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The *Candida albicans ATO* Gene Family Promotes Neutralization of the Macrophage Phagolysosome

Heather A. Danhof, Michael C. Lorenz

Department of Microbiology and Molecular Genetics and the Graduate School of Biomedical Sciences, the University of Texas Health Science Center at Houston, Houston, Texas, USA

Candida albicans **is an opportunistic human fungal pathogen that causes a variety of diseases, ranging from superficial mucosal to life-threatening systemic infections, the latter particularly in patients with defects in innate immune function.** ***C. albicans*** **cells phagocytosed by macrophages undergo a dramatic change in their metabolism in which amino acids are a key nutrient. We have shown that amino acid catabolism allows the cell to neutralize the phagolysosome and initiate hyphal growth. We show here that members of the 10-gene** ***ATO*** **family, which are induced by phagocytosis or the presence of amino acids in an Stp2-dependent manner and encode putative acetate or ammonia transporters, are important effectors of this pH change** ***in vitro*** **and in macrophages. When grown with amino acids as the sole carbon source, the deletion of** ***ATO5*** **or the expression of a dominant-negative** $ATO1^{G53D}$ **allele results in a delay in alkalinization, a defect in hyphal formation, and a reduction in the amount of ammonia released from the cell. These strains also form fewer hyphae after phagocytosis, have a reduced ability to escape macrophages, and reside in more acidic phagolysosomal compartments than wild-type cells. Furthermore, overexpression of many of the 10** ***ATO*** **genes accelerates ammonia release, and an** $ato5\Delta\ ATO1^{G53D}$ **double mutant strain has additive alkalinization and ammonia release defects. Taken together, these results indicate that the Ato protein family is a key mediator of the metabolic changes that allow** ***C. albicans*** **to overcome the macrophage innate immunity barrier.**

Candida albicans is an opportunistic pathogen that colonizes the skin and gastrointestinal and genitourinary tracts of most healthy individuals but also causes a range of diseases, from nonlethal mucosal infections, such as oral thrush and vaginitis, to disseminated hematogenous candidiasis, the latter in immunocompromised individuals (1–3). As the fourth most prevalent cause of hospital-acquired infection, disseminated candidiasis is very difficult to treat, prolongs hospitalization, and has a mortality rate of ~40% (4–6). The high mortality rates and large health care burden associated with *C. albicans* infection highlight the importance of understanding the physiology, virulence factors, and host-pathogen interactions of *C. albicans*.

The healthy immune system is able to effectively prevent systemic candidiasis; however, advances in health care have increased the population of individuals surviving despite immune dysfunctions. Conditions that predispose individuals to disseminated candidiasis include hematological malignancies, genetic immune disorders, HIV/AIDS, and iatrogenic interventions, including organ transplantation, chemotherapy, and invasive procedures (3, 7). The interaction between the innate immune system and *C. albicans* is a primary determinant of disease progression, as those with innate immune defects are more susceptible to serious infection (8). Macrophages, along with other professional phagocytes, are key components of the innate immune response to *C. albicans* (8–11). Mice in which macrophage function has been depleted are more susceptible to mucosal and disseminated candidiasis (8, 11). In counterpoint, *C. albicans* has evolved mechanisms to escape phagocytosis and killing by the macrophage, including differentiation into a filamentous hyphal form that facilitates escape and dissemination. This interaction is highly dynamic and is critical for *C. albicans* virulence and, therefore, remains the subject of intense study (12, 13).

The morphological switch alone does not fully define the response of *C. albicans*, as many studies have revealed large-scale transcriptional reprogramming and proteomic shifts that take place upon phagocytosis, including a shift away from glucose metabolism and toward alternative carbon assimilation (14–17). The importance of metabolic adaptation is underscored by the decreased virulence in animal models of *C. albicans* strains that are defective in the glyoxylate cycle and in β-oxidation (18–21). Furthermore, carbon source utilization plays a significant role in cell wall composition, stress susceptibility, and phagocyte recognition (22, 23).

Our laboratory has demonstrated that *C. albicans* grown *in vitro* in medium that mimics the nutrients predicted to be available after phagocytosis rapidly alkalinizes the extracellular environment (24). This process depends upon the utilization of amino acids as a carbon source and leads to the extrusion of ammonia from the cell, which is derived from amino acid catabolism. This secretion of ammonia counteracts the acidification of the phagolysosome, providing a neutral pH signal that induces *C. albicans* to undergo hyphal morphogenesis, thus facilitating escape from the macrophage (25). Furthermore, a mutant lacking the transcription factor Stp2p, which regulates amino acid permeases, fails to

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Address correspondence to Michael C. Lorenz, Michael.Lorenz@uth.tmc.edu.

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TABLE 1 *C. albicans* strains used

Strain	Relevant genotype	Complete genotype[a]	Reference
SC5314	Wild type	Prototroph	73
SVC17	*stp2*Δ	*stp2*Δ::*FRT*/*stp2*Δ::*FRT*	25
HDC27	*ato5*Δ	*ato5*Δ::*FRT*/*ato5*Δ::*FRT*	This study
HDC30	*ato5*Δ+*ATO5*	*ato5*Δ::*FRT*/*ato5*Δ::*FRT RPS10*/*rps10*::*ATO5-CIp10-SAT1*	This study
HDC33	Wild type *SAT1*	*RPS10*/*rps10*::*CIp10-SAT1*	This study
HDC31	*ato5*Δ *SAT1*	*ato5*Δ::*FRT*/*ato5*Δ::*FRT RPS10*/*rps10*::*CIp10-SAT1*	This study
Can572	$ATO1^{G53D}$	*ura3*/*ura3 RPS10*/*rps10*::*CIp10-ACT1p-*$ATO1^{G53D}$	24
THE1	Wild type TetR	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2*	34
HDC48	Wild type TetR	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2 RPS10*/*rps10*::*CIp10-tetR*	This study
HDC44	*tet-ATO1*	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2 RPS10*/*rps10*::*CIp10-tetR-ATO1*	This study
HDC38	*tet-*$ATO1^{G53D}$	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×*HA-ADE2 RPS10*/*rps10*::*CIp10-tetR-*$ATO1^{G53D}$	This study
HDC39	*tet-ATO2*	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2 RPS10*/*rps10*::*CIp10-tetR-ATO2*	This study
HDC40	*tet-ATO3*	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2 RPS10*/*rps10*::*CIp10-tetR-ATO3*	This study
HDC41	*tet-ATO4*	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2 RPS10*/*rps10*::*CIp10-tetR-ATO4*	This study
HDC42	*tet-ATO5*	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2 RPS10*/*rps10*::*CIp10-tetR-ATO5*	This study
HDC43	*tet-ATO10*	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2 RPS10*/*rps10*::*CIp10-tetR-ATO10*	This study
HDC45	*ato5*Δ *tet-CIp10*	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2 ato5*Δ::*FRT*/*ato5*Δ::*FRT RPS10*/*rps10*::*CIp10-tetR*	This study
HDC49	*ato5*Δ *tet-*$ATO1^{G53D}$	*ade2*::*hisG*/*ade2*::*hisG ura3*::*imm434*/*ura3*::*imm434 ENO1*/*eno1*::*ENO1-tetR-ScHAP4AD-*3×HA-*ADE2 ato5*Δ::*FRT*/*ato5*Δ::*FRT RPS10*/*rps10*::*CIp10-tetR-*$ATO1^{G53D}$	This study

[a] FRT, FLP recombination target; HA, hemagglutinin.

alkalinize both *in vitro* and in the phagolysosome and cannot germinate to escape the macrophage unless the phagosome is neutralized by chemical means (24, 25).

The transcript profiles of cells during alkalinization and following phagocytosis share significant similarities, including the induction of multiple genes of the *ATO* family, which at 10 members is greatly expanded in *C. albicans* relative to its size in other fungi. Though named ATO, for ammonia transport outward (YaaH in bacteria), the molecular function of these plasma membrane proteins is unknown, and there is evidence linking them to transport of acetate in *Saccharomyces cerevisiae*, *Aspergillus nidulans*, and *Yarrowia lipolytica*, as well as release of ammonia in *S. cerevisiae* (24, 26–30). Despite the potential for genetic redundancy in this large family, deletion of *ATO5* alone retards alkalinization *in vitro* (24). This phenotype and the induction following phagocytosis suggest that the Ato proteins modulate the interaction of *C. albicans* with macrophages.

We report here that two alkalinization-defective strains, an *ato5*Δ deletion mutant and a strain expressing an $ATO1^{G53D}$ allele originally identified as a dominant-negative mutation that confers acetate sensitivity in *Y. lipolytica* (29), are impaired in several aspects of the macrophage-fungus interaction. These mutations delay alkalinization *in vitro*, leading to a defect in hyphal formation and a reduction in the amount of ammonia released from the cell. Furthermore, both the *ato5*Δ and $ATO1^{G53D}$ strain reside in more acidic phagolysosomes than do wild-type cells, and as a result, they form fewer hyphae after phagocytosis and have a reduced ability to escape macrophages. Both *ATO* genes are transcriptionally regulated by Stp2, and a double *ato5*Δ $ATO1^{G53D}$ mutant phenocopies the *stp2*Δ strain. Finally, overexpression of multiple *ATO* genes accelerates alkalinization, indicating that this gene family is an important mediator of the host-*Candida* interaction.

MATERIALS AND METHODS

Strains and growth media. *C. albicans* strains were grown under standard conditions in YPD medium (1% yeast extract, 2% peptone, 2% dextrose). For growth on plates, 2% agar was added to the medium. To select for nourseothricin-resistant (Nou^R) transformants, 200 μg/ml of nourseothricin (Werner Bioagents, Jena, Germany) was added to the YPD agar plates (31). Alkalinization experiments were performed in glucose-free minimal yeast nitrogen base (YNB) medium with allantoin as the nitrogen source (0.17% yeast nitrogen base, 0.5% allantoin) supplemented with 2% Casamino acids as the sole carbon source (YAC medium). Ammonia release was measured on solid medium of the same formulation containing 2% agar. NH_3 release by alkalinizing colonies was measured using an acid trap as previously described (24) on YAC plates or GM-BCP medium (27) as described above with the addition of 2% agar.

The strains used are listed in Table 1. *C. albicans* strains lacking *ATO5* were generated using the SAT-flipper method as described previously (31). Briefly, 300 bp of homologous sequence immediately 5′ or 3′ from the *ATO5* open reading frame were amplified by PCR and cloned between the KpnI/XhoI and SacI/SacII sites of pSFS1. The resulting *SAT1*-FLP cassette was used to transform *C. albicans* strain SC5314 by electroporation with selection on YPD-Nou plates. Cassette integration was confirmed in the selected candidates via PCR. To remove the nourseothricin selection marker, the mutant strain was induced to excise the deletion cassette with 1% bovine serum albumin (BSA) in YNB medium for 3 days and the Nou^S colonies were selected. This process was repeated to generate the independently derived homozygous deletion mutants HDC27 and HDC28 (*ato5*Δ::*FRT*/*ato5*Δ::*FRT*). Complementation of the mutant

strain used plasmid pHD-9, which was generated by cloning the *ATO5* open reading frame with 700 bp of 5′ untranslated region (UTR) between the MluI and XhoI sites of pAG6, a *SAT1*-marked version of CIp10 (25). This plasmid was linearized with StuI and used to transform SC5314 or *ato5*Δ mutant cells to generate the strains HDC33 (*ATO5/ATO5 RPS10/rps10::CIp10-SAT1*) and HDC29 and HDC30 (*ato5*Δ::*FRT/ato5*Δ::*FRT RPS10/rps10*::*CIp10-ATO5-SAT1*), respectively.

Constitutive expression of the $ATO1^{G53D}$ allele was achieved as previously described (24). Briefly, ~1,000 bp of the *ACT1* promoter from pAU34 (32) was subcloned between the KpnI and XhoI sites in CIp10 to generate pHZ116. Then, the $ATO1^{G53D}$ mutation was generated by site-directed overlap PCR using complementary oligonucleotides with a single mismatch to encode a change of Gly-53 to Asp, analogous to the *Y. lipolytica GPR1-1* mutant originally identified by Augstein et al. (33), and cloned into pHZ116 to generate pML341. The plasmid was digested with StuI and used to transform CAI4-F2 to uridine prototrophy. Accurate integration at the *RPS10* locus was verified by PCR.

The doxycycline-repressible strains were constructed in the THE1 strain background that was generated by Nakayama (34). Briefly, the doxycycline-repressible promoter from plasmid p97-CAU1 (34) was amplified and cloned between the KpnI and XhoI restriction sites in CIp10 (35) to generate the plasmid pHD72. Subsequently, the *ATO* genes were PCR amplified and cloned between the XhoI and MluI restriction sites in pHD72. Plasmids were sequence verified, digested with StuI, and used to transform THE1 to uridine prototrophy. Accurate integration at the *RPS10* locus was verified by PCR.

Alkalinization and ammonia release assays. Alkalinization experiments were performed as previously described (24, 25), using YAC at pH 4.0, as described above. *C. albicans* cells were grown in YPD medium overnight and diluted to an optical density at 600 nm (OD_{600}) of 0.2 in the alkalinization medium. Cells were incubated at 37°C with aeration for up to 24 h. Growth was measured via optical density at 600 nm, culture pH was measured using a standard pH electrode, and cellular morphology was scored by analyzing photomicrographs of at least 150 cells per condition. Experiments were performed at least in triplicate, and the data were analyzed using Prism 5.0 (GraphPad) software.

Ammonia release by *C. albicans* cells during alkalinization was assessed by using acid traps as previously described (24). In brief, cells were grown in YPD medium overnight, washed in distilled water, and resuspended at an OD_{600} of 1.0 in distilled water. Cells were spotted onto solid YAC medium at pH 4.0, and reservoirs containing 10% citric acid were affixed to the petri dish lid directly underneath the colonies. Cells were incubated at 37°C, and samples from the acid trap collected at 24, 48, or 72 h after initiation of the experiment. Ammonia was quantified using Nessler's reagent, as described previously (24, 36). Experiments were performed in triplicate.

Macrophage cytotoxicity assay. *C. albicans* toxicity on macrophages was assessed by using the CytoTox96 nonradioactive cytotoxicity assay (Promega) as previously described (25). Briefly, RAW264.7 macrophages were seeded at 2.5×10^5 cells per well of a 96-well plate in phenol red-free RPMI medium and incubated overnight at 37°C and 5% CO_2. *C. albicans* cells were grown to log phase in YPD medium, washed in phosphate-buffered saline (PBS), and cocultured with macrophages at a 3:1 ratio for 5 h. The release of lactate dehydrogenase (LDH) by infected macrophages relative to maximum LDH release from lysed macrophages was then calculated according to the manufacturer's protocol and corrected for spontaneous release of LDH by the macrophages or *C. albicans* alone. The experiment was performed in triplicate.

Hyphal formation of phagocytosed *C. albicans*. To assess the interaction of single *C. albicans* cells with the macrophages, we seeded 2.5×10^5 RAW264.7 macrophages to glass coverslips in a 12-well plate and incubated them overnight at 37°C and 5% CO_2. *C. albicans* cells were grown in YPD medium overnight, diluted 1:100 in fresh medium, and grown for 3 h at 30°C. Cells were then washed in distilled water and stained with 1 μM 5-carboxytetramethylrhodamine (Molecular Probes) for 15 min, washed 2 times with PBS, and resuspended in RPMI medium (HyClone). Amounts of 3×10^6 *C. albicans* cells were cocultured with the macrophages at 37°C for 2 h. The cocultures were then washed twice with PBS, and images of the *Candida*-macrophage interaction were taken using an Olympus IX81 automated inverted microscope. Images from 100 phagocytosed cells per experiment were analyzed using SlideBook 6.0 software. The percentage of hyphal morphogenesis during phagocytosis was calculated by obtaining the percentage of phagocytosed cells using the following formula: (germ tubes + hyphal cells/total amount of cells) × 100. Experiments were performed in triplicate.

Endpoint dilution assay. *C. albicans* survival during interaction with the RAW264.7 macrophages was assessed as previously described (25, 37). Briefly, macrophages were seeded at 2.5×10^4 cells/well in 96-well plates and grown overnight at 37°C with 5% CO_2. *C. albicans* cells were grown to log phase and then washed in distilled water and resuspended in fresh RPMI medium. Amounts of 1×10^4 cells/well were added to wells with or without macrophages, followed by six serial 1:5 dilutions. After 48 h, microcolonies of *C. albicans* in wells in which individual colonies could be distinguished were counted using an inverted microscope. The results are presented as 100 times the ratio of the number of colonies in the presence of macrophages to the number of colonies without macrophages. The experiment was performed in triplicate.

LR assay. Assays were performed as previously reported by Vylkova and Lorenz (25). RAW264.7 macrophages were seeded onto glass coverslips in 12-well tissue culture plates at 5×10^5 cells/ml and allowed to adhere overnight 37°C in 5% CO_2. Next, 1 mM LysoTracker red (LR) DM99 (Molecular Probes) was added to fresh RPMI medium and the mixture incubated for 2 h. *C. albicans* cells were grown overnight in YPD, diluted 1:100 in fresh YPD, and grown for 3 h at 30°C. Cells were then washed in distilled water, stained with 1 μM fluorescein isothiocyanate (FITC) for 15 min, and washed in PBS to remove excess dye. Control cells were heat killed by incubation for 60 min at 65°C. Cells were diluted to 1×10^6 cells/ml in phenol red-free RPMI medium and cocultured with macrophages for 60 min. Cultures were stained with Calcofluor white (35 μg/ml for 30 s) to label nonphagocytosed cells and fixed in 2.7% paraformaldehyde. The cocultures were then imaged at ×60 magnification. To estimate the relative phagosomal pH, the signal intensities of both FITC and tetramethyl rhodamine isocyanate (TRITC) were plotted along a line drawn transversely across the short axis of the cell for at least 50 cells per condition using Slidebook 6.0. The average LR signal intensity was calculated for a region of 10 pixels (1 μm) immediately outside the fungal cell, whose boundary was determined by the slope of the FITC signal.

***In vivo* virulence assay.** A murine model of disseminated *C. albicans* infection was performed as described in reference 19. *C. albicans* cells were grown to mid-log phase in YPD, washed, and resuspended in PBS at 5×10^6 cells/ml. Ten female ICR mice (weighing 21 to 25 g) per strain were inoculated via tail vein injection with 100 μl PBS containing 10^6 *C. albicans* cells. Mice were monitored at least twice daily and euthanized when moribund. All mouse experiments were performed under protocols approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston (protocol HSC-AWC-12-099).

RESULTS

The ability of *C. albicans* to cause invasive infections depends on its dynamic and complex interactions with cells of the innate immune system, such as macrophages (12, 38, 39). Strikingly, phagocytosed *C. albicans* cells form hyphae that facilitate their escape, and we have shown that this is induced by a fungus-driven neutralization of the phagolysosome (24, 25). This process is dependent on catabolism of amino acids and is regulated by Stp2p, a transcription factor that activates amino acid permeases (24, 25). We sought to identify whether additional components required for efficient alkalinization also alter interactions with macrophages.

Ato function is required for efficient environmental alkalinization response in *C. albicans*. We previously reported that a mutant lacking *ATO5* has a defect in alkalinization in medium 199, a low-glucose tissue culture medium (24). This mutant was generated using the UAU method, which uses two auxotrophic markers and selects for a chromosomal homozygosis and rearrangement (40), which may be problematic given the importance of amino acids in this phenomenon. Therefore, we constructed an *ato5*Δ mutant in the prototrophic SC5314 strain using the SAT-flipper methodology (31). We have subsequently defined a glucose-free minimal medium, YNB, containing 0.5% allantoin as the nitrogen source and 2% Casamino acids as the sole carbon source (YAC), which supports more robust alkalinization (24, 25), and tested both the new *ato5*Δ mutant and a point mutant in *ATO1* (*ATO1*^{G53D}) that has dominant-negative phenotypes in other systems (33).

These strains were incubated in YAC starting at pH 4.0, where all strains grew at similar rates (Fig. 1A). A rapid increase in the culture pH was observed when the wild-type strain (SC5314) was incubated in aerated culture at 37°C (Fig. 1B), with the pH rising from 4.0 to 6.9 in 8 h, while the increases in pH for the *ato5*Δ and the *ATO1*^{G53D} mutants were significantly retarded (pH 5.19 and 5.25, respectively) in the same time frame (Fig. 1B). This lag was overcome by the 24-h time point, when all cultures had a pH near neutral. The SC5314-derived strains are more robust than those we used previously, but the magnitude of the *ato5*Δ defect relative to the phenotype of the control strains is similar in both backgrounds (data not shown).

We have attributed this environmental alkalinization to the extrusion of ammonia from the cell (24), and we hypothesized that the *ATO* proteins, which have been proposed to facilitate ammonia export (26, 27), may be important effectors of this release. To test this hypothesis, we performed an ammonia release assay in which colonies were allowed to develop on solid defined alkalinization medium (YAC, pH 4) directly apposed across an air interface from an acid trap containing 10% citric acid. Ammonia excreted from the colony is converted to ammonium in the acid trap, where it can be quantified using Nessler's reagent (24, 41). Detectible ammonia from wild-type (SC5314) and reconstituted *ato5*Δ+*ATO5* cells increased significantly over the 72-h period (Fig. 1C). In contrast, the levels of ammonia released from both the *ato5*Δ and *ATO1*^{G53D} cells were significantly reduced (Fig. 1C). Ammonia excretion correlated with the degree of alkalinization, with the *stp2*Δ mutant completely deficient, the *ato* mutants intermediate, and the wild-type and complemented strains releasing abundant ammonia.

Our initial formulation of the minimal Casamino acid medium included ammonium sulfate as the nitrogen source, as is typical in defined yeast media, which we realized may affect our ammonia release results. Indeed, we found that at neutral pH, the presence of ammonium sulfate significantly increased the amount of ammonia present in the trap even in the absence of cells, while no ammonia was released from acidic medium (see Fig. S1 in the supplemental material). This may lead to a feedback loop in which ammonia generated by cellular metabolism raises the pH, which in turn liberates ammonia from the medium, thus overstating the contribution of the cells. To avoid this, we tested additional nitrogen sources and found that no ammonia was released from cell-free medium containing allantoin, urea, or amino acids as the nitrogen source, regardless of pH (see Fig. S1; also data not shown). Allantoin supported optimal growth, and all the assays reported here used allantoin as the nitrogen source. The amount of ammonia released was slightly lower on allantoin than on ammonium sulfate, but the temporal pattern and genetic phenotypes were similar to those we have previously reported and no differences in the rates of pH changes were observed (see Fig. S1; also data not shown).

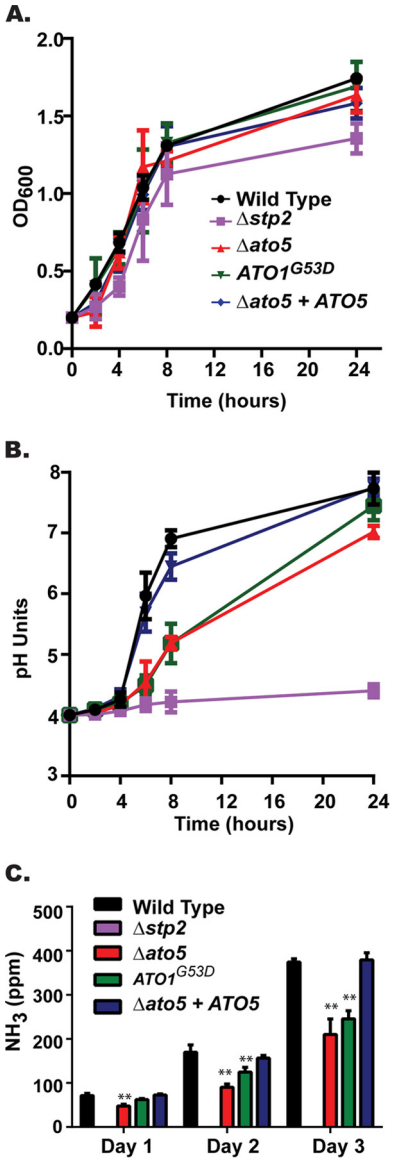

FIG 1 Ato proteins promote environmental alkalinization. The wild-type (SC5314), *stp2*Δ (SVC17), *ato5*Δ (HDC17), *ATO1*^{G53D} (Can572), and *ato5*Δ+*ATO5* (HDC30) strains were incubated in YAC medium initially at pH 4.0 under aerated conditions at 37°C. (A) Growth of the cells was measured by OD_{600} readings at the indicated time points. (B) pH of the cultures from the same experiments whose results are shown in panel A. (C) Ammonia released by *C. albicans* cells was collected in a citric acid trap and quantified using Nessler's reagent as described in Materials and Methods. Results are reported as mean values ± SD from triplicate assays. **, $P < 0.001$.

Mutation of Ato proteins affects autoinduction of hyphal formation. The ability of *C. albicans* to undergo a reversible morphological switch from yeast to hyphal form has been shown to be

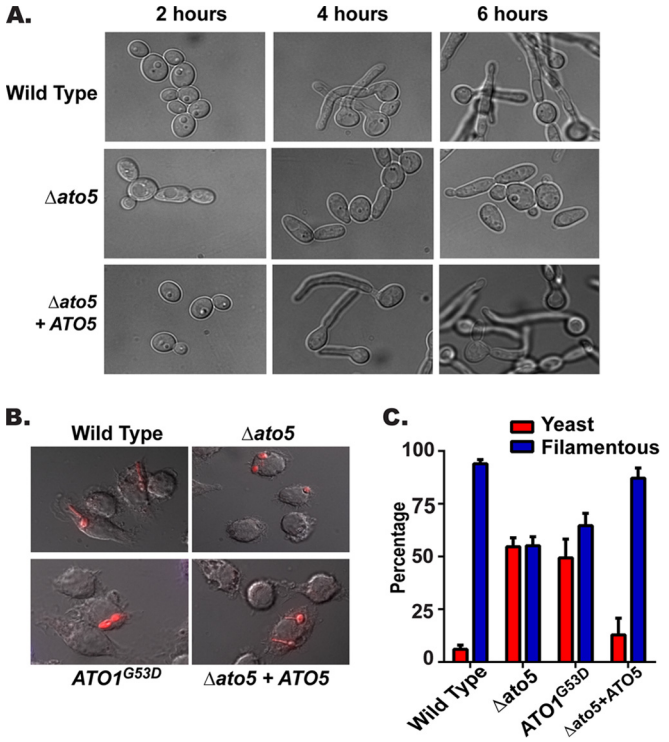

FIG 2 Mutation of Ato proteins reduces autoinduction of hyphal formation. The wild-type (SC5314), *stp2*Δ (SVC17), *ato5*Δ (HDC17), *ATO1*^{G53D} (Can572), and *ato5*Δ+*ATO5* (*ATO5* complemented) strains were assayed for hyphal formation. (A) Strains were grown in YAC medium for 6 h and photographed at ×100 magnification. (B) Cells were labeled with 5-carboxytetramethylrhodamine, cocultured for 2 h with RAW267.4 macrophages, fixed, and photographed at ×60 magnification. (C) Filamentous cells were quantitated in the captured images by counting at least 150 cells per condition. Results are reported as mean values ± SD from triplicate experiments.

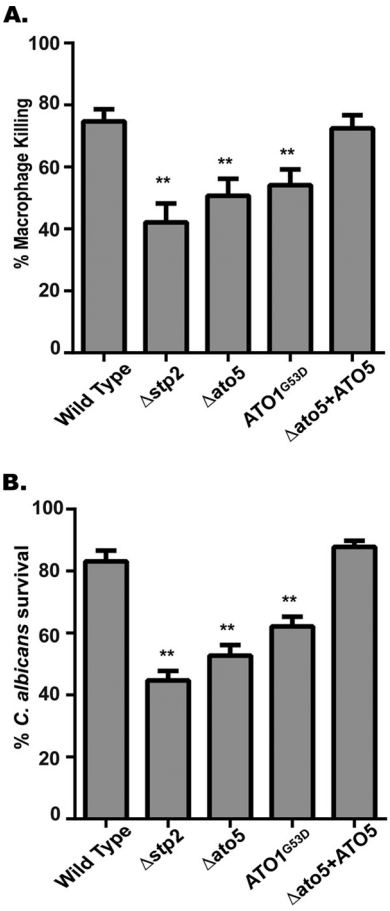

FIG 3 Ato proteins are required for efficient lysis of macrophages and proliferation after phagocytosis. (A) The wild-type (SC5314), *stp2*Δ (SVC17), *ato5*Δ (HDC17), *ATO1*^{G53D} (Can572), and *ato5*Δ+*ATO5* (*ATO5* complemented) strains were cocultured with RAW264.7 macrophages. Macrophage death was assessed based upon lactate dehydrogenase (LDH) release. (B) Strains were cocultured in the presence and absence of RAW264.7 macrophages for 24 h. Fungal survival was calculated as the ratio of microcolonies in the presence versus the absence of macrophages. Results are reported as mean values ± SD from triplicate experiments. **, $P < 0.001$.

critical to virulence of the organism (42, 43). Neutral pH is a key factor that induces this switch, and we have shown that efficient environmental alkalinization is sufficient to promote this morphological switch (24). Therefore, we hypothesized that the *ATO* mutant strains would have a reduced ability to form hyphae due to the defect in alkalinization. The *ato5*Δ and *ATO1*^{G53D} strains were grown in unbuffered alkalinization medium (YAC, pH 4.0) over a period of 6 h and were significantly impaired in hyphal formation compared to that of the wild-type (SC5314) or complemented strains (Fig. 2A). However, when these strains were cultured in the same medium adjusted to pH 7 for 2 h, no defects in hyphal formation were detected (see Fig. S2 in the supplemental material), indicating that the mutants were able to respond to neutral pH cues but could not autoinduce hyphal growth by changing the pH.

We have shown that *C. albicans* blocks the normal acidification of the phagolysosome and that, as *in vitro*, the resulting neutral pH induces hyphal growth; in contrast, an *stp2*Δ mutant does not neutralize this compartment and as a result does not germinate postphagocytosis (25). We hypothesized that the *ato* mutants would also have a defect in the autoinduction of hyphal formation inside the macrophage. In order to test this, strains were cocultured with RAW264.7 macrophages for 2 h, fixed, and assessed for hyphal formation microscopically (Fig. 2B). As expected, the *ATO* mutant strains showed a significant reduction in hyphal formation (~50%), while the wild-type (SC5314) and reconstituted strain were nearly all hyphal (94% and 87%, respectively) (Fig. 3C). Taken together, these results confirm that the ability to efficiently alkalinize the phagolysosome is an important signal for hyphal formation and that the Ato proteins are important effectors of this signaling.

Ato proteins are required for efficient macrophage lysis and escape. We have shown that the ability to alkalinize the phagosome significantly contributes to the ability of *C. albicans* to escape the macrophage (25). As a result of the alkalinization and hyphal formation defects, we predicted that the *ATO* mutant strains would be impaired in the ability to pierce the macrophage and escape. In order to assess this, we utilized a lactate dehydrogenase (LDH) release assay to assess macrophage membrane damage after 5 h of coculture with RAW267.4 macrophages (25). Coculture of macrophages with wild-type (SC5314) and *ATO5*-complemented strains resulted in ~75% maximal LDH release (relative to the results for chemically lysed macrophages). In contrast, the *ato5*Δ and *ATO1*^{G53D} mutants were less able to damage macrophages, with only 50% and 52% of the maximal LDH release, while the *stp2*Δ strain released only 42% of the maximum

(Fig. 3A). These data suggest that functional Ato proteins are necessary for efficient escape from the phagosome.

A reduced ability to escape the macrophage might predict that the *ATO* mutant strains are more susceptible to macrophage killing. To address this, we utilized an established endpoint dilution assay to assess *C. albicans* survival after phagocytosis (37). In good agreement with the LDH release assay, significantly reduced survival was seen in the *ato5*Δ (52%) and *ATO1*^{G53D} (62%) strains, as well as the *stp2*Δ control strain (45%); in contrast, more than 80% of the wild-type cells survived this interaction (Fig. 3B). Thus, we conclude that a defect in macrophage escape also leads to an increased ability of the macrophage to clear the pathogen.

Ato5p is necessary for efficient alkalinization of the phagosome. Taken together, the phenotypes of the *ato* mutant strains strongly suggest that they may have a defect in neutralization of the phagolysosome. To test our hypothesis, we preloaded RAW267.4 macrophages with the acidophilic dye LysoTracker red (LR), which accumulates and fluoresces in acidic organelles, and then cocultured these cells with FITC-labeled *C. albicans* cells. As expected, heat-killed cells strongly colocalized with LR after 60 min of coculture (Fig. 4A). Conversely, wild-type (SC5314) cells were surrounded by a low-level diffuse LR signal, suggesting a more neutral pH (Fig. 4A). *stp2*Δ and *ato5*Δ cells were both frequently contained in acidic compartments (Fig. 4A). To estimate the difference in the pH of phagosomes containing wild-type versus *ato5*Δ or *stp2*Δ cells, we utilized the Slidebook 6 image software to quantitate the signal intensity in the FITC and LR channels along a line drawn through the middle of the *C. albicans* cell on the short axis (Fig. 4B). Plotting the FITC fluorescence intensity clearly delineated the fungal cell (Fig. 4B and C, green). The phagolysosomal membrane is tightly apposed to the fungal cell wall, so LR was confined to the lumen in a narrow band immediately outside the cell (notably, in the heat-killed controls, LR accumulated both in the phagosome lumen and in the permeabilized fungal cell), as seen by the sharp rise in LR intensity as the FITC signal decreased (Fig. 4B and C). This lumenal fluorescence was absent from wild-type and complemented cells but was readily apparent in *stp2*Δ and *ato5*Δ mutants (Fig. 4C). We quantitated the average LR intensity over 10 pixels (1 μm) on each side of the fungal cell (indicated by dashed lines in Fig. 4B). Both the *stp2*Δ and the *ato5*Δ mutant resided in more acidic compartments, as indicated by the LysoTracker red signal being stronger than those of the wild-type and complemented strains (Fig. 4D). From these results, we conclude that *ato* mutants occupy an acidic phagolysosome, indicating that the *in vitro* alkalinization defect we have described extends to the phagocyte as well.

Loss of only *ATO5* does not compromise virulence. The results of the macrophage coculture experiments indicate that *ATO* mutants are less able to tolerate phagocytosis and suggest that this may be an important determinant for virulence of *C. albicans*. We had previously shown that the deletion of *STP2* resulted in a modest but significant attenuation of virulence in this model (25), so we tested whether this was true of an *ATO5* deletion strain by using the standard mouse tail-vein model of disseminated hematogenous candidiasis (see Fig. S3 in the supplemental material) and found no statistically significant attenuation in virulence. This result may be explained by the modest alkalinization phenotype and the potential for functional redundancy with other Ato proteins, whose expression may compensate for the loss of *ATO5 in vivo*.

Expression of *ATO* genes is dependent upon the transcription factor *STP2*. Stp2p is a transcription factor that regulates amino acid permeases (25, 44). Given the similar phenotypes of the *stp2*Δ and *ato5*Δ mutants, we asked whether Stp2p had any role in the regulation of the *ATO* genes by using quantitative real-time PCR to assess the transcript abundance of *ATO1* and *ATO5* in wild-type and *stp2*Δ strains under alkalinizing conditions. *ATO1*, which is highly induced following phagocytosis and in YAC (14, 24), was upregulated 279-fold in wild-type cells compared to its induction under nonalkalinizing conditions. This induction was almost completely abolished in cells lacking *STP2* (Fig. 5A). Similarly, *ATO5* was upregulated 7.5-fold in alkalinizing wild-type cells (Fig. 5B) but only 2-fold in the *stp2*Δ mutant. Taken together, these results indicate that the *ATO* genes are regulated by *STP2*.

Many *ATO* genes affect *C. albicans* alkalinization. The Stp2-dependence of *ATO* gene expression raised the question of whether heterologous *ATO* expression might suppress *stp2*Δ mutant phenotypes. To test this, we generated *ATO* alleles under the control of the constitutive *ACT1* promoter. Surprisingly, *stp2*Δ strains expressing these alleles failed to grow in YAC medium, although growth was unaffected in medium containing glucose (see Fig. S4 in the supplemental material). This suggests that the dysregulation conferred by deletion of *STP2* cannot be suppressed (and might be exacerbated) by overexpression of target genes, perhaps indicative of a careful stoichiometry between Atos and other cellular proteins. To address this, we constructed strains with *ATO* genes under the control of a doxycycline (Dox)-repressible promoter. Overexpression of many *ATO* genes accelerated alkalinization of the environment compared to the rate of alkalinization for the wild-type control (Fig. 6A), while these strains also released more ammonia (Fig. 6B and C). Conversely, overexpression of *ATO3* or the dominant-negative *ATO1*^{G53D} inhibited alkalinization and ammonia release. These data indicate that the *ATO* gene family is broadly involved in the ability of *C. albicans* to alkalinize the extracellular space but that there might be specific interactions between Ato and other proteins that regulate this phenomenon.

Double *ATO* mutation results in additive alkalinization defects. Mutations in *ATO5* (*ato5*Δ) and *ATO1* (*ATO1*^{G53D}) result in strikingly similar phenotypes, and we sought to determine whether a double mutant strain would confer a synthetic phenotype. Indeed, the *in vitro* alkalinization of the double mutant was drastically inhibited compared to that of either single mutant (Fig. 7), despite near-normal growth under these conditions (data not shown). This phenotype is very similar to that of an *stp2*Δ strain (Fig. 7); consistent with this, the double mutant also fails to release ammonia (data not shown). These results provide strong evidence that multiple Ato proteins can facilitate ammonia release, although further studies will be necessary to determine the molecular mechanism through which this process occurs.

DISCUSSION

We show here that members of the *ATO* gene family are important mediators of the ability of *C. albicans* to neutralize its surrounding pH, both *in vitro* and in the macrophage phagolysosome. This conclusion is supported by evidence that mutants with single mutations of *ATO1* (*ATO1*^{G53D}) or *ATO5* (*ato5*Δ) raise the extracellular pH more slowly during *in vitro* growth under amino acid-rich conditions, release less ammonia, and are slower to germinate. In contact with macrophages, *ato* mutants occupy an acidic phagolysosome, which reduces hyphal growth and fungal survival while

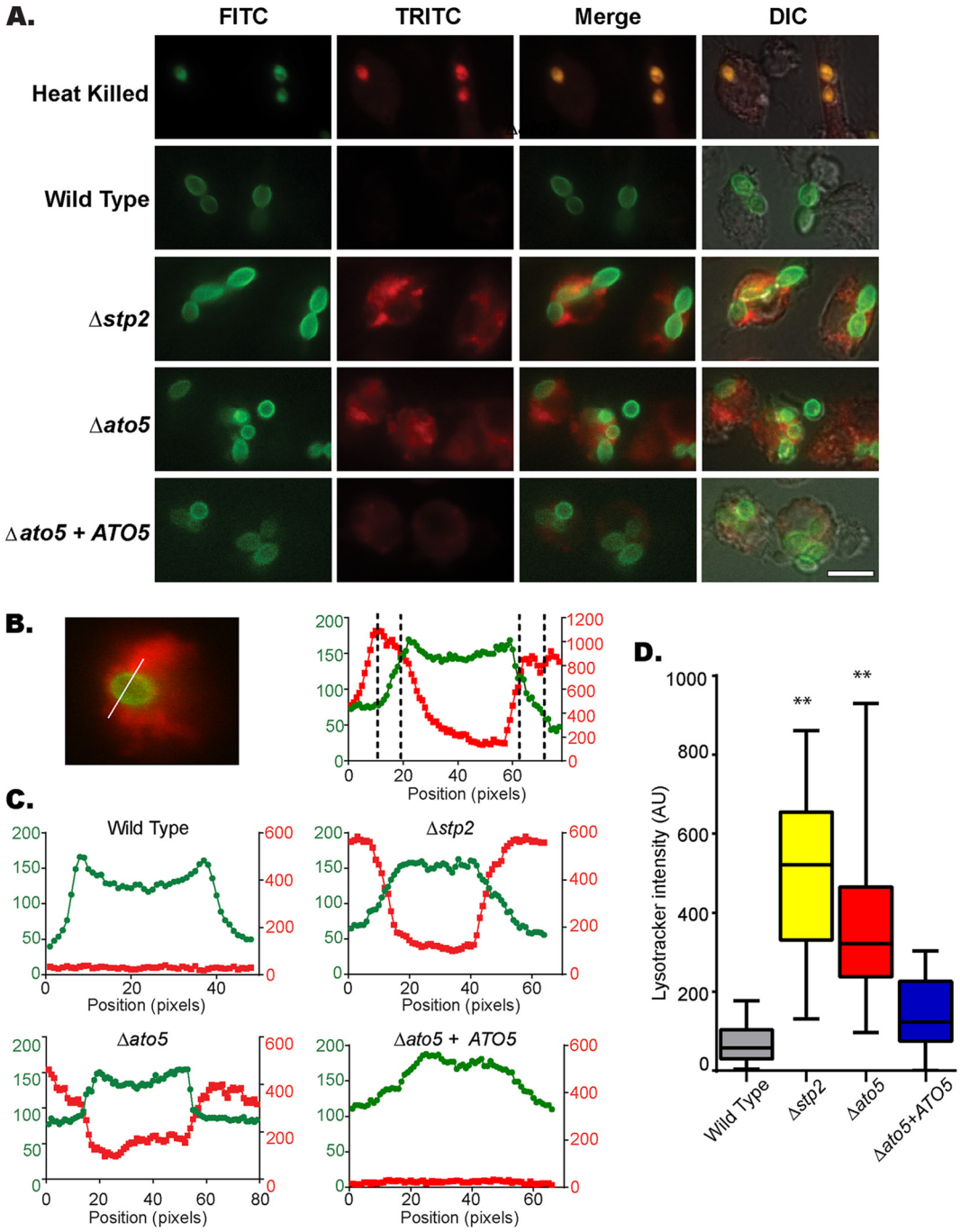

FIG 4 Ato5p is necessary for neutralization of the macrophage phagosome. (A) FITC-stained *C. albicans* cells were cocultured with RAW264.7 macrophages preloaded with LysoTracker red (LR) for 1 h. The cocultures were then fixed and imaged at ×60 magnification. DIC, differential interference contrast. (B) Image analysis of the cocultures shown in panel A was performed using Slidebook 6.0 software. Left, fluorescence intensity for both the FITC and LR channels was plotted along a line drawn through the middle of the *C. albicans* cell on the short axis. Right, dashed lines indicate the regions adjacent to the fungal cell used to quantitate the LR signal (10 pixels = 1 μm). (C) Representative plots from each strain. The FITC signal is plotted on the left axis in green, and the TRITC signal is plotted on the right axis in red. (D) Box (25% to 75%)-and-whisker (minimum to maximum) plot of the average TRITC intensity. At least 50 cells were counted per strain. All assays were performed in triplicate. **, P value < 0.001. AU, arbitrary units.

increasing macrophage integrity. While it is reasonable to infer that the defect in germination is directly responsible for the reduction in macrophage damage, recent publications attribute some of this damage to fungus-dependent induction of pyroptosis (45, 46). It is possible that the aberrant maturation of the *C. albicans*-containing phagosome resulting from the failure to acidify might reduce pyroptosis and, thus, also contribute to improved macrophage survival. Indeed, we have evidence that this is the case (S. Vylkova, H. Danhof, and M. Lorenz, unpublished observations). The phenotypes of a double $ATO1^{G53D}$ *ato5*Δ mutant are additive

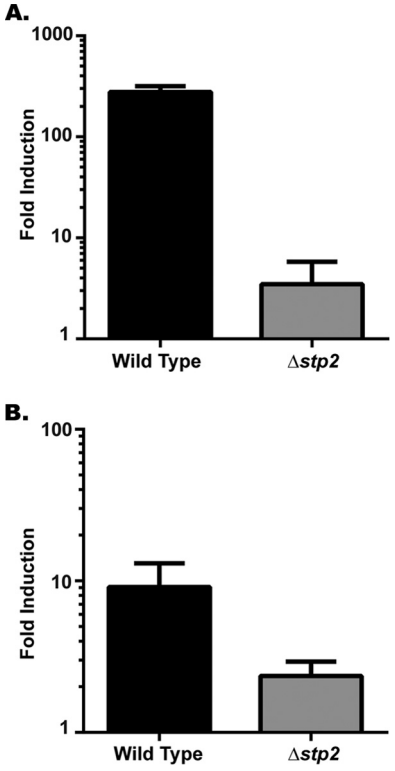

FIG 5 *ATO* gene expression is dependent upon *STP2*. Quantitative real-time PCR analysis of cells grown in alkalinization medium. Cells of both the wild-type (SC5314) and *stp2*Δ (SVC17) strains were grown in YAC (alkalinizing conditions) or the same medium with glucose added (nonalkalinizing conditions), and the transcript levels of *ATO1* (A) and *ATO5* (B) expression were determined. Transcript abundance was normalized to that of *ACT1*, and the expression in glucose was set to 1. Data are expressed as mean values ± SD from triplicate experiments.

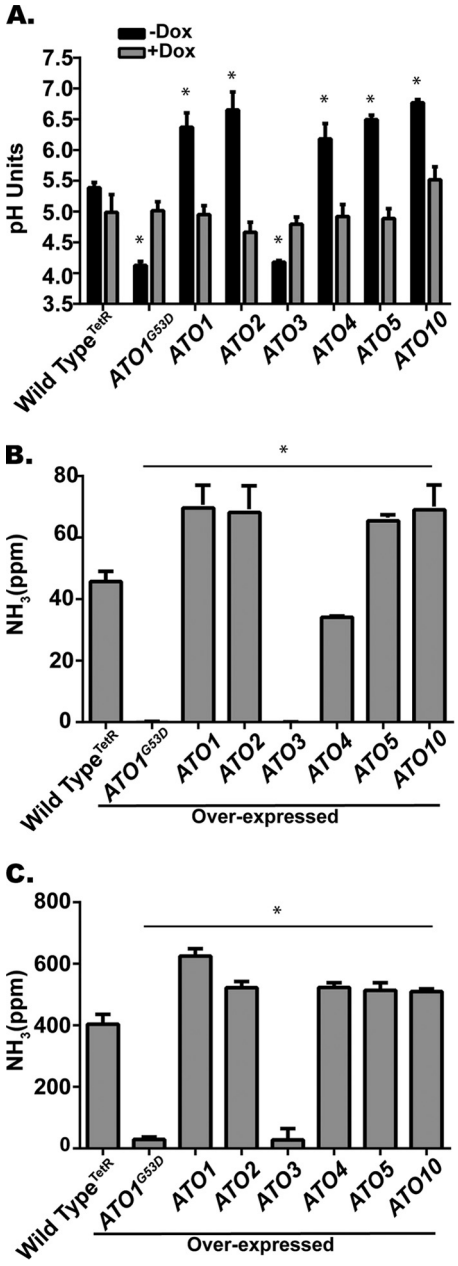

FIG 6 *ATO* overexpression alters alkalinization. (A) The wild type (THE1) and strains with *ATO* genes under the control of a doxycycline (Dox)-repressible promoter were incubated in YAC initially at pH 4.0 under aerated conditions at 37°C with and without Dox. (A) The pH of the cultures after 8 h is shown. The results for all overexpression strains (−Dox) are statistically different from the results for the wild-type control ($P < 0.001$). (B to C) Ammonia released by *C. albicans* cells during alkalinization on solid medium in the absence of Dox after 24 h (B) or 72 h (C). Data are expressed as mean values ± SD from triplicate experiments.

and are similar to those of the previously reported *stp2*Δ strain (24, 25). *ATO1* and *ATO5* are both transcriptionally regulated by Stp2p, though this could be direct or indirect. Furthermore, overexpression of multiple *ATO* genes enhances *in vitro* alkalinization, suggesting that there is a broad role for this family in the pH alteration phenomenon.

Acquisition and utilization of available nutrients from the host is fundamental to the survival and pathogenicity of microorganisms, and the host uses nutrient deprivation and sequestration as a defense mechanism to limit microbial growth. Pathogens have to identify ready sources of nutrients in a host, and it is clear that some are scarce, such as nucleotides and iron, since mutants impaired in the synthesis or uptake of these compounds are avirulent in many pathogens (47–49). In contrast, amino acids appear abundant, since many (but not all) auxotrophic mutants retain full virulence (4, 50, 51). We and others have shown that pathways needed to assimilate amino acids, fatty acids, and other alternative carbon sources are required for virulence (19, 52, 53). We have shown that *C. albicans* efficiently catabolizes amino acids to satisfy carbon requirements and that this results in the neutralization of acidic environments, which has important effects on host-pathogen interactions.

Transcript profiling established that many *ATO* genes are significantly upregulated during environmental alkalinization and that an overlapping but not identical set is also upregulated during phagocytosis (14, 24). We show here that the induction of at least some *ATO* genes is largely or entirely Stp2p dependent, emphasizing the central role of this transcription factor in regulating the metabolic changes that support the fitness of *C. albicans* in contact with phagocytic cells. The dramatic expansion of this gene family strongly suggests that differential functions and/or regulation exists between them, given the potential for redundancy. Indeed, we

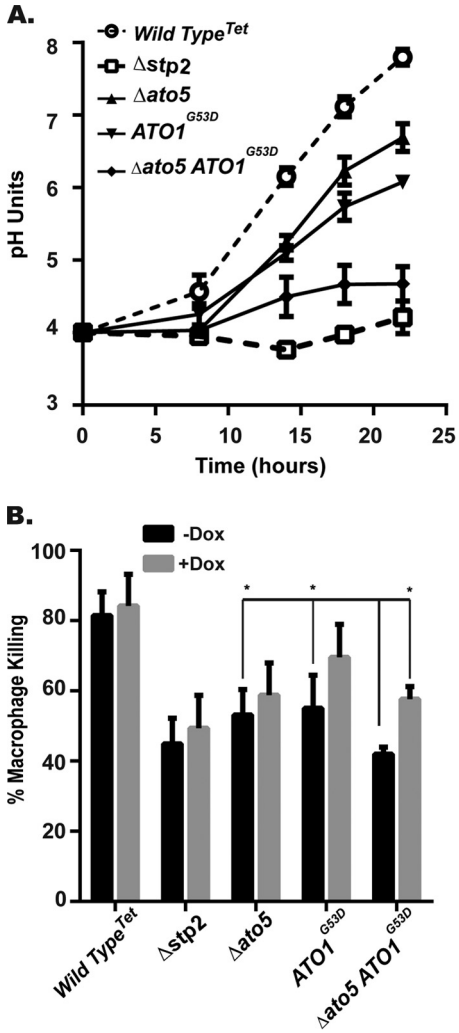

FIG 7 Double *ato* mutation results in additive alkalinization defects. (A) Wild-type (SC5314), *stp2*Δ (SVC17), *ato5*Δ^{Tet} (HDC45), and *ato5*Δ *ATO1*^{G53D} (HDC49) strains were incubated in YAC with an initial pH of 4.0 under aerated conditions at 37°C without Dox. The pH of the cultures was determined at the indicated time points. (B) Indicated strains were cocultured with RAW264.7 macrophages in the presence and absence of 50 μg/ml of Dox in RPMI tissue culture medium. Macrophage death was assessed based upon lactate dehydrogenase (LDH) release. An asterisk indicates a significant difference ($P < 0.01$) relative to the result for the double *ATO1*^{G53D} *ato5*Δ strain grown in the absence of Dox.

found this to be the case, as overexpression analysis showed that many but not all *ATO* genes promote environmental alkalinization under the conditions tested.

We attempted to suppress the phenotypes of the *stp2*Δ mutant by constitutively expressing individual *ATO* genes. We were surprised to find that these overexpression strains failed to grow on medium in which amino acids were the sole carbon source, though they were viable when glucose was present, which suppressed alkalinization. There is evidence for homomultimeric and heteromultimeric interactions between Ato homologs in yeast (54, 55), and our data would support the idea that these proteins form one or more functional complexes. In the *stp2*Δ mutant, then, either the correct stoichiometry is not maintained or a key non-*ATO* target of Stp2p is missing. Elucidating the details of the Ato molecular machine will require further study.

Disruption of Ato function compromises interactions with macrophages but does not attenuate virulence in our mouse model. It stands to reason that pathogens would expand gene families that mediate specific interactions with the host, but this has long been very difficult to demonstrate experimentally because of technical limitations in knocking out multigene families. *C. albicans* has 10 secreted aspartyl proteases (SAPs) with different expression profiles and pH optima, but conclusive evidence of a role in pathogenesis has been elusive (56–58). In *C. glabrata*, a compelling virulence phenotype is not observed until one deletes many members of a cell wall-associated protease family (yapsins) (59). Als3, one of a family of eight adhesins, is required *in vitro* for adhesion, cadherin binding, and iron uptake but is not required for animal virulence (60–62). Thus, we expect that functional redundancy explains the lack of *in vivo* effects and that a more ambitious deletion effort may be necessary; new tools, such as the recent adaptation of the Cas9/CRISPR technology (63), may facilitate this in the future.

The CUG clade of fungi includes the most clinically relevant fungal species, *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, which are predicted to have expanded *ATO* families, and there is a positive correlation between the number of *ATO* homologs and the robustness of alkalinization (24, 64). The correlation does not hold with *C. glabrata*, which is not a CUG species and has only three *ATO* genes but has been shown to alkalinize its environment *in vitro* in the presence of amino acids, as well as in the phagolysosome (65). The only evidence for a connection between Ato function and ammonia export or pH modulation outside our work in *C. albicans* is in *S. cerevisiae*, in which the rudimentary ability of this species to alter extracellular pH is further degraded by deletion of one of the three *ATO* homologs (26, 27). Similar to our findings presented here, the *S. cerevisiae ATO3* is regulated by the SPS plasma membrane amino acid sensor, the downstream target of which is Stp2p (66).

In contrast, there is significantly more support for a role for the Ato protein family in acetate transport. The dominant-negative *ATO1*^{G53D} mutation we use here was originally identified in *Y. lipolytica*, in which it confers sensitivity to acetic acid, as do similar mutations in *S. cerevisiae* (29, 30). Ato proteins are required for active import of acetate in yeast and *Aspergillus nidulans* (28, 67), and the *E. coli* SatP/YaaH is a succinate/acetate transporter (68). Reconciling the biochemical and physiological functions of these proteins will clearly require further study.

All pathogens must identify sources of nutrients before they can elaborate specific virulence attributes within the host; in *C. albicans*, it has become clear that nonfermentable compounds are a key carbon source *in vitro* and that at least some of the pathways required to metabolize fatty acids, amino acids, lactate, acetate, and others are required for full virulence (14, 19, 20, 69, 70). Moreover, the available carbon source regulates morphogenesis, cell wall structure and stress responses (22, 71, 72). Catabolism of amino acids, which appear to be readily available in many host niches, also strongly affects the interaction of *C. albicans* with the host, by allowing the pathogen to modulate the surrounding pH. We demonstrate here that this phenomenon requires the Ato protein family, whose members warrant further study as key mediators of the host-pathogen interaction.

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