Hydrophobic Outer Membrane Pores Boost Testosterone Hydroxylation by Cytochrome P450 BM3 Containing Cells

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The implementation of biocatalytic steroid hydroxylation processes at an industrial scale still suffers from low conversion rates. In this study, we selected variants of the self-sufficient cytochrome P450 monooxygenase BM3 from *Bacillus megaterium* (BM3) for the hydroxylation of testosterone either at the 2β- or 15β-position. Recombinant *Escherichia coli* cells were used as biocatalysts to provide a protective environment for recombinant enzymes and to ensure continuous cofactor recycling *via* glucose catabolism. However, only low initial whole-cell testosterone conversion rates were observed for resting cells. Results obtained with different biocatalyst formats (permeabilized cells, cell-free extracts, whole cells) indicated a limitation in substrate uptake, most likely due to the hydrophilic character of the outer membrane of *E. coli*. Thus, we co-expressed nine genes encoding hydrophobic outer membrane proteins potentially facilitating steroid uptake. Indeed, the application of four candidates led to increased initial testosterone hydroxylation rates. Respective whole-cell biocatalysts even exceeded activities obtained with permeabilized cells or cell-free extracts. The highest activity of 34 U g_{CDW}^{-1} was obtained for a strain containing the hydrophobic outer membrane protein AlkL from *Pseudomonas putida* GPo1 and the BM3 variant KSA14m. Overall, we show that the straightforward application of hydrophobic outer membrane pores can boost whole-cell steroid conversion rates and thus be game-changing with regard to industrial steroid production efficiency.

Keywords: whole-cell biocatalysis, steroid hydroxylation, substrate uptake, cytochrome P450 monooxygenase, hydrophobic membrane pores, AlkL

INTRODUCTION

Steroid-based drugs account for one of the largest and still expanding sectors in the pharmaceutical industry (Tong and Dong, 2009). Their broad spectrum of pharmaceutical activities is reflected in the prominent role they play, e.g., in contraceptives, anti-inflammatory agents, or cancer treatments (Bureik and Bernhardt, 2007; Donova and Egorova, 2012; Donova, 2017). Hydroxylated steroid derivatives frequently show higher biological activities than their more hydrophobic non-hydroxylated analogs, rendering steroid hydroxylations particularly relevant (Donova and Egorova, 2012). As number and position of hydroxyl groups are decisive for the biological activity of steroids, highly selective hydroxylation is crucial (Bureik and Bernhardt, 2007;

Donova and Egorova, 2012). Desired reactions often are not accessible by chemical means, making biocatalysis a promising highly selective and often eco-efficient alternative for steroid functionalization (Bureik and Bernhardt, 2007; Julsing et al., 2008; Donova, 2017).

Several microorganisms show native steroid-modifying activities and are regularly applied at industrial scale. However, respective processes typically suffer from low productivities, defined as amount of product produced per time and volume, and undesired side-reactions (Bureik and Bernhardt, 2007; Fernández-Cabezón et al., 2018). Thus, enzyme discovery as well as enzyme and strain engineering have been of immense interest in recent decades (Donova, 2017; Fernández-Cabezón et al., 2018), giving rise to a huge variety of steroid hydroxylating cytochrome P450 monooxygenases (CYP450s) covering virtually all possible specificities (Bernhardt, 2006; Bureik and Bernhardt, 2007; Donova, 2017; Szaleniec et al., 2018).

Employing CYP450s of mammalian origin appears attractive due to their natural role in steroid synthesis and conversion (Donova and Egorova, 2012; Donova, 2017). However, they often suffer from low enzyme activities severely hampering application (Bernhardt, 2006; Urlacher and Schmid, 2006; Julsing et al., 2008). Bacterial CYP450s typically provide higher activities and high levels of soluble expression in recombinant microorganisms. The vast majority of CYP450s features separate components involved in the shuttling of electrons from NAD(P)H to the monooxygenase subunit (Bernhardt, 2006). Subunits need to be expressed in a stable and active form and in the optimal ratio (Murdock et al., 1993; Staijen et al., 2000) which is likely to confine the biotransformation efficiency (Duetz et al., 2001). Biocatalyst efficiency, namely the enzyme turnover rate k_{cat} (s^{-1}), may therefore benefit from a fusion of subunits as it is the case for native CYP450 BM3 (BM3, in CYP450 nomenclature: CYP102A1) from *Bacillus megaterium* ATC14581 featuring the highest known CYP450 activity (Narhi and Fulco, 1986, 1987; Noble et al., 1999). For this fatty acid monooxygenase, which does not accept steroids as substrates (van Vugt-Lussenburg et al., 2006), Kille *et al.* developed a set of variants with promising activities as well as regio- and diastereoselectivities for testosterone hydroxylation (Kille et al., 2011), constituting an interesting foundation for efficient steroid hydroxylation.

CYP450s are cofactor-dependent and instable in isolated form and thus are preferably applied in metabolically active cells (Duetz et al., 2001; Schrewe et al., 2013), with self- and enzyme regeneration and reactive oxygen species (ROS) degradation as important stabilizing factors (Woodley, 2006). However, microbial cell envelopes typically feature a hydrophilic outer face, established by a lipopolysaccharide layer and the hydrophilic nature of outer membrane porins, e.g., in Gram-negatives (Leive, 1974; Nikaido, 2003; Chen, 2007; Schrewe et al., 2013). This restrains the uptake of hydrophobic substrates (Carter et al., 2003; Schrewe et al., 2011; Grant et al., 2014), including steroids (Zehentgruber et al., 2010a; Zehentgruber et al., 2010b; Putkaradze et al., 2019). The heterologous co-expression of genes encoding hydrophobic

outer membrane pores can facilitate hydrophobic substrate uptake into metabolically active cells, as it has been shown for the bioconversion of alkanes (Julsing et al., 2012; Grant et al., 2014; Call et al., 2016; Hsieh et al., 2018), long-chain fatty acids (Schneider et al., 1998; Jeon et al., 2018), fatty acid methyl esters (Julsing et al., 2012; Ladkau et al., 2016; van Nuland et al., 2016), terpenes (Cornelissen et al., 2013; Ruff et al., 2016), and aromatics (Ruff et al., 2016).

In this study, we evaluate the steroid hydroxylation performance of highly active BM3 variants in living *Escherichia coli* cells and follow up the hypotheses that the outer membrane of Gram-negative bacteria forms an effective barrier for steroids and that this barrier can be relieved by introducing hydrophobic pores. Thereby, 2β- or 15β-hydroxylations of testosterone served as model reactions and the performance of permeabilized and intact cells was compared (**Figure 1**). Further, different outer membrane proteins were evaluated for their potential to improve specific steroid hydroxylation activities of living cells (**Supplementary Table S1**).
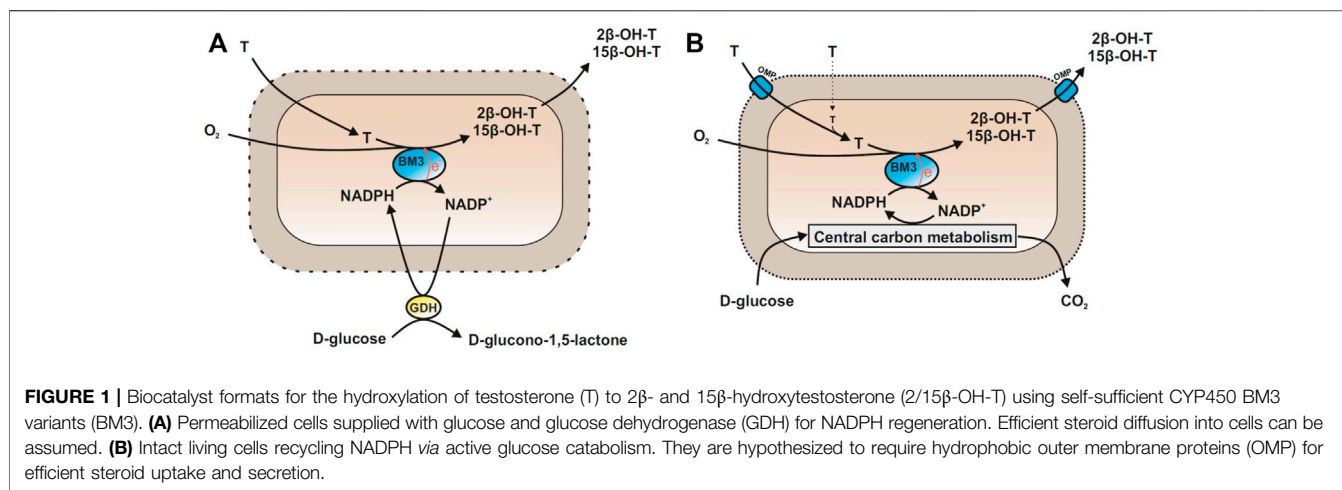
MATERIALS AND METHODS

Gene Synthesis, Oligonucleotides, and Chemicals

Custom synthesized genes and oligonucleotides were purchased from Eurofins (Ebersberg, Germany). 2β- and 15β-hydroxytestosterone were purchased from Steraloids Inc. (Newport, RI, United States). All other chemicals were obtained from AppliChem (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Chemsolute (Renningen, Germany), or Sigma-Aldrich (Steinheim, Germany) in the highest purity available.

Generation of Recombinant Bacterial Strains

Enzymes (Phusion High-Fidelity Polymerase, restriction enzymes, T5 exonuclease, Taq DNA ligase), dNTPS, and the corresponding buffers were purchased from Thermo Scientific Molecular Biology (St. Leon-Rot, Germany) or New England Biolabs (Frankfurt/Main, Germany). Plasmid isolation and purification of DNA from agarose gels was performed according to the manufacturer's instructions of the respective kits from Macherey-Nagel (Düren, Germany). Electro-competent *E. coli* cells were prepared according to standard procedures (Sambrook and Russell, 2001) and transformed *via* electroporation (2500 V, Eppendorf Eporator®, Hamburg, Germany).

Microbial strains and plasmids used in this work are listed in **Supplementary Table S2**. *E. coli* DH5α was used for cloning purposes, whereas *E. coli* BL21-Gold(DE3) was used for expression and biotransformation studies. The plasmids pETM11_KSA1, pETM11_KSA2, pETM11_KSA3, and pETM11_KSA14 were constructed according to the publication of Kille et al. (2011). Plasmids harboring genes encoding the respective outer membrane proteins were

FIGURE 1 | Biocatalyst formats for the hydroxylation of testosterone (T) to 2β- and 15β-hydroxytestosterone (2/15β-OH-T) using self-sufficient CYP450 BM3 variants (BM3). **(A)** Permeabilized cells supplied with glucose and glucose dehydrogenase (GDH) for NADPH regeneration. Efficient steroid diffusion into cells can be assumed. **(B)** Intact living cells recycling NADPH *via* active glucose catabolism. They are hypothesized to require hydrophobic outer membrane proteins (OMP) for efficient steroid uptake and secretion.

constructed as shown in **Supplementary Table S3**. The respective genes were either ordered as gene synthesis constructs or obtained from genomic DNA. Successful cloning was confirmed by sequencing (Genewiz Germany GmbH, Leipzig, Germany).

Cultivation of *E. coli* Strains

For bacterial growth and heterologous protein synthesis, cells were grown in lysogeny broth (LB) medium (Sambrook and Russell, 2001), modified terrific broth (TB) (Kille et al., 2011), or M9 medium (Sambrook and Russell, 2001) supplemented with 0.1% (v/v) US* trace element solution (Panke et al., 1999), 2 mM $MgSO_4$, and 0.5% (w/v) D-glucose as sole carbon and energy source. Cultivations were conducted in a Multitron shaker (Infors, Bottmingen, Switzerland).

Microorganisms from a frozen glycerol stock were cultivated in LB medium (supplemented with 50 µg mL^{-1} kanamycin for plasmid-containing cells) at 37°C and 200 rpm for 6–8 h. From the LB culture, 10 mL of either M9 or TB medium were inoculated and incubated at 30°C for 14–16 h. These precultures were used to inoculate the same media to an optical density of 0.2 at 450 nm (OD_{450}). Heterologous gene expression was induced in the early exponential phase (OD_{450} ~0.6) by addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). In case of cultivation in M9 medium, 0.5 mM of the heme precursor 5-aminolevulinic acid was simultaneously added for enhanced heme synthesis. Cells were harvested 5 h after induction by centrifugation (5,000 g, 5 min, 4°C) for the preparation of permeabilized cells, resting cells, or cell-free extracts. For the isolation of total membrane fractions, cell pellets were stored at −20°C until further processing.

Biotransformation With Permeabilized Cells

Specific activities of permeabilized cells were determined according to a modified version of the protocol described by Kille et al. (2011). Cells were harvested from TB cultures 5 h after induction, washed once with P450 washing buffer (100 mM potassium phosphate, pH 7.4, 5% (v/v) glycerol), and

resuspended to a cell concentration of 1 g$_{CDW}$ L^{-1} in P450 reaction buffer (100 mM potassium phosphate, pH 7.4, 5% (v/v) glycerol, 5% (w/v) glucose, 5 mM EDTA, 0.25 mM NADP$^+$, 1 U mL^{-1} glucose dehydrogenase). The cell suspension was immediately frozen in liquid nitrogen and then thawed at room temperature. One mL of sample was transferred to screw-capped glass tubes (12 mL) and equilibrated in a water bath at 30°C and 250 rpm for 15 min. The biotransformation was then started by adding 10 µL of a 100 mM testosterone stock solution in DMSO, resulting in final concentrations of 1 mM testosterone and 1% (v/v) DMSO. Reactions were terminated after a specific incubation time (given in figures and corresponding descriptions) by adding 12.5 µL HCl (1 M) per 100 µL resting cell suspension. Acetonitrile was added to the samples (50% v/v) to dissolve precipitated steroids, followed by mixing (2000 rpm, 5 min, 4°C) and centrifugation (17,000 g, 5 min, 4°C) for biomass removal. The resulting supernatant was stored at −20°C until further analysis. Specific activities are given in U g$_{CDW}$$^{-1}$, with 1 U defined as the activity forming 1 µmol of product per min, and were retrieved via quantification of accumulated product divided by the biomass applied.

In Vivo Biotransformation With Resting Cells

Fresh cells harvested 5 h after induction from an exponentially growing M9 culture were washed once and resuspended to a cell concentration of 1 g$_{CDW}$ L^{-1} in 100 mM potassium phosphate buffer (pH 7.4) supplemented with 1% (w/v) glucose as the source for energy and reduction equivalents. One mL of resting cell suspension was filled into screw-capped glass tubes (12 mL) and equilibrated at 30°C and 250 rpm for 15 min. Reaction start and termination as well as sampling were performed as described above for biotransformations with permeabilized cells.

In Vitro Biotransformation With Cell-Free Extracts

Cells were harvested from induced exponentially growing M9 cultures, and cell pellets were resuspended in 100 mM potassium phosphate buffer (pH 7.4) to a concentration of 3 g$_{CDW}$ L^{-1}.

Subsequently, cells were disrupted by three passages through a French Press (6.9 MPa, Glenn Mills Inc., Clifton, NJ, United States). Cell debris was removed by centrifugation at 12,000 g for 15 min at 4°C. One mL of supernatant was transferred to screw-capped glass tubes (12 mL), equilibrated at 30°C and 250 rpm for 15 min, and supplied with 10 μL of a 50 mM NADPH stock solution in the same buffer. The reaction was started by adding 10 μL of a 100 mM testosterone stock solution in DMSO, resulting in final concentrations of 0.5 mM NADPH, 1 mM testosterone, and 1% (v/v) DMSO. Reactions were terminated and samples were treated as described above.

Isolation of Total Membrane Fractions

Cell pellets were thawed in ice, resuspended in a 100 mM potassium phosphate buffer (pH 7.4) to a concentration of $3-5$ g_{CDW} L^{-1}, and disrupted by two passages through a French Press (6.9 MPa, Glenn Mills Inc.). The resulting crude cell extract was centrifuged at 12,000 g for 15 min at 4°C followed by centrifugation of the supernatant at 200,000 g and 4°C for 2 h. The resulting pellet was resuspended in the same buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, see below).

Analytical Methods

Biomass concentrations were determined photometrically as the optical density at a wavelength of 450 nm (Libra S11, Biochrom Ltd., Cambridge, United Kingdom) with one OD_{450} unit corresponding to 0.166 g_{CDW} L^{-1} (Blank et al., 2008).

Protein concentrations were analyzed *via* Bradford Dye Reagent (Alfa Aesar, Kandel, Germany) according to the manufacturer's instructions using bovine serum albumin as protein standard. Monitoring of protein synthesis was performed by harvesting 80 μg of cell dry weight (CDW) from cultures followed by SDS-PAGE according to Laemmli (1970). Proteins extracted from 15 $μg_{CDW}$ were loaded per lane and stained with Coomassie Brilliant Blue R-250. For cell-free extract and membrane fraction analysis, 7.5 μg of total protein were loaded per lane. PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, Waltham, MA, United States) was used as reference.

Steroid concentrations were determined by HPLC using a Dionex Ultimate 3,000 system (Thermo Fisher Scientific) equipped with an Accucore C18 column (150 × 3 mm, 2.6 μm particle size, Thermo Fisher Scientific). An eluent consisting of 40% acetonitrile in ultrapure water was used at a flow rate of 0.6 mL min^{-1}. The column oven temperature was set to 40°C and a sample volume of 5 μL was injected. Steroids were detected at 245 nm using an UV detector and quantified based on calibration curves.
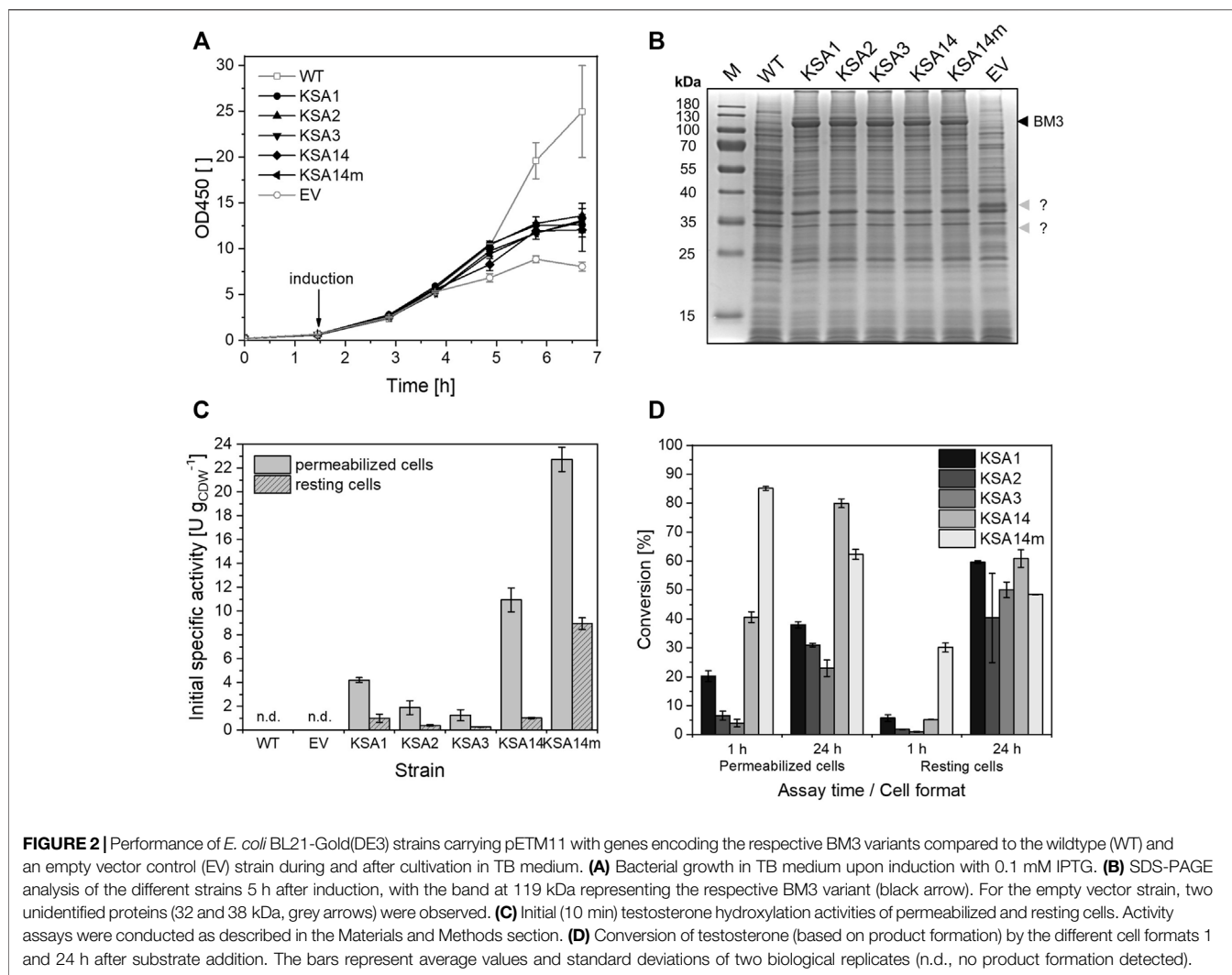
RESULTS

Testosterone Hydroxylation by *E. coli* BL21-Gold(DE3) Harboring Engineered BM3 Variants

In a previous study, Kille *et al.* developed a library of engineered BM3 variants capable of regio- and stereoselective hydroxylation of testosterone either at the 2β- or the 15β-position (Kille et al., 2011). Genes encoding the respective BM3 variants were heterologously expressed in *E. coli* BL21-Gold(DE3) *via* the *lac*-based T7 expression system. Promising variants showed good conversions and regioselectivities, from which volumetric activities of up to 0.58 U L^{-1} can be estimated (**Supplementary Table S4**). An important remark is that cell preparation included the addition of EDTA and a freeze-thawing step which is prone to involve cell membrane permeabilization (Felix, 1982; Chen, 2007). Thus, cells presumably were incapable of intracellular cofactor regeneration (Julsing et al., 2008; Julsing et al., 2012; Schrewe et al., 2013), which is corroborated by the use of an extracellular cofactor regeneration system (Kille et al., 2011). The study presents long-term data (24 h of biotransformation). In order to assess biocatalyst efficiency in detail, we set out to determine initial and specific catalytic rates for permeabilized as well as living cells equipped with the promising BM3 variants KSA1, KSA2, KSA3, or KSA14.

Constructed strains were based on pETM11 and *E. coli* BL21-Gold(DE3) according to Kille et al. (2011) (**Supplementary Table S2, Supplementary Figure S1A**). For cell cultivation and heterologous protein synthesis in TB medium, the protocol for microplate cultivation (Kille et al., 2011) was adapted to shake flask scale. As expected, permeabilized cells containing KSA1, KSA2, and KSA3 primarily produced 2β-hydroxytestosterone, whereas 15β-hydroxytestosterone was detected as main product of KSA14 (**Supplementary Table S5**). Volumetric activities for 24 h of biotransformation were in a similar range as previously reported (**Supplementary Figure S2**). Initial activities within the first 10 min were found to exceed long-term activities by factors of 7.8–19.8, indicating that testosterone hydroxylation rates do not remain constant in the experimental setup chosen. Interestingly, one particular KSA14 clone exhibited a 2-fold higher initial activity and similar 15β-regioselectivity (**Supplementary Table S5**). Sequencing of the plasmid from this clone revealed four additional nucleotide exchanges compared to the original *ksa14* gene, leading to two missense mutations (K224E, V314I) and two silent mutations (L78L, L871L). Both amino acid exchanges are located in the BM3 monooxygenase subunit and thus are likely to affect the catalytic properties of the enzyme. For example, K224 forms salt bridges with the residues involved in the opening and closing of the substrate channel (Whitehouse et al., 2012). This variant is hereafter designated as KSA14m.

Compared to the wildtype strain (specific growth rate μ = 0.85 ± 0.04 h^{-1} at 30°C in TB medium), the recombinant strains after induction showed a shorter phase of exponential growth, followed by a growth rate reduction which may be due to a metabolic burden of BM3 expression (**Figure 2A**). However, growth of the empty vector control was even slower than that of BM3-containing strains and involved the synthesis of two unexpected 32 and 38 kDa proteins (**Figure 2B**). Analysis of open reading frames on the empty vector revealed that the so-called max dimerization protein 1 (NCBI accession number NP_002348) may be synthesized. However, its molecular weight is estimated to be only 25 kDa, which does not fit to the observed protein weights.

FIGURE 2 | Performance of *E. coli* BL21-Gold(DE3) strains carrying pETM11 with genes encoding the respective BM3 variants compared to the wildtype (WT) and an empty vector control (EV) strain during and after cultivation in TB medium. **(A)** Bacterial growth in TB medium upon induction with 0.1 mM IPTG. **(B)** SDS-PAGE analysis of the different strains 5 h after induction, with the band at 119 kDa representing the respective BM3 variant (black arrow). For the empty vector strain, two unidentified proteins (32 and 38 kDa, grey arrows) were observed. **(C)** Initial (10 min) testosterone hydroxylation activities of permeabilized and resting cells. Activity assays were conducted as described in the Materials and Methods section. **(D)** Conversion of testosterone (based on product formation) by the different cell formats 1 and 24 h after substrate addition. The bars represent average values and standard deviations of two biological replicates (n.d., no product formation detected).

The high expression levels of the respective BM3 variants (**Figure 2B**) enabled initial specific activities ranging from 1.25 ± 0.47 to 10.93 ± 1.00 U g_{CDW}^{-1} for permeabilized cells of the different strains (**Figure 2C**), whereas KSA14m hydroxylated testosterone at a significantly higher rate of 22.73 ± 1.02 U g_{CDW}^{-1}. To investigate if intact cells enable similar specific testosterone hydroxylation activities as permeabilized cells, non-growing living (i.e., resting) cells were prepared after cultivation and heterologous gene expression in TB medium and employed in activity assays. Direct comparison revealed 4.2 to 10.7-fold lower initial activities in the range of 0.26 ± 0.01 to 1.02 ± 0.06 U g_{CDW}^{-1} for KSA1, KSA2, KSA3, and KSA14 (**Figure 2C**). KSA14m containing cells again showed the highest testosterone hydroxylation rate (8.94 ± 0.49 U g_{CDW}^{-1}, 2.5-fold lower than in permeabilized cell format). Regioselectivity was not or only slightly reduced compared to permeabilized cells (**Supplementary Table S5**). Furthermore, permeabilized cells generally demonstrated higher conversions than resting cells 1 hour after substrate addition (**Figure 2D**). This however changed during the following 23 h, after which resting cells of some strains showed higher conversions than

permeabilized cells. It has to be mentioned that solubility limits of testosterone and the 15β-hydroxylated product in the aqueous assay setup (RCA buffer supplemented with glucose and 1% (v/v) DMSO) amounted to 66 and 565 μM, respectively. Depicted conversions after 1 and 24 h of biotransformation were calculated based on product formation and need to be interpreted carefully when exceeding the aqueous solubility limit (conversions >56%).

Heterologous BM3 Synthesis in Defined Media

High CYP450 levels led to noticeably impaired growth of the recombinant bacterial strains in TB medium, pointing to metabolic burden effects. High oxygenase levels have been reported to affect host cell physiology (van Beilen et al., 2003; Bühler et al., 2008) and thus do not necessarily lead to higher whole-cell activities (Schäfer et al., 2020). In contrast to complex media like TB, defined media usually lead to slower bacterial growth and lower metabolic activity, including heterologous gene
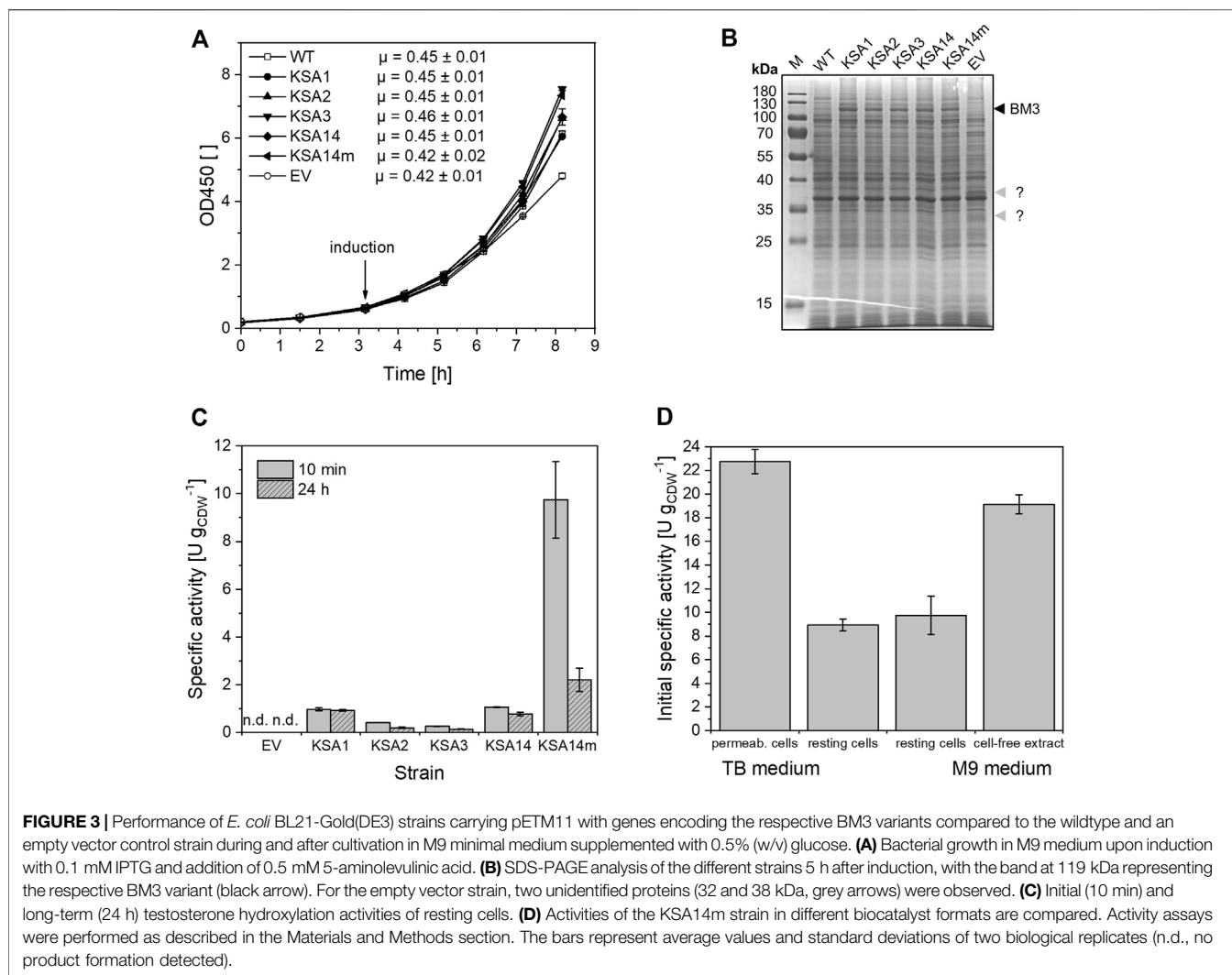
FIGURE 3 | Performance of *E. coli* BL21-Gold(DE3) strains carrying pETM11 with genes encoding the respective BM3 variants compared to the wildtype and an empty vector control strain during and after cultivation in M9 minimal medium supplemented with 0.5% (w/v) glucose. **(A)** Bacterial growth in M9 medium upon induction with 0.1 mM IPTG and addition of 0.5 mM 5-aminolevulinic acid. **(B)** SDS-PAGE analysis of the different strains 5 h after induction, with the band at 119 kDa representing the respective BM3 variant (black arrow). For the empty vector strain, two unidentified proteins (32 and 38 kDa, grey arrows) were observed. **(C)** Initial (10 min) and long-term (24 h) testosterone hydroxylation activities of resting cells. **(D)** Activities of the KSA14m strain in different biocatalyst formats are compared. Activity assays were performed as described in the Materials and Methods section. The bars represent average values and standard deviations of two biological replicates (n.d., no product formation detected).

expression and protein synthesis, but are more feasible for process scale up. We thus also tested M9 medium for cultivation.
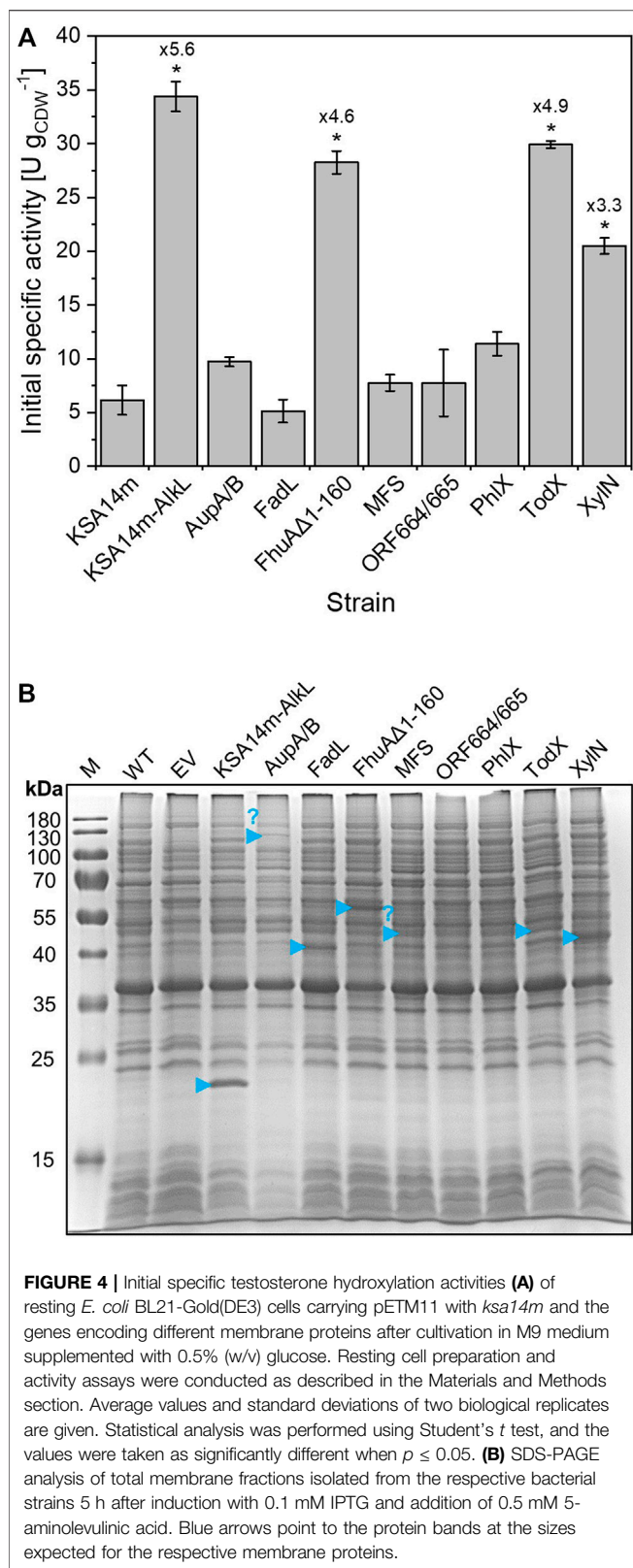
In contrast to modified TB medium, all recombinant strains showed exponential growth in M9 medium, which was not impaired compared to the wildtype, also not upon induction (**Figure 3A**). Even though BM3 levels after 5 h of induction were lower in M9 medium compared to TB medium (**Figure 3B**), resting cells showed similar initial specific activities, ranging from 0.26 ± 0.01 U g_{CDW}^{-1} to 1.06 ± 0.02 U g_{CDW}^{-1}. The KSA14m strain again displayed a noticeably higher activity of 9.75 ± 1.60 U g_{CDW}^{-1} (**Figure 3C**). Interestingly, regioselectivity was slightly lower (**Supplementary Table S5**).

In summary, the choice of cultivation medium did not affect the activity of intact cells. However, selection of a suitable biocatalyst format proved to be pivotal for higher specific testosterone hydroxylation rates. The resting-cell format appears less suitable for BM3-catalyzed testosterone hydroxylation than the permeabilized-cell format, suffering from impaired substrate uptake. It however is clearly superior regarding cofactor regeneration and biocatalyst stability, making

cell engineering for improved substrate uptake a strategy of choice.

Testosterone Hydroxylation by KSA14m in Cell-Free Extracts Versus Whole Cells

As the strain containing KSA14m displayed the highest specific testosterone hydroxylation activity, it was used to investigate the possible substrate uptake limitation. Cells cultivated in M9 medium were either used for the preparation of intact resting cells or disrupted to generate cell-free extracts, for which steroid hydroxylation is not subject to mass transfer limitation over cellular membranes. A specific activity of 34.8 ± 1.5 U per g of total protein was obtained with cell-free extracts, which, assuming that standard *E. coli* cells consist of 55% total protein (Schmidt et al., 2016), is 2-fold higher than that of intact cells (**Figure 3D**). Obviously, the activity obtained for intact cells does not reflect the maximum BM3 activity available in the cells which corroborates the findings with permeabilized cells and clearly indicates steroid uptake limitation.

FIGURE 4 | Initial specific testosterone hydroxylation activities **(A)** of resting *E. coli* BL21-Gold(DE3) cells carrying pETM11 with *ksa14m* and the genes encoding different membrane proteins after cultivation in M9 medium supplemented with 0.5% (w/v) glucose. Resting cell preparation and activity assays were conducted as described in the Materials and Methods section. Average values and standard deviations of two biological replicates are given. Statistical analysis was performed using Student's *t* test, and the values were taken as significantly different when $p \leq 0.05$. **(B)** SDS-PAGE analysis of total membrane fractions isolated from the respective bacterial strains 5 h after induction with 0.1 mM IPTG and addition of 0.5 mM 5-aminolevulinic acid. Blue arrows point to the protein bands at the sizes expected for the respective membrane proteins.
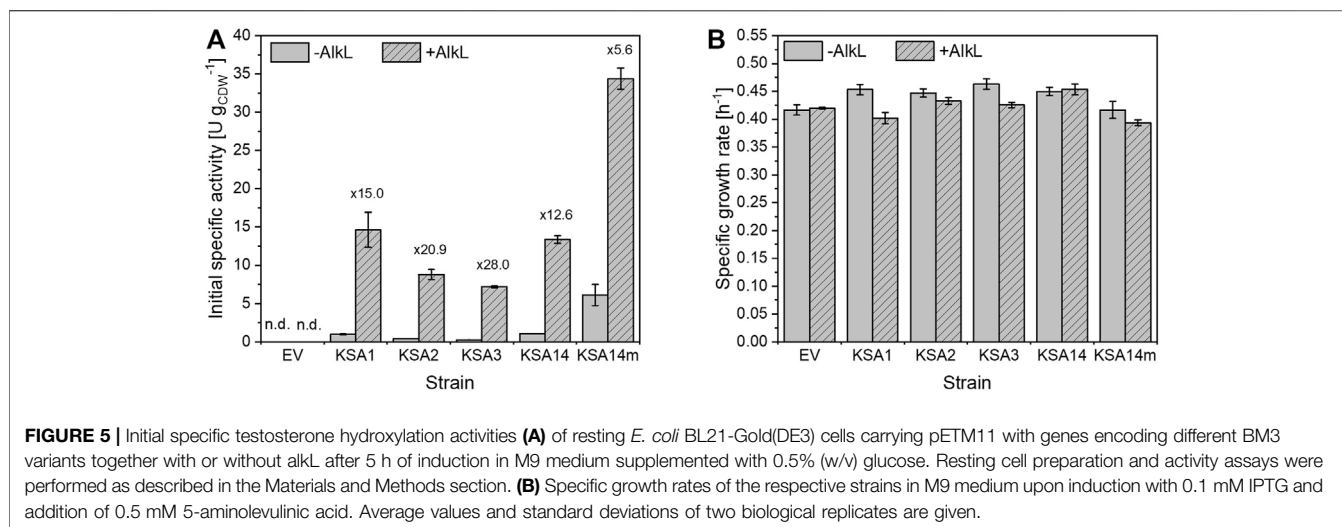
Hydrophobic Outer Membrane Pores Boost Testosterone Hydroxylation by Living Cells

The significant difference between the activities of cell-free extracts, permeabilized, and intact cells indicates that the uptake of testosterone as a large hydrophobic substrate limits its hydroxylation by intact Gram-negative cells. We tested nine different outer membrane proteins for an improvement of testosterone uptake by *E. coli* BL21-Gold(DE3) containing the BM3 variant KSA14m. Candidates were selected from operons that encode pathways enabling growth on or degradation of hydrophobic compounds (Wang et al., 1995; Ayoubi and Harker, 1998; Kasai et al., 2001; Hearn et al., 2008; Leu et al., 2011; Kothari et al., 2016; Mounier et al., 2018; Olivera and Luengo, 2019) or based on previous studies employing hydrophobic outer membrane proteins to facilitate hydrophobic substrate uptake (Julsing et al., 2012; Cornelissen et al., 2013; Grant et al., 2014; Call et al., 2016; Ruff et al., 2016; Jeon et al., 2018). Corresponding genes were placed downstream of the *ksa14m* gene in a bicistronic operon (**Supplementary Figure S1B**).

The co-synthesis of four outer membrane proteins significantly improved the specific whole-cell testosterone hydroxylation activity of living cells. XylN, FhuA Δ1-160, and TodX resulted in 3.3-, 4.6-, and 4.9-fold specific activity increases, respectively (**Figure 4A**). AlkL emerged as the most effective candidate enabling a 5.6-fold increase in whole-cell testosterone hydroxylation activity, corresponding to an even higher protein-specific rate than obtained with KSA14m containing cell-free extract. Induction of heterologous gene expression slightly affected bacterial growth of only the strains carrying *aupA/aupB*, *fhuAΔ1-160*, *todX*, and *xylN* compared to strains without an additional outer membrane protein (**Supplementary Figure S3**). SDS-PAGE analysis of isolated total membrane fractions further confirmed AlkL, FhuA Δ1-160, TodX, and XylN synthesis, whereas bands representing the remaining outer membrane proteins were not clearly visible (**Figure 4B**) which may explain why an effect on whole-cell activity was not observed in these cases. Interestingly, a protein band representing the fatty acid pore FadL was clearly visible, but the respective strain did not show an improved activity. To conclude, whole living cells equipped with hydrophobic outer membrane proteins facilitating testosterone uptake allowed even higher specific turnover rates than obtained with permeabilized cells and cell-free extracts.

Potential of Engineered BM3 Variants for Testosterone Hydroxylation

Incorporation of AlkL into the whole-cell biocatalyst turned out as the most promising strategy to increase whole-cell testosterone hydroxylation activities by a facilitated steroid uptake. Thus, the testosterone hydroxylation capabilities of different BM3 variants were investigated with cells co-expressing *alkL*. Following this

FIGURE 5 | Initial specific testosterone hydroxylation activities **(A)** of resting *E. coli* BL21-Gold(DE3) cells carrying pETM11 with genes encoding different BM3 variants together with or without alkL after 5 h of induction in M9 medium supplemented with 0.5% (w/v) glucose. Resting cell preparation and activity assays were performed as described in the Materials and Methods section. **(B)** Specific growth rates of the respective strains in M9 medium upon induction with 0.1 mM IPTG and addition of 0.5 mM 5-aminolevulinic acid. Average values and standard deviations of two biological replicates are given.

strategy, increased whole-cell testosterone hydroxylation rates also were obtained with the variants KSA1, KSA2, KSA3, and KSA14 (**Figure 5A**) emphasizing the high efficiency, with which these variants can catalyze steroid hydroxylation in living cells. Thereby, AlkL increased specific activities 6- to 28-fold depending on the BM3 variant. Again, bacterial growth was barely affected by additional *alkL* expression (**Figure 5B**). Cells carrying KSA14m and AlkL emerged as the most active whole-cell biocatalysts and enabled almost complete conversion of 1 mM testosterone in the course of 1 h as it is shown in **Supplementary Figure S4**. Substrate depletion appeared to cause a conversion rate decrease towards the end of the biotransformation experiment. Catalyst instability issues possibly caused by inherent enzyme instability and/or substrate/product inhibitions may however also contribute to this effect and will be subject of future research. In conclusion, incorporation of hydrophobic outer membrane proteins, such as AlkL, led to a significant increase of testosterone hydroxylation activities of recombinant *E. coli* cells and thus proved to be a valuable strategy to overcome steroid uptake limitation.

DISCUSSION

Biotechnological steroid hydroxylation at industrial scale with the typically employed wildtype microorganisms still suffers from low transformation rates and poor selectivities (Fernández-Cabezón et al., 2018). Steroid-hydroxylating CYP450s are generally characterized by low catalytic turnover rates, the need for electron transfer partners, as well as limitations in the electron transfer from the redox cofactor to the active site (Bernhardt, 2006; Donova and Egorova, 2012; Donova, 2017). These bottlenecks considerably impede the generation of recombinant production strains for commercial applications (Duetz et al., 2001; van Beilen et al., 2003; Urlacher and Eiben, 2006; Julsing et al., 2008; Bernhardt and Urlacher, 2014). By engineering the self-sufficient and highly active

CYP450 BM3 from *Bacillus megaterium* ATC14581 towards regio- and stereoselective hydroxylation of testosterone, Kille and colleagues laid the foundation for efficient steroid-hydroxylation biocatalysts (Kille et al., 2011). In this study, we set out to evaluate and improve the applicability of selected BM3 variants in intact living cells as the preferred biocatalyst format for oxygenase-based biocatalysis (Duetz et al., 2001; Schrewe et al., 2013) with a focus on increasing the catalytic turnover rates.

Introduction of Hydrophobic Outer Membrane Proteins as Promising Strategy to Improve Whole-Cell Steroid Turnover Rates

Former studies demonstrated that hydrophobic substrate uptake across cellular membranes constitutes a major bottleneck in whole-cell biotransformations (Carter et al., 2003; Fontanille and Larroche, 2003; Zehentgruber et al., 2010b; Schrewe et al., 2011; Grant et al., 2014). Contrary to small hydrophobic molecules crossing the outer membrane of Gram-negatives through hydrophilic porins and readily diffusing through the cytoplasmic membrane, the uptake of larger hydrophobic molecules is restrained by polar and charged lipopolysaccharides on the cell surface and the hydrophilic nature of porins in the outer membrane (Hancock, 1997; Nikaido, 2003). Although strategies targeting membrane destruction or permeabilization may relieve this limitation, they are destructive to the cell and its functionalities and thus not suitable for biocatalytic reactions depending on an active metabolism for cofactor and enzyme regeneration as well as ROS degradation (Julsing et al., 2008; Julsing et al., 2012; Schrewe et al., 2013).

Alternatively, the supply of cells with membrane pores has been established as an approach enabling the conversion of a variety of highly hydrophobic substrates by intact metabolically active cells. **Table 1** shows a comparison of the best results obtained in this study for steroid hydroxylation with those

TABLE 1 | Studies reporting enhanced uptake or biotransformation of hydrophobic substrates *via* heterologous outer membrane proteins.

Outer Membrane Protein	Substrate Converting Enzyme	Substrate (MW in g mol^{-1})	Impact	References
AlkL (*P. putida* GPo1)	alkane monooxygenase AlkBGT (*P. putida* GPo1)	n-octane (114)	4-fold increase of specific activity	Julsing et al. (2012)
		n-nonane (128)	40-fold increase of specific activity	Julsing et al. (2012)
		C$_{12}$-C$_{16}$ alkanes (170–226)	up to 100-fold increase of specific yields	Grant et al. (2014)
		nonanoic acid ethyl ester (186)	1.7-fold increase of specific activity	van Nuland et al. (2016)
		dodecanoic acid methyl ester (214)	28- and 62-fold specific activity increase in single and two-liquid phase systems, respectively	Julsing et al. (2012); Ladkau et al. (2016)
	CYP153A6, Fd, FDR (*Mycobacterium* sp. HXN-1500)	(S)-limonene (136)	2- and 5-fold specific activity increase in single and two-liquid phase systems, respectively	Cornelissen et al. (2013)
	CYP153A, Fd, FDR (*Marinobacterium aquaeolei* VT8)	C$_{12}$-C$_{16}$ alkanes (170–226)	increased induction caused by alkanes	Hsieh et al. (2018)
	BM3 variants KSA1, KSA2, KSA3, KSA14, and KSA14m (*B. megaterium* ATCC14581)	testosterone (288)	15-, 20.9-, 28-, 12.6-, and 5.6-fold increase of specific activity	This study
FadL (*E. coli* BL21)	hydratase (*Stenotrophomonas maltophilia*)	oleic acid (282)	5.5-fold increase of specific activity	Jeon et al. (2018)
	ADH (*Micrococcus luteus*)	12- and 10-hydroxy-octadecanoic acid (300)	5.5-fold increase of specific activity	Jeon et al. (2018)
	ADH (*Micrococcus luteus*), BVMO (*P. putida* KT2440)	ricinoleic acid (298)	2-fold increase of specific activity	Jeon et al. (2018)
	alkane monooxygenase AlkBGT (*P. putida* GPo1)	n-octane (114)	4.5-fold increase of alkane import rate	Call et al. (2016)
FadL (*P. putida* GPo1)	BM3 wildtype (*B. megaterium* ATCC14581)	pentadecanoic acid (242)	up to 2-fold increase of specific activity	Schneider et al. (1998)
FhuA Δ1-160 (*E. coli*)	BM3 variants (*B. megaterium* ATCC14581)	toluene (92)	1.5-fold increase of translocation efficiency	Ruff et al. (2016)
		anisol (108)	1.8-fold increase of translocation efficiency	Ruff et al. (2016)
		pinene (136)	1.8-fold increase of translocation efficiency	Ruff et al. (2016)
		limonene (136)	2-fold increase of translocation efficiency	Ruff et al. (2016)
		7-benzoxy-3-carboxy-coumarin ethyl ester (324)	5-fold increase of translocation efficiency	Ruff et al. (2016)
	BM3 variant KSA14m	testosterone (288)	4.6-fold increase of specific activity	This study
TodX (*P. putida* F1)	BM3 variant KSA14m	testosterone (288)	4.9-fold increase of specific activity	This study
XylN (*P. putida* mt-2)	BM3 variant KSA14m	testosterone (288)	3.3-fold increase of specific activity	This study

Fd, ferredoxin; FDR, ferredoxin reductase; ADH, alcohol dehydrogenase; BVMO, Baeyer-Villiger monooxygenase.

obtained in earlier studies for the conversion of other hydrophobic substrates facilitated by outer membrane proteins. Applied pores range from members of aliphatic and aromatic hydrocarbon degradation pathways to artificially created pores, all with a typically uncharacterized substrate spectrum. In this study, the feasibility of this approach was tested for and extended to steroids. It is clear that such uptake limitation only becomes relevant, if high intracellular steroid conversion activities are established. Our results obtained with the recently published testosterone hydroxylating BM3 variants show that their high activity becomes limited by steroid uptake into intact living cells. The introduction of 4 out of 9 tested outer membrane proteins significantly increased the activity of *E. coli* containing the highly active BM3 variant KSA14m.

However, the introduction of outer membrane proteins also can be harmful to host cell physiology, either due to metabolic burden of the expression of additional heterologous genes and/or energy demanding, inefficient, and/or improper folding and translocation of membrane proteins (van Beilen et al., 2003; Kadisch et al., 2017b). The expression level of pores can be another critical factor as consequentially increased intracellular

substrate concentrations may become toxic (Julsing et al., 2012; Call et al., 2016; Kadisch et al., 2017a). For example, biocatalyst destabilization related to high *alkL* expression has been reported during biotransformation of dodecanoic acid methyl ester (Schrewe et al., 2014) and octane (Grant et al., 2014). Similar findings have been reported for high level *fadL* expression employed for the conversion of octane (Call et al., 2016) and (hydroxy) fatty acids (Jeon et al., 2018). Strains expressing *aupAB*, *fhuAΔ1-160*, *todX*, and *xylN* displayed slightly lower specific growth rates compared to a strain harboring only the BM3 variant. Effects on biocatalyst stability remain to be investigated. A fine-tuning of membrane protein expression levels may become necessary to control substrate influx, for example by the use of tightly controlled expression systems (Grant et al., 2014; Call et al., 2016), variation of promoter strength (Kadisch et al., 2017a), or appropriate gene copy number (Jeon et al., 2018). Furthermore, several technical solutions can be applied to avoid toxic effects of substrate and product molecules, such as the introduction of an organic phase as substrate reservoir and product sink (van Beilen et al., 2003; Schrewe et al., 2014).

Substrate Spectrum of Outer Membrane Proteins

Introduction or overexpression of outer membrane proteins has become an increasingly relevant tool for biotransformations of hydrophobic substrates with whole microbial cells (**Table 1**). Several investigated candidates feature a broad substrate range reaching beyond the natural substrates of the pathways they are associated to. In this study, we report an extension of the so far-known substrate spectrum of several hydrophobic pores towards steroids. Several studies showed that AlkL promotes alkane uptake by *E. coli* (Julsing et al., 2012; Grant et al., 2014). This outer membrane protein has been proposed to be structured as an eight-stranded *β*-barrel traversing the lipopolysaccharide layer similar to OmpW of *E. coli*, with which it shares 27% amino acid sequence homology (van Beilen et al., 1992; Hong et al., 2006). The extracellular domain has a high affinity for hydrophobic molecules so that substrates are easily channeled across the lipopolysaccharide layer into the hydrophobic interior of AlkL in the outer membrane (Grant et al., 2014). As for OmpW, the passage is hindered by a narrowing in the channel, which directs molecules to exit through a putative lateral opening into the outer membrane (Hong et al., 2006). Apparently, this mechanism also works for fatty acid (m)ethyl esters (Julsing et al., 2012; Ladkau et al., 2016; van Nuland et al., 2016) and monoterpenes (Cornelissen et al., 2013). Interestingly, the radius of the lateral opening (1.3 Å) has been described as incompatible with the size of these known AlkL substrates but has recently been shown to be dynamic due to a continuous restructuring of the barrel dimension and release into the membrane through ephemeral openings (Schubeis et al., 2020). In this study, AlkL relieved the constrained testosterone uptake into *E. coli* and boosted specific testosterone hydroxylation activities, thus expanding the AlkL substrate spectrum to steroids, which are even larger than the substrates known so far (**Table 1**).

The pivotal role of FadL as a long-chain fatty acid transporter in *E. coli* has already been reported decades ago (Nunn and Simons, 1978; Black, 1988). In whole-cell biotransformations of long-chain fatty acids, increased FadL levels significantly improved specific rates (Schneider et al., 1998; Jeon et al., 2018). Similar results were obtained for atypical FadL substrates, i.e., long-chain hydroxyl fatty acids (Jeon et al., 2018). Interestingly, overexpression of *fadL* did not lead to improved steroid hydroxylation rates in this study. Even though the FadL basic structure has been described as similar to AlkL (van den Berg, 2010), the proposed uptake mechanism differs significantly. FadL is described as a long barrel composed of 14 antiparallel *β*-strands that is plugged by a central hatch domain and possesses an opening based on an inward-pointing kink in one of the *β*-strands (van den Berg et al., 2004). Between two extracellular loops, a hydrophobic groove comprises a low-affinity binding site for substrates, which then diffuse into a high-affinity binding pocket inside the barrel close the N-terminus. Conformational changes in the N-terminus and the hatch result in an affinity decrease and generate a lateral channel for the substrate to move into the outer membrane (Hearn et al., 2009; van den Berg, 2010). Thereby, substrate binding has been suggested as prerequisite (Black and Zhang, 1995; Lepore et al., 2011), with the affinity strongly varying with fatty acid chain length (Black, 1990) and long-chain fatty acids ($\geq C_{12}$) being preferred over mid-chain fatty acids (C_8-C_{11}) (Maloy et al., 1981; Black, 1990). Generally, electrostatic forces between negatively charged carboxyl groups and positively charged amino acid residues within the FadL binding pocket have been considered necessary for substrate binding (van den Berg, 2005), which would exclude non-charged compounds as FadL substrates. The variation of FadL expression levels however was recently shown to modulate the import of medium-chain alkanes into *E. coli* (Call et al., 2016), suggesting that substrate binding is not necessarily charge-dependent. The missing facilitation of steroid import by FadL observed in this study is in line with a possible charge dependency and/or a rather confined substrate specificity.

In contrast, truncated FhuA was found to facilitate testosterone uptake. The native, complete protein is responsible for the active import of ferric hydroxamate into *E. coli*. Removal of the first 160 amino acids (N-terminal cork domain) converted the active iron transporter into a large passive diffusion channel (i.e., FhuA Δ1-160) differing from AlkL in terms of uptake mechanism (Ruff et al., 2016). FhuA Δ1-160 has been shown to improve the uptake of terpenes and aromatic compounds into *E. coli* up to 5-fold (Ruff et al., 2016). It also was found to facilitate testosterone uptake resulting in a 4.6-fold specific whole-cell activity increase, and thus can be considered a valuable outer membrane protein with a broad substrate spectrum.

Among the putative hydrophobic substrate uptake pores from hydrocarbon-degrading bacteria, TodX from *P. putida* F1 and XylN from *P. putida* mt-2 were found to significantly enhance whole-cell testosterone hydroxylation. Both have been reported to be involved in the uptake of alkylaromatic compounds such as toluene or xylene (Wang et al., 1995; Kasai et al., 2001). Interestingly, they share sequence homology with each other and further putative outer membrane proteins from hydrocarbon-degrading Gram-negative bacteria, e.g., TbuX, CymD, PorA, and CumH (Kahng et al., 2000; Kasai et al., 2001; Hearn et al., 2008), which thus also constitute interesting candidates for the enhancement of hydrophobic substrate uptake.

Few bacteria have been reported to be capable of metabolizing steroids, which generally are considered as highly biodegradation-resistant. Known uptake systems mostly do not involve only one, but a set of proteins (Olivera and Luengo, 2019), e.g., ten ATP-dependent proteins encoded in the actinobacterial *mce4* locus (Mohn et al., 2008). A putative, but not yet characterized Major Facilitator Superfamily Transporter (MFS) originating from the testosterone-degrading strain *Comamonas thiooxydans* (NCBI Reference Sequence WP_041743963) did not increase testosterone hydroxylation rates. While AupA and AupB have been described as outer and inner membrane proteins,

TABLE 2 | CYP450-catalyzed steroid hydroxylation efficiencies.

Enzyme	Redox Partners	Host and Biocatalyst Format	Reaction	Reaction Time	Product Titer [mg L^{-1}]	Volumetric Productivity [mg L^{-1} h^{-1}]	Maximum Specific Activity	References
CYP21 (human)	CYP450 reductase (endogenous)	*Schizosaccharomyces pombe* CAD 18 (permeabilized cells)	21-hydroxylation of 17α-hydroxy-progesterone	10 h	78[a]	7.8[a]	0.05 U g$_{WCW}$$^{-1a,b}$	Zehentgruber et al. (2010a)
CYP3A4 (human)	CYP450 reductase (human)	*Yarrowia lipolytica* H222-S4 (resting cells)	6β-hydroxylation of progesterone	90 h	76[a]	0.84[a]	0.02 U g$_{CDW}$$^{-1}$	Braun et al. (2012)
CYP11B1 G23R_L271M (human)	Ad, ADR (bovine)	*E. coli* C43(DE3) (resting cells)	11β-hydroxylation of 11-deoxycortisol	24 h	843	35	0.09 U g$_{WCW}$$^{-1}$ (first 12 h)[a,b]	Schiffer et al. (2015)
CYP21A2 (bovine)	Fd, ADR (*S. pombe*)	*E. coli* C43(DE3) (resting cells)	21-hydroxylation of medrane	20 h	650	27	0.06 U g$_{WCW}$$^{-1}$ (first 3 h)[a,b]	Brixius-Anderko et al. (2015)
CYP21A2 M210V (bovine)	truncated CYP450 reductase (bovine)	*E. coli* C43(DE3) (permeabilized cells)	21-hydroxylation of medrane	24 h	691	29	0.01 U g$_{WCW}$$^{-1}$ (24 h)[a,b]	König et al. (2020)
CYP106A2 (*B. megaterium*)	Ad, ADR (bovine)	*E. coli* JM109 (growing cells)	15β-hydroxylation of 11-deoxy-corticosterone	48 h	330 (theoretical)[a]	14 (theoretical)[a]	OD or biomass conc. not given	Hannemann et al. (2006)
CYP106A2 (*B. megaterium*)	Ad, ADR (bovine)	*E. coli* BL21 (lyophilized cell extract)	15β-hydroxylation of progesterone	45 min	121[a]	154 (theoretical)	2 U g$_{WCW}$$^{-1}$ (first 5 min)[a,b]	Zehentgruber et al. (2010b)
CYP106A2 (*B. megaterium*)	Ad, ADR (bovine)	*E. coli* BL21 (lyophilized cell extract)	15β-hydroxylation of testosterone	30 min	115[a]	229 (theoretical)	1.08 U g$_{WCW}$$^{-1}$ (first 7 min)[a,b]	Zehentgruber et al. (2010b)
CYP106A2 (*B. megaterium*)	truncated Ad, ADR (bovine)	*Bacillus megaterium* MS941 (resting cells)	7β-hydroxylation of dehydroepiandro-sterone	24 h	2,470[a]	103	0.14 U g$_{WCW}$$^{-1a,b}$	Schmitz et al. (2014)
CYP109B1 (*B. subtilis* 168)	Fd, FDR (*Synechococcus elongates*)	*E. coli* BL21(DE3) (permeabilized cells)	15β-hydroxylation of testosterone	20 h	235[a]	12[a]	0.10 U g$_{CDW}$$^{-1a,c}$	Zhang et al. (2021)
CYP109E1(*B. megaterium*)	truncated Ad, ADR (bovine)	*E. coli* C43(DE3) (permeabilized cells)	24- and 25-monohydroxylation of cholesterol	48 h	1[a]	45	0.01 U g$_{cells}$$^{-1}$ (first 4 h)[a]	Putkaradze et al. (2019)
CYP154C5 (*Nocardia farcinica*)	Pd, PDR (*P. putida*)	*E. coli* C43(DE3) (resting cells)	16α-hydroxylation of pregnenolone	6 h	633[a]	106[a]	0.31 U g$_{CDW}$$^{-1}$ (first 120 min)[a,d]	Bracco et al. (2013)
CYP154C5 (*Nocardia farcinica*)	Pd, PDR (*P. putida*)	*E. coli* C43(DE3) (resting cells)	16α-hydroxylation of progesterone	4 h	629[a]	167[a]	0.86 U g$_{CDW}$$^{-1}$ (first 1 h)[a,d]	Bracco et al. (2013)
CYP154C5 (*Nocardia farcinica*)	Pd, PDR (*P. putida*)	*E. coli* C43(DE3) (resting cells)	16α-hydroxylation of testosterone	8 h	289[a]	36[a]	0.17 U g$_{CDW}$$^{-1}$ (first 1 h)[a,d]	Bracco et al. (2013)
CYP260A1 T224A (*Sorangium cellulosum*)	truncated Ad, ADR (bovine)	*E. coli* C43(DE3) (growing cells)	9α-hydroxylation of 11-deoxycortisone	24 h	250	10	OD or biomass conc. not given	Litzenburger and Bernhardt, (2017)
CYP102A1 (BM3) variant M02	-	*Rhodococcus erythropolis* RG9 (growing cells)	16β-hydroxylation of norandrostane-dione	20 h	350	18	biomass concentration not deducible	Venkataraman et al. (2015)
BM3 variant LG-23	-	*E. coli* BL21(DE3) (permeabilized cells)	7β-hydroxylation of testosterone	5 h	271[a]	54[a]	OD or biomass conc. not given	Li et al. (2020)
BM3 variant LG-23/T438S	-	*E. coli* BL21(DE3) (permeabilized cells)	11α-hydroxylation of estra-4,9-diene-3,17-dione	5 h	267[a]	53[a]	0.19 U g$_{CDW}$$^{-1a}$	Peng et al. (2022)
BM3 variant KSA1	-	*E. coli* BL21-Gold(DE3) (resting cells with AlkL)	2β-hydroxylation of testosterone	1 h	164	164	14.6 U g$_{CDW}$$^{-1}$ (first 10 min)	This study
BM3 variant KSA2	-	*E. coli* BL21-Gold(DE3) (resting cells with AlkL)	2β-hydroxylation of testosterone	1 h	61	61	8.78 U g$_{CDW}$$^{-1}$ (first 10 min)	This study
BM3 variant KSA3	-	*E. coli* BL21-Gold(DE3) (resting cells with AlkL)	2β-hydroxylation of testosterone	1 h	41	41	7.19 U g$_{CDW}$$^{-1}$ (first 10 min)	This study
BM3 variant KSA14	-	*E. coli* BL21-Gold(DE3) (resting cells with AlkL)	15β-hydroxylation of testosterone	1 h	123	123	13.4 U g$_{CDW}$$^{-1}$ (first 10 min)	This study
BM3 variant KSA14m	-	*E. coli* BL21-Gold(DE3) (resting cells with AlkL)	15β-hydroxylation of testosterone	1 h	195	195	34.4 U g$_{CDW}$$^{-1}$ (first 10 min)	This study

Fd, ferredoxin; FDR, ferredoxin reductase, Ad, adrenodoxin; ADR, adrenodoxin reductase; Pd, putidaredoxin, PDR, putidaredoxin reductase.
[a]*Estimated from figures and parameters (conversions, selectivities, biocatalyst concentrations) provided by the respective reference.*
[b]*WCW: wet cell weight.*
[c]*CDW, calculated based on given OD$_{600}$ = 20 and empirical correlation factor 0.34 for* E. coli *(= 6.8 g$_{CDW}$ L^{-1}) (Falcioni et al., 2013).*
[d]*CDW, calculated based on given OD$_{600}$ = 40 and empirical correlation factor 0.34 for* E. coli *(= 13.6 g$_{CDW}$ L^{-1}).*

respectively, involved in alkane uptake in *Marinobacter hydrocarbonoclasticus* SP17 (Mounier et al., 2018), the exact function of proteins encoded by ORF664 and ORF665 in *Acinetobacter venetianus* RAG-1 is currently uncertain, with a possible role in dodecane uptake (Kothari et al., 2016). Besides an unsuitable substrate specificity, expression issues such as improper folding may be the reason for the lacking positive effect on testosterone hydroxylation in these cases.

The increase in testosterone hydroxylation activities not only depended on the type of outer membrane protein applied, but, interestingly, also deferred for different BM3 variants applying the same pore. This high variation may be due to differing K_m values of respective BM3 variants. For example, the K_m of KSA2 for testosterone has been reported to be 86-times higher than the K_m of KSA14 (Kille et al., 2011). Such a high K_m may increase the substrate limitation effect without AlkL. The impact of facilitated substrate uptake *via* an outer membrane protein thus would be much higher as it was the case for KSA2 as compared to KSA14 in combination with AlkL (**Figure 5A**).

Benchmarking BM3-Catalyzed Testosterone Hydroxylation in the Whole-Cell Biocatalyst Format

In this study, we investigated the steroid hydroxylation performance of highly active BM3 variants in living *E. coli* cells equipped with different hydrophobic outer membrane proteins to relieve substrate uptake limitation. *In vivo* testosterone hydroxylation rates obtained with these whole-cell biocatalysts even exceeded activities obtained with permeabilized cells or cell-free extracts (for KSA14m), which can be ascribed to the stabilizing cellular environment and efficient cell-, enzyme-, and cofactor regeneration qualifying such whole-cell biocatalysts for successful future bioprocess development (Duetz et al., 2001; van Beilen et al., 2003; Woodley, 2006; Schrewe et al., 2013). During the past decades, many CYP450s with diverse specificities have been described and engineered for steroid hydroxylations (Bureik and Bernhardt, 2007; Donova and Egorova, 2012; Szaleniec et al., 2018; Zhang et al., 2020), with the discovery of novel products and selectivity improvement as main arguments for high industrial potential (Fernandes et al., 2003; Zhang et al., 2020). However, only few studies went beyond typical screening experiments towards the evaluation of biocatalytic performance in terms of final product titer, volumetric productivity, and maximum specific activity. An overview of the latter is given in **Table 2** based on data provided in references and estimations derived therefrom. The summarized studies differ in employed CYP450 and desired hydroxylation as well as applied redox partner proteins, host organism, and biocatalyst format.

Some reported final product titers exceed the minimum process requirements of 0.001 and 0.1 g L^{-1} h^{-1} for pharmaceuticals and fine chemicals, respectively (Straathof et al., 2002). Mammalian CYP450s provided the least promising results (Zehentgruber et al., 2010a; Braun et al., 2012; Brixius-Anderko et al., 2015; Schiffer et al., 2015; König et al., 2020) as expected based on their discussed drawbacks for biotechnological use (Bernhardt, 2006; Urlacher and Schmid, 2006). With bacterial CYP450s generally acknowledged as more favorable, recent research has focused especially on the families

CYP106, CYP154, CYP109, and CYP260 (Szaleniec et al., 2018). Comparable with mammalian CYP450s, good steroid hydroxylation efficiencies were not reported for wildtype CYP109 and CYP260 or their variants in the chosen setups (Litzenburger and Bernhardt, 2017; Putkaradze et al., 2019; Zhang et al., 2021). In contrast, CYP106A2 and CYP154C5 enabled ca. 10-fold higher final product titers (Hannemann et al., 2006; Zehentgruber et al., 2010b; Bracco et al., 2013; Schmitz et al., 2014). Final product titers in the same range have also been reported for BM3 variants engineered for steroid hydroxylation with differing regiospecificities (Venkataraman et al., 2015; Li et al., 2020; Peng et al., 2022). In the present study, such product titers were reached in a shorter period of time and with lower whole-cell biocatalyst concentrations. This was enabled by specific activities exceeding the so far reported values by factors of 8 up to 40 compared with the turnover rates of progesterone hydroxylation by Bracco and coworkers (Bracco et al., 2013), emphasizing the great potential of these BM3 variants in whole cells equipped with hydrophobic outer membrane pores such as AlkL for steroid hydroxylation. For high volumetric productivities and product titers, extended reaction times and high biomass concentrations are prerequisites and promise highly efficient steroid hydroxylation with the hydrophobic pore-containing whole-cell biocatalysts developed in this study.

CONCLUSION

In this study, we highlight whole-cell biocatalysts as preferable format for testosterone hydroxylation. The application of a highly active steroid-hydroxylating CYP450 such as the BM3 variant KSA14m and a suitable hydrophobic outer membrane protein such as AlkL brings whole-cell steroid hydroxylation activities to a new level (an up to 40-fold increase) with the potential to boost productivities and product titers of microbial steroid conversion processes. Furthermore, this study highlights the broad substrate spectrum of several hydrophobic outer membrane proteins and thus also contributes to the improvement of whole-cell bioconversions of hydrophobic substrates in general. Following studies will assess stability and kinetics of the constructed whole-cell biocatalysts aiming at their highly productive application on bioreactor scale.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CB, MM, RK, AS, and BB were involved in the conception and design of the study. CB and MM performed experimental work and collected data. CB, MM, and BB did data curation and interpretation.

CB wrote the original draft of the manuscript. BB contributed in terms of article structuring and editing. All authors were involved in final editing and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fctls.2022.887458/full#supplementary-material

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