Title: Modular and selective biosynthesis of gasoline-range alkanes

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22 **Abstract:**

23 Typical renewable liquid fuel alternatives to gasoline are not entirely compatible with current
24 infrastructure. We have engineered *Escherichia coli* to selectively produce alkanes found in gasoline
25 (propane, butane, pentane, heptane, and nonane) from renewable substrates such as glucose or glycerol.
26 Our modular pathway framework achieves carbon-chain extension by two different mechanisms. A fatty
27 acid synthesis route is used to generate longer chains heptane and nonane, while a more energy efficient
28 alternative, reverse- β -oxidation, is used for synthesis of propane, butane, and pentane. We demonstrate
29 that both upstream (thiolase) and intermediate (thioesterase) reactions can act as control points for chain-
30 length specificity. Specific free fatty acids are subsequently converted to alkanes using a broad-
31 specificity carboxylic acid reductase and a cyanobacterial aldehyde decarbonylase (AD). The selectivity
32 obtained by different module pairings provides a foundation for tuning alkane product distribution for
33 desired fuel properties. Alternate ADs that have greater activity on shorter substrates improve observed
34 alkane titer. However, even in an engineered host strain that significantly reduces endogenous
35 conversion of aldehyde intermediates to alcohol byproducts, AD activity is observed to be limiting for
36 all chain lengths. Given these insights, we discuss guiding principles for pathway selection and potential
37 opportunities for pathway improvement.

38

1 Introduction

The United States relies heavily on gasoline to fulfill transportation needs. The U.S. consumes roughly 4 billion barrels of gasoline annually, which amounts to 40% of total annual domestic petroleum usage and 47% of all gasoline produced worldwide (International Energy Statistics, 2014; Fichman et al., 2012). Vast gasoline infrastructure exists to facilitate its usage, and prevailing renewable liquid fuel alternatives have limited compatibility. Ethanol requires a different distribution system than gasoline because of its hygroscopicity, corrosivity, and biodegradability (Strogen and Horvath, 2013). Due to dissimilar fuel performance characteristics such as energy density and research octane number (RON) (**Table S1**), renewable gasoline alternatives are blended with gasoline for use in conventional automobile engines. One approach towards addressing the compatibility of renewable fuels is to metabolically engineer a microbe that converts sugars into a product that mimics the composition of gasoline. An added benefit of such a process would be streamlined product separation from the aqueous phase due to increased product hydrophobicity (**Table S1**).

We began the pathway design process by first looking at published chemical composition studies of gasoline. Typical regular unleaded gasoline is a blend of over 30 aliphatic and aromatic hydrocarbons (**Fig. 1A**) (Cline et al., 1991; Johansen et al., 1983; Sanders and Maynard, 1968). C3 (propane) to C9 (nonane) are the most common alkane components in gasoline with C5 (pentane) species predominating. With such targets in mind, we next examined previously observed natural and engineered enzymes involved in alkane synthesis. Initial work on microbial alkane production elucidated natural routes to C15-C17 alkanes produced via fatty acid synthesis (FAS) (Schirmer et al., 2010). In cyanobacteria, acyl-carrier protein (acyl-ACP) reductases (AAR) and aldehyde decarbonylases (ADs, also known as aldehyde-deformylating oxygenases or ADOs (Aukema et al., 2013)) convert growing acyl-ACP chains to long-chain alkanes. In a natural progression, fatty acid reductases and ADs have been co-expressed

62 with acyl-ACP thioesterases in order to terminate FAS at medium-chain lengths and convert the free
63 acids to alkanes (Akhtar et al., 2013; Andre et al., 2013; Harger et al., 2012; Howard et al., 2013;
64 Schirmer et al., 2010). The C12 and longer alkanes reported may serve as alternatives for diesel fuels but
65 not for gasoline.

66 Recent efforts have also targeted the production of short-chain alkanes (< C10). Using a similar
67 FAS termination strategy, introduction of a plant AD, CER1_{At} from *Arabidopsis thaliana*, resulted in
68 production of alkanes as short as nonane from glucose in *E. coli* (Choi and Lee, 2013). Nonane and the
69 longer alkanes produced collectively constitute less than one weight percent of typical regular unleaded
70 gasoline. Other short-chain acyl-ACP thioesterases have been described (Jing et al., 2011), and recently
71 two thioesterases were used to synthesize heptane and propane via FAS (Kallio et al., 2014). Synthesis
72 of these alkanes relied on the use of an AD from *Prochlorococcus marinus* MIT9313 (AD_{Pm}), which has
73 a solved crystal structure (Andre et al., 2013; Khara et al., 2013). Kallio and colleagues achieved
74 increases in titer from < 1 mg/L to 32 mg/L propane by varying process conditions to improve oxygen
75 availability. Like nonane, propane also constitutes less than one weight percent of typical regular
76 unleaded gasoline.

77 Although the report detailing propane production describes a notable achievement in the ability
78 to produce short alkanes, there are drawbacks that provide motivation for additional approaches. One is
79 the limited ability of propane to serve as a drop-in or blended gasoline replacement. As shown in Fig.
80 1A, the range of alkanes bounded in carbon chain length between propane and nonane (C4-C8)
81 constitute a much greater fraction of gasoline. All gasoline-range alkanes (C3-C9) can separate rapidly
82 from aqueous cultures due to their hydrophobicity and volatility, but alkanes longer than propane have
83 greater energy density (**Table S1**). Longer alkanes also require less energy than propane to store and
84 transport in the liquid phase. Given that the number of vehicles that can be powered solely on propane is

85 significantly smaller than the gasoline powered automobile fleet, production of gasoline-range alkanes
86 addresses a larger problem in the transportation fuels sector.

87 Previously published approaches to alkane synthesis have also been dependent on FAS for
88 carbon chain elongation as compared to the use of CoA-dependent, reverse β -oxidation (RBO) pathways
89 (Dekishima et al., 2013; Dellomonaco et al., 2011). RBO routes provide greater theoretical efficiency
90 than FAS for alkane production, in terms of co-factor balancing and energy consumption. The vast
91 majority of FAS reductases that have been described and predicted are likely to strongly prefer an
92 NADPH cofactor (Handke et al., 2011; Javidpour et al., 2014; Ratledge, 2004). In contrast, canonical
93 CoA-dependent pathways contain beta-oxidation enzymes, which are NADH dependent (Kunau et al.,
94 1995; Schulz, 1991). Barring the use of an efficient transhydrogenase to rapidly interconvert reducing
95 equivalents, the increased requirement for NADPH in FAS-based chain elongation necessitates greater
96 carbon flux to the pentose phosphate pathway, which lowers carbon efficiency of alkane production.
97 Furthermore, the carboxylation of acetyl-CoA to produce malonyl-CoA in FAS pathways leads to a
98 higher net ATP consumption than in RBO pathways (Handke et al., 2011) (see **Discussion** for a more
99 detailed analysis).

100 In addition to pathway energetics, the reliance of RBO on the universal CoA molecule rather
101 than the reliance of FAS on organism specific ACPs provides RBO platforms with increased
102 transferability into alternative host organisms. Every reaction in the FAS pathway, including the release
103 of a free acid by the thioesterase, requires the enzyme to interact with an acyl-ACP substrate. While
104 ACP is highly conserved across many organisms, sequence diversity exists which may influence the
105 efficiency of interactions between ACP and FAS enzymes derived from different sources (Byers and
106 Gong, 2007; De Lay and Cronan, 2007). This may already be manifested in the differing performance
107 of thioesterases expressed in *E. coli* (Jing et al., 2011; Lennen and Pfleger, 2012). Differences in

108 productivity may depend to some degree on the efficiency of interaction between *E. coli* acyl-ACP and
109 the given plant acyl-ACP thioesterase. Additionally, transfer of such a pathway to a yeast strain is
110 complicated by the fused structure of the type I FAS of yeast which includes an ACP domain
111 (Schweizer and Hofmann, 2004). Growing acyl chains are linked to the complex and released via a
112 transferase as palmitoyl-CoA. Instead, RBO pathways rely on a universal cofactor, CoA. Additionally a
113 number of natural anabolic, RBO-like pathways have been identified with enzymes tuned to generate
114 medium chain length acids (Anderson and Dawes, 1990; Hoffmeister et al., 2005; Inui et al., 2008;
115 Lütke-Eversloh and Bahl, 2011; Steinbüchel and Lütke-Eversloh, 2003).

116 In constructing a biosynthetic route to gasoline-range alkanes, we first began with an FAS-based
117 pathway towards the longer of these mid-range molecules, heptane and nonane, utilizing an approach
118 similar to previously reported efforts. Next, given our experience constructing modular pathways for
119 synthesis of C4-C6 alcohols via RBO pathways (Nielsen et al., 2009; Sheppard et al., 2014; Tseng and
120 Prather, 2012), we hypothesized that an AD could be harnessed to an ATP-efficient platform for
121 production of associated alkanes. While an RBO pathway presents theoretical advantages, there were
122 several questions associated with the development of such a platform. For example, could the platform
123 be selective for multiple alkanes within the desired range? If so, how would different modules within the
124 pathway contribute to the selectivity? Here, we report selective production of propane, butane, and
125 pentane using a modular RBO pathway approach in metabolically engineered *E. coli*. Modules
126 represent convenient gene groupings that collectively convert one easily detectable metabolite into
127 another. Module 1 variants enable generation and activation of precursors for carbon chain extension
128 using either Acyl Carrier Protein (ACP) or Coenzyme A (CoA). Module 2 variants perform carbon chain
129 extension either by FAS or by Reverse β -Oxidation (RBO). Module 3 variants result in termination of

chain extension and generation of free fatty acids (FFAs) ranging from C4 to C10. Finally, Module 4 variants convert C_n fatty acids into corresponding $C(n-1)$ alkanes via a C_n fatty aldehyde intermediate.

2 Materials and Methods

2.1 Strains and Modules. *E. coli* strains and modules used in this study are listed in Table S2.

Molecular biology techniques were performed according to standard practices (Sambrook and Russell, 2001) unless otherwise stated. Molecular cloning and new vector propagation were performed in DH5 α .

Previously constructed *E. coli* K-12 MG1655(Δ endA Δ recA) and the reduced aromatic aldehyde reduction (“RARE”) Δ endA Δ recA strains (Kunjapur et al., 2014; Tseng et al., 2010) were used as hosts

for experiments testing biosynthesis of C3-C5 alkanes. The *fadD* gene was deleted in MG1655(Δ endA Δ recA) and RARE strains using a donor strain from the Keio collection (Baba et al., 2006) and the method of P1

transduction (Thomason et al., 2001). The vector pCP20 was used to cure the kanamycin resistance cassette (Datsenko and Wanner, 2000). MG1655(Δ endA Δ recA) Δ *fadD* and RARE Δ *fadD* strains were used as

hosts for experiments testing biosynthesis of C7 and C9 alkanes. Oligonucleotides (Sigma, The Woodlands, TX) used as PCR primers are shown in Table S3. Q5 High Fidelity DNA Polymerase (New England Biolabs, Beverly, MA) was used for DNA amplification. A codon-optimized sequence for *P. marinus* MIT9313 AD (*PMT1231*) was purchased as a DNA String (GeneArt, Regensburg, Germany)

(see Supplementary Text for all codon-optimized sequences). Once codon-optimized *PMT1231* was cloned into pACYC-*car-sfp-PMT1231*, the AD_A134F_{pm} mutant sequence was generated using Polymerase Incomplete Primer Extension (PIPE) cloning (Klock and Lesley, 2009). The new sequence replaced the GCA codon at positions 400-402 with a TTT codon. Codon-optimized sequences for the *N. punctiforme* wild-type and mutant ADs were also purchased as DNA Strings. All DNA Strings were

digested with NdeI and AvrII restriction enzymes and cloned into the second multiple cloning site of the pACYC-*car-sfp* plasmid. Construction of pACYC-*car-sfp* was previously reported (Kunjapur et al.,

2014; Sheppard et al., 2014). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. A codon optimized version of the full open reading frame of *C. hookeriana* *FATB2* was purchased from GenScript (Piscataway, NJ). The *FATB2* gene and variants were cloned into the pETDuet-1 vector (Novagen, Darmstadt, Germany) using BamHI and NotI.

2.2 Chemicals. The following compounds were purchased from Sigma: pentane, isopentane, heptane, nonane, butyraldehyde, butanol, 4-methylvalerate, sodium hexanoate, hexanal, hexanol, sodium octanoate, octanoic acid, octanal, and decanoic acid. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was purchased from Denville Scientific (South Plainfield, NJ). Ampicillin sodium salt, chloramphenicol, and kanamycin sulfate were purchased from Affymetrix (Santa Clara, CA).

2.3 Culture Conditions. For all production experiments, 3 mL LB overnight seed cultures in 14 mL round-bottom tubes were used as inocula. All 2 mL production cultures were inoculated with overnight culture at 1% by volume and grown directly in 10 mL GC vials with PTFE Silica Septa screw caps (Supelco, Bellefonte, PA, Cat.#: SU860099 and SU860103). The production medium was LB with either 1.2% (w/v) glucose or 1.2% (v/v) glycerol. Depending on modules used, culture medium was supplemented with 50 mg/L ampicillin (for Modules 3-Oc, 1-Ma, 2-MCC, and 2-BC), 17 mg/L chloramphenicol (for all Module 4 variants), and/or 25 mg/L kanamycin (for Module 1-Pr). Culture vials were placed in tube racks at 45° angles and incubated with agitation at 30°C and 250 rpm. Cultures were induced with 0.5 mM IPTG (final concentration) at OD₆₀₀ values between 0.7 and 0.9. Cultures were incubated for 22-26 hours after induction prior to metabolite analysis. For aldehyde accumulation experiments, 2 mL cultures were similarly inoculated and grown in LB medium supplemented with 34 mg/L chloramphenicol in GC vials. At induction, culture medium was supplemented with either 721 mg/L octanoate or 580 mg/L hexanoate (both equivalent to 5 mM). Cultures were incubated for 22-26 hours after induction prior to metabolite analysis.

176 **2.4 Metabolite Analysis.** Liquid chromatography: Culture samples were pelleted by centrifugation and
177 supernatant was removed for HPLC analysis with an Agilent 1200 series instrument with a refractive
178 index detector. Analytes were separated using the Aminex HPX-87H anion exchange column (Bio-Rad
179 Laboratories, Hercules, CA) with a 5 mM sulfuric acid mobile phase at 35°C and a flowrate of 0.6
180 mL/min. Gas chromatography – fatty acid methyl esters (FAME) as a proxy for free fatty acids in
181 culture medium: Culture samples taken at 48 hours were pelleted by centrifugation and 5 mL of
182 supernatant was removed and added to a 15 mL conical centrifuge tube. The supernatant was acidified
183 with 50 µL of 10 M HCl to increase extraction of acids into the organic phase. After acidification, 5 mL
184 of a 2:1 mixture of chloroform:methanol with 100 µg/ml tridecanoic acid was added and samples were
185 vortexed for 2 hours. After vortexing, the phases were separated by centrifugation (5000g) and the
186 bottom chloroform layer (~3 mL) was transferred to a capped glass vial. The samples were then
187 completely evaporated by flowing compressed air over the samples with a manifold (~30 minutes).
188 Samples were held in a polystyrene tray which kept them partially insulated so they cooled upon
189 evaporation. The remaining solid was resuspended in 1 mL of methanol + 2% (vol) sulfuric acid and
190 incubated in a heating block at 60°C for 2 hours to esterify the acids. After 2 hours, the liquid was
191 transferred to 1.7 mL microcentrifuge tubes and partially evaporated using the same manifold set-up for
192 ~40 minutes until the liquid volume was ~100 µL. Methyl esters were then extracted from this
193 concentrated liquid by addition of 1 mL of hexane and vortexing for 15 mins. Phases were separated by
194 centrifugation and 800 µL of the hexane layer was transferred to vials for GC analysis. Fatty acid methyl
195 esters were analyzed by using a Bruker 450-GC instrument with a flame ionization detector. Compounds
196 were separated using a HP-INNOWAX capillary column (30 m x 0.25 mm). The oven conditions were
197 set at 120°C (2 min) followed by a linear 10 minute ramp up to 180°C and a hold at 180°C (18 min).
198 Fatty acids were quantified by creating standard curves of methyl esters prepared using both the

199 supernatant and pellet esterification methods from known dilutions of acid standards. The ratio of
200 methyl-ester peak area to methyl tridecanoate internal standard peak area was calculated for each
201 concentration to build the curve. Octanoic and decanoic acids were prepared using the cellular lipid
202 protocol, and standard curves were made using the supernatant protocol. Gas chromatography – alkanes
203 in headspace: At 24 hours post induction, vial cultures were placed in a 42°C incubator for 20 minutes in
204 order to drive the volatile alkanes into the gas phase. Gas headspace samples were then taken using a 10
205 mL gas-tight syringe (SGE Ringwood, Victoria, Australia). In order to mix the gas sample and prevent
206 formation of a vacuum, one syringe was used to inject a 1 mL volume of air as 1 mL of sample was
207 drawn. The process was repeated until a 9 mL volume was taken from the vial and an additional 9 mL of
208 air was injected into the vial. The concentration injected into the GC was thus diluted 2-fold. A
209 Shimadzu GC (GC-2014) with a RT-Q bond column (30 m length, 530 µm ID, 20 µm film thickness)
210 and flame ionization detector (FID) was used for the analysis. A 5 µL sample loop was used for sample
211 injection. The method oven conditions were as follows: a 40°C hold for 1 minute followed by a
212 25°C/min ramp up to 280°C with a 5 minute final hold. Quantification of propane, butane, and pentane
213 was based on a one point calibration using a standard gas mixture purchased from AIRGAS. A separate
214 standard curve for heptane was created by adding known volumes to a 1.127 L glass bottle fitted with a
215 septum cap. A heptane standard and the 1.127 L bottle were chilled to 4°C and different known volumes
216 of heptane were added. The bottle was then warmed to room temperature allowing the heptane to fully
217 vaporize. A gas tight syringe was used to inject 8 ml of gas from the bottle into the GC. A similar curve
218 was generated for nonane.

219 **2.5 Western Blot Analysis.** *E. coli* MG1655(DE3)Δ*endA*Δ*recA* was transformed with empty
220 pETDuet-1 or pETDuet-1 with each of the *FatB2Ch* variants and plated on LB Agar + 100 µg/mL
221 ampicillin. Single colonies of each transformant were grown overnight in 5 mL of LB. Shake flask

222 cultures (250 mL flasks) containing 50 mL LB + 1% glucose were inoculated at 1% inoculum from
223 overnight LB cultures and incubated with agitation at 30°C and 250 rpm. Shake flasks were induced
224 with 1 mM IPTG ~2 hours post inoculation when they reached OD₆₀₀ values between 0.6 and 0.8. Four
225 hours after induction 10 mL of each culture was sampled and pelleted by centrifugation. Cell pellets
226 were resuspended in 1 mL of 10 mM Tris-HCl at pH 8.0 and added to 1.7 mL microcentrifuge tubes
227 containing 500 µL of 0.1 mm diameter glass beads (Scientific Industries, Inc. Disruptor Beads,
228 SIBG01). Samples were then vortexed for 10 minutes. After lysis, samples were pelleted by
229 centrifugation (6,000g) and the supernatant was removed as soluble lysate. Total protein was quantified
230 using Bio-Rad Protein Assay Dye Reagent (Cat.#: 500-0006) and the Quick StartTM Bradford Protein
231 Assay (Bio-Rad). A Bio-Rad 10% Mini-PROTEAN TGX gel (Cat.#: 456-1034) was run using the
232 Mini-PROTEAN Tetra Cell electrophoresis set up. Bio-Rad Precision Plus Protein Unstained Standard
233 (Cat.#: 161-0363) and 15 µg of total protein for each sample was loaded on the gel. After running the gel
234 for 33 min at 200 volts, the gel was removed from the casing and washed for 5 mins in 100 mL of
235 deionized water. The washed gel was then equilibrated in Transfer Buffer for 15 minutes (described
236 below) along with blotting paper, sponges, and nitrocellulose used with the Bio-Rad Mini Trans-Blot
237 Module (Cat.#: 170-3935). The Trans-Blot Module was then used for transfer to the nitrocellulose by
238 running the cell at 100 volts for 1 hour. Once transferred, the nitrocellulose was blocked by washing in 5
239 wt% BSA in 1x TBS buffer (described below) for 2 hours at room temperature. The nitrocellulose was
240 then washed twice for 10 minutes with 1x TBST buffer (described below) and incubated overnight at
241 4°C with THETM His Tag Antibody, mAb, Mouse (GenScript Cat.#: A00186-100) diluted in 1x TBS +
242 10% glycerol to a final concentration of 0.5 µg/ml. The blot was then washed three times with 1x TBST
243 for 10 minutes each time at room temperature and incubated for 2 hours with a donkey anti-Mouse
244 IgG-HRP secondary antibody (Santa Cruz Biotechnologies, Dallas, TX, Cat.#: sc-2318) at a 1:5000

dilution in 1x TBS buffer. Following secondary antibody binding, the blot was washed twice for 10 minutes with 1x TBST and then once with 1x TBS at room temperature. The blot was developed using the Western Blotting Luminol Reagent (Santa Cruz Biotechnologies, Cat.#: sc-2048) and imaged using an Alpha Innotech FluroChem imager.

2.6 Western Blot Buffers. One liter of Transfer Buffer was prepared with the following components: 14.4 g glycine, 3.025 g Tris base, 200 mL methanol, 800 mL of deionized water. 500 mL of 10x TBS buffer was prepared with the following: 12.1 g Tris base, 146.2 g sodium chloride, and 500 mL of deionized water. The pH was adjusted to 7.5. 500 mL of 1x TBST was prepared with the following: 50 mL of 10x TBS, 450 mL deionized water, and 250 μ L of Tween-20 (0.05% final concentration).

3 Results

3.1 N-terminally Truncated FatB2_{Ch} Acyl-ACP Thioesterase Results in Synthesis of C8 and C10 FFAs in *E. coli*. We began by investigating whether native FAS could be used to produce alkanes shorter than nonane using a selective acyl-ACP thioesterase. In this approach, alkane product selectivity is primarily determined by the thioesterase reaction. The core FAS reactions drive flux towards long-chain fatty acids while the thioesterase diverts selected acyl-ACP intermediates to free acids. Microbial production of heptane from glucose was realized by using Modules 1-Ma, 3-Oc, and 4-LA (**Fig. 1B**). The thioesterase FatB2_{Ch} from *Cuphea hookeriana* had been observed to generate C8 FFA, but previously published work did not describe the sequence required for mature FatB2_{Ch} protein expression in *E. coli* (Dehesh et al., 1996; Liu et al., 2011). Based on a previous report that identified potential membrane-associated N-terminal sequences for two homologs (Jones et al., 1995), we tested three versions of FatB2_{Ch} for soluble expression, all with N-terminal His-tags: full open reading frame (ORF); ORF truncated before the α -helix (His-FatB2m1_{Ch}); and ORF truncated after the α -helix (His-

267 *FatB2m2_{Ch}*). Because His-*FatB2m2_{Ch}* displayed significantly increased soluble expression (**Fig. S1**),
 268 *FatB2m2_{Ch}* was used in subsequent experiments. We tested for synthesis of FFAs by transforming *E.*
 269 *coli* MG1655 Δ *fadD* (WT Δ *fadD*) to express *FatB2m2_{Ch}* (Module 3-Oc). We chose to use strains lacking
 270 *fadD*, encoding fatty acyl-CoA synthetase, when using FAS for carbon chain elongation because of
 271 increased activity of FadD on C8 and longer FFAs compared to FFAs shorter than C8 (Kameda and
 272 Nunn, 1981). Although FadD is also active on C6 FFA, we did not subsequently use strains lacking
 273 *fadD* when using RBO given previous reports of successful C4-C6 FFA synthesis in the presence of
 274 *fadD* (Sheppard et al., 2014; Tseng and Prather, 2012). Use of Module 3-Oc led to the formation of $43 \pm$
 275 4 mg/L octanoate and 1.2 ± 0.4 mg/L decanoate without detection of longer chain length species (**Fig.**
 276 **2A**). To investigate whether higher titers could result from increased malonyl-ACP precursor, we
 277 overexpressed an artificial operon containing genes encoding the *E. coli* acetyl-CoA carboxylase
 278 complex (Module 1-Ma) (Davis et al., 2000). Combined use of Module 1-Ma and Module 3-Oc led to
 279 increased titers of both FFAs (72 ± 9 mg/L octanoate and 3.2 ± 0.2 mg/L decanoate) (**Fig. 2A**).

280 **3.2 An Engineered Host Strain (RARE) Enables Octanal Accumulation.** After obtaining selective
 281 carbon chain termination, we next focused on conversion of octanoate to heptane (Module 4-LA) (**Fig.**
 282 **1B**). Previous work demonstrated that Car_{Ni} from *Nocardia iowensis*, coexpressed with Sfp_{Bs} from
 283 *Bacillus subtilis*, converts FFAs to fatty aldehydes (Akhtar et al., 2013; Sheppard et al., 2014). However,
 284 fatty aldehydes such as octanal are prone to endogenous conversion to corresponding fatty alcohols (**Fig.**
 285 **2B**) (Akhtar et al., 2013; Howard et al., 2013; Rodriguez and Atsumi, 2014). A recent review has
 286 highlighted several studies that have discussed or demonstrated the potential for improving selectivity of
 287 desired products over undesired alcohol byproducts by engineering *E. coli* to minimize endogenous
 288 aldehyde reduction (Kunjapur and Prather, 2015). Work by Kallio *et al.* demonstrates that propane titers
 289 increase by deletion of two native aldehyde reductases, *yqhD* and *ahr* (Kallio et al., 2014). In a separate

study, Rodriguez and Atsumi reported an engineered *E. coli* strain displaying limited endogenous conversion of exogenously supplied aliphatic aldehydes after thirteen gene deletions (Rodriguez and Atsumi, 2014). Prior to these studies, we reported an *E. coli* strain (RARE) that was originally designed to accumulate aromatic aldehydes and features six deletions of aldehyde reductases (Kunjapur et al., 2014). We hypothesized that the RARE strain may be able to accumulate octanal given that several genes deleted in RARE encode enzymes capable of reducing both aromatic and aliphatic aldehydes (Pick et al., 2013). We used both wild-type (WT) and RARE host strains to express Car_{Ni} in medium supplemented with octanoate. Cultures were grown directly in gas chromatography (GC) vials and octanal concentrations were measured in the headspace (**Fig. 2C**). When RARE was used, 37 ± 12 mg/L of octanal was detected, whereas no octanal was detected using WT.

3.3 Modules 1-Ma, 3-Oc, and 4-LA Result in Heptane and Nonane Biosynthesis. We then tested production of heptane from glucose in WT Δ *fadD* and RARE Δ *fadD* containing Modules 1-Ma, 3-Oc, and 4-LA. We sampled culture headspace 24 hours after induction for formation of possible C3-C9 alkanes (**Figs. 2D, S2, and S3**). In both cases, we observed heptane synthesis. RARE Δ *fadD* produced more heptane than WT Δ *fadD* (0.52 ± 0.03 mg/L versus 0.32 ± 0.05 mg/L, respectively). Given that decanoate was also observed previously (**Fig. 2A**), roughly 0.2 mg/L of nonane was also produced using each strain (**Fig. 2D**). No other alkanes were detected. Based on low alkane titers relative to FFA titers and reported activity of Car_{Ni} on octanoate (Sheppard et al., 2014), the AD-catalyzed reaction was the likely bottleneck. The low observed AD activity *in vivo* suppressed the benefit of increasing aldehyde substrate pool size using RARE Δ *fadD*.

3.4 Substitution of an RBO Module (Module 2-MCC) Results in Biosynthesis of Pentane as the Sole Alkane Product. To achieve selective synthesis of more common gasoline-range alkanes using a more energy efficient platform we next substituted carbon chain extension modules for an RBO module

313 tailored for C6 fatty acids. We recently reported a modular pathway framework selective for synthesis of
314 4-methylvalerate, which is a branched C6 acid (Sheppard et al., 2014). In the absence of isobutyryl-CoA
315 generating modules, use of the extension module described in that report (listed here as 2-MCC) results
316 in the production of hexanoate directly from glucose. As demonstrated in the synthesis of 4-
317 methylvalerate, the use of RBO eliminates the need for a termination module due to endogenous
318 thioesterase activity (Sheppard et al., 2014). As a result, unlike our FAS based pathway, the RBO
319 approach generates alkane product selectivity by the specificity of the thiolase (BktB), reductase (PhaB),
320 dehydratase (PhaJ), and enoyl-CoA reductase (Ter) reactions.

321 Although Car_{Ni} can efficiently convert hexanoate to hexanal (Sheppard et al., 2014), we faced
322 two potential challenges with hexanal. The first was whether we could decrease endogenous conversion
323 of hexanal to hexanol. When we cultured WT and RARE host strains expressing Car_{Ni} in medium
324 supplemented with hexanoate, we observed that only RARE accumulated hexanal (**Fig. 2E**). The second
325 obstacle was the potential inability of ADs to act on aldehydes as short as C6. A previous study found
326 that substitution of alanine with phenylalanine at position 134 of AD_{Pm} (AD_A134F_{Pm}) resulted in
327 increased *in vitro* activity on aldehyde substrates ranging from butyraldehyde to nonanal (Khara et al.,
328 2013). Based on that report, we examined whether use of Car_{Ni} with AD_A134F_{Pm} (Module 4-SA) or
329 AD_{Pm} (Module 4-LA) would lead to higher pentane titers. We tested alkane production expressing
330 Module 2-MCC with either Module 4-SA or 4-LA in either a WT or RARE host (**Fig. 2F**). All four
331 strains produced pentane, and the highest titer (1.6 ± 0.3 mg/L) was observed for the pairing of RARE
332 with Module 4-SA. No other alkanes were detected. The RARE host eliminated butanol and hexanol
333 byproduct formation in the liquid-phase, whereas the WT host produced 31 ± 6 mg/L butanol and $115 \pm$
334 16 mg/L hexanol (**Fig. 2G**). Despite the presence of butyraldehyde and the possibility of AD-catalyzed
335 conversion to propane, the absence of propane in these cultures likely results from the preference of both

Module 4 enzymes (Car and AD) for C6 over C4 substrates. The absence of longer alkanes stems from the highly selective carbon-chain extension module (Module 2-MCC).

3.5 Modules 1-Pr, 2-MCC, and 4-SA Enable Butane Biosynthesis. To investigate whether alkane chain lengths could be further shortened, we added a module previously used for pentanol synthesis (listed here as Module 1-Pr) (**Fig. 1B**) (Tseng and Prather, 2012). We hypothesized that pentanal, the immediate precursor to pentanol, could be converted to butane instead. In this case, we chose to use glycerol rather than glucose as a carbon source given that previously reported pentanol titers were 6-fold higher using glycerol (Tseng and Prather, 2012). When we combined Module 1-Pr with Modules 2-MCC and 4-SA, we observed synthesis of butane and pentane from glycerol (**Fig. 3A**). Butane titers were similar in WT and RARE hosts (0.35 ± 0.12 mg/L and 0.46 ± 0.15 mg/L, respectively). Pentane titers exceeded butane, with up to 1.27 ± 0.08 mg/L pentane using RARE. Pentane production was expected given the preference of Module 4 enzymes for longer substrates and the ability of Module 2-MCC to generate butyryl-CoA and hexanoyl-CoA. A route to propionyl-CoA that does not rely on the PDH complex may offer greater selectivity for butane.

3.6 Modules 2-BC and 4-SA Enable Propane Biosynthesis. Although propane is a minor constituent of gasoline, we were curious about the lower bound of our selective platform and next tested for propane synthesis by attempting to limit carbon chain extension beyond C4. We created Module 2-BC by replacing BktB_{Cn} from *Cupriavidus necator* in Module 2-MCC with Thl_{Ca} from *Clostridium acetobutylicum* based on increased specificity of Thl_{Ca} for condensation of two acetyl-CoA (Slater et al., 1998). WT and RARE hosts, each harboring Modules 2-BC and 4-SA, produced propane from glucose (0.17 ± 0.04 mg/L and 0.13 ± 0.02 mg/L, respectively) (**Fig. 3B**). Although propane titers were similar, the two strains displayed contrasting intermediate and byproduct profiles (**Fig. S4**). Surprisingly, RARE produced more pentane (0.41 ± 0.09 mg/L) than propane, whereas propane titers exceeded pentane titers

359 in WT. The increased pentane could result from increased pools of butyryl-CoA and/or reducing
360 equivalents in the RARE host. The 2-BC module further demonstrates the use of the initial thiolase
361 reaction as a control point for product specificity.

362 **3.7 Alternate ADs Improve Alkane Titters.** Having accomplished proof-of-concept synthesis, we
363 sought to explore whether titers could be improved using alternate ADs. Schirmer *et al.* assayed a
364 variety of AD homologs in *E. coli* for production of C13-C17 alkanes (Schirmer et al., 2010). Although
365 we selected AD_{Pm} based on confirmed *in vitro* activity on aldehyde substrates of interest, AD_{Pm} was
366 among the poorest performing enzymes reported by Schirmer *et al.* However, the AD from *Nostoc*
367 *punctiforme* PCC73102 (AD_{Np}) produced the highest reported titers of over 30 mg/L of both
368 pentadecane and heptadecane and the only detectable tridecane (Schirmer et al., 2010). When we
369 exchanged AD_{Pm} with AD_{Np} to create Module 4-LA' and included Modules 1-Ma and 3-Oc, we
370 observed more than an 8-fold increase in heptane titer (4.3 ± 0.3 mg/L) and more than a 6-fold increase
371 in nonane titer (1.22 ± 0.07 mg/L) (**Fig. 4A**). We then considered the use of AD_{Np} for shorter substrates
372 by examining pentane production as a model. Because the A134F mutation significantly increased the
373 activity of AD_{Pm} for C4-C6 aldehyde substrates, we hypothesized that a similar mutation in AD_{Np} might
374 provide increased activity on shorter substrates. We retrieved an existing ModBase homology model of
375 the AD_{Np} protein (UniProt ID B2J1M1) that was based on the structure of AD_{Pm} (Pieper et al., 2011) and
376 then aligned structures using Chimera (Pettersen et al., 2004). Because the AD_{Pm} structure was solved
377 with a substrate analog bound in the active site, we were able to identify all binding pocket residues
378 within 5 Å of the substrate (**Fig. 4B**). While there is significant sequence variation between the two ADs
379 (56% identity), all binding pocket residues are identical. The high similarity in binding pockets
380 supported the possibility that a functional AD_A122F_{Np} mutant could be created to enhance AD_{Np}
381 activity on short-chain substrates. We found that AD_A122F_{Np} resulted in 1.5-fold more pentane than

AD_{Np} (**Fig. 4C**). When we exchanged AD_A134F_{Pm} with AD_A122F_{Np} to create Module 4-SA' and included Module 2-MCC, we observed a 1.8-fold increase in pentane titer (2.80 ± 0.06 mg/L) (**Fig. 4D**).

3.8 Potential of Platform for Branched Alkane Products. Given the ability of our pathway modules to specifically target the C5 carbon chain length that is more common in gasoline, we next sought to investigate whether downstream modules would enable the production of the most common constituent of gasoline, isopentane. As shown in Fig. 1A, isopentane represents 9.3% of typical regular unleaded gasoline, and its properties more closely resemble those of a standard gasoline mixture than any other molecule. However, production of isopentane would require AD activity on the branched precursor 4-methylvaleraldehyde, and no previous efforts directed towards characterizing ADs *in vivo* or *in vitro* have utilized branched substrates. To directly assess whether branched alkane synthesis would be possible using ADs reported in this study, we chose to simplify the number of modules involved and expressed only Module 4 variants in the RARE host strain. In particular, we compared performance of the two mutant ADs (AD_134F_{Pm} and AD_122F_{Np}) when expressed along with Car_{Ni} (Modules 4-SA and 4-SA', respectively) (**Fig. 5A**). For these experiments, we added 5 mM 4-methylvalerate at induction. After sampling the headspace 24 hours after induction, we observed synthesis of isopentane in both cases (**Fig. 5B, Fig. S5**). Under these conditions, use of the AD_122F_{Np} led to the formation of roughly three times as much isopentane (5.21 mg/L vs. 1.73 mg/L). This encouraging result provides evidence that ADs can be harnessed for synthesis of branched alkanes and supports the possibility that an AD could be coupled to branched FFA generating modules in order to produce branched alkanes from glucose.

4 Discussion

Initial efforts to produce microbial fuels as gasoline alternatives focused on production of ethanol, which remains the dominant biofuel (Fortman et al., 2008). In recent decades, biotechnological advances enabled the design and construction of *de novo* biosynthetic pathways, several of which have resulted in the production of next-generation or advanced biofuels that more closely approximate properties of gasoline (Peralta-Yahya et al., 2012). Unlike many previous attempts to produce biofuels with similar properties as that of gasoline, this study aimed to produce a representative set of gasoline constituents, thereby naturally conferring properties characteristic of gasoline. Although only the *de novo* production of straight chain alkanes have been demonstrated here, and at very small titers, the platform could be further engineered to incorporate branched precursor-generating modules and optimized to achieve higher titers. In particular, we provide a theoretically efficient and transferable platform which could be used to produce alkanes with desired properties for diverse fuel applications. Production of any one of these alkanes alone would likely be insufficient for most transportation uses given the diversity of gasoline constituents.

As stated in the introduction, a detailed analysis reveals that RBO chain elongation pathways have greater theoretical efficiency than their FAS counterparts. To better understand this, we begin by examining co-factor and carbon efficiency tradeoffs in the common upstream portion of the pathways. Both routes require acetyl-CoA building blocks for chain extension and each requires multiple reducing equivalents either as a combination of NADH and NADPH or solely as NADPH. Due to the NADPH requirement, it is likely beneficial for the cell to direct at least a portion of the carbon flux through the pentose phosphate pathway (PPP) (Chemler et al., 2010). These upper (catabolic) pathways are shared for either FAS or RBO routes. For the production of 2 acetyl-CoA, the overall reaction for the upper pathways can be written as:

Glycolysis and Pyruvate Dehydrogenase Complex (PDH)

Glycolysis/PPP and PDH

At one extreme, a route that avoids use of the PPP more efficiently conserves carbon while producing 2 NADH for every acetyl-CoA unit. Conversely, if glucose is shunted exclusively through the PPP, then an additional 0.1 moles of glucose is converted to 0.6 moles CO₂ and 1.2 moles of NADPH.

From acetyl-CoA, FAS and RBO utilize related but distinct mechanisms for carbon chain extension that differ in co-factor and ATP requirements. In the following comparison, we present equations for propane as a target alkane. Despite its inferiority relative to other gasoline-range alkanes, we chose propane for simplicity given the lack of iteration in chain elongation. The overall reaction for the FAS pathway from acetyl-CoA to propane can be written as:

FAS (Acetyl-CoA to Propane)

The FAS pathway begins with the conversion of acetyl-CoA to malonyl-CoA, which is catalyzed by the acetyl-CoA carboxylase and consumes 1 ATP molecule. After transfer of the malonyl unit to ACP, a second acetyl-CoA is condensed with malonyl-ACP to produce β-keto-butyl-ACP and CO₂. The preceding ATP-coupled carboxylation enables a condensation-decarboxylation mechanism for carbon-

444 carbon bond formation with an estimated ΔG of -27 kcal/mol (Caspi et al., 2014). Two NADPH
 445 reducing equivalents are consumed during reduction of the new carbon chain to butyryl-ACP. After
 446 release by the previously mentioned acyl-ACP thioesterase, butyrate is first activated and then reduced
 447 by a carboxylic acid reductase (CAR) consuming 1 ATP (activation) and 1 NADPH (reduction). The
 448 mechanism of butyrate activation hydrolyzes ATP to AMP and pyrophosphate (PP_i). During
 449 deformylation of the resulting butyraldehyde to propane, two additional NADPH are consumed leading
 450 to reduction of O_2 to formate (CHO_2^-). In an *E. coli* host, secondary reactions are likely coupled to this
 451 core pathway. The PP_i produced by CAR is likely hydrolyzed to inorganic phosphate (P_i) by inorganic
 452 pyrophosphatase (Ppa) creating an additional driving force for acid activation. The byproduct AMP is
 453 likely recycled back to ADP via adenylate kinase (Adk) consuming one additional ATP.

454 Taking these background reactions into account, the overall FAS pathway is altered to:

457 For synthesis of propane, the FAS route requires 3 ATP and 5 NADPH. If a purely glycolytic route is
 458 used for acetyl-CoA generation only 2 ATP and 4 reducing equivalents (NADH) are produced. Even if
 459 NADH can be freely interconverted to NADPH additional carbon must be consumed to fulfill the energy
 460 demand. Given the AD-catalyzed formation of the byproduct formate, a formate dehydrogenase could
 461 potentially be employed to convert formate to CO_2 while recovering an additional reducing equivalent,
 462 but additional ATP would still be required (Berríos-Rivera et al., 2002; Gul-Karaguler et al., 2001;
 463 Seelbach et al., 1996). Alternatively, if PPP is used exclusively to produce acetyl-CoA, then 2 ATP and
 464 6.4 reducing equivalents (2.4 NADPH, 4 NADH) are generated from 1.2 moles of glucose. Additional

465 carbon has already been consumed to produce excess reducing equivalents, but as with glycolysis, direct
 466 ATP production is insufficient. Conversion of excess reducing equivalents via respiration is required.
 467 An even split of carbon flux between glycolysis and PPP would nearly balance reducing equivalents, but
 468 still maintain the ATP debt. Pathways to longer chain alkanes via FAS also would require respiration or
 469 byproduct pathways to supply one additional ATP per molecule of product.

470 RBO pathway architecture provides an intriguing alternative. Unlike FAS, chain extension is not
 471 dependent on ATP for carboxylation since acetyl-CoA units are used directly. In addition, beta-
 472 oxidation utilizes NAD^+/NADH cofactors and RBO enzyme variants have been found which utilize
 473 either NADH or NADPH for acyl chain reduction. The overall RBO reaction from acetyl-CoA to
 474 propane described in the current work can be written as:

475 RBO (Acetyl-CoA to Propane, Adk and Ppa reactions)

477 The two key differences between FAS and RBO pathway energetics are the reduction of ATP demand
 478 from 3 to 2 molecules per propane and the change of one reducing equivalent from NADPH to NADH.
 479 The cofactor switch occurs because the enoyl-CoA reductase employed in our described pathway uses
 480 NADH while FAS enoyl-ACP reductases utilize NADPH. With carbon flux split between glycolysis
 481 and glycolysis/PPP, the reducing equivalents can be approximately balanced with sufficient ATP
 482 generated for the downstream pathway. If a formate dehydrogenase is included, the overall pathway
 483 from glucose would be redox neutral via glycolysis only. Interestingly, for longer chain products a
 484 surplus of ATP can be generated with one additional ATP generated for every additional C2 unit. One
 485 might also imagine engineering the pathway in a strain either incapable of respiration or with respiration

486 repressed, similar to *S. cerevisiae* grown on high glucose concentrations (Kobmann et al., 2002; Piškur
487 et al., 2006; Postma et al., 1989; Vemuri et al., 2006). Just as *S. cerevisiae* uses high flux to ethanol to
488 provide ATP for growth, alkane production could be coupled to ATP generation for growth.

489 Further developments will require improvement of key bottleneck reactions within the pathway.
490 As has been observed by others (Kallio et al., 2014), relatively high titers of alcohol or aldehyde
491 byproducts were observed even with ADs expressed. In the context of our RBO platform, utilizing an
492 alternative AD from *N. punctiforme* enabled modest increases in alkane titer. There has been precedent
493 for improved AD activity through additional strain engineering (recombinant ferredoxin and ferredoxin
494 reductase overexpression) and process engineering (Kallio et al., 2014; Schirmer et al., 2010). Both
495 Kallio et al. and Schirmer et al. report between 10 and 100 fold increases in titer by improving process
496 conditions but the maximum titers of both long and short chain alkanes only reached approximately 1
497 mM. Similar strategies could be employed to boost titers via the RBO platform presented in this work.
498 Perhaps further improvements in electron transfer or stability can be made if native protein-protein
499 interacting partners have been hitherto overlooked when recombinantly expressing cyanobacterial ADs.

500 Even with a step-change improvement in AD activity, the tradeoff between alcohol and alkane
501 products must be considered for fuel applications. Consider hexanol and pentane as an example. Both
502 can be synthesized via RBO module 2-MCC when combined with different terminal enzymes (alcohol
503 dehydrogenase or AD). The overall energy efficiency of a pathway (γ^P) can be estimated by considering
504 reaction stoichiometry and the degree of reductance of both substrates and products (Dugar and
505 Stephanopoulos, 2011). 1.5 moles of glucose are required to produce either 1 mole of hexanol or 1 mol
506 of pentane via RBO pathways. The stoichiometric product to substrate ratios are equivalent at 0.66.
507 The degrees of reductance of glucose, hexanol, and pentane are 24, 36, and 32 respectively. Multiplying
508 the ratio of degrees of reductance of product to substrate by the stoichiometric ratios gives $\gamma^P_{\text{hexanol}}=1$

509 and $\gamma^P_{\text{pentane}} = 0.89$. While hexanol synthesis is theoretically as efficient as ethanol synthesis, the route to
510 pentane is inherently less efficient. Under standard conditions the energy density of hexanol (31.7
511 MJ/L) actually exceeds pentane (30.4 MJ/L) which further supports the selection of the alcohol product
512 (Burgess). While alcohol production appears favorable on a theoretical basis, further development of
513 medium chain alkane bioproduction may be driven by overall bioprocess considerations. Increased
514 hydrophobicity could reduce separations costs, enhance *in situ* product removal (reducing product
515 toxicity effects), and reduce capital investment for infrastructure changes.

516 The energy yield deficiencies of alkane biosynthesis could be improved by addressing the source
517 of pathway inefficiencies. In the described pathways the inefficiency is created by the AD reaction
518 where energy is used to reduce O₂ to produce formate while deformylating hexanal. Simple pathway
519 energy analysis also reveals that *any* terminal deformylation or decarboxylation will reduce maximum
520 pathway energy efficiency. Because the loss in efficiency occurs from deformylation, extension to
521 longer chain alkane products creates higher energy efficiencies. An improved energy yield could be
522 obtained by dehydration of an alcohol to an alkene followed by reduction. Such reactions are known for
523 conjugated systems (enoyl-ACP in FAS, bile acid 7 α -dehydroxylase pathway, enoate reductases), but
524 are energetically less favorable for non-conjugated terminal carbons (Hall et al., 2008; Heath and Rock,
525 1995; Parikh et al., 1999; Richter et al., 2011; Wells and Hylemon, 2000).

526 Overall, we have demonstrated that *E. coli* can be metabolically engineered to selectively
527 produce key gasoline-range alkanes (propane, butane, pentane, heptane, and nonane) from simple and
528 renewable carbon sources. We achieved this by utilizing energy efficient RBO modules such that
529 product distribution was tuned towards key gasoline components. We showed that extension by RBO,
530 with increased ATP-efficiency, could be substituted for extension by FAS to selectively produce shorter
531 alkanes. Using RBO extension modules, we were able to achieve production of pentane, blends of

pentane/butane, and blends of pentane/propane. Finally, we showed that use of the A134F mutant of AD_{Pm} resulted in higher titers of the model gasoline-range alkane pentane than the wild-type AD_{Pm} and that an A122F mutant of AD_{Np} resulted in still higher pentane titers than either of those enzymes or the wild-type AD_{Np} . Biosynthesis of gasoline-range alkanes represents a step forward towards the bridging of biofuels research with existing assets of the petroleum and automotive industries.

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Footnotes:

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555 performed experiments; all authors wrote, reviewed, and edited the manuscript.

556 The authors declare no conflict of interest.

557 **Figure Legends:**

558 **Fig. 1:** (A) Composition of typical regular unleaded gasoline displayed in weight percent (wt. %) based
559 on the average of values reported by Sanders and Maynard and by Johansen *et al.* Single asterisk
560 indicates that compounds below 0.5 wt. % are not reported by Johansen *et al.* Double asterisks indicate
561 that wt. % includes contribution from trace compounds in Sanders and Maynard. (B) Modular pathway
562 design used for selective synthesis of key gasoline-range alkanes in engineered *E. coli*. Genes in gray
563 within Modules 1-Pr and 1-Ma are native and were not overexpressed, whereas genes in black were
564 overexpressed. Module names are abbreviations for the following: “Pr” = Propionate; “Ma” = Malonyl-
565 ACP; “BC” = Butyryl-CoA; “MCC” = Medium-Chain-CoA; “Oc” = Octanoate; “SA” = Short Alkanes;
566 “LA” = Long Alkanes.

567 **Fig. 2:** Selective alkane production using either FAS or RBO for carbon chain extension. (A) C8 and
568 C10 FFA titers resulting from Module 3-Oc or Modules 1-Ma and 3-Oc in WT Δ *fadD*. No detectable
569 products were observed for the following longer chain molecules: C12, C14, C16, C16:1, C18, C18:1.
570 (B) Illustration of octanal as a branch-point metabolite to heptane or octanol. (C) Gas-phase titers of
571 octanal observed 24 hours after supplying octanoate to WT and RARE expressing Car_{Ni}. (D) Alkane
572 titers resulting from Modules 1-Ma, 3-Oc, and 4-LA in WT Δ *fadD* and RARE Δ *fadD*. (E) Liquid-phase
573 titers of hexanoate and downstream metabolites observed 24 hours after supplying hexanoate to WT and
574 RARE expressing Car_{Ni}. (F) Alkane titers resulting from Modules 2-MCC and either 4-LA or 4-SA in
575 WT and RARE. (G) Liquid-phase titers of butanol, and hexanol in WT and RARE containing Modules
576 2-MCC and 4-SA. Experiments performed in triplicate with averages as reported values and standard
577 deviation as error bars. All alkane titers are derived from gas-phase sampling.

578 **Fig. 3:** Alternative modules enable synthesis of butane and propane. Titers of alkanes produced by
579 strains MG-C3 (Modules 2-BC and 4-SA in WT), RARE-C3 (Modules 2-BC and 4-SA in RARE), MG-

580 C4 (Modules 1-Pr, 2-MCC, and 4-SA in WT), RARE-C4 (Modules 1-Pr, 2-MCC, and 4-SA in RARE),
581 MG-C5 (Modules 2-MCC and 4-SA in WT), and RARE-C5 (Modules 2-MCC and 4-SA in RARE).
582 Strains MG-C4 and RARE-C4 were supplied glycerol rather than glucose.

583 **Fig. 4:** Increased alkane titers resulting from alternative ADs. (A) Alkane titers resulting from use of
584 AD_{Pm} or AD_{Np} with Car_{Ni} and Modules 1-Ma and 3-Oc in RARE. (B) Protein sequence alignment of
585 wild-type ADs from *P. marinus* and *N. punctiforme*, indicating comparable amino acid candidate for
586 mutation. (C) Relative pentane titers resulting from the use of AD_{Np} or AD_A122F_{Np}, along with Car_{Ni}
587 and Module 2-MCC, in RARE. (D) Alkane titers resulting from use of AD_A134F_{Pm} or AD_A122F_{Np}
588 with Car_{Ni} and Module 2-MCC in RARE.

589 **Fig. 5:** Synthesis of isopentane from 4-methylvalerate (4MV). (A) Illustration of conversion of 4MV to
590 isopentane using variants of Module 4 presented earlier. (B) Titters of isopentane detected in culture
591 headspace 24 hours after addition of 5 mM 4MV to two different cultures expressing alternate ADs.

592

594
595 **Fig. 1.**
596

Fig. 2.

Substrate:			
Glucose	+	-	+
Glycerol	-	+	-
Modules:			
1-Pr	-	+	-
2-BC	+	-	-
2-MCC	-	+	+
4-SA	+	+	+

Fig. 3.

A

B

606

607 **Fig. 5.**

608

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