A neutral trehalase gene from *Candida albicans*: molecular cloning, characterization and disruption

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A neutral trehalase gene, *NTC1*, from the human pathogenic yeast *Candida albicans* was isolated and characterized. An ORF of 2724 bp was identified encoding a predicted protein of 907 amino acids and a molecular mass of 104 kDa. A single transcript of approximately 3.2 kb was detected by Northern blot analysis. Comparison of the deduced amino acid sequence of the *C. albicans* *NTC1* gene product with that of the *Saccharomyces cerevisiae* *NTH1* gene product revealed 57% identity. The *NTC1* gene was localized on chromosome 1 or R. A null mutant ($\Delta ntc1/\Delta ntc1$) was constructed by sequential gene disruption. Extracts from mutants homozygous for neutral trehalase deletion had only marginal neutral trehalase activity. Extracts from heterozygous mutants showed intermediate activities between extracts from the wild-type strain and from the homozygous mutants. The null mutant showed no significant differences in pathogenicity as compared to the wild-type strain in a mouse model of systemic candidiasis. This result indicates that the neutral trehalase of *C. albicans* is not a potential target for antifungal drugs.

Keywords: neutral trehalase, *Candida albicans*, gene disruption, pathogenicity

INTRODUCTION

Trehalases, trehalose-hydrolysing enzymes, have been isolated from many organisms (Hill & Sussmann, 1963; Killick, 1985; Merdinger *et al.*, 1971; Retief & Hewitt, 1973; Arguelles & Gacto, 1985). Fungal trehalases are divided into acid trehalases and neutral trehalases (Thevelein, 1984). Acid trehalases exhibit their maximal activity around pH 4.5. They are localized in the vacuole (Mittenbühler & Holzer, 1988). Neutral trehalases exhibit their maximal activity around pH 7.0. They can be activated by phosphorylation and are localized in the cytosol (App & Holzer, 1989).

The gene encoding the *Saccharomyces cerevisiae* neutral trehalase (*NTH1*) was cloned and characterized by Kopp *et al.* (1993). It has an ORF of 2253 bp (Kopp *et al.*, 1994). The native protein consists of two identical subunits with a molecular mass of 86 kDa (App & Holzer, 1989; Kopp *et al.*, 1993, 1994).

S. cerevisiae neutral trehalase is activated by cyclic-AMP-dependent phosphorylation. Dephosphorylation of the active phosphorylated enzyme by alkaline phosphatase is accompanied by 90% inactivation (App & Holzer, 1989). The phosphorylated amino acid residues were identified as phosphoserines. The amino acid sequence suggested two possible phosphorylation sites close to the N-terminus.

The predicted amino acid sequence of the *NTH1* gene product shows 77% identity to the amino acid sequence of an ORF, *YBR0106*, from *S. cerevisiae* (Wolfe & Lohan, 1994). In contrast to the *NTH1* deletion mutant, a mutant disrupted in the *YBR0106* ORF showed no changes in trehalase activity. Disruption experiments showed that trehalose accumulated during heat stress is hydrolysed by neutral trehalase and not by acid trehalase (Mittenbühler & Holzer, 1988).

Neutral trehalase is of minor importance in humans. Trehalose has not yet been found at a significant level in mammals. The trehalase detected in the brush border membranes of the small intestine is most probably involved in the hydrolysis of ingested trehalose and has

Abbreviations: 5-FOA, 5-fluoroorotic acid.

The EMBL accession number for the sequence reported in this paper is X95099.

no essential functions (Forstner *et al.*, 1968). The physiological role of kidney trehalase is still unclear (George & Kenny, 1973; Kyosseva *et al.*, 1995). It has been suggested that trehalase could be involved in sugar transport in the mammalian kidney (Sacktor, 1968).

Different growth phases (budding, conidiation, germination) of fungi are connected with accumulation or mobilization of trehalose. Trehalose plays a role as a protectant against environmental injuries (Wiemken, 1990) and as a storage carbohydrate (Thevelein, 1984). It accumulates under conditions of restricted growth, during sporulation and upon various stress conditions (heat shock, hyperosmotic shock, dehydration, cold shock). This accumulation correlates with an increase in thermotolerance (Hottiger *et al.*, 1987). Breakdown of storage trehalose is usually one of the main biochemical events during early germination of fungal spores (Thevelein, 1984). Hyphal growth of the phytopathogenic fungus *Rhizoctonia solani* is connected with the mobilization of trehalose by trehalase. Validamycin A, a potent inhibitor of trehalase, is active against sheath blight of rice plants caused by *R. solani* (Asano *et al.*, 1987).

Although trehalose metabolism has been extensively studied in *S. cerevisiae*, our knowledge of its physiological significance in the human pathogenic yeast *Candida albicans* is limited. Trehalases have been isolated from different *Candida* species. Acid trehalases have been found in *C. tropicalis* (Lucas & Phaff, 1952) and *C. albicans* (Arnold & McLellan, 1975). A neutral trehalase was identified in *C. utilis* (Arguelles & Gacto, 1985). This trehalase is similar to the *S. cerevisiae* enzyme activated by cyclic-AMP-dependent phosphorylation and deactivated by phosphatases (Carrillo *et al.*, 1995). For evaluation of neutral trehalase as a target for the screening of antifungal compounds, we cloned and disrupted the *C. albicans* gene which encodes neutral trehalase, *NTC1*, and tested the pathogenicity of strains heterozygous and homozygous for *ntc1* disruption in a mouse model for systemic candidiasis.

METHODS

Strains and culture conditions. *Escherichia coli* XL1-Blue {*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F' [proAB⁺ lacI^q M15 Tn10(Tet)]*} (Stratagene) was used for cloning. *C. albicans* strain BMSY 212, a clinical isolate, was used for cloning of the *NTC1* gene. *C. albicans* CAI-4 (URA3::imm434/URA3::imm434) derived from the wild-type strain SC5314 was used as the parental strain for *NTC1* gene disruption. Strain SC5314 was used as wild-type strain in the growth analysis and pathogenicity tests (Fonzi & Irvine, 1993). Strain 3153a was used for the preparation of a cDNA library in the lambda ZAP II vector (Stratagene) (Swoboda *et al.*, 1993). *C. albicans* was routinely cultured in YPG medium (2% glucose, 2% peptone, 1% yeast extract) at 28 °C. For transformation, *C. albicans* cells were spread on YNB plates [0.7% yeast nitrogen base (Difco Laboratories), 2% glucose, 2% Noble agar]. For the neutral trehalase assay, *C. albicans* cells were grown in YEG medium (2% glucose, 1% yeast extract). Hyphal formation was induced using three different experimental conditions with shift up in temperature to 37 °C

[addition of 10% (v/v) *N*-acetylglucosamine to YPG medium, addition of 1 ml serum to YPG medium and shift from pH 4.5 to pH 6.5 in Soll's medium (Soll, 1986)]. Hyphal growth was monitored by light microscopy after 0, 45, 60, 90, 120 and 150 min. To impose heat shock, *C. albicans* strains (SC5314 and homozygous *ntc1* mutant strain) were grown in YPG medium at 28 °C to mid-exponential growth phase with shaking at 200 r.p.m. Cells were diluted 1:10 (v/v) in fresh YPG medium preheated to 40, 45 and 50 °C and left at this temperature for 10 min. Cells were then returned to 28 °C and the OD₆₀₀ was measured at different times up to 60 min. To compare the growth of the homozygous *ntc1* mutant strain with the growth of the wild-type strain SC5314, equal numbers of cells from overnight cultures were added to preheated (28 °C, 37 °C and 40 °C) YPG medium. The OD₆₀₀ was measured at different times up to 60 min. Growth (at 28 °C, 37 °C and 42 °C) and the effect of heat shock (40 °C, 45 °C and 50 °C) were also determined in trehalose medium (0.7% yeast nitrogen base, 2% trehalose).

Construction of a probe for the *NTC1* gene. Oligonucleotide primers 1 (5' CCATATGCTGTCCAGGTGGTAGATT 3') and 2 (5' GGTGGTTGTGATCTACATAAATAATA 3') were derived using the trehalase consensus sequences of the *S. cerevisiae* *NTH1* gene (Kopp *et al.*, 1993, 1994), the *E. coli* *treA* gene (Gutierrez *et al.*, 1989) and the *Tre* gene from rabbit small intestine (Ruf *et al.*, 1990) (see Fig. 2). The sequences of the primers were adapted to the codon usage in *C. albicans* (Lloyd & Sharp, 1992) because the DNA of *C. albicans* is A/T-rich (third position of triplets mostly contains an A or T). Using primers 1 and 2, a 178 bp fragment of the *NTC1* gene (NTCPS) was amplified by PCR using *C. albicans* genomic DNA and cloned into the pCR-Script Amp SK(+) cloning vector (Stratagene). The nucleotide sequences were determined by the dideoxy chain-termination method with the T7 standard primer using the Sequenase version 2.0 kit (United States Biochemical) (Sanger *et al.*, 1977).

PCR. PCR amplification reactions were carried out in a 100 µl reaction volume containing 10 mM Tris/HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.2 mM dNTP and 0.2 µM of each primer. The reaction was started by addition of 1 unit *Taq* polymerase (Boehringer) and the samples were overlaid with mineral oil. Thirty cycles, consisting of a 30 s incubation at 96 °C, 1 min at 42 °C and 2 min at 72 °C, were performed using an Omn-E Hybaid thermal cyclor.

Cloning and sequence analysis of the *NTC1* gene. Chromosomal DNA from *C. albicans* strain BMSY 212 was fractionated and probed with NTCPS. Hybridizing fragments were isolated and cloned into plasmid pUC18. By screening this size-selected library with NTCPS a single clone was obtained. Furthermore, using the same probe we isolated three cDNA clones from a ZAP II expression library. The nucleotide sequences were determined by the dideoxy chain-termination method with synthetic oligonucleotide primers using the Sequenase version 2.0 kit (United States Biochemical) (Sanger *et al.*, 1977). All other recombinant DNA procedures were carried out according to standard protocols (Sambrook *et al.*, 1989). Alignments were done by the GCG software package (Genetics Computer Group, Madison, WI, USA).

Construction of plasmid pNR1. We amplified the 5' region of *NTC1* by PCR using chromosomal DNA of *C. albicans* strain BMSY 212 and primers 3 and 4 (primer 3, 5' GCGAGCTCCAGCAATTCATTCTGATTCTGAGG 3', position +314 to +337; primer 4, 5' GCGGTACC-GCCAGTATCTTCAATGGTAATTTG 3', position +765 to +742; underlined sequences are complementary to the

Fig. 1. Restriction maps of plasmids pCTX16 and pNR1. (a) The *NTC1* gene (arrow, coding region; boxes, non-coding regions) was cloned into pUC18, resulting in pCTX16. (b) The 5' and 3' regions of *NTC1* obtained by PCR were cloned in front of or behind the *hisG-URA3-hisG* cassette (4 kb) in the disruption vector pMB-7. Arrows (P3, P4, P5, P6) indicate the primers used. X, XbaI; B, BamHI; S, Sall; E, EcoRI; K, KpnI; P, PstI; H, HindIII; Sa, SacI.

genomic sequence of the *NTC1* gene). The resulting 468 bp fragment was digested with *SacI/KpnI* and cloned in front of the *hisG-URA3-hisG* cassette (4 kb) of pMB-7 (Fonzi & Irvine, 1993) to give pMB-7/1. Primer 5 (5' GCCTGCAG-CAAAGGGAGCAGTACCTCATAGAG 3', position +2507 to +2530) and primer 6 (5' GCAAGCTTAAACTCCAA-CTCAAAGGAAGGACT 3', position -49 to -26 downstream of the stop codon) were used to amplify the 3' end of *NTC1*. The resulting 284 bp fragment was digested with *HindIII/PstI* and cloned into pMB-7/1. The resulting plasmid, pNR1, containing the *hisG-URA3-hisG* cassette flanked by the 5' and 3' regions of the *NTC1* gene was used for gene disruption (Fig. 1b).

Transformation of *C. albicans* and selection of heterozygous and homozygous neutral trehalase mutants. Mid-exponential-phase yeast cells from a 150 ml YPG culture of *C. albicans* CAI-4 and the trehalase heterozygous mutant strain CAI-4 *NTC1*/Δ*ntc1*::*hisG* (for the second disruption) were harvested by centrifugation and washed three times with 1 M sorbitol, 10 mM Tris/HCl pH 8.0, 1 mM EDTA at room temperature. The cells were incubated for 10 min in 10 ml 1 M sorbitol, 10 mM Tris/HCl pH 8.0, 50 mM DDT, 1 mM EDTA at room temperature. After washing with 1 M sorbitol, 0.1 M Na citrate pH 5.8, 10 mM EDTA, the cells were resuspended in 10 ml 1 M sorbitol, 0.1 M Na citrate, pH 5.8, 10 mM EDTA, 10 μg Zymolyase ml⁻¹ and shaken for 60 min at 30 °C. Spheroplasts were washed three times with 1 M sorbitol, 10 mM Tris/HCl pH 8.0, 1 mM EDTA at 4 °C and resuspended in 0.5–1.0 ml 1 M sorbitol, 10 mM Tris/HCl pH 8.0, 1 mM EDTA. In a 1.5 ml tube, 100 μl spheroplast suspension was gently mixed with 10 μg plasmid pNR1 digested with *SacI* and *HindIII*. After incubation for 10 min on ice, electroporation was performed with ice-cold 0.2 cm electrode gap cuvettes at 1.5 kV voltage, 100 Ω resistance and 25 μF capacity using a Gene Pulse apparatus (Bio-Rad). The electroporated cell suspension was mixed with 0.5 ml 1 M sorbitol, 1% peptone, 2% glucose and incubated for 60 min at 30 °C. Aliquots of 100 μl were spread on YNB plates supplemented with 1 M sorbitol (Swoboda *et al.*, 1995). Uridine-positive colonies could be detected after 3–4 days incubation at 30 °C. The disruption of trehalase genes was verified by Southern analysis and PCR.

Selection of Ura⁻ auxotrophs. This was done on medium containing 5-fluoroorotic acid (5-FOA) (Boeke *et al.*, 1984). Prior to selection the strains were plated on YPG agar and incubated for 48 h at 30 °C. Individual colonies were suspended in 200 μl H₂O. Aliquots of 50 μl were spread on YNB plates with uridine (25 μg ml⁻¹) and 5-FOA (1 mg ml⁻¹). Ura⁻ cells grew after 3–4 days incubation at 30 °C.

Southern blot analysis. Southern blot analysis of genomic DNA from *C. albicans* strain BMSY 212, mutant strains and the chromosomes of *C. albicans* strain BMSY 212 was

performed according to standard procedures (Sambrook *et al.*, 1989).

Electrophoretic karyotyping. Separation of chromosomes in a CHEF (Bio-Rad) apparatus was performed with a running time of 24 h at 150 V with a pulse time of 120 s, followed by 40 h at 150 V with a pulse time of 240 s. The gel was run using a 120 ° rotation angle at a temperature of 10 °C.

Preparation of total RNA from *C. albicans*. Late-exponential-phase yeast cells from a 150 ml YPG culture were harvested by centrifugation, resuspended in 10 ml 50 mM Na acetate, 10 mM EDTA pH 5.3, 10 ml phenol, 100 μl 10% SDS and incubated twice for 5 min at 65 °C followed by 5 min in liquid nitrogen. After centrifugation, the aqueous phase was extracted with phenol and with phenol/chloroform (1:1, v/v). After ethanol precipitation the RNA was dissolved in 80% ethanol and quantified on the basis of A₂₆₀.

Northern blot analysis. RNA samples were separated in formaldehyde gels containing 1% agarose. The RNA was blotted onto Hybond nylon membranes (Amersham). Membranes were hybridized and washed by standard techniques (Sambrook *et al.*, 1989).

Primer extension analysis. Two hundred nanograms [γ-³²P]ATP end-labelled primer (PEXIRD) 5' GCAACATCA-AATGGGTCATCATCACTTGATGTAG 3' (position +65 to +32 of *NTC1*) was precipitated with 200 μg total RNA prepared from *C. albicans* strain BMSY 212. One hundred microlitres annealing buffer (0.15 M KCl, 10 mM Tris/HCl pH 8.3) was added and the sample was denatured and annealed (15 min at 90 °C, 90 min at 30 °C). After precipitation primer extension was performed with 40 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), 1 μl rRNasin (Boehringer Mannheim), 1 mM dATP, 1 mM dTTP, 1 mM dCTP, 1 mM dGTP, 50 mM Tris/HCl pH 8.5, 8 mM MgCl₂, 30 mM KCl, 1 mM DTT in a total volume of 20 μl. The mixture was incubated for 120 min at 42 °C. Two microlitres 0.5 M NaOH was added and the mixture incubated for 15 min at 65 °C. After addition of 80 μl 10 mM Tris/HCl pH 8.0, 1 mM EDTA and 10 μl 3 M Na acetate pH 5.3, the sample was precipitated and resuspended in 8 μl loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). At the same time, the end-labelled primer PEXIRD was used to prime a DNA sequencing reaction on the genomic *NTC1* clone by using the Sequenase version 2.0 kit (United States Biochemical) (Sanger *et al.*, 1977). The primer extension reaction, sequencing reactions and the end-labelled primer were analysed on a 6% polyacrylamide/urea gel.

Partial purification of neutral trehalase. *C. albicans* cells grown at 30 °C in YEG medium were homogenized (Bead-Beater, BioSpec Products, Bartlesville, USA) with glass beads in extraction buffer (50 mM imidazole-HCl pH 7.0, 1 mM PMSF and 15 μg Pepstatin ml⁻¹). After centrifugation (30 min,

3750

43 000 g), crude extract was obtained from the supernatant (total protein concentration 10–20 mg ml⁻¹). Neutral trehalase in the crude extract was activated by addition of 1/5 vol. phosphorylation solution (1 mM ATP, 10 µM cAMP, 10 mM MgSO₄, 50 mM NaF, 5 mM theophylline in extraction buffer) and incubated for 10 min at 30 °C. Phosphorylation was stopped by transfer to ice. Activated crude extract aliquots containing 25 mg total protein were separated by anion exchange chromatography on a DEAE-cellulose column (Whatman DE-52, 1.6 cm × 10 cm, 50 mM imidazole/HCl buffer/NaCl gradient). Neutral trehalase activity eluted at about 250 mM NaCl, whereas acid trehalase activity was found in the flowthrough. Fractions containing neutral trehalase activity were pooled as partially purified neutral trehalase.

Enzyme assay. Enzymic activity of neutral trehalase was tested at 37 °C. The reaction mixtures contained 100 mM trehalose, 10 mM MnCl₂, 50 mM MES pH 6 and 100 µl partially purified neutral trehalase in a total volume of 400 µl. Reactions were stopped by boiling for 5 min after 30–60 min. The concentration of the released glucose was determined by enzymic Trinder assay (Sigma). Trehalase activities were calculated in units (mg total protein)⁻¹ in crude extracts (1 unit catalyses the hydrolysis of 1 µmol trehalose min⁻¹ and the release of 2 µmol glucose min⁻¹). Values given are means ± standard deviations of at least two measurements.

Pathogenicity assays. The mouse candidiasis model described by Plempel (1984) was used to test the pathogenicity of *C. albicans* mutants. Briefly, wild-type or mutant strains were grown for 24 h at 28 °C in YNB medium. Mice (*n* = 20) (8-week-old, male, strain CFW1; Halan-Winkelmann) were infected by injection of 10⁶ cells in 0.2 ml PBS into the caudal vein. Survival was monitored for 13 d. Survival curves were calculated according to the Kaplan–Meier method using the Prism program (GraphPad Software, San Diego, USA) and compared using the log-rank test. A *P* value ≤ 0.05 was considered as significant.

RESULTS

Cloning of the *C. albicans* gene encoding the neutral trehalase

Using primers 1 and 2 a 178 bp fragment from *C. albicans* strain BMSY 212 genomic DNA was amplified by PCR. The PCR product was cloned into the pCR-Script Amp SK(+) cloning vector. The nucleotide sequences were determined with the T7 standard primer.

Sequence analysis of the cloned PCR product showed 75% identity to the neutral trehalase gene *NTH1* of *S. cerevisiae*. The PCR product (NTCPS) was used as probe in Southern analysis of *C. albicans* strain BMSY 212 genomic DNA digested with *Xba*I. The probe hybridized with a fragment of 4.7 kb. Fragments of approximately 4.7 kb were cloned into plasmid pUC18. After colony hybridization with the NTCPS probe, plasmid pCTX16 was isolated (Fig. 1a).

Sequence analysis of the *NTC1* gene

The 4.7 kb insert of pCTX16 contained one ORF of 2724 bp. This ORF encodes a polypeptide consisting of 907 amino acids with a calculated molecular mass of

104 kDa. At position +2061 of *NTC1* the nucleotide A in the genomic clone (strain BMSY 212) is replaced by the nucleotide C in the cDNA clones (strain 3153a). The triplet AAA in the genomic clone codes for lysine, whereas AAC in the cDNA clone codes for aspartic acid.

Amino acid sequence comparison using the program GAP from the GCG software package revealed a 57% overall identity between the neutral trehalases Nth1p from *S. cerevisiae* and Ntc1p from *C. albicans*, supporting the assumption that the *C. albicans* neutral trehalase gene had been isolated. The *C. albicans* neutral trehalase is 156 amino acids longer than the *S. cerevisiae* enzyme. Most of the additional amino acids are localized in the N-terminus (Fig. 2).

The deduced amino acid sequence showed two possible cAMP-dependent phosphorylation sites: Arg/Arg/Met/Ser at position 7 and Arg/Arg/Leu/Ser at position 210 (Fig. 2) (Rittenhouse *et al.*, 1986).

Three independent cDNA clones were isolated from the ZAP II *C. albicans* 3153a cDNA library (Swoboda *et al.*, 1995) by plaque hybridization with the ³²P-labelled probe NTCPS. The 5' and 3' ends of the three inserts were sequenced. No full-length cDNA was isolated. All three clones have an identical 196 nucleotide sequence between the stop codon and the poly(A) cleavage site.

Mapping the *NTC1* gene transcription unit

The transcriptional start site on the *NTC1* gene was mapped by primer extension. One transcription start site was observed 130 bases upstream from the ATG start codon of the *NTC1* gene. A TATA-like sequence exists 19 bases upstream from the transcription start site. The mRNA poly(A) cleavage site was determined by sequencing the 3' end of three independent cDNA clones. The nucleotide T at position -196 downstream from the stop codon is the last transcribed nucleotide of the chromosomal sequence since the following nucleotide on chromosomal DNA is a T and not an A from the poly(A) tail as in the cDNA clones.

Transcription analysis and chromosomal localization of the *NTC1* gene

Northern analysis was performed with total RNA from *C. albicans* strain BMSY 212 using the internal 1.3 kb *Bam*HI fragment of the *NTC1* gene as probe. A single band of about 3.2 kb was detected (Fig. 3a).

Chromosomes from *C. albicans* strain BMSY 212 were separated by PFGE. Southern analysis showed that the 1.3 kb *Bam*HI fragment of *NTC1* hybridized with the upper chromosome band (Fig. 3b), which consists of chromosome R comigrating with chromosome 1 (Barton

Fig. 3. Northern blot analysis and chromosomal location of *NTC1*. (a) Twenty micrograms total RNA from late-exponential-phase yeast cells from a YPG culture of *C. albicans* strain BMSY 212 was separated on a formaldehyde/1% agarose gel and stained with ethidium bromide (lane 1). The RNA was blotted and hybridized with a [α - 32 P]dATP-labelled 1.3 kb *Bam*HI fragment of the *NTC1* gene (lane 2). (b) Chromosomes from *C. albicans* strain BMSY 212 were separated by PFGE and stained with ethidium bromide (lane 1) or blotted and hybridized with the 1.3 kb *Bam*HI fragment of the *NTC1* gene (lane 2).

& Scherer, 1993). It is not possible to separate these two chromosomes by PFGE. The data of the Southern analysis (Figs 3b, 4a) show that the *NTC1* gene is a single-copy gene.

Disruption of the *NTC1* gene

C. albicans CAI-4 was transformed with the *hisG*–*URA3*–*hisG* cassette flanked by 5' and 3' *NTC1* gene sequences obtained by digestion of pNR1 with *Sac*I/*Hind*III (Fig. 1b). DNA from a representative Ura⁺ colony was isolated and digested with *Hind*III. Southern analysis with the 32 P-labelled 4.7 kb *Xba*I insert of plasmid pCTX16 showed two hybridizing fragments (Fig. 4a, lane 2). One hybridization band is identical to the 7.3 kb *Hind*III band of the parental strain CAI-4 in lane 1. The size of the second hybridizing band of 9.55 kb is consistent with the replacement of 64% of the *NTC1* gene by the *hisG*–*URA3*–*hisG* cassette.

The disruption of the second trehalase allele required as a first step the loss of the selectable marker gene *URA3*. Selection of Ura[−] segregants on 5-FOA plates gave approximately 10³ *C. albicans* CAI-4 *NTC1*/ Δ *ntc1*::*hisG* Ura[−] colonies. Southern analysis of DNA from a representative colony showed two bands (Fig. 4a, lane 3). One hybridizing band is identical to the 7.3 kb band of the parental strain in lane 1. The size of

Fig. 4. Sequential disruption of the *NTC1* gene. (a) Southern blots of *Hind*III-digested chromosomal DNA of the following *C. albicans* strains were hybridized with a [α - 32 P]dCTP-labelled 4.7 kb *Xba*I insert of plasmid pCTX16: CAI-4 (parental strain, lane 1), CAI-4 *NTC1*/ Δ *ntc1*::*hisG*–*URA3*–*hisG* (heterozygous strain, lane 2), CAI-4 *NTC1*/ Δ *ntc1*::*hisG* (heterozygous strain, lane 3), CAI-4 Δ *ntc1*::*hisG*/ Δ *ntc1*::*hisG*–*URA3*–*hisG* (homozygous strain, lane 4) and CAI-4 Δ *ntc1*::*hisG*/ Δ *ntc1*::*hisG* (homozygous strain, lane 5). (b) Confirmation of *NTC1* disruption by PCR. PCR was carried out with primers 3 and 6 (Fig. 1b) and chromosomal DNA from *C. albicans* CAI-4 (parental strain, lane 2), CAI-4 *NTC1*/ Δ *ntc1*::*hisG* (heterozygous strain, lane 3) and CAI-4 Δ *ntc1*::*hisG*/ Δ *ntc1*::*hisG* (homozygous strain, lane 4). Lane 1 contains molecular mass standards (1 kb ladder, Gibco-BRL).

the second band of 6.7 kb is consistent with the loss of the *URA3* gene and one copy of *hisG* from the *hisG*–*URA3*–*hisG* cassette.

The gene replacement was repeated to disrupt the second allele of the *NTC1* gene in *C. albicans* CAI-4, heterozygous for *NTC1* gene disruption. Five presumably homozygous clones (Δ *ntc1*::*hisG*/ Δ *ntc1*::*hisG*–*URA3*–*hisG*) were obtained. Southern analysis of chromosomal DNA from a representative clone is shown in Fig. 4a, lane 4. One hybridizing band is identical to the 6.7 kb band of *C. albicans* CAI-4

NTC1/ Δ *ntc1::hisG* in lane 3. The second band is identical to the 9.55 kb band of *C. albicans* CAI-4 *NTC1*/ Δ *ntc1::hisG*–*URA3*–*hisG* in lane 2. The 7.3 kb fragment of the parental strain CAI-4 disappeared (see lane 1).

After the second 5-FOA selection one of the Ura[−] segregants (Δ *ntc1::hisG*/ Δ *ntc1::hisG*) only showed the 6.7 kb band in Southern analysis (Fig. 4a, lane 5).

These results were confirmed by PCR with primers 3 and 6 (Fig. 1b). Amplification with chromosomal DNA of the parental strain *C. albicans* CAI-4 resulted in a 2.45 kb product that represents a fragment of the wild-type *NTC1* gene (Fig. 4b, lane 2). PCR with DNA of Ura[−] strain *C. albicans* CAI-4 *NTC1*/ Δ *ntc1::hisG* (heterozygous for neutral trehalase mutation) resulted in a 2.45 kb product identical to the product in lane 2 and a 1.85 kb product consistent with the replacement of 64% of the *NTC1* gene by one copy of *hisG* (lane 3). PCR with DNA of Ura[−] strain *C. albicans* CAI-4 Δ *ntc1::hisG*/ Δ *ntc1::hisG* (homozygous for neutral trehalase mutation) showed only the 1.85 kb PCR product (lane 4).

Characterization of the mutant strains

The growth rates of the wild-type strain SC5314 and the homozygous mutant strain were identical at 28 °C, 37 °C and 42 °C and after heat shock at 40 °C, 45 °C and 50 °C in YPG medium. The heterozygous mutant strain grew poorly after heat shock at 50 °C.

In trehalose medium, the growth and the cell number in stationary phase of the homozygous mutant strain were slightly reduced compared with the wild-type strain at 28 °C, 37 °C and 42 °C and after heat shock at 40 °C, 45 °C and 50 °C.

Because the hyphal growth of the phytopathogenic fungus *R. solani* is connected with the mobilization of trehalose by trehalase, we estimated the level of *NTC1* transcription in yeast and hyphal growing cells, but Northern analysis showed no differences of the *NTC1* mRNA level.

Hyphal growth of the homozygous mutant strain after addition of *N*-acetylglucosamine or serum was delayed (by approximately 30 min) compared with the wild-type strain SC5314, but after 2 h no differences were observed. Hyphal growth of the homozygous mutant and the wild-type strain after shift from pH 4.5 to pH 6.5 in Soll's medium was identical.

Neutral trehalase activity in mutant strains of *C. albicans*

Crude extracts of *C. albicans* CAI-4 and trehalase mutant strains CAI-4 Δ *ntc1::hisG*/ Δ *ntc1::hisG* (homozygous for neutral trehalase mutation) and CAI-4 *NTC1*/ Δ *ntc1::hisG* (heterozygous for neutral trehalase mutation) were activated by cAMP-dependent phos-

Fig. 5. Pathogenicity of neutral trehalase mutants. Survival of mice ($n=20$) infected with wild-type *C. albicans* SC5314 (□) and isogenic trehalase deletion mutants CAI-4 *NTC1*/ Δ *ntc1::hisG*–*URA3*–*hisG* (Δ, heterozygous strain) and CAI-4 Δ *ntc1::hisG*/ Δ *ntc1::hisG*–*URA3*–*hisG* (○, homozygous strain).

phorylation and neutral trehalase was partially purified by anion exchange chromatography to avoid cross-contamination with acid trehalase as described in Methods. Extracts from mutants homozygous for neutral trehalase mutation showed 1.4% neutral trehalase activity of the corresponding CAI-4 [0.395 ± 0.085 milliunits (mg protein)^{−1} and 28 ± 2 milliunits (mg protein)^{−1}, respectively]. Extracts from heterozygous mutants showed 52% activity [14.5 ± 2.5 milliunits (mg protein)^{−1}].

Activity of partially purified neutral trehalase was reduced to 50% by validamycin A at $0.5 \mu\text{g ml}^{-1}$.

Pathogenicity of neutral trehalase mutants

C. albicans mutants were tested for pathogenicity in the mouse candidiasis model. No significant differences ($P \leq 0.05$) were observed between survival curves of mice infected with the wild-type strain *C. albicans* SC5314, with *C. albicans* CAI-4 (*NTC1*/ Δ *ntc1::hisG*–*URA3*–*hisG*), heterozygous for neutral trehalase mutation and with *C. albicans* CAI-4 (Δ *ntc1::hisG*/ Δ *ntc1::hisG*–*URA3*–*hisG*), homozygous for neutral trehalase mutation (Fig. 5).

DISCUSSION

Screening systems based on molecular targets of *C. albicans*, the major fungal pathogen of humans, may result in identification of new antifungal drugs. After selection of a potential target, the influence on the pathogenicity of *C. albicans* has to be evaluated by a combination of molecular methods and tests of pathogenicity. Therefore, the *NTC1* gene encoding the

C. albicans neutral trehalase was cloned and disrupted. The resulting heterozygous and homozygous *ntc1* mutant strains were tested for pathogenicity in a mouse model for systemic candidiasis.

The deduced amino acid sequence of the *Candida* neutral trehalase has 57% identity to *S. cerevisiae* Nth1p. Multiple alignment of neutral trehalases from *S. cerevisiae* (Kopp *et al.*, 1993, 1994), *E. coli* (Gutierrez *et al.*, 1989), *Tenebrio molitor* (Takiguchi *et al.*, 1992), rabbit (Ruf *et al.*, 1990) and *C. albicans* shows eight central and C-terminal regions which contain three to seven similar amino acids (Fig. 2). It is unknown whether these conserved sequences are functional regions of neutral trehalase.

A second possible start codon was found at position +25 of the nucleotide sequence of the *NTC1* gene. On the basis of the primer extension analysis results it is not possible to establish which start codon is used. However, considering that a part (Arg/Arg) of a putative phosphorylation site (Arg/Arg/Met/Ser, doubly underlined in Fig. 2) which also exists in the *S. cerevisiae* neutral trehalase (Arg/Arg/Leu/Ser) (Kopp *et al.*, 1994) was identified between the two possible start codons, we assume that the longer ORF is transcribed in *C. albicans*.

A molecular mass of 104 kDa was calculated from the deduced amino acid sequence of the *NTC1* gene. Compared with the calculated molecular mass of the neutral trehalase of *S. cerevisiae* (Kopp *et al.*, 1993, 1994), Ntc1p is 18 kDa larger. In agreement with this calculated molecular mass, preliminary data from ultrafiltration, size exclusion chromatography and SDS-PAGE of partially purified neutral trehalase from *C. albicans* indicate a molecular mass of about 100 kDa for the denatured protein. Under non-denaturing conditions the native enzyme has a molecular mass of about 200–250 kDa. As in *S. cerevisiae*, the active form of the *C. albicans* enzyme therefore seems to be a dimer.

Northern blot analysis showed a single band of approximately 3.2 kb. Considering the 2724 bp of the ORF, upstream (130 bases) and downstream (196 bases) nucleotides and polyadenylation, this value corresponds to the expected size of the mRNA for the neutral trehalase.

The N-terminal part of Ntc1p contains two possible phosphorylation sites (Rittenhouse *et al.*, 1986). The Nth1p from *S. cerevisiae* also has two phosphorylation sites in this region (Kopp *et al.*, 1993, 1994). One or both of these probably mediate the increase in trehalase activity by cAMP-dependent phosphorylation of a serine residue (Van Solingen & van der Plaats, 1975; App & Holzer, 1989). Experiments with crude extract from *C. albicans* wild-type cells showed a similar activation of neutral trehalase by cAMP-dependent phosphorylation. Treatment with alkaline phosphatase destroyed the activity of *C. albicans* neutral trehalase (data not shown).

To determine whether the *NTC1* gene is essential for *C.*

albicans growth and pathogenicity, gene disruption was performed. In *C. albicans*, which is a diploid organism without a detectable sexual life cycle, sequential disruption of the two alleles of the host gene is required. This can be achieved by repeated homologous recombination between the resident genes and truncated alleles using the Ura-blasting procedure developed by Fonzi & Irvine (1993).

Disruption of both alleles was confirmed by Southern blot analysis, PCR and activity assays of neutral trehalase. As the enzyme is probably regulated by phosphorylation, activity measurements in unphosphorylated extracts cannot prove a reduction in enzyme content in mutant cells. However, results of assays with phosphorylated and partially purified samples should correspond to the enzyme content of wild-type and mutant cells.

Our results indicate that the neutral trehalase encoded by the *NTC1* gene is not essential for growth and pathogenicity of *C. albicans*. We assume that *C. albicans* can compensate for the absence of neutral trehalase by an unknown gene or pathway because the trehalose level is not significantly increased in trehalase mutants (data not shown) and, after a slight delay, the growth rate of the homozygous mutant strain was identical with the wild-type strain in trehalose medium at different temperatures and after heat shock.

Under normal conditions we found no neutral trehalase activity in the homozygous mutant strain. However, *C. albicans* could have a second trehalase gene like *YBR0106* in *S. cerevisiae* that is activated under specific conditions. The homology between *NTC1* and this putative second trehalase gene in *C. albicans* must be low, because a low-stringency Southern analysis showed no second signal.

The delay in hyphal formation of homozygous mutant strains after induction with N-acetylglucosamine and serum indicates that the initiation of the dimorphic switch may be connected with the mobilization of trehalose by the neutral trehalase. The constant level of *NTC1* mRNA in yeast and hyphal growing cells indicates that neutral trehalase activity is regulated by protein phosphorylation as in *S. cerevisiae*.

No significant differences between survival curves of mice infected with the wild-type strain SC5314 and with the heterozygous/homozygous *ntc1* mutant strains were observed. We used only Ura⁺ strains because the Ura⁻ strain *C. albicans* CAI-4 showed no pathogenicity in a rat candidiasis model (Cole *et al.*, 1995). However, an effect of the delay in hyphal formation on the pathogenicity of the mutant strains could not be detected using an intravenous injection model of virulence.

Nevertheless, trehalose metabolism remains an interesting target for antifungal drugs. *S. cerevisiae* mutants defective in trehalose synthesis are unable to grow in the presence of glucose at physiologically relevant concentrations (Hohmann *et al.*, 1994). The gene responsible,

GGI1/TPS1, encodes the small subunit of trehalose synthase (Van Aelst *et al.*, 1993). This enzyme could be a target for the development of fungus-specific screening systems.

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