Robust Extracellular pH Modulation by *Candida albicans* during Growth in Carboxylic Acids

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ABSTRACT  The opportunistic fungal pathogen *Candida albicans* thrives within diverse niches in the mammalian host. Among the adaptations that underlie this fitness is an ability to utilize a wide array of nutrients, especially sources of carbon that are disfavored by many other fungi; this contributes to its ability to survive interactions with the phagocytes that serve as key barriers against disseminated infections. We have reported that *C. albicans* generates ammonia as a byproduct of amino acid catabolism to neutralize the acidic phagolysosome and promote hyphal morphogenesis in a manner dependent on the Stp2 transcription factor. Here, we report that this species rapidly neutralizes acidic environments when utilizing carboxylic acids like pyruvate, 2-oxoglutarate (2OG), or lactate as the primary carbon source. Unlike in cells growing in amino acid-rich medium, this does not result in ammonia release, does not induce hyphal differentiation, and is genetically distinct. While transcript profiling revealed significant similarities in gene expression in cells grown on either carboxylic or amino acids, genetic screens for mutants that fail to neutralize 2OG medium identified a nonoverlapping set of genes, including CWT1, encoding a transcription factor responsive to cell wall and nitrosative stresses. Strains lacking CWT1 exhibit retarded 2OG-mediated neutralization *in vitro*, exist in a more acidic phagolysosome, and are more susceptible to macrophage killing, while double *cwt1Δ stp2Δ* mutants are more impaired than either single mutant. Together, our observations indicate that *C. albicans* has evolved multiple ways to modulate the pH of host-relevant environments to promote its fitness as a pathogen.

IMPORTANCE  The fungal pathogen *Candida albicans* is a ubiquitous and usually benign constituent of the human microbial ecosystem. In individuals with weakened immune systems, this organism can cause potentially life-threatening infections and is one of the most common causes of hospital-acquired infections. Understanding the interactions between *C. albicans* and immune phagocytic cells, such as macrophages and neutrophils, will define the mechanisms of pathogenesis in this species. One such adaptation is an ability to make use of nonstandard nutrients that we predict are plentiful in certain niches within the host, including within these phagocytic cells. We show here that the metabolism of certain organic acids enables *C. albicans* to neutralize acidic environments, such as those within macrophages. This phenomenon is distinct in several significant ways from previous reports of similar processes, indicating that *C. albicans* has evolved multiple mechanisms to combat the harmful acidity of phagocytic cells.

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lipases, phospholipases, and superoxide dismutases, cell surface adhesins, and proteins that bind complement regulators (8–10). Many of these factors are strongly induced following contact with host cells, particularly phagocytes. Following phagocytosis by macrophages, \textit{C. albicans} manipulates phagosomal maturation and acidification and inhibits nitric oxide production (11–14). These virulence traits are frequently absent or more limited in closely related but less virulent \textit{Candida} species (15). Accordingly, \textit{C. albicans} mutant strains impaired in these host-associated traits are often less virulent, though genetic redundancy sometimes makes this hard to demonstrate conclusively.

Phagocytosis induces dramatic transcriptional changes in \textit{C. albicans}, with the biggest component being a massive change in carbon metabolism in these cells (16, 17). We and others have shown that the catabolic pathways for a variety of nonsugar compounds are induced by phagocytosis and are required for fungal survival in macrophages and/or virulence in a mouse model of disseminated candidiasis (18–23), suggesting that certain host niches are carbon-limited environments.

Among the nonglucose nutrients potentially present in abundance in the host are fatty acids, \textit{N}-acetylglucosamine (GlcNac), carboxylic acids such as lactate, amino acids, peptides, and proteins; indeed, many amino acid auxotrophs of both \textit{C. albicans} and \textit{Candida glabrata} are fully virulent in mice, suggesting a ready supply of these compounds. We have shown that \textit{C. albicans} can avidly use amino acids as a sole carbon source, releasing the amine groups in the form of ammonia (24). This has the effect of raising the extracellular pH, both in vitro and in the macrophage phagosome (14); this pH manipulation induces the hyphal morphogenesis that characterizes the \textit{C. albicans}-macrophage interaction and is essential for fungal fitness in both tissue culture and whole-animal models (14, 25–27). Growth on GlcNac has also been reported to result in neutralization of the medium, and mutants that cannot utilize this amino sugar are highly attenuated in mouse models, though whether this is linked to the pH phenotype has not been addressed (28). Lactate induces a number of profound changes in fungal cell wall structure, drug resistance, and immune recognition (29–31).

We report here a similar but independent phenomenon in which \textit{C. albicans} is able to rapidly neutralize acidic environments when carboxylic acids such as \textit{\alpha}-ketoglutarate (\textit{\alpha}KG), pyruvate, or lactate are the sole carbon source. Under these conditions, cells do not excrete ammonia and do not germinate, important differences from the amino acid-driven process. Furthermore, genetic mutations that have significant phenotypes in the presence of amino acids, such as deletion of the transcription factor Stp2, do not affect growth or pH changes on carboxylic acids. Through genetic screening, we have identified several mutations that specifically block growth and/or neutralization on carboxylic acids; many of these have known phenotypes in central carbon pathways, morphogenesis, or cell wall biogenesis, one of which encodes the transcription factor Cwt1. Mutants lacking \textit{CWT1} have a modest defect in carboxylic acid-driven alkalization in vitro but occupy a more acidic phagosome and are impaired in survival following macrophage phagocytosis. We conclude that extracellular pH modulation on carboxylic acid substrates is a novel phenomenon that contributes to fungal success in host contexts.

**RESULTS**

Carboxylic acids promote rapid changes in extracellular pH. We have proposed a model in which \textit{C. albicans} cells growing with amino acids as the sole carbon source excrete the amine groups as ammonia, leading to an increase in extracellular pH (14, 24). This model predicts that amino acid-like compounds that lack amine groups should not produce ammonia and, presumably, would not drive changes in pH. To test our model, we grew wild-type \textit{C. albicans} cells on minimal medium adjusted to an initial pH of 4.0 in which the sole carbon sources were glucose, Casamino Acids (CAA), the amino acid glutamate, or the deaminated form of glutamate, \textit{\alpha}KG, in which the amino nitrogen is replaced by a carboxyl group. As we reported previously, \textit{C. albicans} cells grow rapidly in medium with either glucose or CAA (Fig. 1A); in contrast, growth was significantly slower when either glutamate or \textit{\alpha}KG was the sole carbon source. Surprisingly, all of the nonsugar compounds supported robust neutralization of the medium, including \textit{\alpha}KG (Fig. 1B). In fact, the pH rose more rapidly when \textit{\alpha}KG was the carbon source than when either CAA (slightly) or glutamate (significantly) was the carbon source. We also compared serine and pyruvate as a second pair of structurally related compounds and observed that pyruvate also promotes a more rapid rise in medium pH than does serine (data not shown).

\textit{\alpha}KG and pyruvate are C-5 and C-3, respectively, \textit{\alpha}-keto acids. We wondered whether similar carboxylic acids might also promote changes in the extracellular pH and so tested lactate (a C-3 carboxylic acid with a hydroxyl group) and acetate (a C-2 carboxylic acid). Each of these compounds could support modest growth when present as the sole carbon source under initially acidic (pH 4) conditions (Fig. 1C). As for \textit{\alpha}KG, growth on pyruvate was also associated with a very rapid rise in extracellular pH (Fig. 1D), while lactate induced a slower but still robust neutralization. Acetate is somewhat toxic at low pH, further reducing growth. Nevertheless, the pH rose by nearly two units during the course of this experiment. Thus, many carboxylic acids support growth and the modulation of the environmental pH by the fungal pathogen \textit{C. albicans}.

Neutralization on carboxylic acids is a physiologically and genetically distinct phenomenon. The data presented above might be interpreted to suggest that our model of ammonia generation through amino acid catabolism is incorrect. Alternatively, the pH changes we observe during growth on carboxylic acids may represent a phenomenon distinct from the amino acid-driven process. To address this question, we measured the amount of volatile ammonia released from cells grown on carboxylic acids. In this assay, ammonia is collected in a citric acid trap directly applied to a developing colony growing on solid neutralizing medium, as previously described (24). Cells growing on CAA, glutamate, or serine all excreted measurable ammonia in proportion to the speed with which they alkalized the medium, and yet, none of the carboxylic acids lacking amine groups (pyruvate, \textit{\alpha}KG, acetate, or lactate) released any detectable ammonia (Fig. 2). This suggests that the mechanism of neutralization is distinct from that promoted by amino acids.

The rise in extracellular pH during growth on amino acids induces germination (24), as neutral pH is normally a potent inducer of the switch to the hyphal form. Thus, we asked whether cells also begin to form filaments as the pH rises on carboxylic acids. To our surprise, all cells remained as yeast form throughout
the experiment, despite the rapidly neutralizing pH (Fig. 3) and 37°C temperature, a combination that is usually a potent morphogenetic inducer. It is worth noting that cells grow quite slowly on these media, which might retard germ tube emergence. To address whether the carboxylic acids were inhibiting hyphal growth or amino acids were promoting it, we grew cells in medium containing CAA and $\alpha$KG and observed germination similar to that with CAA alone (data not shown). To simplify this experiment, we assayed morphology in medium containing glutamate, $\alpha$KG, or both. Again, $\alpha$KG-grown cells remained exclusively in the yeast form, while those in medium containing glutamate began to germinate, albeit at reduced rates relative to the germination rates in medium containing CAA (data not shown). Thus, carboxylic acids do not induce germination but, also, do not inhibit hyphal formation induced by other stimuli.

We and others have identified several genes required for amino acid-induced pH changes, including the genes encoding the transcription factor Stp2, a membrane sensor of amino acids (SPS, composed of Ssy1, Ptr3, and Ssy5), the acetyl-coenzyme A (CoA) hydrolase ACh1, the urea amidolyase Dur1,2, the putative acetate/ammonia transporters Atol and Atos, and a putative polyamine transporter, Dur31 (14, 24, 27, 32, 33). We asked whether these mutations would similarly affect pH changes stimulated by carboxylic acids. Very similar patterns were seen during growth on both CAA and glutamate, in which the stp2Δ mutation completely abrogated any change in pH, while the remaining mutants we
tested significantly retarded but did not eliminate neutralization (Fig. 4B and D). In contrast, none of the mutants tested affected growth or pH changes in the presence of αKG (Fig. 4E and F) or the other carboxylic acids (data not shown). Thus, the genetic control of extracellular neutralization on carboxylic acids is much different, further supporting the conclusion that these are distinct processes.

Genomic analysis of growth on carboxylic acids. We took both genetic and genomic approaches to understanding the difference in metabolism and pH modulation during growth on amino acids versus carboxylic acids. First, we assayed transcriptional profiles using RNA deep sequencing for cells grown in minimal yeast nitrogen base (YNB) medium containing glucose, CAA, αKG, or α-ketoglutarate; Pyr, pyruvate; Ace, acetate; Lac, lactate. The scale bar in the lowest image on the right is 10 μm.

Genomic analysis of carboxylic acid-induced alkalinization. Because none of our previously identified mutations conferred phenotypes for growth on carboxylic acids, we conducted a screen of two mutant libraries. The Homann library includes 166 homozygous mutations in putative transcriptional regulators (37), while the Noble library contains homozygous deletions in ~674 genes with a wide array of predicted functions (38). Both libraries contain two independently constructed mutants for most genes, increasing the robustness. To perform the screen, we transferred cells of each strain growing in rich yeast extract-peptone-dextrose (YPD) medium to 96-well plates containing YNB medium with 10 mM αKG as the carbon source, adjusted to pH 4 and containing bromocresol purple to visualize the pH. Both of these libraries are auxotrophic for arginine (arg4Δ), so our screening media also contained 40 μM arginine, a concentration empirically determined to be the minimal amount needed to support full growth. These were incubated at 37°C for 24 to 48 h and inspected visually for wells in which the medium remained acidic; growth was not severely impaired, and both independent mutants exhibited similar phenotypes. Secondary screens in aerated tubes validated those with significant alkalinization defects.

We identified mutations in six genes that conferred defects in medium alkalinization during growth on αKG: ALI1, SINV, COX4, PEP8, KIS1, and CPFI (Table 2; see also Fig. S2 in the supplemental material). Mutants that entirely failed to grow were excluded from further analysis. Several of these encode proteins with functions with a clear link to carbon metabolism, such as the cytochrome c oxidase Cox4 and the Snf1-associated protein Kis1,
while others have a less obvious connection, such as Sin3, a transcriptional repressor that promotes histone deacetylase recruitment (39), and Pep8, whose yeast homolog mediates retrograde endosome-to-Golgi vesicle transport (40). Cph1 is a well-studied transcriptional regulator of morphogenesis that is activated by the pheromone-responsive mitogen-activated protein (MAP) kinase pathway (41, 42). Cph1 was previously shown to regulate galactose utilization genes but had not otherwise been associated with metabolic functions (36). Ali1 is a recently described plasma membrane protein with roles in cell wall structure and oxidative stress responses (43).

Notably, all of these mutants confer partial growth defects on medium containing $\alpha$KG (see Fig. S2 in the supplemental material). The linkage of growth and pH changes probably reflects a requirement for metabolism of these acids to effect alkalinization. Furthermore, they have all been reported to confer aberrant filamentation profiles, while several of them have altered sensitivities to agents like Calcofluor white, caspofungin, Congo red, or SDS.

**FIG 4** Amino acid- and carboxylic acid-driven alkalinization are genetically distinct. Strains of the indicated genotypes (Wild-type, SC5314; stp2Δ, SVC17; ATO1*, MLC112; ato5Δ, HDC31; ach1Δ, ACC15) were grown in minimal liquid YNB medium with the indicated carbon source: Casamino Acids (CAA) (A, B); glutamate (Glut) (C, D); or $\alpha$-ketoglutarate ($\alpha$KG) (E, F). Culture density (A, C, E) and pH (B, D, F) were measured at the indicated times. The culture pH for all mutants was significantly different than that of the wild-type control (*, $P < 0.05$) at $t = 6$ h and $t = 8$ h during growth in CAA and glutamate only. Error bars show standard deviations.
that suggest perturbations in cell wall structure and function (38, 41–45). Several of these mutants (ali1Δ and cph1Δ mutants) are also known to be impaired in cell culture or whole-animal models of virulence (25, 43). Only ALI1 was among the genes induced during growth on αKG relative to its expression on glucose (4.1-fold).

We reasoned that mutants with growth defects would be likely to have altered interactions with phagocytes. To separate the effects of these mutations on growth on αKG from the alkalinization phenotype, we reanalyzed our genetic data to identify mutants that might have defects at earlier time points but not at the endpoint of the assay. One such strain carried a deletion of CWT1, and mutants lacking this transcription factor have been reported to be sensitive to nitrosative stress and to cell wall-damaging agents and to be required for full virulence in the systemic model (46–48); in contrast, a systematic survey of transcription factor mutants reported no phenotypes for the cwt1Δ strain (37). To address this discrepancy, we generated an independent homozygous cwt1Δ mutant strain, using the SAT flipper approach (49), along with a complemented strain. As seen by the results in Fig. 6, this new mutant grows well on αKG-containing medium (Fig. 6A) and has a modest delay in neutralization (Fig. 6B). The mutation does not affect growth or pH changes when amino acids are the carbon source (Fig. 6C and D).

To test whether impairing the ability of the cell to modulate pH on amino acids and carboxylic acids is synergistic, we generated a double cwt1Δ stp2Δ mutant by deleting CWT1 in an existing stp2Δ mutant (14). This strain behaved like a cwt1Δ single mutant when grown on αKG (Fig. 6A and B) and like an stp2Δ single mutant on amino acids (Fig. 6C and D), as expected.

Cwt1-mediated pH neutralization contributes to fungal survival in macrophages. We have reported that stp2Δ mutant cells, which are unable to neutralize amino acid-rich media, occupy more acidic phagosomes, germinate less readily, and are more susceptible to macrophage killing than control strains. To test

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### TABLE 1: Significantly induced GO terms (selected terms only)

| GO term                                         | No. of genes in set of total annotated | P value        |
|------------------------------------------------|--------------------------------------|----------------|
| Induced in multiple carbon sources (n = 352)    |                                      |                |
| Tricarboxylic acid cycle                        | 9 of 16                               | 2.6 × 10⁻⁵     |
| Fatty acid oxidation                            | 8 of 14                               | 1.3 × 10⁻⁴     |
| Respiratory chain 1 biogenesis                  | 7 of 13                               | 0.0014         |
| Acetate metabolic process                       | 7 of 13                               | 0.0014         |
| Glycolytic process                              | 11 of 16                              | 5.8 × 10⁻¹³    |
| Amino acid metabolic process                    | 14 of 138                             | 6.0 × 10⁻⁴     |
| Biotin biosynthetic process                     | 4 of 6                                | 0.0013         |
| Amino acid catabolic process                    | 6 of 32                               | 3.9 × 10⁻⁶     |
| Arginine biosynthetic process                   | 7 of 9                                | 4.3 × 10⁻¹²    |
| Amino acid biosynthetic process                 | 13 of 102                             | 2.4 × 10⁻¹²    |
| Sulfur amino acid biosynthetic process          | 5 of 26                               | 1.7 × 10⁻⁴     |
| Induced only in glutamate (n = 96)              |                                      |                |
| Ribosome biogenesis                             | 14 of 298                             | 0.034          |
| Amino acid transmembrane transport              | 5 of 40                               | 0.092          |

| a | GO terms significantly enriched in the genes identified in each category by both rank ordering and K-means clustering, as determined using the GO Term Finder at the Candida Genome Database. Due to extensive overlaps in GO terms, only selected terms are shown. |
| b | Genes in the selected data set that map to that term out of the total number of genes in the genome annotated to that term. |
| c | Though the P value is greater than 0.05, the false discovery rate for this term was 5.0%. |
whether metabolism of carboxylic acids plays a similar role, we cocultured concanavalin A-fluorescein isothiocyanate (FITC)-labeled strains with J774A.1 murine macrophages. To distinguish between phagocytosed and external C. albicans cells at 1 h of incubation, we stained with the membrane-impermeant dye Calcofluor white, which binds to chitin in the fungal cell wall, and then fixed the cells with paraformaldehyde. We detected no significant difference in the rates of phagocytosis of the single or double mutants relative to the rate of phagocytosis for the control (data not shown). However, it was immediately obvious that phagocytosed cwt1/H9004 or stp2/H9004 cells germinated much less readily, and this was confirmed by scoring the morphology of cells in multiple microscopic fields (Fig. 7A). The proportions of both the cwt1/H9004 and stp2/H9004 mutant cells that remained in the yeast form were similar, with a further modest increase in the double mutant. No differences in morphology were observed in nonphagocytosed cells (data not shown).

To ask whether phagosomes containing cwt1Δ cells differed in pH from phagosomes containing a wild-type control, we preloaded macrophages with the acidophilic dye LysoTracker red (LR) before initiating the coculture with FITC-labeled cells. After 1 h, we fixed and stained with Calcofluor and then quantitated the LR intensity surrounding the phagocytosed fungal cell (in the phagosomal lumen), as we have described previously (27). To do so, we averaged the background-subtracted LR fluorescence across the 10 pixels (1 μm) immediately outside the cell, as delineated by the FITC-labeled cell walls, along a line drawn through the short axis of the cell. This was done for at least 50 cells per strain in each of three experiments and, as seen by the results in Fig. 7B, there is a marked difference in the LR accumulations

### TABLE 2 Mutants with pH defects in medium containing α-ketoglutarate

| Gene | Growth defect | Phenotype affecting: | Function |
|------|---------------|----------------------|----------|
| ALI1 | + | + | + | NADH-ubiquinone oxidoreductase |
| COX4 | + | + | + | Cytochrome c oxidase |
| CPH1 | + | + | + | Morphology transcriptional regulator |
| CWT1 | + | + | + | Cell wall transcriptional regulator |
| KIS1 | + | + | + | Snf1 signaling complex |
| PEP8 | + | + | + | Retrograde vesicular transport |
| SIN3 | + | + | + | Transcriptional corepressor |

a, b, c, the gene, when mutated, confers either slow growth on medium containing dextrose or αKG or an aberrant phenotype related to cell wall function of morphology. Phenotypes are from this study or references 38, 43, 45, and 47.

\[ \text{FIG 6} \quad \text{cwt1Δ and stp2Δ mutants have distinct phenotypes.} \]

Strains of the indicated genotypes (Wild-type, SC5314; stp2Δ, SVC17; cwt1Δ, SVC36; cwt1Δ stp2Δ, SVC39) were grown overnight in YPD and then diluted into YNB medium containing α-ketoglutarate (αKG) (A, B) or Casamino Acids (CAA) (C, D). (A, C) Growth was monitored by optical density. (B, D) pHs of the supernatants of the cultures whose growth is shown in panels A and C, respectively. (B) Significant differences (*, P < 0.05) were found for the cwt1Δ and cwt1Δ stp2Δ mutants relative to the WT or complemented controls at t = 6 h and t = 8 h. (C, D) The defects of the stp2Δ and cwt1Δ stp2Δ mutants in CAA medium are also significant. Error bars show standard deviations.
surrounding wild-type cells and stp2Δ mutant cells, with the mutant occupying a much more acidic compartment, as we have reported previously (14). The cwt1Δ/H9004 strain was also found in more acidic compartments, but this effect was not as pronounced as for the stp2Δ/H9004 mutant. The double mutant resembled the stp2Δ/H9004 single mutant strain. Representative microscopic images are shown in Fig. 7C. Thus, Cwt1 contributes to the ability of C. albicans to neutralize the phagosome, but it plays a secondary role relative to Stp2.

Defects in germination and modulation of phagosomal pH may compromise the ability of the cell to survive phagocytosis or to damage macrophages. To address this, we first measured the survival of fungal cells cocultured with macrophages at a very low fungal/host cell ratio using an endpoint dilution assay (14, 50). After 24 h, the difference in the number of microcolonies formed relative to the number in a macrophage-free control was used to calculate fungal survival. As seen by the results in Fig. 8A, deletion of CWT1 compromises survival after phagocytosis to a similar extent as the stp2Δ mutation. A slight further reduction is observed in the double mutant, but this is not statistically significant.

Wild-type C. albicans cells kill macrophages both through mechanical disruption via hyphal growth and through induction of pyroptosis (51, 52). We assessed macrophage membrane damage by measuring the release of cytosolic lactate dehydrogenase into the culture medium after coincubation for 5 h, as we have described previously (14). The single cwt1Δ/H9004 and stp2Δ/H9004 mutants and the double mutant all significantly reduce macrophage cytotoxicity relative to the reduction by control strains, but there is no difference between the single and double mutants (Fig. 8B).

DISCUSSION

The work presented here demonstrates that C. albicans avidly utilizes carboxylic acids like α-ketoglutarate, pyruvate, and lactate as the sole source of carbon and, in doing so, rapidly neutralizes the extracellular environment. Though this is superficially similar to the phenomenon we have described during catabolism of amino acids (14, 24), several lines of evidence indicate that these are unrelated activities. First, pH alkalinization on carboxylic acids does not generate ammonia, nor does it induce cells to switch to the hyphal morphology. While the transcriptional profiles of cells grown on either carbon source are quite similar, mutants that impair amino acid-driven neutralization do not inhibit the carboxylic acid phenomenon and vice versa. A genetic screen identified mutations that reduce growth and/or the ability to manipu-
late pH. We focused on CWT1, encoding a transcriptional regulator with known roles in response to cell wall damage and nitrosative stress, because it dissociated growth and pH defects. Mutants lacking CWT1 are impaired in several aspects of the host-pathogen interaction, as are other nonalkalinizing mutants. Thus, we have identified another independent means by which C. albicans can manipulate the pH of the phagolysosome.

C. albicans uses a variety of nonglucose carbon sources far more efficiently than related nonpathogenic species, such as S. cerevisiae, including amino acids, fatty acids, carboxylic acids, and N-acetylglucosamine, all of which are present in host environments. There is accumulating evidence that cells use multiple carbon sources during growth in the host, including findings of transcriptional activation of the catabolic pathways following phagocytosis and in animals (16–18, 53–55) and attenuated virulence of mutants with mutations that disrupt gluconeogenesis, the glyoxylate cycle, or peroxisomal functions (18, 21, 34, 56, 57).

While utilization of these compounds generates energy and biomass, nonglucose carbon sources also seem to be a signal of specific host niches and result in significant changes to cellular metabolism and/or physiology. One of the most potent inducers of hyphal growth is the presence of the ubiquitous sugar N-acetylglucosamine (58); metabolism of this compound raises extracellular pH, but this is not required for the hyphal induction (28). Similarly, we have shown that lactate, also abundant in the host, promotes alkalization as well. Elegant work from the Brown laboratory has demonstrated that cells grown on lactate are more resistant to stresses, including antifungal drugs, are less readily recognized by the immune system, and have altered cell walls (29–31). Our genetic analysis of growth and pH modulation on carboxylic acids reinforces the connection between alternative carbon metabolism and virulence-related processes. Each of the seven genes identified have been linked to defects in morphogenesis, and a subset of these (including CWT1) have aberrant cell walls that increase susceptibility to various stressors, including clinically relevant antifungal drugs (38, 41, 43–47).

While the cwt1Δ mutant incorporates carboxylic acids equally as well as control strains, it is retarded in neutralization of acidic media. This extends to the phagosome, where cwt1Δ strains occupy a more acidic compartment than wild-type strains, germinate less often, and are more readily cleared by macrophages. While our work is highly correlative, we cannot draw a definitive causal link between these phenotypes. Two possibilities exist, the first being that the inability to neutralize the phagosome is directly responsible for the impairments in macrophages and the second that the cell wall defects might compromise viability in the macrophages, with the failure to modulate phagosomal pH a secondary effect. There is some disagreement in the literature regarding the phenotypes of the cwt1Δ strain (37, 46), and further study will be required to dissect these possibilities. However, we note that there is precedent for a role for Cwt1 in carbon metabolism, as Sellam et al., found that it bound the promoters of a number of genes involved in the utilization of nonpreferred carbon sources (48).

During environmental neutralization induced by amino acid catabolism, the driving force behind the rise in pH is the excretion of ammonia derived from amino and side chain amine groups. We demonstrate here that no ammonia is released during growth on carboxylic acids, as expected since these compounds lack nitrogen. As a result, the chemical mechanism behind the rise in pH remains unclear. These compounds are acids, so their consumption may in itself contribute to the rise in pH, which closely tracks growth in the cultures, supporting this idea. Cells would, however, need some compensatory mechanism to maintain cytosolic pH balance. Part of this could be inherent in the metabolism of the acids: as glycosylation is acidogenic, gluconeogenesis consumes six protons for each glucose molecule generated. There could also be other basic compounds secreted into the medium. Metabolomic or other approaches will be needed to address this question.

While the metabolically driven mechanism we propose here and elsewhere (14, 24) is novel, remodeling of the phagosome is a common strategy of pathogens. The fungal pathogens Cryptococcus neoformans and Histoplasma capsulatum both appear to neutralize the phagosome by permeabilizing the organelle membrane via quite different routes (59–61). C. glabrata also occupies a neutral phagosome but, interestingly, cell viability is not required (62), in contrast to what we observe with C. albicans. A variety of bacterial pathogens also subvert phagosomal maturation and acidification, including Mycobacterium tuberculosis, Legionella pneumophila, Listeria monocytogenes, Salmonella enterica, and others (63–65). Our observations here represent the third separate mechanism—along with catabolism of amino acids and GlcNAc (14, 24, 28)—by which C. albicans can manipulate phagosomal maturation, indicative of the evolutionary pressures during the adaptation of this species to the mammalian host.

MATERIALS AND METHODS

Strains and media. For routine propagation, C. albicans strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% glucose, with or without 2% agar for solid or liquid medium) (66). Experiments in which the carbon source was varied utilized minimal YNB medium containing allantoin as the nitrogen source (YNBA; 0.17% yeast nitrogen base without amino acids or ammonium sulfate, 0.5% allantoin), with various compounds as the sole carbon source as indicated; the medium was adjusted to the starting pH (usually 4.0) using HCl (14, 27). The use of allantoin avoids the spontaneous generation of ammonia from ammonium sulfate seen in neutral- to basic-pH media (27). Selection for nourseothricin-resistant strains used YPD with 200 μg/ml of nourseothricin (YPD-Nou) (Werner Bioagents, Jena, Germany). The murine macrophage-like cell line J774A.1 was propagated in RPMI 1640 with glutamate, HEPES (HyClone), and 10% fetal bovine serum (FBS; VWR International) in a 5% CO2 environment.

The fungal strains used are listed in Table 3. The cwt1Δ and cwt1Δ sp2Δ deletion strains were generated using the SAT flipper methodology (49). Briefly, approximately 300 bp of homology immediately 5’ or 3’ of the CWT1 ORF were amplified by PCR and cloned between the Apal/ Xhol and SacI/Sacl sites, respectively, of pSFS2. The resulting SAT1-FLP cassette was used to transform C. albicans SC5314 and SVC17 (sp2Δ) strains by electroporation, with selection on YPD-Nou. Genomic DNA was isolated, and cassette integration confirmed in the selected candidates via PCR. Nourseothricin sensitivity was restored by inducing the expression of the Mal2p-FLP recombinase gene through growth on yeast extract-peptone-maltose medium. This process was repeated to generate the homozygous disruptants SVC36 (cwt1Δ::FRT/cwt1Δ::FRT) and SVC39 (cwt1Δ::FRT/cwt1Δ::FRT sp2Δ::FRT/sp2Δ::FRT). Complementation of the mutant strain used plasmid pSV-7, a SAT1-marked version of Clp10 expressing CWT1 under its native promoter. pSV-7 was generated by cloning the entire CPW1 ORF into the Apal and Xhol sites of pGAr (14). This plasmid was linearized with Stul and used to transform cwt1Δ mutant cells to generate the CWT1-complemented strain (RPS10/pS10::Clp10-CWT1-SAT1).

Alkalization, morphology, and ammonia release assays. The ability of strains to alter medium pH was assayed as described previously (14).
Briefly, strains were grown overnight in YPD, washed in water, diluted to an initial optical density at 600 nm (OD$_{600}$) of 0.2 in minimal YNB medium with the indicated carbon sources, and then incubated at 37°C in aerated cultures. At the indicated time points, the optical density and pH of the cultures were measured; in certain experiments, cellular morphology was assessed microscopically after fixation with 2.7% paraformaldehyde.

Ammonia generation was assayed as described previously (24). Overnight YPD cultures were washed and resuspended in water at an OD$_{600}$ of 1.0 and then spotted (5 μl) onto solid YNBA medium with the indicated carbon source and incubated at 37°C. A small reservoir (the cap of a microfuge tube) was affixed to the lid of the petri dish directly opposite the acid trap. The sequence quality was very high and averaged 150 fragments per kilobase per million mapped reads (FPKM) for each gene. Using the Tuxedo suite of software (69–72) to generate the number of genome coverage –means clustering with Cluster and TreeView (73, 74). The coregulated clusters were analyzed for common content of this acid trap was determined using Nessler’s reagent as described previously (24). Experimenters were performed at least in triplicate, and the results analyzed using Excel and Prism (GraphPad Software, Inc.).

Phagocytosis rate and hyphal formation of phagocytosed C. albicans. To assess the interaction of C. albicans cells with the macrophages, we seeded 5 × 10$^5$ cells of J774A.1 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 YNB medium, and grown for 3 h at 30°C. Cells were then washed in distilled water (dH$_2$O) and stained with 1 μM concanavalin A-FITC for 15 min, washed two times with phosphate-buffered saline (PBS), and resuspended in RPMI medium (HyClone). Amounts of 1 × 10$^6$ C. albicans cells were cocultured with the macrophages at 37°C for 1 h. The cocultures were then washed twice with PBS, nonphagocytosed cells were stained with 35 μg/ml of Calcofluor white for 1 min, and the excess dye was removed by washing three times in PBS. Next, cells were fixed with 2.7% paraformaldehyde for 20 min at room temperature and washed with PBS. Images of the Candida-macrophage interaction were taken using an Olympus IX81 automated inverted microscope. Images from at least 100 phagocytosed cells per experiment were analyzed using SlideBook 6.0 software. The percentage of cells phagocytosed after 1 h of coculture was calculated using the following formula: (percent internalized cells/total number of cells) × 100. Hyphal morphogenesis during phagocytosis was quantitated by scoring the morphology of phagocytosed cells after 1 h of coculture using the following formula: (germ tubes + hyphal cells/total amount of cells) × 100. Experiments were performed in triplicate.

Assessment of phagosomal pH. Phagosomal pH was assessed as previously described (14, 27). Briefly, 1 × 10$^6$ J774A.1 cells/ml were seeded onto glass coverslips in phenol red-free RPMI in 12-well plates and allowed to adhere for 2 h. Next, 30 nM LysoTracker red DM99 (Molecular Probes) was added to the cells and incubated for 2 h to ensure concentration of the dye in the lysosomes. C. albicans cells were grown overnight in YPD medium, diluted 1:100 in fresh YNB, and grown for 3 h at 30°C. Then, cells were washed in dH$_2$O and stained with 1 μM concanavalin A-FITC for 15 min, and excess dye was removed by washing three times in PBS. Cells were then diluted to 1 × 10$^6$ cells/ml in phenol red-free RPMI medium and cocultured with the macrophages for the indicated times at 37°C and 5% CO$_2$. After washing with PBS to remove cell debris and nonadherent cells, the remaining cells were fixed in 2.7% paraformaldehyde for 20 min and stored at 4°C in PBS before visualization. The cocultures were imaged at ×60 magnification with an Olympus IX81 automated inverted microscope using the appropriate filter sets. To estimate the relative phagosomal pH, the signal intensities of both FITC and LysoTracker Red (LR) 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 software. 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. All experiments were performed at least in triplicate.

Assessment of fungal survival and macrophage cytotoxicity. C. albicans survival during interaction with the J774A.1 macrophages was assessed as previously described (14). Macrophages were collected by cen-

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**TABLE 3 Candida strains**

| Strain     | Description or mutation | Complete genotype                                           | Reference or source |
|------------|--------------------------|-------------------------------------------------------------|---------------------|
| SC5314     | Wild type                | Prototroph                                                  |                     |
| SN250      | Library control          | his1::hisG/his1::hisG/leu2Δ::GdHis1/leu2Δ::CuLeu2 arg4Δ::hisG/arg4Δ::hisG |                     |
| SVC17      | STPΔ                     | stp2Δ::FRT/stop2Δ::FRT                                       |                     |
| MLC112     | ATO1*                    | ura3::ura3::RPS10::ps10::Clp10-Act1::ATO1/G53D             |                     |
| HDC31      | ato5Δ                    | ato5Δ::FRT/ato5Δ::FRT RPS10::ps10::Clp10-SAT1              |                     |
| ACC2       | ach1Δ                    | ach1Δ::hisG/ach1Δ::hisG/ura3::ura3::RPS10::ps10::Clp10-Ura3 |                     |
| SVC6       | cwt1Δ                    | cwt1Δ::FRT/cwt1Δ::FRT                                       | This study          |
| SVC41      | cwt1Δ::CWT1              | cwt1Δ::FRT/cwt1Δ::FRT RPS10::ps10::Clp10-SAT1-CWT1         | This study          |
| SVC39      | cwt1Δ STPΔ               | cwt1Δ::FRT/cwt1Δ::FRT STP2Δ::FRT STP2Δ::FRT                | This study          |

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Both were tested. Candidate mutants were subjected to secondary screens in aerated liquid cultures for both growth and pH, as described above.

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trifugation at 700 × g for 3 min, washed with PBS, and resuspended in fresh RPMI medium. Cells were seeded at 2.5 × 10^4 cells/well in 96-well plates and grown overnight at 37°C and 5% CO_2. Log-phase C. albicans cells were washed in D_2O and resuspended in fresh RPMI medium. Amounts of 1 × 10^4 cells/well were added to wells containing macrophages or medium alone, followed by six serial 1:5 dilutions. After 48 h at 37°C and 5% CO_2, microcolonies of C. albicans were counted, using an inverted microscope, in wells in which individual colonies could be distinguished. The results were presented as the following ratio: (number of colonies in the presence of macrophages/number of colonies without macrophages) × 100. The experiment was performed in triplicate.

C. albicans toxicity on macrophages was assessed using the CytoTox96 nonradioactive cytotoxicity assay (Promega) as previously described (14). Briefly, J774A.1 macrophages were prepared as described above, seeded at 2.5 × 10^4 cells per well in a 96-well plate, and incubated overnight at 37°C and 5% CO_2. C. albicans cells were grown to log phase in YNB medium, washed in PBS, and cocultured with macrophages at a 3:1 ratio for 5 h. To assess macrophage cytotoxicity, the plates were centrifuged at 250 × g for 4 min and 50-μl aliquots of the coculture supernatant were transferred to a fresh plate and mixed with an equal volume of substrate mixture. After 30 min of incubation, the reaction was stopped with 50 μl of Stop solution and the absorbance at 490 nm recorded. The data from spontaneous release of lactate dehydrogenase (LDH) by the macrophages and by the C. albicans cells alone, as well as maximum LDH release from lysed macrophages, were used to calculate C. albicans cytotoxicity on macrophages according to the manufacturer’s protocol. The experiment was performed in triplicate.

Accession number(s). The RNA-seq data set is available through the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo) under GenBank accession number GSE87832.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01646-16/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Table S1, PDF file, 0.1 MB.

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