Disruption of the *Candida albicans* *TPS2* Gene Encoding Trehalose-6-Phosphate Phosphatase Decreases Infectivity without Affecting Hypha Formation

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Deletion of trehalose-6-phosphate phosphatase, encoded by *TPS2*, in *Saccharomyces cerevisiae* results in accumulation of trehalose-6-phosphate (Tre6P) instead of trehalose under stress conditions. Since trehalose is an important stress protectant and Tre6P accumulation is toxic, we have investigated whether Tre6P phosphatase could be a useful target for antifungals in *Candida albicans*. We have cloned the *C. albicans* *TPS2* (*CaTPS2*) gene and constructed heterozygous and homozygous deletion strains. As in *S. cerevisiae*, complete inactivation of Tre6P phosphatase in *C. albicans* results in 50-fold hyperaccumulation of Tre6P, thermosensitivity, and rapid death of the cells after a few hours at 44°C. As opposed to inactivation of Tre6P synthase by deletion of *CaTPS1*, deletion of *CaTPS2* does not affect hypha formation on a solid glucose-containing medium. In spite of this, virulence of the homozygous deletion mutant is strongly reduced in a mouse model of systemic infection. The pathogenicity of the heterozygous deletion mutant is similar to that of the wild-type strain. *CaTPS2* is a new example of a gene not required for growth under standard conditions but required for pathogenicity in a host. Our results suggest that Tre6P phosphatase may serve as a potential target for antifungal drugs. Neither Tre6P phosphatase nor its substrate is present in mammals, and assay of the enzymes is simple and easily automated for high-throughput screening.

Over the last years, the prevalence of *Candida albicans* infections in humans has increased seriously (40). The two main reasons are the increasing number of immunocompromised patients and the increasing resistance against the limited number of antimycotic drugs that are commercially available. These drugs act on a small number of targets. They either bind ergosterol or inhibit its biosynthesis (amphotericin B, terbinafine, nystatin, and the azoles) or interfere with nucleic acid biosynthesis (flucytosine). New drugs still under clinical investigation act on cell wall formation or on protein synthesis (echinocandins, nikkomycins and aureobasidin, rustmicin, and khafrefungin) (23, 35). A major problem for development of new antifungal compounds is the fact that fungi are eukaryotes and therefore have most essential functions in common with mammalian cells.

Recently, much research focus has gone to targets involved in the regulation of the dimorphic shift from yeast cells to hyphae, since it has been shown that the capacity to form hyphae is related to virulence (12, 20, 30). Based on the similarity with pathways involved in the control of pseudohyphal growth in *Saccharomyces cerevisiae* (38), the mitogen-activated protein (MAP) kinase pathway and the Ras-cyclic AMP pathway have been identified as being involved in control of *C. albicans* dimorphism. The MAP kinase pathway includes Cst20, Hst7, Cek1, and Cph1 (14, 16, 27, 33), while the Ras-cyclic AMP pathway includes Ras1, Cap1, Tpk2, and Efg1 (3,

17, 43, 45). Although deletion of these genes renders *C. albicans* cells less virulent or even avirulent in a mouse model, the gene products do not seem to be promising as antifungal targets because homologous components are present in mammals. A similar situation applies to the Hog1 MAP kinase pathway (1, 33, 34). More promising are signaling pathways involved in cell wall formation, and for some of the components clinical studies to investigate their potential as antifungal targets are under way (8, 10, 32, 42, 44, 49). Another class of interesting targets are important for adherence to host cells. Two groups, the secreted acid protease family genes and the cell surface glycoprotein family genes, have been identified, and their deletion results in lower virulence (9, 20, 25, 41).

Trehalose metabolism might be an interesting target for antifungals. It is entirely absent in mammalian cells and makes use of highly specific enzymes. Trehalose ($\alpha,\alpha,1,1$ -diglucose) is synthesized in fungi in a two-step process. Trehalose-6-phosphate (Tre6P) synthase, encoded by *TPS1*, synthesizes Tre6P from glucose-6-phosphate and UDPglucose (4). Tre6P is then hydrolyzed into trehalose by Tre6P phosphatase, encoded by *TPS2* (15). Trehalose is a storage carbohydrate, but it also plays a major role as stress protectant (47, 51, 53). It appears that trehalose has unusual chemical properties which make it more suitable than other sugars to protect proteins and membranes against denaturation under stress conditions (13, 37). It accumulates in large quantities in survival forms of a diverse array of organisms and also accumulates in vegetative cells of fungi under stress conditions (47, 51, 53). Since pathogens are living under adverse conditions in host organisms because of the host defense reactions, insufficient nutrient supply, or high osmolarity, etc., one can assume that their stress response mechanisms are continuously activated. Trehalose accumula-

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TABLE 1. *C. albicans* strains used in this study

Strain	Genotype	Parent strain	Reference
SC5314	Wild type		21
CAI4	<i>ura3Δ::imm434/ura3Δ::imm434</i>	SC5314	18
CC5	<i>ura3Δ::imm434/ura3Δ::imm434 TPS2/tps2Δ::hisG-URA3-hisG</i>	CAI4	This study
KAR5	<i>ura3Δ::imm434/ura3Δ::imm434 TPS2/tps2Δ::hisG</i>	CC5	This study
CC17	<i>ura3Δ::imm434/ura3Δ::imm434 tps2Δ::hisG-URA3-hisG/tps2Δ::hisG</i>	KAR5	This study
EL17	<i>ura3Δ::imm434/ura3Δ::imm434 tps2Δ::hisG/tps2Δ::hisG</i>	CC17	This study
KAR17	<i>ura3Δ::imm434/ura3Δ::imm434 pTPS2-URA3/tps2Δ::hisG</i>	EL17	This study

tion is part of the stress response, and previous work has shown that prevention of trehalose accumulation by deletion of the *C. albicans* *TPS1* gene renders the cells less virulent (54). In *S. cerevisiae*, deletion of the *TPS2* gene encoding Tre6P phosphatase causes hyperaccumulation of Tre6P instead of trehalose under stress conditions (15, 39). As a result, a *tps2Δ* strain is thermosensitive. Tre6P accumulation is toxic because it sequesters phosphate and as a result inhibits ATP generation. Moreover, Tre6P is an inhibitor of hexokinase, causing additional reduction of glycolytic flux and energy generation (6, 48). Energy provision is required for most cellular functions, including the activity of drug efflux pumps. Because of these reasons, it appeared to us that Tre6P phosphatase might be even a better target for antifungals than Tre6P synthase. Moreover, not only is Tre6P phosphatase absent in mammals, its substrate Tre6P is also absent, increasing the chances for design of specific inhibitors.

Disruption of the *C. albicans* *TPS1* gene (*CaTPS1*) impairs the formation of hyphae on glucose-containing medium and decreases virulence in a mouse systemic infection model (2, 54). The reason for the lower virulence is not well understood. There are at least two possibilities. First, deletion of *TPS1* in other yeasts such as *S. cerevisiae* or *Kluyveromyces lactis* results in complete deregulation of glycolysis after addition of glucose and rapid loss of viability (31, 50). In *C. albicans*, a similar deregulation of metabolism is found but only at higher temperatures. Second, the absence of trehalose may result in lower stress resistance and as a result lower virulence. If the absence of trehalose is the main reason for the reduced virulence, it appears that deletion of the *C. albicans* *TPS2* gene (*CaTPS2*), which results in high levels of Tre6P instead of trehalose, should at least give the same reduction in stress resistance and virulence. Because of the toxic effects of Tre6P hyperaccumulation on glycolysis, inactivation of *CaTPS2* might impair cellular functions even more and therefore further reduce virulence. In this work we have cloned the *CaTPS2* gene and constructed hetero- and homozygous deletion mutants. We show that complete inactivation of *CaTPS2* results in a 50-fold increase in Tre6P levels, growth inhibition, and loss of viability during heat stress. Whereas deletion of *CaTPS1* prevented glucose-induced hypha formation (54), deletion of *CaTPS2* did not affect hypha formation under all conditions examined. In spite of this, virulence of the homozygous deletion mutant in a mouse systemic infection model was strongly reduced.

MATERIALS AND METHODS

Yeast strains and growth conditions. The wild-type *C. albicans* SC5314 strain (21) and the genetically marked CAI-4 strain (*ura3Δ::imm434/ura3Δ::imm434*) (18) were kindly provided by Alistair Brown (Aberdeen, United Kingdom). The

construction of the different *C. albicans* *tps2Δ* strains (Table 1) is described further. The yeast cells were grown with shaking at 28°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose). To study the yeast-hypha transition, early-exponential-phase cells (optical density at 600 nm = 0.8) growing at 28°C on YPD medium were supplemented with 10% fetal calf serum (Sigma) and shifted to 37°C. To study colony morphology, stationary-phase *C. albicans* cells were resuspended in fresh YPD medium and diluted to obtain single colonies on plates. After 5 days at 37°C, individual colonies were photographed. The formation of hyphae and colony morphology were tested on different media, including medium containing fetal calf serum (Sigma), Spider medium (1% nutrient broth σ , 0.2% K_2HPO_4 , 1% mannitol), Lee's medium (28), SLAD medium (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, 50 μ M ammonium sulfate), and medium 199 (Sigma; containing Earle's salts and L-glutamine).

Growth curves were determined with a Bioscreen apparatus (Life Sciences). Strains were grown overnight in YPD medium and diluted to an optical density of 0.05 in fresh YPD medium, and 300 μ l was added to the wells of the honeywell plate and incubated at the indicated temperature with 30 s of shaking every minute.

Chromosomal deletion of *TPS2*. The *CaTPS2* gene and its flanking DNA (530 bp upstream and 640 bp downstream) were isolated by PCR amplification with genomic DNA of strain SC5314 as a template. The sequences of the two oligonucleotides used were as follows: primer FOR2, 5'-GAGTCGACCTCACCTGAGGCATCCACATAC3', and REV2, 5'-GAGGTACCGTGTAATCCGGACATTAATCCCG3'. The FOR2 oligonucleotide contains a *SalI* recognition site (underlined), and the REV2 oligonucleotide contains a *KpnI* recognition site. The 3,800-bp fragment was subcloned in pUC19 digested with the same restriction enzymes. The plasmid obtained was then digested with *SnaBI* and *NsiI*, which remove nearly the complete open reading frame of the *CaTPS2* gene, leaving the flanking sequences and material encoding only 7 amino acids at the N terminus and 10 amino acids at the C terminus. The cassette *hisG-URA3-hisG* carrying the *C. albicans* *URA3* gene flanked by two direct repeats of the *Salmonella enterica* serovar Typhimurium *hisG* sequence was obtained by digesting plasmid pMB7-A (18) with the restriction enzymes *BglII* and *PstI*. Before ligation into the plasmid containing the flanking sequences of *CaTPS2*, the *BglII* site was blunted using the Klenow polymerase. To make the final deletion construct, the plasmid obtained was digested with *AvrII* and *SpeI* resulting in the 4.8-kb deletion fragment. This fragment was used to transform the CAI-4 strain. Both PCR analysis and Southern blotting were used to confirm deletion of one copy of the *CaTPS2* gene.

Excision of the disruption cassette from the chromosome was performed by plating the cells on minimal medium containing uridine and 5-fluoro-orotic acid (FOA). The FOA-positive colonies were checked by PCR and Southern blotting, and a colony that showed the correct pattern was used to delete the second copy of the *CaTPS2* gene. The same strategy was used as for the deletion of the first copy. For the reintegration of *CaTPS2* in the homozygous deletion strain, we cloned the gene and promoter sequence into the plasmid pCaEX (11) (kindly provided by P. Sudbury, University of Sheffield, Sheffield, United Kingdom). The *CaTPS2* gene was amplified using oligonucleotides *CaTPS2* FOR4 (5'-GAAGTCTGAAGCTGCCGG 3') and *CaTPS2* REV3 (5'-CGGCATGCCCGAGACTGGAGATTAGGTG 3'). The *CaTPS2* gene was cloned by digesting the PCR product with *XbaI* (in the promoter) and *SphI* (underlined) and subcloned in the pCaEX vector digested with the same enzymes, thereby removing the *MET3* promoter sequence in the plasmid. The plasmid was linearized using *AvrII*, directing the integration at the *CaTPS2* locus in the genome.

Heat shock response and resistance. Cells growing exponentially at 30°C in YPD medium were transferred to a water bath at 44°C, and at different times after the shift, samples were taken and a 10-fold serial dilution was spotted on YPD plates and further incubated at 30°C.

Determination of trehalose, Tre6P, and Tre6P phosphatase activity. Cells from an overnight culture were washed and resuspended in fresh YPD medium for 4 h. Samples for trehalose and Tre6P were taken. The culture was then divided in four aliquots, and the cells were further incubated at either 30, 37, 40, or 43°C. At different times after the shift, samples for trehalose and Tre6P determination were taken. Trehalose levels were determined as described by Neves et al. (36), and Tre6P levels were determined as described by Van Vaeck et al. (52).

Tre6P phosphatase activity was determined according to a method described previously (5). The activity was measured at 30°C, and measurements were repeated five times.

Determination of virulence. Female BALB/c mice weighing 20 g were inoculated in the lateral caudal vein with 10^6 *C. albicans* cells suspended in 150 μ l of saline. Survival was scored over a period of 1 month. A group of 10 mice per condition was tested. The fungal burdens in the kidneys, liver, and lungs were determined by homogenizing the organs in saline and counting the colonies on YPD plates after appropriate dilution.

RESULTS

Cloning of the *C. albicans* TPS2 gene. BLAST analysis of the *C. albicans* genome database (<http://www-sequence.stanford.edu:8080/bncontigs6.html>) with the *S. cerevisiae* TPS2 sequence revealed a homologous sequence in contig 4-3098. Based on the homology, PCR primers were designed to clone the complete open reading frame and flanking sequences in pUC19. The *C. albicans* Tps2 protein contains 878 amino acids and shares 70% sequence similarity with the *S. cerevisiae* Tps2 protein (Fig. 1). The *C. albicans* Tps2 protein also contains the two phosphohydrolase motifs (46) which are present in all Tps2 sequences that have been described (22, 29).

Construction of a *C. albicans* TPS2 deletion strain. We made use of the Ura blaster cassette to construct the *CaTPS2* deletion strain (Fig. 2A). The pUC19/*CaTPS2* plasmid was digested with *Sna*BI and *Nsi*I, which removes nearly completely the *CaTPS2* open reading frame. It leaves 523 bp of promoter sequence and 639 bp of terminator sequence on the plasmid. The Ura blaster cassette was isolated from plasmid pMB7-A (18) as a *Bgl*III-*Pst*I fragment. After ligation of the two fragments, we obtained the plasmid pUC19/*Catps2::hisG-URA3-hisG*. The final deletion construct was made by digesting this plasmid with *Avr*II and *Spe*I (Fig. 2A). After transformation of the *C. albicans* CAI4 Ura⁻ strain with this 4,814-bp fragment, we obtained 14 colonies. PCR analysis and Southern blotting (not shown) indicated that 12 transformants contained a proper deletion of the *CaTPS2* gene. The positive transformants were streaked on FOA medium as explained in Materials and Methods. The colonies that grew on these plates were checked by PCR for the loss of the *C. albicans* *URA3* gene. One of the positive colonies was transformed again with the *CaTPS2* deletion construct. In this second round of transformation, we screened 75 colonies and found only one transformant with the correct pattern in Southern blot analysis (Fig. 2B). The homozygous deletion strain was also checked by Northern blotting using a *CaTPS2* probe, and no *CaTPS2* messenger could be detected (data not shown). For the reintroduction of a wild-type copy of the *C. albicans* TPS2 gene, we cloned the gene and its promoter on a plasmid and integrated it at the *tps2* locus. Correct integration was confirmed by Southern blot analysis (not shown).

Phenotypic characterization of the hetero- and homozygous *CaTPS2* deletion strains. (i) **Tre6P phosphatase activity.** In a first control experiment to check for proper deletion of the *CaTPS2* gene, we measured the Tre6P phosphatase activity.

Cell extracts were prepared from glucose-grown stationary-phase cells. The wild-type SC5314 strain has an activity of 231 nkat/g of protein, whereas the *tps2 Δ /tps2 Δ* strain has virtually no Tre6P phosphatase activity (7 nkat/g of protein). The heterozygous deletion strain has an intermediate activity (147 nkat/g of protein). This result clearly indicates that the homozygous deletion strain has little or no Tre6P phosphatase activity left.

(ii) **Temperature-sensitive growth and survival.** We grew the wild type and heterozygous and homozygous *CaTPS2* deletion mutants in glucose-containing medium at 41 and 43°C in a Bioscreen apparatus (Life Sciences). The growth rate of the heterozygous deletion strain was quite similar to that of the wild type. Only at 43°C was there a minor reduction in growth rate. The homozygous deletion mutant showed a minor growth inhibition at 41°C and a severe inhibition at 43°C compared to the wild-type strain (Fig. 3A). These data show that also in *C. albicans*, deletion of *TPS2* results in temperature-sensitive growth. Subsequently, we incubated exponentially growing cultures of the three strains for different time periods at 44°C, after which serial dilutions were plated on YPD plates and incubated further at 30°C. Figure 3B shows that the wild-type and heterozygous deletion strains were able to survive quite well an incubation of at least 6 h at 44°C. On the other hand, the homozygous deletion strain was clearly much more sensitive to the heat treatment than the other two strains, and after 6 h at 44°C nearly all cells had died. These results show that the homozygous *CaTPS2* deletion strain is heat sensitive not only for growth but also for survival.

(iii) **Trehalose accumulation.** It has been suggested that the reason for the temperature sensitivity of the *S. cerevisiae* *tps2 Δ* strain is the accumulation of large amounts of Tre6P, which is toxic because it results in phosphate sequestration and deregulation of central metabolism (15, 39). To investigate whether the *C. albicans* homozygous *CaTPS2* deletion mutant behaves in the same way, we measured trehalose and Tre6P levels during heat treatment. Wild-type, *CaTPS2/Catps2 Δ* , and *Catps2 Δ /Catps2 Δ* strains were grown in YPD medium at 30°C to exponential phase. The cultures were then divided in four and further incubated at either 30, 37, 40, or 43°C. The results show that during heat stress strong accumulation of trehalose occurs in the wild type and in the heterozygous deletion strain (Fig. 4). However, in the *Catps2 Δ /Catps2 Δ* strain significant amounts of trehalose also were accumulated. At 30°C the level of trehalose in the homozygous deletion strain was even somewhat higher than those in the wild type and the heterozygous deletion strain. At 43°C the largest difference was observed between the wild-type and heterozygous deletion strains on the one hand and the homozygous deletion strain on the other hand. However, at 43°C the *Catps2 Δ /Catps2 Δ* strain still accumulated up to 30 mM trehalose. We have repeated the trehalose determinations with the homozygous deletion strain under various stress conditions, and we could always detect up to 30 mM trehalose. Apparently, *C. albicans* contains nonspecific phosphatases which are able to dephosphorylate Tre6P into trehalose with lower efficiency than Tre6P phosphatase. We have found similar results for *S. cerevisiae* (55). Addition of general phosphatase inhibitors, such as levamisole, to the cells did not prevent the accumulation of trehalose in the *Catps2 Δ /Catps2 Δ* strain (data not shown). Although our results indicate

<i>S. cerevisiae</i> TPS2	-----MTTQAQDNSPKKRQRIINCVTQLPYKIQLGESNDD---WKIS	39
<i>C. albicans</i>	MAPQQVNAFSANGSIPSPKEFEGKGKLLSGRILNVMTSLPLQIISDYDNKTGQYYWDVE	60
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<i>S. cerevisiae</i> TPS2	ATTGNSALYSSLEYLQFDSTEYEQHVVGWGTGEITRTERNLFTREAKEKPDLDLDDPLYLT	99
<i>C. albicans</i>	TVRGNSALYSSQHFLAEN-KEWETHLIAWTGELINKKKDTSSLTADT---LQDDPLYLD	115
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<i>S. cerevisiae</i> TPS2	KEQINGLTTLQDHMKSDKEAKTDTTQTAPVTNNVHPVWLLRKNQSRWRNYAEKVIWPTF	159
<i>C. albicans</i>	EEDKLKIEKKLCDASG-----TPN---IHPVWLLRRDQGRWRKYAENVLPVF	160
	:* : ..* * :* :*****:*.***:***:*.**	
<i>S. cerevisiae</i> TPS2	HYILNPSNEGEQEKNNWYDYVKFNEAYAQKIGEVYRKGDIIWIHDYLLLLPQLLRMKFN	219
<i>C. albicans</i>	HYIQGQPSDGKAETDAWHDYVKFNEAYLNKIKSVYKPGDIIWIHDYLLLLPQLLRMEFP	220
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<i>S. cerevisiae</i> TPS2	DESIIGYFHHAPWPSNEYFRCLPRRKQILDGLVGANRIFQNESFSRHFVSSCKRLDA	279
<i>C. albicans</i>	N--AYIGFHLVFPSPSEYFRCLSKRSQLLDGMLGADKIGFQSDSFQRHFISCCARVLGC	278
	: **:* *.**.*.*****:*.**:*::* **:.**.*.*:*.**..	
<i>S. cerevisiae</i> TPS2	TAKKSKNSSDSQYQVSVDVLDLPIGVNTTQILKDAFT--KDIDSKVLSIKQAYQ	337
<i>C. albicans</i>	EVNR--D-----SVSAYGTTISVETLPIGIDTEKIEHDAFSELGVEEKVQALKQVYK	329
	.:: : .**.* :*:*****:*.**:*::.*** :*.**:	
<i>S. cerevisiae</i> TPS2	NKKIIIGRDLDSVRGVVQKLRAFETFLAMYPEWRDQVVLIVSSPTANRNSPQTIRLEQ	397
<i>C. albicans</i>	GKKLIVGRDLDKVRGVIQKLEGEIFLDMYPEWRETVVLIQVSSPGYS-HSAN--VET	385
	.**:*:*****.***:***.*** ** *****: ***** . :.* :*	
<i>S. cerevisiae</i> TPS2	QVNELVNSINSEYGNLNFSPVQHYMRIPKDVYLSLLRVADLCLITSVRDGMNTTLEYV	457
<i>C. albicans</i>	RVTEIISRINSKYGNLHNPVLHYQMRVAKKEYLALLRVADLALITSVRDGMNTTSEFV	445
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<i>S. cerevisiae</i> TPS2	TVKSHMSNFLCYGNPLILSEFSGSSNVLKDAIVNPNWDSVAVAKSINMALKLDKEEKNL	517
<i>C. albicans</i>	ICQKYN-N----SPLILSEFTGTATVLKDAIMVNPWDSVGAKTINDALMLSTKEKVS	499
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<i>S. cerevisiae</i> TPS2	ESKLWKEVPTIQDWTNKFSSLEKEKASDDDDVERKMTPALNRPVLLNENYKQAKRRLFLD	577
<i>C. albicans</i>	ESKLYEKLVSNTVQNWTSFTICDILSHSIVTHSNSYTPALNRPVLLNENYKQAKRRLFLD	559
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<i>S. cerevisiae</i> TPS2	YD GTLTPIVKDPAAAIIPSAARLYTILQKLCADPHNQIWIISGRDQKFLNKWLGKLPQLGL	637
<i>C. albicans</i>	YD GTLTPIVQDPAAAIIPSDKLNRLDVLSSDPKNQIWIISGRDQAFLEKWMGNKN--VGL	617
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<i>S. cerevisiae</i> TPS2	SAEHGCFMKDVSCQDWNLTTEKVDMSWQVRVNEVMEEFTTRTPGSFIERKKVALTWHYRR	697
<i>C. albicans</i>	SAEHGCFMKDIGSKEWVNLAAFSWQEKVDDIFKYYTEKTPGSNIERKKVALTWHYRR	677
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<i>S. cerevisiae</i> TPS2	TVPELGFEHAKELKEKLLS-FTDDFDLEVMDGKANIEVRPRFVNKGIEIVKRLVWHQHKP	756
<i>C. albicans</i>	ADPDLGNFQAEKCMKELNDTVAKEYDVEVMAGKANIEVRPKFVNKGIEIVKRLVHPHGAK	737
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<i>S. cerevisiae</i> TPS2	QDMLKG--ISEKLPKDEMPDFVLCLG DDFT DEDMFRQLNTIETCWKEKYPDKNQWGNYG	814
<i>C. albicans</i>	QEKHPTGHCTKDPIEELPDFMLCLG DDL TDEDMFNSLNEINKKWKGDN-RPTNKFSGYG	796
	: :.: :*:***:*****:*****.*** ** . :*:**	
<i>S. cerevisiae</i> TPS2	FYPVTVGSASKKTVAKAHLTDPQQVLETLGLLVGDVSLFQSAGTVDLDSRGHVKNSESSL	874
<i>C. albicans</i>	VYPVAVGPASKKTVAIAHLNEPRQVLETLGLLAGLVIFESAGTVDLDDRVTLPVCLPK	856
	.***:*. ***** **.*:*****.****:*****.* : .	
<i>S. cerevisiae</i> TPS2	KSKLASKAYVMKRSASYTGAKV	896
<i>C. albicans</i>	DQTMLYLRQYLYVKLVKVKKKL	878
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FIG. 1. Sequence alignment of the *S. cerevisiae* and *C. albicans* Tps2 proteins. The phosphohydrolase motifs typical for all Tps2 proteins (46) are indicated in boldface.

the presence of other phosphatases able to dephosphorylate Tre6P, we cannot exclude the possibility that these phosphatases act only on unphysiologically high Tre6P levels that accumulate in the *Catps2Δ/Catps2Δ* strain.

(iv) **Tre6P accumulation.** To determine the accumulation of Tre6P during heat stress, we grew wild-type, *CaTPS2/Catps2Δ*, and *Catps2Δ/Catps2Δ* strains in YPD medium at 30°C until exponential phase. The cultures were then divided in two and fur-

FIG. 2. Deletion strategy for *C. albicans* *TPS2*. (A) Genetic organization of the *CaTPS2* locus. The *CaTPS2* open reading frame (black arrow) was replaced with the Ura blaster cassette as described in Materials and Methods. Restriction endonucleases: H, *Hind*III; S, *Spe*I; Sn, *Sna*BI; N, *Nsi*I; A, *Avr*II. (B) Southern blot analysis of *Hind*III-digested *C. albicans* genomic DNA probed with part of the *CaTPS2* terminator as indicated in panel A. Lanes: 1, parental strain CAI4; 2, *CaTPS2/Catps2Δ* strain CC5 (Ura⁺); 3 and 4, *Catps2Δ/Catps2Δ* strains CC17 and EL17 (Ura⁺ and Ura⁻, respectively); 5, *CaTPS2*-complemented strain KAR17. Numbers on the left and right are the lengths of the DNA fragments in basepairs.

ther incubated at either 30 or 43°C. At different time points after the shift, samples were taken for Tre6P determination. At 30°C there was no Tre6P accumulation in the wild type or in the *CaTPS2/Catps2Δ* strain. The estimated Tre6P concentration in these strains was around 100 to 200 μM. This is similar to the level found in wild-type *S. cerevisiae* cells (6, 24, 52). In the *Catps2Δ/Catps2Δ* strain there was a continuous increase in Tre6P at 30°C, and after 4 h more than 5 mM Tre6P was present (Fig. 5). After the shift from 30 to 43°C there was a rapid transient increase in Tre6P levels in the wild type and the heterozygous deletion strain (Fig. 5). The maximum level of about 1 to 2 mM was reached 1 h after the shift. In the heterozygous deletion strain this peak level is twice that in the wild-type strain. The Tre6P increase at 43°C in the homozygous deletion strain was very high and was also more permanent than that in the other strains. Two hours after the shift, the cells contained 35 mM Tre6P, which is more than 1,000-fold higher than the basal level in the wild-type strain (Fig. 5). Sugar phosphates are generally present in concentrations of just a few millimolar in the cytosol. This very high concentration of Tre6P is likely to disturb energy metabolism and is most probably the cause of thermosensitive growth in the homozygous deletion strain.

(v) **Yeast-hypha transition.** One of the major virulence factors of *C. albicans* is its ability to undergo a dimorphic switch from the budding yeast form to the hyphal form (26). We have investigated whether deletion of the *TPS2* gene interferes with induction of hypha formation. For that purpose, we grew the wild-type, *CaTPS2/Catps2Δ* and *Catps2Δ/Catps2Δ* strains as well as the *Catps2Δ/Catps2Δ* strain with reintegrated *TPS2* on different hypha-inducing media. Upon addition of serum to cells in liquid glucose-containing medium at 37°C, there was no difference in hypha formation between the wild type and the mutants. One hour after the shift, 93 to 95% of the cells contained a germ tube, and after 3 h all of the cells underwent the morphological switch. Figure 6 gives an illustration of the morphology 90 min after the addition of fetal calf serum. Germ tube formation is clearly visible in all four strains. Under similar conditions, the *Catps1Δ/Catps1Δ* strain was reported to be impaired in the formation of hyphae (54). Next we monitored the morphology of *C. albicans* colonies on different solid media. Under all conditions tested, we could not see a difference in colony morphology between the wild type and the *TPS2* mutant strains. In Fig. 7 we show the results for colonies on solid YPD medium, Spider medium, Lee's medium, and SLAD medium, all incubated at 37°C. On YPD medium, the colonies of the different strains had the shape and morphology reflecting predominant yeast-like growth. On the three other media, the colonies produced filaments at the periphery, and this filamentation was very similar for the different strains. We have also grown colonies on M199 minimal medium and on YPD medium containing serum, and also in those cases we could not see any difference between the wild type and the *Catps2Δ/Catps2Δ* strain in the extent of filamentation.

(vi) **Virulence.** To determine whether deletion of *TPS2* affects virulence of *C. albicans*, we injected 40 mice (10 per strain) in the caudal vein with 10⁶ cells of the wild-type, *CaTPS2/Catps2Δ*, *Catps2Δ/Catps2Δ*, and reconstituted strains. As shown in Fig. 8, mice injected with wild-type *C. albicans*, with the heterozygous mutant, or with the strain containing the reintegrated *CaTPS2* gene died within a period of 4 to 14 days. On the other hand, 50% of the mice infected with the homozygous deletion strain survived this treatment. We have repeated this experiment three more times and obtained similar results each time (not shown). We also injected the mice with 10⁷ or 10⁵ *C. albicans* cells. When injected with 10⁷ cells, all mice infected with the wild type and the heterozygous deletion strain died within 3 days. Those injected with the homozygous deletion strain were all dead after 5 days. When injected with 10⁵ cells, all of the mice injected with the homozygous deletion strain survived for up to 60 days, whereas 80% of the mice infected with either the wild-type, heterozygous, or *TPS2* reintegrated homozygous deletion strain died (data not shown). We also determined the fungal burdens in the kidneys and livers of the dead mice obtained from the experiment shown in Fig. 8. In each case similar numbers of CFU could be isolated for the four different strains (data not shown).

DISCUSSION

The rationale of this work is based on the following elements: (i) trehalose is known to be an important stress protection compound in fungi, (ii) stress conditions induce the accu-

FIG. 3. The *Catps2Δ/Catps2Δ* strain is thermosensitive. (A) The SC5314 wild-type (circles), *CaTPS2/Catps2Δ* (squares), and *Catps2Δ/Catps2Δ* (triangles) strains were grown in YPD medium at 30°C (filled symbols) or 43°C (open symbols) in a Bioscreen apparatus. OD, optical density. (B) Cells of the three strains were grown exponentially in YPD medium at 30°C and then incubated in a water bath at 44°C for the indicated amounts of time. Afterwards, aliquots were taken and diluted several times 10-fold. Five microliters of each 10-fold dilution was plated on YPD plates and incubated further at 30°C. The colonies were photographed 24 h later. WT, wild type.

mulation of trehalose, (iii) pathogenic fungi infecting a host organism are probably subject to constant stress, and (iv) accumulation of the trehalose biosynthesis intermediate Tre6P is toxic. Moreover, the toxic accumulation of Tre6P might itself elicit the stress response, with further accumulation of Tre6P as a consequence and the initiation of a vicious circle which might kill the pathogen. As a result, the second enzyme of trehalose biosynthesis, Tre6P phosphatase, appeared to be a more interesting target for antifungals than the first enzyme, Tre6P synthase. The potential usefulness of Tre6P phosphatase as a target for antifungals is further supported by its absence and the absence of its substrate in mammals, which

might facilitate the isolation and/or design of specific inhibitors. Tre6P phosphatase is also an enzyme whose activity can easily be determined in high-throughput assays to screen for inhibitors. Hence, for all of these reasons we have explored the potential use of Tre6P phosphatase as a novel target for antifungals by constructing the homozygous deletion mutant in *C. albicans*, characterizing it, and determining its virulence.

We have cloned the *TPS2* gene of *C. albicans* based on the homology with the *S. cerevisiae* gene, and we have constructed the heterozygous and homozygous deletion strains with the Ura blaster method. Several data support proper heterozygous and homozygous deletion of the *CaTPS2* gene, including

FIG. 4. Trehalose levels during heat treatment. Cells of the wild type (●), *CaTPS2/Catps2Δ* (■), and *Catps2Δ/Catps2Δ* (▲) strains were grown in fresh YPD medium at 30°C for 4 h. The cultures were divided in four and incubated further at 30, 37, 40, and 43°C. Samples for trehalose determination were taken at the indicated time points. ww, wet weight.

Southern and Northern blotting results and the determination of Tre6P phosphatase activity. The latter was reduced by about 50% in the heterozygous strain and was negligible in the homozygous strain. The latter result appeared to indicate that *C. albicans* contains very little Tre6P phosphatase activity besides that encoded by the *CaTPS2* gene. In *S. cerevisiae* heat treatment induces dramatic trehalose accumulation in a wild-type strain and Tre6P accumulation in a *tps2Δ* strain (15, 39). As a result the latter strain is temperature sensitive, growth is inhibited, and, as shown in this paper, the cells rapidly die after only a few hours at the high temperature. The latter is an important result, since it indicates that inhibition of Tre6P phosphatase in a fungal pathogen during infection could be fungicidal rather than just fungistatic, at least if there is enough stress response in the fungus to cause a large accumulation of Tre6P. Our results show that the *Catps2* homozygous deletion strain is also temperature sensitive and accumulates large amounts of Tre6P at the high temperature. The growth rate at 43°C is strongly reduced, and incubation for more than 6 h at

44°C results in a dramatic drop in viability. This indicates that also in *C. albicans* hyperaccumulation of Tre6P is probably fungicidal.

Unexpectedly, there was still a significant accumulation of trehalose in the *Catps2* homozygous deletion strain at the high temperature. Similar observations have been made for *S. cerevisiae* and *Schizosaccharomyces pombe*. Upon deletion of the *TPS2* gene in these yeasts, heat-induced trehalose accumulation still amounts to up to 20% of the level accumulated in wild-type cells (19, 55). This indicates that in vivo there must be significant Tre6P phosphatase capacity to sustain this accumulation of trehalose, although in vitro very little Tre6P phosphatase activity can be detected. There are several possible explanations. The alternative, presumably unspecific, phosphatases might be more active at high temperature or at the high Tre6P levels that accumulate in vivo. Also, the depletion of the free phosphate pool in vivo, because of its sequestration into Tre6P, might contribute to higher phosphatase activity in vivo because of reduction in phosphate repression or inhibition of

FIG. 5. Tre6P levels during heat treatment. Cells of the wild type (●), *CaTPS2/Catps2Δ* (■), and *Catps2Δ/Catps2Δ* (▲) strains were grown in fresh YPD medium at 30°C for 4 h. The cultures were then divided in two and incubated further at 30 and 43°C. Samples for Tre6P determination were taken at the indicated time points.

phosphatase enzymes. The significant residual trehalose accumulation in the *Catps2Δ* mutant might explain why the phenotype of the strain was not as stringent as that of the *S. cerevisiae tps2Δ* mutant and less stringent than expected (see below).

We have also investigated whether Tre6P accumulation in itself would evoke the stress response, causing even higher Tre6P accumulation and the initiation of a vicious circle. However, investigation of heat shock protein synthesis in a *Catps2Δ/Catps2Δ* strain at 41 or 43°C did not reveal a faster induction of heat shock proteins in spite of the large Tre6P accumulation (results not shown). Hence, it appears that Tre6P accumulation in itself does not induce a stress response.

The homozygous *Catps2Δ* strain was clearly less virulent than the heterozygous and wild-type strains. This supports the

rationale of the work, as follows. During infection the pathogen is probably under stress, e.g., because of the defense reactions of the host and an inadequate nutrient supply, etc. This causes a stress response, with Tre6P accumulation as a result. Because of the sequestration of free phosphate and inhibition of glycolysis, this compromises energy metabolism and weakens the pathogen. However, virulence of the homozygous deletion strain was not abolished. This can be due to several reasons. First, the residual trehalose accumulation in this strain at high temperature indicates that also during fungal infection nonspecific phosphatases might help to rescue the homozygous *Catps2Δ* strain from the Tre6P accumulation problem. Second, the stress experienced by the fungus might be lower than we anticipated. Perhaps pathogenic fungi have developed ways to

FIG. 6. *tps2Δ/tps2Δ* strains are not defective in hyphal development. Overnight cultures of the wild-type (A), *CaTPS2/Catps2Δ* (B), *Catps2Δ/Catps2Δ* (C), and reconstituted (D) strains were diluted in fresh YPD medium containing 10% fetal calf serum. Cells were photographed after incubation at 37°C for 90 min.

evade stressful reactions and conditions rather than ways to respond more vigorously to such conditions compared to non-pathogenic fungi. An alternative explanation for the reduced virulence of the homozygous *Catps2Δ* strain is possible interference of Tre6P with chitin biosynthesis, as has been shown for *Aspergillus nidulans* (7). At elevated temperatures when

FIG. 8. Survival curves for mice (female BALB/c, 20 g, 10 mice/group) systemically infected with 10^6 cells of the *C. albicans* wild-type (●), *CaTPS2/Catps2Δ* (■), *Catps2Δ/Catps2Δ* (▲), or *Catps2Δ/tps2Δ + pCaTPS2* (◆) strains. Similar results were obtained in three independent experiments.

high levels of Tre6P have accumulated, the activity of the first enzyme in chitin biosynthesis (glutamine:fructose-6-phosphate amidotransferase) is reduced in an *orl4*-disrupted strain (Tre6P phosphatase deficient), and the enzyme itself is labile. For *C. albicans* it has recently been shown that chitin-deficient (*chs3*-disrupted) strains are less virulent (8). The reduced virulence of the homozygous *Catps2Δ* strain might actually be due

FIG. 7. Influence of *CaTPS2* deletion on colony morphology. Cells of the wild-type strain, the heterozygous and homozygous *Catps2Δ* strains, and the reintegrated *CaTPS2* strain were grown in YPD medium and diluted to obtain single colonies on plates. The colonies were plated on YPD medium, Lee's medium, Spider medium, and SLAD medium. After 5 days, the colonies were photographed.

to a combination of impairments in metabolism. Phosphate sequestration and inhibition of hexokinase can impair the flux in glycolysis and proper energy generation, which affects many cellular processes. Moreover, these effects can be exacerbated by the presence of artificially high levels of a metabolite which strongly resembles glucose-6-phosphate and other sugar phosphates in its structure and therefore might also impair processes in which such sugar phosphates are involved.

The dimorphic switch from the yeast form to the filamentous form has been linked to virulence in *C. albicans*, and much attention has recently been paid to gene products required for the formation of hyphae (12, 20, 30). Also, the *Catps1*Δ mutant was deficient in hypha formation in medium containing glucose and calf serum, which might be related to its reduced virulence (54). Interestingly, the *Catps2*Δ mutant was not affected in hypha formation under any condition tested, indicating that either trehalose, Tre6P, or the *C. albicans* Tps1 protein might be required in some way for filamentation. Since the *TPS1* gene product is involved in the control of glucose influx into glycolysis in *S. cerevisiae* and other fungi (48), the deficiency in filamentation might be a side effect of impaired glycolytic control. Our results indicate that strains with proper filamentation capacity (at least under in vitro conditions) can still show strong reduction in virulence in vivo.

Is there a potential for Tre6P phosphatase as a target for antifungals? The results that we have obtained for virulence of the homozygous *Catps2*Δ strain are promising but not entirely convincing. Moreover, the use of a Tre6P phosphatase inhibitor as an antifungal drug will never be able to cause complete inhibition of the enzyme as is the case in the *Catps2*Δ strain. Such inhibitors, however, might also act to some extent on the alternative phosphatases that are able to dephosphorylate Tre6P to trehalose, although this might then also cause interference with phosphatases in the mammalian host cells. On the other hand, the systemic infection test in mice is a very stringent test. It is comparable to systemic infection with *C. albicans* of the bloodstream in humans, which occurs only in terminal patients. It is very well possible that *C. albicans* experiences more stressful conditions during the initial phases of the infection, for instance, the invasion of tissues. The virulence of the *Catps2*Δ strain might be much more reduced under experimental conditions simulating a natural infection. Another potential promising avenue is the stimulation of combination therapy with existing antifungals and inhibitors of Tre6P phosphatase. It is possible that the presence of antifungals elicits a stress response in *C. albicans*, stimulating trehalose accumulation in a wild-type strain and as a result triggering higher Tre6P accumulation in the presence of Tre6P phosphatase inhibitors. Hence, Tre6P phosphatase inhibitors might enhance the efficiency of existing antifungals. Because of these reasons, its absence in mammals, the absence of its substrate in mammals, its high specificity, and its highly convenient assay, it appears that Tre6P phosphatase still holds significant potential as a novel target for antifungals.

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