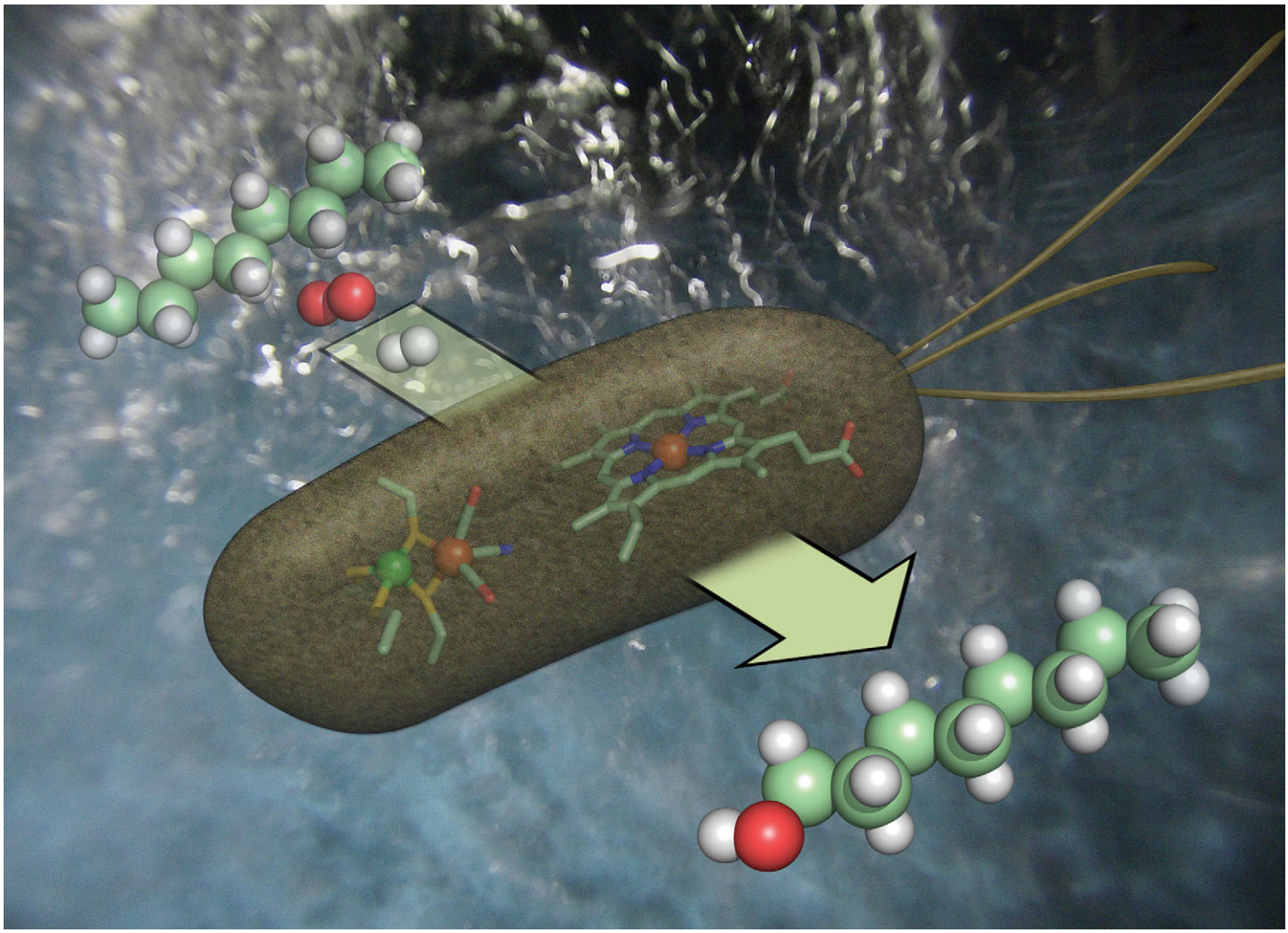

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H_2-driven biotransformation of *n*-octane to 1-octanol by a recombinant *Pseudomonas putida* strain co-synthesizing an O_2-tolerant hydrogenase and a P450 monooxygenase

A recombinant bacterial whole-cell system was designed for hydroxylation of *n*-octane to 1-octanol. An intracellular NAD^+-reducing hydrogenase sustains H_2-driven regeneration of NADH even in the presence of O_2, both are co-substrates of the CYP153A monooxygenase.

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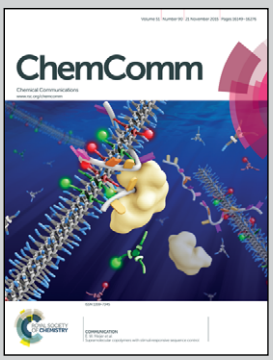

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H_2-driven biotransformation of *n*-octane to 1-octanol by a recombinant *Pseudomonas putida* strain co-synthesizing an O_2-tolerant hydrogenase and a P450 monooxygenase†

Thomas H. Lonsdale,‡[ab] Lars Lauterbach,‡*[a] Sumire Honda Malca,[c] Bettina M. Nestl,[c] Bernhard Hauer[c] and Oliver Lenz*[a]

An *in vivo* biotransformation system is presented that affords the hydroxylation of *n*-octane to 1-octanol on the basis of NADH-dependent CYP153A monooxygenase and NAD^+-reducing hydrogenase heterologously synthesized in a bacterial host. The hydrogenase sustains H_2-driven NADH cofactor regeneration even in the presence of O_2, the co-substrate of monooxygenase.

Cytochrome P450 monooxygenases (CYPs) have the extraordinary capability to introduce oxygen into non-activated C–H bonds in a regio- and stereoselective manner, which is still a challenging task for synthetic catalysts. The continuous discovery of novel CYPs and their modification by protein engineering paves new avenues for their utilization as biocatalysts in the production of pharmaceuticals and fine chemicals.[1–3]

In particular the regioselective terminal hydroxylation of alkanes by CYPs is of significant interest because the corresponding alkanols can be used as surfactants, plasticizers, solvents or polymer precursors.[4] In this study we used the tripartite alkane-oxidizing CYP153A system from *Polaromonas* sp. JS666 for selective oxidation of *n*-octane to 1-octanol.[5] The system is composed of the catalytically active cytochrome (CYP153A), the electron carrier ferredoxin (Fd) and the FAD-containing ferredoxin reductase (FRN). The latter two proteins are necessary for electron transfer from NADH to the P450 active site (Fig. 1, see ESI,† for details). CYP153 enzymes are promising biocatalysts as they can be heterologously produced in active and soluble form.[4] For efficient use of CYPs both *in vitro* and *in vivo*, a sufficient supply of the reduced cofactor NADH is required. In whole-cell systems, NAD(P)H is generated either from the basic metabolic routes or *via* a recombinant regeneration system, generally based on glucose dehydrogenase relying on the supplementation of the growth medium with glucose.[6] Whole-cell catalysis for chemical synthesis is attractive because enzymes are generally more stable in their natural environment. Recently, a hydrogenase that oxidizes dihydrogen (H_2) and transfers the released electrons to NAD^+ has complemented the so-far established systems for NADH regeneration.[7–10] In contrast to glucose, H_2 readily diffuses through cell membranes, and hydrogenase-based NADH recycling has the advantage of proceeding without a carbon-based reducing agent. Moreover, when coupled with CYP-mediated catalysis water is the only by-product (Fig. 1).[11]

Exposure of most hydrogenases to O_2 leads to their irreversible inactivation.[12] However, a few O_2-tolerant [NiFe]-hydrogenases sustain catalytic activity in the presence of O_2. One of the most prominent examples is the NAD^+-reducing hydrogenase (SH) from the "Knallgas" bacterium *Ralstonia eutropha* H16 consisting of two functionally distinct heterodimeric moieties.[13] The H_2-dependent NAD^+ reduction activity of the SH is unaffected even at ambient O_2 concentrations.[14] These features make the SH particularly promising for NADH regeneration in cascade reactions requiring O_2, *e.g.* those involving CYPs.

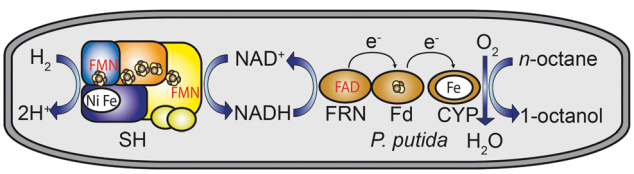

Fig. 1 H_2-driven conversion of *n*-octane to 1-octanol in recombinant *Pseudomonas putida* KT2440 cells producing the soluble NAD^+-reducing hydrogenase (SH) from *Ralstonia eutropha* in addition to CYP153A (CYP), NADH-ferredoxin reductase (FRN), and ferredoxin (Fd) from *Polaromonas* sp. JS666. The SH contains two flavin mononucleotide (FMN) molecules, and FRN contains one flavin adenine dinucleotide (FAD). Electron-conducting iron–sulfur clusters are shown as cluster-forming spheres coloured in brown and yellow.

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† Electronic supplementary information (ESI) available: Construction of SH and CYP153A production systems, GC analysis, CYP153A and SH production in recombinant strains and catabolism assays. See DOI: 10.1039/c5cc06078h

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In this study, we have designed an electron transfer pathway for the H_2-driven ω-hydroxylation of *n*-octane to 1-octanol in recombinant *Pseudomonas putida*.§ Pseudomonads provide an attractive platform for new metabolic pathways due to their solvent resistance, metabolic versatility and simple genetic accessibility.[15] Biosynthesis of the [NiFe] active site of the SH is highly complex and requires a set of auxiliary proteins involved in nickel uptake, metal centre assembly and insertion, site-specific endoproteolysis and transcriptional regulation.[16] The corresponding set of 13 genes is arranged as an operon and was set under control of the *alkB* promoter that controls alkane degradation in *Pseudomonas putida* GPo1.[17] The three genes required for biosynthesis of the CYP153A system from *Polaromonas* sp. JS666 were likewise equipped with the *alkB* promoter. Both recombinant operons were inserted into a broad-host-plasmid replicating in *P. putida* KT2440 (see footnote § and ESI,† for details). In order to obtain the optimal growth conditions for the biotransformations, both the SH activity and the CYP concentration were determined dependent on the length of the induction period. Recombinant *P. putida* cells were first grown for 12 hours in a glucose–glycerol (GGN) minimal medium.

Gene expression was induced through addition of dicyclopropyl ketone (DCPK), which cannot be metabolized by *P. putida.* In the course of the induction period (24 h), the CYP concentration showed an almost continuous increase reaching a final value of 0.58 nmol mg^{-1} (of total protein) after 24 h (Table S1, ESI†). The SH activity, by contrast, showed a considerable increase during the first 4 h of induction and then stayed relatively stable at 0.15 U mg^{-1} for a further 4 h. However, less than 50% of the maximal activity was left after 24 h (Table S1, ESI†). Henceforth, all subsequent biotransformations were carried out with resting cells derived from a recombinant *P. putida* culture grown for 12 hours in GGN minimal medium followed by an induction period of 6 h.

After induction the cells were centrifuged, and the cell pellet was re-suspended in carbon-free H16 minimal medium containing *n*-octane. Biotransformations with 15% (v/v) *n*-octane were performed in gas-tight reaction vessels containing a gas mixture of H_2 and air in the headspace. The same setup without H_2 was used to evaluate the biotransformation capacity of the system without support of the SH.

The addition of H_2 led to a significantly higher yield of 1-octanol (Fig. 2), demonstrating the supportive role of SH-generated NADH during the biotransformation. After 24 h, 101 mg L^{-1} and 36 mg L^{-1} of 1-octanol were formed in the presence and absence of H_2, respectively. The maximum concentration of 1-octanol in the biotransformation mixture was approximately five times higher than that achieved previously in *in vitro* experiments with purified *Polaromonas* sp. JS666 CYP153A.[5]

Further oxidation products such as octanoic acid and 2-octanol/octanal were found to constitute 39% of the total octane oxidation products (Table 1). Overoxidation is frequently observed in biotransformations involving P450 enzymes, and in a previous *in vitro* study, up to 10% of the octane was shown to be overoxidized by CYP153A.[5] In contrast to the latter study, however, no α,ω-diols were detected in our *in vivo* approach.

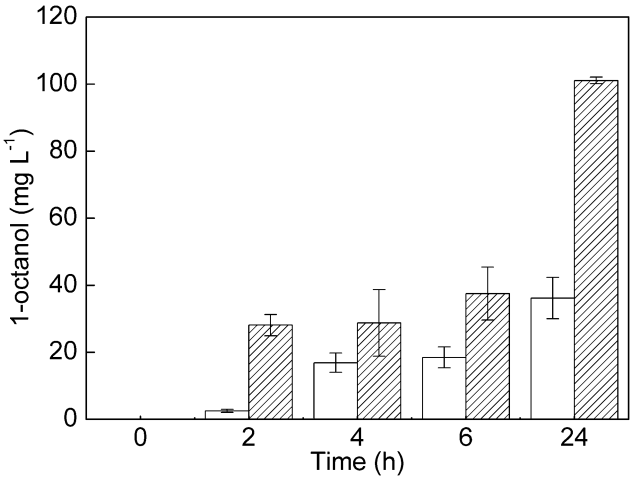

Fig. 2 H_2-supported production of 1-octanol by recombinant *P. putida* KT2440 cells synthesizing CYP153A and SH. Bioconversions were monitored either in the absence (open bars) or presence (dashed bars) of H_2. The bar height represents the mean value of three replicates, and the corresponding standard deviation is indicated.

Table 1 H_2-supported conversion of *n*-octane to 1-octanol, octanoic acid and 2-octanol/octanal. The measurement replicates were done in triplicate. Mean values and the corresponding standard deviations are shown

Time (h)	1-Octanol (mg L^{-1})	2-Octanol/octanal (mg L^{-1})	Octanoic acid (mg L^{-1})
2	28 ± 3	4 ± 0.1	8 ± 3
4	29 ± 10	8 ± 0.6	9 ± 3
6	37 ± 8	15 ± 1	16 ± 5
24	101 ± 1	32 ± 9	32 ± 14

From these observations, it seems likely that the accumulation of octanoic acid and 2-octanol/octanal resulted from a combination of direct overoxidation by CYP153A and native catabolic pathways for 1-octanol degradation present in *P. putida*. Indeed, in contrast to wild-type *P. putida* KT2440, the recombinant strain synthesizing the CYP153A system was able to grow with *n*-octane as the sole carbon and energy source (Fig. S2, ESI†). This clearly indicates catabolic degradation of 1-octanol *in vivo*. Future mutagenesis studies[18] are envisaged to identify potential alcohol dehydrogenases involved in 1-octanol conversion. A subsequent knock-out of the corresponding gene(s) is expected to reduce the amount of overoxidation products.

Recombinant *P. putida* KT2440 cells synthesizing both the CYP153A system and SH grew even slightly faster when H_2 was added (Fig. S3, ESI†). This result in combination with the increased 1-octanol production in the presence of H_2 point out that NADH-regeneration in carbon-free minimal medium is significantly facilitated by the SH. This is in marked contrast to the results obtained for cells grown in glucose-supplemented mineral medium, where *P. putida* is able to adjust its energy and reductant demand according to the metabolic burden.[15,19]

In our proof-of-concept study, we investigated the applicability of the *R. eutropha* SH to increase the NADH supply in whole cell systems. H_2-supported NADH recycling led to an approximately

3-fold increase in the 1-octanol yield in a whole-cell biotransformation system, which uses a dedicated P450 monooxygenase as octane-converting biocatalyst. In order to prevent formation of hazardous gas mixtures during *in vivo* biotransformation, the application of sub-critical gas concentrations, such as <8 vol% O_2 and <5 vol% H_2, is recommended.[20,21] Our study clearly shows that the SH is capable of overcoming potential bottlenecks of cofactor supply in whole-cell systems. Moreover, H_2 represents a viable alternative to carbon-based reductants currently used for *in vivo* cofactor recycling strategies. Thus, our H_2-driven *in vivo* cofactor regeneration system holds considerable potential for application in other cascades reactions that rely on sustainable supply of NAD(P)H as the reducing agent.

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Notes and references

§ The heterologous overproduction system for the SH from *R. eutropha* and the CYP153A system from *Polaromonas* sp. JS666 was assembled as described in the ESI.† Main cultures were grown at 30 °C in baffled 2 L-Erlenmeyer flasks including 300 mL of mineral salts medium[14] containing 0.2% (w/v) glucose and 0.2% (v/v) glycerol as carbon and energy sources (GGN medium). Inoculation was done with 3 mL of a starter culture grown previously for 48 h in mineral salts medium with 0.4% (w/v) glucose. Induction of the *alkB* promoter was carried out by addition of 0.05% (v/v) dicyclopropyl ketone, and CYP production was enhanced through addition of 0.5 mM 5-aminolevulinic acid and 1 mM $FeCl_2$.[5] H_2 oxidation activity in soluble extracts was measured spectrophotometrically at 30 °C by following the conversion of NAD^+ into NADH at 365 nm.[14] Protein concentration was determined using the Bradford assay.[22] The CYP153A concentration was measured in whole cells using the method of Johnston *et al.*[23] Biotransformations were performed in a final volume of 2.2 mL in 110 mL gas-tight, thick-walled glass flasks. Cells obtained from the main cultures were harvested and re-suspended to a final OD_{436} of 20 in carbon-free, five times concentrated, mineral salts medium.[14] After addition of 15% (v/v) *n*-octane and 2% (v/v) DMSO the flasks were sealed with a rubber bung. The headspace of the flasks was filled with a gas mixture of 20% H_2 in air. Control experiments were carried out under air. Aliquots of the biotransformation suspensions were analyzed using GC-FID, see ESI,† for details.

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