HEINRICH HEINE
UNIVERSITÄT DÜSSELDORF

Autodisplay of functional P450 enzymes in *Escherichia coli*

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf
vorgelegt von

Stephanie Dorothee Schumacher

aus Darmstadt

Düsseldorf, Mai 2012

Aus dem Institut für Pharmazeutische und Medizinische Chemie
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Joachim Jose
Korreferent: Prof. Dr. Holger Gohlke

Tag der mündlichen Prüfung: 05.07.2012

MEINER FAMILIE

Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt.

Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 02.05.2012

(Stephanie Schumacher)

Mein Dank gilt:

- Prof. Dr. Joachim Jose für die Möglichkeit der Promotion in seinem Arbeitskreis und das Überlassen eines sehr interessanten Themas. Für ein stets offenes Ohr, wenn es scheinbar nicht wirklich weitergehen wollte, die tolle Unterstützung und das rege Interesse am Fortgang meiner Arbeit.
- Prof. Dr. Holger Gohlke für die Übernahme des Korreferats.
- Prof. Dr. Rita Bernhardt und Dr. Frank Hannemann vom Lehrstuhl für Biochemie der Universität des Saarlandes für die freundliche Aufnahme in die Arbeitsgruppe und die Unterstützung während der ersten Umsetzungsversuche mit CYP106A2.
- Dr. Mark Teese und Dr. Tanja Völker, meinen beiden Postdocs, die es bestimmt nicht immer einfach mit mir hatten, aber nie müde wurden, mit mir über meine Ergebnisse zu diskutieren und hilfreiche Tipps zu geben. Besonders möchte ich Mark auch für die Hilfe bei der Erarbeitung und das anschließende Korrekturlesen der Manuskripte danken.
- Dr. Elisa Winterer, Dr. Klaudia Petermann und Dr. Eva Kranen für die freundliche und sehr herzliche Aufnahme in den Arbeitskreis und die stets sehr lustigen und erholsamen Teepausen, nach denen die Arbeit gleich viel einfacher von der Hand ging.
- Sarah Thömmes und Achim Braukmann, die mich nun schon seit dem ersten Semester begleiten und denen es stets gelungen ist, mir nicht nur fachlich eine große Hilfe zu sein, sondern auch meine Freizeit sehr abwechslungsreich zu gestalten.
- Dr. Andreas Gratz und Dr. Miriam Pein, die mir den Einstieg in das Praktikum „Instrumentelle Analytik“ sehr erleichtert haben und mit denen es stets Spaß gemacht hat die Zeit gemeinsam im Assistentenraum zu verbringen und anschließend das ein oder andere Feierabendbierchen zu trinken.
- Meinen Kollegen Dr. Andreas Gratz, Dr. Mark Teese, Dr. Karsten Rose und Bertan Bopp, für die gute, gemeinsame Betreuung des Praktikums „Biochemie“, das trotz des vorangegangenen Umzugs sehr gut verlaufen ist.
- Allen anderen aktuellen und ehemaligen Kollegen: Dr. Andre Kaeßler, Dr. Christian Detzel, Michael Goblirsch, Claudia Reicheneder, Iris Gawarzewski, Irina Altendorfer, Agne Tubeleviciute, Zoya Orlando, Dr. Tatjana Brossette, Dr. Claas Hundsdörfer, Andre Bollacke, Thorsten Sanger, Wilhelmine

Weckenbrock, Shanna Sichwart, Ilka Rauch, Indra Finger, Winni Scherlitzki, Dr. Dagmar Aichele, Petra Nordlohne, Karin Voigt, Nora Stellisch: Danke für das stets fröhliche, gute und produktive Arbeitsklima und die Kollegialität. Es war wirklich schön, in so einem Arbeitskreis arbeiten zu dürfen, der selbst einen Umzug nach Münster bravourös gemeistert hat. Die Feiern mit Euch bleiben unvergessen.

- Dem Ministerium für Innovation, Wissenschaft und Forschung des Landes Nordrhein-Westfalen, sowie der Heinrich-Heine-Universität Düsseldorf für das Stipendium innerhalb des Graduiertenclusters für industrielle Biotechnologie (CLIB).
- Allen Freunden, die es immer vermocht haben mich abzulenken, aufzumuntern oder einfach nur für mich da zu sein.
- Meiner Familie, inbesondere jedoch meinen Eltern und meinen beiden Geschwistern, die mich währen meines Studiums und meiner Promotion immer unterstützt, aufgebaut und abgelenkt haben, auf die ich mich immer verlassen konnte und kann und die mir in Zeiten, wo es mal stressig war, so viel Arbeit wie möglich abgenommen haben (einen großen Dank nochmal an das Partykomitee für eine unvergessliche Feier).
- David Rönsberg, der mich immer unterstützt hat, egal ob es um einen Umzug oder um das Korrekturlesen der Arbeit ging und gerade in der Endphase viel schlechte Laune ertragen musste. Vielen Dank, dass Du mir den Rücken freigehalten hast und immer für mich da warst und bist.

Table of Content

1 Zusammenfassung

Cytochrom P450 Enzyme spielen aufgrund ihres großen Substratspektrums und ihrer Fähigkeit Sauerstoffatome regio- und stereoselektiv in nicht aktivierte C-H-Bindungen einzufügen sowohl in der chemischen, als auch in der pharmazeutischen Industrie eine große Rolle.

In der vorliegenden Arbeit wurden das aus *Bacillus megaterium* stammende, lösliche CYP106A2 und das humane, membranständige CYP3A4 mittels Autodisplay auf die Oberfläche von *Escherichia coli* gebracht. Nach der Überprüfung der Oberflächenständigkeit beider P450 Enzyme wurden Aktivitätsmessungen durchgeführt. Hierfür wurde ein rekonstituierendes System, bestehend aus NADPH, AdR und Adx zu den CYP106A2 präsentierenden Zellen hinzugefügt, um einen erfolgreichen Elektronentransport zu dem P450 Enzym zu gewährleisten. Im Falle von CYP3A4 wurde statt der Cofaktoren AdR und Adx NADPH-P450-Reduktase eingesetzt. Die Produkte der enzymatischen Reaktionen wurden mittels HPLC-Analyse überprüft. Der CYP106A2-Ganzzellbiokatalysator (OD_{578}=6) hydroxylierte das Substrat Deoxycorticosteron in 15β-Position und zeigte eine Aktivität von 0,3 μU/ml. Das oberflächenexprimierte CYP3A4 zeigte eine Hydroxylierung des Substrates Testosteron zu 6β-OH-Testosteron mit einer Wechselzahl von 5 Molekülen pro Zelle pro Minute. Durch die Etablierung eines photometrischen NADPH Assays konnte sowohl der Umsatz des Substrates Imipramin in das Produkt Desipramin, als auch die Hydroxylierung des Substrates Abietinsäure in das Produkt 12-OH-Abietinsäure durch CYP106A2 nachgewiesen werden.

Beide P450 Enzyme waren aktiv, ohne dass eine externe Zugabe der prosthetischen Gruppe, des Häms, nötig war. Ein Kanal in der äußeren Membran, genannt TolC Kanal, ist für den Export von Häm in *E. coli* verantwortlich. Die Expression von CYP106A2 in einer TolC negativen *E. coli* Mutante führte zu einer deutlich verminderten Aktivität des Enzyms, welche durch die externe Zugabe von Häm wieder hergestellt werden konnte. Dies deutet darauf hin, dass die prosthetische Gruppe der P450 Enzyme durch die *E. coli* Zellen selbst ausgeschleust und dann von außen in das sich auf der Oberfläche befindliche Enzym inkorporiert wird.

2 Abstract

Due to their large variety of substrates and their capability to insert a molecular oxygen regio- and stereoselectively into non-activated C-H-bonds, cytochrome P450 enzymes play an important role in the pharmaceutical and chemical industry.

In this study the soluble, bacterial CYP106A2 from *Bacillus megaterium* and the human, membrane bound CYP3A4 were expressed on the cell surface of *Eschericha coli* by the aid of the Autodisplay system. After successful surface display was confirmed activity measurements were perfomed. A reconstituted system containing NADPH, AdR and Adx was externally added to the whole cell CYP106A2 biocatalyst supplying the enzymes on the surface of *E. coli* with the necessary reducing equivalents for the reaction. In the case of CYP3A4 the cofactors AdR and Adx were replaced by NADPH-P450-Reductase. The obtained reaction products were analyzed by HPLC measurements. The CYP106A2 whole cell biocatalyst (OD_{578}=6) converted the substrate deoxycorticosterone into the product 15β-OH-deoxycorticosterone and showed an activity of 0.3 μU/ml. The surface displayed CYP3A4 converted 5 molecules per cell per minute of the substrate testosterone into the product 6β-OH-testosterone. A newly established NADPH turnover assay revealed the conversion of the substrate imipramine into the product desipramine as well as the conversion of the substrate abietic acid into the product 12-OH-abietic acid by the displayed CYP106A2.

Both P450 enzymes were active without the external addition of the heme prosthetic group. An outer membrane channel called TolC is involved in the export of the heme group. The expression of CYP106A2 in a TolC negative *E. coli* strain lead to a reduced activity, which could be recovered by the external addition of the heme group. This indicates that the prosthetic groups are exported by the *E. coli* cells into the growth medium, where they can be incorporated into P450 enzymes folding at the surface.

3 Introduction

3.1 Biocatalysis

Biocatalysis is the conversion of organic substrates into products through a cell derived catalyst, such as an intracellular or purified enzyme.

Enzymes are ubiquitously distributed proteins which can be found throughout nature. They can catalyze a big variety of biochemical reactions in processes as diverse as metabolism and cell division. The use of enzymes for the production of food such as wine, beer or cheese can be traced back to ancient culture (Ulber and Soyez, 2004). It was not until the early 19th century that the enzyme diastase was discovered, which is capable of the degradation of starch to dextrose and sugar. This finding was the beginning of the scientifically based investigation of enzymes. In the second half of the 19th century Louis Pasteur postulated that microorganisms were responsible for the starch degradation (Pasteur, 1858). 20 years later Wilhelm Friedrich Kühne formed the term „enzyme“ out of the two greek words in (*en*) and yeast (*zyme*), and used the word to describe the process of fermentation. Some time later it was discovered that whole cells were not necessary to carry out alcohol fermentation, and the active substance was named “zymase” (Buchner and Rapp, 1898). At the beginning of the 20th century it was proven that proteins could be enzymes, based on crystallization experiments of urease (Sumner, 1926).

In the 1930´s pectinases were used in the food industry to improve the pressing quality of grapes and clarify fruit juices (Kertesz, 1930). However, the first generation of enzyme preparations was unspecific because no accurate knowledge, neither of the substrate nor the enzymatic activity was available (James and Simpson, 1996). Furthermore, the low stability of enzymes, the short life span and the restriction to aqueous media appeared to be very challenging. It was not until the 1980´s that a major growth in this field was seen (Halling and Kvittingen, 1999). The possibility of recombinant expression in self replicable organisms such as bacteria and yeast in the late 20th century led to the field of biocatalysis as it is known today (Bornscheuer, 2005).

Biocatalysis is becoming a key component in the production of complex molecules in the chemical industry (Pollard and Woodley, 2007). It was estimated that in the year 2010 already 20% of all chemical compounds are produced by biotechnological processes (Festel et al., 2004). In contrast to standard methods of chemical synthesis, the exceptional regioselectivity and specificity of enzymes lower the probability of occuring side products and increase the purity of the desired product (Patel, 2001).

Additionally reaction conditions usually do not require high temperatures or pressures (Schmid et al., 2001), which makes the use of enzymes more environmental friendly. A drawback to the use of natural enzymes is that they have evolved to catalyze a substrate that might be different from the one of interest for the chemical industry. Furthermore they have evolved to work optimal in their cellular environment and temperature, which may not necessarily match the industrial process. In this case directed evolution could help to improve turnover, substrate specificity, or other required properties (Arnold and Volkov, 1999).

Industrial processes often benefit from enzymes of high stability, which can be reused in subsequent process cycles. This can be achieved by an immobilization of the desired enzyme (Torres-Salas et al., 2011), also offering the advantages to separate the product from the enzyme and use it in a continuous fixed-bed operation.

Basically three possible methods of immobilization can be distinguished (Sheldon, 2007):

- Support binding (the physical, ionic or covalent binding to a carrier)
- Entrapment (inclusion of an enzyme into a polymer network)
- Cross-linking (the use of bifunctional reagents to cross-link enzyme aggregates or crystals)

Of particular interest is the regioselective and stereoselective synthesis of drugs or their intermediates in the pharmaceutical sector (Patel, 2001). In the year 2005 the market share of enantiomerically pure drugs was 37%, corresponding to a market volume of 225 Mrd. US dollar (Gadamasetti and Braish, 2007). There are three main reasons why enantiomerically pure drugs are preferred to racemic mixtures. First of all the opposing enantiomers can show different phamacological, pharmacodynamic, toxicological and pharmacokinetical properties and usually only one of the isomers fully contributes to the therapeutic action (Ariens, 1991; Midha et al., 1998). Secondly the US Food and Drug Administration (FDA) enforces the introduction of enantiopure active substances into the market by very strict guidelines. Since 1992 the FDA and the European Committee for Proprietary Medicinal Products expect the individual characterization and description of the physiological action of each enantiomer of a pharmaceutical product. Last but not least a single isomer program was established by the FDA in 1992 to allow already approved racemic drugs to regain market exclusivity if sold as an isomer (Tomaszewski and Rumore, 1994). One example is the use of the proton pump inhibitor omeprazole. The R-isomer is only metabolized by the cytochrome P450 isoform 2C19. In the case of the S-isomer 30% of the drug are metabolized by the isoform 3A4, which shows a lower activity. Therefore the application of the S-isomer leads to a higher bioavailability, slower elimination and a

better therapeutic effect (Abelo et al., 2000). Despite this great demand for enantiomerically pure drugs the isolation of the isomers out of racemic mixtures remains fairly difficult, time consuming and costly (Reddy and Mehvar, 2004). Therefore the use of enzymes for stereospecific synthesis will continue to grow.

3.2 Drug metabolism

Drugs or chemical compounds that enter the mammalian body are usually metabolized by a variety of enzymes. In many cases more polar metabolites are formed, which can then be excreted via the kidneys. In 1959 Williams categorized the metabolic reactions catalyzed by mammalian enzymes into two phases, phase I and phase II (Williams, 1959). Phase I involves oxidations, reductions and/or hydrolysis. The second phase consists mainly of conjugations such as glucuronide-, ethereal sulphate-, thiocyanate- or hippuric acid formation (Nelson and Gordon, 1983). In the first phase biological active compounds might be inactivated, have their activity altered, become active or even toxic (David Josephy et al., 2005). A well-documented example is that of the drug terfenadine, a non-sedative antihistamine. In the oxidative phase I metabolism, in most cases carried out by cytochrome P450s, the drug is rapidly converted into its active metabolite fexofenadine (Mutschler, 2001).

Quantitatively, the smooth endoplasmatic reticulum of the liver is the principal organ of drug metabolism, even though other biological tissues have the ability to metabolize drugs as well. This is due to the fact that the liver is a very large organ and the first, which is perfused by chemicals that were absorbed in the gut. Additionally it shows a very high concentration of drug metabolizing enzymes in contrast to other organs (von Bahr et al., 1980). If a drug is taken into the gastrointestinal tract it is carried through the portal vein into the liver, where it is directly metabolized before it reaches the rest of the body. This effect, whereby the concentration of the drug is greatly reduced is called “First-Pass-Effect” and has to be investigated in clinical trials before a drug candidate can enter the market (Mutschler, 2001).

Through the ability to sequence the human genome researchers were able to discover hundreds of genes that harbor variations contributing to human illness. Additionally they were able to associate certain genetic variations with different metabolization capacities (Hamburg and Collins, 2010). Especially P450 enzymes were shown to be expressed in polymorphical variants, which results in varying metabolic activity. Variation in the activity of these enzymes can result in a faster inactivation of a drug as expected (rapid metabolizer), leading to blood concentrations too low to reach a therapeutic effect. On the other hand patients with reduced activity of metabolic enzymes (slow metabolizer) are at risk of intoxication due to much higher blood levels as aimed for (Autrup, 2000). A genotyping of the patient prior to the

application of a drug comes more and more into focus and is the aim of the new field of “personalized medicine”.

3.3 Cytochrome P450s

Cytochrome P450s are an ubiquitously distributed group of hemeproteins. They were first described during investigations on an enzyme system in the liver microsomes of pigs and rats in the 1950s (Garfinkel, 1958; Klingenberg, 1958). Six years later Omura and Sato were able to identify a hemeprotein and gave it the name ”cytochrome P450“ due to the absorption peak of a complex formed between the reduced heme-iron and carbon monoxide at 450 nm (Omura and Sato, 1964). The shift of absorption from 420 to 450 nm in comparison to other cytochromes leads to a red colour and shows that the electron density at the heme is significantly perturbed. A possible explanation for this might be the thiolate sulphur, which directly binds to the iron in the protoporphyrin-IX and causes this effect (Bernhardt, 2006). In 1996 a cytochrome P450 was isolated from the extreme acidothermophilic archaeon *Sulfolobus solfataricus*. This organism belongs to a very old class of bacteria, giving a major hint that P450 enzymes have ancient roots. Today it is widely known that P450s are present throughout all known biological kingdoms. They occur in mammals, birds, fish, insects, amphibians, plants, bacteria and fungi (Hannemann et al., 2007). A nomenclature of this protein superfamily based on sequence identity was established in 1991 (Nebert and Nelson, 1991). P450 genes are abbreviated with the word CYP followed by a number representing the family (more than 40% sequence identity), a letter designating the subfamily (more than 55% sequence identity) and another number representing the individual gene. Currently there are 977 different CYP families and 2519 subfamilies (for details see: http://drnelson.uthsc.edu/CytochromeP450.html). Cytochrome P450s play an essential role in a lot of research fields, particularly in pharmacology and toxicology (Fig. 1). Also organic chemists, microbiologists and environmental researchers are working with these enzymes to be able to use them for their individual purposes.

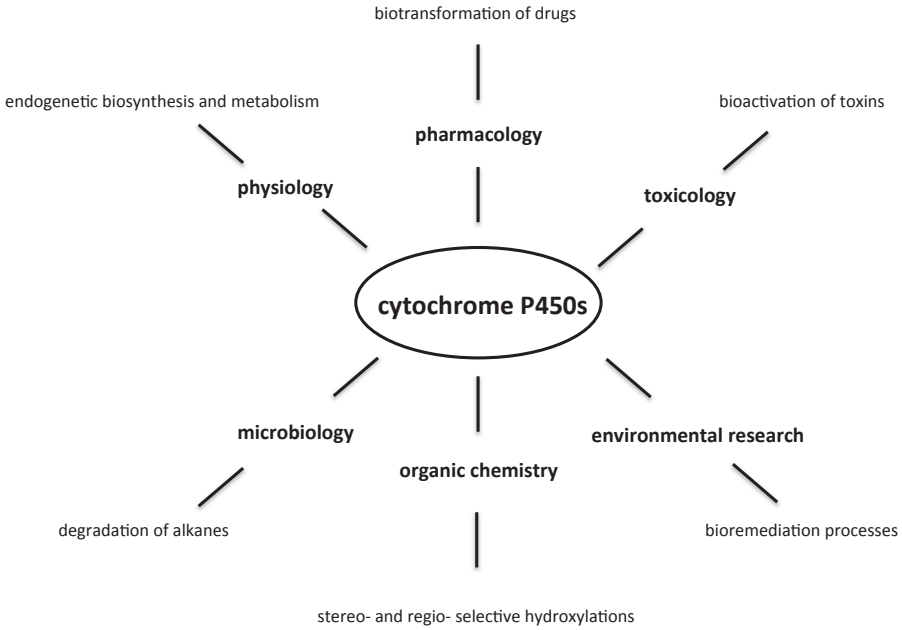

Figure 1: Relevance of P450 enzymes in different research fields.
(Modified, according to Bernhardt, 1996)

3.3.1 Classification of P450 enzymes

Enzymes in general have been classified into six categories depending on the reaction that they catalyse (Fig. 2). P450s belong to the group of external monooxygenases, within the subgroup of oxidoreductases. In contrast to internal monooxygenases, external monooxygenases cannot obtain electrons from the substrate itself and are dependent on external electron donors. However, there are two exceptions. Thromboxane-A2 synthase and Prostacyclin synthase are considered members of the P450 superfamily, even though they function as isomerases (Haurand and Ullrich, 1985; Wu and Liou, 2005).

In the monooxygenase reaction P450s incorporate a single atom of molecular oxygen into a substrate under the reduction of the second atom of oxygen to water. P450s obtain the reducing equivalents needed for their activity from external electron donors. Depending on the P450 enzyme its activity is either NADH or NADPH dependent.

The reaction can be illustrated as follows,

$$NAD(P)H + H^+ + O_2 + RH \rightarrow NAD(P)^+ + H_2O + ROH$$

whereas, RH is the reduced substrate, ROH the oxidized substrate and NAD(P)H the external electron donor.

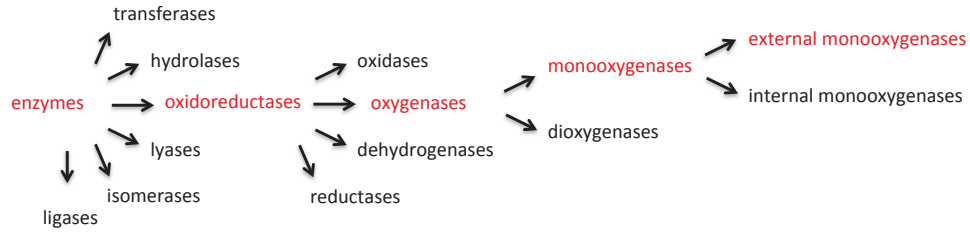

Figure 2: Classification of P450 enzymes. (P450 enzymes belong into the red labeled categories).

NAD(P)H supplies two electrons at a time, however cytochrome P450s can only accept one. Activity therefore requires an electron transport chain to the heme group. The proteins which carry out this function vary widely throughout nature (Hannemann et al., 2007).

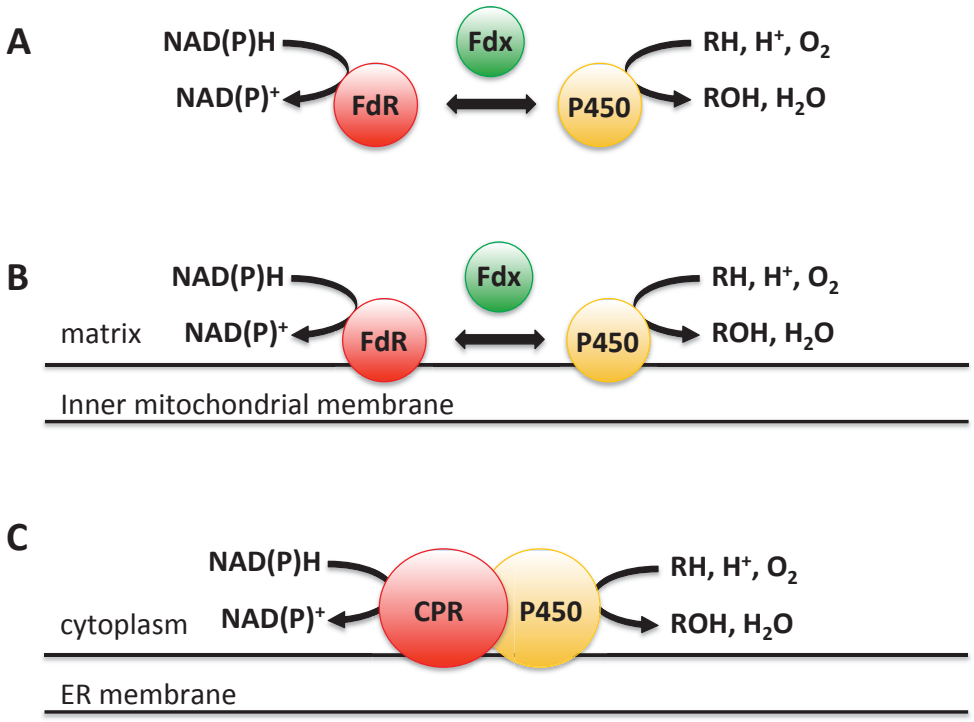

Figure 3: Organisation of P450 enzymes. A: bacterial system, B: mitochondrial system, C: microsomal system (modified, according to Hannemann et al., 2007). FdR: FAD-containing reductase, Fdx: ferredoxin, CPR: NADPH-dependent cytochrome-P450 reductase, ER: endoplasmatic reticulum, RH: substrate, ROH: product.

P450s have been classified into two groups, depending on the additional proteins required for their activity. Class I comprises mitochondrial and bacterial P450s and requires two additional proteins apart from the P450 enzyme (Fig. 3A/B). Class II contains microsomal P450s and requires one additional protein apart from the P450 enzyme (Fig. 3C).

In Class I P450s, a FAD-containing reductase (FdR) transfers the electrons from the external donor NAD(P)H. These electrons are transferred to a ferredoxin protein (Fdx), the second component of the system. Ferredoxins are iron sulfur proteins of the [2Fe-2S] type, which reduce the cytochrome P450 directly (Bernhardt, 1996).

A major difference between the bacterial and the mitochondrial P450s is that in bacteria all three components are soluble, whereas in eucaryotes the P450 and the FdR require membrane surroundings to become active. Microsomal, membrane bound P450s are the most common form found in eucaryotes. Up to now all drug and xenobiotic metabolising cytochrome P450s were shown to belong to this class (Bernhardt, 2006). Class II P450s in the endoplasmatic reticulum of eucaryotes require two integral membrane bound proteins for activity. This is sometimes referred to as a "two component system" due to the requirement for the cytochrome P450 itself, and also a NADPH-dependent cytochrome-P450 reductase (CPR) which contains two prosthetic groups, FMN and FAD, for the transport of both electrons from NAD(P)H to the P450.

3.3.2 The catalytic cycle

The common reductive mechanism of P450 enzymes was proposed in 1968 and is still accepted today (Denisov et al., 2005; Gunsalus et al., 1975) (Fig.4). The sequential two electron-reduction was first characterized in the bacterial CYP101 (P450cam) (Katagiri et al., 1968) and later also in microsomal systems (Estabrook et al., 1971).

Every P450 enzyme contains a heme iron in its active site. In the resting state the iron has a coordination number of six and is tethered to the protein via a thiolate ligand derived from a cysteine residue. In the first reaction step the substrate displaces a water molecule from its distal-axial position of the heme iron and binds to the active site of the enzyme in close proximity to the heme group (1). This changes the low-spin (LS) state of the iron to a high-spin (HS) substrate bound complex (2). In doing so the redox potential of the Fe^{3+} changes to a more positive value and is therefore easier to be reduced to Fe^{2+}(3). The change of redox potential in the presence of the substrate is thought to prevent an uncontrollable electron delivery and minimize NAD(P)H consumption. Molecular oxygen binds to the distal-axial position of the heme iron to form an oxy-P450 complex (4). This is the last relatively

stable intermediate in the catalytic cycle. An additional electron transfer reduces the dioxygen adduct to a negatively charged, instable peroxo group (5a). This peroxo group is rapidly protonated by a local transfer from surrounding water molecules or amino acid side chains to form a hydroperoxo-ferric intermediate (5b). A second protonation leads to the heterolysis of the O-O bond, formation of water and the so-called *compound I* (6), which oxidizes the bound substrate. In the last step of the catalytic cycle the product is released from the active site and the enzyme returns into its original state with a water molecule returning to occupy the distal axial position of the iron.

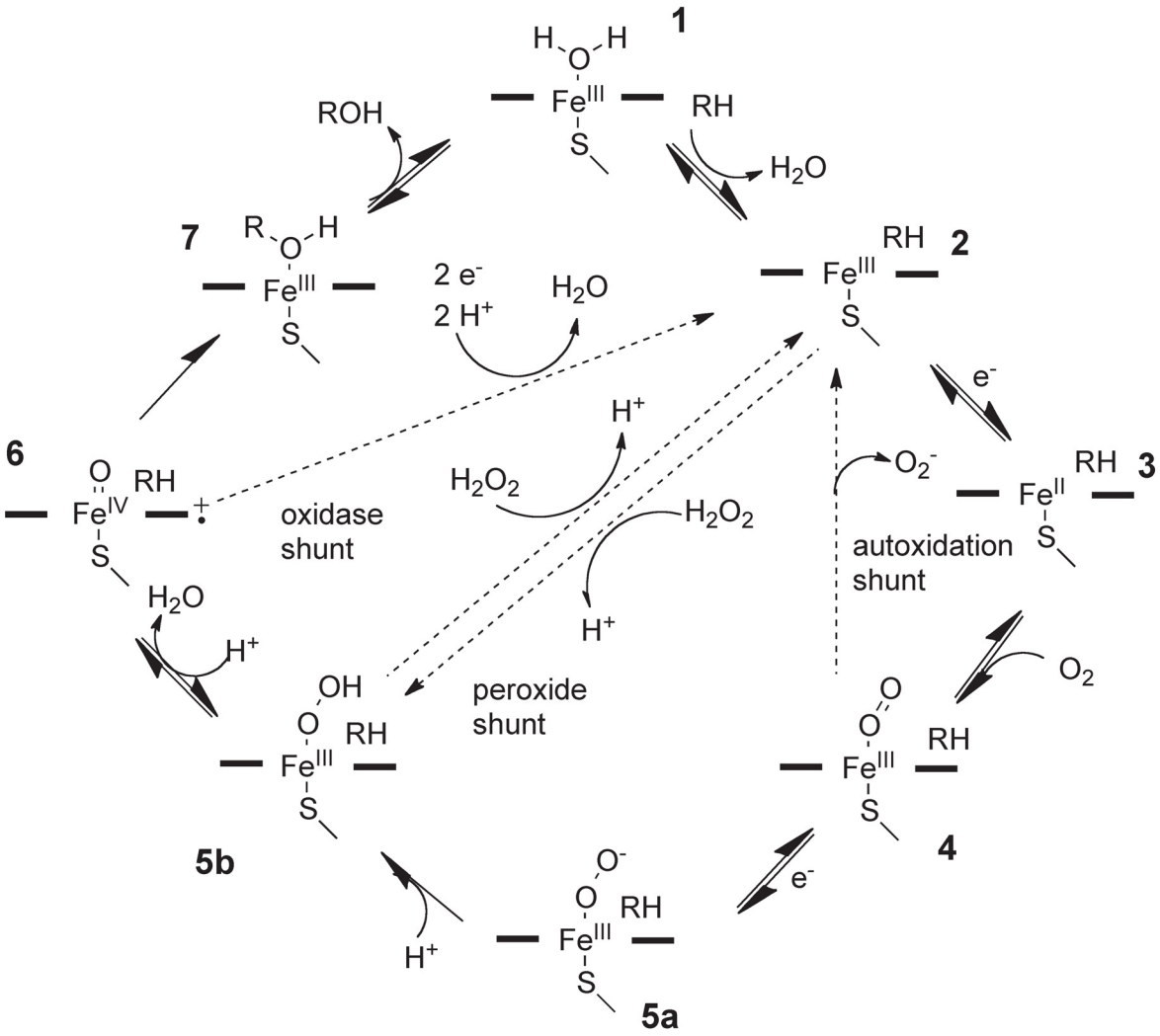

Figure 4: Catalytic cycle of the monoxygenase reaction of P450 enzymes (modified from Denisov et al., 2005)

In addition to those eight intermediates described above the P450 catalytic cycle contains at least 3 branch points, so-called *shunt pathways*, where side reactions are possible. The *autoxidation shunt* describes the release of oxygen from the oxy-P450 (4) complex to return into the substrate bound HS Fe^{3+} complex (2). In the *peroxide*

shunt hydrogen peroxide is separated from the hydroperoxo-ferric intermediate (5b) and returns into the Fe^{3+} complex (2). In the *oxidase shunt* the *compound I* is oxidized to water and returns into the Fe^{3+} complex (2), rather than oxidizing the substrate. Apart from the *shunt pathways* cytochrome P450s are always capable of forming reactive oxygen species, such as superoxide anions. These reactions are referred to as „uncoupling“. Electrons needed for those reactions are consumed without hydroxylation of the substrate.

3.3.2.1 Reactions catalyzed by cytochrome P450 enzymes

The broad variety of reactions of P450 enzymes is related to the different main functions they have in mammals. Figure 5 gives an overview of the most important reactions catalyzed by cytochrome P450 enzymes.

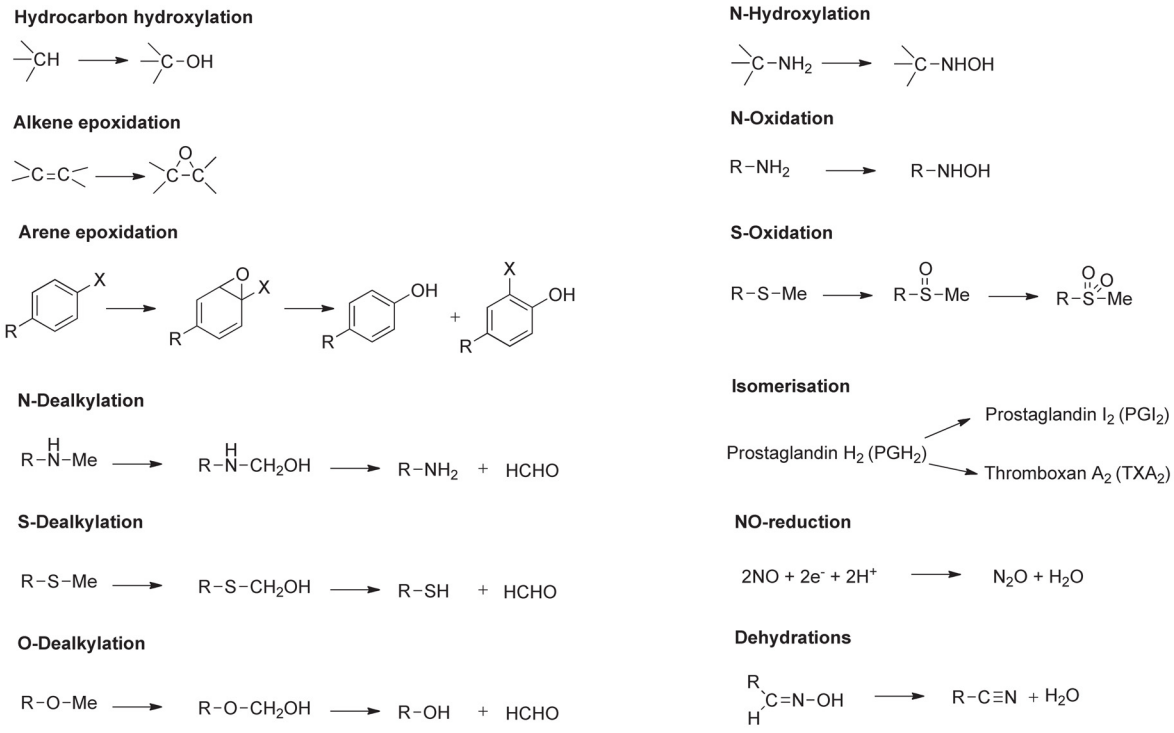

Figure 5: Overview of reactions catalyzed by cytochrome P450 enzymes (modified from Sono et al., 1996)

Those enzymes are responsible for the biotransformation of xenobiotics, the biosynthesis of cholesterol, the biodegradation of cholesterol to bile acid, the synthesis of different steroidal hormones, the inactivation of unsaturated fatty acids and the regulation of intracellular retinoic acid levels. Additionally they are involved in the biosynthesis of different prostaglandines out of the precursor arachidonic acid.

3.3.3 CYP106A2, a model bacterial P450

In 1958 McAleer et al. firstly described the introduction of a 15β-hydroxy group into progesterone by *Bacillus megaterium* ATCC 13368 (McAleer et al., 1958). Almost 20 years later Berg et al. demonstrated that this hydroxylation was performed by a NADPH-dependent cytochrome P450 system (Berg et al., 1979). The electron transport chain in *B. megaterium* consisted of three proteins: a NADPH dependent FMN-containing flavoprotein (megaredoxin reductase), an iron sulfur protein (megaredoxin) and cytochrome $P450_{meg}$ (Berg et al., 1976). The natural electron donor partner proteins of $P450_{meg}$ have never been identified. Nevertheless, its activity can be reconstituted with different electron donor proteins, such as adrenodoxine (Adx) and adrenodoxine reductase (AdR) from bovine adrenal glands (Berg et al., 1979). It was not until 1993 that Rauschenbach et al. were able to clone and completely sequence the gene, which coded for $P450_{meg}$ and its product was named CYP106A2 (Rauschenbach et al., 1993). The gene encoded 410 amino acids and showed activity when expressed in both *E. coli* and *B. subtilis* (Rauschenbach et al., 1993).

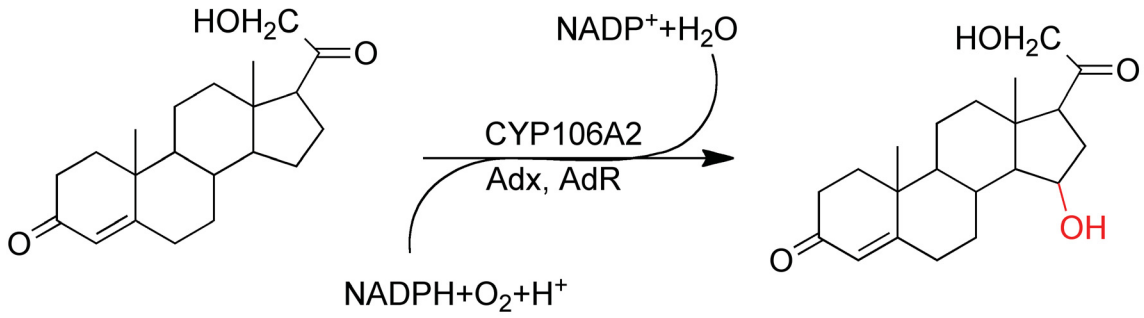

Figure 6: Schematic drawing of the hydroxylation of a 3-oxo-Δ^4-steroid, exemplified by the substrate 11-deoxycorticosterone.

The primary activity of CYP106A2 is the hydroxylation of 3-oxo-Δ^4-steroids, such as progesterone, 11-deoxycorticosterone and testosterone (Fig. 6). In contrast, 3β-hydroxy-Δ^5-steroids are not accepted for conversion. The hydroxylation reaction is mainly carried out in the 15β-position, but other hydroxylation positions have been described as well (Berg et al., 1976; Lisurek et al., 2008).

Even though the natural function of CYP106A2 in *B. megaterium* remains unknown, its ability to hydroxylate various steroids makes it an interesting candidate for industrial purposes. Table 1 gives an overview about substrates tested for CYP106A2 conversion.

Table 1: Substrates of CYP106A2 from *B. megaterium* ATCC 13368

substrate	product	reference
progesterone	6β-hydroxyprogesterone and 15β-hydroxyprogesterone	(Berg et al., 1976; Berg et al., 1979; Kang et al., 2004; Lisurek et al., 2004)
deoxyorticosterone	15β-hydroxydeoxy-corticosterone	(Berg et al., 1976; Berg et al., 1979)
6-fluor-16-methyl-deoxycorticosterone	15β-hydroxy 6-fluor-16-methyl-DOC	(Rauschenbach et al., 1993)
17α-hydroxyprogesterone	15β-17α-dihydroxy-progesterone	(Berg et al., 1976; Berg et al., 1979)
20α-dihydroprogesterone	15β-hydroxy 20α-dihydroprogesterone	(Berg et al., 1976; Berg et al., 1979)
androstendione	15β-hydroxyandrostendione	(Berg et al., 1976; Berg et al., 1979)
testosterone	15β-hydroxytestosterone	(Agematu et al., 2006; Berg et al., 1976; Berg et al., 1979)
corticosterone	15β-hydroxycorticosterone	(Berg et al., 1976; Berg et al., 1979)
aniline	aromatic hydroxylation	(Berg et al., 1979)
imipramine	desipramine	(Berg et al., 1979)
betulinic acid	betulonic acid, 3β-7β-15α-trihydroxy-lup-20(29)-en-28-oic-acid, 3-oxo-11α-hydroxy-lup-20(29)-en-28-oic-acid and 1β-hydroxy-3-oxo-lup-20(29)-en-28-oic-acid	(Chatterjee et al., 2000)
betulonic acid	3-oxo-11α-hydroxy-lup-20(29)-en-28-oic-acid and 1β-hydroxy-3-oxo-lup-20(29)-en-28-oic-acid	(Chatterjee et al., 2000)
4-pregnen-20β-ol-3-on	4 hydroxylated 4-pregnen-20β-ol-3-on species	(Bleif, 2007)

Table 1 (continued): Substrates of CYP106A2 from *B. megaterium* ATCC 13368

substrate	product	reference
17α-methyltestosterone	hydroxylated 17α -methyltestosterone	(Bleif, 2007)
ethisterone	hydroxylated ethisterone	(Bleif, 2007)
4-pregnen-17α,20α,21-triol-3-on	hydroxylated 4-pregnen-17α,20α,21-triol-3-on	(Bleif, 2007)
melengestrol acetate	2 hydroxylated melengestrol acetate species	(Bleif, 2007)
dihydrochinopimaric acid	hydroxylated dihydrochinopimaric acid	(Bleif, 2007)
11-keto-β-boswellic acid	hydroxylated 11-keto-β-boswellic acid	(Bleif, 2007)
abietic acid	12α- and 12β-hydroxy abietic acid	(Bleif et al., 2011)

3.3.4 The human cytochrome P450 3A4

Cytochrome P450 3A4 (CYP3A4) is the most abundant CYP enzyme in humans. It metabolizes more than 50% of all known drugs (Zhang et al., 2005). Interestingly, CYP3A4 is not present in fetal tissue and its role is thought to be carried out by CYP3A7, which acts on a similar range of substrates. By the age of 12 months CYP3A4 is expressed to approximately 70% of adult levels (Johnson et al., 2006). Even though the enzyme is expressed in several tissues, the activity in the liver and small intestine accounts for the conversion of most drugs and xenobiotics (Guengerich, 1999). Human individuals show significant variation in CYP3A4 catalytic activity, and more than 78 nucleotide sequence variations are known (Keshava et al., 2004). The CYP3A4 activity is also known to be induced or inhibited by a number of prescription drugs, which further complicates the prediction of drug-drug interactions. The FDA provides guidelines to simplify this prediction, in which preferrable and acceptable substrates, inhibitors and inducers for in vitro testing of possible CYP3A4 interactions are listed (table 2).

Table 2: **Preferred and acceptable chemical substrates, inhibitors and inducers for in-vitro experiments** (taken from: http://www.fda.gov/Drugs/default.htm)

CYP3A4	preferred	acceptable
inhibitors	ketoconazole	azamulin
	itraconazole	troleandomycin
	-	verapamil
substrates	midazolam	erythromycin
	testosterone	dextromethorphan
	-	triazolam
	-	terfenadine
	-	nifedipine
inducer	rifampin	-

In 1972 Lu et al. characterized properties of an inducible P450 in liver microsomes (Lu et al., 1972). Eight years later an enzyme termed P450p was isolated from the livers of rats, which had been treated with pregnenolone-16α-carbonitrile. In 1985 Watkins et al. not only purified a cytochrome P450 from human liver, but also showed that this enzyme is most probably inducible when the patients are treated with rifampin (Watkins et al., 1985). Only one year later a cDNA fragment was cloned and after sequence determination it was recognized as P450 3A4 (Beaune et al., 1986). Today the entire P450 3A4 gene has been sequenced and the crystal structure of the enzyme was determined (Hashimoto et al., 1993; Williams et al., 2004).

CYP3A4 belongs to the class II of microsomal P450 enzymes. This means that the enzyme needs a membrane surrounding in order to be active. Biochemical studies have been hampered by difficulties in the measurement of its catalytic activities in reconstituted membrane systems. The enzyme can either be used for catalytic reactions in its natural habitat, the endoplasmatic reticulum of the human liver (Jeon et al., 2008), or it needs to be reconstituted with an artificial membrane system, such as micelles, after purification (Shaw et al., 1997), which is time consuming and costly.

While working with mammalian cells Jeon et al. discovered that CYP3A4 could be detected in the soluble cytoplasmic fraction of human liver cells given an N-terminal truncation of the enzyme. The hydrophobic N-terminal region of 20-25 amino acids functions as an signal-recognition-particle-dependent insertion signal and also operates as a stop-transfer signal which anchors the protein to the ER membrane (Sakaguchi et al., 1984). Nevertheless, when large amounts of protein are needed bacterial hosts are preferred over working with mammalian cells in recombinant expression (Gillam et al., 1993). By a truncation of the N-terminus, CYP3A4 could also be expressed in *E. coli* cells at a relatively high level (Gillam et al., 1993).

3.3.5 Biotechnological applications

Since P450 enzymes can accept a wide variety of substrates and are capable of selective oxygenation of C-H bonds, they are of high interest for industrial biocatalysis. Despite their impressive synthetic potential, these enzymes currently enjoy only a very limited biotechnological use due to their limiting complexity and instability.

3.3.5.1 Industrial processes

Table 3 provides an overview of reactions catalyzed by P450 enzymes, which are already implemented in the chemical as well as in the pharmaceutical industry (Zehentgruber, 2009).

Table 3: Industrial applications of cytochrome P450 monooxygenases (modified, according to Zehentgruber, 2009)

reaction	catalysator	product concentration	company	reference
dihydrokaempherol to dihydromyricetin	CYP75A, *Petunia Juss.*	-	Florigene/ Suntory	(Holton et al., 1993)
11-deoxycortisol to cortisol	$P450_{lun}$, *Curvularia lunata*	-	Bayer Schering AG	(Suzuki et al., 1993)
2-phenoxypropionic acid to 2-(4´-hydroxy-phenoxy) propionic acid	*Beauveria bassiana* LU700	7 g/ L	BASF AG	(Dingler et al., 1996)
n-tridecane to α,ω-tridecanicacid	CYP52A1, *Candida tropicalis*	166 g/L	Cognis	(Liu et al., 2004)
amorpha-4,11-dien to artemisinic acid	CYP71AV1, *Artemisia annua L.*	0.12 g/L	Amyris Biotechnol.	(Zeng et al., 2008)
compactin to pravastatine	CYP105A3, *Streptomyces sp. Y 110*	1 g/L	Bristol-Meyer-Squibb, Sankyo Pharma	(Park et al., 2003)
simvastatine to 6β-hydroxy-methylsimvastatine	P450 unknown, *Nocardia autotropica*	0.8 g/L	Merck	(Gbewonyo et al., 1991)
progesterone to 11α-hydroxypro-gesterone	P450 unknown *Rhizopus spec*ies	-	Pharmacia & Upjohn, Pfizer Inc.	(Murray and Peterson, 1952)

In all cases listed reactions were performed in a whole cell biocatalysis system, which is considered to be more suitable than purified enzymes. This is due to the fact that cells contain their own redox-cofactor pool (Urlacher and Eiben, 2006) and an external addition can be circumvented. With the exception of artemisinic acid and dihydromyricetin production, all reactions were performed in organisms where the wildtype already showed the desired activity. To create those two products the genes of the desired cytochrome P450 enzymes were isolated and in the case of artemisinic acid expressed in *E. coli* and *S. cerevisiae* or in the case of dihydromyricetin expressed in carnations.

3.3.5.2 Enzymatic activity and stability

Unfortunately, the group of P450 enzymes show a very low activity compared to other enzymes widely used for industrial biocatalysis. Lipase B out of *Candida antarctica* for example, shows a turnover rate of approximately 6600 min^{-1} (Emond et al., 2010). In contrast, the turnover rate of CYP106A2 from *B. megaterium* is calculated to be approximately 200 min^{-1} (Goni et al., 2009). Microsomal P450s, which can be found in humans, show an even slower turnover rate than bacterial P450 enzymes. CYP3A4 for example only shows a turnover number of 40 min^{-1} (Schmid and Urlacher, 2007).

Apart from their slow activity P450 enzymes rely on an equimolar amount of NAD(P)H for each reaction cycle. This limitation can be overcome by expressing the enzyme in a whole-cell system and making use of its own redox factor pool (Urlacher and Eiben, 2006). Here, however, the reactions carried out by the whole cell biocatalyst are restricted to substrates that can cross the cell membrane (Li et al., 2007). If the substrates are membrane permeable, they or the newly formed products can develop toxicity towards the host cell, in most cases *E. coli* (Urlacher and Eiben, 2006). Additionaly the native enzymes within the host cell could modify or degrade the substrate or product compound in an undesired way, affecting the industrial application. Sometimes the substrate might even be excluded from the cell by an export system to prevent intracellular accumulation, which lowers the efficacy of the whole-cell biocatalyst (Fujii et al., 2009). Most P450 enzymes require proper membrane surroundings to fold into an active form. After the desired enzyme is purified in an elaborate and costly process it needs to be reconstituted into an artificial membrane system for activity.

A new strategy to increase the general stability of enzymes and establish direct contact between them and a substrate is the idea of immobilizing them on the cell surface of either bacteria or yeast (Samuelson et al., 2002). A system with a well

studied secretion mechanism to surface engineer whole cell biocatalysts in Gram-negative bacteria is Autodisplay.

3.4 The Autodisplay system

Autodisplay is defined as the recombinant surface display of a peptide or protein by means of an autotransporter in any Gram-negative bacterium: in most cases *E. coli* (Jose and Meyer, 2007). It was first described in 1997 (Maurer et al., 1997). Autodisplay is based on the type V autotransporter secretion pathway, which was first described in 1987 for the IgA-protease out of *Neisseria gonorrhoeae* (Pohlner et al., 1987). Autotransporters are synthesized as polyprotein precursors. The protein consists of four subdomains and contains the structural requirements necessary for sufficient surface display (Fig. 7A). The exact mechanism of transport across the outer membrane by Autodisplay, remains uncertain. The passenger protein could fold in the periplasm and then be translocated to the cell surface by the aid of the Omp85/ Bam A pathway (Dautin and Bernstein, 2007). However, this is rather unlikely and contradicts recent crystal structures supporting the theory that the protein passes through a 10 Å x 12,5 Å pore in the centre of a β-barrel (Oomen et al., 2004). Since this pore is rather small the protein passes it in an unfolded form and folds extracellularly into its tertiary structure (Ieva and Bernstein, 2009), as originally suggested (Jose et al., 1995; Maurer et al., 1997; Pohlner et al., 1987).

For functional Autodisplay a N-terminally located signal peptide of the cholera toxin β-subunit from *Vibrio cholera* (CTB) is fused to a passenger domain. It functions as a sec-dependent signal recognition particle and aids the way of the precursor across the inner membrane (Henderson et al., 2004). Upon arrival in the periplasm a signal peptidase cleaves off the signal peptide. The C-terminal part of the protein folds as a porin-like structure in the outer membrane, most probably consisting of 14 amphipathic β-strands. The actual passenger is translocated to the cell surface by the aid of this pore, where it folds into its active tertiary structure. A linker between passenger and β-barrel is required to achieve full surface exposure. Both, linker and β-barrel originate from AIDA-I, the adhesin involved in diffuse adherence out of the enteropathogenic *E. coli* strain 2787 (EPEC) (Benz and Schmidt, 1989; Maurer et al., 1997). Figure 7 B illustrates the proposed Autodisplay translocation mechanism.

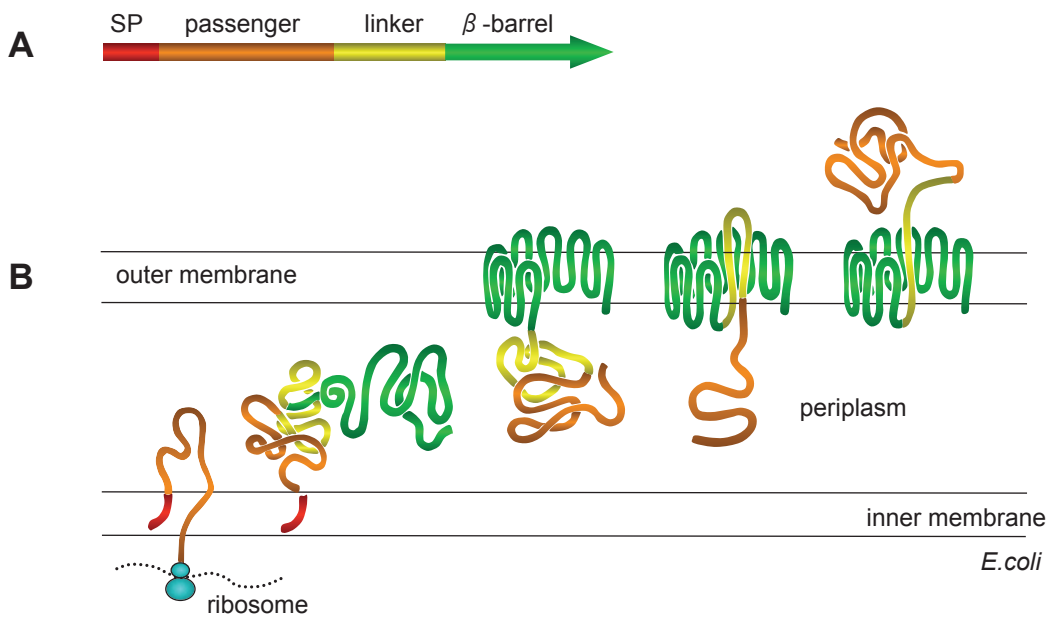

Figure 7: A: Primary structure of an autotransporter fusion-protein. B: Postulated Autodisplay secretion mechanism in *E. coli*: modified, according to Maurer et al., 1997.

Immobilization of the desired passenger in the outer membrane by Autodisplay leads to a better stability and eliminates the need for protein purification. Additionally the protein or peptide can easily be recaptured out of the reaction batch by a simple centrifugation step and separation of the cells displaying the passenger on the cell surface.

By Autodisplay a translocation to the outer membrane of a tremendous number of molecules (> 10^5) is possible (Jose and Meyer, 2007), which is unique among all bacterial surface display systems applied so far. The surface exposure of the recombinant passenger can be demonstrated by several methods, such as an external protease accessibility test (Maurer et al., 1999) and flow cytometry experiments (Petermann et al., 2010).

The passenger domain is variable, so that the initial passenger, the β-subunit of CTB can be replaced by the open reading frame of any other desired protein or peptide, offering a great variety of possible applications for Autodisplay. Peptides can be used to conduct binding studies or as a screening tool for libraries (Jose et al., 2005). The spectrum of enzymes successfully displayed at the cell surface to create a whole cell biocatalyst includes esterases, a sorbitol dehydrogenase, a nitrilase, a NADH-oxidase an isoprenyltransferase, a β-lactamase and a hyaluronidase (Detzel et al., 2011; Jose and von Schwichow, 2004; Kaessler et al., 2011; Kranen, 2011; Kranen et al., 2011; Lattemann et al., 2000; Schultheiss et al., 2002; Schultheiss et al., 2008). A unique

feature of the Autodisplay system is that it is not restricted to monomeric proteins. The AIDA I β-barrel, which serves as a membrane anchor in the outer membrane, is mobile and a spontaneous heteromerization of the displayed passenger molecules is possible. Expression of the homodimeric sorbitol dehydrogenase and the heterotetrameric CK2 lead to functional biocatalysts (Jose and von Schwichow, 2004). The expression of antibody fragments in a variable combinatorial library to screen against a selected target lead to the functional formation of dimers (Blasshofer, 2008). Additionally the surface display of a nitrilase, which is composed of up to 14 subunits was realized (Detzel et al., 2011) proving that Autodisplay provides a powerful tool for the surface display of recombinant proteins.

3.4.1 Autodisplay of enzymes requiring a cofactor

E. coli surface display techniques can also be used to display enzymes requiring a cofactor, such as an iron sulfur cluster [2Fe-2S]. The ferredoxin Adx (14.4 kDa) was successfully exported to the cell surface with Autodisplay. As already observed for SDH, dimeric Adx molecules formed spontaneously on the cell surface (Jose et al., 2002). However, the cells were not active. Electron spin resonance (ESR) measurements conducted with whole cells revealed that the prosthetic group was missing. This lead to the conclusion that apo-Adx was transported in an unfolded state to the cell surface, where it folded into its tertiary structure. Those apo-Adx molecules located on the cell surface could be activated by the incorporation of the iron-sulfur cluster in a one step titration. The titration was conducted under anaerobic conditions and subsequent addition of purified AdR and CYP11A1 yielded a functional whole cell biocatalyst. This was a first hint that the outer membrane of *E. coli* might be sufficient for surface display of a functional P450 enzymes.

The first surface display of a bacterial P450 enzyme on *E. coli* was not achieved by Autodisplay, but with the help of the ice-nucleation protein (Yim et al., 2010), a natural occurring surface protein of *Pseudomonas syringae*. Surface exposure was tested by flow cytometry and activity measurements were performed with photometric assays only. Unfortunately, the translocation mechanism of the ice-nucleation protein is poorly understood and was not investigated, neither for the enzyme, nor for the heme group during this work.

3.5 Aims

3.5.1 Autodisplay of functional P450 enzymes

The goal of the present study was the successful surface display of a bacterial, soluble P450 enzyme, CYP106A2 as a proof of principle, that these enzymes can be translocated to the cell surface of *E. coli* using the Autodisplay system. A sufficient titration method to incorporate the heme group into the surface displayed apoenzym was supposed to be established as well. Interestingly experiments with the autodisplayed CYP106A2 showed that the enzyme was active without the external addition of the heme group. This was rather surprising since previous research showed that autodisplayed Adx was only active after an external titration procedure of the iron sulfur cluster under anaerobic conditions (Jose et al., 2001). Research in the literature gave hints that the outer membrane channel TolC is capable of exporting porphyrins into the supernatant (Tatsumi and Wachi, 2008). TolC is an outer membrane protein in Gram-negative bacteria with a major influence in multidrug efflux and protein export (Fig. 8).

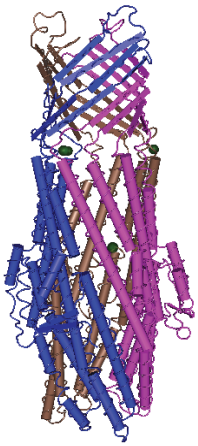

Figure 8: Structure of TolC. Adapted from the protein databank (Berman et al., 2002) and (Koronakis et al., 2000). The structure of TolC was solved to 2.1-Å resolution. TolC consists of three monomers oligomerized to form channel in the outer membrane extending into the periplasm.

Its structure facilitates the direct passage of substrate across the membrane. Recent crystal structures revealed that TolC consists of three monomers forming a β-barrel in the outer membrane. Each monomer of TolC contains four β-strands to form a 12 strand antiparallel β-barrel. An α-helical barrel extends from the outer membrane into the periplasm (Koronakis et al., 2000). Heme belongs to the group of porphyrins and a TolC dependent export seems reasonable.

To elucidate the transport mechanism of the heme group and CYP106A2, surface expression experiments were repeated in an *E. coli* strain lacking the outer membrane channel TolC. **(Publication: 1, 3)**

Subsequently to the Autodisplay of the bacterial CYP106A2, the most abundant human P450 enzyme CYP3A4 was surface displayed, to test the possibility to express a membrane bound enzyme on a bacterial cell surface. The enzyme showed activity, and converted the substrate testosterone into the product 6β-hydroxy testosterone. **(Publication: 2, 3)**

Nevertheless, the approach of using the Autodisplay system for expression of P450 enzymes on the cell surface of *E. coli* is only a first step. To become more attractive for industrial purposes, such as the synthesis of drug metabolites or other biotechnological applications the next step should be the co-expression of the electron supplying enzymes with the P450 enzymes on the surface of a single cell of *E. coli*. In order to facilitate an efficient co-factor regeneration the display of glucose-6-phosphate dehydrogenase on a second cell of *E. coli* and the combination of both cells in one reaction batch should be a goal for future research.

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5 Publication 1: CYP106A2

Published in: „Journal of biotechnology“,

Impact factor: 2.970,

Own proportion of this work: 60 %, 1st author, performance of all experiments, writing of the manuscript

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BIOTEC-6013; No. of Pages 9
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Journal of Biotechnology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Autodisplay of functional CYP106A2 in *Escherichia coli*

Stephanie D. Schumacher[a], Frank Hannemann[b], Mark George Teese[c], Rita Bernhardt[b], Joachim Jose[c,*]

[a] Institut für Pharmazeutische und Medizinische Chemie, Heinrich-Heine-Universität Düsseldorf, D-40225 Düsseldorf, Germany
[b] Institut für Biochemie, Universität des Saarlandes, D-66041 Saarbrücken, Germany
[c] Institut für Pharmazeutische und Medizinische Chemie, Westfälische Wilhelms-Universität Münster, D-48149 Münster, Germany

ARTICLE INFO

Article history:
Received 7 October 2011
Received in revised form 7 February 2012
Accepted 29 February 2012
Available online xxx

Keywords:
Autodisplay
Cytochrome
P450 106A2
Biocatalysis
Surface display
Escherichia coli

ABSTRACT

Cytochrome P450 enzymes catalyse a wide variety of reactions, including the hydroxylation and epoxidation of C—C bonds, and dealkylation reactions. There is high interest in these reactions for biotechnology and pharmaceutical processes. Many P450s require membrane surroundings and have substrates that do not cross biological membranes. To circumvent these obstacles, CYP106A2 from *Bacillus megaterium* was expressed on the outer membrane of *Escherichia coli* cells by Autodisplay. Exposure on the surface was confirmed by a protease accessibility test and flow cytometry after immunolabelling. HPLC assays showed that 0.5 ml of cells displaying the enzyme (OD_{578} = 6) converted 9.13 μmol of deoxycorticosterone to 15β-OH-deoxycorticosterone within 1 h. Imipramine and abietic acid were also accepted as substrates. The number of active enzyme molecules per cell was calculated to be 20,000. Surprisingly, surface-exposed CYP106A2 was active in *E. coli* BL21 without the external addition of the heme group. However, when CYP106A2 was expressed on the surface of an *E. coli* strain lacking the TolC channel protein (JW5503), enzymatic activity was almost completely abolished. The activity of CYP106A2 on the surface of *E. coli* JW5503 could be restored by the external addition of the heme group. This suggests, as has been reported before, that *E. coli* uses a TolC-dependent mechanism to export heme into the growth media, where it can be scavenged by a surface-displayed apoenzyme. Our results indicate that Autodisplay enables the functional surface display of P450 enzymes and provides a new platform to access their synthetic potential.

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1. Introduction

Cytochrome P450s (P450s or CYPs) are a unique family of ubiquitously distributed enzymes that play a major role in primary and secondary metabolic pathways and drug degradation (O'reilly et al., 2011; Urlacher and Eiben, 2006). They are heme-containing monooxygenases which can catalyse the insertion of a single atom of molecular oxygen into a substrate while at the same time reducing a second atom to water (Meunier et al., 2004).

Despite the versatility of reactions carried out by P450 enzymes, their broad use in biotechnology is still limited by several technical problems. Firstly, the vast majority of P450s require membrane contact or a membrane environment to fold into an active form. This limits the amount of enzyme that can be obtained through heterologous expression, and complicates the purification of the expressed enzymes. Secondly, the purified enzymes often show poor stability and may need to be reconstituted into an artificial membrane system for reliable activity (Myasoedova and Arutyunyan, 2007). A simpler and cheaper approach is the use of an expression host such as *Escherichia coli* as a whole cell biocatalyst. For most biocatalysts the enzyme is located within the cell, restricting the reactions to substrates that can cross the cell membrane (Li et al., 2007). A second problem associated with most whole cell biocatalysts is the presence of native *E. coli* enzymes within the cell. These enzymes could modify the substrate or product compound in an undesired way, affecting drug metabolism studies or synthesis applications. Furthermore, cells can also contain export systems that prevent the intracellular accumulation of substrates (Fujii et al., 2009), lowering the efficiency of the whole-cell biocatalyst.

To circumvent these obstacles, the desired P450 can be expressed recombinantly and displayed on the surface of the *E. coli* cell. This strategy eradicates the need for expensive enzyme purification steps, establishes direct contact between substrate and enzyme and results in the immobilisation of the protein in membrane surroundings (Samuelson et al., 2002). The bacterial P450 BM3 has been displayed on the surface of *E. coli* using the ice-nucleation protein from *Pseudomonas syringae* (Yim et al., 2006), a system whose transport mechanism is poorly understood. A simple, proven method with a well studied secretion mechanism to surface engineer whole cell biocatalysts is Autodisplay (Jose and

* Corresponding author. Tel.: +49 251 83 32210; fax: +49 251 83 32211.
E-mail address: joachim.jose@uni-muenster.de (J. Jose).

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doi:10.1016/j.jbiotec.2012.02.018

Please cite this article in press as: Schumacher, S.D., et al., Autodisplay of functional CYP106A2 in *Escherichia coli*. J. Biotechnol. (2012), doi:10.1016/j.jbiotec.2012.02.018

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Meyer, 2007; Maurer et al., 1997). The Autodisplay system provides the possibility to transport a protein, such as a recombinant or natural passenger, to the outer membrane, so long as its coding region lies between a typical signal peptide and a C-terminal "β-barrel" domain (Jose, 2006). It is based on AIDA-I, the adhesin involved in diffuse adherence in enteropathogenic *E. coli* (Benz and Schmidt, 2011), which belongs to the autotransporter family of proteins (Jose et al., 1995). Enzymes expressed using Autodisplay for functional whole cell biocatalysis include esterases, sorbitol dehydrogenase, a nitrilase, an isoprenyltransferase, a β-lactamase and a hyaluronidase (Detzel et al., 2011; Jose and von Schwichow, 2004; Kaessler et al., 2011; Kranen et al., 2011; Lattemann et al., 2000; Schultheiss et al., 2002, 2008). Additionally the human membrane bound CYP3A4, which is very difficult to express in a soluble form, was displayed in an active form on the surface of *E. coli* (Schumacher and Jose, in press).

E. coli surface display techniques have also been successfully used to display enzymes which require an iron–sulfur group. The iron–sulfur protein bovine adrenodoxin was exported to the cell surface with autodisplay (Jose et al., 2001). Electron spin resonance (ESR) measurements conducted with whole cells revealed that the protein lacked the iron–sulfur prosthetic group which was later incorporated by a one step titration under anaerobic conditions. The external addition of CYP11A1 and AdR resulted in a whole cell catalyst for the conversion of cholesterol to pregnenolone.

CYP106A2 is a monooxygenase that was originally isolated from the Gram positive bacterium *Bacillus megaterium* ATCC 13368. It belongs to the class I of bacterial P450 enzymes (Hannemann et al., 2007). The original function of this protein in *B. megaterium* remains unknown, as well as the natural electron partner required for its activity within the cell. It is possible to substitute the bovine proteins adrenodoxin (Adx) and adrenodoxin reductase (AdR) for the natural electron transfer partners, and achieve reasonable amounts of CYP106A2 activity in vitro (Berg et al., 1979a,b). The enzyme has been shown to catalyse the 15β-hydroxylation of several steroids including deoxycorticosterone (Berg et al., 1979a,b). It can also catalyse the hydroxylation of a steroidal compound, diterpene abietic acid, and *N*-demethylation of the antidepressant imipramine (Berg et al., 1979a,b; Bleif et al., 2011). The enzyme has been successfully overexpressed in *E. coli* for use in biotechnology and is one of the P450 enzymes which is active in a soluble form (Rauschenbach et al., 1993), making it a logical candidate to test P450 expression on the cell surface.

In this study, we show that Autodisplay can be used for the surface expression of a bacterial P450 enzyme, leading to the development of a whole cell biocatalyst. The bacterial CYP106A2 enzyme was successfully transported to the surface of *E. coli*, where it showed excellent catalytic activity against the aforementioned substrates.

2. Material and methods

2.1. Materials

11-Deoxycortisol (RSS), deoxycorticosterone, imipramine and abietic acid were purchased from Sigma–Aldrich (Steinheim, Germany). β-Nicotinamide adenine dinucleotide phosphate (NADPH) and hemin chloride were purchased from Carl Roth (Karlsruhe, Germany). Goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) was obtained from Bethyl (Montgomery, AL, USA). The restriction endonucleases were purchased from New England Biolabs (Ipswich, MA, USA). Polystyrene microplates were purchased from Greiner Bio-One (Solingen, Germany). The λ*(DE3)* lysogenisation kit was obtained from Novagen (Darmstadt, Germany).

2.2. Bacterial strains and growth conditions

Expression experiments were conducted with two *E. coli* strains containing the DE3 cassette, for high level inducible transcription of the autotransporter gene under the control of the T7 promoter. BL21 (DE3) (*B*, F^-, *dcm*, *ompT*, *lon*, *hsdS*(rB^- mB^-), *gal*, λ*(DE3)*) and JW 5503 (F-, *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *ΔtolC732::kan*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*) were used, as well as JW 5503, a TolC knockout strain from the Keio collection (Baba et al., 2006), which was distributed by Coli Genetic Stock Center at Yale University, USA. We used a λ*(DE3)* lysogenisation kit to insert the DE3 cassette into JW 5503, according to the manufacturer's instructions. Cells were routinely grown at 37 °C in lysogeny broth (LB) medium, containing 50 mg L^{-1} of carbenicillin, 10 μM ethylenediaminetetraacetate (EDTA) and 10 mM 2-mercaptoethanol.

2.3. Plasmid construction

The plasmid pET-CYP13 was constructed for the expression of the CYP106A2 autotransporter fusion protein under the control of the T7 promoter. The gene encoding CYP106A2 (GenBank accession no. Z21972) was amplified by polymerase chain reaction (PCR) from plasmid pKKHC-MEG (Simgen et al., 2000). The PCR product was digested by the restriction enzymes *XhoI* and *KpnI* and ligated into plasmid pET-SH7 (Petermann et al., 2010). The resulting plasmid pETCYP13 encoded a fusion protein comprising of the CtxB signal peptide, CYP106A2 as a passenger, the autotransporter linker region and the autotransporter β-barrel (Fig. 1). The plasmid was transformed into BL21 (DE3) and JW 5503 (DE3) by electroporation-mediated transformation according to standard techniques (Sambrook and Russell, 2001), and the inserted gene was fully sequenced before use in expression experiments.

2.4. Outer membrane preparation and protein accessibility test

E. coli cells were grown overnight in LB medium and 200 μl were used to inoculate a 20 ml culture. Cells were cultivated at 37 °C with vigorous shaking (200 rpm) until an OD_{578} of 0.5 was reached. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, Roth, Karlsruhe, Germany) to reach a final concentration of 1 mM. After 60 min at 30 °C, induction was stopped by harvesting the cells and washing them with buffer (0.2 M Tris–HCl, pH 8). Outer membrane proteins were prepared according to the rapid isolation protocol of Hantke (1981) with modifications as described previously (Jose and von Schwichow, 2004). A protein accessibility test was used to confirm incorporation of the fusion protein in the outer membrane, based on the protease mediated degradation of the surface-displayed protein (Maurer et al., 1997). *E. coli* cells were harvested, washed and resuspended in phosphate buffered saline (PBS, pH 7.4). Proteinase K was added to a final concentration of 0.2 mg L^{-1} and cells were incubated for 60 min at 37 °C. Digestion was stopped by washing the cells three times with PBS containing 10% fetal calf serum (FCS). After the proteinase K digestion, outer membrane proteins were isolated as described above.

2.5. SDS-PAGE

Proteins isolated from the outer membrane protein were resuspended in two-fold sample buffer (100 mM Tris–HCl, pH 6.8 containing 4% sodium dodecyl sulfate, 0.2% bromophenol blue, 20% glycerol, and 50 mg dithiothreitol). The samples were boiled for 5 min at 95 °C and proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) with a 12.5% acrylamide resolving gel. Proteins were stained with Coomassie Brilliant Blue, and the molecular

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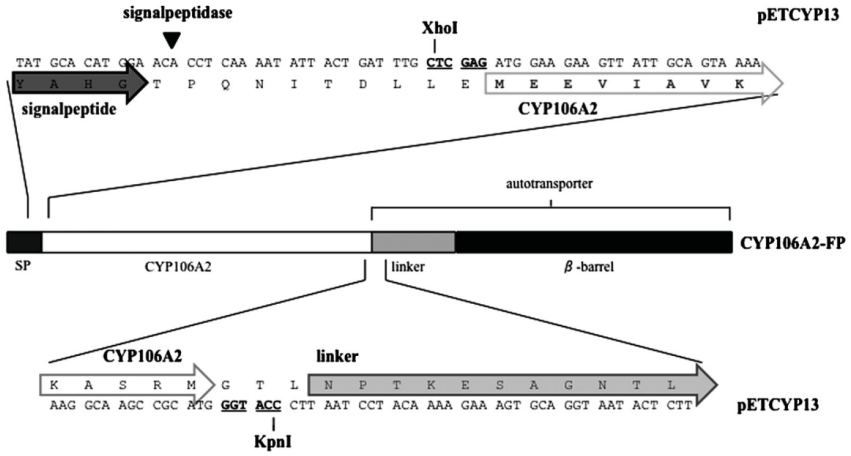

Fig. 1. Structure of the CYP106A2 fusion protein encoded by pETCYP13. Cloning sites are expanded to show DNA and amino acid sequences. Restriction sites added due to the cloning procedure are underlined. The white arrows indicate the boundaries of the passenger domain. The C-terminus of the signal peptide is represented by the dark grey arrow and the light grey arrow depicts the N-terminal region of the autotransporter linker. The signal peptidase is expected to cleave off the signal peptide (SP) at the indicated position after transport across the inner membrane.

weight estimated using a prestained marker as a standard (Fermentas, St. Leon-Rot, Germany).

2.6. Flow cytometer analysis

E. coli cells were grown as described above. Expression of CYP106A2 was induced at an early exponential growth phase (OD_{587} of 0.5) by adding IPTG to a final concentration of 1 mM. Cells were then incubated for 60 min at 30 °C, 200 rpm. Cells were harvested by centrifugation, washed twice with PBS and resuspended to a final OD_{578} of 0.5. 100 μl of the cell suspension was suspended in 500 μl of PBS containing 3% bovine serum albumin (BSA, filter-sterilised), and incubated for 10 min at room temperature (RT). Cells were then pelleted in a microcentrifuge (60 s at 18,000 × *g*, Hettich, Tuttlingen, Germany), resuspended in 100 μl of a solution that contained a polyclonal CYP106A2 antibody (diluted 1:10,000 in PBS + 3% BSA) and incubated for another 30 min at RT. Cells were then washed twice with 500 μl of PBS. The second incubation step was conducted in the dark (30 min, RT) using 100 μl of a goat anti-rabbit IgG antibody conjugated with FITC (diluted 1:25 in PBS [pH 7.4] + 3% BSA). After washing twice in PBS supplemented with 3% BSA, the cell pellet was resuspended in 1.5 ml of PBS. Samples were then analysed using a CyFlow space flow cytometer (Partec GmbH, Münster, Germany) at an excitation wavelength of 488 nm.

2.7. Protein purification

CYP106A2 was expressed in *E. coli* and purified as previously described (Lisurek et al., 2004; Simgen et al., 2000). Protein concentration was estimated by CO-difference spectra of the reduced heme using ε_{450} = 91 mM cm^{-1}. Recombinant adrenodoxin (Adx) and adrenodoxin reductase (AdR) were purified as previously described (Hannemann et al., 2002) and concentrations were calculated using ε_{414} = 9.8 mM cm^{-1} for Adx and ε_{450} = 11.3 mM cm^{-1} for AdR.

2.8. Assay for the hydroxylation of 11-deoxycorticosterone

The enzyme reaction was carried out in a volume of 500 μl in 50 mM Hepes buffer pH 7.4 containing 0.05% Tween20 and 1 mM magnesium chloride. The reaction contained either 0.25 μM of purified, holo CYP106A2 enzyme or *E. coli* pETCYP13 cells at a final OD_{578} of 6 (determined to be approximately 1.5 × 10^8 CFU ml^{-1}). Other substances added to the reaction included 5 μM of Adx, 0.5 μM of AdR, 5 mM of glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase (where a unit is described as the amount of enzyme that will reduce 1.0 μmol of NADP per min at 30 °C, pH 7.8). 50 μmol of 11-deoxycorticosterone were dissolved in ethanol and added to the enzymes, with final ethanol concentrations not exceeding 0.5%. The reaction was initiated by the addition of 180 μM NADPH, proceeded for 60 min at a temperature of 37 °C and was terminated by the addition of 500 μl chloroform. As an internal standard we prepared a 1 mM stock solution of Reichstein's compound S (RSS) in ethanol, and added 2 μl of this was added after termination of the reaction. Samples were extracted twice with chloroform and the layers were separated by centrifugation (60 s at 18,000 × *g* with a microfuge). The organic phases were combined and the solvents removed under vacuum. The dried samples were resuspended in 100 μl of acetonitrile for HPLC analysis (described below).

2.9. Reconstitution of CYP106A2 with hemin

The reconstitution procedure was performed essentially as previously described for cytochrome $P450_{scc}$ (Pikuleva et al., 1992). Cells at an OD_{578} of 10 were resuspended in 16 ng ml^{-1} solution of hemin chloride in 0.1 M His (pH 7.2) containing 20% (v/v) glycerol. After reconstitution, cells were resuspended in 50 mM Hepes buffer pH 7.4 containing 0.05% Tween20 and 1 mM magnesium chloride and the hydroxylation assay was carried out as described above.

2.10. Analytical methods

11-Deoxycorticosterone (DOC) conversion was analysed by HPLC using a reversed phase C18 column with a pore size of 4 μm, a diameter of 3.9 mm and length of 150 mm (Pak Nukleosil, Waters, Milford, MA, USA). Later experiments to investigate heme transport were conducted with a LiChrospher 60 RP-select B column 125-4 (5 μm) from Merck (Darmstadt, Germany) used on a LaChrom Elite System equipped with a diode array detector L-2455. The mobile

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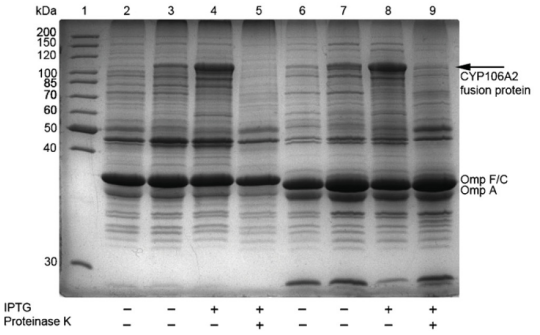

Fig. 2. Expression and surface display of CYP106A2. Outer membrane proteins were isolated from *E. coli* cells as described in materials and methods. Proteins were separated by SDS-PAGE with a 12.5% acrylamide gel, and stained with Coomassie Brilliant Blue G250. Lane 1 contains the molecular weight marker. Surface display is shown for two *E. coli* strains containing the DE3 cassette for IPTG-inducible expression with a T7 promoter, BL21 (DE3) (lanes 2–5) and a strain lacking TolC, JW 5503 (DE3) (lanes 6–9). Lanes 2 and 6: negative control cells (lacking a plasmid). Lanes 3 and 7: cells containing the plasmid for the autodisplay of CYP106A2 (pETCYP13) but without IPTG. Lanes 4 and 8: cells containing the plasmid for the autodisplay of CYP106A2 (pETCYP13) and IPTG for the induction of protein expression. Lanes 5 and 9: cells containing the plasmid for the autodisplay of CYP106A2 (pETCYP13) and IPTG for the induction of protein expression, incubated with proteinase K.

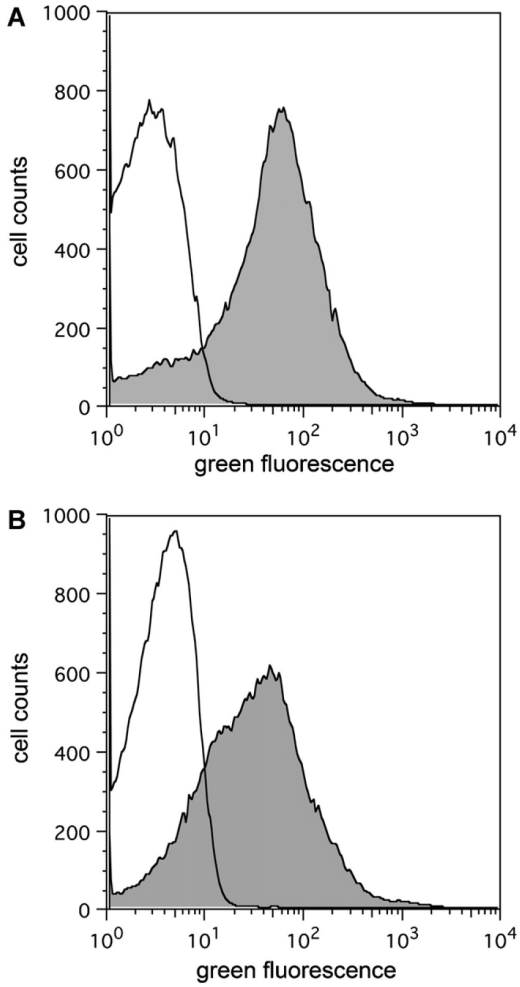

Fig. 3. Flow cytometry analysis of CYP106A2 displaying cells. Cells of *E. coli* were analysed after incubation of the samples with two antibodies, a primary polyclonal anti-CYP106A2 antibody and a secondary FITC- labeled detection antibody. The mean fluorescence (mF) was determined for all four samples; (A): black: BL21 (DE3) cells without plasmid, mF = 3.43; grey: cells displaying CYP106A2 on the surface (containing pETCYP13), mF = 87.76; (B): black: JW 5503 (DE3) TolC negative cells without plasmid (negative control), mF = 4.41; grey: JW 5503 (DE3) TolC negative cells cells displaying CYP106A2 on the surface (containing pETCYP13), mF = 75.51.

phase consisted of acetonitrile and water in a ratio of 40:60 (v:v). The flow rate was 1 ml min^{-1}. The amount of product, 15β-OH-deoxycorticosterone, was determined by comparing the relative peak area to the internal standard, RSS.

2.11. NADPH oxidation assays

A continuous microplate assay was used to investigate the activity of the autodisplayed CYP106A2 against imipramine and abietic acid. The reaction with the whole cells displaying CYP106A2 was performed as described above for the hydroxylation assay. For the analysis of *E. coli* cells expressing CYP106A2 (containing the plasmid pETCYP13), protein expression was induced with IPTG as described above, and cells were resuspended to an OD_{578} of 2.5. The substrates imipramine or abietic acid were dissolved in ethanol and added to achieve a final concentration of 200 μM in a final volume of 200 μl. The final ethanol concentration in the assay did not exceed 1%. The assay was initiated by the addition of 200 μM NADPH. The decrease in the amount of NADPH was detected by measuring absorption at 340 nm with a Mithras LB 940 microplate reader (Berthold Technologies, Bad Wildbad, Germany). NADPH oxidation rates were calculated using $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$.

3. Results

3.1. Display of CYP106A2 on the surface of E. coli

To obtain the CYP106A2 enzyme on the outer membrane of *E. coli*, we created a fusion protein where the enzyme was linked to the C-terminal domain of an *E. coli* autotransporter protein. The corresponding artificial gene contained the sequences encoding an N-terminal signal peptide, the CYP106A2 enzyme, a linker domain and the β-barrel responsible for outer membrane translocation (Fig. 1). The plasmid containing this construct, pET-CYP13, was derived from pKKHC-MEG (Simgen et al., 2000), and contained the T7 promoter for the high-level inducible expression in *E. coli* strains containing the DE3 cassette (Studier and Moffatt, 1986). After transformation of *E. coli* BL21 (DE3) and JW 5503 (DE3) with the plasmid and induction with IPTG, SDS-PAGE of the outer membrane fractions revealed a protein band with an apparent molecular weight of 100 kDa (Fig. 2, lanes 4 and 8), as expected for the CYP106A2 autotransporter fusion protein. A small amount of this protein was visible from cells where protein expression was not induced, consistent with "leaky" expression of the T7 promoter in *E. coli* BL21 (DE3). After induction, the average amount of fusion protein visualised by SDS-PAGE was similar to the natural outer membrane proteins OmpF or OmpC, and roughly three times as great as OmpA (Fig. 2, lanes 4 and 8).

To find out whether the CYP106A2 domain of the fusion protein was indeed exposed at the cell surface, proteinase K was added to whole cells of *E. coli* expressing the fusion protein. Proteinase K cannot cross the cell envelope of *E. coli*, and therefore the degradation of the fusion protein is strong evidence that it is accessible at the

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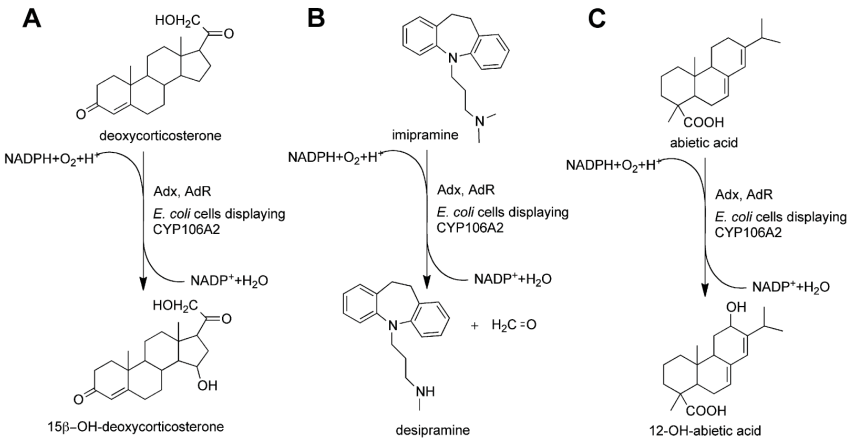

Fig. 4. CYP106A2 mediated conversion reactions. (A): hydroxylation in the 15β- position of the steroid 11-deoxycorticosterone (DOC). (B): N-demethylation of the antidepressant imipramine. (C): hydroxylation in the 12- position of abietic acid. All reactions are catalysed by CYP106A2 displayed on the surface of *E. coli* cells. Redox equivalents are transferred from NADPH via the proteins AdR and Adx to the converting enzyme.

cell surface (Fig. 2, lanes 5 and 9). As a control, we also examined the molecular weight of OmpA, which has a N-terminal extension in the periplasm that is susceptible to proteinase K cleavage. The lack of OmpA digestion (Fig. 2, lanes 5 and 9) is a strong indication that proteinase K had not entered the periplasm.

Flow cytometry experiments were conducted to confirm that CYP106A2 was located at the bacterial surface of *E. coli* BL21 (DE3) and JW 5503 (DE3) and that the lack of the TolC channel does not hamper the transport of the enzyme to the cell surface. Cells with and without the CYP106A2-containing plasmid, pETCYP13, were incubated with a polyclonal serum against CYP106A2. Assuming that the antibodies are too large to cross the outer membrane, the specific binding of the anti-CYP106A2 antibody to the cell surface proves that the proteins are displayed at the cell surface. After washing steps the cells were incubated with a goat anti-rabbit IgG conjugated to FITC and then analysed by flow cytometry. The mean fluorescence of cells expressing the CYP106A2 fusion protein was approximately 25 times higher in BL21 (DE3) and 17 times higher in JW 5503 (DE3) (Fig. 3) than their respective controls (cells without the plasmid). This indicates that the CYP106A2 domain was accessible at the cell surface and, as a consequence, surface display of the P450 enzyme by Autodisplay in *E. coli* was successful.

3.2. Hydroxylation assay

To measure whether CYP106A2 was expressed on the surface in an active form, activity assays were performed. For that purpose cells were prepared as described above and incubated with the 11-deoxycorticosterone substrate for one hour to form the product 15β-OH-deoxycorticosterone (Fig. 4(A)). When cells displaying CYP106A2 at the surface were incubated with 11-deoxycorticosterone, HPLC analysis revealed a product peak at approximately 2 min (Fig. 5(C)). This corresponded to the retention time of the 15β-hydroxylated product produced by the purified enzyme (Fig. 5(A)), indicating that the autodisplayed CYP106A2 was active. There was a small amount of 15β-hydroxylase activity from *E. coli* cells without IPTG for induction of protein expression (Fig. 5(B), Fig. S1), consistent with the "leaky" expression of CYP106A2 fusion protein detected by SDS-PAGE (Fig. 2). The purified enzyme produced a second product peak at an approximate retention time of 1.5 min (Fig. 6(A)). LC–MS analysis suggests this is a hydroxylated version of the first product 15β-OH-deoxycorticosterone (see Supplementary data).

The hydroxylation activity of autodisplayed CYP106A2 was dependent on the external addition of adrenodoxin (Fig. 5(D)), a 15 kDa molecule that is too large to passively enter the cell envelope. Similarly, the activity was completely dependent on the externally applied NADPH (data not shown). Studies of NADPH transport suggest that this molecule also does not cross cellular membranes (Gholson et al., 1969). The dependence of the hydroxylation activity on the external addition of these compounds is further proof that all conversions took place outside the cell, and that none of the observed product was produced by intracellular P450 enzymes (Fig. 5(D)).

The exact number of CYP106A2 molecules on the surface remains unclear, however the amount of catalytic activity was equivalent to an average of ~20,000 enzyme molecules per cell. This was calculated using a standard curve of product (measured by the area under the curve) against the amount of purified enzyme (in nmol) used in the reaction. The rate of turnover by the fusion protein was assumed to be equal to the rate of turnover of the purified enzyme, and the number of colony forming units was assumed to be the number of cells in the assay. This approximation is supported by a visual analysis of the SDS-PAGE (Fig. 2). The intensity of the band corresponding to the fusion protein was approximately 3-fold higher than the band corresponding to OmpA. After accounting for differences in molecular weight (MW of OmpA = 35 kDa, MW of fusion protein = 100 kDa), this suggested that the average number of fusion molecules per cell was similar to OmpA, which has previously been estimated to be 100,000 (Cole et al., 1982). We consider the estimation derived from catalytic activity (20,000 molecules per cell) to be more reliable, although the assumption that the fusion protein and purified CYP106A2 have equal turnover rates needs to be tested. Nevertheless, the display of molecules in a number between 20,000 and 100,000 on the surface of each *E. coli* cell is consistent with previous studies using Autodisplay (Jose and Meyer, 2007).

We used a standard curve based on the substrate conversion of the pure enzyme to quantify conversion rates by the whole cell biocatalyst. It was calculated that 9.13 μmol of the substrate could be hydroxylated within 60 min by 0.5 ml of cells displaying CYP106A2 (OD_{578} = 6) to form the product 15β-OH-deoxycorticosterone.

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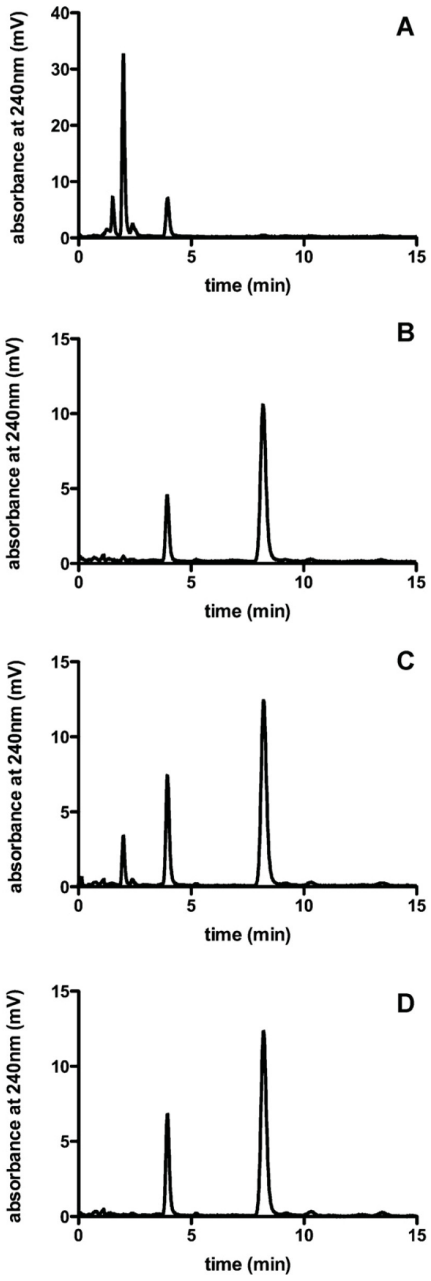

Fig. 5. HPLC chromatograms showing CYP106A2 mediated conversion of 11-deoxycorticosterone to 15β-deoxycorticosterone. Conversion of 11-deoxycorticosterone into 15β-deoxycorticosterone by the purified enzyme and cells displaying the enzyme at the surface. Substrate was incubated with either pure enzyme or cells for 60 min with the required cofactors Adx and AdR, as described in the materials and methods section. (A): purified enzyme, (B): cells without the induction of protein expression (BL21 (DE3) pET-CYP13 – IPTG), (C): cells with the induction of protein expression (BL21 (DE3) pET-CYP13 + IPTG), (D): control to confirm that only surface-expressed enzyme contributed to product formation, consisting of cells with the induction of protein expression, incubated with the substrate without the addition of the electron transfer partner Adx (BL21 (DE3) pET-CYP13 + IPTG – Adx). At the retention time of 4 min the peak of the internal standard RSS appears. The peak at 8 min belongs to the substrate DOC. Incubation of the substrate with the pure enzyme showed a clear product peak at a retention time of 2 min. A smaller amount of this product was visible when the substrate was incubated with cells expressing CYP106A2 at the surface. When the electron transfer partner adrenodoxin (Adx) was omitted from this reaction, no activity was seen.

3.3. Heme transport

We were surprised to find that the activity of CYP106A2 at the cell surface did not require the external addition of a heme group. Previous research by our group found that the external addition of an iron–sulfur cluster was necessary for the activity of autodisplayed adrenodoxin (Jose et al., 2002).

The exact mechanism of transport across the outer membrane by Autodisplay, which requires the AIDA-I β-barrel (Suhr et al., 1996), remains uncertain. However recent structural studies support the theory that the protein passes through the centre of the autotransporter β-barrel in an unfolded form (van den Berg, 2010), as originally suggested (Jose et al., 1995; Maurer et al., 1997; Pohlner et al., 1987). According to this theory, the porphyrin cannot be co-transported with the passenger to the surface, and must be obtained by some other mechanism. Tatsumi and Wachi (2008) have recently provided evidence of a TolC-dependent efflux system in *E. coli* that transports porphyrins into the supernatant. The CYP106A2 apo-protein might therefore fold at the cell surface and scavenge the porphyrin group from the supernatant.

To determine if the TolC-dependent efflux system is reponsible for the heme export, we repeated the CYP106A2 surface expression in *E. coli* JW 5503 (DE3), which lacks the TolC transporter. SDS-PAGE of proteins isolated from the outer membrane suggested an equal amount of protein expression in both the TolC positive and negative strains (Fig. 2). FACS measurements with a primary antibody against CYP106A2 revealed a similar fluorescence level in BL21 (DE3) and JW 5503 (DE3) cells expressing CYP106A2 at the surface (Fig. 3). This not only confirmed that the amount of CYP106A2 at the surface was similar in both strains, but more importantly, that the lack of the TolC channel does not hamper the transport of the enzyme to the cell surface. However after induction of protein expression in these cells, the amount of the 15β-hydroxylated product produced by TolC negative cells (Fig. 6(B), line 2) was significantly lower than in TolC positive cells (Fig. 6(B), line 1). To test whether the heme group was indeed scavenged from the supernatant and then incorporated into the enzyme, we added hemin chloride externally to the cultures of *E. coli* JW 5503 (DE3) expressing CYP106A2 on the surface. This procedure rescued CYP106A2 activity (Fig. 6(C)).

3.4. NADPH oxidation assay

Imipramine is known to be converted by non-recombinant forms of CYP106A2 (Berg et al., 1979a,b) (Fig. 4(B)). To determine if autodisplayed CYP106A2 could also carry out this reaction, we measured the decrease in NADPH in the presence of imipramine via absorbance at 340 nm, as previously established for the Autodisplay of sorbitol dehydrogenase (Jose and von Schwichow, 2004). The decrease in absorbance at 340 nm by cells expressing CYP106A2 at the surface was dependent on the presence of substrate and NADPH (Fig. 8), suggesting that imipramine was indeed converted to desipramine as previously described for the pure enzyme. The decrease in absorbance in samples without substrate addition was not unexpected, as NADPH is susceptible to oxidation (Chave et al., 1982; Olek et al., 2004). The activity towards imipramine could be confirmed by measuring the generation of formaldehyde, a side product of the reaction which is formed in equimolar ratio

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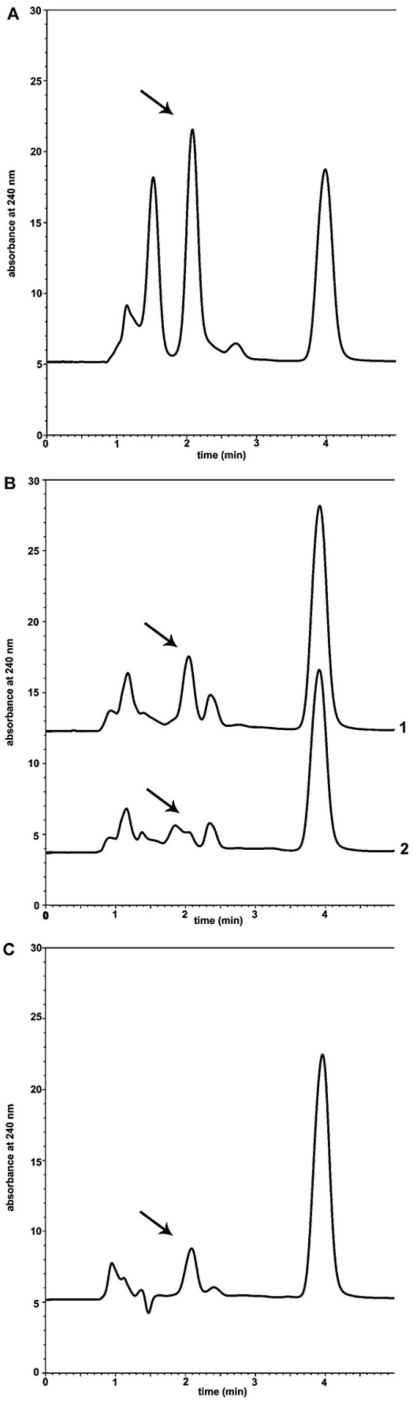

Fig. 6. Effect of TolC on the conversion of 11-deoxycorticosterone to 15β-deoxycorticosterone by the whole cell biocatalyst. The plasmid encoding the CYP106A2 autodisplay fusion enzyme was inserted into cells lacking the TolC channel protein (JW 5503). Protein expression by cells was induced by the addition of IPTG. Substrate was incubated with the cells for 60 min with the required cofactors Adx and AdR, as described in the materials and methods section. The product formation was measured by HPLC. (A) Positive control (purified CYP106A2 enzyme), (B): 1: TolC positive cells (BL21 (DE3) pETCYP13), 2: TolC negative cells (JW 5503 (DE3) pETCYP13), (C): 1: TolC negative cells (JW 5503 (DE3) pETCYP13) reconstituted with hemin chloride. The peak at the retention time of 4 min is the internal standard Reichstein's compound S. The major product, 15β-OH-deoxycorticosterone, was seen at a retention time of 2 min (indicated with an arrow). The amount of product produced by surface displayed CYP106A2 was lower in the *E. coli* strain lacking TolC than the control strain. To determine whether this was due to the lower amount of heme exported by TolC into the growth media, we tested whether the activity could be reconstituted by complementation with the heme prosthetic group. The addition of hemin chloride resulted in an increased turnover of the substrate by CYP106A2 in comparison to the control cells without heme addition.

with desipramine (not shown). For this purpose, formaldehyde was determined by a chromotropic acid assay according to the Pharmacopoea Europaea (Bricker and Johnson, 1945; EDQM, 2008)

A second substrate, abietic acid (Fig. 4(C)), was also associated with a decrease in NADPH levels, and we confirmed the formation of the product 12-OH-abietic acid by HPLC analysis (data not shown). We estimated the turnover of these two substrates by the whole cell biocatalyst from the decrease in absorbance at 340 nm using an area in the linear range (between 20 and 40 min) after accounting for background oxidation (cells without substrate). Using the molar coefficient of NADPH at 340 nm, enzymatic activity of 0.66 μmol min^{-1} was calculated for imipramine and 0.15 μmol min^{-1} for abietic acid.

4. Discussion

In this study, CYP106A2 from the Gram positive bacterium *B. megaterium* was displayed on the *E. coli* cell surface by the autotransporter pathway. Surprisingly, we found that autodisplayed CYP106A2 was active without the addition of an external heme. If the protein is transported across the outer membrane in a folded form, the heme could be incorporated in the periplasm, as described for heme-containing proteins located intracellularly in *E. coli* (Goldman et al., 1996). Some autotransporter passengers may fold in the periplasm, and be transported across the outer membrane in a folded form with the aid of the Omp85/Bam A pathway (Dautin and Bernstein, 2007). However, there is strong evidence that autotransporter passenger proteins are transported to the surface in an unfolded form and fold extracellularly (Ieva and Bernstein, 2009). The heme b is bound to CYP106A2 through non-covalent interactions (Bowman and Bren, 2008), and could therefore not be co-transported with an unfolded CYP106A2. Autodisplay uses the AIDA-I linker and β-barrel (Suhr et al., 1996), and although the translocation mechanism remains uncertain, unfolded transport of the passenger is supported by available crystal structures for other autotransporter proteins. The β-barrel of the NalP autotransporter from *Neisseria meningitidis* contains a 10 Å × 12.5 Å pore, through which a linker was threaded to the outer surface (Oomen et al., 2004). This result has been repeated with a full-length passenger, the esterase EstA from *Pseudomonas aeruginosa*, effectively excluding any other translocation pore for this autotransporter (van den Berg, 2010). The narrow pore sizes suggest that the passenger domains of autotransporter proteins are transported to the surface in an unfolded form.

Based on the clear evidence of P450 activity on the surface, and the assumption that the protein is transported in an unfolded form, we propose that the CYP106A2 apo-protein folds at the cell surface and scavenges a heme group from the supernatant. The heme b is relatively large (616.5 Da) and has a structure that is unlikely to pass through the bacterial membrane through passive mechanisms (Tatsumi and Wachi, 2008; Verkamp et al., 1993). Pathogenic Gram negative bacteria have evolved active mechanisms for the

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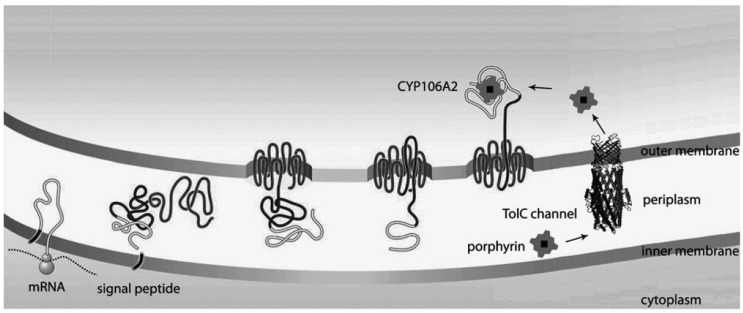

Fig. 7. Autodisplay of CYP106A2 apo-protein and heme incorporation. A Sec signal peptide directs the precursor protein across the inner membrane. In the periplasm, the C-terminal part of the precursor folds into a β-barrel within the outer membrane. CYP106A2 is transmitted to the cell surface by the autotransporter domain, presumably in an unfolded form through the center of the β-barrel. We propose that a TolC-dependent efflux system exports the heme across the outer membrane where it is incorporated into the displayed apo-protein, forming an active enzyme on the cell surface.

uptake of heme into the cell (Burkhard and Wilks, 2007; Cobessi et al., 2010), which support the theory that heme cannot cross the outer membrane by itself. The presence of heme groups in the cell supernatant can be explained by different mechanisms. Firstly, they could originate from lysed cells. We considered it unlikely that enough cells in growth phase spontaneously lyse to provide >20,000 heme molecules per cell, and such spontaneous cell lysis has not been seen in this or previous Autodisplay constructs (Detzel et al., 2011; Jose and Meyer, 2007; Kranen et al., 2011). Secondly the heme groups could have been provided by the *E. coli* growth medium. The LB medium used in this study has been reported to contain 5–6 μM of heme groups (Kaur et al., 2009), as calculated by a pyridine hemochrome assay (Fuhrop and Smith, 1975). It is uncertain if this is enough to account for detectable CYP106A2 activity in the whole cell biocatalyst, and the heme concentration is likely to vary significantly depending on the yeast extract. Thirdly, the heme used by CYP106A2 could be exported by the *E. coli* cells from the periplasm, where it naturally occurs (Goldman et al., 1996; Tommassen, 2010). This is consistent with a recent study showing that porphyrin groups can be exported into the growth media via the outer membrane channel TolC (Fig. 7)(Tatsumi and Wachi, 2008).

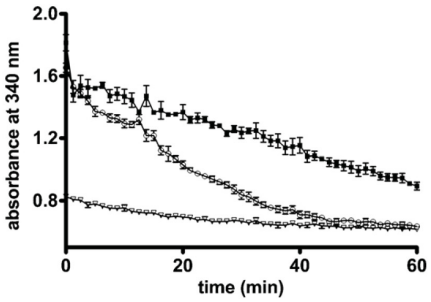

Fig. 8. Photometric assay of NADPH consumption to determine activity of the surface displayed CYP106A2 against imipramine. BL21 (DE3) cells displaying CYP106A2 at the surface were incubated with 200 μM of imipramine. Protein expression was induced with 1 mM IPTG. The substrate turnover was monitored indirectly by measuring the decrease in NADPH corresponding to absorbance at 340 nm. Cells without the external addition of NADPH showed a low level of absorbance (triangles). The reaction mixture in a total volume of 0.2 mL contained Hepes buffer (50 mM, 0.05% Tween20, pH 7.4), imipramine (200 μM), Adx (5 μM), AdR (0.5 μM), NADPH (200 μM) and cells of *E. coli* containing pETCYP13 corresponding to an OD_{578} = 2.5. Data points are the average of triplicate experiments. The bars represent the standard deviation.

To determine whether the export of heme groups from the cell was required for activity, we tested Autodisplay of CYP106A2 in an *E. coli* strain lacking TolC. The strong reduction of enzymatic activity in the TolC mutant supported the theory that TolC is responsible for heme export (Fig. 6(B)). The external addition of heme restored the initial CYP106A2 activity in TolC negative cells displaying the enzyme at the surface, providing evidence that the heme group is scavenged from the growth media (Fig. 6(C)). At this point, however, it should be taken into account, that also other differences between the two *E. coli* strains (BL21 and JW 5503) that are unrelated to heme export could have been of influence. Overall, our results are consistent with the theory that autotransporter passengers are unfolded as they are transported across the outer membrane. The observations made here were obtained with an artificial system, not with a natural autotransporter. Therefore they should be handled with caution in their relevance to the transport mechanism of natural autotransporter proteins in Gram negative bacteria.

In conclusion, the Autodisplay system allows the immobilisation of P450 enzymes on the surface of a living organism, which can then be used as a whole cell biocatalyst. This offers the possibilities for enzyme improvement by random variation. It also enables whole cell biocatalysis with a wide variety of substrates, regardless of their ability to cross biological membranes.

Acknowledgements

We would like to thank the Ministry of Innovation, Science, Research and Technology of the state of North Rhine-Westphalia in Germany (MIWF NRW) and Heinrich-Heine-Universität Düsseldorf for the scholarship of S. Schumacher within the Graduate Cluster Industrial Biotechnology (CLIB). In addition, we thank D. Rönsberg (Heinrich-Heine-Universität Düsseldorf) and J. Fabian (Westfälische Wilhelms-Universität Münster) for their support regarding LC–MS measurements and the technical assistance of I. Rauch during FACS analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2012.02.018.

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Please cite this article in press as: Schumacher, S.D., et al., Autodisplay of functional CYP106A2 in *Escherichia coli*. J. Biotechnol. (2012), doi:10.1016/j.jbiotec.2012.02.018

Supplementary online data

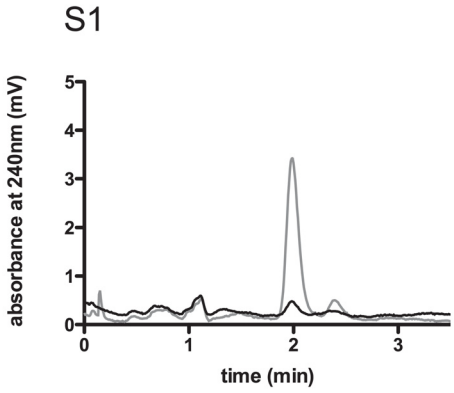

Fig. S1: HPLC analysis to confirm that the 15β-deoxycorticosterone production correlates to CYP106A2 expression

Enlarged picture of the plot areas from figure 5 B and C. *E. coli* BL21(DE3) cells containing pET-CYP13 were incubated for 60 min with 50 µmol of 11-deoxycorticosterone substrate along with the cofactors necessary for activity. The resulting product was analysed by HPLC as described in the materials and methods. Grey: cells with IPTG for the induction of protein expression; Black: cells without IPTG. The product of the reaction appeared at the retention time of 2 min. The small amount of product produced by cells without IPTG is consistent with the "leaky" expression of the T7 promoter in the BL21(DE3) cell line.

Analysis of the products from the conversion of 11-deoxycorticosterone by CYP106A2

The two product peaks produced by the purified enzyme (Fig. 6A) were analysed by HPLC equipped with a diode array detector using a reversed phase C18 column with a pore size of 5 µm, a diameter of 2 mm and a length of 227 mm (Knauer, Berlin,

Germany). The flow rate was 0.25 ml min^{-1}. Methanol was used as mobile phase A, and 0.2 % formic acid (pH2) in water was used as mobile phase B. The HPLC gradient was as follows.

Tab. S1: Solvent gradient for LC-MS analysis

mobile phase A: MeOH

mobile phase B: H_2O + 0.2 % HCOOH pH2

time (min)	**mobile phase A (% v / v)**	**mobile phase B (% v /v)**
0 - 5	10	90
5 - 35	10 → 100	90 → 0
35 - 45	100	0
45 - 55	100 → 10	0 → 90
55 - 60	10	90

Subsequent MS analysis was performed on a Finnigan LCQDeca instrument (Thermoquest, Texas, USA). In order to obtain enough product for LC-MS analysis, multiple reactions with the pure CYP106A2 enzyme were combined. Our attempts to repeat this analysis with the product peak from the whole-cell biocatalyst failed, probably due to insufficient product.

Tab. S2: LC-MS analysis of the products from the conversion of 11-deoxycorticosterone by CYP106A2 purified enzyme

	MS	expected MW	expected substance
	$[M + H]^+$		
Educt	331.1	330 g/mol	DOC
Product 1	347.2	346 g/mol	15β- DOC
Product 2	363.1	362 g/mol	hydroxylated 15β- DOC

The mass spectra of product 1 and 2 showed signals for molecular ions at $[M + H]^+$ 347 and $[M + H]^+$ 363, which suggest the incorporation of one and two oxygen atoms into the substrate.

6 Publication 2: CYP3A4

Published in: „Journal of biotechnology“,

Impact factor: 2.970,

Own proportion of this work: 80 %, 1st author, performance of all experiments, writing of the manuscript.

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Journal of Biotechnology xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Expression of active human P450 3A4 on the cell surface of *Escherichia coli* by Autodisplay

Stephanie D. Schumacher[a], Joachim Jose[b,*]

[a] Institut für Pharmazeutische und Medizinische Chemie, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany
[b] Institut für Pharmazeutische und Medizinische Chemie, Westfälische Wilhelms-Universität Münster, Germany

ARTICLE INFO

Article history:
Received 7 October 2011
Received in revised form 17 January 2012
Accepted 26 January 2012
Available online xxx

Keywords:
Autodisplay
Human Cytochrome P450 3A4
Biocatalysis
Surface display
Phase-1-metabolism

ABSTRACT

The cytochrome P450 enzyme system comprises a large group of enzymes catalyzing a broad diversity of reactions and an extensive substrate specificity, which makes them the most versatile known catalysts. CYP3A4 is one of the important human P450 enzymes and involved in the oxidation of a large range of substrates including toxins and pharmaceuticals. Bottlenecks in studying this enzyme include the difficulty in expressing it in a bacterial host, its need for membrane surroundings and the limited substrate accessibility of enzymes expressed within the cell. To circumvent these difficulties, human CYP3A4 was expressed on the outer membrane of *Escherichia coli* using Autodisplay. Transport of CYP3A4 to the cell surface was monitored by SDS-PAGE and Western blot analysis of outer membrane proteins. Localization on the cell envelope was determined by flow cytometry after immunolabeling, a whole cell ELISA and a protease accessibility assay. A HPLC assay confirmed the catalytic activity of displayed CYP3A4, using testosterone as a substrate. This activity required the external addition of electron supplying enzymes, however surprisingly, we found that the external addition of a heme group was not necessary. Our results indicate that human CYP3A4 can be recombinantly expressed by surface display in a gram-negative bacterium.

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1. Introduction

Cytochrome P450 enzymes play a major role in both, drug discovery research and drug development (Spatzenegger and Jaeger, 1995). In the human body they are the most important enzymes in the phase-1-metabolism. They can catalyze the transformation of a drug from a lipophilic into a more hydrophilic form by hydroxylation, N-, O- and S-dealkylation, sulfoxidation, epoxidation, deamination, dehalogenation, peroxidation, and N-oxide reduction (Bernhardt, 2006; Rushmore and Kong, 2002; Sono et al., 1996). They are of growing importance for the synthesis of drug metabolites and have a well established role in toxicity and metabolic pathways (Nagy et al., 2011). Despite the great interest in P450 enzymes and their important roles in the pharmaceutical and fine chemical industry, their use for wide biochemical studies is still hampered due to several technical problems. The vast majority of these enzymes shows a high lack of stability and needs a membrane environment to become active (Nagy et al., 2011), making a purification process very time consuming and challenging. Secondly, human P450 enzymes tend to show low activity and require several modifications before they can be expressed in significant amounts in bacteria (Gillam, 2008). When expressed in a host such as *Escherichia coli*, whole cell assays can be conducted as a rapid and efficient method to investigate enzyme activity, however the intracellular location of the enzyme limits the set of substrates to those which are able to cross membranes (Li et al., 2007).

One way to overcome these problems and establish an efficient biocatalytic process is to recombinantly express the P450 enzyme on the cell surface. This strategy enables direct contact between enzyme and substrate without the need for the compounds to cross a membrane. It also eliminates expensive enzyme purification steps and results in the immobilization of the protein in membrane surroundings (Samuelson et al., 2002). The Autodisplay system is an elegant way to secrete proteins in gram-negative bacteria to the cell surface (Jose and Meyer, 2007; Maurer et al., 1997). The recombinant passengers can be transported to the outer membrane by simple insertion of their coding region between a signal peptide and a C-terminal domain called β-barrel. The system is based on AIDA-I, the adhesin involved in diffuse adherence in enteropathogenic *E. coli* (Benz and Schmidt, 1992), which belongs to the autotransporter family of proteins (Jose et al., 1995; Jose and Meyer, 2007). Enzymes which have been successfully expressed using Autodisplay include esterases, a sorbitol dehydrogenase, a nitrilase, a isoprenyltransferase, a β-lactamase and a hyaluronidase

* Corresponding author at: Institute of Pharmaceutical and Medicinal Chemistry, Westfälische Wilhelms-Universität, Muenster, Hittorfstraße 58-62, D-48149, Muenster, Germany. Tel.: +49 251 83 32210; fax: +49 251 83 32211.
E-mail address: joachim.jose@uni-muenster.de (J. Jose).

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doi:10.1016/j.jbiotec.2012.01.031

Please cite this article in press as: Schumacher, S.D., Jose, J., Expression of active human P450 3A4 on the cell surface of *Escherichia coli* by Autodisplay. J. Biotechnol. (2012), doi:10.1016/j.jbiotec.2012.01.031

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(Detzel et al., 2011; Jose and von Schwichow, 2004a; Kaessler et al., 2011; Kranen et al., 2011; Lattemann et al., 2000; Schultheiss et al., 2002, 2008). Furthermore the display of rat NADPH-cytochrome P450 oxidoreductase, containing FMN and FAD (Yim et al., 2006) and a heme and diflavin containing P450 BM3 (Yim et al., 2010) using the ice-nucleation protein from *Pseudomonas syringae* have already been reported. During the Autodisplay of bovine adrenodoxin, which serves as an electron donor for mitochondrial P450s, two major observations were made (Jose et al., 2001, 2002). First, it could be shown, that it is possible to incorporate an inorganic, prosthetic group into an apo-protein expressed by Autodisplay at the cell surface by a simple titration step to yield a functional electron donor without loss of cell viability or cell integrity. Second, after external addition of the purified P450s CYP11B1 and CYP11A1, a functional whole cell biocatalyst was obtained for efficient synthesis of different steroids such as pregnenolone and corticosterone (Jose et al., 2001). While working with the soluble, bacterial P450 106A2 we could show that it is possible to display an active P450 enzyme without the external addition of the heme group, which is most likely exported into the supernatant by the outer membrane channel TolC. After this export the porphyrin is incorporated into the enzyme from the outside (Schumacher et al., submitted for publication). However the surface display of any human, membrane bound P450 of clinical relevance in a bacterial background has yet to be shown and is the aim of the present project.

Cytochrome P450 3A4 (referred to here as CYP3A4) is arguably the most important P450 enzyme in humans as it is involved in the oxidation of the largest range of substrates and belongs to the class II microsomal P450 enzymes (Hannemann et al., 2007). In humans it is predominantly found in the liver and often allows prodrugs to be activated and absorbed. Inhibition or induction of CYP3A4 is a major problem in the daily clinical routine, often leading to drug–drug interactions or side effects. Increased activity of CYP3A4 can lead to the fast inactivation of the applied drug, resulting in low plasma levels and a reduced therapeutic effect. In contrast, inhibition of CYP3A4 can lead to intoxication (Guengerich, 1999). To evaluate these possible risks, it is important to determine which drug candidates are accepted as substrates by CYP3A4, and identify the resulting relevant drug metabolites (Schroer et al., 2010). This urgent demand makes CYP3A4 a logical candidate to test whether human P450 enzymes are functional on the cell surface of an *E. coli* cell. In this study, we show experimental evidence that it is possible to translocate an active, human P450 3A4 enzyme to the cell surface of *E. coli* by use of the Autodisplay system.

2. Materials and methods

2.1. Chemicals

Testosterone, 6β-hydroxytestosterone, human Cytochrome P450 reductase, Cytochrome b5 and mouse monoclonal anti-CYP3A4 antibody were purchased from Sigma Chemicals Co (St Louis, MO, USA). Human Cytochrome P450 was obtained from Biozol (Eching, Germany). Nicotinamide adenine dinucleotide phosphate (NADPH) was purchased from Carl Roth (Karlsruhe, Germany). Goat anti-mouse IgG conjugated with DyLight647 was obtained from Thermo Scientific (Waltham, MA, USA). The restriction endonucleases were purchased from New England Biolabs (Ipswich, MA, USA).

2.2. Bacterial strain and growth conditions

E. coli strain UT5600 (DE3) (F$^-$ *ara-14 leuB6 secA6 lacY1 proC14 tsx-67 Δ(ompT-fepC)266 entA403 trpE38 rfbD1 rpsL109 xyl-5 mtl-1 thi-1 λ(DE3)*) was used for the expression of the autotransporter fusion protein (Jose and von Schwichow, 2004b). *E. coli* TOP10 (F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) and the vector pCR4-TOPO which were used for subcloning of PCR products were obtained from Invitrogen (Darmstadt, Germany). *E. coli* Rosetta cells (F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* pRARE (Cam^R)) and the plasmid pRARE to adapt the codon usage were obtained from Novagen (Darmstadt, Germany). Cells were routinely grown at 37 °C in lysogeny broth (LB) medium, containing 50 mg of carbenicillin per liter, 10 μM ethylenediaminetetraacetate (EDTA) and 10 mM 2-mercaptoethanol. Solid media were prepared by the addition of agar (1.5%, w/v).

2.3. Construction of an artificial gene for the surface display of CYP3A4

For construction of the CYP3A4 autotransporter fusion protein, the gene encoding CYP3A4 was amplified by polymerase chain reaction from plasmid pCW-NF14 (Gillam et al., 1993). This PCR product was inserted into vector pCR4-TOPO from which it was recleaved using the two restriction enzymes *XhoI* and *KpnI* before ligation into plasmid pET-SH7 (Petermann et al., 2010), cut with the same enzymes. This yielded an in frame fusion protein consisting of (1) the CtxB signal peptide, (2) CYP3A4 as a passenger, (3) the autotransporter linker region and (4) the autotransporter β-barrel (Fig. 1) under the control of a T7/lac promoter. Construction of the plasmid pST001, used as a control, is described elsewhere (Park et al., 2011). Both plasmids were transformed into UT5600 (DE3) by electroporation-mediated transformation (Sambrook et al., 2001) with standard equipment, and the inserted genes were fully sequenced before use in expression experiments.

2.4. Outer membrane preparation

E. coli cells were grown overnight in LB medium and 20 μl was used to inoculate a 20 ml culture. Cells were cultivated at 37 °C under vigorous shaking (200 rpm) until an OD_{578} of 0.5 was reached. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG, Roth, Karlsruhe, Germany) to reach a final concentration of 1 mM. After 16 h at 30 °C, induction was stopped by harvesting the cells and washing them with buffer (0.2 M Tris–Cl, pH 8). Outer membrane proteins were prepared according to the rapid isolation protocol of Hantke (Hantke, 1981) with modifications as previously described (Jose and von Schwichow, 2004a). A protein accessibility test was used to confirm surface expression, based on the proteinase K mediated degradation of the surface-displayed protein. *E. coli* cells were harvested, washed and suspended in phosphate buffered saline (pH 7.4). Proteinase K was added to a final concentration of 0.2 mg L^{-1} and cells were incubated for 60 min at 37 °C. Digestion was stopped by washing the cells three times with PBS containing 10% fetal calf serum (FCS). After proteinase K digestion, outer membrane proteins were isolated as described above.

2.5. SDS-PAGE

Outer membrane protein isolates were diluted two-fold with sample buffer (100 mM Tris–Cl, pH 6.8 containing 4% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, 20% glycerol, and 50 mg dithiothreitol). The samples were boiled for 5 min at 95 °C and proteins separated by SDS-polyacrylamide gel electrophoresis (PAGE) with a 12.5% acrylamide resolving gel. Proteins were stained with Coomassie Brilliant Blue, and the molecular weight of the proteins estimated using a prestained marker as a standard (Fermentas, St. Leon-Rot, Germany).

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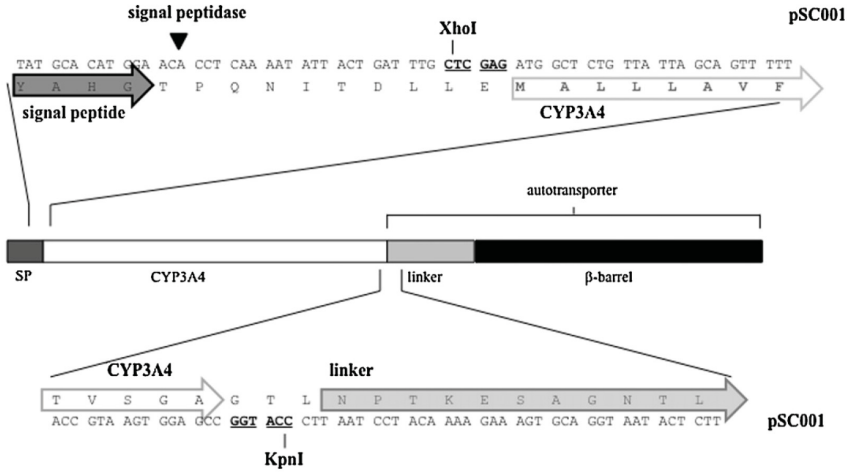

Fig. 1. Structure of the CYP3A4 fusion protein encoded by pSC001. The cloning regions surrounding CYP3A4 are given as sequences. Restriction sites added due to the cloning procedure are underlined. The white arrows indicate the boundaries of the passenger domain. The C-terminus of the signal peptide is represented by the dark gray arrow and the light gray arrow depicts the N-terminus of the linker region. The signal peptidase cleavage site is indicated. SP: signal peptide.

2.6. Western-blot

For Western blot analysis, gels were electroblotted on polyvinylidenefluoride (PVDF) membranes using standard techniques (Tetra Cell, Bio-Rad, USA). Proteins on the membranes were blocked in 25 mM Tris-base pH 7.4 with 140 mM NaCl, 2.7 mM KCl (TBS) and 3% bovine serum albumin (BSA). For immune detection, membranes were incubated for 3 h with the primary anti-CYP3A4 monoclonal antibody diluted 1:5000 in TBS with 3% BSA. Prior to addition of the secondary antibody, blots were rinsed three times in TBS with 0.1% Tween 20. The secondary antibody was then added and the blots were incubated for 2 h at room temperature, before being washed three times in TBS. The secondary antibody, goat anti-mouse IgG conjugated with horseradish peroxidase, was used at a dilution of 1:10,000 in TBS with 3% BSA. Antigen-antibody conjugates were visualized by a color reaction with staining solution consisting of 17 mM 4-chloro-1-naphthol and 0.015% H_2O_2 in TBS.

2.7. Flow cytometer analysis

E. coli cells were grown as described above in Section 2.2. Expression of CYP3A4 was induced at an early exponential growth phase (OD_{587} of 0.5) by adding IPTG to a final concentration of 1 mM. Cells were then incubated overnight at 30 °C, 200 rpm in LB medium. Cells were harvested by centrifugation, washed twice with PBS and resuspended to a final OD_{578} of 0.5. 100 μl of the cell suspension was suspended in 500 μl of PBS containing 3% bovine serum albumin (filter-sterilized), and incubated for 10 min at room temperature (RT). Cells were then pelleted in a microcentrifuge (60 s at 18,000 × *g*, Hettich, Tuttlingen, Germany), resuspended in 100 μl of a solution that contained a monoclonal CYP3A4 antibody (diluted 1:100 in PBS [pH 7.4] + 3% BSA) and incubated for another 30 min at RT. Cells were then washed twice with 500 μl of PBS. The second incubation step was conducted in the dark (30 min, RT) using 100 μl of a goat anti-mouse IgG antibody conjugated with DyLight647 (diluted 1:25 in PBS [pH 7.4] + 3% BSA). After washing twice in PBS supplemented with 3% BSA, the cell pellet was resuspended in 1.5 ml of PBS. Samples were then analyzed using a CyFlow® space flow cytometer (Partec GmbH, Münster, Germany) at an excitation wavelength of 647 nm.

2.8. Whole cell ELISA

For a whole cell ELISA as has been described for surface displayed human Ro/SS-A antigen (SD-ELISA; Petermann et al., 2010), *E. coli* cells were grown and protein expression was induced as described above. Subsequently, cells were washed twice with coating buffer (PBS, pH 7.4) and suspended to a final OD_{578} of 0.5. A 96-well microplate (Maxisorp, Nunc) was coated with 100 μl of the cell suspension overnight at 37 °C. Any nonspecific binding sites were blocked by the addition of 150 μl of PBS (pH 7.4) + 10% FCS for 3 h at RT. After blocking, cells were incubated with a monoclonal anti-CYP3A4 antibody for 1 h at RT. The microplates were rinsed three times with the washing buffer (PBS [pH 7.4] + 0.1% Tween 20), and incubated with a secondary goat anti-mouse IgG conjugated with horseradish peroxidase diluted 1:10,000 in PBS (pH 7.4) + 10% FCS. After a further 45 min of incubation at RT, plates were washed three times, followed by the addition of 100 μl of TMB (3,3′,5,5′-tetramethylbenzidine) to each well. Plates were then incubated in the dark at RT for 25 min, and the enzyme reaction was stopped by adding 100 μl of 2 M H_2SO_4. Absorption at 450 nm was measured with a Mithras LB 940 microplate reader (Berthold Technologies, Bad Wildbad, Germany).

2.9. Assay for the hydroxylation of testosterone

For activity measurements an enzyme premix was prepared and frozen at −80 °C in 1 ml aliquots (Shaw et al., 1997). The 5× protein premix contained 1 μM NADPH-P450-Reductase, 0.5 μM Cytochrome b5, 50 mM Hepes, 0.5 mg/ml CHAPS and 3 mM glutathione. The enzyme reaction was carried out in 50 mM Tris–Cl pH 7.8 containing 200 mM NaCl and 3 mM magnesium chloride. A typical 100 μl biotransformation reaction was prepared by mixing (on ice) 20 μl of 5× protein premix, 5 mM of glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase (where a unit is described as the amount of enzyme that will reduce 1.0 μmol of NADP per minute at 30 °C, pH 7.8) Additionally the reaction contained either 0.2 μM of purified, reconstituted CYP3A4 enzyme or *E. coli* pSC001 cells at an OD_{578} of 10 (approximately 2.5×10^9 cells ml^{-1}). Testosterone was dissolved in ethanol and added to the enzymes to a final concentration of 200 μM. The

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reaction was initiated by the addition of 600 μM NADPH, proceeded for 72 h at a temperature of 37 °C and was terminated by the addition of 500 μl chloroform. Samples were extracted twice with chloroform and the layers were separated by centrifugation (60 s at 18,000 × *g* with a microfuge). The organic phases were combined and the solvents removed under vacuum. The dried samples were resuspended in 100 μl of acetonitrile for HPLC analysis (described below).

2.10. Analytical methods

Testosterone conversion was analyzed using a LiChrospher 60 RP-select B column 125-4 (5 μm) from Merck (Darmstadt, Germany) on a LaChrom Elite System from VWR-Hitachi (Darmstadt, Germany). The hydroxylation product was detected at 254 nm. The mobile phase used in the separation, at a flow rate of 1.0 mL/min, consisted of (A) water (0.02% trifluoroacetic acid and (B) an acetonitrile gradient which decreased from 90% to 40% over 20 min. The column was then reequilibrated for 5 min with 90% acetonitrile. The injection volume was 20 μl. These parameters were also used for commercially available 6β-hydroxytestosterone in order to determine its retention time in this HPLC system and for quantification of the enzyme-derived product.

3. Results

3.1. Expression of the CYP3A4 autotransporter protein

The *E. coli* strains UT5600 (DE3) was transformed with the plasmid pSC001 which encoded the CYP3A4 autotransporter fusion protein. The T7 promoter allowed transcription induced by the addition of IPTG (Studier and Moffatt, 1986). After induction of protein expression the revealed protein band was so weak that it was not visible on the SDS-PAGE. Therefore extracts of outer membrane proteins were applied to Western blot analysis and revealed a protein band with an approximate molecular weight of 110 kDa, as expected for the CYP3A4 autotransporter fusion protein (Fig. 2B, lane 4).

3.2. Protein accessibility test

To test the surface exposure of the CYP3A4 domain, proteinase K was added to whole cells of *E. coli* expressing the fusion protein. Proteinase K is too large to enter the cell envelope and therefore the degradation of the fusion protein is strong evidence that it is accessible at the cell surface (Fig. 2B, lane 5). As a control, we also examined the molecular weight of OmpA, which has a C-terminal extension in the periplasm that is susceptible to protease cleavage. The lack of OmpA digestion (Fig. 2A, lane 5) is a strong indication that proteinase K had not entered the periplasm.

3.3. Effect of codon usage

The intensity of the CYP3A4 band was low in comparison to the bands corresponding to the native *E. coli* outer membrane proteins OmpF/C and OmpA (Fig. 2). This suggests that a relatively low amount of CYP3A4 was present at the cell surface, also in comparison to other enzymes expressed by the Autodisplay system, including, e.g. Adx or nitrilase (Detzel et al., 2011; Jose et al., 2001). The 110 kDa band corresponding to the CYP3A4 autotransporter fusion protein was only visible in the Western blot (Fig. 2B, lane 4) and not in the corresponding SDS-PAGE (Fig. 2A, lane 4), indicating an abundance far lower than the natural outer membrane proteins OmpA or OmpF/C. The human CYP3A4 gene contains seven codons that are rare in *E. coli* (data not shown). To determine if the rare codons were responsible for

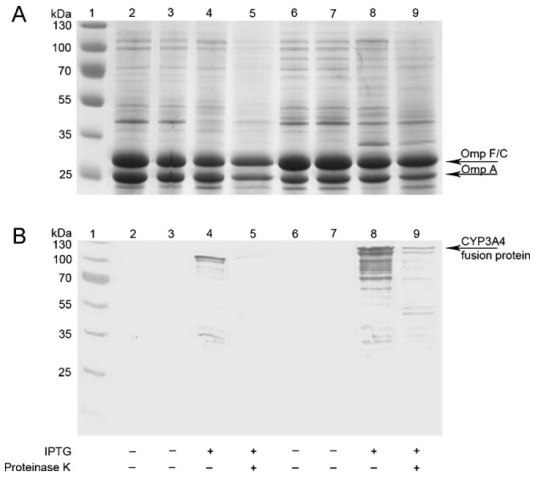

Fig. 2. Surface expression of CYP3A4 in *E. coli*. A: Preparations of outer membrane proteins were prepared as described in Section 2. Proteins were separated by SDS-PAGE (12.5%) and stained with Coomassie Brilliant Blue G250. Expression experiments were performed with the *E. coli* strains UT5600 (DE3) (lanes 2–5) and Rosetta (DE3) (lanes 6–9). 1: marker proteins, 2: cells without plasmid, 3: cells containing pSC001–IPTG, 4: cells containing pSC001 + 1 mM IPTG, 5: cells containing pSC001 + 1 mM IPTG + 1 mM proteinase K for the degradation of surface displaced proteins, 6: cells containing pRARE, which increases the tRNA corresponding to rare codons, 7: cells containing pSC001 + pRARE–IPTG, 8: cells containing pSC001 + pRARE + 1 mM IPTG, and 9: cells containing pSC001 + pRARE + 1 mM IPTG + 1 mM proteinase K. B: Western blot with a monoclonal antibody specific to CYP3A4. Proteins were detected using a secondary antibody conjugated to horseradish peroxidase. Samples were loaded as per A.

the low amount of protein detected, we expressed the protein in cells containing extra tRNAs (Rosetta cells containing pRARE) complementing the observed rare codons. In comparison to UT5600 cells, Rosetta cells expressed a higher amount of the 110 kDa protein in the Western blot analyses, suggesting that the rare codons did indeed have a negative effect on total protein expressed (Fig. 2B, lane 8). In both cases, with and without pRARE, the 110 kDa protein band was only found in cells where expression was induced, providing a strong indication that it was the correct fusion protein. The protein accessibility assay with proteinase K confirmed the surface expression of the CYP3A4 protein in Rosetta cells (Fig. 2B, lane 9).

3.4. Flow cytometer analysis

Flow cytometry has previously been used to confirm the surface expression of proteins, and investigate expression efficiency (Petermann et al., 2010). For the cell labeling, we used a primary monoclonal antibody targeting CYP3A4, and a secondary antibody conjugated to DyLight647. The fluorescence intensity of cells expressing the CYP3A4 fusion protein was shifted to higher intensity in comparison to control cells treated identically (Fig. 3) One population of the cells expressing CYP3A4 could not be marked at all and remained negative. Even though the positive population indicates that the CYP3A4 domain was accessible at the cell surface and, as a consequence, surface display of the P450 enzyme by Autodisplay in *E. coli* was successful, a further method to investigate surface display was performed to confirm these results.

3.5. Whole cell SD-ELISA

A 96-well microplate was coated with *E. coli* UT5600 (DE3) (negative control), *E. coli* UT5600 (DE3) pST001 (displaying streptavidin

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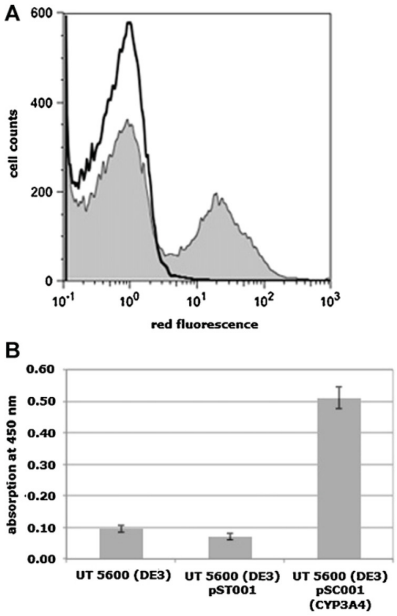

Fig. 3. Surface accessibility of CYP3A4 determined by flow cytometry analysis and whole cell ELISA. For both techniques, cells were incubated with a primary monoclonal anti-CYP3A4 antibody. A: Flow cytometry analysis, after staining cells with secondary antibody labeled with DyLight647. Black: UT5600 (DE3) without plasmid, gray: cells displaying CYP3A4 on the surface (UT5600 (DE3) containing the plasmid pSC001, protein expression induced with IPTG). B: Whole cell ELISA, after incubating cells with a secondary antibody labeled with horseradish peroxidase. Reactions were started by the addition of the dye TMB and stopped with sulfuric acid. Absorption was measured at 450 nm.

on the surface; negative control) (Park et al., 2011) and with *E. coli* UT5600 (DE3) pSC001 (displaying CYP3A4 on the surface). The second negative control, expressing a different protein, was used to eliminate the possibility of false positive results due to a cross reaction against the linker region or the β-barrel. The cells were incubated with a primary monoclonal anti-CYP3A4-antibody and a secondary horseradish peroxidase labeled detection antibody. The dye TMB (3,3′,5,5′-tetramethylbenzidine) was added to the mixture and was immediately oxidized by the horseradish peroxidase. This reaction, after being stopped by the addition of sulfuric acid, yielded a yellow color, which could be measured as absorption at 450 nm. The cells carrying the plasmid encoding for CYP3A4 showed a significant higher absorption after inducing protein expression in comparison to controls, which is strong evidence that the protein is located at the cell surface (Fig. 3B).

3.6. Hydroxylation assay

To test the activity and with it the correct folding of the displayed CYP3A4 a hydroxylation assay was performed with the substrate testosterone (Fig. 4). P450-mediated catalysis required the external addition of Cytochrome b5. Cells were prepared as described above and hydroxylation of testosterone was carried out for 72 h. The activity of the enzyme CYP3A4 was monitored using an HPLC separation system for steroids, which can demonstrate the CYP3A4-dependent conversion of testosterone into 6β-hydroxytestosterone. The purified enzyme produced only a small product peak (Fig. 5A). Despite the low activity, an enlarged picture of the plot areas revealed enzyme activity of whole cells displaying CYP3A4 (Fig. 5B). UT5600 (DE3) cells containing the plasmid encoding the autotransporter fusion protein but without the addition of IPTG did not show 6β-hydroxylase activity (Fig. 5B, graph 2). After induction of protein expression, cells displaying the fusion protein produced the 6β-hydroxylated product (Fig. 5B, graph 3). Since the product peak was only found in cells were expression was induced this is a strong indication for the activity of the displayed enzyme. The retention time of the product produced by the CYP3A4-expressing cells (~12 min) corresponded very well to the retention time of the commercially available 6β-hydroxytestosterone, which was used as a reference compound (see supplementary Fig. S1) and the product produced by the purified enzyme (Fig. 5A and B). The hydroxylation reaction relies on the addition of NADPH which is supposed to be not able to cross cellular membranes (Gholson et al., 1969) giving further proof that all conversions took place on the cell surface. Human CYP3A4 is known to hydroxylate testosterone at several sites, with the 6β-hydroxylated product being usually formed in the highest quantity. The conversion of the substrate testosterone with the purified enzyme yielded indeed an additional product at a retention time of about 13.9 min (Fig. 5B, graph 1). This peak can also be seen in the conversion mediated by the surface displayed CYP3A4 enzyme (Fig. 5B, lane 3) and it appears – by first sight – to be larger than the main product peak. However, this side product peak is overlapping with a metabolite produced by the bacterial cells without CYP3A4 (Fig. 5B, lanes 2 and 4) and by a component or impurity of the reaction buffer (Fig. 5B, lane 5). This clearly indicates that the peak at 13.9 min obtained by HPLC of the reaction sample with cells displaying CYP3A4 is comprised of the reaction side product obtained by hydroxylation of testosterone, the bacterial metabolite and the buffer constituent. As a consequence, there was no indication that the surface display of CYP3A4 resulted in an altered regio- or stereoselectivity in comparison to the reaction with the purified enzyme. Nevertheless such an alteration has been observed in the surface of sorbitol dehydrogenase (Jose and von Schwichow, 2004b) and nitrilase (Detzel et al., 2011).

To increase the activity of the whole cell biocatalyst the same experiment was performed with the strain Rosetta pRARE pSC001, showing a higher expression of the fusion protein (Fig. 2, lane 8).

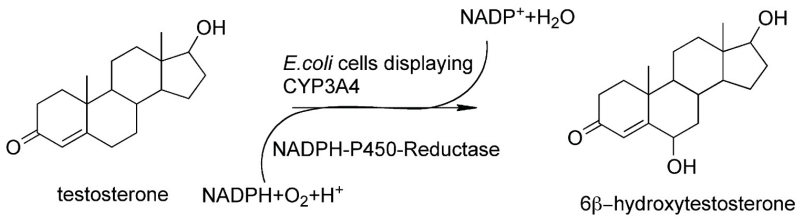

Fig. 4. Hydroxylation of testosterone at the 6β- position by CYP3A. When CYP3A4 is displayed on the surface of *E. coli* cells, redox equivalents from NADPH are transferred to the enzyme by the NADPH-P450-Reductase, included in the protein premix.

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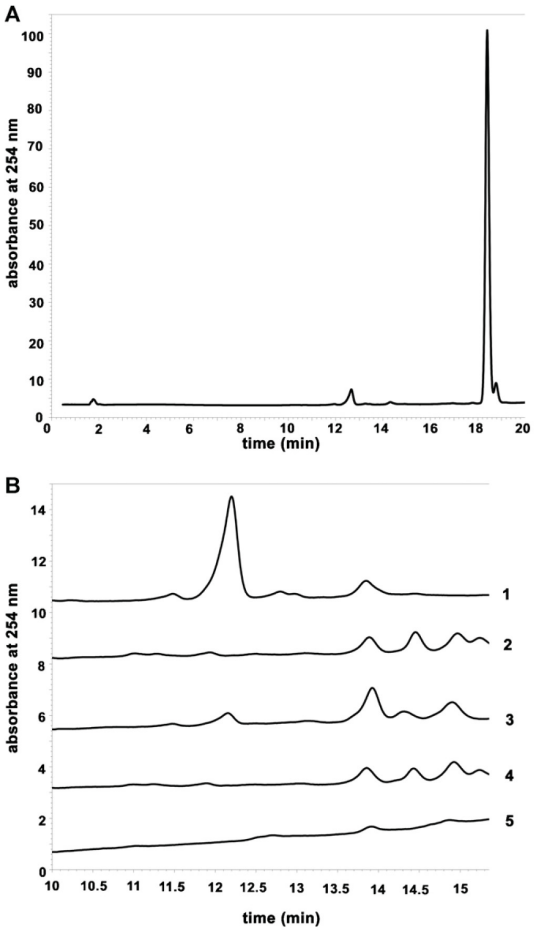

Fig. 5. Activity of the whole cell biocatalyst displaying CYP3A4 by HPLC. A protein premix was added to all samples, which included the NADPH-P450-Reductase required for the supply of reducing equivalents. The substrate, testosterone, is eluted at a retention time of 18 min. The product, 6β-hydroxytestosterone, is eluted at a retention time of 12.2 min. A: Full trace; purified CYP3A4 enzyme. B: Enlarged plot areas for clarity. 1: Purified enzyme, showing only the product peak, 2: cells without protein induction (UT5600 (DE3) pSC001–IPTG), 3: cells with induction of protein expression, displaying CYP3A4 at the cell surface (UT5600 (DE3) pSC001 + IPTG), 4: cells of UT5600 (DE3) without any plasmid, but treated identically, and 5: reaction buffer of the hydroxylation assay.

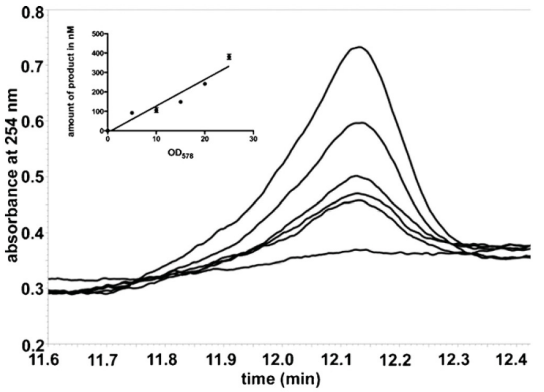

Fig. 6. Activity of the whole cell biocatalyst displaying CYP3A4 at different cell densities in the hydroxylation assay. The amount of product formed was measured using a standard curve of commercially purchased 6β-hydroxytestosterone. The peak height was proportional to the optical density (OD_{578}) of the cells. The highest peak shows the product formed at OD_{578} 25, and in descending order OD_{578} 20, 15, 10, 5 and control cells without induction of protein expression.

Unfortunately the higher expression did not yield a higher enzymatic activity (data not shown).

To verify the functionality of the surface displayed CYP3A4 and to increase the amount of the product 6β-hydroxytestosterone, whole cells of *E. coli* expressing CYP3A4 were incubated with the substrate testosterone in different cell densities. The amount of product formed was measured by HPLC using a standard curve of the commercially available reference compound 6β-hydroxytestosterone product and the results were calculated in nM 6β-hydroxytestosterone as shown by supplementary Fig. S1. The amount of product formed by the whole cell catalyst increased in a linear manner with the optical density (OD_{578}) of the supplied cells (Fig. 6), whereas control cells without a plasmid subjected to this reaction for an identical reaction time did not yield any product peak at all (Fig. 6). This clearly indicated that the product 6β-hydroxytestosterone formed from the substrate testosterone was due to the surface displayed CYP3A4 fusion protein and that the human P450 enzyme was functionally expressed by Autodisplay.

Secondary structure analysis (TMpred program) of the CYP3A4 sequence suggested that the N-terminal part forms a transmembrane helix, which could interfere with the transport of the enzyme across the outer membrane. It could result in a periplasmic degradation of the passenger fusion protein as has been shown before for the Autodisplay passengers CtxB (Jose et al., 1996) and Aprotinin (Jose and Zangen, 2005). To overcome this possible limitation, the N-terminal 17 amino acids were deleted by a PCR based approach and the resulting truncated CYP3A4 autotransporter fusion protein was analyzed for transport and enzymatic activity as described above. As shown in supplementary Fig. S2, expression of this construct under the control of a constitutive promoter (Jose et al., 2001; Maurer et al., 1997) resulted in an almost 50% higher enzymatic activity of the cells displaying CYP3A4, although in all cases the concentration of the product remained low, in the nm range, as generally expected for CYP3A4, which shows the slowest conversion rates of human first pass metabolizing enzymes. In addition, this clearly indicated that human CYP3A4 expressed on the cell surface of *E. coli* by Autodisplay was displayed in an enzymatically active form.

4. Discussion

Microbial cell surface display has a great potential to be used in a wide range of applications and provides several advantages in biotechnological applications (Lee et al., 2005). Therefore a lot of proteins and peptides have already been brought to the cell surface in several bacteria (Daugherty, 2007; Samuelson et al., 2002). So far the display of bovine adrenodoxin, containing an iron sulfur cluster (Jose et al., 2001, 2002), rat NADPH-cytochrome P450 oxidoreductase, containing FMN and FAD (Yim et al., 2006) and a heme and diflavin containing P450 BM3 (Yim et al., 2010) have already been reported. As far as we know this is the first time, that an active human P450 enzyme was successfully displayed on the cell surface of *E. coli*. The recombinant expression of active human P450 enzymes is necessary to allow analyses of drug metabolism and toxicology. Because many mammalian P450s are not glycosylated, *E. coli* is an attractive host for the rapid production of large quantities of enzyme for research purposes. Despite many

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advances in recombinant expression, and in particular recombinant expression in *E. coli*, expression of many P450 enzymes remains difficult (Guengerich et al., 1996). After purification from membranes CYP3A4 is notorious for difficulties in reconstitution of activity (Yamazaki et al., 1995). Additionally the slow 6β-hydroxylase activity of human P450 3A4 is barely detectable after recombinant expression in *E. coli* (Blake et al., 1996). Expression rates in *E. coli* can been improved by modifying the N-terminal part of the enzyme (Gillam, 2008). But even when the enzyme is expressed and active, human P450 3A4 and other P450s require membrane surrounds for activity, increasing the complexity of reconstitution experiments (Hannemann et al., 2007).

We have previously shown proof of concept that a soluble, bacterial P450 can be displayed at the surface of the *E. coli* cell with Autodisplay (Schumacher et al., submitted for publication). This technique solves many of the typical obstacles of a whole-cell monooxygenase system, including the poor stability of the enzymes without proper membrane surroundings, the inability to test substrates that cannot cross membranes, and the necessity for time-consuming enzyme purification. To determine if a mammalian P450 is also functional at the surface of the cell, in this study we attempt the Autodisplay of human CYP3A4, a class II microsomal P450 enzyme that requires a membrane environment for activity. The enzyme showed full activity without the external addition of the prosthetic group as was necessary in other studies (Jose et al., 2001). Expression was verified by SDS-PAGE and Western blot. Flow cytometry, a protease accessibility test and a whole cell SD-ELISA verified surface localization. The functionality of the enzyme was tested by an HPLC assay showing the hydroxylation of the educt testosterone into the product 6β-hydroxytestosterone by the displayed enzyme. Furthermore, the activity was dependent on external addition of substances that are not thought to cross the *E. coli* membrane, including NADPH-P450-Reductase and NADPH (Gholson et al., 1969), supporting the theory that the reaction took place on the cell surface. Unfortunately, the expression of the enzyme as well as the activity of the whole cell biocatalyst obtained thereby was not very high. To improve the expression and with it hopefully the activity the first aim was to improve the codon usage by transforming the plasmid into the strain Rosetta carrying the plasmid pRARE. This strategy yielded into a better expression of the enzyme, as could be seen on the Western blot, but under no circumstances was the fusion protein visible after Coomassie staining of the SDS-PAGE of outer membrane isolations. This clearly indicates that even after improving the codon usage only a small amount of protein was present. The deletion of the N terminal 17 amino acids, which are supposed to form a transmembrane α helix, and therefore interfere with our transport system, resulted in an almost 50% increase of the enzymatic activity of the whole cell biocatalyst displaying human CYP3A4. This indicates that rational enzyme engineering could be a valid tool to improve the activity of CYP3A4 displayed on the surface of *E. coli* (Gillam, 2008).

Nevertheless, towards the synthesis of drug metabolites or for other biotechnological applications, the whole cell biocatalyst displaying CYP3A4 is only the first step. In the present approach, NADPH-P450-Reductase and Cytochrome b5 need to be added from the exterior making this approach rather expensive, and only useful for the production of more expensive metabolites or products in general. The co-factor NADPH can be regenerated by adding glucose-6-phosphate and glucose-6-phosphate dehydrogenase, which is again a burden for a possible commercial use of the system. As a consequence the next steps need to be the co-expression of NADPH-P450-Reductase and Cytochrome b5 with human CYP3A4 on the surface of a single cell of *E. coli* and in order to facilitate efficient co-factor regeneration, to display glucose-6-phosphate dehydrogenase on a second cell of *E. coli* and combine both cell types in one reaction batch. Of course this approach is challenging, but first attempts to co-express enzymes with different functionalities on the surface of *E. coli* were successful (Kranen and Jose, unpublished) and the expansion of this system in this way is under current investigation. The autodisplayed CYP3A4 was active without the addition of an external heme, which has a molecular weight of 616 g mol^{-1} and a structure that is unlikely to pass through the bacterial membrane through passive mechanisms (Tatsumi and Wachi, 2008; Verkamp et al., 1993). The most probable explanation is that CYP3A4 passenger molecules were transported to the cell surface by the autotransporter in an unfolded form, without porphyrin, which is in agreement with the original theory regarding secretion by autotransporters. Thereby the proteins are unfolded and need to pass through a small pore in the β-barrel structure (Jose et al., 1995; Maurer et al., 1997; Pohlner et al., 1987). The protein folds at the cell surface and incorporates heme groups that were present in the cell supernatant. It has been proposed that porphyrins are exported from *E. coli* cells by a TolC-dependent efflux system (Tatsumi and Wachi, 2008) into the supernatant and recently we were able to show, that a CYP106A2 displaying whole cell biocatalyst exhibited much lower enzymatic activity in a TolC negative host background than in the corresponding TolC positive strain of *E. coli* (Schumacher et al., submitted for publication). This makes the export of porphyrins via the outer membrane channel TolC and the incorporation into the CYP3A4 apo-protein at the cell surface very likely und is consistent with the initial model of autotransporter mediated surface translocation of passenger proteins. The heme in the supernatant could also been due to a contamination of the yeast extract used to make LB medium, as described before (Kaur et al., 2009).

It cannot be excluded at this point, that CYP3A4 was transported across the outer membrane in a folded, or partially folded state. Although this appears to be inconsistent with most data regarding the transport mechanism of autotransporters, other studies have suggested that other passenger domains can acquire a stable tertiary structure in the periplasm before transport to the cell surface using the Omp85/Bam A pathway (Bernstein, 2007; Tommassen, 2010). In this case the prosthetic group could have been incorporated in the periplasm, where it occurs naturally (Goldman et al., 1996; Tommassen, 2010). Considering the diversity of autotransporters in gram negative bacteria, it is possible that different mechanisms exist for the transport of passenger proteins to the surface, and indeed, this may be dependent on the passenger itself.

We conclude that it is possible to immobilize a human P450 enzyme on the surface of *E. coli* in a functional form. This is a first step towards establishing a system to screen and synthesize clinical relevant phase-I-metabolites.

Acknowledgments

The authors thank the Ministry of Innovation, Science and Research of North Rhine-Westphalia and Heinrich-Heine-University-Düsseldorf for the scholarship of Stephanie Schumacher within the Graduate Cluster Industrial Biotechnology (CLIB). The provision of plasmid pCW-NF14 by F.P. Guengerich, Vanderbuilt University, is gratefully acknowledged. In addition, the authors thank Mark Teese for critically reading the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2012.01.031.

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Please cite this article in press as: Schumacher, S.D., Jose, J., Expression of active human P450 3A4 on the cell surface of *Escherichia coli* by Autodisplay. J. Biotechnol. (2012), doi:10.1016/j.jbiotec.2012.01.031

Supplementary online data

6β-hydroxytestosterone conversion

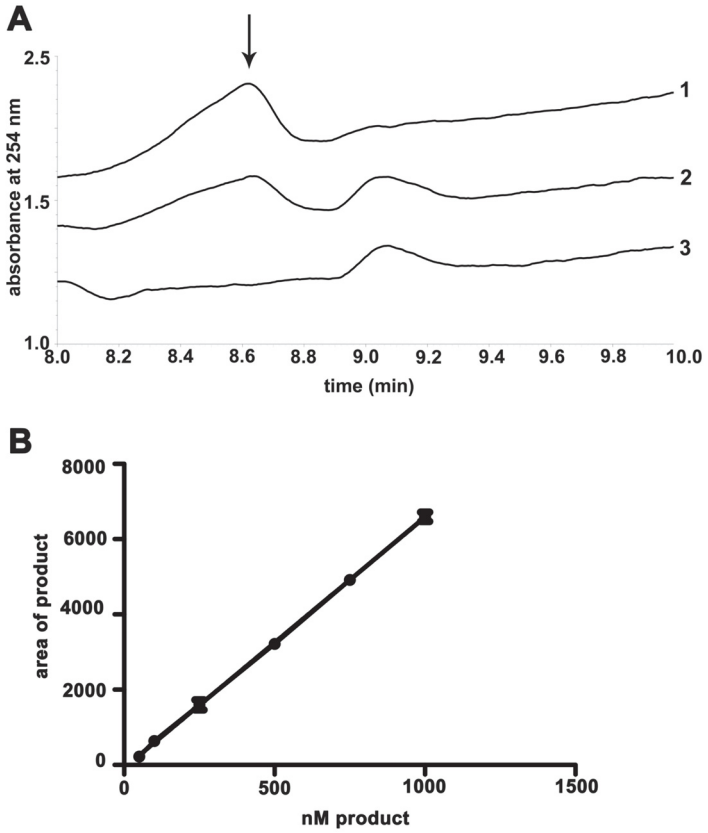

Fig. S1: HPLC analysis of the product of surface displayed CYP3A4.

A: Overlay showing the purified 6β-hydroxytestosterone with the conversion buffer and the product peak after the whole cell assay. The product peak is indicated with an arrow. 1: Commercially purchased 6β-hydroxytestosterone (250nM) dissolved in acetonitrile, 2: cells displaying CYP3A4 at the cell surface (UT5600 (DE3) pSC001 + IPTG) 3: Buffer only. **B:** Calibration curve of the purified product 6β-hydroxytestosterone.

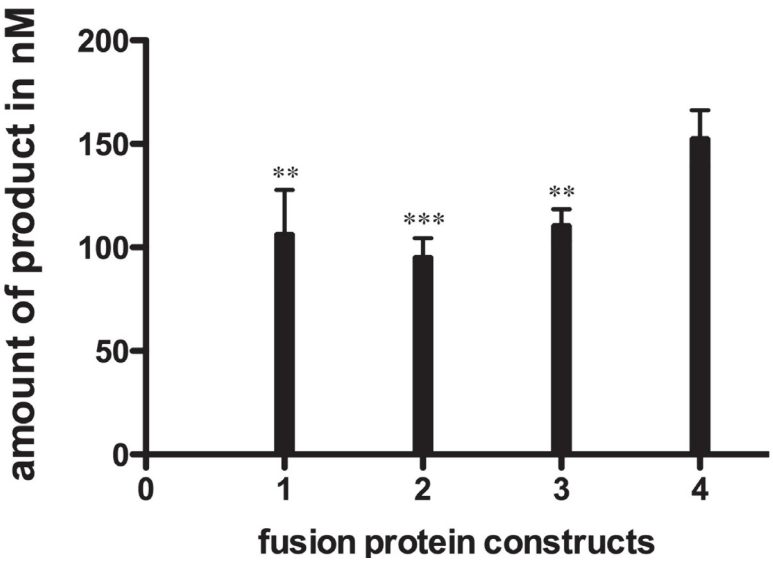

Fig. S2: Comparison of the amount of product formed by different plasmid constructions after HPLC analysis

All cell samples were used at an OD_{578} of 10.

1: UT 5600 (DE3) pSC001. **2:** UT 5600 (DE3) pSC002. The first 17 amino acids at the N-terminal part of of the human CYP3A4 protein were eliminated. **3:** BL 21 (DE3) pAT-SC001. The autotransporter fusion protein with human CYP3A4 as a passenger was expressed under a constitutive promoter. **4:** BL 21 (DE3) pAT-SC002. The first 17 amino acids at the N-terminal part of the human CYP3A4 were eliminated. The construct was expressed under a constitutive promotor.

Values were expressed as means (n = 3) ± SD. Analysis of variance (ANOVA) was followed by the Scheffé test (WinStat, Version 2007.1): $p < 0.05$ and $p < 0.01$ vs. column 4.

Cells were grown, protein expression was induced and incubation with 200 µM of the substrate for 72 hours was performed as described in materials and methods, section 2.9. For expression under a constitutive promoter cells were grown until an OD_{578} of 0.5 was reached. An incubation period at 30°C over night followed, after which the cells were treated as described in section 2.9.

6β-hydroxytestosterone samples were analysed using a LiChrospher 60 RP- select B column 125-4 (5 μm) from Merck (Darmstadt, Germany) on a LaChrom Elite System from VWR-Hitachi (Darmstadt, Germany) equipped with a diode array detector. The detection wavelength was 254 nm. The mobile phase used in the separation, at a flow rate of 1.0 mL/min, consisted of (A) water (0.02% trifluoroacetic acid and (B) an acetonitrile gradient which decreased from 80 % to 40 % over 20 min. The column was then reequilibrated for 5 min. The injection volume was 20 μl.

7 Publication 3: Patent

Own proportion of this work: 20 %, 1st author, performance of all experiments

Surface display of polypeptides containing a metal porphyrin or a flavin

Description

The present invention relates to a method for functionally displaying a recombinant polypeptide containing a prosthetic group on the surface of a host cell, wherein the prosthetic group is selected from metal porphyrins and flavins.

Over the past 30 years, it has become clear that enzymes hold great potential for industry. They are most remarkable biomolecules because of their extraordinary specificity and catalytic power [1]. The specificity and (enantio- and regio-) selectivity of certain enzymatic transformations makes them attractive for the production of fine chemicals and pharmaceutical intermediates. To date, more than 500 products are manufactured by enzymes. Well-known examples are ephedrine, aspartame and amoxicillin [2,3,4].

Cytochromes P450 enzymes have been discovered about 50 years ago and are ubiquitously distributed enzymes, which possess high complexity and display a broad field of catalytic activities. They are hemoproteins, which means, they contain a porphyrin ring system. The P450 enzyme family is involved in the biotransformation of drugs, the bioconversion of xenobiotics, the metabolism of chemical carcinogens, the biosynthesis of physiologically important compounds such as steroids, fatty acids, eicosanoids, fat-soluble vitamins, bile acids, the conversion of alkanes, terpenes, and aromatic compounds as well as the degradation of herbicides and insecticides [5]. Furthermore, there is a broad versatility of reactions catalysed by cytochromes P450, such as carbon hydroxylation, heteroatom oxygenation, dealkylation, epoxidation, aromatic hydroxylation, reduction, and dehalogenation.

Despite their very interesting features for industrial applications, the use of P450 enzymes for wide biotechnology needs is still limited, due to their difficulty in handling. With the exception of a few bacterial P450s, the vast majority needs a

certain membrane contact or environment to fold into an active form. Within those membrane associated P450s, two classes can be identified: a mitochondrial and a microsomal. At the moment there are two different ways to use these enzymes for synthetic purposes. They are either purified after recombinant expression and reconstituted with an artificial membrane system, or they are expressed and used in a whole cell context. Both ways have their limitations. Reconstituted membrane vesicles with P450 enzymes are laborious to produce and they are absolutely not suited for industrial applications. Using whole cells with intrinsic P450s limits the set of substrates to be converted to those which are able to cross membranes [6].

Among other systems for the secretion of proteins in Gram-negative bacteria, the autotransporter pathway represents a solution of impressing simplicity. It is possible to transport a protein, regardless whether it is recombinant or the natural passenger, to the actual outer membrane, as long as its coding region lies between a typical signal peptide and a C-terminal domain called β-barrel. Based on these findings the autodisplay system has been developed by the use of the natural *E. coli* autotransporter protein AIDA-I (the adhesin involved in diffuse adherence) in an *E. coli* host background [9]. Autodisplay has been used for the surface display of random peptide libraries that were successfully screened for the identification of new enzyme inhibitors, and the display of functional enzymes like esterases, oxidoreductases and electron transfer proteins [10].

During the autodisplay of bovine adrenodoxine, which serves as an electron donor for mitochondrial p450s, two major observation were made [11,12]. First, it could be shown, that it is possible to incorporate an inorganic, prosthetic group into an apoprotein expressed by autodisplay at the cell surface by a simple titration step to yield a functional electron donor without loss of cell viability or cell integrity. And second, after external addition of the purified P450s CYP11B1 and CYP11A1, a functional whole cell biocatalyst was obtained for efficient synthesis of different steroids. Therefore the aim of the present invention is, to investigate, whether it is possible to autodisplay a P450 enzyme in a functional form on the surface of *E. coli*. This could provide a new expression platform for the highly interesting group of P450 enzymes with the perspective of being applicable in industrial processes.

Background

Cytochromes P450 are external monooxygenases. Monooxygenases (mixed function oxidases) catalyse the incorporation of a single atom of molecular oxygen into a substrate with the concomitant reduction of the other atom to water. Monooxygenases are divided into two classes: internal and external. Internal monooxygenases extract two reducing equivalents from the substrate to reduce one atom of dioxygen to water, whereas external monooxygenases utilize an external reductant. While initially the microsomal drug and xenobiotic-metabolising enzymes were referred to as mixed function oxidases, in more recent years the term monooxygenase became the widely accepted one.

Cytochromes P450 got their name from their character as hemoproteins as well as their unusual spectral properties displaying a typical absorption maximum of the reduced CO-bound complex at 450 nm: cytochrome stands for a hemoprotein, P for pigment and 450 reflects the absorption peak of the CO complex at 450 nm. The ability of reduced P450 to produce an absorption peak at 450 nm upon CO binding is still used for the estimation of the P450 content (Omura and Sato, 1964). The red shift of about 30 nm as observed in cytochromes P450 means that the distribution of electron density at the heme is significantly perturbed as compared to other cytochromes. It has been documented that the cause of this is the thiolate sulphur, which by means of a direct bond to the iron causes this effect. The Soret band (named after its discoverer) describes the absorption band of hemoproteins at about 380–420 nm.

Cytochrome P450 systems mainly catalyse the following reaction:

$$RH + O_2 + NAD(P)H + H^+ \rightarrow ROH + H_2O + NAD(P)^+$$

They are involved in reactions as diverse as e.g. hydroxylation, N-, O- and S-dealkylation, sulphoxidation, epoxidation, deamination, desulphuration, dehalogenation, peroxidation, and N-oxide reduction. This diversity of catalysed

reactions and, of course, the high amount of acceptable substrates is attractive for biotechnological application in particular when it can be transferred to industrial needs.

CYP106A2 is a bacterial steroid hydroxylase from *Bacillus megaterium* ATCC 13368. Since it is soluble and easy to express it has application for biotechnological purposes. Recently, it was possible to design a whole cell bioconversion system for steroids using a mixed system composed of the bovine mitochondrial electron transfer system AdR and Adx and the bacterial enzyme CYP106A2. This mixed P450 monooxygenase system was expressed in *E. coli* cells. Those successful experiments opened the door to facilitate the application of molecular evolution approaches in order to select mutants of the cytochrome with higher stability, activity, and changed regio- and stereo-speci?city suitable to produce hydroxylated steroid derivatives using a biological transformation process [6]. CYP106A2 catalyzes as main reaction route the 15β-hydroxylation of several steroids, e.g. 11-deoxycorticosterone, testosterone, progesterone, and corticosterone (Fig. 1a) [7]. One disadvantage of this system is the limitation for substrates which are membrane permeable like corticosteroids. A successful expression via autodisplay could broaden the variety of substrates and therefore make the P450 monooxygenase system more valuable for future research.

CYP3A4 is the quantitative most important CYP- enzyme and involved in the oxidation of the largest range of substrates of all CYPs. In humans it is predominantly found in the liver and often allows prodrugs to be activated and absorbed. Inhibition or induction of CYP3A4 is a major problem in the daily clinical routine, since it often causes drug-drug interactions or side effects. Induction can lead to the fast inactivation of the applied drug and in consequence to plasma levels so low, that they do not have the desired therapeutic effect anymore. A commonly used CYP3A4 inductor is the anticonvulsant Carbamazepin. Inhibition instead can cause major intoxications due to plasma levels far beyond the therapeutic dose. On the other hand the capability of inhibition is used in the antiretroviral therapy to lower side effects and make it more bearable for patients. Ritonavir is given in a subtherapeutic dose to inhibit the enzyme and booster the effect of further antiretroviral drugs such as Lopinavir. A well-documented example is that of terfenadine, a nonsedating

antihistamine (Fig. 1b). The oxidation of terfenadine is catalyzed very rapidly by CYP3A4 to its major metabolite fexofenadine which is responsible for the pharmacological activity. [8]

The *E. coli* outer membrane channel-tunnel protein TolC is involved in the exclusion of harmful substances such as antibiotics, dyes, organic solvents, and detergents. The crystal structure of the TolC protein recently has been determined. The TolC protein is composed of a transmembrane domain and a periplasmic domain and forms a homotrimer. The periplasmic barrel structure of TolC is connected to drug efflux pump proteins such as AcrB and AcrE, which are located on the inner membrane. Clamp proteins such as AcrA and ArcF link TolC and pump proteins in the periplasmic space. Pump proteins seem to transport toxic cytoplasmic or periplasmic substances into the extracellular space across the outer membrane via the TolC channel [26].

Porphyrins can act as photosensitizers. If porphyrins accumulate, they can be toxic, as the cells can become sensitive to near-UV irradiation. TolC is involved in porphyrin transport across the cell membrane and provides a mechanism to eliminate superfluous or/and toxic porphyrins. The TolC outer membrane channel-tunnel protein can function together with inner membrane efflux pump proteins. Therefore, an inner membrane pump(s) or exporter(s) is assumed to be involved in porphyrin exclusion in combination with TolC. Porphyrin(ogen) exclusion is considered as a two-step process. In this process, porphyrin(ogen)s are transported to the periplasm by a TolC-independent mechanism and then are transported across the outer membrane by the TolC-dependent efflux system [26].

Autodisplay is based on the secretion mechanism of the autotransporter family or proteins [13]. A concept for this secretion mechanism was proposed concurrently with the first autotranspoter protein, IgA1protease from *Neisseria gonorrhoeae* (Fig. 4a) [14]. With the aid of a typical signal peptide, the precursor is transported across the inner membrane. Arrived in the periplasm, the C terminal part of the precursor forms a porin-like structure, a so-called β-barrel, within the outer membrane and through this pore the N terminally attached passenger (the actual protease) is

translocated to the cell surface. To obtain full surface exposure of the passenger, a linker peptide is required in between the β-barrel and the passenger.

For the development of the autodisplay system the β-barrel and the linker region of AIDA-I were combined in frame with the signal peptide of the cholera toxin β-subunit (CTB) and a strong constitutive promoter (P_{TK}) within a medium copy number plasmid backbone [15]. Into the linker regions used for autodisplay, protease cleavage sites for the sequence specific release of the passenger protein, as well as epitopes for detection by monoclonal antibodies were inserted. An antibody independent detection method, which requires only the addition of a single cysteine in the linker region, was developed for autodisplay and was named “Cystope tagging” [16,17]. A schematic description of the structure of a typical artificial autotransporter protein used for autodisplay is given in Fig. 4b. As mentioned above, the terminal step in autodisplay requires the translocation of the passenger through a size-limited pore formed by the β-barrel. This means that the passenger is not allowed to acquire a stable three dimensional conformation during transport to maintain a transport compatible state [18,19]. In case of stable folding, transport is blocked in the periplasm [19]. As a wide variety of passenger proteins with high biotechnological impact contain disulfide bridges and these bonds are normally formed in the periplasm of *E. coli*, a DsbA-negative mutant strain of *E. coli* (JK321) was constructed and shown to facilitate the autodisplay of such types of proteins as well [19]. In summary, the autodisplay system consists of vectors encoding various artificial autotransporter genes using the β-barrel from AIDA-I and different parts of its linkerregion. Dependent on the application, different modifications of the linker regions, various signal peptides under the control of inducible or constitutive promoters, mutant strains of *E. coli* supporting the transport and the surface display by the autotransporter pathway and detection methods are now available, that allow to follow surface translocation, preferentially independent of the protein domain used as a passenger. It is obvious, that autodisplay is restricted to Gram-negative bacteria i.e. *E. coli* or *Salmonella* as host organisms. Beyond this limitation, the autodisplay system has interesting activa. First, more than 100.000 active enzyme molecules can be displayed per single cell of *E. coli* without loss in cell integrity. Second, dimers or multimers can be formed spontaneously at the cell surface by

subunits expressed from monomeric genes, which is a unique feature of this surface display system and due to the free motility of the anchoring motif, the β-barrel within the outer membrane. Third, EP 02718168 describes that anorganic prosthetic groups (e.g. 2Fe-2S) can be incorporated by a single step/one vial procedure without affecting cell viability, another feature that has not been described for any other surface display system so far. These features have been used in combination for the construction of whole cell biocatalysts displaying functional enzymes which were used as technological tools for the regio- and enantioselective synthesis of products, especially from substrates with several identical reactive groups, including sugars polyalcohols and steroids with high efficiency [12,21,22].

EP 02718168 describes autodisplay of adrenodoxin on an *E. coli* cell. Adrenodoxin belongs to the [2Fe-2S] ferredoxins, a family of small acidic iron-sulfur proteins. When displayed on the surface, the adrenodoxin is present in a non-functional form, because no prosthetic group is present. According to EP 02718168, a functional adrenodoxin attached to the cell surface can be obtained by contacting the adrenodoxin molecule with an exogenous [2Fe-2S] cluster serving as a prosthetic group.

The problem of the present invention is the provision of surface displayed enzymes comprising metal porphyrin-containing or flavin containing prosthetic groups. It was surprisingly found that by recombinant expression of these enzymes by surface display on a Gram-negative bacterium, a functional enzyme comprising the metal porphyrin-containing or flavin containing prosthetic group could be identified on the cell surface without introducing an exogenous prosthetic group, as described for enzymes containing [2Fe-2S] clusters in EP 02718168. In other words, polypeptides comprising prosthetic groups containing a metal porphyrin or a flavin can translocate to the cell surface in a conformation capable of retaining the prosthetic group when crossing the outer membrane, for example by mediation of the Omp85 pathway. The prosthetic group may also be transported to the cell surface independently from the surface-displayed enzyme. Mechanisms are known for elimination of superfluous or toxic compounds, including compounds suitable as prosthetic groups (e.g., porphyrins), from the cell. In the present invention, it has been surprisingly found that metal porphyrins transported across the cell membrane into the extracellular space independently from

the enzyme/autotransporter construct can contact the enzyme displayed on the cell surface to a form an active enzyme (see Example 2).

Thus, a first aspect of the present invention is a method for displaying a recombinant polypeptide containing a prosthetic group on the surface of a host cell, wherein the prosthetic group comprises a metal porphyrin or a flavin, said method comprising the steps:

(a) providing a host cell transformed with a nucleic acid fusion operatively linked with an expression control sequence said nucleic acid fusion comprising:

(i) a portion encoding a signal peptide,

(ii) a portion encoding the recombinant polypeptide to be displayed,

(iii) optionally a portion encoding a protease recognition site,

(iv) a portion encoding a transmembrane linker, and

(v) a portion encoding the transporter domain of an autotransporter, and

(b) culturing the host cell under conditions wherein the nucleic acid fusion is expressed and the expression product comprising the recombinant polypeptide containing the prosthetic group is displayed on the surface of the host cell.

By the method of the present invention, a functional recombinant polypeptide can be displayed. As indicated above, display of the functional recombinant polypeptide of the present invention comprising a prosthetic group containing a metal porphyrin or a flavin does not require an exogenously added prosthetic group. In the present invention, the prosthetic group can be produced by the host cell ("endogenously produced prosthetic group"). In a preferred embodiment, the method of the present invention, in particular step (b), is performed with the proviso that the surface-displayed recombinant polypeptide is not contacted with an exogenous prosthetic group being a metal porphyrin or a flavin. In this context, "exogenous prosthetic group" refers to a prosthetic group not produced by the host cell.

The recombinant polypeptide to be displayed may also be termed "passenger", "passenger polypeptide" or "passenger protein".

Step (a) of the methods of the present invention refers to the provision of a host cell. The host cell used in the method of the present invention is preferably a bacterium, more preferably a Gram-negative bacterium, particularly an enterobacterium such as *E. coli*.

According to the present invention, a host cell, particularly a host bacterium is provided which is transformed with a nucleic acid fusion operatively linked with an expression control sequence, i.e. a promoter, and optionally further sequences required for gene expression in the respective host cell. The skilled person knows suitable promoters and expression control sequences. The promoter or/and the expression control sequence may be homologous or heterologous to the host cell. Preferably, the nucleic acid fusion is located on a recombinant vector, e.g. a plasmid vector. The host cell may be transformed with at least one nucleic acid fusion, for instance two, three, four, five or even more nucleic acid fusions. If two or more nucleic acid fusions are transformed into a host cell, the nucleic acid fusions preferably encode different recombinant polypeptides as described herein. If a host cell transformed with several nucleic acid fusions is used, these nucleic acid fusions may be located on a single vector or on a plurality of vectors.

At least one host cell as described herein, for instance two, three, four, five, six or even more host cells as described herein may be provided in the methods of the present invention. Each of these host cells is transformed with one nucleic acid fusion or at least one nucleic acid fusion, as described herein. Preferably, the nucleic acid fusions transformed in the at least one host cell encode different recombinant polypeptides as described herein.

The different recombinant polypeptides which may be provided in one or at least one host cell may form a functional unit, for instance the subunits of a functional unit,

such as the subunits of an enzyme or the subunits or/and components of an enzyme complex.

The nucleic acid fusion comprises (i) a portion encoding a signal peptide, preferably a portion coding for a Gram-negative signal peptide allowing for transport into the periplasm through the inner cell membrane. The signal peptide may be a signal peptide homologous to the host cell. The signal peptide may also be a signal peptide heterologous to the host cell.

Further, the nucleic acid fusion comprises (ii) a portion encoding the recombinant polypeptide to be displayed.

Further, the nucleic acid fusion optionally comprises a portion encoding a protease recognition site, which may be a recognition site for an intrinsic protease, i.e. a protease naturally occurring in the host cell, or an externally added protease. For example, the externally added protease may be an IgA protease (cf. EP-A-0 254 090), thrombin or factor X. The intrinsic protease may be e.g. selected from OmpT, OmpK or protease X.

Furthermore, the nucleic acid fusion comprises (iv) a portion encoding a transmembrane linker which is required for the presentation of the passenger polypeptide (ii) on the outer surface of the outer membrane of the host cell. A transmembrane linker domain may be used which is homologous with regard to the autotransporter, i.e. the transmembrane linker domain is encoded by a nucleic acid portion directly 5' to the autotransporter domain. Also a transmembrane linker domain may be used which is heterologous with regard to the autotransporter. The length of the transmembrane linker is preferably 30-160 amino acids.

Further, the nucleic acid fusion comprises (v) a transporter domain of an autotransporter. In the context of the present invention, autodisplay may be the recombinant surface display of proteins or polypeptides by means of an

autotransporter in any Gram-negative bacterium. The transporter domain of the autotransporter according to the invention can be any transporter domain of an autotransporter and is preferably capable of forming a β-barrel structure. A detailed description of the β-barrel structure and preferred examples of β-barrel autotransporters are disclosed in WO97/35022 incorporated herein by reference. Henderson et al. (2004) describes autotransporter proteins which comprise suitable autotransporter domains (for summary, see Table 1 of Henderson et al., 2004). The disclosure of Henderson et al. (2004) is included herein by reference. For example, the transporter domain of the autotransporter may be selected from Ssp (P09489, *S. marcescens*), Ssp-h1 (BAA33455, *S. marcescens*), Ssp-h2 (BAA11383, *S. marcescens*), PspA (BAA36466, *P. fluorescens*), PspB (BAA36467, *P. fluorescens*), Ssa1 (AAA80490, *P. haemolytica*), SphB1 (CAC44081, *B. pertussis*), AspA/NalP (AAN71715, *N. meningitidis*), VacA (Q48247, *H. pylori*), AIDA-I (Q03155, *E. coli*), IcsA (AAA26547, *S. flexneri*), MisL (AAD16954, *S. enterica*), TibA (AAD41751, *E. coli*), Ag43 (P39180, *E. coli*), ShdA (AAD25110, *S. enterica*), AutA (CAB89117, *N. meningitidis*), Tsh (I54632, *E. coli*), SepA (CAC05786, S. flexneri), EspC (AAC44731, *E. coli*), EspP (CAA66144, *E. coli*), Pet (AAC26634, *E. coli*), Pic (AAD23953, *E. coli*), SigA (AAF67320, S. flexneri), Sat (AAG30168, *E. coli*), Vat (AAO21903, *E. coli*), EpeA (AAL18821, *E. coli*), EatA (AAO17297, *E. coli*), EspI (CAC39286, *E. coli*), EaaA (AAF63237, *E. coli*), EaaC (AAF63038, *E. coli*), Pertactin (P14283, *B. pertussis*), BrkA (AAA51646, *B. pertussis*), Tef (AAQ82668, *B. pertussis*), Vag8 (AAC31247, *B. pertussis*), PmpD (O84818, *C. trachomatis*), Pmp20 (Q9Z812, *C. pneumoniae*), Pmp21 (Q9Z6U5, *C. pneumoniae*), IgA1 protease (NP_283693, *N. meningitidis*), App (CAC14670, *N. meningitidis*), IgA1 protease (P45386, *H. influenzae*), Hap (P45387, *H. influenzae*), rOmpA (P15921, *R. rickettsii*), rOmpB (Q53047, *R. rickettsii*), ApeE (AAC38796, *S. enterica*), EstA (AAB61674, *P. aeruginosa*), Lip-1 (P40601, *X. luminescens*), McaP (AAP97134, *M. catarrhalis*), BabA (AAC38081, *H. pylori*), SabA (AAD06240, *H. pylori*), AlpA (CAB05386, *H. pylori*), Aae (AAP21063, *A. actinomycetemcomitans*), NanB (AAG35309, *P. haemolytica*), and variants of these autotransporters. Given in brackets for each of the exemplary autotransporter proteins are examples of suitable genbank accession numbers and species from which the autotransporter may be obtained. Preferably the transporter domain of the autotransporter is the *E. coli* AIDA-I protein or a variant thereof, such as e.g. described by Niewert U., Frey A., Voss T., Le Bouguen C., Baljer G., Franke S., Schmidt MA. The AIDA Autotransporter System is Associated

with F18 and Stx2e in Escherichia coli Isolates from Pigs Diagnosed with Edema Disease and Postweaning Diarrhea. Clin. Diagn. Lab. Immunol. 2001 Jan, 8(1):143-149;9.

Variants of the above indicated autotransporter sequences can e.g. be obtained by altering the amino acid sequence in the loop structures of the β-barrel not participating in the transmembrane portions. Optionally, the nucleic acid portions coding for the surface loops can be deleted completely. Also within the amphipathic β-sheet conserved amino exchanges, i.e. the exchange of an hydrophilic by another hydrophilic amino acid or/and the exchange of a hydrophobic by another hydrophobic amino acid may take place. Preferably, a variant has a sequence identity of at least 70%, at least 90%, at least 95% or at least 98% on the amino acid level to the respective native sequence of the autotransporter domain, in particular in the range of the β-sheets.

Step (b) of the methods of the present invention refers to culturing the host cell under conditions wherein the nucleic acid fusion is expressed and the expression product comprising the recombinant polypeptide is displayed on the surface of the host cell. The person skilled in the art knows suitable culture conditions. The method according to the invention allows for an efficient expression of passenger proteins on the surface of host cells, particularly *E. coli* or other Gram-negative bacterial cells up to 100 000 or more molecules per cell by using a liquid medium of the following composition: 5 g/l to 20 g/l, preferably about 10 g/l trypton, 2 g/l to 10 g/l, preferably about 5 g/l yeast extract, 5 g/l to 20 g/l, in particular about 10 g/l NaCl and the remaning part water. The medium should possibly contain as little as possible divalent cations, thus preferably Aqua bidest or highly purified water, e.g. Millipore water is used. The liquid medium may contain in addition preferably EDTA in a concentration of 2 µM to 20 µM, in particular 10 µM. Moreover, it contains preferably reducing reagents, such as 2-mercapto ethanol or dithiotreitol or dithioerythritol in a preferred concentration of 2 mM to 20 mM. The reducing reagents favour a non-folded structure of the polypeptide during transport. The liquid medium can further contain additional C-sources, preferably glucose, e.g. in an amount of up to 10 g/l, in order to favour secretion i.e. transfer of the passenger to the surrounding medium.

For surface display preferably no additional C-source is added. Preferred culture conditions for Gram-negative cells, such as *E. coli*, are described in the Examples.

If the host cell is a Gram-negative bacterium, the polypeptide synthesized in the cytoplasma can be translocated from the cytoplasm into the periplasmic space by crossing the inner membrane. This can be effected by the signal peptide.

While not wishing to be bound by theory, display of a functional polypeptide comprising a metal porphyrin or a flavin on the surface of a Gram-negative cell by autodisplay can be explained as follows. In a first step the prosthetic group comprising a metal porphyrin or a flavin is introduced into the polypeptide of the present invention in the periplasmic space. In a second step, the recombinant polypeptide of the present invention is translocated from the periplasmic space onto the cell surface in a conformation capable of retaining the prosthetic group when crossing the outer membrane, for example via the Omp85 pathway. By this procedure, a functional polypeptide attached to the cell surface can be obtained. In a different mechanism, the prosthetic group, present in the periplasmic space, may be transported independently from the recombinant polypeptide across the outer membrane. A suitable transporter is the outer membrane channel-tunnel protein TolC, in particular for the transportation of metal porphyrins. Both mechanisms may account for transportation of at least a part of prosthetic group transported to the cell surface.

In the present invention, the prosthetic group can be transported to the cell surface by any suitable transport protein, which may be recombinantly expressed in the host cell. This transport can be independent from the autotransporter. The prosthetic group being a metal porphyrin can preferably be transported to the cell surface by a TolC-dependent mechanism. The prosthetic group being a metal porphyrin can also be transported to the cell surface by TolC or/and another suitable transport protein. In Gram negative cells, the prosthetic group being a metal porphyrin can preferably be transported across the outer membrane surface by TolC.

In the present invention, any TolC polypeptide may be employed. For example, an *E. coli* TolC may be employed.

In particular, the TolC polypeptide is homologous to the host cell. For example, an *E. coli* TolC may be employed in an *E. coli* host cell.

The TolC polypeptide may be a recombinant TolC. For example, the TolC polypeptide may be recombinantly expressed in the host cell. TolC may be over-expressed in the host cell. If, for example, the host cell has only low expression of TolC and thus only low capability of porphyrin transport to the cell surface, TolC may be over-expressed.

The TolC polypeptide, as used herein, may comprise a sequence selected from

(a) SEQ ID NO:8, and

(b) sequences having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identity to the sequence of (a).

The TolC polypeptide, as used herein, may be encoded by a sequence selected from

(a) nucleic acid sequences encoding the amino acid sequences of SEQ ID NO:8,

(b) nucleic acid sequences encoding amino acid sequences having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identity to the amino acid sequence of SEQ ID NO:8,

(c) SEQ ID NO:7, and

(d) sequences having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identity to the sequence of (c).

In particular, nucleic acid sequences of (a), (b) and (d) include sequences within the scope of the degeneracy of the genetic code.

The TolC polypeptide, as defined herein, may be a HasF polypeptide, for example from Serratia marcescens.

If the passenger polypeptide is transported together with the prosthetic group to the cell surface, the passenger may acquire the prosthetic group within the cell. In the method of the present invention, in step (b), the prosthetic group endogenously produced in the cell may be introduced into the polypeptide of the present invention within the periplasmic space.

In the method of the present invention, step (b) may involve the omp85 pathway. Step (b) may comprise transportation of the polypeptide of the present invention via the omp85 pathway. Step (b) may comprise translocation of the polypeptide of the present invention from the periplasmic space onto the cell surface by the omp85 pathway, in particular in a conformation capable of retaining the prosthetic group when crossing the outer membrane.

In the present invention, any Omp85 or Omp85 homologue may be employed. "Omp85", as used herein, includes homologues of Omp85. For example, the Omp85 homologue YaeT from *E. coli* may be employed.

The Omp85, in particular YaeT, may comprise a sequence selected from

(a) SEQ ID NO:3 and SEQ ID NO:4, and
(b) sequences having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identity to the sequence of (a).

Also employed may be a nucleic acid encoding an Omp85. The nucleic acid encoding Omp85, in particular YaeT, may comprise a nucleic acid sequence selected from

(a) nucleic acid sequences encoding the amino acid sequence of SEQ ID NO:3 and SEQ ID NO:4, and
(b) nucleic acid sequences encoding amino acid sequences having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identity to the amino acid sequence of SEQ ID NO:3 or/and SEQ ID NO:4,

In particular, nucleic acid sequences of (a) and (b) include sequences within the scope of the degeneracy of the genetic code.

The components (i) to (v) in the nucleic acid fusion of the present invention are preferably oriented from 5' to 3'. In the expression product obtained in step (b), the amino acid sequences encoded by nucleic acid sequences (i) to (v) are preferably arranged N terminal to C terminal.

The method of the present invention may comprise preparing a membrane preparation from the cell obtained in step (b). The membrane preparation may comprise membrane particles. The membrane particles may be membrane vesicles. Preferred membrane particles are outer membrane particles. In particular the method of the present invention may comprise preparing outer membrane particles of cells displaying a recombinant polypeptide on the surface, e.g. of Gram-negative bacterial cells. The person skilled in the art knows suitable conditions (e.g. Hantke, 1981, Schultheiss et al., 2002). Typical conditions for preparing membrane particles are employed in the examples of the present invention. Outer membrane particles from a host cell as described herein may be performed by a method comprising the steps:

(a) treating the host cell with a hydrolase (such as lysozyme) and optionally with a DNAse. This enzymatic treatment may be performed at room temperature. The hydrolase hydrolyses the cell wall within the periplasmic space. The cell wall comprises peptidoglycans to be hydrolyzed.

(b) optionally solubilizing the preparation of (a) with a tenside, such as Triton X-100, or/and with sarcosine, followed by optional centrifugation of cell debris. The thus obtained preparation of outer membrane particles may be centrifuged, washed and resuspended.

The diameter of the membrane particles may be in the range of 1 nm to 1000 nm, in the range of 50 nm to 500 nm, in the range of 75 to 200 nm, or in the range of 90 to 120 nm. At least 80%, at least 90%, at least 95 %, or at least 98% of the membrane particles may have a diameter in a range selected from the ranges described herein.

In a host cell being a Gram-negative bacterium, such as *E. coli*, after translocation, the recombinant passenger remains attached to the surface of the outer membrane by the β-barrel, which is serving as an anchor within the outer membrane. Due to the controlled integration of the β-barrel within the outer membrane, the C terminal part of the β-barrel is directed to the inner side of the outer membrane, whereas the N-terminal part of the linker, to which the recombinant passenger protein is covalently bound, is directed to the outer surface of the outer membrane, i.e. the environment. The recombinant passenger protein has an oriented location after transport, namely it is directed to the cellular surface. The recombinant passenger protein has the identical orientation as the lipopolysaccharide (LPS) layer which may be present in the outer membrane.

Membrane particles of the present invention prepared from the host cell of the present invention comprise the recombinant peptide at the surface directed to the environment. In contrast to the inner membrane which is a unit membrane, the outer membrane of Gram-negative bacteria, in particular *E. coli*, is asymmetric. The outer membrane may comprise an inner layer comprising phospholipids and an outer layer comprising LPS. LPS is hydrophilic and may contain several negative charges. By using outer membrane particles with anchored passenger proteins by a β-barrel for the coating of carriers, the outer side of the outer membrane, in particular the LPS side will be directed to the surface distal to the carrier. As a consequence the recombinant protein or a domain thereof, which are integrated in the outer membrane by autodisplay, will be directed to the surface distal to the carrier as well. The core part of the membrane particles may stabilize the interaction of the outer membrane layer obtained by applying outer membrane particles to the carrier by hydrophobic interactions and may contain lipoproteins or peptidoglycans.

A preferred prosthetic group is a metal porphyrin, as described herein.

The prosthetic group being the metal porphyrin may comprise a heavy metal such as cobalt, nickel, manganese, copper and iron. The metal porphyrin of the present invention in particular comprises a heme group.

Another preferred prosthetic group is a flavin, as described herein.

The prosthetic group being the flavin may be selected from FAD and FMN.

The polypeptide of the present invention comprising a prosthetic group preferable is an enzyme.

The polypeptide comprising the metal porphyrin may be an enzyme. The polypeptide comprising the metal porphyrin may be selected from P450 enzymes (such as P450 reductases) and cytochromes (such as cytochrome b5). The polypeptide comprising the metal porphyrin may be selected from hemoproteins. In particular, the polypeptide comprising the metal porphyrin may be selected from monooxygenases. The polypeptide comprising the metal porphyrin may be selected from CYP11B1, CYP11A1, CYP106A2 and CYP3A4. The polypeptide comprising the metal porphyrin may preferably be selected from CYP106A2 and CYP3A4.

In a preferred embodiment, the polypeptide comprising the metal porphyrin comprises a sequence selected from

(a) SEQ ID NO:2 and SEQ ID NO:6, and
(b) sequences having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identity to the sequence of (a).

The portion (ii) of the nucleic acid fusion of the present invention may encode a polypeptide which, when functional, comprises the metal porphyrin, as described herein. The portion (ii) of the nucleic acid fusion of the present invention may comprise a nucleic acid sequence selected from

(a) nucleic acid sequences encoding the amino acid sequences of SEQ ID NO:2 and SEQ ID NO:6,
(b) nucleic acid sequences encoding amino acid sequences having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identity to the amino acid sequence of SEQ ID NO:2 or/and SEQ ID NO:6,

(c) SEQ ID NO:1 and SEQ ID NO:5, and

(d) sequences having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identity to the sequence of (c).

In particular, nucleic acid sequences of (a), (b) and (d) include sequences within the scope of the degeneracy of the genetic code.

The skilled person knows suitable methods to determine the degree of identity of nucleic acid sequences and amino acid sequences. Known algorithms, such as BLAST (for nucleic acids) or PBLAST (for amino acid sequences) may be used. A nucleic acid or polypeptide comprising sequences having at least 70%, at least 80%, at least 90%, at least 95%, or at least 98% identity to a given sequence includes fragments of the given nucleic acid or polypeptide.

The flavin containing polypeptide of the present invention may be selected from flavoproteins, in particular FAD or FMN containing proteins. Preferred are FAD containing proteins. The flavin containing polypeptide may be selected from enzymes such as oxidoreductases, NADH oxidases, dehydrogenases, and oxidases, especially sugar oxidases, such as pyranose oxidase. The NADH oxidase is in particular an FAD containing enzyme.

The polypeptide of the present invention to be displayed on the surface of the cell may be a multimeric polypeptide. The multimeric recombinant polypeptide may be a homodimer, i.e. a polypeptide consisting of two identical subunits or a homomultimer, i.e. a polypeptide consisting of three or more identical subunits. On the other hand, the multimeric recombinant polypeptide may be a heterodimer, i.e. a polypeptide consisting of two different subunits or a heteromultimer consisting of three or more subunits wherein at least two of these subunits are different. For example, the multimeric polypeptide is comprised of a plurality of subunits which form a "single" multimeric polypeptide or a complex of a plurality of functionally associated polypeptides which may in turn be monomeric and/or multimeric polypeptides. It should be noted that at least one subunit of the multimeric recombinant protein may contain at least one prosthetic group as described herein. Further, is should be noted that the nucleic acid

fusion may encode a plurality of polypeptide subunits as a polypeptide fusion which when presented on the cell surface forms a functional multimeric polypeptide.

Homodimers or homomultimers may be formed by a spontaneous association of several identical polypeptide subunits displayed on the host cell membrane. Heterodimers or heteromultimers may be formed by a spontaneous association of several different polypeptide subunits displayed on the host cell membrane.

On the other hand, a multimeric recombinant polypeptide may be formed by an association of at least one polypeptide subunit displayed on the host cell membrane, as described herein, and at least one soluble polypeptide subunit added to the host cell membrane. The added subunit may be identical to the displayed subunit or be different therefrom.

Yet another aspect of the present invention is a host cell displaying the recombinant polypeptide on the surface. The host cell may be any host cell as described herein, in particular a host cell displaying a recombinant polypeptide on the surface thereof, wherein the recombinant polypeptide contains a prosthetic group comprising a metal porphyrin or a flavin, and wherein the recombinant polypeptide comprises

(I) a portion comprising the recombinant polypeptide to be displayed,

(II) optionally a portion comprising a protease recognition site,

(III) a portion comprising a transmembrane linker, and

(IV) a portion comprising the transporter domain of an autotransporter.

The displayed polypeptide is in particular a functional polypeptide.

The portions (I) to (IV) of the recombinant polypeptide displayed by the host cell of the present invention are encoded in particular by the components (ii), (iii), (iv) and (v) of the nucleic fusion, as described herein.

Yet another aspect of the present invention is a membrane preparation comprising a recombinant polypeptide. The membrane preparation of the present invention may comprise membrane particles, as described herein. The membrane preparation may be obtained from a host cell as described herein. The recombinant polypeptide of the may be any recombinant polypeptide as described herein.

Yet another aspect of the present invention is the use of a membrane preparation comprising a recombinant polypeptide in the manufacture of a carrier comprising a recombinant polypeptide.

The membrane preparation of the present invention may be employed for coating a carrier. The carrier may comprise a membrane preparation of the present invention, as described herein.

The carrier may comprise a hydrophobic surface. The hydrophobic surface may have a contact angle of more than 90°. A increasing surface angle of more than 30° indicates a gradually increasing hydrophobicity of a surface. In the present context, a hydrophobic surface may have a contact angle of at least 40°. The surface preferably has a hydrophobicity described by a contact angle of at least 40°, at least 50°, at least 60°, at least 65°, at least 70°. Contact angles are preferably determined by the sessile drop method. The sessile drop method is a standard method for determining contact angles. Measurements may be performed with a contact angle goniometer. Preferred contact angles of the hydrophobic surface are in a range of 40° to 100°, 50° to 90°, or 60° to 80°.

The surface of the carrier may be a metal surface. A suitable metal surface has a contact angle e.g. in the range of 50° to 80°. A suitable metal may be selected from gold, silver, titanium, aluminium and alloys such as brass. A preferred surface is a gold surface. The gold surface may be employed as it is. An untreated gold surface has a hydrophobicity suitable for the carrier as described herein. A treatment of the gold surface with thiolated hydrocarbons or hydrocarbons with functional groups such as carboxylic acids or hydroxyl groups is not required.

Another preferred surface of the carrier comprises a polymer, for instance a surface usually employed in disposable materials for use in biochemical or/and medical science. The polymer may be an artificial polymer. Examples of artificial polymers include a polymer selected from polystyrenes, polypropylenes, and polycarbonates. The polystyrene may be produced from [2,2]paracyclophane monomers. Polystyrene surfaces may be treated with oxygene plasma introducing OH or/and methylene groups in order to modify the hydrophobicity. Examples of such modified surfaces include Maxi-sorp, Medi-sorp, Multi-sorp, and Poly-sorp surfaces. Another suitable polystyrene surface is Parylene N produced from [2,2]paracyclophane monomers. Yet another suitable surface is Parylene A [Poly(monoamino-p-xylene)]. Especially suitable are surfaces comprising a polymer having a hydrophobicity described by a contact angle of at least 50°. Suitable surfaces are selected from polystyrene, Parylene A, Parylene N, Maxi-sorp, Medi-sorp, Multi-sorp, and Poly-sorp. Preferred surfaces are selected from polystyrene, Parylene A, Parylene N, Maxi-sorp, Medi-sorp, and Poly-sorp.

The surface may comprise a natural polymer. Suitable natural polymers include polybutyrate and cellulose and derivatives thereof. A further surface is provided by latex particles, in particular latex beads.

Yet another surface is provided by C18-modified particles, in particular C18-modified monolithic silica particles. C18 refers to an alkyl group comprising 18 carbon atoms. C18-modified particles are known in the art.

Yet another suitable surface is a glass surface.

The surface may be modified is order to adjust the hydrophobicity. Modification may be performed by chemical treatment (i.e. by oxygen plasma), physical treatment (e.g. by laser irradiation or/and radioactive irradiation), or by mechanical treatment.

The method according to the invention and the host cells according to the invention can be used for a variety of different applications, e.g. as whole cell biofactories or membrane preparation biofactories for chemical synthesis procedures, e.g. for the synthesis of organic substances selected from enzyme substrates, drugs, hormones, starting materials and intermediates for syntheses procedures and chiral substances (cf. Roberts, Chemistry and Biology 6 (1999), R269-R272). Typical CYP106A2 substrates are described in Fig. 12.

In particular, the method according to the invention and the host cells according to the invention, as described herein, can be used in the chemical synthesis, for example in enzymatically catalyzed enantioselective or/and regioselective steps. For example, CYP106A2 displayed on the surface of a cell, as described herein, can be used for the conversion of steroids, for the conversion of abietic acid, or for the preparation of desipramine from imipramine, as exemplified by Examples 1 and 3.

Furthermore, the cell or the membrane preparation of the invention may be used for a directed evolution procedure, e.g. for the development of new biocatalysts for the application in organic syntheses.

This is achieved in a particular embodiment by varying the amino acid sequence of the polypeptide containing a prosthetic group selected from metal porphyrins and flavins, as described herein, via site-specific or random mutagenesis and by testing variant carrying cells or membrane preparations or libraries containing variant carrying cells or membrane preparations thereof using a certain chemical reaction with the help of suitable screening methods, in particular high throughput screening (HTS) methods for the conversion of a certain substrate.

In yet another preferred embodiments libraries of variants of a polypeptide containing a prosthetic group selected from metal porphyrins and flavins, as described herein, are examined in view of the role of defined amino acids during certain functions, in particular catalytic functions.

In general, these particular embodiments concern the production of variants of proteins and/or enzymes and the production of libraries with variants of proteins and/or enyzmes, respectively, which carry a prosthetic group, as described herein, or multimers etc. and which are screened in view of a certain characteristic, i.e. one or optionally several variants fulfilling this desired characteristic perfectly are selected. By selecting the variant the cell is selected, too, and carries the nucleic acid coding the variant. Thus, at the same time both the amino acid sequence and the structural information of the variant can be determined via the nucleic acid sequence. The characteristics in question are particularly enzyme inhibiting, catalytical, toxin degrading, synthesizing, therapeutical etc. characteristics.

Moreover, the host cell or the membrane preparation may be used as an assay system for a screening procedure, e.g. for identifying modulators (activators or inhibitors) of displayed polypeptides, containing a prosthetic group selected from metal porphyrins and flavins, as described herein, which may be used as potential therapeutic agents. The screening procedure may also be used to identify variants of displayed polypeptides having predetermined desired characteristics. For this purpose, libraries of modulators and/or libraries of displayed polypeptides may be used. Further, the host cells or membrane preparations derived therefrom may be used as a system for toxicity monitoring and/or degrading toxic substances in the environment, in the laboratory or in biological, e.g. human, animal, or non-biological systems.

An essential advantage of applying the host cells and membranes according to the invention is enabling correct folding and biological activity of proteins or protein complexes, e.g. of the polypeptide containing a prosthetic group selected from metal porphyrins and flavins, as described herein, which require a membrane environment. Thus, a reconstitution as previously considered to be necessary is no longer required. Thereby the production steps of a functional biocatalytic system are simplified and an increased stability of the system per se is obtained.

Further preferred examples for the recombinant polypeptide to be displayed, i.e. the passenger polypeptides are peptides or proteins selected from the group of drug

metabolizing enzymes, such as CYP1A2 involved in the activation of aromatic amine carcinogenes, heterocyclic arylamine promutagenes derived from food pyrolysates and aflatoxin B1 (Gallagher EP, Wienkers LC, Stapleton PL, Kunze KL, Eaton DL., Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. Cancer Res. 1994, Jan 1;54(1):101-8) or CYP2E1 capable of activating the procarcinogenes N-nitrosodimethylamine and N-nitrosodiethylamin and metabolizes the procarcinogenes benzene, styrene, carbon tetrachloride, chloroform (Yoo JS, Ishazaki H, Yang CS., Roles of cytochrome P450IIE1 in the dealkylation and denitrosation of N-nitrosodimethylamine and N-nitrosodiethylamine in rat liver microsomes. Carcinogenesis. 1990 Dec; 11(12):2239-43; Peter R, Bocker R, Beaune PH, Iwaskai M, Guengerich FP, Yang CS., Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. Chem. Res. Toxicol. 1990 Nov-Dec;3(6):566-73). Further preferred passenger peptides are peptides from the group of steroid biosynthesis enyzmes, such as CYP11B1 involved in the formation of cortisol and aldosterone (Bernhardt R., Cytochrome P450: structure, function and generation of reactive oxygen species. Rev. Physiol. Biochem. Pharmacol. 1996;127:137-221) or CYP19 involved in the conversion of adrostenedione to 19-hydroxyandrostenedione, 19-oxo-androstenedione and estrone (Ryan KJ., Biological aromatization of steroids. J. Biol. Chem. 1959;134:268). Further preferred metal ion containing enzymes are Cu-containing enzymes, such as cytochrome-oxidase, Mn-containing enzymes, such as arginase and ribonucleotide reductase, Mo-containing enzymes, such as dinitrogenase and Se-containing enzymes, such as glutathione peroxidase.

Preferably the P450 enzymes are hepatic P450 enzymes, particularly P450 3A4, 2D6, 2C9 and 2C19. The host cells and/or preparations according to the invention are preferably used sequentially for testing the enzyme inhibition of P450 enzymes. For example, with the help of the host cell and/or membrane preparation according to the invention it can be found out in an early step of drug discovery, the so-called lead identification, whether the new drug lead structure to be tested could possibly have side-effects or lead to the so-called drug-drug interaction.

Further, the present invention shall be further illustrated by the following figures and examples:

Figure 1: **A:** Schematic drawing of the hydroxylation reaction in the 15β-position of the steroid 11-deoxycorticosterone (DOC) catalyzed by CYP106A2. Redox equivalents are transferred from NADPH via the proteins AdR and Adx to the steroid converting enzyme CYP106A2. **B:** Oxidation of terfenadine by CYP3A4 to fexofenadine.

Figure 2: Chromatogram of the CYP106A2 activity assay. Conversion of 11-deoxycorticosterone into 15beta-deoxycorticosterone using the pure enzyme. (Reichstein´s Compound S, RSS, internal standard)

Figure 3: Sequence of CYP106A2. Nucleic acid sequence (SEQ ID NO:1) and derived amino acid sequence (SEQ ID NO:2) of the CYP106A2 insert in plasmid pET-CYP13.

Figure 4: **A:** Secretion mechanism of the autotransporter proteins in Gram-negative bacteria. **B:** Structure of a typical artificial autotransporter protein used in autodisplay (SEQ ID NO:9 and SEQ ID NO:10). **C:** Structure of the CYP106A2 fusion protein. Illustration of the fusion proteins necessary for the expression of CYP106A2. Important restriction sites for cloning are underlined (SEQ IDs NO:11-14).

Figure 5: Expression of CYP106A2. SDS-PAGE (10 %) and Coomassie staining of outer membrane preparations obtained form *E. coli* BL21(DE3) pET-CYP13. 1: marker proteins, 2: control, BL21(DE3) without plasmid, 3: BL21 (DE3) pET-CYP13 – IPTG, 4: BL21 (DE3) pET-CYP13 + 1 mM IPTG.

Figure 6: **a:** Surface display of CYP106A2. Whole cell trypsin digestion and subsequent SDS-PAGE (10 %) and Coomassie staining of outer membrane preparations obtained from *E. coli* BL21(DE3) pET-CYP13. 1: marker proteins, 2: control, BL21(DE3) without plasmid, 3: BL21 (DE3) pET-CYP13 + IPTG - trypsin, 4: BL21 (DE3) pET-CYP13 + 1 IPTG + trypsin. **b:** Proof of successful surface display of CYP106A2 by indirect immune fluorescence A: *E. coli* BL21 (DE3) pETCYP13, abs.: 490nm, em.: 520nm, B: *E. coli* BL21 (DE3) pETCYP13, transmitted light, C: *E. coli* BL21 (DE3), abs.: 490nm, em.: 520nm, D: *E. coli* BL21 (DE3), transmitted light. All samples were prepared with two antibodies: a primary polyclonal anti- CYP106A2 antibody and a secondary FITC-labelled detection antibody. Only the cells containing the expression plasmid showed a positive reaction (see A). **c:** Proof of successful surface display of CYP106A2 by flow cytometry. All samples were prepared with two antibodies: a primary polyclonal anti- CYP106A2 antibody and a secondary FITC- labelled detection antibody. The mean fluorescence (mF) of the labelled cells was determined by FACS analysis; BL21(DE3) (negative control), mF = 27; cells displaying CYP106A2 on the surface (BL21(DE3) pETCYP13), mF = 268.

Figure 7: Chromatogram of the activity assay with whole cells displaying CYP106A2. Conversion of 11-deoxycorticosterone into 15beta-deoxycorticosterone by BL21(DE3) pETCYP13. For that purpose cells were cultivated and half of them induced with 1mM IPTG. Formation of the product only occurs after induced protein expression. (Reichstein´s Compound S, RSS, internal standard)

- - - - BL21(DE3) pET CYP 13 without addition of IPTG;

——— BL21(DE3) pET CYP 13 with addition of 1mM IPTG

Figure 8: Chromatograms of the CYP106A2 activity assay using BL21(DE3) pETCYP13 cells without addition of Adx (additional negative control). Cells were cultivated and protein expression was induced with 1mM

IPTG. To proof that all conversions took only place on the surface of *E. coli* and not inside the cell by other electron supplying systems, substrate conversions were done without the addition of Adx, since it is too large of a molecule to enter the cell envelope. The chromatograms of this conversion assay shows, as expected, no product peak. (Reichstein´s Compound S, RSS, internal standard).

—— BL21(DE3) pET CYP 13 with addition of 1mM IPTG

Figure 9: Schematic drawing of the hydroxylation of abietic acid catalysed by CYP106A2. Redox equivalents are transferred from NADPH via the proteins AdR and Adx to CYP106A2.

Figure 10: Chromatogram of the CYP106A2 activity assay using purified enzyme. Conversion of the non membrane transferrable educt abietic acid into the two products 12-alpha und 12-beta-Hydroxy-abietic acid by the purified enzyme CYP106A2. (Reichstein´s Compound S, RSS, internal standard).

Figure 11: Chromatogram of the activity assay using BL21(DE3) cells displaying CYP106A2. Conversion of the non membrane transferrable educt abietic acid into the two products 12-alpha and 12-beta-hydroxy-abietic acid by BL21(DE3) pETCYP13. For that purpose cells were cultivated and half of them induced with 1mM IPTG. Formation of the product occurs in particular after induced protein expression. (Reichstein´s Compound S, RSS, internal standard).

- - - - BL21(DE3) pET CYP 13 without addition of IPTG;

—— BL21(DE3) pET CYP 13 with addition of 1mM IPTG

Figure 12: Table of known CYP106A2 substrates from *Bacillus megaterium* ATCC 13368.

Figure 13: Sequence of YaeT (Outer membrane protein assembly factor, Omp85 homologue) in E. coli strains Bl21 (SEQ ID NO:3) and K-12 (SEQ ID NO:4).

Figure 14: Sequence of CYP3A4**.** Nucleic acid sequence (SEQ ID NO:5) and derived amino acid sequence (SEQ ID NO:6) of the CYP3A4 insert in plasmid pSC001 used for autodisplay of CYP3A4.

Figure 15: Surface display of CYP106A2 in TolC negative cells. Whole cell proteinase k digestion and subsequent SDS-PAGE (12.5 %) and Coomassie staining of outer membrane preparations obtained from *E. coli* BL21(DE3) pET-CYP13 and JW 5503 (DE3) pETCYP13. **1:** marker proteins, **2:** control, BL21(DE3) without plasmid, **3:** BL21 (DE3) pET-CYP13 - IPTG – proteinase k **4:** BL21 (DE3) pET-CYP13 + IPTG – proteinase k, **5:** BL21 (DE3) pET-CYP13 + 1 IPTG + proteinase k, **6:** control, JW 5503 (DE3) without plasmid, **7:** JW 5503 (DE3) pET-CYP13 - IPTG – proteinase k **8:** JW 5503 (DE3) pET-CYP13 + IPTG – proteinase k, **9:** JW 5503 (DE3) pET-CYP13 + 1 IPTG + proteinase k.

Figure 16: HPLC chromatograms showing CYP106A2 conversion of 11-deoxycorticosterone to 15b-deoxycorticosterone **A:** positive control (purified CYP106A2 enzyme). **B:** TolC positive cells (BL21 (DE3) pETCYP13) induced with IPTG. **C:** TolC negative cells (JW 5503 (DE3) pETCYP13) induced with IPTG. **D:** Overlay of the 3 graphs. The major product, 15b-deoxycorticosterone, was seen at a retention time of 2 min. The amount of this product decreased when CYP106A2 was expressed in the *E. coli* strain lacking the TolC channel protein. The peak at the retention time of 4 min is the internal standard Reichstein´s compound S.

Figure 17: Schematic drawing of the N-demethylation of the antidepressant imipramine into desipramin. The reaction is catalyzed by CYP106A2

displayed on the surface of *E. coli* cells. Redox equivalents are transferred from NADPH via the proteins AdR and Adx to the converting enzyme.

Figure 18: NADPH consumption of cells by *E. coli* BL21(DE3) pETCYP13. Data points are the average of triplicate experiments. The bars represent the standard deviation. Squared symbols represent BL21(DE3) cells and round symbols represent BL21(DE3) pETCYP13 cells induced with 1 mM IPTG.

Figure 19: Sequence of E. coli TolC. SEQ ID NO:7 describes a nucleotide sequence encoding TolC. SEQ ID NO:8 describes a TolC amino acid sequence.

Example 1

In this example, mainly two P450 enzymes shall be expressed by autodisplay, CYP106A2 and CYP3A4.

To establish an efficient HPLC analytic method experiments using the purified enzyme were conducted and retention times of the educt, product and internal standard determined. (Fig.1a and Fig. 2) The gene of CYP106A2 was amplified by PCR and inserted into a plasmid encoding the domains needed for autodisplay. Successful expression was shown in an SDS- PAGE (Fig. 5) To find out whether the CYP106A2 domain of the fusion protein was indeed exposed at the cell surface, trypsin was added to whole cells of E. coli Bl21(DE3) pET-CYP13 after incubation with 1 mM IPTG for one hour. Trypsin is too large of a molecule to enter the cell envelope of *E. coli*. This means, if the CYP106A2 is degraded by trypsin, when added to whole cells, it must be accessible at the cell surface. Because OmpA, which has a N terminal extension in the periplasm, was not digested at all, it could be excluded, that trypsin had entered the periplasm due to cell leakyness. (Fig. 6a) Two additional methods to proof successful surface display came into operation as well; fluorescence microscopy (Fig. 6b) and FACS (Fig. 6c).

To measure if CYP106A4 was indeed expressed on the surface of the cells in a functional form activity tests were conducted. For that purpose cells were cultivated and protein expression induced using 1mM Isopropyl-beta-D-thiogalactopyranosid (IPTG). In a conversion assay it was tested whether the cells displaying CYP106A2 on the surface have the ability to efficiently convert 11-deoxycorticosterone (DOC) into 15beta-DOC. To enhance the activity of the displayed enzyme adrenodoxin (Adx) and adrenodoxin reductase (AdR) from bovine adrenals, supplying this enzyme with the reducing equivalents necessary for steroid hydroxylation activity, were added. The use of whole *E.coli* cells only resulted in a product peak if protein expression was induced with 1 mM IPTG (Fig. 7). To proof that all conversions took only place on the surface of *E. coli* and not inside the cell by other electron supplying systems, substrate conversions were done without the addition of Adx, since it is too large of a molecule to enter the cell envelope. The chromatogram of this conversion assay shows, as expected, no product peak (Fig. 8).

Further experiments using a non-membrane transferrable substrate, abietic acid, were conducted. (Figs. 9-12) and it was shown that a product occurred at the expected retention time if protein expression was induced with 1 mM IPTG.

A similar approach starting from amplification of the gene by PCR to successful HPLC activity measurements was performed for the human enzyme CYP3A4.

We succeeded to display the P450 enzymes CYP106A2 and CYP3A on the surface of *E. coli* in a functional form by using the autodisplay system. Functional expression was achieved by a one step procedure after induction of protein expression. Without wishing to by bound by theory, the prosthetic group was incorporated during transport in the periplasm, and the folded protein was translocated to the cell surface by the aid of the Omp85 pathway.

Example 2

This example refers to the role of TolC present in the outer membrane of *E. coli* cells displaying CYP106A2 on the surface. TolC is involved in porphyrin transport across the cell membrane [26].

Functional expression was determined by the CYP106A2-dependent conversion of 11-deoxycorticosterone to 15b-deoxycorticosterone, employing the experimental condition as described in Example 1.

The strain BL21 (DE3) employed in Example 1 expresses TolC on the outer membrane. Therefore, this strain has the capability to transport porphyrins, including P450, to the outer membrane surface.

The strain JW5503-1 (DE3) is a TolC defective mutant, with a reduced capability of transporting porphyrins (including P450) onto the outer membrane surface. JW5503-1 was obtained from the Keio collection distributed by *Coli* Genetic Stock Center at Yale University.

Figure 15 indicates that CYP106A2 is expressed by pET-CYP13 to the same extent on the surface on TolC negative strain JW5503-1 (DE3) and on TolC postive BL21 (DE3) *E. coli* cells.

Figure 16 shows HPLC chromatograms of CYP106A2 conversion of 11-deoxycorticosterone to 15b-deoxycorticosterone. By comparison of Figure 16C and B, it can be seen that the amount of 15b-deoxycorticosterone decreased when CYP106A2 was expressed in the *E. coli* strain lacking the TolC channel protein.

It is concluded that TolC can provide porphyrins, in particular P450, on the cell surface, so that the porphyrins can be introduced into a recombinant surface-displayed polypeptide of the present invention, such as CYP106A.

Figure 16 indicates that there is still a CYP106A activity in the absence of TolC. Thus other transporters different from TolC may be present in the outer membrane so that porphyrins, in particular P450, can be provided on the cell surface so that the prosthetic group can be introduced into a recombinant surface-displayed polypeptide of the present invention, such as CYP106A.

Example 3

N-demethylation of the antidepressant imipramine into its active metabolite, desipramine.

The reaction as illustrated by Figure 17 is catalyzed by CYP106A2 displayed on the surface of *E. coli* cells. The cells were prepared as described in Example 1. Redox equivalents are transferred from NADPH via the proteins AdR and Adx to the converting enzyme.

The reaction mixture contained in a total volume of 0.2 mL Hepes buffer (50 mM, 0.05 % Tween20, pH 7.4), imipramine (2.5 mM), Adx (5 mM), AdR (0.5 mM), NADPH (200 mM) and cells of *E. coli* BL21(DE3) or BL21(DE3) pETCYP13 corresponding to an OD_{578}= 2.5.

Figure 18 shows the kinetics of NADPH consumption of cells by *E. coli* BL21(DE3) pETCYP13 expressing CYP106A2 cells and control BL21(DE3) cells in the presence of imipramine, as indicated above. The by-product formalin (cf. Figure 17) has been photometrically identified to be produced by the cells displaying CYP106A2, but not in the control cells (data not shown). Thus, the difference of CYP106A2 expressing cells and control cells in NADPH consumption indicates conversion of imipramine into desipramine.

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Claims

1. A method for displaying a recombinant polypeptide containing a prosthetic group on the surface of a host cell, wherein the prosthetic group comprises a metal porphyrin or a flavin, said method comprising the steps:

   (a) providing a host cell transformed with a nucleic acid fusion operatively linked with an expression control sequence said nucleic acid fusion comprising:

      (i) a portion encoding a signal peptide,

      (ii) a portion encoding the recombinant polypeptide to be displayed,

      (iii) optionally a portion encoding a protease recognition site,

      (iv) a portion encoding a transmembrane linker, and

      (v) a portion encoding the transporter domain of an autotransporter,

      and

   (b) culturing the host cell under conditions wherein the nucleic acid fusion is expressed and the expression product comprising the recombinant polypeptide containing the prosthetic group is displayed on the surface of the host cell.

1. The method according to claim 1, wherein the prosthetic group is transported to the cell surface independently from the expression product comprising the recombinant polypeptide.

2. The method according to claim 1 or 2 wherein metal porphyrin comprises one selected from cobalt, nickel, manganese, copper and iron.

3. The method according to any one of claims 1 to 3, wherein the metal porphyrin comprises a heme.

4. The method according to any one of the preceding claims wherein the

polypeptide comprising the metal porphyrin is selected from hemoproteins, P450 enzymes, P450 reductases, cytochromes, and monooxygenases.

5. The method according to any one of the preceding claims, wherein the prosthetic group being a metal porphyrin is transported to the cell surface by a TolC-dependent mechanism.

6. The method according to claim 6, wherein the cell is a Gram-negative cell, and the prosthetic group is transported across the outer membrane by TolC.

7. The method according to claim 6 or 7, wherein the TolC is a recombinant TolC.

8. The method according to any one of the claims 6 to 8, wherein the TolC polypeptide is homologous to the host cell.

9. The method according to claim 1 wherein the polypeptide comprises a flavin selected from FAD and FMN.

10. The method according to claim 10 wherein the polypeptide comprising a flavin is selected from flavoproteins.

11. The method according to any one of the preceding claims wherein the host cell is a bacterium, particularly a Gram-negative bacterium, moreparticularly an enterobacterium, e.g. *E. coli*.

12. The method according to any one of the preceding claims wherein the transporter domain of the autotransporter forms a ß-barrel structure.

13. The method according to any one of the preceding claims wherein the transporter domain of the autotransporter is selected from Ssp, Ssp-h1, Ssp-h2, PspA, PspB, Ssa1, SphB1, AspA/NalP, VacA, AIDA-I, IcsA, MisL, TibA, Ag43, ShdA, AutA, Tsh, SepA, EspC, EspP, Pet, Pic, SigA, Sat, Vat, EpeA,

EatA, EspI, EaaA, EaaC, Pertactin, BrkA, Tef, Vag8, PmpD, Pmp20, Pmp21, IgA1 protease, App, Hap, rOmpA, rOmpB, ApeE, EstA, Lip-1, McaP, BabA, SabA, AlpA, Aae, NanB, and variants thereof.

14. The method according to any one of the preceding claims wherein the transporter domain of the autotransporter is the *E. coli* AIDA-I protein or a variant thereof.

15. The method according to any one of the preceding claims wherein in step (b), the prosthetic group endogenously produced in the cell is introduced into the recombinant polypeptide within the periplasmic space.

16. The method according to any one of the preceding claims wherein step (b) comprises transportation of the recombinant polypeptide via the Omp85 pathway.

17. Host cell displaying a recombinant polypeptide on the surface thereof wherein the recombinant polypeptide contains a prosthetic group comprising a metal porphyrin or a flavin, and wherein the recombinant polypeptide comprises

    (I) a portion comprising the recombinant polypeptide to be displayed,

    (II) optionally a portion comprising a protease recognition site,

    (III) a portion comprising a transmembrane linker, and

    (IV) a portion comprising the transporter domain of an autotransporter.

18. The host cell of claim 18 wherein the recombinant polypeptide is displayed by the transporter domain of an autotransporter.

19. The host cell according to claim 18 or 19, wherein the prosthetic group is transported to the cell surface independently from the expression product comprising the recombinant polypeptide.

20. The host cell of any one of the claims 18 to 20, wherein metal porphyrin comprises one selected from cobalt, nickel, manganese, copper and iron.

21. The host cell according to any one of the claims 18 to 21, wherein the metal porphyrin comprises a heme.

22. The host cell according to any one of the claims 18 to 22, wherein the polypeptide comprising the metal porphyrin is selected from hemoproteins, P450 enzymes, P450 reductases, cytochromes, and monooxygenases.

23. The host cell according to any one of the claims 18 to 23, wherein the prosthetic group being a metal porphyrin is transported to the cell surface by a TolC-dependent mechanism.

24. The host cell according to claim 24, wherein the cell is a Gram-negative cell, and the prosthetic group is transported across the outer membrane by TolC.

25. The host cell according to claim 24 or 25, wherein the TolC is a recombinant TolC.

26. The host cell according to any one of the claims 24 to 26, wherein the TolC polypeptide is homologous to the host cell.

27. The host cell according to any one of the claims 18 to 20 wherein the polypeptide comprises a flavin selected from FAD and FMN.

28. The host cell according to any one of the claims 18 to 20 and 28 wherein the polypeptide comprising a flavin is selected from flavoproteins.

29. The host cell according to any one of the claims 18 to 29 wherein the host cell is a bacterium, particularly a Gram-negative bacterium, more particularly an enterobacterium, e.g. *E. coli*.

30. Membrane preparation which is derived from a host cell of any one of the claims 18 to 30, wherein the membrane preparation comprises in particular membrane particles.

31. Use of a cell of any one of the claims 18 to 30 or a membrane preparation of claim 31 for a chemical synthesis procedure.

32. Use of claim 32 for the synthesis of organic substances selected from enzyme substrates, drugs, hormones, starting materials and intermediates for synthesis procedures and chiral substances.

33. Use of a cell of any one of the claims 18 to 30 or a membrane preparation of claim 31 for a directed evolution procedure.

34. Use of a cell of any one of the claims 18 to 30 or a membrane preparation of claim 31 as an assay system for a screening procedure, e.g. for identifying modulators of metal porphyrin containing enzymes.

35. Use of a cell of any one of the claims 18 to 30 or a membrane preparation of claim 31 as a system for toxicity monitoring.

36. Use of a cell of any one of the claims 18 to 30 or a membrane preparation of claim 31 as a system for degrading toxic substances.

Abstract

The present invention relates to a method for the display of recombinant functional polypeptides containing a prosthetic group selected from metal porphyrin and flavin containing groups on the surface of a host cell using the transporter domain of an autotransporter.

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Figure 1a

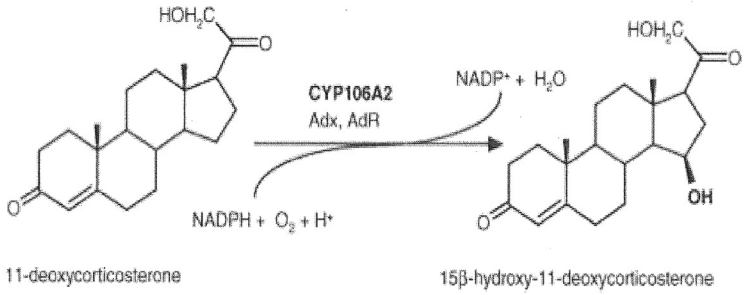

Figure 1b

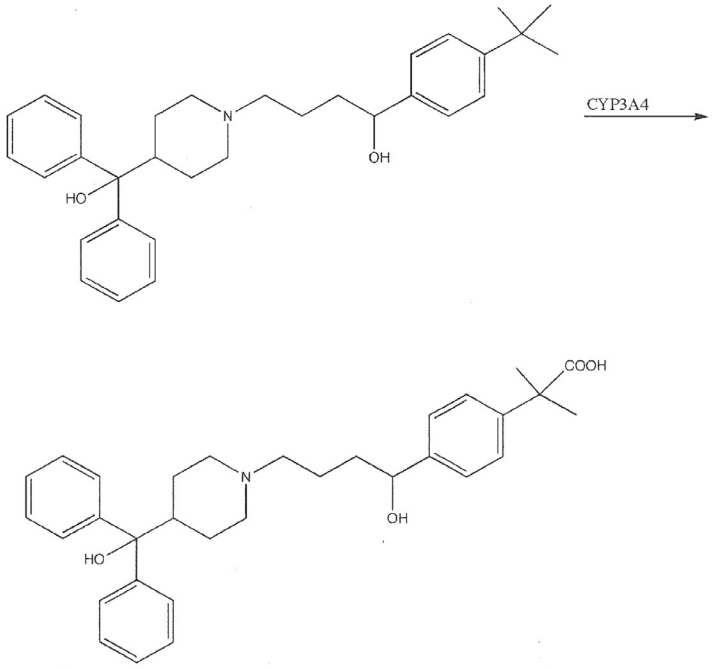

Figure 2

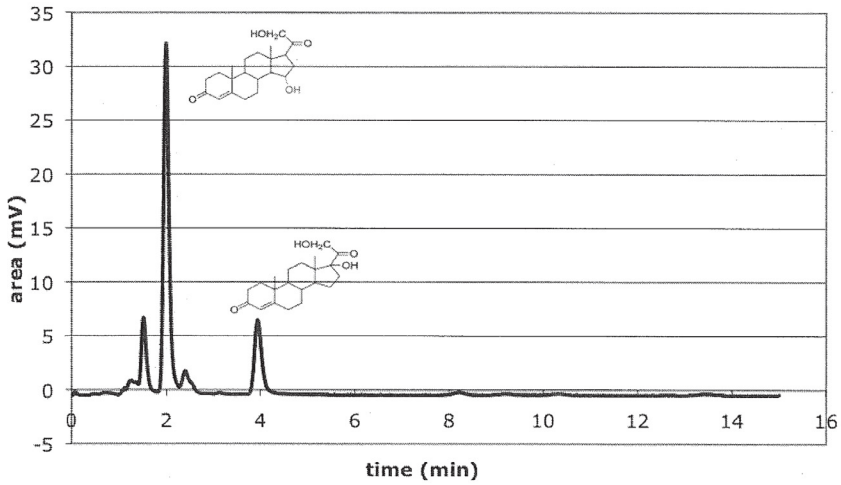
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Figure 3

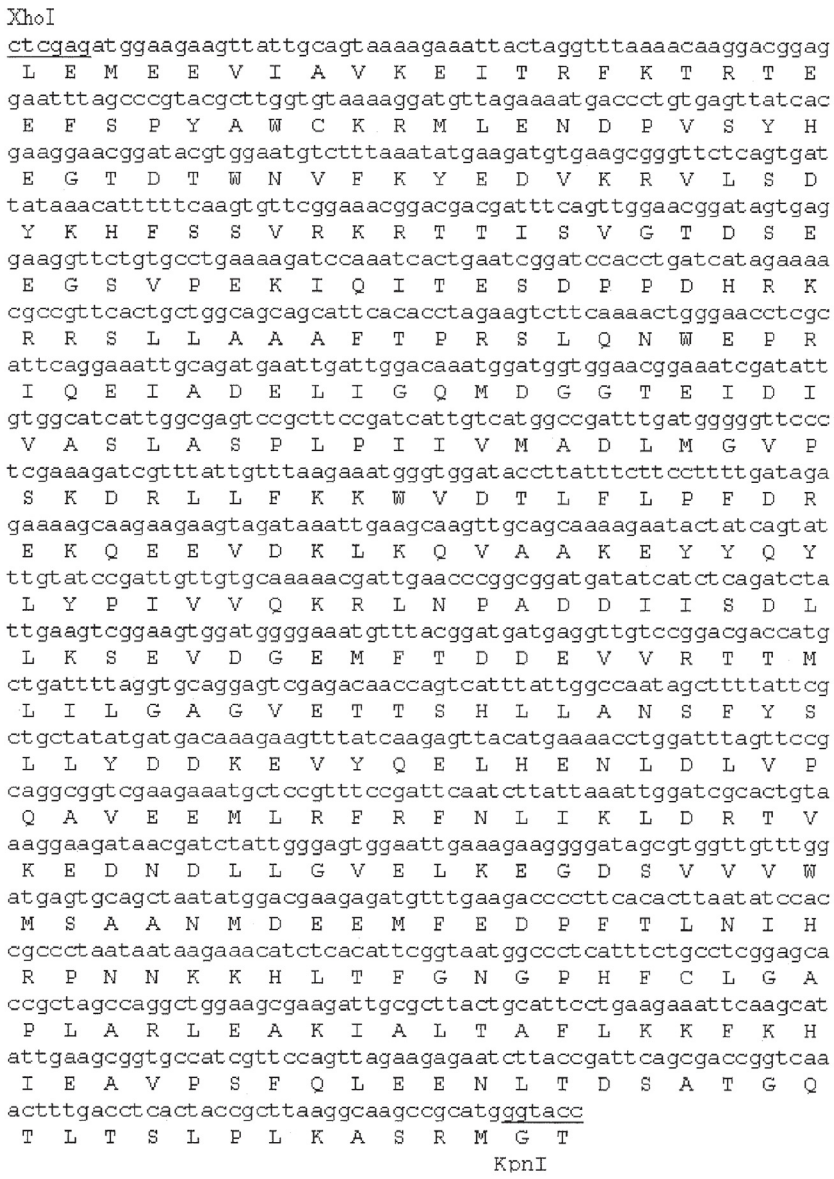
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Figure 4a

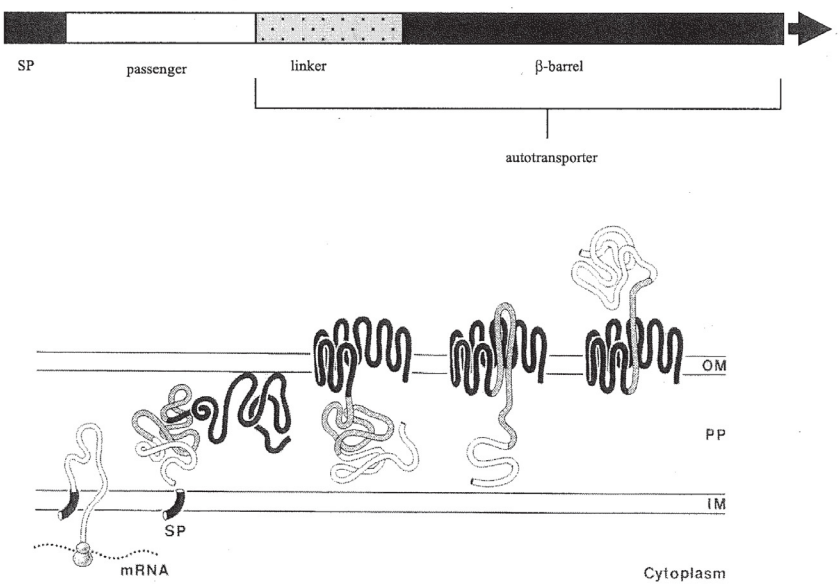

Figure 4b

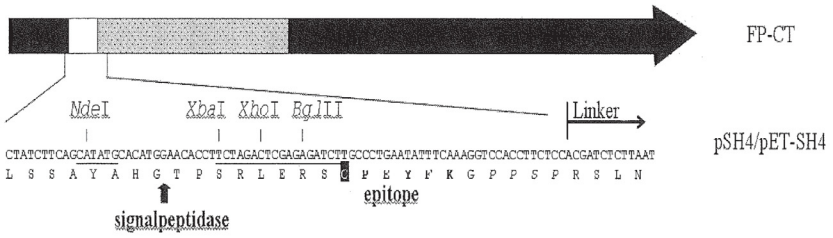

Figure 4c

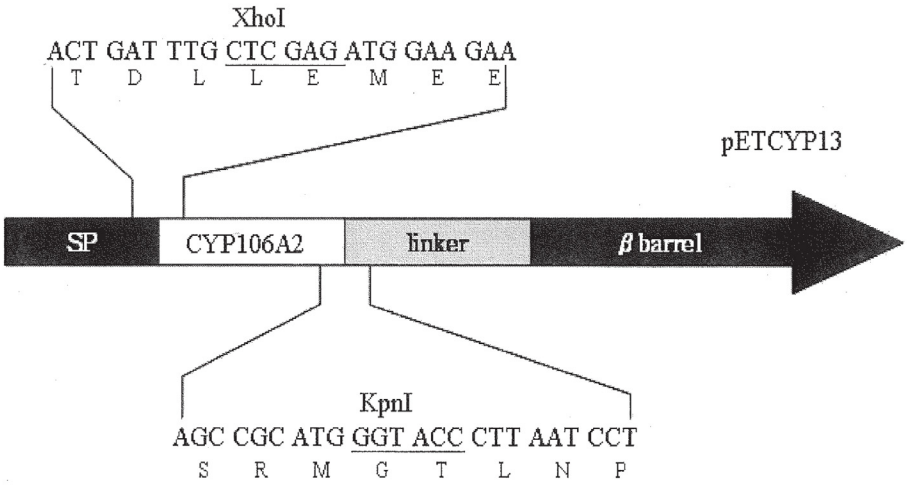
XhoI
ACT GAT TTG CTC GAG ATG GAA GAA
T D L L E M E E
pETCYP13
SP
CYP106A2
linker
β barrel
KpnI
AGC CGC ATG GGT ACC CTT AAT CCT
S R M G T L N P

Figure 5

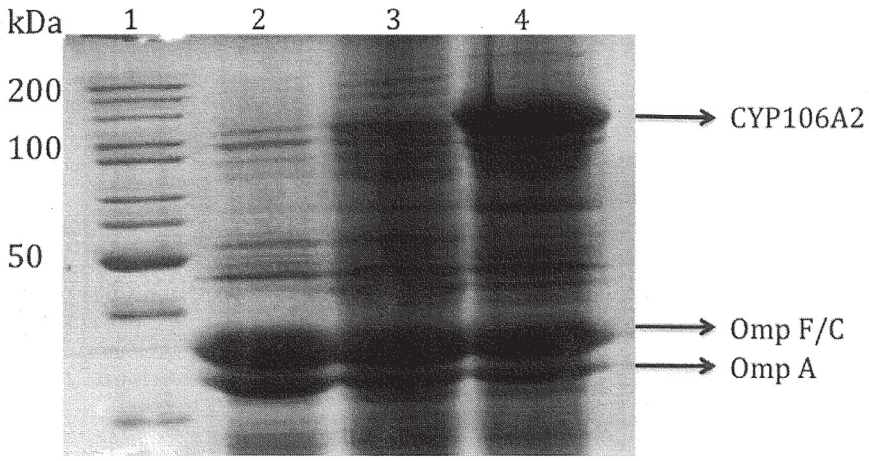

Figure 6a

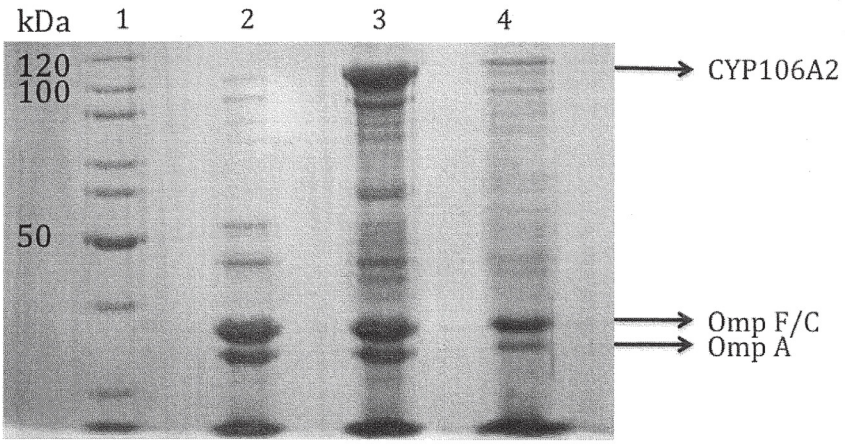

Figure 6b

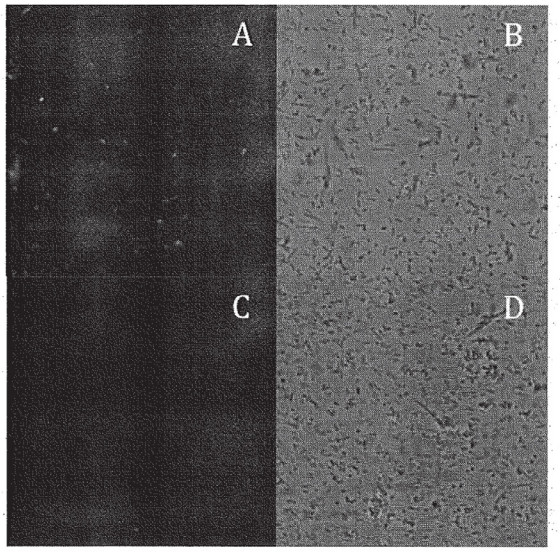

Figure 6c

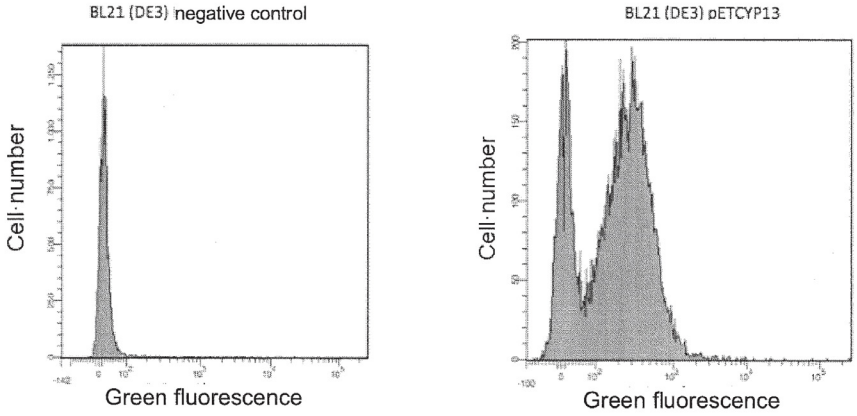
BL21 (DE3) negative control
Cell number
Green fluorescence
BL21 (DE3) pETCYP13
Cell number
Green fluorescence

Figure 7

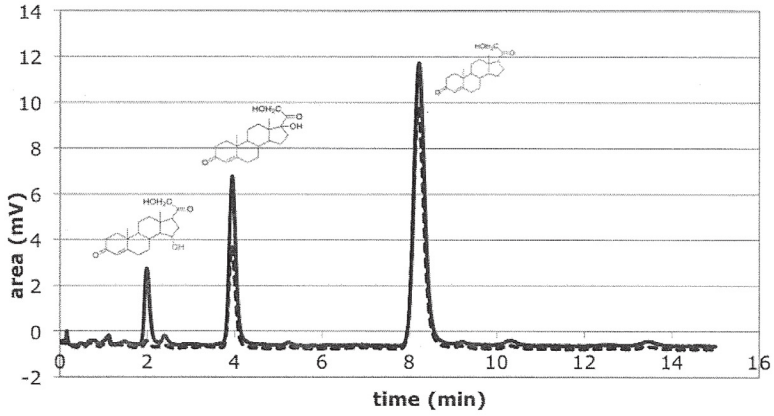

Figure 8

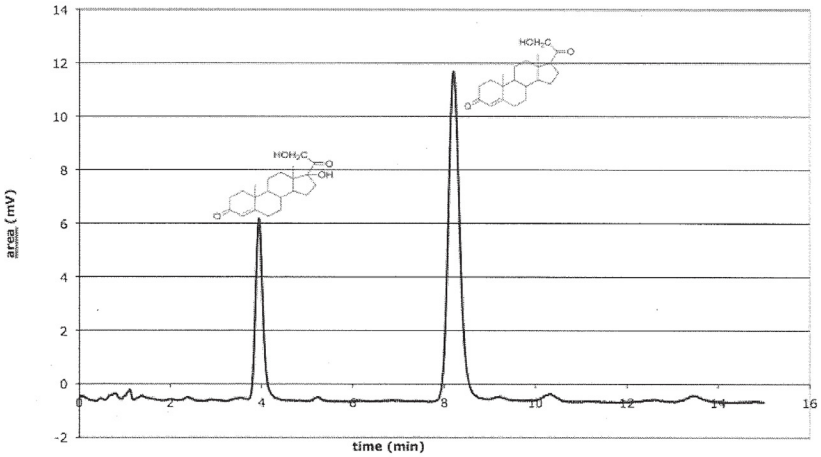

Figure 9

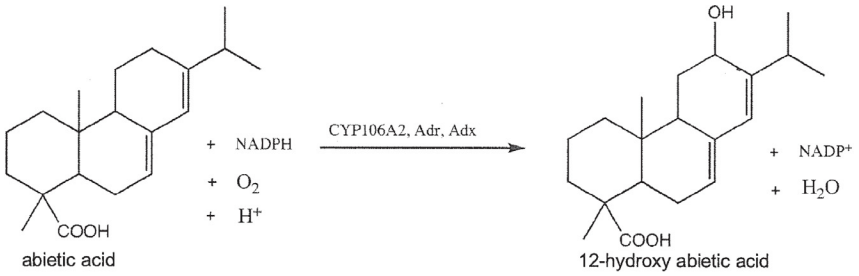

Figure 10

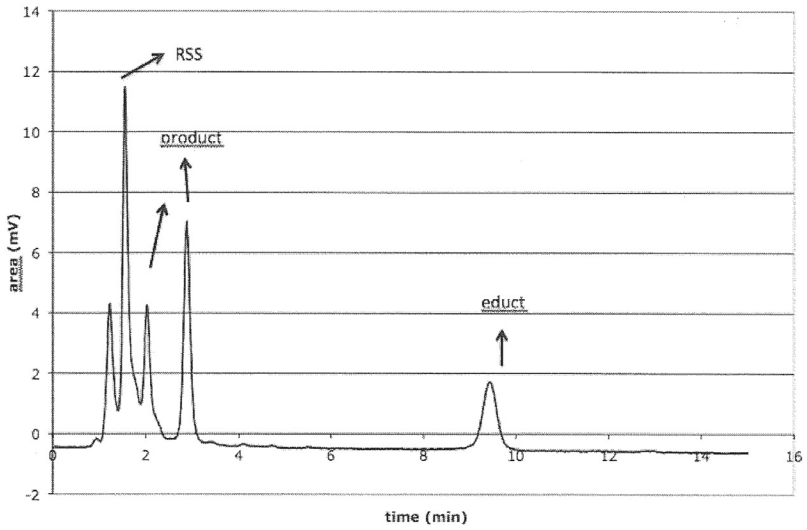

Figure 11

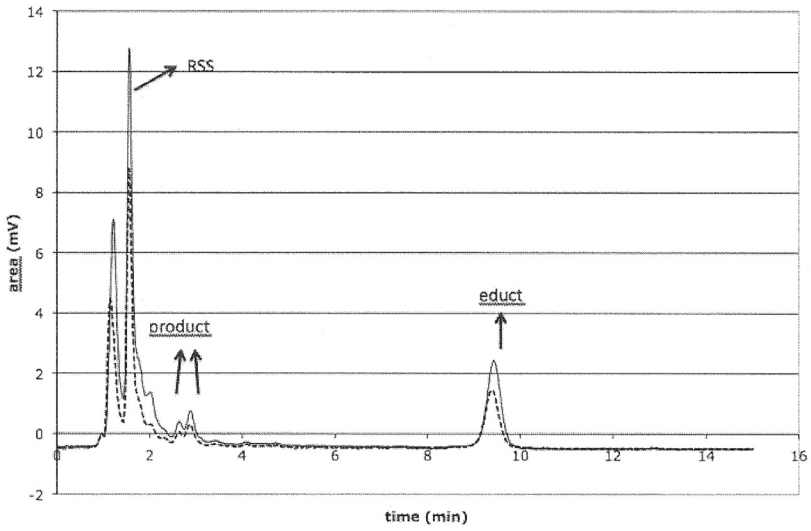
14
12
10
8
6
4
2
0
-2
area (mV)
RSS
product
educt
0
2
4
6
8
10
12
14
16
time (min)

Figure 12

Substance	Conversion	Reference
Progesterone	+	Berg et al., 1976, 1979b; Kang et al., 2004 ; Lisurek *et al.*, 2004
DOC	+	Berg *et al.*, 1976, 1979b
6-Fluor-16-methyl-DOC	+	Rauschenbach, 1993
17α-Hydroxyprogesterone	+	Berg *et al.*, 1976, 1979b
20α-Dihydroprogesterone	+	Berg *et al.*, 1976, 1979b
Androstendione	+	Berg *et al.*, 1976, 1979b
Testosterone	+	Berg *et al.*, 1976, 1979b; Agematu *et al.*, 2006
Corticosterone	+	Berg *et al.*, 1976, 1979b
Aniline	+	Berg and Rafter, 1981
Estrone	-	Berg *et al.*, 1976
Estradiol	-	Berg *et al.*, 1976
Estriol	-	Berg *et al.*, 1976
Dehydroepiandrosterone	-	Berg *et al.*, 1976
Pregnenolone	-	Berg *et al.*, 1976
5α-Androstan-3α,17β-diol	-	Berg *et al.*, 1976
5α-Dihydrotestosterone	-	Berg *et al.*, 1976
5α-Androstan-3α,17β-diol	-	Berg *et al.*, 1976, 1979b
5α-Androstan-3α,17β-diol-3,17-disulfate	-	Berg *et al.*, 1976
5β-Pregnan-3α,20α-diol	-	Berg *et al.*, 1976
Cholesterine	-	Berg and Rafter, 1981
Desoxycholesterine	-	Berg and Rafter, 1981
Cholic acid	-	Berg and Rafter, 1981
Desoxycholic acid	-	Berg and Rafter, 1981
β-Sitosterol	-	Berg and Rafter, 1981
Lithocholic acid	-	Berg and Rafter, 1981
Hexadecanoic acid	-	Berg and Rafter, 1981
Octadecanoic acid	-	Berg and Rafter, 1981
Prostaglandin $F_{2}\alpha$	-	Berg and Rafter, 1981
Biphenyl	-	Berg and Rafter, 1981
Benzo[a]pyrene	-	Berg and Rafter, 1981
Lidocaine	-	Berg and Rafter, 1981
Ethylmorphine	-	Berg and Rafter, 1981
Aminopyrine	-	Berg and Rafter, 1981
7-Ethoxyresorufin	-	Berg and Rafter, 1981
Imipramine	+	Berg and Rafter, 1981
Betulin acid	+	Chatterjee *et al.*, 2000
Betulon acid	+	Chatterjee *et al.*, 2000
4-Pregnen-20β-ol-3-on	+	Bleif, 2007
17α-Methyltestosterone	+	Bleif, 2007
Ethisterone	+	Bleif, 2007
4-Pregnen-17α,20α,21-triol-3-on	+	Bleif, 2007
Melengestrol acetate	+	Bleif, 2007
Dihydrochinopimaric acid	+	Bleif, 2007
11-Ketoβ-boswellic acid	+	Bleif, 2007

Figure 13

YaeT E. coli Bl21 (Outer membrane protein assembly factor, Omp85 homologue) (SEQ ID NO:3)

```
  1 mamkkllias llfssatvyg aegfvvkdih feglqrvavg aallsmpvrt gdtvndedis
 61 ntiralfatg nfedvrvlrd gdtllvqvke rptiasitfs gnksvkddml kqnleasgvr
121 vgesldrtti adiekgledf yysvgkysas vkavvtplpr nrvdlklvfq egvsaeiqqi
181 nivgnhaftt delishfqlr devpwwnvvg drkyqkqkla gdletlrsyy ldrgyarfni
241 dstqvsltpd kkgiyvtvni tegdqyklsg vevsgnlagh saeieqltki epgelyngtk
301 vtkmeddikk llgrygyayp rvqsmpeind adktvklrvn vdagnrfyvr kirfegndts
361 kdavlrremr qmegawlgsd lvdqgkerln rlgffetvdt dtqrvpgspd qvdvvykvke
421 rntgsfnfgi gygtesgvsf qagvqqdnwl gtgyavging tkndyqtyae lsvtnpyftv
481 dgvslggrlf yndfqaddad lsdytnksyg tdvtlgfpin eynslraglg yvhnslsnmq
541 pqvamwryly smgehpstsd qdnsfktddf tfnygwtynk ldrgyfptdg srvnltgkvt
601 ipgsdneyyk vtldtatyvp idddhkwvvl grtrwgygdg lggkempfye nfyaggsstv
661 rgfqsntigp kavyfphqas nydpdydyec atqdgakdlc ksddavggna mavaslefit
721 ptpfisdkya nsvrtsffwd mgtvwdtnwd ssqysgypdy sdpsnirmsa gialqwmspl
781 gplvfsyaqp fkkydgdkae qfqfnigktw
```

YaeT E. coli K-12 (SEQ ID NO:4)

```
  1 mamkkllias llfssatvyg aegfvvkdih feglqrvavg aallsmpvrt gdtvndedis
 61 ntiralfatg nfedvrvlrd gdtllvqvke rptiasitfs gnksvkddml kqnleasgvr
121 vgesldrtti adiekgledf yysvgkysas vkavvtplpr nrvdlklvfq egvsaeiqqi
181 nivgnhaftt delishfqlr devpwwnvvg drkyqkqkla gdletlrsyy ldrgyarfni
241 dstqvsltpd kkgiyvtvni tegdqyklsg vevsgnlagh saeieqltki epgelyngtk
301 vtkmeddikk llgrygyayp rvqsmpeind adktvklrvn vdagnrfyvr kirfegndts
361 kdavlrremr qmegawlgsd lvdqgkerln rlgffetvdt dtqrvpgspd qvdvvykvke
421 rntgsfnfgi gygtesgvsf qagvqqdnwl gtgyavging tkndyqtyae lsvtnpyftv
481 dgvslggrlf yndfqaddad lsdytnksyg tdvtlgfpin eynslraglg yvhnslsnmq
541 pqvamwryly smgehpstsd qdnsfktddf tfnygwtynk ldrgyfptdg srvnltgkvt
601 ipgsdneyyk vtldtatyvp idddhkwvvl grtrwgygdg lggkempfye nfyaggsstv
661 rgfqsntigp kavyfphqas nydpdydyec atqdgakdlc ksddavggna mavaslefit
721 ptpfisdkya nsvrtsffwd mgtvwdtnwd ssqysgypdy sdpsnirmsa gialqwmspl
781 gplvfsyaqp fkkydgdkae qfqfnigktw
```

Figure 14

Figure 15

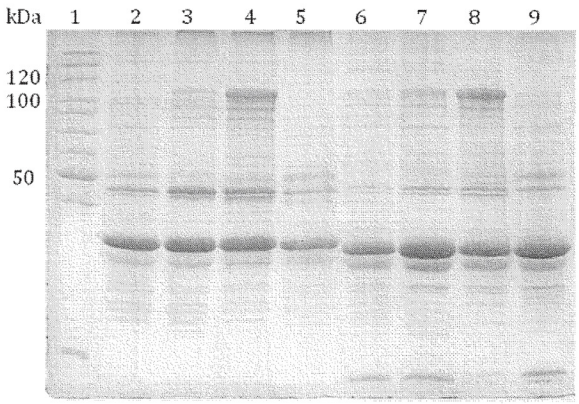

Figure 16

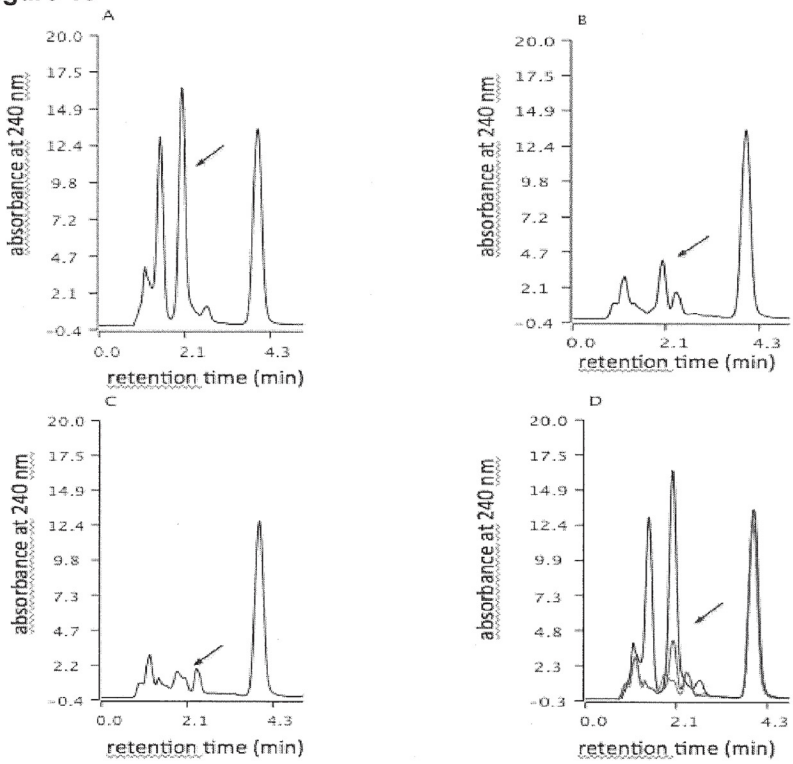

Figure 17

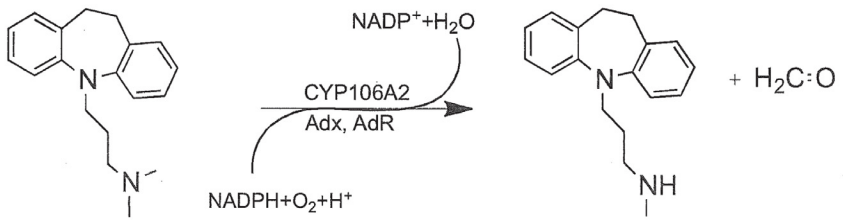

Figure 18

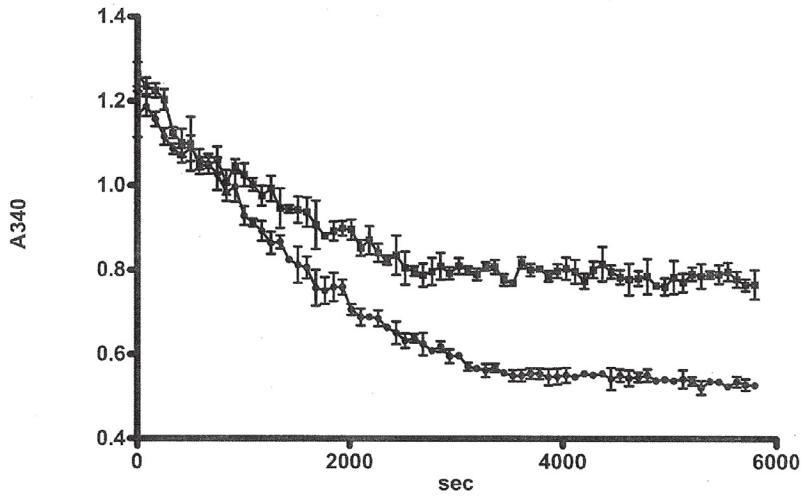

Figure 19

Nucleotide sequence of the *tolC* gene of *E. coli* (SEQ ID NO:7)

ATGAAGAAATTGCTCCCCATTCTTATCGGCCTGAGCCTTTCTGGGTTCAGTTCGTTGAGC
CAGGCCGAGAACCTGATGCAAGTTTATCAGCAAGCACGCCTTAGTAACCCGGAATTGCGT
AAGTCTGCCGCCGATCGTGATGCTGCCTTTGAAAAAATTAATGAAGCGCGCAGTCCATTA
CTGCCACAGCTAGGTTTAGGTGCAGATTACACCTATAGCAACGGCTACCGCGACGCGAAC
GGCATCAACTCTAACGCGACCAGTGCGTCCCTGCAGTTAACTCAATCCATTTTTGATATG
TCGAAATGGCGTGCGTTAACGCTGCAGGAAAAAGCAGCAGGGATTCAGGACGTCACGTAT
CAGACCGATCAGCAAACCTTGATCCTCAACACCGCGACCGCTTATTTCAACGTGTTGAAT
GCTATTGACGTTCTTTCCTATACACAGGCACAAAAAGAAGCGATCTACCGTCAATTAGAT
CAAACCACCCAACGTTTTAACGTGGGCCTGGTAGCGATCACCGACGTGCAGAACGCCCGC
GCACAGTACGATACCGTGCTGGCGAACGAAGTGACCGCACGTAATAACCTTGATAACGCG
GTAGAGCAGCTGCGCCAGATCACCGGTAACTACTATCCGGAACTGGCTGCGCTGAATGTC
GAAAACTTTAAAACCGACAAACCACAGCCGGTTAACGCGCTGCTGAAAGAAGCCGAAAAA
CGCAACCTGTCGCTGTTACAGGCACGCTTGAGCCAGGACCTGGCGCGCGAGCAAATTCGC
CAGGCGCAGGATGGTCACTTACCGACTCTGGATTTAACGGCTTCTACCGGGATTTCTGAC
ACCTCTTATAGCGGTTCGAAAACCCGTGGTGCCGCTGGTACCCAGTATGACGATAGCAAT
ATGGGCCAGAACAAAGTTGGCCTGAGCTTCTCGCTGCCGATTTATCAGGGCGGAATGGTT
AACTCGCAGGTGAAACAGGCACAGTACAACTTTGTCGGTGCCAGCGAGCAACTGGAAAGT
GCCCATCGTAGCGTCGTGCAGACCGTGCGTTCCTCCTTCAACAACATTAATGCATCTATC
AGTAGCATTAACGCCTACAAACAAGCCGTAGTTTCCGCTCAAAGCTCATTAGACGCGATG
GAAGCGGGCTACTCGGTCGGTACGCGTACCATTGTTGATGTGTTGGATGCGACCACCACG
TTGTACAACGCCAAGCAAGAGCTGGCGAATGCGCGTTATAACTACCTGATTAATCAGCTG
AATATTAAGTCAGCTCTGGGTACGTTGAACGAGCAGGATCTGCTGGCACTGAACAATGCG
CTGAGCAAACCGGTTTCCACTAATCCGGAAAACGTTGCACCGCAAACGCCGGAACAGAAT
GCTATTGCTGATGGTTATGCGCCTGATAGCCCGGCACCAGTCGTTCAGCAAACATCCGCA
CGCACTACCACCAGTAACGGTCATAACCCTTTCCGTAACTGA

Amino acid sequence of the *tolC* gene of *E. coli* (SEQ ID NO:8)

MKKLLPILIGLSLSGFSSLSQAENLMQVYQQARLSNPELRKSAADRDAAFEKINEARSPL
LPQLGLGADYTYSNGYRDANGINSNATSASLQLTQSIFDMSKWRALTLQEKAAGIQDVTY
QTDQQTLILNTATAYFNVLNAIDVLSYTQAQKEAIYRQLDQTTQRFNVGLVAITDVQNAR
AQYDTVLANEVTARNNLDNAVEQLRQITGNYYPELAALNVENFKTDKPQPVNALLKEAEK
RNLSLLQARLSQDLAREQIRQAQDGHLPTLDLTASTGISDTSYSGSKTRGAAGTQYDDSN
MGQNKVGLSFSLPIYQGGMVNSQVKQAQYNFVGASEQLESAHRSVVQTVRSSFNNINASI
SSINAYKQAVVSAQSSLDAMEAGYSVGTRTIVDVLDATTTLYNAKQELANARYNYLINQL
NIKSALGTLNEQDLLALNNALSKPVSTNPENVAPQTPEQNAIADGYAPDSPAPVVQQTSA
RTTTSNGHNPFRN

8 Scientific output

8.1 Publications

Schumacher S D, Hannemann F, Teese M G, Bernhardt R, Jose, J (2012). Autodisplay of functional CYP106A2 in *Escherichia coli*. J. Biotechnol. DOI: 10.1016/j.biotec.2012.02.018

Schumacher S D, Jose, J (2012). Expression of active human P450 3A4 on the cell surface of *Escherichia coli* by Autodisplay. J. Biotechnol DOI: 10.1016/j.biotec.2012.01.031

Schumacher S, Hannemann F, Bernhardt R, Jose J (2010) Autodisplay funktioneller P450-Enzyme in *Escherichia coli*. Chemie Ingenieur Technik (CIT), 82:1521

8.2 Patent

Schumacher S D, Hannemann F, Bernhardt R, Jose J (2010). Surface display of polypeptides containing a metal porphyrin or a flavin. EP10179060.8

8.3 Talks

Schumacher S, Hannemann F, Bernhardt R, Jose J (2011) Funtional display of CYP106A2 and CYP3A4 on *Escherichia coli.* 2nd CLIB International Conference, 5.- 6. April 2011, Düsseldorf

Schumacher S, Hannemann F, Bernhardt R, Jose J (2010) Autodisplay of functional P450 enzymes in *Escherichia coli.* (Ref.: V.7.69, *CIT* 9:1521), DECHEMA Jahrestagung der Biotechnologen, 21.-23. September 2010, Aachen

8.4 Poster

Schumacher S, Teese M G, Hannemann F, Bernhardt R, Jose J (2012) Autodisplay of P450 monooxygenases for the conversion of testosterone, deoxycorticosterone, imipramine and abietic acid. Symposium “Forschung der chemischen Industrie”, 18. April 2012, Münster

Schumacher S, Hannemann F, Bernhardt R, Jose J (2010) Autodisplay of CYP106A2 and CYP3A4 in *Escherichia coli.* (Ref.: C109, ISBN: 978-3-927115-65-1), DPhG Jahrestagung, 4.-7. Oktober 2010, Braunschweig