Supplementary Information

Green Synthesis of ω-Hydroxydodecanoic Acid by Engineering *C. viswanathii* with Cas13d

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Running Title: Metabolic engineering for HDA production

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(A)

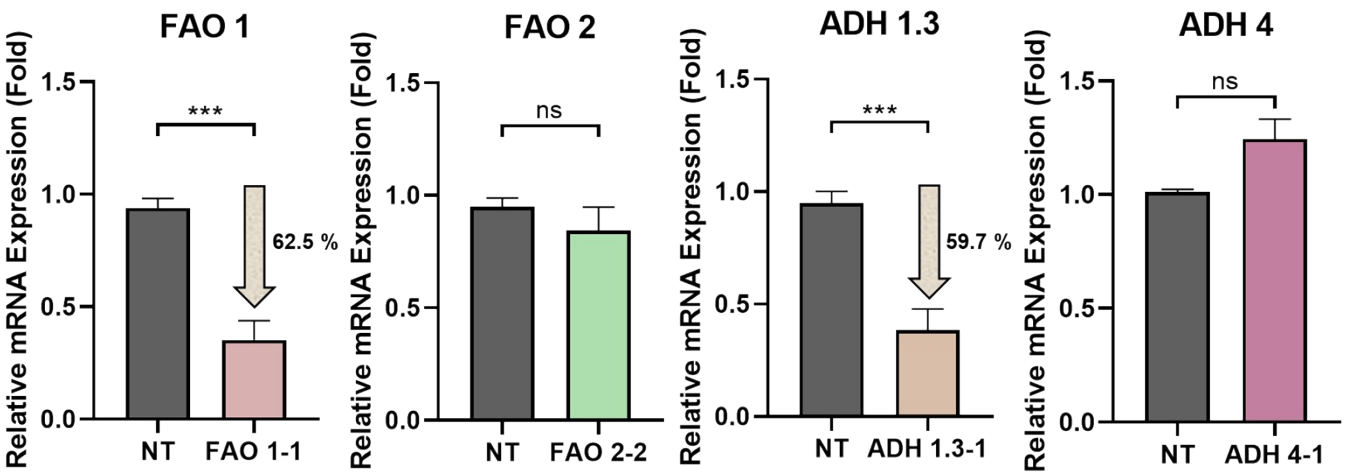

Fig. S1. Incomplete multiplex gene knockdown by the Array plasmid. The Array plasmid was electroporated into cells and the recombinants were selected. The recombinants were cultured in the shake flasks and induced by dodecane addition for HDA production. The mRNA levels were measured after 24 h by qRT-PCR and normalized to those of the NT plasmid. The data showed that only FAO1 and ADH 1.3 were significantly knocked down.

(A)

Strain	Integrated gene
ATCC 20962	None
P19	CYP52A19, POS5
P19C	CYP52A19, CPRb, POS5
aFP19C	CYP52A19, CPRb, POS5, antiFAO2

(B)

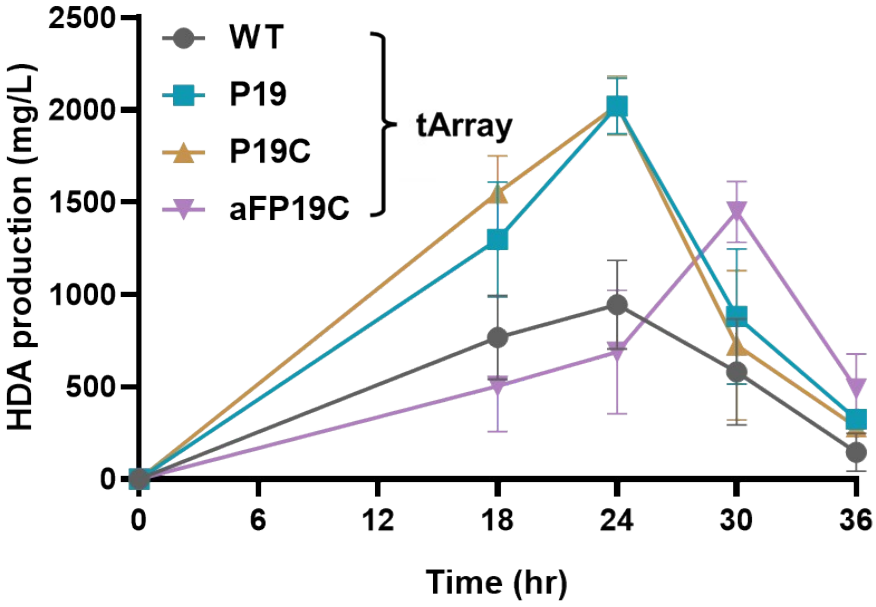

Fig. S2. HDA production in different *C. viswanathii* strains. ATCC 20962 was the parent *C. viswanathii* strain we used for engineering. Pox4 and pox5 were deleted in this strain. P19 strain was the engineered strain in which we inserted CYP52A19, Nrs^R and POS5 genes. P19C was the strain in which we inserted CYP52A19, Nrs^R, CPRb and POS5 genes. aFP19C was the strain in which we inserted CYP52A19, Nrs^R, CPRb and POS5 genes and an anti-sense gene for FAO2. The tArray plasmid was electroporated into these strains and HDA production was induced in the shake flasks. The data showed that only P19C resulted in similar HDA titers compared to P19, which peaked at 24 h and then declined. Other strains gave poorer HDA production.

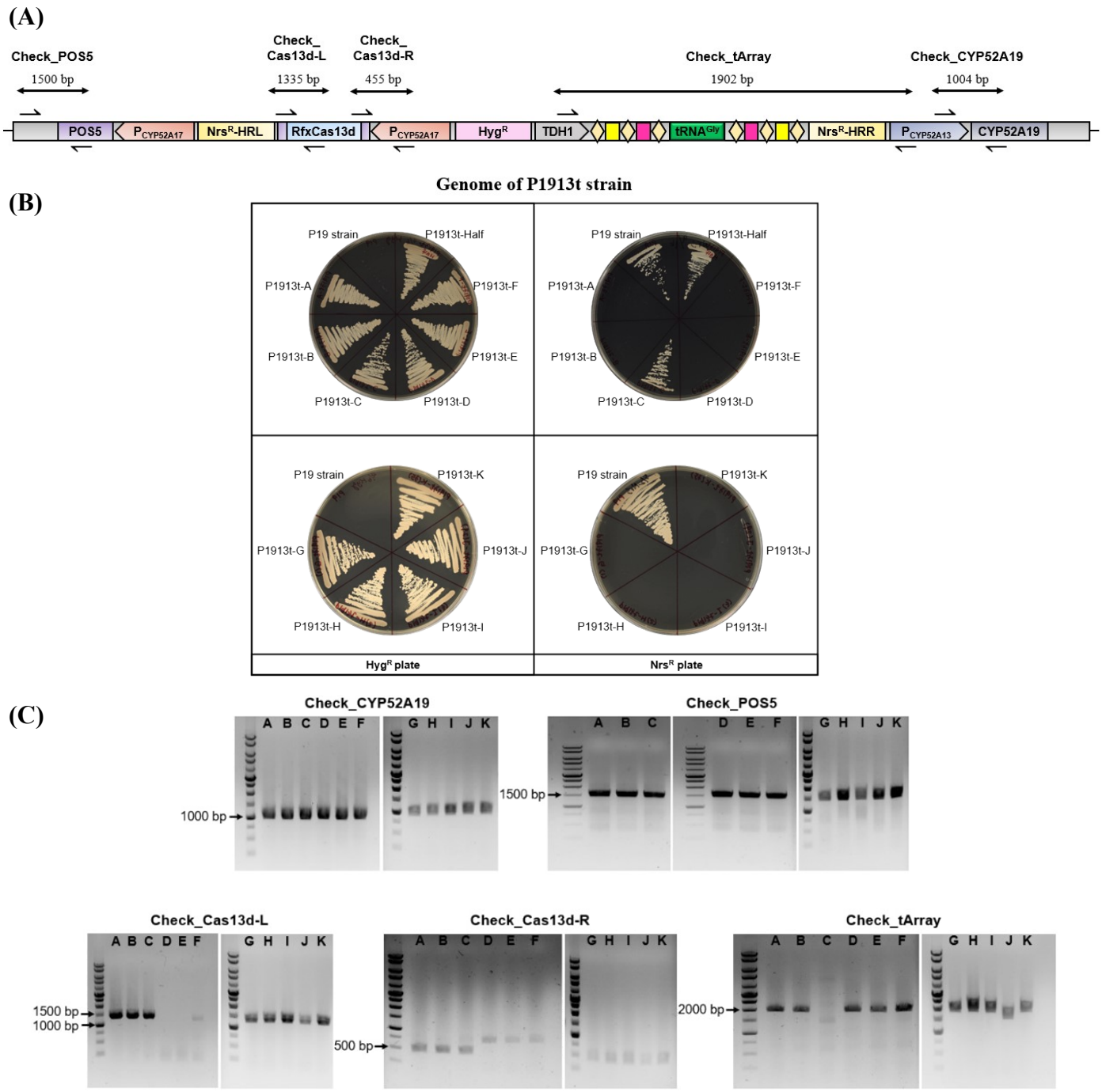

Fig. S3. Successful integration of the CRISPR/Cas13d repression module into *C. viswanathii* P19 strain. (A) Schematic illustration of the genome containing the integrated CRISPR/Cas13d module and the primer pairs for colony PCR check. (B) Colonies after antibiotic selection. (C) Results of colony PCR confirmation. P19 strain was the engineered strain in which we inserted CYP52A19, Nrs^R and POS5 genes. The genome contained an Nrs^R gene as the selectable marker and integration site. We linearized the pHRR-tArray and pHRL-17Cas13d plasmids with restriction enzymes and co-electroporated both linear templates into P19 strain, followed by antibiotic selection. *C. viswanathii* is a diploid so that homozygous integration (integrated with 2 copies) would result in Nrs-sensitive (Nrs^s) and Hyg^R phenotype while heterozygous integration (integrated with only 1 copy) would lead to Nrs^R and Hyg^R phenotypes. Non-engineered cells would be Hyg^s and Nrs^R. After recovery, we spread the cells onto YPD agar plate containing hygromycin to select Hyg^R colony groups A to K, among them >50 colonies were picked for second round of Nrs selection. Except colony group C, most of the colony groups were Hyg^R and Nrs^s (B). Colony PCR confirmed that P1913t clones A, B, G, H, I and K possessed correct genotypes.

Supplementary Tables

Table S1. Methods and titers of HDA Production using Biological Methods

Host strain	Substrate	Key enzyme / genetic modification	HDA titer (g/L)	References
E. coli	Dodecane	CYP153AM.aq-CYP102A1 fusion protein	1.2	(Scheps, *et al.*, 2013)
E. coli	Glucose	ACC, TesA, and CYP102A1	0.0587	(Cao, *et al.*, 2016)
E. coli	Decanoic acid	AlkBGT from P. putida GPo1	~~10.32~~0.2490	(He, *et al.*, 2019)
E. coli	Dodecanedioic acid	CYP153AL.m, ferrodoxin, and ferrodoxin reductase	2	(Joo, *et al.*, 2019)

Table S2. Formula of CM^{Flask} (Culture Medium for Flask)

component	concentration (g/L)
K_2HPO_4	1.18
KH_2PO_4	4.88
$(NH_4)_2SO_4$	8
Yeast extract	3
Glycerol	30
YNB (Yeast Nitrogen Base without amino acid)	1.7

Table S3. Formula of RM^{Flask} (pH~8.0, Reaction Medium for Flask)

component	concentration (g/L)
Glucose	20
Urea	8
Tris-base	60.5
NP-40	0.5
YNB (Yeast Nitrogen Base without amino acid)	1.7

Table S4. Formula of CM (Culture Medium for Bioreactor)

component	concentration (g/L)
Soy Peptone	5
Yeast Extract	3
K_2HPO_4	1.18
KH_2PO_4	4.88
$(NH_4)_2SO4$	8
Glucose	90
NaCl	0.1
$CaCl_2$	0.1
$MgSO_4$	0.5

*The medium was supplemented with 10 μl of each component of Trace Elements (Table S4) to finalize the formulation.

Table S5. Formula of FM (Feeding Medium for Bioreactor)

component	concentration (g/L)
Tween80	0.9
Urea	27
Glucose	300
NaCl	0.1
$CaCl_2$	0.1
$MgSO_4$	0.5

*The medium was supplemented with 10 μl of each component of Trace Elements (Table S4) to finalize the formulation.

Table S6. Formula of Trace Elements (TE)

component	concentration (g/L)
H_3BO_3	50
$CuSO_4 \cdot 5H_2O$	4
KI	10
$FeCl_2 \cdot 7H_2O$	20
$MnCl_2$	40
$NaMoO_4 \cdot 2H_2O$	20
$ZnSO_4 \cdot 7H_2O$	40

Table S7. Spacer sequences

Target gene name	Spacer Sequence (5'-3')
EGFP-1	AATTTACCGTAAGTAGCATCACCTTCACCT
EGFP-2	AATTCAACCAAAATTGGGACAACACCAGTG
EGFP-3	ACAGAAAATTTGTGACCATTAACATCACCA
FAO1-1	ACATAATAACATAAGGGTGTCGACGTGTTT
FAO1-2	CACCAAAAGTGGAACCAGCAAGAACAAACA
FAO1-3	ACAACAAGTCAGCAGTGACCAACAATGCCT
FAO2-1	TGTTTGTATTCGAGCACGTCTGGCAAGAAG
FAO2-2	AATACCGTACTTACAGCCCAAGTAACAGAA
FAO2-3	GTAGGCAAAACACTAGCATCAGCAACATAG
ADH0-1	GCCTTGTAAACAGTGACACCAGCACACAAG
ADH0-2	GTGTAAATCAGTGTGGCAGACACCGGAGTA
ADH0-3	AGGAAATCAACAAAGACCTCAGCGCCCAAA
ADH1.3-1	AACCATTCAACCACTTGATACCAGCCAAGT
ADH1.3-2	CAGCACCAGAGATAGCCACCCATTGGCCAG
ADH1.3-3	TTTGTATTCTAACTTGCCACCATTGGTTTC
ADH4-1	AATCAACGAAAACTTCAGCACCCAACGATT
ADH4-2	TTCAACATCTTTATACCTGCCAAGTCACCT
ADH4-3	GCTTTGTAAACAGTAACACCGGCGCAGGAT
FALDH3H1-1	TCCCGATTTACCAATACCACCGAACGGAGC
FALDH3H1-2	AAGCACTATCCATTTTACCACCGATGATTA
FALDH3H1-3	GTCAGTATAAGTGAGAACCGGCAAGATAGG
FALDH3A1-1	CTCACCAAGCAAATGGAAGACACCAGTGAA
FALDH3A1-2	GACCGCAAAGTAAAGGTTACGCAATTGGTT
FALDH3A1-3	AGATGATCAACACGACCCCCAATGGGATTC
FALDH3A2-1	CCAGCATTAGCATATCTCCCCAAGCAATAC
FALDH3A2-2	TCATCGACTGTTGAAGCAAGCGACTTTGAC
FALDH3A2-3	TCCACTTGTGCAATTGCGACATCGTGTACA

Table S8. Primer sequences used for qRT-PCR

Primers	Sequence (5'-3')
18s rRNA-F	CATGGCCGTTCTTAGTTGGT
18s rRNA-R	ATTGCCTCAAACTTCCATCG
Q-EGFP-F	ATGTCTAAAGGTGAAGAATTATT
Q-EGFP-R	AACTGGCAATTTACCAGTA
Q-FAO1-F	GCTCCATTTTTGCCCG
Q-FAO1-R	GGGTTTGGTGAATGTCC
Q-FAO2-F	AAGGAGGTAAGAAGTTGG
Q-FAO2-R	TGAACTTGGACCCGTG
Q-ADH1.3-F	TCAAGGGCTGGAAGGTG
Q-ADH1.3-R	TCCAAGCTGCCAAGATCC
Q-ADH4-F	GACGAAAGAGGAGTGTTTG
Q-ADH4-R	TATTCAACAGACTGGTTGATGG

F: forward; R: reverse

Table S9. Calculation of green chemistry metrices.

Name	Formula	Calculation
Theoretical Atom Economy (%)	$\frac{MW\ of\ product}{MW\ of\ reactant} \times 100$	**CH_3-$(CH_2)_{10}$-CH_3 + 2 O_2 → OH-CH_2-$(CH_2)_{10}$-COOH + H_2O** MW:170.34 g/mol MW:31.99 g/mol MW:216.32 g/mol $\frac{216.32}{234.32} \times 100$ = 92.3%
Experimental Atom Economy (%)	$\frac{mass\ of\ product}{mass\ of\ reactant} \times 100$	$\frac{23.8}{300} \times 100$ = 7.9%
E-Factor	$\frac{Mass\ of\ wastes\ (kg)}{Mass\ of\ Product\ (kg)}$	$= \frac{(0.3845\ (chemicals) \times 0.3\ (dodecane) \times 0.1278\ (dry\ biomass)) - }{0.0238\ (HDA)}$ = 33.1 %
Percentage Yield (%)	$\frac{Actual\ yield}{Theoretical\ yield} \times 100$	$\frac{23.8}{1.761 \times 216.32} \times 100$ = 6.25%

References

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He Q, Bennett GN, San KY, Wu H. 2019. Biosynthesis of medium-chain omega-hydroxy fatty acids by AlkBGT of *Pseudomonas putida* GPo1 with native FadL in engineered *Escherichia coli*. Front Bioeng Biotechnol 7: 273.

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