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Decaffeination and Measurement of Caffeine Content by Addicted *Escherichia coli* with a Refactored *N*-Demethylation Operon from *Pseudomonas putida* CBB5

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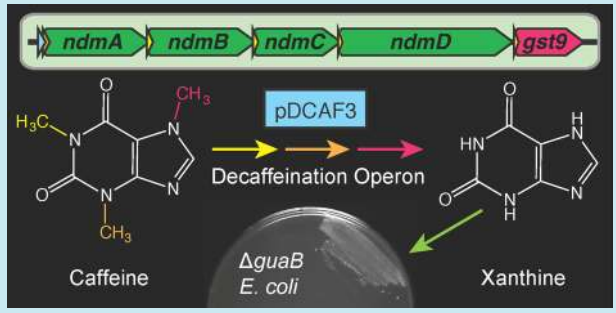
Supporting Information

ABSTRACT: The widespread use of caffeine (1,3,7-trimethylxanthine) and other methylxanthines in beverages and pharmaceuticals has led to significant environmental pollution. We have developed a portable caffeine degradation operon by refactoring the alkylxanthine degradation (Alx) gene cluster from *Pseudomonas putida* CBB5 to function in *Escherichia coli*. In the process, we discovered that adding a glutathione *S*-transferase from *Janthinobacterium* sp. Marseille was necessary to achieve N_7-demethylation activity. *E. coli* cells with the synthetic operon degrade caffeine to the guanine precursor, xanthine. Cells deficient in *de novo* guanine biosynthesis that contain the refactored operon are "addicted" to caffeine: their growth density is limited by the availability of caffeine or other xanthines. We show that the addicted strain can be used as a biosensor to measure the caffeine content of common beverages. The synthetic *N*-demethylation operon could be useful for reclaiming nutrient-rich byproducts of coffee bean processing and for the cost-effective bioproduction of methylxanthine drugs.



KEYWORDS: *decaffeination, N-demethylation, bioremediation, xanthine alkaloid, theophylline*

Caffeine and other methylxanthines are found in foods and beverages such as chocolate, sodas, energy drinks, tea, and coffee. As a result of their widespread use, these compounds have become common pollutants in wastewater and surface waters around population centers, to the extent that caffeine levels serve as a marker of human impact in some areas.[1,2] Caffeine is toxic to a wide variety of organisms. For example, caffeine pollution can alter natural bacterial flora and inhibit the germination and growth of certain plants.[3] Byproducts from processing and brewing coffee beans are often rich in carbohydrates, proteins, and other nutrients, but may be unsuitable as agricultural or biofuel feedstocks due to toxic levels of caffeine. The ability to decaffeinate this waste could alleviate this problem and transform what is currently a cost of production into a valuable resource.[4] Methylxanthines also have medical applications, not all of which are directly related to their neurologic effects. For example, they are used to treat asthma[5] and to prevent apnea in preterm infants.[6] Therefore, the bioproduction of methylxanthines and their derivatives could make it possible to economically produce new families of drugs.

Bacteria capable of degrading caffeine have been found in several studies that enriched microorganisms from soil samples.[7−10] *Pseudomonas putida* CBB5 was isolated in this way by using caffeine as a sole carbon and nitrogen source.[10] CBB5 has a nitrogen demethylation pathway that can convert caffeine to xanthine and formaldehyde. The proteins responsible for this activity are encoded within a gene cluster containing at least nine putative reading frames. The *N*-demethylation pathway is known to require four genes: *ndmA*, *ndmB*, *ndmC*, and *ndmD*.[11,12] NdmA and NdmB are Rieske nonheme iron monooxygenases that remove the methyl groups from the N_1 and N_3 positions of caffeine, respectively. NdmC is a nonheme iron monooxygenase that is predicted to remove the N_7-methyl group from 7-methylxanthine to form xanthine. All of these reactions are dependent upon the Rieske reductase, NdmD.

Porting novel biological functions out of their original context into microorganism chassis that are better-understood,

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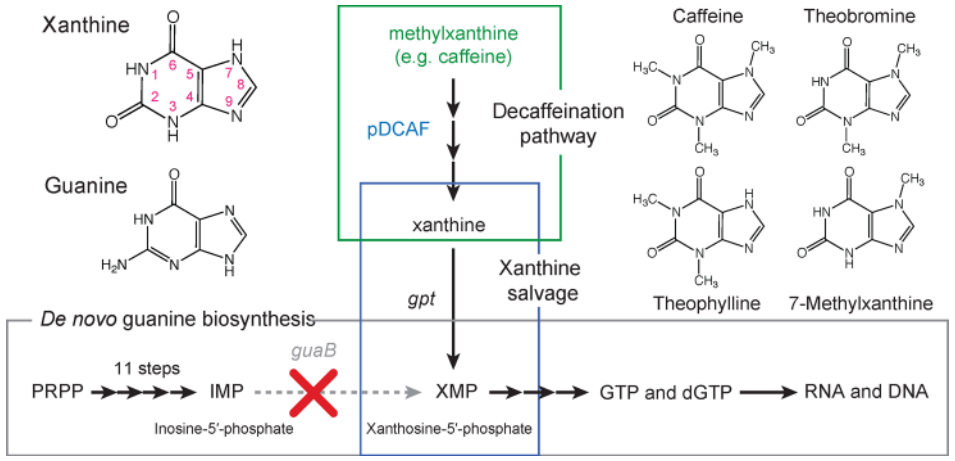

Figure 1. Scheme for complementing guanine auxotrophy with decaffeination activity. Without a source of guanine, the Δ*guaB* strain is unable to synthesize DNA and RNA and cannot replicate. The decaffeination activity provided by pDCAF3 enables conversion of caffeine and other methylxanthines to xanthine, which can be converted to guanine by a salvage pathway, and rescues growth of these cells in minimal media.

simplified, or more amenable to genetic engineering and industrial applications is an important strategy for understanding and improving these functions.[13] It is not without its challenges, however. First, one must be sure that the inventory of all of the genetic parts required for the new function is truly complete. Second, the new activity may be subject to poorly understood or complex regulation in the native organism that cannot be transferred readily to a new context. For example, regulatory proteins, promoters, terminators, ribosome binding sites, and codon usage may function poorly or not at all in a different organism. Genetic refactoring approaches seek to alleviate the latter problems by removing extraneous parts and replacing existing regulatory elements and reading frames with new sequences that are optimized for tuned, predictable performance in the destination chassis.[14,15]

We used a genetic refactoring approach to port caffeine degradation functionality from *P. putida* CBB5 to *Escherichia coli*. In the process, we discovered that adding a glutathione *S*-transferase homologue was necessary to complete the function of the synthetic operon. By moving the final refactored operon into an auxotrophic *E. coli* host, we were able to create an "addicted" bacterium that can act as a biosensor to measure the caffeine content of sodas and energy drinks.

RESULTS AND DISCUSSION

In order to quickly and reliably assay for a functional decaffeination pathway in *E. coli*, we devised a genetic selection whereby the xanthine produced by the complete demethylation of caffeine restores the growth of a guanine auxotroph (Figure 1). The pathway for *de novo* guanine biosynthesis in *E. coli* involves xanthosine-5′-phosphate (XMP) as an intermediate. The enzyme responsible for the formation of XMP from inosine-5′-phosphate (IMP) is IMP dehydrogenase, which is encoded by the *guaB* gene. If *guaB* is knocked out, the cell cannot synthesize guanine and is unable to grow in minimal media. However, if xanthine is supplied to a Δ*guaB* cell, it can be converted to XMP by xanthine-guanine phosphoribosyltransferase (*gpt*), bypassing the need for the *de novo* pathway and restoring growth. Using the Δ*guaB* strain as a host allowed us to select for the presence of a functional decaffeination pathway because only the conversion of caffeine or another methylxanthine to xanthine could supply these cells with enough guanine precursor to support growth in minimal media.

We first attempted to directly clone and express the entire *Pseudomonas putida* CBB5 decaffeination operon in *E. coli*. Plasmid pDCAF1, containing the full 13.2 kb known operon sequence cloned into the low copy number vector pACYC184 (Figure 2), was found to be unable to support the growth of Δ*guaB E. coli* on either caffeine or theophylline (data not shown). We hypothesized that the lack of sufficient demethylation activity to complement growth of the Δ*guaB* auxotroph was not due to an inability of the enzymes in this pathway to function in *E. coli*, but rather due to genetic incompatibilities between *P. putida* CBB5 and *E. coli*. Specifically, the promoters or ribosome binding site (RBS) sequences native to CBB5 may not function in *E. coli*. Protein expression might also be prevented due to regulation by uncharacterized genes located within or outside the cloned region. Additionally, the use of suboptimal non-ATG start codons in several of the open reading frames could interfere with efficient initiation of translation in *E. coli*.

Therefore, we developed a strategy to refactor the decaffeination gene cluster to address each of these potential issues (Figure 2). A strong constitutive transcriptional promoter (BBa_J23100) and associated RBS were chosen from the Registry of Standard Biological Parts to begin the operon (composite part BBa_K515105). The open reading frames of the CBB5 *ndmA*, *ndmB*, *ndmC*, and *ndmD* genes were PCR-amplified with primers that replaced the GTG start codons of *ndmB* and *ndmD* with ATG start codons and changed each of the subsequent ribosome binding sites to the same strong *E. coli* RBS (BBa_B0034). The altered transcriptional unit containing these genes was assembled in one isothermal reaction with five input PCR fragments[16] into the high copy number BioBrick vector pSB1C3 to produce plasmid pDCAF2.

The refactored operon pDCAF2 was found to support growth of Δ*guaB E. coli* on minimal media agar plates supplemented with theophylline but not caffeine (Figure 3). This result indicated that the operon had N_1- and N_3-demethylation activity but was apparently unable to remove the N_7-methyl group, an activity that has been ascribed to the NdmC protein.[12] Indeed, we found that 7-methylxanthine accumulated in the media when liquid cultures of resting cells with pDCAF2 were exposed to caffeine (Figure 4A) and were

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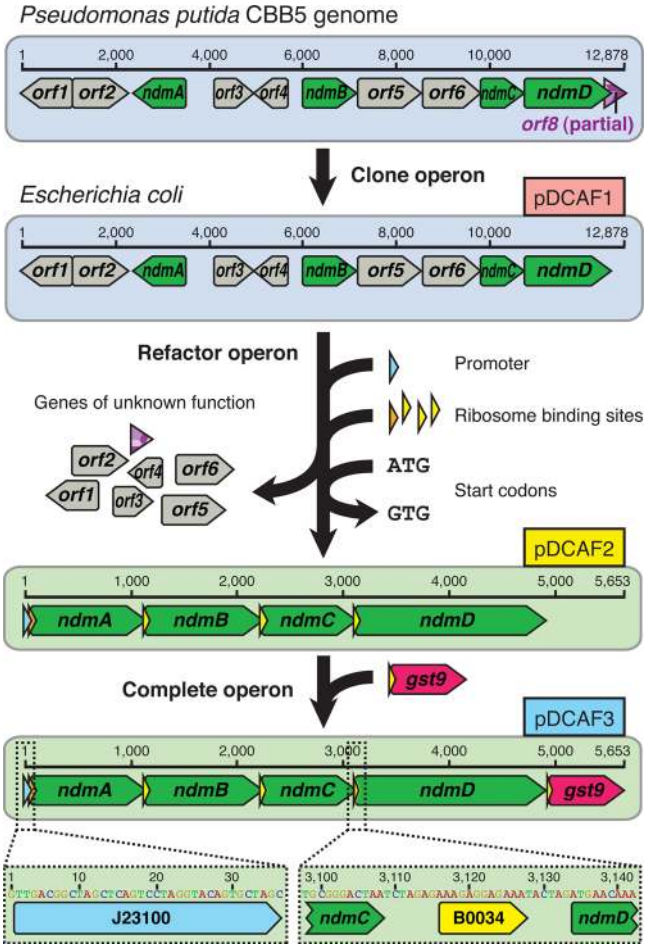

Figure 2. Design of refactored decaffeination operon. The entire known sequence of a *Pseudomonas putida* CBB5 decaffeination gene cluster was cloned onto an *E. coli* expression vector to create plasmid pDCAF1. The CBB5 sequence was refactored in plasmid pDCAF2 by removing genes of unknown function, adding a strong constitutive promoter, substituting well-characterized ribosome binding sites, and changing GTG start codons to ATG. A *Janthinobacterium* sp. Marseille gene (*gst9*) homologous to the partial sequence of the putative glutathione *S*-transferase reading frame (*orf 8*) from the original CBB5 gene cluster was added to plasmid pDCAF3.

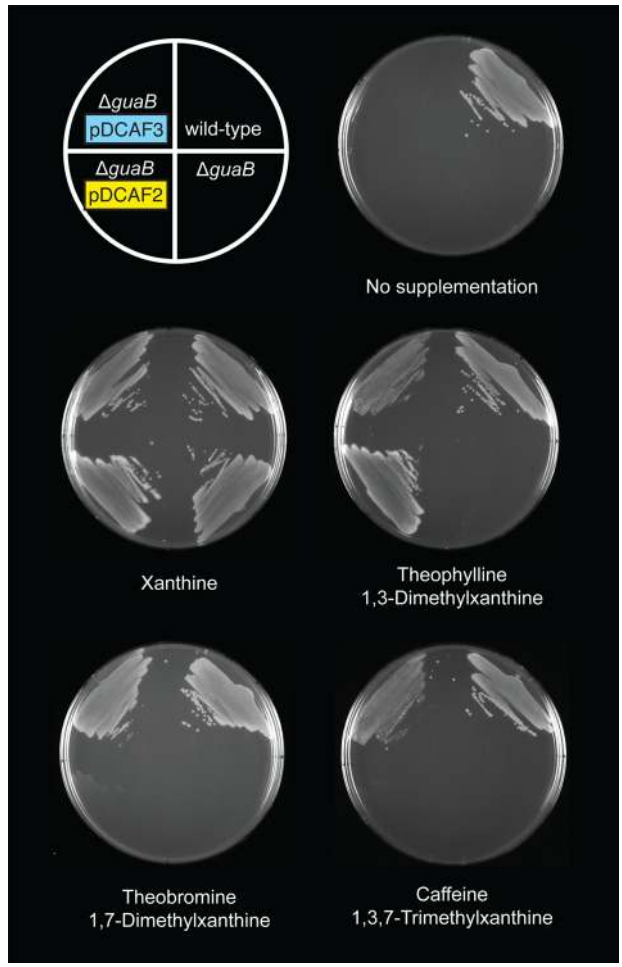

Figure 3. Refactored decaffeination operon restores growth of Δ*guaB E. coli* on media supplemented with caffeine and other methylxanthines. To assay for degradation of caffeine and other methylxanthines to xanthine, agar plates containing minimal media supplemented with a single xanthine compound were each streaked with four equal sectors in the same orientation of wild-type *E. coli,* the Δ*guaB* mutant, or the Δ*guaB* mutant with a pDCAF plasmid as indicated. Slight growth on the edges of the areas streaked with certain strains on some plates was apparently due to diffusion of demethylated products from nearby cells capable of growth on a given xanthine compound (e.g., Δ*guaB* + pDCAF2 edge near Δ*guaB* + pDCAF3 on theobromine).

further able to show that these cells were unable to convert 7-methylxanthine to xanthine at any appreciable rate (Figure 4C).

Previous attempts to express and characterize NdmC in *E. coli* were also unsuccessful.[12] We reasoned that a missing activity supplied by another protein could be essential for N_7-demethylation activity, explaining the lack of full functionality of our synthetic operon in *E. coli*. NdmC had previously been found to copurify with an uncharacterized putative glutathione *S*-transferase (GST) encoded by *orf 8* in the CBB5 gene cluster.[12] Unfortunately, the complete DNA sequence of *orf 8* was not available: this reading frame was truncated in the plasmid insert containing the decaffeination genes that was originally isolated from a CBB5 genomic library (Figure 2). A protein homology search was performed using the available partial sequence to find potential homologues that might substitute for the function of the missing *orf 8*. The search revealed that an uncharacterized gene, *gst9*, from *Janthinobacterium* sp. Marseille had a modest degree of sequence homology (66% nucleotide identity) to *orf 8*. We hypothesized that adding *gst9* to our operon might restore the N_7-demethylation activity of NdmC. The *gst9* gene was synthesized and cloned into the end of the pDCAF2 decaffeination operon to generate pDCAF3.

The addition of *gst9* to the refactored operon enabled growth of the Δ*guaB* strain on minimal media agar plates supplemented with either caffeine or theobromine (Figure 3). We were also able to confirm that caffeine was fully demethylated to xanthine (Figure 4B) in media added to resting Δ*guaB E. coli* cells harboring pDCAF3. These cells were also able to convert 7-methylxanthine to xanthine (Figure 4D), which more directly shows that the addition of *gst9* completed the decaffeination operon by making high-efficiency N_7-demethylation possible.

In liquid media at lower concentrations, caffeine appeared to be the nutrient limiting the final density to which Δ*guaB* pDCAF3 cells could grow. To assess how efficiently caffeine was being utilized, we measured the final optical densities

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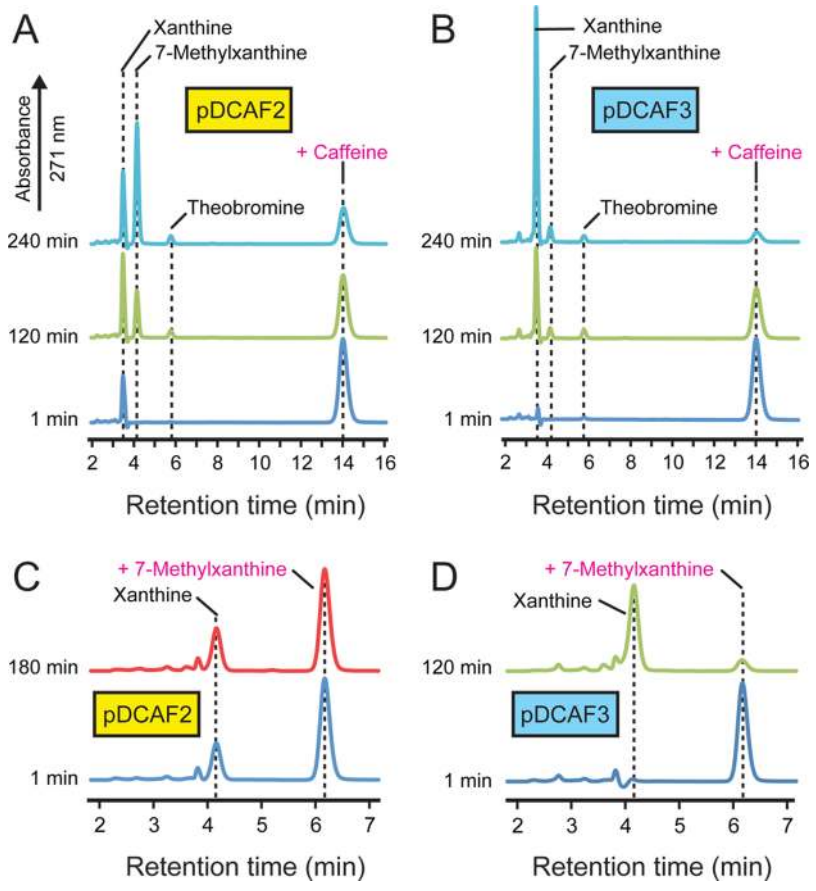

Figure 4. Refactored decaffeination operon converts caffeine to xanthine. Cultures of resting *E. coli* cells were supplemented with caffeine or 7-methylxanthine, and the concentrations of xanthine and methylated xanthine species in the culture medium over time were monitored by HPLC with detection of absorbance at 271 nm. Cells with the initial refactored decaffeination plasmid (pDCAF2) convert caffeine to 7-methylxanthine (A) but do not convert 7-methylxanthine to xanthine (C). Cells with the refactored decaffeination operon plasmid containing the *Janthinobacterium gst9* gene (pDCAF3) convert caffeine to xanthine (B) and 7-methylxanthine to xanthine (D). Xanthine peaks in the initial (1 min) samples (especially noticeable in the pDCAF2 assays) were due to carryover of xanthine from the growth media.

reached by cultures of this strain after 24 h of growth in minimal media supplemented with a range of caffeine concentrations. Growth was measurable with as little as 10 μM of added caffeine and saturated above ~250 μM. At caffeine concentrations above ~5 mM growth appeared to be inhibited, presumably due to the toxicity of methylxanthines at this concentration.[17] Dilutions of cultures grown in 40 μM caffeine were plated on minimal agar to estimate the number of caffeine molecules required for the replication of each *E. coli* cell. If we assume colony-forming units are roughly equivalent to cell numbers under these conditions, then one additional cell is produced per 29 ± 5 million molecules of caffeine added to these cultures (95% confidence limits).

Assuming demethylated caffeine is used only for guanine synthesis in the Δ*guaB* pDCAF3 strain, we can estimate the approximate efficiency with which caffeine is utilized. The genome of *E. coli* is roughly 4.6 Mb. Since this is double-stranded DNA and approximately 50% consists of G-C base pairs, there are about 2.3×10^6 guanines needed per cell to replicate DNA. The dry weight of a typical *E. coli* cell is approximately 280 fg and ~20% of this is RNA.[18] Given that the molecular weight of a typical RNA nucleotide is roughly 330 g/mol and that ~1/4 of these bases are guanine, this means that there are about 2.55×10^7 guanines needed per cell to replicate its RNA. So, overall the number of guanine molecules in RNA is about 10 times the amount in DNA, and there are roughly 2.8×10^7 total guanines incorporated into nucleic acids per cell. The concentration of guanine in free nucleotides, including signaling molecules such as (p)ppGpp, is less than 0.2 mM during stationary phase, which corresponds to a negligible 1.2×10^5 molecules per ~1 fL cell.[19] So, the total estimate of 28 million molecules of guanine needed for replicating a single *E. coli* cell is very close to the 29 ± 5 million molecules of caffeine added per cell produced. Therefore, we conclude that caffeine is nearly stoichiometrically converted to the guanine needed for cell growth under these conditions.

The combination of the Δ*guaB* knockout and the decaffeination operon results in *E. coli* that can be considered "addicted" to caffeine. We were able to show that these *E. coli* could grow in minimal media supplemented with various sodas and energy drinks containing caffeine, but not a caffeine-free soda (Figure 5). Because there was a strong correlation between the saturating cell optical density and the amount of added caffeine, we reasoned that this strain could be used as a quantitative biosensor for measuring the total concentration of guanine, xanthine, and methylxanthines in an unknown sample. In fact, we were able to correctly estimate (within error) the concentrations of caffeine in several beverages by using the standard curve we constructed and growth of the Δ*guaB* pDCAF3 strain in dilutions of these beverages (Table 1).

We have demonstrated that both refactoring the decaffeination gene cluster from *Pseudomonas putida* CBB5 and

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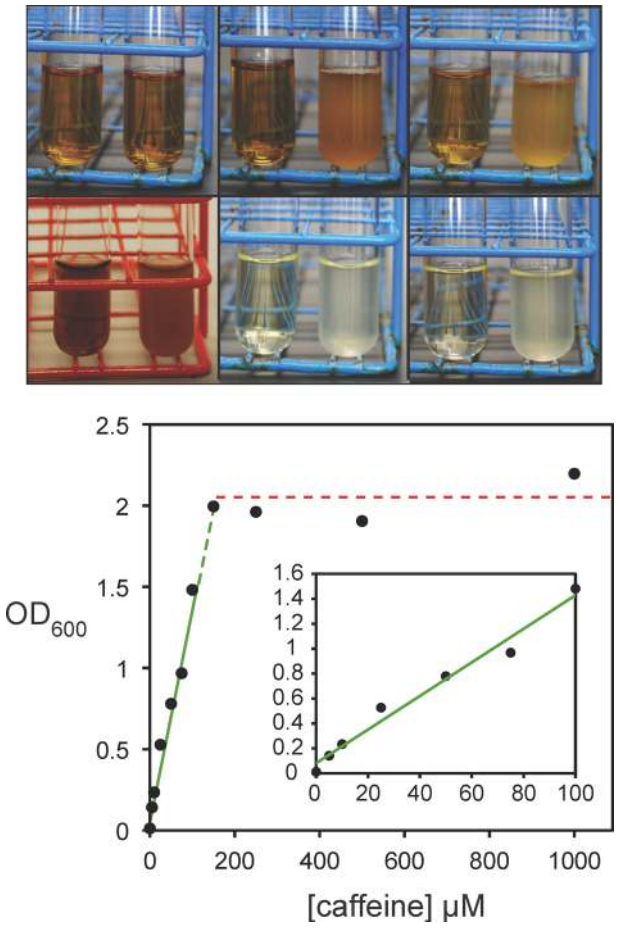

Figure 5. Refactored decaffeination operon enables growth and measurement of caffeine content in media supplemented with common sodas and energy drinks. In each photograph, cultures of Δ*guaB E. coli* without (left) and with (right) the pDCAF3 plasmid were grown in minimal media supplemented with a dilution of the pictured beverage. Top row, from left to right: Caffeine-Free Coca-Cola, Coca-Cola, Diet Coca-Cola. Bottom row, from left to right: Starbucks Espresso, Monster, Red Bull. The standard curve used to determine the caffeine content of these beverages from *E. coli* growth is shown below. The final optical densities at 600 nm (OD_{600}) achieved by Δ*guaB* + pDCAF3 cultures grown in media supplemented with various concentrations of caffeine are plotted. The inset shows the linear fit to the data for caffeine concentrations ≤100 μM. Cultures containing more dilute samples of the beverages than those in the photographs were used to determine the caffeine content values presented in Table 1.

completing its N_7-demethylation function with a recombinant glutathione *S*-transferase gene were necessary to create a decaffeination operon that functions efficiently in *E. coli*. An "addicted" *E. coli guaB* knockout strain with this operon can be used to measure the caffeine content of an unknown sample. In connection with other synthetic biology efforts, it might be possible to reduce the fitness cost of carrying the operon by making it inducible under the control of a theophylline riboswitch.[17,20,21]

Microorganisms with derivatives of the engineered decaffeination operon could be used for decontaminating wastewater and recapturing byproducts from coffee processing or for the bioproduction of specific methylxanthines from caffeine for use as building blocks for new pharmaceutical drugs. The concentration of caffeine in typical byproducts of processing coffee berries may be as high as ∼15 mM,[22] which would inhibit the growth of the *E. coli* strain that we used. *E. coli* B mutants that grow in the presence of >16 mM caffeine have been reported in the past.[23] Therefore, it would potentially be possible to evolve a host strain of *E. coli* with a higher methylxanthine tolerance for some of these applications.

METHODS

Plasmid Assembly. pDCAF1, pDCAF2, and pDCAF3 were constructed using Gibson isothermal assembly.[16] In each case, primers were designed to introduce ∼35−45 base homology overlaps to adjacent pieces and to add short synthetic sequences as required (Supplementary Table S1). DNA fragments were amplified in standard PCR reactions with Phusion polymerase (New England Biolabs). Amplification reactions from plasmid templates were digested overnight with DpnI. PCR products were cleaned-up, combined, and assembled at 50 °C for 1 h. Each assembly reaction was desalted, transformed into Top10 electrocompetent *E. coli* cells (Invitrogen), and plated on LB agar with 34 μg/mL chloramphenicol (Cam). Cultures grown from colonies were archived as glycerol stocks after the expected assemblies were verified by Sanger sequencing. The sequences of each plasmid have been deposited in GenBank (accessions KC619528-KC619530).

For pDCAF1, a backbone fragment amplified from plasmid pACYC184 and the 13.2 kb caffeine gene cluster amplified from *P. putida* CBB5 genomic DNA were combined. To construct pDCAF2, part BBa_K515105 was used as PCR template to amplify a fragment containing the pSB1C3 backbone, BBa_J23100 promoter, and adjacent RBS. CBB5 genomic DNA was used as PCR template to separately amplify each of the genes *ndmA*, *ndmB*, *ndmC*, and *ndmD* with primers

Table 1. Caffeine Content of Sodas and Energy Drinks Estimated from the Final Densities Achieved by Cultures of Δ*guaB E. coli* with the pDCAF3 Decaffeination Operon Plasmid

			caffeine content (μM)			
beverage	dilution	OD_{600}[a]	reported	predicted	[95% CI]	% error
Caffeine-Free Coca-Cola	10.2	0.036	0	−25	[−150, 101]	NA
Coca-Cola	10.2	0.797	508	536	[411, 659]	+5.5
Diet Coca-Cola	13.3	0.771	667	679	[517, 842]	+1.7
Starbucks Espresso	261	0.257	5600−39500[b]	3540	[333, 6750]	NA
Monster	33.2	0.634	1740	1900	[1480, 2330]	+9.2
Red Bull	34.8	0.824	1660	1360	[954, 1760]	−18

[a]Optical density at 600 nm. [b]Caffeine concentrations in espresso vary widely depending on preparation so percent error was not calculated for this beverage. Previous values reported for Starbucks espresso are 9970 and 9730 μM.[26,27]

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that introduced homology for assembly, as above, but also added the new ribosome binding sites (part BBa_B0034) to all genes other than *ndmA* and changed GTG start codons to ATG. These five fragments were assembled in one reaction. For pDCAF3, plasmid pDCAF2 was PCR amplified and assembled with two synthetic dsDNA gBLOCKs (Integrated DNA Technologies) that encoded BBa_B0034 followed by the *gst9* reading frame from *Janthinobacterium* sp. Marseille (GenBank: NC_009659.1).

Genetic Selection for Caffeine Demethylation. *E. coli* strains BW25113, BW25113Δ*guaB*,[24] and BW25113Δ*guaB* carrying the pDCAF2 or pDCAF3 plasmids were revived from glycerol frozen stocks in LB supplemented with 30 μg/mL kanamycin (Kan) for selection of the *guaB* gene replacement and 34 μg/mL Cam for maintenance of pDCAF plasmids, where applicable. Cultures were grown at 30 °C and 250 rpm shaking overnight to saturation. Overnight cultures were then diluted 1:1000 for preconditioning in mineral M9 media plus 2 g/L glucose and 2 g/L casein (M9CG) for BW25113, M9CG supplemented with 500 μM xanthine for BW25113Δ*guaB*, 500 μM theophylline for BW25113Δ*guaB* plus pDCAF2, or 500 μM caffeine for BW25113Δ*guaB* plus pDCAF3 and incubated overnight again. Then, the cultures were washed twice with 1× M9 salts and streaked on M9CG agar plates supplemented with 500 μM xanthine, theophylline, theobromine, caffeine, or no supplement. Plates were grown for 48 h at 30 °C and photographed.

Methylated Xanthine Conversion. *E. coli* BW25113Δ*guaB* carrying pDCAF plasmids were inoculated into 5 mL of M9CG medium with 30 μg/mL Kan, 34 μg/mL Cam, and 1 mM xanthine and grown overnight at 37 °C with 225 rpm shaking. Cells were then inoculated into 50 mL of M9CG with the same antibiotic concentrations and 1 mM xanthine for pDCAF2 or 1 mM caffeine for pDCAF3. Both cultures were incubated at 18 °C and 250 rpm for 2 days. Upon reaching an optical density at 600 nm (OD_{600}) above 3.0, cells were harvested by centrifugation at 4,000 × *g* for 10 min at 4 °C. The pelleted cells were washed twice with 25 mL of 50 mM potassium phosphate (KP_i) buffer (pH = 7.5), and the final pellet was suspended in 7 mL of 50 mM KP_i buffer.

Degradation of caffeine and 7-methylxanthine by cells with pDCAF2 or pDCAF3 was monitored in resting cell assays. Each 1-mL assay contained freshly harvested and washed cells (OD_{600} = 5.0) and either 1 mM caffeine or 0.5 mM 7-methylxanthine in KP_i buffer. Reactions were incubated at 30 °C with 400 rpm shaking in a microplate shaker (VWR), and aliquots were periodically removed and mixed with an equal volume of acetonitrile to stop the reaction. A Shimadzu LC-20AT HPLC system equipped with a SPD-M20A photodiode array detector and a Hypersil BDS C18 column (4.6 mm × 125 mm) was used to detect caffeine, 7-methylxanthine, and their degradation products in these samples. For reactions with caffeine as substrate, methanol/water/acetic acid (15:85:0.5, v/v/v) was used as the mobile phase. For reactions with 7-methylxanthine as substrate, the mobile phase was changed to methanol/water/acetic acid (7.5:92.5:0.5, v/v/v) for better resolution of the 7-methylxanthine and xanthine peaks.

Caffeine Content Measurements. *E. coli* BW25113Δ*guaB* carrying the pDCAF3 plasmid were revived from glycerol stocks into 2 mL LB cultures supplemented with 62.5 μg/mL Kan and 25 μg/mL Cam and grown overnight at 30 °C with 225 rpm shaking. Revived cells were diluted 1:1000 into triplicate 2 mL cultures of M9CG media with 50 μg/mL Kan and 20 μg/mL Cam, as well as a range of caffeine concentrations from 0 to 1000 μM. After growth to saturation at 30 °C over 24 h with 225 rpm shaking in 18 mm test tubes, the final OD_{600} for each culture was measured relative to tubes of uninoculated media. Final OD_{600} values as a function of supplemented caffeine concentration (Figure 5) were fit to a linear model with slope and intercept terms using the R statistical computing package version 2.15.0[25] to create a standard curve for predicting caffeine content. For fitting this standard curve, only measurements between 0 and 100 μM caffeine were considered.

The number of caffeine molecules utilized per cell was determined from a separate set of six replicate cultures all grown as above with 40 μM caffeine. Saturated cultures were serially diluted by transferring 5 μL of culture or dilution into 495 μL of M9CG media, creating three dilutions of 10^{-2}, 10^{-4}, and 10^{-6} of each culture. From the third dilution of each culture 200 μL was plated on individual LB agar plates supplemented with 50 μg/mL Kan and 20 μg/mL Cam. Colonies were counted after overnight incubation at 37 °C and converted to estimates of the number of caffeine molecules present per *E. coli* cell replicated.

Growth in Caffeinated Beverages. *E. coli* BW25113Δ*guaB* with the pDCAF3 plasmid were revived from frozen glycerol stocks into 2 mL LB cultures supplemented with 62.5 μg/mL Kan and 25 μg/mL Cam. Cultures were grown overnight at 30 °C with 225 rpm shaking. For the photographs, a revived Δ*guaB* pDCAF3 culture was diluted 1:1000 into a 1:9 mixture of beverage and M9CG media for espresso and a 1:1 mixture of beverage with this media for all other sodas and energy drinks. Each culture contained final antibiotic concentrations as above and was grown for 3 days under the same conditions. For measuring caffeine content, a revived Δ*guaB* pDCAF3 culture was diluted 1:1000 into triplicate 5 mL cultures of M9CG media with a dilution of each beverage expected to give a final OD_{600} of ∼0.8 from the standard curve and the same concentrations of antibiotics. Each beverage dilution was calculated from the linear model of supplemented caffeine concentration versus OD_{600} measurements using reported caffeine values from literature and manufacturer sources. Cultures were grown overnight as before, and OD_{600} was measured with respect to uninoculated tubes. Maximum likelihood estimates of the caffeine content of each beverage and prediction intervals were determined from the average OD_{600} value and the standard curve using R.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Primer and gBlock sequences used to construct pDCAF plasmids. This information is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

E.M.Q. designed, constructed, and tested the pDCAF plasmids. R.M.S. performed HPLC experiments. P.B.O. and R.N.A. performed caffeine content measurements. J.E.B., E.M.Q., M.J.H., B.S., R.M.S., P.B.O, and R.N.A. wrote the manuscript, analyzed data, and created figures. A.D. and J.L.B. were involved

in designing the project and background research. J.E.B. and M.V.S. supervised and coordinated the study.

Notes

The authors declare no competing financial interest.

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