. albicans</i> LOZ200	<i>ura3::imm434 his1::hisG tps2::hisG-CaURA3-hisG</i> <i>ura3::imm434 his1::hisG tps2::HIS1</i>	This work
<i>C. albicans</i> LOZ250	<i>ura3::imm434 his1::hisG tps2::hisG</i> <i>ura3::imm434 his1::hisG tps2::HIS1</i>	This work
<i>C. albicans</i> LOZ252	<i>ura3::imm434 his1::hisG tps2::hisG/pOZ11-6</i> <i>ura3::imm434 his1::hisG tps2::HIS1</i>	This work

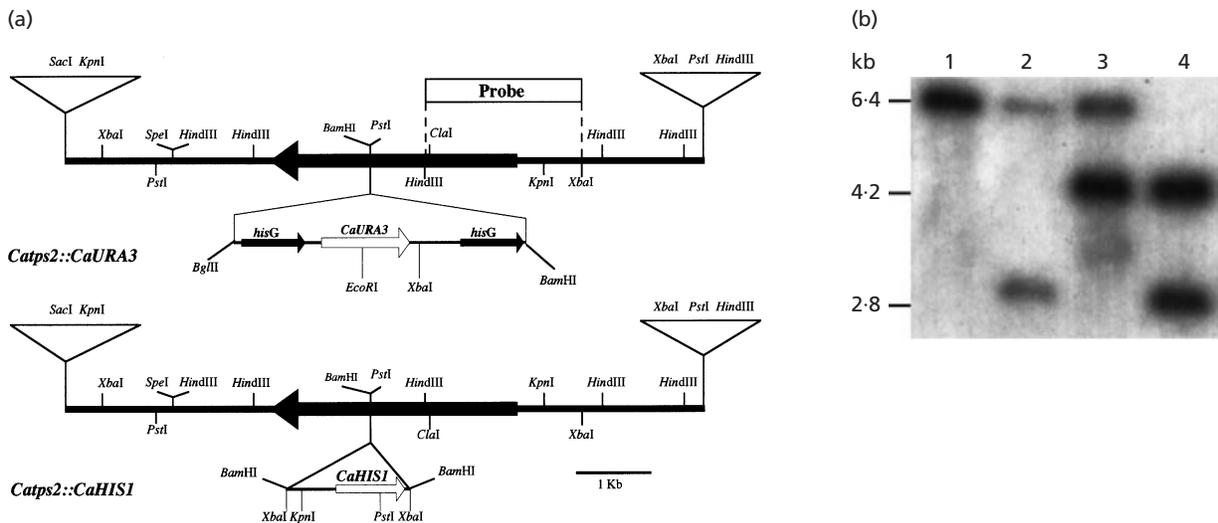


Fig. 1. Disruption of the *CaTPS2* gene and Southern analysis of *Catps2/Catps2* disruptants. (a) Disruption of *CaTPS2* with *CaURA3* and *CaHIS1* (see Methods for details of construction). The *CaTPS2* gene and its direction of transcription is indicated by the thick black arrow. (b) Southern analysis of the chromosomal disruption of *CaTPS2*. The 2 kb *Clal*-*XbaI* fragment indicated in (a) was used as probe. Genomic DNA was digested with *XbaI*. Lanes: 1, *CaTPS2/Catps2* (SC5314); 2, *CaTPS2/Catps2::CaHIS1* (LOZ183); 3, *CaTPS2/Catps2::hisG-CaURA3-hisG* (LOZ184); 4, *Catps2::CaHIS1/Catps2::hisG-CaURA3-hisG* (LOZ200). Sizes of the fragments are indicated on the left side of the figure.

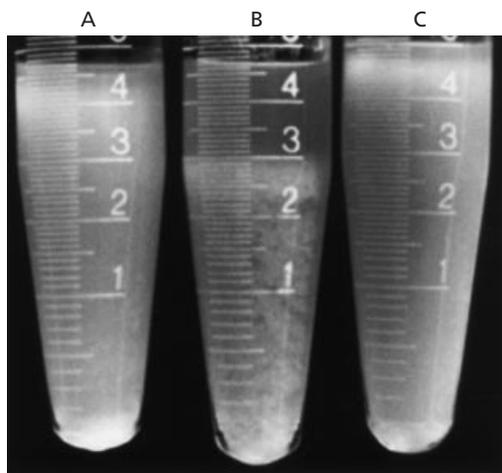


Fig. 2. Aggregation of *C. albicans Catps2/Catps2* mutants. Cultures from (A) a wild-type strain (SC5314), (B) a *Catps2/Catps2* mutant (LOZ200) and (C) a *Catps2/Catps2* mutant (LOZ250; C) with a reintroduced *CaTPS2* gene (pOZ11-6) were grown in YPD at 37 °C until stationary phase. Aliquots of each culture were transferred to graduate tubes and photographed after 10 min standing at room temperature.

mutant, plasmid pOZ11-6 was constructed. Briefly, plasmid pOZ11-2 was obtained by ligation of a 4 kb *SpeI* (blunt-ended)-*XbaI* fragment from plasmid YEp352-TPS2-1 into YEp352 (Hill *et al.*, 1986) digested with *XbaI* and *SmaI*. The *CaTPS2* gene was excised by digestion with *SacI* and *XbaI* and subcloned into YCplac111 (Gietz & Sugino, 1988) digested with the same enzymes, to yield pOZ11-5. A 4 kb *XbaI*-*KpnI* fragment was ligated into the *C. albicans* vector pLC14

carrying the *CaURA3* marker (J. F. García-Bustos unpublished) digested with the same enzymes. The resulting plasmid, pOZ11-6, was introduced into *C. albicans* LOZ250 using protoplast transformation to give strain LOZ252.

Determination of trehalose and T6P. Trehalose was assayed after hydrolysis with trehalase as described by Blázquez *et al.* (1994a), and T6P by measuring the specific inhibition of *Yarrowia lipolytica* hexokinase as described by Blázquez *et al.* (1994b). The *Y. lipolytica* hexokinase needed was obtained from *Sac. cerevisiae* CJM291 (Petit *et al.*, 1996), a strain lacking all glucose-phosphorylating enzymes carrying plasmid pDB20-*YlcHXX1* (Petit & Gancedo, 1999). In some cases, T6P was assayed by HPLC as described by De Virgilio *et al.* (1993).

Analysis of proteins in culture medium. Cultures were centrifuged and the supernatants collected. Trichloroacetic acid was added at a final concentration of 10% and the mixture was kept at 4 °C for 15 min, centrifuged at 13000 r.p.m. for 15 min in an Eppendorf microcentrifuge and the precipitate resuspended in Laemmli reagent (Laemmli, 1970). The samples were applied to an SDS-PAGE (10%) gel and stained with Coomassie blue. To detect glycosylated proteins, PAS staining was performed as described by Kapitany & Zebrowsky (1973).

For analysis of intracellular proteins, cell-free extracts were obtained as described by Blázquez *et al.* (1993) and proteins were assayed using the commercial Pierce reagent.

Propidium iodide staining. A 25 µl sample of a culture was centrifuged and the cells were resuspended in 200 µl PBS (Sambrook *et al.*, 1989) to which 13 µl propidium iodide (50 µg ml⁻¹) was added. The cells were then examined under a fluorescence microscope.

Preparation of samples for electron microscopy. For scanning electron micrographs, samples were prepared as described by

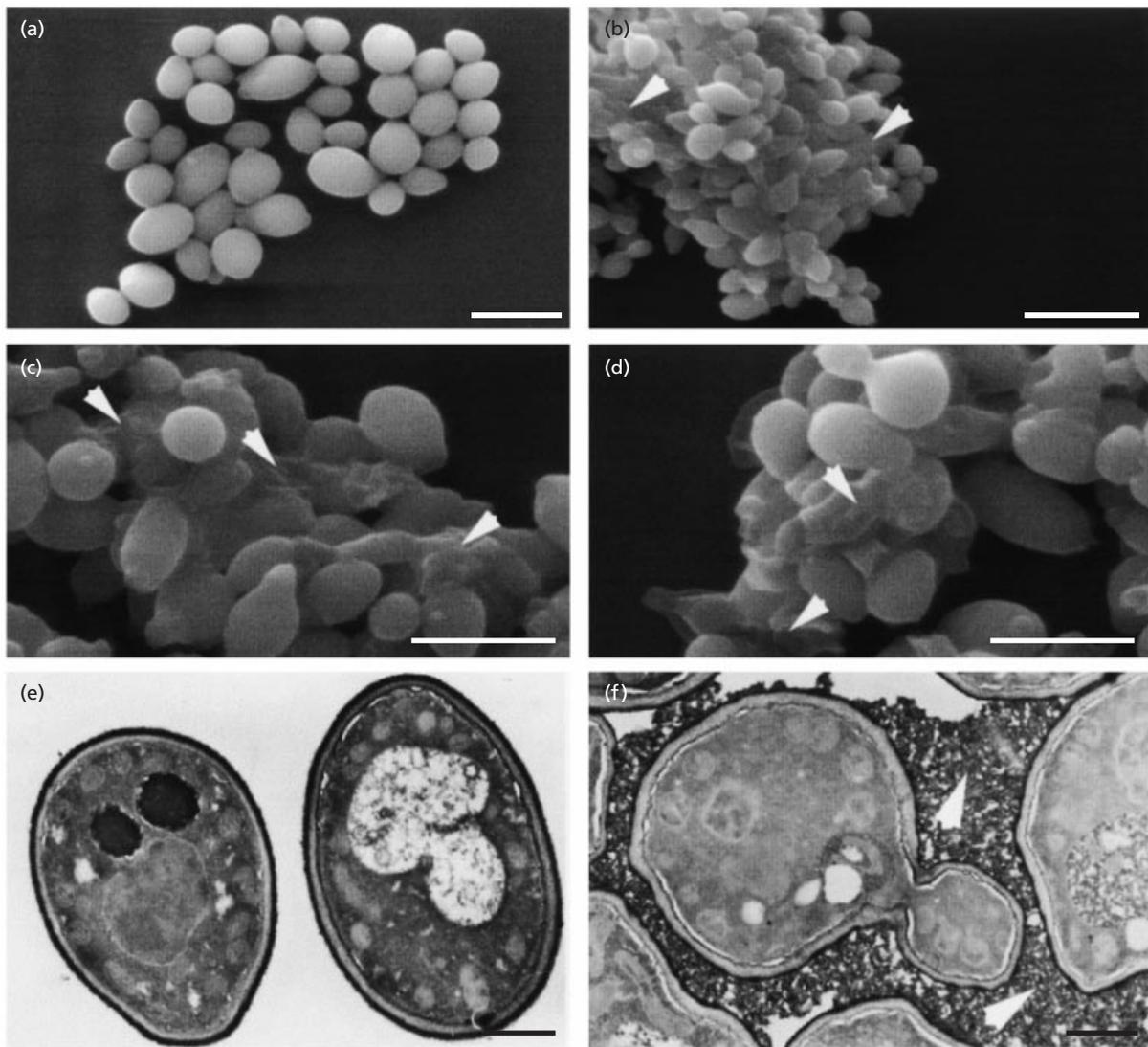


Fig. 3. Electron microscopy of a *C. albicans* *Catps2/Catps2* mutant. Cultures of a wild-type strain (SC5314) and a *Catps2/Catps2* mutant (LOZ200) were grown in YPD at 37 °C until stationary phase. Scanning electron microscopy (a–d) and transmission electron micrographs (e, f) were performed as described in Methods. (a, e) Wild-type; (b–d, f) *Catps2/Catps2* mutant. Observe the material surrounding the mutant cells (marked with white arrows in c, d and f). Bars: a, c, d, 5 µm; b, 10 µm; e, 800 nm; f, 700 nm.

Herrero *et al.* (1999) and for transmission electron micrographs as described by Wright *et al.* (1988).

Virulence in mice. Yeasts were grown on Sabouraud Dextrose agar (Difco) at 37 °C for 24 h, washed with 0.85% NaCl and resuspended in the same solution. The number of cells to be inoculated in the mice was determined by counting the cells in an haemocytometer and the viability (c.f.u.) was checked by plating the suspension onto Sabouraud Dextrose Agar plates. In these conditions no appreciable aggregation was observed and a good agreement between c.f.u. data and haemocytometer counting was observed. Groups of 10 female mice (BALB/c, 6–8 weeks old, 25–30 g wt) were inoculated in the lateral caudal vein with 1.5×10^6 viable *C. albicans* cells of each strain and observed for 1 month. To determine the c.f.u. in the mice, each organ was removed, weighed and homogenized in 0.85% NaCl. Dilutions were plated on Sabouraud Dextrose at 30 °C and colonies were counted after 48 h.

Differences in c.f.u. were analysed by the statistical Mann–Whitney *U* test and considered statistically significant at a $P < 0.05$.

RESULTS

Isolation and characterization of the *CaTPS2* gene

The *C. albicans* *TPS2* gene was isolated by complementation of the temperature-sensitive growth of an *Sac. cerevisiae* *tps2* mutant (see Methods). The complementation brought about a drastic decrease in the intracellular concentration of T6P in the transformed cells (in stationary phase from 0.104 mM in the *tps2* mutant to 0.015 mM in the transformant) and in cells subjected to a heat shock at 42 °C (from 0.143 mM in the *tps2* mutant to 0.022 mM in the transformant). These results indicate

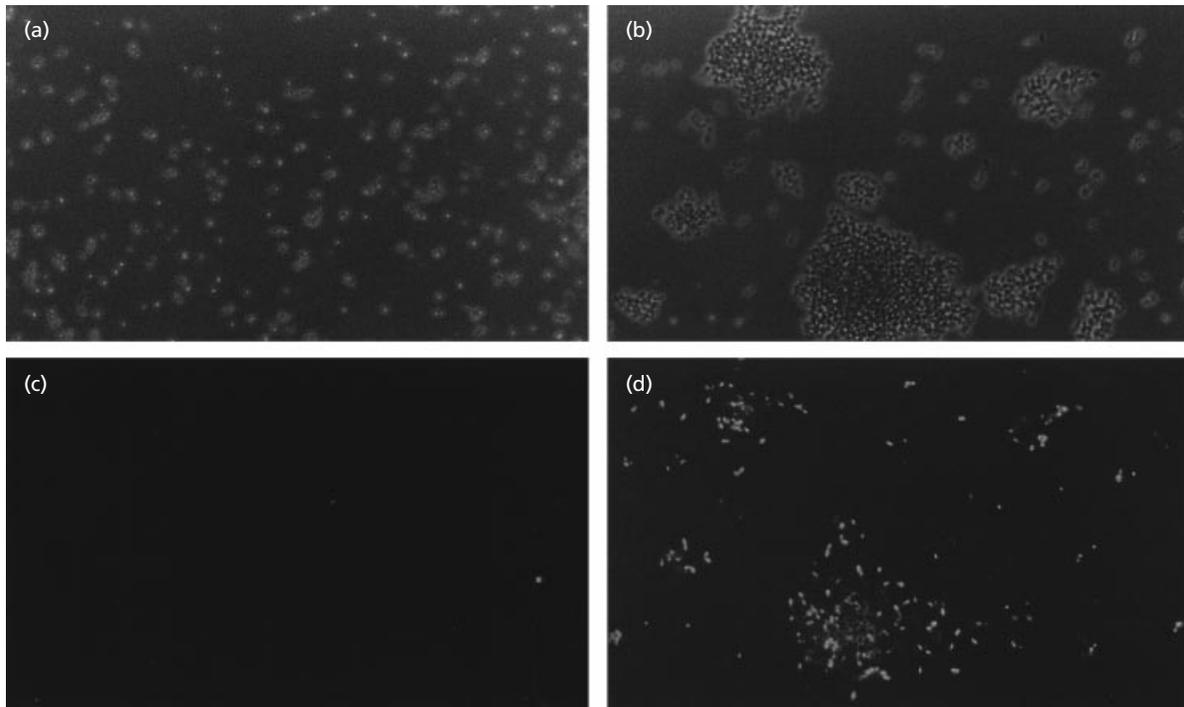


Fig. 4. Uptake of propidium iodide by *Catps2/Catps2* mutants. Wild-type (SC5314) (a, c) and *Catps2/Catps2* mutant (LOZ200) (b, d) cells were grown in YPD until stationary phase and treated with propidium iodide as described in Methods. (a, b) Phase-contrast micrographs; (c, d) propidium iodide fluorescence micrographs.

that the cloned fragment encodes a protein with phosphatase activity against T6P. When the DNA inserted in the plasmid was sequenced, we found an ORF whose predicted protein sequence had high similarity with T6P phosphatases from other organisms: 67% with the protein of *Sac. cerevisiae* (De Virgilio *et al.*, 1993), 53% with that of *Schizosaccharomyces pombe* (Franco *et al.*, 2000) and 73% with that of *A. nidulans* (Borgia *et al.*, 1996). Analysis of the DNA sequence up to 700 bp upstream of the putative *CaTPS2* start codon revealed the existence at positions -91 and -267 of CCCCT motifs that are found in most *Sac. cerevisiae* genes with stress-controlled transcription (Kobayashi & McEntee, 1993; Marchler *et al.*, 1993). The orthologous *ScTPS2* gene has four to five such stress responsive elements (STRE) in its promoter (Gounalaki & Thireos, 1994; Varela *et al.*, 1995).

Effects of the disruption of the *CaTPS2* gene

The *Catps2/Catps2* disrupted strain did not accumulate trehalose after a heat shock at 42 °C. Throughout growth, the concentration of T6P in the mutant was 1 nmol (mg dry wt) $^{-1}$ during the exponential phase and 3 nmol (mg dry wt) $^{-1}$ in the stationary phase, while in the wild-type it was below our detection level (0.5 nmol). These results strongly indicate that there is only one gene encoding T6P phosphatase in *C. albicans*.

The *Catps2/Catps2* mutant grew at 37 °C and even at 42 °C in YP-glucose (YPD), although slower than the wild-type. Generation times at 37 °C were 60 min for

the wild-type and 80 min for the mutant, and 80 min for the wild-type and 130 min for the mutant at 42 °C. In contrast to the wild-type, the mutant cells formed aggregates in stationary phase (Fig. 2). These aggregates were not dispersed by washing with 0.25 M EDTA, a treatment that disperses flocks in *Sac. cerevisiae* (Stratford, 1989), but they were dispersed by a mild sonication or by a 10 min treatment with 0.2 mg Zymolyase ml $^{-1}$ that did not affect cell integrity. Reintroduction of an intact *CaTPS2* caused the disappearance of the aggregation phenotype, showing that the effects observed were due to the absence of a *CaTPS2* gene (Fig. 2). Electron microscopic examination of the mutant cells did not show marked morphological abnormalities in the aggregated cells, but a material surrounding them was observed (Fig. 3). This material was not seen in preparations from exponential-phase cells when aggregation was not observed (results not shown).

Aggregation occurred independently of the carbon source in the media. We observed that whenever aggregation occurred, the final pH of the media was >7 . If media were buffered at pH 5, aggregation was not observed. We did not observe aggregation during the exponential phase of growth, even in media buffered at pH 7.5.

More than 50% of stationary-phase *Catps2/Catps2* cells were permeable to propidium iodide, while less than 1% wild-type cells from the same growth phase took up the dye (Fig. 4), indicating that the mutant had

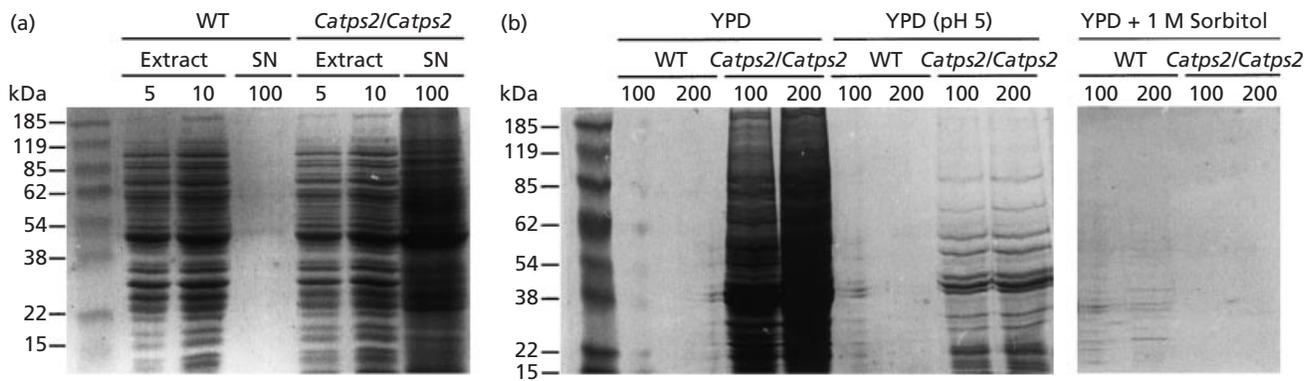


Fig. 5. Analysis of proteins in culture medium. (a) Wild-type (SC5314; WT) and a *Catps2/Catps2* mutant (LOZ200) were grown in YPD at 37 °C until stationary phase and samples were harvested by centrifugation. Proteins in 100 µl of supernatant (SN) were precipitated as described in Methods. The cells were extracted and proteins were determined in the extracts as described in Methods. Intracellular proteins (5 and 10 µg) from the corresponding yeast were applied to the gel. (b) Wild-type (SC5314) and a *Catps2/Catps2* mutant (LOZ200) were grown under the conditions indicated and treated as described in (a). Proteins in 100 and 200 µl of the supernatants were precipitated as described in Methods. The figures over each lane indicate the amount of supernatant precipitated. Proteins were stained with Coomassie blue.

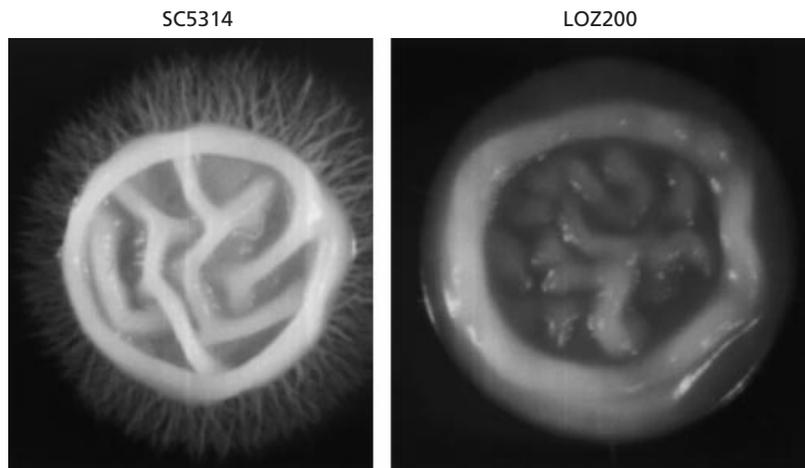


Fig. 6. Colony morphology of *C. albicans* wild-type and *Catps2/Catps2* mutant cells. *C. albicans* wild-type (SC5314) and a *Catps2/Catps2* mutant (LOZ200) were grown in YPD and spread on Spider medium as described in Methods. Photographs were taken after 7 days at 37 °C.

a defect in cell integrity. This idea was also supported by the finding of a large amount of proteins in the medium when mutant cells had grown to stationary phase (Fig. 5a). The pattern of proteins from the medium was similar to that of a cell-free extract (Fig. 5a), making it unlikely that the proteins found in the medium were a selected set preferentially secreted by the mutant. Leakage of proteins took place, although at a decreased level in media buffered at pH 5 (Fig. 5b), suggesting that a certain loss of cell integrity already occurred at this pH.

Addition of 1 M sorbitol to the culture medium prevented aggregation, reduced permeability to propidium iodide (not shown) and leakage of proteins (Fig. 5b), suggesting an osmoprotective effect. However, it also lowered the final pH of the medium to values < 5, so the prevention of aggregation could not unequivocally be ascribed to osmoprotection. Addition of 1 M KCl did not decrease the pH and prevented the cells aggregating

and taking up propidium iodide, thus demonstrating that osmotic stabilization can remediate the phenotype caused by mutation. This result suggests that the *Catps2/Catps2* mutant has a defect in the cell wall. Consistent with this idea was the increased sensitivity of the mutant to Zymolyase (results not shown).

Cells bearing the double *Catps2/Catps2* disruption formed colonies with a smooth border on plates of Spider medium (Fig. 6) and when challenged with serum at 37 °C showed a delay in the formation of hyphae. Both facts indicate that the mutation interferes with the correct filamentation process.

Disruption of *CaTPS2* decreases infectivity in mice

Mice inoculated with the same amount of the *Catps2/Catps2* disruptant and the wild-type showed a striking difference in their survival (Fig. 7). After 7 days no

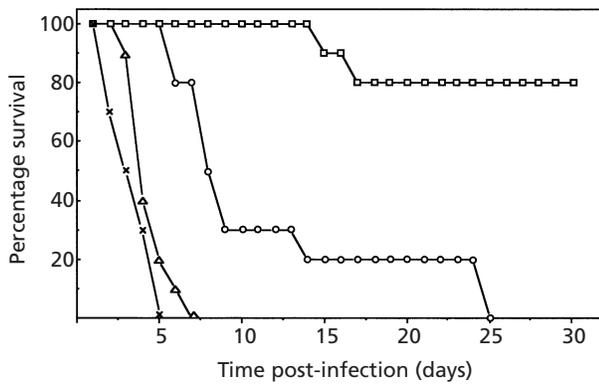


Fig. 7. Infectivity of *C. albicans* wild-type and *Catps2/Catps2* mutant cells. Mice were inoculated as described in Methods with 1.5×10^6 c.f.u. of either wild-type (SC5314; Δ), the *CaTPS2/Catps2* heterozygote (LOZ184; \circ), the *Catps2/Catps2* mutant (LOZ200; \square) or a *Catps2/Catps2* mutant in which the *CaTPS2* gene was reinserted (LOZ252; \times).

survivors were found in the group of mice inoculated with the wild-type, while after 1 month, 80% of those having received the injection of the double disruptant were alive (Fig. 7). When the *CaTPS2* gene was reintroduced by way of plasmid pOZ11-6 (see Methods) in the *Catps2/Catps2* strain, the strain regained infectivity and mice inoculated with the transformed strain died like those inoculated with the wild-type (Fig. 7). Mice inoculated with the heterozygous strain showed an intermediate behaviour (Fig. 7). The fate of the yeast in several organs was followed in mice inoculated in parallel. As shown in Table 2, after 1 month only a low fungal burden was found in the mice inoculated with the double disruptant. These mice were able to eliminate an important proportion of the infecting cells and those remaining did not significantly affect their survival.

DISCUSSION

We have cloned the *CaTPS2* gene that encodes a T6P phosphatase and appears to play an important role in the determination of cell-surface properties and virulence. In *Sac. cerevisiae* and *A. nidulans* only one gene encoding this activity has been found and this also appears to be the case in *C. albicans* as shown by the lack of measurable trehalose and the increase in intracellular T6P concentration in the *Catps2/Catps2* mutant.

The disruption of *CaTPS2* did not cause a strong thermosensitive growth phenotype, even at 42 °C, and produced an aggregation of cells in the stationary phase. This is an important difference with the phenotypes reported for the *tps2* mutant of *Sac. cerevisiae* (De Virgilio *et al.*, 1993) and of *Sch. pombe* (Franco *et al.*, 2000) where no flocks have been observed and indicates that the accumulation of T6P acts differently in these organisms. The fact that aggregation in the *Catps2/Catps2* mutant was apparent only in the stationary phase suggests that the increased levels of T6P influence processes taking place at this phase of growth when an important number of not yet well understood changes occur that affect different cell properties (Werner-Washburne *et al.*, 1996). Reports of flocculation in *C. albicans* mutants are scarce; among them one concerns a mutant in a two-component histidine kinase, *CaHK1* (Calera & Calderone, 1999), and another one, a mutant affected in the gene *BGL2* encoding a glucosyl transferase (Sarthy *et al.*, 1997). In the first case the aggregation is due to interactions between the hyphal surfaces, while in the second case no information about how the aggregation occurred is available. In the case of the aggregation observed in the *Catps2/Catps2* mutant, hyphae were not seen when aggregation took place. Expression of *ALS1*, a gene that encodes an agglutinin (Hoyer *et al.*, 1995), was similar in the wild-type and in the mutant (results not shown), suggesting that changes

Table 2. Distribution of *C. albicans* wild-type and *Catps2/Catps2* mutant cells in different organs of mice at different times following inoculation

Mice were infected as described in Methods with either the wild-type (SC5314), the *Catps2/Catps2* mutant (LOZ200) or a *Catps2/Catps2* mutant in which the *CaTPS2* gene was reinserted (LOZ252). At the times indicated the number of *C. albicans* cells per g tissue was determined as described in Methods. Data represent the mean \pm standard error of at least three mice.

Organ	log (No. of <i>C. albicans</i> cells per g tissue)			
	3 days			30 days
	Wild-type	<i>Catps2/Catps2</i>	<i>Catps2/Catps2</i> with <i>CaTPS2</i>	<i>Catps2/Catps2</i>
Kidney (right)	5.76 \pm 0.42	4.16 \pm 0.18	6.65 \pm 0.151	0.52 \pm 0.52
Kidney (left)	6.54 \pm 0.35	3.74 \pm 0.57	6.67 \pm 0.125	2.26 \pm 0.92
Spleen	3.53 \pm 0.14	3.59 \pm 0.34	3.76 \pm 0.31	1.20 \pm 0.59
Liver	2.42 \pm 0.13	1.48 \pm 0.88	3.33 \pm 0.40	1.09 \pm 0.70
Lung	2.63 \pm 0.20	2.41 \pm 0.95	3.19 \pm 0.67	0.91 \pm 0.60

in Als1 levels are not involved in the aggregation. A modification in hydrophobic properties of the cells could also occur in the mutant and determine its aggregation. The material that surrounded the aggregated cells of the *Catps2/Catps2* mutant could have adhering properties that contribute to the aggregation of the cells. We do not know the nature of this material, but it is not too far fetched to think that its presence is a consequence of a defective cell wall. This idea is supported by the deaggregation of the flocks by a mild Zymolyase treatment.

The pH influence on aggregation could be explained by a difference in the charge of some proteins whose levels are changed in the *Catps2/Catps2* mutant. The accumulated T6P could also have an effect on the expression of some pH-regulated genes or on the activity of their products. It is well known that pH controls expression of different genes whose products are implicated in morphogenesis in *C. albicans* and that acidic pH favours the yeast form (El Barkani *et al.*, 2000; Fonzi, 1999; Ramon *et al.*, 1999). It is noteworthy that aggregation of the *CaHK1* mutant also occurred at pH 7.5 and not at acidic pH (Calera & Calderone, 1999). In *Sac. cerevisiae* it has been found that cell wall organization is altered when the organism is faced with pH changes; at acidic pH values higher levels of certain cell-wall proteins were produced and resistance to lysis by β -1,3-glucanase increased (Kapteyn *et al.*, 2001). The cell wall architecture of *C. albicans* has been shown to be similar to that of *Sac. cerevisiae* (Kapteyn *et al.*, 2000), therefore it is not illogical to think that these changes may also occur in *C. albicans* and that they are altered in the *Catps2/Catps2* mutant. That the changes are specifically related to the absence of an intact *CaTPS2* and not to a lack of trehalose is shown by the fact that *Catps1/Catps1* mutants did not aggregate nor showed changes similar to those shown by the *Catps2/Catps2* mutant (Zaragoza *et al.*, 1998).

Two facts support the conclusion that when *Catps2/Catps2* cells enter stationary phase, they suffer an important loss of viability: the leakage to the culture medium of high amounts of proteins, most of them intracellular, and the permeability to propidium iodide. We could not quantify the loss of viability by colony counting because the aggregation produced highly variable counts. Again, lack of trehalose was not the cause of loss of viability as *Catps1/Catps1* cells did not take up propidium iodide (unpublished results). The effects of the mutation are likely to be a consequence of defects in the cell wall as they are suppressed by osmotic stabilizers. Sorbitol also suppressed the phenotype of the *CaHK1* mutant (Calera & Calderone, 1999) and remediated the germination defect of conidia of *A. nidulans orlA* mutants defective in T6P phosphatase (Borgia *et al.*, 1996). However, its action in the first case could be related to an inhibition of hyphal development (Alex *et al.*, 1998), while in the second case it appears to be due to a stabilization of the cell wall weakened by a decreased chitin content. A decrease in chitin does not seem to be the cause of the increased permeability of the

Catps2/Catps2 mutant, since a decrease in chitin does not affect cell stability in *C. albicans* (Bulawa *et al.*, 1995; Gow *et al.*, 1994).

Our hypothesis is that the accumulation of T6P in the mutant is responsible for alterations in the architecture of the cell wall, a structure implicated in the maintenance of cell integrity and in interactions with other cells. Up to now the only role demonstrated for T6P outside the trehalose biosynthetic pathway is its inhibition of hexokinase (Blázquez *et al.*, 1993); our results suggest other regulatory roles for this metabolite. In *Sac. cerevisiae*, *tps1* mutants are not thermosensitive in permissive sources (unpublished data; Elliott *et al.*, 1996), showing that a lack of trehalose is not the cause of the thermosensitive phenotype of *tps2* mutants. These latter mutants accumulate T6P and it has been shown that a decrease in T6P intracellular accumulation suppresses their thermosensitive phenotype (Elliott *et al.*, 1996), indicating that a high concentration of this metabolite interferes with normal cell growth as we have shown for the *Catps2/Catps2* mutant. The possibility that T6P plays a role in the regulation of a cell integrity pathway cannot be ruled out.

The altered colony morphology found on Spider medium may be due to interference of the accumulated T6P with changes in cell wall composition that occur during this process (Schwartz & Larsh, 1980). However, we cannot exclude that the protein Tps2 itself may have a direct effect on components of the pathway(s) that control hyphae formation.

The decreased infectivity of the *Catps2/Catps2* mutant is clearly due to the lack of *CaTPS2* and not to a secondary effect originated during the construction of the disrupted strain, as shown by the regain of infectivity of that strain when transformed with a plasmid bearing the *CaTPS2* gene. Several causes may explain the greatly decreased infectivity of the *Catps2/Catps2* mutant: a somewhat slower growth rate, defects in the cell integrity or impaired hyphae formation. Since a *Catps1/Catps1* mutant also has a lower infection potential (Zaragoza *et al.*, 1998), the possibility that trehalose may be an important factor that protects *C. albicans* from some host defence mechanisms cannot be discarded. In fact, the *Catps1/tps1* mutant appears to be highly sensitive to free radicals (J. C. Argüelles, personal communication), compounds that have been proposed to act as a host defence (Murphy, 1991).

Although the disruption of *CaTPS2* did not cause a thermosensitive phenotype in *C. albicans*, the properties of the *Catps2/Catps2* mutant and the absence of a trehalose biosynthetic pathway in mammals still make inhibitors of T6P phosphatase attractive candidates for antifungal therapy.

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