# Comparison of Sterol Import under Aerobic and Anaerobic Conditions in Three Fungal Species, *Candida albicans*, *Candida glabrata*, and *Saccharomyces cerevisiae*

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Sterol import has been characterized under various conditions in three distinct fungal species, the model organism *Saccharomyces cerevisiae* and two human fungal pathogens *Candida glabrata* and *Candida albicans*, employing cholesterol, the sterol of higher eukaryotes, as well as its fungal equivalent, ergosterol. Import was confirmed by the detection of esterified cholesterol within the cells. Comparing the three fungal species, we observe sterol import under three different conditions. First, as previously well characterized, we observe sterol import under low oxygen levels in *S. cerevisiae* and *C. glabrata*, which is dependent on the transcription factor Upc2 and/or its orthologs or paralogs. Second, we observe sterol import under aerobic conditions exclusively in the two pathogenic fungi *C. glabrata* and *C. albicans*. Uptake emerges during post-exponential-growth phases, is independent of the characterized Upc2-pathway and is slower compared to the anaerobic uptake in *S. cerevisiae* and *C. glabrata*. Third, we observe under normoxic conditions in *C. glabrata* that Upc2-dependent sterol import can be induced in the presence of fetal bovine serum together with fluconazole. In summary, *C. glabrata* imports sterols both in aerobic and anaerobic conditions, and the limited aerobic uptake can be further stimulated by the presence of serum together with fluconazole. *S. cerevisiae* imports sterols only in anaerobic conditions, demonstrating aerobic sterol exclusion. Finally, *C. albicans* imports sterols exclusively aerobically in post-exponential-growth phases, independent of Upc2. For the first time, we provide direct evidence of sterol import into the human fungal pathogen *C. albicans*, which until now was believed to be incapable of active sterol import.

*C*andida albicans and *Candida glabrata* are two medically important pathogens in humans, which cause disease predominantly in immunocompromised individuals (resulting from AIDS, transplants, and cancer treatment). *C. albicans* accounts for more than 50% of all *Candida* infections, causing oral, vaginal, and systemic disease, the last with a high morbidity rate between 30 and 50%. *C. glabrata* is the second or third most common species causing *Candida* infections (1). Azole antifungals are one of the most commonly used classes of drugs used to treat fungal infections. These drugs target the biosynthesis of ergosterol, the fungal equivalent of the sterol found in higher eukaryotes, cholesterol. Azoles inhibit 14α-lanosterol demethylase, the product of the *ERG11* gene. In general, sterols are essential components of eukaryotic cell membranes, and therefore cells tightly regulate sterol levels and sterol metabolism. Alteration of sterol levels can have an effect on the susceptibility of fungal cells to antifungal drugs, and to a variety of stresses, including osmotic and oxidative pressures.

**Saccharomyces cerevisiae.** Sterol metabolism in *S. cerevisiae*, a eukaryotic model organism, has been intensively studied (reviewed in references 2, 3, and 4). The fungal sterol ergosterol is synthesized in the endoplasmic reticulum (ER), the cellular membrane with otherwise the lowest levels of ergosterol. From there, ergosterol is transported through the Golgi bodies to the plasma membrane (PM), the membrane with the highest ergosterol content. Mitochondria also contain low levels of ergosterol, mostly in its inner membrane (5). In addition to its structural role in the membranes, a minimal level of ergosterol is required for cell cycle progression and for cell viability in *S. cerevisiae*, a process known as "sparking" (6, 7).

Under anaerobic conditions, or in mutants with defects in heme biosynthesis (heme biosynthesis is oxygen dependent, and its decreased levels are thought to be the primary sensor of oxygen availability [8]), *S. cerevisiae* requires exogenous methionine, sterols, and unsaturated fatty acids, since the synthesis of these requires both molecular oxygen and heme (9–11). Oxygen is necessary for several ergosterol biosynthesis reactions, including those performed by squalene epoxidase, sterol demethylase, and sterol desaturase. *S. cerevisiae* cells import exogenous sterols under anaerobic conditions, but not under aerobic conditions, a phenomenon known as "aerobic sterol exclusion" (12). Anaerobic sterol uptake in *S. cerevisiae* is found to be inversely proportional to the concentration of sterol already present in the cell. Cells that are saturated for free sterols stop accumulating exogenously supplied sterols (13–15). In *S. cerevisiae*, anaerobic sterol uptake is mediated by two ABC transporters: the more active ScAus1 and its paralog ScPdr11 (16, 17). Both proteins are localized to the plasma membrane, with portion of ScAus1 localized intracellularly (18).

The transcription factor ScUpc2 regulates expression of most of the genes in sterol biosynthesis, as well as sterol import, and up to one-third of anaerobically expressed genes. Thus, ScUpc2 is important for to adaptation to hypoxia (19). Constitutively active

mutants of ScUpc2, or its paralog ScEcm22, induce sterol uptake under aerobic conditions, overcoming the aerobic sterol exclusion (20, 21).

Sterol uptake in *S. cerevisiae* is not limited to ergosterol; various sterols from the environment, including cholesterol, can also be imported. Import of cholesterol and other sterols results in alterations in phospholipids, fatty acid compositions, and sterol to phospholipid ratios within the cells (22). Before incorporation into the *S. cerevisiae* plasma membrane, ergosterol, but not cholesterol, binds tightly to the cell wall and is resistant to detergent wash (16, 23). The ScUpc2-regulated cell wall protein ScDan1 is involved in sterol uptake associated with the cell wall (17, 24). After sterol saturation of membranes, excess imported cholesterol is esterified via ScAre1p and ScAre2p (25), while excess ergosterol remains unesterified (15, 18, 26). Many other genes involved in sterol uptake have been described, including several related to mitochondrial metabolism (23).

**Candida glabrata.** *C. glabrata* is more closely related to *S. cerevisiae* than to *C. albicans*, and the two species share some features of sterol import. *C. glabrata* requires the same anaerobic supplements as *S. cerevisiae*. However, *C. glabrata* differs in the conditions of sterol import. Recent studies (27, 28) revealed that *C. glabrata* wild-type strains import sterol under aerobic conditions, independent of the mutations in heme synthesis or the Upc2 orthologs that are required for *S. cerevisiae* aerobic sterol uptake. Wild-type *C. glabrata* responds to sterol-containing serum and bile, but not free cholesterol, whereas Cg*ERG* mutants can utilize free sterols, as well as components containing sterol. In addition, wild-type *C. glabrata* continuously synthesizes ergosterol, even while importing exogenous cholesterol from serum (29, 30).

The *C. glabrata* sterol transporter CgAus1 was identified based on homology to ScAus1, although a *C. glabrata* ortholog of Sc*PDR11* was not found. CgAus1 is transcriptionally induced in the presence of serum, a process dependent on the *C. glabrata* paralogs Cg*UPC2A* and Cg*UPC2B* (28).

**Candida albicans.** Previous work suggests that *C. albicans*, unlike the two species mentioned above, depends solely on the synthesis of endogenous ergosterol. No mechanism of sterol uptake was identified in previous work (27, 31, 32). Unlike *S. cerevisiae* and *C. glabrata*, there is lack of response to exogenous sterols by *C. albicans* wild-type, Ca*HEM1*-null mutant, or sterol auxotrophs (27). Moreover, no clear orthologs of Sc*AUS1* or Sc*PDR11* can be identified in the *C. albicans* genome. However, *C. albicans* is able to grow anaerobically without need for exogenous sterols or even unsaturated fatty acids, both of which are crucial for *S. cerevisiae* and *C. glabrata* growth, although addition of oleic acid to the medium significantly increases growth (33). Sterols on the other hand have no such effect. *C. albicans* can grow without ergosterol in the membranes (34). Unfortunately, sterol levels in anaerobically grown *C. albicans* have never been determined. There is some indirect evidence for *C. albicans* being able to import sterols, published more than 30 years ago, where a nystatin-resistant *C. albicans* strain, with no ergosterol in its membranes, increased its susceptibility after cultivation in medium with the presence of ergosterol (35).

In the present study, we compare and contrast sterol import into three fungal species: *C. albicans*, *C. glabrata*, and *S. cerevisiae*. We determine sterol import in various conditions using two substrates, cholesterol, the major sterol of higher eukaryotes, and ergosterol, the typical fungal sterol. Moreover, for the first time we

provide direct evidence of active sterol import into the fungal pathogen *C. albicans*. Understanding sterol metabolism, including import, in the medically important fungi *C. albicans* and *C. glabrata* will help us to understand the behavior of these fungi in infection, especially in response to treatment with azoles and other drugs targeting sterol biosynthesis pathway. This deeper understanding could help us to design better treatment strategies for these infections.

## MATERIALS AND METHODS

**Strains and growth conditions.** The strains used in the present study are listed in Table 1. *C. albicans*, *C. glabrata*, and *S. cerevisiae* strains were propagated on YPD agar plates (1% yeast extract, 2% Bacto peptone, 2% glucose, and 2% agar) and otherwise were grown in liquid complete synthetic medium (CSM; 0.17% yeast nitrogen base without amino acids [Difco], 0.5% ammonium sulfate, 2% glucose, 0.79-g/liter CSM complete supplement mixture [Sunrise Science Products]) supplemented with 50 mg of uridine/liter. For microaerophilic growth, the GasPak EZ anaerobe gas generating pouch system with indicator (Becton Dickinson) or the MCG AnaeroPack System (Mitsubishi Gas Chemical Company, Inc.) with anaerobic indicator (Oxoid) were used according to the manufacturer's instructions.

**Construction of *C. glabrata* Δ*upc2A*/Δ*upc2B* mutant.** Since *S. cerevisiae* paralogs, ScUpc2 and ScEcm22, can partially complement for each other, we expected the same for *C. glabrata*. The Cg*UPC2A* allele was deleted in the *C. glabrata* Δ*upc2B* strain (36) in order to obtain the *C. glabrata* Δ*upc2A*/Δ*upc2B* double mutant. The dominant selectable marker *SAT1* form plasmid pSFS2A (37) was amplified by PCR using Phusion polymerase (Thermo Scientific) with the oligonucleotides CgUPC2A_SAT1_F (5′-TAAGTCTTAGT TGTTTATCATCAAAACCACTGTTGGGGTTTATTTTGGGTGTGATCA ATGAAAATTTCGGTGATCCC-3′) and CgUPC2A_SAT1_R (5′-ACAG TATACCATAAATGTATCTTTGAAAGAACTACAAAAGAACAATAAA CTCATTAGGCGTCATCCTGTGCTC-3′). The purified PCR product was transformed into *C. glabrata* using the standard lithium acetate transformation protocol for *S. cerevisiae* (38). After the transformation, cells were kept for additional 4 h in YPD in order to express the integrated *SAT1* gene and then grown on YPD plus 100 μg of nourseothricin/ml. Positive transformants were verified by PCR (see Fig. S1 in the supplemental material) using the oligonucleotides SAT1_F (5′-CATCTCGG ATGATGACTCTG-3′), SAT1_R (5′-CCAGTACCAGTACATCGCTG-3′), CgUPC2A_up_F (5′-AGTAATAGCACATCGTATAG-3′), and CgUPC2A_down_R (5′-TTGATTTCAAACTGACCTAG-3′).

**Construction of *C. glabrata* Δ*aus1* mutant.** Cg*HIS3* gene deletion cassette was originally amplified by using the oligonucleotides CgAUS1_HIS3_F (5′-TAGAATAAAATTTTTTTAAACTTAACTTGTT GCCGCGCTATAGCCCATATACAAGTTCTCCTAACTCCTTGTTCAA CAGTG-3′) and CgAUS1_HIS3_R (5′-GTGTTAAATTTAAGAATAAAA TGGAATTGTTATTTCATTAAAAGCTTGTAGGAGTCACTCTGGTA GCTGTGGGCTGTGTT-3′), both carrying 60 bases homologous to Cg*AUS1* upstream and downstream regions. However, the transformation in this case did not result in a strain with correct integration of the cassette. For this reason, three additional PCRs were performed in order to increase the length of the homologous region of the deletion cassette. The Cg*AUS1* upstream region was amplified using the oligonucleotides CgAUS1_uF (5′-CCCGTTTCACCATCCCATTC-3′) and CgAUS1_uR (5′-GGAGAACTTGTATATGGCTA-3′). Cg*AUS1* downstream region was amplified using oligonucleotides CgAUS1_dF (5′-GAGTGACTCCT ACAAGCTTT-3′) and CgAUS1_dR (5′-TTTCTTGGTCAGAATAGGA G-3′). All three of the resulting PCR products were then used together as templates to create a single large PCR cassette that was amplified using oligonucleotides CgAUS1_uF and CgAUS1_dR. After transformation, the correct integration of the deletion cassette was verified by PCR (see Fig. S1 in the supplemental material) using oligonucleotides CgHIS3_F (5′-CGCACTTCTTGGAGAGTTTC-3′), CgHIS3_R (5′-TACACTCCA

TABLE 1 Strains used in this study

| Strain | Designation | Genotype | Source or reference |
|---|---|---|---|
| *C. albicans* | | | |
| BWP17 | TW19412 | *ura3::λimm434/ura3::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG* | 51 |
| S-7 | TW19413 | *ura3::λimm434/ura3::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG UPC2/upc2::URA3* | 43 |
| D-6 | TW19414 | *ura3::λimm434/ura3::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG upc2::URA3/upc2::ARG4* | 43 |
| EC-2 | TW19415 | *ura3::λimm434/ura3::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG upc2::ARG4/upc2::UPC2-HIS1* | 43 |
| *S. cerevisiae* | | | |
| W303-1a | TW19404 | *MAT**a** ade2-1 leu2-3,112 his3-1 ura3-52 trp1-100 can1-100* | Jasper Rine |
| JRY7179 | TW19405 | *MAT**a** ade2-1 leu2-3,112 his3-1 ura3-52 trp1-100 can1-100 upc2::HIS3* | 44 |
| JRY7180 | TW19406 | *MAT**a** ade2-1 leu2-3,112 his3-1 ura3-52 trp1-100 can1-100 ecm22::TRP1* | 44 |
| JRY7181 | TW19407 | *MAT**a** ade2-1 leu2-3,112 his3-1 ura3-52 trp1-100 can1-100 ecm22::TRP1 upc2::HIS3* | 44 |
| BY4742 | TW19408 | *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0* | 52 |
| YRS1945 | TW19410 | *MAT? his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 aus1::kanMX4 pdr11::kanMX4* | 23 |
| *C. glabrata* | | | |
| KUE200 | TW19037 | *trp1::Scura3 his3::ScURA3 ura3 FRT-YKU80* | 53 |
| upc2AΔ | TW19038 | *trp1::Scura3 his3::ScURA3 ura3 FRT-YKU80 upc2A::HIS3* | 36 |
| upc2AΔ/UPC2A | TW19039 | *trp1::Scura3 his3::ScURA3 ura3 FRT-YKU80 upc2A::HIS3-UPC2A-TRP1* | 36 |
| upc2BΔ | TW19040 | *trp1::Scura3 his3::ScURA3 ura3 FRT-YKU80 upc2B::HIS3* | 36 |
| upc2BΔ/UPC2B | TW19041 | *trp1::Scura3 his3::ScURA3 ura3 FRT-YKU80 upc2B::HIS3-UPC2B-TPR1* | 36 |
| upc2A/upc2B | TW20017 | *trp1::Scura3 his3::ScURA3 ura3 FRT-YKU80 upc2B::HIS3 upc2A::SAT1* | This study |
| aus1 | TW20047 | *trp1::Scura3 his3::ScURA3 ura3 FRT-YKU80 aus1::CgHIS3* | This study |

GGATCAGAGAC-3′), CgAUS1_up_F (5′-CCACTGCAGTTACTTGCA TG-3′), and CgAUS1_down_R (5′-GTAATGGGTCATGTGAGTTC-3′).

**Sterol uptake experiments.** For sterol uptake experiments, CSM plus uridine (CSM+Uri) was supplemented with 0.5 μg (1:4,000) of unlabeled ergosterol or cholesterol/ml (both as 2-mg/ml stocks in Tween 80-ethanol (EtOH) at 1:1 [vol/vol]), together with radiolabeled [³H]ergosterol (1 mCi/ml in EtOH; specific activity, 20 Ci/mmol; American Radiolabeled Chemicals) or [¹⁴C]cholesterol (40 μCi/ml in EtOH; specific activity, 49.78 mCi/mmol; Perkin-Elmer NEN Radiochemicals) to reach an activity of 5 nCi/ml of media. Prior to medium supplementation, the radioactively labeled sterol was mixed together with an appropriate volume of the stock of the identical cold sterol in the Tween 80-EtOH mixture in order to ensure that the sterols are properly dissolved in the CSM+Uri medium. 1 ml of media in 14-ml conical tube was inoculated to optical density at 600 nm ($OD_{600}$) of 0.01 with overnight cultures grown in CSM+Uri. Heat-killed control was always included (80°C for 30 min before incubation). Cultures were grown for 48 h in normoxic or microaerophilic conditions at 30°C with constant shaking at 180 rpm. After 48 h, the cultures were vortexed, and their $OD_{600}$ values were determined (1:20 dilution) to determine the cell number. Samples were filtered on glass microfiber filters (Whatman, 24-mm GF/C) prewet with PBSC/PBSE (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$ [pH 7.4]) supplemented with 1.25% Tween 80 and 50 μg of cold cholesterol (PBSC) or ergosterol (PBSE)/ml. Conical tubes were washed with an additional 5 ml of PBSC/PBSE and filtered with the sample. Filters were transferred to 7-ml scintillation vials, and 5 ml of scintillation cocktail Ecoscint XR (National Diagnostics) was added. After 24 h, the radioactivity associated with the filter was measured with a liquid scintillation counter (Beckman Coulter, LS 6500 multipurpose scintillation counter) and normalized to $10^8$ cells. Absolute numbers of sterol import cannot be compared among separate experiments due to day-to-day variation. All data obtained from one experiment are thus presented as a separate graph.

For the experiments presented in Results, we used the parental strains CaBWP17 and CgKUE200, in which all subsequent mutations were derived. We have analyzed sterol import in the sequenced wild-type strains (CaSC5314 and CgCBS138) and the auxotrophic parental strains (CaBWP17 and CaKUE200). We did not observe any statistical differences.

**Drop test analysis.** Microdilution assays were performed in order to test growth phenotypes in the presence of various supplements. CSM+Uri plates containing 2% agar and additional supplements were used. Fluconazole (FLC) plates contained 256 μg of fluconazole (Sigma-Aldrich)/ml. If the plates contained cholesterol or ergosterol (20 μg/ml), these sterols were added from their stocks (2 mg/ml in Tween 80-EtOH at 1:1 [vol/vol]), in which Tween 80 provides a source of oleic acid.

In a 96-well plate, 1:10 dilution series of strains starting from an $OD_{600}$ of 1.0 in sterile-distilled water ($dH_2O$) were prepared. Dilution series of strains were then transferred from a 96-well plate to the agar plates by using a 48-Replica Plater (DAN-KAR Corp.), transferring ~3 μl of the suspension. Agar plates were incubated at 30°C for 2 days in normoxic or microaerophilic conditions and documented by photography.

**Sterol isolation and thin-layer chromatography (TLC) separation.** Parental strains of *C. albicans* (BWP17), *C. glabrata* (KUE200), and *S. cerevisiae* (W303-1a) were grown in CSM+Uri plus cold ergosterol-cholesterol (0.5 μg/ml; 2 mg/ml stock in Tween 80-EtOH at 1:1 [vol/vol]) and [³H]ergosterol (resulting in a specific activity of 500 nCi/ml of medium) or [¹⁴C]cholesterol (resulting in a specific activity of 5 nCi/ml of medium). Prior to medium supplementation, the radioactively labeled sterol was mixed together with the appropriate volume of the identical cold sterol in Tween 80-EtOH, in order to ensure its proper dissolution into the CSM+Uri. Then, 2 ml of medium in 14-ml conical tubes was inoculated to an $OD_{600}$ of 0.01 with overnight cultures grown in CSM+Uri. Three conditions were used: 48-h cultivation in normoxic or microaerophilic conditions or 5-day cultivation in normoxic condition at 30°C, all with constant shaking of 180 rpm. At the end of cultivation, the cells were washed twice with PBS. Sterol isolation was based on previously published protocols (39). Briefly, the pellet was resuspended in 200 μl of methanol, and 200 μl of acid-washed glass beads (0.5 mm; BioSpec Products) were added. The suspension was vortexed at room temperature for 10 min. Next, 400 μl of chloroform was added, and the suspension was incubated at room temperature for additional 1 h, with a 10-s vortex every 10 min. Finally, the suspension was spun down (16,000 × *g*, 5 min), the supernatant was transferred into a new Eppendorf tube, and the solvent was evaporated in a heating block set at 60°C within a fume hood. The dried pellet was dissolved in 25 μl of methanol-chloroform at 1:1 (vol/vol) and spotted onto precoated TLC plates (silica gel on glass [thickness, 250 μm;

particle size, 2 to 25 μm; mean pore diameter, 60 Å]; Aldrich Chemical Co., Inc.). Sterols were separated in mobile phase of petroleum ether-diethyl ether-acetic acid at 70:30:2 (vol/vol/vol), and the plates were dried out. Radioactivity was detected using phosphor screen and tritium phosphor screen (GE Healthcare) and scanned by Typhoon 9400, Variable mode imager (Amersham Biosciences). For band quantification, ImageJ software (Wayne Rasband, National Institutes of Health) was used.

**Cholesteryl-BODIPY import on plates.** Solid medium CSM+Uri supplemented with 0.25 μg of cholesteryl-BODIPY 542/563 (Molecular Probes)/ml dissolved in Tween 80-EtOH at 1:1 (vol/vol) as a 1-mg/ml stock was used for characterization of sterol import on solid media. Overnight cultures in CSM+Uri were diluted to $OD_{600}$ of 1.0, and 3 μl was spotted onto the plate. Two parallel plates were cultivated for 48 h at 30°C under normoxic and microaerophilic conditions. After the incubation, the fluorescence from the plates was read on a Typhoon 9400 variable-mode imager (Amersham Biosciences) with excitation and emission wavelengths of 532 and 555 nm, respectively. Images were processed with Photoshop CS5 (Adobe).

**MIC.** Normoxic and microaerophilic MICs of the yeast strains for fluconazole were determined by using a protocol based on the CLSI broth microdilution protocol that determines the MIC of drug needed to inhibit 80% of cell growth. Strains were grown in a 96-well plate containing a gradient of drug by using serial 2-fold dilutions. Media used for cultivation were based on CSM+Uri plus 2% glucose and eventually supplemented with 0.5% Tween 80, 10% fetal bovine serum (FBS; Gibco), or 20 μg of cholesterol/ml (a 2-mg/ml stock in Tween 80-EtOH at 1:1 [vol/vol]). The plates were incubated for 48 h at 30°C with constant shaking at 180 rpm. Wells containing no drug served as a positive control for growth (100%), and cell growth in wells containing drug dilutions were standardized to the positive control.

## RESULTS

**Aerobic and anaerobic sterol uptake in three fungal species.** In the present study, we compare sterol uptake in three fungal species *C. albicans*, *S. cerevisiae*, and *C. glabrata*, using both radioactively labeled cholesterol and ergosterol. Cholesterol uptake and metabolism within cells is more thoroughly characterized due to its better commercial availability as a fluorescently or radiolabeled substrate; thus, it is used for most of the uptake studies. Although there are several structural differences between cholesterol and ergosterol, they both efficiently support the growth of *S. cerevisiae* and *C. glabrata* incapable of ergosterol biosynthesis (15, 27). The third species, *C. albicans*, has never been directly analyzed for sterol import. Moreover, most studies characterizing sterol import in *S. cerevisiae* use strains with Sc*HEM1* deleted, which is inducing cell response to hypoxia and promoting aerobic sterol import. In our studies, we use microaerophilic conditions and Sc*HEM1* prototrophs in order to get closer to the true *in vivo* hypoxic situation.

First, we analyzed cholesterol and ergosterol import into the three fungal species (Fig. 1A). For each species, we used a mutant strain deleted for *UPC2* homologs, as well as the standard parental strain. It has been described previously that mutations of Sc*UPC2* or Sc*ECM22* can promote aerobic sterol uptake (20, 21, 40, 41). However, the deletion of Sc*UPC2* and Sc*ECM22* together leads to anaerobic inviability (our observation). The deletion of *UPC2* and its paralogs has the same effect in *C. albicans* (42), *S. cerevisiae*, and *C. glabrata* (Fig. 1A). Upc2 is believed to be involved in the sensing of intracellular sterol levels. In *C. albicans* and *S. cerevisiae*, the deletion of *UPC2* and its paralogs lead to the decrease of cellular ergosterol levels (43, 44).

As seen in Fig. 1A, induction of sterol import under microaerophilic conditions is observed for *S. cerevisiae* (parental strain W303-1a; cholesterol, 25.4-fold upregulation, $P = 0.06$; ergosterol, 5.7-fold

upregulation, $P = 0.05$) and *C. glabrata* (parental strain KUE200; cholesterol, 4.5-fold upregulation, $P < 0.01$; ergosterol, 1.7-fold upregulation $P < 0.01$), while *C. albicans* displays a downregulation (parental strain BWP17; cholesterol, 1.4-fold downregulation, $P = 0.01$; ergosterol, 4.7-fold downregulation, $P < 0.01$).

In *C. albicans* the Ca*UPC2* heterozygous deletion has a similar phenotype to its parent, i.e., no induction of microaerophilic sterol uptake. However, the deletion of both Ca*UPC2* alleles leads to an inability to grow under microaerophilic conditions.

In *S. cerevisiae*, the deletion of either Sc*UPC2* or Sc*ECM22* does not have a significant effect on cholesterol or ergosterol import compared to the parental strain ($P > 0.05$). The deletion of both Sc*UPC2* and Sc*ECM22* leads to an inability to grow under microaerophilic conditions.

In *C. glabrata*, the deletion of either of the *UPC2* paralogs, Cg*UPC2A* or Cg*UPC2B*, does not have any significant effect on microaerophilic cholesterol or ergosterol import. However, deletion of both *UPC2* paralogs, as in *S. cerevisiae* and *C. albicans*, leads to a strain incapable of growth under microaerophilic conditions.

In all cases, heat-killed cells were associated with higher amounts of radiolabeled sterols than any of the aerobically grown strains. We suggest that this is an artifact associated with the heat killing, which disrupts cellular membranes. Heat killing makes cells permeable also to propidium iodide and thus is likely to have significant effects on membrane structure and function (45).

To determine the effect of exogenous sterols on strain growth, we characterized aerobic and microaerophilic growth of *C. albicans*, *S. cerevisiae*, and *C. glabrata* on agar media supplemented with various microaerophilic supplements and drugs affecting ergosterol biosynthesis (Fig. 2). We did not see any significant growth of any of the three fungal species under microaerophilic conditions on media that did not contain both necessary anaerobic supplements for *S. cerevisiae* and *C. glabrata*—oleic acid (in the form of Tween 80) and sterol (cholesterol or ergosterol).

For *C. albicans*, we did not observe microaerophilic growth for the parental or the Δ*upc2*/Δ*upc2* mutant on solid media with or without supplementation with either Tween 80 alone (data not shown) or with Tween 80 and either cholesterol (Fig. 2) or ergosterol (data not shown). In the previous experiment, we saw *C. albicans* growing under microaerophilic conditions in liquid CSM+Uri supplemented with Tween 80 and either cholesterol or ergosterol. Comparing solid and liquid media, we see significantly slower growth on the agar plates of the same composition. Observation of any growth differences on agar media might require significantly longer incubation time. The difference between liquid and agar media is probably caused by a lower rate of oxygen level decrease in liquid media compared to the surface of agar media, directly exposed to the microaerophilic atmosphere. Thus, cells may utilize the limited amounts of oxygen dissolved in liquid medium.

The *S. cerevisiae* parental strain and *S. cerevisiae* Δ*upc2* or Δ*ecm22* single mutants were able to grow microaerophilically if the medium was supplemented with Tween 80 and either cholesterol (Fig. 2) or ergosterol (data not shown). This restoration of microaerophilic growth in the presence of Tween 80 and either cholesterol or ergosterol was observed even in the presence of high concentrations of fluconazole (256 μg/ml), which compromises normoxic growth of these *S. cerevisiae* strains. However, the *S. cerevisiae* Δ*upc2*/Δ*ecm22* double mutant is inviable under microaerophilic conditions, regardless of the plate supplements.
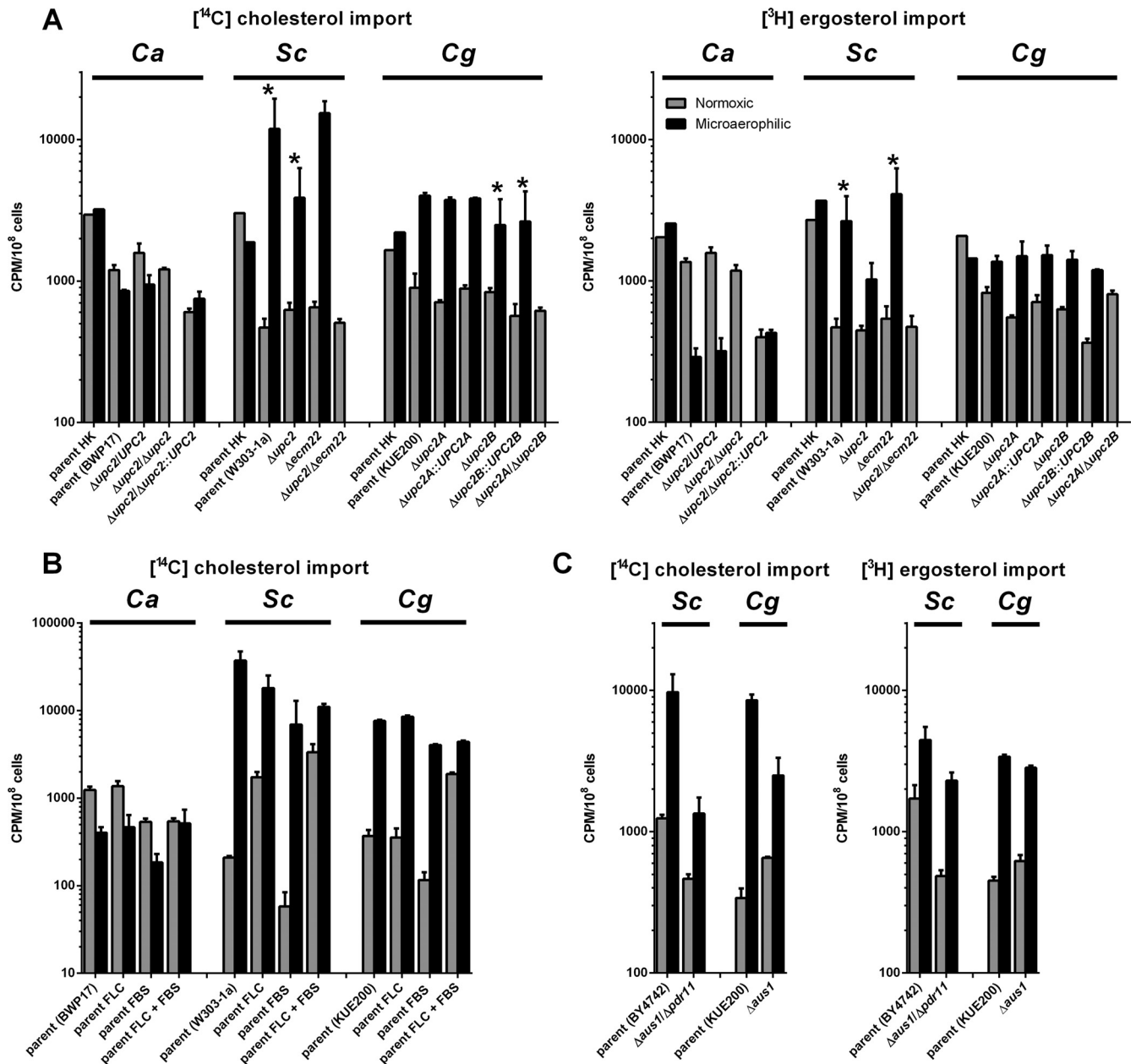
**FIG 1** Sterol uptake in *C. albicans*, *S. cerevisiae*, and *C. glabrata* strains under various conditions. Cholesterol (A, B, and C) and ergosterol (A and C) uptake under normoxic and microaerophilic conditions into the strains of three fungal species. Sterol import was measured in parental strains and their *UPC2*-deficient derivatives (A), parental strains in the presence of fluconazole (FLC) and/or fetal bovine serum (FBS) (B), or in parental strains and their *AUS1*- and *PDR11*-deficient derivatives (C), comparing heat-killed (HK) cells with actively growing strains. The graphs in panel C represent separate experiment from those in panel A and thus are presented separately. In all cases strains were grown for 48 h under normoxic (gray bars) and microaerophilic condition (black bars) in CSM complete supplemented with uridine, oleic acid in the form of Tween 80, and the appropriate sterol (cholesterol or ergosterol) with its radiolabeled form. In the experiment in panel C, fluconazole (FLC; each strain's $MIC_{80}$) and/or fetal bovine serum (FBS; 10%) was added as noted. Samples were normalized to $cpm/10^8$ cells. Graphs represent the average of three biological replicates with the standard errors. All differences between normoxic and microaerophilic conditions are statistically significant as determined by using the Student *t* test ($P < 0.05$), and data pairs marked with asterisks are also significant (to $P < 0.1$). In panel B the difference between *C. albicans* cholesterol uptake in the presence of FLC and FBS is not statistically significant. Heat-killed controls were only performed once due to the number of samples in the experiment. The heat killing causes significant disruption of the cell membranes, as proven by an increase in propidium iodide permeability (45).

The *C. glabrata* parental strain and single mutants can grow microaerophilically if supplemented with Tween 80 and sterols. However, the susceptibility of *C. glabrata* to fluconazole is much lower than *S. cerevisiae* ($MIC_{80}$ of 256 μg/ml for the *C. glabrata* parent versus 32 μg/ml for the *S. cerevisiae* parent). This high MIC allows some growth in the presence of 256 μg of fluconazole/ml. However, all of the *C. glabrata* strains, except *C. glabrata* Δ*upc2A*/Δ*upc2B* double mutant, display increased microaerophilic growth
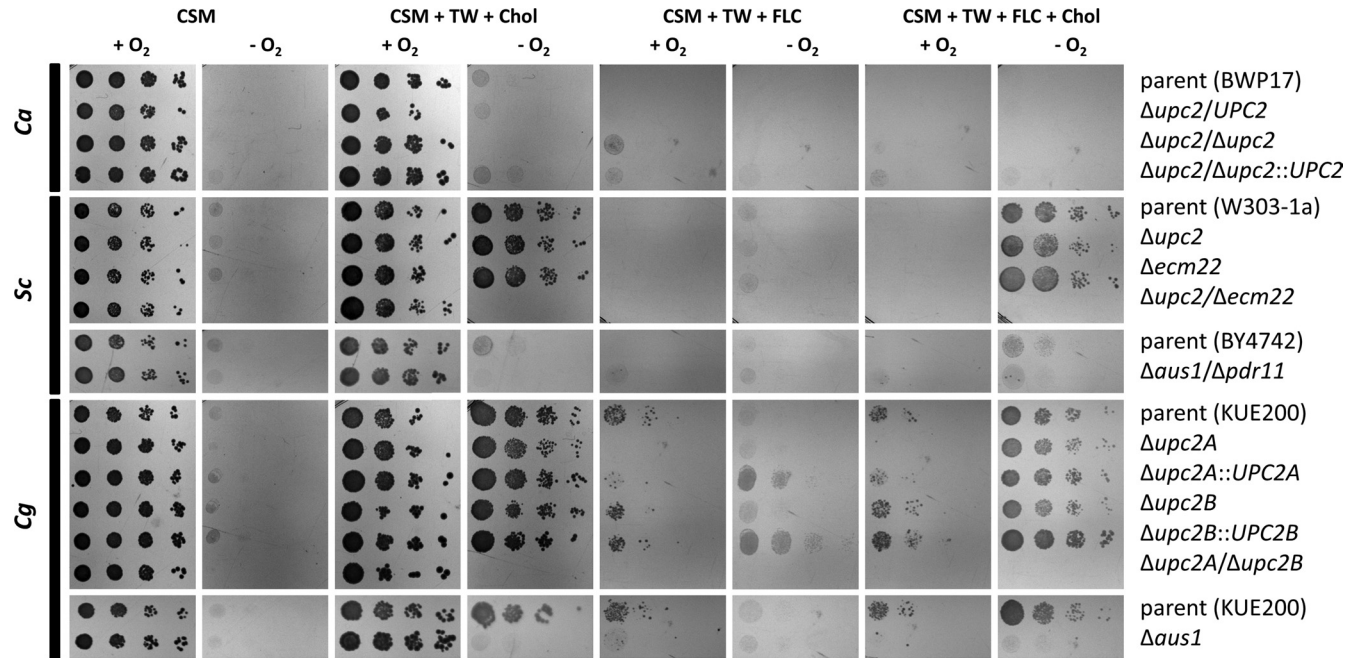
**FIG 2** Normoxic and microaerophilic growth of *C. albicans*, *S. cerevisiae*, and *C. glabrata* strains. A dilution series (10-fold) of cells were grown for 48 h under normoxic and microaerophilic conditions at 30°C on various agar media. CSM (CSM complete supplemented with uridine), TW (Tween 80, 0.1%), Chol (cholesterol, 20 μg/ml), and FLC (fluconazole, 256 μg/ml) were used. *S. cerevisiae* parental strains differ for the *UPC2* mutants (W303-1a) and the *AUS1/PDR11* mutant (BY4742) and thus are presented separately with the appropriate mutants. CSM supplemented with Tween 80 was performed, and it was not significantly different from CSM alone (data not shown).

when sterols are present in the medium, even in the presence of high levels of fluconazole. This is proof that sterol uptake occurs in *S. cerevisiae* and *C. glabrata* under microaerophilic conditions, and they can overcome high concentrations of fluconazole, blocking *de novo* intracellular ergosterol biosynthesis. This is not observed for *C. albicans*.

**Characterization of sterol import in later growth phases.** Interestingly, in some cases we saw an increase of sterol uptake with long-term cultivation under normoxic conditions. Thus, sterol import was analyzed over 5 days of growth both aerobically and microaerophilically and normalized to the cell count (Fig. 3A to F). In general, we see an increase of sterol import associated with aerobically grown *C. albicans* and *C. glabrata* cells, while for *S. cerevisiae* after 1 day, the amount of cell-associated sterols remain constant, or even decreases (Fig. 3A and D). This is the first direct evidence for *C. albicans* sterol import, which has not been previously described.

Under microaerophilic conditions (Fig. 3B and E), *S. cerevisiae* and *C. glabrata* displayed rapid uptake of both sterols with saturation within 2 days. In some cases for these two fungi we observed a decrease in sterols associated with the cells after 4 or 5 days. We do not know the reason for this, but under these conditions sterols could be metabolized and the metabolic products secreted into the medium. In contrast to the other two fungal species, *C. albicans* shows minimum microaerophilic uptake of sterols, although the levels do increase slowly with time.

Because microaerophilic and normoxic growth lead to different efficiencies of carbon and nitrogen source utilization and each yeast species reaches different cell density under these conditions, we also present the percentage of cholesterol taken up from the medium (Fig. 3G and H).

The *UPC2*-null mutants of each of the three fungal species were also tested aerobically in order to determine whether aerobic sterol uptake in later growth phases is Upc2 dependent (Fig. 3C and F). The behavior of all *UPC2* mutants was consistent with their respective parents. Due to a delay in growth, *C. albicans* shows a lag of 24 h before sterol import begins. Thus, sterol uptake in later growth phases is exclusive to the two fungal pathogens, *C. albicans* and *C. glabrata*, and is independent of the presence of Upc2.

Under normoxia, *C. albicans* and *C. glabrata* take cholesterol from the medium relatively slowly but with constant increases over time. These species might use sterols as a nonpreferable carbon source. On the other hand, microaerophilically *S. cerevisiae* and *C. glabrata* take up most of the available sterol in the media within 2 days. This termination of cholesterol uptake might happen when the cells run out of other nutrients in the medium and sterols are no longer needed for cell growth.

**Esterification of radiolabeled sterols and uptake of fluorescently labeled sterol.** The radioactive sterols associated with the cells may have been actively taken up by the cell and incorporated to the plasma membrane, or they may just be bound to the cell wall. Recently, it was shown that the *S. cerevisiae* Δ*aus1*/Δ*pdr11*/Δ*hem1* mutant associates with significant amounts of dehydroergosterol. However, it remains bound to the cell wall and never gets incorporated in the plasma membrane (16). In order to exclude such effect, we grew the parental strains of *C. albicans*, *S. cerevisiae*, and *C. glabrata* under different conditions and in the presence of radioactively labeled cholesterol or ergosterol. Phospholipids and sterols were isolated from the cells and separated on TLC (Fig. 4). The proof of sterol membrane incorporation is their
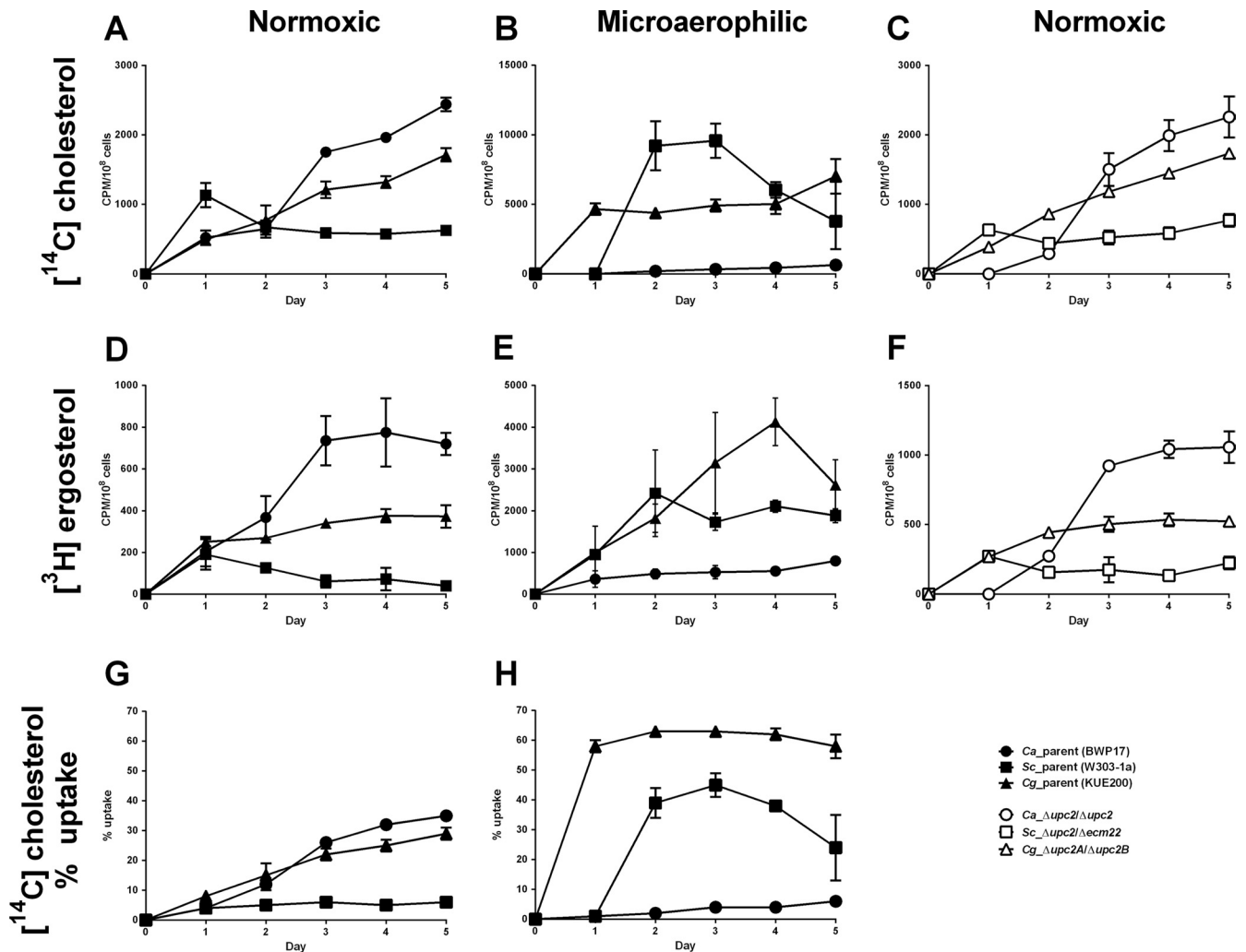
**FIG 3** Time course experiments of sterol uptake into *C. albicans*, *S. cerevisiae*, and *C. glabrata*. Cholesterol (A, B, and C) and ergosterol (D, E, and F) uptake by *C. albicans* (circles), *S. cerevisiae* (squares), and *C. glabrata* (triangles) under normoxic (A, C, D, and F) and microaerophilic (B and E) conditions. Parental strains are represented by solid symbols and *UPC2*-null mutants by open symbols. Strains were grown for 5 days in CSM complete supplemented with uridine, Tween 80, and a mix of cold and radioactively labeled cholesterol-ergosterol. Samples were analyzed every 24 h. The graphs represent average of three biological replicates with standard deviation. (G and H) Cholesterol uptake in three fungal species presented as a percentage of all available cholesterol taken up from the medium under normoxic (G) and microaerophilic (H) conditions. The graphs in panels G and H were created from data presented also in panels A and B.

esterification inside the cells. This can be clearly demonstrated by [¹⁴C]cholesterol uptake (Fig. 4A).

Both *C. albicans* and *C. glabrata* take substantial amounts of radiolabeled cholesterol under normoxic conditions (Chol in Fig. 4A), and some of that labeled cholesterol is esterified (CE in Fig. 4A), which is strong evidence for sterol import. The amounts taken by *S. cerevisiae* are significantly reduced under these conditions. For all three species, the Chol and CE bands are increased after 5 days of normoxic growth.

On the other hand, under microaerophilic conditions, only *S. cerevisiae* and *C. glabrata* take up significant amounts of radiolabeled cholesterol and both of them esterify a portion of it. Very little Chol or CE is observed for *C. albicans*. These findings are consistent with the earlier assays characterizing each species (Fig. 1A and 3), which show that *C. albicans* has reduced import of sterols microaerophilically.

Ergosterol, on the other hand, is poorly esterified after uptake for any species (Fig. 4B). However, the import characteristics of the unmodified ergosterol, in terms of the amounts associated with each fungal species, are consistent with the cholesterol levels. Exogenous ergosterol, in comparison to cholesterol, is known to be poorly esterified in *S. cerevisiae* (18, 26). This appears to be true for all three fungal species.

Another method to show sterol import is to monitor the uptake of fluorescently labeled sterol available in the agar media (Fig. 5). Cell cultures were spotted on the CSM complete agar plates supplemented with uridine and cholesteryl-BODIPY, which is fluorescently labeled cholesterol. The cells were grown for 48 h under normoxic and microaerophilic conditions. The uptake of the fluorescently labeled sterol was indicated by zone of clearing in close proximity of the colony. This study characterizes uptake of sterols by the cells, rather than their fate within the cell. As proposed previously (16), before incorporation to the PM the sterols seem to be bound to the cell wall first. Thus, in this assay we
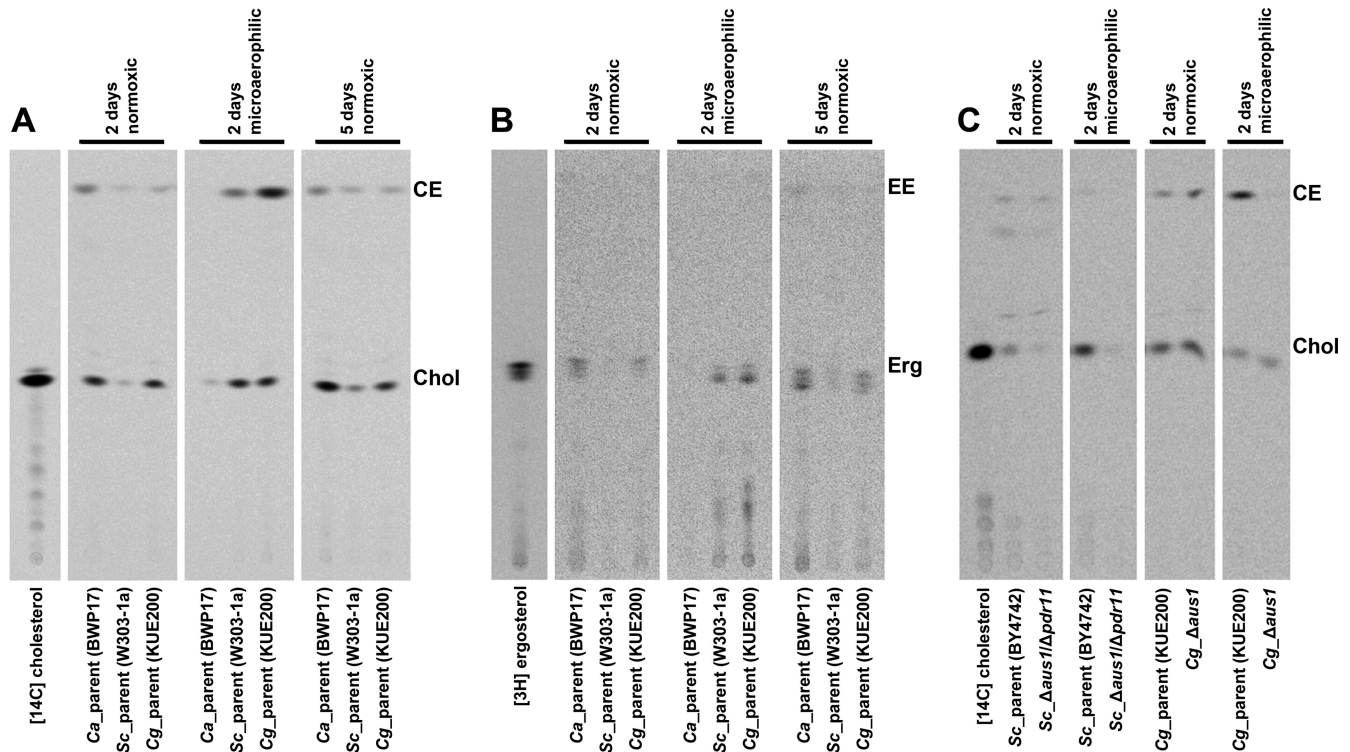
FIG 4 TLC separation of radioactively labeled cholesterol and ergosterol associated with the cells of *C. albicans*, *S. cerevisiae*, and *C. glabrata*. Set of parental strains of the three fungal species were compared to each other under normoxic (2 and 5 days) and microaerophilic (2 days) conditions in the presence of radioactively labeled cholesterol (A) or ergosterol (B). *AUS1/PDR11* mutants were compared to their parents only in the presence of radioactively labeled cholesterol (C). Total cell lipids were extracted from the cells and separated by TLC. The samples represent the amount of cells grown in equal volume of culture medium. Chol, cholesterol; CE, cholesteryl ester; Erg, ergosterol; EE, ergosteryl ester.

cannot distinguish whether the sterols were incorporated into the PM or are just bound to the cell wall.

As expected for *S. cerevisiae* grown in normoxia, no evidence for the sterol uptake can be seen. On the other hand, under microaerophilic conditions, the *S. cerevisiae* parental strain and the *S. cerevisiae* Δ*upc2* and Δ*ecm22* single mutants are capable of sterol uptake from the medium. The anaerobically nonviable *S. cerevisiae* Δ*upc2*/Δ*ecm22* double mutant displays no uptake.

In *C. glabrata*, we see sterol uptake in all strains studied, both in aerobic and microaerophilic conditions. This was quite surprising for the *C. glabrata* Δ*upc2A*/Δ*upc2B* double mutant under microaerophilic conditions, where the cells grow very poorly. *C. glabrata* appears to be the most tolerant species to the low oxygen levels, and the double mutant could probably undergo a limited number of divisions on the agar medium before all of the available oxygen was removed by the anaerobic pouch. The same strain under microaerophilic conditions displayed no measurable growth in liquid medium.

*C. albicans* displays sterol uptake under normoxic conditions and reduced levels under microaerophilic conditions, which is in agreement with previous experiments (Fig. 1A and 3). As expected, the *C. albicans* Δ*upc2*/Δ*upc2* mutant displays no microaerophilic growth, and thus no uptake can be observed, while under normoxic conditions it does not differ from the parental strain, since it is capable of sterol import in later growth stages (Fig. 3C and F).

**Effect of fluconazole and fetal bovine serum on sterol import.** In *S. cerevisiae*, the depletion of ergosterol in the fungal membranes is thought to activate the Upc2-dependent pathway and induce *de novo* sterol synthesis under normoxic conditions or sterol uptake under hypoxia. Drugs such as azoles block ergosterol biosynthesis and activate Upc2 (46). For this reason, we focused on fluconazole and its possible role in sterol uptake in the three fungal species. Fetal bovine serum (FBS) and bile have also been described as substrates that may induce sterol uptake in *C. glabrata* (28). Thus, we analyzed these compounds and their relationship to sterol uptake in the three fungal species.

We tested the parental strains of all three species and used unsupplemented medium, medium supplemented with fluconazole (an $MIC_{80}$ for BWP17 of 1 μg/ml, an $MIC_{80}$ for W303-1a of 32 μg/ml, and an $MIC_{80}$ for KUE200 of 512 μg/ml), 10% FBS, or both (10% FBS and fluconazole [FLC] $MIC_{80}$s) (Fig. 1B). In several cases, there is a small decrease in sterol import in media containing FBS (aerobically and microaerophilically grown *C. albicans*, and aerobically grown *S. cerevisiae* and *C. glabrata*). This is probably caused by the presence of additional cholesterol in the FBS and thus a dilution of the specific activity of [$^{14}$C]cholesterol.

In *C. albicans*, there is little effect on sterol uptake by serum or fluconazole. *S. cerevisiae* responds to the presence of fluconazole in normoxic condition by increased sterol uptake in the absence or presence of serum. However, serum itself has no stimulating effect on *S. cerevisiae* (compared to medium without supplements, FLC increases cholesterol uptake 8.3-fold, $P < 0.01$, FBS decreases it 3.6-fold, $P < 0.01$ and for FLC plus FBS increase 16.0-fold, $P = 0.01$). In the case of FLC-stimulated sterol uptake in *S. cerevisiae*, this increase does not have any effect on the strain's MIC (Fig. 6B).
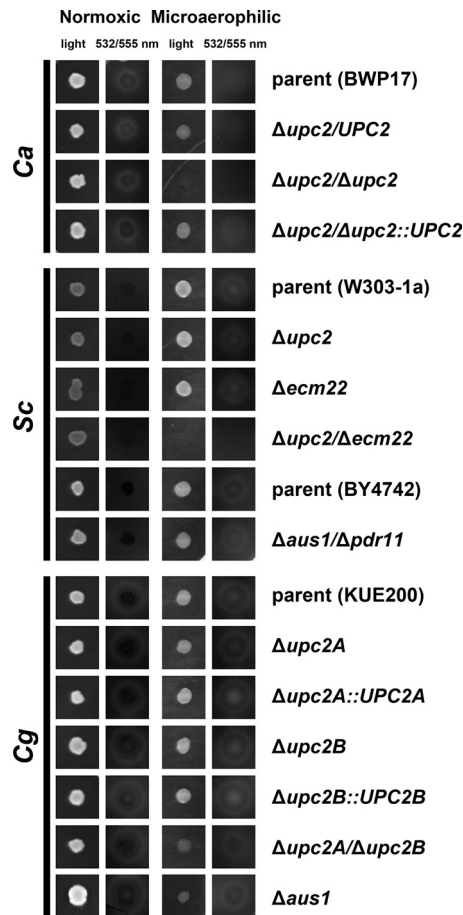
**FIG 5** Uptake of fluorescently labeled cholesterol from agar medium. Cell cultures of *C. albicans*, *S. cerevisiae*, and *C. glabrata* strains spotted on CSM complete supplemented with uridine and cholesteryl-BODIPY (fluorescently labeled cholesterol) were incubated for 2 days under normoxic and microaerophilic conditions and scanned. The zone of clearance (white area) around colonies is indicative of decreased local concentration of cholesteryl-BODIPY and thus active uptake of this sterol. *S. cerevisiae* parental strains differ for the *UPC2* mutants (W303-1a) and *AUS1/PDR11* mutant (BY4742) and thus are presented both separately with the appropriate mutants.

When *S. cerevisiae* is grown anaerobically, it imports sterols from all of the media.

*C. glabrata* does not respond under normoxic conditions to the presence of serum or fluconazole if supplemented separately (compared to unsupplemented medium, no statistically significant change with FLC alone, $P > 0.05$, FBS does downregulate the uptake 3.2-fold, $P < 0.01$). Aerobically, however, the presence of serum and fluconazole together induces sterol uptake (5.1-fold, $P < 0.01$). Anaerobically, *C. glabrata* behaves similarly to *S. cerevisiae* and takes up sterols regardless of other media supplements.

To complement these uptake studies, we determined the MICs of all three species under normoxic and hypoxic conditions in the presence of Tween 80, Tween 80-cholesterol, and Tween 80-cholesterol-FBS (Fig. 6), using both the parental strains and strains deleted for *UPC2* (and its paralogs). For the *C. albicans* parental strain, there is no significant effect of sterol supplementation on the MIC under normoxic or hypoxic conditions. However, supplementation with FBS does increase the strain's resistance under both conditions (Fig. 6A and D). This is surprising, since we do

not see any significant increase in sterol uptake in the presence of serum (Fig. 1B). This does not seem to be an effect of the nutrient components of the medium that would allow better growth but is likely related to the presence of Ca*UPC2*, since this growth advantage is lost in the *C. albicans* Δ*upc2* mutant (Fig. 6G).

Under normoxic conditions *S. cerevisiae* does not increase resistance in the presence of serum, as seen for *C. albicans*, but rather serum reduces resistance, both for the parental strain and *S. cerevisiae* Δ*upc2*/Δ*ecm22* mutant (Fig. 6B and H). Under microaerophilic conditions, the *S. cerevisiae* wild-type becomes more resistant to fluconazole in the presence of cholesterol (Fig. 6E), with no additional effect due to supplementation with serum.

*C. glabrata* behaves as described previously (28). In normoxic conditions the parental strain is able to overcome ergosterol biosynthesis block caused by fluconazole only in the presence of serum, while the supplementation with cholesterol does not provide any advantage (Fig. 6C). This effect, similar to the effect seen with *C. albicans*, is eliminated in the *C. glabrata* Δ*upc2A*/Δ*upc2B* mutant, showing that the effect is Upc2 related (Fig. 6I). However, in *C. glabrata* wild-type, we can link the rescue of growth to the active sterol uptake. Under microaerophilic conditions *C. glabrata* takes up cholesterol regardless of the presence of serum (Fig. 6F). Surprisingly, in the MIC assays, *S. cerevisiae* and *C. glabrata* are able to grow under microaerophilic conditions in medium containing no cholesterol. It is likely that the environment is not completely without oxygen and low oxygen levels allows these strains to synthesize small amounts of their own ergosterol. The other required anaerobic supplement, oleic acid, is present as Tween 80, which has no effect on the microaerophilic MICs compared to the normoxic values.

**Characterization of *S. cerevisiae* Δ*aus1*/Δ*pdr11* and *C. glabrata* Δ*aus1* strains.** Two *S. cerevisiae* ABC transporter paralogs, ScAus1 and ScPdr11, were characterized previously (16–18, 23) and described as responsible for sterol import into fungal cells. Unfortunately, most of the work with these proteins was done in strain backgrounds deleted for Sc*HEM1*, a gene whose deletion turns on a hypoxic response under normoxic conditions and thus not representing true hypoxia. We characterized *S. cerevisiae* Δ*aus1*/Δ*pdr11* mutant under normoxic and microaerophilic conditions. In addition, we characterized the *C. glabrata* Δ*aus1* mutant, deleted for the only known homolog of the ScAus1/ScPdr11 ABC sterol transporters. No homolog has been identified in *C. albicans*.

We determined the cholesterol/ergosterol import into the *S. cerevisiae* Δ*aus1*/Δ*pdr11* and *C. glabrata* Δ*aus1* deletion strains (Fig. 1C). In *S. cerevisiae*, we observed a decrease in cholesterol and ergosterol uptake in the *S. cerevisiae* Δ*aus1*/Δ*pdr11* mutant under both normoxic and microaerophilic conditions. In *C. glabrata*, deletion of Cg*AUS1* under microaerophilic conditions exhibited a decrease in cholesterol uptake, but not ergosterol uptake (compared to the parent in hypoxia, for cholesterol, a 3.4-fold decrease [$P < 0.01$], and for ergosterol, a 1.2-fold decrease [$P = 0.02$]). In normoxia, the amount of cholesterol or ergosterol taken up from the medium by *C. glabrata* Δ*aus1* mutant actually slightly increased (1.9-fold for cholesterol [$P < 0.01$] and 1.4-fold for ergosterol [$P = 0.01$]).

We also checked esterification of the transported cholesterol (Fig. 4C), an indication of its incorporation into the PM. Consistent with the uptake studies, the amount of cholesterol taken up by the *S. cerevisiae* Δ*aus1*/Δ*pdr11* mutant decreased, both under normoxic and microaerophilic conditions. Under normoxic conditions, a fraction of the cholesterol imported by the *S. cerevisiae*
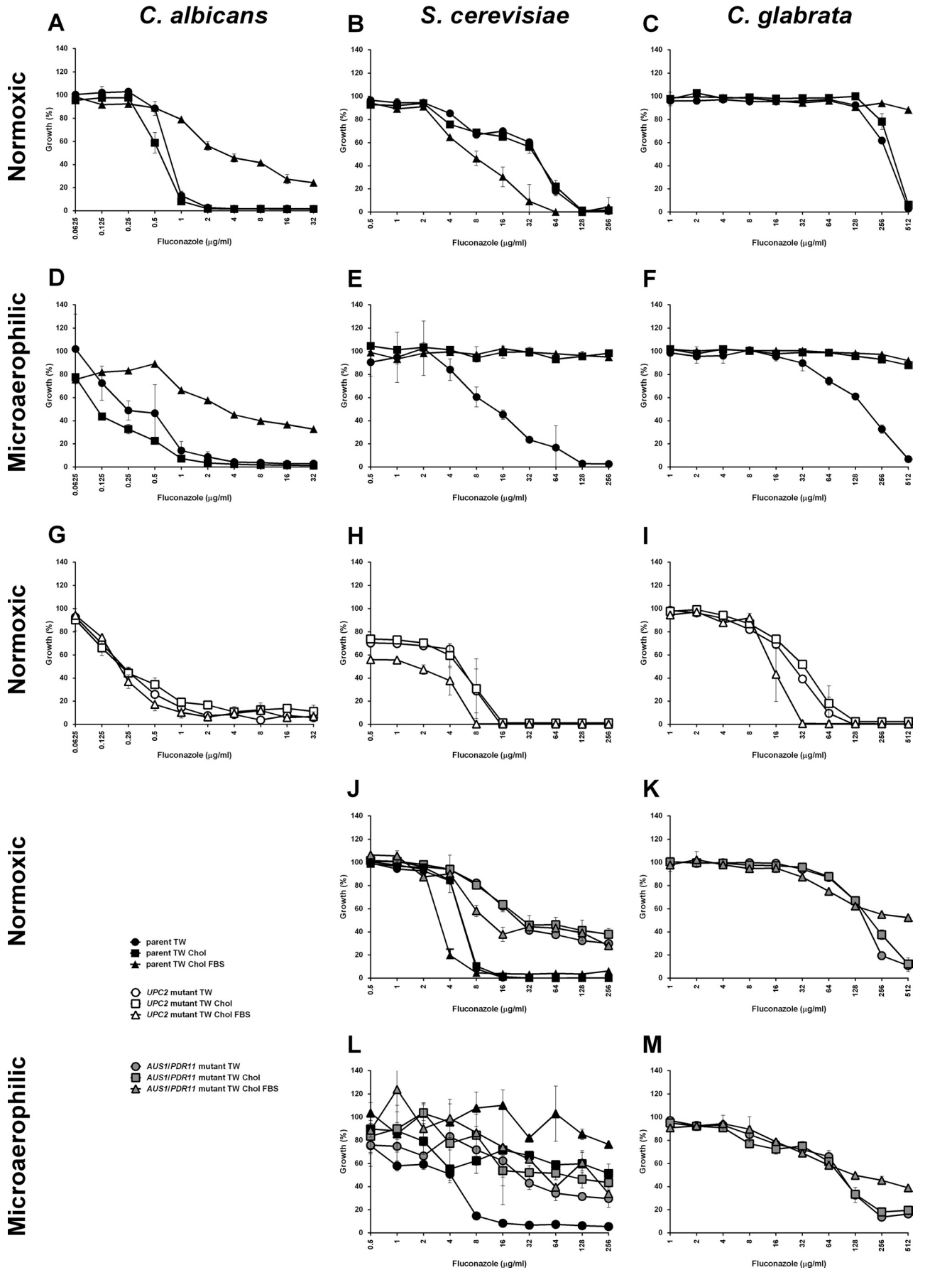
**TABLE 2** Sterol uptake overview

| Condition[a] | Sterol uptake | | |
|---|---|---|---|
| | *S. cerevisiae* | *C. albicans* | *C. glabrata* |
| Aerobic exclusion | ± | − | − |
| Anaerobic exclusion | − | + | − |
| Tween 80 supports microaerophilic growth | ± | − | ± |
| Tween 80 and sterols support microaerophilic growth | + | − | + |
| Sterol supplement overcomes FLC inhibition microaerophilically | + | − | + |
| FBS stimulates sterol uptake | − | − | − |
| FLC stimulates sterol uptake | + | − | − |
| FBS plus FLC stimulates sterol uptake | + | − | + |
| Anaerobic growth of *UPC2* mutant | − | − | − |
| Upc2 involved in serum-stimulated sterol uptake in the presence of FLC | − | − | + |
| Upc2 involved in normoxic post-exponential-phase sterol uptake | − | − | − |
| Aus1/Pdr11 required for aerobic sterol uptake* | + | NH[b] | − |
| Aus1/Pdr11 required for microaerophilic sterol uptake* | + | NH | + |

[a] FBS, fetal bovine serum; FLC, fluconazole. *, In *S. cerevisiae* both ScAus1 and ScPdr11 paralogs are required, while in *C. glabrata*, only one ortholog, CgAus1, is present.
[b] NH, no homolog.

Δ*aus1*/Δ*pdr11* mutant was still esterified (28%). In addition, there are two additional bands on the TLC, which are probably minor forms of chemically modified cholesterol, e.g., acetylated cholesterol. Under microaerophilic conditions, there was no evidence for cholesterol esterification for the *S. cerevisiae* Δ*aus1*/Δ*pdr11* mutant, although the amount of uptake is very low.

In *C. glabrata*, normoxic cholesterol uptake and esterification were clearly evident. The *C. glabrata* Δ*aus1* mutant displayed no decrease in cholesterol uptake under normoxic conditions, and esterified this sterol extensively (30% compared to 22% in the parent), while under microaerophilic conditions, there is no evidence for esterified cholesterol (compared to 68% in parent), and the unmodified cholesterol levels are comparable to those for the parent.

Using drop tests (Fig. 2), we could see a decrease in microaerophilic growth for both *S. cerevisiae* Δ*aus1*/Δ*pdr11* and *C. glabrata* Δ*aus1* mutants. However, *S. cerevisiae* parental strain BY4742 (S288C derived) displays worse microaerophilic growth in the presence of sterols than the other parent, W303-1a, background for the Sc*UPC2* and Sc*ECM22* deletions. The strain S288C grows worse under microaerophilic conditions, because it has a Ty1 insertion in the carboxy terminus of Sc*HAP1* gene, a transcription factor important for the regulation of genes dependent on heme/oxygen levels (47).

MICs of *S. cerevisiae* Δ*aus1*/Δ*pdr11* and *C. glabrata* Δ*aus1* mutants in presence or absence of cholesterol and serum were also checked (Fig. 6J, K, L, and M). Surprisingly, the deletion of Sc*AUS1* and Sc*PDR11* in *S. cerevisiae* led to tolerance of high fluconazole concentrations under normoxic and microaerophilic conditions, regardless of the supplements. However, this strain does not grow on agar media supplemented with sterols (Fig. 2).

This is again most likely related to low oxygen levels available in the liquid medium. Deletion of Cg*AUS1* in *C. glabrata* led to more predictable results. Under microaerophilic condition, there is no positive effect of supplemented cholesterol on growth, and also the effect of FBS supplementation seems to be somewhat reduced, although it still provides little growth advantage.

Last, when sterol import of the fluorescently labeled cholesterol was investigated using these strains (*S. cerevisiae* Δ*aus1*/Δ*pdr11* and *C. glabrata* Δ*aus1*), there was no change from their parents under normoxic or microaerophilic conditions (Fig. 5). Thus, it seems that the collecting of the fluorescently labeled sterol from agar medium in the colony proximity is not affected by the presence of *AUS1*/*PDR11* in these two species.

## DISCUSSION

In this study, we have characterized sterol import in three fungal species: *C. albicans*, *S. cerevisiae*, and *C. glabrata*. We have used radioactively labeled versions of the fungal sterol, ergosterol, as well as the major sterol in higher eukaryotes, cholesterol. In general, for all three fungal species, we have not seen any clear difference in import of these two sterols. However, we observed their different fates within the cells. In *S. cerevisiae*, cholesterol gets extensively esterified, while ergosterol remains mostly intact (18, 26). We have confirmed this observation in *S. cerevisiae*, and expanded this analysis to two additional fungi, *C. albicans* and *C. glabrata*. In addition, esterification of cholesterol proves that these sterols are incorporated into the membranes, and not bound to the cell wall unmodified, as reported previously for some cases (16). Overall, growth and sterol uptake under all tested conditions are summarized in Table 2.

**FIG 6** MICs determined for *C. albicans*, *S. cerevisiae*, and *C. glabrata*. MICs determined under normoxic (A, B, C, G, H, I, J, and K) and microaerophilic (D, E, F, L, and M) conditions for *C. albicans* (A, D, and G), *S. cerevisiae* (B, E, H, J, and L), and *C. glabrata* (C, F, I, K, and M). Parental strains (CaBWP17, ScW303-1a/ScBY4742, and CgKUE200) are represented by black solid symbols, *UPC2*-null mutants (CaΔ*upc2*/Δ*upc2*, ScΔ*upc2*/Δ*ecm22*, and CgΔ*upc2A*/Δ*upc2B*) by open symbols and *AUS1*/*PDR11* mutants (ScΔ*aus1*/Δ*pdr11* and CgΔ*aus1*) by gray solid symbols. Circles represent medium supplemented with Tween 80, squares represent medium supplemented with Tween 80-cholesterol, and triangles represent medium supplemented with Tween 80-cholesterol-FBS. *S. cerevisiae* parental strains differ for the *UPC2* mutants (W303-1a; graphs B, E, and H) and *AUS1*/*PDR11* mutant (BY4742; graphs J and L) and thus are presented both in separate sections of the figure. Parental strain BY4742 is presented together with its ScΔ*aus1*/Δ*pdr11* derivative (J and L). Fluconazole concentrations did not exceed 256 μg/ml, with the exception of 2-fold increase for *C. glabrata*, in order to prevent reaching of critical micelle concentration for this compound. Graphs represent the average of three biological replicates with standard errors.

*S. cerevisiae* is the best-characterized species in terms of sterol import of these three fungi. *S. cerevisiae* takes up sterols exclusively under hypoxic conditions and not under normoxia. This is known as "aerobic sterol exclusion" (12). The uptake of sterols under hypoxia is the only option due to the inability of *S. cerevisiae* to synthesize ergosterol in the absence of molecular oxygen and the inability to grow without sterols present in its membranes. However, we do see limited amounts of aerobically imported sterols during the initial 24 h of growth, with evidence of esterification for cholesterol. When we tested sterol import in later growth stages in normoxia, we did not seen any increase in sterol uptake over time, which is in agreement with previous studies (15). Interestingly, we saw a slight stimulation of sterol import in *S. cerevisiae* in the presence of fluconazole. This is in agreement with the finding that treatment of *S. cerevisiae* with lovastatin also increases cholesterol import and blocks its esterification (13). However, the sterol import we observed was insufficient to substitute for endogenous ergosterol biosynthesis or alter the strain MIC in the presence or absence of sterols.

The second organism, *C. glabrata*, is often discussed in the literature as an organism with low fluconazole susceptibility due to active sterol import. However, its low susceptibility is demonstrated even in media without sterols available. Under hypoxia, *C. glabrata* behaves similar to *S. cerevisiae* and takes up exogenous sterols, because it has the same need for hypoxic supplements. On the other hand, *C. glabrata* is capable of slow sterol import under normoxic conditions, mostly in the postexponential phases of growth. When grown in the presence of fluconazole and/or exogenous sterols in liquid medium, no increase in sterol uptake can be detected, as described previously (28). Moreover, although the sterol transporter Cg*AUS1* was described to be transcriptionally induced by the presence of serum in the liquid medium within a matter of hours (28), we did not observe any alteration in sterol import. Only when serum was present together with high concentrations of fluconazole was sterol import induced. For the induction of aerobic sterol uptake, *C. glabrata* appears to require both a block in ergosterol biosynthesis and the presence of serum. We have some indications that this induction of sterol uptake is not limited to serum or bile but could also be promoted in media containing peptone, such as YEPD (data not shown). The nature of the inducing compound has not yet been identified.

In *C. albicans* grown under normoxic conditions, there is a clear increase in sterol uptake over time, with evidence of esterification. Therefore, it is successfully incorporated in the membranes and reaches the ER. When grown under microaerophilic conditions, little sterol uptake is seen. We suggest that this is "anaerobic sterol exclusion," the opposite of aerobic sterol exclusion as seen in *S. cerevisiae*. The growth of *C. albicans* in the presence of high fluconazole levels is increased when the liquid media is supplemented with FBS and is dependent on Upc2. Despite this increase in growth, there is no evidence for increased sterol uptake. Interestingly, the growth of *C. albicans* on agar media is severely reduced under microaerophilic conditions, regardless of the medium supplements, suggesting significant differences between growth in liquid and on solid supports. This is probably caused by the rate of oxygen level decrease in air versus liquid. We have also checked sterol import in *C. albicans* hypha-stage-locked mutants Ca*nrg1* and Ca*tup1*. We do not see any alterations in sterol import for these mutants (data not shown).

For all three species, the *UPC2* genes are linked to sterol uptake.

In *S. cerevisiae* Sc*UPC2* transcription is induced by treatment with statins or azoles and by growth in microaerophilic conditions (40, 46, 48). Constitutively active mutants in *S. cerevisiae* can promote sterol uptake (20, 21, 40, 41). In *C. albicans*, the deletion of both *UPC2* alleles results in reduced normoxic cholesterol uptake in RPMI medium (43), although we have not observed this for CSM in the present study. Moreover, in *C. glabrata* both the presence of serum and ergosterol depletion caused by lovastatin induce Cg*UPC2B* expression (36). Deletion of *UPC2* and its paralogs results in anaerobic inviability in all three organisms. However, it has not been determined that the lack of anaerobic growth is solely due to impaired sterol import. In support of this, *C. albicans* wild-type does not appear to depend on sterol import under microaerophilic conditions, and *AUS1/PDR11* mutants of *S. cerevisiae* and *C. glabrata* seem to be microaerophilically viable.

By characterizing *S. cerevisiae* Δ*aus1* Δ*pdr11* and *C. glabrata* Δ*aus1* mutants, we confirmed the importance of these ABC transporters for sterol uptake. In *S. cerevisiae* the deletion of the *AUS1* and *PDR11* genes negatively affected sterol uptake under both normoxic and microaerophilic conditions. However, in *C. glabrata*, the deletion of *AUS1* only had an effect on the uptake of cholesterol under microaerophilic conditions. The deletion of *AUS1* and *PDR11* in *S. cerevisiae* has also an interesting effect; it improves growth under high fluconazole concentrations. We can only speculate on this. The partial intracellular localization of ScAus1 (18) may have an effect on membrane composition and sterol distribution within cells. Overall, this phenomenon remains to be properly characterized. The deletion of *AUS1* and *PDR11*, both in *S. cerevisiae* and *C. glabrata*, does not negatively affect their microaerophilic growth in medium supplemented with Tween 80 and sterols. It is possible that the uptake of unsaturated fatty acids (Tween 80) and low oxygen levels allow the synthesis of some ergosterol, sufficient for their growth. However, the oxygen levels may not be enough to allow for growth of the *UPC2* mutants. We can speculate that at least in *C. glabrata* there is an alternative method of normoxic sterol import. This might be also the case in *C. albicans*, which is also capable of normoxic sterol import, although it has no Sc*AUS1*/Sc*PDR11* homolog.

Compared to *S. cerevisiae* (20), constitutively active mutation of Upc2 in *C. albicans* increases the strains MIC (49), but it does not lead to an increase of sterol import (our unpublished observation). Moreover, the deletion of *HEM1* in *C. albicans* does not promote sterol uptake as in *S. cerevisiae* and *C. glabrata* (27, 50). Sterol uptake in *S. cerevisiae* and *C. glabrata* under these situations is most likely linked to the Aus1/Pdr11 proteins, and the absence of the ortholog in *C. albicans* may be responsible for its missing sterol uptake under these conditions. On the other hand, *C. albicans* and *C. glabrata* can take up significant amounts of sterol under normoxia, even in the absence of Aus1/Pdr11 orthologs. Passive diffusion is not likely, since it is not observed in *S. cerevisiae* (Fig. 3). These organisms, unlike *S. cerevisiae*, accumulate increasing amounts of sterols as the growth of their culture progresses. The amount of sterols taken up after 5 days of cultivation is significant, ca. 30%, while in case of *S. cerevisiae* it is 6%. This suggests that sterols may be imported by facilitated diffusion through an as yet unidentified transporter. There is evidence that both organisms, *C. albicans* and *C. glabrata*, "collect" sterols from agar medium in their proximity under normoxic conditions, whereas *S. cerevisiae* does not. Overall, this area of transport remains yet to be explored.

It is still unclear how *C. albicans* grows under microaerophilic or anaerobic conditions. Anaerobic growth has been partially characterized (33) and the authors speculated that the early oxygen-dependent step in ergosterol biosynthesis, squalene epoxidation, might use oxygen from water. However, the significantly decreased susceptibility of anaerobically grown *C. albicans* to both azoles and polyenes in their work indicate decreased levels of synthesis or complete absence of ergosterol in membranes. Unfortunately, the ergosterol levels of anaerobically grown *C. albicans* have never been determined. In our microaerophilic system, we could see a decrease in ergosterol levels by >50% (data not shown). However, the growth under true anaerobic conditions still could lead to total absence of ergosterol or alteration of its synthesis.

This study brings new understanding of the response of two pathogenic fungi, *C. albicans* and *C. glabrata*, to treatment with drugs affecting sterol biosynthesis, the azoles. Although clinically this class of drugs works well against *C. albicans*, their effect against *C. glabrata* is limited. The explanation would be that while *C. albicans* relies mostly on internal ergosterol biosynthesis, which can be negatively affected by azole treatment, *C. glabrata*, besides its high azole resistance, can overcome the block in ergosterol biosynthesis by active transport of exogenous sterols. Although azoles work against *C. glabrata in vitro*, their importance *in vivo* seems to be limited, since human body provides conditions favoring the stimulation of sterol import from the fungal environment.

## ACKNOWLEDGMENTS

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