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The Production of Reactive Oxygen Species Is a Universal Action Mechanism of Amphotericin B against Pathogenic Yeasts and Contributes to the Fungicidal Effect of This Drug

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Amphotericin B (AMB) is an antifungal drug that binds to ergosterol and forms pores at the cell membrane, causing the loss of ions. In addition, AMB induces the accumulation of reactive oxygen species (ROS), and although these molecules have multiple deleterious effects on fungal cells, their specific role in the action mechanism of AMB remains unknown. In this work, we studied the role of ROS in the action mechanism of AMB. We determined the intracellular induction of ROS in 44 isolates of different pathogenic yeast species (*Candida albicans*, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Cryptococcus neoformans*, and *Cryptococcus gattii*). We also characterized the production of ROS in AMB-resistant isolates. We found that AMB induces the formation of ROS in all the species tested. The inhibition of the mitochondrial respiratory chain by rotenone blocked the induction of ROS by AMB and provided protection from the killing action of the antifungal. Moreover, this phenomenon was absent in strains that displayed resistance to AMB. These strains showed an alteration in the respiration rate and mitochondrial membrane potential and also had higher catalase activity than that of the AMB-susceptible strains. Consistently, AMB failed to induce protein carbonylation in the resistant strains. Our data demonstrate that the production of ROS by AMB is a universal and important action mechanism that is correlated with the fungicidal effect and might explain the low rate of resistance to the molecule. Finally, these data provide an opportunity to design new strategies to improve the efficacy of this antifungal.

The antifungal drug amphotericin B (AMB) has successfully been used for the treatment of the most common invasive fungal diseases since it was first licensed in the 1950s (1). Although new antifungals have been developed in the last 2 decades, AMB is the first choice for the treatment of diseases such as cryptococcosis and zygomycosis (2, 3) and is an alternative therapy for aspergillosis, invasive candidiasis, and other systemic fungal infections (4–7). In addition, it is also widely used among pediatric patients (8–10). Conventional AMB produces renal toxicity (11–15), and therefore new lipidic combinations have been developed to improve the delivery of the drug and decrease its toxicity (16–21).

The classical action mechanism involves AMB binding to membrane ergosterol, with subsequent pore formation and ion leakage (22–26). However, different findings suggest that this is not the main action of this molecule. For example, potassium loss after AMB exposure does not correlate with fungal death (27). The chemistry of AMB has been widely studied, with particular emphasis on how chemical modifications of the molecule affect its biological activity (see seminal reviews in references 28–32). Some of these molecular changes that result in the inability to form pores still retain antifungal activity (33), suggesting that AMB acts through ergosterol sequestration (34).

In addition, it has been shown that AMB induces the intracellular accumulation of reactive oxygen species (ROS) (35–38). The addition of free radical scavengers, such as catalase and/or superoxide dismutase, protects *Candida albicans* protoplasts from AMB (35). Furthermore, a genome-wide expression analysis confirmed that AMB not only affects the expression of genes involved in ergosterol synthesis but also promotes the expression of stress genes (39). AMB has immunomodulatory properties in mammalian cells, which have been shown to be related to its toxicity and proinflammatory responses (see reviews in references 13, 40).

The role of oxidative damage after AMB treatment of fungal cells remains to be elucidated. Under physiological conditions, free radicals are produced in the mitochondria as natural by-products of the respiratory chain (see review in reference 41), and they contribute to signaling, metabolic adaptation, and immunity (42). However, under conditions for which the endogenous concentration of ROS increases (i.e., in the presence of oxidants, UV light, or other external stimuli), they produce alterations in different macromolecules (proteins, lipids, and DNA), which eventually lead to cell damage and death. For these reasons, ROS have been associated with apoptosis and aging (43, 44). The complex I of the electron respiratory chain (NADH/ubiquinone dehydrogenase) plays a crucial role in the regulation of mitochondrial activity and ROS production. In fact, if this activity is bypassed, it produces an increase in the life span of *Drosophila melanogaster* (45). Protection

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against ROS is achieved by the presence of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase, and peroxiredoxins. Several studies demonstrate that AMB can autooxidize, and as consequence, oxidative stress is induced (46). On the other hand, it has been demonstrated that AMB can also act as an antioxidant, similar to carotenoids and retinoids (47, 48). In agreement, the killing effect of AMB is enhanced by the addition of prooxidants (49, 50).

In this work, we studied how AMB stimulates the formation of ROS in pathogenic yeast species to investigate if the induction of oxidative burst is a general phenomenon after treatment with AMB. Moreover, we investigated the mechanism of resistance to this antifungal. We conclude that ROS are important mediators in the fungicidal effect of AMB.

MATERIALS AND METHODS

Yeast strains and growth conditions. The strains used in this study are shown in Table 1. We chose several strains (4 to 7) from the main species of pathogenic yeasts and 44 isolates in total (see Table 1). In addition, we used two *Candida tropicalis* isolates with reduced susceptibility to AMB: ATCC 200956 and CL-6835 (from the yeast collection of the Mycology Reference Laboratory of the Spanish National Centre for Microbiology [51]). Another *C. tropicalis* strain, TP-13650, was resistant to azoles but susceptible to AMB (51). Yeast cells were routinely grown in liquid Sabouraud medium (Oxoid, Ltd., Basingstoke, Hampshire, England) at 30°C with moderate shaking (150 rpm). In some experiments, yeast nitrogen base (YNB) medium (Difco) was used.

Antifungal susceptibility testing. Antifungal susceptibility testing (AFST) was performed according to the EUCAST protocol (52, 53). AMB (Sigma-Aldrich, Madrid, Spain) was used at concentrations between 0.03 and 16 mg/liter. RPMI medium was supplemented with 2% glucose. The yeasts were inoculated in flat-bottom 96-well plates at 1×10^5 to 5×10^5 cells/ml and then incubated at 35°C without agitation. The optical density of the plates was measured after 24 h, and the MIC_{90} (for AMB) was defined as the antifungal concentration that produced a >90% decrease in the optical density (OD) of the well compared to the OD of the control well.

Amphotericin B exposure and detection of ROS with fluorescent probes. To expose the cells to AMB, we used the protocol described in Sangalli-Leite et al. (37). Briefly, the cells were grown overnight in 10 ml of liquid Sabouraud medium, and then they were washed twice with sterile phosphate-buffered saline (PBS). A suspension of 10^7 cells/ml (2×) from each strain was prepared in PBS. Several AMB concentrations (ranging from 16 mg/liter to 0.03 mg/liter) were added to the samples, which were incubated at 37°C for different time periods (1 and 3 h). The production of ROS was measured using dihydrofluorescein diacetate (DHF), which produces fluorescence after being attacked by reactive oxygen species (ROS) that can be easily visualized as green fluorescence by microscopy and quantified by flow cytometry. Therefore, after the incubation period with AMB, DHF was added at a final concentration of 40 μM. After 30 min of incubation at 37°C, fluorescence was measured by flow cytometry in the FL1 channel. A total of 10,000 cells were recorded using a FACSCalibur cytometer (FL-1 channel, CellQuest; BD Biosciences), and the percentage of stained cells was obtained. The data were processed using the FlowJo 7.1.6. software (OR, USA). Control cells nonexposed to AMB or H_2O_2, in addition to nonstained cells, were also included. Two different parameters were evaluated, with the first being the percentage of cells that produced ROS. For this analysis, control histograms from the untreated samples were subtracted from the histograms of each AMB-treated aliquot. In this way, it was possible to define the proportion of cells that accumulated higher fluorescence than that of the control cells. The other parameter was the intensity of the cell fluorescence, which is an estimate of the amount of free radicals produced inside the cells. For this purpose, a histogram of the positive cells was obtained by subtracting the histogram of the control cells from the histogram of the cells exposed to AMB, using the histogram tools from the CellQuest software. Thus, we excluded the cells that did not produce ROS from the population of cells exposed to AMB, and we concentrated only on the cells that responded to the antifungal. Next, we obtained the geometric mean (GM) of the fluorescence intensity from this population and compared it with the geometric mean of the control cells not exposed to AMB. In this way, we obtained a fold change in fluorescence ($GM_{positive\ cells}/GM_{control\ cells}$), which gives an estimation of the increase of ROS after the addition of AMB.

TABLE 1 List of pathogenic yeasts used for determination of ROS

Species	Strain	Source or reference	MIC to AMB (mg/liter)
C. albicans	SC5314	89	0.12
	CL-8752	YC-SNCM[a]	0.06
	CL-8756	YC-SNCM	0.06
	CL-8790	YC-SNCM	0.06
	CL-8191	YC-SNCM	0.06
C. glabrata	CL-8164	YC-SNCM	0.03
	CL-8754	YC-SNCM	0.12
	CL-8753	YC-SNCM	0.12
	CL-8196	YC-SNCM	0.06
C. parapsilosis	CL-8717	YC-SNCM	0.12
	ATCC 22019	ATCC	0.25
	CL-8722	YC-SNCM	0.06
	CL-8708	YC-SNCM	0.12
	CL-8707	YC-SNCM	0.12
C. tropicalis	CL-8698	YC-SNCM	0.12
	CL-8715	YC-SNCM	0.12
	CL-8796	YC-SNCM	0.12
	CL-8747	YC-SNCM	0.25
	CL-8759	YC-SNCM	0.06
	ATCC 750	ATCC	0.25
	CL-7099	YC-SNCM	0.25
	CL-7119	YC-SNCM	0.25
C. krusei	CL-8059	YC-SNCM	0.25
	CL-8076	YC-SNCM	0.25
	CL-8197	YC-SNCM	0.5
	CL-8285	YC-SNCM	0.25
	ATCC 6258	ATCC	0.25
	CL-8827	YC-SNCM	0.5
C. neoformans	CL-5707	YC-SNCM	0.06
	B3501	90	0.06
	KN-99	91	0.06
	ATCC 24067	ATCC	0.06
	H99	92	0.03
C. gattii	CBS-10514 (R265)	93	0.06
	NIH 191	94	0.06
	NIH 198	94	0.03
	CBS-10865 (R272)	93	0.06
	NIH 34	94	0.06
T. asahii	CL-6331	YC-SNCM	8
	CL-6579	YC-SNCM	>16
	CL-6809	YC-SNCM	>16
	CL-7717	YC-SNCM	8
	CL-7784	YC-SNCM	>16

[a] YC-SNCM, yeast collection from the Spanish National Centre for Microbiology.

Protein carbonylation. The cells were grown as described above. The strains were treated for 3 h with AMB (2 mg/liter) or H_2O_2 (2.5 mM). Total cell extracts were obtained, and the protein concentrations were determined using the Bradford method with the protein assay kit (Bio-Rad, Munich, Germany). The damage of free radicals on the cells was evaluated by protein carbonylation analysis by Western blotting after the derivatization of carbonyl groups with dinitrophenylhydrazine (DNPH) (Sigma), as described previously (54, 55). The blotted membranes were incubated with a rabbit anti-DNPH as the primary antibody (Sigma) at a 1:2,000 dilution for 1 h, followed by the addition of a secondary anti-rabbit antibody at a 1:10,000 dilution (Amersham). The experiment was repeated twice on different days, with similar results.

The relative intensity of the carbonylated protein bands and the respective control gel stained with Coomassie blue was calculated using the ImageJ software (National Institutes of Health; see http://rsb.info.nih.gov/ij). Three representative areas of the wells were selected, and a relative plot of the intensity was estimated as the ratio of the intensity between the well of the carbonylated protein and the intensity of the well of the Coomassie blue gel. The control well (without any treatment) was given an arbitrary value of 100, and the value for the treated samples was expressed as a percentage relative to the value of the control well. Parallel untreated controls were carried out.

Catalase activity. Catalase was assessed as described elsewhere (56). Briefly, the cell samples were harvested, washed, and suspended at known densities (10 to 15 mg/ml [wet weight]) in 100 mM 2-morpholine-ethanesulfonic (MES) buffer (pH 6) supplemented with 5 mM cysteine and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were broken after adding 1.5 g of Ballotini glass beads (0.45-mm diameter) by vigorous shaking in a vortex (6 cycles of 45 s) alternating with periods on ice between each cycle. The extracts were centrifuged, and enzymatic activity was determined in the supernatants. Catalase was assessed spectrophotometrically at 240 nm following H_2O_2 decay (57). For the catalase assay, 25 μl of extract was added to 1.475 ml of a solution containing 10.6 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7). The protein concentration was determined using bovine serum albumin as the standard. Specific activity was expressed as nmol/min/mg of protein.

Growth curves. To perform growth curves, the yeast cells were suspended at a concentration of 10^5 cells/ml in YNB supplemented with 2% glucose or a mixture of 3% glycerol plus 2% ethanol, and 200 μl was placed in 96-well plates. The plates were incubated at 35°C without shaking for 48 h, and the optical density at 530 nm was monitored every hour in a Labsystems iEMS reader MF spectrophotometer. The results were plotted as OD versus time.

In some experiments, the yeast cells were suspended in Sabouraud liquid medium (2×) at 2×10^5 cells/ml, and 75 μl was placed in 96-well plates. Next, 75 μl of H_2O was added. In some cases, rotenone (Sigma-Aldrich, St. Louis, MO, USA) was added at different concentrations to test the optimal amount that inhibited cell growth (58, 59). From a 100 mM stock prepared in 100% dimethyl sulfoxide (DMSO), different rotenone working stocks (2× [5 to 0.32 mM]) were prepared by diluting the inhibitor in H_2O. Parallel tubes containing the equivalent concentration of DMSO without rotenone were prepared. Next, 75 μl of the rotenone (2×) or DMSO solvents was mixed in the 96-well plates with 75 μl of yeast cells, prepared as described above. The microtiter plate was incubated at 35°C in a Labsystems iEMS reader MF spectrophotometer, and a growth curve was performed as described above. From those wells in which rotenone inhibited cell growth, we took 7 μl and placed it on a Sabouraud agar plate. Similar samples from the control wells and wells containing DMSO were also placed on the same Sabouraud agar plate, which was incubated at 30°C for 2 days.

Detection of ROS in the presence of rotenone. The effect of rotenone on the cells was assessed as adapted from published protocols (58, 59), with the following modifications. To evaluate the role of rotenone in the production of ROS induced by AMB, we chose inhibitor concentrations that inhibited cell growth but did not kill the cells. These concentrations were obtained from the growth curves described in the section above. Rotenone was again prepared as 2× stocks from a 100 mM solution in 100% DMSO. Five hundred microliters from these 2× stocks was mixed with 500 μl of yeast cells prepared at 10^7 cells/ml in PBS, as described above. After sample incubation at 37°C for 1 h, AMB was added at different final concentrations (0.5, 1, and 2 mg/liter) from a stock solution (800 mg/liter in DMSO), and the incubation was continued at 37°C for another hour. After this incubation, DHF was added to the cells, and the production of ROS was evaluated 30 min later as described above. In parallel, and prior to the addition of DHF, serial 1:10 dilutions of the cells were prepared, and 7 μl was placed on Sabouraud agar plates, which were incubated at 30°C for 48 h.

Measurement of oxygen consumption. Oxygen consumption was measured using an oxygen electrode (Hansatech Instruments), as described in Alonso-Monge et al. (59). Briefly, the cells were grown overnight in YPD medium at 30°C, recovered, washed twice with cold water, and suspended in 1 mM potassium phosphate buffer (pH 7.0). The cells were introduced into the electron chamber. The maximal respiratory capacity was determined from the stimulation of respiration induced by the uncoupling reagent 2,4-dinitrophenol (DNP). Respiration due to the cytochrome oxidase pathway was inhibited with KCN, while respiration due to alternative oxidase (AOX) was inhibited with 5 mM salicylhydroxamic acid (SHAM) (Sigma-Aldrich). The respiration rates are expressed as nmol O_2 min^{-1} per 10^6 cells, while respiration is expressed as a percentage of the basal rate of respiration. The experiments were performed twice on two independent days, with almost identical results.

Mitochondrial membrane potential. Mitochondrial membrane potential was evaluated using the fluorescent probes rhodamine 123 and MitoTracker green (Invitrogen) (60, 61), according to the manufacturer's recommendations. Yeasts were grown in Sabouraud liquid medium as described above and suspended at 10^7 cells/ml. When rhodamine 123 was used, the cells were suspended in 50 mM sodium citrate (pH 5.0) supplemented with 2% glucose. For MitoTracker green, 10 mM HEPES (pH 7.4) supplemented with 5% glucose was used. Rhodamine 123 was added at a final concentration of 35 μM and MitoTracker green at 50 nM. Parallel samples without any probe were carried out as negative controls. In both cases, the cells were incubated for 20 min at room temperature and protected from light, and the fluorescence of 10,000 cells was quantified using a FACSCalibur cytometer (BD) in the FL1 and FL2 channels for MitoTracker green and rhodamine 123, respectively. The data were processed with the FlowJo 7.1.6 software (Tree Star, Inc., OR, USA). The median intensity of the fluorescence of the control cells was subtracted from the fluorescence of the samples treated with the probes, and the data are presented as the means ± standard deviations.

Statistics. The statistical test analysis of variance (ANOVA), followed by Bonferroni's correction to allow for multiple comparisons, were applied to evaluate differences in catalase activity and to estimate changes in the mitochondrial membrane potential among the strains. To analyze differences in the percentage of ROS-producing cells among species and the fold change of the fluorescence of the cells after the addition of AMB, the nonparametric Kruskal-Wallis test was used. Statistics were performed with GraphPad and SPSS software. In all cases, a P value of <0.05 was considered statistically different.

RESULTS

Induction of oxidative stress in a different yeast species. We evaluated a variety of pathogenic yeasts to investigate if the production of ROS is a universal phenomenon after challenge with AMB. For this purpose, we selected strains from some of the main pathogenic yeasts (see Table 1). We first investigated if there was any difference in the number of yeast cells able to produce ROS. In all the species tested, there was a significant number of cells that displayed an accumulation of free radicals after adding AMB (2 or 8 mg/liter; Fig. 1A and B). When the data were statistically analyzed, it was found that all the species responded in a similar way,

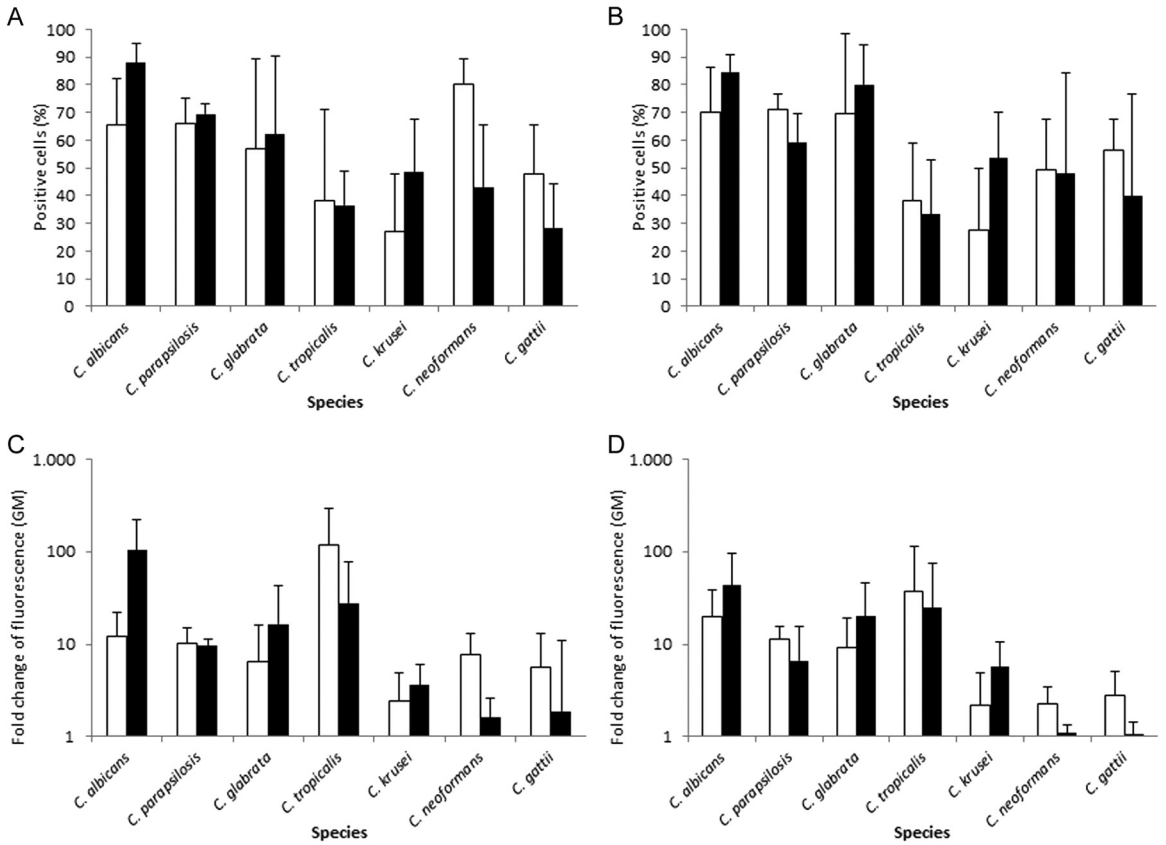

FIG 1 Production of ROS by different pathogenic yeast species. Shown are the percentage of cells that produce ROS in the presence of 2 mg/liter (A) or 8 mg/liter (B) AMB after 1 (white bars) or 3 h (black bars) of incubation. The experiments were performed as described in Materials and Methods using the strains shown in Table 1. The graph represents the average and standard deviation values. (C and D) Fold change in the fluorescence intensity of the cells after addition of AMB. After selecting the population of positive cells (see Materials and Methods and Results), the geometric mean (GM) of the fluorescence intensity was obtained from the cells exposed to 2 mg/liter (C) and 8 mg/liter (D) AMB after 1 (white bars) and 3 h (black bars). In parallel, the GM of the control cells was also obtained. The fold change of the GM was calculated as described in Materials and Methods for all strains. The graph shows the average and standard deviation values. The results for *T. asahii* are not included, because DHF provided a high background even in untreated cells, so it was not possible to evaluate the effect on AMB with this approach.

except *C. tropicalis* and *Candida krusei*, for which the proportion of cells that produced ROS was lower. In *C. tropicalis*, a high variation was recorded between the checked strains, and the difference in the proportion of ROS-producing cells was more noticeable at high AMB concentrations (see Fig. 1A and B; see also Table S1 in the supplemental material for significance). In the case of *C. krusei*, the difference with other species was related to a kinetic effect, because after 3 h of incubation, the proportion of ROS-producing cells increased, suggesting that AMB induced cell death at a lower rate.

Concerning *Trichosporon asahii*, the induction of oxidative damage was not evaluated by flow cytometry in this species, because even in the control cells (i.e., those not exposed to AMB or any oxidant agent), the fluorescent probe (dihydrofluorescein diacetate) already produced a strong signal.

We then analyzed the magnitude of the oxidative stress produced in the cells that showed AMB-induced accumulation of ROS. For this purpose, we measured the fluorescence intensity of the cells that produced ROS. For this analysis, the fluorescence intensity of the cells exposed to AMB that did not produce ROS was excluded (see Materials and Methods). We also calculated the fluorescence intensity of the control cells not exposed to AMB, and then we calculated the fold change (ratio) of the fluorescence between the AMB-treated and untreated cells. In this way, we estimated how much the fluorescence of the cells increased after AMB treatment. Notable variations in the intensity of the fluorescence were found, even between different strains from the same species (Fig. 1C and D). For this reason, we did not find statistical differences between the species at short incubation times with 2 mg/liter AMB (Fig. 1C; see also Table S1 in the supplemental material). In the case of *Cryptococcus* spp., the fold change of the fluorescence decreased after 3 h of incubation (Fig. 1B and C; see also Table S1), most probably due to the high mortality of the cells after prolonged incubation. These data indicate that when AMB induced oxidative damage in the cells, there were no significant differences in the concentration of free radicals accumulated.

Effect of inhibition of respiratory chain on the AMB-induced oxidative burst. ROS are mainly produced in the mitochondria as by-products of the electron respiratory chain. We wanted to evaluate the role of the respiratory chain in the production of ROS after exposure to AMB. For this purpose, we used rotenone, which is a specific inhibitor of complex I. We first standardized the rotenone concentrations that induced a decrease in cell growth but did not kill the cells. Growth curves were performed in the pres-

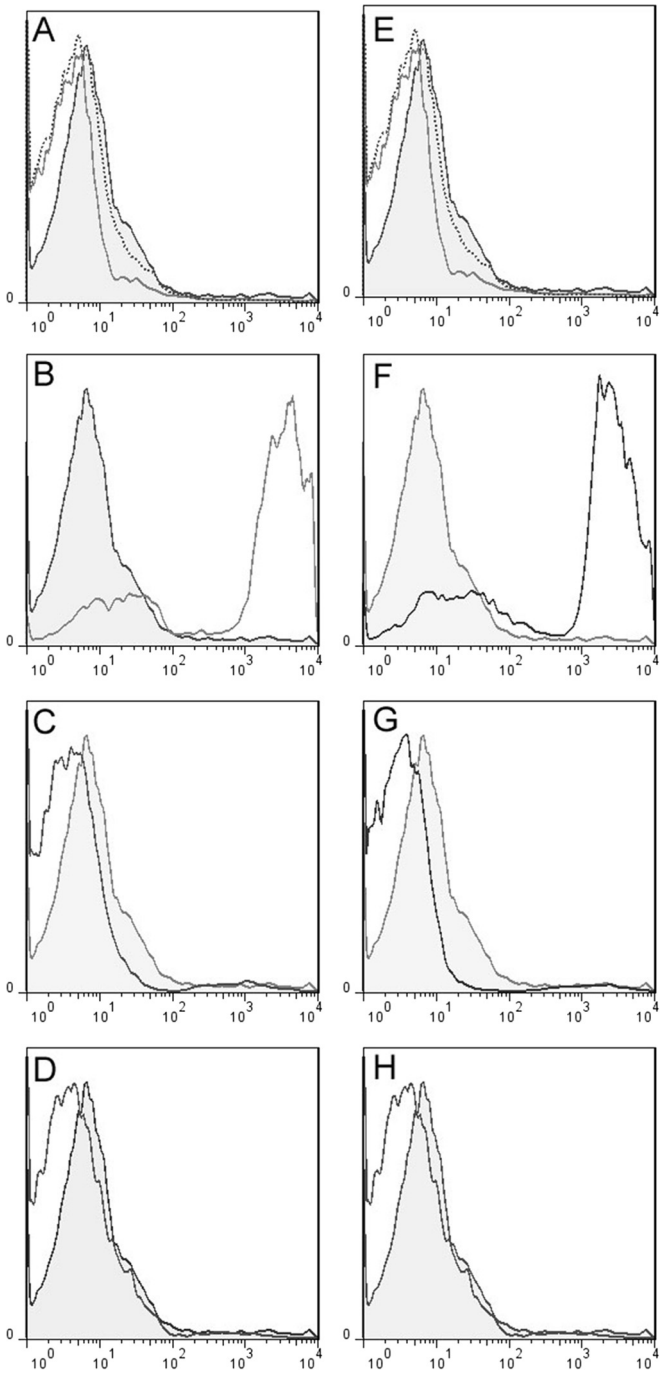

FIG 2 Effect of rotenone on accumulation of ROS after AMB treatment. *C. tropicalis* ATCC 750 was grown in liquid Sabouraud medium overnight and then suspended in PBS. The cells were treated with rotenone at concentrations of 0.156 mM (A, C, E, and G) or 0.31 mM (A, D, E, and H) for 1 h and then exposed to AMB. ROS production was evaluated by flow cytometry as described in Materials and Methods. Controls without rotenone (B and F) or AMB (A and E) were carried out in parallel. (A and E) Control cells (filled area) or cells treated only with 0.31 mM (solid line) or 0.156 mM (dotted line) rotenone. (B and F) Cells treated with 0.5 mg/liter (B) or 2 mg/liter (F) AMB. (C and G) Control cells (filled area) or cells treated with 0.156 mM rotenone and 0.5 mg/liter (C) or 2 mg/liter (G) AMB. (D and H) Control cells (filled area) or cells treated with 0.31 mM rotenone and 0.5 mg/liter (D) or 2 mg/liter (H) AMB.

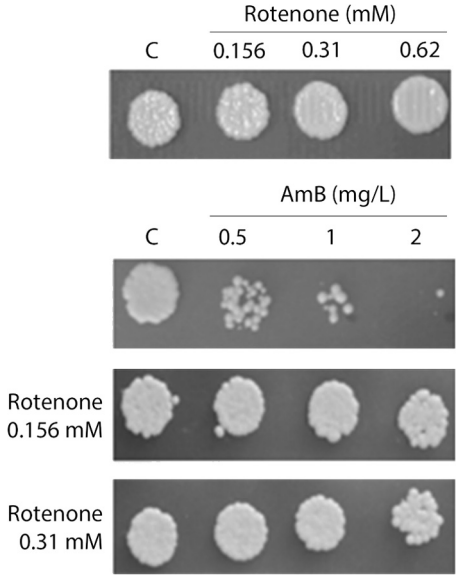

FIG 3 Effect of rotenone on yeast survival after AMB treatment. The cells from the experiment described in Fig. 2 were placed on Sabouraud agar plates, as described in Materials and Methods. C, control cells treated only with PBS.

ence of different rotenone concentrations, including a control with an equivalent concentration of the solvent in which rotenone is prepared (DMSO). In this experiment, we used *C. tropicalis* as the model (strain ATCC 750) because we had two AMB-resistant isolates available from this species, which have been extensively characterized below and in previous findings from our group (51). Rotenone inhibited *C. tropicalis* growth at concentrations between 0.62 and 0.156 mM (data not shown). To ensure that rotenone had a fungistatic but not fungicidal effect, the cells from the wells after the end of the growth curve were placed on Sabouraud agar plates. In all cases, the cells were viable, even in wells in which there was a significant inhibition of cell growth (data not shown).

We next evaluated if rotenone inhibited the production of ROS after the addition of AMB. As shown in Fig. 2, AMB did not induce an accumulation of ROS in cells preincubated for 1 h with rotenone.

Since ROS have deleterious effects on proteins, lipids, and DNA, we investigated whether rotenone protected the cells from the fungicidal effect of AMB by placing them on Sabouraud agar plates. AMB induced killing of the cells after 1 h of incubation at concentrations of >1 mg/liter (Fig. 3). However, when the cells were preincubated with rotenone, AMB did not significantly kill the cell population (Fig. 3), supporting the hypothesis that the induction of ROS by the antifungal is important for its fungicidal effect.

Characterization of strains with acquired resistance to amphotericin B. We then evaluated if AMB induced ROS accumulation in two *C. tropicalis* strains that were resistant to AMB. We first confirmed the susceptibility profile of *C. tropicalis* strains using the EUCAST protocol. *C. tropicalis* strains ATCC 200956 and CL-6835 presented MIC values to AMB of 1 to 2 mg/liter (51), which are significantly higher than the MICs regularly measured for *C. tropicalis* (i.e., between 0.03 to 0.25 mg/liter; data not shown). The resistant strains also showed reduced susceptibility in the time-killing assays (data not shown).

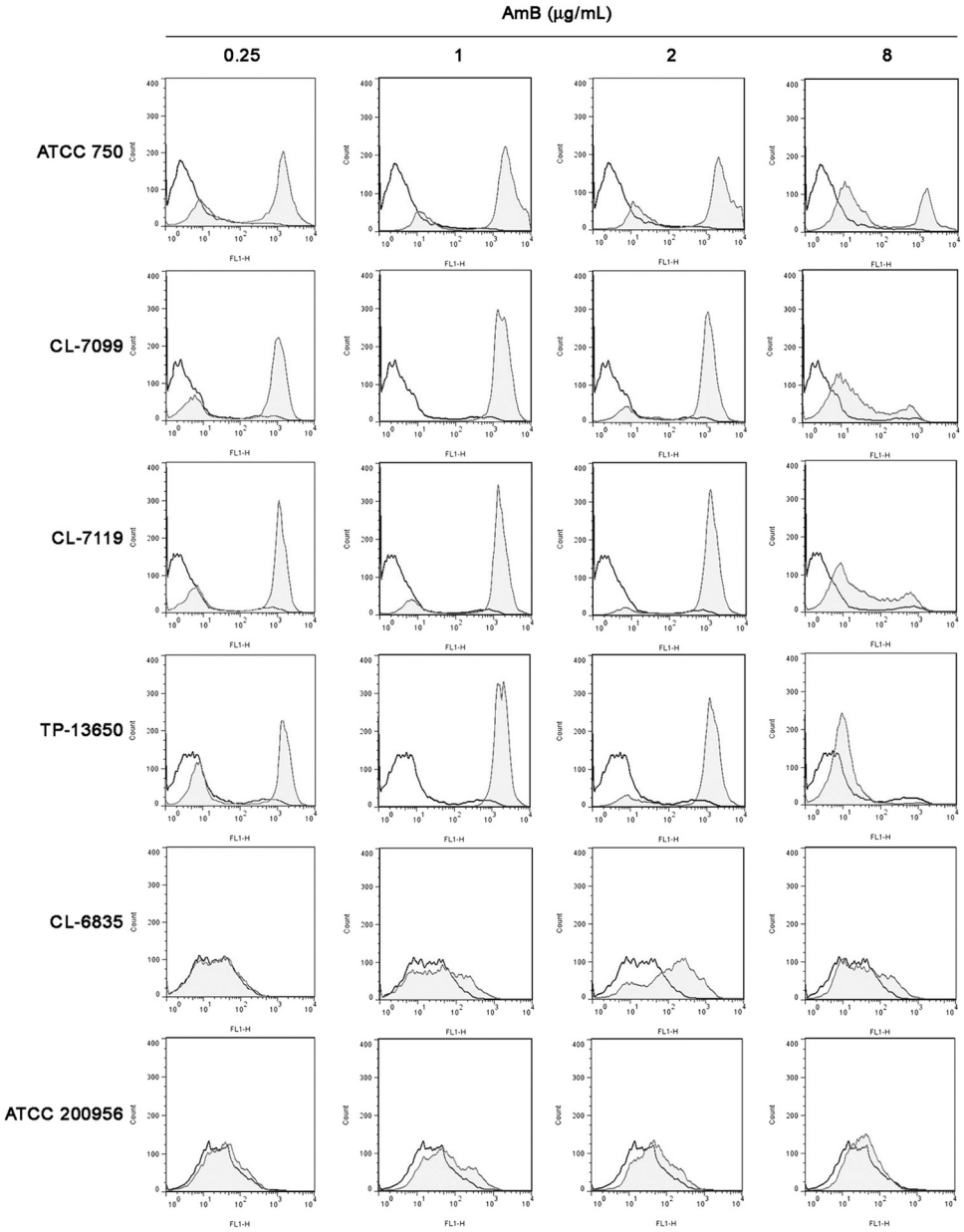

FIG 4 ROS production by susceptible and resistant *C. tropicalis* strains after addition of AMB. Different *C. tropicalis* strains were treated with 0.25, 1, and 2 mg/liter AMB for 1 h, and ROS accumulation was detected by flow cytometry.

To correlate the resistant phenotype with the AMB-induced oxidative burst, we measured the production of ROS by flow cytometry. The strains that were susceptible to azoles and AMB (*C. tropicalis* strains ATCC 750 and CL-7099) treated with this fungicide (2 mg/liter) accumulated significant amounts of ROS (Fig. 4). An azole-resistant isolate (*C. tropicalis* TP-13650) also produced large levels of ROS. However, this ROS increase was not observed in the two AMB-resistant strains (Fig. 4). This result suggests that the AMB resistance phenotype is associated with a defect in the production of ROS or the activation of antioxidant mechanisms. Similar results were observed with the other concentrations of AMB tested (data not shown).

Western blot analyses and protein carbonylation assay. Carbonylation is a consequence of oxidative damage on the proteins and can be used as a marker of protein damage by oxidants (55). Thus, we evaluated the effects of AMB (2 mg/liter) and H_2O_2 (5 mM) on protein carbonylation in an AMB-resistant and a susceptible strain of *C. tropicalis* by Western blotting. AMB induced oxidative damage on proteins in the susceptible strain (Fig. 5), as shown in *Cryptococcus neoformans* (37). In contrast, AMB did not induce protein carbonylation in the resistant strain, *C. tropicalis* ATCC 200956 (Fig. 5). In both strains, protein carbonylation was observed after treatment with H_2O_2.

Oxygen consumption and mitochondrial membrane potential measurement. Since ROS are produced in the mitochondria, and inhibition of the respiratory chain by rotenone protected the

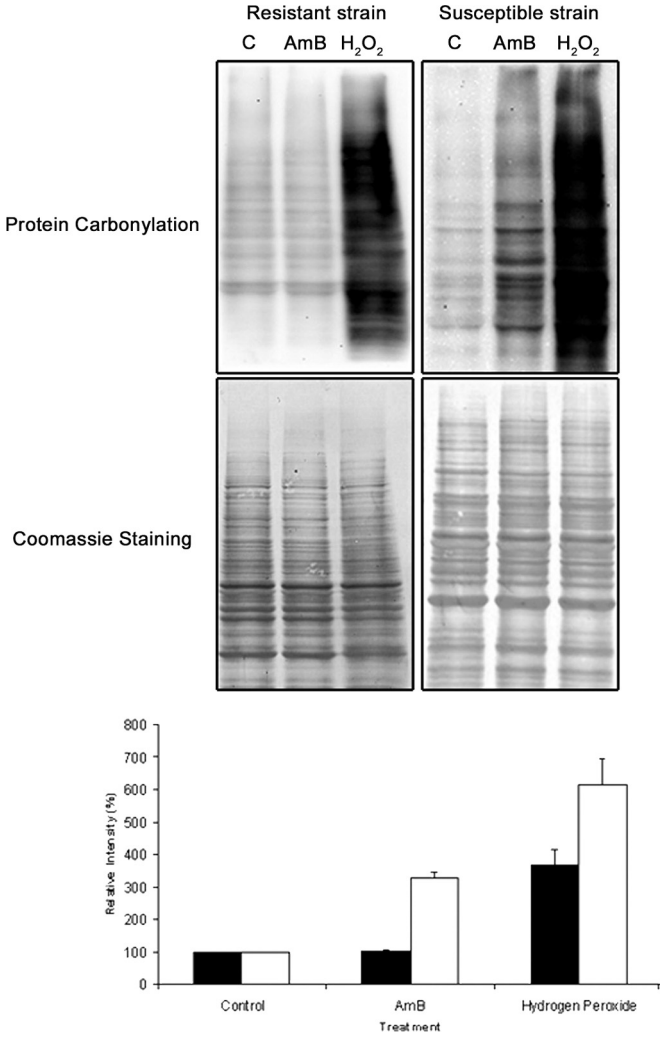

FIG 5 Protein carbonylation induced by AMB in a susceptible and resistant strain. *C. tropicalis* ATCC 750 (susceptible strain) and ATCC 200956 (resistant strain) were treated for 3 h with AMB (2 mg/liter) or H_2O_2 (2.5 mM). The untreated cells were covered in parallel as a control. Protein extracts were then prepared, and carbonylation was analyzed by Western blotting as described in Materials and Methods. Upper panel, protein carbonylation; middle panel, same gel stained with Coomassie blue as loading control; lower panel, quantification of the signal of three different areas of the gel of the upper panel, normalized by the intensity of the same areas of the Coomassie blue staining.

cells from the killing effect induced by AMB, we wanted to know whether the *C. tropicalis* strains studied had any defect in their mitochondrial activity. We first measured the respiration rate by detecting O_2 consumption. In addition to the classical respiratory chain, *Candida* species possess an alternative route of electron transfer and oxygen reduction, mainly based on alternative oxidases (62, 63), which are sensitive to the inhibitor SHAM. As shown in Fig. 6A, the basal respiration of the AMB resistant strain (ATCC 200956) was significantly lower than that of the susceptible isolate (ATCC 750), which confirmed that this strain presents a defect in mitochondrial activity. Both strains increased the respiration rate after the addition of the respiratory uncoupler DNP. However, we also observed a difference in the effects of respiratory inhibitors. When SHAM was added to the cells (which inhibits alternative oxidases), strain ATCC 750 showed a partial reduction in respiration (Fig. 6B). However, this inhibitor reduced the oxygen consumption in the AMB-resistant isolate ATCC 200956 by >90% (Fig. 6C), indicating that in this case, the classical respiratory chain through the mitochondrial complexes is almost absent. Finally, respiration in both strains is completely blocked after the addition of KCN. These results confirm that AMB resistance in ATCC 200956 is associated with a defect in the respiration rate of the cells.

We then analyzed if there was any difference in the mitochondrial membrane potential using two specific probes, rhodamine 123 and MitoTracker green, which are fluorescent and accumulate in the mitochondria, depending on its membrane potential. As shown in Fig. 6D and E, the two resistant strains accumulated significantly larger amounts of the probes than the rest of the AMB-susceptible strains tested (including the azole-resistant isolate), suggesting that in these strains, there were alterations in the mitochondrial membrane potential.

Catalase activity in AMB-resistant strains. We also investigated whether resistance to AMB was associated with an increase in ROS-detoxifying enzymes. For this purpose, we measured the activity of catalase. As shown in Fig. 6F, the catalase activity was 2.0 to 2.5 times higher in the two resistant strains compared to that in the susceptible strains. We also tested enzymes from the glutathione system, such as glutathione peroxidase, but found no statistical differences between the susceptible and resistant strains (data not shown).

Growth on gluconeogenic carbon sources. Mitochondrial activity is required for growth on respiratory carbon sources, such as glycerol or ethanol. Thus, we reasoned that if the AMB-resistant strains had mitochondrial defects, their growth on these respiratory carbon sources would be impaired. The growth curves of *C. tropicalis* AMB-susceptible and -resistant strains were carried out in medium with glucose or a mixture of glycerol plus ethanol. As shown in Fig. 7, the two AMB-resistant strains had their growth partially affected in glucose, but this defect was more pronounced in the glycerol-and-ethanol-containing medium.

DISCUSSION

Although AMB has been used for decades to treat fungal infections, its mechanism of action is still not completely understood. Although the primary mechanism of action studied is the binding to ergosterol and pore formation, it has been established in the last few years that AMB has other effects on the cells (35, 37, 64). However, the contribution of each mechanism to the fungicidal effect of the antifungal is not known.

We have demonstrated that the induction of ROS accumulation is a universal action mechanism of AMB. Interestingly, there were differences in the production of an oxidative burst among the species. In *C. tropicalis* and *C. krusei*, AMB induced a smaller proportion of ROS-producing cells. The rate of ROS accumulation does not directly correlate with the conditions under which regular susceptibility is measured (i.e., 24 h of incubation and determination of 90% of growth inhibition). Remarkably, *C. krusei* displayed lower susceptibility among the more prevalent *Candida* species (65). Regarding *C. tropicalis*, we do not know why this yeast showed lower ROS accumulation after longer incubation times than that of other *Candida* species with the same susceptibility. This might be related to differences in the membrane and cell wall compositions that affect the binding of AMB to the cell membrane. In any case, there were no significant differences in the

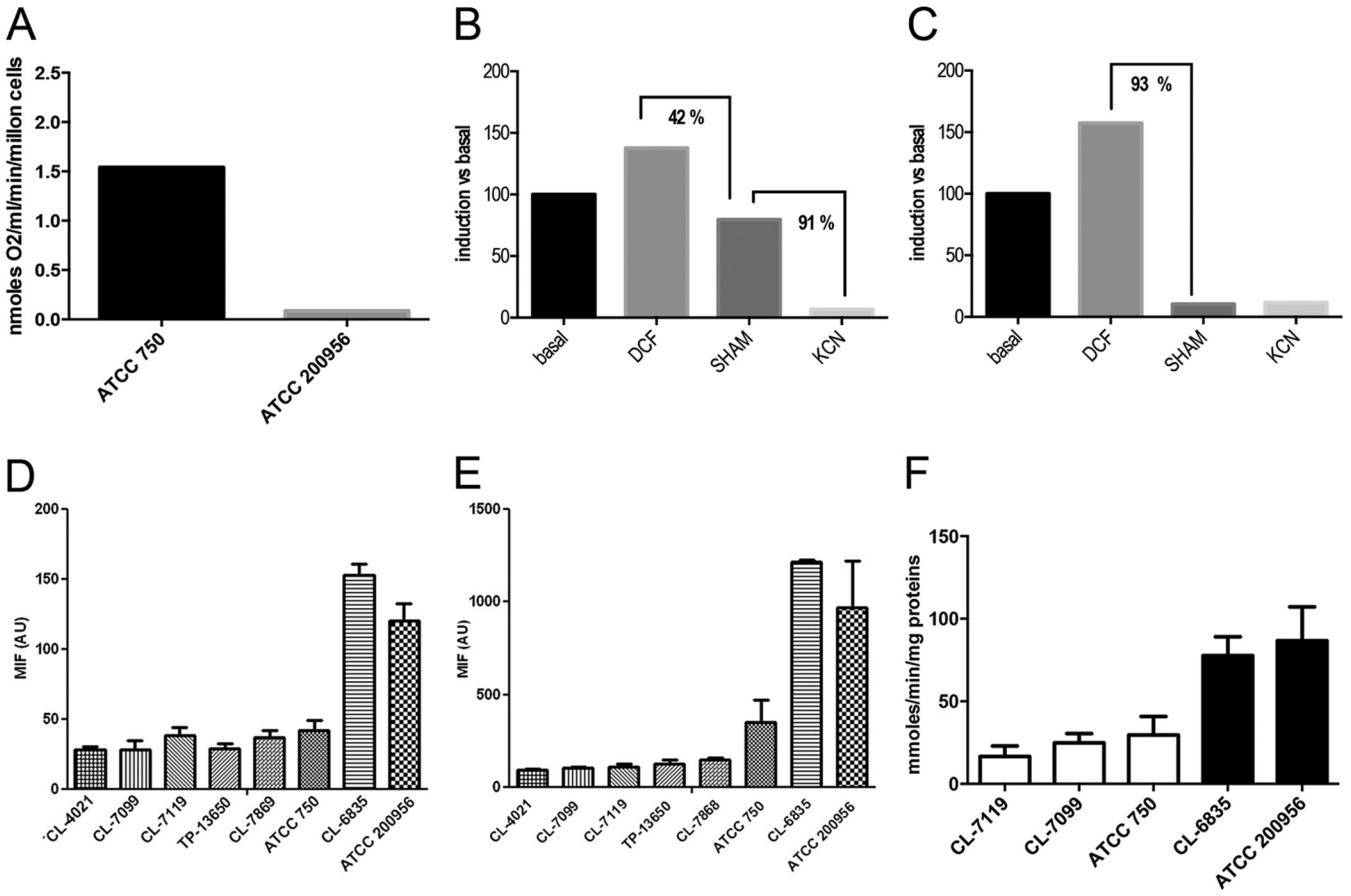

FIG 6 Changes in mitochondrial activity and catalase between AMB-resistant and -susceptible isolates. (A to C) Oxygen consumption by *C. tropicalis* ATCC 750 and ATCC 200956 strains. Oxygen consumption was measured as described in Materials and Methods. (A) Total oxygen consumption of both strains. (B and C) Relative oxygen consumption of ATCC 750 (B) and ATCC 200956 (C) strains after addition of the respiratory uncoupler DCP, followed by SHAM (which inhibits alternative oxidases) and KCN (which blocks both the classical electron transfer and alternative oxidases). The numbers represent the increase or decrease (percentage) in the relative oxygen consumption. The experiments were performed twice on two independent days, obtaining similar results. A representative graph is presented. (D and E) Estimation of mitochondrial membrane potential. *C. tropicalis* strains were grown in liquid Sabouraud agar, and the mitochondrial membrane potential was estimated after the addition of rhodamine 123 (D) and MitoTracker green (E). The fluorescence intensity was measured by flow cytometry, as described in Materials and Methods. The graph shows the average and standard deviation from three different repetitions. (F) *C. tropicalis* strains susceptible (white) or resistant (black) to AMB were grown in liquid Sabouraud medium, and catalase activity was measured as described in Materials and Methods. The graph shows the average and standard deviation of four different repetitions.

fold change increase of the fluorescence after short incubation times, suggesting that once the cells begin to accumulate ROS after the addition of AMB, all the species accumulate comparable amounts of this type of free radical.

In this work, we used *T. asahii* as a model of intrinsic resistance to AMB, but ROS production was not evaluated in this species due to the background of the DHF probe. This result suggests that *T. asahii* produces large amounts of endogenous ROS, and as a consequence, this species might have an induction of antioxidant mechanisms. Further studies in other species with reduced sus-

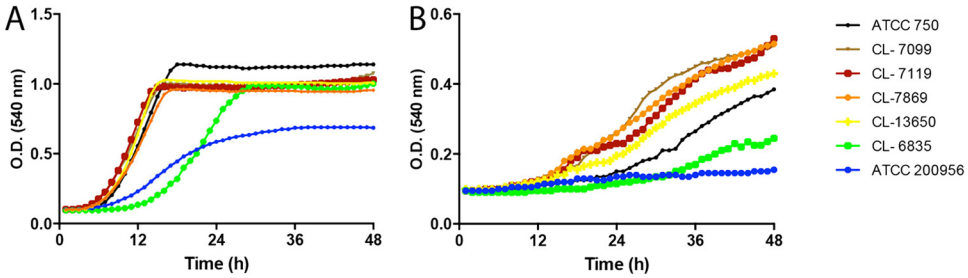

FIG 7 Growth curves of different *C. tropicalis* strains in fermentable or nonfermentable carbon sources. The growth of different *C. tropicalis* strains was performed in YNB plus glucose (A) or YNB glycerol plus ethanol (B) as described in Materials and Methods for 48 h at 35°C. Optical density was monitored every hour.

ceptibility to AMB, such as *Candida lusitaniae*, are warranted in the future.

Binding to ergosterol is the classical action mechanism, but pore formation might not be the only way in which AMB induces cell killing. Recent findings suggest that the AMB effect on the cell membrane is due to ergosterol sequestration (34). Recent data have demonstrated that *C. tropicalis* strains with reduced susceptibility to AMB do not contain ergosterol in their membranes (51, 66); therefore, they are also resistant to azoles. Ergosterol depletion from the cell membrane poses a problem, since this molecule is required to maintain an optimal membrane structure. In these strains, there is an accumulation of other sterols (51) that complement the ergosterol deficiency. Additionally, the depletion of ergosterol activates compensatory signals through the chaperone Hsp90. In this context, AMB resistance is abolished by the addition of Hsp90 inhibitors (66). Furthermore, the expression of several genes encoding proteins related to oxidative stress is increased after the addition of AMB(67). Our findings are in agreement with these data, since we also observed that AMB-resistant isolates have increased catalase levels.

We also evaluated how resistance to AMB correlates with the production of ROS. We observed that in the resistant yeasts, there was a significant reduction of ROS production after the addition of AMB compared to that in the susceptible isolates. Interestingly, we also found other differences associated with AMB resistance, such as alterations in mitochondrial membrane potential and reduced oxygen consumption. These results confirm that alterations in mitochondrial activity affect the production of ROS in response to AMB. In this sense, the addition of AMB to the cells results in increased mitochondrial respiration (38). At the moment, we do not know which complexes are specifically affected in the AMB-resistant strains, although preliminary results discard an alteration in cytochrome *c* oxidase (complex IV; data not shown). Superoxide dismutase, another enzyme that detoxifies ROS, also plays a role in protection against AMB, since the inhibition of this enzyme with *N*,*N*′-diethyldithiocarbamate (DDC) enhances the antifungal activity of AMB against *C. albicans* (68). We have demonstrated that the inhibition of complex I and ROS production by rotenone protected the yeast from the killing effect of AMB. This is in agreement with data obtained from mitochondrial respiratory mutants that exhibit decreased susceptibility to AMB (69). These mutants also had a reduction in ergosterol content, so the authors argued that resistance to AMB was associated with this decrease. In contrast, *Kluyveromyces lactis* mutants affected in the electron respiratory chain show decreased susceptibility to AMB, although in this case, AMB resistance was not associated with the absence of ergosterol (70).

Our findings confirm that AMB exerts multiple effects on the fungal cell, but how these different mechanisms interact is not known. Although speculative, two plausible mechanisms can be envisaged for the AMB action mechanism. Ergosterol might be needed to bind AMB and induce intracellular effects, with pore formation being a minor contribution to the killing action. Using fluorescent probes, it has been shown that AMB can translocate into human cells but not into yeast cells, where it localizes mainly at the plasma membrane (71). This finding suggests that in yeasts, AMB triggers a signal that produces an oxidative burst and stress responses. However, liposomal AMB formulations, such as AmBisome, can be found inside the cell (72). If ROS production by AMB depends on the presence of ergosterol, the depletion of this sterol might explain the changes observed in the resistant strains. Ergosterol is also present in the mitochondrial membrane (73), so its depletion might affect the functionality of this organelle. For this reason, cells would induce increased amounts of antioxidant enzymes to counteract the toxicity caused by an abnormal increased production of ROS at the mitochondria. On the other hand, if ROS accumulation occurs through an ergosterol-independent process, several adaptation mechanisms are required to become resistant (i.e., ergosterol depletion, an increase in catalase, and defects in the mitochondria). Any of these possibilities would explain why resistance to AMB is rarely found. In addition, *C. tropicalis* strains with AMB resistance show decreased fitness and reduced virulence (51, 66), which might also explain why those strains are difficult to isolate from patients. However, AMB-resistant strains can produce persistent infections in immunocompromised patients (74), which emphasizes the need to monitor yeast susceptibility profiles to AMB.

The role of ergosterol in AMB resistance is unclear. Different approaches have demonstrated that mutants resistant to AMB have reduced ergosterol content (74–82). In contrast, resistance to AMB has also been reported in isolates without any ergosterol defect (83–85). In this sense, intrinsic resistance to AMB in filamentous fungi, such as *Aspergillus terreus*, does not correlate with changes in ergosterol content but with increases in catalase and Hsp90 activities (85–88). Therefore, future work is warranted to clarify the action mechanism of AMB.

Our findings open new perspectives in the clinical management of invasive fungal diseases. The alternative contributory intracellular mechanism of action of AMB should be taken into account in the rationale of combined therapy. Combinations of AMB and triazoles can be based on that additional effect, as therapy can target both the membrane and the oxidative burst of the fungal cells. In addition, the induction of oxidative stress and the accumulation of free radicals are universal effects of AMB, which provides an explanation of the near absence of secondary resistance to this antifungal.

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