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

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Supplementary Material

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Supplementary Figures and Tables

1.1 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	H8WEC1 H8WEC0	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_004877570 none 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:
MPSITPVTRSIGLPSVPPVMPSITPVTRSIGLPSVPPVIPSITPVTKSIGLPSVPPVIPSITPVTKSIGLPSVPPVIPSMTPVTRSIGLPSVPPVMPSITPVTKSIGLPSVPPV
MPSITPVTRSIGLPSVPPVIPSITPVTKSIGLPSVPPVMPSITPVTKSIGLPSVPPVMPSMTPVTRSIGLLSVPPVIPSITPVTISSGVPSVRLPKLPRSLPMCWTTSVAV
EPLLINVSTTPDTESIAPLKSAPAKSETASKEPFTVSREVSKVSVPRSAVSPIGCSD

Table S2. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Strain abbreviation	Reference
Strains			
E. coli DH5α	*sup*E44 Δ*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*(r_B^- m_B^-) *dcm*$^+$ *Tet*^{R} *gal*λ(DE3) *endA* Hte	WT	Agilent Technologies, Santa Clara, USA
Plasmids			
pETM11	pMB1 ori, *lac*-regulatory system (*lacI*, P_{T7}), Km^R, 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_alkL pETM11_KSA2_alkL pETM11_KSA3_alkL pETM11_KSA14_alkL pETM11_KSA14m_alkL pETM11_alkL	AlkL_for	GTAAGAGCTCCGTCGACAAGCT TGCGGCCGC**TAACTTTAAGAA GGAGATATACC**ATGAGTTTTTC TAATTATAAAGTAATCG	pCW3A4LS (derived from pLAFR3-AlkL (Cornelissen et al., 2013))	inserted by *in vitro* assembly
		AlkL_rev	ATCTCAGTGGTGGTGGTGGTGG TGCTCGAGTTAGAAAACATATG ACGCACC		
AupA/B	pETM11_KSA14m_aupBA	AupB_for	GTAAGAGCTCCGTCGAC	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
		AupB_rev	**TATCTCCTTCTTAAAGTTA**CCT AGGTTATTCATACTGCGCCGGA ATTTC		
		AupA_for	CCTAGG**TAACTTTAAGAAGG**		
		AupB_rev	TCTCAGTGGTGGTGGTGGTGGT GCCTCGAGTTAAAACTTCAGGG TGATGC		
FadL	pETM11_KSA14m_fadL	FadL_for	GTAAGAGCTCCGTCGACAAGCT TGCGGCCGC**TAACTTTAAGAA GGAGATATACC**ATGAGCCAGA AAACCCTG	genomic DNA from *Escherichia coli* BL21-Gold(DE3)	inserted by *in vitro* assembly
		FadL_rev	ATCTCAGTGGTGGTGGTGGTGG TGCTCGAGTCAGAACGCGTAGT TAAAG		
FhuAΔ1-160	pETM11_KSA14m_fhuAΔ1-160	FhuA_for	GTAAGAGCTCCGTCGACAAGCT TGCGGCCGC**TAACTTTAAGAA GGAGATATACC**ATGGCGCGTT	genomic DNA from *Escherichia coli* MG1655	after amplification of the DNA fragments encoding the signal

			CCAAAACTG		sequence (SS) and FhuA Δ1-160, both fragments were combined in a fusion PCR
		FhuA_SSrev	CGGCTTTAAACTGAACTTCTTTCAGTGCCTGTGCATAAACAGAC		
		FhuA_afterSS	CTGAAAGAAGTTCAGTTTAAAG		inserted by *in vitro* assembly
		FhuA_rev	ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTAGAAACGGAAGGTTGC		
		FhuA_fusion_for	GTAAGAGCTCCGTCGAC		
		FhuA_fusion_rev	ATCTCAGTGGTGGTGGTG		
MFS NCBI Reference Sequence: WP_041743963.1	pETM11_KSA14m_mfs	MFS_for	GTAAGAGCTCCGTCGACAAGCTTGCGCGGCCGC**TAACTTTAAG**	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
		MFS_rev	ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTACGCCGCATTCATCTGCAG		
ORF664/665	pETM11_KSA14m_orf	664_for	GTAAGAGCTCCGTCGACAAGCTTGCGGCCGC**TAACTTTAAGAAGGAGATATACC**ATGCTTTTTAAAAACATCC	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
		664_rev	**TATCTCCTTCTTAAAGTTA**CCTAGGTTAAAACAGATTCAGGCTATGAAACAGC		
		665_for	CCTAGG**TAACTTTAAGAAGG**		
		665_rev	ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTAATCGCTGCAGCCAATC		
PhlX	pETM11_KSA14m_phlX	PhlX_for	GTAAGAGCTCCGTCGACAAGCTTGCGGCCGC**TAACTTTAAGAAGGAGATATACC**ATGCCCCGCGCTTCTTTATC	PhlX gene synthesis product (codon-optimized for *E. coli* B strains)	inserted by *in vitro* assembly

		PhlX_rev	ATCTCAGTGGTGGTGGTGGTGG TGCTCGAGTTAAAAGCGTTTGC GATACGC		
TodX	pETM11_KSA14m_todX	TodX_for	GTAAGAGCTCCGTCGACAAGCT TGCGGCCGC**TAACTTTAAGAA GGAGATATACC**ATGAAGATTG CCAGCGTG	genomic DNA from *Pseudomonas putida* F1	inserted by *in vitro* assembly
		TodX_rev	ATCTCAGTGGTGGTGGTGGTGG TGCTCGAGTTAAAAATTTTTGCT ATAGGAAACCACTGC		
XylN	pETM11_KSA14m_xylN	XylN_for	GCGGCCGC**TAACTTTAAGAAG GAGATATACC**ATGAAAATAAA AAATTTACCCAATAAAG	genomic DNA from *Pseudomonas putida* mt-2_pWWO	inserted by restriction and ligation
		XylN_rev	CTCGAGTCAGAATGAATAATTA TAGGCC		

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.

1.2 Supplementary Figures

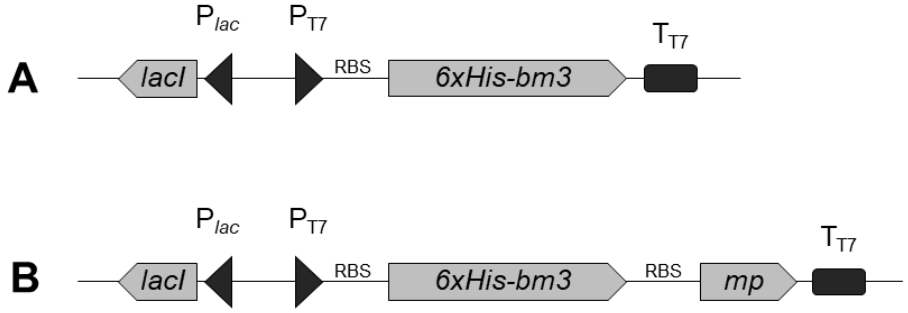

Figure S1. Genetic constructs applied **(A)** for expression of the genes encoding respective BM3 variants under control of the *lacI*-based T7 expression system and **(B)** for co-expression of the genes encoding respective BM3 variants in combination with outer membrane proteins (mp) in a bicistronic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

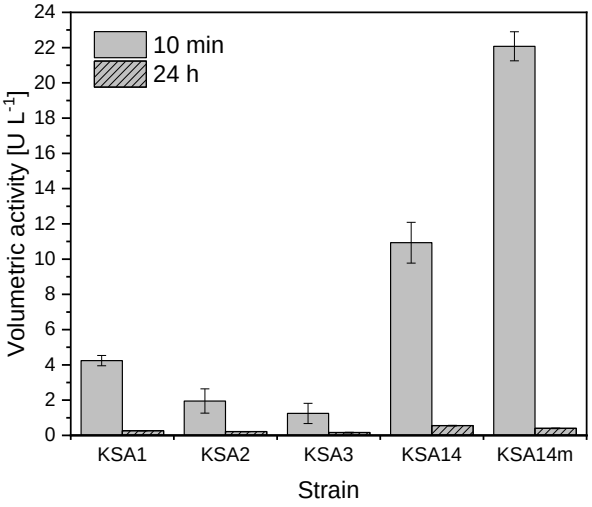

Figure S2. Volumetric activities of permeabilized *E. coli* BL21-Gold(DE3) cells carrying the BM3 variants of interest. Cell cultivation and heterologous protein synthesis was conducted in modified TB medium (Kille et al., 2011). For permeabilization, cells were resuspended in P450 reaction buffer and 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.

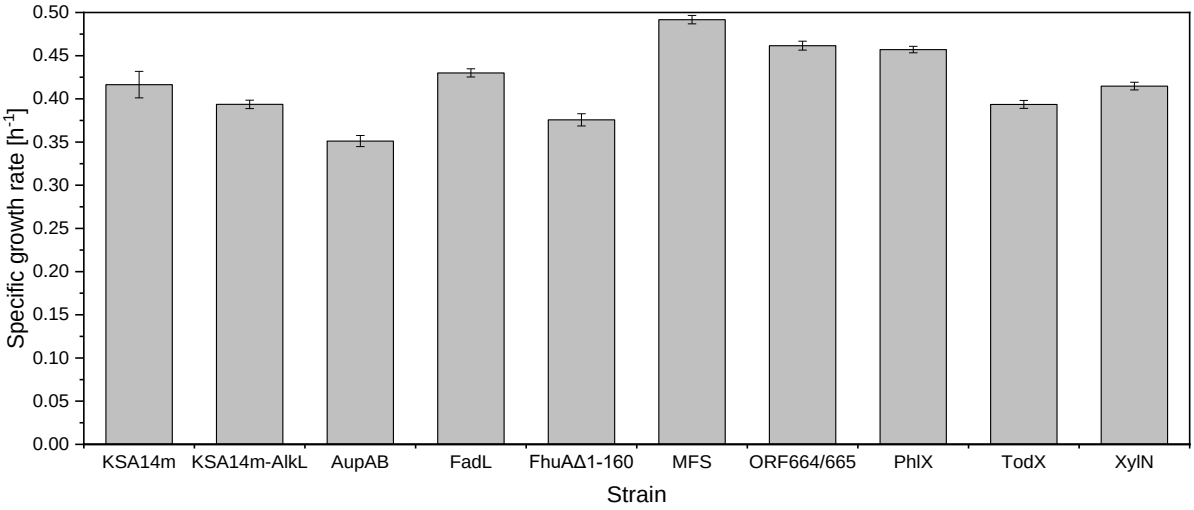

Figure S3. Specific growth rates of *E. coli* BL21-Gold(DE3) strains carrying pETM11 with *ksa14m* and the genes encoding different membrane proteins during cultivation in M9 medium supplemented with 0.5% (w/v) glucose upon induction with 0.1 mM IPTG and addition of 0.5 mM 5-aminolevulinic acid. Average values and standard deviations of two biological replicates are given.

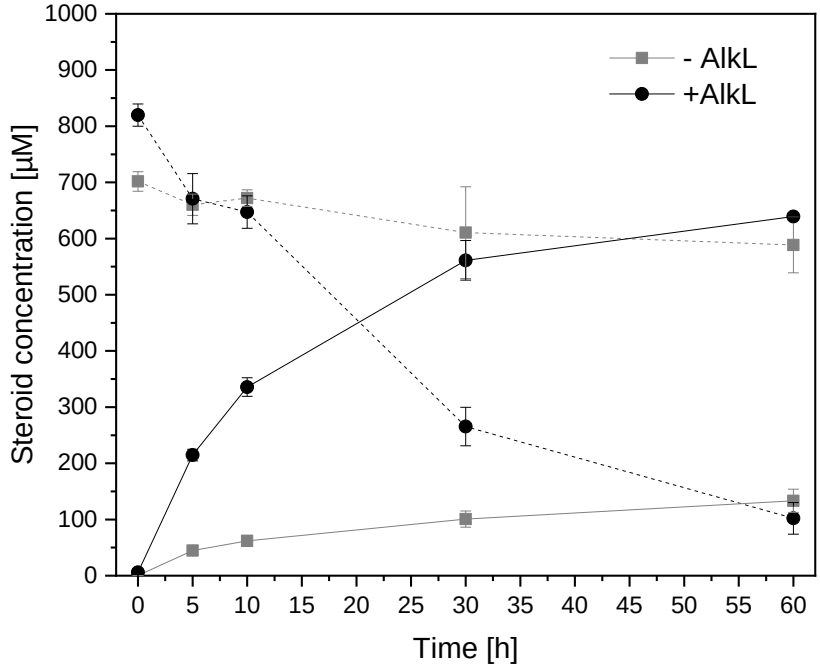

Figure S4. Time courses of testosterone biotransformation by *E. coli* BL21-Gold(DE3) whole-cell biocatalysts carrying pETM11 with *ksa14m* together with or without *alkL*. The substrate testosterone (dashed lines) is converted to 2β- and 15β-hydroxytestosterone, respectively (sum of products: solid 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.

2 References

Ayoubi, P.J., and Harker, A.R. (1998). Whole-cell kinetics of trichloroethylene degradation by phenol hydroxylase in a *Ralstonia eutropha* JMP134 derivative. *Appl Environ Microbiol* 64(11)**,** 4353-4356. doi: 10.1128/AEM.64.11.4353-4356.1998.

Black, P.N. (1988). The *fadL* gene product of *Escherichia coli* is an outer membrane protein required for uptake of long-chain fatty acids and involved in sensitivity to bacteriophage T2. *J Bacteriol* 170(6)**,** 2850-2854. doi: 10.1128/jb.170.6.2850-2854.1988.

Call, T.P., Akhtar, M.K., Baganz, F., and Grant, C. (2016). Modulating the import of medium-chain alkanes in *E. coli* through tuned expression of FadL. *J Biol Eng* 10**,** 5. doi: 10.1186/s13036-016-0026-3.

Cornelissen, S., Julsing, M.K., Volmer, J., Riechert, O., Schmid, A., and Bühler, B. (2013). Whole-cell-based CYP153A6-catalyzed (*S*)-limonene hydroxylation efficiency depends on host background and profits from monoterpene uptake via AlkL. *Biotechnol Bioeng* 110(5)**,** 1282-1292. doi: 10.1002/bit.24801.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166(4)**,** 557-580. doi: 10.1016/s0022-2836(83)80284-8.

Hearn, E.M., Patel, D.R., and van den Berg, B. (2008). Outer-membrane transport of aromatic hydrocarbons as a first step in biodegradation. *Proc Natl Acad Sci U S A* 105(25)**,** 8601-8606. doi: 10.1073/pnas.0801264105.

Jeon, E.-Y., Song, J.-W., Cha, H.-J., Lee, S.-M., Lee, J., and Park, J.-B. (2018). Intracellular transformation rates of fatty acids are influenced by expression of the fatty acid transporter FadL in *Escherichia coli* cell membrane. *J Biotechnol* 281**,** 161-167. doi: 10.1016/j.jbiotec.2018.07.019.

Kasai, Y., Inoue, J., and Harayama, S. (2001). The TOL plasmid pWW0 *xylN* gene product from *Pseudomonas putida* is involved in m-xylene uptake. *J Bacteriol* 183(22)**,** 6662-6666. doi: 10.1128/JB.183.22.6662-6666.2001.

Kille, S., Zilly, F.E., Acevedo, J.P., and Reetz, M.T. (2011). Regio- and stereoselectivity of P450-catalysed hydroxylation of steroids controlled by laboratory evolution. *Nat Chem* 3(9)**,** 738-743. doi: 10.1038/nchem.1113.

Kothari, A., Charrier, M., Wu, Y.-W., Malfatti, S., Zhou, C.E., Singer, S.W., et al. (2016). Transcriptomic analysis of the highly efficient oil-degrading bacterium *Acinetobacter venetianus* RAG-1 reveals genes important in dodecane uptake and utilization. *FEMS Microbiol Lett* 363(20)**,** fnw224. doi: 10.1093/femsle/fnw224.

Liu, Z., Ghai, I., Winterhalter, M., and Schwaneberg, U. (2017). Engineering enhanced pore sizes using FhuA Δ1-160 from *E. coli* outer membrane as template. *ACS Sens* 2(11)**,** 1619-1626. doi: 10.1021/acssensors.7b00481.

Mounier, J., Hakil, F., Branchu, P., Naïtali, M., Goulas, P., Sivadon, P., et al. (2018). AupA and AupB are outer and inner membrane proteins involved in alkane uptake in *Marinobacter hydrocarbonoclasticus* SP17. *mBio* 9(3)**,** e00520-00518. doi: 10.1128/mBio.00520-18.

Olivera, E.R., and Luengo, J.M. (2019). Steroids as environmental compounds recalcitrant to degradation: genetic mechanisms of bacterial biodegradation pathways. *Genes (Basel)* 10(7)**,** 512. doi: 10.3390/genes10070512.

Ruff, A.J., Arlt, M., van Ohlen, M., Kardashliev, T., Konarzycka-Bessler, M., Bocola, M., et al. (2016). An engineered outer membrane pore enables an efficient oxygenation of aromatics and terpenes. *J Mol Catal B Enzym* 134**,** 285-294. doi: 10.1016/j.molcatb.2016.11.007.

van Beilen, J.B., Eggink, G., Enequist, H., Bos, R., and Witholt, B. (1992). DNA sequence determination and functional characterization of the OCT-plasmid-encoded *alkJKL* genes of *Pseudomonas oleovorans*. *Mol Microbiol* 6(21)**,** 3121-3136. doi: 10.1111/j.1365-2958.1992.tb01769.x.

Wang, Y., Rawlings, M., Gibson, D.T., Labbé, D., Bergeron, H., Brousseau, R., et al. (1995). Identification of a membrane protein and a truncated LysR-type regulator associated with the toluene degradation pathway in *Pseudomonas putida* F1. *Mol Gen Genet* 246(5)**,** 570-579. doi: 10.1007/BF00298963.