

Chapter 4

***In Vivo* Evolution of Butane Oxidation by AlkB and CYP153A6 Terminal Alkane Hydroxylases**

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A. Abstract

Enzymes of the AlkB and CYP153 families catalyze the first step in the metabolism of medium chain-length alkanes, selective oxidation of alkanes to the 1-alkanols, and enable their host organisms to utilize alkanes as carbon sources. Small gaseous alkanes, however, are converted to alkanols by members of the evolutionarily unrelated methane monooxygenase (MMO) family. Propane and butane can be oxidized by CYP enzymes engineered in the laboratory, but these produce predominantly the 2-alkanols. Here we report the *in vivo* directed evolution of two medium chain-length terminal alkane hydroxylases, the integral-membrane di-iron enzyme AlkB from *Pseudomonas putida* GPo1 and the class I soluble CYP153A6 from *Mycobacterium* sp. HXN-1500, for enhanced activity on small alkanes. We established a *P. putida* evolution system that enables selection for terminal alkane hydroxylase activity and used it to select propane- and butane-oxidizing enzymes based on enhanced growth complementation of an adapted *P. putida* GPo12 (pGEc47ΔB) strain. The resulting enzymes exhibited higher rates of 1-butanol production from butane and maintained their preference for terminal hydroxylation. This *in vivo* evolution system could be generally useful for directed evolution of enzymes that hydroxylate small alkanes.

B. Introduction

Microbial utilization and degradation of alkanes was discovered almost a century ago (1). Since then, several enzyme families capable of hydroxylating alkanes to alkanols, the first step in alkane degradation, have been identified and categorized based on their preferred substrates (2). The soluble and particulate methane monooxygenases (sMMO and pMMO) and the related propane monooxygenase and butane monooxygenase (BMO) are specialized on gaseous small-chain alkanes (C_1 to C_4), while medium-chain (C_5 to C_{16}) alkane hydroxylation seems to be the domain of the CYP153 and AlkB enzyme families.

Conversion of C_1 to C_4 alkanes to alkanols is of particular interest for producing liquid fuels or chemical precursors from natural gas. The MMO-like enzymes that catalyze this reaction in nature, however, exhibit limited stability or poor heterologous expression (2) and have not been suitable for use in a recombinant host that can be engineered to optimize substrate or cofactor delivery. Alkane monooxygenases often co-metabolize a wider range of alkanes than those which support growth (3). We wished to determine whether it is possible to engineer a medium-chain alkane monooxygenase to hydroxylate small alkanes, thereby circumventing difficulties associated with engineering MMO-like enzymes as well as investigating the fundamental question of whether enzymes unrelated to MMO can support growth on small alkanes.

The most intensively studied medium-chain alkane hydroxylases are the AlkB enzymes (4 – 6), especially AlkB from *Pseudomonas putida* GPo1 (7 – 10). While most members of the AlkB family act on C_{10} or longer alkanes, some accept alkanes as small as C_5 (2). A recent study (3) indicated that AlkB from *P. putida* GPo1 may also be involved in propane and butane

assimilation. AlkB selectively oxidizes at the terminal carbon to produce 1-alkanols. No systematic protein engineering studies have been conducted on this diiron integral membrane enzyme, although selection and site-directed mutagenesis efforts identified one amino acid residue that sterically determines long-chain alkane degradation (9).

The most recent addition to the biological alkane-hydroxylating repertoire is the CYP153 family of heme-containing cytochrome P450 monooxygenases. Although their activity was detected as early as 1981 (11), the first CYP153 was characterized only in 2001 (12). Additional CYP153 enzymes were identified and studied more recently (13 – 15). These soluble, class I-type three-component P450 enzymes and the AlkB enzymes are the main actors in medium-chain-length alkane hydroxylation by the cultivated bacteria analyzed to date (15). CYP153 monooxygenases have been the subject of biochemical studies (12 – 13, 16), and their substrate range has been explored (14, 17). Known substrates include C₅-C₁₁ alkanes. The best characterized member, CYP153A6, hydroxylates its preferred substrate octane predominantly (> 95%) at the terminal position (13).

Recent studies have shown that high activities on small alkanes can be obtained by engineering other bacterial P450 enzymes such as P450cam (CYP101, camphor hydroxylase) and P450 BM3 (CYP102A1, a fatty acid hydroxylase) (18 – 19). The resulting enzymes, however, hydroxylate propane and higher alkanes predominantly at the more energetically favorable subterminal position; highly selective terminal hydroxylation is difficult to achieve by engineering a subterminal hydroxylase (20). We wished to determine whether a small-alkane terminal hydroxylase could be obtained by directed evolution of a longer-chain alkane hydroxylase that exhibits this desirable regioselectivity. For this study, we chose to engineer

AlkB from *P. putida* GPo1 and CYP153A6 from *Mycobacterium* sp. HXN-1500 (13, 21) to enhance their activity on butane. Because terminal alkane hydroxylation is the first step of alkane metabolism, we reasoned that it should be possible to establish an *in vivo* evolution system that uses growth on small alkanes to select for enzyme variants exhibiting the desired activities.

The recombinant host *Pseudomonas putida* GPo12 (pGEc47ΔB) was engineered specifically for complementation studies with terminal alkane hydroxylases and was used previously to characterize members of the AlkB and CYP153 families (15, 22). This strain is a derivative of the natural isolate *P. putida* GPo1 lacking its endogenous OCT-plasmid (octane assimilation) (23), but containing cosmid pGEc47ΔB, which carries all genes comprising the *alk* machinery necessary for alkane utilization, with the exception of a deleted *alkB* gene (24). We show that this host can be complemented by a plasmid-encoded library of alkane hydroxylases and that growth of the mixed culture on butane leads to enrichment of novel butane-oxidizing terminal hydroxylases.

C. Results

C.1. *P. putida* GPo12 (pGEc47ΔB) growth on short-chain 1-alkanols

P. putida GPo12 (pGEc47ΔB) was shown in previous studies to grow on medium chain-length alkanols like 1-octanol and on the corresponding alkane only when complemented by a terminal alkane hydroxylase (15, 22). To determine whether this strain could be used to select or screen for improved terminal alkane hydroxylation activity, we tested its ability to grow with the primary and secondary C₁-C₈ alkanols as sole carbon sources (Figure 4.1). No growth was observed on any of the secondary alcohols or on methanol during the 18 -day period. Ethanol, 1-propanol and 1-butanol supported relatively strong growth, comparable to that of the positive control grown on glucose. Slow growth was observed on 1-pentanol, 1-hexanol, and 1-octanol.

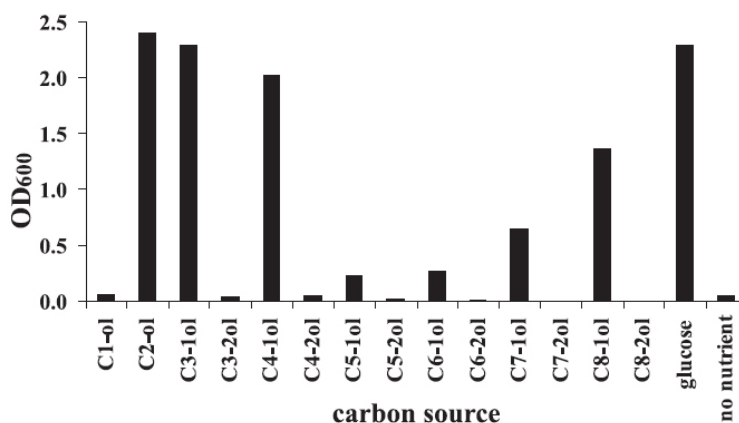


Figure 4.1: Growth of *P. putida* GPo12(pGEc47ΔB) with primary and secondary linear short and medium chain-length alkanols. The OD₆₀₀ of the cultures was measured after 18 days of growth in liquid M9 minimal medium with 0.5% (vol/vol) primary (C_x-1ol; X = number of carbon atoms) or secondary (C_x-2ol) alcohols as carbon source dissolved in a 5% (vol/vol) organic layer of heptamethylnonane. Alcohols smaller than five carbon atoms were added without organic solvent. Cultures with 0.5% (wt/vol) glucose or no added nutrient served as controls.

The results indicated that the terminal hydroxylation products of all the short-chain *n*-alkanes except methane are readily utilized as carbon sources, while subterminal oxidation products (the *sec*-alkanols) are not. Thus, this strain should be suitable for growth-based

screening and selection for terminal hydroxylation of long, medium, and short chain-length alkanes.

C.2. Creation of gene libraries through random plasmid mutation

It was not efficient to use error-prone PCR to randomly mutate the target genes, as cloning of PCR products into the pCom vector yielded fewer than 2,000 transformants, much less than the hundreds of millions of mutants that can be used for evolution with a growth selection. Mutant libraries were therefore constructed by complementing *P. putida* GPo12 (pGEc47ΔB) strains with randomly mutated plasmids encoding AlkB or CYP153A6. The drawbacks of including mutations that affect the vector (antibiotic resistance and origin of replication) rather than just the inserted genes were compensated by the large library size and ease of library construction. Mutator strains were used to generate the plasmid libraries, and to increase diversity both available mutator strains were used, *Escherichia coli* XL1Red (Stratagene) and *E. coli* JS200 pEP Pol I (25). *E. coli* XL1Red has deficiencies in the DNA repair mechanism that lead to a 5,000-fold increase in the general mutation rate (26) (Stratagene manual). *E. coli* JS200 pEP Pol I expresses an engineered mutator DNA polymerase I, which mainly amplifies plasmid DNA with lower reliability, thus introducing mutations in the plasmid DNA (25). The nucleotide mutation level in XL1Red after two weeks of continuous culturing was approximately 0.1/kb, while four rounds of mutation in JS200 pEP Pol I yielded up to 0.4/kb. Cultures of both mutator strains were combined, and the mutated plasmids were transformed into *P. putida* GPo12 (pGEc47ΔB) through triparental mating with the helper strain *E. coli* CC118(pRK600) (15). The growth selection was performed by culturing the resulting

strain library in minimal medium with an alkane as sole carbon source for up to three weeks, as described in Chapter 8.E.6.

C.3. *P. putida* growth on butane through complementation with *AlkB* and *CYP153A6* variants

A mixed culture containing alkane hydroxylase variants will become enriched in strains best adapted to use alkanes as their sole carbon source. However, not only can beneficial mutations in the hydroxylase gene lead to improved growth, but adaptations of the host and vector will do so as well. For *P. putida* GPo12 (pGEC47ΔB) complemented by CYP153 genes, it had been observed that the host had to be adapted through prolonged cultivation on alkanes to obtain significant growth on these substrates (15) without any mutations occurring in the *cyp153* genes themselves. To test whether host adaptation was also occurring in our experiments, 21 single colonies obtained from the first round of enrichment cultures were compared to the parent strain by plate growth tests (data not shown). Solid media growth tests were chosen over liquid media due to the growth instability of liquid minimal media cultures of *P. putida* GPo12 (pGEC47ΔB), which often showed different growth rates between replicates or, occasionally, failure to grow at all. The host, vector, and operons of the best mutants were analyzed individually by comparing them in growth tests to their wild-type counterparts. To identify and analyze potentially adapted hosts, the adapted recombinant strain was cured of the plasmid and transformed with the appropriate wild-type plasmid. Adapted vectors were isolated from the strains and the *alkB* gene or *cyp153A6 fdrA6 fdxA6* operon was replaced by the wild-type sequence by cloning, before being mated into the wild-type host. Furthermore, potentially improved hydroxylase genes were recloned into a wild-type vector and transferred into a wild-type host.

Comparison of all the resulting strains in growth assays led to the identification of several improved hosts and vectors. Strain Pcyp1, an adapted *P. putida* GPo12 (pGEc47ΔB) strain, showed faster growth on pentane than its parent when complemented by the wild-type pCom8_cyp153A6 plasmid. Further adaptation of Pcyp1 led to Pcyp2, which again grew faster on pentane. Similarly, strain Palk1 showed improved growth on propane and butane when transformed with wild-type pCom10_alkB. For the CYP153A6 system, adapted plasmid pCom8* enabled faster growth of *P. putida* GPo12 (pGEc47ΔB) on pentane, even when it contained the wild-type operon. Sequencing showed no mutation of the CYP operon in pCom8*_cyp153A6 or in the sequence 500 nucleotides up- and downstream from the operon. For the AlkB system, no improved vectors were obtained. The adapted host and vector components were specific for the particular system used, i.e., strain Palk1 did not show improved growth on short chain-length alkanes compared to the wild-type host when complemented with CYP153A6. Likewise, Pcyp1, Pcyp2, and pCom8* were only adapted for their specific systems. The nature of the mutations and how they benefit growth on short-chain alkanes is unknown.

In addition to creating these adapted hosts and plasmids, the first rounds of *in vivo* directed evolution generated enzyme mutants AlkB-BMO1 (butane monooxygenase) and CYP153A6-BMO1, both of which conferred improved growth on butane. Sequencing revealed a single nucleotide mutation in each: mutation of the codon CTA to GTA led to single amino acid substitutions L132V in AlkB-BMO1 and, through a GCA to GTA change, the substitution A94V in CYP153A6-BMO1 (A97V in the published sequence reported in reference 13). All the adapted components were combined and evaluated in plate growth tests (Table 4.1, Figure 4.2).

Table 4.1: Relative growth of adapted *P. putida* GPo12 (pGEc47ΔB) strains, expressing CYP153A6 and AlkB variants, on minimal media plates with alkanes as the sole carbon source

Complementing alkane monooxygenase	Days required for growth to a full lawn with selected carbon source				
	Ethane	Propane	Butane	Pentane	Octane
AlkB wild-type	NG ^[a]	5	5	3	2
AlkB-BMO1	NG	5	3	6	7
AlkB-BMO2	NG	5	2	4	8
CYP153A6 wild-type	NG	NG	NG	2	2
CYP153A6-BMO1	NG	NG	5	1.5	5

^[a]NG: No growth detected during 3 -week observation

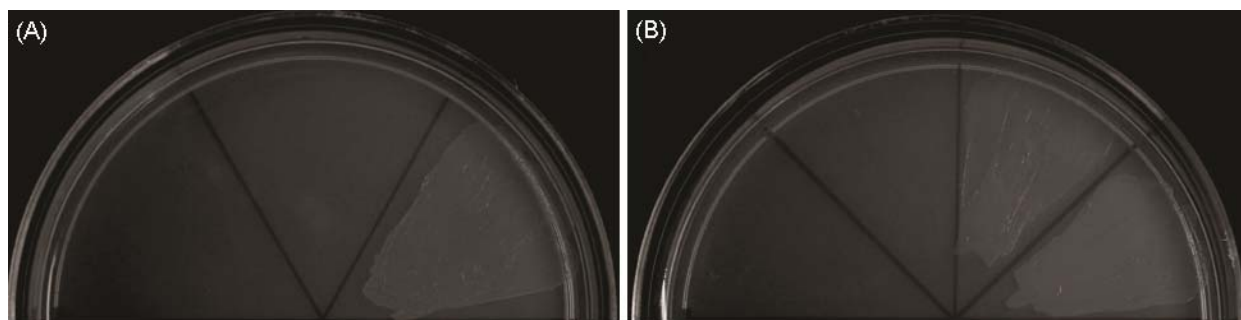


Figure 4.2: Growth of *P. putida* GPo12 (pGEc47ΔB) strains on alkanes. (A) Strains of adapted *P. putida* GPo12 (pGEc47ΔB) complemented by an empty plasmid, or expressing CYP153A6 wild type or CYP153A6-BMO1 (left, middle, and right section) were grown for 5 days with butane as sole carbon source. (B) Growth of adapted *P. putida* GPo12 (pGEc47ΔB) strains complemented by an empty plasmid, expressing the AlkB wild type, or its mutants BMO1 and BMO2, on butane after 2 days

Palk1 expressing AlkB-BMO1 showed a significant increase in rate of growth on butane compared to Palk1 expressing wild-type AlkB. In contrast, growth rates on pentane and octane were reduced. No significant growth improvement on propane was observed, and neither enzyme supported growth on ethane. These results suggest that the L132V mutation in AlkB-BMO1 specifically improves activity toward butane. A similar result was found for Pcyp2 (pCom8*_cyp153A6-BMO1), which grew more slowly on octane than Pcyp2 (pCom8*_cyp153A6) but faster on pentane. The CYP153A6-BMO1 variant also supported growth on butane, which wild-type CYP153A6 did not. Thus the A94V mutation appears to improve activity on the smaller alkanes.

Plasmids pCom8*_cyp153A6-BMO1 and pCom10_alkB-BMO1 were subjected to a second round of mutagenesis and mated into Pcp2 and Palk1, respectively. A further-improved AlkB monooxygenase, AlkB-BMO2, was obtained after enrichment and screening. Sequencing revealed a total of three nucleotide mutations, all resulting in amino acid substitutions in AlkB-BMO2: V129M (GTG to ATG), L132V (CTA to GTA), and I233V (ATC to GTC). To ensure comparison in identical genetic backgrounds, the mutated gene was recloned into a wild-type pCom10 vector, mated into fresh Palk1, and compared in growth tests to Palk1 expressing AlkB wild-type and AlkB-BMO1 (Table 4.1, Figure 4.2). Compared to its parent AlkB-BMO1, AlkB-BMO2 performed even better in growth complementation studies with butane. Growth on pentane and octane was also improved, but was still inferior to that obtained with the wild-type enzyme. Thus, the mutations V129M and I233V improved the overall activity of AlkB-BMO2 compared to its parent, AlkB-BMO1. Enrichment and screening yielded no additional improvement in the CYP153A6 system.

C.4. Whole-cell butane bioconversions of AlkB-BMO1, -BMO2, and CYP153A6-BMO1

In order to quantify the effects of the mutations on enzyme performance, whole-cell bioconversions were performed using growth-arrested *E. coli* BL21(DE3) cells containing the CYP153A6 variants expressed from pCom8*. Although pCom plasmids are not efficient expression platforms, we nonetheless observed functional CYP153A6 expression in *E. coli*, as indicated by the CO difference spectral peak at 450 nm (Figure 4.3). In contrast, no 450 nm signal was observed for cells harboring the empty pCom8* plasmid. Cytochrome P450s are notoriously difficult to express, and it has been reported that the CO binding activity of CYP153A6 is lost shortly after cell disruption at room temperature, even when the enzyme is

isolated from its native host (21). However, we found CYP153A6 to express well in *E. coli* DH5 α and showed a stable CO difference spectrum for hours at room temperature, if protease inhibitor was added before cell disruption. Using *E. coli* BL21(DE3) cells, which are deficient in the Lon and OmpT proteases, eliminated the need to add protease inhibitor for stable CYP153A6 expression. Cell extract from these cultures, expressing CYP153A6 or CYP153A6-BMO1, retained full CO-binding capacity for 24 h when stored at 25 °C. At 45 °C, CO-binding capacity decreased with time, showing a half-life of 638 ± 68 min for cell extract containing CYP153A6-BMO1 and 367 ± 58 min with CYP153A6.

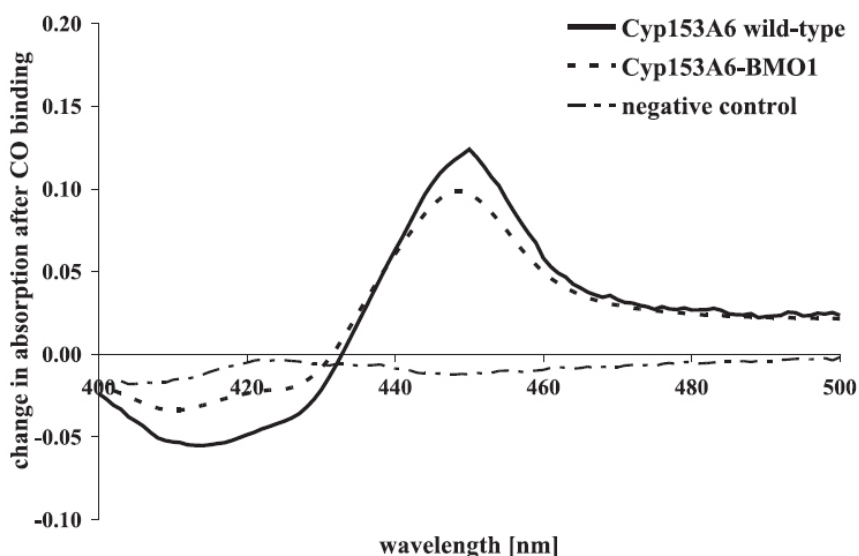


Figure 4.3: CO difference spectra of lysed *E. coli* BL21(DE3) cell suspensions. *E. coli* BL21(DE3) cultures expressing the CYP153A6 variant were concentrated 5:1 in phosphate buffer, and UV-VIS spectra were obtained from the cell-free extracts after CO saturation. The peaks correspond to 0.21 mM and 0.17 mM folded P450 for the CYP153A6 and CYP153A6-BMO1 samples, respectively. Cells carrying the empty vector treated in the same way served as the negative control.

The typical concentration of folded CYP153A6 in the cell suspensions used for bioconversions was 0.1 – 0.2 μ M, with CYP153A6-BMO1 usually expressing ~ 20% less than its parent, CYP153A6. For both enzymes, we observed no significant decrease in apparent P450

concentration after the 60 -min bioconversion reactions. Bioconversion studies showed significantly altered activity and selectivity for CYP153A6-BMO1 that closely followed its growth complementation performance (Figure 4.4 (A)). Control bioconversions performed under the same conditions but using transformants of empty pCom8 vector did not produce alkanols. Butane bioconversions yielded an average total of 393 μM 1-butanol in the aqueous phase after 60 minutes with CYP153A6-BMO1, versus 277 μM with wild-type CYP153A6. From the concentration of product formed divided by the concentration of folded P450, the average turnover rate of CYP153A6-BMO1 in 1-butanol production was 49 min^{-1} , a 75% increase compared to 28 min^{-1} for CYP153A6. Interestingly, the selectivity for terminal hydroxylation also increased with the mutant, from 78% to 89% of total alkanol product. The A94V mutation also improved activity and selectivity for conversion of pentane to 1-pentanol, but had the opposite effect with propane and octane.

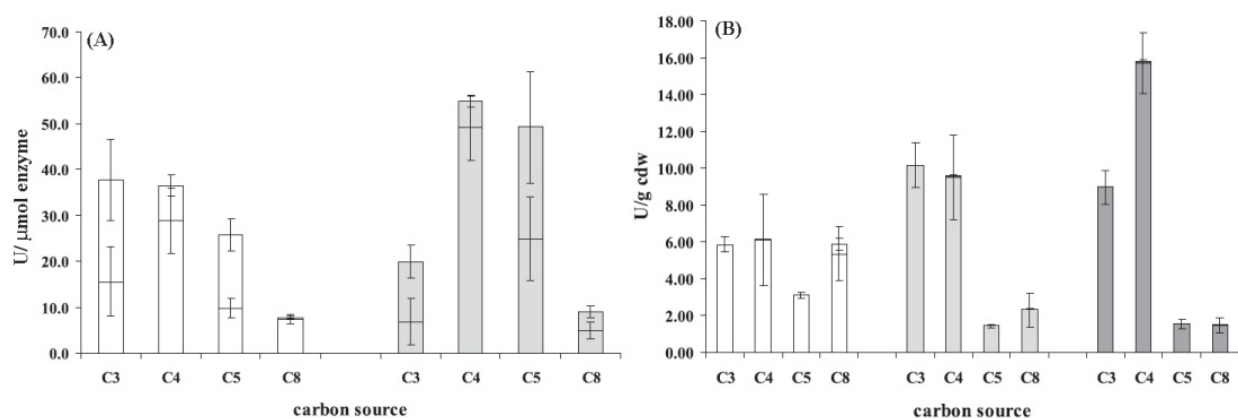


Figure 4.4: Whole-cell bioconversions were carried out with at least two replicates using resting *E. coli* BL21(DE3) cells expressing CYP153A6 (A) and AlkB (B) variants. Relative enzymatic activities in the aqueous cell suspension were calculated from the alcohol product formed per minute and the enzyme concentration (A) or total cell dry weight (B). The lower graphs represent relative activities for 1-alkanol, and the upper part shows 2-alkanol production (if detected). Solely added carbon sources were propane (C3), butane (C4), pentane (C5), and octane (C8). White graphs depict wild-type activity, light gray shows the BMO1 activity, and dark gray shows the BMO2 variant activities

Since *E. coli* cells expressing only AlkB showed no product formation, bioconversions were performed using cells transformed with pCom8_alkBFG. Minak-Bernero et al. (27) demonstrated that the AlkBFG system including the monooxygenase AlkB and the non-essential and essential rubredoxins AlkF and AlkG is functional in *E. coli*, without the need for the rubredoxin reductase AlkT. Results of bioconversions using AlkB, AlkB-BMO1, and AlkB-BMO2 (Figure 4.4 (B)) showed that the activity of the mutants was greater than that of wild type on butane, the substrate used for *in vivo* evolution, as well as on propane. As with CYP153A6, performance on pentane and octane decreased. The evolved mutants also showed increased activity on propane, the only change in activity that was not reflected in a similar observable change in growth complementation performance. This could reflect an activity that is too low or sub-optimal growth conditions during enrichment, such as insufficient substrate concentration or toxicity of the substrate.

In 30 minutes, butane bioconversions utilizing AlkB, AlkB-BMO1, or AlkB-BMO2 produced on average 630 μM , 1030 μM , and 1580 μM 1-butanol in the aqueous phase, respectively. The dry cell weights of the cell suspension used for the bioconversions ranged from 3.0 to 4.0 g/L. The activities (in μmol 1-butanol min^{-1} g [cell dry weight] $^{-1}$) thus increased from 6.1 to 9.5 and 15.7 units, respectively. Bioconversions with AlkB variants were highly regioselective, producing no detectable 2-alkanol from propane and pentane and very little 2-alkanol from butane and octane.

D. Discussion

D.1. *Advantages and drawbacks of in vivo evolution of terminal alkane hydroxylases*

An *in vivo* directed evolution system with selection for terminal alkane hydroxylase activity has been developed and applied to engineering enzymes from the AlkB and CYP153 families. The goal of this work was to increase hydroxylase activity on short-chain alkanes while maintaining these enzymes' remarkable preference for the thermodynamically disfavored terminal position, thereby taking a first step toward engineering small-alkane hydroxylases that can be expressed in a recombinant bacterial host amenable to further engineering.

Evolution *in vivo* using a growth selection enables searches through libraries as many as 10^8 mutants in a simple flask culture while exerting selection pressure for many useful enzyme characteristics simultaneously (e.g., substrate specificity, regioselectivity, specific activity, coupling of cofactor utilization to product formation, and expression level). In this particular case, the *Pseudomonas* host used for the directed evolution only utilizes primary alcohols, allowing selection directly for terminal hydroxylation. This is the only method we know of for efficient, high throughput selection or screening of this important activity. Previous efforts to engineer P450 BM3 or P450cam for alkane hydroxylation resulted in enzyme variants mainly targeting energetically favored subterminal positions (18 – 19). The present system promises to be useful for the directed evolution of a range of alkane hydroxylases, since *P. putida* GPo12 (pGEc47ΔB) can be complemented for growth on alkanes by many different AlkB and CYP153 genes (15, 22). Furthermore, growth tests showed that the host can utilize a range of 1-alkanols down to ethanol, potentially enabling enrichment for activity on alkanes as small as ethane.

This powerful growth selection system could handle a mutation rate perhaps ten times greater than the 0.1 to 0.4 nucleotide changes per kb obtained using the mutator strains. The *P. putida* GPo12 organism and the components used in this study, however, are somewhat difficult to handle with regards to growth stability, expression, mutation rates, cloning, and transformation efficiency. Furthermore, *in vivo* evolution also has limits for identifying moderately improved mutants, since enrichment to pure culture may require weeks or months of continuous culturing, depending on the actual doubling time on a given substrate and the level of improvement over the parent. This makes the stepwise accumulation of small improvements more challenging.

D.2. Selection for growth on propane and butane improves hydroxylase function

Our results prove that propane and butane are substrates of AlkB from *P. putida* GPo1, verifying earlier assumptions (3). No growth complementation was observed on ethane, and the *E. coli* system utilized in the bioconversion reactions could not be used to determine whether ethane is converted to ethanol by AlkB variants, because the glycerol added to allow NADH regeneration was fermented to ethanol by the *E. coli* BL21(DE3) host. Quantification of AlkB activity on ethane would require purification and reconstitution of AlkB and its electron transport system or genetic modification of the strain used for whole-cell bioconversions.

Second-generation variant AlkB-BMO2 supported significantly better growth and butane bioconversion, more than double that of wild-type AlkB. Although specific activity (mol product/mol enzyme/minute) could not be determined for the AlkB variant, absolute 1-butanol production rates in bioconversion reactions were significantly higher (up to 1.6 mM in 30 min) than that of the evolved CYP153A6-BMO1 system (0.4 mM in 60 min). The higher product

formation with the AlkB system was surprising, in view of the fact that it relied on a non-native *E. coli* host reductase, while the CYP153A6 system contained the complete native electron transfer chain composed by its ferredoxin and ferredoxin reductase. In earlier studies, AlkB activity in *E. coli* cells increased from 3 U to 20 U, when the specific reductase, encoded by *alkT*, was present (6). However, the significantly higher *E. coli* whole-cell bioconversion rates with AlkB variants did not translate into better growth complementation of adapted *P. putida* GPo12 (pGEc47ΔB) strains. It is known that AlkB expresses two-to tenfold better in *E. coli* than *P. putida*. On the other hand, AlkB has a five- to sixfold-higher specific activity in *P. putida* (8). Thus, a possible explanation for the growth complementation is that CYP153 variants express better or have a higher specific activity in *P. putida* compared to AlkB variants. Furthermore, the twice-adapted host Pcyp2 might contain chromosomal improvements that are able to compensate for the lower apparent butane oxidation activity of CYP153A6-BMO1 and thus achieve the same growth rate on butane as Palk1 expressing AlkB.

Although no crystal structures are available for any enzyme of the AlkB family, a topology model for AlkB (9, 28) has been published. Mapping of the evolved AlkB variant mutations onto that model (Figure 4.5 (A)) shows that two of the three mutations generated in AlkB-BMO2, V129M and L132V, are close to the histidine-containing sequence motif A (H₁₃₈EXXHK₁₄₃), which is one of four highly conserved histidine-containing motifs in AlkB believed to coordinate the iron ions and presumed to be part of or close to the active site (9, 29). Thus, a direct effect of V129M and L132V on the active site of AlkB seems possible. The third amino acid substitution, I233V, is located close to the periplasm in the sixth predicted transmembrane domain, distant from the active site.

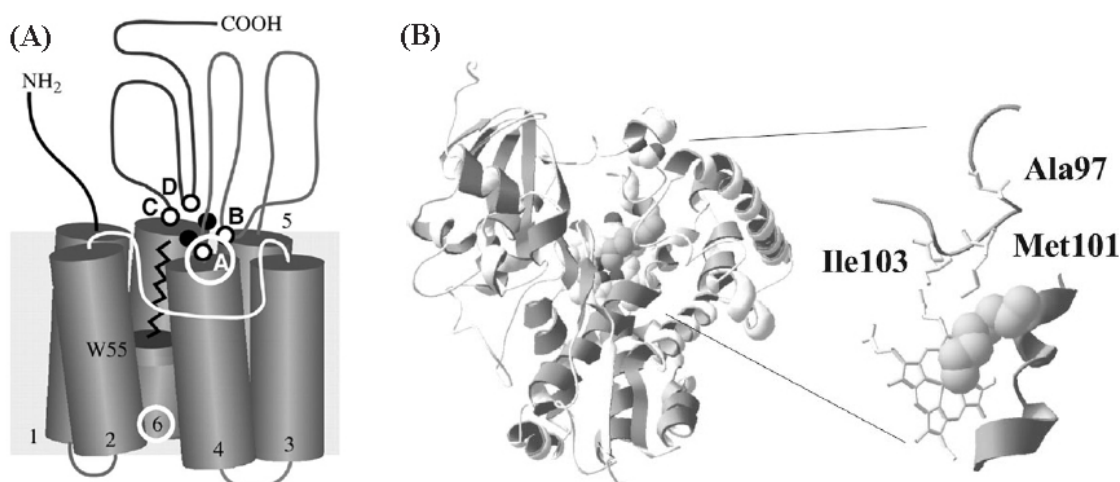


Figure 4.5: Models for (A) AlkB and (B) CYP153A6. (A) Topology model of AlkB, taken from van Beilen et al. (34). Variants AlkB-BMO1 and AlkB-BMO2 with increased activity on butane carry L132V and V129M+L132V+I233V mutations (marked by white circles), respectively. Amino acids 129 and 132 are close to the first of four histidine-containing sequence motifs (A – D) that contribute to coordinating the Fe ions. Position 233 is located in the periplasmic end of the sixth transmembrane domain (numbers 1 – 6). (B) CYP153A6 structural model with bound substrate octane (spheric object), modified from Funhoff et al. (8). CYP153A6-BMO1 carries a mutation corresponding to A97V in the model.

Evolved CYP153A6-BMO1 is the first CYP153 shown to hydroxylate butane and enable its host to grow on this short chain-length alkane. The gaseous alkanes are usually processed by distinct, specialized enzymes, like the di-iron methane and butane monooxygenases (2) that are unrelated to the cytochrome P450s. Complementation of *P. putida* GPo12 (pGEc47ΔB) with various CYP153 wild-type enzymes resulted in growth on alkanes ranging from pentane to decane (15), but never on butane. And, until now, biotransformations using CYP153 enzymes have only demonstrated activity for hexane and longer alkanes (13 – 14, 17). Here, we demonstrate that CYP153A6 wild type and CYP153A6-BMO1 act on butane and even propane, but only the BMO1 variant was able to complement growth of Pcyp2 on butane. The activity for 1-butanol formation of 49 min^{-1} (0.8 s^{-1}) measured in CYP153A6-BMO1 assay is still less than that reported for the methane monooxygenase from *Methylosinus trichosporium* OB3b (8.8 s^{-1}) (30), although these values were generated under very different reaction conditions. Not only

does CYP153-BMO1 exhibit increased total activity on butane compared to its wild-type parent, but its preference for terminal hydroxylation also increased, resulting in only 11% 2-butanol formation compared to 22% for the wild-type enzyme. CYP153A6-BMO1 was converted into a genuine butane monooxygenase by *in vivo*-directed evolution.

CYP153A6-BMO1 contains a single amino acid substitution, which corresponds to A97V in the published structure model (13). This mutation, which has a slightly negative effect on expression level (the CYP153A6-BMO1 variant usually showed ~ 80% of the CO-binding activity of the wild type) stabilized CYP153A6 and nearly doubled its half-life at 45 °C. However, this effect is unlikely to explain the higher observed bioconversion rates, since both enzymes were stable at 25 °C, and no loss of folded P450 was observed at the end of the bioconversions. These findings argue for a direct positive effect of the A97V substitution on the butane hydroxylation activity. Based on a proposed model of CYP153 (13), however, A97 is predicted to be located in a loop distant from the active site, pointing toward the protein surface and not the substrate channel (Figure 4.5 (B)). Why the activity of CYP153A6 and CYP153A6-BMO1 on propane, as observed in the *E. coli* bioconversion experiments, is not accompanied by growth complementation of P_{cyp2} on propane remains unknown. A minimum activity required for growth may not have yet been reached. It is also possible that the substrate is not available at a sufficient concentration or is toxic.

The appearance of improved variants of AlkB and CYP153A6 demonstrated that *in vivo* evolution in adapted *P. putida* GPo12 (pGEc47ΔB) can be applied to very different monooxygenase enzymes. Despite the higher absolute activity and better regioselectivity of AlkB variants in the bioconversion experiments, the CYP153A enzymes nonetheless offer some

important advantages for further engineering. CYP153 enzymes are soluble, and accurate determination of functional expression and concentration is easier due to observable CO binding. Furthermore, CYP153 proteins have been shown to function in whole-cell bioconversions as single-component, self-sufficient fusion proteins with the P450RhF reductase domain (17, 31). Further directed evolution of terminal alkane hydroxylases like AlkB or CYP153A6 should help us to better understand and utilize their remarkable catalytic activities.

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