

# Trehalose is required for the acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*

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**Trehalose is a non-reducing disaccharide found at high concentrations in *Aspergillus nidulans* conidia and rapidly degraded upon induction of conidial germination. Furthermore, trehalose is accumulated in response to a heat shock or to an oxidative shock. The authors have characterized the *A. nidulans* *tpsA* gene encoding trehalose-6-phosphate synthase, which catalyses the first step in trehalose biosynthesis. Expression of *tpsA* in a *Saccharomyces cerevisiae* *tps1* mutant revealed that the *tpsA* gene product is a functional equivalent of the yeast Tps1 trehalose-6-phosphate synthase. The *A. nidulans* *tpsA*-null mutant does not produce trehalose during conidiation or in response to various stress conditions. While germlings of the *tpsA* mutant show an increased sensitivity to moderate stress conditions (growth at 45 °C or in the presence of 2 mM H<sub>2</sub>O<sub>2</sub>), they display a response to severe stress (60 min at 50 °C or in the presence of 100 mM H<sub>2</sub>O<sub>2</sub>) similar to that of wild-type germlings. Furthermore, conidia of the *tpsA* mutant show a rapid loss of viability upon storage. These results are consistent with a role of trehalose in the acquisition of stress tolerance. Inactivation of the *tpsA* gene also results in increased steady-state levels of sugar phosphates but does not prevent growth on rapidly metabolizable carbon sources (glucose, fructose) as seen in *Saccharomyces cerevisiae*. This suggests that trehalose 6-phosphate is a physiological inhibitor of hexokinase but that this control is not essential for proper glycolytic flux in *A. nidulans*. Interestingly, *tpsA* transcription is not induced in response to heat shock or during conidiation, indicating that trehalose accumulation is probably due to a post-translational activation process of the trehalose 6-phosphate synthase.**

**Keywords:** trehalose 6-phosphate synthase, spore germination, glycolysis, heat stress, oxidative stress, hexokinase

## INTRODUCTION

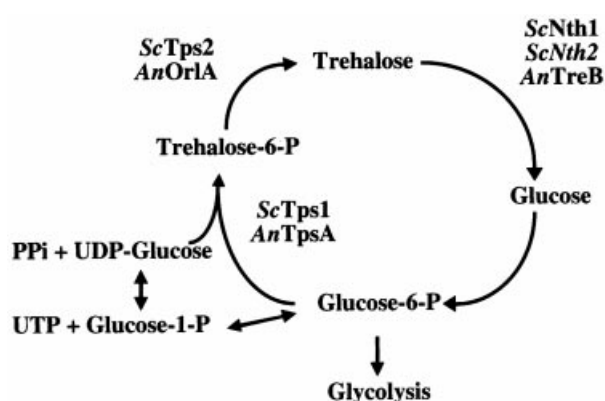
Trehalose ( $\alpha$ -D-glucopyranosyl- $\alpha$ -D-glucopyranoside) is a non-reducing disaccharide found in a wide variety of organisms (bacteria, fungi, protozoa, plants). In lower eukaryotes, it constitutes up to 15 % of the dry weight of

stationary-phase cells or spores (Elbein, 1974). The universal mobilization of trehalose during growth resumption from resting stages supports a role as a storage carbohydrate (Arguelles, 2000; Thevelein, 1984, 1996). However, because of a number of physico-chemical properties, including high hydrophilicity and chemical stability, non-hygroscopic glass formation and the absence of internal hydrogen bond formation, trehalose is apparently able to serve a unique role as stress metabolite (Arguelles, 2000; Thevelein, 1996).

In the yeast *Saccharomyces cerevisiae*, biosynthesis of trehalose is mediated by a multi-protein complex that

**Abbreviations:** EST, expressed sequence tag; 5-FOA, 5-fluoro-orotic acid; T6P, trehalose 6-phosphate; T6PP, trehalose-6-phosphate phosphatase; T6PS, trehalose-6-phosphate synthase.

The GenBank accession number for the sequence reported in this paper is AF043230.



**Fig. 1.** Trehalose metabolism in fungi. ScTps1 and AnTpsA, trehalose-6-phosphate synthase; ScTps2 and AnOrlA, trehalose-6-phosphate phosphatase; ScNth1, ScNth2 and AnTreB, neutral trehalase; Sc, *S. cerevisiae*; An, *A. nidulans*.

contains a trehalose-6-phosphate synthase (T6PS; Fig. 1) encoded by *TPS1* and a trehalose-6-phosphate phosphatase (T6PP; Fig. 1) encoded by *TPS2* (Bell *et al.*, 1992, 1998; de Virgilio *et al.*, 1993). This multi-protein complex contains regulatory subunits, the products of the redundant *TSL1* and *TPS3* genes, which share a conserved amino-terminal domain with Tps1 and Tps2 (Bell *et al.*, 1998; Reinders *et al.*, 1997; Vuorio *et al.*, 1993). Mobilization of the trehalose pool in response to various stimuli is mediated by a neutral trehalase encoded by *NTH1* (Kopp *et al.*, 1993) while the role of a second neutral trehalase encoded by *NTH2* remains uncertain (Nwaka *et al.*, 1995). Studies using mutants in the different genes of trehalose metabolism in *S. cerevisiae* and in other yeasts support the protective role of trehalose and more specifically a role in the acquisition of stress tolerance (Arguelles, 2000). However, additional functions have been proposed for trehalose 6-phosphate (T6P), which is the first intermediate in the biosynthesis of trehalose (Fig. 1). In particular, analysis of *S. cerevisiae* mutants impaired in the biosynthesis of T6P has indicated a role for T6P and the T6PS in the control of the influx of glucose into glycolysis (Blazquez *et al.*, 1993; Bonini *et al.*, 2000; Hohmann *et al.*, 1996; Thevelein & Hohmann, 1995).

Several studies have been conducted to determine the role of trehalose and T6P in filamentous fungi but they have remained inconclusive. Two genes encoding T6PS have been identified in *Aspergillus niger* (Wolschek & Kubicek, 1997). However, disruption of only one of these genes has been achieved and therefore an unambiguous role for T6P or trehalose in filamentous fungi could not be deduced from this study (Wolschek & Kubicek, 1997). Arisan-Atac *et al.* (1996) have nevertheless shown that T6P participates in the control of citrate production in *A. niger*. In *A. nidulans*, T6PP is encoded by the *orlA* gene, the inactivation of which results in an osmoremediable thermosensitive growth phenotype that can be explained by an inhibitory role of

T6P in the control of chitin biosynthesis (Borgia *et al.*, 1996). However, *orlA* mutants produce normal levels of trehalose and are therefore not suited to determine the role of trehalose in filamentous fungi (Borgia *et al.*, 1996). T6P is a potent inhibitor of hexokinase also in *A. nidulans* and *A. niger*, suggesting a role in the control of glycolytic flux in these species (Panneman *et al.*, 1998; Ruijter *et al.*, 1996). Finally, neutral trehalases have also been identified in filamentous fungi and a role in the control of trehalose mobilization has been demonstrated during growth resumption stages, in particular spore germination, in a manner similar to that observed in yeast (d'Enfert *et al.*, 1999).

Interestingly, analysis of *A. nidulans* mutants devoid of neutral trehalase has suggested that trehalose has a minor role as a storage carbohydrate and could function in the protection of germinating conidia against thermal stress (d'Enfert *et al.*, 1999).

Here, we report the characterization of the *tpsA* gene of *A. nidulans*, encoding a T6PS, and the construction of an *A. nidulans tpsA*-null mutant. This mutant fails to accumulate trehalose in response to a variety of stress conditions but this defect is not associated with an increased sensitivity to a short exposure of the same stress conditions. In contrast, this mutant is defective for growth at high temperature, shows an increased sensitivity to long exposure to sublethal doses of reactive oxygen species and has reduced spore viability, thus suggesting a role for trehalose in the resistance of *A. nidulans* to sustained exposure to various stress conditions, including starvation.

## METHODS

**Strains and growth conditions.** *A. nidulans* strains FGSC28 (*pabaA6 biA1*) and FGSC773 (*wA3; pyroA4; pyrG89*) were obtained from the Fungal Genetics Stock Centre (University of Kansas, Kansas City, KA, USA). *A. nidulans* strains CEA150 (*wA3; pyroA4; pyrG89; tpsA-AfpyrG-tpsAA*) and CEA152 (*wA3; pyroA4; pyrG89; tpsAA*) are derivatives of FGSC773 obtained in the course of this study. Growth conditions for *A. nidulans* strains have been described (d'Enfert & Fontaine, 1997).  $H_2O_2$  was added to solid culture media in a 0–5 mM range. Cultures of *A. nidulans* strains for the assay of heat or oxidative-shock sensitivity were inoculated at  $2 \times 10^7$  conidia  $ml^{-1}$  and grown for 3 h at 30 °C in minimal glucose medium containing 0.01% Tween-20. Following heat shock (0–60 min at 50 °C) or oxidative shock (100 mM  $H_2O_2$  for 0–60 min), an aliquot of each culture was withdrawn and serially diluted in PBS (150 mM NaCl, 10 mM sodium phosphate pH 7.2)/0.1% Tween-20. Heat- or  $H_2O_2$ -shocked cells were then plated on complete medium containing 0.1% Triton X-100 in order to limit the growth of the colonies. Colonies were counted after a 2 d incubation at 37 °C. The same cultures were used to measure trehalose content in the conidia with previously described procedures (d'Enfert & Fontaine, 1997). Alternatively, trehalose, T6P, sugar phosphates and ATP were measured using extracts from mycelia grown at 30 °C in minimal glucose medium for 4 h using the procedure of Ruijter & Visser (1996). Conidium viability was monitored using duplicates of two independent stocks of FGSC773 and CEA152 conidia stored at either 4 °C or 20 °C in

**Table 1.** Oligonucleotides used in this study

Oligonucleotide	Sequence
tps1F	5'-TGGCCNCTNTTCCAYTACCA-3'
tps1B	5'-GGNACNCCYTTRATGTARTC-3'
tps5'Bgl	5'-TGCAGATCTCCCGCGTTGAGAAATCC-3'
tps3'Not	5'-TTGATGCGGCCGCTACTGTGACGAAGTCTC-3'
tps4	5'-GTTGCGAGCCAAGTTCAG-3'
tps5	5'-CCCTGGAATTCTATCCCA-3'
tps6	5'-CATTGTGATGTTGAACA-3'
tps9	5'-GGTTGGCAGTCCCAGCAA-3'
tps10	5'-AGGAGATCCCGACTCTG-3'
tps11	5'-TACTCGGTCAGACCAGG-3'

PBS/0.1% Tween-20. At different time intervals, an aliquot of each stock was serially diluted and plated in duplicate on complete medium containing 0.01% Triton X-100. Colonies were counted after a 2 d incubation at 37 °C. Conidiospore germination was monitored by microscopic examination of slides coated with minimal glucose medium and spot-inoculated with approximately  $10^4$  freshly harvested conidia. The percentage of germinated spores was recorded at different times.

The *S. cerevisiae* strains used in this study were W303.1A (Thomas & Rothstein, 1989; *MATa leu2-3,112 ura3-1 trp1-92 his3-11,15 ade2-1 can1-100 GAL SUC mal*) and the isogenic YSH290 strain containing the *tps1Δ* mutation (Neves *et al.*, 1995). Yeast cells were routinely grown on a rotary shaker at 30 °C in yeast nitrogen base medium (YNB; Sherman, 1991) containing 2% glucose or 2% galactose (YSH290) as carbon source.

*Escherichia coli* strains PAP105 [ $\Delta(lac-pro)$  F'(*lacI*<sup>q1</sup>  $\Delta(lacZ)$ M15 *pro*<sup>+</sup> Tn10)] and DH5 $\alpha$  (Woodcock *et al.*, 1989) were used for plasmid propagation. The  $\beta$ -lactam antibiotic carbenicillin (100  $\mu$ g ml<sup>-1</sup>) and tetracycline (15  $\mu$ g ml<sup>-1</sup>) were added to the growth medium when required.

#### PCR amplification of a segment of the *A. nidulans tpsA* gene.

The genomic DNA of *A. nidulans* FGSC28 prepared according to Girardin *et al.* (1993) was used as template to amplify a segment of genes potentially encoding a T6PS. The sense and antisense primers (tps1F and tps1B, Table 1) were based on amino-acid sequences (WPLFHYH and DYIKGVP, respectively) conserved in several fungal T6PS (Bell *et al.*, 1992; Blazquez *et al.*, 1994; Luyten *et al.*, 1993; Wolschek & Kubicek, 1997). The amplification protocol consisted of a denaturation step at 94 °C for 5 min followed by 35 cycles of the following steps: denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 2 min. A last elongation step was carried out at 72 °C for 10 min. An approximately 650 bp amplification product was gel purified and cloned in Bluescript SK<sup>+</sup> (Stratagene) using standard cloning procedures. Two plasmids, pTPS1 and pTPS2, were obtained that carry the amplification product in opposite orientations.

**DNA and RNA manipulations.** General recombinant DNA techniques and Southern-blot analyses were essentially performed according to Sambrook *et al.* (1989) and Ausubel *et al.* (1992). Transformation of calcium-manganese-treated *E. coli* was as described by Hanahan *et al.* (1991). Oligonucleotides used in this study were obtained from Genset (Paris, France) and are listed in Table 1. A *Bam*HI–*Eco*RV fragment

corresponding to the cloned PCR product was labelled with [ $\alpha$ -<sup>32</sup>P]dCTP using the Megaprime kit (Amersham) and used to probe a replica of a genomic library (a gift from C. Scazzocchio) made in plasmid pFB39, a derivative of pUC18 carrying the *A. nidulans argB* gene. This screening yielded a single positive plasmid, pTPS4, which lacked the 3' end of the *tpsA* gene as deduced from DNA sequencing. To identify a clone carrying the entire *tpsA* gene, the chromosome-specific libraries of *A. nidulans* genomic DNA (Brody *et al.*, 1991) that had been obtained from the Fungal Genetic Stock Center and transferred onto nylon membranes (ZetaProbe, Bio-Rad) were probed using a 400 bp *Eco*RI fragment located at the 3' end of the cloned *tpsA* region and labelled as above. The plasmids pTPS6 and pTPS7 are derivatives of pUC18 and pBLSN<sup>+</sup> (d'Enfert, 1996), respectively, that carry a 1.75 kb *Sall*–*Sma*I fragment containing the region of *tpsA* carried by pTPS4. The plasmids pTPS11 and pTPS12 are derivatives of pBLSN<sup>+</sup> which carry a 2.4 kb *Eco*RI fragment of cosmid L24E04 in opposite orientations. DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) on double-stranded plasmids derived from pTPS6, pTPS7, pTPS11 and pTPS12 by internal restriction-enzyme-mediated deletions and using a set of appropriate oligonucleotide primers. The sequence of a 2699 bp fragment was read at least twice on each strand and is deposited in the GenBank database under accession number AF043230.

Plasmid pTPS13 was obtained by subcloning the 0.8 kb *Sall*–*Clal*I fragment of pTPS7 into *Sall*–*Clal*I-digested pTPS11 $\Delta$ P, a derivative of pTPS11 with an internal deletion of a *Pst*I fragment. Plasmid pTPS17 was then obtained by subcloning a *Ksp*I–*Eco*RV fragment carrying the *A. fumigatus pyrG* gene (Weidner *et al.*, 1998) into *Ksp*I/*Sma*I-digested pTPS13. Plasmid pTPS17 was used to transform protoplasts of *A. nidulans* strains (Osmani *et al.*, 1987). Genomic DNA from 15 prototrophic transformants was prepared according to Mol *et al.* (1998) and screened by PCR using primers tps10 and tps11 (Table 1). While a 2006 bp product is expected in transformants carrying an ectopic integration of pTPS17 or a *tpsAΔ*–*Afp*yrG–*tpsA* allele, a 1462 bp fragment is expected in transformants with a *tpsA*–*pyrG*–*tpsAΔ* allele. Putative merodiploids with a *tpsA*–*Afp*yrG–*tpsAΔ* allele were confirmed by Southern blot analysis of *Eco*RI/*Sall*-digested genomic DNA prepared according to Girardin *et al.* (1993) and probed with a 0.8 kb *Clal*–*Sall*I fragment of pTPS13 that had been labelled with the Rediprime labelling kit (Amersham). Washed membranes were exposed to X-omat films (Kodak). While strains carrying only a wild-type *tpsA* allele show a 1.15 kb hybridizing fragment, *tpsA*–*Afp*yrG–*tpsAΔ* merodiploids show

1.15 kb and 0.8 kb hybridizing fragments corresponding to the wild-type and mutant allele respectively. Conversion of the *tpsA*-*AfpYrG*-*tpsAA* allele to the *tpsAA* allele was obtained by plating conidia of strain CEA150 on minimal glucose plates containing 1 mg ml<sup>-1</sup> 5-fluoro-orotic acid (5-FOA), uridine, uracil and pyridoxine.HCl, thus promoting the excision of the *A. fumigatus* *pyrG* gene through recombination between the two *tpsA* alleles (d'Enfert & Fontaine, 1997). The nature of the *tpsA* allele in 5-FOA-resistant clones was checked by PCR using primers *tps10* and *tps11* (Table 1), which can discriminate between wild-type *tpsA* and *tpsAA*, and with primers *tps4* and *tps5* (Table 1), which yield only a 363 bp product if the wild-type *tpsA* gene is present. Excision of the *A. fumigatus* *pyrG* gene was confirmed by Southern blot analysis as described above. In this case a single *EcoRI*-*SalI* fragment could be detected corresponding to either the wild-type allele (1.15 kb) or the mutant *tpsAA* allele (0.8 kb).

Preparation of total RNA from conidia, germinating conidia, heat-shocked germinating conidia, mycelia and developing cultures of *A. nidulans* strain *pabaA1* and FGSC773 has been previously reported (d'Enfert *et al.*, 1999). RT-PCR experiments were achieved using the Reverse Transcription System according to the manufacturer's instructions (Promega). Approximately 1 µg total RNA was used for each oligo-dT primed reverse transcription. An aliquot of the reaction was then subjected to the following amplification protocol using primers *tps4* and *tps5* (Table 1): a denaturation step at 93.5 °C for 5 min followed by 20 cycles of the following steps: denaturation at 93.5 °C for 30 s, annealing at 58 °C for 1 min, extension at 71 °C for 1 min. Amplification was limited to 20 or 25 cycles in order to remain in a linear range and therefore produce semi-quantitative data. The *tps4* and *tps5* oligonucleotides are sense and anti-sense primers, respectively, that are located on both sides of an intron in the *tpsA* gene. Therefore amplification from genomic DNA yields a 363 bp product while amplification from reverse-transcribed mRNA yields a 308 bp product. Alternatively, primers *tps4* and *tps9* were used that yield a 282 bp fragment corresponding only to reverse-transcribed mRNA because *tps9* overlaps with an intron in *tpsA*.

**Expression of *tpsA* in *S. cerevisiae*.** To obtain a cDNA encompassing the full *tpsA* ORF, total RNA prepared from the mycelium of a *A. nidulans* *pabaA1* strain was reverse transcribed as described above. Reverse transcription products were then amplified using primers *tps5'*Bgl and *tps3'*Not (Table 1) and the following amplification procedure. A denaturation step at 93 °C for 5 min, 30 cycles of the following steps: denaturation at 93 °C for 30 s, annealing at 54 °C for 1 min, extension at 72 °C for 5 min and a final extension step of 10 min at 72 °C. The amplification product was subcloned using the TA cloning kit according to the supplier's instructions (Invitrogen), yielding plasmid pTPS15. Following sequencing of the cloned *tpsA* cDNA, the *BglII*-*NotI* fragment of pTPS15 was subcloned into the yeast expression vector pCM190L (Llorente *et al.*, 1999) cut by *Bam*HI and *NotI*. Controlled expression in yeast is achieved by a tetracycline-repressible promoter. Furthermore, the protein is produced as a fusion with an HA-epitope and a (His)<sub>6</sub> tail that allows quantification of protein production. Two independent recombinants, pTPS16-1 and pTPS16-2, were selected for transformation into the *S. cerevisiae* strain YSH290 along with pCM190L and pCM190L::X, a derivative of pCM190L carrying a *S. cerevisiae* ORF unlinked to trehalose metabolism. Yeast transformation was performed using the one-step method (Chen *et al.*, 1992). Trehalose levels in the transformants were measured using cells that had been grown into

stationary phase on galactose as described by Neves *et al.* (1994).

## RESULTS

### Isolation of the *A. nidulans* *tpsA* gene encoding a T6PS

To investigate the role of trehalose in *A. nidulans*, we set out to identify genes encoding T6PS using a two-step strategy. First, two degenerate primers, *tps1F* (sense primer; Table 1) and *tps1B* (antisense primer; Table 1), were designed that correspond to two regions conserved in the T6PS of yeast (Bell *et al.*, 1992; Blazquez *et al.*, 1994; Luyten *et al.*, 1993) and in the *A. niger* TpsA T6PS (Wolschek & Kubicek, 1997). Using these primers, a 624 bp fragment was amplified by PCR from genomic DNA of *A. nidulans* strain FGSC28. The nucleotide sequence of this PCR product was determined and the deduced amino acid sequence revealed significant identity to corresponding regions of *A. niger* and *S. cerevisiae* T6PS (94.2% and 66.8% identical amino acids, respectively). These results suggested that the PCR product corresponded to an *A. nidulans* T6PS-encoding gene referred to as *tpsA*.

In a second step, the PCR product was used to probe different libraries of *A. nidulans* genomic DNA. Using chromosome-specific libraries (Brody *et al.*, 1991), three positive cosmids, L09H05, L14G06 and L24E04, were identified. These cosmids have been assigned to the same region of *A. nidulans* chromosome V (Prade *et al.*, 1997), suggesting that the *tpsA* gene is located on this chromosome.

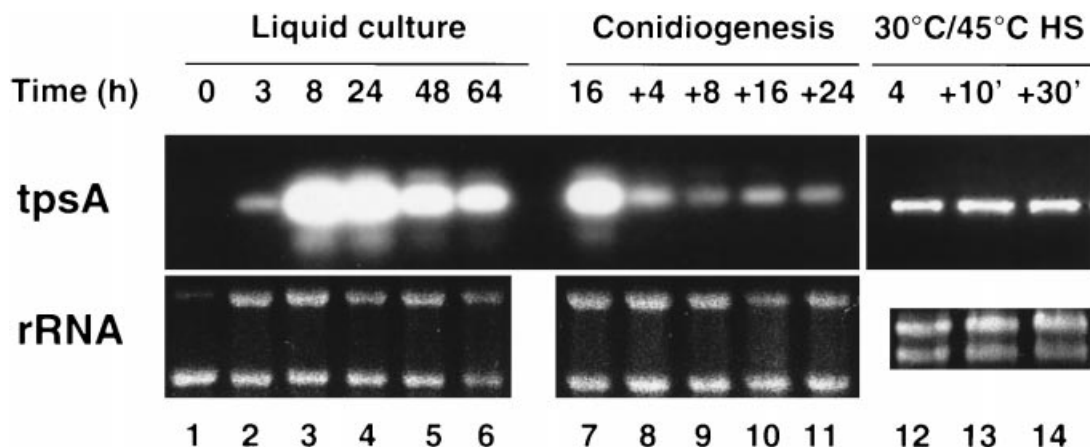
### Sequencing of the *A. nidulans* *tpsA* gene

DNA sequencing of an approximately 1.8 kb *SalI*-*SmaI* fragment derived from plasmid pTPS4 (see Methods) and of an approximately 1.2 kb *EcoRI*-*PstI* fragment derived from cosmid L24E04 yielded a nucleotide sequence of 2699 bp (GenBank accession number AF-043230; data not shown). Analysis of this DNA sequence revealed an ORF of 1512 bp interrupted by four putative introns of 63, 55, 48 and 55 bp, respectively. The location of these introns was confirmed by sequencing a cDNA of *tpsA* obtained by amplification of reverse-transcribed mRNAs using primers *tps5'*Bgl and *tps3'*Not (Table 1). Furthermore, analysis of several *A. nidulans*-expressed sequence tags (c3e03, m7e02, c5f08) determined within the *A. nidulans* EST sequencing program (D. Kupfer & B. Roe, <http://www.genome.ou.edu/fungal.html>) confirmed the location of these introns and revealed the occurrence of an additional intron of 200 bp located in the 5'-untranslated region of the gene and extending from position -302 to -103 relative to the *tpsA* start codon (data not shown).

The *A. nidulans* 1512 bp ORF identified in the cloned DNA region encodes a 504 amino acid protein with a molecular mass of 56.8 kDa. This protein shares a minimum of 62.2% identical amino acids and 74.1% similar amino acids with known fungal T6PSs and its

[illegible]

**Fig. 2.** Alignments for maximal amino acid similarities of the *A. nidulans* TpsA protein (antpsA) with the *S. cerevisiae* (sctps1; Bell *et al.*, 1992), *K. lactis* (klggs1; Luyten *et al.*, 1993), *Schiz. pombe* (sptps1; Blazquez *et al.*, 1994) and *A. niger* (AgtpsA and AgtpsB; Wolschek & Kubicek, 1997) T6Ps. This alignment was produced using the PILEUP program of the UWGCG package version 9 (Devereux *et al.*, 1984). Conserved residues (identical, upper case letters; similar, lower case letters) in all six proteins have a black background.



**Fig. 3.** Expression of the *tpsA* gene under different growth conditions. RNA was prepared from *A. nidulans* cultures grown under the following conditions: rich liquid medium for 0–64 h (samples 1–6); rich liquid medium for 16 h and transfer to rich solid medium for 0–24 h (samples 7–11); minimal glucose medium at 30 °C for 4 h and transfer to 45 °C for 10 and 30 min (samples 12–14). Total RNA was reverse transcribed and subjected to an amplification using oligonucleotides that yield a PCR product specific to the *tpsA* transcript. Amplification was reduced to 20 cycles in order to obtain semi-quantitative data.

closest known homologue is the *A. niger* TpsA protein (87.4 % identical amino acids and 90.1 % similar amino acids; Fig. 2).

### Regulation of the *tpsA* gene

Expression of the *A. nidulans tpsA* gene under different culture conditions was monitored using semi-quantitative RT-PCR. Results presented in Fig. 3 show that

transcription of the *tpsA* gene is induced during spore germination in rich medium (Fig. 3, lane 2), is maximal during exponential growth (Fig. 3, lanes 3–5 and lane 7) and decreases during the stationary phase of growth (Fig. 3, lane 6) and during conidiogenesis (Fig. 3, lanes 8–11). As a consequence, an RT-PCR product corresponding to the *tpsA* transcript could not be detected in non-germinating conidia of *A. nidulans* (Fig. 3, lane 1). When 4 h germlings of *A. nidulans* were exposed to

**Table 2.** Trehalose levels in transformants of the *S. cerevisiae tps1Δ* strain

Transforming plasmid	Cloned gene	Trehalose (% of wet weight)*
pCM190L	—	0.33
pCM190L::X	Control	0.31
pTPS16-1	<i>A. nidulans tpsA</i>	2.10
pTPS16-2	<i>A. nidulans tpsA</i>	2.13

\*Trehalose levels in wild-type *S. cerevisiae* grown in minimal galactose medium are 1.5–2.0% of wet weight.

a heat shock at 45 °C (Fig. 3, lanes 12–14) expression of *tpsA* was not altered.

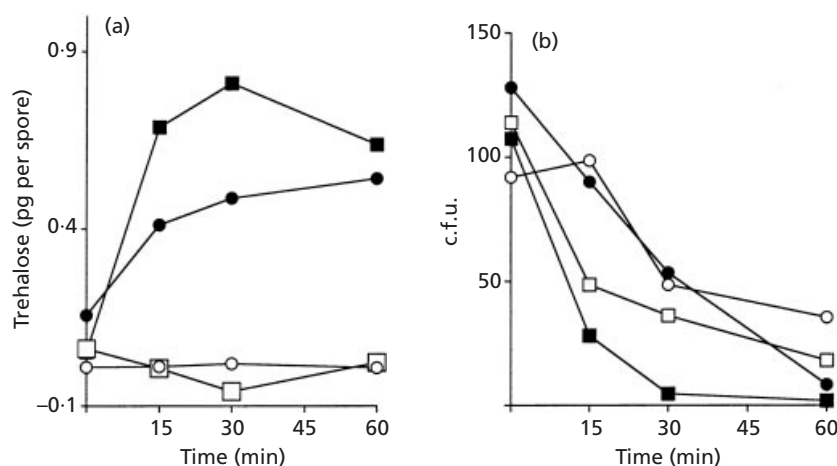
#### Expression of the *tpsA* gene complements the various defects of a *S. cerevisiae tps1Δ* mutant

*S. cerevisiae* strains lacking the *TPS1* gene are deficient in the synthesis of trehalose in response to various stress conditions and are unable to grow on glucose or other rapidly fermentable sugars because of an uncontrolled influx of sugar into glycolysis, causing rapid ATP depletion (Van Aelst *et al.*, 1993). To test whether *tpsA* could functionally complement the various defects of a *S. cerevisiae tps1Δ* strain, the *tpsA* ORF was amplified and cloned into the yeast expression vector pCM190L (Llorente *et al.*, 1999), yielding plasmids pTPS16-1 and

pTPS16-2. After transformation of the plasmids into the yeast *tps1Δ* mutant, growth of the transformants on glucose and galactose media was monitored. Expression of the *A. nidulans tpsA* gene fully restored growth of the *S. cerevisiae tps1Δ* mutant on glucose (data not shown). Furthermore, trehalose production in the *S. cerevisiae* transformants expressing *A. nidulans tpsA* was restored to wild-type levels (Table 2). These results indicate that the *A. nidulans* TpsA protein can fulfil all of the functions of the yeast Tps1 T6PS, including the control of hexokinase function.

#### Disruption of the *tpsA* gene in *A. nidulans*

A pop-in/pop-out strategy was used to inactivate the *tpsA* gene. First, a strain carrying both the wild-type *tpsA* gene and a mutant allele separated by the *A. fumigatus pyrG* gene was constructed (CEA150: *wA3 pyroA4 pyrG89 tpsA-pyrG-tpsAΔ*) by transformation of *A. nidulans* strain FGSC773 with plasmid pTPS17. The *tpsAΔ* mutant allele removes 146 internal amino acids (90–235) in the 504 residue TpsA polypeptide, including stretches of amino acids that are highly conserved within fungal T6PS (Fig. 2). Excision of the *A. fumigatus pyrG* gene by recombination between the two *tpsA* alleles was then forced in the presence of 5-FOA. PCR analysis of random 5-FOA-resistant clones using primers *tps10* and *tps11* or *tps4* and *tps5* (Table 1), which yield products of different sizes according to the *tpsA* allele, revealed that segregation between the wild-type and mutant alleles occurred randomly under these conditions (data not shown). Interestingly, all segregants with a *tpsAΔ* allele were thermosensitive for growth while the CEA150



**Fig. 4.** *A. nidulans tpsAΔ* mutants are defective for trehalose accumulation in response to a heat or oxidative stress. (a) Trehalose accumulation in response to a heat shock (30 °C → 50 °C) or an oxidative shock (100 mM H<sub>2</sub>O<sub>2</sub>) in germinating conidia of *A. nidulans* strains FGSC773 (*wA3 pyroA4 pyrG89*) (■, heat shock; ●, oxidative shock) and CEA52 (*wA3 pyroA4 pyrG89 tpsAΔ*) (□, heat shock; ○, oxidative shock). Conidia were germinated for 3 h in minimal glucose medium at 30 °C and subjected to the shock for the indicated times. (b) Conidia survival following a heat shock or an oxidative shock in germinating conidia of *A. nidulans tpsA+* and *tpsAΔ* strains. Duplicates of the samples analysed in (a) were diluted and plated on complete medium containing 0.01% Triton X-100. Colonies were counted after 2 d at 37 °C. Results are representative of two independent experiments. Symbols as in (a).

**Table 3.** Metabolites accumulated in the wild-type and *tpsAΔ* strains during vegetative growth at 30 °C

Values are in  $\mu\text{mol (g dry wt)}^{-1}$  and are means of assays of two independent cultures and standard deviations are indicated. The ratios between values obtained for the mutant and wild-type strains are shown in parentheses.

Metabolite	FGSC773 (wt)	CEA152 ( <i>tpsAΔ</i> )
Trehalose	46.5 ± 2.5	< 1.0
Trehalose 6-phosphate	2.39 ± 0.65	< 0.2
Glucose 6-phosphate	0.54 ± 0.10	2.49 ± 0.48 (4.6 ×)
Fructose 6-phosphate*	0.13	0.48 (3.7 ×)
Fructose 1,6-bisphosphate	0.33 ± 0.04	0.79 ± 0.53 (2.4 ×)
ATP	1.37 ± 0.22	2.13 ± 0.42 (1.5 ×)

\* Values for fructose 6-phosphate were obtained from a single culture.

parental strain and *tpsA*<sup>+</sup> progenies were not, suggesting that thermosensitive growth resulted from the inactivation of *tpsA* (see below). The occurrence of the *tpsAΔ* allele in two of the 5-FOA-resistant clones was confirmed by Southern analysis of *EcoRI*/*SalI*-digested genomic DNA. One of the clones carrying the *tpsAΔ* allele was designated CEA152. A comparison of strains FGSC773 (*pyrG89 wA3 pyroA4*) and CEA152 (*pyrG89 wA3 pyroA4 tpsAΔ*) is reported below.

#### ***tpsA* is required for trehalose accumulation in response to various stress conditions**

In *A. nidulans*, trehalose is known to accumulate during conidiogenesis (d'Enfert & Fontaine, 1997) as well as in response to heat shock (Noventa-Jordao *et al.*, 1999). In other fungal species, trehalose has been shown to accumulate in the stationary phase of growth and in response to an oxidative or osmotic shock (Hounsa *et al.*, 1998; Lewis *et al.*, 1995; Lingappa & Sussman, 1959; Van Laere, 1989; Wiemken, 1990). Accumulation of trehalose was therefore monitored in wild-type and *tpsAΔ* conidia as well as in germinating conidia that were subjected to a heat shock or an oxidative shock. Trehalose could not be detected in mutant conidia (data not shown). When conidia of the wild-type strain FGSC773 were germinated for 3 h at 30 °C and subsequently subjected to a 50 °C heat shock, a rapid increase in trehalose levels was observed (Fig. 4a). Similarly, addition of 100 mM H<sub>2</sub>O<sub>2</sub> to wild-type germlings resulted in trehalose accumulation, although to a lesser extent (Fig. 4a). Addition of 1 M NaCl did not result in a significant increase in trehalose levels (data not shown). In contrast to these results, neither heat shock nor 100 mM H<sub>2</sub>O<sub>2</sub> resulted in trehalose accumulation in germlings of the *tpsAΔ* strain (Fig. 4a). Furthermore, T6P and trehalose were undetectable in the mycelium of a *tpsAΔ* strain (Table 3). We conclude

that *tpsA* encodes a T6PS essential for biosynthesis of T6P and trehalose in *A. nidulans* under various conditions, including conidiogenesis, heat shock and oxidative shock.

#### **Inactivation of *tpsA* does not increase sensitivity of germinating conidia to heat or oxidative shock**

It has previously been shown that viability of wild-type conidia is significantly decreased following a heat shock and that the loss of viability can be prevented by accumulated trehalose (d'Enfert *et al.*, 1999). This would suggest that the inability to accumulate trehalose in response to stress may further increase the stress sensitivity of germinating conidia. To test this hypothesis, the viability of germinating wild-type or *tpsAΔ* conidia that had been subjected to a 50 °C heat shock or a 100 mM H<sub>2</sub>O<sub>2</sub> shock (see above) was monitored. Results in Fig. 4(b) show that wild-type and *tpsAΔ* germlings display a similar sensitivity to heat and oxidative shock independent of the ability to accumulate trehalose. It therefore appears that the rapid accumulation of trehalose in response to a heat or oxidative stress does not contribute significantly to cell survival during short-term exposure to a stress.

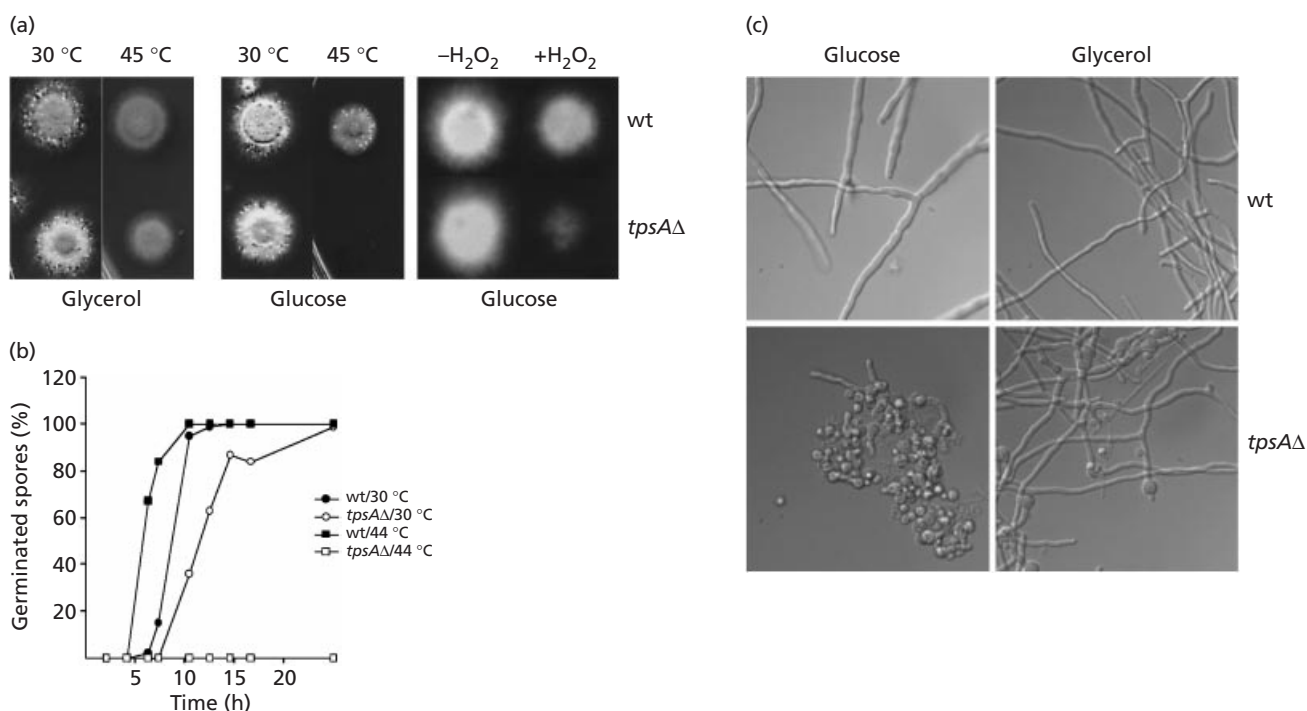
#### **Inactivation of *tpsA* results in thermosensitive growth**

To investigate the consequence of the inactivation of the *tpsA* gene in *A. nidulans*, we first compared the growth of strains FGSC773 (wild-type) and CEA152 (*tpsAΔ*) on various media and at different temperatures. The *tpsAΔ* mutant was unable to form colonies at temperatures above 44 °C when glucose (Fig. 5a) or fructose (data not shown) were used as a carbon source. Thermosensitive growth was also observed when glucose was replaced by glycerol (Fig. 5a) although to a lesser extent. The *tpsAΔ* mutant also showed reduced growth on media containing sublethal doses (1–2 mM) of H<sub>2</sub>O<sub>2</sub> (Fig. 5a).

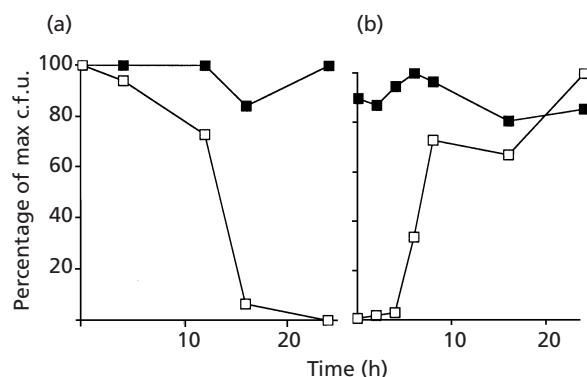
Microscopic examination of conidia grown on minimal glucose medium revealed that the inability of strain CEA152 to establish a colony at temperatures above 44 °C was due to a block of conidia germination (Fig. 5b, c). Only a few spores had produced a germ tube after 24 h of growth at 44 °C (Fig. 5c). At lower temperatures, conidia of strain CEA152 were able to produce germ tubes at a frequency similar to that observed for the wild-type strain FGSC773, although with slower kinetics (Fig. 5b). When conidia were germinated on minimal glycerol medium at 45 °C germination was only partially delayed (Fig. 5c).

Results presented in Fig. 6 show that the thermosensitive growth defect of strain CEA152 became irreversible after prolonged incubation at the non-permissive temperature and was limited to the developmental stages extending from spore germination to early filamentous growth. Indeed, when conidia of strain CEA152 were germinated at the non-permissive temperature (45 °C)





**Fig. 5.** Inactivation of *tpsA* results in a thermosensitive growth defect. (a) Comparison of the growth of the *tpsAΔ* mutant (CEA152, *wA3 pyroA4 pyrG89 tpsAΔ*) and the wild-type strain (FGSC773, *wA3 pyroA4 pyrG89*) on minimal medium with 1% glucose or 1% glycerol as carbon source in the presence or absence of 2 mM H<sub>2</sub>O<sub>2</sub>. Incubation was for 48–64 h at 30 °C or 45 °C. (b) Germination frequency of the *tpsAΔ* mutant and the wild-type strain at 30 °C or 45 °C in glucose-containing minimal medium. Results are representative of two independent experiments. (c) Microscopic examination ( $\times 570$ ) of germinated spores after 24 h at 45 °C in liquid minimal medium with the indicated carbon sources.



**Fig. 6.** The thermosensitive phenotype of the *tpsAΔ* mutant is dependent on the developmental stage. Shift experiments from restrictive (45 °C) to permissive (37 °C) temperature (a) and vice versa (b). Between 100 and 200 spores of the *A. nidulans* wild-type (FGSC773, *wA3 pyroA4 pyrG89*; ■) and *tpsAΔ* (CEA152, *wA3 pyroA4 pyrG89 tpsAΔ*; □) strains were inoculated on complete medium and incubated for the indicated time periods before the shift. Counts represent the percentage of the maximal count. Results are representative of two independent experiments.

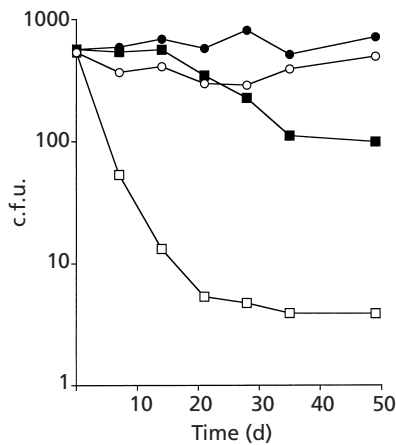
and shifted to the permissive temperature (37 °C) after various times, they were only able to form a colony when incubation at the non-permissive temperature was

restricted to 12 h (Fig. 6a). In contrast, transfer to the non-permissive temperature of conidia germinated at the permissive temperature did not block colony formation when the transfer was performed after 10–12 h of germination (Fig. 6b).

#### Inactivation of *tpsA* results in reduced conidium viability during prolonged storage

Since the results presented above appeared to confirm a protective role of trehalose against sustained exposure of *A. nidulans* cells to various stress conditions, we evaluated the role of trehalose for the survival of conidia of *A. nidulans* during prolonged storage. Conidia of strains FGSC773, which contain trehalose, and CEA152, which are devoid of trehalose (data not shown), were maintained at 4 °C or 20 °C for several weeks and the number of viable spores was estimated at different times. Results presented in Fig. 7 show that the conidia of the *tpsAΔ* mutant strain lost viability much more rapidly than wild-type conidia when incubated at 20 °C. In contrast, no difference in viability between the wild-type and mutant conidia was observed upon incubation at 4 °C over a period of up to 7 weeks (Fig. 7). These results indicate that trehalose might play an important role in the survival of conidia during prolonged storage, either as a protective molecule or reserve carbohydrate.





**Fig. 7.** Trehalose is required for conidium viability during prolonged storage. Viability of conidia from *A. nidulans tpsA*<sup>+</sup> (FGSC773, *wA3 pyroA4 pyrG89*) or *tpsA*Δ (CEA152, *wA3 pyroA4 pyrG89 tpsA*Δ) strains upon prolonged incubation at 4 °C [*tpsA*<sup>+</sup> (●) and *tpsA*Δ (○)], or 20 °C, *tpsA*<sup>+</sup> (■) and *tpsA*Δ (□)]. Conidia were resuspended in PBS/0.1% Tween-20 at a density of 10<sup>9</sup> conidia ml<sup>-1</sup> and maintained at room temperature or 4 °C for the indicated times. Spore viability was determined by the number of c.f.u. after plating on complete medium and growth at 37 °C.

## DISCUSSION

In this paper we have reported the cloning of the *A. nidulans tpsA* gene and physiological characterization of the *tpsA*Δ mutant. Our results demonstrate unambiguously that the *tpsA* gene product is a T6PS that is essential for trehalose biosynthesis in *A. nidulans*. The finding that *tpsA* is the unique T6PS-encoding gene in *A. nidulans*, in contrast to the situation in *A. niger*, where two T6PS genes have been identified (Wolschek & Kubicek, 1997), and that inactivation of *tpsA* results in a complete loss of trehalose biosynthesis, in contrast to the inactivation of the *A. nidulans* T6PP-encoding *orlA* gene (Borgia *et al.*, 1996), has allowed us for the first time to investigate the physiological role of trehalose in a filamentous fungus.

In the yeast *S. cerevisiae*, trehalose is synthesized by a large multi-subunit complex (Bell *et al.*, 1998). The subunits of this complex share a domain which is similar to T6PS although devoid of T6PS activity. Our results show that the *A. nidulans* T6PS can fulfil all of the functions of its yeast counterpart, thus suggesting that it can associate in the multi-subunit trehalose synthase complex in yeast. Our reinvestigation of the sequence of the *orlA* gene shows that the *A. nidulans* T6PP is larger than previously proposed (Borgia *et al.*, 1996) with an amino-terminal domain which is homologous to *A. nidulans* T6PS (C. d'Enfert & A. Antczak, unpublished data). Furthermore, cDNAs encoding a homologue of *S. cerevisiae* Tps3 have been identified in the course of expressed sequence tag (EST) sequencing of *A. nidulans* cDNAs (D. Kupfer & B. Roe, <http://www.genome.ou.edu/fungal.html>;

C. d'Enfert, unpublished data) thus suggesting that the *A. nidulans* trehalose synthase is also present in a multi-subunit complex in *A. nidulans*.

In *A. nidulans* trehalose has been shown to accumulate under a variety of conditions including nutrient starvation, conidiospore differentiation and heat stress (d'Enfert & Fontaine, 1997; Noventa-Jordao *et al.*, 1999; this study). Here we have shown that trehalose accumulation is also stimulated in response to an oxidative stress (Fig. 4a) consistent with the results of Noventa-Jordao *et al.* (1999), who showed a link between heat shock recovery and the cellular response to oxidative stress in *A. nidulans*. Results presented in this paper show that trehalose accumulation is mediated by TpsA under the different conditions tested, i.e. heat stress, oxidative stress and conidiogenesis. Furthermore, TpsA is responsible for the basal levels of trehalose and T6P that are produced during mycelial growth (data not shown). Although these results would suggest that trehalose biosynthesis in response to temperature and oxidative stress could contribute to the resistance of the germlings to these stress conditions, results presented in Fig. 4(b) show that this is not always the case: wild-type and *tpsA*-null germlings are similarly sensitive to a heat or an oxidative shock. In contrast, the absence of trehalose and/or trehalose biosynthesis results in reduced spore viability and a reduced ability to grow upon constant exposure to sublethal stress, including prolonged exposure to high temperature and growth in the presence of reactive oxygen species. Taken together, these results suggest that trehalose is mainly involved in the resistance of *A. nidulans* to progressive exposure to lethal stress or prolonged exposure to sublethal stress rather than rapid exposure to lethal stress. This is in agreement with the previous observations that germlings of *A. nidulans* maintaining a high level of trehalose due to a defect in the TreB neutral trehalase are less sensitive to heat stress than wild-type germlings (d'Enfert *et al.*, 1999) and that viability of the conidia of an *A. niger tpsA* mutant is reduced (Wolschek and Kubicek, 1997). This is also consistent with the role of trehalose in the acquisition of thermotolerance and halotolerance demonstrated in *S. cerevisiae* (Hounsa *et al.*, 1998; Lewis *et al.*, 1995) and in *Schizosaccharomyces pombe* (Ribeiro *et al.*, 1998). Results presented in Fig. 6 show that this protective role of trehalose is most important during *A. nidulans* conidial germination as opposed to later developmental stages, including mycelial growth, suggesting that additional mechanisms of adaptation to stress operate following germ tube formation.

Analysis of the expression of the *tpsA* gene in response to a heat shock or during conidiogenesis, when trehalose is synthesized, did not reveal induction of the transcription of *tpsA* under these conditions (Fig. 3). In contrast, expression of *tpsA* is induced during the early stages of mycelial growth, when trehalose biosynthesis appears minimal. Our results contrast with those of Wolschek & Kubicek (1997), who showed that the *A. niger tpsA* and *tpsB* genes are respectively down-regulated and up-regulated by a heat shock. While

activation of trehalose biosynthesis in *A. niger* appears to be controlled in part at the transcriptional level, our data suggest that, in *A. nidulans*, an inactive form of T6PS is accumulated during phases of rapid growth to prepare for induction of trehalose biosynthesis in response to stress, nutrient starvation, or developmental transitions by means of post-transcriptional control mechanisms. In *S. cerevisiae* T6PS activation is mediated both at the transcriptional level through an STRE-dependent activation mechanism (de Virgilio *et al.*, 1993; Winderickx *et al.*, 1996) and at the post-translational level by the protein kinase Rim15 (Reinders *et al.*, 1998). A homologue of Rim15 has been identified by systematic sequencing of *A. nidulans* cDNAs (D. Kupfer & B. Roe, <http://www.genome.ou.edu/fungal.html>; C. d'Enfert, unpublished data). It is a possible candidate for post-translational control of TpsA, allowing rapid activation of trehalose synthesis under stress conditions.

In *S. cerevisiae*, inactivation of the T6PS results in an inability to grow on rapidly fermentable sugars such as glucose and fructose because of an uncontrolled influx of the sugars into glycolysis, causing rapid ATP depletion (Van Aelst *et al.*, 1993). Recent results suggest that both T6P inhibition of hexokinase and a direct involvement of T6PS are responsible for this phenomenon (Bonini *et al.*, 2000). In *A. nidulans*, T6P is also known to inhibit hexokinase (Ruijter *et al.*, 1996) and our results show that TpsA is able to fulfil all the functions of the yeast T6PS, including its control on glucose influx into glycolysis, thus suggesting that similar mechanisms of glucose influx could operate in yeast and *A. nidulans*. On the other hand, the *A. nidulans* *tpsA*-null mutant is able to grow on glucose or fructose as a carbon source. Although increased levels of sugar phosphates could be detected in the mycelium of the mutant strain grown at 30 °C, this increase was not associated with a decrease of the ATP pool (Table 3). This suggests that T6P is a physiological inhibitor of hexokinase in *A. nidulans* but that the increase in glycolytic flux resulting from T6P depletion has only minor consequences in this fungus compared to what has been observed in *S. cerevisiae*. This physiological role of T6P is also supported by the poor growth on fructose of the *A. nidulans* *orlA* mutant which accumulates T6P and consequently should have reduced hexokinase activity (S. Fillinger & C. d'Enfert, unpublished results). Interestingly, the thermosensitive growth defect of the *A. nidulans* *tpsAΔ* strain appeared less pronounced on minimal glycerol medium than on minimal glucose medium (Fig. 5). Although this may reflect a more stringent role of T6P on the control of glycolytic flux at high temperature or the replacement of trehalose as a stress metabolite by intracellular glycerol resulting from glycerol uptake, the thermosensitive growth defect might also be less pronounced on slowly metabolizable carbon substrate because of a slower growth rate and the resulting presence of a higher intrinsic stress resistance (see Thevelein & de Winde, 1999, for a recent review). Further analysis of metabolic fluxes at different temperatures in wild-type and tre-

halose biosynthesis mutants is needed to assess precisely the role of T6P glycolytic control in filamentous fungi.

In summary, results presented in this study show that T6P, in addition to its role in the control of chitin biosynthesis (Borgia *et al.*, 1996), appears to play only a minor role in the control of the glycolytic flux in *A. nidulans*, in contrast to what has been observed in *S. cerevisiae* and some phylogenetically close yeast species (*Kluyveromyces lactis*, *Candida albicans*). More importantly, our results show that trehalose is a major stress metabolite in *A. nidulans* and is probably involved in the acquisition of resistance to a variety of stress conditions, including heat and oxidative stress, as well as in the survival of conidia during prolonged storage.

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