

# Inhibition of Mitochondrial Complex III in *Candida Albicans* *ADH1* Deletion Mutant Attenuates Its Pathogenicity Significantly

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## Research Article

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# Abstract

Fermentation and aerobic respiration in mitochondria are coordinately regulated and compensated either when *C. albicans* grows in vitro or in the hosts, and the creature gain the strong viability. It's insufficient to influent the growth, reproduction and pathogenicity of *C. albicans* by inhibiting the electron transport chain (ECT) CI, CII, CIII, CV, or fermentation related gene ADH1. Our study showed that the induction of AA (inhibitor of complex III) rather than SHAM (alternative oxidase inhibitor) abolishes the mitochondrial function completely (96% less ATP generation, 59% reduction in MMP), and increases ROS production significantly in ADH1-deleted mutant (*adh1Δ/ adh1Δ*) that in turn becomes hypersensitive to azole and apoptosis, less viable and more difficult to form hyphae. At the same time, the expression of virulence related genes ALS3 and HWP1 were significantly lower than that of WT under AA induction. Under the induction of AA, the mitochondrial function of WT was slightly damaged and cell apoptosis increased slightly (ROS production and sensitivity of azoles increased significantly, but mycelium formation and the growth of cells were not affected). Under aerobic growth, we observed an ADH1 - dependent mitochondrial effect in *C. albicans* demonstrated by 64% less ATP generation, 58% reduction in MMP and significant elevations of the ROS and apoptosis in ADH1 -deleted mutant. However, mycelium formation and azole susceptibility are not affected. Our results suggested that ADH1 plus CIII played an important role in antifungal activity by damaging mitochondrial function, inhibiting cell growth and hyphae formation, promoting apoptosis and reducing pathogenicity.

## 1. Introduction

*C. albicans* is one of most common opportunistic pathogens in human beings. Its transformation from harmless commensal status into invasive infection often occurs in immunocompromised patients or at mucosal sites (Pfaller and Diekema, 2007). Azoles have been used for a few decades to treat these and other fungal infections. This organism can obtain strong viability and pathogenicity through the mutual compensation and regulation of anaerobic digestion and mitochondrial aerobic phosphorylation in the host. That is the main reason why the disease is difficult to treat and the problem of drug resistance (especially azole drugs) becomes serious.

Two strategies have been considered to combat azole resistance. One is to identify new antifungal targets and the second one is to locate azole synergists. Yan and others (Yan et al., 2009; Ruy et al., 2006; Sun et al., 2013a) found that inhibition of mitochondrial electron transport chain (ETC) complex CI-CV (except CII) could increase the azole susceptibility, or inhibit the growth of cells. And the inhibition of the growth was most obvious in *C. albicans* by antimycin A (AA, the inhibitor of CIII). Our previous study also found that the fluconazole susceptibility of *C. albicans* were increase while the activity of ETC complexes (CI, CIII and CV) were inhibited by drugs (Guo et al., 2013). It was also found that inhibition of CV can reduce the virulence, but has little effect on the growth of *C. albicans* in our study (Li et al, 2017). These results suggest that ECT complex inhibitors have shortcomings in inhibiting the growth, reproduction and pathogenicity of *C. albicans*. However, the results were inconsistent under conditions of respiration suppression or deficiency with different species of fungi. For example, suppression of mitochondrial

electron transport chain (ETC) Complex I (CI) makes *C. albicans* more susceptible to azole (Bambach et al., 2009; Sun et al., 2013b) and yet causes *Aspergillus fumigatus* to resist azoles (Bromley et al., 2017). The increasing susceptibility to azoles is also found in *Rhizopus oryzae* when two mitochondrial respiratory pathways are blocked by inhibitors, but *Candida parapsilosis* behaves differently from *C. albicans* and becomes more susceptible to caspofungin when respiratory capacity is blocked (Chamilos et al., 2006).

Therefore, the effect of anti *C. albicans* therapy by inhibiting one of the pathways (or one complex) of aerobic respiratory energy metabolism may not be ideal. Similar strategies based on alternation of metabolism are also applied for other therapeutic purposes. For example, atovaquone has been used as an anti-malarial drug due to its inhibitory role on ETC cytochrome *bc1*, having been repurposed from its anticancer activities (Fiorillo et al., 2016). Finally, the competitive inhibitors of alcohol dehydrogenase (ADH) such as berberine (herbal extracts) that has been used for alcoholism treatment (Wang et al., 2015) can restore azole susceptibility of azole resistant fungi (Xu et al., 2009). Indeed, this connection of ADH with azole susceptibility in *C. albicans* has also been observed in our previous studies. We have found that *ADH1* is highly expressed in azole resistant clinical strains, which is more consistent with higher expressions of *CDR1/CDR2/MDR1* in these strains (Guo et al., 2013). Previous study suggested that *ADH1* deletion may reduce *C. albicans* virulence, but has no effects on the growth and cell viability in the study, and *ADH1* plays an important role in the pathogenicity of *C. albicans* by affecting the oxidative phosphorylation activity in mitochondria (Song et al., 2019).

We hypothesized that inhibition of key targets for both anaerobic fermentation and mitochondrial aerobic respiration would be a new strategy for the treatment of refractory *Candida* infections. In this study, *ADH1* knockout strain (EX2) and its parent strain (the wild-type strain SC5314) were selected, and the mitochondrial function, virulence related genes, cell growth and reproduction, mycelial formation and cell apoptosis were observed under different growth conditions (with or without respiratory inhibitors). The aim of our study was to investigate the effects of key anaerobic fermentation gene *ADH1* and mitochondrial CIII on energy metabolism and pathogenicity of *C. albicans*.

## 2. Materials And Methods

### 2.1 *C. albicans* Strains and Growth Conditions

*C. albicans* standard strain SC5314 (provided by William A Fonzi, Department of Microbiology and Immunology, Georgetown University, Washington, DC, USA), its *ADH1* knockout strain EX2 (*adh1Δ/adh1Δ*), and strain RE1(*adh1Δ/ADH1*) were used throughout the study. And MIC<sub>80</sub> of FLC was 0.25μg/mL in all strains. After the concentration of those activated strains were adjusted to (2.5 ~ 5) ×10<sup>6</sup> cells /mL, strains were incubated without or with AA (antimycin A, inhibitor of CIII, Enzo Life Sciences, Inc.) or SHAM (salicyl hydroxamic acid, inhibitor of AOX pathway)(Sigma, USA) using YPD liquid medium at 37 °C for 6 h.

## 2.2 Determination of drug sensitivity in vitro

According to the CLSI M27-A3 (2008) program, the MIC of FLC was determined by micro dilution method for the strains treated with different respiratory inhibitors. The quality control strain was *C. albicans* ATCC 90028 recommended by the American Society for clinical and laboratory associations (CLSI). The final concentration of FLC was 0.125-32 µg/mL (Ltd. CIPLA, India, purify, 99%). Each strain was added a standard concentration of either AA (10 µM), SHAM (5 mM), or AA (5µM AA) plus SHAM (1.25 mM).

## 2.3 Spot assay

YPD solid medium containing different inhibitors was prepared, and the concentrations of inhibitors were 8µM for AA, 2mM for SHAM. And YPD solid medium without drug was also formulated as a control(Li and Calderone, 2016). Each strain from YPD plate was cloned into YPD liquid medium. After culturing at 30°C, 200 rpm shaking overnight, the *C. albicans* cells were collected, and washed with PBS Buffer, then the concentration of strains was adjusted to  $1.0 \times 10^6$  cells/mL. The cell suspension was diluted to 5 times successive concentration gradients with PBS Buffer ( $1.0 \times 10^6$ ,  $1.0 \times 10^5$ ,  $1.0 \times 10^4$ ,  $1.0 \times 10^3$ ,  $1.0 \times 10^2$  cells/mL). 5µL cells suspension of different concentration were pointed to YPD containing without or with inhibitors, and the cultures were incubated static at 30°C after the culture plates dry. After cultured for 48h, colonies were observed and photographed after generation status.

## 2.4 Growth of *ADH1* mutant strain

After the activated strain cells were washed with PBS Buffer, the cells were collected and inoculated on 250mL fresh YPD liquid medium, and the initial concentration was  $OD_{600} = 0.015-0.020$ . Each strain was cultured with or without AA (10 µM) or SHAM (5 mM) at 30°C, 200 rpm shaking. The  $OD_{600}$  value of each strain was determined every 1h, until a stable period. The culture doubling time of the cell logarithmic growth phase was calculated by the following formula:  $T = t \times \ln 2 / (\ln a - \ln b)$  (T: doubling time of cell logarithmic growth phase, t: time interval; a: initial cell concentration; b: terminal cell concentration).

## 2.5 Determination of morphological transition

After the activated strain cells were washed with sterilized PBS Buffer, the cells were collected and inoculated with Spider, Lee's, YPD + FBS liquid culture medium, and the cell concentration was adjusted to  $0.5 \times 10^6$  cells/mL respectively. The strain cell suspension was added to the 12 hole-plate, 2 mL/ holes. Then the culture holes were added with AA (10µM) or SHAM (5 mM) or AA (10µM) + SHAM (5 mM), and the blank control added without inhibitors. The 12 hole-plates were static cultured at 37 °C. The formation of hyphae was observed under microscope after 4 hours.

## 2.6 Measurement of intracellular ATP levels

*C. albicans* cells incubated in the presence or absence of AA (10µM) and/or SHAM(5 mM) were adjusted to a concentration of  $1 \times 10^7$  cells/mL. A total of 100 mL cell suspension was mixed with the same volume of BacTiter-Glo reagent (Promega) and incubated for 5 min at room temperature as described previously (Zhang et al., 2013). Luminescent signals were determined on a full wavelength

multifunctional enzyme mark instrument (Safire2; Tecan) after the cells were slightly oscillating and mixed. A control tube without cells was used to obtain a value for background luminescence. A standard curve of incremental ATP concentrations (from 1 mM to 10 pM) was constructed. Signals represented the mean of three separate experiments and the ATP content was calculated from the standard curve.

## 2.7 Assessment of $\Delta\Psi_m$

Mitochondria Membrane Potential ( $\Delta\Psi_m$ , MMP) an indicator of the energetic state of mitochondria and cells, was used to assess the activity and depolarization of the mitochondrial permeability in *C. albicans*. To determine the effect of each inhibitor on  $\Delta\Psi_m$ , fluorescence was measured using a Yeast Cell Mitochondria Membrane Potential Fluorescent Assay kit (GenMed Scientifics) as described previously (Misra et al., 2009). *C. albicans* strains ( $1 \times 10^7$  cells/mL) incubated in the presence or absence of AA (10  $\mu$ M) and/or SHAM (5mM) for 6 h were stained with 10 mg/mL JC-1 (5,59,6,69-tetrachloro-1,19,3,39-tetraethylbenzimidazol-carbocyanine iodide) at 30 °C for 20 min in the dark. Cells were washed twice, re-suspended in PBS buffer, and measured on the full wavelength multifunctional enzyme mark instrument with an excitation wavelength of 490 nm and emission wavelength shifting from green (~ 530 nm) to red (~ 590 nm).  $\Delta\Psi_m$  was determined as a ratio of red to green FI.

## 2.8 ROS measurement

ROS production was measured using a Yeast Cell Oxidative Stress Active Oxygen Fluorescence Assay kit (GenMed Scientifics) as described previously (Xiao et al., 2010). *C. albicans* strains ( $1 \times 10^7$  cells /mL) incubated in the presence or absence of AA (10  $\mu$ M) and/or SHAM (5mM) for 6 h were stained with 10 mg/mL CM-H2DCFDA (6-chloromethyl- 29,79-dichlorofluorescein diacetate, acetyl ester) at 30 °C for 20 min. Following staining, the cells were washed twice, re-suspended in PBS buffer, and measured on the full wavelength multifunctional enzyme mark instrument with an excitation wavelength of 490 nm and emission wavelength of 530 nm. ROS production was calculated by subtracting the FI value of cells in the absence of CM-H2DCFDA from that of cells with CM-H2DCFDA.

## 2.9 FACS Analysis of Apoptosis

*C. albicans* cells were stained with FITC-labeled Annexin V and PI using the FITC-Annexin V apoptosis detection kit (BD Pharmingen) (Hwang et al., 2012). Cells ( $2.5 \times 10^6$  cells/mL) were digested for 1 h at 30°C in a potassium phosphate Buffer (pH 6.0, containing 50U/mL Lyticase enzyme and 1 M sorbitol). After washed with PBS Buffer, cells were suspended with YPD culture liquid medium, adding AA (10  $\mu$ M) and/or SHAM (5mM) at 30°C, and shock cultured overnight. Washed cells were incubated in 200  $\mu$ L Annexin-binding buffer containing 5  $\mu$ L FITC–Annexin V and 5  $\mu$ L PI at room temperature in the dark for 20 min. Cells were then examined by a Gallios flow cytometer (Beckman Coulter, USA).

## 2.10 Real-time reverse transcription (RT)-PCR of virulence related genes

Total RNA was extracted from the cells using the E.Z.N.A. Yeast RNA kit (Omega Bio-tek) according to the manufacturer's protocol. cDNA was synthesized according to the manufacturer's instructions (TaKaRa,

Biotechnology, Dalian, P. R. China). Real-time RT-PCR was performed using SYBR Green I to visualize and monitor the amplified product in Applied Biosystems 7500 Real-Time PCR System (Life Technologies). Gene-specific primers were designed as described previously (Feng et al., 2016; Samaranayake et al., 2013). The PCR protocol (Wang et al., 2006) consisted of a denaturation step (95 °C for 10 s), and 40 cycles of amplification and quantification (95 °C for 10 s, 55 °C for 20 s and 72 °C for 15 s, with a single fluorescence measurement). The change in the fluorescence of the SYBR Green I dye in every cycle was monitored using the Light Cycler system software (Roche Diagnostics) and the threshold cycle (Ct) above the background for each reaction was calculated. The Ct value of 18S rRNA was subtracted from that of the gene of interest to obtain a  $\Delta\text{CT}$  value. The gene expression level relative to that of the calibrator was expressed as  $2^{-(\Delta\Delta\text{Ct})}$ . Each strain in the absence of drug was used as the baseline control strain and the expression levels of the treated strains were quantified relative to that of the control strain.

## 2.11 Statistics

Experiments were performed three times. SPSS 13.0 was used for the analyses and the results were expressed as the mean  $\pm$  SD. Differences between groups of the same strain were measured using one-way ANOVA. Statistical significance was defined as  $P < 0.05$ .

## 3. Results

### 3.1 Effects of respiratory inhibitors on biological characteristics and mitochondrial metabolism of *C. albicans*

#### 3.1.1 Respiratory inhibitors increase the azole sensitivity but single inhibitor has little effects on hyphae formation and growth

According to CLSI M27-A3 (2008) protocol, we tested the drug sensitivity of *C. albicans* WT strain SC5314 to FLC by adding CRC (CIII) or/and AOX inhibitor. The  $\text{MIC}_{100}$  of strain SC5314 to FLC was significantly reduced (the  $\text{MIC}_{100}$  was reduced to 2  $\mu\text{g}/\text{mL}$ , 1  $\mu\text{g}/\text{mL}$ , 1  $\mu\text{g}/\text{mL}$ ) when the strain was treated with respiratory inhibitor (AA 10  $\mu\text{M}$  / SHAM 5mm / AA 5  $\mu\text{M}$  + SHAM 1.25 mM). Without any inhibitor, the  $\text{MIC}_{100}$  of SC5314 to FLC was  $> 32 \mu\text{g}/\text{mL}$ . It is suggested that respiratory inhibitors (CIII or / and AOX inhibitors) can increase the azole sensitivity of *C. albicans*.

The growth of strain SC5314 was almost not suppressed by respiratory inhibitors (CIII or / and AOX inhibitors) (Fig. 1AB). AA or SHAM had no significant effect on the hyphal formation of strain SC5314, but the hyphae formation was suppressed by the combination of AA and SHAM (Fig. 2A).

## 3.1.2 Effects of respiratory inhibitors on mitochondrial metabolism of *C. albicans*

With the treatment of AA (CIII inhibitor), the mitochondrial function of *C. albicans* strain SC5314 was damaged (ATP decreased by 37%, mitochondrial membrane potential (MMP) decreased by 79% (Fig. 3AB). With regard to dysfunctional mitochondria, endogenous ROS levels were particularly enhanced when challenged with AA (ROS levels were increased 3.4-fold,  $P < 0.05$ ) (Fig. 3C). And the mitochondrial function was also damaged in strain SC5314 treated with SHAM (or AA combined with SHAM). Meanwhile, cell apoptosis associated with oxidative stress was also induced in strain SC5314. As shown in Fig. 3D (in top panel), the percentage of necrotic cells in strain SC5314 treated with AA accounts for 3.01% of the cell population compared to 0.29% in WT without any respiratory inhibitor, 2.41% in strain SC5314 with SHAM and 34.48% in strain SC5314 with AA combined with SHAM ( $P < 0.05$ ).

## 3.1.3 Respiratory inhibitors can induce high expression of Virulence Related Genes of *C. albicans*

The three testing genes -*ALS3*, *PLB1* and *HWP1*- are well known to relate to virulence of *C. albicans*, particularly with regard to tissue invasion and hyphal formation. As shown in Fig. 2B, the expression levels of gene *ALS3* and *HWP1* were up-regulated (14 times and 16 times of strain WT respectively,  $P < 0.05$ ) in strain WT treated with AA (CIII inhibitor). The expression levels of gene *HWP1* and *PLB1* were also up-regulated (5.9 times and 6.6 times of WT, respectively,  $P < 0.05$ ) in strain WT treated with SHAM (AOX pathway was inhibited). Respiratory inhibitors can induce high expression of virulence related genes *ALS3*, *PLB1* and *HWP1* of *C. albicans* strain SC5314.

## 3.2 Effects of *ADH1* knockout on biological characteristics and mitochondrial metabolism of *C. albicans*

### 3.2.1 Deletion of *ADH1* decelerates growth but has subtle effects on hyphal formation and azole sensitivity

The gene encoding *ADH1* was disrupted from the *C. albicans* strain WT (SC5314) by means of the *SAT1*-flipper method. In YPD medium with 2% glucose, the null mutant (EX2) cells grow slower than the corresponding strain WT and reconstituted strain RE1 during exponential phase, as shown in Fig. 4A. In contrast to a longer generation time of EX2 (DT = 120 min) in YPD, the DTs for SC5314 and RE1 are 96 min and 104 min, respectively. Consistent with this deceleration of growth, at 48 h strain EX2 forms smaller colonies than do the control strains on the YP agar plate supplemented with 2% glucose (YPD), 2% oleic acid (YPO), 2% glycerol (YPG) or 2% ethanol (YPE) as shown in Fig. 4B. However, the growth of *ADH1* deleted strain with other possible (i.e., non-glucose) carbon sources are not any stronger than with glucose.

The hyphal formation for each strain was measured with hyphal induced media as indicated in Fig. 4C in a 12-well plate. The hyphal growth was monitored at 37°C. Both WT strain and reconstituted strain are able to germinate post 1 h incubation and hyphae fully develop at 4 h. Consistent with a slower growth phenotype, deletion of *ADH1* in strain EX2 leads to a slightly suppressed hyphal population as shown in Fig. 4C.

Mitochondrial mutants of fungi often affect azole sensitivity.<sup>4</sup> When three strains were tested for their FLC sensitivities, the results showed that MIC<sub>100</sub> of FLC for *ADH1* mutant is 64 µg/ml, comparable to SC5314 (32 µg/mL) and reconstituted RE1 (64 µg/mL). At MIC<sub>80</sub> of FLC scale, all three strains have the same 0.25 µg/mL sensitivity.

### **3.2.2 Deletion of *ADH1* compromises ATP generation and mitochondrial function**

The total cellular ATP content is significantly reduced in *C. albicans ADH1* mutant. When compared with SC5314 and reconstituted strain RE1, mutant strain EX2 generates 2.7-fold less ATP (230 nM vs. 639.9 nM of WT,  $P < 0.05$ ) as shown in Fig. 5A. In order to understand how this ethanolic fermentation enzyme interferes with energetic metabolism in mitochondria, the mitochondrial function of *ADH1* mutant were evaluated by measurement of mitochondrial membrane potential, ROS level and apoptosis as well. We find that mitochondrial membrane potential in EX2 decreased 2.4-fold (1.7 vs. 4.1 of WT red/green ratio,  $P < 0.05$ ) as shown in Fig. 5B. As a consequence, the endogenous ROS production in EX2 is 2.83-fold higher than WT ( $P < 0.05$ , Fig. 5C) and the percentages of necrotic (top-right quadrant in Fig. 5D) and apoptotic (top left quadrant in Fig. 5D) cells are significantly higher in EX2 (11.25% and 4.56%) versus SC5314 (0.29% and 0.14%) or RE1 (4.22% and 0.78%) with  $P < 0.05$ .

### **3.2.3 Pathogenesis-related genes in *ADH1* deleted mutant are less expressed**

Consistent with the slightly decreased growth, the deletion of *ADH1* gene of *C. albicans* results in the downregulation of *ALS3* and *HWP1* when grew in YPD medium. As shown in Fig. 4D, *ADH1*-deleted mutant at 6 h growth displays a compatible level of *PLB1* ( $P > 0.05$ ), but the expression levels of *ALS3* and *HWP1* are only 41% and 26% of the WT strain.

## **3.3. Effects of *ADH1* on mitochondrial CRC, AOX and PAR pathways**

### **3.3.1 Respiratory inhibitors decreased ATP production and suppress mitochondrial function in *ADH1*-deleted mutant**

To determine the target pathway of *ADH1* on mitochondrial respiration, we measured mitochondrial activities of *ADH1* mutant under AA (inhibitor of complex III of CRC), SHAM (inhibitor of AOX pathway), and a combination of SHAM and AA conditions, respectively, in order to uncover the relative contribution

of the AOX, CRC and PAR respiratory pathways (Yan et al., 2009; Sun et al., 2013b). The concentrations used here (10  $\mu$ M AA and 5 mM SHAM) can effectively suppress mitochondrial function of *C. albicans*, including control strains. As shown in Fig. 3A, intracellular ATP levels in WT are decreased by approximately 48% with SHAM or AA + SHAM and by 37% with AA alone when compared to untreated WT. Meanwhile, the overall ATP generation in mutant strain EX2 becomes further compromised compared to the two control strains no matter which respiratory inhibitor is used. However, the effectiveness of AA treatment seems greater than that of SHAM in this mutant with regard to ATP level, as demonstrated by an 89% reduction with AA over a 39% reduction with SHAM when compared to its untreated cells, respectively (Fig. 3A). This ATP deficiency of mutant EX2 in response to AA suggests that the CRC pathway may be vulnerable when *ADH1* is deleted.

For mitochondrial membrane potential, the reduction pattern with each ETC inhibitor regimen of mutant strain EX2 is similar to that of WT. Both strains are more sensitive to the CIII inhibitor AA than to SHAM when compared to untreated cells that is similar to ATP changes mentioned above. However, the scale of reduction of EX2 in presence of AA seems to be less than that of WT (59% vs. 79%), particularly in AA + SHAM treatment where the membrane potential of EX2 is even higher than WT (Fig. 3B). The relative insensitivity to SHAM in both strains can be explained by the lesser contribution of AOX pathway on mitochondrial membrane potential and ATP production as expected. The quite comparable levels of mitochondrial membrane potentials in EX2 under AA treatment on the other hand suggest that AOX and/or PAR pathways had been up-regulated in cells with *ADH1* deletion. This possibility is supported by the insensitivity to SHAM in mutant strain in Fig. 3B.

With regard to dysfunctional mitochondria, endogenous ROS production increased in three strains when treated with respiratory inhibitors ( $P < 0.05$ ). As shown in Fig. 3C, the ROS levels are particularly enhanced when challenged with AA to inhibit CIII of CRC pathway. Meanwhile, cell apoptosis associated with oxidative stress is also increased in *ADH1*-deleted mutant. As shown in Fig. 3D (middle panel), the percentage of necrotic cells in EX2 strain without any respiration inhibitor accounts for 11.25% of the cell population compared to 0.29% in WT (in top panel) and 4.22% in reconstituted RE1 strain (bottom panel) ( $P < 0.05$ ). The suppression of CRC pathway by AA treatment causes a slight increase of necrotic and apoptotic cells in WT strain (3.01% and 1.49%) and reconstituted strain RE1, but AOX inhibitor similarly results in 2.41% and 11.23% necrotic cells in WT and reconstituted strains. Compared to WT and reconstituted strains, the respiratory inhibitors AA and SHAM alone remarkably promote the necrotic cells by 80.85% and 29.79% respectively in mutant strain as shown in the middle panel of Fig. 3D ( $P < 0.01$ ). The addition of SHAM treatment shows a similar percentage of necrotic cells (79.03%) under AA treatment alone in the EX2 strain. We find it interesting that, while SHAM increases apoptotic cells population (top left quadrant) by 11.03% in WT and necrotic cells (top right quadrant) by 11.23% in reconstituted strain, two types of apoptotic cells are greatly increased with 6.12% and 29.79%, respectively. Like the mitochondrial function measurements above, the reconstituted strain can partially restore the function of mitochondrial related phenotypes. The insalubrious function of the mutant mitochondria merely renders the *ADH1*-deleted death more complete.

### 3.3.2 Growth and morphological switching are suppressed in *ADH1* mutant in the presence of respiratory inhibitors

Growth of mutant strain EX2 is more suppressed under respiratory inhibitors. As shown in Fig. 1A, the growth rates in WT and reconstituted strain RE1 are not remarkably affected under either treated condition when compared to their untreated cells. The rate of *ADH1* mutant strain in the presence of SHAM is the same as for untreated EX2 cells during 12 h growth; however, the growth is fully arrested in the presence of AA or AA + SHAM conditions. At 48 h, no trace of growth on YPD is observed with AA or with AA + SHAM, as shown in Fig. 1B. We find it likely that this cell death phenotype of EX2 under AA treatment, together with a ~ 80% necrotic and 10.99% apoptotic cell population in Fig. 3D explain a ~ 89% reduction in ATP yield (Fig. 3A).

The yeast-to-hyphae transition is an important virulence factor of *C. albicans*. In the absence of respiratory inhibitors, hyphal formation is slightly affected in the *ADH1*-deleted mutant EX2 even though its ATP level is 2.7-fold lower than that of WT. To determine whether this rate of morphological switching is correlated with ATP abundance or insufficiency, or alternatively, is simply a reflection of the mutants' slower growth in general (Fig. 4C), the yeast-to-hyphae transition under respiratory inhibitors condition was studied cross all three strains at 4 h for control strains and 6 h for the mutant strain. We find that AA or SHAM alone (and especially the former) can effectively inhibit hyphal formation in all three strains, grown in hyphae inducible conditions (Fig. 2Aa – 2Ac). The complete abolition of this yeast-to-hyphae transition occurs when two respiratory inhibitors are combined at 4 h growth of WT and reconstituted strain regardless of their nearly 50% ATP remaining (Fig. 3A), which is easily found in mutant EX2 when treated with AA alone for 6 h. For mutant strain, the overall effect of SHAM on hyphal formation is much smaller than AA. Some degree of germination in mutant strain can be seen in Fig. 2Ab and Fig. 2Ac under SHAM treatment while the nearly 25% WT-ATP is remaining (Fig. 3A), however, no hyphal formation is corresponding to a ~ 10% ATP remaining under AA treatment. The artifact of filamentous formation based on ATP arisen from mutant strain is not observed in WT. These results suggest that ATP insufficiency is not the only determinant factor for hyphal formation even though functional energetic pathways like CRC, AOX are required for this morphological switching event.

### 3.3.3 Azole tolerance mediated by CRC respiratory pathway in *ADH1* mutant

As we mentioned above, deletion of *ADH1* has no effect on azole sensitivity, which are often found in the mutants from mitochondrial CRC and AOX pathways (Yan et al., 2009; Sun et al., 2013a). The insufficient ATP yield seems not to be the cause of azole sensitivity in our case because *ADH1*-deleted mutant EX2 shows equal tolerance of control strains to FLU ( $MIC_{100}=64 \mu\text{g/mL}$ ) when its ATP content is only 36% of WT level (Fig. 5A). For WT and reconstituted strains, the  $MIC_{100}$  values of azole in culture with 10  $\mu\text{M}$  AA, 5 mM SHAM or 5  $\mu\text{M}$  AA + 1.25 mM SHAM are 1, 2 and 1  $\mu\text{g/mL}$  following to CLSI M27-A3 (CLSI, 2008)<sup>15</sup> protocol, which are similar to those previous reports (Costa-de-Oliveira et al., 2012; Yan et al., 2009).

Compared to control strains, the MIC<sub>100</sub> value of FLC in mutant became a 2 µg/ml with 5 mM SHAM treatment, meanwhile, no sign of EX2 growth can be detected in culture wells in the presence of AA. To limit the remarked effects of high dose AA on mutant's growth and energetic phenotypes, we retested FLC sensitivity in EX2 using 0.125 µM AA (1/80 strength of WT) as well. Under this low concentration of AA, we find that mutant was still hypersensitive to FLC ( $\geq$  8-fold vs. WT with 0.125 µM AA). These results suggest that CRC pathway is main contributor of FLC tolerance in this mutant.

### 3.3.4 Mitochondrial inhibitors induce *ALS3* and *HWP1* gene expression in *ADH1* mutant

In the presence of the CIII inhibitor AA, the expression of *ALS3* increases 14 times ( $P < 0.05$ ) in WT and 5.7 times in reconstituted strain as shown in Fig. 2B, but expression of *PLB1* is much less affected. Similar to *ALS3* response, *HWP1* is also up-regulated under AA treatment in both control strains. Unlike *ALS3*, however, the *HWP1* gene also responds to the AOX inhibitor SHAM treatment even though the magnitude is larger than *ALS3* expression. Again, *PLB1* seems unaffected with AA treatment alone in both control strains, and this is similar to EX2 mutant without any respiration inhibitor. However, SHAM can induce 6.6- and 4.0- fold increases of *PLB1* in WT and reconstituted strains, along with a 2.8-fold increase in mutant strain as well.

Compared with WT and reconstituted strains, the expression levels of *ALS3* and *HWP1* in EX2 mutant are not seen to elevate when treated with AA. Meanwhile, the *HWP1* level is 10-fold higher than WT strain under SHAM treatment. We find it interesting that the reconstituted strain constitutes a reasonable intermediate for *HWP1* and *PLB1* expression when SHAM is introduced into the medium. Also, *HWP1* transcribed in reconstituted strain under AA alone treatment is nearly twice as that in WT, suggesting that some compensatory mechanism may be responsible for this excess *HWP1* response. Furthermore, the lack of response with SHAM when combined with AA, as shown in *ADH1*-deleted mutant, also indicates some compensatory mechanism at work.

We found that compared with WT (or reconstituted strain), the expression level of virulence related genes *ALS3* and *HWP1* decreased significantly in *ADH1*-deleted mutant under the treatment of CRC inhibitor AA (Fig. 2B). We consider that the inhibition of both *ADH1* gene and CRC pathway has a certain effect on the virulence of the *C. albicans*. In brief, the expressions of three genes vary with either AA or SHAM or combined treatments, and none of respiration pathways are positively correlated with hyphal formation. While *ALS3* only responds to AA inhibited CRC pathway, *HWP1* responds to both CRC and SHAM inhibited AOX pathway as well in control strains. The similar levels of *PLB1* in the three strains under respiratory inhibitors indicate a lack of association of *PLB1* with *ADH1*-related mitochondrial function. Divergence of virulence gene profiling and hyphae formation between AA and AA/SHAM treatment may imply that PAR respiration, if *C. albicans* has such, and fermentative energetic pathway may also participate in these biological phenotypes as graphed in Fig. 6. Therefore, when AOX pathway is inhibited by SHAM or together with CRC suppression at the same time by AA in WT, the upgraded CRC and/or PAR may also regulate these genes expression and promote therein under gene-related biological processes. However,

the mutant strain with an inborn vulnerable CRC fails to respond to these inhibitor-inducible upregulation of virulence genes.

## 4. Discussion

Anaerobic fermentation and mitochondrial aerobic respiration of *C. albicans* compensate and regulate each other during the metabolic process, which makes it difficult to treat fungus infection clinically. In recent years, mitochondrial respiratory chain (MRC) has been considered as a new target for clinical antifungal therapy (Shirazi and Kontoyiannis, 2013; Chamilos et al., 2006).

However, it is not ideal to restrain the growth, reproduction and pathogenicity of *C. albicans* by inhibiting a complex of mitochondrial ECT (CI, CIII, CIV or CV). In the search for ideal drug targets that can affect multiple energy metabolisms, *C. albicans* ADHs have earned our particular attention, since this gene family is the key connection to link two major energy metabolisms in non-pathogenic *S. cerevisiae*. And we found that there was a certain relationship between ADH1 and the azole-resistance genes (Guo et al., 2013). In addition, ADH1 deletion can reduce the virulence of *C. albicans*, but has no effect on the growth and cell viability (Song et al., 2019).

There are few studies on the combined inhibition of *C. albicans* fermentation and mitochondrial respiratory pathway. Through comparisons among growth, hyphal formation phenotypes, apoptosis and mitochondrial function between *ADH1* null mutant and WT with or without different respiratory inhibitors, we characterize the biological function and pathological roles of *ADH1* in this study.

Relevant study showed that the inhibition of CIII or AOX on the growth of yeast cells is obvious, but rotenone (inhibitor of CI) has no obvious effect on proliferation of *C. albicans* cell (Ruy et al., 2006). We find that, the mitochondrial function of strain WT (SC5314) was slightly damaged by AA (inhibitor of CIII in classical pathway), including ATP and MMP, and AOX was the main respiratory pathway of mitochondria at present. In addition, ROS increased significantly and apoptosis increased slightly. AA also can increase the azole sensitivity and up-regulate the expression of virulence related genes *ALS3* and *HWP1* in strain WT. It is worth noting that even in the case of a 37% reduction in ATP production, the hyphal formation and cell growth rate did not change significantly. There was a similar situation in WT induced by SHAM (inhibitor of AOX), and CRC was the main respiratory pathway. Only with the combined action of AA and SHAM (PAR is the main respiratory pathway of mitochondria), hyphal formation was obviously limited, and the cell growth was slightly slowed down in strain WT. At this time, the decrease of ATP was similar to that induced by AA.

These results suggest that even if the mitochondrial function of *C. albicans* is slightly damaged (treated with AA or SHAM), the CRC or AOX pathway can compensate each other, which shows that the cell growth, reproduction and hyphal formation are less affected. Unlike *S. cerevisiae*, *C. albicans* is a typical facultative anaerobe that can switch between aerobic respiration and anaerobic pathways depending on the availability of oxygen. The latter allows it to gain more ATP out of glucose when oxygen is available. Also, the multiple-branched metabolic pathways afford *C. albicans* a more competitive advantage in

adapting to different host environments. In spite of several studies focusing on *ADH1* contributions to biofilm, virulence and immune response (Klotz et al., 2001; Mukherjee et al., 2006), the roles of *C. albicans ADH1* and other *ADH* isomers in energy metabolism have not yet received much attention, due at least part to the assumption that respiration dominates energy generation in this organism.

The gene encoding *ADH1* was disrupted from the *C. albicans* WT strain SC5314 by means of the *SAT1*-flipper method, and the *ADH1* mutant (EX2) was used in this study. Deletion of *ADH1* in this study showed subtle effects on biological phenotypes such as a WT-level azole susceptibility and a slightly delayed germination, perhaps due to a longer generation time observed by us and others (Kwak et al., 2014). We find that, except in cases where growth is truly constrained by ATP availability, all of these phenotypes depend more or less on mitochondrial respiration, since these phenotypes can be remarkably suppressed in both WT and mutant as long as mitochondrial respiration inhibitors are present.

On the other hand, ATP yield is only 35% of WT in *ADH1* mutant, due largely to an impaired mitochondrial function. The latter is demonstrated by a 41% reduction of mitochondrial membrane potential, a 2.8-fold higher level of ROS, and more than 10% apoptosis in untreated *ADH1* mutant over those of WT (under aerobic conditions). After this impaired respiration was further suppressed by AA, the ATP generating process is nearly abolished in the mutant strain, suggesting that *ADH1* is a critical element for ATP generation from the CRC respiration pathway. In an earlier study (Bertram et al., 1996), *ADH1* expression reached its maximum at late exponential growth in *C. albicans*. Under non-glucose media such as glycerol, galactose, lactate, *ADH1* mRNA levels were high, but low in glucose or ethanol growth medium. The same authors also speculated that *ADH1* is regulated at both the transcriptional and translational levels. We find that a slightly reduced growth of *ADH1* mutant on 2% glucose YPD agar is also seen in ethanol, glycerol or oleic acid medium under aerobic conditions in this study. To date we have no answer for carbon dependency of *ADH1* activity under aerobic and anaerobic growth conditions. Similar growth behavior on glucose and non-glucose media are perhaps the consequence of inter-substitution by other *ADH* isomers such as *C. albicans* Adh2p, which shows a 76 ~ 77% identity to *S. cerevisiae* Adh1p, Adh2p and Adh3p as well.

The above results suggest that it can't effectively inhibit *C. albicans* cells by deleting *ADH1* or inhibiting one mitochondrial respiratory pathway. We found that the hyphal formation of *ADH1* mutant was obviously limited under the induction of respiratory inhibitor (AA). Even at very low concentrations of AA (0.25  $\mu$ M), the mutant cells showed no signs of growth (MIC detection and spot assay). And AA could reduce the expression of *ALS3* and *HWP1* in strain EX2. These results suggest that CIII inhibitors can significantly reduce the pathogenicity of *C. albicans ADH1* mutant. And the mutant became hypersensitive to azoles and apoptosis, which may be closely related to the severe damage of respiratory function.

Because *ADH1* null mutant strain is highly sensitive to respiratory inhibitors, it is difficult to survive in large numbers in animals. Therefore, the cytotoxicity of both *ADH1* and CIII on *C. albicans* cells is not further studied in rats.

The direct link between ethanol fermentation and mitochondrial metabolism is still something of a mystery in this organism. We have no evidence on which to build an interpretation of this *ADH1*-dependent respiration event. The divergent metabolic behavior of *C. albicans* compared to *S. cerevisiae* suggests that *ADH* is likely to have a different biological significance in *C. albicans*. Further characterization of each *C. albicans ADH* isomer with enzymatic kinetics, location determination and gene regulation in different growth conditions will eventually be needed in order to fully understand how alcohol fermentation links to other energy metabolic pathways in colonization and growth *in vivo*.

Anaerobic fermentation and mitochondrial aerobic respiration are involved in the energy metabolism of *C. albicans*. Each pathway contributes to the survival of the fungi and the pathogenicity to the host. Our results indicate that inhibition of anaerobic fermentation (*Adh1*) and CIII of CRC pathway can significantly attenuate the pathogenicity, thus effectively killing *C. albicans* cells. This discovery will provide a scientific basis for clinical search for better antifungal drugs and their targets.

## Declarations

### Disclosure of interest

The authors declare that they have no competing interest.

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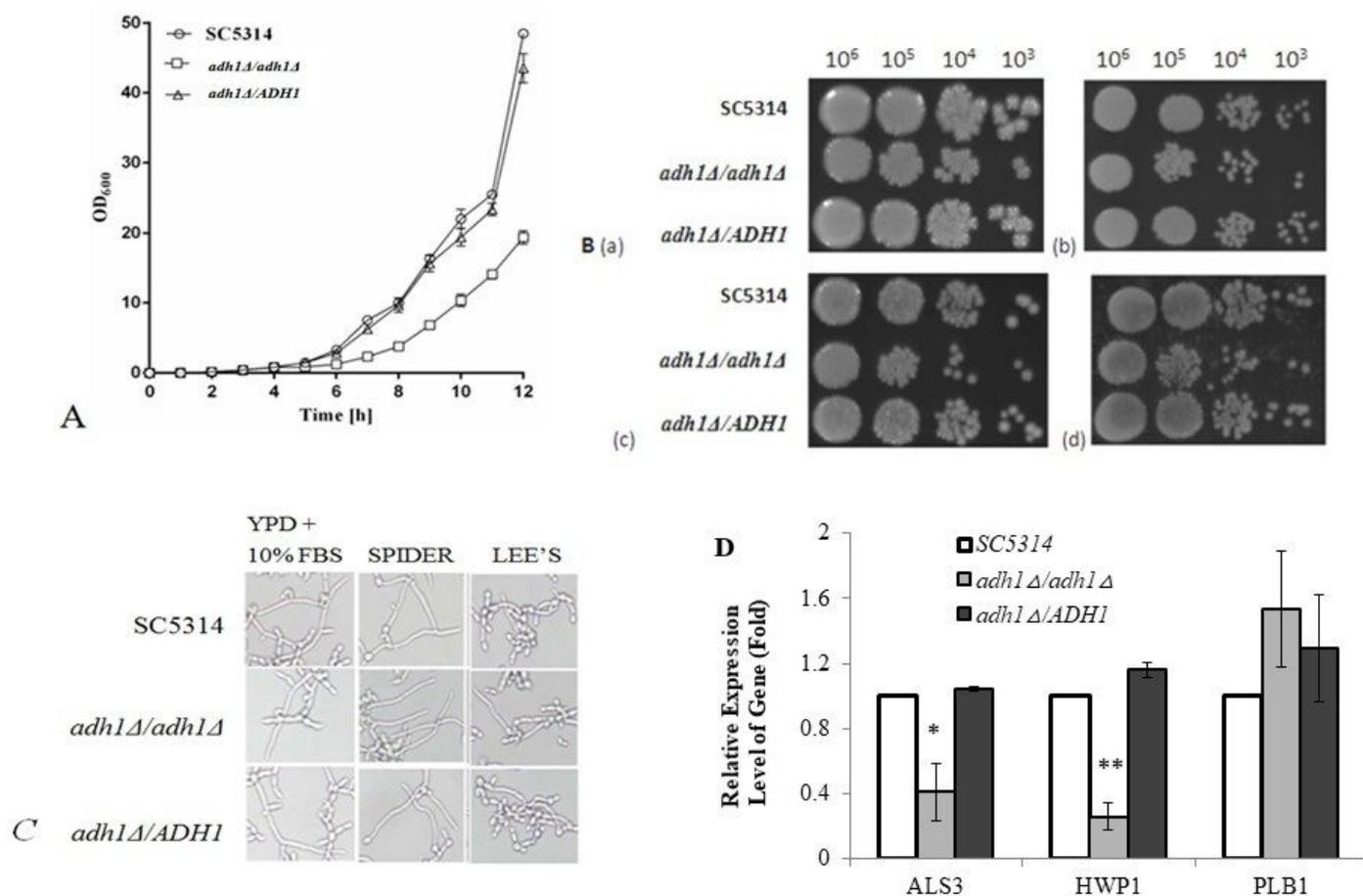
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## Table

**Table 1** Primer sequences used in RT-PCR

Primer name	Sequence(5'-3')
SAP2-F	TTGGATTTGGTGGTGTTCG
SAP2-R	ATTATTTGTCCCGTGGCAG
ALS3-F	CTGGACCACCAGGAAACT
ALS3-R	ACCTGGAGGAGCAGTGAAAG
PLB1-F	GGTGGAGAAGATGGCCAAA
PLB1-R	AGCACTTACGTTACGATGCAACA
HWP1-F	CGGAATCATGTGCTGTCGTCTCT
HWP1-R	CGCACTTGAGTAATTGGCAGATG

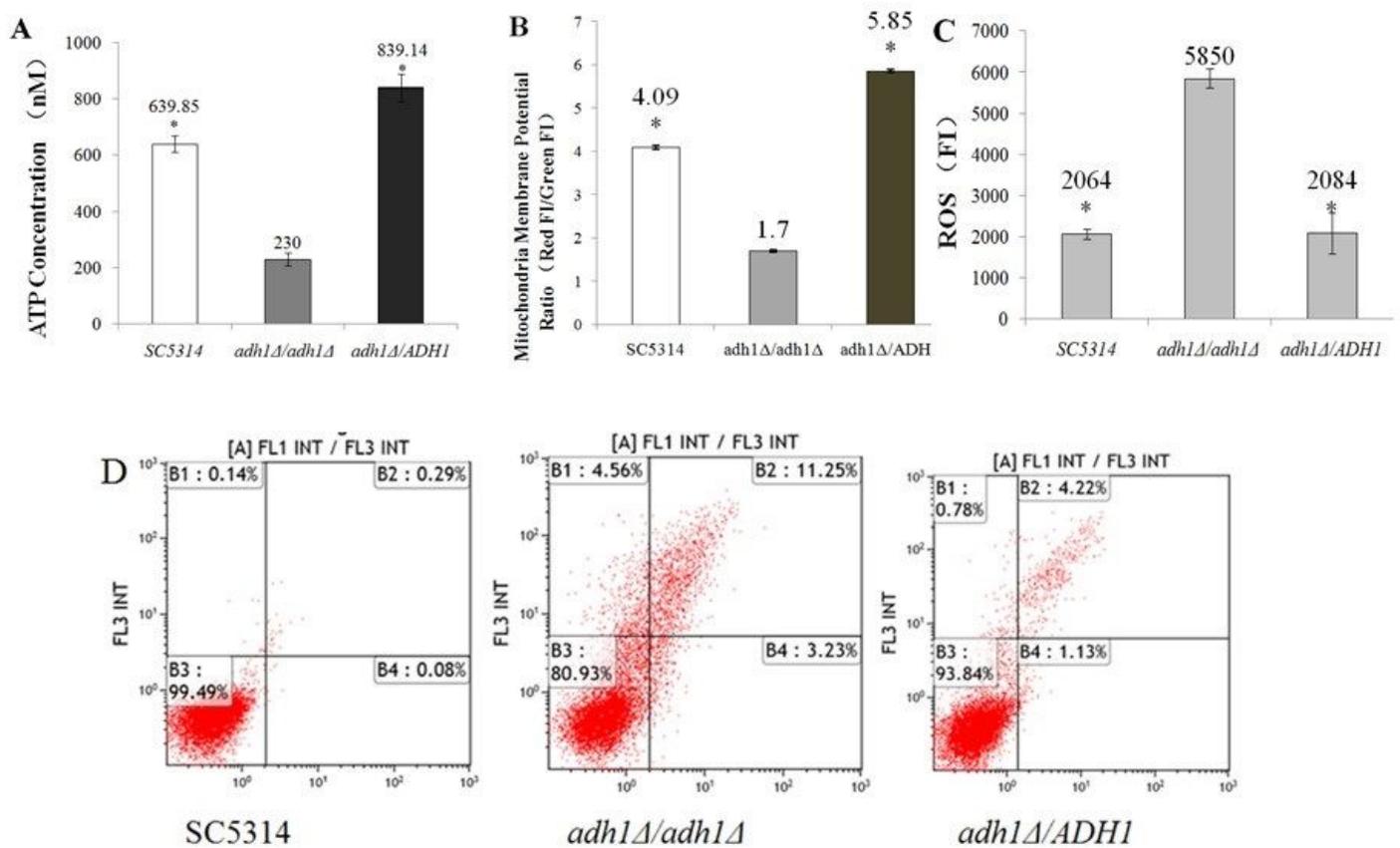
## Figures



# Figure 1

**Figure 1**

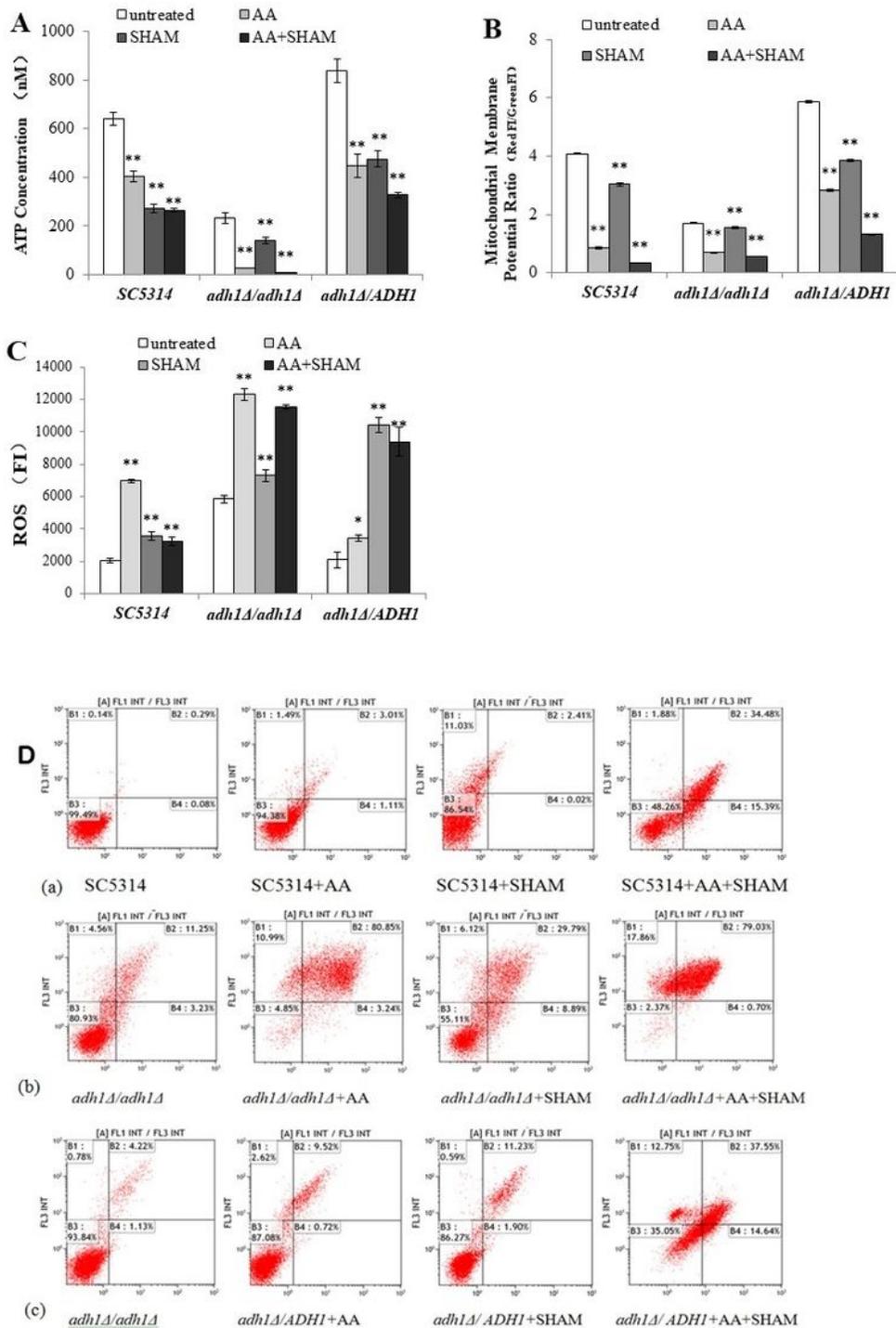
Deletion of ADH1 results in a longer generation time and less pathogenic gene expression. (A) The growth curves of strains SC5314 (WT), ADH1-deleted mutant EX2 (*adh1Δ/adh1Δ*) and reconstituted strain RE1 (*adh1Δ/ADH1*) are shown in YPD at 30°C without compounds. (B) Spot assays of SC5314, EX2 and RE1 strains grow on YPD (a. 2% glucose), YPE (b. 2% ethanol), YPG (c. 2% glycerol) and YPO (d. 2% oleic acid) agar plates. The growth was recorded after 48 h incubation at 30 °C. (C) Germination of three strains grown in YPD + 10% FBS broth, Spider and Lee's media at 37°C for 4 or 6 h. (D) Relative expression levels of pathogenesis related genes (ALS3, HWP1 and PLB1) in the three strains without respiration inhibitors. "\*" and "\*\*" indicate P < 0.05 and P < 0.01 (vs. SC5314).



**Figure 2**

**Figure 2**

Changes of mitochondrial activities under respiratory inhibitors in *C. albicans* ADH1-deleted mutant strain. Measurements of (A) intracellular ATP content, (B) mitochondrial membrane potential, (C) endogenous ROS and (D) apoptosis, were obtained from strain SC5314, EX2 (*adh1Δ/adh1Δ*) and RE1 (*adh1Δ/ADH1*) in the absence or the presence of 10  $\mu$ M AA, 5 mM SHAM and 10 $\mu$ M AA+ 5 mM SHAM for 12 h, respectively. “\*” and “\*\*” indicate  $P < 0.05$  and  $P < 0.01$  (vs. untreated WT cells) for each strain.



**Figure 3**

**Figure 3**

Decreases of mitochondrial activities in ADH1-deleted mutant strain. (A) Determination of intracellular ATP concentration by luminescent signals with BacTiter-Glo reagent. Flow cytometric analyses for (B) mitochondrial membrane potential and (C) endogenous ROS production. (D) Phosphatidylserine externalization determination with 5.0  $\mu$ l of FITC-labeled Annexin V and 5.0  $\mu$ l PI staining in untreated

fungal cells of SC5314, EX2 (*adh1Δ/adh1Δ*) and RE1 (*adh1Δ/ADH1*) strains, by a FACS Calibur flow cytometry.

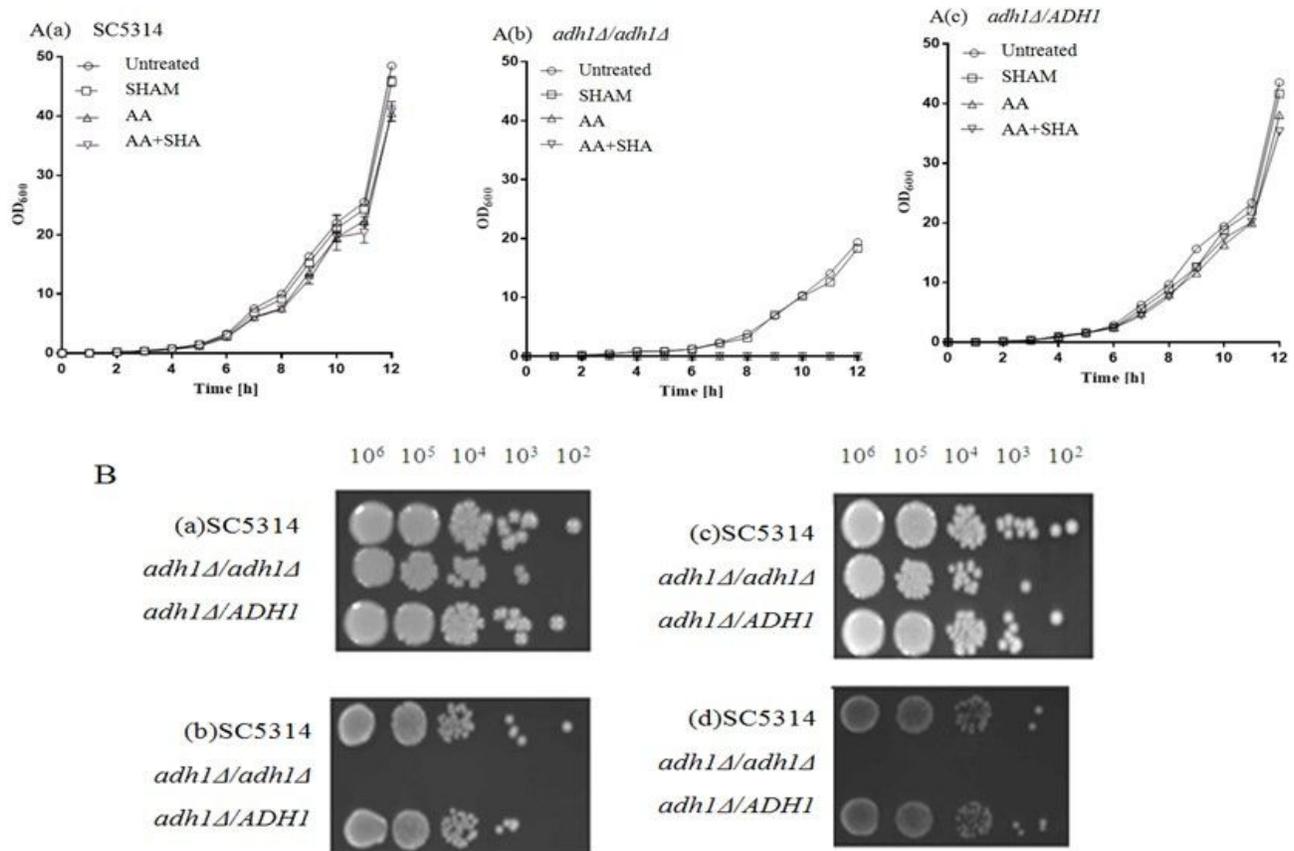


Figure 4

Figure 4

The CIII inhibitor AA abolishes growth of ADH1-deleted mutant completely. (A) the OD values obtained at every 1 h interval from 12 h cultures of *C. albicans* SC5314, EX2 (*adh1Δ/adh1Δ*) and RE1 (*adh1Δ/ADH1*) with or without treatment of respiratory inhibitors as indicated in the diagram. No growth can be detected from AA- or AA+SHAM- treated ADH1-deleted EX2 strain. (B) Spot assay of strain SC5314, EX2 and RE1 in (a) YPD, (b) YPD + AA, (c) YPD + SHAM, and (d) YPD + AA + SHAM. The growth was monitored after 48 h incubation at 30 °C. AA: 10 μM, SHAM: 5 mM.

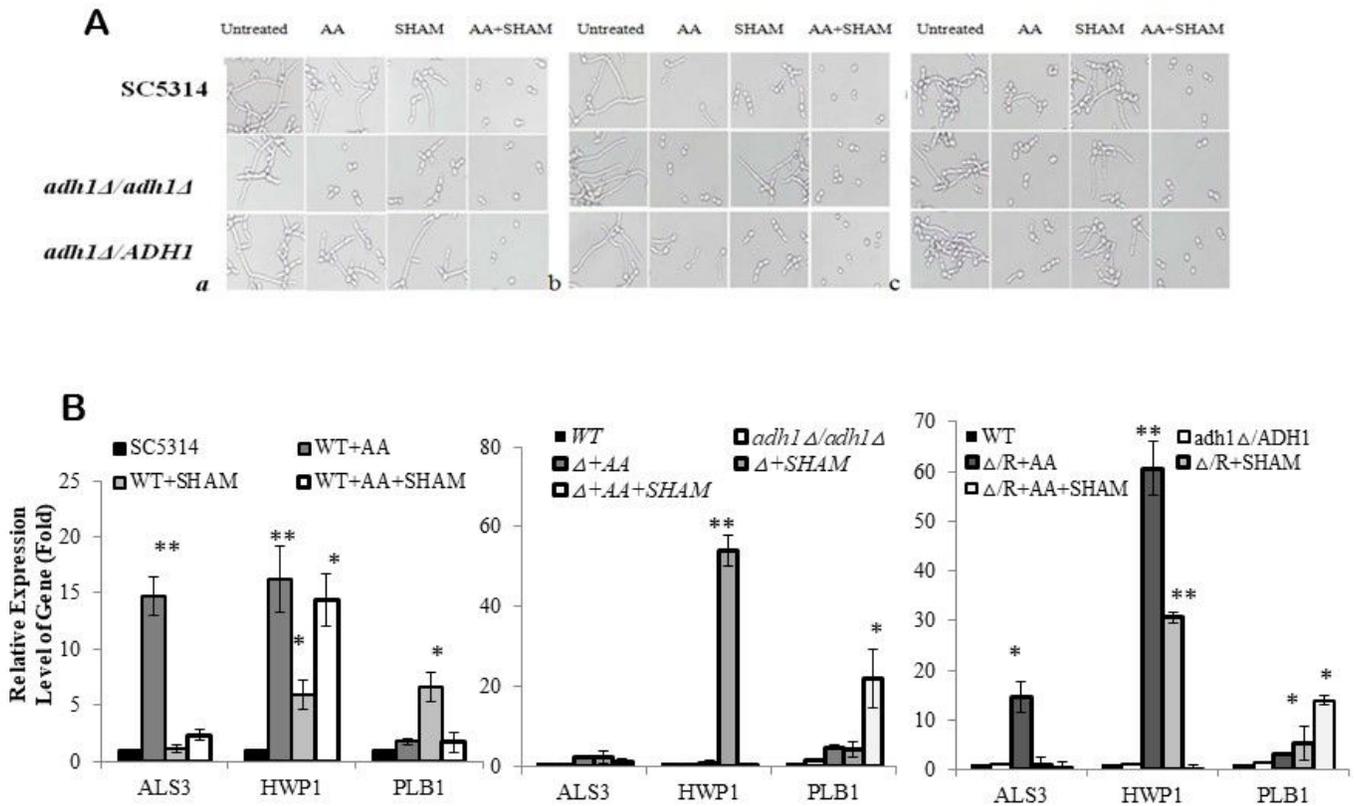


Figure 5

Figure 5

Changes of virulence-related hyphal formation and genes expression in ADH1-deleted mutant under mitochondrial respiratory inhibitors. (A) Filamentous growth of strains SC5314, EX2 (*adh1Δ/adh1Δ*) and reconstituted RE1 (*adh1Δ/ADH1*) under respiratory inhibitors are examined after cultured in YPD +10% FBS, SPIDER and LEE'S media at 37 °C for 4 ~6 h. (B) Expression Levels of gene ALS3, HWP1 and PLB1 in *C. albicans* SC5314, ADH1-deleted mutant (EX2) and reconstituted strains treated and untreated with respiration inhibitors. "\*" and "\*\*" indicate  $P < 0.05$  and  $P < 0.01$  (vs. untreated cells) for each strain.

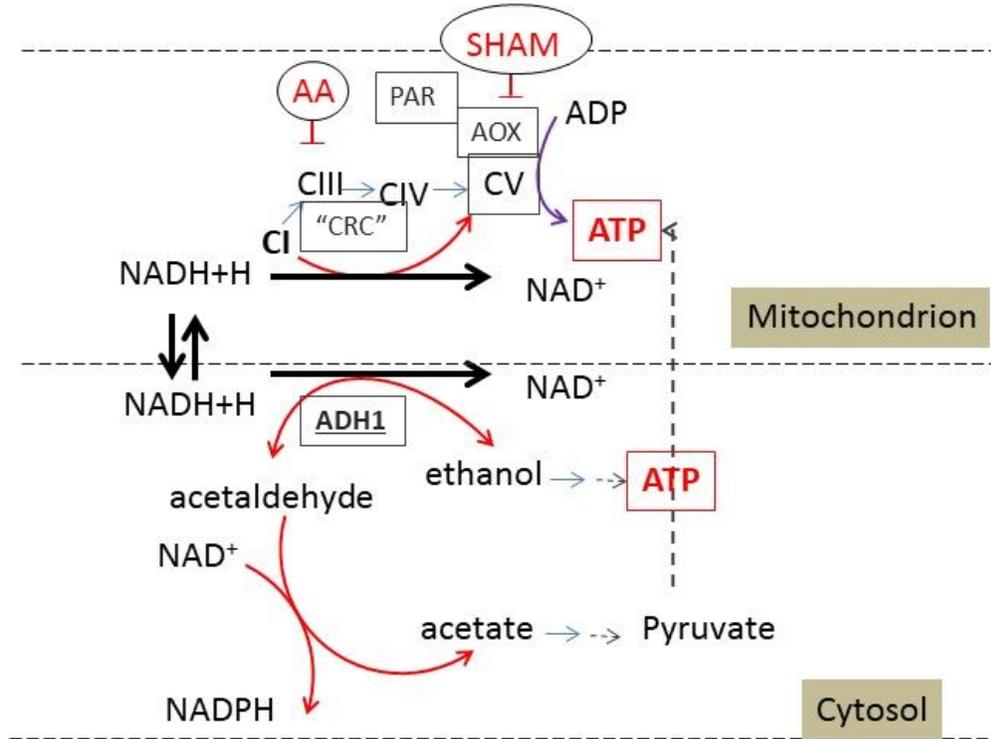


Figure 6

Figure 6

Divergence of virulence gene profiling and hyphae formation between AA and AA/SHAM treatment may imply that PAR respiration, if *C. albicans* has such, and fermentative energetic pathway may also participate in these biological phenotypes as graphed.