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ORIGINAL PAPER

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Disruption of mitochondrial function in *Candida albicans* leads to reduced cellular ergosterol levels and elevated growth in the presence of amphotericin B

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Abstract A respiratory-deficient mutant of *Candida albicans* MEN was generated by culturing cells in medium supplemented with ethidium bromide at 37 °C for 5 days. The respiratory-deficient mutant *(C. albicans* MMU11) was incapable of growth on glycerol, had a reduced oxygen uptake rate and demonstrated an altered mitochondrial cytochrome profile. Respiratory-competent cybrids were formed by mitochondrial transfer following fusion of protoplasts with those of *C. albicans* ATCC 44990. Mutant MMU11 possessed lower levels of ergosterol than the parental isolates and the cybrids, and demonstrated a small but statistically significant increase in tolerance to amphotericin B. The results demonstrated that disruption of mitochondrial function in *C. albicans* increases the tolerance to amphotericin B, possibly mediated by a reduction in cellular ergosterol content.

Keywords Amphotericin B · *Candida* · Ergosterol · Mitochondrion

Introduction

The dimorphic yeast *Candida albicans* is an opportunistic fungal pathogen and the dominant cause of candidosis in humans (Pfaller et al. 1998). There has been a considerable increase in the incidence of disease attributable to this yeast in recent years due to the spread of AIDS, the widespread use of immuno-suppressive therapy and the prolonged survival of patients with critical illnesses (Lunel et al. 1999). The emergence of *C. albicans* isolates resistant to anti-fungal drugs has serious implications for the continued success of conventional anti-fungal therapy. A number of *Candida* isolates manifesting anti-fungal drug resistance have been characterised as being respiratory-deficient i.e. incapable of aerobic respiration (Defontaine et al. 1999). Respiratory-deficient isolates of *Candida glabrata* demonstrate tolerance to polyene (Geber et al. 1995) and azole anti-fungal drugs (Bouchara et al. 2000).

Respiratory-deficient mutants (frequently referred to as *petite* mutants) are difficult to isolate in *C. albicans* (Aoki and Ito-Kuwa 1987; Abu-Hatab and Whittaker 1992) whereas they arise spontaneously in *Saccharomyces cerevisiae* at a rate of approximately 0.1% of cells per generation. *S. cerevisiae petite* mutants grow at a lower rate than their parents and are incapable of metabolising glycerol, have disrupted cytochromes and reduced rates of oxygen consumption (Gyurko et al. 2000; Aoki et al. 1990).

Respiratory competency can be restored to respiratory-deficient cells by transfer of the mitochondrion from a donor strain through protoplast fusion (Kavanagh and Whittaker 1996). A mitochondrion donor from a closely related strain (intra-specific transfer) or, in some cases, a mitochondrion from a different species (inter-specific transfer) can restore some respiratory function (Spirek et al. 2000). Mitochondrial transfer opens the possibility of examining the role of the mitochondrion in yeast virulence and anti-fungal drug susceptibility by allowing the creation of novel strains with the same nuclear background as the parent strain but with a different mitochondrial genome.

The objective of the work presented here was to establish the role of the mitochondrion in regulating the anti-fungal drug susceptibility of *C. albicans* by firstly creating a respiratory-deficient mutant and then restoring respiratory function by mitochondrion transfer following protoplast fusion.

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Materials and methods

Yeast isolates

Candida albicans MEN (serotype B, originally isolated from an eye infection and a gift from Dr. D. Kerridge, Cambridge, UK) and ATCC 44990 *(ade, thr)* (from the American Type Culture Collection, Manasas, Va., USA) were used as the parental isolates. A respiratory-deficient mutant, MMU11, was created using ethidium bromide mutagenesis of *C. albicans* MEN. Three cybrids (numbers 3, 26 and 27) were created by fusion of protoplasts of *C. albicans* MMU11 and 44990.

Culture conditions

Cultures were grown in YEPD broth [2% (w/v) glucose (Sigma-Aldrich), 2% (w/v) bactopeptone (Difco), 1% (w/v) yeast extract (Oxoid] or minimal medium (MM) [2% (w/v) glucose, 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulfate) (Difco) and 0.5% (w/v) ammonium sulfate]. Where appropriate, adenine and threonine were added to MM to allow growth of *C. albicans* 44990. Media were solidified by the addition of 2% (w/v) agar (Oxoid). YEPG plates had the same constituents as YEPD but with 2% (w/v) glycerol replacing 2% (w/v) glucose.

Isolation of respiratory-deficient mutants

Respiratory-deficient mutants were obtained by culturing *C. albicans* MEN for 5 days at 37 °C and 200 rpm in an orbital incubator in YEPD broth supplemented with 25 µg ethidium bromide (Sigma-Aldrich) ml^{-1} as described (Aoki and Ito-Kuwa 1987). Respiratory-deficient mutants were selected by their inability to grow on glycerol.

Protoplast isolation and fusion

Protoplasts were isolated by enzymatic digestion of the cell walls of *C. albicans* MMU11 and 44990 as described (Kavanagh et al. 1991). Protoplasts of *C. albicans* MMU11 and 44990 were fused by resuspending 1×10^7 protoplasts of each strain in 60% (w/v) PEG 3350 (Sigma-Aldrich) and 0.1 M calcium acetate as described (Kavanagh et al. 1991). The negative control consisted of a similar number of protoplast pairs, but mixed in the absence of PEG and calcium acetate. Protoplasts (1×10^6) were embedded in molten (47 °C) osmotically stabilised MM containing glycerol as the carbon source and incubated at 30 °C for 7 days. Cybrids were identified as being capable of metabolising glycerol (unlike mutant MMU11) and of growing in the absence of the requirements of *C. albicans* 44990. The cybrid fusion frequency was expressed as the number of cybrids formed as a percentage of the number of pairs of parental protoplasts in the fusion mixture.

Amphotericin B susceptibility testing

Yeast cultures were grown to the stationary phase in antibiotic medium 3 (AB 3) (Oxoid) overnight at 30 °C and 200 rpm, harvested by centrifugation (2,220x*g* for 5 min in a Beckmann GS-6 centrifuge) and diluted to 1×10^6 cells ml^{-1}. Cells (1×10^5 in 100 µl) were added to each well of a 96-well plate containing amphotericin B (Sigma-Aldrich) ranging from 0.0048 to 2.5 µg ml^{-1} in AB3. The plates were incubated at 37 °C for 24 h and the optical density was read at 540 nm using a microplate reader. The MIC_{90} was determined to be the lowest concentration of amphotericin B required to reduce growth by 90% relative to the control (Moran et al. 1997).

Cytochrome analysis

Stationary phase cells (2×10^{10}) were harvested by centrifugation and washed twice in phosphate-buffered saline (PBS, pH 7.2). Half of the sample was oxidised by suspension in 20 ml of 0.3% (w/v) sodium hypochlorite (Sigma-Aldrich). The cells were harvested by centrifugation and resuspended in 50% (v/v) glycerol. The remaining 1×10^{10} cells were resuspended in 50% (v/v) glycerol to which a few crystals of sodium dithionite (Merck, Darmstadt, Germany) were added to reduce the cells. Reduced-oxidised differential spectra were measured on a dual-beam Cary IE UV-Visible spectrophotometer over the range 500–650 nm.

Measurement of oxygen uptake

Stationary phase cells (approximately 1.5×10^8 cells ml^{-1}) grown in YEPD broth at 30 °C overnight were harvested, washed with PBS and resuspended in 25 mM phosphate buffer (pH 7.2) at a density of 5×10^8 ml^{-1}. Oxygen uptake measurements were made at 30 °C using a Clark-type oxygen electrode. Oxygen uptake rates were calculated as µmol oxygen consumed $(10^8 \text{ cells})^{-1}$ $(\text{min})^{-1}$.

Sterol extraction and analysis

Sterols were extracted using the method of Arthington-Skaggs et al. (1999) with slight modifications. Stationary phase cells (1 g wet weight) were harvested and washed with PBS. Cells were resuspended in 2 ml of 20% (w/v) KOH and 60% (v/v) ethanol and placed in a shaking water bath (80–90 °C) for 90 min. N-heptane was added to the solution, which was agitated for 10 s and the aqueous layer was removed. The sterol content was quantified using a gas chromatograph (Hewlett Packard 5890 Series II) with a flame ionisation detector and a Chromapack capillary column (Chromapack International BV, Middelburg, The Netherlands) operated isothermally at 300 °C. Injector and detector temperatures were 320 °C and the carrier gas was N_2.

Statistical analysis

Data accumulated for each figure were analysed by *t* tests (normality and equal variance tests) and the Kruskal Wallis test. The SigmaStat Statistical Analysis System (version 1.00) was used throughout. Each experiment was carried out on three independent occasions and results are presented as the mean±standard deviation.

Results

Isolation of respiratory-deficient mutant and generation of cybrids

Growth of *C. albicans* MEN in YEPD broth supplemented with ethidium bromide (25 µg ml^{-1}) at 37 °C was used to obtain a respiratory-deficient mutant. Approximately 2,500 potential respiratory-deficient colonies were tested for respiratory status by screening on YEPD and YEPG plates. One isolate (MMU11) was identified as being respiratory-deficient by its inability to grow on glycerol. Replacing the damaged mitochondrion of a respiratory-deficient mutant with a fully functional mitochondrion from a donor strain results in a respiratory competent cybrid that resembles the original parent (Maraz and Ferenczy 1980; Richards et al. 1987). Protoplasts of *C. albicans* MMU11 and 44990 were prepared by enzymatic degradation of the cell walls

and fused using PEG and calcium cations. Cybrids were selected on MM containing glycerol as the carbon source. The cybrid fusion frequency was calculated to be 1.64×10^{-2} % while the reversion rate of *C. albicans* 44990 to protrophy on the same medium was 2.33×10^{-7} %. The three fastest-growing putative cybrids (numbered 3, 26 and 27) were selected for further analysis. The parental strains *(C. albicans* MEN and 44990), mutant MMU11 and the cybrids were assessed for their ability to grow on glycerol. *C. albicans* MEN and 44990 and the three cybrids grew on this carbon source while the respiratory-deficient mutant, MMU11, was incapable of growth. In all subsequent work, stocks of the three cybrids were kept on MM-glycerol in order to maintain selection pressure and prevent reversion or selective loss of the mitochondrion.

Determination of respiratory status of *C. albicans* strains

The mitochondrion is the site of oxidative phosphorylation within the eukaryotic cell, and one component of this process is the electron transfer chain, which involves a number of cytochromes. Cytochromes may be nuclear and/or mitochondrially encoded. For example, three of the seven subunits of cytochrome aa_3 are mitochondrially encoded (Whittaker and Danks 1978), so it is conceivable that a respiratory-deficient mutant with disrupted mitochondrial function might have an altered cytochrome profile. Reduced *minus* oxidised difference spectra were obtained using a dual-beam spectrophotometer to record alterations in the relative amounts of the cytochromes in *C. albicans* MEN, mutant MMU11 and the three cybrids. Cytochromes aa_3 (602 nm), *b* (564 nm) and *c* (550–554 nm) are present in C. *albicans* MEN, but major alterations are evident in mutant MMU 11 (Fig. 1). While the cybrids possessed restored cytochrome profiles some alterations are still evident, for example in cybrid 26, but the alterations are less dramatic than those seen in mutant MMU11.

Disruption of the mitochondrial cytochrome content of a cell has the potential to reduce its respiratory efficacy, while restoration of respiratory function following transfer of a mitochondrion by protoplast fusion has the capacity to restore, partially or completely, respiratory status (Ferenczy and Maraz 1977; Maraz and Ferenczy 1980). The oxygen uptake rate demonstrated by mutant MMU11 was 9.3±0.54 µmol of oxygen consumed $(10^8$ cells$)^{-1}$ min^{-1} while that of *C. albicans* MEN was 54.2±6.1 µmol of oxygen consumed $(10^8$ cells$)^{-1}$ min^{-1} *C. albicans* 44990 had an oxygen uptake rate of 26.2±5.58 µmol of oxygen consumed $(10^8$ $cells^{-1})min^{-1}$. The cybrids demonstrated a respiration rate intermediate between that of *C. albicans* MEN and 44990, ranging from 32.4±1.5 (cybrid 3) to 45.7± 1.56 (cybrid 27) µmol of oxygen consumed $(10^8$ $cells^{-1})$ min^{-1} (Fig. 2). Analysis of these results using the normality test indicated a statistically significant difference at $p<0.001$ between the oxygen uptake rate of *C. albicans* MEN and that of the other strains tested.

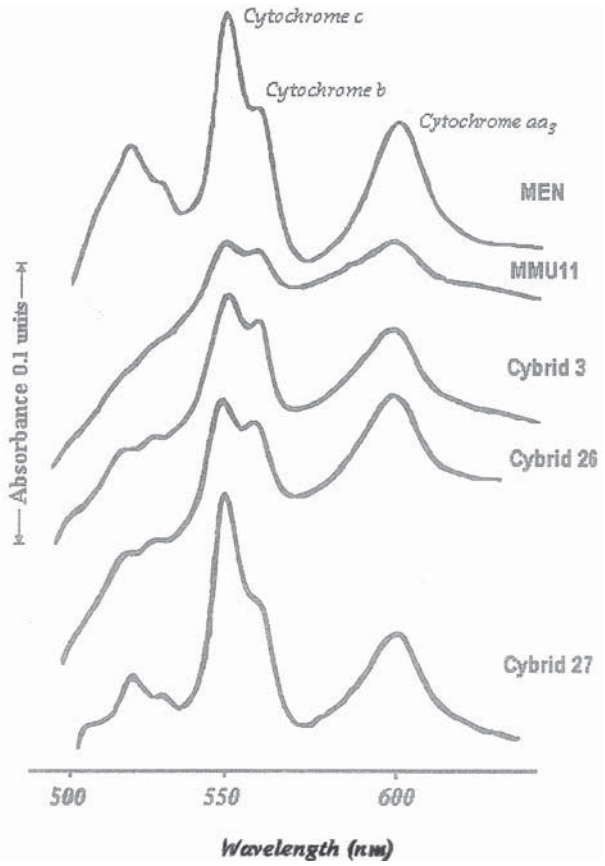

Fig. 1 Cytochrome profiles of *Candida albicans* strains. Differential cytochrome profiles of stationary-phase cells of *C. albicans* were obtained as described by scanning in a dual-beam spectrophotometer from 500–650 nm

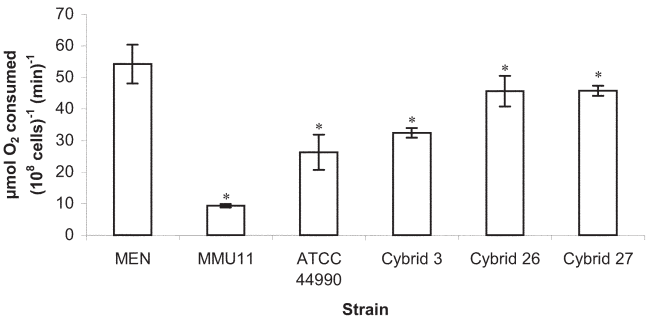

Fig. 2 Oxygen consumption of C. *albicans* strains. Oxygen consumption was measured using a Clark-type oxygen electrode and expressed as µmol oxygen consumed $(10^8$ cells$)^{-1}$ min^{-1}. All values are the mean of three independent determinations±standard deviation of the mean. A statistically significant difference compared to *C. albicans* MEN is indicated by *

Amphotericin B susceptibility of *C. albicans* strains

Respiratory-deficient cells of *C. glabrata* have previously been shown to manifest an increased tolerance to azole anti-fungal drugs (Defontaine et al. 1999; Bouchara et al. 2000), and respiratory-deficient mutants of *C. albicans* show enhanced tolerance to the anti-microbial peptide histatin 5

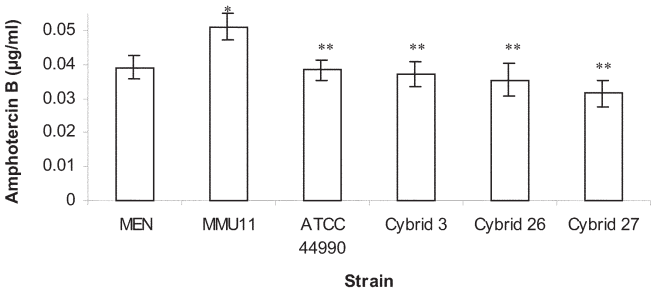

Fig. 3 Susceptibility of C. *albicans* strains to amphotericin B. The MIC_{90} was calculated as the concentration of amphotericin B that inhibited growth by 90% relative to the control. All values are the mean±standard deviation of three independent assays. A statistically significant difference relative to *C. albicans* MEN is indicated by *. The absence of a statistically significant difference compared to *C. albicans* MEN is indicated by **

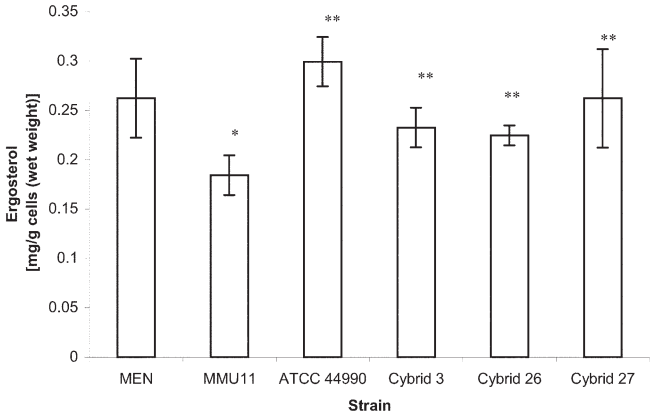

Fig. 4 Ergosterol content of C. *albicans* strains. Ergosterol was extracted from cells as described and quantified by GC analysis. Values are expressed as ergosterol [mg/g cells (wet wt.)]. All values are the mean±standard deviation of three independent assays. A statistically significant difference relative to *C. albicans* MEN is indicated by *. The absence of a statistically significant difference is indicated by **

(Gyurko et al. 2000). The appearance of azole resistance in *C. glabrata* has been attributed to the up-regulation of CgCDR1 and CgCDR2 in response to the absence of a functional mitochondrion (Sanglard et al. 2001). The responses of *C. albicans* MEN, 44990, mutant MMU11 and the cybrids to the anti-fungal drug amphotericin B were measured. An amphotericin B concentration of 0.051± 0.004 µg ml^{-1} corresponded to the MIC_{90} value for mutant MMU11, while the respiratory competent strains MEN and 44990 had MIC_{90} values of 0.039±0.003 and 0.038± 0.003 µg ml^{-1}, respectively (Fig. 3). The amphotericin B MIC_{90} values for the three cybrids ranged from 0.031± 0.004 (cybrid 27) to 0.037±0.003 (cybrid 3) µg ml^{-1}. Statistical analysis of these results using both *t* tests (i.e. normality and equal variance tests) indicated a statistically significant difference between the MIC_{90} value obtained for MEN and mutant MMU11 at p=0.01 but no significant difference at p=0.174 between the value obtained for MEN and those obtained for *C. albicans* 44990 and the cybrids. This indicates that, in the absence of a functional mitochondrion, the respiratory-deficient mutant shows a statistically significant increase in tolerance to amphotericin B, but that in normally respiratory-competent isolates (MEN, 44990) and in those constructed by protoplast fusion (cybrids 3, 26 and 27), this tolerance is absent.

Determination of ergosterol content of *C. albicans* strains

The anti-fungal agent amphotericin B acts by binding to ergosterol, the chief sterol found in the fungal cell membrane. This results in the formation of channels in the membrane, which leads to metabolite leakage, acidification of the cytoplasm and cell death (Abu Salah 1996). A reduction in sterol production may represent a mechanism for tolerance of amphotericin B since a diminution in ergosterol results in fewer binding sites for the drug (Kelly et al. 1997). The ergosterol content of the parents, mutant MMU11 and the three cybrids was assessed as described. The results reveal the lowest levels of ergosterol in mutant MMU11 [0.18±0.02 mg (gwet wt.)$^{-1}$, Fig. 4]. In contrast, the parent strains *C. albicans* MEN and 44990 exhibited levels substantially greater than that of MMU11 at 0.26±0.04 and 0.29±0.02 mg (gwet wt.)$^{-1}$, respectively, while the cybrids possessed ergosterol levels ranging from 0.22±0.01 to 0.26±0.05 mg (gwet wt.)$^{-1}$. Examination of these results revealed a significant difference between the ergosterol content of *C. albicans* MEN and that of mutant MMU11 at p=0.028 using a normality *t* test. In contrast, there was no significant difference at p=0.258 between *C. albicans* MEN and *C. albicans* 44990 and the cybrids. This latter comparison also failed the Kruskal-Wallis test.

Discussion

A respiratory-deficient mutant (MMU11) of *C. albicans* was obtained by culturing cells at 37 °C for 5 day in the presence of 25 µg ethidium bromide ml^{-1}. Such culture conditions are capable of rendering entire populations of *S. cerevisiae* respiratory deficient (Ferguson and von Borstel 1992), but the isolation of respiratory-deficient mutants of *C. albicans* is a low-frequency event (Abu Hatab and Whittaker 1992), possibly because the mutation proves lethal for the majority of such *C. albicans* mutants. Ethidium bromide is a DNA-intercalating agent that inhibits mitochondrial DNA replication, leading to a cell incapable of metabolising glycerol as a carbon source and of respiring and with a dysfunctional electron transport chain (Goldering et al. 1970; Ferguson and Von Borstel 1992). Disruption of mitochondrial function results in an altered cytochrome profile since three of the seven subunits of cytochrome aa_3 are mitochondrially encoded (Whittaker and Danks 1978). Disruption of cytochromes leads to an

inefficient electron transfer pathway and a concomitant reduction in respiration efficiency.

Respiratory competency was restored to mutant MMU11 by fusion with protoplasts of *C. albicans* 44990. Protoplast fusion has been employed previously to restore respiratory competency to *petite* cells of *S. cerevisiae* (Maraz and Ferenczy 1980) and offers the possibility of transferring intact organelles (e.g. the mitochondrion) between cells (Ferenczy and Maraz 1977). The selection conditions employed here screened for the transfer of mitochondria in the construction of the cybrids, rather than the transfer of chromosomes, which is a far rarer event (Kavanagh and Whittaker 1996). Cybrids demonstrated a restored respiratory status, but some characteristics (e.g. oxygen uptake) were intermediate between those evident in both parents. The cybrids were capable of growing on glycerol, had a respiration rate greater than that of mutant MMU11 but intermediate between *C. albicans* 44990 and MEN and the three cybrids demonstrated partially restored cytochrome profiles.

The response of *C. albicans* MEN, 44990, MMU11 and the cybrids to amphotericin B was assessed. Mutant MMU11 was the most tolerant of this anti-fungal (MIC_{90} 0.051 μg ml^{-1}) while the cybrids were the most sensitive, having MIC_{90} values of 0.031–0.037 μg ml^{-1} (Fig. 3). The parents (MEN and 44990) had amphotericin B MIC_{90} values of 0.039 and 0.038 μg ml^{-1}, respectively. Reductions in sterol levels in *C. albicans* have been identified as a mechanism for increased growth in the presence of amphotericin B (Kelly et al. 1997). Amphotericin-B-tolerant *C. albicans* has been described previously and tolerance was attributed to a reduction in the ergosterol content of the fungal cell membrane (White et al. 1998). Disruptions of the genes in the ergosterol biosynthesis pathway leads to decreased ergosterol in *C. glabrata* and an increase in drug tolerance, particularly to amphotericin B (Geber et al. 1995; Vazquez et al 1996). Analysis of the ergosterol content of the strains constructed here demonstrated that mutant MMU11 had the lowest level of ergosterol compared to *C. albicans* 44990 and MEN. The cybrids demonstrated an amount of ergosterol greater than mutant MMU11 but lower than that of the parents. The involvement of a functional mitochondrion in ergosterol biosynthesis is well characterised and arises from the provision of NADPH for squalene dimerisation (Parks and Casey 1995). In addition, *ERG1* encodes squalene epoxidase which converts squalene to 2, 3 oxidosqualene. This is an oxygen-dependent step and in a respiratory-deficient mutant there would be little synthesis of ergosterol (Daum et al. 1998) thus leading to the reduced ergosterol content evident in mutant MMU11. While mutant MMU11 was characterised as respiratory-deficient, it still retained some respiratory capacity (Fig. 2), which may explain the presence of reduced levels of ergosterol (Fig. 4) within the cell. The complete destruction of respiratory function would lead to a depletion of NADPH required for ergosterol biosynthesis and a concomitant loss of ergosterol from the cell membrane. This could have the effect of giving an enhanced tolerance to amphotericin B.

These results demonstrate that the mitochondrion plays a central role in governing the susceptibility of *C. albicans* to amphotericin B by regulating the ergosterol content of the plasma membrane. Respiratory-deficient mutants of *C. glabrata* have been isolated clinically and implicated in the failure of anti-fungal therapy due to elevated resistance to azole anti-fungal drugs (Defontaine et al. 1999; Bouchara et al. 2000). Resistance in this case was explained by elevated expression of genes coding for drug efflux pumps in response to the loss of a functional mitochondrion (Sanglard et al. 2001). While the respiratory-deficient mutant isolated here demonstrated elevated tolerance to amphotericin B, those isolated by Defontaine et al. (1999) and Bouchara et al. (2000) did not. This may be attributable to the fact that ethidium bromide mutagenesis can yield a range of different respiratory-deficient mutants in yeast and that, while the majority of the genome may be destroyed, small fragments can be retained and amplified to give a mitochondrial DNA content equivalent in size to that of the wild type (Whittaker and Danks 1978). As a consequence, different mitochondrial genes can be retained even in a cell incapable of respiration. It is possible that the respiratory-deficient mutants of Defontaine et al. (1999) and Bouchara et al. (2000) manifested no alteration in cellular ergosterol content while the one generated here (mutant MMU11) manifested a decreased ergosterol content and a concomitant increase in tolerance to amphotericin B. In addition, while the respiratory-deficient mutants of *C. glabrata* isolated by Defontaine et al. (1999) were relatively easily isolated, the equivalent mutant produced here required exposure to ethidium bromide for 5 days and was the only mutant isolated from 2,500 potentially respiratory-deficient colonies. This indicates that the mitochondria of *C. glabrata* and *C. albicans* have different sensitivities to ethidium bromide mutagenesis and possibly yield different types of respiratory-deficient mutants.

In the work presented here, a small but statistically significant elevation in the tolerance of a respiratory-deficient mutant of *C. albicans* to amphotericin B was achieved and this tolerance could be removed by restoration of respiratory function following mitochondrial transfer via protoplast fusion. The ability to create respiratory-deficient mutants and to restore respiratory competency through protoplast fusion opens the possibility of examining the interaction of the nuclear and mitochondrial genomes in regulating fungal pathogenicity and drug susceptibility.

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