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Investigation of Differentially Expressed Proteins of *Candida tropicalis* Biofilm in Response to Citral

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Abstract

Candida tropicalis is an opportunistic human pathogen with an ability to cause superficial as well as systemic infections in immunocompromised patients. *C. tropicalis* biofilms can cause persistent infections which are difficult to treat due to acquired resistance. Citral has been used as antifungal agents against *Candida* species and biofilms. In the present study, we used one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/TOF-MS) to identify the changes in the protein expression of *C. tropicalis* in response to the sub-lethal concentration of citral. A total of six differential proteins involved inoxidative stress (Tsa1p, Psa2p), amino acid biosynthesis (Met6p, Gln1p), heme biosynthesis (Hem13p) and glucose metabolism (Eno1p) pathways were detected. Our results revealed citral-induced proteins of *C. tropicalis biofilm*. This study will further help in the interpretation of mode of action of citral and development of novel antifungal agents against these potential protein targets.

Keywords: *Candida tropicalis*; Citral; Antifungal

Introduction

Candida tropicalis is the most prevalent *non-albicans Candida* species in tropical countries responsible for high mortality rate due to candidiasis [1,2]. *C. tropicalis* exhibits an intrinsic ability to form the biofilmon the biotic as well as abiotic surfaces [2]. The infected devices isolated from the patients have revealed biofilms formed by fungal species which seed furtherrecurrent infections [3]. The *C. tropicalis* biofilm has shown elevated resistance towards conventional drugs, including fluconazole and amphotericin B as well as other antifungal agents [4,5]. *Candida* species have evolved numerous mechanisms; including alteration of targets, reduction in uptake and active extrusion, in order to combat the effects of standard antifungal agents [6]. The use of the natural products is common from earliest civilizations and well known for their antiseptic and medicinal properties [7]. In recent decades, the development of new bioactive compounds based on modified natural products is suggestively considered to overcome the emerging resistance [8].

Citral, a major component of the essential oil of *Cymbopogon citratus* (~ 75%), has shown to exhibit potent activity against *Candida* species and its biofilm [9-11]. A recent study on *C. tropicalis* by Sousa et al. has demonstrated the likely mechanism of the action of citral does not involve cell walls or direct binding to ergosterol but is mediated through the inhibition of membrane ergosterols biosynthesis; however, the molecular mechanism remains unclear [12]. "Hypothesis-free" system biology tools such as genomics, proteomics and bioinformatics can be utilized to investigate the molecular machinery of the antifungal resistance and drug development. The identification of the potential drug targets which are frequently involved in essential signalling pathways is required to explore the molecular mechanism of action which can be used for the development of the novel therapeutics and optimization of the existing agents. To the best of our knowledge, this is the first study on the variation of protein profile of *C. tropicalis* biofilm when exposed to citral. In the present work, the differences in the expression of the proteins of *C. tropicalis* biofilm in the presence of citral were identified using proteomics approach.

Materials and Methods

Phytoconstituents, standard antifungal and substances

Citral and standard antifungal drugs; amphotericin B (AmpB) and fluconazole (FLU) were commercially acquired from Sigma-Aldrich, USA. The stock solution of citral and standard drugs were prepared in dimethylsulfoxide (DMSO, HiMedia, India). Culture media Sabouraud dextrose broth (SDB), Sabouraud dextrose agar (SDA) and RPMI-1640-L-glutamine (without sodium bicarbonate) were purchased from Hi Media, India.

Fungal strain and growth conditions

C. tropicalis strain (NCIM-3118) used in the present study was procured from National Collection of Industrial Microorganism, Pune. The strain was streaked on SDA and incubated at 37°C for 24 h. Glycerol stock of the strain was prepared in SDB and frozen at -80°C.

Antifungal and anti-biofilm susceptibility testing

The minimum inhibitory concentration (MIC_{50}) of citral and antifungal drugs (AmpB and FLU) were determined by broth micro dilution following reference protocols M27-A3 as recommended by Clinical and Laboratory Standards Institute (CLSI, 2008) [13]. Serially double-diluted concentration of citral and standard drugs (0–1,024 µg/mL) were added in 96-well microtiter plates to provide 0.5-2.5 × 10^3 cells/mL with 5% DMSO as the negative control. The plates were then incubated at 37°C for 24 h and growth of cells was measured by microtiter plate reader (Spectra Max, Molecular Devices, USA) at 600 nm and MIC_{50} was determined. The anti-biofilm susceptibility assay was performed in 96-well polystyrene microtiter plates to determine biofilm inhibitory (BIC_{50}) and biofilm eradicating concentrations (BEC_{50}) as described earlier [14]. The serial double diluted concentration of citral/

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drugs (0–1,024 µg/mL) were made in RPMI-1640 and added to each well as treatment while acquiring a final concentration of cells as 1 × 10^6cells/ml with 5- DMSO in RPMI-1640 as a negative control. The plates were incubated at 37°C for 24 h and the metabolic activity of biofilm was quantitatively determined by colourimetricXTT [2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium- 5-carboxanilide sodium salt] reduction assay at 492 nm. Further, the BIC_{50} and BEC_{50} values were determined.

Scanning electron microscopy (SEM)

The effect of citral and Amp B on biofilm was qualitatively evaluated by scanning electron microscopy (SEM, Carl Zeiss, AG, EVO 40) as described in an earlier study [15], with slight modifications. The biofilm was formed on sterile fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific) pre-treated silicon elastomer discs in 12 well culture plates at 37°C for 24 h. The preformed biofilms were treated in the presence citral and AmpB at their respective BEC_{50} values and incubated for 24 h at 37°C. After incubation, the biofilm were washed with phosphate buffered saline (PBS, 0.01 M, pH 7.4) and subsequently fixed in 2% (v/v) glutaraldehyde (HiMedia, India) followed by dehydration in a series of 25%, 50%, 75% and 100% of the ethanol (Merck). Finally, the samples were dried and sputtered with gold for visualization under SEM in high-vacuum mode at 20 kV.

Whole cell protein extraction

C. tropicalis biofilm was cultured without and with citral treatment (BEC_{50}) on silicon elastomer sheets for 24 h at 37°C. After incubation, the sheets were washed with PBS to remove non-adherent cells and resulting biofilm was scraped off, centrifuged (5 mins, 4000 g) and washed thrice with PBS. Cell pellets were re-suspended in lysis buffer (20 mMTris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and protease inhibitor cocktail) and disrupted using glass beads with alternate vortexing on the ice every 30 sec. The supernatant was collected after centrifugation at 12000 g for 20 mins at 4°C. Further, the protein concentration was measured using BCA assay (Pierce BCA Protein Assay Kit).

One-dimensional (1D) gel electrophoresis and protein identification

The whole cell protein lysates (75 µg) of treated and untreated biofilm were separated on 10% 1D sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) at constant 90 V. Then, the gel was fixed, stained, destained and imaged using a Gel Doc XR+system (Bio-Rad) and differentially expressed proteins were selected for further identification. Protein bands of interest were excised and in-gel digested with trypsin (Promega, Madison, WI, USA) as previously described [16]. In brief, each excised band were destained using the ammonium bicarbonate/acetonitrile (1:1, v/v) and acetonitrile (100%) followed by trypsin digestion for 16 h at 37°C. The digested peptides were recollected in extraction buffer (1:2 (v/v) 5% formic acid/acetonitrile) at room temperature. The peptides were dried in Speedy Vac (Labconco) and stored at -20°C for mass spectrometry. The samples were mixed with HCCA (α-cyano-4-hydroxy-cinnamic acid) matrix and spotted on the MALDI plate. The mass spectrometric analysis was carried out using Ultraflextreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) equipped with a 60-Hz nitrogen laser in positive mode. Background ions from trypsin autolysis and keratin contamination were cleared from mass lists. Protein identification was performed by searching the generated peak list files to NCBI database using the Mascot Server 2.4(http://www.matrixscience.com). The MASCOT search parameters were: 1) allowed number of missed cleavage: 1; 2) fixed modification: carbamidomethyl cysteine; 3) variable modification: methionine oxidation; 4) peptide tolerance: ± 200 ppm; 5) MS/MS tolerance: ± 0.7 Da; and 6) peptide charge: +1. Probabilistic MASCOT scoring was used to evaluate the identified peptides and proteins. $P \leq 0.05$ was considered significant for peptide identification. The false discovery rate (FDR) was less than 2%, by searching against a decoy database. All experiments were performed in three replicates to minimize the error.

Results and Discussion

Candida biofilms have shown more resistance to the antifungals agents than their planktonic counterparts. Biofilms usually develop resistance to cope the stress caused by antifungal drugs [17]. The MIC_{50}, BIC_{50} and $BEC_{50}$are defined as the concentration which inhibits 50% cell growth in contrast to the control. AmpB, FLU and citral have shown a significant effect against *C. Tropicalis* but less effective during biofilm formation and biofilm eradication. TheBIC_{50} and BEC_{50}were two and four folds higher than planktonic cells, respectively (Table 1). The results were in corroboration with the previous studies in which *C. tropicalis* biofilm wasresistant during treatment [18]. The formation of biofilms was visualized through SEM imaging as shown in (Figure 1). Control biofilms displayed confluent cells, whereas the treated biofilm were ensuring distorted porous cells. These morphological alterations of the cells could be associated with the loss of cell membrane integrity caused by bursting of the cells and subsequent oozing out of intracellular components ultimately resulting in cell death [19]. Fungal biofilms have exhibited changes in the expression of various proteins associated with the antifungal tolerance [20]. The differentially expressed proteins in the biofilm during the presence and absence of citral were examined through protein profiling of the whole cell extract of *C. tropicalis* biofilm. The use of 1D gel electrophoresis has successfully been implemented in the studying the protein profiling [21]. 1D gelimages (Figure 2) indicated four distinct bandsat ~ 85 kDa, ~ 45 kDa, ~ 37 kDa and ~ 21 kDa, respectively, in the presence of citral when compared with untreated biofilm. The obtained protein bands were identified by high throughput mass spectrometry. Tandem Mass Spectrometry (MS/MS) has identified a reference protein and six proteins with differential expression through MASCOT Server. A reference protein: Glyceraldehyde-3-phosphate dehydrogenase (XP_002551368.1, Mw: 36.209 kDa, Score: 87) with three peptides (R.DPINIPWG.K, K.EATYEEICAAV.K, K.IHVFQE.R) was observed with similar expression in both the samples. On the other hand out of the six differentially expressed proteins; two were associated with oxidative stress (Tsa1p, Psa2p), two with amino acid biosynthesis (Met6p, Gln1p), one with heme biosynthesis (Hem13p) and one with glucose metabolism (Eno1p). The differentially expressed proteins and their respective peptides are documented in (Table 2).

Oxidative stress is concurrently prominent during the antifungal resistance. The thiol-specific antioxidant 1 (Tsa1) protein plays an important role in the survival of cells under oxidative stress created by the host immune response. Shin et al. reported that *C. albicans* TSA1 mutants show higher levels of H_2O_2 which indicated the Tsa1p ability to neutralize this peroxide [22]. Tsa1p is also expressed in *C. tropicalis*

S. No.	Drugs	MIC_{50} (µg/ml)	BIC_{50} (µg/ml)	BEC_{50} (µg/ml)
1	Amphotericin B	0.5	1	2
2	Fluconazole	8	16	32
3	Citral	32	64	128

Table 1: IC_{50} values of drugs against *Candida tropicalis.* Values represent the arithmetic means ($p<0.05$) of the effective concentration against planktonic cells and biofilms.

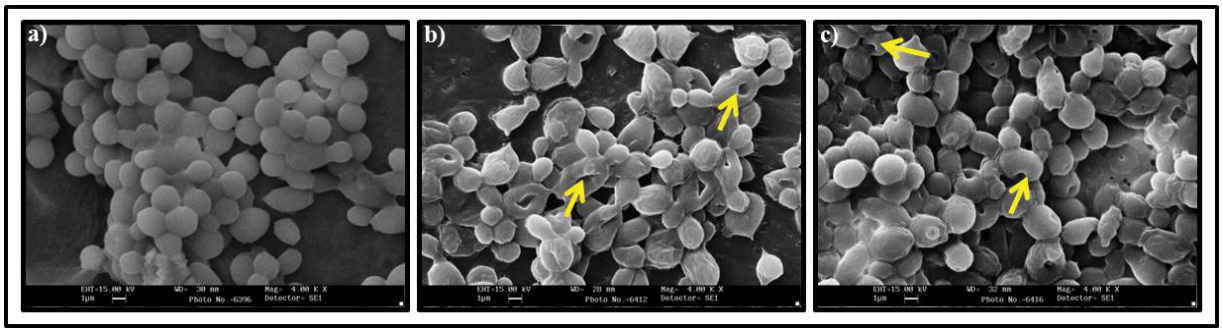

Figure 1: Scanning electron microscopyimages of *C. tropicalis* biofilm confluence grown on substrate reveals the surface topology a) Untreatedcells (0 µg/ml), b) With AmpB (0.5 µg/ml), and c) With citral (16 µg/ml). Yellow arrows denote cell damage (Scale: 1µm).

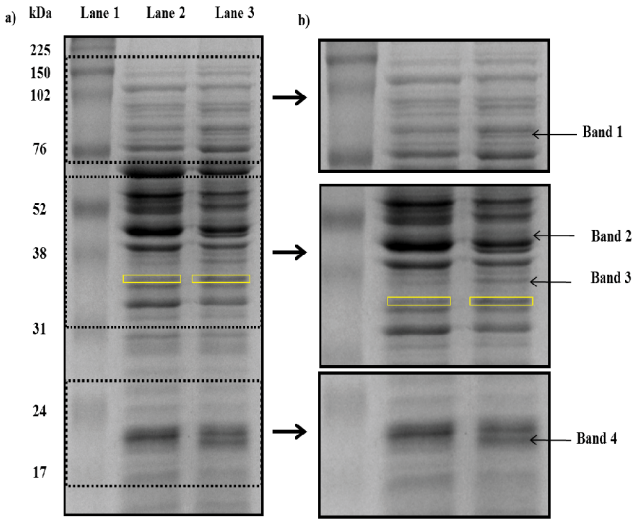

Figure 2: *C. tropicalis* proteins resolved using 1D gel electrophoresis. a) 1D gel image: Lane 1: Molecular weight marker (kDa) as indicated on the left side; Lane 2: Control biofilm; and Lane 3: Citral (16 µg/ml) b) Zoomed subsets of the dotted box on the gel with distinct bands. Reference protein is shown in the yellow box. Differentially expressed proteins that were identified by mass spectrometer are correspondingly numbered in Table 2.

Band No.	Accession No.[a]	Protein[b]	Functional description	Mw (kDa)	Score[c]	Identified peptides[d]
1	XP_002546146.1	Methionine-synthesizing 5-methyl tetrahydro-pteroyltriglutamate-homocysteine methyltransferase (Met6)	Amino-acid biosynthesis	85.69	69	K.GQITAEEYEAFINKEIETVVR.F M.VQSSVLGFPR.M R.YVRPPIIVGDVDRPK.A K.YDLAPIDVLFAMGR.G K.AGVDVIQVDEPALR.E R.SDYLNWAAQSFR.V K.AIENLPVAGFHFDFVR.V
2	XP_002548866.1	Enolase1 (Eno1)	Glucose metabolism	46.984	118	R.SGETEDTFIADLSVGLR.N K.IQIVGDDLTVTNPIR.I K.VNQIGTLTESIQAANDSYAAGWGVMVSHR.S
2	XP_002545775.1	Glutamine synthetase (Gln1)	Amino acid biosynthesis	42.090	60	K.VLAEYVWIDAEGNTR.S
3	XP_002548314.1	Coproporphyrinogen III oxidase (Hem13)	Heme biosynthesis	37.087	41	M.VSPDQIHDTSFPIRER.M K.GGVNISIVHGKLPPQAVTR.M
4	XP_002547929.1	Peroxiredoxin (Tsa1)	Oxidative-stress	21.839	76	R.DYGVLIEEEGVALR.G K.DAQVLFASTDSEYTWLAWTNVAR.K
4	XP_002545791.1	Protoplast secreted protein 2 precursor (Pst2)	Oxidative-stress	21.228	160	K.MHAPAKPDYPIASAETLTQYDAFLFGIPTR.F K.IAIIEYSTYGHITQLSR.A K.AFALQSNLEEIHGASAYGAGTFAGADGSR.Q K.VAIIIYSLYHHVAQLAEEEK.K K.AFWDTTGGLWAQGALHGK.Y

[a]Protein accession numbers according to NCBI protein database; [b]Protein named and description according to the *C. tropicalis*genomic database (*Candida*DB); [c]Score based on NCB Inr database using the MASCOT server as MALDI-TOF data ($p < 0.05$); [d]Peptides generated through Tandem Mass Spectrometry

Table 2: Summary of the identified proteins with differential expressions in response to citral.

in response to citral suggesting that it may have caused the inhibition of biofilm by developing the oxidative stress condition. Similarly, the up regulation of Tsa1p in *C. glabarata* represented one of the rescue mechanisms during oxidative stress [21]. In *C. albicans*, the up regulation of the various oxidative stress-related proteins has indicated that the higher antifungal resistance is contributed by anti-oxidant biomarkers including alkyl hydroperoxide reductase, thioredoxin peroxidase and thioredoxin [23]. The four eisosome proteins (Pst1, Pst2, Pst3 and Ycp4) have recently shown novel antioxidant function in *C. albicans* [24]. Pst2pis also identified with over expressionin the presence of citral.

The expression of methionine-synthesizing, 5-methyl tetrahydropteroyltriglutamate homocysteine methyltransferase (Met6p) and glutamine synthetase (Gln1p), which are essential enzymes of amino acid biosynthesis pathways, upregulated in the presence of citral. In higher plants, fungi and some prokaryotes represent cobalamin-independent methionine synthases, whereas human methionine synthase is cobalamin-dependent. Therefore, the development of novel antifungal agents which targets cobalamin-independent methionine synthase can be an effective approach for specific inhibition [25]. Similarly, the over-expression of Gln1 pin the present study also directs to further elaborate diverse aspects in hindering these pathways. This stimulates the development of specific novel inhibitory molecules against these enzymes. Several enzymes of these pathways are already proven drug targets [26].

Coproporphyrinogen III oxidase (Hem13p) involved in heme biosynthesis is also expressed during citral treatment. A previous study has suggested that heme plays a vital role in sterol biosynthesis and transcriptional regulation of several processes related genes [27]. Sterol biosynthesis such as ergosterol maintains the cell membrane integrity. The hindrance of ergosterol biosynthesis has constantly been a major target pathway for antifungals including azoles.

Citral has induced the expression of enolase 1 protein (Eno1p), indicating its usage for *in vivo* antibody generation during infections in correspondence to the investigations when mice vaccinated with Eno1p of *C. albicans*, showed an increase in antibody resulting higher survival rate than non-vaccinated mice [28]. Clinical use of citral in combination with conventional drugs might escalate their efficacy through antibody production by the host cells.

Conclusion

The biofilm lifestyle, stress response and antifungal tolerance are directly related to the endurance of the fungus in a hostile milieu. From this study, it is concluded that the biofilm not only causes antifungal resistance but also help in the survival of *C. tropicalis* in distributed habitats. Detailed experiments are required to explore more proteins which are expressed during the exposure of citral to devise the probable targets.

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Conflict of Interest

None declared.

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