The ATC1 Gene Encodes a Cell Wall-linked Acid Trehalase Required for Growth on Trehalose in Candida albicans*

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After screening a Candida albicans genome data base, the product of an open reading frame (IPF 19760/CA2574) with 41% identity to Saccharomyces cerevisiae vacuolar acid trehalase (Ath1p) was identified and named Atc1p. The deduced amino acid sequence shows that Atc1p contains an N-terminal hydrophobic signal peptide and 20 potential sites for N-glycosylation. C. albicans homozygous mutants that lack acid trehalase activity were constructed by gene disruption at the two ATC chromosomal alleles. Analysis of these null mutants shows that Atc1p is localized in the cell wall and is required for growth on trehalose as a carbon source. An Atc1p endowed with acid trehalase activity was obtained by an in vitro transcription-translation coupled system. These results strongly suggest that ATC1 is the structural gene encoding cell wall acid trehalase in C. albicans. Determinations of ATC1 mRNA expression as well as acid trehalase activity in the presence and absence of glucose point out that ATC1 gene is regulated by glucose repression.

The non-reducing disaccharide trehalose (α-1-glucopyranosyl α-1-glucopyranoside) has been identified and characterized in a wide variety of organisms ranging from bacteria and fungi to plant and mammals (1–3). Trehalose plays a set of different functions, depending on the specific biological system analyzed. Thus, in prokaryotes (4), it can be used as an external carbon source (Escherichia coli, Bacillus subtilis) (5, 6), compatible solute (Ectothiorhodospira halochloris) (7), or structural component of the “cord factor” in mycobacteria (8). However, in yeasts and fungi, trehalose is mainly used as a reserve carbohydrate as well as a cell protectant against several physiological or environmental stimuli (9, 10). In recent years, biotechnological applications of trehalose have increased in the last few decades, due to the rise of the immunocompromised population (22–24). The incidence of candidiasis has dramatically increased in the last few decades, due to the rise of the immunocompromised population (25, 26). Furthermore, C. albicans is a dimorphic organism, which exists as unicellular budding yeast (blastoconidia) or as mycelial structures (hyphae and/or pseudohyphae). Morphological transition has been associated with pathogenicity, and the mycelial forms seem to be predominant during host tissue colonization (22, 27–29). In recent years, trehalose metabolism has been intensively investigated in C. albicans in connection with dimorphism and as a possible contributory factor to its virulence. In this respect, disruption of TPS1 indeed impairs hypha formation and decreases infectivity (30), whereas TPS2 is involved in cell integrity and pathogenicity but is not required for dimorphic transition (31, 32). However, the neutral trehalase (Nt1p) lacks a specific role during dimorphism and pathogenicity (33).

In yeasts, trehalose synthesis takes place in a sequential two-step reaction: trehalose 6-phosphate is synthesized from UDP-glucose and glucose 6-phosphate, a reaction catalyzed by a magnesium-dependent trehalose 6-phosphate synthase (coded by the TPS1 gene). Subsequent dephosphorylation by a specific trehalose phosphatase (coded by the gene TPS2) renders free trehalose (3, 12, 13). In turn, hydrolysis of the disaccharide points to amply differences among the species of fungi studied. As a general rule, two different trehalases are present, which differ in location, catalytic properties, and regulation: (i) a cytosolic enzyme, which exhibits maximal activity at neutral pH (7.0) and which is activated by Ca2+ or Mn2+ and regulated by cAMP-dependent protein kinases; (ii) an acidic trehalase (optimum pH about 4.5), located inside the vacuoles or associated to the cell wall, whose activity is independent of divalent cations (2, 12, 14, 15). Whereas neutral trehalases (Nt1p) hydrolyze the intracellular pool of trehalose, the role of acid trehalases (Ath1p) remains more elusive. Ath1p appears to be required for growth on exogenous trehalose (14) and its regulation by catabolite repression has been postulated (17, 18).

In Saccharomyces cerevisiae and Candida utilis, both kinds of cytosolic and vacuolar trehalases are present (2, 3, 10). However, in fungi such as Neurospora crassa and Mucor rouxii, acid trehalase activity is linked to the cell wall of ascospores and conidiospores (19, 20). In Emericella nidulans, an acid trehalase has been purified, and the corresponding treA gene has been cloned, being the TreAp located in the cell wall of conidiospores (21).

Candida albicans is an opportunistic pathogen fungus in humans, which can cause either septicaemic or mucosal infections (22–24). The incidence of candidiasis has dramatically increased in the last few decades, due to the rise of the immunocompromised population (25, 26). Furthermore, C. albicans is a dimorphic organism, which exists as unicellular budding yeast (blastoconidia) or as mycelial structures (hyphae and/or pseudohyphae). Morphological transition has been associated with pathogenicity, and the mycelial forms seem to be predominant during host tissue colonization (22, 27–29). In recent years, trehalose metabolism has been intensively investigated in C. albicans in connection with dimorphism and as a possible contributory factor to its virulence. In this respect, disruption of TPS1 indeed impairs hypha formation and decreases infectivity (30), whereas TPS2 is involved in cell integrity and pathogenicity but is not required for dimorphic transition (31, 32). However, the neutral trehalase (Nt1p) lacks a specific role during dimorphism and pathogenicity (33).

Preliminary enzymatic data suggest that acid trehalase in C. albicans is associated to the external surface (34) and is not strictly necessary for serum-induced dimorphism (35). The whole picture of trehalose biosynthesis and hydrolysis in
**Characterization of C. albicans Acid Trehalase**

**Table 1**

| Strain          | Genotype                  | Source  |
|-----------------|---------------------------|---------|
| CA14 (C. albicans) | ura3Δ::imm434/ura3Δ::imm434 | Ref. 41 |
| ATC1-1 (C. albicans) | atc1Δ::hisG-URA3::hisGATC1 | This work |
| ATC1-2 (C. albicans) | atc1Δ::hisGATC1 | This work |
| ATC2-1 (C. albicans) | atc1Δ::hisGATC1 | This work |
| ATC2-2 (C. albicans) | atc1Δ::hisGATC1 | This work |
| ATC2-3 (C. albicans) | atc1Δ::hisGATC1 | This work |
| DH5α (E. coli) | gyrA96, thiA1, relA, supE44, relA, deoR, ∆lacZYA-argF/U169 | Ref. 37 |

**Strains, Growth Conditions, and Transformations**—The *C. albicans* and *Escherichia coli* strains used in this study are listed in Table I. *C. albicans* cells were grown in YM medium (1% yeast extract, 2% bactopeptone) (36) or MM medium (0.7% yeast nitrogen base without amino acids) supplemented with the appropriate nutrients in amounts specified by Sherman (36) and with the appropriate carbon source (2% glucose or 2% trehalose). YPD, MMD, and MMT media were solidified with 2% agar. *C. albicans* transformation was performed according to Gietz et al. (37). *E. coli* DH5α, which was grown routinely in LB medium (0.5% yeast extract, 1% tryptone, 0.5% NaCl) supplemented with 100 μg/ml ampicillin (LBA) when required, was used for plasmid propagation. *E. coli* was transformed as described by Hanahan (38).

### Nucleic Acid Manipulations and Analysis—Genomic DNA preparation from *C. albicans* was carried out using the method described by Fujimura and Sakuma (39); total RNA isolation was carried out as previously described (40). Plasmid purification was performed using the FlexiPrep Kit commercial system (Amersham Biosciences). Standard DNA manipulation procedures were followed using standard protocols (41). DNA probes (amplons JCE12 and JCE35) for Southern analysis were labeled by random primed incorporation of digoxigenin-labeled deoxyuridine triphosphate (DIG-labeled DNA) using a DIG DNA-labeling kit (Roche Applied Science) according to the manufacturer’s instructions. Southern hybridization was performed as described by Ramón et al. (40). DNA and RNA concentrations were determined by measuring absorbance (A260) in a GeneQuant II RNA/DNA calculator spectrophotometer (Amersham Biosciences).

### Plasmid Construction for Disruption and Reintegration of the ATC1 Gene—The strategy followed for disrupting the ATC1 gene was carried out by replacing part of the ORF (from amino acid 29 to 128) with an *hisG::URA3::hisG* (42). The disruption cassette construction was made by PCR amplification in two steps using genomic DNA as a template. In the first step, an amplicon of 619 bp was obtained (JCE 35) was digested with BamHI and SacI and then subcloned into p5921 (42), containing the *hisG::URA3::hisG* cassette; the resulting plasmid, which was named pATH1, contained 619 bp of the ORF (positions +1895 to +2602 with respect to the start codon). In the second step, an ampiclon of 650 bp by the ORF (positions +238 to +868 with respect to the start codon) was obtained by using the sense primer JCE1 (TCT-GACCTGCGAGCAAATGCTCTGATC) and the antisense primer JCE2 (CACAGCGCGAGAAACAAATGCTTGACG) containing engineered restriction sites HindIII and PstI, respectively (underlined). The amplicon obtained (JCE12) was ligated into the HindIII and PstI sites of pATH1 to create plasmid pATH51 in which 1100 bp (29%) of the coding region were deleted.

To reintegrate the ATC1 gene into the genome of the mutant strain, ATC2-2, an integrative plasmid, using CIp10-MAL2p (43), was constructed. The construction of a derivative of CIp10-MAL2p, in which the ATC1 gene was placed under the control of the MAL2 promoter, was as follows. The ATC1 open reading frame was obtained by PCR amplification using genomic DNA as template, and the sense primer YEJ3 (CACAGCGCGAGAAACAAATGCTTGACG) and the antisense primer YEJ2 (CACACCGGTATTTACTTTATAAAAACACTTCT-GCG) containing engineered restriction sites EcoRV and MluI, respectively (underlined). The amplicon obtained (YEJ32) was ligated into the EcoRV and MluI sites of CIp10-MAL2p to create plasmid CIpMAL2p-ATC1.

To target integration of CIp10MAL2p-ATC1 to the *C. albicans* RP10 locus, it was first digested with NcoI, which cuts plasmid uniquely within the RP10 sequence. Digested plasmid was used to transform *C. albicans* atc1Δ null mutant.

### Isolation of ATC1 Null Mutant—Disruption of ATC1 was achieved as described by Fanzi and Irwin (42). *C. albicans* CA14 cells were transformed into *Ura* protohyb with 10 μg of an HindIII/SacI fragment from plasmid pATH1. Transformed cells were selected as *Ura* in an MMD medium lacking uridine and checked for integration of the cassette at the ATC1 locus by Southern blot analysis. One of the heterozygous disruptants recovered (designated *C. albicans* ATC1-1) was used to select spontaneous *Ura* derivatives in MMD medium containing 5-fluoro-orotic acid (44). These clones were analyzed by Southern blot hybridization to identify those that had undergone intrachromosomal recombination between *hisG* repeats. One of these *Ura* derivatives (termed *C. albicans* ATC1-2) was used for replacement of the second ATC1 allele in a similar way, using the HindIII/Sacl fragment from plasmid pPATH1. Transformed cells were selected as *Ura* and integrated into the correct allele was verified by Southern blot analysis. One of these *Ura* transformants (designated *C. albicans* ATC2-1) was used for 5-fluoro-orotic acid selection to *Ura* auxotrophy. *Ura* segregants were screened by Southern blot analysis using digestion with BglII to identify those carrying both disrupted ATC1 alleles. One of these ATC1 null mutants was designated *C. albicans* ATC2-2.

### Cell Wall Purification and Solubilization of Wall Proteins—To purify cell walls, washed cells were resuspended in a small volume of cold 1 mM phenylmethylsulfonyl fluoride. Glass beads were added in a proportion of 1.5 g/ml of dry cells, and the mixture was vortexed at room temperature for eight periods of 1 min each with intermediate periods of 1 min on ice. Using this method, the whole cell population was broken up, as monitored in the phase-contrast microscope. The purification procedure was continued by repeated washing of the cell wall pellet in cold phenylmethylsulfonyl fluoride (1 mM). The pellet was collected and operationally defined as the cell wall.

Conditions for the solubilization of cell wall proteins with SDS or zymolase 20T have been described (45, 46); growth media and β-mer-
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Protein synthesis was produced using the RTS100 E. coli HY Kit (Roche Applied Science). Reactions were performed mainly according to the manufacturer’s manual, using ~200 ng of DNA per reaction. Reactions were incubated at 30 °C for 6 h. The resulting lysates were directly used for the acid trehalase assay. To act as a control, GFP was synthesized according to the manufacturer’s recipe.

RESULTS

Silico Screening for Potential Acid Trehalases in C. albicans—Taking as template the acid trehalase from S. cerevisiae (Atp1p), a C. albicans genome database (available on the World Wide Web at genolist.pasteur.fr/CandidaDB/) was blasted. One ORF protein (IPF 19760/CA2574) was found and presented a significant homology with the S. cerevisiae Ath1p. Comparison of the amino acid sequence with the sequences found in protein data bases using the BLAST search algorithm (54) revealed a significant homology with both the S. cerevisiae Ath1p and E. nidulans TreAp (Fig. 1). CA2574 shares 41% identical and 59% similar amino acids with Ath1p and 29% identical and 47% similar amino acids with TreAp, both comparisons being made over their entire length. These results suggest that IPF 19760 could encode C. albicans acid trehalase, for which reason we called it Atc1p (for acid trehalase of Candida albicans).

Structural Analysis of the Amino Acid Sequence Encoded by ATC1—The ORF encodes a putative polypeptide of 1040 amino acids with a calculated molecular weight of 116,260 and a pI of 4.81. Analysis of the predicted amino acid sequence revealed an N-terminal region with characteristics of a signal peptide (55) and a predicted cleavage site between positions 22 and 23 (CSG↓AP) (Fig. 2B). Hydroxylation analysis (56) of the deduced amino acid sequence showed that the hydrophobic signal sequence is followed by a neutral region representing the mature protein (Fig. 2A). Assuming the cleavage site is at position 22, the mature protein has 1019 amino acid residues with a calculated molecular mass of 113.7 kDa. Nineteen potential N-glycosylation sites (Asn-Xaa-Ser/Thr) were identified at amino acid positions 113, 147, 205, 250, 264, 313, 495, 554, 564, 624, 633, 792, 800, 867, 885, 904, 908, 966, and 1000. The difference between the predicted size of Atc1p (113.7 kDa) and the size deduced from the motility in SDS-PAGE (170 kDa) (Fig. 5) can be accounted for by glycosylation of one or more of the potential N-glycosylation sites.

A search of protein motifs in Atc1p revealed a match to the conserved sequence of glycosyl hydrolase family 65, N-terminal domain (amino acids 89–350) and glycosyl hydrolase family 65 central catalytic domain (amino acids 351–753) (Fig. 2B). This domain is believed to be essential for catalytic activity, although its precise function remains unknown. The catalytic domain also forms most of the dimerization interface.

In Vitro Synthesis of Acid Trehalase—The in vitro transcription-translation coupled system (Fast Translation System RTS 100 E. coli HY Kit; Roche Applied Science) was utilized for the synthesis of acid trehalase from the PCR-amplified ATC1 gene carrying the T7 promoter sequence. To generate linear DNA, two PCRs were performed using the Linear Template Generation Set, HA tag (Roche Applied Science) following the manufacturer’s instructions and the specific primers ATC-C-N-FOR (CTTTAAGAAGAGATATAACCTACGTGTGGAGCTAATTTAACCAACCTTAGA) and ATC-N-REV (ATCTGATGGTGAGCTGTTCCGATAAAAAACAATCTTACGTA), which allow amplification of the entire, HA-tagged, ATC1 gene.

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an α-helical region (residues 1–88), except for one short β-sheet (residues 72–75) on the basis of secondary structure prediction. 

The second domain includes residues 89–350 in a β-strand region, entirely made up of antiparallel β-strands. This region was recognized as the N-terminal domain for glycosyl hydrolase family 65 (52). The third domain (residues 351–753) is an α-helical region including a short β-strand linker region (residues 552–578). Basically, this is the glycosyl family 65 central catalytic domain. From residue 754 to 930, the prediction program suggests a β-strand region. The C-terminal region was not threaded by the prediction program. It is reasonable to assume that an α-helical sheet region (residues 931–1019) exists, as suggested by the secondary structure prediction.

The structural resemblance of the central domain of Atc1p to LbMP leads us to suggest a structural interpretation for that protein. The two crucial catalytic residues, Glu570 and Asp442, are situated at the center of the pocket. Both residues overlap with totally conserved residues in LbMP. Glu570 (Atc1p) overlaps with Glu487 (LbMP), and Asp442 (Atc1p) overlaps with Asp359 (LbMP) (Fig. 2C, 2). The carboxylate OE1 atom of Glu570 is hydrogen-bonded to the carboxylate group of Glu506, whereas its OE2 atom points toward Lys639. The putative active site pocket in Atc1p is also walled by residues that are very well conserved in family 65 (52). Tyr435, which is positioned around the rim of the pocket, provides side chains that could interact with hydroxyl groups of the substrate. Asp570 is also surrounded by a cluster of hydrophobic residues that would be good candidates for interaction with hydrophobic parts of the trehalose molecule (Leu587, Lys639, Pro649, and Leu650).

Disruption of the ATC1 Gene—To investigate the function of the Atc1p, null mutants were constructed by targeted gene disruption, and the resulting phenotypes were analyzed. The ATC1 gene was disrupted by using a strategy originally developed for S. cerevisiae (57) and modified for use in C. albicans (42). This method uses a cassette consisting of the C. albicans URA3 flanked by direct repeats of the Salmonella typhi-

murium hisG (see “Materials and Methods” for details). This cassette was used to replace 1097 bp of the ATC1 ORF. A linear HindIII/SacI fragment (see “Materials and Methods”) including the cassette flanked by ATC sequences was used to transform C. albicans CAI4 to Ura−.

Fourteen of the resulting Ura− transformants were analyzed, and nine of them were seen to contain the desired insert at the ATC1 locus (data not shown). Southern analysis of a representative isolate, C. albicans ATC1-1, after digestion with PstI, revealed that the cassette had integrated in the correct allele, originating a 5.88- and a 0.75-kb fragment when ampli-

cons JCE12 and JCE35 were used as a probe and a fragment of 5.88 kb when the cassette was used as a probe (Fig. 3, A, step 2, and B, lanes 2); the 0.75-kb fragment appeared because a
new PstI restriction site had been formed in the disruption cassette construction process. This is consistent with the replacement of one allele of the ATC with the transforming DNA. The 1.94- and 1.04-kb PstI fragments correspond to the other allele that was still present in the Ura⁺/H11001 transformants. Ura⁺/H11002 segregants of strain C. albicans ATC1-1 were selected on a medium containing 5-fluoro-orotic acid (44) and examined by Southern blot analysis. Five of the eight independent segregants examined had undergone intrachromosomal recombination between the hisG repeats, resulting in excision of the URA3 marker and one copy of hisG, whereas three of them had experienced an interchromosomal recombination event, reverting to the parental genotype (data not shown). Southern blot analysis of a representative intrachromosomal recombinant, strain C. albicans ATC1-2, is shown in Fig. 3B (lanes 3). The 5.88-kb PstI fragment in strain C. albicans ATC1-2, containing the atc1Δ::hisG-URA3-hisG disruption was absent, and a new band of 2.88 kb was present, indicating the loss of both URA3 and one copy of hisG (Fig. 3A, step 3).

The homozygous atc1Δ null mutant was generated after transformation of strain C. albicans ATC1-2 with the same disruption cassette. Fifteen Ura⁺ transformants were examined by Southern blot analysis, and only four of them showed the hybridization pattern corresponding to the integration of the cassette in the undisrupted allele of ATC1, whereas the other 11 transformants showed replacement of the first disrupted allele. The Southern blot analysis of a representative Ura⁺ isolate that exhibited the correct hybridization pattern, strain C. albicans ATC2-1, is shown in Fig. 3B (lanes 4). The parental 1.94- and 1.04-kb PstI fragments were absent, with a 5.88-kb fragment appearing instead, indicating correct integration. C. albicans ATC2-1 was plated on 5-fluoro-orotic acid-containing medium to select Ura⁺ segregants. Five Ura⁺ segregant isolates were screened by Southern analysis, and each exhibited the correct hybridization pattern. The Southern blot analysis of one of these segregants, strain C. albicans ATC2-2, is shown in Fig. 3A (lanes 5). The 5.88-kb PstI fragment was absent, giving rise to a 2.88-kb fragment, which results from the loss of URA3 and one copy of hisG, indicating that both alleles of the ATC1 gene were disrupted. Northern blot analysis demonstrated that no mRNA was present when hybridized with the amplicons JCE12/JCE35 probe in RNA samples from the null mutant C. albicans ATC2-2.

C. albicans atc1Δ Lacks Acid Trehalase Activity and Does Not Grow on Trehalose as Carbon Source—Two trehalase ac-
activities, neutral and acid enzymes, have been characterized in S. cerevisiae and related yeasts (3). Neutral trehalase is responsible for the physiological hydrolysis of trehalose in intact cells (12, 14, 33), and acid trehalase is necessary to grow on trehalose as a carbon source (16, 21). This led us to search for conditions under which ATC1 deletion shows a phenotype.

C. albicans atc1/H9004 null mutant lacked acid trehalase activity, an effect that appears to be gene dosage-dependent (Table II). Of note is the fact that substitution of glucose by trehalose as an exogenous carbon source induced a marked activation of Atc1p, either in exponential or stationary cultures (Table II). In turn, atc1/H9004 cells presented the same neutral trehalase activity as the parental strain (data not shown). As Fig. 4A shows, C. albicans strains CAI4, ATC1-2, and ATC2-2 exhibited normal growth on glucose, but when a replica plating was made on a medium containing trehalose as carbon source, the null mutant strain C. albicans ATC2-2 showed no growth.

In addition to the studies using solid medium, growth in liquid medium was also analyzed. No differences were found when the different strains were grown in liquid MMD medium (Fig. 4B). When cells were grown in liquid MMT medium, no growth of the atc1Δ null mutant was observed, and the parental and the heterozygous mutant strain grew as the same rate (Fig. 4B).

To assess whether the absence of acid trehalase activity in the C. albicans atc1Δ null mutant was a consequence of the ATC1 gene disruption, the ATC1 open reading frame placed under the control of the MAL2 promoter was integrated into the genome of the atc1Δ/ATC1 Δ mutant. When these transformed cells were grown on glucose as a carbon source, the activity of acid trehalase was virtually undetectable (<0.3 nmol min⁻¹ (mg of protein)⁻¹). However, when maltose was used instead of glucose as carbon source, an acid trehalase activity of 6.5 nmol min⁻¹ (mg of protein)⁻¹ was measured in the transformed C. albicans atc1Δ null mutant.

The ATC1 Null Mutant Lacks a 170-kDa Cell Wall Protein—To identify the gene product encoded by ATC1, the different protein species present in different subcellular fractions were analyzed by Western blotting, using antibodies raised against a synthetic oligopeptide containing 15 amino acid res-

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**TABLE II**

| Carbon Source | CAI4 | ATC1-2 | ATC2-2 |
|---------------|------|--------|--------|
| Glucose       | 5.76 | 1.40   | <0.3   |
| Galactose     | 2.26 | 1.57   | <0.3   |
| Trehalose     | 68.00| 33.30  | —      |
| Exponential phase |  |  |  |
| Galactose     | 7.02 | 6.94   | <0.3   |
| Trehalose     | 1.62 | 1.21   | <0.3   |
| Stationary phase |  |  |  |

“—” indicates that the culture was unable to grow on exogenous trehalose as a carbon source.
idues of the predicted protein (pAb-Yol1) (see “Materials and Methods”). As shown in Fig. 5, the antibody mainly detected a 170-kDa species in SDS extracts of the cell wall from parental strain but not in the null mutant. A band of 120 kDa appeared in both the parental strain and the null mutant (Fig. 5) that could be the consequence of this protein cross-reacting with the antibody. No differences were observed in the protein pattern of other cell wall extracts, spent media, and cytosolic fraction (data not shown). It was therefore concluded that ATC1, in C. albicans, codes a cell wall protein necessary to grow on trehalose as a carbon source.

Expression Patterns of ATC1 and Activity in Presence of Glucose—The expression of ATC1 was examined by RT-PCR. Fig. 6 shows the RT-PCR amplification of an ATC1-specific DNA fragment (685 bp) from first strand cDNA derived from cells growing on glucose or trehalose as a carbon source. Different samples, containing the same quantity of first strand cDNA, were prepared and subjected to different cycles of amplification. Fig. 6 shows that the quantity of EFB-specific amplicon (526 bp) was approximately the same in each of the first strand cDNA samples. The presence of one intron in the corresponding region of the EFB genomic DNA allows differentiation between bands amplified from cDNA and any potentially contaminating genomic DNA (890 bp) (58). The results indicate that ATC1 shows a differential expression pattern as a function of the carbon source, being more strongly expressed when cells were grown on trehalose as a carbon source than on glucose. In order to elucidate whether acid trehalase activity is subjected to catabolite repression by glucose, cells growing in trehalose as carbon source (MMT medium) were supplemented with 4% glucose. As is shown in Fig. 7A, acid trehalase activity decreased significantly upon glucose addition and remained at a low level after 3 h. To rule out the possibility that the presence of glucose may alter the Atc1p function, but not necessarily its mRNA abundance, an RT-PCR was performed at different times after glucose addition to cells growing in trehalose as the sole carbon source. As is shown in Fig. 7B, the abundance of ATC1 mRNA underwent a significant decrease after the glucose addition. This decrease ran parallel to a rapid loss of acid trehalase activity (Fig. 7). These results strongly suggest that ATC1 is a glucose-repressible gene.

Protein Analysis of in Vitro Translation Products—In order to verify that the ATC1 gene actually encodes a structural acid trehalase and not a transcription factor necessary for expression of another putative acid trehalase gene in the C. albicans genome, Atc1p was in vitro synthesized from a PCR-amplified ATC1 gene carrying the T7 promoter sequence (see “Materials and Methods”). Western blot analysis of the resulting lysates showed that a correct synthesis of Atc1p function, but not necessarily its mRNA abundance, an RT-PCR was performed at different times after glucose addition to cells growing in trehalose as the sole carbon source. As is shown in Fig. 7A, acid trehalase activity decreased significantly upon glucose addition and remained at a low level after 3 h. To rule out the possibility that the presence of glucose may alter the Atc1p function, but not necessarily its mRNA abundance, an RT-PCR was performed at different times after glucose addition to cells growing in trehalose as the sole carbon source. As is shown in Fig. 7B, the abundance of ATC1 mRNA underwent a significant decrease after the glucose addition. This decrease ran parallel to a rapid loss of acid trehalase activity (Fig. 7). These results strongly suggest that ATC1 is a glucose-repressible gene.
Enzymes involved in trehalose metabolism must be considered as a potential target in the search for new antifungal compounds (31, 32), since the sugar is absent from mammalian cells, whereas trehalase is located in the brush border membranes of epithelial cells and in the kidney proximal tube (59). Previous work showed that in C. albicans, TPS1 and TPS2 genes are factors of virulence (30–32). In addition, it is also conceivable that proteins located in the external surface should be preferential targets for antifungal drugs. On the basis of this rationale approach, we have carried out the cloning and functional characterization of a cell wall-linked acid trehalase.

To identify ORFs showing homology with S. cerevisiae Ath1p (acid trehalase) a sequence approach was followed, screening the database of C. albicans. In this way, we found the prospective protein code for one ORF (IPF 19760/CA2574) that has high homology with Ath1p of S. cerevisiae and TreAp of E. nidulans, which we therefore called Atc1p.

The results obtained revealed that ATC1 encodes a cell wall protein, since its disruption leads to the absence of a 170-kDa protein band only in the material released from isolated cell walls by SDS. The deduced amino acid sequence reveals the presence of a signal peptide at the N terminus of the protein, which is characteristic of proteins that transit through the

**DISCUSSION**

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Characterization of C. albicans Acid Trehalase

secretory pathway. The theoretical molecular mass of mature Atc1p is 113.7 kDa, but by SDS-PAGE it shows an apparent motility of 170 kDa. This experimental motility could be due to N-glycosidic modification, as happens in S. cerevisiae and E. nidulans; Atc1p has 20 potential N-glycosylation sites.

To obtain information about the possible function of Atc1p, we analyzed the phenotype of the atc1Δ mutant. The only phenotype that we could demonstrate when studying this mutant was the absence of acid trehalase activity as well as its inability to grow on a medium containing trehalose as a single carbon source, suggesting that Atc1p is required for the hydrolysis of exogenous trehalose (Table II, Fig. 4). This phenotype is similar to that observed for the S. cerevisiae ath1Δ strain (15, 16, and 60) and for E. nidulans treAΔ strains (21). It is possible that acid trehalases form a family involved in the assimilation of external trehalose. The fact that Atc1p is localized in the cell wall, as was previously shown for E. nidulans (21), that it is probably N-glycosylated, and that it contains a signal peptide (as deduced from the amino acid sequence) is in agreement with this conclusion. In contrast to the E. nidulans and C. albicans acid trehalases, S. cerevisiae Ath1p has been localized in vacuoles (61), a difference that might reflect the distinct mechanisms that fungal species use to assimilate exogenous trehalose.

Comparison of the amino acid sequence of C. albicans Atc1p with E. nidulans TreA and S. cerevisiae Ath1p showed that it shares 29% identity and 41% similarity with TreA and shares 41% identity and 59% similarity with Ath1p over their entire length. A more detailed analysis of the alignment between the three proteins reveals stretches of amino acids (15–20 residues) that are up to 75% identical and could constitute the catalytic domain of these enzymes. This region (amino acids 404–757) of maximum identity matches the central catalytic domain of the glycosyl hydrolase family 65. The two crucial catalytic residues, Glu570 and Asp442 that overlap with totally conserved residues in a domain of three proteins reveals stretches of amino acids (15–20 residues) that are up to 75% identical and could constitute the catalytic domain of these enzymes. This region (amino acids 404–757) of maximum identity matches the central catalytic domain of the glycosyl hydrolase family 65. The two crucial catalytic residues, Glu570 and Asp442 that overlap with totally conserved residues with Ath1p has been localized in vacuoles (61), a difference that might reflect the distinct mechanisms that fungal species use to assimilate exogenous trehalose.

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