RESEARCH ARTICLE

The Fungal Pathogen *Candida albicans* Autoinduces Hyphal Morphogenesis by Raising Extracellular pH

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ABSTRACT pH homeostasis is critical for all organisms; in the fungal pathogen *Candida albicans*, pH adaptation is critical for virulence in distinct host niches. We demonstrate that beyond adaptation, *C. albicans* actively neutralizes the environment from either acidic or alkaline pHs. Under acidic conditions, this species can raise the pH from 4 to >7 in less than 12 h, resulting in autoinduction of the yeast-hyphal transition, a critical virulence trait. Extracellular alkalinization has been reported to occur in several fungal species, but under the specific conditions that we describe, the phenomenon is more rapid than previously observed. Alkalinization is linked to carbon deprivation, as it occurs in glucose-poor media and requires exogenous amino acids. These conditions are similar to those predicted to exist inside phagocytic cells, and we find a strong correlation between the use of amino acids as a cellular carbon source and the degree of alkalinization. Genetic and genomic approaches indicate an emphasis on amino acid uptake and catabolism in alkalinizing cells. Mutations in four genes, *STP2*, a transcription factor regulating amino acid permeases, *ACH1* (acetyl-coenzyme A [acetyl-CoA] hydrolase), *DUR1,2* (urea amidolyase), and *ATO5*, a putative ammonia transporter, abolish or delay neutralization. The pH changes are the result of the extrusion of ammonia, as observed in other fungi. We propose that nutrient-deprived *C. albicans* cells catabolize amino acids as a carbon source, excreting the amino nitrogen as ammonia to raise environmental pH and stimulate morphogenesis, thus directly contributing to pathogenesis.

IMPORTANCE *Candida albicans* is the most important fungal pathogen of humans, causing disease at multiple body sites. The ability to switch between multiple morphologies, including a rounded yeast cell and an elongated hyphal cell, is a key virulence trait in this species, as this reversible switch is thought to promote dissemination and tissue invasion in the host. We report here that *C. albicans* can actively alter the pH of its environment and induce its switch to the hyphal form. The change in pH is caused by the release of ammonia from the cells produced during the breakdown of amino acids. This phenomenon is unprecedented in a human pathogen and may substantially impact host physiology by linking morphogenesis, pH adaptation, carbon metabolism, and interactions with host cells, all of which are critical for the ability of *C. albicans* to cause disease.

The ability of microorganisms to sense and adapt to changes in the environment is essential for their survival; this is particularly important for species with an intimate association with host organisms, such as pathogens, symbionts, or commensals. One environmental factor to which microorganisms must respond is extracellular pH. In the human body, pH can vary widely, from highly acidic (pH ~2) in the stomach to mildly acidic (skin and vagina), to neutral (bloodstream and parts of the gut), and even alkaline (some parts of the gut). *Candida albicans*, which is both the most common fungal commensal of humans and the most important fungal pathogen of humans, thrives in most of these sites and is highly tolerant to a wide range of environmental pH conditions, from pHs of <2 to pHs of >10. Extracellular pH is also a potent morphogenetic signal (reviewed in reference 1), as neutral-to-alkaline pH promotes a switch from the budding yeast to the filamentous hyphal form, a differentiation event that is essential for virulence in this species (2, 3).

Adaptation to pH in different host niches has been shown to be critical for virulence in many pathogens. For many bacterial pathogens acquired by an oral route, the highly acidic stomach is a formidable challenge, and the molecular details of the "acid tolerance response" (ATR) that enables them to survive transient passage through the stomach have been elucidated for several species (reviewed in reference 4). Transcriptional profiling and molecular genetics confirm the complexity of the ATR, which is required for the earliest stages of infection in many species, including *Vibrio cholerae*, *Listeria monocytogenes*, and *Salmonella enterica* serovar Typhimurium (5–7).

Fungi are generally more acidophilic than the common pathogenic bacteria, and most are acquired via nonoral routes, so a more important response for these organisms is adaptation to neutral or alkaline pH. As for the ATR, this response is complex and is required for full virulence in several species. Alkaline adaptation has been well studied in *Saccharomyces cerevisiae*, *Aspergil-*

lus nidulans, and *C. albicans*, where the central mediators are the Rim101p/PacC transcription factors, which are activated by a proteolytic cleavage event at neutral-alkaline pH (8–10). In both *C. albicans* and *Aspergillus fumigatus*, Rim101p/PacC is required for full virulence, and in *C. albicans* it is required for hyphal induction by neutral pH (8, 11). A compelling example of the importance of host niche pH in *C. albicans* is found in Phr1p and Phr2p, homologous glycosidases that are inversely regulated by pH (12, 13). Mutants lacking the acid-expressed *PHR2* gene are unable to grow *in vitro* at pHs of <5 and are avirulent in a rodent model of vaginitis, a body site with a low ambient pH, yet remain fully virulent in a bloodstream sepsis model (12, 14). *PHR1*, induced at neutral pH by Rim101p, is required for bloodstream infections but not during growth at low pH or in the vagina (8, 12, 15).

A much less well understood aspect of pH regulation is the ability to actively alter extracellular pH. *Helicobacter pylori* tolerates the highly acidic pH of the stomach by expressing a urease, found in part on the cell surface, that produces ammonia to create microenvironments of more hospitable pH; mutants that lack urease activity are unable to colonize the stomach and are avirulent (16, 17). Several fungal species have limited ability to alkalinize the extracellular milieu, including *Neurospora crassa*, *A. fumigatus*, *Metarhizium anisopliae*, and *S. cerevisiae* (18, 19). Dermatophytic fungi are identified based on a slow alkalinization phenomenon assayed over 10 to 14 days (20). More significantly, phytopathogenic species of *Colletotrichum* elevate both the local ammonia concentration and the pH in plant tissue, and this regulates expression of pectin lyase, a key virulence factor (21, 22). In some of these species, the rise in pH has been associated with the release of ammonia, a highly basic compound (18, 19, 21, 22). The mechanisms behind ammonia production are mostly unknown, but in *Colletotrichum coccodes* it has been associated with nitrogen catabolite repression and reduction of nitrate/nitrite to ammonia (23).

C. albicans has also been reported to raise extracellular pH over the course of 10 to 14 days in a minimal medium containing keratin (24). In contrast, we report here that *C. albicans* has a remarkable ability to alter extracellular pH, creating a neutral environment from either acidic or alkaline starting conditions, with changes of >3 pH units in 12 h. This phenomenon occurs in both solid and liquid media, is glucose repressible, and requires exogenous amino acids. The rise in pH induces hyphal morphogenesis, a key virulence trait of this species, and is correlated with release of volatile ammonia from the cells. By using a combination of genetic and genomic approaches, we have identified several components required for alkalinization, including regulators of amino acid uptake (Stp2p and Csh3p), putative ammonia transporters (the Ato family), and amino acid catabolic enzymes (Dur1,2p and Ach1p). Our findings link carbon metabolism, hyphal growth, and pH responses, each important contributors to virulence in *C. albicans*.

RESULTS

Candida albicans neutralizes the environmental pH under glucose-limiting conditions.

The wide range of pH values in which *C. albicans* can grow has prompted numerous studies on how this species adapts to such extreme conditions (reviewed in references 25 and 26), often using medium 199, an amino acid-rich, glucose-poor tissue culture medium with phenol red as a colorimetric readout of pH. *C. albicans* grows well in this medium over a wide range of initial pHs, from 2 to 10 (Fig. 1A). In unbuffered medium, we observed a dramatic increase in the culture pH when the wild-type strain SC5314 was incubated in aerated (shaking) cultures at either 37 or 30°C (Fig. 1B and data not shown). From an initial pH of 4.0, the pH was noticeably higher within 6 h (pH 4.9), reached 7.2 within 24 h (Fig. 1B), and peaked at 7.5 by 48 h (data not shown). The ability to alter pH was limited: from pH 3 there was only a small rise in culture pH by 24 h (to 3.7) (Fig. 1B), and no change was seen from pH 2 after extended incubation, despite overall growth similar to that obtained with the other pH conditions (Fig. 1A).

From alkaline pH (pH 8 to 10), the pH dropped rapidly and then slowly rose again to 8.15 to 8.70 by 24 h (Fig. 1B). Medium 199 contains 0.1% glucose, and we believe that the transient acidification is related to the breakdown of this limited glucose, a well-known effect of glycolysis, though we have been unable to definitively prove this, as *C. albicans* grows very poorly at very alkaline pH in the presence of nonfermentable carbon sources. We subsequently focused on the alkalinization of low-pH environments.

GM-BCP medium, containing glycerol, yeast extract, and the pH indicator bromocresol purple, has been used to study alkalinization in *S. cerevisiae* (18). *C. albicans* alkalinizes this medium very rapidly (see Fig. S1 in the supplemental material; see also Fig. 3C); the process is essentially complete in *C. albicans* after 2 to 3 days, while *S. cerevisiae* strain BY4742 has been reported to require ~2 weeks for significant change to be shown (29) (in fact, we have not observed BY4742 to alkalinize even after 4 weeks of incubation, but we do see alkalinization with an ancestor, EM93; data not shown). Substantial pH changes are seen in both GM-BCP and medium 199 inoculated with other *Candida* species as well (Fig. S1 and data not shown). *C. albicans*, *Candida tropicalis*, *Candida dubliniensis*, and *Candida parapsilosis* all alkalinized to similar degrees, as did the related species *Lodderomyces elongisporus* and *Debaromyces hansenii*; *Candida guilliermondii* and *Candida lusitaniae* show a delay in alkalinization. The taxonomically distinct *Candida glabrata* and *S. cerevisiae* showed little or no pH change under these conditions (Fig. S1).

Environmental alkalinization in *C. albicans* is glucose-repressible.

We sought to better understand the environmental conditions that stimulate the extracellular pH changes we observed. One key difference between both medium 199 and GM-BCP and more standard media used to propagate *C. albicans* (e.g., yeast extract-peptone-dextrose [YPD]) is the level of glucose: most yeast media have 2% (wt/vol) glucose, while medium 199 has 0.1% glucose and GM-BCP has no added sugars. Supplementing medium 199 (pH 4.0) to give a final concentration of 0.3 to 2.0% glucose promoted more-rapid growth (Fig. 1C) but either greatly slowed (0.3%) or abolished (>0.5%) alkalinization (Fig. 1D). Addition of 2% glucose also inhibited alkalinization on GM-BCP medium (see Fig. 3C). These results indicate that alkalinization is glucose repressible.

Amino acids promote alkalinization.

To further understand the environmental conditions that promote alkalinization, we developed an assay based on the minimal defined medium YNB, which contains salts, trace minerals and cofactors, and ammonium sulfate but no source of carbon (see Materials and Methods). The addition of glucose, ethanol, or glycerol supported growth without any rise in pH (Fig. 2A). In contrast, addition of
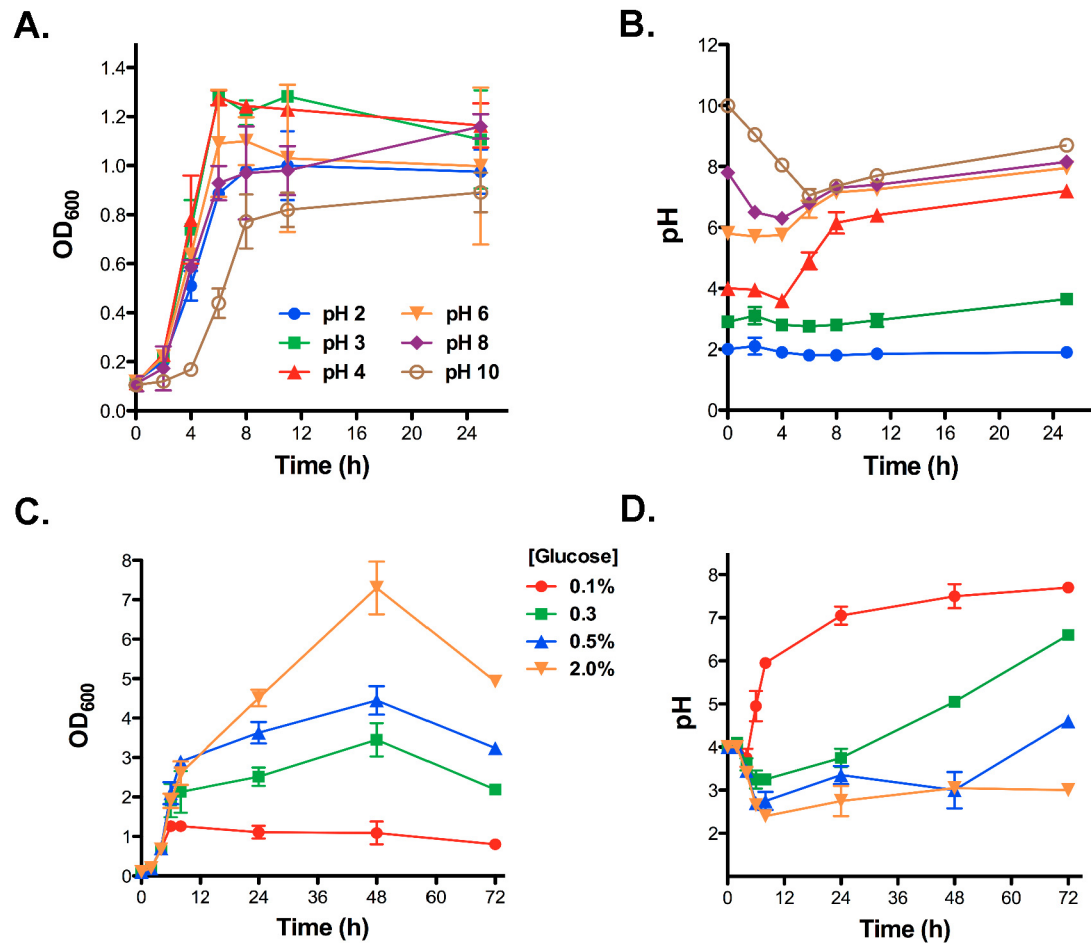
FIG 1 *C. albicans* effects a glucose-repressible change in environmental pH on medium 199. (A) *C. albicans* wild-type strain SC5314 was grown in medium 199 with an initial pH between 2.0 and 10.0 at 37°C in aerated Erlenmeyer flasks. Growth of the cells was assessed by measuring the OD_{600} at the indicated time points. (B) The pH of the same cultures was measured using a pH electrode. (C) SC5314 was grown in medium 199, pH 4.0, supplemented with glucose to give a final concentration between 0.1% (no added glucose) and 2%. Growth was assayed by measuring the OD_{600} at the indicated time points. (D) The pH of the same cultures was measured using a pH electrode. The data in all panels are the averages of results from triplicate experiments.

1% Casamino Acids (an acid hydrolysate of casein) permitted growth accompanied by a significant alkalinization, from pH 4 to pH ~6.3, over 24 h (Fig. 2A). Alkalinization was also seen on synthetic complete (SC) medium (YNB plus a mixture of most of the amino acids), and this was repressed by the addition of glucose (data not shown). Thus, alkalinization is stimulated by the presence of exogenous amino acids.

We assayed growth and pH changes in YNB medium at pH 4 containing 1 mM each amino acid individually as the sole carbon source (Fig. 2B). At this level, only a subset of amino acids allowed any increase in culture density (Ala, Arg, Asn, Gln, Glu, Pro, and Ser), and there was a strong correlation between the ability of *C. albicans* to metabolize individual amino acids as a carbon source and the degree of alkalinization (Fig. 2B), suggesting that metabolism of amino acids is required for the change in pH.

Alkalinization autoinduces hyphal morphogenesis. *C. albicans* cells shifted to neutral pH media rapidly initiate germ tube formation and hyphal growth. We hypothesized that the gradual neutralization under these conditions would also promote this morphological transition. We tested this by growing cells of the wild-type strain SC5314 in liquid YNB medium with 2% (wt/vol)

Casamino Acids as the sole carbon source. In this medium, the pH rises gradually from 4 to 7.2 over 12 h (Fig. 3B). By 7 h, when the pH of the medium was 5.9, 78% of cells were hyphal, compared to 4.2% of cells in the same medium supplemented with 2% glucose, where the pH was 3.8 (Fig. 3A; representative images are shown in Fig. S2 in the supplemental material). Buffering the medium with 0.1 M Tris and 0.1 M MOPS (morpholinepropanesulfonic acid) greatly slowed the rise in pH (4.3 at 7 h) and reduced the proportion of hyphal cells (13%). By 24 h, the pH of the medium containing Casamino Acids as the sole carbon source was 7.3, and 60% of the cells remained in the hyphal state; no hyphae were observed in the glucose or buffered control cultures, despite small rises in pH (to 5.5 and 5.4, respectively). Initial germ tube growth was observed in all cultures at an early time point (3 h), as has been previously reported following dilution into fresh medium (30), but hyphal induction is maintained only in the alkalinizing culture.

Alkalinizing colonies on solid GM-BCP medium also switched to hyphal growth. As shown in Fig. 3C, the alkalinizing colony had a border of highly filamentous cells, and both alkalinization and filamentous growth were blocked by the addition of glucose. Thus,
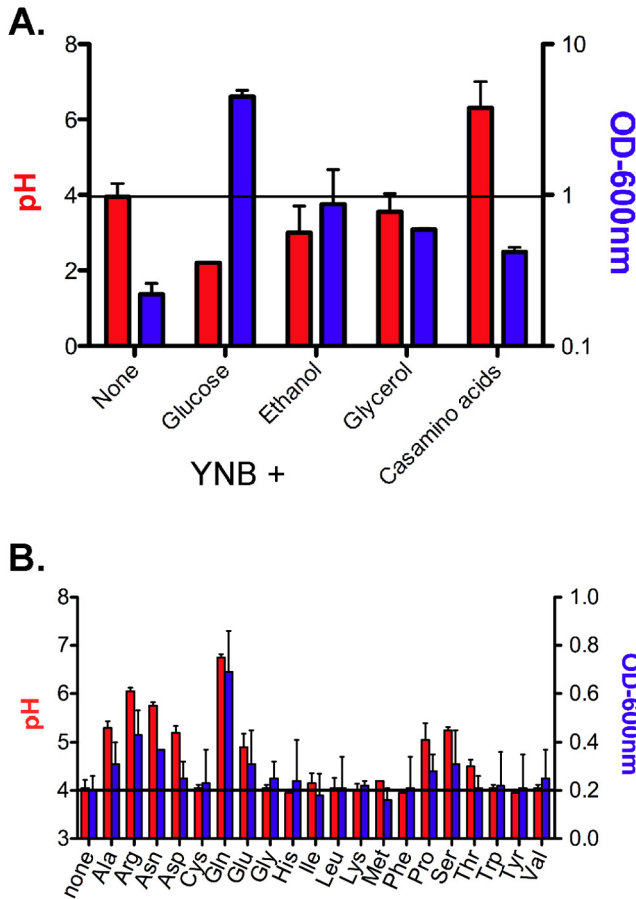
FIG 2 Amino acids are required for alkalinization. (A) Wild-type strain SC5314 was grown in liquid YNB medium at pH. 4.0 (see Materials and Methods), with the indicated compound as the sole carbon source, present at 1% (wt/vol) (for Casamino Acids) or 2% (wt/vol) (for other compounds). Growth (blue bars) and pH (red bars) were measured after 24 hours at 37°C. The horizontal black line is present to indicate the starting pH of the cultures. (B) The wild-type strain (SC5314) was grown in liquid YNB medium at pH 4.0, supplemented with the indicated amino acid present at 1 mM as the sole carbon source. Growth (blue bars) and pH (red bars) were measured after 24 hours at 37°C. The horizontal black bar indicates the starting pH and OD_{600} of the cultures for reference.

C. albicans appears to induce its own morphogenesis via raising extracellular pH.

Alkalinization is not controlled by the Rim101p or Mnl1p transcription factors. In *C. albicans*, two transcription factors have been linked to pH responses: the Rim101p/PacC pathway is required for growth at alkaline pH (reviewed in reference 25), and Mnl1p mediates adaptation to the presence of several weak acids, such as acetic and formic acids (31). Strains lacking *RIM101* were obtained from D. Davis (8, 11), and *mnl1Δ* mutants were derived from a transcription factor mutant library (a gift from D. Sanglard; see below). Neither of these mutants affected the alkalinization phenomenon (see Fig. S3 in the supplemental material). We also tested strains lacking several other components of the Rim101p pathway or the ESCRT complex, and none altered alkalinization (data not shown). The pH changes that we observe are therefore independent of the previously identified pH adaptation machinery.
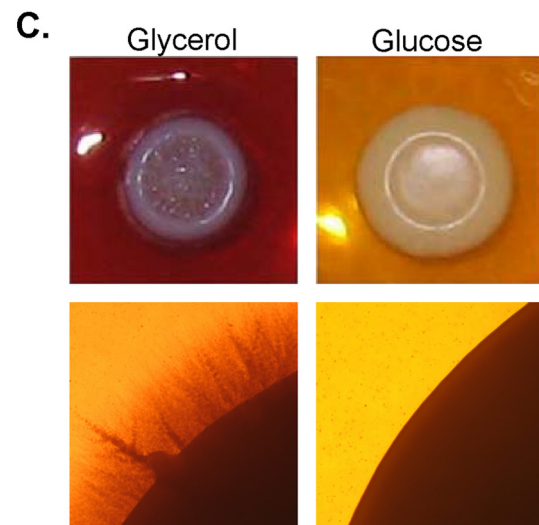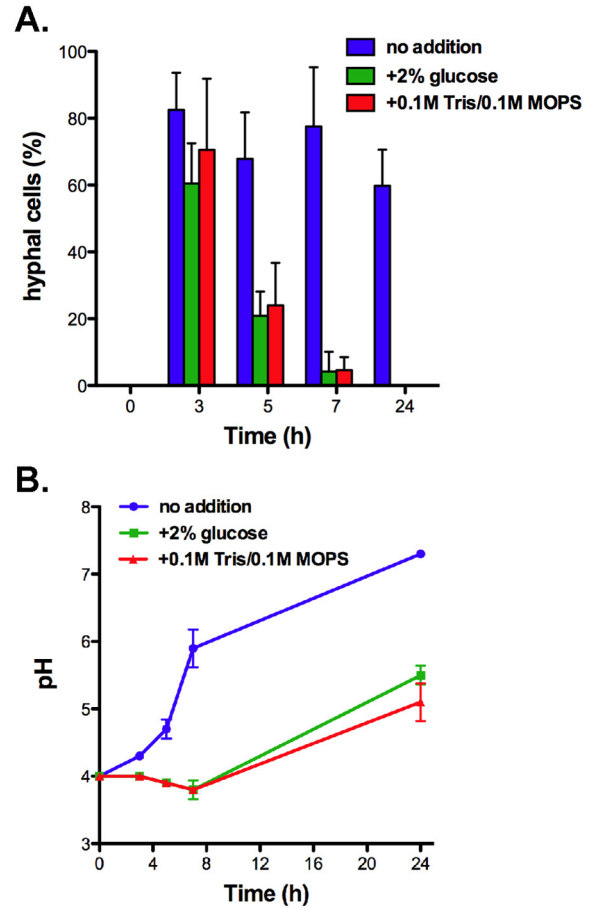
FIG 3 Alkalinization induces hyphal morphogenesis. The wild-type strain (SC5314) was grown overnight in YPD and then washed in water and diluted to an OD_{600} of ~0.2 in YNB plus 2% Casamino Acids at pH 4.0 or in this medium supplemented with 2% glucose or 0.1 M Tris and 0.1 M MOPS to block alkalinization. At the time points indicated, samples were collected and photographed (see Fig. S2 in the supplemental material). (A) Hyphal induction was quantified by counting cells from the photomicrographs. (B) The pH of the same cultures was measured at the same time points. (C) *C. albicans* wild-type cells were grown in YPD, washed in water, and diluted to an OD_{600} of 1.0, and then 2 μl was spotted onto GM-BCP, pH 4.0, medium with or without 2% glucose. The alkalinization of the medium and the morphology of the colony were observed at 48 h.
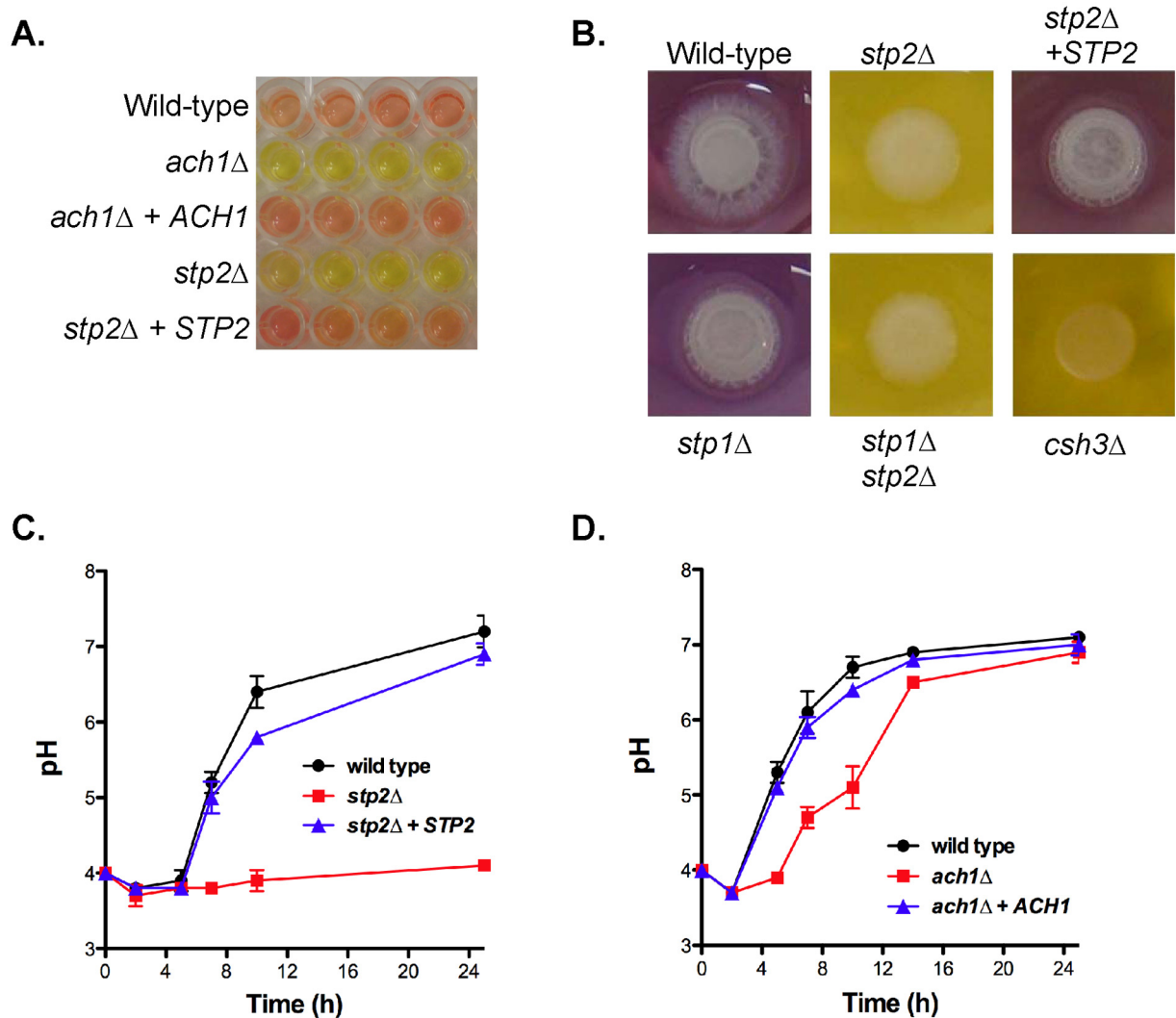
FIG 4 *C. albicans* Ach1p and Stp2p are required for efficient alkalinization. (A) *C. albicans* SC5314, *ach1*Δ (ACC16), and *stp2*Δ (PMRCA57) mutants and their complemented controls (*ach1*Δ plus *ACH1* [ACC17] and *stp2*Δ plus *STP2* [PMRCA96]) were grown overnight in YPD, collected by centrifugation and washed with water, resuspended in medium 199, pH 4.0, at an OD$_{600}$ of 1.0, and then serially diluted (1:5 dilutions) in 96-well plates. Alkalinization was observed colorimetrically as the orange-red color of the medium (pH > 6.0) after 24 h of incubation at 37°C. (B) Strains were prepared as described in the legend to Fig. 3C, spotted onto GM-BCP, pH 4.0, medium, and grown at 37°C. Plates were photographed after 3 days. Strains were wild type (SC5314), *stp2*Δ (PMRCA57), *stp1*Δ (PMRCA59), *stp1*Δ *stp2*Δ (PMRCA94), *stp2*Δ plus *STP2* (PMRCA96), and *csh3*Δ (PMRCA12). (C) The *C. albicans* wild-type, *stp2*Δ (PMRCA57), or *stp2*Δ complemented (PMRCA96) strain was grown in medium 199, pH 4.0. The growth (not shown) and pH of the medium were assessed at the indicated time points. (D) Alkalinization of the *C. albicans* wild-type, *ach1*Δ (ACC16), or *ach1*Δ complemented (ACC17) strain was tested on medium 199, pH 4.0. All the experiments were done at least in triplicate.

Identification of *C. albicans* genes involved in environmental alkalinization. To identify factors that mediate extracellular alkalinization, we performed a genetic screen for *C. albicans* mutants unable to change the pH of the medium using libraries containing ~500 strains enriched for mutations in transcription factors, signaling components, and cell wall proteins generated in the laboratories of A. Mitchell (32, 33) and D. Sanglard (personal communication). The mutants were screened in 96-well plates containing medium 199, pH 4.0, to identify wells in which the medium remained yellow (acidic) but abundant yeast growth was observed. This screen identified two alkalinization-deficient mutants: *ach1*, encoding acetyl-coenzyme A (acetyl-CoA) hydrolase, and *stp2*, encoding a transcription factor that regulates the expression of amino acid permeases.

We confirmed the results from the library screen using previously published mutants of *ACH1* and *STP2*. First, we plated serial dilutions of both strains in 96-well plates in medium 199, pH 4, and incubated them for 24 h at 37°C. Neither mutant alkalinized the medium, while the wild-type (WT) strain and complemented versions of both mutants did (Fig. 4A). The *stp2*Δ strain was also unable to alkalinize solid GM-BCP medium (Fig. 4B), while the *ach1*Δ mutant did alkalinize this medium, albeit with a variable delay relative to the level for wild-type controls (data not shown). Similarly, we observed no pH change in cultures of the *stp2*Δ mutant in aerated medium 199 (Fig. 4C). The *ach1*Δ mutant retarded the rise in pH but eventually reached the same pH as the wild-type or complemented strains (Fig. 4D). The *ach1*Δ mutants grew at the same rate as the wild-type strain in medium 199, while

the $stp2\Delta$ mutant had a moderate growth retardation, presumably as a result of impaired amino acid uptake (data not shown).

Stp2p is a transcription factor that activates amino acid permeases in response to extracellular amino acids (34). Many of these permeases require Csh3p, an endoplasmic reticulum (ER)-resident chaperone, for proper folding and sorting to the cell surface (35); a $csh3\Delta$ mutation also blocked alkalinization (Fig. 4B), reinforcing the role of amino acid import and metabolism in this phenomenon. Conversely, Stp1p, homologous to Stp2p, has no effect (Fig. 4B). Stp1p regulates the protease Sap2p and the oligopeptide permease Opt1p when protein is present in the medium (34); we were unable to detect alkalinization when protein (bovine serum albumin [BSA]) was the sole source of carbon. Collectively, these results suggest that general amino acid uptake and amino acid catabolism are aspects of intermediate metabolism essential for the extracellular pH changes we observe.

Microarray analysis of neutralization in *C. albicans*. In order to further understand the molecular basis for alkalinization, we assayed transcript profiles of cells actively alkalinizing the medium by growing wild-type cells in medium 199, initially at pH 4.0, until the pH of the medium reached pH 5.0. This profile was compared to two control conditions that do not alkalinize (the wild-type strain in the same medium supplemented with 2% glucose or the $ach1\Delta$ mutant in medium 199), focusing on genes whose expression was increased or decreased under the alkalinizing conditions but not under the nonalkalinizing conditions (see Materials and Methods for details). Using a significance cutoff of 3-fold change and a P value of less than 0.01 (Student's t test), we identified 60 genes, 34 overexpressed and 26 underexpressed, under the alkalinizing conditions. Uncharacterized open reading frames (ORFs) comprised the largest set of regulated genes (22/60). None of the genes identified genetically, *STP2* or *CSH3* (under both conditions) or *ACH1* (versus the glucose culture), were differentially regulated in a statistically significant manner. The remainder of the data showed an emphasis on amino acid metabolism and transmembrane transport, as described below and in Table 1.

Differential regulation of amino acid metabolism was apparent in the microarray data, particularly for arginine and methionine. Genes for arginine synthesis, such as *ARG1* and *ARG3*, are repressed in alkalinizing cultures (Table 1). Conversely, alkalinizing cells induce arginine import via genes encoding basic amino acid permeases (*CAN1* and *CAN2*) and degradation via genes for arginiase (*CAR1*) and ornithine transaminase (*CAR2*). One product of arginase is urea, and the urea amidolyase gene *DUR1,2* is strongly repressed in the alkalinizing culture relative to the level for the high-glucose conditions (Table 1). Together, there is a strong transition during alkalinization from synthesis to catabolism of arginine. Methionine synthesis is also repressed during alkalinization: *MET1*, *-2*, *-3*, *-4*, and *-14* are each repressed in the alkalinizing culture, and two other *MET* genes (*MET10* and *MET15*) were underexpressed in comparison to the level for only one nonalkalinizing condition (Table 1).

Other transmembrane transporters were also affected during alkalinization (Table 1). Alkalinizing cultures upregulated genes encoding transporters for glucose (HGT2, HGT13, and HGT16), lactate (*JEN1*), phospholipids (*CDR3*), phosphoinositol (*GIT2*), and water (*AQY1*). Genes for two ammonia permeases, *MEP1* and *MEP2*, were significantly induced in the nonalkalinizing WT-glucose cells but not in the $ach1\Delta$ culture. Four other putative

transporters, homologs of the *S. cerevisiae* Ato proteins (see Table S1 in the supplemental material), are discussed below.

Finally, we observed several notable changes in carbon metabolism (Table 1). Genes encoding the glyoxylate cycle components Icl1p and Mls1p, acetyl-CoA synthase Acs1p, and the carnitine acetyltransferases Ctn1p and Ctn3p and several putative β-oxidation genes (*FOX2*, *PXP2*, and *FAA2*) were expressed at lower levels under one or both of the nonalkalinizing conditions.

These data suggested additional candidate effectors of the pH changes we observe. We assayed alkalinization on both medium 199 and GM-BCP medium of mutant strains lacking *ICL1*, *FOX2*, *CTN1*, *INO1*, *MET15*, *MEP1*, *MEP2*, and *DUR1,2* either generated in our laboratory or generously provided by others (28, 36–40). We also constructed strains lacking *ARG1*, *ARG3*, *JEN1*, or *JEN2* (see Materials and Methods). Only a strain with a mutation in *DUR1,2*, which degrades urea to CO_2 and ammonia, showed a defect in alkalinization, with a slower pH change than its wild-type strain A72 (see Fig. S4 in the supplemental material). It is notable that while most of the genes tested are involved in either transport or anabolic functions, *DUR1,2* and *FOX2* are the only genes in this set that are catabolic and $dur1,2\Delta$ has an alkalinization phenotype under these conditions.

In *Colletotrichum coccodes*, a pathogen of tomatoes, three genes have been associated with alkalinization (23). One of these, *AreA*, encodes a transcription factor that mediates nitrogen catabolite repression. Its homolog in *C. albicans*, *GAT1*, was upregulated 4.2-fold relative to the level in one nonalkalinizing culture, but a $gat1\Delta$ mutant (41) had no effect on alkalinization in *C. albicans* (data not shown). The other *C. coccodes* genes, *Nit1* and *Nit3*, encode nitrate and nitrite, respectively, reductases, and have no *C. albicans* homologs. It is interesting to note that extracellular pH changes in *C. coccodes* appear tightly linked to nitrogen metabolism, while our data indicate that it is associated with carbon metabolism in *C. albicans*.

Role of Ato proteins in environmental alkalinization by *C. albicans*. Four of the genes highly induced during alkalinization relative to the levels for both control conditions encode homologs of a family of fungus-specific transmembrane proteins that have been associated with the limited extracellular pH changes observed in *S. cerevisiae*. It was proposed that they actively extrude ammonia and hence were named Ato, for "ammonia transport outward" (29). The precise function of these proteins is not clear, but in addition to pH changes, they have been associated with acetate utilization in *S. cerevisiae*, *A. nidulans*, and *Yarrowia lipolytica* (29, 42–45) and sporulation in *S. cerevisiae* (46) and have a variety of aliases. Most fungal species have two or three predicted homologs (A. J. Carman and M. C. Lorenz, unpublished observations) and we were surprised to identify 10 *ATO* homologs in *C. albicans* (see Table S1 in the supplemental material). The regulation of *ATO1* was particularly striking: it is expressed 79- to 177-fold higher in alkalinizing cells than in the *ach1* or glucose-repressed cultures, respectively (Table S1). Quantitative reverse transcription-PCR (qRT-PCR) on independent RNA samples indicated that this was actually an underestimate of the magnitude of regulation (data not shown).

S. cerevisiae mutants lacking any one of the *ATO* homologs show modest reductions in alkalinization (29). We attempted to test the role of the larger *C. albicans ATO* family in a similar manner, by constructing several *ATO* mutants using the UAU-mediated gene disruption method (47) using constructs created as

TABLE 1 Genes differentially regulated during alkalinization[a]

Annotation and gene	Expression (fold)			Function of encoded protein
	Orf19	*ach1Δ*	WT-Glu	
Arginine metabolism				
CAN2	111	*8.9*	*66.6*	Basic amino acid permease
CAR1	3,934	*8.1*	*3.3*	Arginase (Arg → urea-ornithine)
CAR2	5,641	*10.3*	*3.1*	Ornithine transaminase (Orn → Glu)
CAN1	97	*4.2*	*3.0*	Basic amino acid permease
ARG1	7,469	**0.18**	**0.31**	Argininosuccinate synthetase
ARG4	6,689	0.63	**0.31**	Argininosuccinate lyase
ARG3	5,610	**0.11**	**0.14**	Ornithine carbamoyltransferase
DUR1,2	780	1.3	**0.12**	Urea amidolyase
DUR3	781	0.97	**0.03**	Putative urea transporter
Methionine/cysteine/sulfate metabolism				
MET2	2,618	**0.15**	**0.29**	Homoserine acetyltransferase
MET10	4,076	0.59	**0.28**	Sulfite reductase
MET4	5,312	**0.28**	**0.28**	Transcription factor for MET/CYS
MET15	5,645	1.1	**0.19**	Acetylhomoserine aminocarboxypropyl transferase
MET14	946	**0.33**	**0.18**	Adenylyl-sulfate kinase
MET1	5,842	**0.26**	**0.12**	Uroporphryin methyltransferase
MET3	5,025	**0.25**	**0.10**	ATP sulfurylase
Transporters/channels				
JEN1	7,447	*15.7*	*99.9*	Lactate transporter
HGT13	7,093	*3.4*	*12.2*	Glucose transporter
HIP1	3,195	2.1	*10.3*	Similar to amino acid permeases
CDR3	1,313	*17.7*	*9.4*	ABC transporter of phospholipids
HGT2	3,668	*3.3*	*5.6*	Glucose transporter
DIP5	2,942	0.94	*4.0*	Dicarboxylic acid permease
GIT2	1,978	*5.6*	*4.0*	Putative glycerophosphoinositol permease
HGT16	6,141	*2.7*	*3.2*	Glucose transporter
AQY1	2,849	*22.7*	*3.1*	Aquaporin water channel
	34	1.9	**0.31**	Putative glycerophosphoinositol permease
	3,120	0.38	**0.27**	Putative PDR family transporter
HAK1	6,249	0.35	**0.19**	Putative potassium transporter
MEP1	1,614	1.8	**0.12**	Ammonium permease
MEP2	5,672	0.49	**0.05**	Ammonium permease
Alternative carbon utilization				
ACS1	1,743	*49.7*	*19.1*	Acetyl-CoA synthetase
FDH1	638	0.82	*11.7*	Formate dehydrogenase
PXP2	1,655	*15.1*	*9.5*	Acyl-CoA oxidase (β-oxidation)
FAA2	7,379	*4.6*	*6.8*	Fatty acyl-CoA ligase (β-oxidation)
ICL1	6,844	0.88	*5.3*	Isocitrate lyase (glyoxylate cycle)
MLS1	4,833	0.90	*4.4*	Malate synthase (glyoxylate cycle)
FOX2	1,288	0.69	*3.3*	3-Hydroxyacyl-CoA epimerase (β-oxidation)

[a] Ratios given are the expression levels in alkalinizing cultures relative to the levels for the nonalkalinizing *ach1Δ* mutant (*ach1Δ* column) or the nonalkalinzing high-glucose condition (WT-Glu column). Shown are >3-fold changes up (bold) or down (italic).

part of a large-scale project for three of these genes. Surprisingly, given the potential for redundancy, mutation of *ATO5* (orf19.6997) significantly retarded alkalinization in all three of our assays (Fig. 5; see also Fig. S5 in the supplemental material), while insertional mutations in *ATO1* and *ATO8* had no effect. The *ato5Δ* mutant strain grows at the same rate as the wild-type control DAY286 (data not shown). In a parallel approach, we constructed an *ATO1* allele carrying a mutation in a highly conserved motif (FGGTLN) in the amino-terminal part of the protein that has previously been identified as critical for some Ato functions and was predicted to be dominant negative (42). This allele, *ATO1** (Gly53Asp, a mutation of the second glycine in the FG-GTLN motif), was expressed under the control of the strong, constitutive *ACT1* promoter in a wild-type strain, where it mildly retarded alkalinization in medium 199 (Fig. S5). Expression of ATO8, which is naturally nonconsensus in this region (Table S1), also slightly reduced alkalinization. Further, we find a correlation

between the number of *ATO* homologs and the degree of alkalinization in the *Candida* genus: the six species that alkalinize effectively (Fig. S1) have 4 to 10 *ATO* homologs each (median = 6.5), while the three that do not have 2 to 4 (median = 3). Our results are consistent with a role for the Ato family in ammonia extrusion, but the phenotypes that we have observed are moderate, probably due to redundancy in this large gene family.

Alkalinization is associated with extrusion of ammonia. Localized alkalinization in several fungal species has been associated with the release of volatile ammonia, a strong base, from the cells. To track the release of ammonia during this pH change, we grew strains on GM-BCP plates under alkalinizing and nonalkalinizing conditions (with or without 2% glucose) and placed an "acid trap" containing 10% citric acid below the cells in the beginning of this process (see Materials and Methods). Over 24 to 72 h, the pH rose (Fig. 6A), and we analyzed the acid trap for the presence of ammonium, released as volatile ammonia from the cells, via a color-
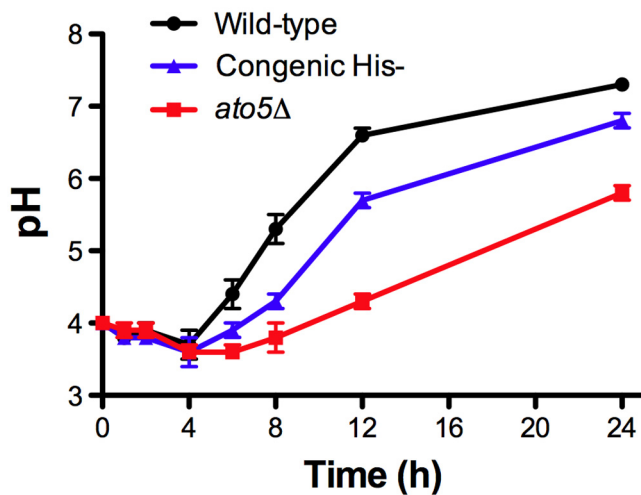
FIG 5 Mutation of *ATO5* retards alkalinization. The *ato5*Δ strain (HDC1), its congenic His⁻ control (P 17), and the prototrophic wild-type (SC5314) were grown overnight in YPD and then diluted to an OD_{600} of 0.1 in medium 199 at pH 4. These cultures were incubated at 30°C with aeration, and the pH was measured at the indicated time points. Culture density was also measured (not shown). A second, independent *ato5*Δ strain behaved identically (not shown).

imetric assay based on Nessler's reagent, as previously described (48). Detectible ammonia increased significantly (Fig. 6B) over the 72-h period, correlating with the pH change observed on the plates (Fig. 6A). Very little volatile ammonia was released by non-alkalinizing cultures with the use of either the *stp2*Δ mutant or a glucose-repressed wild-type strain. We attempted to measure ammonia release from liquid cultures, aerated or not, and were unsuccessful using several approaches, possibly as a result of the volatile nature of this compound. On solid media, however, extrusion of ammonia correlates with the observed pH changes, providing a probable explanation for extracellular alkalinization, consistent with reports of ammonia release from *Colletotrichum* during its alkalinization *in planta* (23).

DISCUSSION

We show here that *C. albicans* is able to actively modulate the environmental pH to a remarkable degree, neutralizing media initially at pH 4 to 10. We have focused mostly on the alkalinization of acidic media, which occurs rapidly, with the pH changing from 4 to >7 in less than 24 h in aerated liquid cultures. This glucose-repressible phenomenon occurs on both solid and liquid media of several compositions but requires the presence of exogenous amino acids. Neutral pH has been long recognized as an inducer of hyphal morphogenesis, and cells in alkalinizing media shift to the hyphal form; this is blocked by buffering the medium or addition of glucose. Thus, *C. albicans* effectively autoinduces morphogenesis under these conditions. The rise in pH is associated with the release of ammonia, a highly basic compound, as has been observed in other fungi.

Genetic and genomic approaches have begun to identify the mechanism behind this phenomenon, and these have confirmed the importance of amino acid import and metabolism: the amino acid permease regulators Stp2p and Csh3p have strong phenotypes, while the catabolic enzymes Ach1p and Dur1,2p have more mild effects. This is consistent with the microarray analysis of

alkalinizing cells in which induction of amino acid uptake and catabolism and repression of amino acid biosynthesis were general trends.

Figure 7 presents a model for the metabolic functions that contribute to alkalinization, based on the utilization of amino acids as the primary source of carbon. Amino acid uptake via transmembrane permeases is required, and while there is significant redundancy in permease specificity, a deletion of the Stp2p transcription factor reduces expression of multiple permeases (34), and this ablates alkalinization. The close correlation of pH changes and growth indicates that catabolism of these amino acids is required. Amino acids are catabolized through several different routes, resulting in acetyl-CoA, succinyl-CoA, α-ketoglutarate, or oxaloacetate, each of which involves deamination steps. Under carbon-rich conditions nitrogen is generally stored in the form of glutamate and glutamine, but the capacity for doing so when amino acids are needed as a carbon source is presumably limited. High cytosolic ammonia concentrations are toxic (49), and excretion of the excess ammonia would both detoxify the cytosol and raise the extracellular pH. The Ato family of proteins has been proposed to be ammonia exporters—the name comes from "ammonia transport outward" (29)—and we find that deletion of one of these genes, *ATO5*, slows alkalinization. The Ato family is significantly enlarged in *C. albicans* (10 members) and other alkalinizing species relative to species that alkalinze poorly. We note that the most pathogenic CUG *Candida* species (*C. albicans*, *C. tropicalis*, and *C. parapsilosis*) have large Ato families, reminiscent of the expansion of gene families for a number of virulence-related traits, particularly those on the cell surface or secreted, such as secreted aspartyl proteases, lipases, and phospholipases, and GPI-anchored cell wall proteins such as the ALS agglutinins (27).

The model in Fig. 7 is based on simple metabolic activities and should occur in any fungal cell capable of using amino acids to satisfy cellular carbon requirements (which is true for at least most fungi) and, indeed, has been observed in several other species. Ammonia has been reported to occur in alkalinized halos surrounding colonies of *Metarhizium anisopliae*, *A. fumigatus*, *N. crassa*, *Coccidioides immitis*, and *S. cerevisiae* (19, 29, 50, 51), and alkalinzation by several dermatophyte species also occurs (20). One previous report has also shown ammonia-dependent pH changes in *C. albicans* in media containing keratin; as with the other species mentioned, this process occurred slowly over the course of ~2 weeks (24). Under these defined conditions of glucose limitation and amino acid excess, *C. albicans* alters the pH much more rapidly than previously seen. Of the other fungal species examined, only *Colletotrichum* species raise pH to a similar degree in the tissues of decaying fruit, though the process is slower than in *Candida* (21, 22). The metabolic basis for the pH change in *Colletotrichum* appears linked to nitrogen metabolism, as opposed to amino acid catabolism in *C. albicans*.

The rise in pH resulting from *Colletotrichum in planta* induces expression of the key virulence factor pectin lyase (22). Similarly, we show that the pH change caused by *C. albicans* induces hyphal morphogenesis, a key virulence trait in this species (2, 3). Indeed, the conditions that promote alkalinization are at least superficially similar to those encountered within phagocytes, as determined by transcriptional profiling (52–54), and it has been suggested that *C. albicans* might interfere with normal phagosomal trafficking such that it is not always in an acidified phagolysosome (55, 56). If *C. albicans* were to neutralize the phagolysosome, it would

FIG 6 Ammonia extrusion by alkalinizing *C. albicans* colonies. (A) The wild-type strain, the *stp2Δ* mutant, and its complement were prepared as described in the legend to Fig. 4 and spotted to GM-BCP, pH 4.0 (plus 2% glucose where indicated). Plates were photographed at the indicated time points. At 4 days, a stereomicroscope was used to photograph the colony borders at ×20 magnification. Hyphal growth is indicated in the alkalinizing samples by the brush border at the colony edge. (B) Ammonia release from the same strains was measured as described in Materials and Methods at the indicated time points. A strong correlation between medium color (indicating alkalinization) and ammonia concentration was observed. The measurements in panel B are not from the same plates shown in panel A, as the arrangement of the acid trap to collect ammonia does not facilitate manipulation of the plates, but the time course in panel A is representative.
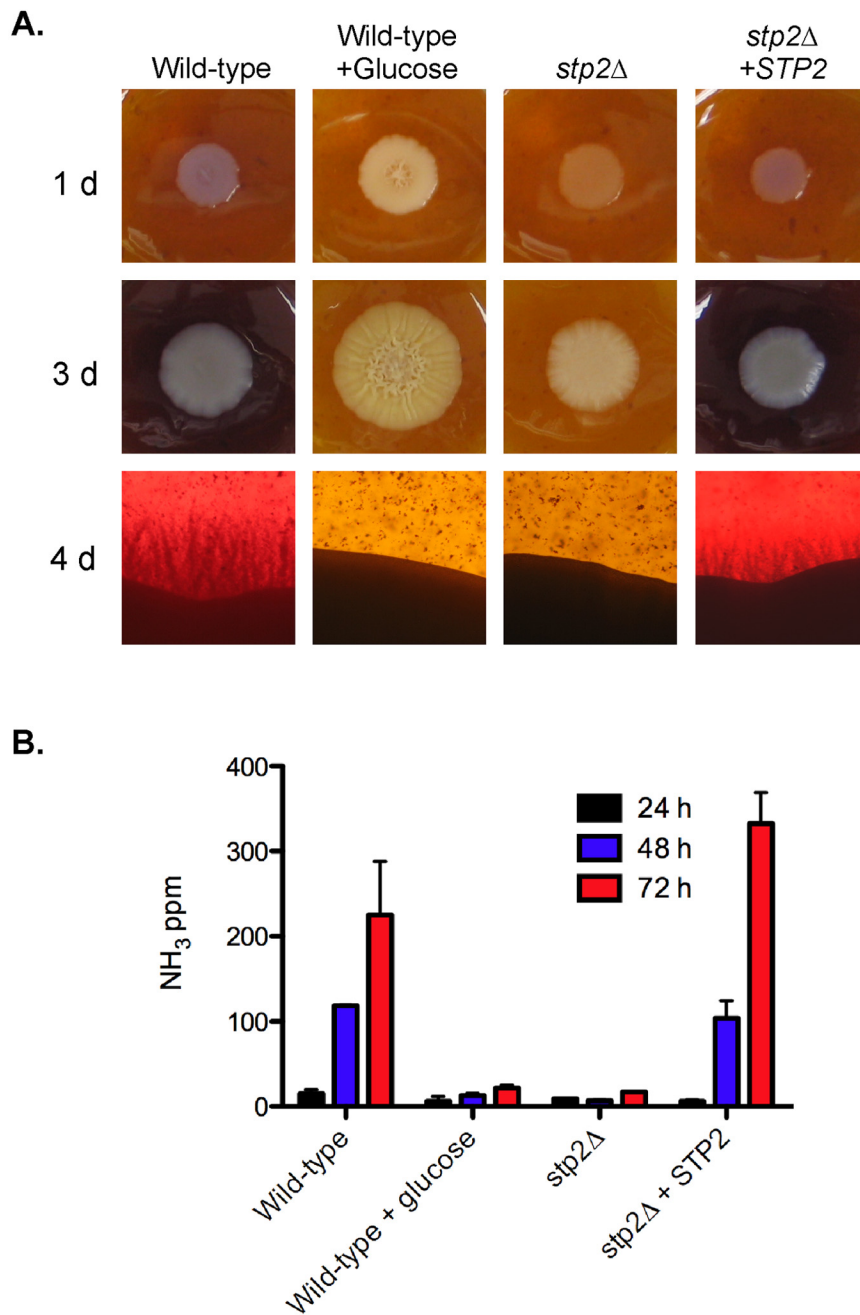
genesis in this manner, though a conclusive proof of this idea poses several technical and biological challenges.

C. albicans has a uniquely intimate association with the mammalian host amongst fungi. Virulence in these species appears to be the result of a gradual adaptation of common aspects of fungal physiology to the host environment rather than the acquisition of specific virulence factors such as toxins or secondary metabolites. Amongst these adaptations are a robust response to changes in extracellular pH, rapid metabolic shifts after changes in nutritional status, and, of course, avid hyphal morphogenesis; all of these are required for virulence in *C. albicans* (2, 3, 11, 57–59). Our findings link these phenomena, as the extracellular pH changes require a carbon-limited environment and result in hyphal growth. In conclusion, we suggest that the ability to control extracellular pH by *Candida* species may be an evolutionary adaptation that contributes to fitness within the host.

MATERIALS AND METHODS

Strains and media. The *C. albicans* strains used in this study are presented in Table S2 in the supplemental material. Strains were routinely maintained on YPD (1% yeast extract, 2% peptone, 2% glucose) or YNB (1.7% yeast nitrogen base without amino acids, 0.5% ammonia sulfate, 2% glucose) medium, as previously described (60). Neutralization assays with liquid media were performed either with medium 199 (Invitrogen), prepared according to the manufacturer's instructions, without buffering, or with YNB prepared without glucose. Amino acids or other carbon sources were added to this medium as indicated in the text and figures. For neutralization on solid media, strains were grown on GM-BCP (1% yeast extract, 30 mM $CaCl_2$, 3% glycerol, 0.01% bromocresol purple, 2% agar) (29). The media were adjusted to the desired pH using HCl or NaOH. *C. albicans* mutant libraries were obtained from A. Mitchell (32, 33) and D. Sanglard (unpublished) and contain mutations in approximately 500 unique genes.
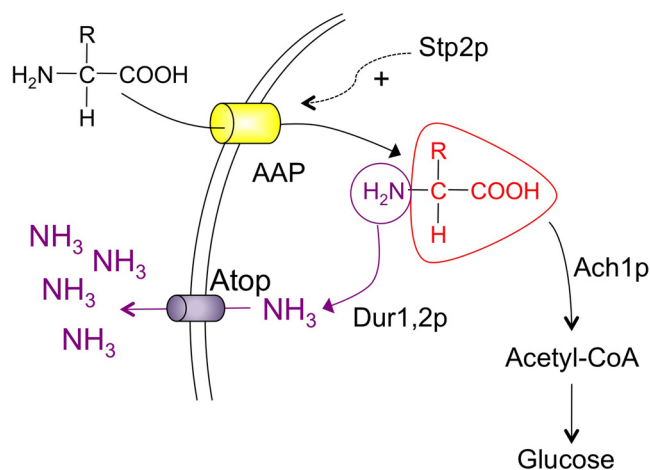
Strain and plasmid construction. Strains lacking *ATO1*, *ATO5*, *ATO8* (see Table S1 in the supplemental material), *JEN1*, or *JEN2* were generated via the UAU disruption method (47) using transposon insertion constructs created by Q. Zhao and W. Nierman (Institute for Genomic Research) and provided by F. Smith and A. Mitchell (Columbia University) as part of a large-scale project. Linearized constructs were used to transform strain BWP17 by electroporation (61), selecting for arginine prototrophy. Candidates were passaged twice in YPD at 30°C and then plated to SD-Arg-Ura medium to select for mutants generated by gene

promote fungal survival both by inhibiting acid hydrolases and by enabling escape via hyphal growth. It is intriguing to speculate that the activity we have identified here contributes to fungal patho-

FIG 7 Model of environmental alkalinization by *C. albicans*. Under conditions in which amino acids are metabolized as carbon sources, the cell upregulates transmembrane transporters for various amino acids, facilitated by the Stp2p transcription factor. Amino acids are converted into tricarboxylic acid (TCA) cycle intermediates via several routes, many of which require acetyl-CoA production and intracellular transport mediated by acetyl-CoA hydrolase (Ach1p), and all of which remove the amine group(s). In mammals, excess nitrogen is excreted as urea, whereas we propose that in *C. albicans* this is converted into ammonia and CO_2 by urea amidolyase (Dur1,2p) and exported from the cell in a process involving the Ato proteins.

conversion and recombination. Loss of the wild-type allele was confirmed by PCR.

Mutations in *ARG1* and *ARG3* were generated using the SAT-flipper method, as previously described (61). Briefly, 5′ and 3′ untranslated regions of the two genes were cloned to flank the FRT-SAT1-FLP-FRT cassette. These were used to transform strain SC5314, with selection on YPD plus 100 μg/ml nourseothricin (Werner Bioagents, Jena, Germany). Integration was confirmed by PCR, and the SAT-FLP was excised as previously described (61) to generate nourseothricin-sensitive colonies. The second allele was disrupted in the same manner.

Constitutive expression of *ATO1* alleles was accomplished by cloning the ORF (or a point mutant generated by overlap PCR) under the control of the *ACT1* promoter and integrating it at the *RPS10* locus. Briefly, ~1,000 bp of the *ACT1* promoter from pAU34 (62) was subcloned between the KpnI and XhoI sites in CIp10 (63) to generate pHZ116. Next, the *ATO1* ORF was PCR amplified and cloned as the XhoI-HindIII fragment into pHZ116 to generate *ACT1p-ATO1* (pML342) plasmids. The *ATO1** mutant was generated by site-directed overlap PCR using complementary oligonucleotides with a single mismatch to change Gly-53 to Asp, analogous to the *Y. lipolytica GPR1-1* mutant identified by Barth and colleagues (42), and cloned into pHZ116 to generate pML341. Both plasmids, plus pHZ116, were digested with StuI and used to transform CAI4-F2 to uridine prototrophy. Accurate integration at the *RPS10* locus was verified by PCR.

Neutralization assays. Three forms of neutralization assays were used. For assays with aerated (shaking) cultures, strains were grown overnight in YPD, collected by centrifugation, washed in water, and transferred at a final optical density at 600 nm (OD_{600}) of 0.05 to 0.1 in medium 199, pH adjusted as indicated. Cells were incubated at 37°C with aeration and growth, and pH measurements were taken at the indicated time points by drawing 5-ml samples. Glucose or amino acids were added in some experiments as described in the text and figure legends. Assays were also performed with 96-well plates (nonaerated), in which cells were grown in YPD overnight, collected by centrifugation, washed in water, and transferred to medium 199, pH 4, at a final OD_{600} of 1.0 and then serially diluted 1:5.

Assays with solid media were performed using 12-well plates to isolate strains from potential intercolony communication as has been described for *S. cerevisiae* (18), in which each well contained ~2 ml of GM-BCP or SC-BCP, supplemented or not supplemented with glucose, as indicated. Strains were grown overnight in YPD, collected by centrifugation, washed in water, and diluted to an OD_{600} of 1.0 in distilled water (dH_2O), and 7 μl of this dilution was spotted into the wells, one strain/well. Cells were incubated at 30°C, and alkalinization observed as a purple halo around the colonies was documented after 48 to 72 h unless otherwise indicated.

The effects of individual amino acids on alkalinization were tested using YNB medium, pH 4.0, supplemented with individual amino acids at 1 mM. *C. albicans* strain SC5314 was grown as previously described and added to the medium to give a final OD_{600} of 0.2. Cells were incubated for 24 h, at which time point the pH and OD_{600} of the medium were recorded.

Assessment of hyphal induction in alkalinizing cultures. Wild type SC5314 cells were grown into YPD medium overnight, washed, and diluted to give a final OD_{600} of 0.2 in YNB medium supplemented with 2% Casamino Acids as the sole carbon source, and the pH was adjusted to 4.0. For the control conditions, this medium was either supplemented with 2% glucose or buffered with 0.1 M Tris-0.1 M MOPS, pH 4.0. Cells were incubated at 37°C, and samples were drawn at 0 h, 3 h, 5 h, 7 h, or 24 h. Cellular morphology was accessed by counting cells in photomicrographs; at least 150 cells were counted per time point. The pH of the medium was taken using a standard pH meter.

Screen for genes involved in neutralization in *C. albicans*. Mutant libraries of *C. albicans* generously provided by A. Mitchell and D. Sanglard were screened to identify nonalkalinizing mutants. The libraries, in 96-well-plate format, were grown in YPD at 30°C for 2 days, and then 5 μl from each well was transferred to unbuffered medium 199, pH 4.0, in 96-well plates. Strains were incubated at 37°C, and neutralization was detected colorimetrically after 24 h. Candidate nonalkalinizing mutants were retested individually in triplicate for neutralization on medium 199 and GM-BCP medium as described above.

Microarray analysis of *C. albicans* during neutralization. To determine the transcript profiles of *C. albicans* cells as cultures alkalinize, strains SC5314 and ACC229 (*ach1Δ/ach1Δ*) were grown overnight in YPD, collected by centrifugation, washed with water, and diluted to an OD_{600} of 0.1 in 50 ml unbuffered medium 199 with or without 2% glucose at pH 4.0. Cells were incubated at 37°C to the point where SC5314 cultures had reached pH 5.0. The pH of the *ach1Δ* cultures remained ~4.0. Cells were collected and RNA was extracted using the hot acidic phenol method as described previously (64). Duplicate cultures were prepared for all conditions.

RNA samples were converted to labeled cDNA via oligo(dT) priming using an aminoallyl protocol (53) and hybridized to *C. albicans* complete genome microarrays generated from a 70-nucleotide (nt) oligonucleotide set (Qiagen) and printed on glass slides (Microarrays, Inc., Huntsville, AL). Experimental samples were hybridized against a reference RNA pool composed of a mixture of all the samples. We used technical replicates as dye-swap pairs for all RNA samples. Arrays were scanned with an Axon Instruments 4000B scanner, and data were extracted using the Axon Instruments GenePix program. Data processing and analysis basically followed our standard protocols (53, 65). The data from each array were normalized such that the median spot intensity from both the Cy3 and the Cy5 channels were equalized, the data were floored to a minimum value of 5% of the slide median, and then the ratio of the level for the experimental condition to that for the reference RNA pool was calculated. Genes with a P value of >0.01 as determined by Student's t test were discarded.

Three analyses were conducted, each involving a neutralizing condition compared to a nonneutralizing one: WT versus *ach1Δ*, WT versus WT-glucose, and *ach1Δ* versus WT-glucose. Analyses included rank ordering genes by fold regulation, identification of overrepresented gene ontology (GO) annotations using the term finder tool at the *Candida* Genome Database (http://www.candidagenome.org), and hierarchical

clustering using the Cluster and TreeView algorithms (66). The complete data set can be downloaded at http://www.lorenzlab.org.

Colorimetric analysis of volatile NH₃ during alkalinization. Ammonia released by *C. albicans* cells during alkalinization was collected using acid traps as previously described (48). Briefly, cells were grown in YPD medium overnight, washed in water, and resuspended to an OD_{600} of 1.0. Cells were spotted onto GM-BCP plates or GM-BCP plates containing 2% glucose, and 100 μl of 10% citric acid was placed underneath the colonies. After cells were incubated for 24 to 72 h at 37°C to allow alkalinization to occur, the acid trap was removed and the NH₃ concentration evaluated colorimetrically. For NH₃ quantification, 20 μl of the samples was diluted with dH₂O to 200 μl and combined with 800 μl Nessler's reagent. Samples were incubated at room temperature for 30 min, and the OD_{400} was measured. NH₃ quantity in the samples was calculated based on an ammonia standard.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00055-11/-/DCSupplemental.

Table S1, PDF file, 0.060 MB.
Table S2, PDF file, 0.102 MB.
Figure S1, TIF file, 1.272 MB.
Figure S2, TIF file, 0.809 MB.
Figure S3, TIF file, 0.492 MB.
Figure S4, TIF file, 0.184 MB.
Figure S5, TIF file, 1.586 MB.

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