ACS Synthetic Biology

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Technical Note

Improving the Transformation Efficiency of *Synechococcus* sp. PCC 7002 via Methylome-Guided Premethylation of DNA

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Cite This: https://doi.org/10.1021/acssynbio.5c00370

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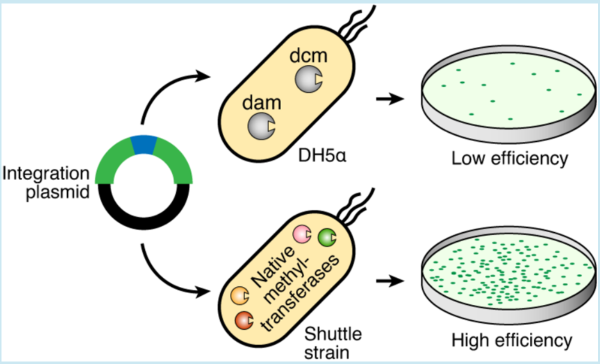
ABSTRACT: Cyanobacteria are promising microbial platforms for a diverse set of biotechnology applications, from living materials to photosynthetic chemical production, but are less well characterized than commonly engineered microbes such as *Escherichia coli*. This study facilitates genetic engineering in *Synechococcus* sp. PCC 7002, a fast-growing, halotolerant, and naturally competent strain, by identifying ten native methylation motifs and designing shuttle strains that mimic the native methylation state by expressing a subset of heterologous methyltransferases. DNA methylation in *E. coli* with as few as two active methyltransferases increased transformation efficiency up to 30-fold across four distinct integration sites in PCC 7002. This work provides an experimental framework to bypass native restriction-modification systems for efficient genome editing and metabolic engineering in nonmodel bacteria.



KEYWORDS: *cyanobacteria, transformation efficiency, genetic engineering, DNA methylation, restriction modification*

INTRODUCTION

Cyanobacteria are the simplest organisms capable of oxygenic photosynthesis and have served as model systems for studying biological carbon fixation. *Synechococcus* sp. PCC 7002 (also known as ATCC 27264, hereafter PCC 7002), a marine species isolated from Magueyes Island in Puerto Rico,[1] is a promising engineering strain due to its fast doubling time[2] and tolerance to high light intensities[3] and salt concentrations.[4] It has been used to convert waste streams to value-added products[5] and to generate microbial biomass as a renewable feedstock for heterotrophic conversion to bioproducts.[6,7] PCC 7002 is naturally transformable[8] and can integrate DNA from plasmids or linear fragments into its genome via homologous recombination.[9−11] Recent studies have used replicating vectors in PCC 7002,[12] though genomic integration remains the preferred method for stable gene expression and knockout. Unfortunately, transformation efficiencies vary widely across genomic sites.[13] Increasing the length of homologous regions can improve efficiencies,[14] but the extent of improvement varies by site. A site-agnostic approach to improve transformation efficiencies could unlock the genome of PCC 7002 and other species capable of natural transformation for diverse applications in science and engineering.

Methods to map and exploit the unique methylomes of bacteria can provide a path to evade native restriction-modification (R-M) systems and improve transformation efficiencies. R-M systems are prokaryotic immune systems that protect bacteria against foreign genetic elements.[15] They are comprised of restriction enzymes that hydrolyze specific DNA sequences and methyltransferases that methylate those sequences in the host genome to prevent cutting. Bases can be methylated on the N6 of adenine (6-methyladenine, 6 mA), the N4 of cytosine (4-methylcytosine, 4mC), or the C5 of cytosine (5-methylcytosine, 5mC). Foreign DNA lacks these signatures and is rapidly degraded.[16] R-M systems limit transformation efficiencies in many microbes, though their effects can be minimized by methylating DNA prior to transformation via heterologous expression of the corresponding R-M methyltransferases in cloning strains of *Escherichia coli*.[17] In prior work, the heterologous expression of methyltransferases native to *Synechocystis* sp. PCC 6803, a well-studied freshwater cyanobacterium, increased transformation efficiencies of harvested DNA by up to 2 orders of magnitude in both *Synechocystis*[18] and in PCC 7002.[19] As the number and composition of active R-M systems within organisms vary,[20] even among closely related species,[21,22] the development of similar cloning strains specific to PCC 7002

Received: May 22, 2025
Revised: July 14, 2025
Accepted: July 24, 2025

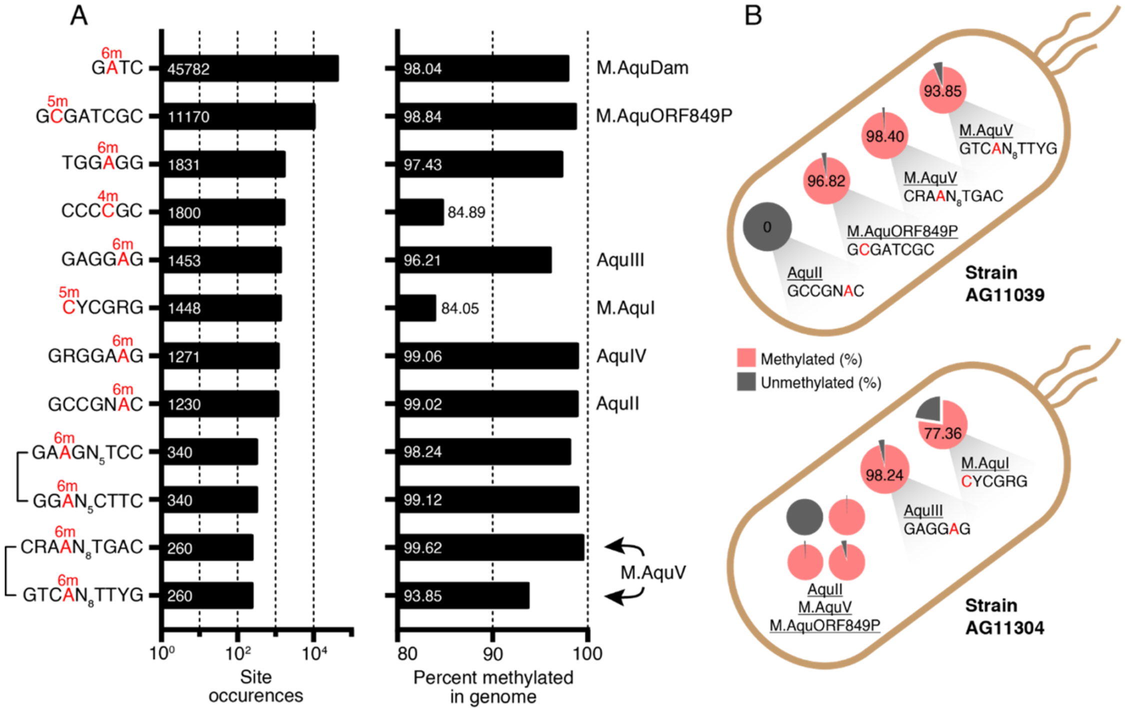


Figure 1. Heterologous methyltransferase expression mimics native methylation patterns. (A) Frequency and percent methylation of sequence motifs in the genome of PCC 7002, as detected via ONT sequencing and the MIJAMP pipeline. Methyltransferases that are predicted to methylate the corresponding motif, as predicted by REBASE, are listed on the right. The reverse complement of a motif is included when differential methylation is observed (e.g., M.AquV; motif pairs are bracketed). (B) Methylated sites within *E. coli* cloning strains expressing three (AG11039) or five (AG11304) methyltransferases native to PCC 7002. Each circle is a pie chart illustrating the percent methylation for each motif. Methylation of the predicted AquII motif was not detected in either strain. The four motifs from strain AG11039 are represented by smaller circles in AG11304.

could enhance efficiencies further by recapitulating native methylation patterns.

In this study, we sought to improve the efficiency of integrative transformation in PCC 7002 by using the methyltransferases from its active R-M systems to build cloning strains that methylate recombinant DNA to match the native genome. Our approach uses methylome analysis, a technique extensible to any species with long-read nanopore sequencing, to classify active methyltransferases that can help bypass native R-M systems. This work expands the pool of accessible integration sites for high-throughput techniques in PCC 7002 and provides a generalizable workflow to create species-specific cloning strains for nonmodel bacteria.

■ RESULTS AND DISCUSSION

Methylome Analysis Identifies Methylated Motifs in PCC 7002. R-M systems are highly variable, even between closely related bacterial strains, so we used orthogonal methods to identify active methylation motifs in PCC 7002. First, we examined methyltransferases encoded in the chromosome by using New England Biolab's Restriction Enzyme Database, or REBASE,[23] which lists predicted methyltransferase-encoding genes and potential methylation sites based on comparisons to known methyltransferases. We tested these predictions by using nanopore sequencing of extracted DNA and the methylome discovery software package MIJAMP,[24] which detects methylation motifs in DNA sequencing data from Oxford Nanopore Technologies (ONT) systems. The results of the two analyses were largely consistent with notable differences (Figure 1A). REBASE predicted 11 methyltransferases in PCC 7002, eight of which have predictions of the target methylation motif. The motif associated with one enzyme (M.AquORF12P), which REBASE predicted to methylate YGGCCR, was not identified during motif discovery with MIJAMP. Direct query of this motif in our experimental data set (MIJAMP "query" function) showed no methylation at either cytosine, suggesting that the associated methyltransferase may not be active in PCC 7002. Interestingly, two of the methyltransferases with confirmed methylation at their predicted motifs (M.AquORF849P and M.AquDam) had no predicted cognate restriction endonuclease. These enzymes may function similarly to dam, an orphan methyltransferase from *E. coli* that helps regulate DNA replication and gene expression via G(6 mA)TC methylation.[25] The MIJAMP analysis also suggests an alternative methylation pattern for M.AquORF849P; for this enzyme, G(5mC)GATCGC exhibited a genome-wide methylation (GWM) score of almost 99% (i.e., ∼ 99% of those motifs were methylated in this data set), while the predicted (5mC)GATCG from REBASE showed only ∼ 92% GWM. Finally, methylome analysis detected three additional methylation motifs (TGG(6 mA)GG, CCC(4mC)GC, and GG(6 mA)N$_5$CTTC) not predicted by REBASE.

The methylated motifs of PCC 7002 deviate substantially from other model cyanobacteria: seven of the ten detected motifs are not methylated in either *Synechocystis* sp. PCC 6803[22] or Anabaena PCC 7120,[21] and PCC 7002 contains twice as many active methyltransferases as PCC 6803, despite a smaller genome size (3.5 vs 3.9 Mb). The limited overlap across cyanobacteria highlights the role of R-M systems as genomic fingerprints, enabling hosts to differentiate their own DNA from that of even closely related species. Two motifs

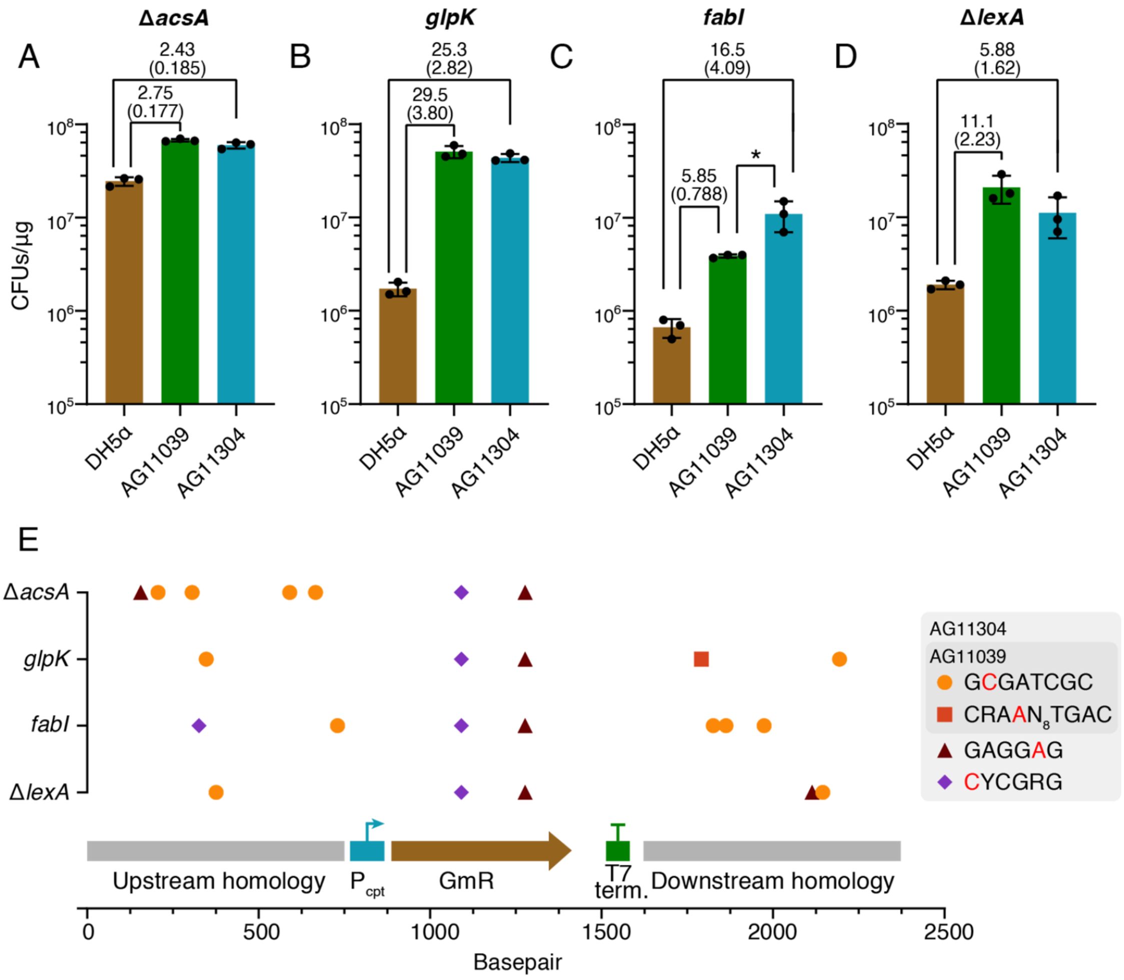


Figure 2. Methylation of DNA with PCC 7002 methyltransferases in *E. coli* dramatically improves transformation efficiency. Wild-type PCC 7002 was transformed with plasmids containing a gentamicin resistance cassette and homologous DNA regions targeting (A) Δ*acsA*, (B) *glpK*, (C) fabI, and (D) Δ*lexA*. All strains exhibited statistically significant increases in transformation efficiency when plasmids were methylated, as calculated via an unpaired Student's *t* test ($p < 0.0001$). Only *fabI* displayed a significant difference in efficiency between AG11039 and AG11304 (*; $p < 0.05$). Enumerated data above each bar cluster indicate the fold-change in transformation efficiency and its standard error. (E) Location of methylated motifs from AG11039 and AG11304 (Table S3) are shown for each cassette tested in A-D.

(CCC(4mC)GC, (5mC)YCGRG) had notably lower GWM scores than others in our strain. Motifs with methylation rates less than 90% have been observed in bacteria using both PacBio SMRT[26] and ONT sequencing technologies.[27] Further investigation is needed to determine if inaccurate sequencing or a biological mechanism, such as lower enzyme expression or activity, could explain these results.

DNA Methylation with Heterologous Methyltransferases in *E. coli* Increases PCC 7002 Transformation Efficiency. To determine if DNA methylation would protect plasmid DNA from restriction enzymes during transformation in PCC 7002, we generated strains of *E. coli* (AG11039 and AG11304, Figure 1B) that are deficient in native DNA methyltransferases and instead express PCC 7002 methyltransferases that have both (i) motifs detected in our methylome analysis and (ii) associated restriction enzymes (Figure 1A; additional strains are listed in Table S7). We confirmed the activity of the heterologous methyltransferases in *E. coli* via ONT sequencing and subsequent analysis using MIJAMP (Figure 1B). In both strains, we detected the expected methylation motifs for all methyltransferases except for AquII (GCCGN(6 mA)C). Successful PCR confirmation and sequencing of the AquII cassette in the engineered strains suggests poor functional expression of AquII in *E. coli*. The (5mC)YCGRG motif had a lower GWM score in AG11304 (∼77%) than the other motifs, though this corresponds with the low GWM score (∼84%) in PCC 7002. These data highlight the importance of validating methylation targets when expressing DNA methyltransferases in *E. coli*.

We transformed PCC 7002 with test plasmids isolated from either our *E. coli* strains expressing methyltransferases or DH5*α*, a standard cloning strain. These plasmids contain 750 bp homology arms to place a gentamicin resistance cassette at either conventional or previously uncharacterized integration sites (Δ*acsA* and *glpK*; *fabI* and Δ*lexA*) by replacing the gene of interest (as indicated by "Δ") or by adjacent insertion. We used an optimized transformation protocol, where cells in exponential phase are concentrated to high densities (Methods Section; Figure S1), and measured transformation efficiency with serial dilutions. For each plasmid, the methyltransferases in AG11039 and AG11304 significantly increased transformation efficiency relative to the native methyltransferases of DH5*α* (e.g., dam and dcm; Figure 2A−D).

Analysis of the methylated motifs in the tested cassettes (Figure 2E) provides some insight into the observed trends. The Δ*acsA* locus exhibited a modest increase (<3-fold) in transformation efficiency for both strains, but integration downstream of *glpK* afforded a 30-fold improvement. We hypothesize that the presence of the CRA(6 mA)N_8TGAC motif in the downstream *glpK* homology region contributes to the large increase in efficiency; when methylated, it can evade restriction that would severely reduce homology arm length. A similar mechanism of evasion is also supported by the improved transformation afforded by AG11304 relative to AG11039 for the *fabI* plasmid, whose homology arms contain a (5mC)YCGRG motif, which is methylated only in AG11304. Interestingly, methylation of GAGG(6 mA)G does not impart the same boost in efficiency for the Δ*acsA* and Δ*lexA* constructs, suggesting a higher tolerance (i.e., lower cleavage activity) of unmethylated GAGG(6 mA) sites. Overall, our data suggest that the observed improvements to transformation efficiency are dependent on methylation of select—yet not all—motifs detected by our analyses. Inconsistencies in the influence of each motif on efficiency may stem from differences in governing mechanism (e.g., Type I vs II),[28] stringency of methylation (i.e., genome methylation percentage), or susceptibility to restriction—for example, the reduced activity or complete absence of a cognate restriction endonuclease (i.e., an orphan methyltransferase).[29] Combining multiple methyltransferases into a single "cocktail" strain eliminates the need to characterize each enzyme individually and accelerates the construction of effective shuttle strains. In our experiments, methylation from either AG11039 or AG11304 significantly enhanced transformation efficiency relative to plasmids derived from *E. coli* DH5*α*. By consistently achieving efficiencies greater than 10^7 CFUs/μg at different sites, our methylation workflow promises to enable high-coverage transformation of large libraries (e.g., > 10^5 variants) across the PCC 7002 genome.

Direct Validation Expands Integration Site Pool in PCC 7002. We observed discrepancies in baseline transformation efficiency across our tested sites (Figure 2A−D), in line with previous work.[13] To further characterize these sites and others, we examined how locus of integration affected strain growth and gene expression via insertion of a fluorescent reporter into nine sites (Figures S2−S4). Integration into the native plasmid pAQ3 yielded a 4-fold increase in maximum expression over the conventional Δ*acsA* site with no detectable effect on growth, likely a consequence of the higher copy number relative to the chromosome (Figure S4).[30] Others varied in maximum expression, dynamic range, and neutrality. Our improved transformation toolkit facilitates the use of sites with different transcriptional properties (i.e., those tested here and others yet to be characterized) in high-throughput screens.

CONCLUSIONS

In this work, we characterized the methylation patterns of *Synechococcus* sp. PCC 7002 and identified discrepancies between the predictions by REBASE and the motifs identified experimentally with MIJAMP, including three novel motifs with no associated enzyme. Using these insights, we developed two strains of *E. coli* that express methyltransferases from PCC 7002. Plasmids purified from these strains demonstrate an order of magnitude higher transformation efficiency with PCC 7002 compared to controls. This work provides new strains and a companion transformation protocol that will facilitate the construction of large, unbiased libraries of PCC 7002 mutants. New neutral integration sites with high maximum expression levels provide additional optionality for genome engineering. These additions to the PCC 7002 toolkit will enable high-efficiency genetic manipulation of this powerful cyanobacterial system and, through straightforward extension to any species capable of natural transformation, facilitate genetic engineering of new varieties of nonmodel organisms.

METHODS

Genome Assembly and Methylome Analysis of PCC 7002. Genomic DNA was extracted from wild-type PCC 7002 grown in standard conditions (SI Methods) using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA) and then prepared for sequencing using the Native Barcoding Sequencing Kit 24 V14 (SQK-NBD114.24); manufacturer's instructions were followed with the exception of an omitted bead cleanup between end repair and barcode ligation. The library was loaded onto an R10.4.1 minION flowcell, generating 1.83 M reads with an N50 of 8.3 kbp. Live basecalling was performed within MinKnow using an HP Z8 workstation equipped with two NVIDIA RTX A6000 GPUs. Reads were filtered using Filtlong (https://github.com/rrwick/Filtlong) to remove reads under 1 kbp in length and to return the top 1 Gbp worth of reads by quality score. Genome assembly was conducted on this 1 Gbp read set using the Trycycler v0.5.4 workflow[32] together with Raven v1.5.3,[33] Miniasm/Minimap v0.3,[34] Flye v2.9.4,[35] and Medaka v1.5 (https://github.com/nanoporetech/medaka). The resulting genome is 3.4 Mbp in size and consists of six circular molecules: one chromosome, four megaplasmids, and one small plasmid.

MIJAMP[24] was used to detect methylated motifs within the data set. Briefly, the 1 Gbp read set was further reduced with Filtlong to a final size of 350 Mbp, or ∼ 100x coverage of the genome. Read IDs from the resulting FASTQ file were extracted via the readIDExtract.py script included in MIJAMP, then the specified read IDs were used to rebasecall the data set via Dorado v0.7.1 + 80da5f5 (https://github.com/nanoporetech/dorado) with v5 all-context modified base models for 6 mA, 5mC, and 4mC modifications. Following the MIJAMP workflow, the resulting BAM file and previously assembled genome were preprocessed (preprocess command), then motifs were detected (motif command), revealing seven 6 mA, two 5mC, and one 4mC modified motifs, with genome-wide methylation of each motif ranging from 85% to 99% (Table S1). The reported proportion of explained modified bases (diagnostic command) was 99.9%, 81.9%, and 80.3% for 6 mA, 5mC, and 4mC modified bases, respectively.

Construction of *E. coli* Methylating Shuttle Strains. Genes encoding predicted DNA methyltransferases from *Synechococcus* sp. PCC 7002 were codon optimized to *E. coli* K12 (IDT DNA) and cloned in the nonreplicating integration plasmid backbone pMTV210 by GenScript (Nanjing, China). The plasmid pMTV210 was also constructed by GenScript (Nanjing, China). In this backbone, the methyltransferases genes are expressed from an arabinose-inducible promoter in *E. coli*. The Serine-integrase Assisted Genome Engineering (SAGE) method[36] was used to integrate all the PCC 7002 methyltransferases. First, pMTV1133 was integrated in AG5589. This base strain, AG5589, was engineered from AG4277[37] by removal of the *aadA* gene using Lambda Red recombineering.[38] A temperature sensitive plasmid expressing the BL3 integrase (pLAR051) was used to aid integration. 200 ng of each plasmid was transformed into 70 μL of electrocompetent cells. Transformation was recovered in 1 mL SOC at 30 °C for 40 min to allow replication of pLAR051 and moved to 42 °C for another 40 min for plasmid curing. The transformation was plated onto LB with 30 μg mL^{-1} kanamycin at 37 °C. At least 6 colonies were picked and grown in 2 mL LB with 30 μg mL^{-1} kanamycin at 42 °C overnight. Colony PCR with primers oMTV2234/oMTV2235 and oMTV24/oMTV27 were used to confirm integration of SYNPCC7002_C0003-SYNPCC7002-C0004 and the kanamycin marker in AG5589.

To remove the kanamycin marker, competent cells were made from the confirmed integration strain. Another temperature sensitive plasmid expressing the PhiC31 integrase (pLAR047) was transformed into 50 μL of competent cells and recovered in SOC at 30 °C. Transformation was plated on LB with 100 μg mL carbenicillin^{-1}. Colonies were picked into LB and grown at 42 °C overnight. About 2 μL of the outgrowth was streaked on LB and grown overnight at 37 °C. Colonies were then patched onto LB with 30 μg mL^{-1} kanamycin, LB with 100 μg mL^{-1} carbenicillin, and LB only to screen for the removal of the kanamycin resistance marker and loss of helper plasmid pLAR051. Colony PCR was used to confirm SYNPCC7002_C0003-SYNPCC7002-C0004 and the loss of the kanamycin marker to generate strain AG10460. Electrocompetent cells from AG10460 were made to integrate SYNPCC7002_A0849 (pMTV1128) using the procedure described above with pLAR058 as the recombinase helper plasmid resulting in AG10965. Similarly, SYNPCC7002_A0358 (pMTV1127) was integrated into AG10460 with pLAR074 as the helper recombinase to generate AG11039. To generate AG11078, SYNPCC7002_A2132 (pMTV1131) with helper plasmid pLAR060 was integrated in AG11039. For AG11304, SYNPCC7002_A1188-SYNPCC7002_A1188−2 (pMTV1130) was integrated using pLAR056 to aid integration.

Methylome Analysis of *E. coli* Strains. Methylating strains were grown in 5 mL of LB with 1 mM arabinose to induce methyltransferase expression followed by high molecular weight genomic DNA extraction. Library preparation and nanopore sequencing were performed as before with PCC 7002 to produce 340 Mb and 422 Mb of reads for AG11039 and AG11304, respectively. As these data sets were under 100× coverage for the *E. coli* genome, the entire read pool for each strain was used in the analysis. MIJAMP was used as with PCC 7002 before to confirm methylation capability of the heterologous methyltransferases, however methylome discovery by MIJAMP failed to discover multiple motifs. Instead of discovering methylation *de novo*, we used the query function to check for each motif present in PCC 7002 in case an enzyme's associated motif was incorrect (Table S3).

***In Vivo* Plasmid Methylation in *E. coli*.** Strains of modified *E. coli* BW25113 (Table S2) were transformed with gentamicin integration plasmids (Table S4) via electroporation. Briefly, cells were grown from freezer stock in LB overnight, then spun down to pellet. The supernatant was removed, then cells were resuspended in one-half the culture volume of sucrose solution (300 mM) to wash. After two additional wash steps, pelleted cells were resuspended in 1:50th of the culture volume of sucrose solution. Concentrated cells were electroporated at 1.8 kV in 0.2 cm cuvettes at a 1:1 DNA (ng) to cell (μL) ratio and plated on selective plates (LB agar; 35 μg mL^{-1} gentamicin). Transformed strains were grown overnight in 5 mL LB with 1 mM arabinose to express the integrated methyltransferases. Methylated plasmids were purified using an Omega EZNA Plasmid Mini Kit I and used in the Standardized Transformation protocol for PCC 7002.

Standardized Transformation of PCC 7002. Wild-type PCC 7002 was inoculated into 25 mL Media A+ (initial OD$_{730}$ = 0.05; Thermo Scientific Genesys 10S Vis) and grown overnight in a 125 mL Erlenmeyer flask (37 °C, 150 μmol photons m^{-2} s^{-1} white light, air, 200 rpm) until an OD$_{730}$ of 0.2−0.3 was reached. Cultures were centrifuged at 4300 rcf for 10 min, the supernatant was decanted, and then pellets were resuspended in Media A+ to an OD$_{730}$ of 1.0−9.0 (standard = 9.0; Figure S1). In a 1.5 mL microcentrifuge tube, 300 μL of concentrated cells were combined with 0.1−1 μg of plasmid DNA (standard = 1; quantified by Thermo Scientific NanoDrop) and vortexed to mix, then incubated at 37 °C and 150 μmol photon m^{-2} s^{-1} white light for 1−24 h (standard = 24). The tube was vortexed briefly to resuspend settled cells, then plated directly onto selective Media A+ plates or serially diluted with Media A+ and spotted (5 μL) to quantify transformants. Plates were incubated at 37 °C with ambient air and white light (100−150 μmol photon m^{-2} s^{-1}) until colonies were visible. Transformation efficiency η was calculated as the number of colony forming units (CFUs) per μg of DNA, incorporating spot volume V_s, dilution factor d, total transformation volume V_t, and mass of DNA added m, as described in the equation below

$$\eta = \mathrm{CFU}/m \times d \times (V_t/V_s)$$

■ ASSOCIATED CONTENT

Data Availability Statement

The data that support the findings of this study and relevant DNA sequences are available from the corresponding authors upon reasonable request.

SI Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.5c00370.

> Methods for culturing, cloning, and gathering fluorescence data; transformation protocol optimization; integration site context and constituent motifs; EYFP expression across integration sites and promoters; growth impact of integration at tested sites; tabulated methylation analysis data; tabulated strain and oligo details (PDF)

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Author Contributions

A.H., J.P.A., B.F.P., J.M.F., and C.A.E.: Conceived of research and design of experiments. J.P.A. and A.H.: Constructed and tested heterologous cassettes in PCC 7002. M.P.T.: Constructed *E. coli* methyltransferase expression strains. M.P.T., W.G.A., M.M.V., A.M.G., and A.H.: Performed methylome analyses. A.H., J.P.A., W.G.A., J.M.F., and C.A.E.: Wrote the manuscript.

Notes

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The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Dylan Courtney for his work developing pDC011 from the original, published CRISPR/Cas12a system[31] and Gregory Donovan for his advice and support. This work was supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, under Award Numbers DE-SC0018368 and DE-SC0019404. This research used resources of the Compute and Data Environment for Science (CADES) at the Oak Ridge National Laboratory, which is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC05-00OR22725.

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