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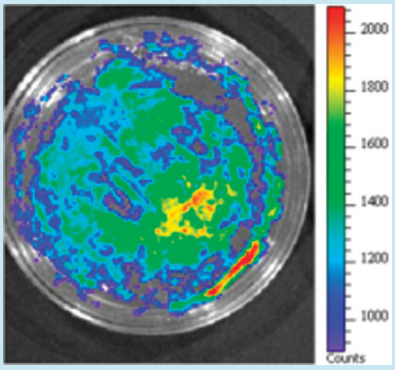
Monitoring Alkane Degradation by Single BioBrick Integration to an Optimal Cellular Framework

Suvi Santala,* Matti Karp, and Ville Santala

Department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland

ABSTRACT: Synthetic biology enables rewiring and reconstruction of desirable biochemical routes using well-characterized BioBricks. One goal is to optimize these biological systems in terms of robustness, functionality, and simplicity. Thus, in addition to optimizing the molecular level of the metabolic network, choosing an optimal "chassis" can have a great significance in the constructed system. As an example, this study presents a simplified system for monitoring and studying long-chain *n*-alkane degradation in *Acinetobacter baylyi* ADP1 online, provided by a single BioBrick insertion, bacterial luciferase *luxAB*. The system exploits the natural alkane degradation machinery of ADP1 and a sensitive response of bacterial luciferase to a specific intermediate, providing important aspects to natural alkane degradation kinetics. The study suggests the monitoring system to be applicable in the field of environmental biotechnology and emphasizes the utility of ADP1 as a host in both model systems and applications.



KEYWORDS: *bioluminescence, Acinetobacter baylyi ADP1, alkane degradation, synthetic biology*

One goal of synthetic biology is to provide a sufficient toolbox of well-characterized, compatible BioBricks enabling new types of cell design in a straightforward, global, and even an automated manner.[1,2] As a well-known work horse, *Escherichia coli* has served as the obvious host for research and applications of synthetic biology. However, depending on the objective and the engineered pathway, introducing a new host, or "chassis", can significantly change the nature of the constructed system as the natural biochemical pathways can be exploited. When integrated to the host's metabolism, the role and function of a biocomponent can be altered and even multiplied, expanding the possibilities of a single BioBrick. Thus, the optimal combination of genetic tools and cellular framework should be chosen on the basis of the goal, taking into account the host's natural metabolism and characteristics.

Acinetobacter baylyi ADP1 has been recognized as a new potential model organism for genetic and metabolic studies on microbes, providing a promising cellular platform for applications and model systems of various fields of biotechnology.[3,4] The unique and diverse metabolism and the exceptional competence for natural transformation and homologous recombination make ADP1 a compelling alternative for *E. coli* as a synthetic biology host.[5] The genetic similarity between ADP1 and *E. coli* enables the exploitation of the knowledge applied to *E. coli*,[6] also providing compatibility with the existing BioBrick standards. Gene inactivation, promoter studies, and working with large linear DNA fragments can be carried out effortlessly. Also, a constraint-based metabolic model and a single gene knockout mutant library for ADP1 have been constructed.[7,8]

Long-chain hydrocarbon utilization of several *Acinetobacter* species has been reported,[9−11] and the efficient degradation of *n*-alkanes has been also brought up in the context of bioremediation.[12,13] *Acinetobacter* species have been frequently found in environments contaminated with petrochemicals and other oil-derived pollutants.[14,15] The pathway of *n*-alkane degradation involves several enzymatic steps and intermediates,[9] of which a corresponding long-chain aldehyde, a suitable substrate for bacterial luciferase LuxAB,[16] is one representative. For sensor applications, whole-cell biosensors for detection of long-chain hydrocarbons exploiting bacterial luciferase and heterogeneous catabolic genes or specific transcription regulation system have been constructed in recombinant *E. coli*.[17,18] However, many of these systems require a construction of an artificial response system and/or use of external substrate. Exploiting a natural alkane-degrading host provides the simplest possible model system and improves the understanding of dynamics and kinetics of oil and *n*-alkane degradation in variable conditions. For example, *Acinetobacter* species produce bioemulsifiers that significantly affect the bioconversion dynamics by improving the bioavailability of *n*-alkanes, petroleum, and other hydrocarbon pollutants.[19]

In this study, a simple monitoring system for *n*-alkane metabolism using a single BioBrick was constructed in a new potential synthetic biology chassis, *A. baylyi* ADP1, serving as a model platform for studies regarding microbial *n*-alkane metabolism and hydrocarbon degradation potential in contaminated soils.

In order to study the *n*-alkane metabolism in *A.baylyi* ADP1, a synthetic gene cassette[4] containing bacterial luciferase *luxAB* was constructed using well-characterized biocomponents: a constitutive strong promoter, transcription termination loop,

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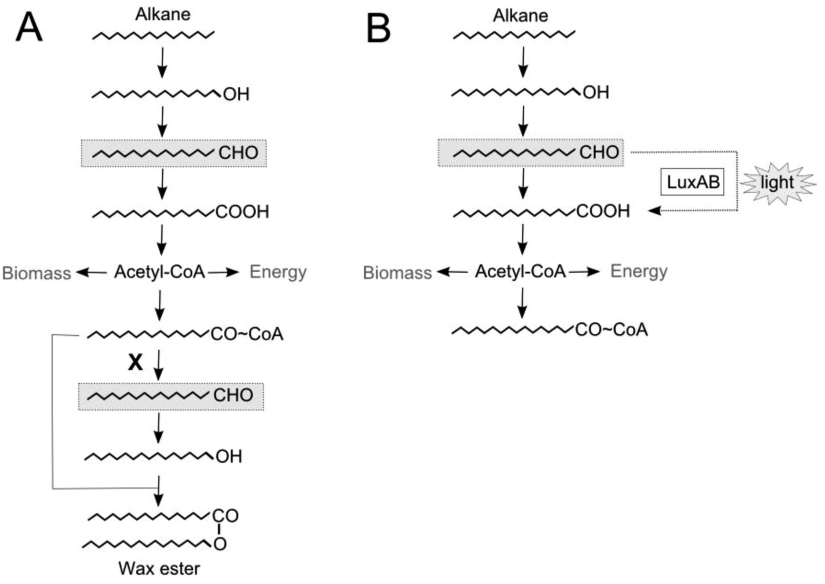

Figure 1. Schematic representation of the *n*-alkane metabolism of (A) *Acinetobacter baylyi* ADP1 wild type and (B) the monitoring system in the engineered strain Oab+. The fatty aldehydes (substrate for LuxAB) are marked with gray. By a single gene knockout the strain's natural anabolic aldehyde producing pathway (X) is shut down, resulting in an unambiguous and simplified monitoring system when bacterial luciferase *luxAB* is inserted.

selection marker (cm^r), and homologous sequences from ADP1 (downstream and upstream of ACIAD3381, which was shown to be a neutral deletion in terms of hydrocarbon metabolism). The gene cassette designated as *iluxAB* was introduced to the genome of *A. baylyi* ADP1Δ*acr1*::Kan^r/*tdk*[8] by natural transformation, as described previously.[3] The single gene knockout mutant of ADP1 lacking the fatty acyl-CoA reductase *acr1* was used in the study in order to obtain a specific response to external long-chain hydrocarbons; the strain's natural anabolic aldehyde producing pathway related to storage lipid metabolism is shut down by the deletion, resulting to an unambiguous and simplified monitoring system (Figure 1). The resulting strain ADP1Δ*poxB*:: *iluxAB*,Δ*acr1*:: Kan^r/*tdk* was designated as Oab+.

For observation of cell growth and the response of the luminescent construct to hydrocarbons of chain lengths C12–C18 as a carbon and energy source, cultivations in MA/9 minimal salts medium were carried out at 30 °C and 300 rpm for 48 h. Cultivation with glucose as a carbon source was used as a control. The results for the end-point measurement are presented in Figure 2. Luminescence signal response could be detected for all tested carbon chain lengths, thus showing relatively wide substrate range and stability for LuxAB protein originated from *Photorhabdus luminescens.* For the alkanes there was a correlation between carbon chain length and growth, indicating a preference of utilization for the higher alkanes C16 and C18. The same trend in carbon utilization was observed with control cultivations carried out with the strain ADP1Δ*acr1*:: Kan^r/*tdk* without the expression of LuxAB, indicating that expression of LuxAB does not affect the alkane usage preference. Slight decrease in overall growth rate was detected for LuxAB-expressing cells growing on alkanes; this can be partly explained by the loss of oxidation energy in form of luminescence. For glucose-grown cells, no differences in growth patterns were observed between the strains, indicating that expression of LuxAB does not cause any genetic load in terms of ADP1 growth (data not shown).

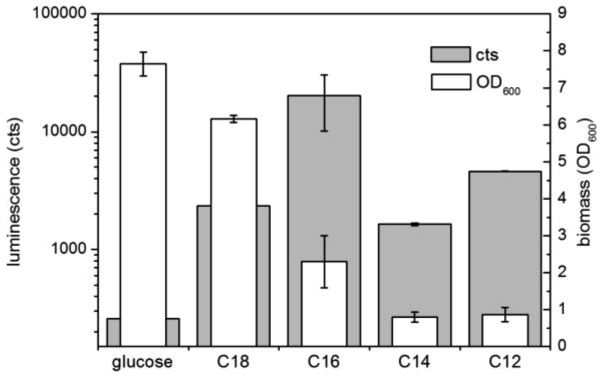

Figure 2. End-point biomass (OD_{600}) and the corresponding luminescent signal (counts, cts) of the strain ADP1Δ*poxB*:: *iluxAB*,Δ*acr1*:: Kan^r/*tdk* (designated as Oab+) grown for 48 h in MA/9 medium supplemented with a long-chain hydrocarbon (C12, C14, C16, or C18) or glucose as a carbon and energy source.

For studying the kinetics of cellular and molecular response to the long-chain *n*-alkanes and diesel oil, cells grown overnight on agar plates were exposed to glucose, alkane, and diesel oil samples; droplets of glucose solution, alkanes C12–C18, and petrodiesel were applied on cell lawns on individual plates, after which the luminescent signal data were monitored for 13 h in static conditions without sampling. For visualization and quantification of luminescence an IVIS Lumina was used (Figure 3). During the incubation, the maximal radiance for a pixel within the plate was measured for the plate exposed to petrodiesel sample. The light-emission data collected from each time point for the petrodiesel-containing plate and the for glucose-containing plate, respectively, are presented in Figure 4A. According to the kinetic data shown in Figure 4B, the most rapid relative response was obtained for C12, which can be simply related to the more preferred carbon chain length of the substrate for LuxAB. Typically petrodiesel contains mainly saturated hydrocarbons of average chain length C12, thus serving as an appropriate target compound for the monitoring

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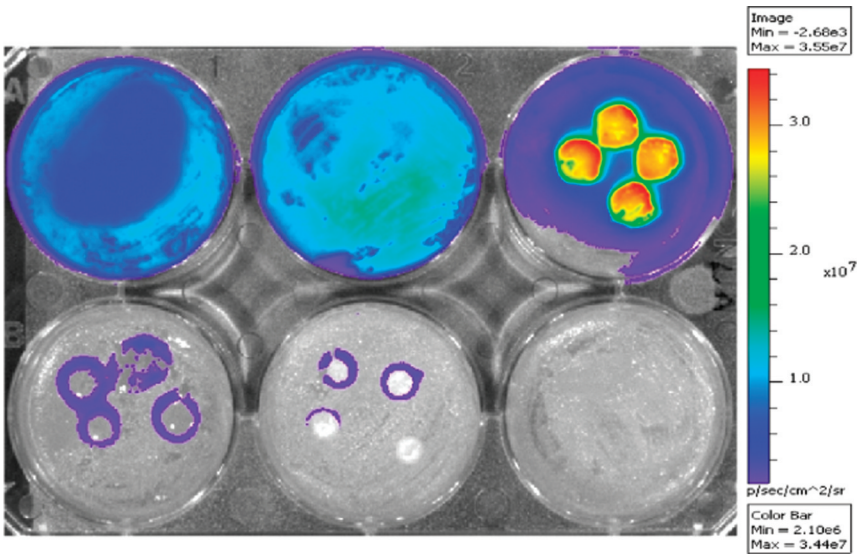

Figure 3. Visualization of the LA-grown Oab+ cells after 13 h of incubation at room temperature with long-chain hydrocarbon samples and glucose. Upper row: C12, C14, petrodiesel. Lower row: C16, C18, glucose (control). Due to different viscosity properties of alkane samples the droplets are dispersed unevenly on the plate surface, affecting the signal distribution.

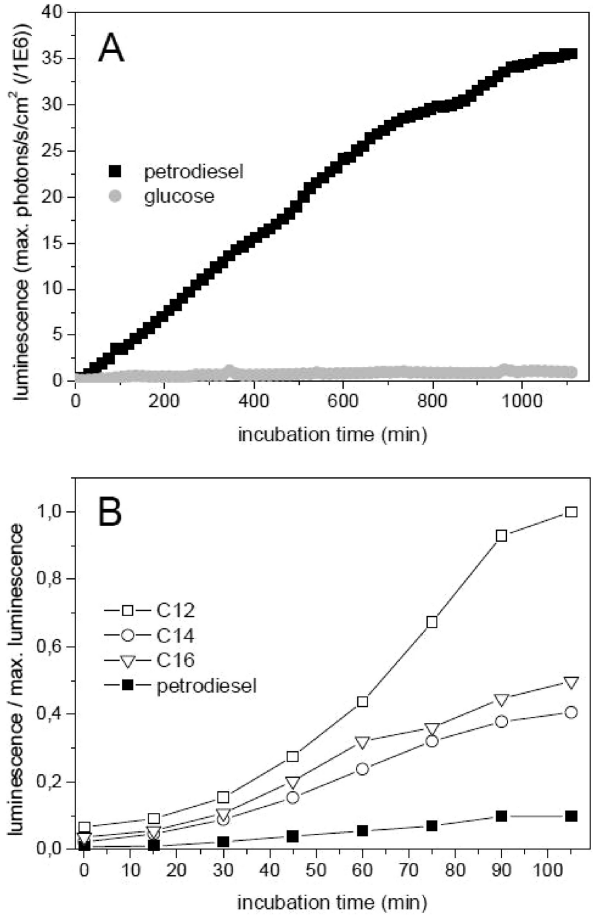

Figure 4. Kinetics of the cellular luminescent response to different *n*-alkanes and petrodiesel of LA-grown Oab+ cells. (A) Maximal light emission obtained for petrodiesel and glucose as a function of incubation time. (B) Kinetics of the relative response time to different hydrocarbon samples as a function of incubation time (the signals are proportioned to the maximum signals of the time-scale used).

system in natural samples. The delay in the signal increase compared to the pure *n*-alkane C12 might be due to the slightly toxic nature of other constituents of petrodiesel, such as aromatic hydrocarbons and impurities. For C18, solidification of droplets was assumed to affect the signal rates. In the case of glucose used as negative control, no detectable signal was observed.

The analysis of the alkane-degrading pathway and synthetic biology approach enabled the construction of a simple real-time method for monitoring long-chain hydrocarbon degradation in a natural oil-degrading model host. The luminescence response for long-chain hydrocarbons was tested for both actively alkane-growing cells and LA-grown cells at stationary growth phase exposed to long-chain *n*-alkane and petrodiesel samples. According to the results, a specific luminescent signal could be obtained for all tested hydrocarbon samples regardless of the cell growth phase and the initial carbon and energy source. The suitability and stability of LuxAB for a new type of monitoring application was demonstrated, imposing no significant genetic load on ADP1 growth. Thus, it was shown that by combining the metabolic engineering approach, a well-characterized and widely used BioBrick, and an appropriate host with efficient biodegradation capabilities, a more robust and stripped-down tool for kinetic studies regarding bacterial hydrocarbon catabolism can be developed. However, for interpretation of the system functionality and competitiveness in actual applications, further tuning of the genetic construct should be carried out, e.g., by testing other potential bacterial luciferases for the monitoring system. Also, deeper analysis on the metabolic effect of different LuxAB enzymes on alkane metabolism is required.

Microbial catabolic pathways on biotechnologically relevant molecules are widely studied via molecular tools and enzymatic assays, but the methods are somewhat laborious and time-consuming. Some luminescence-based tools for biosensing applications, mainly relying on recombinant *E. coli*, exist. These systems, however, often require a complex construction of an artificial degradation system or transcription regulation and possibly the use of external substrate and are thus not sufficient

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in terms of modeling the natural hydrocarbon catabolism. *A. baylyi* ADP1 serves as an excellent cellular framework for studying the catabolism and degradation potential in a natural hydrocarbon degrading host; the exceptional competence for natural transformation and metabolic engineering, the broad biodegradation capabilities, and abundance in nature and actual application sites are highly valued also in several other fields of biotechnology. Furthermore, this monitoring system potentially enables rapid and high-throughput screening for efficient alkane degraders. This is the first time that ADP1 hydrocarbon metabolism is described and studied via a luminescence-based built-in monitoring system. This proof of principle case study emphasizes the ease of use, simplicity, and potentiality of ADP1 to be exploited in applications and model systems regarding metabolic engineering and synthetic biology.

METHODS

Strains. A single gene knockout mutant of *A.baylyi* ADP1 (gene deletion ACIAD3383, accession number for the wild-type strain DSM24193) was used in the study, kindly provided by Dr. Veronique de Berardinis (Genoscope, France). In the single gene knockout mutant, the gene in question is replaced with a gene cassette containing a kanamycin resistance gene (Kan^r).[8] The strain was transformed with the construct *iluxAB* as described in the next section.

Genetic Engineering. The molecular work was carried out by using methods described by Sambrook et al.[20] For digestions and ligations, the enzymes and buffers were provided by Fermentas (Lithuania) and used according to manufacturer's instructions. PCR reagents were provided by Finnzymes (Finland) (DNA polymerase PhusionTM and buffer) and Fermentas (nucleotides). Primers were ordered from Thermo-Fisher Scientific (USA) with appropriate restriction sites.

The natural transformation of ADP1Δ*acr1*:: Kan^r/*tdk* was carried out by methodology described previously.[4] The colonies were selected on LA plates containing chloramphenicol (50 μg/mL) and glucose. The construct in the obtained strain was verified with colony PCR and further by sequencing. A synthetic gene cassette described by Santala et al.[4] was used as a scaffold for the construction of gene cassette *iluxAB* with flanking regions from neutral target knockout gene ACIAD3381. The bacterial luciferase gene *luxAB* (the corresponding BioBrick part BBa_K216008) was amplified from the plasmid pCGLS1[21] with primers ab39 (5′-ATATCATATGAAATTTGGAAACTTTTTGCTTAC-3′) and ab40 (5′-ATATCTCGAGTTAGGTATATTC-CATGTGGTACTTCTTAATATTATC-3′) and inserted to the gene cassette using restriction sites *Nde*I and *Xho*I. The flanking region Gene_I′ (downstream of ACIAD3381) was amplified from ADP1 genome with primers ab59 (5′-AATACCTAGGAGGTCAATTCTCCAGCTTTTTTATC-3′) and ab60 (5′-AATAGGCCCCCGAGGCCTGTCAAAAGCA-TAGGAAGTGG-3′) and cloned to the gene construct using restriction sites *Avr*II and *Sfi*I. The final gene cassette was amplified by PCR with primers ab57 (5′-ATATGGTACCCA-CACCAATTTTAGCACCCGGAAAAAATG-3′) and ab60, the final product being 4112 bp long. Purification of the PCR products was carried out in every step using PCR purification kit (Fermentas) or gel extraction kit (Fermentas) for agarose gel.

Cultivations and Luminescence Determination. For observation of cell growth and the response of the luminescent construct to different carbon chain lengths, duplicate cultivations in 5 × 5 mL MA/9 minimal salts medium[4] were carried out at 30 °C and 300 rpm. The medium contained 0.05% casein amino acids and 2.0% of glucose, dodecane (C12), tetradecane (C14), hexadecane (C16), or octadecane (C18) as an energy and carbon source. The *in vivo* luminescence was measured from parallel 200 μL samples after 48 h using a Victor 2 plate reader (Perkin-Elmer Life Sciences, Finland), and growth was determined by optical density measurement at 600 nm wavelength, respectively. For controlling the effect of *luxAB* expression on alkane utilization and strain performance, the same cultivations with the strain ADP1Δ*acr1*:: Kan^r/*tdk* were carried out.

For studying the kinetics of cellular and molecular response to the long-chain *n*-alkanes and diesel oil, the cells were plated from actively growing culture and grown overnight on LA plates (tryptone 10 g/L, yeast extract 5 g/L, NaCl 1 g/L, and agar 15 g/L), after which droplets (total 50 μL) of glucose solution (40%), alkanes C12−C18, and petrodiesel (obtained from fuel company St1, Finland) were applied on individual plates. The plates were incubated at room temperature for 13 h. An IVIS Lumina (Caliper Life Sciences, USA) system was used for visualization and quantification of luminescent signal; overlaid photo and light-emission data were collected every 15 min with 10 min exposure time, and the maximal radiance for a pixel within the plate was determined.

AUTHOR INFORMATION

Corresponding Author

*Tel: +358 3 3115 11. Fax: +358 3 3115 2869. E-mail: suvi.santala@tut.fi.

Author Contributions

S.S. and V.S. designed the study. S.S. performed the molecular and microbiological work and wrote the manuscript. S.S. and V.S. performed the experimental analyses. V.S. and M.K. supervised and coordinated the study. All authors approved the final manuscript.

Notes

The authors declare no competing financial interests.

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