Disruption of the *Candida albicans* *ATC1* gene encoding a cell-linked acid trehalase decreases hypha formation and infectivity without affecting resistance to oxidative stress

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In *Candida albicans*, the *ATC1* gene, encoding a cell wall-associated acid trehalase, has been considered as a potentially interesting target in the search for new antifungal compounds. A phenotypic characterization of the double disruptant *atc1Δ/atc1Δ* mutant showed that it was unable to grow on exogenous trehalose as sole carbon source. Unlike actively growing cells from the parental strain (CAI4), the *atc1Δ* null mutant displayed higher resistance to environmental insults, such as heat shock (42 °C) or saline exposure (0.5 M NaCl), and to both mild and severe oxidative stress (5 and 50 mM H₂O₂), which are relevant during *in vivo* infections. Parallel measurements of intracellular trehalose and trehalose-metabolizing enzymes revealed that significant amounts of the disaccharide were stored in response to thermal and oxidative challenge in the two cell types. The antioxidant activities of catalase and glutathione reductase were triggered by moderate oxidative exposure (5 mM H₂O₂), whereas superoxide dismutase was inhibited dramatically by H₂O₂, where a more marked decrease was observed in *atc1Δ* cells. In turn, the *atc1Δ* mutant exhibited a decreased capacity of hypha and pseudohypha formation tested in different media. Finally, the homozygous null mutant in a mouse model of systemic candidiasis displayed strongly reduced pathogenicity compared with parental or heterozygous strains. These results suggest not only a novel role for the *ATC1* gene in dimorphism and infectivity, but also that an interconnection between stress resistance, dimorphic conversion and virulence in *C. albicans* may be reconsidered. They also support the hypothesis that Atc1p is not involved in the physiological hydrolysis of endogenous trehalose.

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INTRODUCTION

The opportunistic yeast *Candida albicans* has become the most prevalent fungal pathogen in humans (Verduyn Lunel *et al.*, 1999; Berman & Sudbery, 2002; Akins, 2005; Chauhan *et al.*, 2006). Superficial candidiasis is common in skin, oral cavity, gastrointestinal tract and vagina in otherwise healthy individuals (Verduyn Lunel *et al.*, 1999; Calderone & Fonzi, 2001; Eggimann *et al.*, 2003). However, *C. albicans* also causes life-threatening septicaemic infections in debilitated patients (Patterson, 2005; Chauhan *et al.*, 2006). Furthermore, the incidence of nosocomial bloodstream candidiasis

has increased dramatically in recent years (McNeil *et al.*, 2001; Patterson, 2005). Together with some well-characterized virulence factors, the low selective toxicity of currently available antifungal therapies and the increase in resistant strains might account for this high prevalence (Akins, 2005; Mukherjee *et al.*, 2005).

Some specific physiological features of *C. albicans* have been invoked as contributory factors of pathogenicity (Odds, 1994; Calderone & Fonzi, 2001; Gow *et al.*, 2002). *C. albicans* is a dimorphic organism capable of switching from yeast to mycelial (hypha and pseudohypha) morphology. Although both forms are present in infected hosts (Cannon *et al.*, 1994; Gow *et al.*, 2002; Sudbery *et al.*, 2004), a predominance of filamentous structures is apparently required for effective

Abbreviations: GR, glutathione reductase; ROS, reactive oxygen species; SOD, superoxide dismutase.

tissue invasion (Lo *et al.*, 1997). During the progress of an *in vivo* infection, the microbial pathogens should combat or adapt to the stresses imposed by the host defence machinery (Vázquez-Torres & Balish, 1997; Chauhan *et al.*, 2006). Following ingestion by active phagocytes, for instance, *C. albicans* encounters high levels of oxidants that must be counteracted. In fact, this fungus is relatively resistant to oxidative stress and has evolved a proficient antioxidant response to survive after phagocytosis (Vázquez-Torres & Balish, 1997; Chauhan *et al.*, 2006).

In the search for novel antifungal targets, the non-reducing disaccharide trehalose has emerged as a very promising candidate, as this sugar is present in bacteria, fungi and plants, but not in mammals (Argüelles, 2000; Elbein *et al.*, 2003). Trehalose acts mainly as an energy source and a stress protector in yeast and fungi (Thevelein, 1996; Argüelles, 2000; Elbein *et al.*, 2003). Trehalose metabolism has been investigated intensively in *C. albicans* in connection with fungal dimorphism, oxidative stress and protection, as has its possible involvement in virulence. In fact, the genes implicated in the biosynthesis of this disaccharide, namely *TPS1* (trehalose-6-phosphate synthase) and *TPS2* (trehalose-6-phosphate phosphatase), have been cloned and characterized, and the corresponding homozygous null mutants have been constructed. Disruption of *TPS1* impairs hypha formation and decreases infectivity (Zaragoza *et al.*, 1998), whereas *TPS2* is involved in cell integrity and pathogenicity, but is not required for dimorphic transition (Van Dijk *et al.*, 2002; Zaragoza *et al.*, 2002). Furthermore, the role of trehalose-catabolism enzymes remains unclear. The *NTC1* gene, encoding the neutral trehalase, lacks a specific function during dimorphic transition and pathogenicity (Eck *et al.*, 1997). In turn, trehalose appears to play a main protective role, specifically against oxidative stress rather than other environmental challenges (Alvarez-Peral *et al.*, 2002).

We have recently cloned the *ATC1* gene, which encodes a cell wall-linked acid trehalase (Pedreño *et al.*, 2004) in

C. albicans. The *atc1Δ* null mutant was unable to grow on trehalose as sole carbon source, confirming that Atc1p is required to hydrolyse exogenous trehalose (Pedreño *et al.*, 2004). In the present paper, we used this *atc1Δ/atc1Δ* mutant to analyse the putative changes in trehalose metabolism (synthesis, hydrolysis and endogenous content) in response to different stress challenges during the development of hyphal structures, and to test the hypothetical implication of the *ATC1* gene in the infectivity of this fungus. Our results suggest a new role for Atc1p in hypha formation and virulence, but they dismiss the interest of trehalose as a potentially useful antifungal target.

METHODS

Yeast strains, culture conditions and induction of hypha formation. *C. albicans* strains employed in this study are listed in Table 1. A detailed description of the constructions and procedures followed to obtain this set of mutants is given elsewhere (Pedreño *et al.*, 2004). A new strain, CEY-1 (CAI4-URA3), was constructed. The vector Clp10 (Murad *et al.*, 2000) was utilized to integrate the *URA3* gene into the genome of CAI4; to target the integration of Clp10, it was digested with *Nco*I, which cuts uniquely within the *RP10* sequence. Digested plasmid was used to transform *C. albicans* CAI4 according to Gietz *et al.* (1995). Transformed cells were selected as Ura⁺ in minimal medium without uridine; three independent positive transformants were examined and the correct integration of the *URA3* marker was confirmed by Southern blot.

Yeast-cell cultures were grown at 28 °C. They were shaken in a medium consisting of 2 % peptone, 1 % yeast extract and 2 % glucose (YPD). Strains were maintained by periodic subculturing in solid YPD. Cultures were supplemented directly with 10 % human blood serum at 37 °C to induce germ tubes. The serum was sterilized by filtration (0.22 µm). Filamentation was also examined in two ways: in Lee's medium by using starved resting cultures (OD₆₀₀ = 5.0), as described by Elorza *et al.* (1985), and on Spider medium plates. Germ-tube formation was monitored by means of a phase-contrast light microscope with a haemocytometer. When required, clumped cells were dispersed prior to microscopic examination by mild sonication

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

Strain	Genotype	Reference
CAI4	<i>ura3Δ::imm434/ura3Δ::imm434</i>	Fonzi & Irwin (1993)
ATC1-1	<i>atc1Δ::hisG-URA3-hisG/ATC1</i>	Pedreño <i>et al.</i> (2004)
	<i>ura3Δ::imm434/ura3Δ::imm434</i>	
ATC1-2	<i>atc1Δ::hisG/ATC1</i>	Pedreño <i>et al.</i> (2004)
	<i>ura3Δ::imm434/ura3Δ::imm434</i>	
ATC2-1	<i>atc1Δ::hisG/atc1Δ::hisG-URA3-hisG</i>	Pedreño <i>et al.</i> (2004)
	<i>ura3Δ::imm434/ura3Δ::imm434</i>	
ATC2-2	<i>atc1Δ::hisG/atc1Δ::hisG</i>	
	<i>ura3Δ::imm434/ura3Δ::imm434</i>	Pedreño <i>et al.</i> (2004)
ATC2-3	<i>atc1Δ::hisG/atc1Δ::hisG, RP10::ATC1::URA3</i>	Pedreño <i>et al.</i> (2004)
	<i>ura3Δ::imm434/ura3Δ::imm434</i>	
CEY-1	<i>ura3Δ::imm434/ura3Δ::imm434</i>	This work
	<i>RP10::URA3</i>	

(10–15 s). At least 200 cells were counted each time and the percentage of dimorphism was represented as the ratio of germ tube-forming cells to the total number of cells.

Heat-shock, oxidative and osmotic stress treatments. Cultures were grown in YPD until the exponential phase (OD_{600} = 0.8–1.0) and were then divided into several identical aliquots, which were treated with different H_2O_2 concentrations (5–50 mM), 0.5 M NaCl or 42 °C for oxidative, osmotic or heat-shock treatments, respectively, or maintained without treatment as a control and incubated at 28 °C for 1 h.

Viability was determined after samples had been diluted appropriately with sterile water by plating in triplicate on solid YPD after incubation for 2–3 days at 28 °C. Between 30 and 300 colonies were counted per plate. Survival was normalized to control samples (100 % viability).

Preparation of cell-free extracts. After exposure to different stresses, samples from the cultures were harvested and resuspended at known densities (10–15 mg ml⁻¹, wet weight) in MES extraction buffer (100 mM), pH 6.0, containing 5 mM cysteine and 0.1 mM PMSF. The cellular suspensions were transferred into small, pre-cooled tubes (1.0 cm diameter) with 1.5 g Ballotini glass beads (0.45 mm diameter). Cells were broken by vibrating the tubes vigorously in a vortex mixer. The tubes were cooled quickly on ice. The crude extract was then centrifuged at 10 000 g for 5 min and the pellet was resuspended in the same buffer at the initial density. For antioxidant assays, the supernatant fraction was filtered through Sephadex G-25 NAP columns (Amersham Biosciences), previously equilibrated with 50 mM sodium phosphate buffer, pH 7.8, to remove low-molecular-mass compounds.

Enzymic assays. The assay for acid trehalase was performed by incubating 50 µl cell-wall pellet with 200 µl trehalose (200 mM) prepared in 200 mM sodium citrate, pH 4.5, containing 2 mM EDTA. The reaction for neutral trehalase activity contained 50 µl cell-free extract (25–100 µg protein) and 200 µl trehalose (200 mM) prepared in 25 mM MES, pH 7.1, 125 µM CaCl₂. The reactions were incubated at 30 °C for 30 min and stopped by heating in a water bath at 100 °C for 5 min. The amount of glucose released was determined by using the glucose oxidase–peroxidase method. Specific activity is expressed as nmol glucose min⁻¹ (mg protein)⁻¹. Trehalose-6-phosphate synthase activity was measured at 40 °C in the supernatants of cell-free extracts as described previously (Argüelles *et al.*, 1999). Specific activity is expressed as nmol trehalose min⁻¹ (mg protein)⁻¹.

Catalase activity was determined at 240 nm by monitoring the removal of H_2O_2 , as described elsewhere (González-Párraga *et al.*, 2003). Glutathione reductase (GR) activity was assayed by measuring the glutathione disulfide (GSSG)-dependent oxidation of NADPH after Hernández *et al.* (1999). Measurements of superoxide dismutase (SOD) were carried out spectrophotometrically by the ferricytochrome *c* method using xanthine/xanthine oxidase as the source of O_2^- radicals (González-Párraga *et al.*, 2003). Data of enzymic activity were normalized in relation to a control measurement (100 %).

Other measurements. Intracellular trehalose was measured by following the method described by Blázquez *et al.* (1994). Briefly, cell samples (20–50 mg, wet weight) were washed, resuspended in 2 ml water and boiled for 30 min with occasional shaking. The concentration of trehalose released in the supernatant was determined with commercial trehalase (Sigma). The assay contained 90 µl 25 mM sodium acetate buffer, pH 5.6, 100 µl cell-free supernatant and 10 µl trehalase (2 units ml⁻¹). After incubation overnight at 37 °C, the amount of glucose produced was estimated by the glucose oxidase–peroxidase procedure. Parallel controls were run to correct the basal glucose levels.

Growth was monitored by measuring the OD_{600} of cultures in a Shimadzu UV spectrophotometer. Protein was estimated by the Lowry method, with BSA as standard.

Virulence in mice. Overnight yeast cultures were refreshed for 4 h at 28 °C in YPD to an OD_{600} of 0.8. Groups of eight female SWISS CD-1 mice (6–8 weeks old), weighing 21–25 g each, were inoculated in the lateral caudal vein with 1.0×10^6 viable *C. albicans* cells suspended in 150 µl PBS. Survival was monitored daily over a 1 month period. Survival curves were calculated according to the Kaplan–Meier method by using the PRISM program (GraphPad Software) and compared by using the log-rank test. A *P* value of ≤ 0.05 was considered significant.

Statistical analysis. All experiments were repeated at least three times, with the exception of the viability analysis and the infectivity assay in mice, which were performed twice with consistent results. Differences between the values recorded were tested for significance in accordance with Duncan's multiple range test.

RESULTS

Level of cell viability after several stress treatments

We analysed the degree of cell killing caused by several stress treatments (H_2O_2 , heat-shock and saline exposures) on exponential-phase blastoconidia obtained from both the parental strain CAI4 and its congenic null mutant ATC2-2 (*atc1Δ/atc1Δ*). Fig. 1 shows that CAI4 performed as the most sensitive strain under all environmental conditions tested. In the case of oxidative treatments, exposure to a moderate concentration of H_2O_2 (5 mM) resulted in a higher percentage of survival in the mutant after 1 h incubation (an almost 3-fold increase) than in parental cells, whilst a high concentration of H_2O_2 (50 mM) was responsible for a dramatic loss of viability in both strains. Again, ATC2-2 sustained a better resistance to oxidative stress (Fig. 1).

Similar results were obtained when these exponential-phase cultures were submitted to saline exposure (0.5 M NaCl) for 1 h. CAI4 cells suffered an appreciable loss of viability (approx. 40 %), whereas the ATC2-2 mutant only underwent a mortality of 12 % (Fig. 1). In contrast, a heat shock at 42 °C for 1 h triggered an approximately equivalent rate of cell death in both strains (approx. 25 %; Fig. 1b). When the treatment was prolonged to 3 h, however, the *atc1Δ/atc1Δ* cells exhibited a greater capacity to withstand heat shock than CAI4, whereas the sensitivity against saline and oxidative stress remained practically identical (Fig. 1c).

Changes in trehalose metabolism and trehalose content during stress treatments

The same cultures submitted to several stress conditions were used to monitor simultaneously possible changes in the enzymic activities involved in both trehalose metabolism and the intracellular content of this disaccharide. Acute oxidative stress (50 mM H_2O_2) promoted a significant synthesis of intracellular trehalose in both strains, whilst low H_2O_2 levels (5 mM) induced only a weak increase in

Fig. 1. Level of cell survival under different stress treatments in the *C. albicans* CAI4 strain (parental) and its congenic ATC2-2 mutant, deficient in acid trehalase activity. Exponential-phase YPD-grown cultures were adjusted to a cell density of between 1.2×10^6 and 1.5×10^6 cells ml^{-1} and subjected to the following stress challenges for 1 h (b) or 3 h (c): 5 and 50 mM H_2O_2 , 0.5 M NaCl or heat shock at 42°C . Identical, untreated samples were maintained at 28°C as a control (a). Viability data were normalized with regard to the control measurement (100%). The experiment was repeated twice in triplicate with consistent results and the values shown are the mean $\pm$ SD. The distinction between the mean values obtained was statistically significant to $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) according to Duncan's multiple range test.

disaccharide storage (Table 2). This increase in trehalose appears to be due to the partial inhibition of neutral trehalase after the addition of 5 mM H_2O_2 and the concomitant activation of trehalose synthase (Table 3). On the other hand, it is likely that the marked accumulation of sugar in response to 50 mM H_2O_2 exposure is due to a dramatic inhibition of Ntc1p (80 %), as Tps1p activity also underwent a degree of inactivation (around 20 %; Table 3). As expected, acid trehalase was virtually undetectable in the mutant (Table 3). A notable fact is that this activity followed the same trend in response to oxidative stress as that recorded for neutral trehalase (Table 3). However, Atc1p makes no significant contribution to the endogenous content of trehalose (Alvarez-Peral & Argüelles, 2000; Pedreño *et al.*, 2004).

The saline treatment imposed by the addition of 0.5 M NaCl induced a clear elevation (from 1.5- to 2.5-fold; Table 3) in

the two trehalase activities, with the exception of acid trehalase in the mutant, where this enzyme is not functional (Table 3). Interestingly, trehalose synthase activity was inactivated in both ATC2-2 and CAI4 cells, albeit to a lower extent in the latter strain (20 % decrease; Table 3). This could account for the lack of variability of the endogenous trehalose content upon saline stress in relation either to the control assay in CAI4 cells or to the slight decrease in the mutant (Table 2).

A pronounced increase (6-fold) of intracellular trehalose in both parental and mutant cultures was observed when a heat shock at 42°C was applied to exponential-phase cells (Table 2). This net synthesis arises from a 2-fold activation of trehalose synthase activity compared with the control assay (Table 3). The heat shock also triggered an increase in neutral trehalase activity (Table 3). The apparently contradictory parallel rise in endogenous trehalose and its hydrolase Ntc1p has been explained convincingly as the need of trehalose mobilization to yield enough energy to ensure proper cellular recovery after heat shock (Singer & Lindquist, 1998).

Induction of antioxidant activities in response to H_2O_2 treatments

The metabolic study was completed by analysing the hypothetical changes recorded in a set of activities that play an antioxidant role, such as catalase, GR and total SOD. For this purpose, exponential-phase cultures of the ATC2-2 and CAI4 strains were subjected to identical H_2O_2 exposures. The presence of a non-lethal H_2O_2 concentration (5 mM) promoted a clear rise in catalase activity (almost 40 %) in parental cells after incubation for 1 h; however, the increase was more conspicuous (2-fold) in the ATC2-2 cells (Fig. 2a). In both cell types, treatment with a potentially lethal H_2O_2 concentration (50 mM) caused a partial decrease in catalase activity in relation to the values recorded in control assays

Table 2. Effect of different stress treatments on the intracellular content of trehalose stored by exponential-phase cultures of the parental strain (CAI4) and its congenic mutant deficient in acid trehalase (ATC2-2)

Cultures were grown at 28°C in YPD, harvested in the exponential phase ($\text{OD}_{600} = 0.8\text{--}1.0$) and exposed to the indicated stress challenges for 60 min. Control samples were maintained at 28°C . Values are nmol trehalose (mg wet weight) $^{-1}$; the results are the mean $\pm$ SD of three independent measurements.

Treatment	CAI4	ATC2-2
Control	5.2 ± 0.3	4.8 ± 0.3
5 mM H_2O_2	6.9 ± 0.4	6.4 ± 0.3
50 mM H_2O_2	14.3 ± 0.8	13.5 ± 0.7
0.5 M NaCl	5.5 ± 0.3	3.7 ± 0.2
42°C	31.3 ± 1.8	27.3 ± 1.5

Table 3. Levels of enzymic activities corresponding to acid (Atc1p) and neutral (Ntc1p) trehalases, as well as trehalose-6-phosphate synthase (Tps1p), in exponential-phase cultures of the strain CAI4 and ATC2-2 (*atc1Δ*) null mutant submitted to different stress treatments

Numbers in parentheses represent the relative activity normalized in relation to the control for each parameter, where this control treatment is taken as 1.0.

Treatment	CAI4			ATC2-2		
	Atc1p*	Ntc1p*	Tps1p†	Atc1p*	Ntc1p*	Tps1p†
Control	2.5 (1.0)	19.2 (1.0)	15.0 (1.0)	<0.3	18.3 (1.0)	17.8 (1.0)
5 mM H ₂ O ₂	1.2 (0.5)	8.1 (0.4)	24.2 (1.6)	<0.3	6.9 (0.4)	29.0 (1.6)
50 mM H ₂ O ₂	0.6 (0.2)	3.2 (0.2)	11.6 (0.8)	<0.3	2.0 (0.1)	9.5 (0.5)
0.5 M NaCl	6.4 (2.6)	30.2 (1.6)	9.1 (0.6)	<0.3	28.6 (1.6)	12.7 (0.8)
42 °C	3.6 (1.4)	33.6 (1.8)	30.0 (2.0)	<0.3	35.8 (2.0)	31.5 (1.8)

*Values are nmol glucose min⁻¹ (mg protein)⁻¹.

†Values are nmol trehalose min⁻¹ (mg protein)⁻¹.

(Fig. 2a). As regards GR, the results shown in Fig. 2(b) support the fact that this activity was strikingly high when either mild or acute oxidative exposure (5 or 50 mM H₂O₂) was applied for 1 h (Fig. 2b). These data are consistent with the oxidative stress-induced GR activation observed in a *tps1Δ* mutant deficient in trehalose synthesis (González-Párraga *et al.*, 2003).

In turn, total SOD activity behaved in a completely opposite way to catalase and GR activities in response to oxidative stress. This enzyme has been reported to be an important virulence factor in *C. albicans* (Hwang *et al.*, 2002). Measurements of total SOD activity after incubation with 5 mM H₂O₂ revealed a 40 % decrease of the initial activity in CAI4 cells (Fig. 2c) and a slightly higher loss of activity in mutant cells (Fig. 2c). Likewise, toxic H₂O₂ concentrations (50 mM) were harmful for this enzymic activity, which suffered a larger reduction in ATC2-2 cultures (80 %) than in parental-type cells (60 %; Fig. 2c).

Effect of *ATC1* gene disruption on dimorphic conversion

The relationship between hypha formation and trehalose metabolism has been disputed in the past (Ram *et al.*, 1984; Argüelles *et al.*, 1999). Genetic evidence demonstrated

Fig. 2. Changes in the enzymic activities of catalase (a), glutathione reductase (b) and superoxide dismutase (c) in response to both gentle and intense oxidative stress triggered by H₂O₂ for 1 h. Exponential-phase cultures (OD₆₀₀ = 0.8–1.0) of the two *C. albicans* strains were harvested and processed as described in Methods. Data of activity were normalized with regard to a control measurement (100 %). The values represent the mean ± SD of three determinations. The distinction between the mean values obtained was statistically significant to *P* < 0.01 (**) and *P* < 0.001 (***) according to Duncan's multiple range test.

Fig. 3. Colony morphologies shown by the parental strain (CAI4) and single (ATC1-2) and double (ATC2-2) disruptant mutants in the *ATC1* gene on solid Spider medium. Plates were incubated for 7 days at 37 °C. The upper panel corresponds to a whole colony and detail of the border is shown in the lower panel.

conclusively that neither the *TPS1* (trehalose-6-phosphate synthase) nor the *NTC1* (neutral trehalase) gene is involved in this process (Eck *et al.*, 1997; Zaragoza *et al.*, 1998). In order to analyse the hypothetical role of the *ATC1* gene in dimorphism, exponential-phase cultures from the ATC1-2

(*ATC1/atc1Δ*), ATC2-2 (*atc1Δ/atc1Δ*) and CAI4 strains were subjected to a set of conditions under which efficient hyphal growth may be induced, e.g. Spider medium (Fig. 3) or human serum at 37 °C (Fig. 4a), whereas stationary-phase cells were induced in Lee's medium, as reported by Elorza *et al.* (1985) (Fig. 4b). All of these procedures caused a considerable delay in the degree of germ-tube formation in the ATC2-2 null mutant compared with its isogenic parent CAI4 (Figs 3 and 4). The heterozygous ATC1-2 mutant exhibited an intermediate percentage of filamentous growth in Spider and Lee's media (Figs 3 and 4b), although it was similar to the value measured in CAI4 cells in YPD supplemented with human serum (Fig. 4a). When equivalent experiments were performed with the corresponding *URA*⁺ instead of *URA*[−] strains, using CEY-1 strain as control, the results obtained revealed that the homozygous *atc1Δ* mutant (ATC2-1) was also defective in germ-tube formation (data not shown).

Measurements of intracellular trehalose carried out at 37 °C in human serum-supplemented cultures revealed a progressive increase in the disaccharide content when the time of dimorphic induction was prolonged (Table 4). ATC2-2 cultures, which exhibited a lower capacity to produce germ tubes (Fig. 4), accumulated more endogenous trehalose

Fig. 4. (a) Percentage of germ-tube formation in human serum at 37 °C. Exponential-phase cultures grown in YPD at 28 °C were supplemented with human serum and transferred to 37 °C as described in Methods. Controls at 28 and 37 °C without serum were run in parallel. (b) Percentage of germ-tube formation in Lee's medium. Cultures were incubated in liquid Lee's medium at 28 °C and harvested in the stationary phase, subjected to metabolic starvation for 24 h at 4 °C and then incubated in Lee's medium at 37 °C. Values represent the mean ± SD of three determinations.

Table 4. Changes in trehalose content during hypha formation induced by addition of serum at 37 °C in the ATC2-2 (*atc1Δ/atc1Δ*) and CAI4 strains

Cultures were grown in YPD at 28 °C, harvested in the exponential phase and supplemented with human serum (10 %) at 37 °C. Controls were maintained in parallel at 28 and 37 °C without serum addition. Samples were taken after 1 and 7 h serum supply. Values [nmol trehalose (mg wet weight)^{−1}] represent the mean ± SD of three independent determinations. The percentages of filamentation after 7 h induction were 47.5 % for CAI4 and 38.7 % for ATC2-2 cells. For other details, see Fig. 4(a).

Treatment	Serum supply			
	1 h		7 h	
	CAI4	ATC2-2	CAI4	ATC2-2
Control (28 °C)	4.3 ± 0.3	4.8 ± 0.2	4.7 ± 0.3	5.1 ± 0.2
37 °C −serum	6.1 ± 0.3	7.2 ± 0.4	6.2 ± 0.4	6.4 ± 0.5
37 °C +serum	7.7 ± 0.5	9.7 ± 0.6	17.4 ± 1.0	20.4 ± 1.2

than CAI4 cells (Table 4). Hence, there is a clear lack of correlation between the degree of hyphal formation and the concentration of stored trehalose, which supports the hypothesis that disaccharide is not required to enter dimorphic transition (Zaragoza *et al.*, 1998; Argüelles *et al.*, 1999).

Disruption of *ATC1* decreases infectivity in mice

As *ura3* auxotrophy has an effect on infectivity in certain genetic *C. albicans* backgrounds (Shepherd, 1985), the corresponding *URA*⁺ transformants were obtained from the strains used in this study. In order to determine whether deletion of *ATC1* affects the virulence of *C. albicans*, groups of selected mice were injected in the lateral caudal vein with 1 × 10⁶ cells of the following strains: CEY-1 (CAI4-*URA*⁺), the ATC1-1 (*ATC1/atc1Δ URA*⁺) and ATC2-1 (*atc1Δ/atc1Δ URA*⁺) mutants, as well as the reconstituted strain with the intact *ATC1* gene (ATC2-3). The pattern of mouse survival is shown in Fig. 5. After 16 days, no survivors were found in

the group of mice inoculated with parental cells, whereas > 75 % of those inoculated with the ATC2-1 mutant were still alive after at least 1 month (Fig. 5). Mice infected with the heterozygous mutant (ATC1-1) showed an intermediate behaviour between mice infected with the CEY-1 cells and those infected with the homozygous mutant (Fig. 5), whereas injection of the ATC2-3 strain (with the reintroduced *ATC1* gene) evidenced a behaviour similar to that recorded in parentally and heterozygously infected mice (Fig. 5). The experiment was repeated once more with consistent results. These data support the idea that at least one *ATC1* copy is required to regain infectivity.

DISCUSSION

The enzymes involved in trehalose metabolism have emerged as potential antifungal targets, according to the following pieces of evidence: (i) the non-reducing sugar is present in bacteria, fungi, plants and invertebrates, but is absent in mammals (Argüelles, 2000; Elbein *et al.*, 2003); (ii) trehalose accumulation may be a specific protective mechanism of pathogenic fungi during *in vivo* infection (Alvarez-Peral & Argüelles, 2000); (iii) the genes encoding trehalose-6-phosphate synthase (*TPS1*) and trehalose-6-phosphate phosphatase (*TPS2*) are determinants of virulence, and the corresponding *tps1Δ* and *tps2Δ* null mutants show high sensitivity to severe oxidative stress (Zaragoza *et al.*, 1998, 2002; Alvarez-Peral *et al.*, 2002; Van Dijck *et al.*, 2002). The design of antifungals against the cell wall has received priority, and the echinocandins, which act as potent inhibitors of glucan synthases, are currently in clinical use (Denning, 2003).

The cloning of the *ATC1* gene (Pedreño *et al.*, 2004) has led to it being considered as a promising candidate for the development of antifungal compounds, as its product, acid trehalase, combines both features, namely *Atc1p* is linked to the cell wall and it is required for the hydrolysis of exogenous trehalose (Pedreño *et al.*, 2004). To explore this possibility, we performed a phenotypic analysis of the *atc1Δ* null mutant to study its resistance to several stresses, its ability to enter dimorphism and its degree of virulence. Thus, *atc1Δ/atc1Δ*

Fig. 5. Infectivity of the *C. albicans* parental strain and the corresponding *Ura*⁺ mutants for *ATC1*. Mice (SWISS CD-1) were inoculated with 1.0 × 10⁶ cells of the CEY-1 (CAI4-*URA*⁺) strain or the ATC1-1 mutant, the ATC2-1 mutant or an *atc1Δ/atc1Δ* mutant in which the *ATC1* gene was reinserted (ATC2-3, *URA*⁺). Control mice were inoculated with PBS and survival was checked daily. The percentage of animals surviving is plotted against time. Similar results were obtained in an independent experiment.

cells showed a greater capacity to withstand oxidative and osmotic challenges than those of its parental strain (Fig. 1). Of the antioxidant enzymes monitored, only catalase and GR appear to act as cell protectors against oxidative exposure, whereas SOD was inhibited (Fig. 2). As Atc1p is a cell wall-linked protein, the stress resistance in *atc1Δ* cells is presumably due to structural modifications associated with *ATC1* disruption, as the endogenous trehalose content accumulated in mutant and parental cultures was similar (Table 2).

The need for trehalose mobilization as a putative factor involved in dimorphic transition and the hydrolytic role played by acid trehalase in this process have been disputed (Ram *et al.*, 1984; Zaragoza *et al.*, 1998; Argüelles *et al.*, 1999). Available data support the hypothesis that human serum-induced dimorphism is independent of trehalose hydrolysis (Argüelles *et al.*, 1999), although no conclusive genetic evidence exists. For this reason, we analysed the ability of the *ATC2-2* (*atc1Δ*) null mutant to enter dimorphic conversion in response to various stimuli. The morphogenetic programme that induces hyphal outgrowth underwent a slight delay in growing *atc1Δ* blastoconidia (Figs 3–5). This phenotype of retardation in dimorphism might, once again, be the consequence of modifications in the cell-wall structure after the *ATC1* double disruption, rather than to changes in stored trehalose. However, a direct effect on the signalling pathways governing cell morphology cannot be ruled out (Berman & Sudbery, 2002).

In comparison to the parental and heterozygous strains, the homozygous *atc1Δ* mutant showed a significant reduction in pathogenicity during long-term infection in mice (Fig. 5). This attenuated virulence is clearly comparable with similar phenotypes caused by the double disruption of the *TPS1* and *TPS2* genes involved in trehalose biosynthesis (Zaragoza *et al.*, 1998, 2002; Van Dijck *et al.*, 2002). However, our data discount the possibility that this lower infectivity could be due to differences in trehalose accumulation; whereas the *tps1Δ* and *tps2Δ* null mutants exhibited a negligible or clearly diminished reduction of endogenous trehalose (Zaragoza *et al.*, 1998, 2002; Van Dijck *et al.*, 2002), the *atc1Δ* cells strongly accumulated disaccharide upon exposure to several stresses (Table 2). Therefore, we conclude preliminarily that the expectations raised concerning the potential usefulness of the *ATC1* gene as an antifungal target are ill-founded, because *atc1Δ* cells simultaneously displayed reduced filamentation and virulence, but enhanced resistance to heat shock and oxidative stress.

Why is the *atc1Δ* mutant more resistant to stress *in vitro*, but less virulent *in vivo*? The success of an opportunistic pathogen during infection stems, in part, from its capacity to counteract the defence reactions from the host, including the high levels of reactive oxygen species (ROS) released after ingestion by phagocytes (Vázquez-Torres & Balish, 1997). The *atc1Δ* cells might withstand the damage provoked by endogenous ROS, as they store a large amount of intracellular trehalose when incubated at 37 °C with human

serum (Table 4) and the disaccharide in *C. albicans* acts as a specific protectant against oxidative stress (Alvarez-Peral *et al.*, 2002). On the other hand, it is well known that structural perturbations of cell-wall integrity affect the capacity of host recognition, adhesion and further infection of host cells (Gow *et al.*, 2002; Bates *et al.*, 2006). Furthermore, recent evidence has demonstrated the critical importance of *N*-glycans embedded in the outer layer of the cell wall in host–fungal interaction and virulence (Bates *et al.*, 2006), and that Atc1p contains 20 potential *N*-glycosylation sites (Pedreño *et al.*, 2004). Hence, disruption of *ATC1* probably alters the external surface stability, with a subsequent reduction in virulence.

The connection between hypha formation and virulence in *C. albicans* is controversial (Cannon *et al.*, 1994; Kobayashi & Cutler, 1998; Gow *et al.*, 2002). In general, mycelium shape appears to be associated with infectivity, and Lo *et al.* (1997) demonstrated how mutants unable to filament are avirulent. However, all cellular types are present in clinical lesions caused by other dimorphic fungi (*Histoplasma*, *Blastomyces*), where the yeast-like form is predominant in pathogenesis (Cannon *et al.*, 1994). With regard to the virulence of mutants altered in trehalose metabolism, the link between dimorphic conversion and virulence is unclear. Thus, whilst disruption of the *TPS1* gene impairs the yeast-to-hypha transition and decreases infectivity (Zaragoza *et al.*, 1998), *TPS2* is apparently necessary for proper cell integrity (Van Dijck *et al.*, 2002; Zaragoza *et al.*, 2002). However, the homozygous mutant showed strongly reduced virulence, without affecting the capacity to produce germ tubes and subsequent hypha development (Van Dijck *et al.*, 2002; Zaragoza *et al.*, 2002). The performance of the *atc1Δ* mutant is quite similar to that of *tps1Δ*, although the *atc1Δ* cells synthesize a high quantity of intracellular trehalose during supply of human serum at 37 °C (Tables 2 and 4). Indeed, such a level of trehalose might be relevant in the course of an *in vivo* infection.

On the other hand, the *NTC1* gene, which encodes the cytosolic neutral trehalase, does not seem to play a significant role in growth and infectivity (Eck *et al.*, 1997) or to participate in the protective response against oxidative stress (Pedreño *et al.*, 2006). Hence, *ATC1* is the first gene involved in trehalose catabolism that is necessary for virulence in *C. albicans*.

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