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Candida parapsilosis produces prostaglandins from exogenous arachidonic acid and *OLE2* is not required for their synthesis

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Prostaglandins are C20 fatty acid metabolites with diverse biological functions. In mammalian cells, prostaglandins are produced from arachidonic acid (AA) via cyclooxygenases (*COX1* and *COX2*). Although fungi do not possess cyclooxygenase homologues, several pathogenic species are able to produce prostaglandins from host-derived arachidonic acid. In this study, we characterized the prostaglandin profile of the emerging human pathogen *Candida parapsilosis* with HPLC-MS and compared it to that of *C. albicans*. We found that both species synthesized prostaglandins (mainly PGD_2 and PGE_2) from exogenous AA. Furthermore, as *OLE2* has been associated with prostaglandin synthesis in *C. albicans*, we generated homozygous *OLE2* deletion mutants in *C. parapsilosis* and examined their PGE_2 production. However, the PGE_2 production of the *OLE2* KO strain was similar to that of wild type (WT), indicating that *OLE2* is not required for prostaglandin synthesis in *C. parapsilosis*. Interestingly, analyses of the fatty acid composition of WT and *OLE2* KO cells by gas chromatography (GC) highlighted the accumulation of palmitoleic and oleic acid in the *OLE2* deletion mutant. The *OLE2* KO cells were killed more efficiently by human monocytes-derived macrophages (MDMs) as well as induced higher interleukin-10 (IL-10) secretion, indicating that *OLE2* affects the virulence of *C. parapsilosis*. Taken together, these results contribute to the better understanding of fatty acid biosynthesis pathways in *C. parapsilosis*.

Introduction

Candida parapsilosis is an emerging opportunistic human fungal pathogen, and it is currently one of the leading causes of invasive candidiasis.[1-3] The populations with the highest risk for nosocomial infections with *C. parapsilosis* are the low-birth-weight neonates, elderly and immunocompromised patients, such as individuals with AIDS or organ transplantation.[1,2,4] Unlike *Candida albicans, C. parapsilosis* is frequently transmitted horizontally, and it can cause invasive disease without colonizing the host before dissemination.[4] The use of prosthetic devices and indwelling catheters also increases the risk for *C. parapsilosis* infection.[4] However, despite its high clinical relevance, the pathogenesis of *C. parapsilosis* has remained largely obscure.

Prostaglandins (PGs) are biologically active polyunsaturated fatty acids (PUFAs) that have important signaling and immunomodulatory functions.[5] In mammalian cells, they are synthesized *de novo* from membrane-derived arachidonic acid (AA) via cyclooxigenases (COX).[5,6] Prostaglandins play an important role in the regulation of immune responses by modulating phagocytosis, cytokine and chemokine production and release, and lymphocyte proliferation.[7] In particular, PGE_2 inhibits T helper (Th) 1 and promotes Th2 responses by modulating the cytokine production of lymphocytes.[8] Numerous pathogenic fungi, such as *C. albicans* and *Cryptococcus neoformans*, are able to produce prostaglandins along with other arachidonic acid metabolites.[9] Importantly, *C. albicans* PGE_2 is biologically active on mammalian cells, as it inhibits the proliferation of splenocytes and decreases their TNFα production while stimulating IL-10 secretion.[7] Furthermore, PGE_2 modulates the morphogenesis of *C. albicans*, further supporting the role of fungal prostaglandins as potential virulence factors.[7] Although little information is available about the biosynthesis of prostaglandins in *Candida* spp., one study has described that the multicopper oxidase gene *FET3* and the stearyl-CoA desaturase gene *OLE2* are involved in PGE_2 synthesis in *C. albicans*.[10] This finding indicates a direct link between the production of fatty acids and bioactive lipid mediators, underscoring the role of *de novo* fatty acid biosynthesis in pathogenic yeasts.

Saturated and unsaturated fatty acids (SFA and UFA, respectively) have an essential role in eukaryotic cells as building blocks of membranes and lipid storage.[11,12] *De novo* fatty acid biosynthesis is essential for growth and virulence of both *C. albicans* and *C. parapsilosis*.[13-15] As in *Saccharomyces cerevisiae*, the

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biosynthesis of fatty acids in *C. albicans* and *C. parapsilosis* starts with the generation of malonyl-CoA from acetyl-CoA and CO_2, via the biotin-bound enzyme acetyl-CoA carboxylase (AccI).[11,16,17] In the second step, the elongation of the carbon chain is catalyzed by fatty acid synthases (Fas1 and Fas2) and elongase 1 (Elo1), resulting in long-chain SFA.[18,19] To produce UFA, a subsequent desaturation reaction catalyzed by stearyl-CoA desaturase (Ole1) is necessary to introduce a double bond into saturated fatty acyl-CoA substrates.[17,20] In *C. albicans* and *C. parapsilosis*, there are 2 homologues with the *S. cerevisiae OLE1, OLE1* and *OLE2*.[21] While *OLE1* is essential in *C. albicans*, deletion of *OLE1* in *C. parapsilosis* results in UFA auxotrophic mutants.[17,21] Importantly, Ole1 plays a crucial role in virulence in both species, as demonstrated by *in vivo* murine infection models.[17,22] However, while *OLE2* has been associated with prostaglandin synthesis in *C. albicans*, the role of *OLE2* in *C. parapsilosis* is unknown.

In this study, our main objective was to examine whether *C. parapsilosis* is able to synthesize prostaglandin-like molecules from exogenous AA. Furthermore, to assess the potential role of *OLE2* in prostaglandin production, we generated an *OLE2* "knock-out" (KO) *C. parapsilosis* strain. To gain further insights into the role of *OLE2* in the pathobiology of *C. parapsilosis*, we also analyzed the fatty acid composition and the virulence properties of the mutant strain.

Results

C. parapsilosis produces prostaglandins from exogenous arachidonic acid

First we wanted to examine whether *C. parapsilosis* is able to synthesize prostaglandins from exogenous AA. To characterize the prostaglandin profiles of *C. parapsilosis* and *C. albicans*, wild type yeast cells were grown overnight in YPD medium and subsequently incubated in PBS with or without 0.5 mM AA for 24 hours. The levels of different prostaglandin derivatives (13,14-dihydro-15-keto-PGF2α, 11β-PGF2α, PGE1, PGF1α, PGH2, PGE2, PGF2α, PGD2, PGF2β, PGA2, PGJ2, PGB2) in cell-free supernatants were determined by HPLC-MS analysis (**Fig. 1**). Following incubation with AA, we detected a variety of prostaglandin compounds in the supernatants of *C. albicans* and *C. parapsilosis* cultures. In both species, the main secreted prostaglandins were PGD2 and PGE2. Furthermore, we also detected compounds from other prostaglandin classes, such as PGH2, PGF2β and PGA2, in the supernatants of both *C. albicans* and *C. parapsilosis* cultures. However, we did not detect any prostaglandin production in our experimental system when *Candida* cells were incubated solely in PBS. These results clearly demonstrate that, *C. parapsilosis* synthesizes prostaglandins from exogenous AA.

CpOLE2 is not required for prostaglandin synthesis

The biosynthetic pathway of fungal eicosanoid production is still not fully understood. However, several candidate enzymes, including cytochrome P450s and multicopper oxidases have been implicated in prostaglandin production.[23] The disruption of the putative Δ9-desaturase gene *OLE2* in *C.* albicans results in significantly decreased PGE2 production.[10] Therefore, in order to examine whether *OLE2* is involved in prostaglandin synthesis in *C. parapsilosis*, we constructed homozygous *OLE2* deletion mutants (**Fig. S1**), and tested their PGE2 production using HPLC-MS as well as a monoclonal PGE2 EIA kit. However, we found that *OLE2* mutants secreted PGE2 in amounts that were similar to that of the WT parental strain (**Fig. 2**). Therefore, *OLE2* is not required for PGE2 biosynthesis in *C. parapsilosis*.

Role of *CpOLE2* in *de novo* fatty acid biosynthesis

After the finding that *OLE2* is not required for PGE_2 production in *C. parapsilosis*, we asked the question whether it has a role in *de novo* fatty acid biosynthesis. To investigate this possibility, we compared the fatty acid profile of *OLE2* mutants with that of WT *C. parapsilosis*. The amount of different fatty acids (palmitic acid, palmitoleic acid, palmitolinoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid) in fungal cell extracts was determined by gas chromatography. **Figure 3** shows the fatty acid composition of *OLE2* KO *C. parapsilosis* cells relative to that of WT cells. Interestingly, we found that *OLE2* mutants accumulated significantly more palmitoleic and oleic acid compared to WT *C. parapsilosis* cells (mean ± SEM, palmitoleic acid, 615.0 ± 117.5 vs. 228.1 ± 29.1 ng/mg dry weight, $P < 0.05$; oleic acid, 16.3 ± 0.5 vs. 9.2 ± 0.9 μg/mg dry weight, $P < 0.01$). These monounsaturated fatty acids are synthesized from palmitic and stearic acid, respectively, via Δ9-desaturases, and are further converted into palmitolinoleic (16:2 n-4) and linoleic acid (18:2) by Δ12-desaturases. However, we found that the cellular levels of palmitic and stearic acids as well as that of palmitolinoleic and linoleic acids were unaffected by the absence of *OLE2*. Taken together, these results suggest that *C. parapsilosis* Ole2 is involved in fatty acid biosynthesis and the action is presumably not or not exclusively a Δ9-desaturase.

OLE2 C. parapsilosis mutants have decreased virulence *in vitro*

Next we sought to determine whether the lack of *OLE2* affected the virulence of *C. parapsilosis*. First we examined the growth of *OLE2* mutants in comparison to WT on different media (YPD, YNB, YCB/BSA; **Fig. S1**), at different temperatures (20°C, 30°C, 37°C; **Fig. S2**), as well as at different pH ranges (pH 4-8; **Fig. S3**). We also assessed the growth of the mutant strain under different oxidative, membrane and cell wall stress-inducing conditions (H2O2, SDS, calcofluor white, congo red, caffeine; **Fig. S4**). However, the growth of the mutant strain was similar to the WT for all circumstances tested. Also, *OLE2* deletion did not affect the formation of pseudohyphae (**Fig. S5**). Next we investigated the phagocytosis and killing of *OLE2* mutants by primary human monocyte-derived macrophages (MDMs) using an acridin orange staining method (**Fig. 4**). We found that more MDMs phagocytosed the *OLE2* mutants (phagocytosis%, mean ± SEM, 69.2 ± 5.7% vs. 52.7 ± 4.9%, $P < 0.05$, **Fig. 4C**), and *OLE2* deficient cells were killed with significantly higher efficiency compared to WT yeasts (killing

efficiency, mean ± SEM, 50.2 ± 8.7% vs. 41.2 ± 8.4%, $P < 0.05$, Fig. 4D). We also examined the killing of *OLE2* mutant *C. parapsilosis* cells by CFU determinations, which confirmed the results of the fluorescence microscopic analysis (**Fig. 4E**). As fungal lipids can modulate the immune response,[7] we also examined the cytokine secretion of MDMs following stimulation with *OLE2* mutants or WT *C. parapsilosis* cells. We found that while the level of TNFα, IL-1β and IL-6 were similar in MDMs stimulated with *OLE2* mutant or WT *C. parapsilosis* (not shown), *OLE2* mutants stimulated significantly higher IL-10 production than WT cells (mean ± SEM, 581.8 ± 292.2 pg/mL vs. 437.0 ± 246.9 pg/mL, $P < 0.05$, n = 5). **Fig. 4F** shows the cytokine secretion of MDMs stimulated with *OLE2* KO or WT *C. parapsilosis* (cytokine levels were normalized for each donor to cytokine levels induced by WT cells [100%] to exclude donor-to-donor variability). Altogether, these results suggest that *OLE2* affects the virulence of *C. parapsilosis*, although further research is warranted to determine its specific role during infection.

Discussion

Several pathogenic *Candida* spp., including *C. albicans, C. tropicalis* and *C. dubliniensis* produce prostaglandins.[7,24] In this report, we demonstrate that *C. parapsilosis* is also capable of synthesizing prostaglandins from exogenously added AA. We show that the prostaglandin profile of *C. parapsilosis* is similar to that of *C. albicans*, with PGE_2 and PGD_2 being the main prostaglandins produced. In mammals, prostaglandins are important signaling molecules that are crucial for the regulation of the inflammatory response.[5] They are synthesized from AA which is liberated from membrane phospholipids by phospholipase A_2 upon inflammatory stimuli.[8] Subsequently, AA is converted to PGH_2 by cyclooxigenases, and PGH_2 is further processed by tissue-specific prostaglandin synthases to give rise to a series of different classes of prostaglandins.[8] Secretion of fungal prostaglandins during infection may substantially influence the host's immune response, promoting the survival of the pathogen. Notably, we found that the

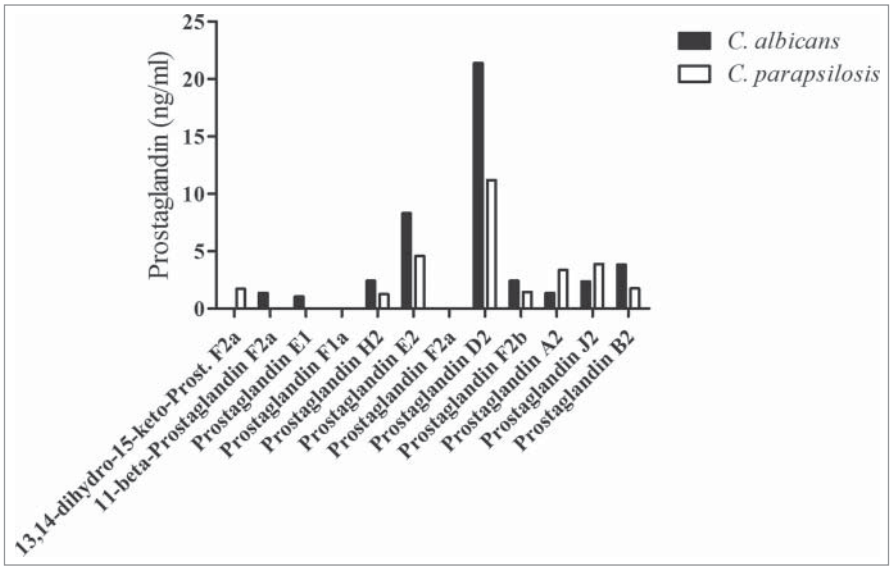

Figure 1. *C. albicans* and *C. parapsilosis* produce prostaglandins from exogenous AA. *C. albicans* and *C. parapsilosis* WT strains were cultured in YPD medium overnight, washed, and subsequently incubated in PBS for 24 h with 500 μM AA. The concentration of different prostaglandin compounds in culture supernatants was determined by HPLC-MS analysis. Results are representative of 2 independent experiments.

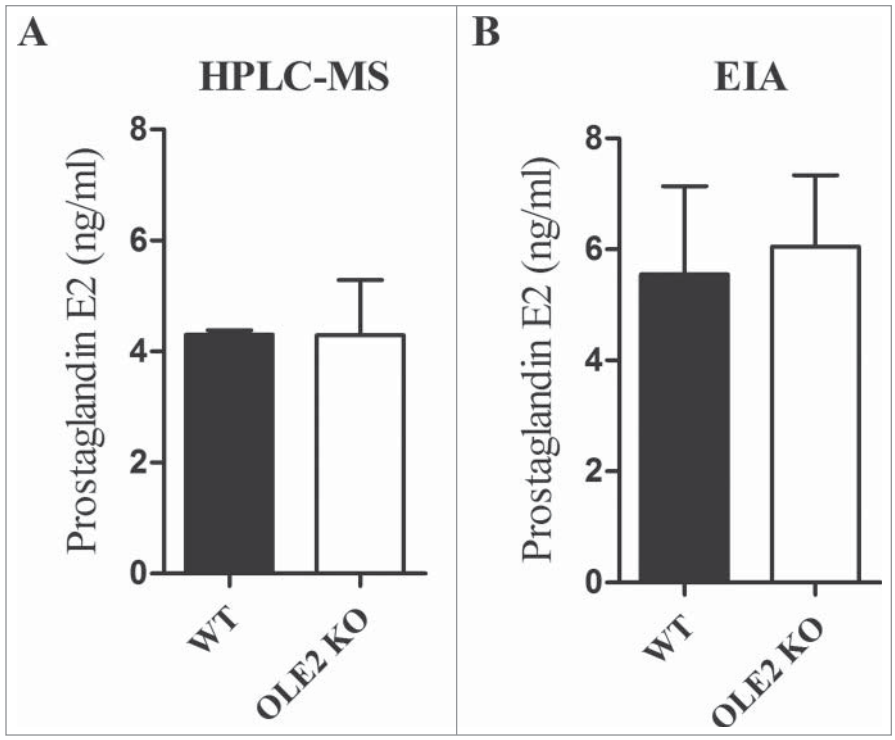

Figure 2. *CpOLE2* is not required for PGE_2 synthesis. *C. parapsilosis* WT and *OLE2* KO strains were cultured in YPD medium overnight, washed, and subsequently incubated in PBS for 24 h with or without 500 μM AA.The concentration of PGE_2 in culture supernatants was determined by HPLC-MS (**A**) or EIA (**B**). Results are mean ± SEM and represent the average of 3 independent experiments.

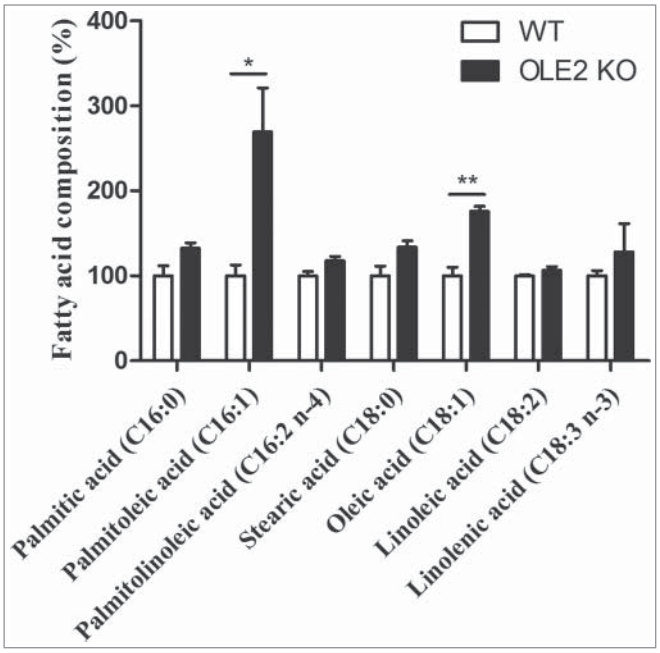

Figure 3. *OLE2* KO *C. parapsilosis* cells have altered fatty acid composition. WT and *OLE2* KO *C. parapsilosis* cells were cultured in YPD medium overnight, washed, lyophilized, and subjected to analysis by gas chromatography. For better comparison, fatty acid levels were normalized to those of WT cells and expressed as %. The actual concentration of palmitoleic and oleic acid (expressed as ng/mg or μg/mg dry weight) is described in Results. Data are mean ± SEM and represent the average of 3 independent experiments. *$P < 0.05$, **$P < 0.01$ by unpaired t-test.

most abundant prostaglandin produced by both *C. albicans* and *C. parapsilosis* was PGD_2, which exerts both pro-and anti-inflammatory properties, and is also involved in thermoregulation, hormone release and pain responses in mammals. Furthermore, PGD_2 is converted by a non-enzymatic reaction to 15-deoxy-Δ12,14-PGJ_2, which is a potent anti-inflammatory lipid mediator and has been shown to inhibit the NFκB signaling pathway.[25] As a potent pro-inflammatory response is required for the successful clearance of invasive *Candida*, the anti-inflammatory effects of 15-deoxy-Δ12,14-PGJ_2 may be beneficial for the pathogen. PGE_2 was also produced in high concentrations by *C. albicans* and *C. parapsilosis*, and PGE_2 mediates T-cell responses and influences other physiological functions.[8] Due to its ability to block Th1-type responses and promote Th2-type immunity,[8] fungal PGE_2 may also help the pathogen survive in the host.

Although the pathway of eicosanoid production in mammalian cells is well studied, little is known about the biosynthesis of prostaglandins in lower eukaryotes. While *C. albicans* does not possess cyclooxigenases, several enzymes, such as the fatty acid desaturase Ole2 and the multicopper oxidase Fet3 have been shown to contribute to prostaglandin production.[10] The presence of a conserved Δ9-desaturase domain in Ole2 protein suggested that it has Δ9-desaturase activity, while its cytochrome B domain was thought to be responsible for prostaglandin synthesis.[10] However, the role of *OLE2* in *C. parapsilosis* has never been previously investigated. To examine whether *OLE2* is involved in prostaglandin synthesis in *C. parapsilosis*, we generated *OLE2* deletion mutants using a previously described gene disruption technique.[26] However, we found that there was no difference between the PGE_2 secretion of WT and *OLE2* KO *C. parapsilosis* cells. The fact that *OLE2* is not required for PG synthesis in *C. parapsilosis* is unexpected, but can be explained considering that *C. albicans* and *C. parapsilosis* have crucial differences in their fatty acid metabolism. Interestingly, we also found that *OLE2* mutants had a different fatty acid composition compared to WT cells, containing higher amounts of palmitoleic and oleic acids. These UFAs are produced from palmitic and stearic acids, respectively, by Δ9-desaturases and are further converted to PUFAs via Δ12-desaturases.[27] The accumulation of palmitoleic and oleic acids in *OLE2* KO cells argues against the role of *OLE2* as an exclusive Δ9-desaturase and suggests that it may have other enzymatic activities as well. These results are in line with the findings of Krishnamurthy et al., who demonstrated that heterologous expression of *CaOLE2* could not reconstitute the phenotype of *S. cerevisiae OLE1* (Δ9-desaturase) mutants, implicating that Ole2 is not a Δ9-desaturase.[21] Moreover, UFA auxotrophic *OLE1 C. parapsilosis* mutants are unable to grow in SFA-supplemented media without the presence of UFA, indicating that Ole1 is the only enzyme in *C. parapsilosis* responsible for the synthesis of these essential fatty acids.[17] Further supporting these results, we found that Ole2 proteins from different *Candida* species (including *C. parapsilosis* and *C. albicans*) form a phylogenetically distinct group which is far from both Δ9− and Δ12− fungal desaturases (**Fig. S6**).

Interestingly, we also found that *OLE2* KO *C. parapsilosis* cells were phagocytosed and killed more efficiently by human macrophages compared to WT cells. One potential explanation for this decreased virulence is that the accumulated UFAs are toxic to yeast cells; however, we found no difference between the growth of *OLE2* KO and WT strains in medium conditions, making this possibility unlikely. On the other hand, as it has been shown with *OLE1* KO *C. parapsilosis* cells,[17] *OLE2* mutants could become hypersensitive to cellular stress. However, we found that the lack of *OLE2* did not impact the capacity of *C. parapsilosis* to cope with extracellular stressors such as H_2O_2 or SDS (data not shown). In addition to increased phagocytosis and killing, *OLE2* mutants also stimulated higher IL-10 production in macrophages compared to WT cells. IL-10 has been shown to increase the phagocytic capacity of macrophages[28]; therefore, suppression of IL-10 secretion may also be beneficial for the pathogen during infection.

There is an increasing amount of evidence showing that the integrity of fatty acid biosynthesis pathways in pathogenic fungi is essential for growth and virulence. For example, the downregulation of fatty acid synthase genes *FAS1* and *FAS2* substantially affects the virulence of *C. neoformans* in a murine model of pulmonary cryptococcosis.[29] Similarly, fatty acid synthase 2 (*FAS2*) mutant *C. parapsilosis* cells are defective in biofilm formation, are highly sensitive to human serum and have decreased virulence *in vivo* compared to WT cells.[30] Moreover, the stearyl-CoA desaturase Ole1 regulates the stress response and cellular morphology of *C. albicans* and *C. parapsilosis*, and it plays a major role in the

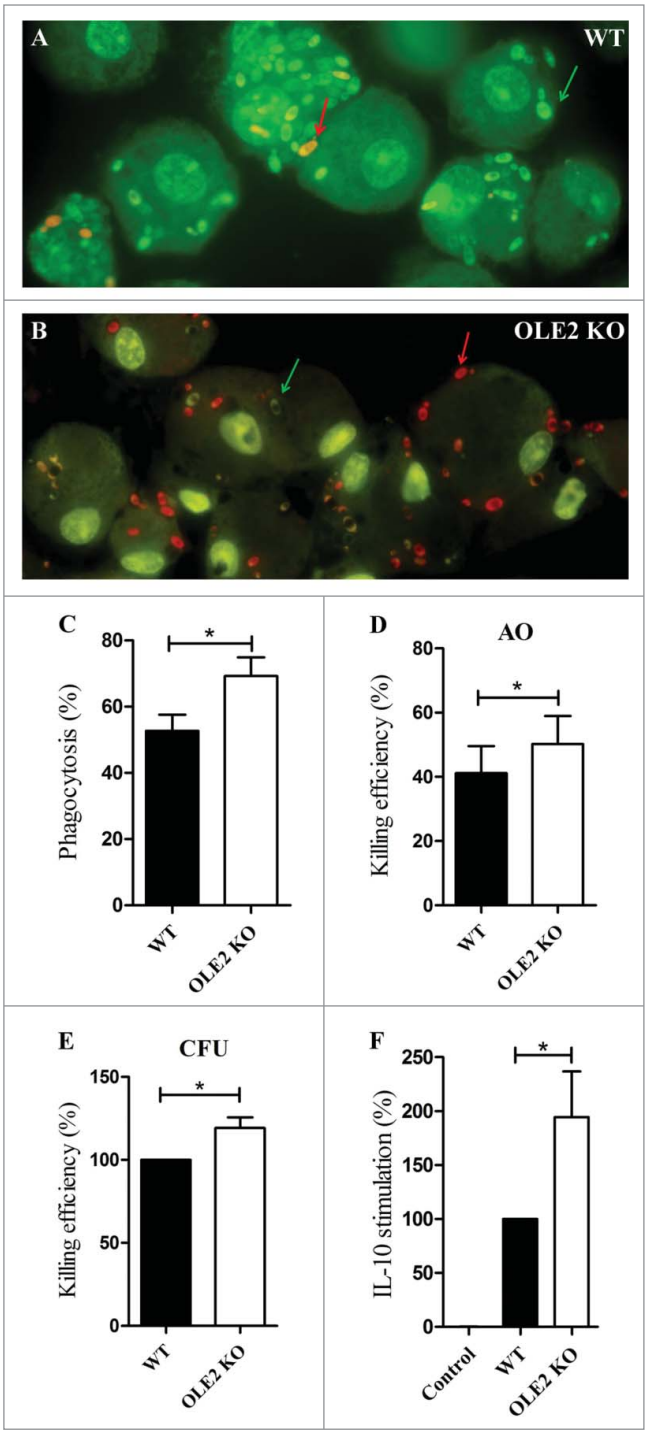

Figure 4. The lack of *OLE2* affects the virulence of *C. parapsilosis*. (**A–B**) Acridine orange staining of MDMs co-cultured for 3 h with WT (**A**) or *OLE2* KO (**B**) *C. parapsilosi* s. Live cells show green fluorescence (green arrow), dead cells show red fluorescence (red arrow). (**C**) Phagocytosis of WT and *OLE2* KO *C. parapsilosis* by human MDMs as determined by fluorescent microscopic analysis. Phagocytosis is expressed as the percent of macrophages that have ingested at least one yeast cell (phagocytosis%). Data represent mean ± SEM for 4 donors. (**D**) Intracellular killing of WT and *OLE2* KO *C. parapsilosis* by human MDMs as determined by fluorescent microscopic analysis. Results represent the percent of dead yeast cells ± SEM for 4 donors. AO, acridin orange. (**E**) Killing of WT and *OLE2* KO *C. parapsilosis* by human MDMsas measured by CFU determinations. Data were normalized to WT and represent mean ± SEM for 4 donors. (**F**) MDMs were stimulated for 24 h with WT or *OLE2* KO *C. parapsilosis* and the concentration of IL-10 in cell culture supernatants was determined by ELISA. Data were normalized for each donor to cytokine levels induced by the WT strain (100%) and are expressed as mean ± SEM for 5 donors. Actual cytokine levels are described in Results. $*P < 0.05$ as determined by paired t-test using the GraphPad Prism 5 software.

pathobiology of both species.[17,22] In accordance with these findings, our results further support the role of fatty acid homeostasis in the virulence of pathogenic fungi.

Taken together, we have shown here that *C. parapsilosis* is capable of producing prostaglandins similarly to other *Candida* spp. Furthermore, we generated a *OLE2* KO *C. parapsilosis* strain to assess the role of this gene in prostaglandin synthesis. Although we found that *OLE2* is not required for PGE_2 synthesis in *C. parapsilosis*, we show that *OLE2* deficient *C. parapsilosis* cells have altered fatty acid composition, which is accompanied by decreased virulence *in vitro*. Although further investigation is needed to reveal the exact function of *OLE2* during infection, our results contribute to the better understanding of fatty acid biosynthesis pathways in *Candida parapsilosis*.

Materials and Methods

Strains and growth conditions

Unless otherwise specified, *C. albicans* SC5314, *Candida parapsilosis* strains (GA1 wild type [WT][31] and *OLE2* mutant [*ΔCpole2/ΔCpole2::FRT*]) were grown overnight in liquid YPD medium (1% yeast extract, 2% bactopepton, 2% glucose) at 30°C. Cells were harvested by centrifugation, washed twice with PBS (phosphate-buffered saline; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4; pH 7.4) and counted in a Bürker-chamber prior to experiments.

Generation of the disruption constructs pSFS2Ole2 and pSFS2NewOle2

The pSFS2Ole2 and pSFS2NewOle2 plasmids were used to disrupt the open reading frame of the *OLE2* gene, consisting of 1 653 nucleotides. In order to construct the pSFS2Ole2 vector, a 599bp upstream and a 594bp downstream fragment was amplified from *C. parapsilosis* GA1 genomic DNA. For the amplification of the upstream PCR product "*Cp*OLE2 upstKpnIfrw" (5′-ttttttggtaccGAAAAATGAAGCTAAATCCTCCAAGGGAC-3′; [KpnI site underlined]) and "CpOLE2 upstXhoI rev" (5′-tttttctcgagATAGGAAAAGTAGTTGAAAATGCGCACAC-3′; [XhoI]) primers were used. For the amplification of the downstream PCR product "CpOLE2 down NotIfrw" (5′-tttttgcggccgcGCGTTGTTTCTCATCCATTTGAGGAAATTC-3′; [NotI]) and "CpOLE2 down SacI rev" (5′-tttttgagctcGGTTTATACTCAAATGGATGAATAGTCTTCTG-3′; [SacI]) primers were used. To construct the pSFS2NewOle2 vector, only the upstream region was changed for easier detection of the elimination

of the second allele. For the amplification of the upstream 417 bp PCR product "CpOLE2 NewupstKpnIfrw" (5′-ttttttggtaccC-TATTTACACTAAGCAGCTTCGGCAG-3′; [KpnI]) and "*Cp*OLE2 NewupstXhoI rev" (5′-ttttttctcgagGGCACACCAA-TATCTAAACTACCATTG-3′; [XhoI]) primers were used. The PCR fragments were ligated into pSFS2[32] appropriate cloning sites (KpnI/XhoI and NotI/SacI) to generate the pSFS2Ole2 and pSFS2NewOle2 disruption plasmids.

Candida parapsilosis transformation and generation of _Cp_Ole2 deletion mutants

C. parapsilosis WT cells were transformed by electroporation as previously described,[31] with 5 μg KpnI-SacI digested and purified fragment from the pSFS2Ole2 plasmid. For the disruption of the second allele, heterozygous mutants (*OLE2*/Δ *ole2* :: FRT) were transformed with 5 μg KpnI-SacI digested and purified fragment from the pSFS2NewOle2 plasmid. Transformants were regenerated and analyzed by Southern blotting (**Fig. Supplementary 1**). As our primary focus was to measure the production of prostaglandins and we did not find any difference between the WT and *OLE2* strain, we did not include the reconstituted strain in our study.

Determination of prostaglandin profile by HPLC-MS

For prostaglandin measurements, 100 ml PBS (control) or 100 ml PBS+AA (500 μM AA [Sigma, A9673] in PBS) was inoculated with 2×10^9 yeast cells and incubated for 24 h at 30°C. Subsequently, cells were harvested by centrifugation and the supernatants were collected by sterile filtration. Samples were analyzed with HPLC-MS technique using an Agilent 1100 liquid chromatograph and an Agilent 6410 triple quadrupole mass spectrometer (Palo Alto, USA). For the HPLC separation, a YMC Pack ProC18 (150 mm × 2.1 mm, 5 μm) (Dinslaken, Germany) column was applied with a guard column (20 mm × 2.1 mm, 5 μm). The A eluent was water, while the B eluent was acetonitrile/methanol (95:5) and both of them were supplemented with 0.005% acetic acid. The flow rate of the mobile phase was 0.3 ml/min with the gradient program progressing from 25% to 40% at 10 minutes and 50% at 20 minutes, and then reduced to 25% for an additional 15 minutes. For ionization, an ESI ion source was used with negative ionization at the following mass spectrometric parameters: nitrogen drying gas temperature, 325°C; drying gas flow, 12 l/min; capillary voltage, 3800 V; fragmentation voltage, 110 V. The analyzer operated in the multiple reaction monitoring (MRM) mode using 2 transitions for each components with the following transition and collosion energy (CE) parameters: 13,14-dihydro-15-keto-prostaglandin F2α (DKPGF2α, 353.2→309.1-20V, 291.2-20V), 11β-Prostaglandin F2α (11βPGF2α, 353.2→309.1-20V, 291.2-20V), Prostaglandin E1 (PGE1, 353.2→309.1-20V, 273.3-20V), Prostaglandin F1α (PGF1α, 355.5→311.2-20V, 293.2-20V), Prostaglandin H2 (PGH2, 351.3→271.2-15V, 180.1-15V), Prostaglandin E2 (PGE2, 351.3→271.2-15V, 315.1-15V), Prostaglandin F2α (PGF2α, 353.2→317.2-20V, 273.3-20V), Prostaglandin D2 (PGD2, 351.3→271.2-15V, 189.1-15V), Prostaglandin F2β (PGF2β, 353.2→291.2-20V, 317.2-20V), Prostaglandin A2 (PGA2, 333.3→271.2-20V, 189.2-20V), Prostaglandin J2 (PGJ2, 333.3→271.2-20V, 189.2-20V), Prostaglandin B2 (PGB2, 333.3→235.0-20V, 175.0-20V). For method development and quantifications, the Prostaglandin HPLC mixture (10002), Cyclopentenone Prostaglandin HPLC mixture (10000), Prostaglandin Metabolite HPLC mixture (10005) and Prostaglandin H2 (17020, all from Cayman Chemical) were used. All measurements were carried out in triplicates.

Determination of fatty acid composition of _C. parapsilosis_ strains by gas chromatography

For fatty acid measurements, strains were grown as described in 1 l YPD medium. After washing with PBS, yeast cells were lyophilized. The sample preparation was carried out according to Wei et al.[33] with minor modifications. Approximately 100 mg of a lyophilized sample was supplemented with 100 μg heptadecanoic acid (Sigma, H3500) as internal standard (1000 μg/ml). Samples were suspended in 5 ml of 5% methanolic solution of potassium hydroxide and saponified at 70°C for 1 hour. Subsequently, the pH of the mixture was adjusted to 2 with HCl. A mixture of 4 ml water and 2 ml chloroform was added and the samples were vigorously shaken. The chloroform phase was collected after centrifugation (4000 rpm, 4°C, 15 min) and the extraction was repeated again from the upper phase with 1 ml chloroform. The collected chloroform phases were pooled and evaporated under a stream of nitrogen. Four ml of 14% methanolic solution of boron-trifluoride was added to the saponified samples, and incubated at 70°C for 1.5 hour. The fatty acid methyl-esters (FAMEs) were partitioned with hexane, which was evaporated to dryness under nitrogen. Finally, the derivatized samples were reconstituted in 50 μl hexane before chromatographic analysis. The samples were analyzed with an Agilent 6890N (Palo Alto, USA) gas chromatograph (GC) and fatty acid methyl esters were identified by comparison of their retention times with those of standards (methyl palmitate, Sigma, P5177; methyl palmitoleate, Sigma, P9667; 9(Z),12(Z)-hexadecadienioc-acid methyl ester, Larodan AB, 20-1620-4; methyl stearate, Sigma, S5376; methyl oleate, Sigma, 311111; methyl linoleate, Sigma, L1876; methyl linolenate, Sigma, L2626). The instrument was equipped with an FID detector and an HP-Innowax (60 m*0.25 mm*0.5 μm) (Hewlett-Packard, USA) column. The injected volume was 1 μl of sample in split mode (split ratio was 50:1) and the injector and detector temperatures were 250°C. The temperature gradient was 50°C for 2 min, then increased to 200°C by 20°C/min, and then followed by increases of 3°C/min to reach 240°C where it was maintained for 50 min. For the separations, constant pressure mode (32 psi) was used and the flow of the detector gases were 30 ml/min, 300 ml/min and 20 ml/min for the hydrogen, air and nitrogen, respectively. The samples were analyzed under the same conditions at least 3 times.

Isolation of human peripheral blood mononuclear cells (PBMCs) and differentiation of monocyte-derived macrophages (MDM) (under approval of the institutional ethical review board of Szeged University)

PBMC isolation and differentiation was carried out as described[34] with minor modifications. Following isolation, PBMCs were plated on different cell culture plates: 96-well for killing assay (5×10^5cell/well); tissue culture coverslips (13mm, SARSTEDT, 83.1840.002) for acridine orange staining (5×10^5cell/coverslip) or 12-well for cytokine measurement (10^7cell/well). To differentiate macrophages, isolated monocytes were cultured for 7 d in X-VIVO 15 medium (Lonza, 04-744Q) supplemented with 10 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, Sigma-Aldrich, SRP3050) and 1% 100ċ penicillin–streptomycin solution (Sigma-Aldrich, P0781).

Fluorescent microscopy

MDMs were cultured on tissue culture coverslips (13 mm, SARSTEDT) in 12-well cell culture plates. The in vitro infection of the macrophages was carried out at a 1:5 macrophage:yeast ratio. After 3 h incubation at 37°C, the samples were stained with 0.01% acridine orange dye (Sigma, 235474) as described.34 This method allows differentiation between live and dead yeast cells. When acridine orange binds to intact (ds) DNA, it emits green fluorescence, while binding to damaged (ss) DNA it shows red fluorescence, indicating dead cells. For quenching the fluorescence of non-phagocytosed yeast cells, 0.05% crystal violet (Sigma, C3886) dye was used. The killing efficiency was calculated as follows: (number of dead yeast cells / number of engulfed yeast cells) × 100. Phagocytosis% was calculated as follows: (phagocytosing macrophages / all macrophages) × 100. In every experiment, 10 fields in each well were counted, and approximately 1000 MDMs were analyzed in each well.

Killing assay

Human macrophages were cultured on 96-well tissue culture plates. The *in vitro* infection of the macrophages was carried out at a 1:5 macrophage:yeast ratio. As a control, the same number of yeast cells were incubated in cell culture medium without macrophages. The killing assays were carried out as described.[34]

Cytokine measurements

Human macrophages were cultured on 12-well tissue culture plates. The *in vitro* infection of the macrophages was carried out at a 1:5 macrophage:yeast ratio. Cell culture supernatants were collected after 24 h and stored at −20°C until assayed for cytokine production. The concentration of cytokines in cell culture supernatants was determined by DuoSet ELISA Kits (R&D Systems; TNFα, DY210; IL-1β, DY201; IL-6, DY206; IL-10, DY217B) according to the manufacturer's instructions.

PGE_2 EIA

For PGE_2 measurement, the strains were grown as described above. Subsequently, 100 ml PBS (control) or 100 ml PBS + AA (500 μM AA [Sigma] in PBS) was inoculated with 2×10^9 yeast cells and incubated for 24 h at 30°C. Cells were harvested by centrifugation and the supernatants were sterile filtered. Samples were purified on a PGE_2 Affinity Column (Cayman Chemical, 400056) and analyzed with a Prostaglandin E_2 EIA Kit – Monoclonal (Cayman Chemical, 514010) according to the manufacturer's instructions.

Statistical analysis

GraphPad Prism 5 software was used for statistical analysis. Data were analyzed by paired or unpaired t-test (see Figure legends for details) and differences were considered statistically significant at $P < 0.05$. All experiments were performed at least twice (see Results and figure legends for details).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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