Molecular Microbiology (2008) 68(6), 1428–1437 ■
doi:10.1111/j.1365-2958.2008.06245.x
First published online 7 May 2008

# The role of laccase in prostaglandin production by *Cryptococcus neoformans*

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## Summary

**Recently, it has been demonstrated that the opportunistic fungal pathogen *Cryptococcus neoformans* can synthesize authentic immunomodulatory prostaglandins. The mechanism by which this takes place is unclear as there is no cyclooxygenase homologue in the cryptococcal genome. In this study, we show that cryptococcal production of both $PGE_2$ and $PGF_{2\alpha}$ can be chemically inhibited by caffeic acid, resveratrol and nordihydroguaiaretic acid. These polyphenolic molecules are frequently used as inhibitors of lipoxygenase enzymes; however, BLAST searches of the cryptococcal genome were unable to identify any homologues of mammalian, plant or fungal lipoxygenases. Next we investigated cryptococcal laccase, an enzyme known to bind polyphenols, and found that either antibody depletion or genetic deletion of the primary cryptococcal laccase (*lac1*Δ) resulted in a loss of cryptococcal prostaglandin production. To determine how laccase is involved, we tested recombinant laccase activity on the prostaglandin precursors, arachidonic acid (AA), $PGG_2$ and $PGH_2$. Using mass spectroscopy we determined that recombinant Lac1 does not modify AA or $PGH_2$, but does have a marked activity toward $PGG_2$ converting it to $PGE_2$ and 15-keto-$PGE_2$. These data demonstrate a critical role for laccase in cryptococcal prostaglandin production, and provides insight into a new and unique fungal prostaglandin pathway.**

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## Introduction

The production of oxidized fatty acids (oxylipins) from single-celled eukaryotes is an emerging area of research that has defined new mechanisms by which these organisms sense environment and community structure, signal morphological differentiation and regulate virulence (Noverr *et al.*, 2003a; Erb-Downward and Huffnagle, 2006; Shea and Del Poeta, 2006; Tsitsigiannis and Keller, 2007). Among these discoveries is evidence that many fungi are capable of producing 20-carbon oxylipins known as prostaglandins (Noverr *et al.*, 2002; Tsitsigiannis *et al.*, 2005a). In particular, *Cryptococcus neoformans* and *Candida albicans* have been identified as capable of producing authentic prostaglandin-$E_2$ ($PGE_2$) (Erb-Downward and Huffnagle, 2007; Erb-Downward and Noverr, 2007), a potent signalling molecule that can regulate inflammation in higher eukaryotes (Harris *et al.*, 2002).

Prostaglandins are 20-carbon fatty acid metabolites of arachidonic acid (AA). In higher eukaryotes, the synthesis of prostaglandins is dependent upon the action of cyclooxygenase enzymes which first generate the common precursor of prostaglandin synthesis, $PGH_2$ (Smith *et al.*, 2000). This precursor is then converted to the various prostaglandins by the action of prostaglandin synthases. The absolute dependence of prostaglandin synthesis in higher eukaryotes upon a cyclooxygenase has led to a biochemical paradox in that there is no cyclooxygenase homologue in either the *C. neoformans* or *C. albicans* genome (Tsitsigiannis *et al.*, 2005b; Erb-Downward and Huffnagle, 2007; Erb-Downward and Noverr, 2007) and classical inhibitors of prostaglandin synthesis, which are known to broadly inhibit cyclooxygenases, do not inhibit prostaglandin synthesis (Erb-Downward and Huffnagle, 2007; Erb-Downward and Noverr, 2007). Recently, a family of fatty acid dioxygenase enzymes (PpoA, PpoB and PpoC) which possess homology to the catalytic domains of mammalian cyclooxygenases were identified in *Aspergillus nidulans* and *Aspergillus fumagatis* (Tsitsigiannis *et al.*, 2005b). Further analysis showed that the cultures produced prostaglandins (as detected by immunoassay) and ppo mutants produced less (Tsitsigiannis *et al.*, 2005a). But these enzymes do not exist in the *C. neoformans* genome (Tsitsigiannis *et al.*, 2005b). The current work details our

investigation into the enzymes involved in cryptococcal prostaglandin synthesis, and the discovery that a cryptococcal laccase is required for prostaglandin synthesis in *C. neoformans.*

## Results

### *Chemical inhibition of cryptococcal prostaglandin synthesis*

To examine the effects of chemical inhibition on cryptococcal prostaglandin production we first established a lysate system by which multiple species of prostaglandins could be measured. Briefly, lysates were generated through mechanical disruption of stationary phase *C. neoformans* cells followed by a 2 h incubation with AA. Figure 1 demonstrates that multiple prostaglandin species can be efficiently generated using this method including $PGF_{2\alpha}$ and $PGE_2$. These also can be measured using a pan-specific prostaglandin immunoassay that detects multiple prostaglandin species (Fig. 1A). In this lysate system, proportionally more $PGF_{2\alpha}$ is synthesized (Fig. 1C) than $PGE_2$ (Fig. 1B). Furthermore, production of prostaglandins is an enzymatic process (i.e. heat denaturable), because boiling of the lysates prior to incubation with AA results in a significant reduction in prostaglandin production.

As we have previously demonstrated that cyclooxygenase enzymes are not present in *C. neoformans* (Erb-Downward and Huffnagle, 2007), we investigated whether other classes of oxygenases could be involved by using the inhibitors resveratrol, caffeic acid and nordihydroguaiaretic acid (NDGA) to inhibit prostaglandin synthesis in cell-free lysates. Caffeic acid (Fig. 2B) and NDGA (Fig. 2A) are well characterized for their ability to inhibit lipoxygenase enzymes (Koshihara *et al.*, 1984; Salari *et al.*, 1984; Whitman *et al.*, 2002; Tanaka *et al.*, 2003) whereas resveratrol (Fig. 2A), which shares a similar structure, inhibits both cyclooxygenase and lipoxygenase enzymes (Kimura *et al.*, 1985; Kimura *et al.*, 1995; Pinto *et al.*, 1999; Tanaka *et al.*, 2003). All of the polyphenols tested significantly decreased the total prostaglandin production (Fig. 2). For resveratrol and NDGA, the inhibition was equivalent to boiling the lysates prior to the addition of AA. These findings indicate that polyphenols are capable of inhibiting cryptococcal prostaglandin production.

BLAST searches of the cryptococcal genome for homologues of 5-, 12- or 15-lipoxygenases from humans, mice or rats did not reveal a lipoxygenase homologue (data not shown). Similarly, searches for homologues of plant lipoxygenases (1-LO from soy-beans, and 13-LO from *Arabidopsis thaliana*) did not yield any protein with significant homology. Even the 1-lipoxygenase found in the fungus *Neurospora crassa* does not have a homologue in *C. neoformans* (data not shown). Thus, we shifted our focus from lipoxygenases and cyclooxygenases to other enzymes known to interact with polyphenols (Li *et al.*, 2005).

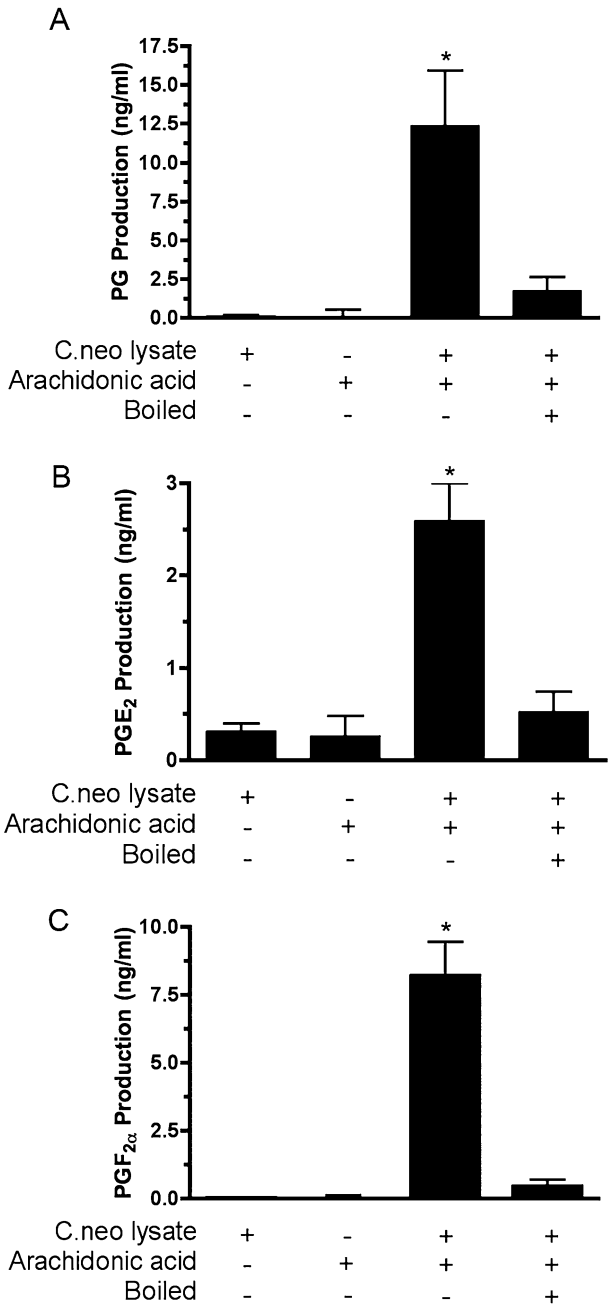


**Fig. 1.** Prostaglandin production in cryptococcal lysates. *C. neoformans* cells were lysed and the lysates incubated ± AA for 2 h at 37°C. *C. neoformans* (C.neo) lysates were incubated with AA and total prostaglandins were measured using a prostaglandin screening EIA (A: $n = 11$; $^*P < 0.001$); $PGE_2$ were levels assayed using a $PGE_2$ monoclonal EIA (B: $n = 7$; $^*P < 0.001$); and $PGF_{2\alpha}$ levels were assayed using a $PGF_{2\alpha}$ monoclonal EIA (C: $n = 5$; $^*P < 0.001$).

 

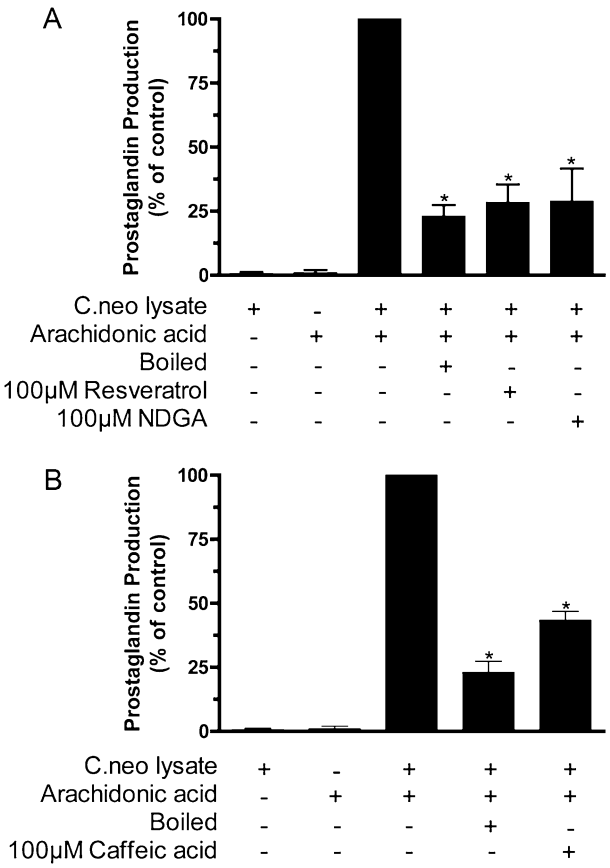


**Fig. 2.** The effect of inhibitors on total prostaglandin production from *C. neoformans* lysates *C. neoformans* (C.neo) cells were lysed, the lysates incubated ± AA for 2 h at 37°C and total prostaglandin production was measured using a prostaglandin screening EIA. (A) and (B) document the effect of various polyphenols on cryptococcal prostaglandin production. The results are normalized as a per cent inhibition (where the lysate + AA was set to 100%; A: $n = 5$ and B: $n = 2$; $^*P < 0.001$).

### *A role for laccase in cryptococcal prostaglandin production*

Cryptococcal laccase (*lac1*) is a multicopper oxidoreductase having a broad specificity that includes Fe(II), aminophenols and polyphenols (Williamson, 1994; Liu *et al.*, 1999). Additionally, we have previously noted that growth conditions that promote prostaglandin expression also result in increased levels of prostaglandin production (Noverr *et al.*, 2001). To test whether this polyphenol-binding enzyme plays a role in cryptococcal prostaglandin synthesis, lysates were depleted of laccase prior to incubation with AA by the addition of a monoclonal antibody (G3P4D3) raised against the N-terminal portion of cryptococcal laccase. The laccase-depleted supernatants were then incubated with AA, and prostaglandin levels were measured by enzyme immunoassay (EIA; Fig. 3A). Lysates treated with control antibody produced prostaglandins at the same levels as untreated lysates. However, immunoprecipitation of laccase from cryptococcal lysates significantly diminished the ability of the lysates to produce prostaglandins from AA.

Recently, a second laccase gene (*LAC2*) was identified that shares 65% identity with *LAC1* at the nucleotide level (Zhu and Williamson, 2004). Sequence analysis suggests that the mAb directed against the protein product of *LAC1* might also recognize the product of *LAC2*. To assess the relative contribution of each of these enzymes to cryptococcal prostaglandin production, *LAC1* and *LAC2* deletion strains were incubated in the presence of exogenous AA, the prostaglandins purified from the supernatants using organic extraction followed by Reverse-phase high-

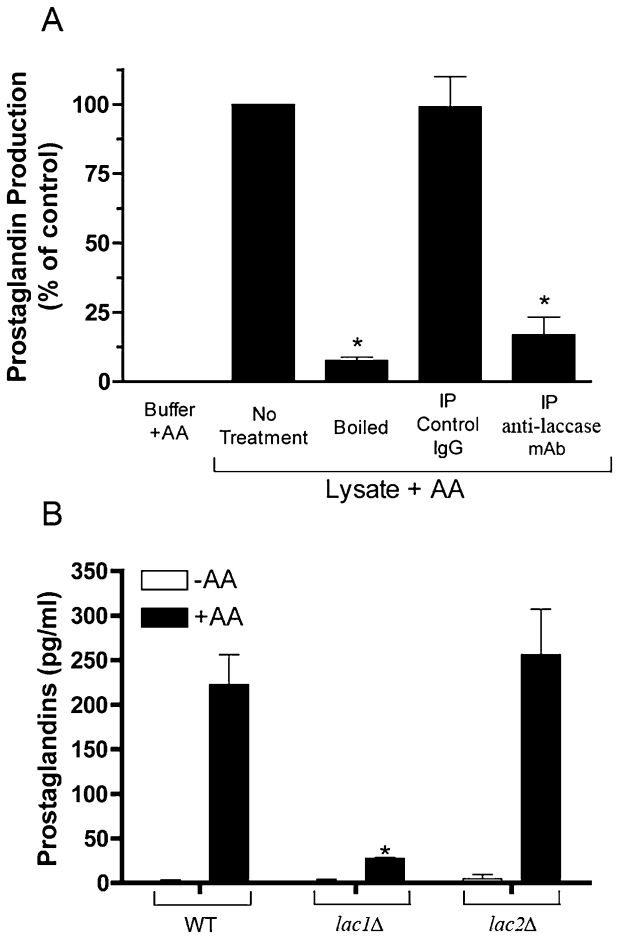


**Fig. 3.** The effect of the removal of laccase on cryptococcal prostaglandin production.
A. The effect of the immunoprecipitation (IP) of laccase from cryptococcal lysates prior to the addition of AA. Inhibitory effect is displayed as a per cent of control (the non-immunodepleted lysate + AA), which was set to 100% ($n = 4$; $^*P < 0.001$).
B. The effect of deleting the cryptococcal laccase genes on prostaglandin production into culture (when incubated with AA). Culture supernatants were taken from wild-type *C. neoformans*, *lac1Δ* or *lac2Δ* strains that had been incubated for 2 h at 37°C in the presence of AA. Fungal prostaglandins were extracted from the culture supernatant into ethyl acetate and purified using RP-HPLC. Total prostaglandins were measured by EIA ($n = 2$; $^*P < 0.05$).

 

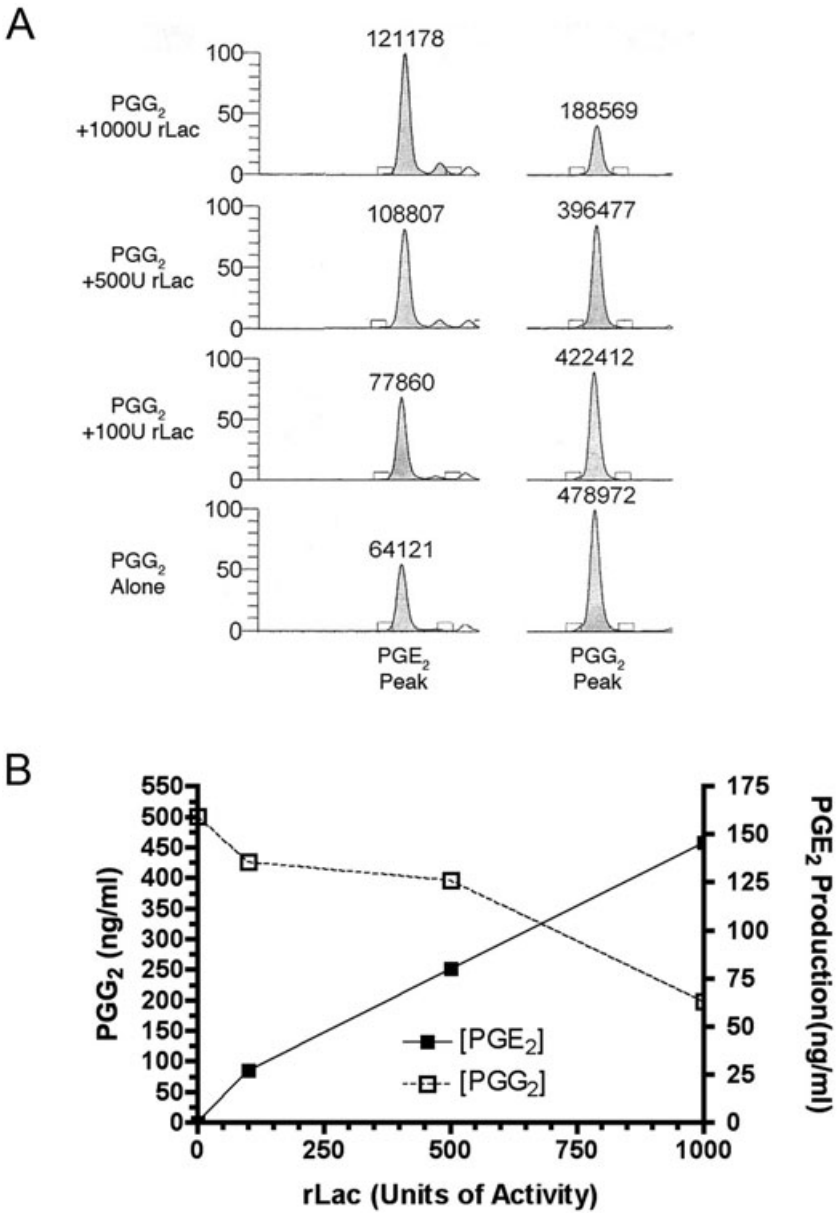


**Fig. 4.** The effect of recombinant Lac1 on $PGG_2$ to $PGE_2$ conversion. Increasing concentrations of rLac1 were incubated with $PGG_2$, and the products were separated and analysed using LC-MS as described in *Experimental procedures*.
A. The extracted ion chromatograms of $PGE_2$ (m/z range = 351–352 $[M\text{-}H]^-$ ion) and $PGG_2$ (m/z range = 367–368 $[M\text{-}H]^-$ ion) with increasing concentrations of rLac1. The numbers above each peak denote the area under the peak.
B. The increase in $PGE_2$ production over background levels and the decrease in $PGG_2$.

performance liquid chromatography (RP-HPLC) fractionation, and the levels assayed by EIA (Fig. 3B). The *LAC2*-deficient strain produced the same levels of prostaglandins as the wild-type strain upon the addition of AA. However, consistent with the lysate immunodepletion experiments, the *LAC1*-deficient strain of *C. neoformans* produced significantly lower levels of prostaglandins. Thus, loss of *LAC1*, but not *LAC2*, decreases cryptococcal prostaglandin production

We next investigated how Lac1 is involved in prostaglandin synthesis. To address the question, recombinant Lac1 was tested for whether prostaglandins or intermediates in prostaglandin synthesis could be generated from AA, $PGG_2$ or $PGH_2$. If Lac1 functions as a cyclooxygenase, $PGG_2$ and $PGH_2$ should be generated from AA. Alternatively, if the activity of Lac1 is similar to a prostaglandin synthase, then Lac1 should convert $PGH_2$ to individual prostaglandin species such as $PGE_2$ or $PGF_{2\alpha}$. To this end, increasing concentrations of recombinant Lac1 (rLac) were incubated with 50 μM of substrate (AA, $PGG_2$ or $PGH_2$) at 37°C for 3 h. The products were then extracted into ethyl acetate (EtOAc), dried down and analysed by tandem liquid chromatography-mass spectroscopy. Incubation of rLac with either AA or $PGH_2$ alone did not result in the generation of new prostaglandins (data not shown). However, increasing amounts of rLac incubated with 50 μM $PGG_2$ resulted in a dose-dependent decrease in $PGG_2$ and a concomitant increase in $PGE_2$ (Fig. 4). Thus,

 

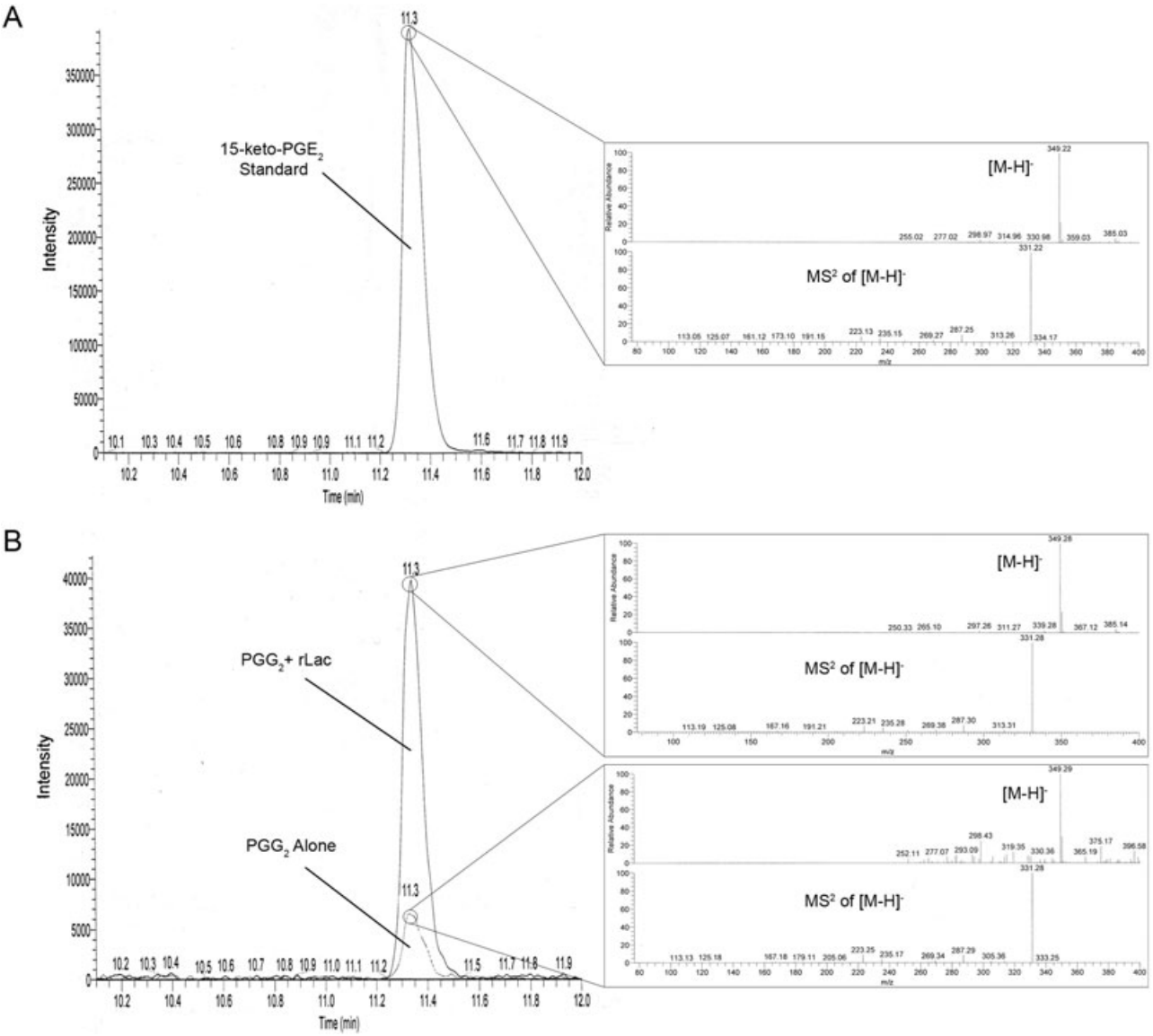


**Fig. 5.** The effect of recombinant Lac1 on 15-keto-$PGE_2$ levels. Chromatograms of samples containing 15-keto-$PGE_2$ standard (A) or $PGG_2$ ± 1000 U recombinant Lac1 (B) focusing on the m/z range = 349.1–349.5. Panel insets indicate the parent $[M-H]^-$ ion mass and $MS^2$ of this ion.

Lac1 does not carry out the cyclooxygenase reaction or convert $PGH_2$ to a prostaglandin like a prostaglandin synthase, but it does possess a unique activity catalyzing the conversion of $PGG_2$ to $PGE_2$.

In addition to $PGE_2$, several other peaks within the m/z range of 340–370 were notably different in the samples that contained $PGG_2$ +rLac when compared with those that contained $PGG_2$ alone. The most significant of these occurred with an elution time of 11.3 min and possessed a m/z = 349 $[M-H]^-$. Using R-package XCMS (Smith *et al.*, 2006) and the METLIN database (http://metlin.scripps.edu/) we were able to narrow down the possible related compounds to $PGE_3$, 15-keto-$PGE_2$ and $PGK_2$. Commercial standards of these compounds were obtained and assessed by LC-MS. Based on retention times, MS and $MS^2$ this peak was identified as the $PGE_2$ metabolite 15-keto-$PGE_2$ (Fig. 5). Thus, while Lac1 did not form the endocyclic peroxide that a cyclooxygenase generates, Lac1 carries out the unique conversion of $PGG_2$ to $PGE_2$ and 15-keto-$PGE_2$ which clearly distinguishes it from a prostaglandin synthase.

## Discussion

*Cryptococcus neoformans* can synthesize authentic $PGE_2$ that is identical to mammalian $PGE_2$; however, this

 

process proceeds via unique enzymatic pathways which do not involve a cyclooxygenase enzyme (Erb-Downward and Huffnagle, 2007). We have identified the *C. neoformans* laccase Lac1 as a critical enzyme in this new pathway based on evidence that either the depletion or disruption of Lac1 all but eliminates specific cryptococcal prostaglandin production, and that the recombinant enzyme can convert $PGG_2$ to $PGE_2$ and 15-keto-$PGE_2$. In addition to Lac1, these studies demonstrate that there are likely at least two other enzymes in the cryptococcal prostaglandin pathway: one upstream of Lac1 activities and the other downstream. The evidence for the existence of the upstream enzyme is that cryptococcal lysates can produce prostaglandins from exogenous AA, but Lac1 can only produce prostaglandins from $PGG_2$, an oxidized product of AA. Similarly, the lysate data indicate that in addition to $PGE_2$, cryptococcal lysates supplemented with exogenous AA synthesize $PGF_{2\alpha}$, but if Lac1 is removed from the system the enzymatic production of $PGE_2$ and $PGF_{2\alpha}$ ceases. However, Lac1 does not synthesize $PGF_{2\alpha}$, indicating that another enzyme must act on the products of Lac1 to synthesize $PGF_{2\alpha}$. Together, these data indicate that the cryptococcal prostaglandin pathway is unique not only in the enzymes involved, but also the structure of the pathway, which the evidence suggests is linear. This clearly differentiates it from the common precursor model that is seen in cyclooxygenase-dependent prostaglandin production (Fig. 6).

The demonstration that cryptococcal laccase is critical to the production of cryptococcal prostaglandins provides the first association between a laccase and prostaglandin synthesis. Classically, laccases are thought of as enzymes involved in the oxidation of polyphenols or the degradation of plant lignin (Leonowicz *et al*., 2001). In *C. neoformans*, laccase catalyses the formation of a polymer melanin from 2,4- or 3,5-dihydroxy polyphenolic precursors. Melanin, in turn, enhances the ability of the organism to survive the oxidative burst within a macrophage and ultraviolet radiation induced damage out in the environment (Gomez and Nosanchuk, 2003). However, recent lines of study have begun to explore the action of this class of enzymes on non-polyphenolic molecules (Casa *et al*., 2003). In one report investigators studied the ability of a mushroom laccase to degrade olive oil mill waste effluents, a mixture of polyphenols, fatty acids and other compounds. They found that in addition to polyphenols, fatty acids were also oxidized by this enzyme (Karlsson *et al*., 2001). Further study revealed that the oxidation of long-chain poly-unsaturated fatty acids by the mushroom laccase was specific and proceeded via a mechanism that was remarkably similar to that of a lipoxygenase (Zhang *et al*., 2002). A recent study comparing enzymes from different organisms that, based on DNA sequence homology, are considered laccases, found that many members of the family do not possess the ability to degrade lignin or oxidize polyphenols (Valderrama *et al*., 2003). This suggests that alternative activities for this enzyme might be more common than was previously believed. Our studies provide additional support for the activity of laccase enzymes beyond the oxidation of polyphenolic compounds and lignin degradation: the generation of oxylipins from poly-unsaturated fatty acids.

In recent years much has been discovered about the critical importance of lipid mediators in single-celled eukaryotic pathogens. These effects include the stimulation or inhibition of sexual development (depending on the mediator), quorum sensing and the regulation of virulence (Angeli *et al*., 2001; Noverr and Huffnagle, 2004; Tsitsigiannis *et al*., 2005a; Tsitsigiannis and Keller, 2006; Erb-Downward and Noverr, 2007; Tsitsigiannis and Keller, 2007). Some of the prostaglandin production in these organisms is attributable to the presence of a cyclooxygenase homologue (Tsitsigiannis *et al*., 2005a). However, there also exists a body of literature relating to prostaglandin production in non-vertebrate organisms that has attempted to fit, unsuccessfully, what is found in these organisms into model of cyclooxygenase-dependent prostaglandin production (reviewed in Lamacka and Sajbidor, 1995; Noverr *et al*., 2003a). Recent studies have examined *Trypanosoma brucei*, *Trypanosoma cruzii* and *Plasmodium falciparum*, in the absence of a host, and found that they produce prostaglandins that are not inhibited by aspirin or indomethacin (Kilunga Kubata *et al*., 1998; Kubata *et al*., 2000; 2002). This production also occurs in the absence of an identifiable cyclooxygenase enzyme. In these organisms, a $PGF_{2\alpha}$ synthase has been identified which is homologous to the Old Yellow enzyme found in *Saccharomyces cerevisiae* (Kubata *et al*., 2000; 2002). It has also been reported that the parasite-derived prostaglandins of *Schistosoma mansoni* actively suppress the immune system to facilitate infection of the host (Herve *et al*., 2003). $PGE_2$ is well known for its ability to suppress inflammation (Harris *et al*., 2002), and 15-keto-$PGE_2$ has been recently identified as a ligand for PPAR$\gamma$ (Chou *et al*., 2007), a nuclear receptor involved in the regulation of macrophage activation. Thus, it is tempting to speculate that cryptococcal production of prostaglandins may help downregulate local inflammatory responses. The recent finding that cryptococcus carries out its sexual cycle in association with plants (Xue *et al*., 2007) is another area where these oxylipins may be involved since oxylipin regulation of sexual cycles of other fungi has been well established (Tsitsigiannis *et al*., 2005b; Tsitsigiannis and Keller, 2006; Tsitsigiannis and Keller, 2007). To date only two enzymes involved in cryptococcal prostaglandin synthesis have been identified: phospholipase B (PLB; Cox *et al*., 2001; Noverr *et al*., 2003b) and now laccase (Lac1). Both are known virulence

 

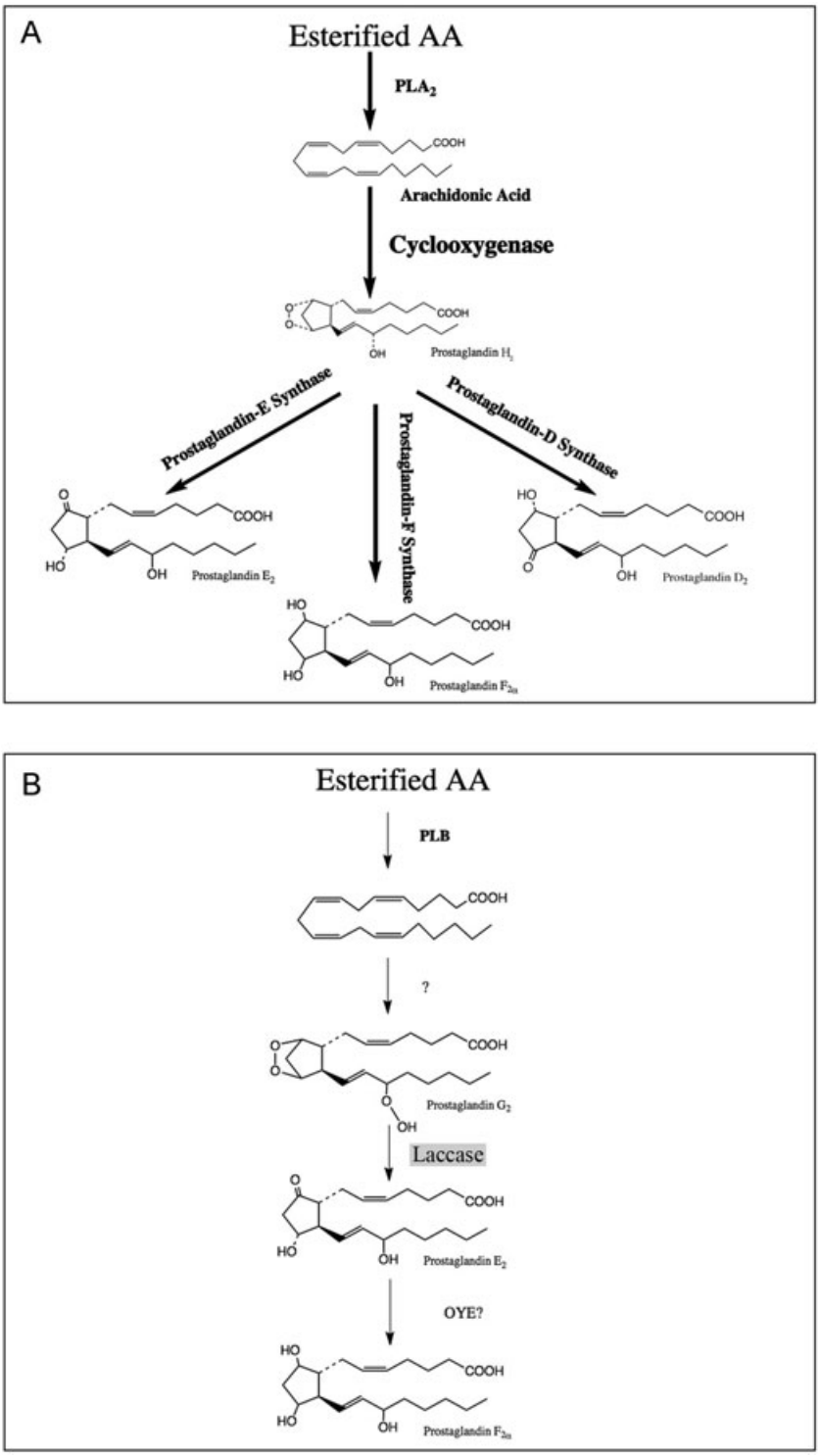


**Fig. 6.** The common precursor versus a linear model of cryptococcal prostaglandin synthesis.
A. The common precursor model of prostaglandin synthesis where cyclooxygenase enzymes act on free arachidonic acid to produce $PGH_2$, which then serves as the common substrate for the individual prostaglandin synthases.
B. The proposed linear model of cryptococcal prostaglandin synthesis, where each step is dependent upon the product of the step before. Question marks denote steps where the enzymes are not yet known, or a likely candidate whose activity has yet to be demonstrated.

factors and the currently proposed mechanisms of virulence do not account for their role in prostaglandin synthesis. It is our belief that as additional enzymes involved in cryptococcal prostaglandin synthesis are discovered, the contribution of prostaglandins to the pathogenesis of *C. neoformans* will likely become clear.

## Experimental procedures

### *Strains and culture conditions*

Throughout this study, *C. neoformans* strain 24067 was used except where *C. neoformans* deletion mutants were used. For these latter studies, the *LAC1* deletion mutant (*lac1*Δ),

the *LAC2* deletion mutant (*lac2*Δ) and the parent strain (H99) were utilized (Zhu and Williamson, 2004). All strains were grown shaking at ~37°C in 30 ml Sabouraud dextrose broth (1% neopeptone and 2% dextrose; Difco, Detroit, MI) until stationary phase was reached (72 h or until the cells reached a concentration $> 2 \times 10^8$ ml$^{-1}$).

### C. neoformans *lysis protocol*

A total of $1.2 \times 10^9$ cells were transferred to a 50 ml conical tube, spun down at 3000 r.p.m. and washed 2× with 40 ml sterile saline. Next, 1.2 ml of cold phosphate buffer (pH 7.1) was added to the tube without disturbing the pellet. This was followed by the addition of 0.5 μm glass beads until the beads just began to dry. The tube was then subjected to a vigorous regimen of vortexing 5× for 1 min followed by 1 min on ice. The liquid was then transferred to another tube, and the beads washed 2× with 600 μl cold phosphate buffer, vortexing and transferring the liquid portion with each wash. This crude lysate was then spun down at 13 000 r.p.m. for 45 min to remove debris and unlysed cells. From these tubes the supernatant (lysate) was transferred to a fresh 15 ml conical and kept on ice.

### *Prostaglandin production and measurement*

Prostaglandins were generated from cryptococcal lysates by incubating 150 μl of lysate in a 1.5 ml Eppendorf tube with 500 μM AA (Cayman Chemical) at 37°C for 2 h. The requirement for functional enzymes was tested by boiling a portion of *C. neoformans* lysate for 10 min followed by spinning down the precipitated proteins prior to the addition of AA. The lipoxygenase inhibitors caffeic acid and NDGA (Cayman Chemical) and resveratrol (Sigma Chemical) were dissolved in DMSO. Inhibition studies were performed by adding inhibitor or an equivalent amount of DMSO to lysate samples just prior to incubation with AA.

Prostaglandins were generated in whole-cell supernatants by incubating $1 \times 10^7$ cells in 1 ml phosphate buffer, pH 7.1, with 500 μM AA or control at 37°C for 2 h. Following incubation, the cells were spun down and the supernatant removed and assayed for prostaglandin content.

Prostaglandins were measured utilizing $PGE_2$ specific, $PGF_{2\alpha}$ specific or the Prostaglandin Screening EIA (Cayman Chemical) according to manufacturer's protocols. EIA plates were read at 405 nm, and the concentrations of prostaglandin were calculated based on a bound versus free ($B/B_0$) measurement. Background values in the absence of lysate were subtracted from each corresponding sample condition to obtain a specific measurement of prostaglandin production owing to the conversion of AA.

### *Organic extraction and reverse-phase HPLC*

To assay the organic compounds produced in cryptococcal supernatants, samples were transferred to a silianized $12 \times 75$ cm glass test tube, the organic components extracted twice into 500 μl HPLC grade EtOAc (Aldrich Chemical), and the organic phase transferred to a silianized conical screw-top vial. The contents of the vial were dried down in a water bath under a stream of grade 5 nitrogen.

Reverse-phase high-performance liquid chromatography analysis was carried out using a Waters 600 HPLC system, with a 5 μl sample loop and a Waters Symmetry $2.1 \times 150$ mm analytical column. The compounds were separated using a gradient elution starting at 75:25:0.1 (water : acetonitrile : acetic acid) for 10 min followed by a linear shift to 0:100:0.1 (water : acetonitrile : acetic acid) over 75 min. The dry sample was brought up in 25 μl HPLC grade EtOH, and 12.5 μl was (5 μl) injected. The remainder was dried down under nitrogen and stored at –20°C for not longer than 1 week. Fractions were collected, and the organic compounds were once again extracted twice into 500 μl EtOAc, followed by drying down under nitrogen. These samples were then brought up in 150 μl prostaglandin EIA buffer (Cayman Chemical) and assayed according to manufacturer's protocols as above.

The elution times of cyclooxygenase-derived prostaglandin standards (Cayman Chemical) were obtained using the same elution gradient.

### *Purified enzyme studies*

The recombinant Lac1 used in these studies was generated as previously described (Zhu *et al*., 2001). Lyophilized recombinant Lac1 (rLac) was reconstituted in 50 mM phosphate buffer pH 6.5. Enzyme activity was determined by adding 1 μl of rLac to 1 ml of 1 mM epinephrine bitartrate for 30 min at 37°C and measuring the absorbance at 475 nm. One unit of enzyme activity was equal to change of 0.001 AU. To test rLac activity on prostaglandin relevant substrates 100, 500 or 1000 U of rLac was added to 50 μM AA or $PGG_2$ in a 30 μl reaction volume for 3 h at 37°C. Reactions were stopped by the addition of 150 μl of EtOAc, the organic phase was removed and dried down under N2.

LC-MS/MS was carried out using a ThermoFinnigan Surveyor HPLC (San Jose, CA) interfaced directly to the electrospray ionization source of a ThermoFinnigan LTQ linear ion-trap mass spectrometer (San Jose, CA). Samples were re-suspended in a methanol : water solution (1:1, v/v) and 20 μl aliquots were injected onto a Phenomenex Luna $2.00 \times 150$ mm 3 μm C-18(2) column (Torrence, CA). Mobile phase solvents were water + 0.1% acetic acid (A) and acetonitrile + 0.1% acetic acid (B). Compounds were separated and eluted from the analytical column with a linear gradient of 25–100% B over 40 min at a flow rate of 0.3 ml min$^{-1}$. The sample tray was cooled to 4°C throughout the analysis. Concentrations of compounds were calculated by taking the ratio of area of peak to the area of the internal standard peak (15-deoxy-$\Delta^{12,14}$-PGJ2) and multiplying by the response factor. The response factor was calculated as a ratio of the peak areas of known concentrations of commercial standards of $PGG_2$, 15-keto-$PGE_2$ and $PGE_2$ to the peak area of a 1 μg ml$^{-1}$ 15-deoxy$\Delta^{12,14}$-PGJ2.

### *Immunoprecipitation*

Immunoprecipitation of laccase from cryptococcal lysates was carried out by incubating 600 μl lysates on ice with

15 μg ml$^{-1}$ anti-laccase mouse monoclonal antibody (clone G3P4D3) raised against full-length recombinant laccase enzyme or control mouse IgG, for 2 h on ice prior to the addition of 50 μl of protein-A beads (Sigma). Tubes were set to rock overnight at 4°C and the following day, beads were spun down at 13 000 r.p.m. for 1 min and the supernatant transferred to a fresh tube. This depleted lysate was then treated as above to generate prostaglandins.

### *Statistical analysis*

Statistical analysis of results was performed using Prism 4.0 (GraphPad Software). Statistical significance was determined using either a one-way ANOVA with Bonferroni's multiple comparison post-test. For the purposes of comparing the degree of inhibition of the various inhibitors, the data were represented as a percentage of control (Lysate + AA).

## Acknowledgements

We wish to thank Dr Arturo Casadevall of Albert Einstein College of Medicine, Yeshiva University for providing the anti-laccase mAb. We also want to thank the other members of the Huffnagle lab for their insight and evaluation and Dr Galen Toews for his ever thought-provoking discussions. Also, we wish to thank Kate Noon at the University of Michigan Department of Pharmacology's Biomedical Mass Spectrometry Facility for all her help and mass spectrometry expertise. This work was supported by funding from NIAID Grants 1R01AI059201, AI45995, AI49371 and NIH-NHLBI T32 HL007749-11.

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