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# Cytochrome $b_5$ reductase- Cytochrome $b_5$ as an active P450 redox enzyme system in *Phanerochaete chrysosporium*: Atypical properties and *in vivo* evidence of electron transfer capability to CYP63A2

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## Abstract

Two central redox enzyme systems exist to reduce eukaryotic P450 enzymes, the P450 oxidoreductase (POR) and the cyt $b_5$ reductase-cyt $b_5$. In fungi, limited information is available for the cyt$b_5$ reductase-cyt $b_5$ system. Here we characterized the kinetic mechanism of (cyt $b_5$r)-cyt $b_5$ redox system from the model white-rot fungus *Phanerochaete chrysosporium* (Pc) and made a quantitative comparison to the POR system. We determined that Pc-cyt $b_5$r followed a "ping-pong" mechanism and could directly reduce cytochrome c. However, unlike other cyt $b_5$ reductases, Pc-cyt $b_5$r lacked the typical ferricyanide reduction activity, a standard for cyt $b_5$ reductases. Through co-expression in yeast, we demonstrated that the Pc-cyt $b_5$r-cyt $b_5$ complex is capable of transferring electrons to Pc-P450 CYP63A2 for its benzo(a)pyrene monooxygenation activity and that the efficiency was comparable to POR. In fact, both redox systems supported oxidation of an estimated one-third of the added benzo(a)pyrene amount. To our knowledge, this is the first report to indicate that the cyt $b_5$r-cyt $b_5$ complex of fungi is capable of transferring electrons to a P450 monooxygenase. Furthermore, this is the first eukaryotic quantitative comparison of the two P450 redox enzyme systems (POR and cyt $b_5$r-cyt $b_5$) in terms of supporting a P450 monooxygenase activity.

### Keywords

*Phanerochaete chrysosporium*; Cytochrome $b_5$ reductase; Cytochrome $b_5$; P450 oxidoreductase; Cytochrome P450 monooxygenase; *Pichia pastoris*; Ping-pong Kinetic mechanism; Redox enzymes; Reductase



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## 1. Introduction

The whole genome sequencing era has unveiled the presence of an extensive cytochrome P450 enzyme repertoire (P450ome) in fungi. In contrast to the high number of P450s in their genome, these organisms typically contain a single P450 oxidoreductase (POR)1 with some exceptions [1]. The presence of a single POR may not be sufficient to accommodate the entire electron transfer needs of their extraordinarily large P450ome. Consequently, alternative redox system proteins, such as cytochrome $b_5$ reductase (cyt $b_5$r)-cyt $b_5$, likely play a significant role in this process.

Cyt $b_5$ and cyt $b_5$r are ubiquitous proteins found throughout biology, from yeasts to insects to seed plants to animals. These proteins are typically involved in NADH-dependent electron transport, where cyt $b_5$r transfers two reducing equivalents from NADH to cyt $b_5$. Cyt $b_5$ has a heme prosthetic group that lacks a free coordination position, and consequently, it serves as a physiological electron transfer component facilitating a number of reactions, including P450 monooxygenation [2], fatty acid desaturation [3] and fatty acid elongation [4]. In addition, cyt $b_5$r can independently reduce ferric-chelates such as $Fe^{3+}$-EDTA and $Fe^{3+}$-citrate [5, 6].

Cyt $b_5$ and cyt $b_5$r proteins from higher eukaryotes have been a subject of biochemical research due to their importance in drug metabolism [7]. Cyt $b_5$ reductases, apart from reducing the natural substrate cyt $b_5$, also reduce the synthetic substrate ferricyanide, which is typically used for enzyme assay measurements. However, cyt $b_5$r exhibits species-specific differences in the reduction of cyt *c*, a heme protein found in the mitochondria. Cyt $b_5$r from humans and certain plants has the ability to directly reduce cyt *c* [6, 8-10], whereas cyt $b_5$r from rats, sheep and calves requires cyt $b_5$ as an intermediate electron acceptor that in turn reduces cyt c [11-15]. Reduction of cyt c, either directly or indirectly (*via* cyt $b_5$) by cyt $b_5$r, has been utilized as a basis for measurement of cyt $b_5$r activity [6, 8-15]. Furthermore, reduction of cyt $b_5$ by cyt $b_5$r has been shown to occur through either a “ping-pong” [16, 17] or “sequence order” [5, 18, 19] type mechanism depending on the species. Despite characterization of the mammalian and plant cyt $b_5$ reductases, no information is available on fungal cyt $b_5$r in terms of cyt c and cyt $b_5$ specificity and enzyme kinetic mechanism. Initial studies on fungal cyt $b_5$r from the zygomycetes *Mortierella alpina* 1S-4 [20, 21] and *Mucor racemosus* PTCC 5305 [22] have been limited to recombinant expression and activity assessment against ferricyanide.

[1]Abbreviations used

| | |
|---|---|
| **cyt $b_5$ or $b_5$** | Cytochrome $b_5$ |
| **cyt $b_5$r or $b_5$r** | Cytochrome $b_5$ reductase |
| **Pc-cyt $b_5$r** | *Phanerochaete chrysosporium* cytochrome $b_5$ reductase |
| **Pc-cyt *b*5** | *Phanerochaete chrysosporium* cytochrome $b_5$ |
| **CYP or P450** | Cytochrome P450 |
| **POR** | P450 oxidoreductase |
| **cyt c** | Cytochrome c |
| **NADH** | Nicotinamide adenine dinucleotide |
| **NADPH** | Nicotinamide adenine dinucleotide phosphate |
| **AOX** | Alcohol oxidase |

The role of cyt $b_5$r-cyt $b_5$ in the P450 monooxygenation reaction has been characterized in mammals [23-25] and also in yeast [26]. These studies were based on either *in vitro* analysis of the reconstituted P450 reaction using purified cyt $b_5$r, cyt $b_5$ and P450 [24, 26] or *in vivo* analysis using a deletion mutant (POR) [25, 27, 28]. One study utilized a bacterial co-expression strategy to study this phenomenon [23]. However, the study lacked quantitative data on the expression levels of these proteins due to the difficulty of assessing membrane-bound mammalian proteins in a bacterial system [23]. Studies on a POR knock-out strain of the ascomycete *Fusarium fujikuro* showed a low level of NADH-dependent P450 monooxygenation activity, indicating that auxiliary mechanisms might exist to reduce P450 [28]. However, there is no direct evidence in filamentous fungi that the cyt $b_5$r-cyt $b_5$ complex can promote the P450 monooxygenation reaction and complement the POR system.

A recent study from our laboratory on the model white rot basidiomycete fungus *Phanerochaete chrysosporium* has led to the cloning, heterologous expression and initial characterization of its cyt $b_5$r and cyt $b_5$ proteins [29]. The current study was aimed at characterizing the substrate specificity and kinetic mechanism of the cyt $b_5$r enzyme and investigating whether the *P. chrysosporium* cyt $b_5$r-cyt $b_5$ system can replace the POR for supporting the redox activity during P450 monooxygenation. Considering that genetic manipulation in *P. chrysosporium* is difficult, the redox role of cyt $b_5$r-cyt $b_5$ in the P450 monooxygenation reaction was investigated using a quantitative *in vivo* co-expression strategy in *Pichia pastoris*. The results revealed that the fungal cyt $b_5$r-cyt $b_5$ system alone provides a POR-independent system for P450 monooxygenation reaction. The study also indirectly revealed the PAH-oxidizing capability of CYP63A2 in *P. chrysosporium*.

## 2. Materials and Methods

The microbial strains, plasmids and constructs used in this study are listed in Table 1.

### 2.1. Recombinant expression and purification of *P. chrysosporium* cyt $b_5$r and cyt $b_5$ proteins

Expression and purification of recombinant cyt $b_5$r and cyt $b_5$ proteins were carried out as described previously using *Escherichia coli* Rosetta Blue DE3 clones containing pET30a(+)-*cyt* $b_5$*r* and pET30a(+)-*cyt* $b_5$ plasmids [29]. Purified cyt $b_5$r and cyt $b_5$ proteins were stored at −80°C. Protein concentrations were estimated using the Bio-Rad *DC* Protein Assay Kit II (Cat. No. 500-0112) according to the manufacturer's protocol.

### 2.2. Enzyme assays

All spectroscopic measurements were performed using a Spectramax UV plus 384 spectrophotometer (Molecular Devices, CA, USA) at 25°C. P450 quantification based on CO-difference spectrum was done as described elsewhere [30, 31]. Quantitative analyses for POR, cyt $b_5$r and cyt $b_5$ activities were performed as described previously [29]. In the cyt c reduction assay for cyt $b_5$r, cyt c (50 μM) derived from equine heart (Sigma-Aldrich, Cat. No. C-2506) was used as a substrate. The activity of cyt $b_5$r with ferricyanide as a substrate was assayed as described elsewhere [16].

### 2.3. Kinetic properties of cyt $b_5$r

The steady-state kinetic properties of the recombinant Pc-cyt $b_5$r were determined using Pc-cyt $b_5$, cyt *c* and NADH as substrates using the concentration range 1-50 μM and the assay conditions described above. Kinetic constants for NADH utilization were determined using either of the cytochrome substrates (cyt $b_5$ or cyt *c*) and monitoring the decrease in absorbance at 340 nm. The enzyme concentration was adjusted such that the reaction was

linear for at least a 2 min time period. For each substrate, three independent experiments were carried out at each enzyme concentration. Kinetic parameters were calculated using the GraphPad Prism Software (GraphPad Software, Inc, CA, USA). One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 nmol of the substrate (cyt $b_5$ or cyt c) per min. Substrate saturation experiments were performed by varying the cyt $b_5$ concentration at five different fixed NADH concentrations (5, 10, 20, 30, and 40 μM) and by varying the NADH concentration at five different fixed cyt $b_5$ concentrations (7.5, 15, 25, 35, and 45 μM). Reaction velocities were expressed as nmol cyt $b_5$ reduced per min per mg of cyt $b_5$r, and the data were presented as Lineweaver-Burk plots.

### 2.4. *In vivo* co-expression of the *P. chrysosporium* redox enzyme systems and its P450 (CYP63A2) in the yeast *Pichia pastoris*

**2.4.1. Construction of *PC2-POR* (*PP*) binary construct—***PC*2 cDNA was amplified from our previously isolated clone [32] using gene-specific primers such that a *Not*I site was built in at both the N-terminal and C-terminal ends. The amplified *PC*2 cDNA was cloned into the TA cloning vector pCR2.1 and subsequently cloned into our previously generated POR-construct [33] using *Not*I. Proper orientation of the *PC*2 insert in the POR-construct was confirmed by sequencing, and the final construct *(POR-PC2)* was designated *PP* (Fig. 1A). A similar strategy was used to clone *PC2* into the Pichia vector pPICZB at the *Not*I site as described above, and a construct showing the right orientation of the cDNA insert (pPCIZB-*PC2*), designated as *PC2*, was used for comparison studies.

**2.4.2. Construction of $b_5r$-$b_5$-*PC2* (*BBP*) ternary construct—**The *cyt* $b_5r$ and *cyt* $b_5$ cDNAs were amplified from our previously isolated clones [29] using gene-specific primers such that an *Eco*RI site and a *Not*I site were built in at the N-terminal and C-terminal ends, respectively. The amplified *cyt* $b_5r$ and *cyt* $b_5$ cDNAs were cloned into the original (pPICZB) and restriction site-modified (M-pPICZB) vectors [33], respectively. The recombinant clones were named pPICZB-*Cyt* $b_5r$ and M-pPICZB-*Cyt* $b_5$. The M-pPICZB-*Cyt* $b_5$ construct was digested with *Bgl*II-*Bam*HI to excise the *cyt* $b_5$ cDNA, along with the promoter (AOX) and terminator (TT) sequences, and subcloned into the pPICZB-*Cyt* $b_5$ construct at the *Bam*HI site to generate pPICZB-*Cyt* $b_5r$-*Cyt* $b_5$. To construct the ternary plasmid, a *PC*2 cDNA fragment containing AOX and TT flanking sequences (liberated from the M-pPICZB-*PC*2 clone) was cloned into pPICZB-*Cyt* $b_5r$-*Cyt* $b_5$ at the *Bam*HI site and named *BBP* ($b_5r$-$b_5$-*PC*2) (Fig. 1B).

**2.4.3. Transformation and co-expression of the fungal P450 redox proteins and a homologous P450 monooxygenase (PC2) in *P. pastoris*—***Pme*I-linearized *PC2*-, binary (*PP*)-, and ternary (*BBP*)-constructs were transformed into *P. pastoris* using the *Pichia* transformation kit (Invitrogen). Genomic DNA PCR on the zeocin-resistant transformant colonies confirmed the presence of the individual gene inserts (*PC2*, *POR-PC*2 or $b_5r$-$b_5$-*PC*2) in the genome, expected as a result of integrative transformation. The colonies which showed positive amplification for all the inserted cassette genes were selected for further studies (Fig. S1). A negative control, designated vector-only control (C), was generated by transforming *P. pastoris* with the empty vector (pPICZB). Co-expression of the recombinant redox enzymes along with the homologous Pc-P450 protein, PC2, in *P. pastoris* was carried out as described in our recent study [33]. Briefly, 24 h after induction, *P. pastoris* cells were pelleted and resuspended in a lysis buffer (50 mM potassium phosphate buffer, pH 7.4, 20% glycerol, 1 mM PMSF, and protease inhibitor cocktail from Sigma; cat no. P-8465). The cells were disrupted by vortexing (10 cycles of alternate pulsing and cooling for 30 s each on ice) using an equal volume of acid-washed glass beads and the lysate was centrifuged at 5,000g for 10 min at 4°C. Supernatant (whole cell extract) was either, stored at −80°C for analyzing expression levels of cyt $b_5$, or further processed by

centrifugation at 20,000g for 30 min at 4°C to pellet the mitochondria. The supernatant was collected and re-centrifuged at 100,000g for 3h. The supernatant [soluble protein fraction (SPF)] was transferred into a clean tube and the pellet [microsomal protein fraction (MPF)] was resuspended in the lysis buffer. Both the protein fractions were aliquoted and stored at −80°C until used for analyzing the expression levels (cyt $b_5$, reductase, and P450) and for the enzyme assays [29, 33].

### 2.5. Benzo(a)pyrene oxidation activity of the recombinant *P. pastoris* clones

Benzo(a)pyrene oxygenation activities of the recombinant *P. pastoris* clones for the *PC2*, *PP* and *BBP* constructs were compared using the vector-only clone as a control (C) in yeast whole cell assays, as described in our recent study [33]. Briefly, a single colony from each recombinant clone (*PC2*, *PP* and *BBP*) and the negative control clone (C) were inoculated into buffered minimal glycerol (BMG) medium (100 ml) and the cultures incubated (30°C, 250 rpm) until the absorbance ($A_{600}$) reached 2.0. The cells were pelleted by centrifugation (5000g for 5 min), washed once with buffered minimal solution and resuspended in buffered minimal methanol (BMY) medium (21 ml) containing 0.5% methanol and aminolevulenic acid (2 mM). The cell suspension was subdivided equally (7 ml each) into three conical flasks. Each flask was spiked with benzo(a)pyrene dissolved in acetone at a 20 ppm final concentration. To estimate the initial level of benzo(a)pyrene and the degree of any abiotic degradation, a flask prepared using the same medium was left untreated and compared to the experimental flasks. All treatments were run in triplicate. The cultures were incubated (30°C and 250 rpm) for 24 h. After incubation, 5 ml aliquots of the cultures were removed aseptically and extracted (3X) with equal volumes of methylene chloride. The solvent extracts were dried on sodium sulfate and resuspended in acetonitrile. The organic extract samples were filtered through 0.45-μm glass fiber filters and analyzed using a Prostar 210/215 HPLC system (Varian, Inc.) equipped with $C_{18}$ reverse-phase column and UV detector. HPLC separation was achieved using a 20-min linear gradient of acetonitrile in water (60% to 100% acetonitrile) as the mobile phase at a flow rate of 2 ml/min. Benzo(a)pyrene was detected at 254 nm and quantified based on a standard curve generated using known concentrations of this compound. Simultaneously, after incubation, 1 ml of the culture was centrifuged and the pellet was used for dry biomass weight measurement as described elsewhere [34].

## 3. Results and Discussion

### 3.1. Substrate specificity of Pc-cyt $b_5$r

To characterize the substrate specificity of the purified recombinant Pc-cyt $b_5$r, we performed a cyt $b_5$r enzymatic assay utilizing cyt $b_5$, cyt c as substrates and in the presence of co-factors, NADH and NADPH. Pc-cyt $b_5$r successfully reduced both the purified cyt $b_5$ (Pc-cyt $b_5$) and cyt c independently. Pc-cyt $b_5$r showed strict specificity towards NADH as an electron donor as no activity was observed using NADPH. The specificity towards NADH matches that reported for cyt $b_5$r from other organisms [20, 35-37].

The Pc-cyt $b_5$r enzyme obeyed classical Michaelis-Menten kinetics for all tested substrates (Fig. 2A). The apparent steady-state kinetic parameters are shown in Table 2. The enzyme showed similar affinity for the three tested substrates (cyt $b_5$, cyt c, and NADH). It also showed comparable affinity for NADH in the presence of cyt $b_5$ versus cyt c. The affinity constant ($K_m$) values for cyt $b_5$ and NADH were comparable to those for higher eukaryotic cyt $b_5$r enzymes from sheep liver and calf liver [15, 38]. In a majority of the eukaryotes, cyt $b_5$r is only able to reduce cyt c in the presence of cyt $b_5$ [11-15]. Here we show that Pc-cyt $b_5$r can reduce cyt c in the absence of cyt *b*5, similar to the observation in humans and some plant sources [6, 8-10]. However, contrary to the cyt $b_5$r enzymes from humans, maize, and

other fungal organisms, Pc-cyt $b_5$r did not show any NADH-ferricyanide reduction activity, a typical property of cyt $b_5$ reductases. Taken together, our results indicated that Pc-cyt $b_5$r is somewhat unique among the eukaryotic cyt $b_5$r enzymes in that it can directly donate electrons to cyt c but lacks the ability to reduce ferricyanide.

### 3.2. Kinetic mechanism of Pc-cyt $b_5$r

Substrate saturation effect on the Pc-cyt $b_5$r activity was studied by varying the concentration of cyt $b_5$ at various fixed concentrations of NADH. A Lineweaver-Burk (LB) plot of the initial enzyme activities against cyt $b_5$ resulted in a series of parallel lines, as shown in Fig. 2B, consistent with a “ping-pong” type kinetic mechanism. The LB plot of the Pc-cyt $b_5$r activity for varying NADH concentrations at fixed cyt $b_5$ concentrations (Fig 2C) also showed a series of parallel patterns, suggesting a “ping-pong” kinetic mechanism for both cyt $b_5$ and NADH with Pc-cyt $b_5$r. This is consistent with other cyt $b_5$r enzymes from sheep lung and sipunculid worm (*Phascolopsis gouldii*) erythrocytes that show a similar “ping-pong” type kinetic mechanism, although these other eukaryotic cyt $b_5$r proteins can not directly reduce cyt c without cyt $b_5$ [16, 17]. Interestingly, maize cyt $b_5$r, which directly reduces cyt c, exhibits a “sequence order” kinetic mechanism [5]. Therefore, Pc-cyt $b_5$r is unique in that it shows an unusual combination of properties, the “ping-pong” type kinetic mechanism and direct reduction of cyt c.

### 3.3. *In vivo* co-expression of *P. chrysosporium* redox enzyme systems (POR and cyt $b_5$r-cyt $b_5$) and the homologous P450 CYP63A2 in *P. pastoris*

To assess the ability of the *P. chrysosporium* cyt $b_5$r-cyt $b_5$ redox system to substitute for the Pc-POR in a P450 monooxygenation reaction, we relied on the two constructs *PP* and *BBP* (Fig. 1) to achieve *in vivo* co-expression of either of the two fungal redox systems (POR or $b_5$r-$b_5$) along with the homologous Pc-P450 CYP63A2 in *P. pastoris*.

**3.3.1. Screening of the whole cell extracts—**In the co-expression cultures of *P. pastoris*, whole cell extracts from the $b_5$r-$b_5$-PC2 cells appeared red orange in color (Fig. 3A). However, no such color was observed for the cell extracts or supernatants from the control and POR-PC2 cells (Fig. 3A). The red orange appearance of $b_5$r-$b_5$-PC2 cell extracts may be due to the high levels of cyt $b_5$ expressed, as observed in our previous study on cyt $b_5$ expression in *E. coli* [29]. This is supported by SDS-PAGE, where an intense band at the expected MW of cyt $b_5$ is only present in the $b_5$r-$b_5$-PC2 cell extracts (Fig. 3B).

Interestingly, the total protein content in the cell extracts was 37.6% less in the $b_5$r-$b_5$-PC2 sample (111 mg) as compared to the vector-only control extract (178 mg). In contrast, the observed protein content in the POR-PC2 cell extracts (153 mg) showed only 14% loss and that in the PC2 extracts (171 mg) showed negligible loss compared to the control sample. This indicated that expression of the $b_5$r-$b_5$-PC2 triad is more deleterious to yeast cells as compared to POR-PC2 and that expression of the P450 protein alone is not deleterious to the cells. A similar trend was observed in terms of cell biomass yield during the benzo(a)pyrene oxidation experiment (Fig. 5B). The whole cell extracts were further separated into SPF and MPF. Interestingly, SPF and MPF from $b_5$r-$b_5$-PC2 cells showed pink color and dark yellow color, respectively. However, no such colors were observed for the fractions prepared from the C, PC2, and POR-PC2 cells.

#### 3.3.2. Quantification of P450, reductase and cyt $b_5$ content in cytosolic versus microsomal compartments in recombinant *P. pastoris*

**3.3.2.1. Soluble expression:** A pink color of the $b_5$r-$b_5$-PC2 SPF sample indicated the possible presence of the expressed cyt $b_5$. Sodium dithionite-reduced difference spectrum analysis on the SPF samples from C, PC2, POR-PC2 and $b_5$r-$b_5$-PC2 clones confirmed the

presence of cyt $b_5$ in the $b_5$r-$b_5$-PC2 SPF (Fig. 3C). Spectroscopic analysis showed 757 pmol cyt $b_5$ protein per mg of SPF protein (Table 3). The SPF samples from the different clone types showed no detectable levels of P450, POR and cyt $b_5$r proteins.

**3.3.2.2. Microsomal expression:** A typical P450 CO-difference spectrum was observed in the microsomal fractions prepared from the PC2, POR-PC2 and $b_5$r-$b_5$-PC2 cells as compared to the control microsomes derived from the C cells (Fig. 4A). This indicated that the cloned P450 PC2 was expressed in an active form. The Soret peaks for the PC2, POR-PC2, and $b_5$r-$b_5$-PC2 microsomes corresponded to 446 nm, 448 nm, and 452 nm, respectively (Fig. 4A). The POR-PC2 microsomal sample showed a higher P450 content compared to the PC2 and $b_5$r-$b_5$-PC2 samples (Table 3); the latter two samples showed a comparable P450 content.

High levels of reductase activity in the POR-PC2 and $b_5$r-$b_5$-PC2 microsomes compared to the baseline levels in C and PC2 microsomes confirmed the heterologous expression of the cloned fungal reductase proteins (Table 3). Direct spectral quantification of cyt $b_5$ is not possible in the presence of P450. But since the P450 levels were comparable (Table 3) in the PC2 and $b_5$r-$b_5$-PC2 samples, we subtracted the reductase content (contributed by the reduced P450) from PC2 from the $b_5$r-$b_5$-PC2 reductase content to eliminate the P450 contribution and determine the absolute cyt $b_5$ protein levels. Based on these calculations, the $b_5$r-$b_5$-PC2 sample showed a net 175 pmol of cyt $b_5$ per mg of microsomal proteins (Table 3).

Selection of a eukaryotic heterologous host (*P. pastoris*) for co-expression of the *P. chrysosporium* redox enzyme system proteins along with its P450 seemed to be advantageous because previous workers failed to proportionately co-express and precisely quantify these individual eukaryotic membrane-bound proteins in prokaryotic cells [29]. Moreover, eukaryotic expression of these proteins would closely mimic the native reaction conditions, thereby providing an ideal scenario for understanding their relative role in the overall monooxygenation reaction.

### 3.5. *In vivo* analysis of the role of redox enzyme systems in P450 catalysis—

The *P. chrysosporium* P450 CYP63A2, first cloned in our laboratory [32], was shown to effectively oxidize a number of polyaromatic hydrocarbon (PAH) compounds including benzo(a)pyrene. Hence we utilized CYP63A2 and its benzo(a)pyrene oxidation property to investigate the potential of each redox enzyme system to transfer electrons to a P450 monooxygenase. The catalytic activity toward benzo(a)pyrene was assessed using whole cell assays based on HPLC measurement of the remaining unoxidized benzo(a)pyrene in the cultures (Fig. 5). HPLC analysis of the organic extracts of the whole cultures showed an estimated one-third reduction of the benzo(a)pyrene peak in the POR-PC2 (35.6 ± 2.3%) and $b_5$r-$b_5$-PC2 (32.3 ±3.2 %) extracts compared to the PC2 sample (Fig. 5A) and formation of an oxidation metabolite. There was no change in benzo(a)pyrene level in the vector-only control cells compared to the untreated control, suggesting that the host yeast does not have the required P450s to oxidize benzo(a)pyrene. Lack of oxidation activity in PC2 cells further confirmed that the yeast native redox enzymes are not supporting the electron transfer to the expressed CYP63A2 (Fig. 5B). This is in contrast to a previous study that showed *P. pastoris* native redox partners were sufficient to support heterelogously expressed P450 [39]. These observations collectively suggest that the Pichia native redox partners show differential specificity in terms of their ability to transfer electrons to foreign P450s.

Comparable benzo(a)pyrene oxidation by the $b_5$r-$b_5$-PC2 cells and POR-PC2 cells (Figure 5), despite ~1.5 times higher P450 content in the latter (Table 3), suggested that the $b_5$r-$b_5$ complex can efficiently substitute POR for transfer of electrons to the P450 (PC2) during

benzo(a)pyrene oxidation. Comparing the expression data (Table 3) to benzo(a)pyrene oxidation data (Figure 5) shows that a higher reductase activity in $b_5$r-$b_5$-PC2 cells than in POR-PC2 cells might have contributed to the higher turnover of the P450 enzyme activity for benzo(a)pyrene oxidation in the former. This is the first convincing demonstration in fungi that $b_5$r-*b*5 is capable of transferring electrons to a P450. This indicates that the $b_5$r-$b_5$ protein pair can act as an effective redox enzyme system in P450 oxidation reactions in *P. chrysosporium*.

This observation that cyt $b_5$r-$b_5$ is capable of transferring electrons to P450 is significant considering the electron needs of the extraordinarily large P450ome (~150 P450s) in *P. chrysosporium* and opens up avenues for manipulation of the native organism for improved biocatalysis and biotechnological applications.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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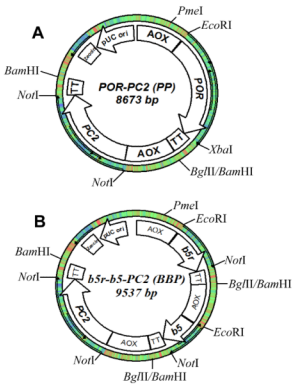


**Figure 1.**

Schematic illustration of the binary (A) and ternary (B) constructs for co-expression of the *P. chrysosporium* redox enzyme system proteins (POR and cyt $b_5$r-cyt $b_5$) along with a homologous P450 (PC2) in *Pichia pastoris*. Restriction sites used for the cloning of cDNAs and for linearization of the constructs (*Pme*I) are shown.

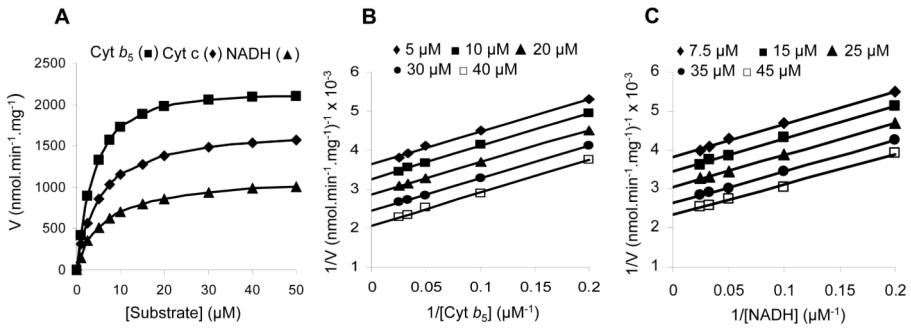


**Figure 2.**

Kinetic properties of *P. chrysosporium* cyt $b_5$ reductase (Pc-cyt $b_5$r). (A) Substrate saturation curves of Pc-cyt $b_5$r for cyt $b_5$, cyt c, and NADH substrates. Since the affinity of the Pc-cyt $b_5$r enzyme for NADH was comparable for the two cytochrome substrates (cyt $b_5$ and cyt c), only one saturation curve is presented. The data points represent means of the triplicates. (B&C) Double reciprocal plots of the initial velocity for the reduction of Pc-cyt $b_5$ by the purified Pc-cyt $b_5$r as a function of Pc-cyt $b_5$ at various fixed concentrations of NADH (B) and as a function of NADH at various fixed concentrations of Pc-cyt $b_5$ (C).

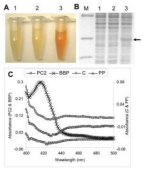

**Figure 3.**

Heterologous co-expression of the *P. chrysosporium* redox enzyme system proteins (POR, cyt $b_5$r-$b_5$) and a homologous Pc-P450 (PC2) in *Pichia pastoris*. (A) Visual color appearance of the yeast whole cell extracts. (B) SDS-PAGE analysis of the cell extracts. Lane marked M represents protein molecular weight marker. Lanes 1 to 3 in panels A and B represent the extracts for vector-only control (C), POR-PC2 (PP) and $b_5$r-$b_5$-PC2 (BBP) cells, respectively. Cyt $b_5$ band corresponding to the 30 kDa size marker is indicated by an arrow. (C) Sodium dithionite-reduced difference spectrum of the soluble protein fraction (SPF) prepared from the C, PC2, PP (POR-PC2) and BBP ($b_5$r-$b_5$-PC2) cells.

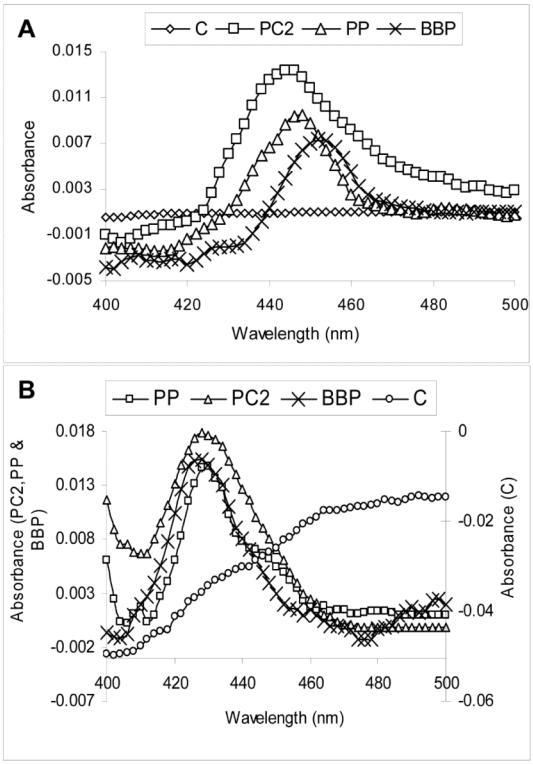


**Figure 4.**

Spectral analysis of the expression levels of P450 protein and cyt $b_5$ protein in the microsomal protein fraction (MPF) from different yeast clones, viz. control (C), PC2, PP (POR-PC2) and BBP ($b_5$r-$b_5$-PC2). (A) Characteristic P450 CO-difference spectra to estimate the total P450 content. (B) Sodium dithionite-reduced difference spectrum to estimate the cyt $b_5$ content.

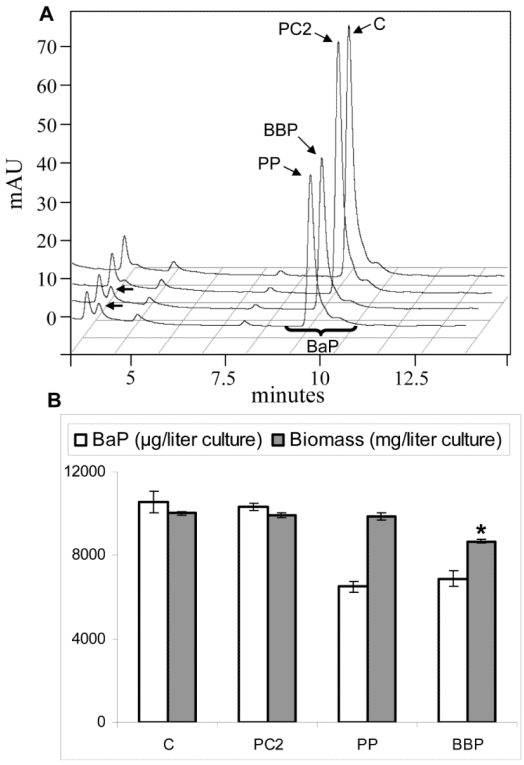


**Figure 5.**

Benzo(a)pyrene oxidation activity and dry biomass yield in yeast whole cell assays. (A) HPLC chromatograms of the organic extracts from the control (C), PC2, PP (POR-PC2) and BBP ($b_5$r-$b_5$-PC2) cells after 24 h of induction. The major peak represents the parent compound benzo(a)pyrene whereas the minor peak shown with an arrow indicates a metabolite. (B) Benzo(a)pyrene oxidation activity of the recombinant *P. pastoris* cultures (PC2, PP, and BBP) represented by the remaining benzo(a) pyrene levels as compared to the vector-only control (C) culture. The values represent means ± standard deviations for three biological replicates. The difference between the dry biomass values for the PP and BBP cells was statistically significant ($P \leq 0.05$) as indicated by an asterisk.

## Table 1

Strains and plasmids used in this study.

| Strain/Plasmid | Genotype/Phenotype | Reference or source |
|---|---|---|
| **Strain name** | | |
| *E. coli* TOP 10 | F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL($Str^R$) endA1 $\lambda^-$ | Invitrogen |
| *E. coli* Rosetta Blue DE3 | endA1 hsdR17 ($r_{K12}^-K_{12}^+$) supE44 thi-1 recA1 gyrA96 relA1 lac (DE3) F'[$proA^+B^+$ $lacI^qZ\Delta M15$::Tn10] pLacIRARE ($Cam^R$, $Tet^R$) | EMD biosciences |
| *Pichia pastoris* KM71H | $Mut^s$, $Arg^+$ | Invitrogen |
| **Plasmid ID** | | |
| pPICZB | Yeast expression vector (*Pichia pastoris*), $Zeocin^r$ | Invitrogen |
| pCR2.1-*PC2* | *P. chrysosproium* P450 PC2 cDNA cloned in pCR2.1 vector | 32 |
| pET30a(+)-*Cyt* $b_5r$ | *Cyt* $b_5r$ cDNA cloned in *E. coli* pET30a(+) vector | 29 |
| pET30a(+)-*Cyt* $b_5$ | *Cyt* $b_5$ cDNA cloned in *E. coli* pET30a(+) vector | 29 |
| M-pPICZB | pPICZB vector mutated at *Pme*I site | 33 |
| POR construct | *Bgl*II-*Bam*HI digested M-pPICZB fragment containing *AOX1* promoter-MCS-terminator region cloned in pPICZB-*MPor* at *Bam*HI site in Head-to-tail orientation | 33 |
| pPICZB-*Cyt* $b_5r$ | *Cyt* $b_5r$ cDNA cloned in pPICZB vector at *Eco*RI-*Not*I site | This work |
| M-pPICZB-*Cyt* $b_5$ | *Cyt* $b_5$ cDNA cloned in M-pPICZB vector at *Eco*RI-*Not*I site | This work |
| pPICZB-*Cyt* $b_5r$-*Cyt* $b_5$ | *Cyt* $b_5$ cDNA cloned in pPICZB-*Cyt* $b_5r$ construct at *Bam*HI site | This work |
| pPICZB-*CYP63A2* (*PC2*) | *CYP63A2* cloned in pPICZB at *Not*I site | This work |
| *PP* | Binary construct (pPICZB-*POR-PC2*) | This work |
| *BBP* | Ternary construct (pPICZB-*Cyt* $b_5r$-*Cyt* $b_5$-*PC2*) | This work |

## Table 2

Kinetic properties of *P. chrysosporium* cytochrome $b_5$ reductase (Pc-cyt $b_5$r).

| Substrate | $K_m$ (μM) | $V_{max}$ (nmol.min$^{-1}$.mg$^{-1}$ of cyt $b_5$r) | $K_{cat}$ ($s^{-1}$) |
|---|---|---|---|
| Cyt $b_5$ | 4 ± 0.2 | 2318 ± 32 | 381 ± 5.2 |
| NADH [a] | 6 ± 0.1 | 1125 ± 9 | 185 ± 1.4 |
| Cyt c | 5 ± 0.1 | 1727 ± 10 | 284 ± 1.7 |

[a], For NADH, almost similar kinetic constant values were observed in the presence of cyt $b_5$ versus cyt c and hence only one value (corresponding to cyt $b_5$) is presented here

**Table 3**

Co-expression analysis of the fungal redox proteins and P450 in *Pichia pastoris*.

| Sample Name | P450 content (pmol.mg$^{-1}$ of microsomal protein) | Reductase activity[a] (nmol.min$^{-1}$.mg$^{-1}$ of microsomal protein) | | Cytochrome $b_5$ content (pmol.mg$^{-1}$ protein) | |
|---|---|---|---|---|---|
| | | NADPH | NADH | Soluble protein Fraction | Microsomal protein fraction |
| C | ND | 189 ± 21 | 87 ± 5.5 | ND | ND |
| PC2 | 41 | 230 ± 5 | 79 ± 4 | ND | ND |
| PP | 64 | 1616 ± 3.5 | NA | ND | ND |
| BBP | 43 | NA | 1957 ± 7 | 757 | 175 |

[a], Reductase activity represents either POR or cyt $b_5$r activity. The POR activity was measured at 550 nm wavelength using cyt c as the electron acceptor and NADPH as the electron donor. The cyt $b_5$ reductase activity was measured at 424 nm wavelength using NADH as the electron donor and purified Pc-cyt $b_5$ as an electron acceptor. Enzyme assays were performed in triplicate; means and standard deviation were calculated. Abbreviations: ND, not detected; NA, not applicable.