SUPPORTING INFORMATION

H_2-driven biotransformation of *n*-octane to 1-octanol by a recombinant *Pseudomonas putida* strain co-synthesizing an O_2-tolerant hydrogenase and a P450 monooxygenase

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SUPPLEMENTARY METHODS AND RESULTS

Construction of the CYP153A production system in *P.putida* KT2440

The CYP153A system from *Polaromonas* sp. JS666 is encoded by three genes: *bpro_5301* (CYP153A *P.* sp.), *bpro_5300* (a ferredoxin reductase) and *bpro_5299* (a ferredoxin). Three gene fragments were created: Gene Fragment 1 (GF1), comprising only the gene of the CYP enzyme; Gene Fragment 2 (GF2), consisting of the CYP and ferredoxin reductase genes and; Gene Fragment 3 (GF3), containing the genes for CYP, ferredoxin reductase (FRN) and ferredoxin (Fd).

Gene Fragments 1, 2 and 3 were amplified from genomic DNA using a standard PCR protocol with primers containing NdeI and HindIII restriction sites as follows: Forward primer for GF 1-3 5'-GGTcatatgAGATCATTAATGAGTGAAGCGATTGTGGTAAACAACC-3, reverse primer for GF 1 5'-ATTaagcttTCAAGCGTTGATGCGGACGGG-3', reverse primer for GF 2 5'-AGCTaagcttTCAGCCGGCCAGGTCCTTCGCT-3' and reverse primer for GF 3 5'-AGCTaagcttTCAGTGCTGGCCGAGCGG-3' (sequences for newly introduced restriction sites are indicated by lower case letters).

The resulting amplicons were cut with NdeI and HindIII and subsequently inserted into the same restriction sites of pCom-10-.alkB-BMO2[1] that was previously amended with a NdeI site by using the QuikChange site-directed mutagenesis protocol with primer 5'-TAA AAA TTG GAG AAT TCA TAT GCT TGA GAA ACA CAG AGT TC-3 and the complementary primer 5'-GAA CTC TGT GTT TCT CAA GCA TAT GAA TTC TCC AAT TTT A-3'. Correct insertion of the CYP153A genes into the vector was verified by sequencing.

The resulting plasmids were named pCYP1, pCYP2, and pCYP3. Plasmid pCYP1 harboured solely the CYP153A-encoding gene under control of the *alkB* promoter. Plasmid pCYP2 carried the CYP153A gene in combination with the Fd gene, and pCYP3 contained all three genes. The plasmids were transferred from *E. coli* S17-1 to *P. putida* KT2440 via conjugation, resulting in the recombinant strains *P. putida* KT-GF1 (pCYP1, CYP153A), KT-GF2 (pCYP2, CYP153A, Fd,) and KT-GF3 (pCYP3, CYP153A, Fd, FRN).

Construction of the SH production system in *P. putida* KT2440

Heterologous production of the NAD^+-reducing hydrogenase (SH) from *R. eutropha* in *P. putida* KT2440 was achieved as follows. In order to equip the SH operon with the *alkB* promoter, an *alkB* promotor fragment was amplified with primers 5'-TTAAAGCTTCACTTAAGTGTAGTCGGCATG-3' and 5'-GCGCTCGAGTATTGTCGTGATACGACTATCCATGGGGTGTTCTCCAATTTTTATTAAATTAG-3' using plasmid pSPZ10 as the template.[2] The resulting PCR product was digested with XhoI and HindIII and subsequently inserted as a 0.9 kbp fragment into plasmid pCH1613, which contains the 5' region of *hoxF* equipped with a *Strep*-tag II-encoding sequence at the 5' end ($_{Strep}$*hoxF*).[3] From the resulting plasmid, pCH1614, a 1.8 kbp HindIII-EcoRV fragment was transferred to 4.46 kbp HindIII-EcoRV fragment of pTBu#1085.[3] This yielded plasmid pJH#3180 harbouring a P_{alkB}-$_{Strep}$*hoxFUY*' fragment, which was transferred as a 3.4 kbp BamHI-HindIII to Litmus28 (New England Biolabs) resulting in plasmid pCH1615. Plasmid pCH1615 was cut with XbaI and SapI and the resulting 3.0 kbp fragment was ligated with a XbaI-SapI-cut 12.55 kbp fragment of pJH#2904, which contained the *hoxFUYHWIhypA2B2F2CDEX* genes under control of the native SH promotor.[3] This resulted in plasmid pCH1616 harbouring the artificial *hox*$_{Strep}$*FUYHWIhypA2B2F2CDEX* operon under control of the *alkB* promoter. A 15.50 kbp SpeI-XbaI fragment was transferred from pCH1616 to XbaI-cut pCM62.,[4] yielding the broad-host-range plasmid pJH#3257 carrying the *hox*$_{Strep}$*FUHYWIhypA2B2F2CDEX* gene cassette whose transcription is controlled by the *alkB* promoter from *P .putida*. Finally, the *alkS* gene, encoding the transcriptional activator of the *alkB* promoter, was cut from pSPZ10 and inserted as a 3.32 kbp BstZ17I-SmaI fragment into EcoRV-cut Litmus 28. From the resulting plasmid, pJH#3325, a 3.32 kbp EcoRI fragment containing *alkS* was transferred to pCM62, yielding plasmid pJH#3363. In the last step, a 15.5 kbp HindIII fragment of pCH1616 was ligated with the 9.5 kbp HindIII fragment of pJH#3363. The expression of the *hox*$_{Strep}$FUHYWIhypA2B2F2CDEX operon on the resulting plasmid, pJH#3382, is controlled by the P_{alkB} promotor whose activity regulated by the transcription activator *alkS*. Plasmid pJH#3382, which confers tetracycline resistance, was transferred via conjugation from *E. coli* S17-1 to *Pseudomona putida* KT2440.[5]

Strain cultivation and sample preparation

Strain cultivation and sample preparation was done as described in the Experimental Section of the main manuscript.

Analysis of octane oxidation products by gas chromatography

Aliquots of the biotransformation suspensions were analysed using GC-FID (GC-2010 Plus, Shimadzu equipped with an AOC-20i auto injector, Shimadzu), 1 mL of sample was extracted with 2 x 0.5 mL ethyl acetate containing 0.2 mM 1-hexanol (ISTD). Analysis was carried out using an Agilent DB-5 column (30 m x 0.25 mm x 0.25 μm) with H_2 as the carrier gas (flow rate 1.1 ml min^{-1}; linear velocity, 32 cm s^{-1}), with the limitation that it was not possible to separate 2-octanol/octanal. The injector and detector temperatures were set at 250°C and 310°C, respectively. The column oven was set at 40°C for 2 min, then the temperature was raised to 160°C at a rate of 10°C/min, and finally the oven was heated to 300°C at 80°C/min. Products were measured from calibration curves estimated from a series of standard solutions treated in the same manner as the samples.[6]

CYP153A and 1-octanol production in SH-free recombinant *P. putida* KT2440 strains

Strains *P. putida* KT-GF1 (CYP), KT-GF2 (CYP, Fd,) and KT-GF3 (CYP, Fd, FRN) strains were grown in glucose-glycerol mineral medium (GGN), and after a 6-h induction with

DCPK, cell-free extracts were prepared. The P450 monooxygenase content was quantified by measuring the characteristic absorption peak of the CO adduct at 450 nm of the reduced cytochrome P450 (ε_{450} = 91 $mM^{-1} \cdot cm^{-1}$).[7]. The 1-octanol content was measured by GC analysis of the cell free extracts as described in "Analysis of octane oxidation products by gas chromatography". Cell-free extracts of the strains *P. putida* KT-GF1 (CYP), KT-GF2 (CYP, Fd,) and KT-GF3 (CYP, Fd, FRN) displayed $A_{(450-490)}$ values equivalent to 0.06, 0.11 and 0.35 nmol, respectively, of active CYP per mg total protein. When the artificial reductant sodium dithionite was added to the extracts, CYP concentrations of 0.59, 0.59 and 0.4 nmol active CYP per mg total protein were measured for the three strains, which is in the range of a comparable CYP system.[8] Cell-extracts from strain KT-NC contained negligible CYP concentrations. The results clearly indicate that only the cell-free extract of *P. putida* (KT-GF3) does contain all proteins required to deliver metabolism-derived electrons to the CYP153A enzyme. Biotransformation of *n*-octane was carried out using induced resting cells at a final concentration of 50 g wet biomass per litre. CYP concentrations measured for extracts of *P. putida* KT-GF1, KT-GF2 and KT-GF3 cells amounted to 431, 404 and 261 µg CYP per g_{cww}. After 20 h, the whole-cell biotransformation with strain KT-GF1 did not result in significant accumulation of octanol. The corresponding biotransformation with strains KT-GF2 and KT-GF3 yielded a maximum of 0.8 and 8.5 mg of octanol/L, respectively. This results shows that Fd is crucial for the hydroxylation activity of CYP153A. Furthermore, the result implies that native redox proteins present in *P. putida* KT2440 are not capable in activating recombinant CYP153A.

SH production in recombinant *P. putida* KT2440 strains

Strain *P. putida* KT2440 (pJH#3382) was grown in glucose/glycerol minimal medium (Experimental Section) in the absence or presence of the inducer 0.05% dicyclopropylketone (DCPK). The corresponding soluble protein extracts showed an SH-mediated, H_2-driven NAD^+ reduction activity of 0.18 ± 0.06 and 0.40 ± 0.05 U/mg of protein for the non-induced and induced strain, respectively. The latter corresponds to approximately 50 % of the activity measured in *R. eutropha* extracts.[9] Figure S1 demonstrates the successful heterologous production of the four structural SH proteins, HoxF, HoxU, HoxY and HoxH, in *P. putida*. A basal SH production is detectable also in the absence of DPCK. Induction with DCPK, however, led to a considerable increase in SH production.

The utilisation of glucose/glycerol minimal medium led to reproducible results, *i.e*. relatively high enzyme activities and good cell yields. Glucose alone allowed high growth rates but resulted in catabolite repression of the *alkB* promoter used for gene expression.

SUPPLEMENTARY TABLE

Table S1: SH activities and CYP153A concentrations in recombinant cell cultures held under biotransformation conditions.

Time after induction (h)	CYP concentration (nmol mg^{-1})[a]	SH activity (U mg^{-1}) [a]
0	0.00	0.075 ± 0.016
4	0.30	0.16 ± 0.02
6	0.35	0.15 ± 0.03
8	0.35	0.15 ± 0.016
24	0.58	0.071 ± 0.03

[a]Biotransformations and subsequent measurements of CYP concentration and SH activity were done as described in the Experimental Section. Values refer to the total protein content. The mean value of the SH activity is given with its standard deviation of three repeated measurements

SUPPLEMENTARY FIGURES

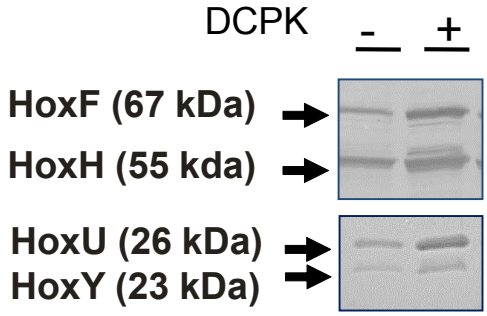

Figure S1. Immunological detection of the HoxFUHY subunits of the SH in recombinant P. putida KT2440 (pJH#3382) cells. The strain was grown for 42 h in glucose/glycerol minimal medium following by an 6 h period without (-) or with (+) 0.05% dicyclopropylketone (DCPK). Aliquots of soluble extracts (20 µg per lane) were electrophoretically separated on a 12% SDS-polyacrylamide gel and an immunoblot with polyclonal antibodies against all four SH subunits (indicated by arrows) performed.

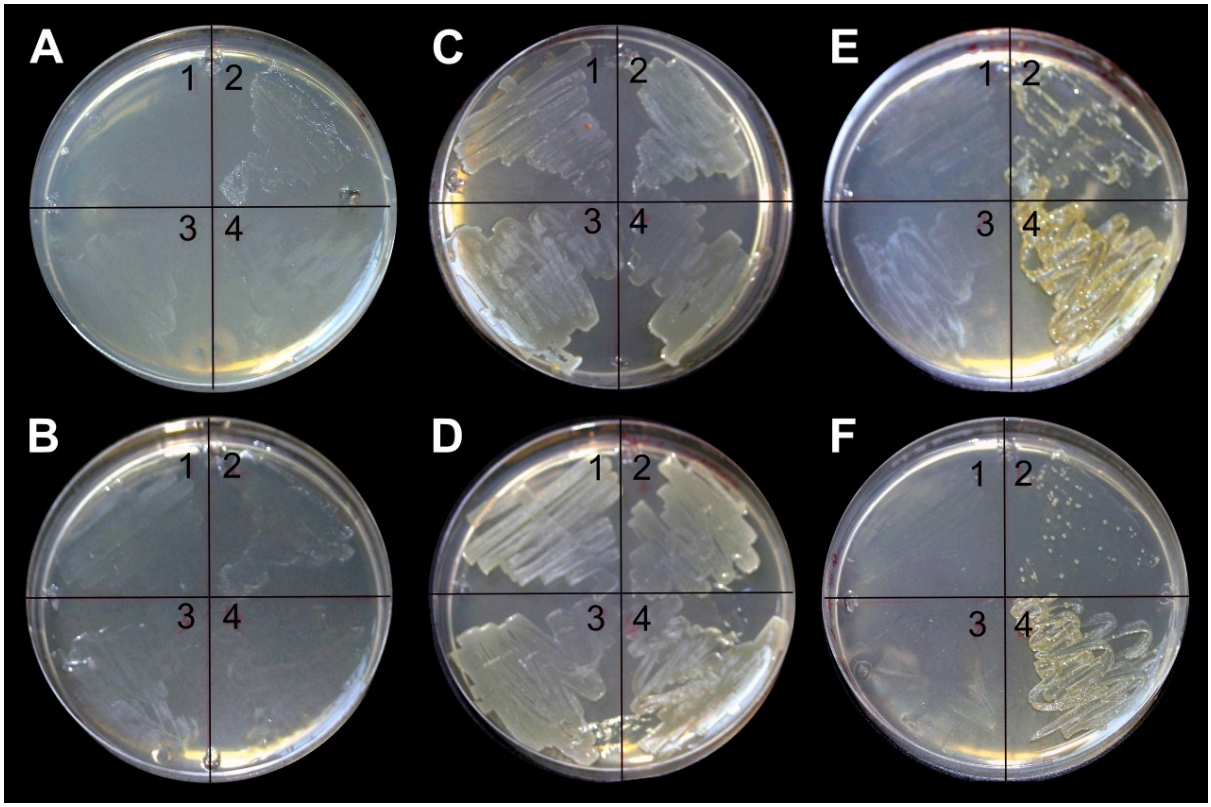

Figure S2. Production of 1-octanol supports growth of recombinant *P. putida* KT2440 producing the CYP153A system and SH. Strains *P. putida* KT2440 (wild type) (1), *P. putida* KT2440 (pCYP3) (2), *P. putida* KT2440 (pJH#3382) (3) and *P. putida* KT2440 (pCYP3, pJH#3382) (4) were incubated at 30°C on mineral agar plates either in the absence of a carbon source (A + B) or in the presence of a saturated *1*-octanol (C + D) or a saturated *n*-octane atmosphere (E + F). Plates A, C and E were incubated under 20 % O_2, 80 % N_2 and plates B, D and F were incubated under 20 % H_2, 20 % O_2, 60 % N_2. To maintain saturated *n*-octane and 1-octanol atmospheres, 25-mL glass beakers containing 2 mL of the respective hydrocarbons were deposited in the 5-L gas-tight reaction vessels containing the agar plates. As expected, all four strains showed no growth in the absence of a carbon source (A+B), but they were able to grow when the atmosphere was supplemented with 1-octanol (C+D). The latter result demonstrates that the *P. putida* KT2440 wild-type strain contains the enzymatic machinery for metabolizing 1-octanol (similar results have previously been reported for a derivative of *P. putida* GPo1 lacking the *alkB* gene[10]). Only those *P. putida* recombinants that harbor pCYP3 (E-2/4, F-2/4) were able to grow in the presence of *n*-octane. Growth was considerably enhanced when both pCYP3 and the SH-producing plasmid pJH#3382 were present at the same time (E-4, F-4).

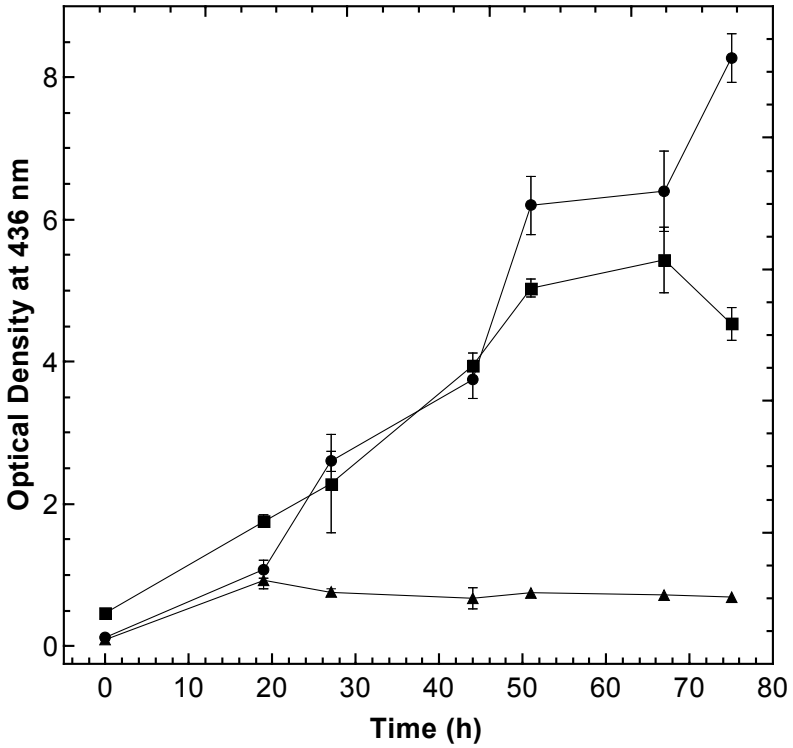

Figure S3. Growth of *P. putida* KT2440 (pCYP3, pGE#3382) in liquid cultures containing *n*-octane either in the presence (circles) or absence of H_2 (squares). Cultures were grown in sealed 1-L bottles containing of 50 mL mineral salts medium[11] supplemented with 15 % (v/v) *n*-octane and 2 % (v/v) DMSO. A control culture without *n*-octane supplementation showed no growth (triangles). After 50 h of growth, the culture incubated with 20 % H_2 showed significantly higher optical densities than the H_2-free culture. Individual values represent the mean of three three biological replicates and the corresponding standard deviation.

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