Hydrophobic outer membrane pores boost testosterone hydroxylation by cytochrome P450 BM3 containing cells

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Supplementary Material

# Supplementary Figures and Tables

## Supplementary Tables

Table S1. Outer membrane pores or import systems potentially facilitating steroid uptake by *E. coli*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Protein(s) of interest** | **Original organism** | **GenBank accession number or NCBI reference sequence** | **Molecular weight (kDa)** | **(Putative) function/ natural substrate** | **References** |
| **AlkL** | *Pseudomonas putida* GPo1 | CAB54056 | 23 | alkanes | (van Beilen et al., 1992) |
| **AupA****AupB** | *Marinobacter hydrocarbonoclasticus* SP17 | H8WEC1H8WEC0 | 49\*113\* | alkanes (C19-C31) | (Mounier et al., 2018) |
| **FadL** | *Escherichia coli* BL21-Gold(DE3) | CAD6007925 | 49 | long-chain fatty acids | (Black, 1988; Call et al., 2016; Jeon et al., 2018) |
| **FhuA Δ1-160** | *Escherichia coli* MG1655 | WP\_000124402(without deletion) | 65\* | iron(III)-hydroxamate | (Ruff et al., 2016; Liu et al., 2017) |
| **MFS (**Major Facilitator Superfamily Transporter) | *Comamonas thiooxydans* | WP\_041743963 | 48\* | testosterone | (Olivera and Luengo, 2019) |
| **ORF664****ORF665** | *Acinetobacter venetianus* RAG-1 | WP\_004877570none deposited for ORF665\*\*  | 163\*29\* | dodecane | (Kothari et al., 2016) |
| **PhlX** | *Ralstonia eutropha* JMP34 | AAC77387 | 49\*\* | phenol | (Ayoubi and Harker, 1998) |
| **TodX** | *Pseudomonas putida* F1 | WP\_012052603 | 48 | toluene | (Wang et al., 1995; Hearn et al., 2008) |
| **XylN** | *Pseudomonas putida* mt-2\_pWWO | WP\_011005927 | 47 | xylenes | (Kasai et al., 2001) |

\* estimated *via* https://www.sciencegateway.org/tools/proteinmw.htm

\*\* amino acid sequence ORF665: MPSITPVTRSIGLPSVPPVMPSITPVTRSIGLPSVPPVIPSITPVTKSIGLPSVPPVIPSITPVTKSIGLPSVPPVIPSMTPVTRSIGLPSVPPVMPSITPVTKSIGLPSVPPVMPSITPVTRSIGLPSVPPVIPSITPVTKSIGLPSVPPVMPSITPVTKSIGLPSVPPVMPSMTPVTRSIGLLSVPPVIPSITPVTISSGVPSVRLPKLPRSLPMCWTTSVAVEPLLINVSTTPDTESIAPLKSAPAKSETASKEPFTVSREVSKVSVPRSAVSPIGCSD

Table S2. Bacterial strains and plasmids used in this study

|  |  |  |  |
| --- | --- | --- | --- |
| **Strain or plasmid** | **Characteristics** | **Strain abbreviation** | **Reference** |
| **Strains** |  |  |  |
| *E. coli* DH5α | *sup*E44Δ*lacU*169(Φ80 *lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 |  | (Hanahan, 1983) |
| *E. coli* BL21-Gold(DE3) | F– *ompT hsdS(rB– mB–) dcm+* *TetR gal*λ(DE3) *endA* Hte | WT | Agilent Technologies, Santa Clara, USA |
| **Plasmids** |  |  |  |
| pETM11 | pMB1 ori, *lac*-regulatory system (*lacI*, *PT7*), KmR, pETM11 RBS | EV | EMBL Vector collection, Heidelberg, Germany |
| pETM11\_KSA1 | pETM11 derivative with 6xHis-tagged BM3 mutant gene *ksa1* | KSA1 | (Kille et al., 2011) |
| pETM11\_KSA2 | pETM11 derivative with 6xHis-tagged BM3 mutant gene *ksa2* | KSA2 | (Kille et al., 2011) |
| pETM11\_KSA3 | pETM11 derivative with 6xHis-tagged BM3 mutant gene *ksa3*  | KSA3 | (Kille et al., 2011) |
| pETM11\_KSA14 | pETM11 derivative with 6xHis-tagged BM3 mutant gene *ksa14*  | KSA14 | (Kille et al., 2011) |
| pETM11\_KSA14m | pETM11 derivative with 6xHis-tagged BM3 mutant gene *ksa14m*  | KSA14m | This study |
| pETM11\_KSA14m\_aupBA | pETM11\_KSA14m derivative with codon-optimized *aupA* and *aupB* genes from *Marinobacter hydrocarbonoclasticus* SP17 | AupA/B | This study |
| pETM11\_KSA14m\_fadL | pETM11\_KSA14m derivative with *fadL* gene from *E. coli* BL21-Gold(DE3) | FadL | This study |
| pETM11\_KSA14m\_fhuAΔ1-160 | pETM11\_KSA14m derivative with *fhuAΔ1-160* gene from *E. coli* MG1655 | FhuAΔ1-160 | This study |
| pETM11\_KSA14m\_mfs | pETM11\_KSA14m derivative with codon-optimized *mfs* gene from *Comamonas thiooxydans* | MFS | This study |
| pETM11\_KSA14m\_orf | pETM11\_KSA14m derivative with codon-optimized *orf664* and *orf665* from *Acinetobacter venetianus* RAG-1 | ORF664/665 | This study |
| pETM11\_KSA14m\_phlX | pETM11\_KSA14m derivative with codon-optimized *phlX* gene from *Ralstonia eutropha* JMP34 | PhlX | This study |
| pETM11\_KSA14m\_todX | pETM11\_KSA14m derivative with *todX* gene from *P. putida* F1 | TodX | This study |
| pETM11\_KSA14m\_xylN | pETM11\_KSA14m derivative with *xylN* gene from *P. putida* mt-2\_pWWO | XylN | This study |
| pETM11\_KSA1\_alkL | pETM11\_KSA1 derivative with *alkL* gene from *P. putida* GPo1 | KSA1-AlkL | This study |
| pETM11\_KSA2\_alkL | pETM11\_KSA2 derivative with *alkL* gene from *P. putida* GPo1 | KSA2-AlkL | This study |
| pETM11\_KSA3\_alkL | pETM11\_KSA3 derivative with *alkL* gene from *P. putida* GPo1 | KSA3-AlkL | This study |
| pETM11\_KSA14\_alkL | pETM11\_KSA14 derivative with *alkL* gene from *P. putida* GPo1 | KSA14-AlkL | This study |
| pETM11\_KSA14m\_alkL | pETM11\_KSA14m derivative with *alkL* gene from *P. putida* GPo1 | KSA14m-AlkL | This study |
| pETM11\_alkL | pETM11 derivative with *alkL* gene from *P. putida* GPo1 | pETM11-alkL | This study |

Table S3. Primer sequences and PCR templates used for the construction of pETM11 plasmids that, besides different CYP450 BM3 variants, carry genes encoding membrane proteins. Genes encoding the respective outer membrane proteins were amplified from various sources using suitable primers. Primers contained the pETM11 ribosomal binding site (bold), NotI and XhoI restriction sites (underlined) and 25 bp overhangs complementary to pETM11 to be introduced adjacent to the amplified gene unless stated otherwise in the “PCR template” column. The resulting amplicons were inserted into the different pETM11\_KSA vectors (excised with NotI and XhoI) by either *in vitro* assembly (Gibson et al., 2009) or restriction and digestion. Final plasmid constructs were then introduced into *E. coli* BL21-Gold(DE3).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Protein(s) of interest** | **Plasmid** | **Primer** | **Sequence (5’🡪3’)** | **PCR template** | **Additional information** |
| **AlkL** | pETM11\_KSA1\_alkLpETM11\_KSA2\_alkLpETM11\_KSA3\_alkLpETM11\_KSA14\_alkLpETM11\_KSA14m\_alkLpETM11\_alkL | AlkL\_forAlkL\_rev | GTAAGAGCTCCGTCGACAAGCTTGCGGCCGC**TAACTTTAAGAAGGAGATATACC**ATGAGTTTTTCTAATTATAAAGTAATCGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTAGAAAACATATGACGCACC | pCW3A4LS (derived from pLAFR3-AlkL (Cornelissen et al., 2013)) | inserted by *in vitro* assembly |
| **AupA/B** | pETM11\_KSA14m\_aupBA | AupB\_forAupB\_revAupA\_forAupB\_rev | GTAAGAGCTCCGTCGAC**TATCTCCTTCTTAAAGTTA**CCTAGGTTATTCATACTGCGCCGGAATTTCCCTAGG**TAACTTTAAGAAGG**TCTCAGTGGTGGTGGTGGTGGTGCCTCGAGTTAAAACTTCAGGGTGATGC | AupA/B gene synthesis product (codon-optimized for *E. coli* B strains);already contained NotI site and RBS upstream of *aupB* and RBS upstream of *aupA*, therefore primer AupB\_for binds in a region not relevant for expression and AupB\_rev/AupA\_for partly binds to the RBS sequence | inserted by *in vitro* assembly |
| **FadL** | pETM11\_KSA14m\_fadL | FadL\_forFadL\_rev | GTAAGAGCTCCGTCGACAAGCTTGCGGCCGC**TAACTTTAAGAAGGAGATATACC**ATGAGCCAGAAAACCCTGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTCAGAACGCGTAGTTAAAG | genomic DNA from *Escherichia coli* BL21-Gold(DE3) | inserted by *in vitro* assembly |
| **FhuAΔ1-160** | pETM11\_KSA14m\_fhuAΔ1-160 | FhuA\_forFhuA\_SSrevFhuA\_afterSSFhuA\_revFhuA\_fusion\_forFhuA\_fusion\_rev | GTAAGAGCTCCGTCGACAAGCTTGCGGCCGC**TAACTTTAAGAAGGAGATATACC**ATGGCGCGTTCCAAAACTGCGGCTTTAAACTGAACTTCTTTCAGTGCCTGTGCATAAACAGACCTGAAAGAAGTTCAGTTTAAAGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTAGAAACGGAAGGTTGCGTAAGAGCTCCGTCGACATCTCAGTGGTGGTGGTG | genomic DNA from *Escherichia coli* MG1655 | after amplification of the DNA fragments encoding the signal sequence (SS) and FhuAΔ1-160, both fragments were combined in a fusion PCRinserted by *in vitro* assembly |
| **MFS**NCBI Reference Sequence: WP\_041743963.1 | pETM11\_KSA14m\_mfs | MFS\_forMFS\_rev | GTAAGAGCTCCGTCGACAAGCTTGCGCGGCCGC**TAACTTTAAG**ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTACGCCGCATTCATCTGCAG | MFS gene synthesis product (codon-optimized for *E. coli* B strains);already contained RBS upstream of *mfs*, therefore primer MFS\_for partly binds to the RBS sequence and not the gene | inserted by *in vitro* assembly |
| **ORF664/665** | pETM11\_KSA14m\_orf | 664\_for664\_rev665\_for665\_rev | GTAAGAGCTCCGTCGACAAGCTTGCGGCCGC**TAACTTTAAGAAGGAGATATACC**ATGCTTTTTAAAAACATCC**TATCTCCTTCTTAAAGTTA**CCTAGGTTAAAACAGATTCAGGCTATGAAACAGCCCTAGG**TAACTTTAAGAAGG**ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTAATCGCTGCAGCCAATC | ORF664 and ORF665 gene synthesis products (codon-optimized for *E. coli* B strains);already contained RBS upstream of *orf665*, therefore primers 664\_rev and 665\_for partly bind to the RBS sequence and not the gene | inserted by *in vitro* assembly |
| **PhlX** | pETM11\_KSA14m\_phlX | PhlX\_forPhlX\_rev | GTAAGAGCTCCGTCGACAAGCTTGCGGCCGC**TAACTTTAAGAAGGAGATATACC**ATGCCCCGCGCTTCTTTATCATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTAAAAGCGTTTGCGATACGC | PhlX gene synthesis product (codon-optimized for *E. coli* B strains) | inserted by *in vitro* assembly |
| **TodX** | pETM11\_KSA14m\_todX | TodX\_forTodX\_rev | GTAAGAGCTCCGTCGACAAGCTTGCGGCCGC**TAACTTTAAGAAGGAGATATACC**ATGAAGATTGCCAGCGTGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTAAAAATTTTTGCTATAGGAAACCACTGC | genomic DNA from *Pseudomonas putida* F1 | inserted by *in vitro* assembly |
| **XylN** | pETM11\_KSA14m\_xylN | XylN\_forXylN\_rev | GCGGCCGC**TAACTTTAAGAAGGAGATATACC**ATGAAAATAAAAAATTTACCCAATAAAGCTCGAGTCAGAATGAATAATTATAGGCC | genomic DNA from *Pseudomonas putida* mt-2\_pWWO | inserted by restriction and ligation |

Table S4. Performance of permeabilized *E. coli* BL21-Gold(DE3) cells carrying the BM3 variants of interest (Kille et al., 2011).

|  |  |  |  |
| --- | --- | --- | --- |
| **Strain** | **Conversion of 1 mM testosterone in 24h [%]** | **Regioselectivity [%]** | **Volumetric activity [U L-1]\*** |
| KSA1 | 79 | 97[a] | 0.55 |
| KSA2 | 67 | 94[a] | 0.47 |
| KSA3 | 53 | 94[a] | 0.37 |
| KSA14 | 85 | 96[b] | 0.58 |

\* Volumetric activities were estimated based on reported conversions in 24 h.

[a] towards 2β-hydroxylated product; [b] towards 15β-hydroxylated product

Table S5. Regioselectivity of *E. coli* BL21-Gold(DE3) harboring different BM3 variants.

|  |  |  |
| --- | --- | --- |
|  | Cultivation in TB medium | Cultivation in M9 medium |
| Strain | Permeabilized cells | Resting cells | Resting cells |
| KSA1[a] | 100\* | 98.0 ± 0.6 | 97.3 ± 0.1 |
| KSA2[a] | 100\* | 100\* | 96.9 ± 1.1 |
| KSA3[a] | 100\* | 100\* | 97.6 ± 1.4 |
| KSA14[b] | 97.7 ± 0.1 | 92.9 ± 0.2 | 92.0 ± 0.0 |
| KSA14m[b] | 96.6 ± 0.4 | 90.4 ± 0.3 | 84.9 ± 0.5 |

[a] towards 2β-hydroxytestosterone; [b] towards 15β-hydroxytestosterone; \* 15β-hydroxytestosterone not detected.

Cells were prepared as permeabilized and living resting cells after growth in TB or M9 medium as described in the materials and methods section. Biotransformations were carried out for 1 h.

## Supplementary Figures



Figure S1. Genetic constructs applied (A) for expression of the genes encoding respective BM3 variants under control of the *lacI*-based T7 expression system and (B) for co-expression of the genes encoding respective BM3 variants in combination with outer membrane proteins (mp) in a bicistronic operon.



Figure S2. Volumetric activities of permeabilized *E. coli* BL21-Gold(DE3) cells carrying the BM3 variants of interest. Cell cultivation and heterologous protein synthesis was conducted in modified TB medium (Kille et al., 2011). For permeabilization, cells were resuspended in P450 reaction buffer and prepared via freeze-thawing and EDTA addition. Reactions were started by adding testosterone in DMSO (to final concentrations of 1 mM and 1% (v/v), respectively). Average values and standard deviations of two biological replicates are given.



Figure S3. Specific growth rates of *E. coli* BL21-Gold(DE3) strains carrying pETM11 with *ksa14m* and the genes encoding different membrane proteins during cultivation in M9 medium supplemented with 0.5% (w/v) glucose 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.



Figure S4. Time courses of testosterone biotransformation by *E. coli* BL21-Gold(DE3) whole-cell biocatalysts carrying pETM11 with *ksa14m* together with or without *alkL*. The substrate testosterone (dashed lines) is converted to 2β- and 15β-hydroxytestosterone, respectively (sum of products: solid lines). Cell cultivation and heterologous protein synthesis were performed 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. Reactions were started by the addition of testosterone in DMSO (1 mM and 1% (v/v) final concentrations, respectively). Average values and standard deviations of two biological replicates are given.

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