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Development of a Vanillate Biosensor for the Vanillin Biosynthesis Pathway in *E. coli*

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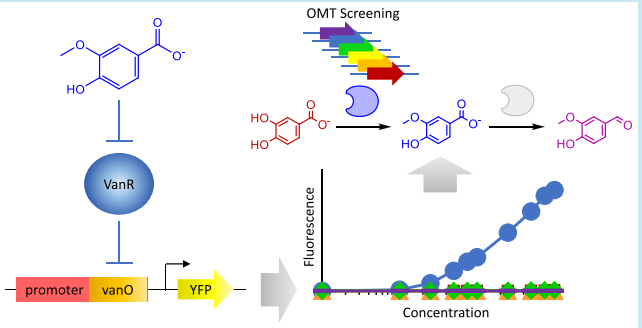
Supporting Information

ABSTRACT: The engineered *de novo* vanillin biosynthesis pathway constructed in *Escherichia coli* is industrially relevant but limited by the reaction catalyzed by catechol *O*-methyltransferase, which is intended to catalyze the conversion of protocatechuate to vanillate. To identify alternative *O*-methyltransferases, we constructed a vanillate sensor based on the *Caulobacter crescentus* VanR-VanO system. Using an *E. coli* promoter library, we achieved greater than 14-fold dynamic range in our best rationally constructed sensor. We found that this construct and an evolved variant demonstrate remarkable substrate selectivity, exhibiting no detectable response to the regioisomer byproduct isovanillate and minimal response to structurally similar pathway intermediates. We then harnessed the evolved biosensor to conduct rapid bioprospecting of natural catechol *O*-methyltransferases and identified three previously uncharacterized but active *O*-methyltransferases. Collectively, these efforts enrich our knowledge of how biosensing can aid metabolic engineering and constitute the foundation for future improvements in vanillin pathway productivity.



KEYWORDS: *biosensor, metabolic engineering, vanillin, vanillate, catechol O-methyltransferase, bioprospecting*

Nature evolved a myriad of genetically encoded small molecule biosensors for detection and response to chemical stimuli. Allosteric transcription factors are a common form of biosensor and are commonly employed in inducible gene expression systems[1] and in engineered genetic circuits.[2] The potential value of biosensors to metabolic engineers, who seek to maximize production of value-added products by manipulating cellular metabolism, is immense and multifaceted.[3,4] Genetically encoded biosensors facilitate metabolic engineering by enabling high-throughput detection of metabolite concentrations,[5−9] directed evolution of host and pathway enzymes,[10−12] and dynamic regulation.[13−15] During the past decade, sensors have been used to increase titers in engineered pathways based on detection of molecules as diverse as short-chain alcohols such as butanol,[5] CoA-tethered pathway intermediates such as malonyl-CoA,[6] and aromatic molecules such as *L*-3,4-dihydroxyphenylalanine,[7] *p*-coumaric acid,[8] and *L*-phenylalanine.[9]

The *E. coli de novo* vanillin biosynthesis pathway[16,17] is a highly industrially relevant pathway for which genetically encoded biosensors could be developed for all three natural heterologous metabolites, and its potential as a testbed for multiplexed biosensing increases its scientific relevance. Vanillin is the primary molecule responsible for vanilla flavor and is the largest flavor additive by volume, with an annual global market worth $650 million and a total volume of 18 000 tons.[18] It is also used as an intermediate for pharmaceuticals and specialty chemicals.[19] Though the vanillin biosynthesis pathway consists of just three heterologous enzymes, numerous potential byproducts can be formed, which can be difficult to separate (Figure 1). The enzyme AsbF from *Bacillus thuringiensis* catalyzes the conversion of an endogenous intermediate of the aromatic amino acid biosynthesis pathway, 3-dehydroshikimate, to protocatechuate (also referred to as 3,4-dihydroxybenzoate).[20] The soluble catechol O-methyltransferase from *Homo sapiens* (OMT_{Hs}) catalyzes the next desired conversion of protocatechuate to vanillate.[21−23] Finally, the carboxylic acid reductase from *Nocardia iowensis* (Car_{Ni}) catalyzes the final desired conversion of vanillate to vanillin.[24,25] Use of an engineered *E. coli* host (the *E. coli* RARE strain) minimizes rapid endogenous conversion of vanillin to vanillyl alcohol.[16] However, other products are formed downstream of the protocatechuate branch point due to the broad substrate specificity of OMT_{Hs} and Car_{Ni}. OMT_{Hs} catalyzes the formation of isovanillate by transferring a methyl group to the hydroxy group at the para rather than meta position. Car_{Ni} can also convert protocatechuate to protocatechualdehyde, which is a toxic intermediate, though it is possible for OMT_{Hs} to subsequently convert protocatechualdehyde into vanillin.

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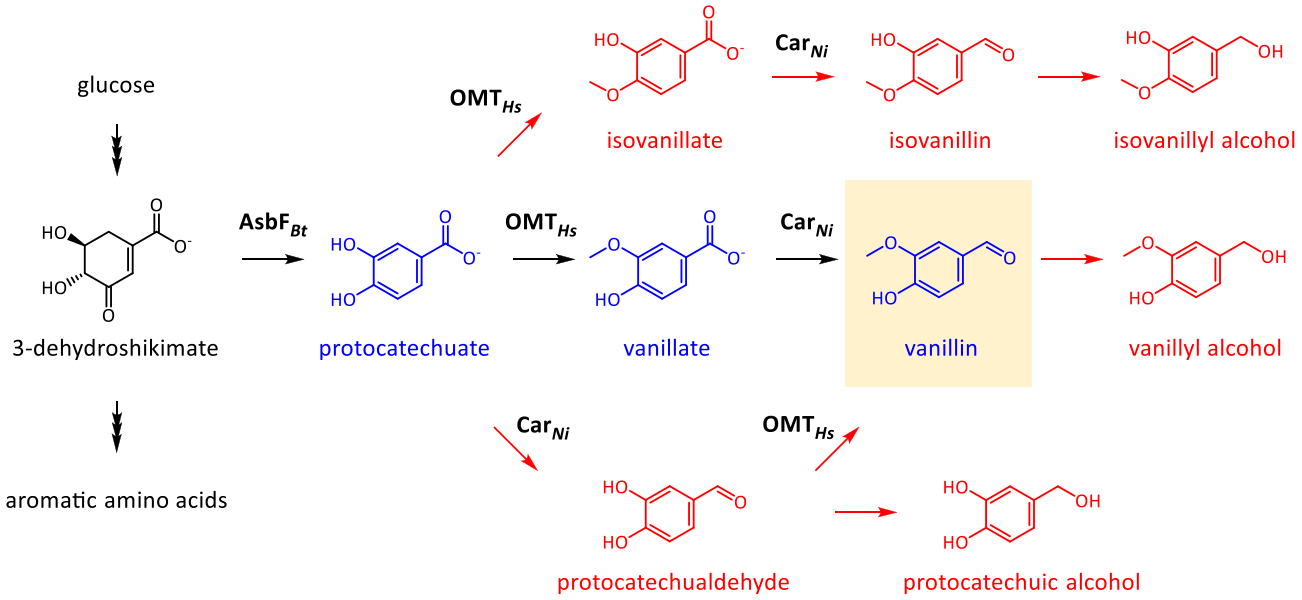

Figure 1. Metabolic pathway diagram of the engineered *de novo* vanillin biosynthesis pathway and potential byproducts in *E. coli*. Heterologous steps begin with AsbF-catalyzed conversion of endogenous 3-dehydroshikimate into protocatechuate. Protocatechuate, vanillate, and vanillin form desired heterologous metabolites and are shown in blue. Undesired metabolites that form due to low specificity of Car and OMT enzymes or due to endogenous aldehyde reductase activity in wild-type *E. coli* strains are shown in red.

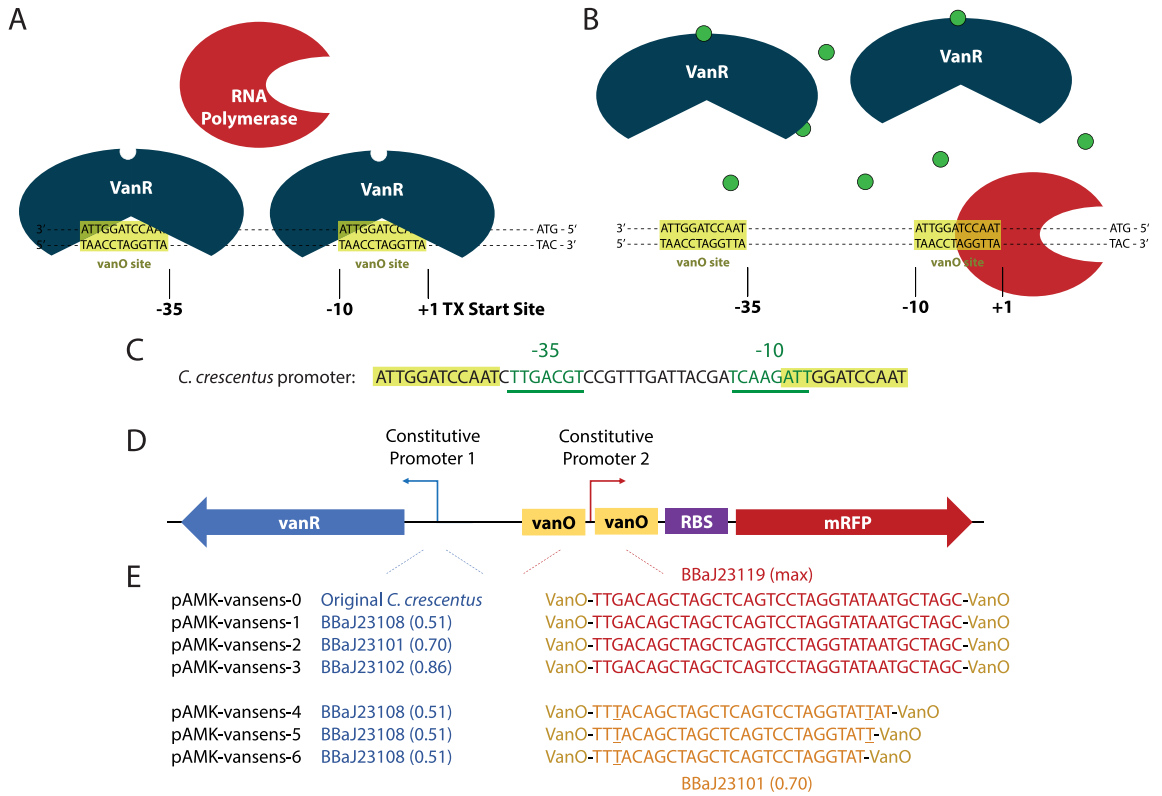

Figure 2. Original vanillate biosensor components and design. (A) Cartoon illustrating desired binding of vanillate repressor VanR to vanillate operator VanO sites. VanO sites are adjacent to −35 and −10 regions upstream of transcriptional start site, thereby hindering binding of sigma factor and RNA polymerase complex. (B) Cartoon illustrating desired unbinding of allosterically regulated VanR from VanO in the presence of vanillate, thereby enabling transcription. (C) Sequence of the native pVanAB promoter regulated by VanR in *Caulobacter crescentus*, showing that one of the two VanO sites overlaps with the −10 region. The consensus −10 region in *E. coli* is TATAAT and the consensus −35 is TTGACA. (D) High-level abstraction of vanillate sensor design. (E) Candidate promoter sequences tested experimentally in this manuscript. The two underlined "T"s in BBaJ23101 reflect the sequence difference between it and BBaJ23119.

The ability to sense vanillate could improve vanillin titers through several means. On the basis of the observation that protocatechuate accumulates, we previously found that OMT$_{Hs}$ steadily loses activity during the first 48 h after induction and

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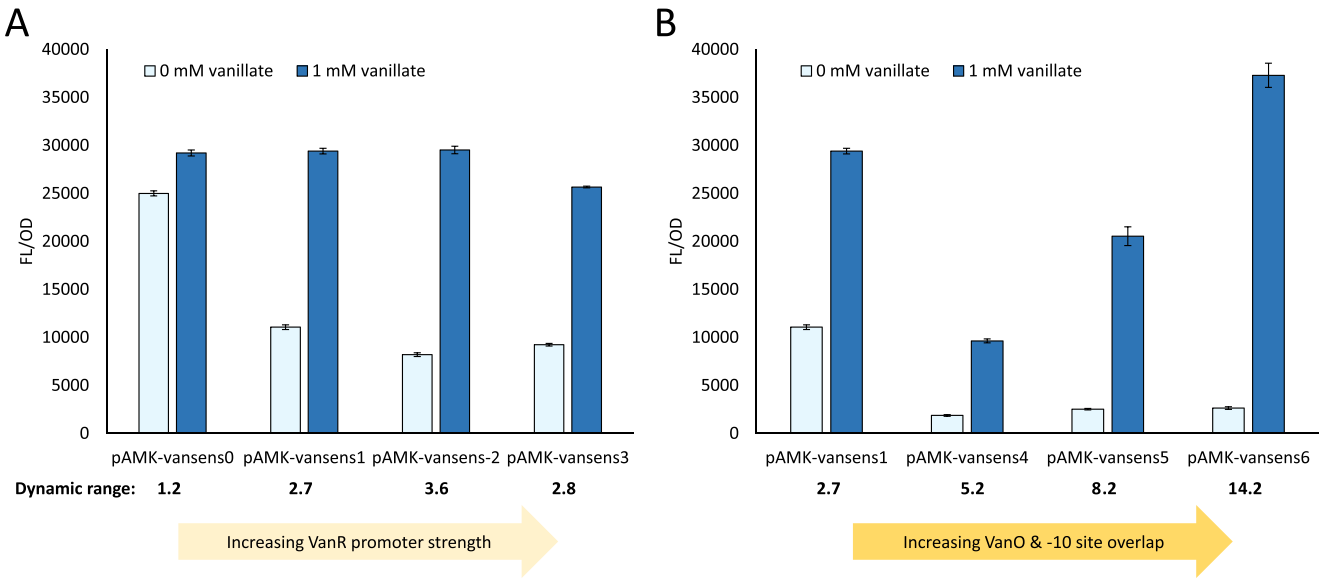

Figure 3. Dynamic range characterization of candidate vanillate sensor constructs. (A) Dynamic ranges observed using candidate vanillate sensor constructs expressing different VanR promoter strengths. (B) Dynamic ranges observed using constructs containing different extents of overlap between VanO and *E. coli* −10 site.

that the cofactor *S*-adenosyl-methionine (SAM), which is the required methyl donor, appears to become scarce as fermentation time proceeds.[17] A vanillate sensor could be used to improve OMT_{Hs} activity, to engineer a host for increased SAM pool size, or to rapidly screen the natural diversity of OMTs in search of a better variant to relieve this pathway bottleneck. In principle, a vanillate sensor could also be used to delay expression of Car_{Ni} until vanillate accumulated, which should favor flux through desired heterologous reaction steps and decrease formation of toxic protocatechualdehyde.

We examined the literature for allosteric transcription factors that respond to the three desired heterologous metabolites—protocatechuate, vanillate, and vanillin—given that they are all naturally obtained through lignin degradation (Figure 1). An *E. coli* sensor for protocatechuate was previously constructed by importing the PcaU activator and associated promoter sequence from *Acinetobacter*.[26] This sensor was reported to exhibit a graded (or analog) response across millimolar concentrations of protocatechuate, with high substrate specificity and approximately 10-fold dynamic range. In addition to the protocatechuate sensor, multiple efforts have been undertaken to develop an *E. coli* sensor specific for vanillin. The QacR transcriptional repressor was computationally redesigned to bind to the non-native substrate vanillin and led to variants that exhibited dose-dependent derepression *in vitro* and *in vivo*.[27] Additionally, recent work has revealed that a native *E. coli* promoter may be responsive specifically to vanillin.[28] However, we found no dedicated effort to construct and characterize a sensor responsive to vanillate in *E. coli*.

Here, we report the development, characterization, and application of a genetically encoded sensor for vanillate in *E. coli* based on naturally occurring transcriptional repressor-operator sequences found in *Caulobacter crescentus*. Our initial results obtained through rational modifications demonstrate the respective influence of repressor promoter strength and operator site positioning on sensor dynamic range. Our subsequent results showcase the exquisite molecular specificity of the VanR transcription factor through the dose−responses obtained for original and evolved vanillate biosensor constructs. We conclude by illustrating the value of biosensor-based bioprospecting by screening the natural diversity of catechol *O*-methyltransferases sampled across all domains of life. Our approach rapidly identifies previously uncharacterized *O*-methyltransferases that successfully convert protocatechuate to vanillate but also highlights challenges associated with using biosensors to rank order candidate enzymes for metabolic pathway applications.

RESULTS AND DISCUSSION

Vanillate-responsive transcriptional repressors (VanR) exist in several bacteria, including *Acinetobacter*,[29] *Caulobacter*,[30] *Corynebacterium*,[31] *Myxococcus*,[32] and *Pseudomonas*.[33] These VanR variants bind to different operator (VanO) sites consisting of inverted repeats ranging from AACTAACTAA-(N_4)TTAGGTATTT in *Cornyebacterium glutamicum* to ATTGGATCCAAT in *Caulobacter crescentus* (Figure 2A−C). We sought to import the *C. crescentus* system into *E. coli* given the relatively small operator site and because a vanillate-inducible expression system was successfully developed for use in *C. crescentus*.[30] At first, we considered the three base pair overlap of the natural VanO sequence with the *C. crescentus* −10 site a potential obstacle to direct promoter transfer and sought to test rational alternatives for *E. coli* (Figure 2C). Placement of the VanO site at the same position in relation to the *E. coli* −10 site (leading to TATATT instead of the consensus TATAAT[34]) is predicted to significantly disfavor interaction with the *E. coli* sigma factor 70.[35]

We aimed to construct a functional vanillate sensor in *E. coli* using the *C. crescentus* VanR-VanO system to regulate expression of a red fluorescent protein (mRFP) (Figure 2D). The complete list of candidate promoter sequences used for expression of codon-adjusted variants of VanR and mRFP are shown in Figure 2E. Our initial vanillate sensor construct (pAMK-vansens-0) used the native *C. crescentus* promoter upstream of VanR to drive its expression. A strong Anderson library promoter[36] flanked by VanO sequences was placed upstream of mRFP. *E. coli* harboring this construct showed

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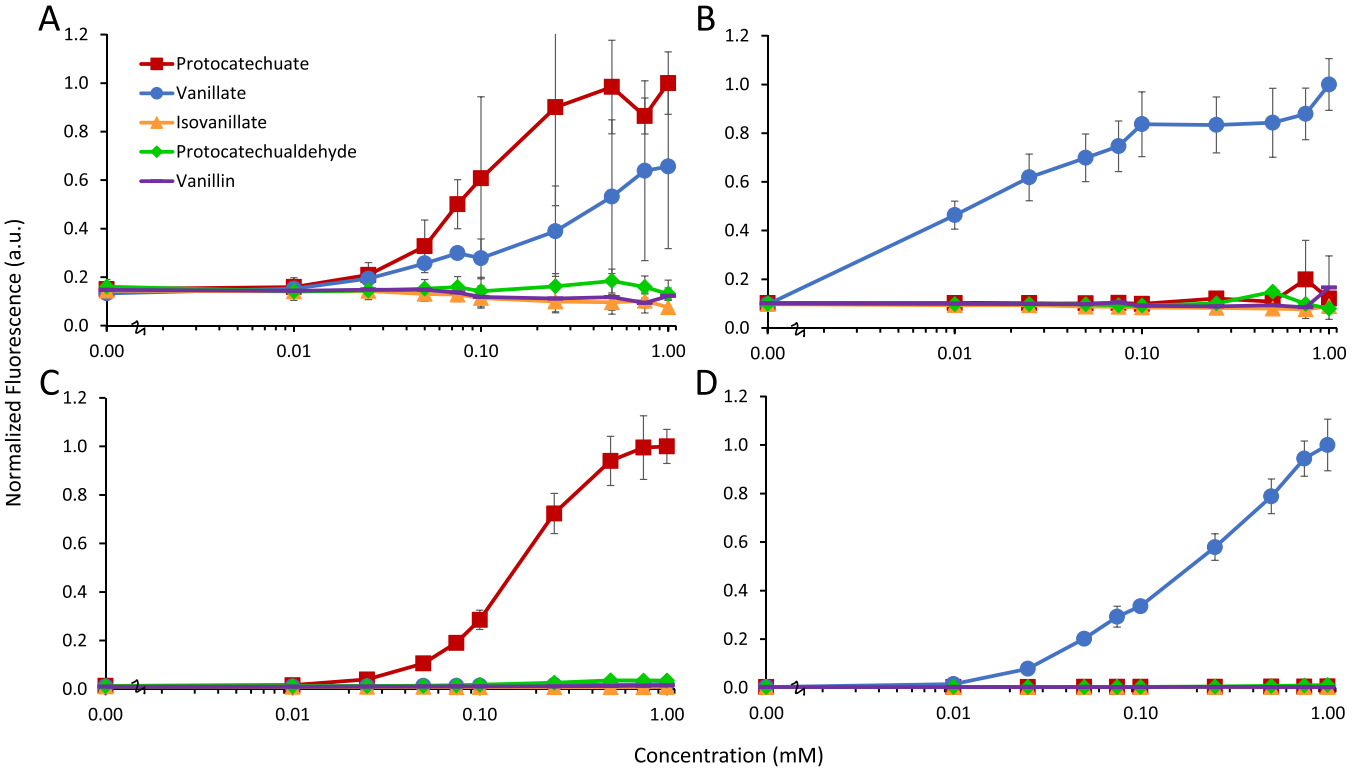

Figure 4. Dose−response characterization of original and evolved sensors intended for protocatechuate and vanillate, respectively. (A) Original protocatechuate sensor (from ref 26 and subcloned into pCDFDuet-1 as pAMK-protosens). (B) Original vanillate sensor (pAMK-vansens-6). (C) Evolved protocatechuate sensor (pAJM.690, from ref 39). (D) Evolved vanillate sensor (pAJM.773, from ref 39).

little to no change in mRFP fluorescence upon addition of 1 mM vanillate. As we began to increase expression of VanR using Anderson library promoters instead of the native *C. crescentus* promoter (previously valued at 0.51/0.70/0.86 expression strength−pAMK-vansens-1/2/3, respectively), we observed an increased ratio of fluorescence in the presence of vanillate to fluorescence in the absence of vanillate (Figure 3A). However, we observed substantial leaky fluorescence (Figure 3A). After realizing that the downstream VanO site was neither adjacent to nor overlapping with the −10 site, we generated three additional constructs with the VanO site placed adjacent (pAMK-vansens-4) or with 1 or 2 bp overlap (pAMK-vansens-5 or pAMK-vansens-6, respectively). *E. coli* harboring these constructs exhibited low fluorescence in the absence of vanillate and increasing dynamic range as overlap of VanO and −10 site increased (Figure 3B). The best performing construct, pAMK-vansens-6, exhibited a dynamic range of approximately 14-fold and was chosen for further characterization.

We characterized the dose−response behavior of this vanillate sensor for five relevant substrates that are typically produced after coexpression of vanillin pathway genes in *E. coli*: protocatechuate, vanillate, isovanillate, protocatechualdehyde, and vanillin. These substrates were provided at concentrations ranging from 0.01 mM to 1 mM, which are the orders of magnitude of observed accumulation of protocatechuate and vanillate in the absence of Car_{Ni} expression[17] in minimal and rich media conditions. Furthermore, we compared vanillate sensor performance with the best available sensor reported for protocatechuate as a reference and to explore future potential for dual sensing. We cloned the published protocatechuate sensor (PcaU and promoter region) upstream of mRFP in place of equivalent components in the vanillate sensor to create an otherwise identical construct (pAMK-protosens-1). On the basis of an end point assay 24 h after substrate supplementation, we observed graded dose−response to protocatechuate consistent with that in the literature[26] (Figure 4A). Consistent with what is reported in the literature, the protocatechuate sensor also activated in the presence of vanillate. In contrast, our best vanillate sensor (pAMK-vansens-6) exhibited high sensitivity to vanillate, with half-maximal activation occurring around 10 μM and full activation below the 100 μM range (Figure 4B). The high sensitivity and more quickly saturating response were concerning given our aspirations to ascertain the relative concentration resulting from biosynthesis in different strains rather than merely whether vanillate was present. Our concerns were mitigated by the excellent specificity of the vanillate sensor, which exhibited minimal response to protocatechuate and remarkably no response to the regioisomer isovanillate. Many catechol *O*-methyltransferases, including OMT_{Hs}, possess the undesired capacity to methylate a hydroxy group on the para rather than meta position of the aromatic ring relative to the carboxylic acid group, which gives rise to isovanillate from protocatechuate.[22,37] Isovanillate is particularly problematic given that Car_{Ni} will catalyze conversion to isovanillin, and these two regioisomers add hurdles to the separation from vanillate and vanillin for accurate quantification or product purification.

We next tested the vanillate sensor in cells that expressed the vanillin biosynthesis pathway. *E. coli* RARE cells harboring pathway constructs and pAMK-vansens-6 exhibited a low level of fluorescence in LB medium and several-fold additional activation in response to externally supplied vanillate

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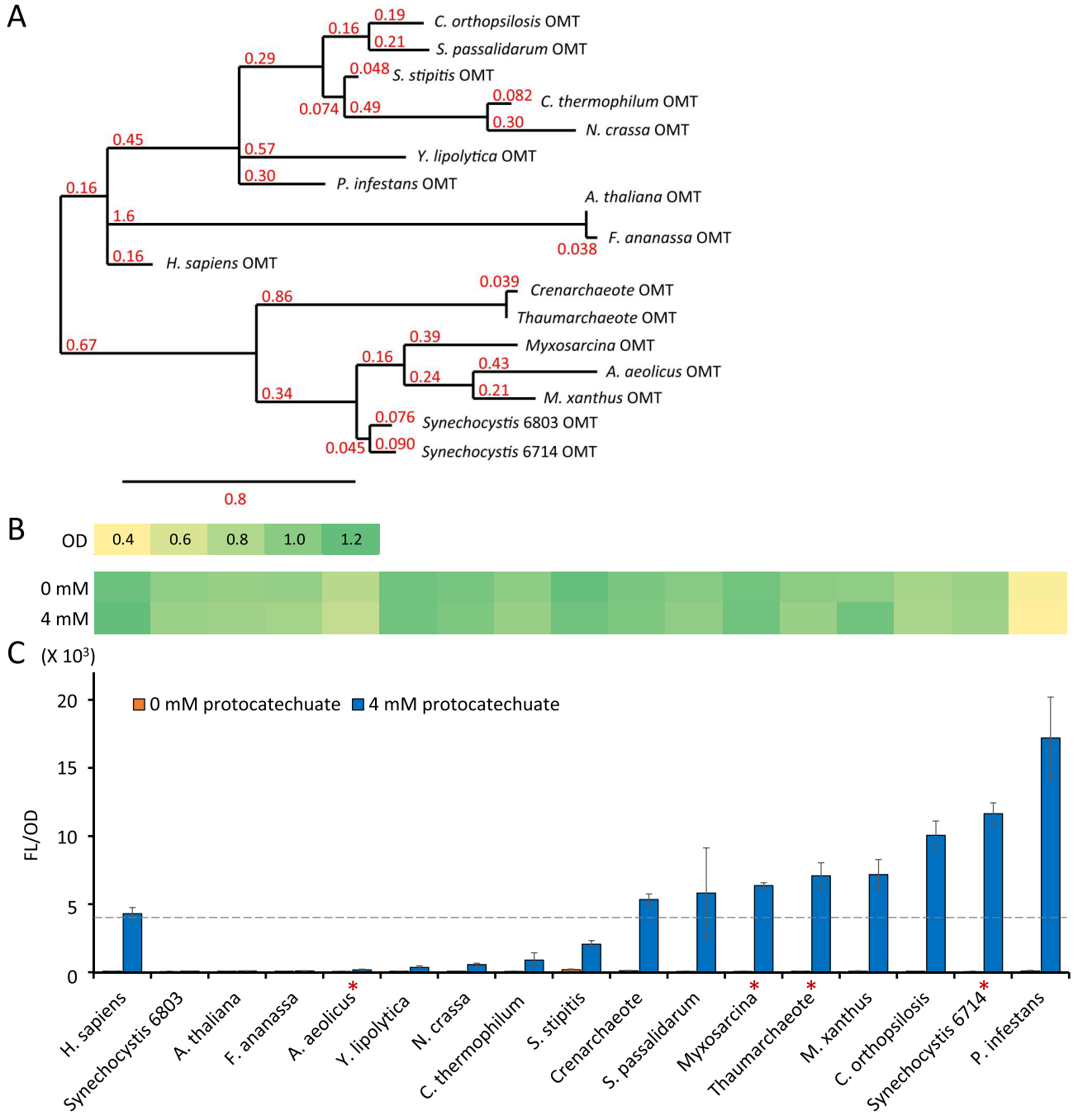

Figure 5. Biosensor-based bioprospecting of naturally occurring *O*-methyltransferase variants. (A) Sixteen variants from diverse sources were chosen for rapid screening of *in vivo O*-methyltransferase activity on exogenously supplemented protocatechuate. Phylogenetic tree was generated using Phylogeny.fr and protein sequence inputs. Red values indicate branch lengths. (B) Average final optical densities (OD) of cultures that harbor evolved vanillate biosensor and that express OMT variants in the absence (0 mM) or presence (4 mM) of exogenously supplied protocatechuate at the 24 h end point of the assay. (C) End point OD-normalized fluorescence of cultures that harbor vanillate biosensor and that express OMT variants in the absence or presence of exogenously supplied protocatechuate. Dashed line demarcates performance of reference OMT (Hs) to facilitate identification of active variants. Variants with red asterisk above species name were not previously characterized.

(Supporting Information (SI) Figure 1A). These cells also demonstrated endogenous activation of the vanillate sensor in M9 minimal media supplemented with glucose and IPTG for induction of pathway genes (SI Figure 1B). However, the sensor exhibited full activation under this condition and full activation when no IPTG was added, suggesting that the sensor is saturated by leaky uninduced pathway expression in the presence of glucose.

To overcome these challenges, we pursued further engineering of our vanillate sensor. We provided colleagues in the laboratory of Professor Christopher Voigt (MIT Department of Biological Engineering, Cambridge, MA) this sensor as well as the recloned protocatechuate sensor for evolution by compartmentalized partnered replication,[38] which was successfully used to improve the dynamic range of these two sensors as well as 10 others as very recently reported.[39] In brief, the process of compartmentalized partnered replication encapsu-

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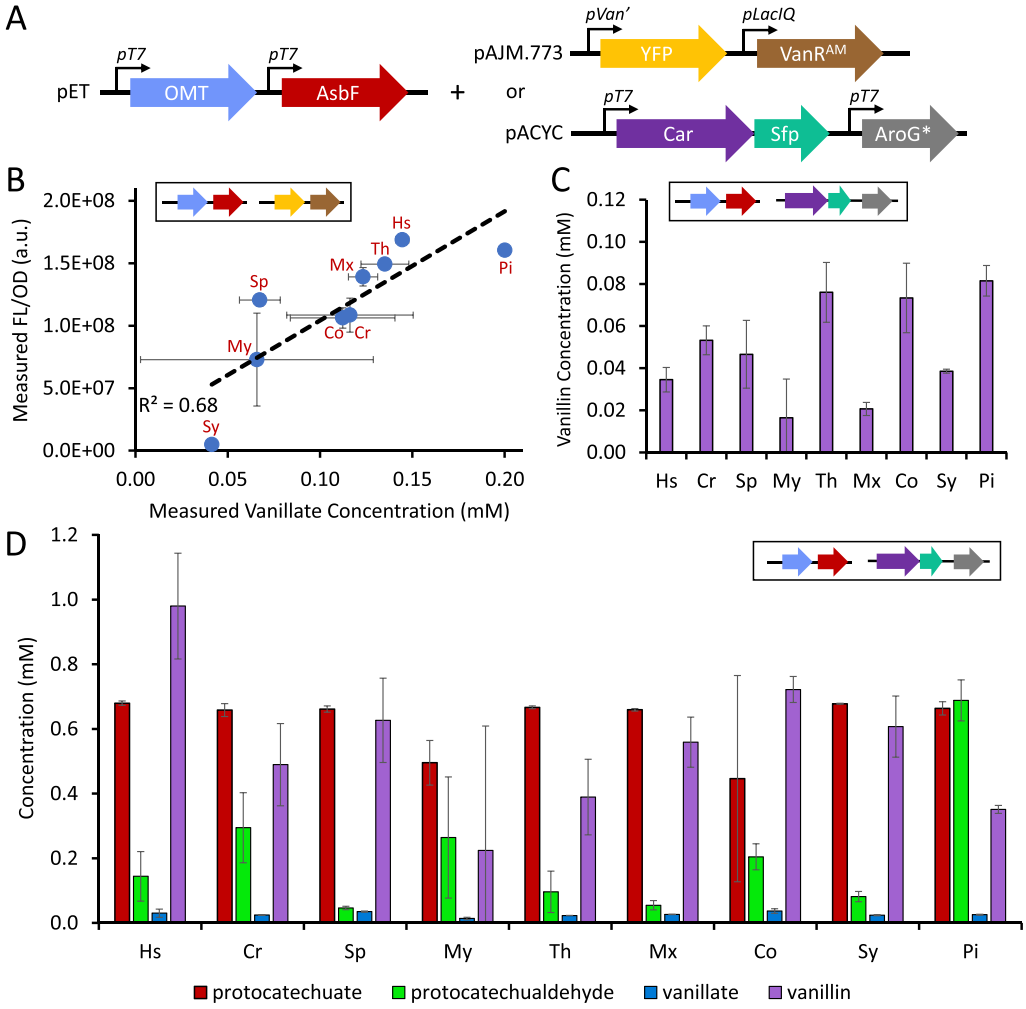

Figure 6. OMT performance in *E. coli* vanillin biosynthesis pathway. (A) Schematic of constructs used for experiments shown in Figure 6. pET plasmid was paired with either biosensor plasmid pAJM.773 or full pathway plasmid pACYC. (B) Correlation between measured vanillate titer in supernatant and OD-normalized fluorescence from cells cotransformed with pET-OMT$_i$-AsbF and pAJM.773, cultured in M9 minimal media supplemented with 1% glucose. Two letter abbreviations used to denote variants are first letter of genus and species with the exceptions of Sy (*Synechocystis* sp. PCC 6714), My (Myxosarcina sp. GI1), Cr (Crenarchaeote), and Th (Thaumarchaeote). (C) Concentration of vanillin 24 h after induction of *aroG**, *asbF*, *car*, and candidate OMT gene expression in cultures cotransformed with pET-OMT$_i$-AsbF and pACYC-Car-Sfp-AroG* grown in M9+glucose medium as measured by HPLC. (D) Concentration of vanillin and other key heterologous metabolites 24 h after induction in LB+glucose medium.

lates individual sensor constructs within water-in-oil compartments with primers that flank the sensor library. Positive selection features sensor-controlled transcription of a thermostable polymerase such that sensors responsible for most polymerase expression are most amplified by emulsion PCR. Negative selection based on transcription of a promiscuous phenylalanine aminoacyl-tRNA synthetase and provision of a toxic nonstandard amino acid substrate was also used to minimize leaky sensor transcription. To standardize preparation of these constructs, changes were made to the promoter sequence, including addition of a RiboJ insulator between promoter and RBS and slight modification of the pAMK-vansens-6 promoter sequence (from "VanO-TTTACAGC-TAGCTCAGTCCTAGGTAT-VanO" to "VanO-TTGA-CAGCTAGCTCAGTCCTAGGTATA-VanO"; single underline showing −35 and −10 regions, double underline highlighting changes). The evolved construct featured a further modified −10 region (from TATA-VanO to TACC-VanO; *pVan′*) and three mutations to VanR (T49I/A111 V/P179S).[39] Finally, these "Marionette" biosensors drive expression of YFP rather than mRFP.

As before, we investigated the dose−response and substrate specificity of these evolved sensors (Figure 4C and 4D). We observed improvements in dynamic range and protocatechuate sensor specificity consistent with recent work.[39] Fortunately, the evolved vanillate sensor, which includes mutations to VanR, maintains an absence of response to isovanillate. Although the evolved vanillate sensor was originally found to have increased cooperativity relative to the original vanillate sensor, we found across repeated experiments that the dose−response of the evolved vanillate sensor is more graded than the original vanillate sensor and saturates at a much higher vanillate concentration (SI Figure 2A). Both features increase biosensing efficacy for metabolic engineering contexts. The discrepancy in vanillate sensor dose−response compared to recent work[39] may be a function of media conditions, supplementation time, and/or end point time used in these assays. Finally, protocatechuate and vanillate have low toxicity,

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thereby increasing their attractiveness as chemical inducers of gene expression (SI Figure 2B).

Given that the evolved vanillate sensor can generate proportional fluorescence at submillimolar vanillate concentrations, has extremely low background fluorescence, and does not respond to related vanillin pathway molecules including isovanillate, we harnessed it to demonstrate the potential for vanillin pathway improvements by biosensor-based bioprospecting. We were curious to screen other *O*-methyltransferases that may be better than OMT_{Hs} at catalyzing vanillate formation, especially in a bacterial host. As a starting point for sampling the natural diversity of *O*-methyltransferases, we considered all relevant literature including published patent applications. A patent application filed by Evolva S.A. and International Flavors and Fragrances, Inc. (IFF) describes the testing of different *O*-methyltransferases expressed in yeast and displays data for roughly 20 natural variants.[37] We compiled a shortlist consisting of the best *O*-methyltransferases reported and then used protein BLAST on the NCBI nucleotide collection to identify putative enzymes and other *O*-methyltransferases that had not been previously tested. We sampled across many domains of life but biased our final candidate list of 16 toward bacterial, fungal, and archaeal sources given our bacterial host (Figure 5A).

We screened these *O*-methyltransferases by cotransforming constructs harboring the evolved vanillate sensor and constructs harboring each of the library members into the *E. coli* RARE Δ*metJ* strain.[17] Deletion of the MetJ regulator of methionine biosynthesis is one of several modifications shown to modestly improve conversion of protocatechuate to vanillate, presumably due to increased SAM pool size. We tested these cultures in LB Lennox medium with 1 mM IPTG provided at inoculation to induce *O*-methyltransferase expression and with or without 4 mM protocatechuate supplementation. We then measured final OD and normalized fluorescence (YFP/OD) 24 h after inoculation. Cells transformed with one variant grew substantially more slowly than the rest and achieved a lower final OD by approximately 2- to 3-fold (Figure 5B). This variant, the *O*-methyltransferase from *Phytophthora infestans* or OMT_{Pi}, also exhibited the highest normalized fluorescence despite its potential toxicity. Because the expression of OMT_{Pi} resulted in a lower final OD independent of exogenous protocatechuate provision, the toxicity of OMT_{Pi} expression may be unrelated to its high enzymatic activity or may be due to activity on other endogenous substrates. *O*-Methyltransferases exhibiting potential toxicity may be better suited to *in vitro* strategies for vanillin biosynthesis.[40]

The results of this preliminary screen suggested that eight *O*-methyltransferases could have comparable or greater desired *in vivo* activity on protocatechuate when expressed in *E. coli* relative to our starting OMT_{Hs}, and that three previously uncharacterized O-methyltransferases may have utility for this pathway and for broader biotechnology applications (Figure 5C). Functional OMT variants from *Phytophthora infestans* and *Candida orthopsilosis* are described in the patent application filed by Evolva and IFF.[37] Another top performing OMT from the fluorescence screen is from *Synechocystis* sp. PCC 6714 and is previously uncharacterized to the best of our knowledge. Reports describing the draft and complete genome sequence of PCC 6714 identified a genomic island containing a "predicted O-methyltransferase YrrM" based on comparative genome analysis of the related *Synechocystis* sp. PCC 6803 strain and function prediction from protein sequence similarity.[41,42] Our finding that the PCC 6714 variant appears active on protocatechuate is particularly interesting given the lack of activity observed from the PCC 6803 variant, which shares 86% protein sequence identity. When previously expressed in *E. coli*, the PCC 6803 variant exhibited preference for trihydroxylated flavones and reduced affinity for protocatechuate (k_{cat}/K_M = 37 M^{-1} s^{-1}).[43] The OMT from *Myxococcus xanthus* is known to prefer protocatechuate as a substrate ($k_{cat}/K_M = 5.5 \times 10^3$ M^{-1} s^{-1})[44] and to have high regioselectivity for the meta position of protocatechuate despite having high regioselectivity for the para position of other substrates.[45,46] Like the OMT from *Synechocystis* sp. PCC 6714, the fifth and sixth best performing OMTs, which are from uncultured marine thaumarchaeote KM3_66_E12[47] and from Myxosarcina sp. GI1, respectively, have not been characterized and are annotated as "predicted O-methyltransferase YrrM".

To determine the utility of these OMTs for the vanillin pathway, we subcloned the corresponding genes into constructs harboring other genes required for vanillin production: *asbF*, *car*, and *aroG** (Figure 6A). AroG* is a feedback-resistant variant of the endogenous enzyme AroG that catalyzes the first committed step to aromatic acid biosynthesis.[48] For host strains, we evaluated the RARE strain and a derivative containing other modifications to endogenous pathways, such as deletion of the phosphotransferase system and upregulation of galactose permease (PTS^- glu^+) as well as upregulation of phosphoenolpyruvate (PEP) synthase (*ppsA*) and transketolase (*tktA*). These modifications are all designed to increase availability of precursors to aromatic amino acid biosynthesis.[49−51] We transformed the two RARE strains with two different sets of plasmids to identify the best starting constructs/host pair for screening in microtiter plate cultures and determined that neither overexpression of endogenous genes nor use of an alternative glucose transport system improved titers under the conditions tested (SI Figure 3).

Given that our new pathway constructs featured OMT variants paired with AsbF and that our ultimate goal was to improve production from glucose as a sole carbon source, we opted to reevaluate our screening of the top eight OMTs in the new constructs. In this case, we used M9+glucose minimal medium with 0.5 mM protocatechuate and 1 mM IPTG supplemented to cultures at mid log. After 24 h, we measured fluorescence and vanillate titers (by high-performance liquid chromatography (HPLC)) to investigate how well these correlated (Figure 6B). Our data demonstrate reasonable correlation between vanillate titer measured in culture supernatant and OD-normalized fluorescence, with most points falling along a regression line with an R^2 value of 0.68. Consistent with expected biosensor dynamics, there appears to be a minimum vanillate concentration required for biosensor activation that the *Synechocystis* sp. PCC 6714 (Sy) variant did not achieve, which affects the correlation coefficient of this linear regression. We observed that titers do not correlate as well to total fluorescence (SI Figure 4). In this experiment, several variants performed similar to one another (five variants generated titers within 0.10 and 0.15 mM), and the rank order varies from the previous screen in LB media with expression of OMT alone. Thus, we observe that, within this range of measured extracellular vanillate concentrations and OMT activities, the biosensor is predictive of activity but can generate false negatives and may not accurately rank order variants.

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Bearing these differences in apparent OMT activity as a function of context in mind, we cotransformed strains with the full pathway constructs and sought to measure vanillin production resulting from expression of different OMTs. Our test of cells that were cultured in M9+glucose medium within deep 96-well plates resulted in detectable vanillin biosynthesis 24 h after induction across all tested strains (Figure 6C). Though titers were low (<0.1 mM) in this experiment, several OMT variants achieved statistically significant improvements in vanillin production compared to OMT_{Hs}: OMT_{Th} (P = 0.0094), OMT_{Co} (P = 0.018), and OMT_{Pi} (P < 0.001; all determined by two-tailed unpaired t tests). The latter OMT variant had also generated the highest vanillate titer as measured by HPLC during the biosensor screen in minimal media. However, the difference between rank order in screen and in vanillin production was unclear for other OMTs. We investigated vanillin production further in LB+glucose medium to achieve higher titers and more clearly track metabolic intermediates (Figure 6D). However, in LB+glucose medium under these culture conditions and at this time point, vanillin titers remained highest when the original OMT_{Hs} was expressed. Our work highlights the context dependence of vanillin pathway components and the benefit of having alternative OMTs as well as a versatile biosensing tool for future analyses.

This work seeks to overcome a limitation of the industrially relevant vanillin biosynthesis pathway through the development, characterization, and application of a genetically encoded biosensor for the heterologous intermediate vanillate. Biosensing in *E. coli* was successfully achieved by importing transcriptional regulator and operator components from *C. crescentus* and by introducing rational modifications in promoter sequences. The vanillate biosensor was then further evolved by directed evolution, and these biosensors along with similar biosensors for protocatechuate were evaluated for their substrate specificity and dose−response. Three of these sensors—the original vanillate sensor and the evolved vanillate, and protocatechuate sensors—each exhibited sufficiently high specificity to enable their future use for improving the vanillin pathway. We demonstrated the value of the vanillate biosensor by using it to rapidly bioprospect alternative *O*-methyltransferases and revealed three previously uncharacterized proteins with desired activity. The effort described here lays the foundation for subsequent vanillin pathway improvements and other biotechnological applications pertaining to catechol *O*-methyltransferases.

METHODS

Strains and Plasmids. *E. coli* strains and plasmids used in this study are listed in SI Table 1. Molecular biology techniques were performed according to standard practices[52] unless otherwise stated. Molecular cloning, vector propagation, and the majority of the biosensor characterization were performed in DH5α. The host strain used for vanillin biosynthetic pathway coexpression experiments was the *E. coli* RARE strain (Addgene Catalog no. 61440),[16] which is derived from *E. coli* K-12 MG1655(DE3). Oligonucleotides were purchased from Sigma and are listed in SI Table 2. Q5 High Fidelity DNA Polymerase (New England Biolabs, MA) was used for DNA amplification.

The VanR and PcaU genes were synthesized as codon-adjusted gBlocks (Integrated DNA Technologies, CA) and their sequences are included in SI Table 3. Original sensor constructs were cloned into the Duet vector system (Novagen, WI) using restriction digest-based cloning. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Propagated constructs were purified using a QIAprep Miniprep Kit (Qiagen, CA) and agarose gel fragments were purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research, CA). All constructs were confirmed to be correct by nucleotide sequencing (Genewiz, NJ).

Constructs for biosensor-based screening of natural *O*-methyltransferase diversity were generated using Gibson assembly[53] from homemade assembly mixtures (enzymes from New England Biolabs, NTPs and other chemicals from Sigma). *O*-Methyltransferase protein sequence accession numbers and corresponding codon-adjusted nucleotide sequences that were ordered as IDT gBlocks are also included in SI Table 3.

Additional description of methods (chemicals, culture conditions, fluorescence measurements, and metabolite analysis) can be found in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.9b00071.

Additional description of methods, data figures, and tables of plasmids, oligonucleotides, and synthetic gene (gBlock) sequences (PDF)

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Notes

The authors declare no competing financial interest.

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