Co-expression of active human cytochrome P450 1A2 and cytochrome P450 reductase on the cell surface of *Escherichia coli*

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**Abstract**

**Background:** Human cytochrome P450 (CYP) enzymes mediate the first step in the breakdown of most drugs and are strongly involved in drug–drug interactions, drug clearance and activation of prodrugs. Their biocatalytic behavior is a key parameter during drug development which requires preparative synthesis of CYP related drug metabolites. However, recombinant expression of CYP enzymes is a challenging bottleneck for drug metabolite biosynthesis. Therefore, we developed a novel approach by displaying human cytochrome P450 1A2 (CYP1A2) and cytochrome P450 reductase (CPR) on the surface of *Escherichia coli*.

**Results:** To present human CYP1A2 and CPR on the surface, we employed autodisplay. Both enzymes were displayed on the surface which was demonstrated by protease and antibody accessibility tests. CPR activity was first confirmed with the protein substrate cytochrome c. Cells co-expressing CYP1A2 and CPR were capable of catalyzing the conversion of the known CYP1A2 substrates 7-ethoxyresorufin, phenacetin and the artificial substrate luciferin-MultiCYP, which would not have been possible without interaction of both enzymes. Biocatalytic activity was strongly influenced by the composition of the growth medium. Addition of 5-aminolevulinic acid was necessary to obtain a fully active whole cell biocatalyst and was superior to the addition of heme.

**Conclusion:** We demonstrated that CYP1A2 and CPR can be co-expressed catalytically active on the cell surface of *E. coli*. It is a promising step towards pharmaceutical applications such as the synthesis of drug metabolites.

**Keywords:** Surface display, Autotransporter, Autodisplay, Cytochrome P450 1A2, Cytochrome P450 reductase, Whole cell biocatalysis

**Background**

Microsomal cytochrome P450 monooxygenases (CYPs, P450s) are the major enzymes in human drug metabolism as they are involved in the breakdown of almost all marketed drugs [1]. As part of the phase-I-metabolism these heme-containing proteins catalyze a huge variety of oxidation reactions accepting a broad range of endogenous and xenobiotic substrates [2]. They hereby convert lipophilic into more reactive and hydrophilic metabolites as first step for their elimination from the body. Of all 57 human CYPs known, five (CYP3A4, 2C9, 2C19, 2D6 and 1A2) catalyze the vast majority of drug oxidations. For these class II type CYPs the required electrons are supplied from the cytochrome P450 reductase (CPR, also referred to as CYPOR) which transfers them from nicotinamide adenine dinucleotide phosphate (NADPH) through its cofactors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) to the heme of the monooxygenase [3]. CYP enzymes are an essential determinant for the half-life and safety of a drug and its metabolic products. Consequently, they represent a key factor in drug discovery and development. For the complete pharmacological characterization of a drug candidate, its metabolites must be produced on a preparative scale [4, 5]. There is a natural interest in exploiting recombinant...
CYPs for the synthesis of drug metabolites, in particular since classical chemical synthesis routes are often very challenging and costly [6]. However, as membrane-bound proteins, their heterologous expression and purification is challenging, laborious and often requires membrane preparations to yield active enzymes [7, 8]. For large scale applications, the usage of purified CYPs suffers from low catalytic activities, purification costs and poor enzyme stability.

Many of these challenges can be solved by the application of a bacterial whole cell catalyst with surface displayed enzymes. The approach is illustrated in Fig. 1. Anchorage of the enzyme in the outer membrane of *Escherichia coli* provides a membrane environment and circumvents mass transfer limitations due to the membrane barrier. Further major advantages are the cheap and easy cultivation, feasibility of large-scale applications and reusability of the biocatalyst. Additionally, common expression hosts like *E. coli* have no own CYP background. As a biotechnological tool for surface display of recombinant proteins so-called autotransporters have been widely employed [9]. They are derived from natural outer membrane proteins in gram-negative bacteria and their translocation mechanism and structure have been intensively studied [10–14]. The technique has been successfully applied for the display of a variety of enzymes such as nitrilase [15], lipase and foldase [16], protein kinase CK2 [17] as well as other proteins like VHH antibody fragments [18], affibodies [19] and peptides [20]. In this study, we employed the two *E. coli* autotransporters AIDA-I [21] and EhaA [22, 23]. For surface display, the protein of interest (“passenger”) is combined with an N-terminal signal peptide and the C-terminal β-domain (also referred as autotransporter unit) of the autotransporter which consists of the β₁ (“autochaperone”) domain, α-helix and β-barrel domain [12, 22]. After translation the protein is transported through the Sec-pathway across the inner membrane [14]. The signal peptide is cleaved off and the protein kept in an unfolded confirmation by periplasmic chaperones such as Skp and SurA. The β-barrel is then inserted into the outer membrane with assistance of the Omp85/Bam complex while the passenger is translocated to the extracellular space.

Human CYP1A2 has a molecular weight of 58 kDa including a 29 amino acid long N-terminal transmembrane domain and contains heme b in its catalytic center [24]. Known substrates like phenacetin, paracetamol, caffeine and imipramine are mostly planar polyaromatic amides and amines. CYP1A2 catalyzes about 9% of CYP related drug metabolism [1]. The redox partner protein, the 77 kDa sized human CPR, is composed of a 55 amino acid N-terminal transmembrane domain, a FMN and a FAD/NADPH binding domain which are connected through a flexible hinge region [25, 26]. The CPR undergoes conformational changes between an open and

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**Fig. 1** Illustration of the biocatalysis by CYP1A2 and CPR on the cell surface. Two electrons are shuttled by the outer membrane (OM) anchored CPR from NADPH via the cofactors FAD and FMN in single-electron steps to the heme group of surface displayed CYP1A2. The monooxygenase catalyzes the oxidative breakdown of a substrate by inserting one oxygen atom into the chemical compound while reducing the other one to water. IM inner membrane.
closed form during its redox-cycle, but only the open form can transfer electrons to all microsomal CYPs. CPR is also able to supply electrons to other redox partners such as heme oxygenase and squalene monoxygenase.

Previously, it has been shown that human CYP3A4 can be displayed in an active form on the surface of *E. coli* using the AIDA-I autotransporter [27]. The obtained whole cell biocatalyst was able to convert testosterone into 6β-hydroxytestosterone with externally added CPR and cytochrome b5. Furthermore, soluble bacterial CYP enzymes such as BM3 [28] and CYP106A2 [29] have been expressed on the surface of bacteria and used for biocatalytic studies. Rat CPR alone has been surface displayed on *E. coli* using ice-nucleation protein from *Pseudomonas syringae* and was active towards cytochrome c [30]. Displayed on *Bacillus subtilis* spores rat CPR was able to transfer electrons to externally added CYP1A2 which was shown by 7-ethoxyresorufin- O-deethylation [31]. Belonging to the class I P450 system, mitochondrial bovine adrenodoxin has been brought to the surface and was active with its externally added redox partners [32] and in electrochemical analyses [33] when the iron-sulfur protein was reconstituted with supplemented [2Fe–2S] clusters. Belonging to the class I P450 system, mitochondrial bovine adrenodoxin has been brought to the surface and was active with its externally added redox partners [32] and in electrochemical analyses [33] when the iron-sulfur protein was reconstituted with supplemented [2Fe–2S] clusters. Belonging to the class I P450 system, mitochondrial bovine adrenodoxin has been brought to the surface and was active with its externally added redox partners [32] and in electrochemical analyses [33] when the iron-sulfur protein was reconstituted with supplemented [2Fe–2S] clusters. In this study, we report on the first successful co-expression of CYP1A2 and CPR on the surface of *E. coli*. We present experimental evidence that this whole cell biocatalyst is functionally active and able to catalyze three typical CYP1A2 oxidation reactions.

**Results**

**Vector design**

Two compatible expression vectors were constructed comprising the human CPR and CYP1A2 coding sequences in combination with the necessary autotransporter elements under control of a rhamnose inducible promoter. The promoter is tightly regulated and expression level is well titratable [34]. The obtained fusion protein consists of an N-terminal CtxB signal peptide for translocation through the Sec pathway, the CPR or CYP1A2 passenger without the transmembrane domains (amino acids 1–56 and 1–29, respectively) and the C-terminal autotransporter unit for outer membrane anchorage (Fig. 2). The CPR was combined with the EhaA and CYP1A2 with the AIDA-I autotransporter unit. For clarity, we address these fusion proteins as CPR and CYP1A2, respectively. Protein expression was always conducted with 1 mmol L\(^{-1}\) rhamnose in the OmpT negative *E. coli* strain BL21(DE3).

**Evaluation of surface display by protease accessibility test**

To examine surface expression of the two autotransporter fusion proteins, an outer membrane protein isolation (OMPI) was performed and analyzed by SDS-PAGE. Additionally, the same portion of the cells was treated 1 h at 37 °C with proteinase K prior to the OMPI procedure to investigate the protease accessibility of the passengers. Protease accessibility is a simple experiment to test the surface display of passengers as the protease is regarded to be too large to pass the outer membrane and digests only proteins exposed on the cell surface. The results are shown in Fig. 3. Samples of cells expressing either the CYP1A2 or CPR fusion protein contained a distinct protein band around the expected apparent molecular weight of 103 and 127 kDa, respectively. No comparable band was found in the control sample. In the OMPI sample of cells co-expressing the CPR and CYP1A2 fusion proteins both protein bands were also detectable. CYP1A2 is moderately higher expressed likely due to its smaller size and the higher plasmid copy number of the pBR322 origin of replication (ORI) in comparison to the P15A ORI (15–20 to ~10 copies) used for CPR expression. This would suggest a CYP1A2:CPR ratio above 1, if all enzymes were active. Both bands were substantially reduced when cells were treated with proteinase K prior to OMPI. This demonstrates a clear protease accessibility of the passengers which is a strong indication for surface exposure of the passengers. The native outer membrane protein OmpA functions as an internal control for excessive protease treatment. If proteinase K had entered the periplasm, the periplasmic domain of OmpA would have been cleaved off. This can be detected by disappearance of the OmpA protein band, which, however, was unchanged in our experiments. In conclusion, the degradation of autotransporter fusion proteins and the intactness of OmpA suggest a successful surface display of CYP1A2 and CPR.

**Evaluation of surface display by flow cytometry and immunofluorescence microscopy**

Flow cytometry is a common technique to investigate surface display of a protein of interest. An antibody does not penetrate the outer membrane due to its size and only binds to proteins exposed on the cell surface. Only for this assay the myc-tag had been genetically introduced in between the passenger and the autotransporter unit (Fig. 2). Cells were treated with a myc-tag specific primary antibody and a Dylight488 conjugated secondary antibody and fluorescence intensities of the cells analyzed subsequently via flow cytometry. Fluorescence intensities of cells expressing the CYP1A2 (pPQ62) or CPR (pPQ61) (Fig. 3b) were significantly higher in comparison to the fluorescence intensity of host cells without plasmid indicating the surface exposure of both proteins. To confirm these results immunofluorescence microscopy analysis of these cells were performed. For this purpose, mere host cells, cells expressing CPR with...
myc-tag (pPQ61, Fig. 2) and cells expressing CYP1A2 with myc-tag (pPQ62, Fig. 2) were treated with a mouse anti-myc antibody and a secondary Dylight488 conjugated anti-IgG antibody and subsequently subjected to confocal laser scanning microscopy. Whereas mere host cells did not exhibit immunofluorescence after this treatment, CPR and CYP1A2 expressing cells clearly showed such fluorescence (Additional file 1: Figure S1). When the fluorescence exhibiting cells were treated with proteinase K before the antibodies were added, immunofluorescence almost completely disappeared (S1). This strongly supported the results obtained by flow cytometry and indicated that CPR and CYP1A2 were indeed surface-expressed. Introduction of the myc-tag did not change
the level of protein expression as assessed by OMPI. Introduction of the myc-tag did also not influence interaction of CPR and CYP1A2. For simplification, however, all following experiments were performed with the passengers without myc-tag.

**Cytochrome c activity**

First, we investigated the activity of single expressed CPR. The reduction of the 11.8 kDa substrate protein cytochrome c is a standard assay to investigate the enzymatic activity of a CPR [35]. Following NADPH oxidation the CPR transfers one electron to cytochrome c which can be detected by an absorbance increase at 550 nm. The assay was conducted with washed cells (OD$_{578}$ nm 0.25), 100 µmol L$^{-1}$ NADPH and 50 µmol L$^{-1}$ cytochrome c. The results are shown in Fig. 4. Cells expressing CPR exhibit an activity of about 0.32 µmol L$^{-1}$ min$^{-1}$ whereas neither the host cells without plasmid nor cells expressing CYP1A2 on the surface without CPR showed any significant activity in comparison to the NADPH control without cells. This demonstrates that surface displayed CPR binds its cofactors FAD and FMN and hence is capable to shuttle electrons from NADPH to cytochrome c. Moreover, cytochrome c is not membrane permeable. Thus, these results support that the CPR was displayed on the cell surface.

**7-Ethoxyresorufin-O-deethylation activity**

CYP1A2 catalyzes the conversion of 7-ethoxyresorufin to the highly fluorescent product resorufin, if electrons are supplied by the redox partner protein CPR. We first determined resorufin by HPLC analysis to ensure detection of the product in the assay at low concentrations. Cells were cultivated as described in a sodium/potassium phosphate buffered LB-medium for 19.5 h at 23 °C. Assays were conducted with washed cells (OD$_{578}$ nm 0.40), 1 µmol L$^{-1}$ 7-ethoxyresorufin and a glucose-6-phosphate dehydrogenase NADPH regeneration system in 0.1 mol L$^{-1}$ potassium phosphate (pH 7.4) for 40 h. An exemplary chromatogram is depicted in Fig. 5a with resorufin appearing at 7.4 min. From the areas under the curve resorufin concentrations were calculated and are shown in Fig. 5b. For host cells without surface displayed enzymes and cells with surface displayed CPR alone resorufin concentration was below 2 nmol L$^{-1}$ at all conditions examined. For cells with co-expression of CYP1A2 and CPR, significant amounts of resorufin were detectable, with 124 nmol L$^{-1}$ resorufin in case of an expression at 23 °C. This clearly indicates that surface displayed CYP1A2 is catalytic active when it is co-expressed with surface displayed CPR. An almost tenfold decrease in activity was observed at an expression temperature of 30 °C (14 nmol L$^{-1}$ resorufin), indicating that enzymatic activity depended strongly on the temperature during expression. A lower expression temperature was favorable which has been reported for CYP enzymes expressed in bacteria in several studies before and appeared to contribute to better folding [7]. The protein expression level of CYP1A2 was obviously increased at 30 °C in comparison to 23 °C as shown by the OMPI samples (Additional file 2: Figure S2 A), demonstrating that the lower activity as observed is not caused by a lower expression rate, but rather due to an unfavorable protein folding. In contrast, expression of CPR was unchanged in comparison to expression at 23 °C. Both passengers were completely accessible by proteinase K, indicating that these proteins were displayed at the cell surface. To our surprise, cells expressing CYP1A2 alone were also active but on a low level from 9 nmol L$^{-1}$ (when expressed at 30 °C) to 19 nmol L$^{-1}$ (at 23 °C).

**Effect of growth medium ingredients on activity**

It has been reported for purified CYP enzymes that the concentrations of mono- and divalent cationic ions and the ionic strength can induce conformational changes in the protein structure, affect membrane insertion and influence the interaction between partner proteins with a significant impact on activity [36–41]. In case of our novel biocatalyst, folding takes place outside the cell on the outer membrane and could be influenced by the surrounding medium. In consequence, the salt composition of the growth medium would have an effect on activity. To investigate this effect, potassium and sodium phosphate buffered LB-medium as well as several buffered LB medium variants with different NaCl concentrations were tested and—as a read out—the resorufin production
of cells grown in these different media was determined. Standard LB-medium is composed of 10 g L\(^{-1}\) NaCl, 5 g L\(^{-1}\) yeast extract and 10 g L\(^{-1}\) peptone. We determined the reaction product resorufin in the corresponding sample supernatants using a microplate reader and set the results into relation to the components added to LB (Fig. 6). As shown in Fig. 6a, there was a severe effect of the buffer used on resorufin production. Cells cultivated in LB medium buffered with 50 mmol L\(^{-1}\) sodium phosphate buffer were fourfold less active (44 nmol L\(^{-1}\) resorufin) than the equivalent cells grown with potassium phosphate buffer (182 nmol L\(^{-1}\) resorufin), and still 2.5 fold less active than the equivalent with a mixed sodium/potassium phosphate buffer (112 nmol L\(^{-1}\) resorufin). In this case, increasing the Na\(^+\) concentration appeared to have a negative effect. However, as shown in Fig. 6b, varying the NaCl content of potassium phosphate buffered LB medium resulted in the highest product titer with 5 g L\(^{-1}\) NaCl. Less than 5 g L\(^{-1}\) NaCl led to a significant reduction in resorufin production. These results suggest

![Graph of fluorescence over time with different conditions](image)

**Fig. 5** Conversion of known CYP1A2 substrates. a HPLC chromatogram of samples from the 7-ethoxyresorufin-O-deethylation assay. Samples were diluted 1:1 with methanol prior to HPLC analysis. (1) Resorufin standard (10 nmol L\(^{-1}\)), (2) co-expression sample without substrate in the assay, (3) host cells, (4) cells displaying CPR, (5) cells displaying CYP1A2, (6) cells expressing CYP1A2 and CPR. b Resorufin formation in the whole assay determined by HPLC following expression at 23 or 30 °C. c HPLC chromatogram of samples of the phenacetin-O-deethylation assay. (1) Paracetamol standard (10 µmol L\(^{-1}\)) with 8.0 min retention time, (2) 3-acetamidophenol internal standard (10 µmol L\(^{-1}\)) with 10.5 min retention time, (3) control sample with co-expression cells without substrate, (4) host cells, (5) cells displaying CPR, (6) cells displaying CYP1A2, (7) cells expressing CYP1A2 and CPR. d MultiCYP activity measured by luciferin formation. Blank: Control sample without cells.
that the NaCl concentration is a critical parameter for defining an optimal growth medium. In our experiments potassium buffered LB medium containing 5 g L$^{-1}$ NaCl turned out to be the growth medium yielding the highest enzymatic activity of the whole cell biocatalyst towards resorufin.

The effects of 1 mmol L$^{-1}$ CaCl$_2$ and/or MgCl$_2$ as medium additives were also studied. The results are shown in Fig. 6c. Addition of MgCl$_2$ to LB medium had no effect on product titer and thus CYP1A2 activity. In contrast, supplementation of the growth medium with CaCl$_2$ led to an increase in resorufin production by approximately 30 %. None of the tested conditions altered the level of protein expression as analyzed by OMPI (Additional file 2: Figure S2 B). In summary, our results indicate that the activity of surface displayed CYP enzyme is strongly influenced by the concentrations of Na$^+$, and Ca$^{2+}$, as well as the buffer used for the growth medium. One possible reason could be that these alterations influence protein folding or membrane insertion as it has been reported before for purified CYPs [41, 56]. Similar observations have not been reported yet for any other recombinant surface displayed enzyme.

Secondly, we were interested how heme b needs to be provided for a fully functional CYP1A2. The outer membrane is considered to be an impermeable barrier for heme. Although LB medium contains about 5 µmol L$^{-1}$ heme [42], E. coli BL21(DE3) is also incapable of actively taking it up due to a lack of heme uptake transporters in the outer membrane [43–45]. It was previously found that surface displayed CYP3A4 and CYP106A2 were active without external addition of heme [27, 29]. Hence, we investigated if supplementation of the precursor 5-aminolevulinic acid (5-ALA) affects the enzymatic activity of the whole cell biocatalyst and if it is a limiting factor. Heme is synthesized in the cytoplasm from eight 5-ALA molecules and can be translocated to the periplasm (e.g. for periplasmic cytochrome c maturation [46]) and to the cell exterior through the TolC channel [47, 48]. For this experiment, potassium phosphate buffered LB medium (as control) or potassium phosphate buffered LB either supplemented with 62.5 µmol L$^{-1}$ hemin chloride, or with 500 µmol L$^{-1}$ 5-ALA or with the standard supplementation of 500 µmol L$^{-1}$ 5-ALA and 4 µmol L$^{-1}$ hemin chloride was used. Hemin chloride is the corresponding salt of heme b. As shown in Fig. 6d, resorufin was detectable using co-expression cells grown in LB medium without supplementation. Addition of hemin led to a 25 % increase in activity in comparison to non-supplemented LB medium. In contrast, addition of the equivalent amount of 5-ALA increased the activity by 400 % to 169 nmol L$^{-1}$ resorufin. Thus, CYP1A2 activity was effectively enhanced through the route of endogenous biosynthesis of heme but hardly through addition of external heme. Although there is little knowledge about the cellular mechanism of cofactor insertion into surface displayed enzymes, the results of this approach clearly indicated that 5-ALA is a key medium component and should be considered in case of future bioprocess optimization.

MultiCYP activity
To test a second common substrate, we applied the so-called MultiCYP assay (Promega). The measurement of activity relies on the O-demethylation of the MultiCYP
substrate to a proluminescent d-luciferin ester, which can then be detected by luminescence after the addition of an esterase and a luciferase containing detection reagent (LDR). Washed cells expressing surface displayed CPR, CYP1A2 or both were suspended in the MultiCYP substrate. After 24 h of incubation the formation of D-luciferinester was determined (Fig. 5d). For this purpose the resulting luminescence was compared to the luminescence values of a d-luciferin calibration curve (not shown). Cells with surface displayed CYP1A2 and CPR produced a significant higher luminescence signal in comparison to cells with only CYP1A2 or CPR, and also in comparison to cells with no plasmid, or control samples without any cells. These results underline that CYP1A2 and CPR were functionally co-expressed and it indicates that the MultiCYP assay represents a viable method for measuring whole cell activity with surface displayed CYPs.

Phenacetin-O-deethylation activity

The oxidation of phenacetin to the analgesic drug paracetamol is a common first pass reaction and can be used to monitor CYP1A2 activity. To examine the reaction, a whole cell assay was performed with 100 µmol L\(^{-1}\) phenacetin as substrate. After the reaction was stopped, samples were spiked with 10 µmol L\(^{-1}\) 3-acetamidophenol as internal standard, subsequently extracted with diethylether and analyzed by HPLC. The chromatogram is shown in Fig. 5c. Only the sample of cells co-expressing CYP1A2 and CPR showed a peak at the expected retention time for paracetamol (8 min). Paracetamol concentration was calculated from the calibration curve to be 1.2 µmol L\(^{-1}\). No peak was observed at this retention time in any control. It could be a hint that the substrate spectrum of the whole cell biocatalyst was not altered in comparison to soluble CYP1A2, as at least three known substrates of the free enzyme were converted by the whole cell biocatalyst co-displaying CYP1A2 and CPR.

Time dependent 7-ethoxyresorufin-O-deethylation by the whole cell biocatalyst

Finally, the formation of resorufin was analyzed over a time course of 40 h (Fig. 7). For this purpose the assay was conducted in 100 mL shake flasks containing 8 mL starting volume with 1 µmol L\(^{-1}\) of substrate under vigorous shaking at 200 rpm at 37 °C. Cells had been cultivated in potassium phosphate buffered LB-medium supplemented with heme, 5-ALA and CaCl\(_2\). At the different time points, resorufin concentration was determined in the supernatant of sample aliquots after depleting the cells. The whole cell biocatalyst turned out to be active for about 20 h. The final product concentration was 320 nmol L\(^{-1}\) and as such moderately higher than the product concentration obtained in the 15 mL tube-based assay as shown in Fig. 6b. This could have been due to a better oxygen supply in the 100 mL shake flasks. When cells were harvested after 40 h, washed, adjusted to the correct OD and reused in a completely fresh assay, they did not show any enzymatic activity at all.

Discussion

In our proof of concept study, we report the first successful co-expression of active human CYP1A2 and CPR on the surface of \textit{E. coli} using autodisplay. We found that the cells co-expressing CYP1A2 and CPR on the surface were able to catalyze the conversion of three structurally different substrates (7-ethoxyresorufin, MultiCYP and phenacetin). However, the observed activities were quite low. In our case, the biocatalytic activity would be equivalent to an average of 175 active CYP1A2 enzymes per cell for the phenacetin-O-deethylation. This calculation is based on several assumptions. First it was assumed the measured end-point concentration at 20 h corresponded to the initial maximal velocity of the reaction, and second, that the turnover number was unchanged during this course. For purified human wild type CYP1A2, a \(k_{cat}\) value of 1 min\(^{-1}\) with a \(K_M\) of 10 µmol L\(^{-1}\) for the phenacetin-O-deethylation has been reported [49]. The number of cells in our assay was calculated to be \(3.44 \times 10^9\) cells mL\(^{-1}\) based on the previously published number of \(0.86 \times 10^9\) cells per OD and mL [17]. Hence, the turnover per cell would equal \(2.9 \times 10^{-13}\) nmol min\(^{-1}\) for a 20 h reaction time. This is only a rough estimation, but it can help to
evaluate the efficiency of the whole cell biocatalyst. In an analogous calculation, the 7-ethoxresorufin-O-deethyl-
ylation activity would equal 22 active CYP1A2 enzymes per cell. In comparison to other whole cell biocatalyst systems, reported values for a whole cell assay with intra-
cellularly expressed CYP1A2 and CPR were 88 mg L⁻¹ 
paracetamol with 100 g L⁻¹ cell dry weight within 40 h at 
28 °C which is roughly about 70 fold better than our sys-
tem [50]. In contrast, the observed cytochrome c activity 
for single expressed CPR would equal about 3000–4700 
enzymes per cell. For the cytochrome c reduction by the 
CPR, a $k_{\text{cat}}$ value of 3000 min⁻¹ had been reported for 
the wild type enzyme and a $k_{\text{cat}}$ value of 1900 min⁻¹ for the anchorless CPR [51]. This number lies within the usual 
range from $10^3$ to $10^5$ enzymes per cell reported for auto-
display [52, 53]. Nevertheless, cytochrome c activity of 
CPR can obviously not serve as a predictor for the cata-
ytic activity of CPR with CYP1A2, because of the dif-
f erent binding sites for both proteins with CPR [25, 26, 
37]. When comparing Fig. 3 with Fig. 7, there is a clear 
discrepancy between the amount of protein expressed 
and CYP1A2 enzymatic activity. This could have been 
due either to a reduced functionality of surface displayed 
CYP1A2 molecules or to a restricted interaction between 
CPR and CYP1A2 on the cell surface. But indeed both 
would represent major bottlenecks for the application of 
cells with surface displayed CPR and CYP1A2. An indi-
cation for an altered CYP–CPR interaction would be the 
finding, that truncated and hence soluble CPR is almost 
unable to interact with CYPs, but is almost as active 
towards cytochrome c as the complete enzyme [26, 51]. 
Several reasons need to be taken into account for the presumably reduced activity of CYP1A2, independent 
of CPR binding. First, the C-terminus is attached to the 
autotransporter domains which could influence fold-
ing. Second, deletion of the N-terminal transmembrane 
domain, which is necessary in the autodisplay approach 
could influence folding. And finally, CYP1A2 could mis-
fold to some extent during transport leading to a reduced 
number of enzymatically active molecules on the cell surface.

Modifying or omitting the N-terminal transmembrane 
domain is a common step in increasing the expression 
and solubility of CYPs [8]. It has been proposed that the transmembrane domain does not take part in catal-
ysis, but has the important role to bring CYP and CPR 
close to the phospholipid interface [54, 55]. The mem-
brane surrounding modulates enzymatic activity as CYP 
enzymes are partially immersed in the membrane [56, 
57]. Several studies about CYP1A2 investigated the effect 
of a truncated or modified transmembrane domain and 
did not observe a loss of function, but in some cases 
different catalytic rates [49, 55, 58]. In contrast, it has 
been reported for CPR that the soluble truncated form 
is not able to supply electrons to the monooxygenase 
[59]. Here, we omitted the N-terminal transmembrane 
domain of both enzymes to achieve high expression lev-
els. We did not observe a loss of function of the enzymes 
which supports the hypothesis that the transmembrane 
domain is not essential for the catalytic activity. Further-
more, CYP1A2 and CPR form a catalytically active unit, 
although membrane anchorage has been altered from the 
N- to the C-terminus in both enzymes because of the 
C-terminal membrane anchoring β-barrel used in 
autodisplay. Hence, the unusual C-terminal fusion is fea-
sible for both proteins, but our results indicate that this alteration could have modified catalytic properties of the enzymes.

The catalytic activity of the whole cell biocatalyst 
implies that CPR is able to interact with CYP1A2 and 
to supply electrons through a transient protein–protein 
binding. This supports the theory that autotransporter facilitated anchorage allows lateral movement of the pas-
sengers on the outer membrane and quaternary complex 
assembly of protein partners. For other surface displayed 
proteins, it has been reported that multimerization is fea-
sible [15, 17]. Conclusively, we demonstrated that bacte-
rial surface display facilitates also transient interaction 
between protein partners.

We identified several factors for expression which 
influences activity of the whole cell biocatalyst and 
could be promising parameters for bioprocess optimiza-
tion. The whole cell biocatalyst was tenfold more active 
when grown at 23 °C instead of 30 °C. It has been often 
observed for CYP enzymes that the choice of expression 
temperature was highly important [7]. For surface dis-
played proteins, lower temperatures can also be favorable 
[60]. Furthermore, we found that Na⁺ and Ca²⁺ concen-
trations in the growth medium affected the product titer 
of washed cells without affecting the protein expression 
level. Addition of 1 mmol L⁻¹ of CaCl₂—but not MgCl₂—
was beneficial as the product titer of resorufin increased 
by roughly 30 %. Na⁺ concentration in the growth medium strongly affected CYP1A2 activity. As intracellu-
lar Na⁺ and Ca²⁺ concentrations are actively maintained 
in living E. coli cells, it appears likely that processes in 
the periplasm or on the outer membrane are affected 
et al. Microb Cell Fact (2016) 15:26
a reliable bioprocess with surface displayed enzymes will be to determine the optimal medium for cell cultivation.

To investigate if the supply of cofactors is a limiting factor for the activity of the whole cell biocatalyst, we focused exemplarily on heme as it is derived from only one precursor which can be easily added to the medium. *E. coli* BL21(DE3) is able to completely synthesize heme from glutamate via 5-ALA in the cytosol and to transport it to the periplasm [46]. The precursor 5-ALA is a common additive for expression of CYP enzymes [7]. Cells co-expressing CYP1A2 and CPR showed resorufin activity already in unmodified LB medium which was also found for surface displayed CYP3A4. However, medium supplementation with 5-ALA had a strong positive effect on CYP activity and our data suggests that the intracellular biosynthesis of the prosthetic group is a highly limiting factor. In our study, the route of endogenous heme was by far more effective than external addition of heme. 500 µmol L⁻¹ 5-ALA increased CYP activity by 400 %. In contrast, the equivalent amount of exogenous heme had a positive effect of merely 25 %. Secondly, it indicates that *E. coli* is metabolically limited during expression in terms of producing sufficient heme from glutamate. Therefore, this could be a promising starting point for strain engineering.

It remains an open question at what stage during translocation the surface displayed CYP1A2 and CPR incorporate their respective cofactors. One hypothesis is that they are incorporated after translocation onto the outer membrane and taken from the extracellular medium. A second is that they are bound by an at least partially folded passenger in the periplasm and then translocated. It is controversially discussed whether a partially folded passenger can be translocated across the outer membrane. Two mechanisms for translocation—the hairpin model and the Omp85 model—have been proposed whereas only the latter would be compatible with partial protein folding [10]. We observed that heme derived from cells is way more effective for increasing CYP activity than externally added heme. This could indicate that heme is incorporated into CYPs in the periplasm and not scavenged from the medium. However, it has been suggested that the TolC efflux pump provides intracellularly produced heme by exporting it to the extracellular medium from where it can be incorporated into CYPs. The outer membrane protein TolC forms a channel with efflux pumps in the inner membrane [61]. Thereby, porphyrins can be exported from the cytosol to the extracellular space which has been observed when natural heme homeostasis was disrupted [47, 48]. Nevertheless, this hypothesis does not explain why only heme provided by the TolC channel but not by medium supplementation would be effectively scavenged from the extracellular medium. In case of the CPR, there is little knowledge about efflux mechanisms for flavins to the cell exterior and periplasm. Although present in LB-medium, *E. coli* cannot take up flavins [62] and synthesizes them instead in the cytosol from GTP and ribulose-5-phosphate [63]. There are no reports on flavin transporters to the periplasm as periplasmic flavoenzymes are uncommon. However, the YeeO multidrug efflux transporter has been reported to export FAD and FMN to the medium [64]. Thus, it is imaginable that cofactor incorporation takes place on the cell surface after translocation. Still, the mechanism of incorporation needs further investigation.

Nevertheless, for preparative drug metabolite synthesis purposes several issues need to be addressed. First, the optimal CYP/CPR ratio should be determined and the influence of the N-terminal transmembrane domain further tested to improve the biocatalytic activity. Moreover, it could be investigated if the usage of inversed autotransporter (type Ve) is beneficial in terms of the catalytic properties of surface displayed enzymes. In this case the passenger is linked by its N-terminus to the anchor- age protein which corresponds to the orientation of the natural transmembrane domain in the endoplasmic reticulum. Secondly, we hypothesize that the activity of the whole cell biocatalyst can be reasonably increased by a thorough bioprocess optimization in terms of growth medium, oxygen supply and NADPH regeneration. Thirdly, the co-expression of cytochrome b5 could be beneficial for the activity of at least some monoxygenases [65]. Fourthly, CYP1A2 is involved in the metabolism of only a small fraction of all drugs. Therefore, an important step will be to establish co-expression of the CPR with all other major drug-metabolizing CYPs.

**Conclusion**

For the first time, human CYP1A2 has been successfully co-displayed with its reductase CPR on the surface of *E. coli* using the autotransporter system. It demonstrates that bacterial surface display is a viable tool for obtaining active CYP enzymes. This is an important step toward metabolite screening and preparative drug metabolite synthesis in a whole cell approach regardless of their membrane permeability. It also offers the chance to apply flow cytometry for human CYPs for applications such as enzyme library screening or to develop CYP based biosensors.

**Methods**

**Vector construction**

The codon-optimized open reading frames (ORF) of CYP1A2 [Uniprot:P05177-2] and CPR [Uniprot:P16435] were obtained by artificial gene synthesis (GeneArt, Regensburg, Germany). For cloning the ligase-free
In-Fusion technique was used [66]. *Escherichia coli* strain DH5α was used for all subcloning work. To construct the plasmid encoding the CYP1A2-autotransporter fusion protein the sequence of the autotransporter unit of AIDA-I including the sequence for N-terminal cholera toxin B signal peptide (CtxB) was amplified via PCR from pSC001 [27] and cloned behind the rhamnose-inducible promoter into the pOE2775 vector [34, 67]. The coding sequence of CYP1A2 without the transmembrane domain (amino acid residues 1–29) was then fused between the CtxB and the AIDA-I autotransporter unit DNA sequence yielding pPQ33. An overview about the fusion protein constructs is given in Fig. 2. For the CPR autotransporter fusion protein expression vector, the origin of replication (ORI) and ampicillin selection marker of pPQ33 were exchanged by the P15A ORI from pKE19 and a kanamycin selection marker from the broad-host range vector pBBR1-MCS2 [68]. The CPR ORF without transmembrane domain (amino acid residues 1–56) was inserted between the CtxB and the codon-optimized EhaA autotransporter unit (amino acids 839–1327 of the native protein) DNA sequence. The latter was taken from pMATE-MT004 [23] and modified to contain a TEV, Fxa and OmpT cleavage site, the PEYFK epitope and an additional flexible (G4S)3 hinge region. The plasmid was termed pPQ29. By inserting the sequence for the anti-myc epitope tag between the passenger and the autotransporter unit into pPQ29 and pPQ33 the plasmids pPQ61 and pPQ62 were obtained. The expression cassettes of all plasmids were checked by sequencing. *E. coli* BL21(DE3) was used for expression experiments. The coding sequence of CYP1A2 without the transmembrane domain (amino acids 839–1327 of the native protein) DNA sequence. The latter was taken from pSC001 [27] and cloned behind the rhamnose-inducible promoter into the pOE2775 vector [34, 67]. The coding sequence of CYP1A2 without the transmembrane domain (amino acid residues 1–29) was then fused between the CtxB and the AIDA-I autotransporter unit DNA sequence yielding pPQ33. An overview about the fusion protein constructs is given in Fig. 2. For the CPR autotransporter fusion protein expression vector, the origin of replication (ORI) and ampicillin selection marker of pPQ33 were exchanged by the P15A ORI from pKE19 and a kanamycin selection marker from the broad-host range vector pBBR1-MCS2 [68]. The CPR ORF without transmembrane domain (amino acid residues 1–56) was inserted between the CtxB and the codon-optimized EhaA autotransporter unit (amino acids 839–1327 of the native protein) DNA sequence. The latter was taken from pMATE-MT004 [23] and modified to contain a TEV, Fxa and OmpT cleavage site, the PEYFK epitope and an additional flexible (G4S)3 hinge region. The plasmid was termed pPQ29. By inserting the sequence for the anti-myc epitope tag between the passenger and the autotransporter unit into pPQ29 and pPQ33 the plasmids pPQ61 and pPQ62 were obtained. The expression cassettes of all plasmids were checked by sequencing. *E. coli* BL21(DE3) was used for expression experiments.

**Growth medium and culture conditions**

Cultivation were performed in lysogeny broth (LB) medium containing 10 g L⁻¹ peptone, 5 g L⁻¹ yeast extract and 10 g L⁻¹ NaCl. Solid media were prepared by addition of 1.5 % (w/v) agar. Kanamycin and carbenicillin were supplemented to ensure plasmid stability to a final concentration of 62.5 and 125 µg mL⁻¹, respectively. Precultures were performed in unmodified LB medium at 37 °C overnight. For expression studies LB medium was modified. Depending on the experiment LB was buffered with either a sodium phosphate buffer, a 37:12 mixed sodium/potassium phosphate buffer or a potassium phosphate buffer, each set to pH 7 with a final concentration of 50 mmol L⁻¹ phosphate. Protein expression was induced with 1 mmol L⁻¹ L-rhamnose. Using an autoinduction approach, 0.5 g L⁻¹ glucose as catabolite repressor was added delaying expression until approximately OD₅₇₈ₐₚ₉ 0.5 was reached. Filter sterilized solutions of 5-ALA and hemin chloride were added to a final concentration of 500 and 4 µmol L⁻¹, respectively. Unless otherwise stated, main cultures were inoculated with 1 mL preculture broth in 100 mL medium in a 500 mL shake flask and vigorously shaken at 200 rpm. Depending on the experiment, induced cells were either cultivated at 23 °C for 19.5 h or at 30 °C for 10 h.

**Outer membrane protein protein isolation and protease accessibility**

Outer membrane protein isolation was conducted as described in literature [69]. To investigate surface exposure of the passenger the protease accessibility test was used. Cells were grown in 100 mL potassium phosphate buffered LB medium at 23 °C for 19.5 h and harvested. Cells from 33 mL culture broth were re-suspended in phosphate buffered saline (PBS). After addition of 50 mAnson U proteinase K the suspension was kept at 37 °C for 1 h. The digest was stopped by addition of 1 mmol L⁻¹ PMSF and washing three times with 5 mL of pre-cooled 0.2 mol L⁻¹ Tris buffer (pH 8). The cells were then re-suspended in 1.5 mL 0.2 mol L⁻¹ Tris buffer and further processed in the outer membrane protein preparation. Samples from the OMPI were boiled 20 min at 95 °C in SDS-PAGE sample buffer with 30 mmol L⁻¹ dithiothreitol and separated with a 10 % acrylamide gel. Proteins were stained with Coomassie Brilliant Blue G-250.

**Flow cytometry analysis**

To probe surface exposure of the passenger, cells were analyzed by flow cytometry after treatment with fluorescence labelled antibodies. Cells were grown in 20 mL potassium phosphate buffered LB medium in a 100 mL shake flask at 23 °C for 19.5 h. After harvest cells were washed tree times with filter-sterilized PBS. 1 mL of cells (OD₅₇₈ₐₚ₉ 0.2) were treated with 10 µg mL⁻¹ monoclonal mouse Anti-myc antibody (ThermoFisher Scientific) for 30 min. Cells were washed three times and then treated with 20 µg mL⁻¹ secondary DyLight 488 conjugated goat anti-mouse IgG (H + L) Antibody in the dark for 30 min. After a final round of washing and re-suspension the fluorescence of 50,000 cells was analyzed using a FACSARia flow cytometer (BD Biosciences, USA) at an excitation wavelength of 488 nm.

**Immunofluorescence microscopy**

Cells were prepared as described for the flow cytometry analysis. Pretreatment with proteinase K was conducted as described for OMPI. After antibody incubation and subsequent washing steps, cells were re-suspended in 30 µL filter-sterilized PBS, 10 µL applied on a slide and the cover slip sealed with nail polish. Fluorescence images were obtained with a confocal laser scanning microscope TCS SP2 (Leica, Wetzlar, Germany) at 488 nm excitation using an Argon laser.
Cytochrome c assay
The whole cell assay was performed as described for purified enzymes [35]. Cultivation was performed with 20 mL medium without hemin and 5-ALA at 23 °C in 100 mL shake flask for 19.5 h. Cells were harvested and washed three times in potassium phosphate buffer (pH 7.4). The enzyme reaction was carried out in a 15 mL reaction tube with 0.8 mL total volume containing whole cells (OD_{578nm} 40), 1 μmol L\(^{-1}\) 7-ethoxyresorufin and a NADPH regeneration system in 0.1 mol L\(^{-1}\) potassium phosphate buffer (pH 7.4). The reaction was terminated by addition of 3 mL diethylether to 800 μL L\(^{-1}\) of assay volume. The samples were vortexed for 10 min, centrifuged and the water phase frozen. The organic layer was collected, 65 μL 1 mol L\(^{-1}\) HCL added to the water phase and the extraction procedure repeated. The collected diethylether was removed under N\(_2\) stream and samples were dissolved in 150 μL 30 % methanol/70 % 0.1 mol L\(^{-1}\) acetate buffer pH 4.

Phenacetin-O-deethylation measurement
Cells were cultivated in potassium phosphate buffered LB medium additionally supplemented with 1 mmol L\(^{-1}\) CaCl\(_2\). Assay conditions were the same as for the 7-ethoxyresorufin-O-deethylation except that 100 μmol L\(^{-1}\) of phenacetin were used. After 40 h incubation 10 μmol L\(^{-1}\) of the internal standard 3-acetamidophenol was added and the reaction terminated by addition of 4 mL diethylether to 800 μL L\(^{-1}\) of assay volume. The samples were vortexed for 10 min, centrifuged and the water phase frozen. The organic layer was collected, 65 μL 1 mol L\(^{-1}\) HCL added to the water phase and the extraction procedure repeated. The collected diethylether was removed under N\(_2\) stream and samples were dissolved in 150 μL 30 % methanol/70 % 0.1 mol L\(^{-1}\) acetate buffer pH 4.

Resorufin measurement
For HPLC analysis of resorufin formation a LiChrospher 60 RP-select B column 250-4 (5 μm) from Merck (Darmstadt, Germany) was used in a LaChrom Elite System equipped with a fluorescence detector L-2485. The mobile phase consisted of 50 % methanol and 50 % 0.05 mol L\(^{-1}\) potassium phosphate buffer (pH 7). The system was run at a flow rate of 0.6 mL min\(^{-1}\). Injection volume was 40 μL. The retention time for resorufin was 7.4 min and for 7-ethoxyresorufin 29 min. Fluorescence was measured at 590 nm using excitation at 550 nm. To save measurement time a sample run was 10 min. Prior to analysis, sample supernatant were diluted with methanol 1:1, centrifuged 10 min at 10,000g to remove precipitates and then filtered through a 0.2 μm PTFE syringe filter. Resorufin was quantified with a standard curve ranging from 1 to 200 nmol L\(^{-1}\).

For microtiter plate analysis 100 μL of the sample supernatant were analyzed fluorometrically in a 96 well plate using Infinite 200 Pro reader (Tecan Group, Männedorf, Swiss). Fluorescence was determined at 590 nm with excitation at 550 nm. Resorufin was quantified using a standard curve made in the supernatant from co-expression assay samples without substrate treated as described before.

Luciferin-MultiCYP assay
The luciferin-MultiCYP assay represents a method for the measurement of CYP activity. MultiCYP is a non-selective luminescent substrate which is convertible to n-luciferin ester by at least 21 CYPs, including CYP1A2 [70]. Protected from light, a 50 mmol L\(^{-1}\) MultiCYP stock solution in acetonitrile and a 2 mmol L\(^{-1}\) stock solution of n-luciferin in water were made and stored at −20 °C. Cells were grown as described, harvested and washed two times in 0.2 mol L\(^{-1}\) potassium phosphate buffer (pH 7.4). Cells (OD_{578nm} 20) were incubated under continuous shaking in 2 mL reaction tubes at 37 °C in 0.1 mol L\(^{-1}\) potassium phosphate buffer (pH 7.4) with 50 μmol L\(^{-1}\).
MultiCYP and 200 μmol L⁻¹ NADPH with a total volume of 120 μL. After 24 h, cells were sedimented (2 min, 14,000 rpm), the supernatant was collected and incubated at 90 °C for 10 min (to prevent ATP hydrolysis by remaining cell components). Denatured cell components were removed by centrifugation (2 min, 14,000 rpm) and 50 μL supernatant was transferred to white microtiter plates, where 50 μL luciferin detection reagent was added. After 20 min of incubation at 30 °C, luminescence was measured for 10 min and compared to a luciferin calibration curve (0–2 μmol L⁻¹). Uptake of luciferin by cells was excluded by incubation of luciferin with or without cells under assay conditions and subsequent measurement of the resulting luminescence. Heat stability of luciferin was determined by incubation of luciferin under assay conditions at 90 °C for 10 min.

### Additional files

- **Additional file 1**: Figure S1. Surface localization of CPR and CYP1A2 analyzed by immunofluorescence microscopy.
- **Additional file 2**: Figure S2. Influence of growth conditions on protein expression level.

### Abbreviations

ATP: adenosine triphosphate; 5-ALA: 5-aminolevulinic acid; CtxB: cholera toxin B; CYP: cytochrome P450; CYP1A2: cytochrome P450 1A2; CPR: cytochrome P450; fXa: factor Xa protease; HPLC: high-performance liquid chromatography; LB: lysogeny broth; NADPH: nicotinamide adenine dinucleotide phosphate; OD: optical density; OMPl: outer membrane protein isolation; Ompt: outer membrane protease T; ORF: open reading frame; ORI: origin of replication; RhaP: rhamnose inducible promoter; TEV: tobacco etch virus.

### Authors' contributions

JJ, RM and TB conceived the study, guided its coordination and participated in designing the study, evaluating the results and writing the manuscript. JS purified the dehydrogenase, paved the way for the myc-tag and participated in designing the study, evaluating the results and writing the manuscript. HPLC: lysogeny broth; NADPH: nicotinamide adenine dinucleotide; fXa: factor Xa protease; HPLC: high-performance liquid chromatography; LB: lysogeny broth; NADPH: nicotinamide adenine dinucleotide phosphate; OD: optical density; OMPI: outer membrane protein isolation; Ompt: outer membrane protease T; ORF: open reading frame; ORI: origin of replication; RhaP: rhamnose inducible promoter, TEV: tobacco etch virus.

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### Competing interests

The authors declare that they have no competing interests.
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