ACS SyntheticBiology

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Engineering the Autotroph *Methanococcus maripaludis* for Geraniol Production

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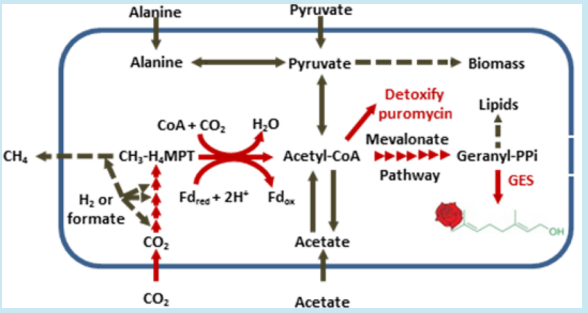
Supporting Information

ABSTRACT: The rapid autotrophic growth of the methanogenic archaeon *Methanococcus maripaludis* on H_2 and CO_2 makes it an attractive microbial chassis to inexpensively produce biochemicals. To explore this potential, a synthetic gene encoding geraniol synthase (GES) derived from *Ocimum basilicum* was cloned into a *M. maripaludis* expression vector under selection for puromycin resistance. Recombinant expression of GES in *M. maripaludis* during autotrophic growth on H_2/CO_2 or formate yielded geraniol at 2.8 and 4.0 mg g^{-1} of dry weight, respectively. The yield of geraniol decreased 2−3-fold when organic carbon sources were added to stimulate heterotrophic growth. In the absence of puromycin, geraniol production during autotrophic growth on formate increased to 4.6 mg g^{-1} of dry weight. A conceptual model centered on the autotrophic acetyl coenzyme A biosynthetic pathway identified strategies to divert more autotrophic carbon flux to geraniol production.



KEYWORDS: *carbon dioxide, hydrogen economy, archaea, methanogen, isoprenoid, synthetic biology*

Isoprenoids are a large family of natural products that serve as excellent starting materials for making drugs, fragrances, biofuels and commodity chemicals.[1] Organisms from all three domains of life synthesize isoprenoids by either the mevalonate pathway or the methylerythritol phosphate (MEP) pathway.[2,3] The former pathway has been found in most eukaryotes, all archaea and a few bacteria. The latter is mainly present in bacteria as well as some photosynthetic eukaryotes.[4] The biosynthetic platforms for isoprenoids have been engineered in eukaryotes and bacteria for making high-value biochemicals. However, the archaeal platform has so far remained uncharted. The rationale for using an archaeal platform is 4-fold. One, archaeal lipids are composed of isoprenoids instead of fatty acids. Thus, archaea already biosynthesize relatively high levels of isoprenoids as part of their normal metabolism.[5] Two, many archaea are autotrophs and derive all their cellular components, including isoprenoids, from CO_2.[6] Thus, there is the potential to make high value chemicals from an inexpensive substrate like CO_2. Three, archaea have evolved many mechanisms to cope with energy stress. Thus, they are generally very energy-efficient.[7] Lastly, many archaea are extremophiles, allowing them to make isoprenoids at high temperature, high salinity, or acidic pHs. These conditions may have advantages for some industrial fermentations.[8]

Methanococcus maripaludis is a genetically tractable model organism for archaea and an excellent chassis for exploring the potential for isoprenoid biosynthesis. Isolated from anaerobic sediments in salt marshes, it is a strictly anaerobic, mesophilic, and hydrogenotrophic methanogen that utilizes H_2/CO_2 or formate as sole carbon and energy sources for methanogenic growth.[9,10] Certain metabolic features make *M. maripaludis* even more attractive. One, autotrophic growth is rapid, with a 2−3 h generation time that allows for a rapid accumulation of biomass. Two, anaerobic product formation offers higher yields and lower costs compared to aerobic systems.[11] Three, its substrates H_2 and formate can be generated by electrical processes powered by solar energy and are an alternative to fossil fuels in the future energy economy.[12,13] Four, the major metabolic waste is CH_4, which is a biofuel already in wide use, and contributes to an efficient energy cycle.

The physiology, biochemistry, and genetic regulation have already been extensively studied in *M. maripaludis*.[14−17] Genome-scale metabolic models, transcriptome and quantitative proteome databases, and transposon maps of essential genes have been recently generated.[18−21] The basic understanding of the microorganism thus generated will contribute greatly to the development of a robust platform for isoprenoid production in *M. maripaludis*. To test this strategy, geraniol was targeted as a compound of interest. Geraniol (3,7-dimethylocta-trans-2,6-dien-1-ol) is a high-value isoprenoid already widely used in fragrance, food and pest control industries due to its pleasant flavor and insect repellent properties.[22] Geraniol is also finding potential applications in the pharmaceutical industry as it exhibits anticancer, antioxidant, anti-inflammatory and antimicrobial activities.[23] However, natural production of geraniol is limited, and metabolic engineering could be a feasible strategy to fulfill

Received: December 9, 2015

DOI: 10.1021/acssynbio.5b00267
ACS Synth. Biol. XXXX, XXX, XXX−XXX

the increasing demand.[24] Here, we present the first study of engineering *M. maripaludis* to autotrophically produce geraniol from CO_2.

Geranyl diphosphate is a key intermediate in the mevalonate pathway for isoprenoid biosynthesis. It can be converted to geraniol by geraniol synthase (GES) in the following reaction:

$$\text{geranyl diphosphate} + H_2O \leftrightarrow \text{geraniol} + \text{pyrophosphate}$$

GES was first characterized in the sweet basil *Ocimum basilicum*, but no such homologue has been identified in *M. maripaludis*.[25] Therefore, recombinant expression of GES would likely be necessary for successful geraniol production in *M. maripaludis*.

Two-phase systems where an organic solvent overlays the culture broth can efficiently harvest toxic or volatile biochemicals from engineered microorganisms.[26] Thus, a two-phased system for growth of methanococci was tested with hexane and decane. A hexane layer completely inhibited growth in minimal formate medium (McF). Although somewhat inhibitory, a decane layer allowed for good growth (Figure 1). When two-phased decane

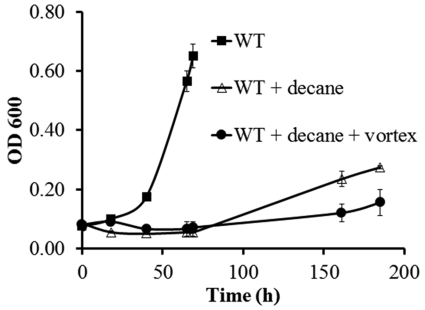


Figure 1. Inhibition of growth of *M. maripaludis* by decane. Growth of strain S0025 (WT) was tested in McF medium. For the vortex treatment, cultures were briefly vortexed to fully mix the decane and broth phases whenever the absorbance was measured. The mean and range of two cultures are shown.

cultures were periodically mixed, growth was further inhibited. For that reason, two-phase decane cultures were grown with minimum mixing in subsequent experiments.

In preliminary experiments, geraniol production was tested in a rich formate medium McFA2 (McF plus acetate and alanine) from recombinant strains S0025, S0026, and S0027, which carried the vector pMEV4 alone, pGESa, and pGESb plasmids, respectively. Although growth of all three strains were indistinguishable in the two-phased medium with decane, geraniol was only detected in S0027 cultures (0.3 mg L^{-1}), indicating that production of geraniol did not inhibit growth of S0027 in McFA2. *M. maripaludis* does not encode a homologue to geraniol synthase, and the failure to detect geraniol in cultures of S0025 was consistent with a requirement for the recombinant GES. The failure to detect geraniol in cultures of S0026 was unexpected given the higher translation initiation rates predicted for GESa, *i.e.*, 1.4-fold and 4.4-fold higher than GESb calculated by RBS calculator and UTR-designer, respectively. GESa and GESb differ in the 14 bp region including 3 bp of upstream of the RBS, the 6 bp of the RBS, and the 5 bp of spacer sequence between the RBS and the initiation codon. Because the difference are more extensive than just the RBS, it is not possible to conclude from these results that ribosome binding was less efficient in pGESa than pGESb. Nevertheless, this result illustrates the difficulty in extrapolating biophysical models for RBS predictions from bacterial to archaeal systems.[27−29]

Geraniol production from strain S0027 was examined further under both autotrophic and heterotrophic growth conditions (Figure 2). In formate medium, growth of S0027 was stimulated

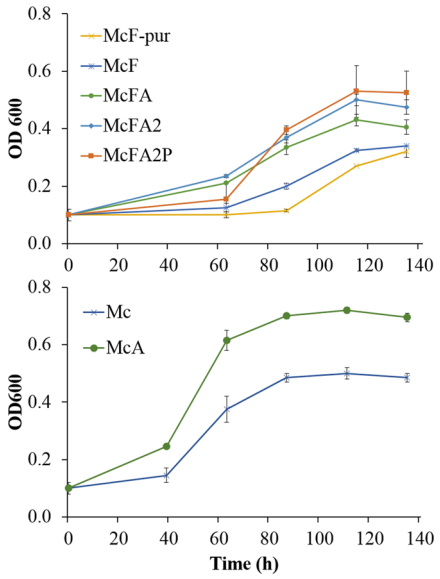


Figure 2. Growth of recombinant *M. maripaludis* strain S0027 with formate (upper) or H_2/CO_2 (lower) as the substrates for methanogenesis. Media were McF, minimal formate medium; McFA, McF+10 mM acetate; McFA2, McFA+1 mM alanine; and McFA2P, McFA2 + 20 mM pyruvate. Mc, minimal H_2/CO medium, McA, Mc+10 mM acetate. All cultures were grown in the presence of 5 μg/mL of puromycin except McF-pur. The mean and range of two cultures are shown.

by addition of acetate (McFA). The growth was further stimulated by addition of alanine (McFA2) and pyruvate (McFA2P). Likewise, the growth yield increased in H_2/CO_2 medium when acetate was included. Removal of puromycin did not stimulate growth in McF. Because resistance depends upon the acetylation of puromycin, these results suggest that acetylation was not a significant burden to growth. Geraniol yields varied substantially among the different growth conditions (Figure 3). In general, conditions which stimulated growth lowered geraniol production. Control experiments indicated that this correlation was not due to geraniol toxicity. In minimal H_2/CO_2 (Mc) medium without decane, growth was not inhibited by 180 mg L^{-1} geraniol, equivalent to 530 mg g^{-1} of dry weight at an absorbance of 1.0. In the absence of puromycin in the minimal formate medium, the geraniol yield was the highest at 4.6 mg g^{-1} of dry weight or 0.5 mg L^{-1}, indicating a negative effect of puromycin in geraniol production. Even under these growth conditions, geraniol was not detected in cultures of S0025 at a detection limit of 0.02 mg L^{-1}, demonstrating that the recombinant GES was necessary.

On the basis of known metabolic pathways in *M. maripaludis*, we propose a conceptual model for autotrophic geraniol biosynthesis in strain S0027 (Figure 4).[14,15,30] Briefly, once CO_2 is fixed into methyl-tetrahydromethanopterin (CH_3-H_4MPT) *via* the methanogenesis pathway, the majority of CH_3-H_4MPT is converted to CH_4 for energy conservation. However, a portion is diverted to autotrophic CO_2 fixation by carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) for biosynthesis of biomass. Thus, acetyl-CoA enters the mevalonate pathway for isoprenoid and lipid biosynthesis, where some of the intermediate geranyl diphos-

DOI: 10.1021/acssynbio.5b00267
ACS Synth. Biol. XXXX, XXX, XXX−XXX

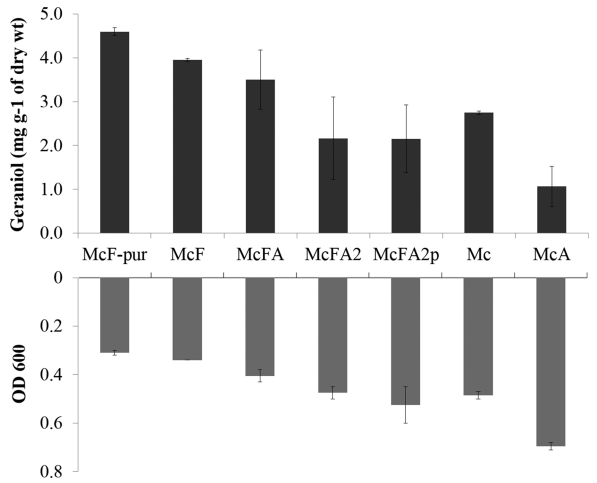


Figure 3. Effect of medium components on geraniol production and growth yield of recombinant *M. maripaludis* strain S0027. Geraniol production and growth yields were measured after 125 h in a two phase culture with decane. See Figure 2 for definition of the media. The mean and range of two cultures are shown.

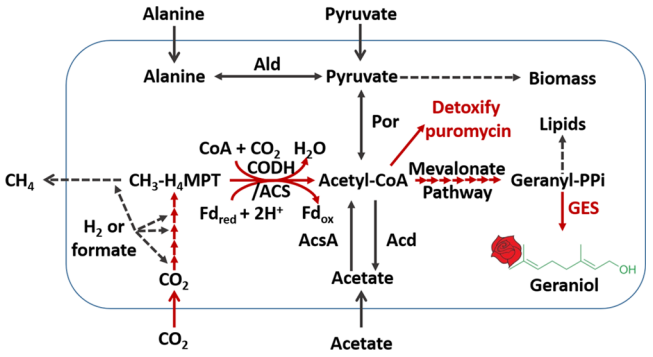


Figure 4. Model for the pathway of geraniol production by the recombinant *M. maripaludis* under autotrophic and heterotrophic growth conditions. The autotrophic pathway is highlighted in red. Growth in minimal H_2/CO_2 or formate medium is fully autotrophic, and all 10 geraniol carbons are derived from CO_2. For heterotrophic growth, carbon from organic substrates, such as acetate, alanine and pyruvate, are assimilated into mevalonate pathway through acetyl-CoA. Carbon for biomass biosynthesis comes from acetyl-CoA and pyruvate. CH_3–H_4MPT: methyl-tetrahydromethanopterin; Fd_{red} and Fd_{ox}: reduced and oxidized ferredoxin, respectively; CODH/ACS (MMP0979–0985): carbon monoxide dehydrogenase/acetyl-CoA synthase; Ald (MMP1513), alanine dehydrogenase. Por (MMP1502–1507), pyruvate oxidoredutase; AcsA (MMP0148) and Acd (MMP0253): AMP and ADP-dependent acetyl-CoA synthetases, respectively; GES, geraniol synthase; Geranyl-PPi: geranyl diphosphate.

phate is diverted to geraniol. Since detoxification of puromycin consumes acetyl-CoA, omission of puromycin releases some of the acetyl-CoA flux into the mevalonate pathway and stimulates geraniol synthesis. Under heterotrophic conditions, uptake of exogenous organic carbons (*i.e.*, acetate, alanine and pyruvate) stimulates protein and biomass synthesis and increases the demand for isoprenoid lipids. As a result, more metabolic flux is pulled away from geraniol synthesis and into lipid biosynthesis. This hypothesis predicts that culture conditions or mutations that limit the flow of carbon into amino acids would increase the yield of geraniol.

Compared to tobacco cell suspension cultures, strain S0027 produced up to 15-fold higher yield of geraniol (*i.e.*, 4.6 *versus* 0.3 mg g^{-1} of dry weight, assuming a fresh weight/dry weight ratio of 10:1 in tobacco cells).[31] However, recombinant yeast cells expressing the *O. basilicum* GES produced even higher yields of 8 mg g^{-1} of dry weight, assuming a dry weight of yeast cells at 0.62 g L^{-1} OD^{-1}.[32] Therefore, further optimization is needed to increase the geraniol yield in *M. maripaludis* to the levels already achieved in yeast. Three strategies could be employed. First, in yeast the yield of geraniol was increased 7-fold by overexpression of key rate-limiting enzymes of the mevalonate pathway necessary for geranyl diphosphate biosynthesis.[33] Overexpression of the same enzymes in *M. maripaludis* may have a similar effect. Second, the *O. basilicum* GES gene also encodes an N-terminal signal peptide for targeting the enzyme to the plastid. Truncating the gene to remove the signal peptide increased the geraniol yield in *E. coli*, presumably by increasing the amount of soluble enzyme.[34,35] In addition, our results suggest that limiting the flux of carbon into other components of biomass, such as protein or nucleic acids, might increase the availability of intermediates of the mevalonate pathway for geraniol biosynthesis without increasing demand for cellular lipids. Further experiments will be necessary to explore these possibilities.

In summary, a synthetic geraniol synthase gene was successfully expressed in *M. maripaludis*, which diverted carbon from cellular isoprenoids synthesis into geraniol production. Geraniol was produced during both autotrophic and heterotrophic growth. This first proof-of-concept study demonstrates the feasibility of fixing CO_2 into high-value biochemicals by taking advantage of the unique autotrophic metabolism of *M. maripaludis*.

METHODS

Microbial Strains and Culture Conditions. Strains used in this study are listed in Table 1. The minimal formate and H_2/CO_2 broth and solid media for cultivation of *M. maripaludis* have been described previously.[17,18,36] Whenever needed, 10 mM of acetate, 1 mM of alanine and 20 mM of pyruvate were added into the minimal medium for stimulating growth. Puromycin, 2.5 μg/mL, was included in the medium to select growth of the recombinant cells, unless otherwise mentioned. For the two-phase growth system, 20% (v/v) of either hexane or decane was added to the top of the broth medium after inoculation. For prescreening positive recombinant colonies for geraniol production, 1.2×10^8 cells were inoculated into 5 mL of broth and then overlaid by decane at 37 °C without shaking. For geraniol production under different growth conditions, cells were first adapted in the two-phase system with minimal formate medium and 10% (v/v) of decane for 3 generations at 30 °C without shaking. After growth, 6×10^7 of the adapted cells were inoculated into 5 mL of broth containing 5 μg/mL of puromycin and 20% decane under various growth conditions. All cultures were prepared at least in duplicate and incubated at 30 °C in a shaker at 150 rpm unless otherwise mentioned.

Plasmid and Recombinant Strain Construction. PCR primers and plasmids are listed in Table 1, and cloning was performed in *E. coli* Top10. Derived from the pAW42 vector, the pMEV4 vector was transformed into *M. maripaludis* S0001 to yield S0025.[17] The GES gene encoding geraniol synthase from *Ocimum basilicum* was codon optimized and synthesized for expression in *M. maripaludis* (Figure S1). Primers GSF-mrtE/GS-R were used to prefix ribosomal binding site A (RBS-A) before GES by PCR amplification, and the resulted RBS-A-GES module was cut at the XbaI/*Pst*I sites and cloned into pMEV4 at the SpeI/*Pst*I sites, producing the pGESa plasmid (Figure S2).

DOI: 10.1021/acssynbio.5b00267
ACS Synth. Biol. XXXX, XXX, XXX–XXX

Table 1. Microbial Strains, Primers, and Plasmids Used in This Study

names	descriptions[a]	references
Primers		
Mpur-F	GCATCTAGATGAATTCTCTGCAGATAAAAAACGCCCTATTCGT	This study
Mpur-R	CCATGGTTATGCTCCTGGTTTTCTTGT	This study
Pmv-F	GCAGAATTCGCGCCCGGTTCTAGACGAGATAAGAATTACTAGATAATTC	This study
Pmv-R	AGCCTGCAGCGGCCGCTACTAGTATTAGTTATCTATAAAATTATAATATCAA	This study
GSF-mrtE	GGGAAATCTAGATGTGAGGTGAAATAATGTCATGTGCAAGAATAACAGTTA	This study
GSF-2	GGGAAATCTAGAAATAGGTGAAATGCATGTCATGTGCAAGAATAACAGTTA	This study
GS-R	GGGAAACTGCAGCGGCCGCTACTAGTTTATTGAGTAAAGAAAAGTGCATCTAC	This study
Plasmids		
pAW42	Expression vector chassis for developing pMEV4	39
pMEV4	Vector made by making pAW42 BioBrick compatible	This study
pGESa	Synthetic RBS-A-GES module cloned into pMEV4	This study
pGESb	Synthetic RBS-B-GES module cloned into pMEV4	This study
Strains		
S0001	Expression host containing ORF1 from pURB500 integrated into the *M. maripaludis* S2 genome	39
S0025	Recombinant *M. maripaludis* strain hosting the pMEV4 vector	This study
S0026	Recombinant *M. maripaludis* strain hosting the pGESa vector	This study
S0027	Recombinant *M. maripaludis* strain hosting the pGESb vector	This study

[a]Restriction sites are either underlined or bolded (NotI only).

Transformation of this plasmid into *M. maripaludis* S0001 yielded the recombinant strain S0026. Likewise, a RBS-B-GES module was made using primers GSF-2/GS-R and cloned into pMEV4 using the same restriction sites as for making pMEV4-GESa, producing the pGESb plasmid. Transformation of this plasmid into *M. maripaludis* S0001 yielded the recombinant strain S0027. RBS-A (5′- TGTGAGGTGAAATA-3′) and RBS-B (5′-AATAGGTGAAATGC-3′) were derived from the 5′-region immediately upstream of the initiation codon for the *mtrE* gene (MMP1560) in *M. maripaludis* or modified from the *hmvA* gene (X69792) in *M. voltae* by replacing the last four base pairs with ATGC, respectively.[37,38] This modification introduced a NsiI restriction site, which would allow convenient editing of the RBS region if needed. Translation initiation rates for RBS-A-GES and RBS-B-GES were predicted by either RBS calculator or UTR designer.[27,28] Colonies harboring the recombinant plasmids were purified under the selection of puromycin.

Analysis of Geraniol. Preliminary experiments indicated that most of the geraniol found in cultures was associated with the culture broth and not the cells (data not shown). For that reason, after growth in the two-phase cultures, the decane phase was collected by centrifugation at 13 800*g* for 10 min at 4 °C and examined for geraniol. The decane phase was concentrated under reduced pressure using the Thermo Savant SpeedVac 121P at 35 °C, unless otherwise mentioned. The recovery efficiency of this concentration step was determined to be 47.1 ± 2.5% by concentrating known amounts of geraniol (0.9 mg L^{-1} in decane) in parallel to samples derived from growth experiments. Geraniol was quantified by injecting 4 μL of each sample into a HP6890 gas chromatograph connected to a HP5973 mass spectrometer. The sample was separated on a 19091J-433 HP-5 column (length, 30 m; diameter, 0.25 mm; film thickness, 0.25 μm). The oven temperature was initially held at 80 °C for 1 min and sequentially increased at a rate of 10 °C min^{-1} to 280 °C. Helium was the carrier gas with an inlet pressure of 1.63 psi. Geraniol (98% purity, Aldrich Chemical Co.) standard was subjected to full mass scan to verify the mass profile. The four most abundant fragment ions 41, 69, 93, and 123 were selected for monitoring with higher sensitivity and construction of a standard curve. The extraction efficiency of geraniol from formate and H_2/CO_2 media in the two-phase system with decane was determined to be 16.5 ± 2.3% and 13.5 ± 2.7%, respectively. The final geraniol yield for each culture was corrected by taking this extraction efficiency into consideration.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00267.

Alignment of the optimized (GES-opt) and original GES (accession number = AY362553) DNA sequences. The optimization increased the Codon Adaptation Index (CAI) value from 0.4 to 0.9 predicted for *M. maripaludis*. (PDF)

Cloning scheme of GES into pMEV4. Geraniol synthase was cloned into the Biobrick sites of the *Methanococcus* expression vector pMEV4. P_{hmvA}: *Methanococcus* promoter. RBS: *Methanococcus* ribosomal binding site. GES-opt: codon optimized geraniol synthase. The module is flanked by the Biobrick sites (*Eco*RI/XbaI and SpeI/NotI/*Pst*I). (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Professor Brahm P. Verma (College of Engineering), Jessica O. Calkins (College of Engineering), Annika McNally (2012 UGA-iGEM alumni), Michael Hampton and Marquise Westbrook (2013 UGA-iGEM alumni), as well as all previous and current members of the iGEM club at University of Georgia for their passionate participation and support for this research project. This project was partially supported by the Office of Vice President for Research of the University of Georgia.

DOI: 10.1021/acssynbio.5b00267
ACS Synth. Biol. XXXX, XXX, XXX−XXX

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DOI: 10.1021/acssynbio.5b00267
ACS Synth. Biol. XXXX, XXX, XXX−XXX