Video Article

A Toolkit to Enable Hydrocarbon Conversion in Aqueous Environments

Eva K. Brinkman[1], Kira Schipper[1], Nadine Bongaerts[1], Mathias J. Voges[1], Alessandro Abate[2], S. Aljoscha Wahl[1]

[1]Department of Biotechnology, Delft University of Technology

[2]Delft Center for Systems and Control, Delft University of Technology

Correspondence to: S. Aljoscha Wahl at s.a.wahl@tudelft.nl

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Abstract

This work puts forward a toolkit that enables the conversion of alkanes by *Escherichia coli* and presents a proof of principle of its applicability. The toolkit consists of multiple standard interchangeable parts (BioBricks)[9] addressing the conversion of alkanes, regulation of gene expression and survival in toxic hydrocarbon-rich environments.

A three-step pathway for alkane degradation was implemented in *E. coli* to enable the conversion of medium- and long-chain alkanes to their respective alkanols, alkanals and ultimately alkanoic-acids. The latter were metabolized via the native β-oxidation pathway. To facilitate the oxidation of medium-chain alkanes (C5-C13) and cycloalkanes (C5-C8), four genes (*alkB2*, *rubA3*, *rubA4*and *rubB*) of the alkane hydroxylase system from *Gordonia* sp. TF6[8,21] were transformed into *E. coli*. For the conversion of long-chain alkanes (C15-C36), the*ladA* gene from *Geobacillus thermodenitrificans* was implemented. For the required further steps of the degradation process, *ADH* and *ALDH (*originating from *G. thermodenitrificans*) were introduced[10,11]. The activity was measured by resting cell assays. For each oxidative step, enzyme activity was observed.

To optimize the process efficiency, the expression was only induced under low glucose conditions: a substrate-regulated promoter, pCaiF, was used. pCaiF is present in *E. coli* K12 and regulates the expression of the genes involved in the degradation of non-glucose carbon sources.

The last part of the toolkit - targeting survival - was implemented using solvent tolerance genes, PhPFDα and β, both from *Pyrococcus horikoshii* OT3. Organic solvents can induce cell stress and decreased survivability by negatively affecting protein folding. As chaperones, PhPFDα and β improve the protein folding process *e.g.* under the presence of alkanes. The expression of these genes led to an improved hydrocarbon tolerance shown by an increased growth rate (up to 50%) in the presences of 10% *n*-hexane in the culture medium were observed.

Summarizing, the results indicate that the toolkit enables *E. coli* to convert and tolerate hydrocarbons in aqueous environments. As such, it represents an initial step towards a sustainable solution for oil-remediation using a synthetic biology approach.

Video Link

The video component of this article can be found at http://www.jove.com/video/4182/

Introduction

Oil pollution is among the most serious causes of environmental contamination, and greatly affects ecosystems, businesses and communities [3]. Solutions are for example required to battle the continuous oil pollution originating from the oil sands tailing waters in Alberta, Canada. During the process of oil extraction from oil sands, bitumen, a semi-solid oxidized form of oil, is removed using thermal recovery techniques that consume about 3.1 barrels of water per single barrel of oil [1]. Oil contaminated process water, mainly originating from a local river, is stored in tailing ponds after bitumen extraction. A more effective recycling of process water in order to reduce the need for freshwater uptake is needed. To facilitate the bitumen extraction and to ensure that downstream sites meet water quality guidelines for the protection of aquatic ecosystems, process water treatments are rapidly evolving [3].

To treat pollution of organic compounds, bioremediation technologies employing microorganisms are presently encouraged [1]. Alkanes are the most abundant family of hydrocarbons in crude oil, containing 5 to 40 carbon atoms per molecule [7, 21]. Many bacteria are known to degrade alkanes of various lengths via sequential oxidation of the terminal methyl group forming first alcohols, then aldehydes and finally fatty acids [8]. Within this iGEM project several enzymes from different organisms were expressed and characterized, and made available via the BioBrick standard and Registry of Standard Biological Parts.

The well-studied alkane hydroxylase system of *Gordonia* sp. TF6 facilitates the initial oxidation step of C_5-C_{13} alkanes along with that of C_5-C_8 cycloalkanes using a minimum of four components: alkB2 (alkane 1-monooxygenase), rubA3, rubA4 (two rubredoxins) and RubB (rubredoxin

reductase) [8, 21]. Oxidation of long-chain alkanes (ranging from C_{15} up to C_{36}) is reported to be performed by ladA, a flavoprotein alkane monooxygenase from *Geobacillus thermodinitrificans NG-80-2* [7, 15, 18, 22]. LadA forms a catalytic complex with flavin mononucleotide (FMN) that utilizes atomic oxygen for oxidation. This results in the conversion of alkanes into the corresponding primary alkanol. The alcohols are further oxidized by alcohol and aldehyde dehydrogenases to fatty acids, which readily enter the β-oxidation pathway [7, 21]. A zinc-independent alcohol dehydrogenase from the thermophillic bacterium *Geobacillus thermoleovorans B23* oxidizes medium-chain alkanols into their respective alkanals, using NAD^+ as a cofactor [10]. Aldehyde dehydrogenase from the same bacterium is able to catalyze the NAD^+-dependent final step in the medium-chain oxidation [11].

In order to reduce induction costs and to maintain optimal proliferation of the bacterial system, the promoter pCaiF from *E.coli* was characterized. This promoter can regulate expression of the hydrocarbon degradation pathway components, and is regulated by cAMP-Crp levels, which in turn depend on glucose levels [6]. At high extracellular glucose concentrations in the environment the cellular cAMP (cyclic Adenosine Mononucleotide Phosphate) level was low through the inhibition of adenylyl cyclase as a side effect of PTS mediated glucose transport. Conversely, during limitation (low glucose concentrations) the cAMP level increased and Crp bound to cAMP forming the complex, cAMP-Crp, which bound pCaiF and activated transcription of the downstream components [6, 14].

Wildtype *E. coli* can only tolerate moderate concentrations of hydrocarbons. To complete the toolkit, tolerance to hydrocarbons had to be addressed. Several organic solvent-tolerant bacteria are known to survive in water-solvent two-phase systems [12]. Molecular components known to increase tolerance are chaperones that facilitate the correct folding of proteins. The prefoldin system from *Pyrococcus horikoshii* OT3, consisting of the proteins phPFDα and phPFDβ, was shown to increase hydrocarbon-tolerance [17].

The alkane conversion toolkit was constructed following the BioBrick principle, which is documented at the Registry of Standard Biological Parts [9]. BioBricks are plasmids containing a specific functional insert that is flanked by 4 predefined restriction sites. The BioBrick inserts can be extended flexibly, allowing the construction of biological systems with new functions.

Protocol

1. BioBrick Assembly

1. BioBricks from the Registry of Standard Biological parts are provided by iGEM headquarters. To construct a new BioBrick from existing BioBricks, digest the donor BioBrick (up to 1.0 µg) with the enzymes EcoRI and SpeI for positioning the donor part downstream of the acceptor part. Digest with XbaI and PstI for positioning the donor part upstream of the acceptor part. Add a third appropriate restriction enzyme that cuts in the backbone of the donor. Perform the digestions in a total volume of 20-25 ml with the appropriate buffer, according to the supplier (final concentration 1x). Use 5 units/µg DNA for the restriction enzymes.
2. Digest the acceptor BioBrick with either EcoRI and XbaI or SpeI and PstI.
3. Incubate the digestions for (at least) one hr at 37 °C. Inactivate the restriction endonucleases by heat, incubation at 80 °C for 10 min and centrifuge shortly.
4. Ligate the digested BioBrick parts (donor and acceptor) together. Since XbaI and SpeI generate compatible DNA ends, a mixed site is created that cannot be cut with any restriction enzyme resulting in a new 'combined' BioBrick that flanked by the 4 standard restriction sites. In the ligation mixture the final DNA concentration is preferably ~100 ng/µl. Perform the ligation reaction in a total volume of 10-15 ml with the T4 ligation buffer (final concentration 1x) and T4 ligase (1 unit/µg DNA).
5. Incubate the ligation mixture at 16 °C for at least 3 hr.
6. Perform transformation reaction with circa half of the ligation mix.
7. Confirm the BioBrick to be correct with sequencing.
8. To construct a new BioBrick from synthesized DNA, modify the genes for synthesis for optimal expression in *E. coli* by means of the JCat website tool (http://www.jcat.de/). Make further modifications in accordance with the requirements of the BioBrick standard. This standardization implies that every BioBrick is composed of a DNA sequence of interest preceded by a prefix and followed by a suffix. The prefix and suffix are sequences that contain predefined restriction sites, that are absent in the remaining plasmid sequence. These standard restriction sites make it possible to interchange and extend the BioBrick inserts flexibly [9]. The prefix and suffix have the following sequences:

Name	Sequence	Comment
Prefix	5' GAATTCGCGGCCGCTTCTAG3'	
	5' GAATTCGCGGCCGCTTCTAGAG 3'	If the following part is a coding sequence or any part that starts with "ATG"
Suffix	5' TACTAGTAGCGGCCGCTGCAG 3'	

2. Alkane Conversion Resting Cell Assay, *In Vivo*

This assay was performed based on the method described by Fujii *et al.* (2004).

1. Culture *E. coli* cells expressing the Alkane Hydroxylase system (BBa_K398014) and cells carrying an empty vector (BBa_J13002) in 5 ml LB medium with appropriate antibiotics overnight.
2. Transfer 500 µl of the overnight culture into 50 ml of fresh LB (with antibiotic) and incubate until the cell turbidity reached an OD (optical density) of 0.3 at 600 nm.
3. Centrifuge for 10 min at 4,000 rpm and resuspend the pellet in 5 ml 0.1 M phosphate buffer (pH 7.4).
4. Centrifuge again for 10 min at 4,000 rpm and resuspend the pellet in 5 ml 0.1 M phosphate buffer now containing E2 salts and 0.66% v/v glycerol (nitrogen-deficient medium).
5. Measure the cell turbidity (OD_{600}).

6. Prepare cell-mixture aliquots of 6 ml in 25 ml closed-cap glass flasks and no-cell controls (E2 salts + 0.66% v/v glycerol).
7. Add 100 nmol of alkane to each flask.
8. Incubate the mixtures at 37 °C for 24 hr (shorten when higher rates are obtained).
9. Measure the OD_{600} after incubation.
10. Extract the hydrocarbons in the culture media by ethyl acetate and determine hydrocarbon concentration in cell culture by gas chromatography (see protocol 3).
11. Calculate the degradation per unit of biomass by dividing the total amount of alkane converted by the total biomass present in each flask (convert OD_{600} to dry weight). Dividing the result by the experimental duration yields the degradation activity per unit biomass per time unit. The average is taken from three individual runs.

3. Alkane Conversion Enzyme Assay, *In Vitro*

This assay was performed essentially according to the method described by Li *et al.* (2008).

1. Culture *E. coli* cells expressing the *ladA* gene (BBa_K398017) and cells carrying an empty vector (BBa_J13002) in 50 ml LB medium with appropriate antibiotics overnight.
2. Transfer 500 µl of the overnight culture into 50 ml of fresh LB (with antibiotic) and incubate until the cell turbidity reached an optical density of 0.6 at 600 nm.
3. Centrifuge 10 min at 4,000 rpm (4 °C) and resuspend the pellet in 5 ml of 50 mM Tris buffer.
4. Sonicate (Cell disrupter, LA Biosystems) the cells at 40% duty cycle with an output control of 4, keep the solution on ice for the entire duration.
5. Centrifuge the resulting mixture for 5 min at 4,000 rpm at 4 °C to remove the cell debris. Transfer the supernatant to a fresh vial.
6. Determine the total protein concentration of the cell extracts by Bradford assay. Note: Use glass vials to prevent the increase of background and/or loss of protein.
7. Prepare a 100 ml mixture containing 0.1% v/v alkane and 50 mM Tris-HCl buffer.
8. Heat the mixture at 100 °C for 5 min. Note: Perform this step only for medium-long chain alkanes with high boiling point. (*e.g.* C_{16}: 287 °C).
9. To achieve an optimal solubility of the alkane, sonicate for 1 min while still warm until a homogenous, viscous mixture is obtained.
10. Add 1mM of NADH, 1 mM FMN, 1 mM $MgSO_4$ and 0.01 v/v Triton X-100.
11. Prepare 6 ml aliquots in 25 ml closed-cap flasks.
12. Add adequate amounts of cell extract (depends on Bradford assays, final concentration of 5 mg protein/l). Prepare a no-protein control.
13. Incubate at 60 °C (for optimal enzymatic activity) for 24 hr.
14. Extract the hydrocarbons in the culture media by ethyl acetate and determine hydrocarbon concentration in cell culture by gas chromatography (see protocol 3).
15. Calculate the degradation per unit of biomass by dividing the total amount of alkane converted by total protein added to each flask (by Bradford calibration curve). Further dividing by experiment duration yields the degradation activity per unit of cellular protein per time unit. The average is taken from three individual runs.

4. Ethyl Acetate Hydrocarbon Extraction and Concentration Measurements

1. Alkanes are extracted from the aqueous solution by adding 2.5 ml ethyl acetate (apolar solvant) to 6 ml experimental solution. An internal standard is added to the solvent at a concentration of 0.1 % (v/v). The standard (*e.g.* cyclo-decan) varied depending on the expected range of the peaks of interest.
2. Optional: Add Triton X-100 to the aqueous mixture and centrifuge the samples for 10 min at 4,000 rpm in order to get a proper bi-phasic system.
3. Vortex the mixture for 5 sec (1,500 rpm) and incubate at room temperature until the two phases separate.
4. Remove a maximal amount of the organic layer (top) and dry the solvent using anhydrous magnesium sulphate.
5. Remove $MgSO_4$ by filtration (0.2 µm) and transfer the filtrate into gas chromatograph vials for measurements, or store at -20 °C.
6. Determine the concentration by gas chromatography using a CP-SIL 5CB column (length = 5 m). Inject 10 µl of sample in split mode (1:10, 230 °C). Set the column gas flow to 1 ml/min (Helium). The following oven temperature program is used:

Rate	Temperature [°C]	Time [min]
0	50	7.5
50	90	1.0
50	110	2.0
50	130	2.0
50	145	2.0
50	160	2.0
50	170	2.0
50	185	2.0
50	210	2.0
50	250	2.0
50	320	2.0

7. Integrate peaks and correct the concentrations with respect to the internal standard.

5. Alcohol/aldehyde Dehydrogenase Activity Assay

This assay was performed essentially according to the method described by Kato *et al.* (2010).

1. Culture *E. coli* cells expressing the *ADH* (BBa_K398018) or *ALDH* (BBa_K398030) gene and cells carrying an empty vector (BBa_J13002) in 50 ml LB medium with appropriate antibiotics overnight.
2. Transfer 500 µl of the overnight culture into 50 ml of fresh LB (with antibiotic) and incubate until the cell turbidity reached an optical density of 0.6 at 600 nm.
3. Centrifuge 10 min at 4,000 rpm at 4 °C and resuspend the pellet in 5 ml of 50 mM Tris buffer.
4. Sonicate the cell solution at 40% duty cycle with an output control of 4 (keep the solution on ice during the sonication).
5. Centrifuge the resulting mixture for 5 min at 4,000 rpm at 4 °C to remove cell debris.
6. Determine the total protein concentration of the cell extracts by Bradford assay (Note: use a glass vial to prevent protein binding).
7. Load a 96 well plate with 180 µl 57 mM glycine buffer containing NAD (final concentration 1 mM, pH 9.5) in each well.
8. Add 5 µl of the alcohol (aldehyde) to be tested to the wells. Note: Heat long chain alcohols before the start of the assay to have a liquid. For each alcohol (aldehyde) a control without substrate should be added (negative control). In addition, prepare a blank containing a mixture of buffer and the substrate without cell extract.
9. Preheat the plate of the plate reader (Tecan Magellan v7.0) for 15 min at 37 °C to allow equilibration of the system.
10. Add adequate amounts of cell extract (depends on Bradford assays, final concentration of 5 mg protein/l). Prepare a no-protein control.
11. Measure the NADH production using a spectrophotometer at a wavelength of 340 nm every 2-3 min for 1 hr at 37 °C.
12. Calculate the NADH production rate from the slope of the OD (340 nm). Take into account the light path length and the extinction coefficient of NADH of 6220 $M^{-1}cm^{-1}$. Divide the NADH production rate by the total amount of protein added to express the activity of the dehydrogenase reaction in the cell extract (U/mg whole cell protein). Calculate the mean and the standard deviation from three independent runs.

6. pCaiF Characterization

1. Culture *E. coli* cells expressing the *pCaiF-GFP* construct (BBa_K398331) and cells carrying the promoter alone (BBa_K398326) overnight in 5 ml LB medium the appropriate antibiotics overnight.
2. Inoculate 5 ml M9 containing 10 g/l glucose and antibiotics with 50 µl of the overnight culture and grow overnight.
3. Subculture 50 µl of the overnight outgrowth into 5 ml of fresh M9 with10 g/l and incubate until the cell turbidity reached an optical density of 0.2 at 600 nm.
4. Load a 96 well plate with 100 µl of fresh M9 medium containing the desired amount of carbon source for testing in each well. Perform triplicate experiments for statistical evaluation and add the respective negative controls (wild-type *E. coli* K12).
5. Add 5 µl of the overnight culture to the medium containing wells.
6. Measure the growth curve (OD_{600}) and GFP fluorescence (485 nm excitation and 520 emission) using a plate reader every 10 min for 18 hr at 37 °C with constant shaking.
7. Calculate the growth rate and the specific GFP content from the respective measurements and compare to the control. Calculate the mean and the standard deviation of at least three independent experiments.

7. Tolerance Assay

1. Culture *E. coli* expressing PhPFDα and β gene (BBa_K398406) overnight in 5 ml LB medium and the appropriate antibiotics. *E. coli* expressing *ladA* gene (BBa_K398017) is used as negative control.
2. Subculture 10 µl of the overnight outgrowth into fresh 5 ml of LB (with antibiotic) and incubate until the cell turbidity reached an optical density of 0.3 to 0.4 at 600 nm.
3. Dilute the cultures with fresh medium until an OD_{600} of 0.1 is reached.
4. Load a 96 well plate with 180 µl M9 medium containing the appropriate antibiotics and the proper final concentration of the toxic compound (*e.g.* 0, 4, 8, 10% of *n*-hexane) in triplicate. Because alkane-water mixtures could lead to two-phase systems it is essential to have appropriate controls on the plate (*e.g.* different strains and the respective blank experiments).
5. Add 20 µl of the culture (control) into the wells.
6. Measure the biomass concentration (OD_{600}) using a plate reader every 10 min for 24 hr at 37 °C with constant shaking.
7. Calculate the growth rates from the respective measurements for the different agent concentrations and compare to the negative control. Calculate the mean and the standard deviation of at least three independent runs.

8. Homolog Interaction Mapping

1. The application HIM was developed to perform protein queries on a PostgreSQL server running the STRING database (free for academic use) [20]. The homologue interaction mapping application identifies interacting proteins in the original host organism using the STRING database. The sequences of the respective interacting genes are used in a BLAST search to find homologous genes in the target organism. Cytoscape [4] is used to visualize the result of the mapping. The HIM software tool can be downloaded at: https://github.com/jcnossen/InteractionHomologMapping.
2. To perform a mapping, (1) enter the BioBrick ID and the application will automatically download the part sequence data from the Registry of Standard Biological Parts [9], or (2) enter (paste) the sequence data in the application.
3. Use the STRING Database website to find the STRING protein ID for the entered amino acid sequence.
4. A protein with high homology is determined using BLAST. Subsequently the application lists each known interacting protein in the source organism and searches for homologs in the host organism (*e.g. E. coli*).

5. Export resulting putative interaction list to text or Cytoscape.

Representative Results

Alkane conversion

The activity of the three oxidation steps from the alkane to the respective fatty acid was evaluated using resting cell assays and enzyme activity measurements. The results are presented following the pathway reactions (1) alkane hydroxylase, (2) alcohol dehydrogenase and (3) aldehyde dehydrogenase.

For the first step, different plasmids were constructed for medium and long-chain alkanes. The plasmid BBa_K398014 contains the four genes of the alkane hydroxylase (AH) system for medium chain alkane oxidation: *alkB2*, *rubA3*, *rubA4* and *rubB* under the control of a single constitutive promoter (BBa_J23100). As negative control, *E. coli* K12 with the plasmid BBa_J13002 was used. The activity was measured by an enzyme activity assay using *n*-octane as short-chain hydrocarbon source. The consumption of alkane, respectively formation of the corresponding alkanol was analyzed using gas chromatography. Based on hydrocarbon consumption, the specific enzymatic activity of the system was determined. For cell extracts from *E. coli* K12 carrying BBa_K398014 an enzymatic activity of 4.49×10^{-2} U/mg was obtained (1-octanol production from octane). For the negative control an activity of 0.12×10^{-2} U/mg was found (**Figure 2B**).

The *ladA* gene from *Geobacillus thermodenitrificans* was synthesized and cloned downstream of the constitutive promoter BBa_J23100 and expressed in *E. coli* (BBa_K398027). The enzyme activities of cells transformed with recombinant ladA protein or cells containing the vector control were determined *in vitro* by a resting cell assay using cell extracts and hexadecane as long-chain hydrocarbon substrate. GC-analysis indicated an enzyme activity of 3.33×10^{-3} U/mg of the *E. coli* carrying ladA while the activity of the negative control was 0.55×10^{-3} U/mg (**Figure 2B**).

The following two oxididation steps were performed using NAD dependent alcohol and aldehylde dehydrogenases (*ADH* and *ALDH* genes, BioBricks BBa_K398018, BBa_K398030 respectively). The degradation activity was measured using a resting cell assay with *E. coli* overexpressing ADH, *E. coli* overexpressing ALDH and *E. coli* carrying the vector control (BBa_J13002). For the ADH system, octanol-1 and dodecanol-1 were used as substrates, for the ALDH system octanal and dodecanal. No difference was observed between *E. coli* transformed with ADH, ALDH plasmids and the control (data not shown). We speculate that the alcohols and aldehydes did not cross the cell membrane *in vivo* and were therefore not metabolized. The activity of the respective enzyme was also measured in cell extracts (*in vitro*). Since the alcohol dehydrogenases use NAD as co-factor, the dehydrogenase activity can be determined from the reduction of NAD to NADH [11].

For cell extracts of the *E. coli* control strain, the activity for dodecanol-1 was measured at 0.58×10^{-3} U/mg. The recombinant strain, expressing ADH, had an activity of 1.76×10^{-3} U/mg, which is slightly higher (2-fold) than the wild type activity (**Figure 2B**). The expression of ALDH increased the dodecanal dehydrogenase activity of *E. coli* cell extracts 3-fold compared to the control strain, activities of respectively 1.75×10^{-2} U/mg and 0.52×10^{-2} U/mg (**Figure 2B**) were obtained. This suggests that the recombinant strains *E. coli* carrying ALDH can functionally convert octanal and dodecanal.

Sensing

The aim of the sensing module is to have an optimal use of cellular resources, *e.g.* only produce the alkane degradation pathway enzymes when required. To characterize and monitor the activity of the pCaiF promoter, GFP was cloned downstream the pCaiF promoter and the fluorescence signal of GFP in time was measured. *E. coli* cells with pCaiF-GFP and a control construct containing only the promoter were monitored at different glucose concentrations (2 g/l, 5 g/l and 10 g/l) and substrate mixtures containing lauric acid. At lower glucose concentration, the substrate was depleted more rapidly leading to a decreased growth rate, respectively no growth, after about 8 hours. At the same time, an increase in the GFP signal was observed (**Figure 3B**). Strains grown in high glucose medium did not produce GFP, because nitrogen limitation is reached before carbon limitation (data not shown). These results showed that pCaiF promoter is activated at low glucose levels. The result suggests that the promoter can be used to enable catabolic shifts from glucose to new degradation pathways. To further analyze the pCaiF promoter a mathematical model was derived using the following hypotheses.

1. *cAMP* levels are coupled to the *carbon influx:*

   It was assumed that the concept of flux sensing, as shown recently by Kotte *et al.* [13], can be applied to 'measure' the flux for the two carbon degradation pathways respectively glucose and fatty acid degradation. Kremling *et. al.* [14] has shown that scrY that is also dependent on the transcription factor Crp has a hyperbolic Hill relation (with n=6) to the $EIIA^{Crr}$ ratio. Assuming that the that $EIIA^{Crr}$ ratio is related to the cAMP levels, we used following relation between cAMP and the uptake flux q_{Glc}:

$$cAMP^{glc} = 1 - \frac{q_{glc}^3}{K_{glc,cAMP}^3 + q_{glc}^3}$$

   Similarly, a function was defined for cAMP levels in dependence of the lauric acid uptake:

$$cAMP^{lau} = 1 - \frac{q_{lau}^2}{K_{lau,cAMP}^2 + q_{lau}^2}$$

To combine the two levels, a multiplication was applied: in the presence of only one high flux, the level of cAMP will be close to zero, while in a situation with two low fluxes, the cAMP level will be high:

$$cAMP = cAMP^{lau}\ cAMP^{glc}$$

2. *Growth Stoichiometry: Yield, maximal uptake rates*:

The stoichiometric coefficients were taken from Lin *et al.*, with a slight adaptation to enable reaching the observed biomass concentrations [16]:

$$Y_{X/glc} = 0.6\ \frac{\mathrm{g_{CDW}}}{\mathrm{g_{glc}}}$$

Since for lauric acid no explicit value was found, it was assumed to be comparable to glucose:

$$Y_{X/lauric} = 0.5\ \frac{\mathrm{g_{CDW}}}{\mathrm{g_{lauric}}}$$

The maximal uptake rate on glucose was determined by Lin *et al.* [16] as:

$$q_{glc} = \frac{q_{glc,max}\ glc}{K_{glc} + glc},\quad q_{glc,max} = 1.3\ \frac{\mathrm{g_{CDW}}}{\mathrm{g_{lauric}\,h}},\quad K_{glc} = 0.035\ \frac{\mathrm{g}}{\mathrm{L}}$$

For lauric acid, a hyperbolic uptake relation was assumed and the pathway activity was directly modeled assuming a 'pathway enzyme' E_{lauric} with a specific activity $k_{E,lauric}$:

$$q_{lauric} = \frac{q_{lauric,max}\ lau}{K_{lauric} + lau},\ K_{lauric} = 0.15,\ \ q_{lauric,max} = k_{E,lauric}\ E_{lauric},\ k_{E,lauric} = 1000\ \frac{U}{\mu mol}$$

The growth rate depends on the uptake rate of both substrates:

$$\mu = Y_{X/glc}\left(q_{glc} - m\right) + Y_{X/lau}\ q_{lau}$$

Since in some experiments nitrogen limitation is reached before carbon limitation, growth was additionally assumed to depend on the nitrogen availability:

$$\mu = \mu\ N_{lim},\quad N_{lim} = 1 - \frac{X^2}{X_{0.5max}^2 + X^2},\quad X_{0.5max} = 0.12\ \frac{g_{CDW}}{g_{CDW}}$$

Notice that this represents a simplification of the real mechanism. In the experiments saturation was observed at OD=0.45, which corresponds to about 0.13 gCDW/l. The balances for biomass and substrates then read:

$$\frac{dX}{dt} = \mu X - k_{lysis} X$$

$$\frac{d\ glc}{dt} = q_{glc}\ X$$

$$\frac{d\ lau}{dt} = q_{lau}\ X$$

3. *Enzyme expression:*

$$\beta = \beta_{max} \frac{cAMP^{1.5}}{K_{E,cAMP} + cAMP^{1.5}}, K_{E,cAMP} = 0.9, \beta_{max} = 50 \frac{\mu mol}{gCDW\,h}$$

The expression of GFP under the control of pCaiF depends on the intracellular cAMP levels with a hyperbolic relationship (n=1.5, the concentration of Crp was assumed to be constant and not included in the model) as:

The parameters were chosen based on estimates found in Alon *et al.* [2]. With the dilution by growth and estimated protein degradation (k_{deg}), the balance for the enzyme read:

$$\frac{dE_{lauric}}{dt} = \beta - (\mu + k_{deg})E_{lauric} \quad , k_{deg} = 0.03\ 1/h$$

4. *Measurement Model:*

The model state variables biomass concentration X and GFP levels (intracellular) were measured using OD and fluorescence (GFP):

$$OD = X / 0.37 * 0.33$$

$$GFP = r_{GFP}\, GFP_{intra}\, X$$

The factor for OD/biomass was taken from Debreczeny and Davies [5]. Because the wells were filled with 100 µl a correction for the shorter light pass was used. The response factor for GFP was estimated from parameter optimization.

Data from several experiments was used to parameterize the model described above (parameter estimation problem). The reproduction of the experimental results is shown in **Figure 3**. Please note that with the same parameters different experimental conditions (10 g/l glucose, 2g/l glucose, no lauric acid) can be reproduced (**Figure 3B**). From the model parameters it is concluded that the response of the pCaiF has a 'sharp' response especially to the glucose uptake rate, the PTS system activity. Given that the alkane degradation might reduce the cAMP levels, an auto-regulation is obtained. The enzyme is then produced in amounts required to sustain growth, which is also favorable for the purpose of alkane degradation in as much active cells as possible. More simulation results can be found at the following URL:

http://2010.igem.org/Team:TU_Delft#page=Modeling/pcaif-model

Hydrocarbon tolerance

To increase hydrocarbon tolerance, genes from various organisms were cloned into a plasmid and expressed in *E. coli.* The selection of genes was accompanied by an *in silico* approach to identify possible unwanted protein-interactions in the host organism. The novel software HIM was applied to search for potential homolog interaction partners of the cloned proteins in the *E. coli* host. We blasted the solvent tolerance protein, prefoldin from *P. horikoshii* in HIM (**Figure 4A**), which resulted in a list of interaction partners (**Figure 4B**). The proteins depicted in green are *E. coli* homologs of *P. horikoshii* proteins that interact with prefoldin (based on interactions described in the STRING database). The proteins depicted in red are interacting proteins in *P. horikoshii* that do not have a homolog counterpart in *E. coli.* An interaction with proteins for RNA processing was found in the host and in the target organism, hinting that recombinant prefoldin interacts similarly (**Figure 4B**).

We constructed a prefoldin BioBrick containing the phPFDα and phPFDβ genes. This solvent tolerance cluster (BBa_K398406) was expressed in *E. coli* K12. As a control, *E. coli* expressing BBa_J13002-was used. Experimentally, the prefoldin expressing cells were challenged by growth experiments in the presence of different amounts of *n*-hexane. The BBa_K398406 BioBrick clearly improved the growth rate under high *n*-hexane conditions. At 10% *n*-hexane, the *E. coli* strain expressing prefoldin showed a 50% growth rate increase compared to the control (**Figure 5**). Since prefoldin is functional in *E. coli*, one could argue that this might be a result of interacting with homologue proteins identified *in silico*, though no proof is available yet (**Figure 4B**).

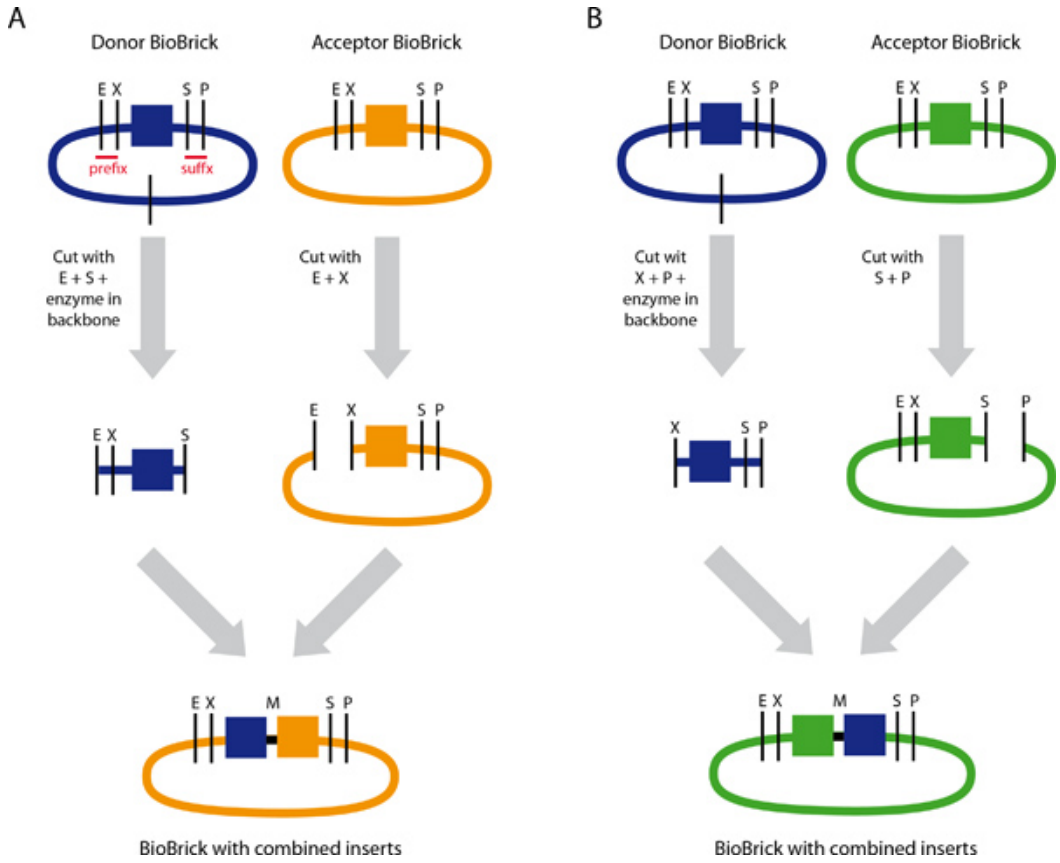

Figure 1. The BioBrick assembly method for combining two BioBrick inserts. The donor BioBrick is digested with either EcoRI and SpeI for positioning the donor part downstream of the acceptor part (**A**) or with XbaI and PstI for positioning the donor part upstream of the acceptor part (B). A third appropriate restriction enzyme is added that cuts the backbone of the donor to improve the success rate of the subsequent ligation reaction. The acceptor BioBrick is digested with either EcoRI and XbaI (A) or SpeI and PstI (**B**). The BioBrick parts are ligated together forming a BioBrick containing both inserts are again flanked by the 4 standard restriction sites.

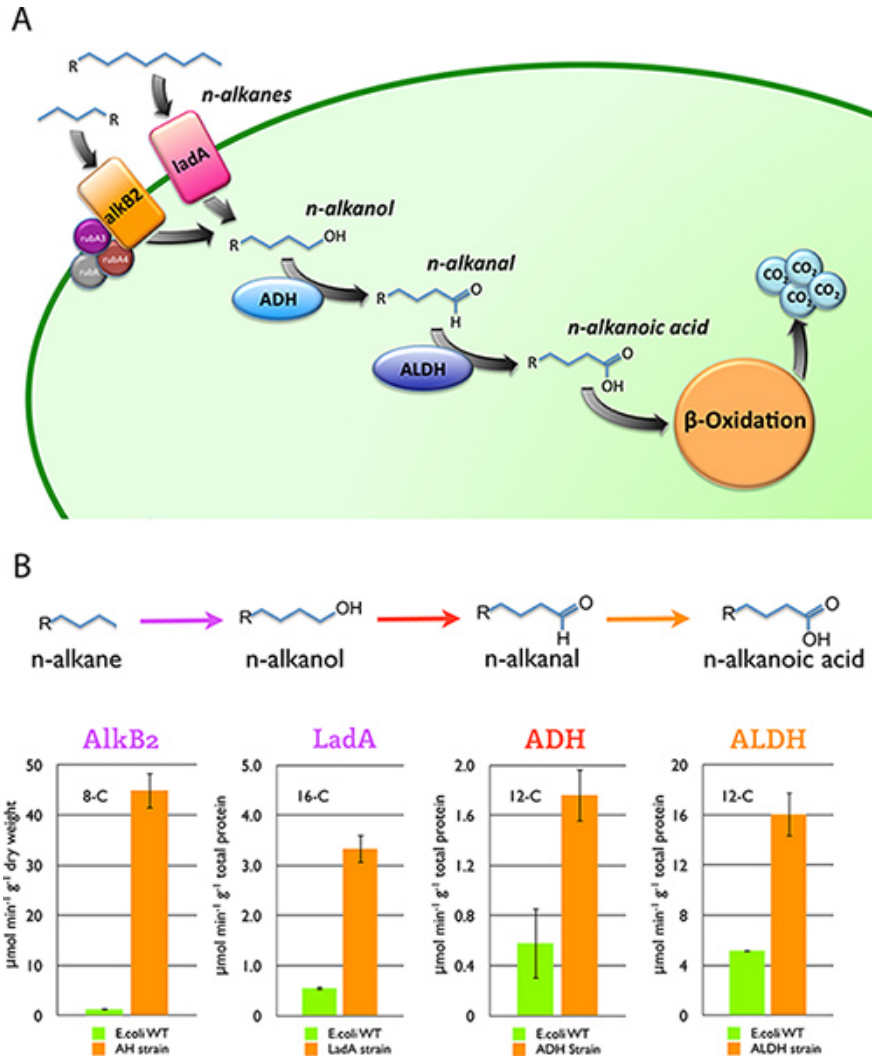

Figure 2. A three-step pathway for alkane oxidation was implemented in *E. coli.* (**A**) Flow chart of the implemented genes. Four genes (*alkB2*, *rubA3*, *rubA4* and *rubB*) of the alkane hydroxylase (AH) system of *Gordonia* sp. TF6 were used to facilitate the oxidation of the medium chain alkanes to their respective alkanols. The ladA gene from *Geobacillus thermodenitrificans* was implemented for the conversion of long-chain alkanes. To improve the degradation process for the alcohols or aldehydes, respectively *ADH* and *ALDH* genes (alcohol and aldehylde dehydrogenase) were expressed in *E. coli.* The resulting alkanoic-acids were metabolized via the host's β-oxidation pathway. (**B**) Enzyme activities of alkane conversion proteins for medium- and long-chain alkanes. The biotransformation of *n*-octane was monitored using resting cells assays in *E. coli* K12 carrying the BBa_K398014 plasmid, the BBa_K398017 plasmid, the BBa_K398018 plasmid, the BBa_K398030 plasmid or control plasmid BBa_J13002. The enzyme activities of ADH and ALDH were tested in cell extracts *in vitro* by monitoring the reduction of NAD to NADH. Error bars represent the SEM of three independent experiments. Click here to view larger figure.

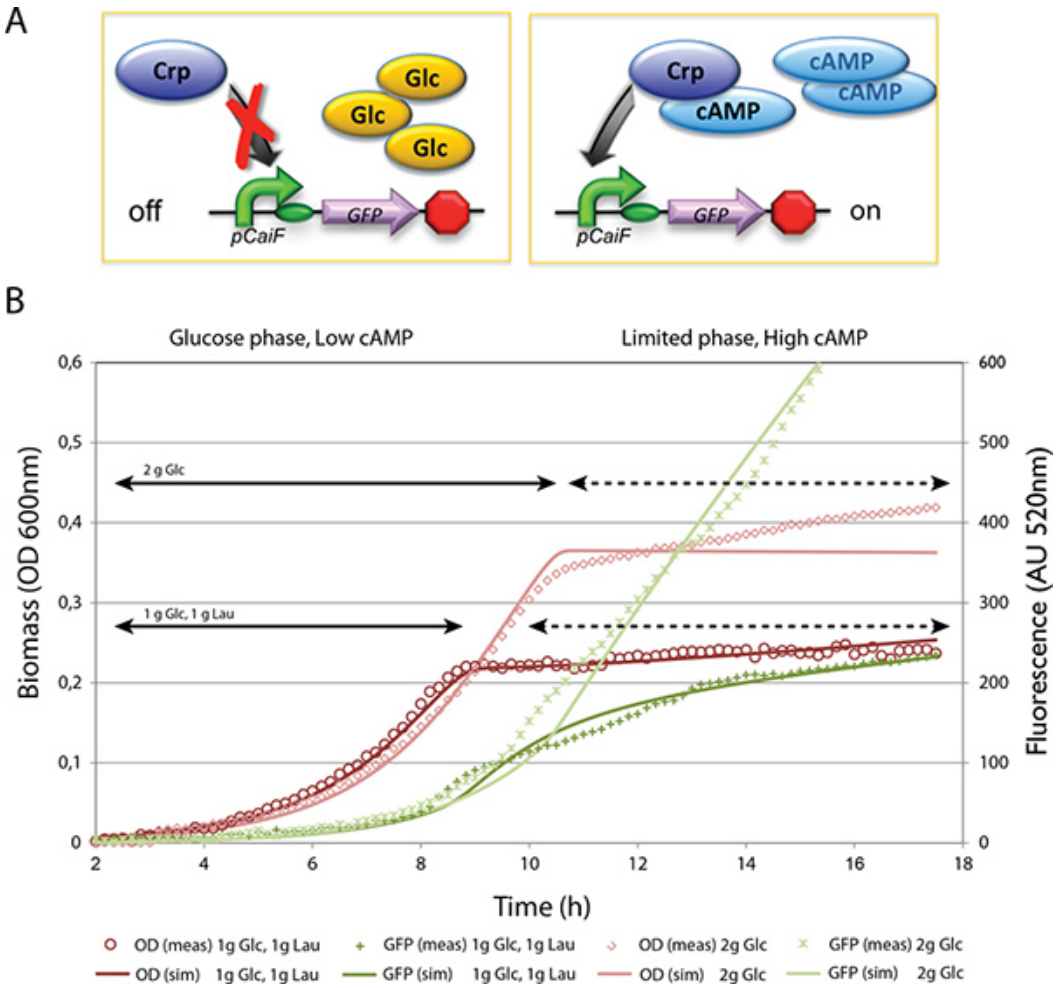

Figure 3. Mechanism and characterization experiment of the pCaiF promoter. (**A**) Mechanism of the regulation of the pCaiF promoter by cAMP-Crp levels. When the glucose concentration in the environment is high, cAMP levels are low. During depletion the cAMP level increases and so does the concentration of the complex cAMP-Crp. This complex will bind and activate the pCaiF promoter. (**B**) The biomass concentration (OD) and the fluorescence signal of *E. coli* K12 carrying pCaiF-GFP with 2g/l glucose. Click here to view larger figure.

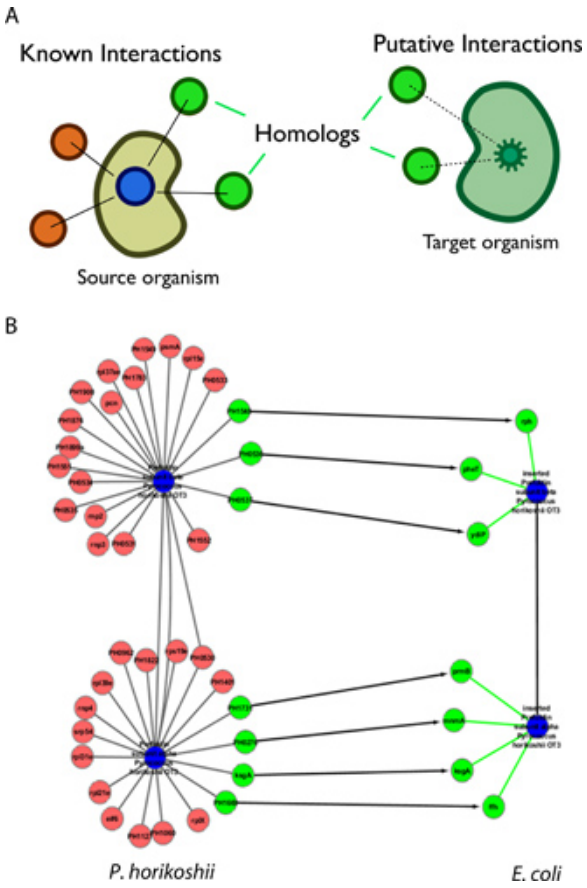

Figure 4. Homolog Interaction Mapping. (**A**) Schematic view of the HIM application. HIM generates a list of putative interactions of proteins in the host organism. Protein sequences can be entered in HIM and the software searches for the corresponding proteins in the STRING database. The application lists each known interacting protein in the source organism and searches for homologs in the host organism, resulting in a map of putative interactions partners for the cloned gene in the host organism. (**B**) HIM of the prefoldin protein from *P. horikoshii.* The proteins depicted in green are *E. coli* homologs of a *P. horikoshii* protein that interacts with prefoldin, according to the STRING database. The proteins depicted in red are interacting proteins in *P. horikoshii* that do not have a homolog in *E. coli.*

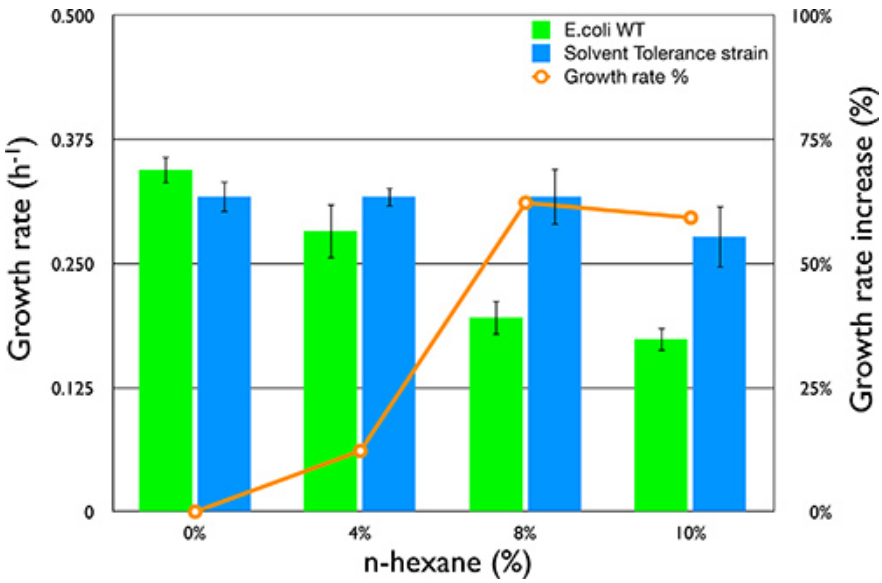

Figure 5. Solvent tolerance. The growth rate (OD_{600}) was measured of *E. coli* strains with a periodicity of 10 min. *E. coli* K12 expressing the solvent tolerance cluster (BBa_K398406) was tested by different amounts of *n*-hexane (0 ,4 ,8, 10%). As a control, *E. coli* expressing BBa_J13002-*ladA* was used. Error bars represent the SEM of three independent experiments.

Discussion

The BioBrick principle is used to construct a chassis for the degradation of alkanes and a proof of principle for the single components of the toolkit was obtained. Several assays are proposed to measure the *in vivo* and *in vitro* activity of alkane degrading pathway enzymes. The presented work successfully demonstrates a number of methods that can be used to determine enzyme activities and expression in the host organism *E. coli* after implementation of suitable BioBricks. Furthermore, it is shown that the BioBrick principle can be used to design an organism that expresses proteins necessary for the degradation of alkanes, provides a regulatory system that produces these enzymes only when necessary and augments the tolerance of the host organism in the presence of hydrocarbons. Once further developed, this chassis could be used for the biological degradation of residual oil, *e.g.* in oil sands tailing waters, or for the treatment of wastewater from the oil industry.

With regards to the alkane degradation, assays were performed for the characterization of the enzymes involved in the first step of alkane degradation (AH-system and ladA). It was demonstrated that an *E. coli* strain carrying the AH-system of *Gordonia* sp. TF6 was able to convert octane *in vivo.* This finding is in agreement with the results of Fujii *et. al.* [8], where biotransformation of *n*-alkanes using *E. coli* expressing the minimal component genes of the AH-system was achieved. The conversion activity was measured by a comparative study (wildtype vs. mutant) after a given time. For a full characterization, additional studies have to be performed on the activity of the AH-system over time and the kinetics of the system.

For the conversion of long-chain alkanes (C_{15}-C_{36}), the *ladA* gene from *Geobacillus thermodenitrificans* was implemented. The enzyme activity was tested *in vitro* using hexadecane as long-chain hydrocarbon source. This modified *E. coli* strain showed an increased enzyme activity compared to the control strain, demonstrating the recombinant ladA protein enables the conversion of hexadecane by *E. coli.* In addition to the property of converting alkanes to alkanols, *E. coli* strains were developed with an improved degradation process for the alcohols and aldehydes by the implementation of *ADH* and *ALDH* genes respectively. The enzyme activity in cell extracts was tested *in vitro* by measuring the production of NADH from NAD^+. The *E. coli* strain expressing ADH showed a two-fold increase in alcohol dehydrogenase activity compared to the wild type activity. Compared to the control strain, the expression of ALDH protein increased the dodecanal dehydrogenase activity in *E. coli* cell extracts three-fold. Since these assays clearly demonstrated increased enzyme activity *in vitro*, it can be speculated that the transformed *E. coli* strains have elevated levels of activity *in vivo* as well.

To explore the possibility of host-induced expression (for instance: non-dependence on induction chemicals and low burden during growth), the degradation pathway was expressed under the control of the pCaiF promoter. This promoter activates gene expression when glucose levels are low, *e.g.* at the end of a batch growth phase. As a proof of principle, this promoter was tested via the production of GFP under difference glucose regimes. It was demonstrated that the pCaiF-GFP transformed *E. coli* produced more GFP during stationary (glucose-limited) phase than in the exponential phase (high glucose levels). In the presence of a secondary carbon source (*e.g.* lauric acid), the GFP production rate decreased again due to the catabolism of the secondary carbon-source.

The experiment showed that this promoter can be effectively used to enable catabolic shifts from glucose to new degradation pathways. This enables to rapidly grow a vast amount of organisms in rich medium before the degradation pathway is activated. We propose to use the pCaiF promoter upstream the AlkS transcriptional regulator. In *Pseudomonas putida*, AlkS recognizes C_5-C_{10} *n*-alkanes as effectors. Subsequently high levels of AlkS-alkane complex activate the PalkB promoter resulting in the expression of the alkBFGHJKL operon encoding proteins involved in the conversion of *n*-alkanes to fatty acids [19].

Sensing and conversion of hydrocarbons is not enough for the cell to be viable. At increasing hydrocarbon levels in the environment, the growth of wild type *E. coli* is inhibited through the presence of hydrocarbons in the membrane and in the cell, which can cause protein misfolding. *P. horikoshii* prefoldin is a chaperone aiding the correct folding of protein in the presence of alkanes [17]. Using HIM, it was predicted that this protein interacts with homolog *E. coli* chaperone proteins, suggesting that overexpression of prefoldin in *E. coli* could improve solvent tolerance. With the BioBrick BBa_K398406 (consisting of *phPFDα* and *phPFDβ* genes), the survivability of *E. coli* in the presence of alkanes was improved up to 50%. HIM is a suitable tool to select potential functional homologs.

Considering the toolbox as a whole, it can be concluded that it provides a first step in the construction of a chassis for bioremediation of hydrocarbons in aqueous environments. This represents a small, autonomous, self-replicating and relatively inexpensive method. The results were mainly acquired through enzyme assays and shake flash cultures and thus need to be validated on a larger scale. Further research on this system should focus on the coupling of the different enzymes, which have been shown here to work individually in order to generate the envisioned system for hydrocarbon degradation. Furthermore, addition of alternative pathways for the degradation of other pollutants could as well expand the use of this chassis beyond the bioremediation of hydrocarbons alone to a broader scope of pollutants, and possibly the implementation of product pathways could even utilize this system for the production of valuable chemicals. Our results emphasize the suitability of the BioBricks assembly strategy in synthetic biology to construct new organisms that can deal with oil pollution, and may additionally be useful to develop a wide variety of other applications.

Disclosures

No conflicts of interest declared.

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References

1. Allen, E.W. Process water treatment in Canada's oil sands industry: I: Target pollutants and treatment objectives. *J. Environ. Eng. Sci.* **7**, 123-138 (2008).
2. Alon, U. An Introduction to Systems Biology: Design Principles of Biological Circuits. *CRC Press.*, (2007).
3. Center, O.P. Understanding Oil Spills and Oil Spill Response. (1999).
4. Cytoscape. An Open Source Platform for Complex Network Analysis and Visualization. http://www.cytoscape.org/ (2012).
5. Debreczeny, M.P. & Davies E.T. Non-Invasive Biomass Monitor with Wide Linear Range. http://www.buglab.com/docs/OnLineODMeasurement.pdf (2012).
6. Eichler, K., Buchet, A., Lemke, R., Kleber, H.P., & Mandrand-Berthelot, M.A. Identification and characterization of the caiF gene encoding a potential transcriptional activator of carnitine metabolism in *Escherichia coli*. *J. Bacteriol.* **178**, 1248-1257 (1995).
7. Feng, L., Wang, W., Cheng, J., Ren, Y., Zhao, G., Gao, C., Tang, Y., Liu, X., Han, W., Peng, X., *et al.* Genome and proteome of long-chain alkane degrading *Geobacillus thermodenitrificans* NG80-2 isolated from a deep-subsurface oil reservoir. *Proc. Natl. Acad. Sci. U.S.A.* **104** (13), 5602-5607 (2007).
8. Fujii, T., Narikawa, T., Takeda, K., & Kato, J. Biotransformation of various alkanes using the *Escherichia coli* expressing an alkane hydroxylase system from Gordonia sp. TF6. *Biosci. Biotechnol. Biochem.* **68** (10), 2171-2177 (2004).
9. iGEM. Registry of Standard Biological Parts. http://partsregistry.org/Main_Page (2012).
10. Kato, T., Miyanaga, A., Haruki, M., Imanaka, T., Morikawa, M., & Kanaya, S. Gene cloning of an alcohol dehydrogenase from thermophilic alkane-degrading *Bacillus thermoleovorans* B23. *J. Biosci. Bioeng.* **91** (1), 100-102 (2001).
11. Kato, T., Miyanaga, A., Kanaya, S., & Morikawa, M. Gene cloning and characterization of an aldehyde dehydrogenase from long-chain alkane-degrading *Geobacillus thermoleovorans* B23. *Extremophiles.* **14**, 33-39 (2010).
12. Kieboom, J. & De Bont, J.a.M. Bacterial Stress Responses. Washington, D.C., *ASM Press.*, (2000).
13. Kotte, O., Zaugg, J.B., & Heinemann, M. Bacterial adaptation through distributed sensing of metabolic fluxes. *Mol Syst Biol.* **6** 355 (2010).
14. Kremling, A., Bettenbrock, K., & Gilles, E.D. Analysis of global control of *Escherichia coli* carbohydrate uptake. *BMC Syst. Biol.* **1**, 42 (2007).
15. Li, L., Liu, X., Yang, W., Xu, F., Wang, W., Feng, L., Bartlam, M., Wang, L., & Rao, Z. Crystal structure of long-chain alkane monooxygenase (LadA) in complex with coenzyme FMN: unveiling the long-chain alkane hydroxylase. *J. Mol. Biol.* **376** (2), 453-465 (2008).
16. Lin, H.Y., Mathiszik, B., Xu, B., Enfors, S.O., & Neubauer, P. Determination of the maximum specific uptake capacities for glucose and oxygen in glucose-limited fed-batch cultivations of *Escherichia coli*. *Biotechnol. Bioeng.* **73** (5), 347-357 (2001).
17. Okochi, M., Kanie, K., Kurimoto, M., Yohda, M., & Honda, H. Overexpression of prefoldin from the hyperthermophilic archaeum *Pyrococcus horikoshii* OT3 endowed *Escherichia coli* with organic solvent tolerance. *Appl. Microbiol. Biotechnol.* **79** (3), 443-449 (2008).
18. Rehm, H.J. & Reiff, I. Mechanisms and occurrence of microbial oxidation of long-chain alkanes. *Adv. Biochem. Eng. / Biotechnol.* **19**, 175-215 (1981).
19. Rojo, F. Degradation of alkanes by bacteria. *Environ. Microbiol.* **11** (10), 2477-2490 (2009).
20. STRING 9.0 Known and Predicted Protein-Protein Interactions. http://string-db.org/newstring_cgi/show_download_page.pl (2012).
21. Van Beilen, J.B., Panke, S., Lucchini, S., Franchini, A.G., Rothlisberger, M., & Witholt, B. Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. *Microbiology.* **147** (Pt. 6), 1621-1630 (2001).
22. Wang, L., Tang, Y., Wang, S., Liu, R.L., Liu, M.Z., Zhang, Y., Liang, F.L., & Feng, L. Isolation and characterization of a novel thermophilic Bacillus strain degrading long-chain n-alkanes. *Extremophiles.* **10** (4), 347-356 (2006).

