Lipidomics and *in Vitro* Azole Resistance in *Candida albicans*

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Abstract

We have shown earlier that fluconazole (FLC) stress induces global changes in the lipidome of *Candida albicans* in clinically adapted isolates. However, several laboratories have developed adapted *in vitro* FLC resistant strains of *C. albicans* to study azole resistance mechanisms. This study aimed to identify the lipid changes associated with FLC resistance in these *in vitro* adapted isolates. Using comparative lipidomics and principal component and discriminant analyses, we observed gradual changes in several lipid classes and molecular species upon FLC exposure of *in vitro* resistant *C. albicans* strains. Although the lipid imprint of FLC *in vitro* resistant isolates was very distinct from that of clinical isolates of *C. albicans*, the overall changes in lipid class compositions were similar in both cases. For example, an increased sterol content and depleted sphingolipid levels were the salient features of FLC resistance in both conditions. Taken together, it appears that the overall cellular lipid homeostasis is a critical factor in the observed FLC resistance and in handling FLC stress in both clinical and laboratory situations. The new observations reported herein have implications for more efficacious antifungal drug development as well as understanding host–infectious agent interactions in postgenomics microbiology practice.

Introduction

IN RECENT YEARS, THE EMERGING CASES of drug resistance in *Candida albicans* have mainly been attributed to prolonged and excessive usage of antifungals. This has caused serious problems in their effective treatment (Clark and Hajjeh, 2002; Pfaller and Diekema, 2007; Richardson, 2005; Tortorano et al., 2006). Reportedly, *C. albicans* cells develop multidrug resistance (MDR) via multiple resistance mechanism(s) during the treatment. In *C. albicans*, reduced intracellular accumulation of drugs that is caused by rapid efflux is the major mechanism of resistance. Accordingly, a transcriptional activation of genes encoding membrane efflux pumps, such as ATP binding cassette (ABC) drug transporters, particularly CaCdr1p or CaCdr2p, or the major facilitator super family (MFS) efflux pump protein, particularly CaMdr1p, is observed in the resistant isolates of *C. albicans* (Prasad and Kapoor, 2005; Sanglard and White, 2005; Sanglard et al., 2009; White et al., 1998).

Lipids play a crucial role in drug resistance in *C. albicans*. Alterations of the target of azole, such as lanosterol 14- α demethylase (*ERG11*), is a main resistance mechanism in *C. albicans* (Prasad and Kapoor, 2005; White et al., 1998). Certain efflux pumps like CaCdr1p show selectivity for lipids and preferentially localize in the “lipid rafts” within plasma membrane (Pasrija et al., 2005). The raft constituents, namely ergosterol and sphingolipids (SLs), are known to interact

closely; disruption in either of these results in altered drug susceptibility profiles (Mukhopadhyay et al., 2004; Pasrija et al., 2005). Therefore, any alternations in sterol or SL biosynthetic genes may cause mislocalization of CaCdr1p (Pasrija et al., 2005). Interestingly, certain efflux pumps such as CaMdr1p are not dependent on raft lipid components and are localized properly throughout the plasma membrane (Pasrija et al., 2008). These observations are supported by the fact that genes which govern MDR and the lipid metabolism genes are co-regulated (Marie and White, 2009; Morschhauser, 2002). Further, perturbations in membrane lipid environment affect azole resistance in *Candida* cells (Kohli et al., 2002). In *C. albicans*, drug susceptibility cell depends on membrane lipid environment, drug extrusion, and diffusion (Mukhopadhyay et al., 2002). Apparently, lipids do contribute towards the acquisition of drug resistance in *C. albicans*. However, exact changes in the lipid metabolism that occur during this drug adaption are not completely known.

In our previous studies, we performed high-throughput lipidomics of isogenic clinical azole-susceptible (AS) and -resistant (AR) isolates of *C. albicans*. There we discussed certain general commonalities among lipid species in AR isolates and summarized a list of molecular lipid species that could be correlated with azole resistance (Singh and Prasad, 2011; Singh et al., 2012).

Although studies using *in vivo* models have provided valuable information regarding the molecular changes linked

with MDR, the precise time point when these changes result in a resistant phenotype is difficult to determine. To overcome such problems, *in vitro* systems have been developed by several groups by exposing *C. albicans* cells to increasing thresholds of fluconazole (FLC) (Albertson et al., 1996; Cowen et al., 2000). Earlier Kohli et al., (2002), by using the sequentially adapted *in vitro* FLC-resistant strains of *C. albicans*, were able to show a stepwise increase in the development of MDR characteristics upon FLC exposure. For example, the upregulation of efflux pump-encoding genes, specifically *CDR1* and *CDR2* as well as the FLC target enzyme *ERG11*, is observed in a FLC-dependent manner in *C. albicans* (Kohli et al., 2002). That study provided initial evidence that an altered lipid homeostasis might be associated with drug resistance. However, complete understanding to this interaction required a closer look.

Therefore, the present study continues the characterization of the role of lipids in the resistance mechanisms present in the series of isolates used by Kohli et al. (2002). Here, by employing comparative lipidomics, we have quantified over 240 molecular lipid species and evaluated the lipid composition changes among the isolates of this series. In this study we present a complete lipidome picture in response to FLC resistance in *C. albicans* cells. We demonstrate that each isolate possesses a unique molecular lipid species profile. However, there is a correlation between the changes occurring gradually the level of molecular species and the increasing FLC resistance in laboratory-adapted strains of *C. albicans*. Apparently, a tight regulation in the amounts of lipids such as PGLs, SLs, and SEs, as well as other lipid classes, might be contributing to the overall FLC resistance observed in *C. albicans*.

Materials and Methods

Strains and culture conditions

Candida albicans strains used in this study are: YO1-16 sensitive to FLC ($MIC_{80} \leq 16 \mu\text{g/mL}$), YO1-32 that shows intermediate sensitivity to FLC ($MIC_{80} \leq 32 \mu\text{g/mL}$), and YO1-64 resistant to FLC ($MIC_{80} \leq 64 \mu\text{g/mL}$) (Kohli et al., 2002). Media and culture conditions used in this study are consistent with our previous work (Singh et al., 2010).

Lipid analysis

Lipids were extracted from *C. albicans* cells using a slight modification of the method of Bligh and Dyer as described previously (Bligh and Dyer, 1959; Singh et al., 2010). The mass spectrometry-based lipidome analysis employed in the present article draws from, and is consistent with our earlier work (Singh et al., 2010; Singh and Prasad, 2011). PGLs, SLs, and SEs were quantified exactly as described previously (Singh et al., 2010; Singh and Prasad, 2011; Singh et al., 2012).

Free sterol analysis by gas chromatography-mass spectrometry

Sterols were extracted and analyzed using the slight modification of the method described previously (Michael and Kelly, 2008; Sharma et al., 2012). Briefly, the saponified sterols were extracted in hexane and derivatized using N, O-Bis (trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA/TMCS) (Sigma). The derivatized sterols were then analyzed using gas chromatography-mass

spectrometry (GCMS) (Shimadzu QP2010 Plus, Japan) with the conditions as described previously (Sharma et al., 2012).

Statistical analysis

All experiments were performed in two or more replicates and are represented as the mean $\pm$ standard deviation (SD)/standard error mean (SEM). Statistically significant lipid changes were highlighted by the pattern recognition tools such as principal component (PCA) and discriminant analysis using the software SYSTAT, version 10 (Systat Software Inc., Richmond, CA, USA). Processing to the datasets for the PCA was done as described previously (Singh et al., 2010). Statistically critical differences were identified using the student *t*-test. Significance level of 0.05 was employed.

Results and Discussion

Shotgun screening of lipidome of adapted *in vitro* *C. albicans* strains

Candida cells when exposed to increasing FLC concentration often develop tolerance towards it. In *C. albicans*, FLC stress induces overproduction of membrane efflux pump proteins such as Cdr1, Cdr2, and Mdr1 (Prasad and Kapoor, 2005; Sanglard and White, 2005; Sanglard et al., 2009; White et al., 1998), which in turn demand many compensatory adjustments such as modifications in lipid homeostasis (Kohli et al., 2002). Lipids play an important physiological role in MDR phenomenon in *C. albicans* (Kohli et al., 2002; Marie and White, 2009; Morschhauser, 2002; Mukhopadhyay et al., 2002, 2004; Pasrija et al., 2005, 2008) and studies evaluated the contribution of lipids in development of FLC tolerance during clinical trials. Our earlier study on several FLC-resistant clinical isolates of *C. albicans* showed that lipids do acclimatize to increasing FLC concentrations (Singh et al., 2011, 2012). However, in certain clinical scenarios of FLC resistance, often several different populations are obtained (Lopez-Ribot et al., 1999). To overcome this drawback, Kohli et al. (2002) generated a series of two isolates YO1-32 and YO1-64, which evolved by growing FLC-susceptible isolate YO1-16 on FLC in a dose-dependent manner *in vitro*. These three isolates (YO1-16, YO1-32, and YO1-64) which have been characterized by Kohli et al., show an increase in expression of CDR genes linked to the acquisition of FLC resistance (Kohli et al., 2002). These isolates together not only provided us with an opportunity to understand the manner in which lipids respond to FLC stress *in vitro* but also to assess the exact time points when these changes take place.

The availability of laboratory-adapted sequential FLC tolerant isolates allowed us to perform a comparative analysis of lipid modifications occurring upon development of FLC resistance. For lipidome analysis, extracted lipids were subjected to ESI-MS/MS and the total lipid content (PGL (phosphoglycerides)+SL+SE (sterol ester)) was found to range between ~ 777 and $801 \text{ nmol per mg dry weight}$ in the *Candida* isolates analyzed (Supplementary Table S1; supplementary data are available online at www.liebertpub.com/omi).

We analyzed nine PGLs, including phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl glycerol (PG), phosphatidic acid (PA), lysoPC, lysoPE, and lysoPG. In

addition, as described previously, lipid molecular species were identified and quantified (Han and Gross, 1994; Singh and Prasad, 2011). We also studied four major SL groups, namely ceramides (CER), inositolphosphorylceramide (IPC), mannosylinositolphosphorylceramide (MIPC), and mannosyldiinositolphosphorylceramide (M(IP)₂C). The identified lipid changes were statistically validated by PCA and discriminant analysis.

PGL levels are altered in resistant isolates

FLC stress necessitates compensatory changes in various lipid classes. We observed that PGLs are partially accumulated upon increasing FLC resistance between YO1-32 to YO1-64 (Fig. 1A). At the compositional level, the order of the abundance of PGLs was PE > PC > PI > PS > PG > PA, which is different than that observed for clinically adapted *C. albicans* isolates, and did not change upon FLC exposure (Fig. 1B). Apparently, PE and PC are the most abundant PGL among the *in vitro* and the clinically adapted *C. albicans* isolates, respectively. Together PC, PI, and PE contributed as much as ~80% of the PGLs.

The slightly increased levels of PGL classes, namely PC, PI, PG, and PA in YO1-64 compared to YO1-32 (Fig. 1B), is responsible for the overall increase (~14%) observed in YO1-64

(Fig. 1A). Notably, a partial decrease in amino-PGL classes like PE and PS upon FLC exposure was compensated by simultaneous increase in PC, PI, PG, and PA classes (Fig. 1B). Among the lyso-PGLs, a significant increase in the levels of lysoPC and lysoPG was observed in YO1-64, compared to sensitive counterparts. Reportedly, the changes in PGL homeostasis have been previously linked directly to cell wall integrity, mitochondrial function, virulence, and drug tolerance in *Candida* (Chen et al., 2010; Dagley et al., 2011; Shingu-Vazquez and Traven, 2011). Chen et al. (2010) demonstrated that upon disruption of *cho1Δ/Δ* gene (PS synthase) of *C. albicans* results in defects in cell wall, mitochondria, and hyphae formation. Also, Dagley et al. showed that both mitochondria and PL homeostasis are essential to the cell wall of *C. albicans* (Dagley et al., 2011). Together, these studies provide evidence that PLs are crucial for maintenance of the mitochondria and therefore for the growth on nonfermentable carbon sources and at variable temperatures. However, we found the cell wall integrity is not affected in laboratory-adapted FLC-resistant strains as determined by observing their growth on various cell wall perturbing agents such as TX-100, SDS, congo red, and calcofluor white (data not shown). Also, we did not find any defect in mitochondrial function of laboratory-adapted FLC-resistant strains as determined by observing their growth on various

FIG. 1. PGL composition of the laboratory-adapted isolates of *C. albicans*. **(A)** Total PGLs (as normalized total PGL mass spectral signal). **(B)** Relative abundance of PGL classes (as % of normalized total PGL mass spectral signal). Values are mean of three independent analyses. Data can be found in Supplementary Table S2A. Significant differences are shown by their *p* values. [#]*p* value of YO1-32 vs. YO1-64 is <0.05.

nonfermentable carbon sources such as glycerol, ethanol, acetate, sorbitol, and xylose, and their ability to grow at different temperatures ranging from 20°C to 42°C (data not shown). Therefore, the fact that we did not observe any drastic changes in PGL composition upon FLC exposure *in vitro* is not very surprising and can be correlated with the proper functioning of the mitochondria.

FLC exposure leads to depletion of SLs

In *C. albicans*, depletion of either membrane SLs or ergosterol content leads to reduced drug tolerance, and the interaction between the two is critical for membrane rafts integrity (Mukhopadhyay et al. 2004; Pasrija et al., 2005, 2008). Therefore, we evaluated whether FLC stress causes any alteration in SL homeostasis among *in vitro* adapted isolates. We observed depleted SL levels in YO1-64 isolate in comparison to its susceptible counterparts YO1-16 and YO1-32 (Fig. 2A). Using MS analysis, we quantified the CER, IPC, MIPC, and M(IP)₂C levels (Fig. 2B). CER and IPCs were the

most significantly variable SLs that were depleted up to 2.8-fold in YO1-32 or YO1-64, as compared to its susceptible counterpart, YO1-16. MIPC levels did change in these isolates, but overall levels did not change between YO1-16 and YO1-64 (Fig. 2B). Of note, the M(IP)₂C was accumulated (as much as ~5-fold) in YO1-64 compared to the sensitive isolates. However, its overall abundance was not more than 2% (% of total normalized mass spectral signal of PGL+SL+SE) (Fig. 2B).

The marked depletion observed in the SL content of the resistant isolate (YO1-64) due to low CER and IPC levels upon FLC exposure *in vitro* appears to be unusual. However, recently Klappe et al. (2010) demonstrated that localization and efflux function of *MRP1*, another ABC transporter localized in lipid rafts, remained unaffected even after depleting the SL to critical levels. We assume that the loss in SLs upon FLC stress is not enough to affect the raft integrity and that with high abundance of ergosterol (discussed below), rafts continue to perform their physiological function, including the localization of Cdr1p. SL depletion has been reported in several other

FIG. 2. SL composition of the laboratory-adapted isolates of *C. albicans*. **(A)** Total SLs (as normalized total SL mass spectral signal). **(B)** Relative abundance of SL classes (as % of normalized total SL mass spectral signal). Values are mean of three independent analyses. Data can be found in Supplementary Table S2B. Significant differences are shown by their *p* values. [#]*p* value of YO1-16 vs. YO1-64 is <0.05.

clinical FLC resistant isolates of *C. albicans* as well (Singh and Prasad, 2011; Singh et al., 2012).

FLC exposure leads to accumulation of sterols

Ergosterol and SLs are the integral constituents of membrane microdomains or “lipid rafts.” These rafts not only provide a localization platform for membrane proteins but serve as an important signaling component as well (Pasrija et al., 2008). Free sterols contents were analyzed as described in Methods, and ranged between ~193 and 549 $\mu\text{g}/\text{mg}$ dry wt. of the cells (Fig. 3A). The overall free sterol content was more than 2.3-fold higher in YO1-64 as compared to YO1-16 and YO1-32 (Fig. 3A). We found as much as ~3-fold higher ergosterol and ergostetraenol in YO1-32 and YO1-64, as compared to YO1-16 (Fig. 3B). Sterols were also analyzed as SEs. SEs ranged between ~8%–15% (% of total normalized mass spectral signal of PGL + SL + SE) (Supplementary Table S3). We found that the ergostetraenol and ergosterol esters levels were increased as much as ~2-fold in YO1-32 and YO1-64, as compared to YO1-16. This accumulation of SEs in YO1-32 and YO1-64 was accompanied by simultaneously lowered levels of intermediate metabolites of sterol biosynthetic pathway, namely lanosterol, zymosterol, episterol, and fe-

costerol, which were depleted up to ~4-fold, as compared to less tolerant strain YO1-16 (Fig. 3C). When we examined the SEs based on their FA-chain compositions, we found that C18:1-FA containing SEs were elevated while C18:2-FA containing SEs were depleted in YO1-64 as compared to the other FLC sensitive isolates (Supplementary Table S2D). The observed increase in ergosterol content in resistant isolates YO1-32 and YO1-64 (Fig. 3) is probably necessary to accommodate the overproduced efflux pumps such as Cdr1p, which preferentially is localized within the lipid rafts (Pasrija et al., 2008). Moreover, certain vacuolar ATPases require ergosterol for their proper function and form the basis of azole cytotoxicity in *C. albicans* (Zhang et al., 2010). Also, sterols have been found to be accumulated many clinical FLC resistant isolates of *C. albicans*, with levels maintained by an upregulation of sterol biosynthetic pathway genes (Kohli et al., 2002; Singh and Prasad, 2011; Singh et al., 2012).

Molecular species respond to FLC stress

Various lipid classes are subcategorized into an extraordinarily large number of molecular species on the basis of fatty acid (FA) position and composition (Shevchenko and Simons, 2010). In yeasts, the interactions of these molecular lipid

FIG. 3. Sterol composition of the laboratory-adapted isolates of *C. albicans*. **(A)** Total free sterol content (as $\mu\text{g}/\text{mg}$ dry wt. of cells). **(B)** Abundance of sterol classes (as $\mu\text{g}/\text{mg}$ dry wt. of cells). **(C)** Relative abundance of SE classes (as % of normalized total SE mass spectral signal). Values are mean of 2 or more independent analyses ($n > 2$). SE data can be found in Supplementary Table S2C. Significant differences are shown by their p values.

species among themselves and with the proteins provide an extra edge towards regulating various cellular processes (Shevchenko and Simons, 2010). In *Candida*, the molecular lipid species diversity is large (Singh et al., 2010). Since the majority of major PGL classes did not show significant change in FLC-resistant isolates, we evaluated whether FLC exposure affects lipid molecular species. Using MS analysis, we detected about 240 species molecular lipid species among the PGLs, SLs, and SEs (Fig. 4, and Supplementary Tables S1 and S3) and observed that while adjusting to the rising FLC concentration *in vitro*, *C. albicans* isolates show significant changes

in the lipid species. For example, upon comparing YO1-16 vs. YO1-32 and YO1-32 vs. YO1-64, significant changes were found to be in about 50 and 98 lipid species, respectively. Upon comparing YO1-16 (most sensitive) with YO1-64 (most resistant), about 53 lipid species showed significant alterations (Fig. 4, and Supplementary Tables S1 and S3). These results suggest that maximum changes in the lipid metabolic flux occur when the cells are adapting to a higher FLC concentration, viz. 64 $\mu\text{g}/\text{mL}$. It is possible that many of these lipid species changes are not directly linked to FLC resistance and may be caused by additional compensatory mechanisms occurring inside the cell.

Odd-chain FA, membrane unsaturation, and long-chain FA (LCFA) levels respond to *in vitro* development of FLC resistance

The majority of the lipid species changes correlates with increasing FLC stress *in vitro*. For example, the compositional changes in molecular lipid species resulted in high membrane order, evident from low unsaturation index in FLC-tolerant isolate YO1-64 (Supplementary Fig. S1B). Further, we found that the LCFA- containing ≥ 35 -carbon PGL species were abundant, while the ≤ 34 -carbon containing PGL species were scarce in FLC-tolerant strains (Supplementary Fig. 1C and 1D). Most of the analyzed PGLs followed a similar pattern, and confirm that FA chain lengths remodel in order to compensate for the compositional changes within the membranes upon increasing the FLC stress. Reportedly, FAs with shorter chain lengths and polyunsaturation are fluid in nature (Janmey and Kinnunen, 2006).

Odd-chain FA containing PGLs have earlier been detected in several FLC tolerant isolates of *C. albicans*, and 31-, 33-, 35-, and 37-carbon containing PGL species form a repertoire of the odd chain FAs (Singh et al., 2010; Singh and Prasad, 2011). Interestingly, the levels of odd-chain FA containing PGL species increased upon increasing the FLC exposure (i.e., from YO1-32 to YO1-64; Supplementary Fig. S1A). This accumulation was more prevalent in the PE, PI, and PC classes (Supplementary Tables S1 and S3). This result is opposite to that obtained for clinically adapted *C. albicans* isolates where depletion in odd-chain FA containing PGL species was observed in the resistant isolates (Singh et al., 2012). Considering the fact that the physiological need of these changes might be very different in laboratory and clinical adaptive conditions, the observed differences in the adaptive pattern of odd-chain FA containing PGLs is not very surprising. Odd-chain FAs serve as useful biomarkers in several food web and environmental studies and, if studied in detail, could serve the purpose in *C. albicans* drug resistance as well.

Overall, these variations in molecular lipid species composition might be directly contributing to membrane homeostasis in FLC *in vitro* resistant isolates. Although, the physiological role of odd-chain FAs and LCFAs is well established in plant systems, a complete understanding of the physiological relevance of these odd-chain FAs and LCFAs in the human pathogenic *C. albicans* will require further validation (Řezanka and Sigler, 2009). Nonetheless, the critical determinants of MDR development such as rate of FLC import and efflux depend on the order of the plasma membrane lipid bilayer; hence this apt membrane environment would facilitate emergence of FLC-resistant *C. albicans in vitro*.

FIG. 4. Molecular lipid species composition of laboratory-adapted isolates of *C. albicans*. Data in the heat map is represented as % of total PGL+SL+SE mass spectral signal normalized to the internal standards. *Candida* strains were cultured in YPD medium at 30°C as described in Materials and Methods. Values are means $\pm$ SD ($n=3$, for all *Candida* strains). Green, yellow, and red color depicts the highest, mid, and lowest values respectively. Data taken from Supplementary Table S3.

PCA and discriminant analysis distinguishes sensitive and resistant isolates

PCA plots provide a statistical tool to visualize and determine changes among data sets and also help to determine grouping among them (Devaiah et al., 2006; Ferreira et al., 2010; Ringner, 2008; Singh et al., 2010; Singh and Prasad, 2011). To confirm whether the observed differences in the lipid imprints represent statistically significant variations between the *in vitro* adapted isolates of *C. albicans*, the PCA was performed using the molecular species percentage composition of PGL+SL+SE (Supplementary Table S1).

As depicted in the plot of principal component 1 versus 2 (Fig. 5A), principal component 1 describes the separation of YO1-16 from YO1-32 (intermediate susceptibility to FLC) and YO1-64 (resistant to FLC). The lipid species with minimum and maximum loading values are the most important determinants of each principal component (Table 1). Upon examining the principal component 1 loadings (Table 1), we found that the content of 34 and 36 carbon PGLs with two or more double bonds (PG 36:3 and 36:5; PS 34:2 and 36:3; PE 34:3, 36:2, 36:3, and 36:5) along with some SL and SE species are important for the segregation of various isolates along the negative principal component 1 axis, while the content of 28 to 34 carbon PGLs with one or two double bonds (PC 28:1, 30:1, 30:2, 32:1, 32:2, 34:1 and 34:2; PE 30:1 and 32:2; PI 32:1, and PS 32:1) are important for the segregation of various isolates along the positive principal component 1 axis. Interestingly, the amounts of all molecular lipid species associated with the lowest and highest loading values of principal component 1 axis showed decreasing and increasing trend respectively, from YO1-16 to YO1-32 to YO1-64 (viz. increasing FLC concentration).

Principal component 2 also differentiates various isolates (Fig. 5A); for example, it describes the variation between YO1-16 as compared to YO1-32 and YO1-64. The lowest loadings for principal component 2 point to decreasing trends in amounts of several SL species (CER 44:0;3 and 46:0;3; IPC 42:0;5) and >34 carbon PGLs (PI 35:1, 36:3, and 36:4; PC 36:3, 38:3, and 38:5, etc.), upon increasing FLC resistance with the

highest amounts observed in YO1-16. The highest loadings for principal component 2 point to increasing trends in amounts of ergosterol and ergostatetraenol esters and SL species (MIPC 42:0;3 and M(IP)₂C 42:0;4) from YO1-16 to YO1-64, while the amounts of some of the molecular species show slightly lower levels in YO1-64 compared to YO1-32. Evidently, the loadings of principal component 2 effectively separate YO1-16 from YO1-32 and YO1-64 (Fig. 5A).

Principal component 3 distinguishes between YO1-32 and YO1-64 (Fig. 5A). The principal component 3 loadings show higher levels of several lyso-PC's (18:0, 18:2, and 18:3), PE (33:2, 34:4, and 35:0), PG 34:4, PC 31:0, etc. in YO1-64 and many PGL species (PE 33:0, 34:2, and 36:6; PC 36:4; PI 26:0) and lyso-PGLs (lysoPE 18:1 and 18:2; lysoPG 16:1) in YO1-32. Low amounts of MIPC 40:0;5 species is present in YO1-64.

Overall, PCA could validate statistically significant variations in the molecular lipid species of isolates used in this study. Also, PCA clearly demarcates YO1-16, YO1-32, and YO1-64 into 3 different clusters based on their molecular lipid imprints. Further PCA highlights the molecular species that mark the transition from YO1-16 → YO1-32 → YO1-64.

Our PCA result that a certain set of molecular species could segregate one isolate from the other was also re-confirmed by the discriminant analysis (another statistical tool) (Fig. 5B). Like PCA, discriminant analysis also showed that the three isolates used in these study possess a distinct lipid profile.

Further, we compared the molecular lipid species imprint of FLC *in vitro* adapted with that previously reported for the clinical AS/AR isolates of *C. albicans* (Singh and Prasad, 2011; Singh et al., 2012), using PCA (Fig. 6A) and discriminant (Fig. 6B) analysis. We found that lipid imprint of FLC *in vitro* adapted strains are distinct from that of clinical isolates (Fig. 6 and Supplementary Table S4). Examination of the loadings associated with principal components 1, 2, and 3 (Supplementary Table S4), shows that the content of SL species (particularly IPC and MIPC species), lyso-PCs (15:0, 15:1, 17:0, 17:1) and odd-chain PGLs (PE 31:2, 33:2, 35:1, 35:2, 37:2; PS 33:1, 33:2, 35:1, 35:2; PG 32:1; PC33:2), PGLs with one or two double bonds (PS 31:1 and 32:2; PC 31:2; PE 31:2; PS 36:1), saturated PGLs (PI 26:0 and 30:0; PG 32:0), monounsaturated

FIG. 5. PCA and discriminant analysis of lipid species amongst laboratory-adapted isolates of *C. albicans*. The figure shows the 3D-PCA (A) and discriminant (B) score plots for the laboratory-adapted isolates. The scores for the first three principal components, explaining >40% of the variance, are plotted. Each point in the PCA plot represents the principal component score of the individual replicate. Data taken from Supplementary Table S3. Loading values are indicated in Table 1.

TABLE 1. LOADINGS OF PRINCIPAL COMPONENTS 1, 2, AND 3 FROM PCA ANALYSIS OF LIPIDS SPECIES OF FLU *IN VITRO* ADAPTED ISOLATES USED IN THIS STUDY

Lipid species 1	PC1	Lipid species 2	PC2	Lipid species 3	PC3
<i>12 Lowest loading values</i>					
Ep- +Feco-sterol ester	-0.962	LysoPC 17:0	-0.929	PI 26:0	-0.642
PG 36:3	-0.939	PI 36:4	-0.818	PG 34:4	-0.622
PS 34:2	-0.930	PI 32:0	-0.817	PE 33:0	-0.607
PE 36:3	-0.927	PC 38:3	-0.815	PA 32:0	-0.546
PE 36:2	-0.923	PS 34:1	-0.764	PE 33:2	-0.524
MIPC 44:0;4	-0.910	PC 36:3	-0.747	LysoPG 16:1	-0.512
PE 36:5	-0.903	PI 35:1	-0.745	PS 36:5	-0.475
PG 36:5	-0.893	IPC 42:0;5	-0.738	PI 38:1	-0.458
Lanosterol ester	-0.887	PC 38:5	-0.719	PA 34:6	-0.458
MIPC 44:0;3	-0.887	PI 36:3	-0.695	PE 34:2	-0.434
PE 34:3	-0.878	CER 44:0;3	-0.670	PE 35:0	-0.430
PS 36:3	-0.876	CER 46:0;3	-0.666	MIPC 40:0;5	-0.414
<i>12 Highest loading values</i>					
PI 32:1	0.953	PA 34:3	0.484	PS 32:0	0.681
PE 30:1	0.954	PG 36:1	0.512	PS 38:6	0.681
PE 37:2	0.957	PA 32:1	0.524	PE 36:6	0.683
PE 32:2	0.959	PS 34:3	0.543	LysoPE 18:1	0.688
PS 32:1	0.963	Ergostatetraenol ester	0.549	LysoPE 18:2	0.694
PC 30:2	0.964	PE 36:1	0.557	LysoPC 18:2	0.697
PC 30:1	0.968	MIPC 42:0;3	0.572	PC 31:0	0.705
PC 34:1	0.969	M(IP) ₂ C 42:0;4	0.579	PC 36:4	0.720
PC 32:1	0.969	Ergosterol ester	0.637	LysoPC 18:3	0.723
PC 34:2	0.975	PA 36:5	0.666	LysoPC 18:0	0.739
PC 28:1	0.984	PG 32:0	0.716	PE 34:4	0.810
PC 32:2	0.986	PG 32:1	0.727	PI 36:6	0.832

The 12 highest and 12 lowest values are indicated.

PGLs with < 32 carbons (PE 28:1; PC 28:1 and 30:1; PS 30:1; PI 30:1 and 32:1), polyunsaturated PGLs with > 34 carbons (PC 36:4, 36:5, 38:4, 38:5 40:3; PE 36:4; PS 36:4, 36:5; PI 36:4, 36:5; PA 36:4, 36:5), etc. are crucial for the separation of *in vitro* adapted and clinical isolates of *C. albicans*. Apparently, the lipid species imprints of AR isolates of both clinical and laboratory conditions are different (Fig. 6). Thus, these results indicate that molecular lipid species response to FLC stress

is different in clinically and *in vitro* adapted isolates of *C. albicans*.

Conclusion

Taken together, this study evaluates the lipid metabolic changes that occur upon *in vitro* FLC exposure in *C. albicans*. We confirmed several compositional changes in molecular

FIG. 6. PCA and discriminant analysis of lipid species amongst laboratory-adapted and clinical AS/AR isolates of *C. albicans*. The figure shows the 3D-PCA (A) and discriminant (B) score plots for the laboratory adapted as well as AS/AR isolates. The scores for the first three principal components, explaining > 40% of the variance, are plotted. Each point in the PCA plot represents the principal component score of the individual replicate. Data taken from Supplementary Table S3. For comparison, the lipid data for clinical AS/AR isolates was taken from the previously published study by Singh and Prasad (2011) and Singh et al., (2012). Loading values are indicated in Supplementary Table S4.

lipid species upon *in vitro* FLC exposure. Although the molecular lipid imprint of *in vitro* FLC-adapted isolates is much distinct from that of previously reported clinical isolates, the changes in critical lipid classes like sterols and SLs are the same under both conditions. Overall, we emphasize that metabolic lipid remodeling is necessary for the sustenance of *C. albicans* in FLC stress. Moreover, the new observations reported herein have relevance for more efficacious antifungal drug development, as well as understanding host–infectious agent interactions in postgenomics microbiology.

Acknowledgments

The work presented in this article has been supported in part by grants to RP from Department of Biotechnology (BT/PR13641/Med/29/175/2010, BT/PR14879/BRB10/885/2010, BT/01/CEIB/10/III/02) and Department of Science and Technology (SR/SO/BB-34/2008). The ESI-MS/MS analyses described in this work were performed at the Kansas Lipidomics Research Center Analytical Laboratory, where equipment acquisition and method development were funded by National Science Foundation (EPS 0236913, MCB 0455318, DBI 0521587), Kansas Technology Enterprise Corporation, K-IDEA Networks of Biomedical Research Excellence (INBRE) of National Institute of Health (P20RR16475), the Johnson Cancer Center, and Kansas State University. GCMS analysis was performed at the AIRF, JNU and assisted by Dr. Ajay Kumar.

Author Disclosure Statement

The authors declare that no competing financial interests exist.

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Abbreviations Used

ABC	= ATP binding cassette
AR	= azole resistant
AS	= azole susceptible
CER	= ceramide
ESI-MS/MS	= electrospray ionization tandem mass spectrometry
FA	= fatty acyl
FLC	= fluconazole
IPC	= inositolphosphorylceramide
MFS	= major facilitator superfamily
MIPC	= mannosylinositolphosphorylceramide
M(IP) ₂ C	= mannosyldiinositolphosphorylceramide
PA	= phosphatidic acid
PC	= phosphatidyl choline
PCA	= principal component analysis
PE	= phosphatidyl ethanolamine
PG	= phosphatidyl glycerol
PGL	= phosphoglyceride
PI	= phosphatidyl inositol
PS	= phosphatidyl serine
SD	= standard deviation
SDS	= sodium dodecyl sulfate
SE	= sterol ester
SL	= sphingolipid