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Arginine-Induced Germ Tube Formation in *Candida albicans* Is Essential for Escape from Murine Macrophage Line RAW 264.7▽†

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The opportunistic fungal pathogen *Candida albicans* is a part of the normal flora but it also causes systemic candidiasis if it reaches the bloodstream. Upon being phagocytized by macrophages, an important component of innate immunity, *C. albicans* rapidly upregulates a set of arginine biosynthetic genes. Arginine, urea, and CO_2 induced hyphae in a density-dependent manner in wild-type, *cph1/cph1*, and *rim101/rim101* strains but not in *efg1/efg1* or *cph1/cph1 efg1/efg1* strains. Arginase (Car1p) converts arginine to urea, which in turn is degraded by urea amidolyase (Dur1,2p) to produce CO_2, a signal for hyphal switching. We used a *dur1,2/dur1,2* mutant (KWN6) and the complemented strain, KWN8 (*dur1,2/dur1,2::DUR1,2/DUR1,2*) to study germ tube formation. KWN6 could not make germ tubes in the presence of arginine or urea but did in the presence of 5% CO_2, which bypasses Dur1,2p. We also tested the effect of arginine on the interaction between the macrophage line RAW 264.7 and several strains of *C. albicans*. Arginine activated an Efg1p-dependent yeast-to-hypha switch, enabling wild-type *C. albicans* and KWN8 to escape from macrophages within 6 h, whereas KWN6 was defective in this regard. Additionally, two mutants that cannot synthesize arginine, BWP17 and SN152, were defective in making hyphae inside the macrophages, whereas the corresponding arginine prototrophs, DAY286 and SN87, formed germ tubes and escaped from macrophages. Therefore, metabolism of arginine by *C. albicans* controls hyphal switching and provides an important mechanism for escaping host defense.

In immunocompromised patients, such as those with AIDS, the innate immune system has an increased role in resisting infectious diseases. However, the opportunistic fungal pathogen *Candida albicans* has evolved mechanisms to evade innate immunity, which is an important reason that candidiasis is a major complication in AIDS patients. *C. albicans* resists macrophage phagocytosis via a mechanism that does not stimulate apoptosis in macrophages (22). *C. albicans* induces hyphae inside macrophages, thereby penetrating the cell membrane and escaping macrophages (21). *C. albicans* cells that are defective in making germ tubes, such as *cph1/cph1 efg1/efg1* (21) and *cdc35/cdc35* (22), cannot escape the macrophages following phagocytosis and are killed. Thus, the interaction between *C. albicans* and macrophages is critical in determining its pathogenicity in immunocompromised patients.

Lorenz et al. (21) used DNA arrays to follow the transcriptional response by *C. albicans* to internalization in macrophages. Their transcriptional analysis suggested that once inside the macrophage *C. albicans* shifts from glycolysis to gluconeogenesis, activates fatty acid degradation, downregulates transcription, and upregulates arginine biosynthesis. In the later stages following internalization, hyphal growth is important for piercing the macrophage cell membrane, and at that time the cells resume glycolytic growth (21). Clearly, switching from yeast to hypha is a critical factor in escaping from macrophages after phagocytosis. Thus, one important question is: what triggers the morphological switch in *C. albicans* inside the macrophage?

In *C. albicans* the yeast-to-hypha switch has been very well studied (3, 4, 18, 38). It is a carefully coordinated event which is regulated by multiple factors and several signal transduction pathways. The environmental triggers for hyphal development include growth at 37°C, the presence of serum or *N*-acetylglucosamine (GlcNAc), neutral pH, CO_2, and nitrogen starvation (3, 4, 18, 38). These environmental stimuli act by turning on one or more signal transduction pathways that either stimulate or repress hypha-specific genes. These pathways include the Cph1p-mediated mitogen-activated protein kinase (MAPK) pathway and the Efg1p-mediated cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway, which has two isoforms of PKA, Tpk1p and Tpk2p, with differential effects on hyphal morphogenesis. Two other hyphal regulators, Rim101p and Czf1p, may function through Efg1p or act in parallel with Efg1p, while another transcription factor, Tec1p, is regulated by Efg1p and Cph1p. The MAPK cascade includes Cst20p (MAPK kinase kinase), Hst7p (MAPK kinase), Cek1p (MAPK), and the downstream transcription factor, Cph1p, which is a homolog of the *Saccharomyces cerevisiae* transcription factor Ste12p. *C. albicans* also has negative regulators of the hyphal transition. Chief among these is Tup1p, which acts in concert

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with Rfg1p, Nrg1p, or Rbf1p (3, 4, 17, 18, 38). The downstream targets of these environmental sensing pathways include the hyphal wall protein Hwp1p, adhesins of the ALS family, and extracellular hydrolytic enzymes (secreted aspartyl proteases and phospholipases) (3, 4, 18, 38).

Another unusual feature of *C. albicans* is that it uses the cytoplasmic enzyme urea amidolyase, encoded by *DUR1,2*, to hydrolyze urea. Dur1,2p (degradation of urea) is a multifunctional, biotin-dependent enzyme (33) that was first characterized in the yeast *Candida utilis* (32). It is also present in *Saccharomyces cerevisiae* (7, 39). Catabolism of urea involves a single protein with two enzymatic activities. The first is an avidin-sensitive urea carboxylase (EC 6.3.4.6); urea is carboxylated in an ATP-dependent reaction, forming allophanate, also known as urea carboxylate. The second is allophanate hydrolase or allophanate amidohydrolyase (EC 3.5.1.54), which releases two NH_3 and two CO_2 (39).

This paper addresses how the macrophage signal for hyphal switching relates to previously known signaling pathways. The transcriptional response analysis reported by Lorenz et al. (21) showed that at an early stage arginine biosynthesis was strongly upregulated. In this report we link arginine biosynthesis to the hyphal switch necessary for escape from the macrophage. The link is mediated by the enzyme urea amidolyase, encoded by *DUR1,2*. Biosynthesis of arginine, which is metabolized by *C. albicans* cells producing CO_2, is essential and acts as a signal to activate the cAMP-dependent PKA pathway, thereby regulating the yeast-to-hypha switch inside the macrophage. This series of events is critical for hyphal development inside the macrophage at the initial phase after phagocytosis, thereby piercing the macrophage and escaping.

MATERIALS AND METHODS

Strains, media, and growth condition. The *C. albicans* strain A-72 was obtained from Patrick Sullivan, University of Otago, Dunedin, New Zealand. Wild-type clinical isolate SC5314, CAF2-1 (*ura3*::*imm434*/*URA3*) (11), CAI4 (*ura3*::*imm434*/*ura3*::*imm434*) (12), SN152 (*URA3*/*ura3*::*imm434 his1*/*his1 arg4*/*arg4 leu2*/*leu2 IRO1*/*iro1*::*imm436*) (28), and SN87 (*URA3*/*ura3*::*imm434 his1*/*his1 leu2*/*leu2 IRO1*/*iro1*::*imm436*) (28) were obtained from Alexander Johnson, University of California at San Francisco. BWP17 (*ura3*::*imm434*/*ura3*::*imm434 arg4*::*hisG*/*arg4*::*hisG his1*::*hisG*/*his1*::*hisG*) (27) and DAY286 (*ura3*::*imm434*/*ura3*::*imm434 pARG4*::*URA3*::*arg4*::*hisG*/*arg4*::*hisG his1*::*hisG*/*his1*::*hisG*) (10) were obtained from Aaron Mitchell's collection. JKC19 (*ura3*::*imm434*/*ura3*::*imm434 cph1*::*hisG*/*cph1*::*hisG URA3*::*hisG*) (20), HLC52 (*ura3*::*imm434*/*ura3*::*imm434 efg1*::*hisG*/*efg1*::*hisG URA3*::*hisG*) (20), and HLC54 (*ura3*::*imm434*/*ura3*::*imm434 cph1*::*hisG*/*cph1*::*hisG efg1*::*hisG*/*efg1*::*hisG URA3*::*hisG*) (20) were obtained from Gerald R. Fink, Cambridge, MA, and CAR2 (*rim101*::*hisG*/*rim101*::*hisG-URA3-hisG ura3*::*imm434*/*ura3*::*imm434*) (30) was obtained from Fritz A. Muhlschlegel, Canterbury, United Kingdom. *GTC41* (*ura3*::*imm434*/*ura3*::*imm434 GCN4*/*gcn4*::*hisG-URA3-hisG*) (37), *GTC43* (*ura3*::*imm434*/*ura3*::*imm434 gcn4*::*hisG-URA3-hisG*/*gcn4*::*hisG*) (37), and *GTC45* (*ura3*::*imm434*/*ura3*::*imm434 gcn4*::*hisG*/*gcn4*::*hisG* CIp10-*GCN4*) (37) were obtained from Alistair J. P. Brown, Aberdeen, United Kingdom. The construction of KWN2 (*dpp3*::*HIS1*/*dpp3*::*LEU2 his1*/*his1 leu2*/*leu2 arg4*/*arg4*) and KWN4 (*dpp3*::*DPP3*/*dpp3*::*DPP3 his1*/*his1 leu2*/*leu2 arg4*/*arg4*) was described previously (25). KWN6 (*dur1,2*/*dur1,2*), KWN7 (*dur1,2*/*dur1,2*::*DUR1,2*), and KWN8 (*dur1,2*/*dur1,2*::*DUR1,2*/*DUR1,2*) were made in this study as described below.

For routine growth and maintenance of the *C. albicans* strains, YPD medium (10 g of yeast extract, 5 g of peptone, and 20 g of glucose per liter) at 30°C was used. Auxotrophic mutants were grown in YPD supplemented with 40 μg/ml of required amino acids. RAW 264.7 cells were grown in complete culture medium (500 ml of Dulbecco's modified Eagle's medium plus 50 ml of fetal bovine serum plus 0.55 ml of 50-mg/ml gentamicin) at 37°C in the presence of 5% CO_2.

Construction of urea amidolyase (*dur1,2*) knockout and complementation mutants. To knock out the *DUR1,2* gene we adapted the strategy reported by Reuß et al. (31) using wild-type strain A72 (16). The pSFS2A plasmid was kindly provided by Joachim Morschhäuser (University of Würzburg, Würzburg, Germany). The *DUR1,2* gene deletion cassette was constructed as follows. An ApaI-XhoI fragment of the *C. albicans DUR1,2* (0.45-kb) upstream flanking sequence from −497 to −47 was amplified using primers 5′-cacacatggGCCCtagaaagctatt ttg-3′ and 5′-tgttgtCtCgaGttggttctatgcaa-3′. A SacII-SacI *DUR1,2* downstream fragment from position +40 to +468 was amplified with primers 5′-ttgataatacc GCGGatgttgttgtaagga-3′ and 5′-aaatctttatGaGcTccatagattgtttcg-3′. The underlined regions are restriction sites, and the capital letters indicate changes from the perfect primers needed to create the restriction sites. Numbers are relative to the first base of the *DUR1,2* translation start codon. The *DUR1,2* upstream and downstream fragments were cloned on the left and right sides of the SAT1 flipper, respectively, to generate pSFS2ADUR1,2.

The *DUR1,2* complementation cassette was constructed as follows. An ApaI-XhoI fragment of the complete *C. albicans DUR1,2* sequence as well as 0.57 kb of upstream and 0.15 kb of downstream flanking sequences for *DUR1,2* were amplified using primers 5′-tctagctaGggcCCttgatggtc-3′ and 5′-cattagtgcttcCTCg agacgacat-3′. A NotI-SacII *DUR1,2* downstream fragment from position kb 0.046 to 0.472 was amplified with primers 5′-taataccatgcggccgcaaggacttaga-3′ and 5′-aatctttataccgcggattgtttcg-3′. *DUR1,2* together with up- and downstream fragments was cloned on the left side of the *SAT1* flipper, and the *DUR1,2* downstream fragment was cloned on the right side of the *SAT1* flipper to generate pDURCOMP. The *SAT1* flipper cassette and the cloned fragments were sequenced at the UNL sequencing facility to confirm accurate cloning.

Transformation for gene knockout was done according to the methods of Reuß et al. (31) after the *DUR1,2* knockout cassette from pSFS2ADUR1,2 had been excised and gel purified as an ApaI-SacI fragment. Transformation for the gene complementation was done in the same manner using a gel-purified ApaI-SacII fragment of pDURCOMP. The cells were spread on YPD plates containing 200 μg/ml of nourseothricin and grown at 30°C. Resistant colonies were picked after 1 day of growth and inoculated in YPD liquid medium containing 200 μg/ml of nourseothricin for DNA isolation. In parallel, the transformants were streaked on YPD plates with 200 μg/ml of nourseothricin for further use. The heterozygous mutant was named KWN5 (*dur1,2*/*DUR1,2*), and the double knockout mutant was designated KWN6 (*dur1,2*/*dur1,2*). The single-copy reconstituted strain was named KWN7 (*dur1,2*/*dur1,2*::*DUR1,2*), and both copies of the reconstituted strain were named KWN8 (*dur1,2*/*dur1,2*::*DUR1,2*/*DUR1,2*). Nourseothricin was obtained from Werner BioAgents (Jena, Germany).

Genomic DNA from A72 and KWN5-8 was isolated for Southern hybridization. Both alleles of *DUR1,2* have ClaI restriction sites at nucleotides 2934 and 3101. Approximately 10 μg of DNA was digested with ClaI, separated on 1% agarose gels, and transferred to a membrane. Two gel-purified fragments, the *DUR1,2* up- and downstream fragments, were used as probes to confirm gene knockout. These were the ApaI-XhoI upstream fragment (450 bp) and the SacII-SacI downstream fragment (418 bp) generated for subcloning to construct pSFS2ADUR1,2. Southern hybridization confirmed the correct insertion of all cassettes (data not shown).

GTF assays. *C. albicans* cells from stationary phase were transferred to GlcNAc-imidazole-Mg buffer, pH 6.8 (11 mM imidazole, 3 mM $MgSO_4$, and 2.6 mM *N*-acetyl-D-glucosamine) (16) at 37°C for 4 h. Germ tube induction by arginine and urea was performed by using 0.004% glucose and 20 mM arginine or 20 mM urea in distilled water at 37°C. There was no germ tube formation (GTF) in the glucose-only controls, i.e., those with no added arginine or urea. GFT assays in the presence of 5% CO_2 were performed in two ways. The first paralleled the arginine and urea experiments in that it used screw-cap flasks containing 0.004% glucose, whereas the second method transferred *C. albicans* cells growing in YPD in six-well plates at 37°C from air to air with 5% CO_2. All the assays except the GTF assay in the presence of CO_2 were conducted in 25-ml Erlenmeyer flasks using *C. albicans* inocula, which had been stored at 4°C in 50 mM potassium phosphate buffer (pH 6.5). The cells were added in aliquots to prewarmed (37°C) assay medium to give a final cell density of 10^5 to 10^7 cells/ml. The flasks were shaken on a New Brunswick Scientific G2 shaker at 37°C and 225 rpm for 4 to 6 h and examined for GTF by confocal microscopy. At time zero, the inoculated cells were >98% undifferentiated, with 0% germ tubes and 0 to 2% budding yeasts.

Coculture conditions and macrophage ingestion assay. The murine RAW 264.7 macrophage line was grown in Dulbecco's modified Eagle's medium culture medium that contained 10% fetal bovine serum and 50 μg/ml gentamicin. One day prior to the experiment, RAW 264.7 cells that reached confluence in culture medium were collected, washed, and counted with a hemacytometer. A total of 10^6 cells were plated in culture medium in six-well plates and grown overnight in 5% CO_2 at 37°C to allow adherence to the surface. On day zero the nonadherent cells were removed from the plates by aspiration and fresh prewarmed complete culture medium was added. Two forms of *C. albicans* yeast

cells were used: either up to 1-week-old resting cells or actively growing, mid-log-phase cells. The resting-phase cells were prepared by growing *C. albicans* strains overnight in YPD at 30°C, washing three times with 50 mM potassium phosphate buffer (16), and storage in the same buffer. In the second case, these yeast cells were diluted 1:100 and grown for 6 to 8 h in YPD at 30°C, whereupon the log-phase cells were harvested by centrifugation. Cultures were washed with phosphate-buffered saline, and concentrations were measured using a Spectronic 20 spectrophotometer. A total of 10^6 or 2×10^6 cells was added to each well (1:1 or 2:1 *C. albicans*/macrophage ratio), and the plates were incubated for 6 h at 37°C. At the 1-h time point the plates were washed with prewarmed phosphate-buffered saline and fresh prewarmed complete culture medium was added to minimize *C. albicans* cells that were not phagocytized. The coculture conditions, germ tube formation, and escape from macrophages were examined by phase-contrast microscopy at different time points. Microscopic examination revealed that a small number of *C. albicans* cells remained that were not phagocytized but adhered to the surface.

RESULTS

Arginine, urea, and CO_2 stimulate hyphae by a cell-density-dependent pathway. Wild-type *C. albicans* A72 formed hyphae in the presence of 2.6 mM GlcNAc, 20 mM arginine, 20 mM urea, or 5% CO_2 within 4 to 6 h (Fig. 1B). High (20 mM) levels of arginine or urea consistently induced germ tube formation in 80 to 90% of the cells (Fig. 1B), whereas lower (5 mM) levels stimulated only ca. 30% of the *C. albicans* cells. Germ tube induction by arginine, urea, or CO_2 was cell density dependent in that the efficiency of GTF was 80 to 90% at $\leq 10^6$ cells/ml, ca. 40% at 10^7 cells/ml, and even lower at higher cell densities (data not shown). Interestingly, 5% CO_2 stimulated GTF under both nutrient-rich conditions (YPD) (see Fig. S1 in the supplemental material) and nutrient-poor conditions (0.004% glucose) (data not shown), and in both cases GTF was cell density dependent. Also, for both arginine and urea, GTF was blocked by ammonium sulfate; 5 mM ammonium sulfate reduced GTF to 10 to 35% and 10 mM blocked GTF completely. In contrast, CO_2-stimulated GTF was not blocked by 10 mM ammonium sulfate. These results are consistent with one or more steps in arginine- and urea-stimulated GTF being subject to nitrogen catabolite repression (NCR). In *S. cerevisiae*, both arginase (36) and urea amidolyase (8) are subject to NCR. Finally, arginine stimulated biofilm formation in *C. albicans* A72 (data not shown).

Germ tube formation is induced by arginine, urea, or CO_2 in an *efg1*-dependent way. We also examined GTF using four strains of *C. albicans* that lack transcription factors responsive to Rim101p-dependent signaling (CAR2) (30), MAP kinase signaling (JKC19) (20), cAMP signaling (HLC52) (20), or both (HLC54) (20). These strains are all derived from CAI4, and they are particularly useful in determining the pathway(s) responsible for germ tube induction by any stimulant. The CAI4 parent exhibited GTF with 2.6 mM GlcNAc, 20 mM arginine, 20 mM urea, or 5% CO_2 (Fig. 2). Significantly, the JKC19 (*cph1/cph1*) and CAR2 (*rim101/rim101*) mutants could respond to arginine, urea, or 5% CO_2 (Table 1), whereas the HLC52 (*efg1/efg1*) and HLC54 (*cph1/cph1::efg1/efg1*) mutants could not (Fig. 2 and Table 1). These results suggest that arginine, urea, and 5% CO_2 induce GTF by an Efg1p-dependent mechanism (Fig. 2). In *C. albicans* external CO_2 is transported inside the cell, either by diffusion or by transporters, and converted to HCO_3^- by carbonic anhydrase, thus activating adenylyl cyclase to synthesize cAMP, which in turn triggers the morphogenetic switch from yeast to hyphae (2).

Urea amidolyase mutants (*dur1,2/dur1,2*) cannot utilize urea as a sole nitrogen source. Arginine can be converted to urea and L-ornithine by the enzyme arginase (Car1p) (24, 36), and urea is converted to CO_2 and ammonia by urea amidolyase (Dur1,2p) (7). To explore whether arginine, urea, and CO_2 are parts of a pathway stimulating GTF or if they act separately, we created a *C. albicans dur1,2* knockout mutant (KWN6) and the homozygous reconstituted strain (KWN8). The effects of *DUR1,2* knockout and reconstitution on the ability to use urea as a nitrogen source are shown in Fig. 1A. The parent strain, A72, and the reconstituted strain (KWN8) were able to grow on defined minimal media with L-proline, urea, or L-arginine as the sole nitrogen source, whereas the *dur1,2/dur1,2* knockout strain (KWN6) was unable to grow on urea at either 30°C (Fig. 1A) or 37°C (not shown). However, KWN6 grew as well as its A72 parent on four media: YPD (not shown) and the three defined media, GPP (L-proline), GPR (L-arginine), and GPPU (L-proline and urea). It is not surprising that KWN6 grew on GPR; Car1p breaks arginine down to urea and L-ornithine, and even though KWN6 cannot use the nitrogens in urea, they can still use the nitrogens in L-ornithine. Also, all three strains grew on proline and urea together, showing that the inability of KWN6 to grow on urea only (Fig. 1A) was not due to the accumulation of toxic components derived from urea. None of the strains grew on thiourea, and thiourea did not inhibit the growth of A72 on either L-proline or urea (data not shown).

Arginine, urea, and CO_2 induce germ tube formation in a single sequential pathway. A72 and the reconstituted KWN8 strain behaved identically under all GTF-inducing conditions, i.e., GlcNAc, arginine, urea, and 5% CO_2. However, KWN6 was defective in GTF in the presence of arginine or urea (Fig. 1B), even though it exhibited unimpaired GTF in the presence of 5% CO_2 or GlcNAc (Fig. 1B) or 10% serum (data not shown). The 6-h GTF assay results for KWN6 in 5% CO_2 (Fig. 1B) are somewhat misleading in that they show many budding yeasts along with the hyphae. The 1- and 2-h samples showed that ≥98% of the cells underwent GTF (see Fig. S1 in the supplemental material); the budding yeasts only appeared later, 2 to 6 h after inoculation. This shift to the yeast morphology is likely a cell-density-dependent phenomenon (26). These results suggest a pathway whereby arginine is converted to urea and then to CO_2, with CO_2 acting as a common signal for GTF in *C. albicans*. These results are summarized in Table 1. They are consistent with a single sequential pathway for stimulating germ tube formation (Fig. 3). This pathway merges our data for arginine and urea with the CO_2-, cAMP-, and Efg1p-dependent pathway developed by the Muhlschlegel laboratory (2).

Arginine biosynthesis is essential for the escape of *C. albicans* from the RAW 264.7 macrophage cell line. We used two types of mutants to test whether the arginine-to-urea-to-CO_2 signal operates inside macrophages. The first type (described here) cannot convert arginine or urea to CO_2 (*dur1,2/dur1,2*), while the second type (next section) cannot synthesize arginine. *C. albicans* A72 (*DUR1,2/DUR1,2*), KWN6 (*dur1,2/dur1,2*), and KWN8 (*dur1,2/dur1,2::DUR1,2/DUR1,2*) all formed hyphae within 1 h at 37°C in the complete macrophage growth medium with a 5% CO_2 atmosphere. This observation shows that KWN6 is not defective in its hypha-forming ability (Table 1). Cells from both resting-phase and log-phase cultures behaved

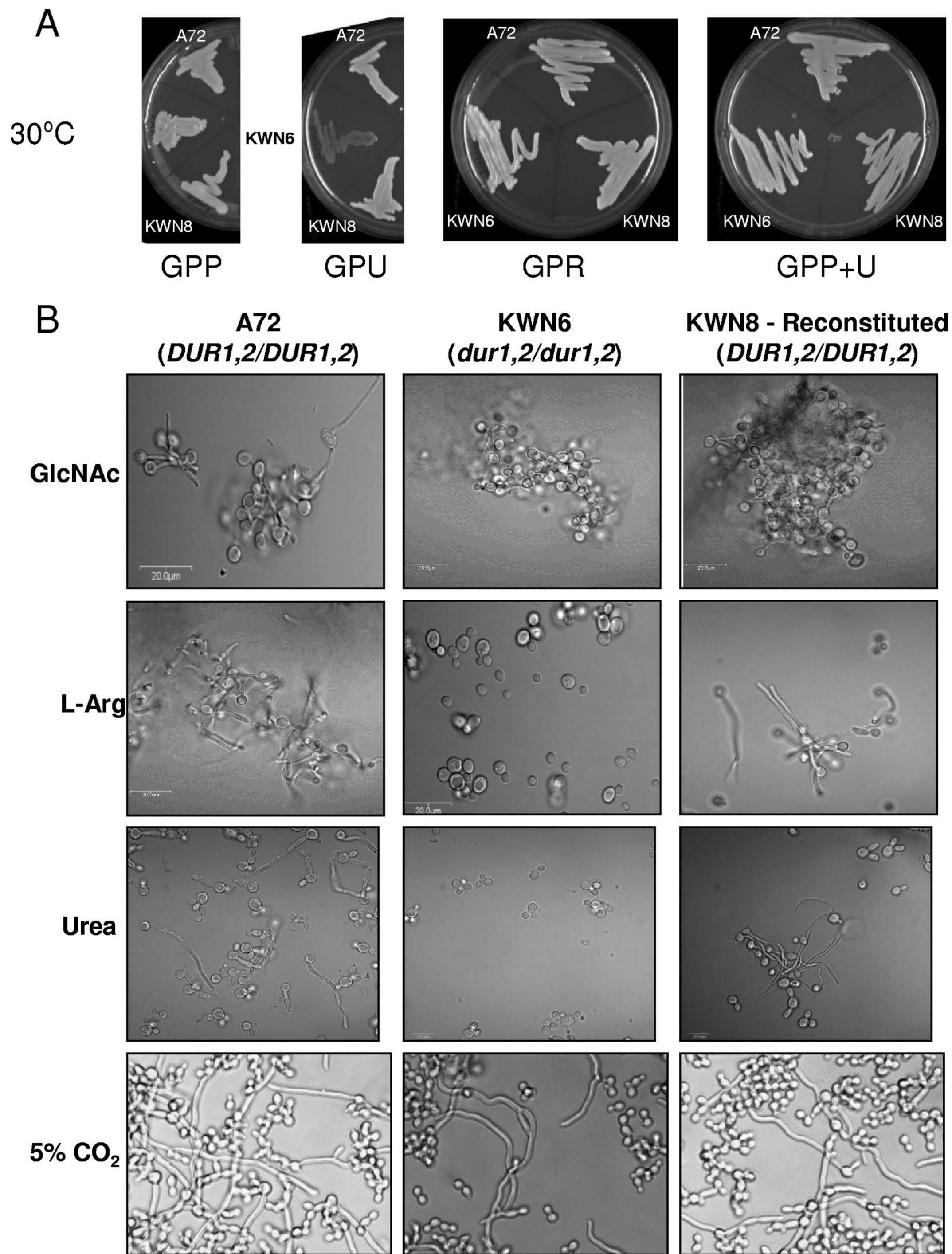


FIG. 1. Urea amidolyase mutants. A. Growth of the *dur1,2/dur1,2* mutant on urea. Parent strain A72 (*DUR1,2/DUR1,2*), the urea amidolyase mutant KWN6 (*dur1,2/dur1,2*), and *DUR1,2*-reconstructed strain KWN8 (*dur1,2/dur1,2*::*DUR1,2/DUR1,2*) were streaked on defined media with proline (GPP), urea (GPU), arginine (GPR), and proline plus urea (GPP+U) as sole nitrogen sources and incubated at 30°C. B. Germ tube formation induced by GlcNAc, arginine, urea, and CO_2 in *dur1,2/dur1,2* mutants. Photomicrographs show germ tube assay results for A72 (*DUR1,2/DUR1,2*), KWN6 (*dur1,2/dur1,2*), and KWN8 (*dur1,2/dur1,2*::*DUR1,2/DUR1,2*) strains in the presence of 2.6 mM GlcNAc (first row), 20 mM arginine (second row), and 20 mM urea (third row), all at 37°C after 4 h, and with 5% CO_2 (fourth row) at 37°C after 6 h. Photomicrographs in the first three rows were taken with a confocal microscope, and the image in the fourth row is with differential interference contrast in a bright-field microscope.

similarly in terms of GTF in the complete culture medium. These observations are not surprising, since this culture medium contains a powerful trigger of GTF, 10% serum, and the cells are incubated in an atmosphere that contains 5% CO_2, another trigger for GTF. Thus, hyphal growth was also observed in coculture experiments for any *C. albicans* that had not been ingested by the RAW 264.7 macrophage cells.

Wild-type *C. albicans* A72 was fully engulfed by the macro-

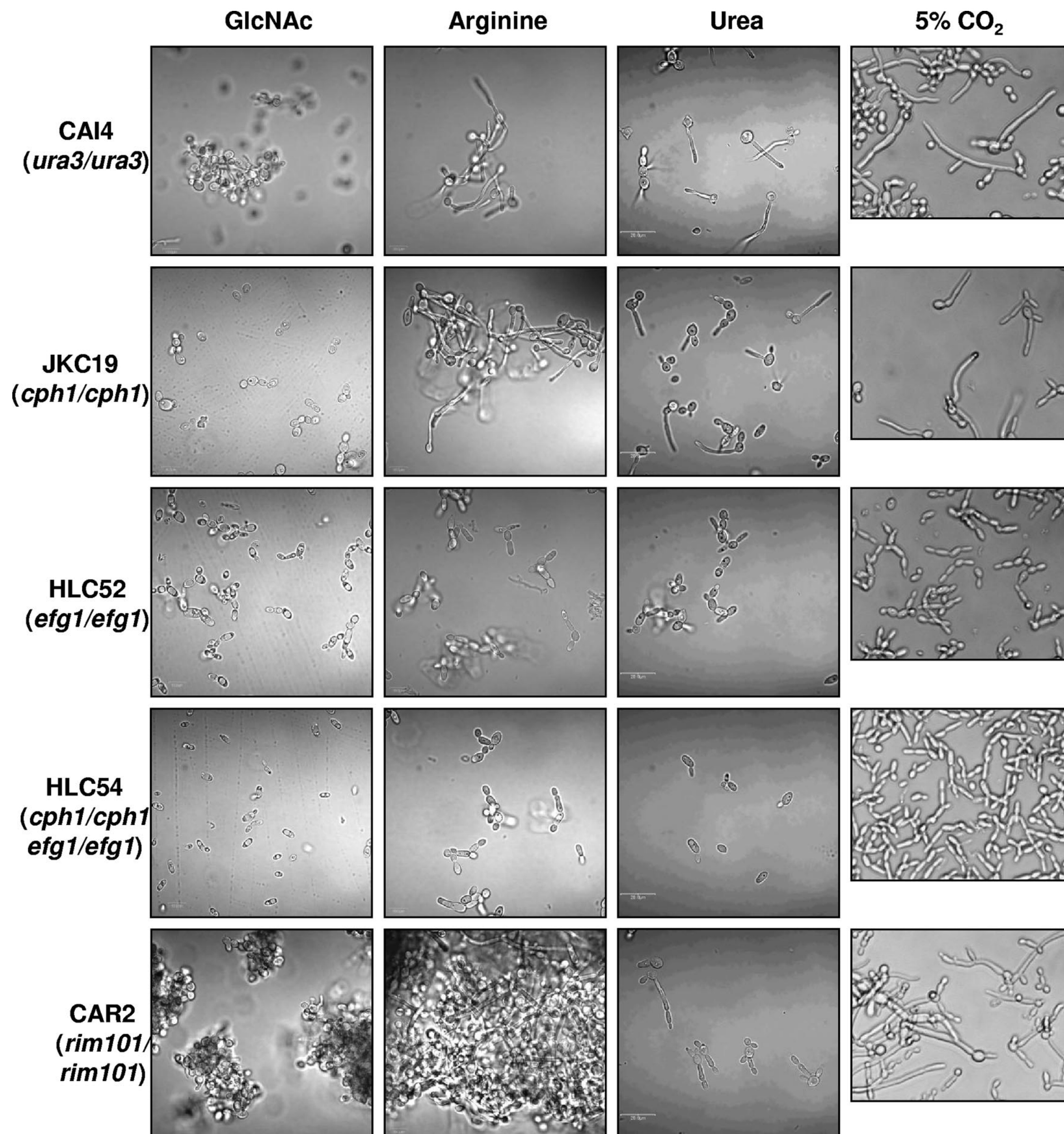


FIG. 2. Germ tube formation induced by GlcNAc, arginine, urea, and CO_2 in nonfilamentous mutants. Photomicrographs show germ tube assay results for CAI4, JKC19 (*cph1*/*cph1*), HLC52 (*efg1*/*efg1*), HLC54 (*cph1*/*cph1 efg1*/*efg1*), and CAR2 (*rim101*/*rim101*) in the presence of 2.6 mM GlcNAc (first column), 20 mM arginine (second column), 20 mM urea (third column), and 5% CO_2 (fourth column) at 37°C after 4 h. Representative photomicrographs in the first three columns were taken with a confocal microscope, and the fourth column was created with differential interference contrast with a bright-field microscope.

TABLE 1. Germ tube formation in wild-type and mutant *C. albicans*

Strain (relevant genotype)	GTF result with:			
	GlcNAc	Arginine	Urea	CO_2
A72 (wild type)	+	+	+	+
KWN6 (*dur1,2*/*dur1,2*)	+	−	−	+
KWN8 (*dur1,2*/*dur1,2*::*DUR1,2*/*DUR1,2*)	+	+	+	+
CAI4 (*ura3*/*ura3*)	+	+	+	+
JKC19 (*cph1*/*cph1*)	−	+	+	+
HLC52 (*efg1*/*efg1*)	−	−	−	−
HLC54 (*cph1*/*cph1 efg1*/*efg1*)	−	−	−	−
CAR2 (*rim101*/*rim101*)	+	+	+	+

phages by 1 h, but within 4 h the fungus had made hyphae inside the macrophage cells, and by 6 h it had penetrated the membranes and emerged or escaped from the RAW 264.7 cells. In contrast, KWN6 (*dur1,2*/*dur1,2*), the urea amidolyase knockout mutant, exhibited delayed hypha formation; by 4 h there were mostly yeast cells and very few hyphae inside the RAW 264.7 cells. Similarly, by 6 h the percentage and length of germ tubes were much less for KWN6 than for the wild-type A72. Finding a few hyphae on KWN6 cells likely means that those cells had been triggered for GTF by the serum and CO_2 and remained committed (26) for GTF even after ingestion. The ability for GTF inside RAW 264.7 macrophage cells was

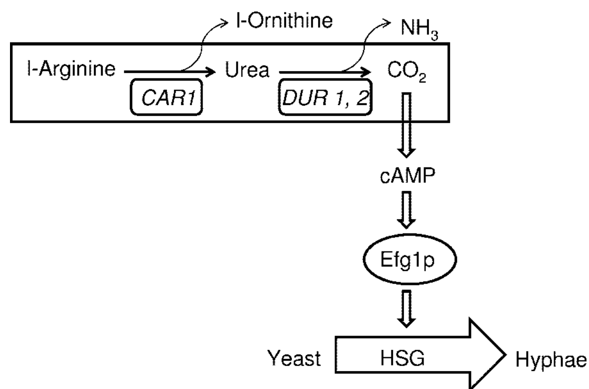


FIG. 3. Suggested pathway for arginine-induced germ tube formation. Arginine is metabolized to ornithine and urea by arginase (Car1p); urea is degraded to CO_2 and NH_3 by the enzyme urea amidolyase (Dur1,2p); CO_2 activates adenyl cyclase and the cAMP-dependent protein kinase A pathway, thereby activating Efg1p, which triggers the yeast-to-hypha switch inside macrophages. The two steps catalyzed by Car1p and Dur1,2p are under NCR. L-Ornithine can be used as an alternative nitrogen source by *C. albicans*.

fully restored in both the *DUR1,2*-complemented strains, the singly *DUR1,2*-reconstituted KWN7 strain (see Fig. S2 in the supplemental material), and the doubly *DUR1,2*-reconstituted KWN8 strain (Fig. 4A).

The wild-type clinical isolate SC5314 was also tested and found positive for the yeast-to-hypha switch and escape from RAW 264.7 cells (Fig. 4B), as was CAF2-1 (*ura3*/*URA3*), but CAI4 (*ura3*/*ura3*) was unable to stimulate hyphae and penetrate the RAW 264.7 cells (Fig. 4B). This defect might be because of the lack of *iro1* (13), which is required to acquire iron.

Arginine auxotrophic mutants are defective in escaping from RAW 264.7 macrophage cells. To test our hypothesis further, we selected two genetically related pairs of amino acid auxotrophic mutants. BWP17 requires His, Arg, and Ura (27), whereas DAY286 requires only His (10). Similarly, SN152 requires His, Leu, and Arg, whereas SN87 requires only His and Leu (28). We found that BWP17 and SN152 could not stimulate hyphae inside the RAW 264.7 cells; they remained inside the macrophages even after 6 h (Fig. 4C). In contrast, DAY286 and SN87 penetrated the membranes and emerged from the macrophages by 6 h (Fig. 4C). These data strongly suggest that arginine biosynthesis is a key regulator for the yeast-to-hypha switch inside macrophages. This view was confirmed by the inability of two *arg4* mutants (25), KWN2 (*dpp3*/*dpp3 arg4*/*arg4*) and KWN4 (*dpp3*/*dpp3*::*DPP3*/*DPP3 his1*/*his1 leu2*/*leu2 arg4*/*arg4*), to escape from RAW 264.7 cells (see Fig. S3 in the supplemental material).

Arginine biosynthesis and escape from macrophages are not regulated by Gcn4 and the general amino acid control pathway. When eukaryotic cells are starved for nitrogen, the cells respond by activating Gcn4p, a transcription factor that targets roughly 500 genes, including most of the amino acid biosynthetic genes (15). The macrophage phagosome environment is likely to be nutritionally poor (5). If the phagosome is nitrogen starved, then it should activate Gcn4p, thereby inducing many amino acid biosynthetic genes as well as morphogenesis (37). Thus, we tested a series of *gcn4*-related mutants of *C. albicans* (Fig. 4D). Significantly, all four strains, CAF2-1 (*GCN4*/*GCN4*), GTC41 (*gcn4*/*GCN4*), GTC43 (*gcn4*/*gcn4*), and GTC45 (*ura3*/*ura3 gcn4*/*gcn4*::CIP10-*GCN4*) (37), switched from yeasts to hyphae and were able to escape from the RAW 264.7 cells (Fig. 4D). Thus, our results are consistent with the DNA array results of Lorenz et al. (21). They found that apart from arginine no other amino acid biosynthetic genes were upregulated (21). Taken together, these data suggest that arginine biosynthesis inside the macrophage is not regulated by Gcn4p but by some other pathway, possibly Arg82p and the Arg80p-Mcm1p-Arg81p complex, which are known to regulate arginine biosynthesis in *S. cerevisiae* (23). This pathway, which specifically induces the *ARG* genes just to breach the macrophage membranes, is of enormous importance, as this breach might lead to systemic candidiasis.

DISCUSSION

We have elucidated the signaling pathway whereby *C. albicans* initiates hyphal growth after being ingested by macrophages. Lorenz et al. (21) showed that the genes for L-arginine biosynthesis were induced following internalization by macrophages, and Sims (35) and Bahn and Muhlschlegel (2) showed that elevated CO_2 triggered hyphal growth. We have connected these two observations via the enzyme urea amidolyase (Dur1,2p). The key role of urea amidolyase is shown by the inability of a *dur1,2*/*dur1,2* mutant (KWN6) to escape from mouse macrophages, while this ability is restored in the reconstituted strains KWN7 and KWN8. The suggested signaling pathway is shown in Fig. 3.

A critical role for arginine following macrophage internalization was implied by DNA microarray studies (21). We confirmed that hypothesis by using two sets of paired mutants (Fig. 4C). Two strains with arginine auxotrophy could not escape from the macrophages, whereas the corresponding strains without arginine auxotrophy could. Interestingly, the strains that were able to escape were auxotrophic for amino acids other than arginine. These findings strongly suggest that the induction of germ tube formation, which is essential for escape from macrophages, requires biosynthesis of arginine but not other amino acids inside macrophages. Also, there is an apparent paradox between the inability of SN152 to escape from macrophages within 6 h (Fig. 4C) and its pathogenicity in a mouse tail vein model (28). This continued pathogenicity may just demonstrate the artificial nature of the tail vein model, or it could reflect the eventual escape of some SN152 from macrophages after a longer period of time.

The last half of the proposed signaling pathway (Fig. 3) is similar to that described by Bahn and Muhlschlegel (2). Those authors showed that *C. albicans* can induce germ tubes in the presence of CO_2 by activating adenylate cyclase (2). Production of CO_2 is important, because *C. albicans* can convert CO_2 to bicarbonate inside the cell by the enzyme carbonic anhydrase (Nce103p). Bicarbonate then activates adenylate cyclase (Cdc35p), which in turn activates cAMP-dependent protein kinase A, thereby activating hypha-specific genes in an Efg1p-dependent manner (2). We confirmed that arginine, urea, and CO_2 induce hyphae in an Efg1p-dependent manner (Fig. 2). It

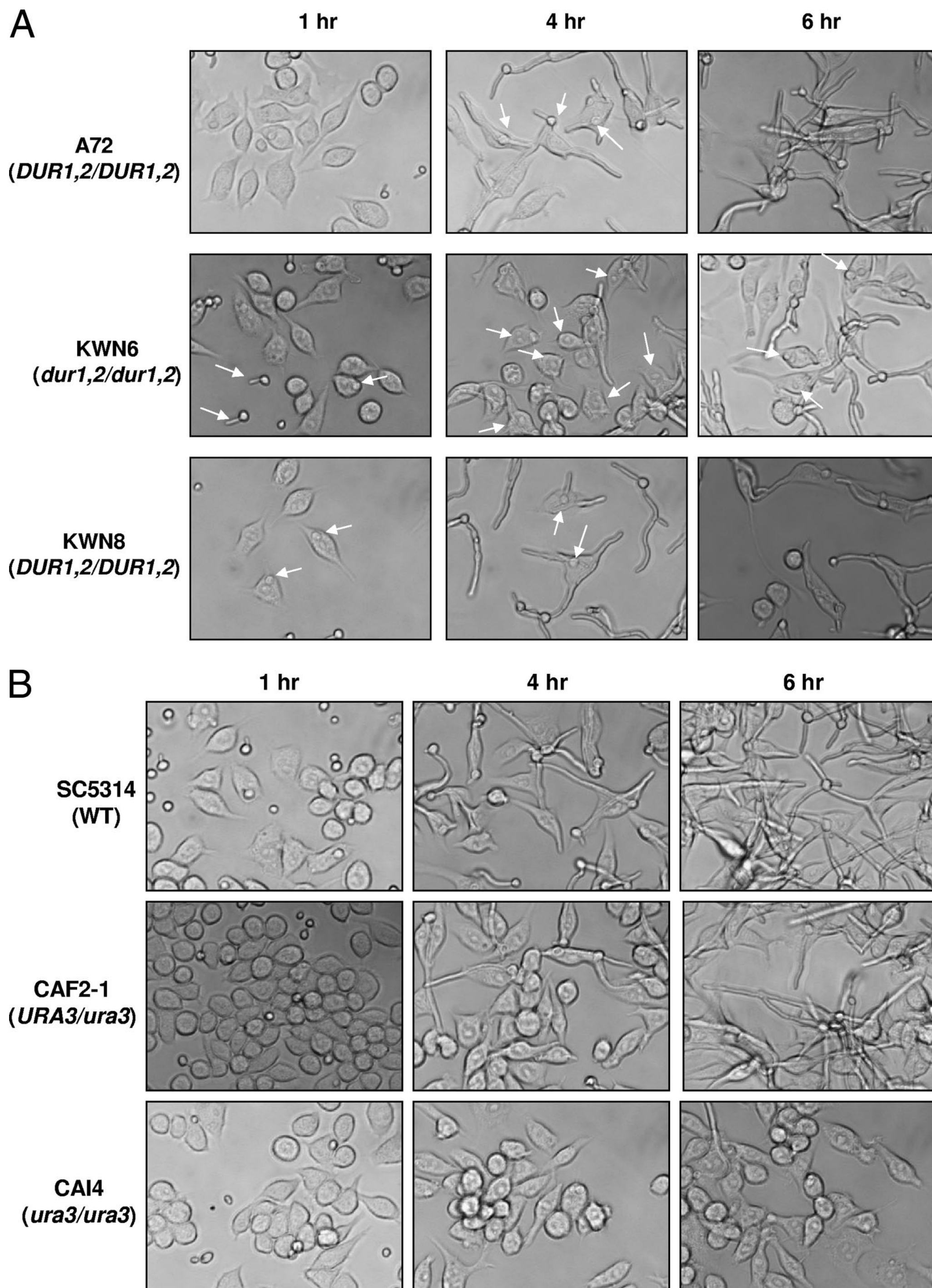
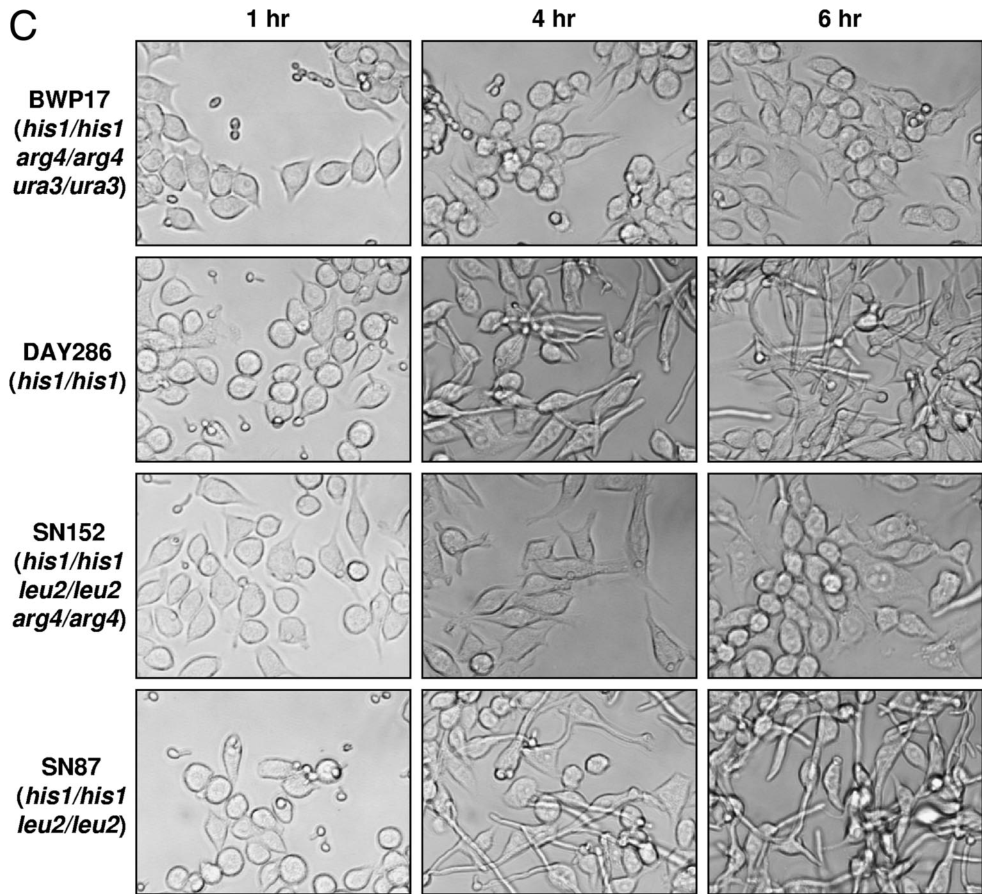


FIG. 4. Interaction of *C. albicans* with macrophages. Yeast cells were incubated ex vivo with RAW 264.7 cells in complete culture medium (with 10% serum) at 37°C in 5% CO_2, and the differential interference contrast photomicrographs were taken at 1 h (first column), 4 h (second column), and 6 h (third column). A. *C. albicans* A72 (*DUR1,2*/*DUR1,2*; parental strain [first row]), KWN6 (*dur1,2*/*dur1,2* [second row]), and KWN8 (*dur1,2*/*dur1,2*::*DUR1,2*/*DUR1,2* [third row]). Arrows at 1 h show three *C. albicans* cells which were phagocytized by macrophages and two noningested *C. albicans* cells which already have visible germ tubes. The arrows at 4 h for A72 and KWN8 show *C. albicans* cells with visible germ tubes in the process of escaping, whereas the 4- and 6-h arrows for KWN6 show *C. albicans* yeast cells within the macrophages. B. SC5314 (*URA3*/*URA3*; wild type [first row]), CAF2-1 (*ura3*/*URA3* [second row]), and CAI4 (*ura3*/*ura3 iro1*/*iro1* [third row]). C. Auxotrophic mutants BWP17 (*his1*/*his1 arg4*/*arg4 ura3*/*ura3* [first row]), DAY286 (*his1*/*his1* [second row]), SN152 (*his1*/*his1 arg4*/*arg4 leu2*/*leu2* [third row]), and SN87 (*his1*/*his1 leu2*/*leu2* [fourth row]). D. *gcn4* mutants CAF2-1 (*GCN4*/*GCN4*; parent strain [first row]), GTC41 (*GCN4*/*gcn4* [second row]), GTC43 (*gcn4*/*gcn4*; [third row]), and GTC45 (*ura3*/*ura3 gcn4*/*gcn4*::CIp10-*GCN4* [fourth row]).



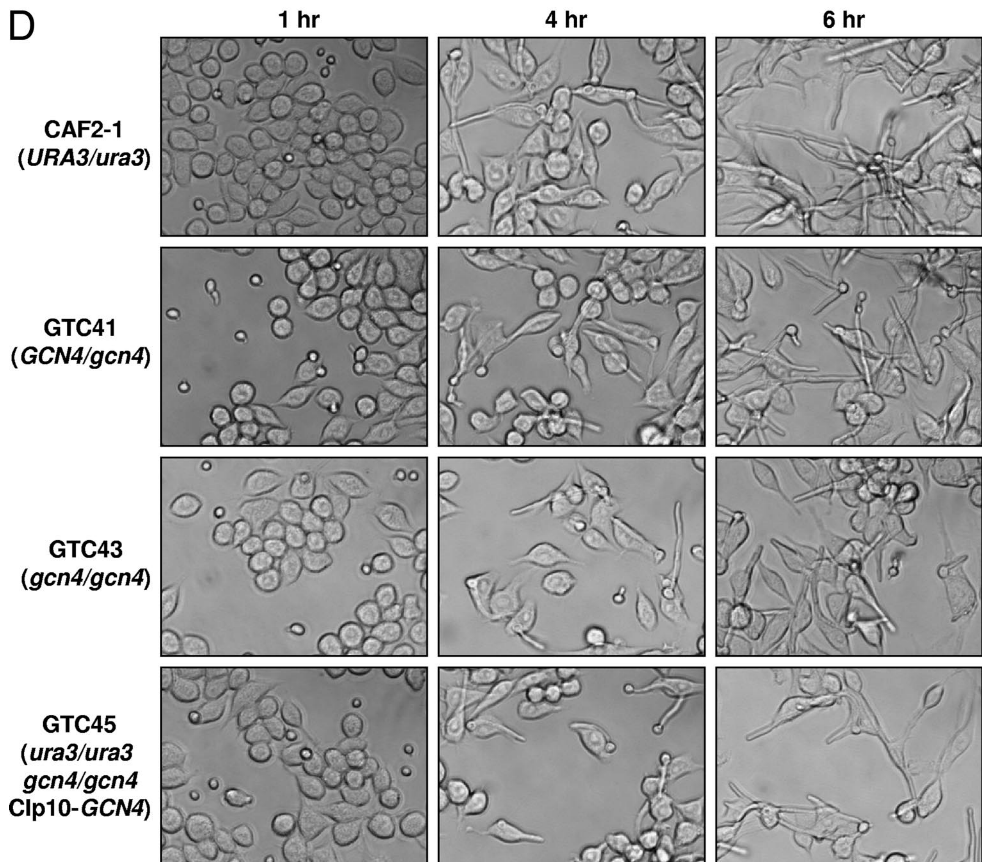


FIG. 4—*Continued.*

already has been established that yeast-to-hypha switch is a critical virulence factor in *C. albicans* (26).

Once inside a macrophage, arginine is converted to L-ornithine and urea by the enzyme arginase (Car1p) (24), and urea is converted to CO_2 and NH_3 by the enzyme urea amidolyase (Dur1,2p) (7). From microarray data, Lorenz et al. (21) observed that 1 h after ingestion *CAR1* (open reading frame [ORF] 19.3934) and two other related arginase genes (ORFs 19.10922 and 19.5862) were upregulated 3.2-, 4.7-, and 5.1-fold, respectively. *DUR1,2* (ORF 19.780) was also upregulated after 1 h but only by 1.4-fold (see the supplementary data of reference 21). These results suggest that inside the macrophage *C. albicans* not only synthesizes arginine but also utilizes arginine. The *dur1,2/dur1,2* mutant could not use arginine or urea for GTF but was able to respond to its downstream product, 5% CO_2 (Fig. 1). The inabilities of *arg4* and *dur1,2* mutants to escape from macrophages suggest that, even though the RAW 264.7 cells were grown in 5% CO_2, the phagosomes contained significantly less CO_2. Alternatively, the phagosome environment might be altered in an unknown manner that prevents *C. albicans* from responding to high CO_2.

Furthermore, we observed that 5 to 10 mM ammonium salts prevented GTF induced by GlcNAc, argininine, or urea, but not that induced by 5% CO_2. The explanation for these differences probably resides in the realm of NCR. Comparative study of *C. albicans* and *S. cerevisiae* often sheds light on the genetic mechanisms by which regulatory mechanisms work. In the case of *S. cerevisiae*, both *CAR1* (31, 36) and *DUR1,2* (8) are under the control of NCR. When rich nitrogen sources such as ammonia or asparagine are available the cells are designed to utilize these sources first and to repress other genes that are responsible for breaking down poorer nitrogen sources, like proline, arginine, or urea (40). When cells are starved for nitrogen, these NCR-regulated genes are induced. In *C. albicans* we found several GAT(A/T)(A/G) sites in the 1,000 bp upstream of the open reading frames for both *CAR1* and *DUR1,2*. These are the putative binding sites for the GATA transcription factors Gln3p and Gat1p, which can mediate NCR in *C. albicans* (9). This regulation makes sense, because in the presence of arginine and urea *C. albicans* will induce the NCR-regulated genes *CAR1* and *DUR1,2*, which in turn will make enough CO_2 to induce hyphae by the cAMP/PKA pathway (Fig. 3). The use of 5% CO_2 bypasses the steps subject to NCR, as shown in Fig. 3. As a final thought on the significance of *CAR1* and *DUR1,2* being NCR regulated, macrophage phagosomes are acidic (21), whereas neutrophil phagosomes are more basic (34). If the greater alkalinity in neutrophils were ammonia mediated, the resulting repression of *CAR1* and *DUR1,2* would partially explain why macrophages kill *C. albicans* less effectively than do neutrophils (1, 34).

DNA array analysis after phagocytosis by human neutrophils revealed that both *C. albicans* and *S. cerevisiae* induced genes for methionine and arginine biosynthesis but still could not escape from neutrophils (34). In addition to the NCR-based explanation provided in the previous paragraph, this situation may arise because neutrophils kill, or at least influence, the *C. albicans* cells quickly through a more potent oxidative and nitrosative burst, thus preventing hyphal formation. This explanation is consistent with the roles of macrophages in innate and adaptive immune responses, which in addition to directly killing the invading microbes include presenting antigens to T cells and to produce many different cytokines and chemokines that in turn attract other innate and adaptive immune components. For *C. albicans* cells ingested by neutrophils, only about 70% of the cells were still alive by 60 min (34), whereas for macrophages all of the cells had formed hyphae and escaped by 6 h (21). In a separate study, Arana et al. (1) found that only 24% of *C. albicans* cells survived after 2 h inside neutrophils, whereas 234% (cell replication) had survived after 2 h inside macrophages (1). These results along with our own data reported here suggest that macrophages kill *C. albicans* less effectively than do neutrophils.

C. albicans has at least three putative arginases (encoded by *CAR1*, ORF 19.3418, and ORF 19.5862), all of which are strongly induced in macrophages (21). Why are three arginases needed, and do they serve the same function? Murine macrophages, including RAW 264.7 cells, primarily kill microbes via nitrosative stress (6, 19, 29). This NO production is mediated by the enzyme inducible NO synthase, which requires arginine as a substrate. Some bacteria are known to avoid macrophage killing by inducing arginase in the host macrophages (19), and a similar protection against macrophage killing has been attributed to arginase (RocF) production by *Helicobacter pylori* (14). Therefore, induction of arginase upon ingestion by macrophages may provide a second survival benefit to *C. albicans* by depriving macrophages of the substrate required for synthesis of NO. In this regard, it is significant that Car1p and Dur1,2p are both cytoplasmic; they do not have predicted N-terminal signal peptides (http://www.cbs.dtu.dk/services/SignalP/). In contrast, the proteins encoded by ORF 19.3418 (361 amino acids) and ORF 19.5862 (418 amino acids) have predicted signal peptides with probabilities of 1.00 and 0.97, respectively. Thus, it seems likely that the three arginases serve at least two functions. Car1p is cytoplasmic, working with Dur1,2p in a pathway for GTF (Fig. 3), whereas the other two arginases are excreted. The excreted arginases may curb nitrosative stress in some fashion. This suggestion predicts that mutants defective in the arginases will have reduced survival in macrophages. Arginase induction would not affect killing by neutrophils, which rely instead on myeloperoxidase.

Gcn4p is a transcription factor that activates most of the amino acid biosynthetic genes under nitrogen-starved conditions (15). Strains lacking Gcn4p were examined to see if it regulated arginine biosynthesis during the initial phase after phagocytosis. It is clear from our data (Fig. 4D) that Gcn4p is not essential for hypha formation, because *gcn4/gcn4* mutants were fully capable of forming hyphae inside macrophages (Fig. 4D). However, the fact that Gcn4p does not appear to be needed increases the interest in finding the activator/pathway which does induce the arginine biosynthetic genes after phagocytosis. This regulation may be via Arg82p and the Arg80p-Mcm1p-Arg81p complex, which is known to regulate arginine biosynthesis in *S. cerevisiae* (23), or it might be unique to *C. albicans*, in which case it would be a candidate target for future drugs in case of candidiasis.

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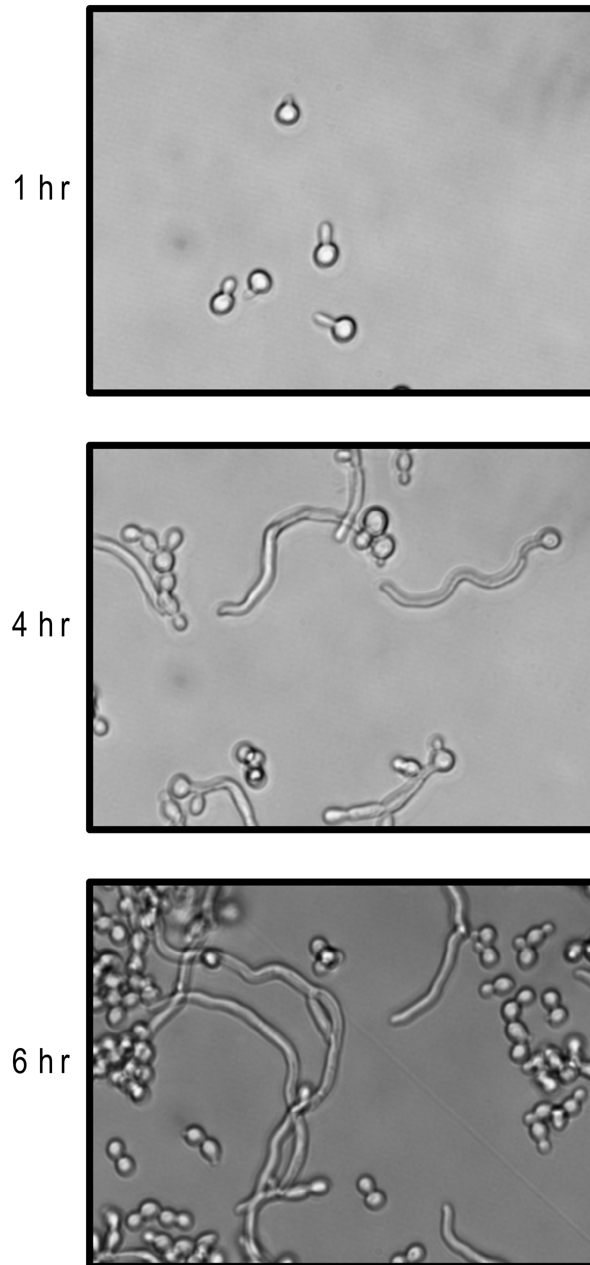
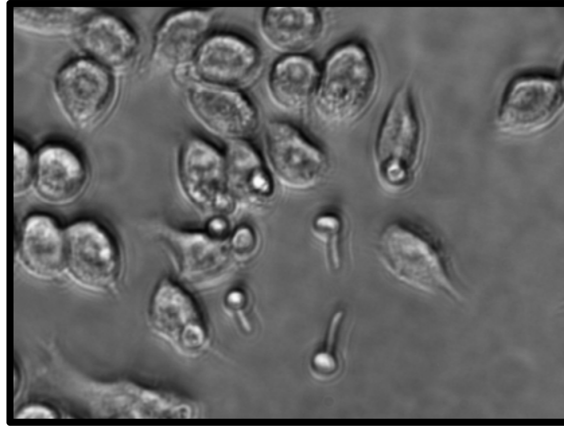


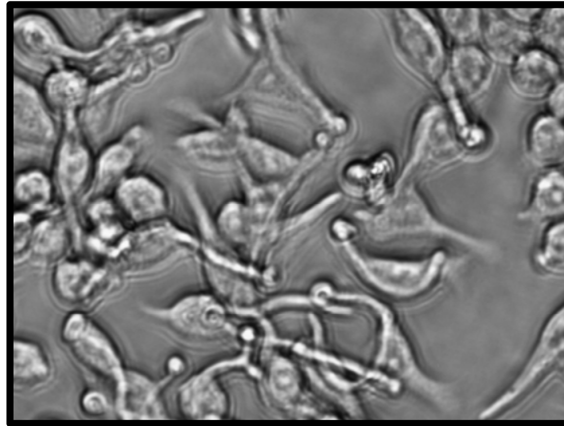
Figure S1. Germ tube formation in KWN6 (*dur1,2/dur1,2*) as induced by 5% CO_2. KWN6 cells were inoculated into YPD in the presence and absence (not shown) of 5% CO_2 and shaken at 37°C and 200 rpm for the indicated time. After 1 hr the cells grown with 5% CO_2 had already induced hyphae whereas those without CO_2 had not. By 4 and 6 hrs budding yeasts had developed from the hyphae, probably as the result of cell growth during that time and a cell density dependent conversion to yeast growth.

KWN7
(*dur1,2; DUR1,2*)

1 hr



4 hr



6 hr

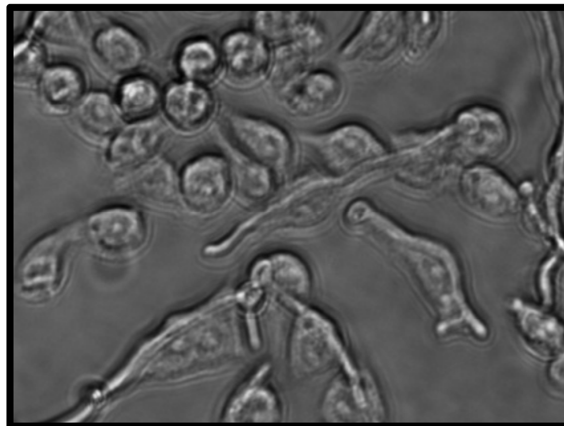


Figure S2. Interaction of *C. albicans* with macrophages. KWN7 (*dur1,2/dur1,2*::*DUR1,2*) cells were incubated *ex vivo* with RAW264.7 cells in complete culture medium (with 10% serum) at 37ºC in 5% CO_2 and the DIC photomicrographs were taken at 1 hour, 4 hour and 6 hour time points.

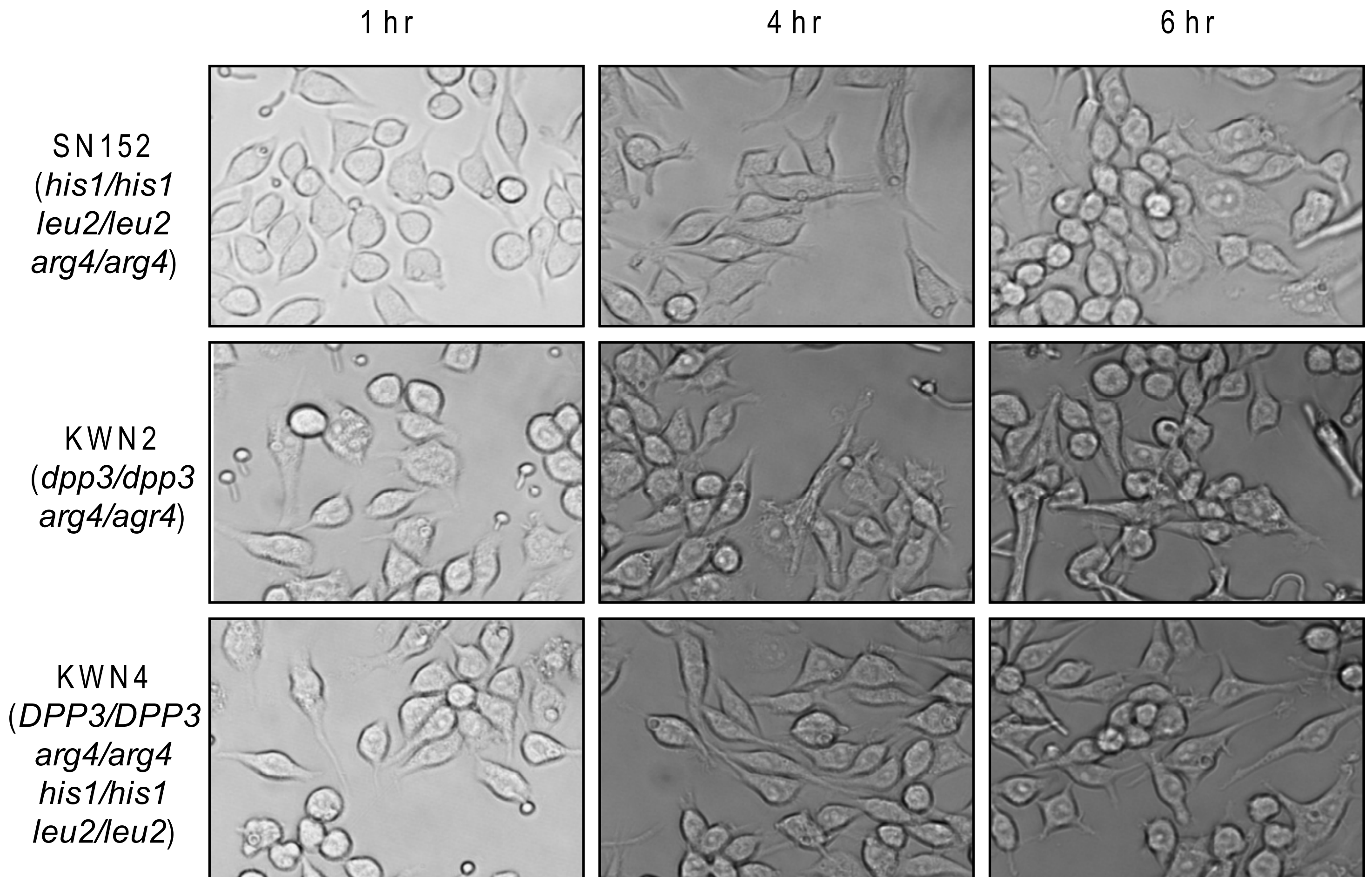


Figure S3. Interaction of *C. albicans* with macrophages. Yeast cells were incubated *ex vivo* with RAW264.7 cells in complete culture medium (with 10% serum) at 37ºC in 5% CO_2 and the DIC photomicrographs were taken at 1 hour (first column), 4 hour (second column) and 6 hour (third column) time points. SN152 (*his1/his1*, *leu2/leu2*, *arg4/arg4*) (first row), KWN2 (*dpp3/dpp3*, *arg4/arg4*) (second row), and KWN4