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An engineered outer membrane pore enables an efficient oxygenation of aromatics and terpenes

Anna Joëlle Ruff[a,1], Marcus Arlt[a,1], Maike van Ohlen[a], Tsvetan Kardashliev[a], Monika Konarzycka-Bessler[b], Marco Bocola[a], Alexander Dennig[a], Vlada B. Urlacher[b,**], Ulrich Schwaneberg[a,*]

[a] Lehrstuhl für Biotechnologie, RWTH Aachen University, Worringerweg 3, 52074 Aachen, Germany and Bioeconomy Science Center (BioSC), Germany
[b] Institute of Biochemistry, Heinrich-Heine University Düsseldorf, Universitaetstr. 1, 40225 Düsseldorf, Germany and Bioeconomy Science Center (BioSC), Germany

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ABSTRACT

Biocatalysis with cytochrome P450 enzymes are important for the industrial production of fine chemicals, pharmaceuticals, fragrance and flavor compounds since chemoselective hydroxylation of aromatics and terpenes are chemically difficult to achieve. A few P450 based industrial processes have been developed based on whole cell catalysis. However, the outer membrane of microbial cells forms an effective barrier, which reduces the uptake of hydrophobic substrates. The coexpression of outer membrane proteins in *E. coli* such as the ferric hydroxamate uptake protein (FhuA) can provide alternative solutions to chemical or physical methods for increasing compound flux through the outer membrane of *E. coli* and thereby to boost productivities. In this study we employed an engineered FhuA Δ1-160 variant in which the "cork domain" was removed (first 160 residues are deleted); FhuA Δ1-160 has a cross-section of 39–46 Å with a "free" inner diameter of about 14 Å that serves as passive diffusion channel. FhuA WT and Δ1-160 were coexpressed on a bicistronic system with two P450 BM3 variants for regiospecific hydroxylation of aromatic compounds toluene and anisole as well as for oxidation of two terpenes (α)-pinene and (*R*)-(+)-limonene. The presence of FhuA Δ1-160 resulted in a doubled product concentration for toluene (35 μ to 50 μM), anisole (25 μM to 45 μM), pinene (12 μM to 20 μM) and limonene (12 μM to 25 μM) and five times higher for the coumarin derivative BCCE. In order to characterizes and compensate for expression variations a quantification method based on Chromeo546-labled StrepTactinII was developed to quantify the number of FhuA Δ1-160 in the outer *E. coli* membrane (~44000 of FhuA Δ1-160 per cell). Morphology studies showed that a 6% *E. coli* surface coverage can be achieved with FhuA Δ1-160 without significantly influencing the *E. coli* rod shape. In summary, FhuA Δ1-160 efficiently increases uptake of hydrophobic aromatics and terpenes for whole-cell biotransformations and can likely be used for other enzymes and/or substrates.

1. Introduction

Biocatalysis is important for the industrial production of fine chemicals, pharmaceuticals, fragrance and flavor compounds [1]. Phenols, alkylphenols and their derivatives experience a constantly growing demand as intermediates in flavor industry and common antioxidants for anti-tumor drugs, cardiovascular disorder treatments, and Parkinsons and Alzheimers diseases as well as resins, plastics, and bisphenol A [2,3]. Most phenols are chemically produced through the "Hock Process" despite the low product yields (about 5% conversion to phenol) [4] while novel synthetic routes mainly involve metal catalysts generating highly reactive oxygen species [3–5]. Terpenes and their more valued oxygenated

* Corresponding author at: RWTH Aachen University, Lehrstuhl für Biotechnologie, Worringerweg 3, 52074 Aachen, Germany.
** Corresponding author at: Heinrich-Heine University Düsseldorf, Institute of Biochemistry, Universitätstr. 1, 40225 Düsseldorf, Germany.
E-mail addresses: aj.ruff@biotec.rwth-aachen.de (A.J. Ruff), m.arlt@biotec.rwth-aachen.de (M. Arlt), m.vanohlen@biotec.rwth-aachen.de (M. van Ohlen), t.Kardashliev@biotec.rwth-aachen.de (T. Kardashliev), Monika.Konarzycka-Bessler@uni-duesseldorf.de (M. Konarzycka-Bessler), m.bocola@biotec.rwth-aachen.de (M. Bocola), a.dennig@biotec.rwth-aachen.de (A. Dennig), Vlada.Urlacher@uni-duesseldorf.de (V.B. Urlacher), u.schwaneberg@biotec.rwth-aachen.de (U. Schwaneberg).
[1] Both authors contributed equally.

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derivatives show organoleptic properties, thus they are attractive for manufacturers of perfumes, soaps, cosmetics and fine chemicals [6]. Chemoselective synthesis of hydroxylated terpenes is very challenging by chemical means. For example (+)-perillyl alcohol has been synthesized in four steps with 39% overall yield from limonene oxide using palladium(0)-mediated oxy-isomerization [7]. Chemo- and/or regioselective hydroxylation of aromatics and terpenes can directly be achieved by cytochrome P450 enzymes which represent therefore attractive and sustainable alternatives to chemocatalytic processes with significantly reduced purification steps, waste generation and process energy demands [8].

Cytochrome P450 (P450s) is heme b containing monooxygenases [9] that catalyze chemical "dream" reactions such as the selective hydroxylation of fatty acids, alkanes, terpenes, and monosubstitued benzenes at ambient temperature and by employing oxygen [10] as oxidant [11–13]. P450 BM3 is a self-sufficient NADPH-dependent monooxygenase from *Bacillus megaterium* (CYP102A1) which consists of a monooxygenase and a reductase domain fused in one polypeptide chain [14]. Recently, P450 BM3 variants M2 (R47S/Y51W/I401 M) for regioselective o-hydroxylation of monosubstituted benzenes [11,15] and VVF (A264 V/A328 V/L437F) for regioselective oxidation of (R)-(+)-limonene [16] were engineered.

Substrates of P450s are typically hydrophobic compounds, and their limited transfer through the outer membrane of *E. coli* hinders effective whole-cell biocatalysis. Strategies to increase outer member permeability of bacteria comprise mainly the pre-treatment with chemical and physical methods with organic co-solvents (e.g. DMSO [17,18]), detergents (e.g. Triton-X100 [19]) or membrane permeabilizing reagents (e.g. EDTA [20–22] or short peptides such as Polymyxin B/Polymyxin B-nonapeptide[23–25], Polyethyleneimine (PEI) [26], Polyphosphates [27]) or electroporation[28] or a combination thereof [29]. Quite often the cell viability is reduced by chemical and physical methods [30]. Recently developed concepts comprise genetic modulation of an efflux pump [31], coexpression of membrane-active peptides [32] and an outer membrane protein, AlkL, by Bühler and coworkers to enhance hydroxylation of mostly long linear alkanes (up to 28 fold) [33]. Besides that, the coexpression of AlkL in *E. coli* enhanced the hydroxylation of octane up to 4 fold, nonane up to 40 fold [33], and the monoterpene (*S*)-limonene up to 25 fold in two-liquid phase biotransformations [34]. These findings indicate a somehow broad substrate scope for AlkL, but the structure and mode of action remains unclear. AlkL shows sequence homology (~28%) with the outer membrane protein OmpW [35], which is an 8-stranded β-barrel outer membrane protein (~17–12 Å elliptical cross-section, pdb 2F1 V) of *E. coli* with a small hydrophobic channel of less than 4 Å in diameter [36]. Nevertheless, AlkL seemed to be specialized in the membrane transfer of long linear alkanes and is less beneficial in whole cell biotransformations of cyclic or aromatic compounds.

FhuA is a native outer membrane protein of *E. coli* (FhuA wildtype = FhuA WT). In the engineered variant FhuA Δ1-160 the globular N-terminal "cork" domain (formed by amino acid residues 1 to 160) was removed such that the active iron transporter becomes in contrast to AlkL a large passive diffusion channel (cross-section 39–42 Å; "free" inner diameter of about 14 Å). Previous reports on FhuA Δ1-160 have shown that its presence in the OM of *E. coli* conferred increased susceptibility to large antibiotics [37] and even allowed the translocation of single stranded DNA (incorporated into polymer vesicles termed Synthosomes) [38]. Uptake of lipophilic compounds through the outer membrane is at least 10- to 100-fold slower compared to cytoplasmic membrane translocation because of the highly charged lipopolysaccharides that are stabilized by divalent cations [39,40].

A prerequisite f compare productivity increases is to quantify number of expressed passive diffusion channels in the outer membrane of *E. coli* and to compensate for variations in expression of FhuA Δ1-160 or other membrane pores. In this study, we report a likely universal quantification method based on an engineered FhuA Δ1-160 variant and applied the developed method to quantify the increase of regiospecific aromatic hydroxylations for two aryl substrates and a bulky fluorogenic substrates catalyzed by P450 BM3 M2 (R47S/Y51W/I401 M) and for oxidation of pinene catalyzed by P450 BM3 M2 and of limonene catalyzed by P450 BM3 VVF (A264 V/A328 V/L437F). In all the cases a bicistronic system [41] was employed in which the engineered FhuA Δ1-160 variant and the respective P450 BM3 variants were coexpressed.

2. Experimental

All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) or ABCR (Karlsruhe, Germany) unless mentioned otherwise. All reagents were of the highest purity grade available. PfuS polymerase was homemade. Salt-free oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany). Enzymes were purchased from New England Biolabs (Frankfurt, Germany). Plasmids were extracted with NucleoSpin Plasmid Extraction Kit (Macherey Nagel, Düren, Germany) and PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). DNA concentrations were determined using the NanoDrop photometer from NanoDrop Technologies (Wilmington, USA). DNA sequencing was done by Eurofins MWG-Operon (Ebersberg, Germany). Sequencing data were analyzed with Clone Manager 9 Professional Edition Software (Scientific & Educational Software, Cary, NC, USA).

2.1. Design of FhuA WT_Strep and FhuA Δ1-160_Strep

The position of the StrepTactinII recognition sequence (WSHPQFEK) [42] was identified based on the crystal structure of the FhuA WT (pdb: 1by3). The StrepTactinII sequence (WSHPQFEK) was introduced in the outer membrane loop 5 (residues 393–419) at different positions using YASARA Structure (version 14.7.17) [43]. The flanking amino acids before and after the inserted loop (best insertion position: proline 405 and valine 406) were included in the loop building routine [44–47] to ensure their correct orientation after sequence insertion. After insertion, the FhuA molecule was protonated [48] at pH 7.4 and energy minimized using the AMBER03 [49]-force field (filled simulation-cell 15 Å around the protein with TIP3P water and 0.9% NaCl). To remove bumps and correct covalent geometry, the structure was energy-minimized using a 8 Å force cutoff and the Particle Mesh Ewald algorithm [50] to treat long-range electrostatic interactions. After removal of conformational stress by a short steepest descent minimization, the procedure continued by simulated annealing (time step 2 fs, atom velocities scaled down by 0.9 every 10th step) until convergence was reached, i.e. the energy improved by less than 0.05 kJ/mol per atom during 200 steps. Stabilization energy and surface accessibility of the inserted StrepTactinII sequence were calculated to assess the optimal insertion position.

2.2. Cloning of FhuA WT and FhuA Δ1-160 in pALXtreme-1a-P450 BM3 M2 and – P450 BM3 VVF

Expression vectors pALXtreme-1a harboring P450 BM3 M2 and without an insert were available from previous in-house study [11]. Cloning of the gene encoding FhuA Δ1-160 in pALXtreme-1a P450 BM3 M2 (R47S/Y51W/I401 M) was carried out by PLICing [51] such that both genes were under the control of a single T7 promoter and a ribosome binding site was placed in front of each