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Expanding the Product Profile of a Microbial Alkane Biosynthetic Pathway

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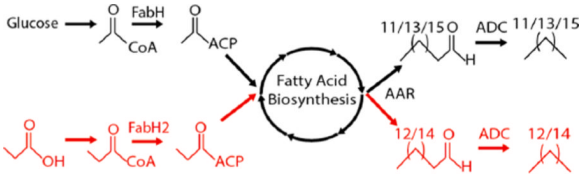
Abstract



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ASSOCIATED CONTENT

Supporting Information

Complete materials and methods as well as supporting tables and figures. This information is available free of charge via the Internet at http://pubs.acs.org/.

Author Contributions

M.H. and L.Z. performed the molecular and microbiological work, analytical chemistry, designed the study, and wrote the manuscript. A.M., C.A., J. A, C.C., Y. L., B. M., D. Z., M.D.S., R.G.E., J.H.M., and D.B., were involved in experimental planning and writing the manuscript. I.S.P. and J.B.S supervised and coordinated the study.

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Microbially produced alkanes are a new class of biofuels that closely match the chemical composition of petroleum-based fuels. Alkanes can be generated from the fatty acid biosynthetic pathway by the reduction of acyl-ACPs followed by decarbonylation of the resulting aldehydes. A current limitation of this pathway is the restricted product profile, which consists of *n*-alkanes of 13, 15, and 17 carbons in length. To expand the product profile, we incorporated a new part, FabH2 from *Bacillus subtilis*, an enzyme known to have a broader specificity profile for fatty acid initiation than the native FabH of *Escherichia coli*. When provided with the appropriate substrate, the addition of FabH2 resulted in an altered alkane product profile in which significant levels of *n*-alkanes of 14 and 16 carbons in length are produced. The production of even chain length alkanes represents initial steps toward the expansion of this recently discovered microbial alkane production pathway to synthesize complex fuels. This work was conceived and performed as part of the 2011 University of Washington international Genetically Engineered Machines (iGEM) project.

Keywords

alkane biosynthesis; biofuels; fatty acid biosynthesis; synthetic biology

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It has been recently demonstrated that the production of alkanes in *Escherichia coli* can be achieved through the expression of two genes: *aar* and *adc*, from the cyanobacterium *Synechococcus elongatus* PCC7942.[1] Acyl-ACP reductase (AAR) reduces fatty acyl-ACPs to the corresponding aldehydes, which are then decarbonylated by aldehyde decarbonylase (ADC) to produce alkanes that are one carbon shorter than the original fatty acyl-ACPs (Figure S1, Supporting Information). Using this system, the production of the odd chain length *n*-alkanes tridecane, pentadecane, and heptadecane has been demonstrated in *E. coli*.[1] However, petroleum based fuels are composed of a significantly more diverse set of alkanes ranging from 10 to 20 carbons in length and composed of linear and branched alkanes with varying degrees of oxidation.[2] Expanding the product profile of this alkane biosynthesis pathway to more closely match petroleum based fuels will be critical for the utilization of microbially produced alkanes as “drop-in” replacement biofuels.

One approach to broaden the downstream alkane production profile is to modify upstream intermediates in fatty acid biosynthesis. The initiation step of fatty acid biosynthesis is controlled by a single enzyme, FabH, and therefore represents a highly attractive target for the modification of upstream fatty acid composition (Figure S1, Supporting Information). FabH catalyzes the condensation of acyl-CoA and malonyl-ACP to form 3-ketoacyl-ACP, as the first step in fatty acid elongation.[3] Native *E. coli* FabH has high specificity for the two-carbon unit acetyl-CoA,[4] resulting in the production of even chain length fatty acids, which are converted into odd chain length alkanes by the AAR and ADC alkane biosynthetic pathway. The introduction of a new enzyme into this system with relaxed substrate specificity for fatty acid biosynthesis initiation could enable the production of a different spectrum of alkanes. FabH2, from *Bacillus subtilis*, represents a variant of this class of enzyme that has been shown to have higher levels of activity than *E. coli* FabH on acyl-CoAs with chain lengths longer than two carbons.[4] In this study, we demonstrate that incorporation of FabH2 into the AAR and ADC alkane production system results in an expanded alkane product profile when the appropriate CoA starter unit is present.

It bears mentioning that this project was conceptualized and conducted by a team of University of Washington undergraduate students participating in the 2011 international Genetically Engineered Machines competition (iGEM). These students attended an undergraduate synthetic biology course during winter and spring quarters to gain exposure to synthetic biology concepts and techniques and planned projects that would use these

techniques to address current problems. A subgroup of students identified the recently discovered AAR and ADC-produced alkanes pathway as a system that they could potentially enhance by expanding product profile using synthetic biology principles. This new biological system was constructed and characterized by these students during the summer quarter under the supervision of graduate student, postdoctoral, and faculty mentors. The work presented here demonstrates that undergraduate students with training in synthetic biology techniques can make significant contributions to the field of synthetic biology.

METHODS AND RESULTS

The previously reported alkane production system was not constructed in a standardized format. Therefore, we first reconstructed it from its constituent parts such that it conforms to the synthetic biology BioBrick standards.[5] The *aar* and *adc* genes from *S. elongatus* PCC7942 were codon optimized for expression in *E. coli*, and synthesized from oligonucleotides. The two genes were cloned adjacent to each other in a high copy number, constitutive expression vector to form part BBa_K590025. The resulting plasmid was transformed into *E. coli* BL21(DE3) cells, and alkane production was assessed using gas chromatography mass spectroscopy (GC–MS, Supporting Information). As previously observed, we found that *E. coli* was only capable of producing alkanes in the presence of the AAR and ADC biosynthetic pathway. The resulting alkane profile was similar to previous reports in that only odd chain length alkanes were produced at significant levels (Table 1 and Figure S2, Supporting Information). One noteworthy difference in the alkane production profile between this reconstituted system and previous reports is in the distribution of the observed products. Previous reports identify heptadecane as the primary product, whereas we observed pentadecane as the major alkane produced (Figure 1 and Table 1). This difference could have been due to a number of factors such as cell line, growth conditions, or pathway enzyme expression levels, and warrants further investigation.

To determine whether the introduction of FabH2 to the AAR and ADC alkane production system would result in a modification of the observed alkane production profile, *fabH2* was synthesized as described above, and cloned into a low copy number, IPTG-inducible BioBrick expression vector to form part BBa_K590064 (Supplemental Methods, Supporting Information). Alkane production was then analyzed in cells expressing AAR, ADC, and FabH2. The addition of FabH2 to the AAR and ADC alkane production system resulted in a 2- fold increase in total alkane production, from 39.4 to 81.3 mg/L (Table 1). This unexpected increase suggests that a ratelimiting step in fatty acid biosynthesis is 3-ketoacyl-ACP production by FabH. In addition to an overall increase in yield, new peaks were now observed on the gas chromatogram. The novel compounds were identified as tetradecane and hexadecane on the basis of their fragmentation pattern and by comparison of elution times to those of authentic product standards (Figure S3, Supporting Information).

Although the production of new even chain length alkanes was observed in the presence of FabH2, the yields were close to detection limits, at approximate levels of 3.7 mg/L of tetradecane and 1.2 mg/L of hexadecane (Table 1 and Figure S2, Supporting Information). Therefore, introduction of FabH2 alone did not have the expected effect of significantly changing the product profile, as hoped. In the presence of FabH2, even chain length alkanes represented only a minor component of the alkane product profile, comprising only 6% of total alkanes produced. It is possible that the levels of even chain length alkanes produced in the presence of FabH2 remained low because of limited availability of propionyl-CoA, the required starter unit for initiation of even chain length alkane production. In *E. coli,* propionyl-CoA is normally present at concentrations that are 10 to 100-fold lower than

acetyl-CoA and is unlikely to be present in quantities sufficient to enable a significant alteration in fatty acid composition.[6]

It has been previously demonstrated that the addition of propanoate into the growth media results in a significant increase of propionyl-CoA levels in *E. coli*.[6] Therefore, we supplemented our production media with 6.5 mM propanoate. When extracellular propanoate was added to cells expressing AAR and ADC alone, even chain length alkane production increased significantly, from trace levels to 3.5 ± 0.3 mg/L tetradecane and 1.8 ± 0.1 mg/L hexadecane (Tables 1 and S1, Supporting Information). This increase in even chain length alkane production in the absence of FabH2 suggests that the addition of extracellular propanoate enables propionyl-CoA utilization by native *E. coli* FabH for fatty acid biosynthesis. This is consistent with previous reports that native *E. coli* FabH can utilize alternate CoA substrates.[4]

The addition of propanoate to cells expressing AAR, ADC, and FabH2 resulted in a 4-fold increase in hexadecane production, from 3.7 ± 0.2 to 14.3 ± 1.9 mg/L, and a 10-fold increase in tetradecane production, from 1.2 ± 0.3 to 11.9 ± 2.5 mg/L (Table 1 and Figure 1). In addition to increasing the total yield of even chain length alkanes, the proportion of even chain alkane yield was increased from 6 to 27% of the total alkanes produced (Table 1 and Figure 1). This supports the hypothesis that overexpression of a 3-ketoacyl-ACP synthase (such as FabH2) enables the expansion of the alkane product profile when sufficient levels of alternative acyl-CoA substrates are present. This effect could be due to either the *in vivo* specificity of FabH2 or an increase in total 3-ketoacyl-ACP synthase expression.

In this study, we have reconstructed a previously reported alkane production system in the BioBrick format and have demonstrated that the introduction of FabH2 from *Bacillus subtilis* into this system results in the production of even chain length alkanes (Figure S5, Supporting Information). In addition, we have established that extracellular addition of propanoate in the presence of FabH2 results in both an increase of even-chain alkane yield of up to 10-fold and 27% of the total fraction of alkanes produced being even-chain length. This work demonstrates that novel alkanes can be produced through the modification of upstream components of the alkane production pathway. Future efforts to further diversify alkane profiles to produce compounds, such as branched-chain alkanes, may be accomplished through overproduction of the appropriate metabolites for incorporation by FabH2. Finally, as part of an iGEM project, this study demonstrates the power of synthetic biology to simplify complex biological systems into accessible engineering problems that students can readily address.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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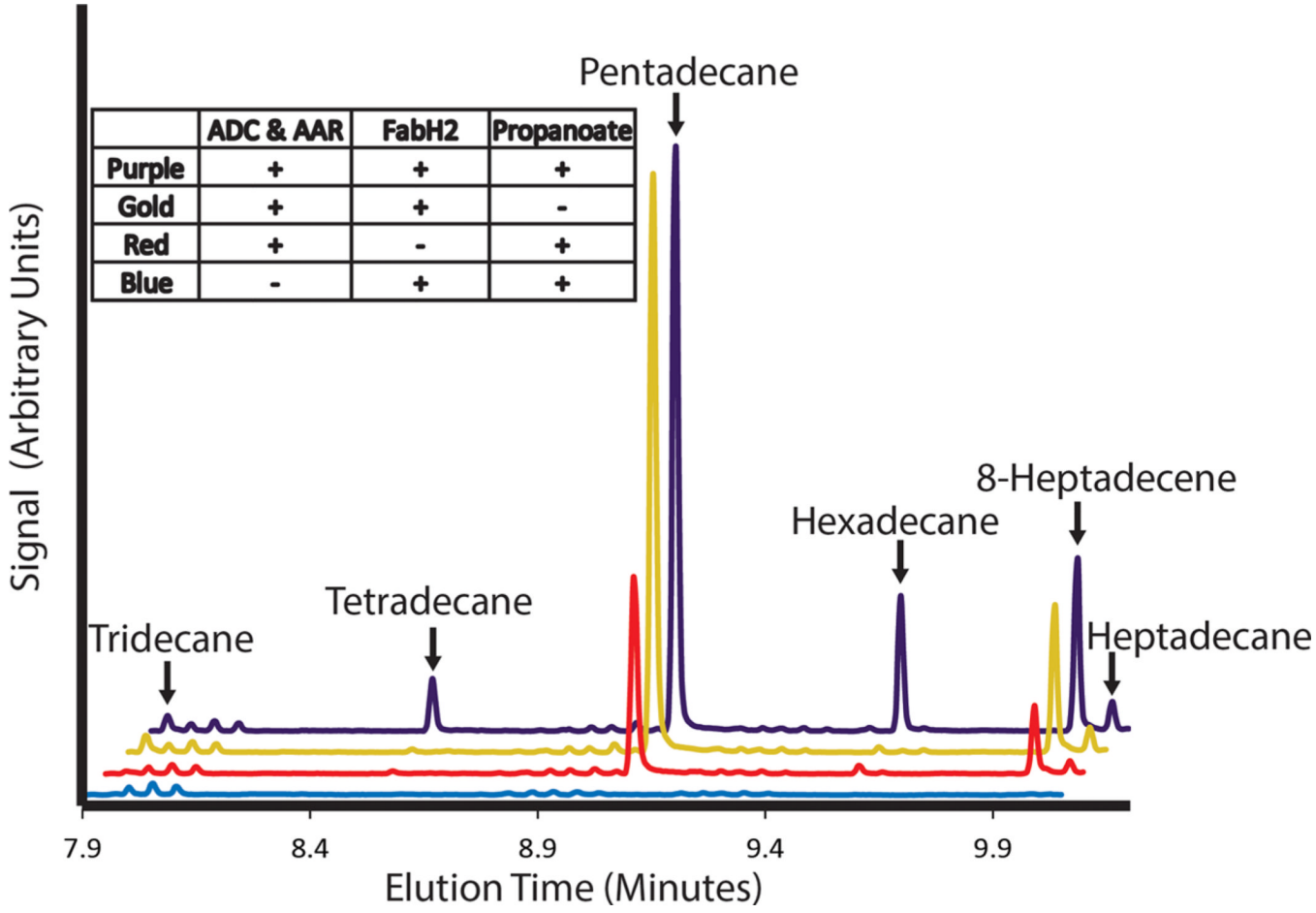

Figure 1.

Gas chromatograms of alkane production in *E. coli* BL21(DE3) cells expressing AAR and ADC, FabH2, or both enzymes. Cultures were supplemented with 6.5 mM propanoate as indicated. Peak identities were confirmed by spectral comparison to the NIST spectral database as well by comparison of elution time to that of known product standards.

Table 1

Alkanes produced under various conditions (mg/L)[a]

	0 mM propanoate		6.5 mM propanoate	
	AAR + ADC	**AAR + ADC + FabH2**	**AAR + ADC**	**AAR + ADC + FabH2**
tridecane (C13)	6.3 ± 0.2	14.9 ± 0.4	6.4 ± 0.7	13.6 ± 1.4
tetradecane (C14)	trace	3.7 ± 0.2	3.5 ± 0.3	14.3 ± 1.9
pentadecane (C15)	23.7 ± 2.1	45.2 ± 7.3	24.2 ± 1.6	41.9 ± 5.8
hexadecane (C16)	trace	1.2 ± 0.3	1.8 ± 0.1	11.9 ± 2.5
8-heptadecene (C17)	7.4 ± 2.9	13.2 ± 3.2	8.2 ± 0.4	13.5 ± 2.7
heptadecane (C17)	2.1 ± 0.4	3.0 ± 0.5	2.1 ± 0.1	3.2 ± 0.4
total yield	39.4 ± 0.4	81.3 ± 12.4	46.3 ± 2.1	98.3 ± 13.7

[a] *E. coli* BL21(DE3) cells expressing the AAR and ADC alkane production pathway were grown in the presence or absence of FabH2 and in the presence or absence of 6.5 mM propanoate. Alkane titers were quantified using GC–MS and are reported as mean ± standard deviation of three independent cultures. Detailed growth conditions and alkane analysis are described in the Supplemental Methods (Supporting Information).