Enabling Plasmid-based Expression in *Clostridium kluyveri* using a Biparental Methylation-Conjugation System

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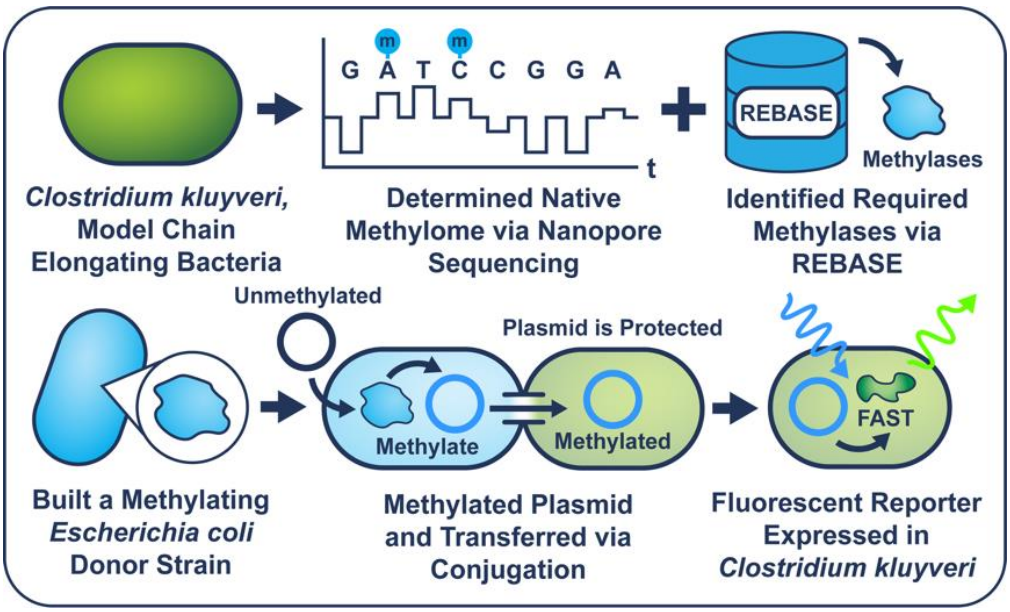
Keywords: *Clostridium kluyveri*, methylomics, conjugation, anaerobic fermentation, microbial chain elongation, restriction-modification systems, non-model microbes

Lay Summary

We developed a streamlined method for genetically modifying *Clostridium kluyveri*, a promising strain for industrial anaerobic biomanufacturing, and used it to express a fluorescent protein useful for downstream applications.

Graphical Abstract



Abstract

Clostridium kluyveri is a promising biocatalyst for producing medium-chain fatty acids (MCFAs) from waste-derived carbon via chain elongation. MCFAs are platform chemicals with diverse applications across agriculture, food, cosmetics, and fuels, and could support efforts towards tandem resource recovery and sustainable chemical production. However, genetic intractability has hindered efforts to engineer *C. kluyveri* for improved product yields, control over chain length and selectivity, and production of non-native oleochemicals. Here, we report a streamlined, biparental methylation-conjugation system developed for *C. kluyveri* DSM555^{T} to bypass the organism's restriction-modification barriers and enable stable plasmid delivery. We use this system to demonstrate heterologous expression of the Fluorescence-Activated absorption-Shifting Tag (FAST), an anaerobic fluorescent reporter. This system supports advances in metabolic engineering of *C. kluyveri* and the broader adoption of genetic tools in chain elongating bacteria to expand the applications of anaerobic chain elongation in industrial biomanufacturing.

Introduction

Anaerobic biotechnologies that transform waste carbon into high-value chemicals offer a sustainable platform for industrial biomanufacturing.[1,2] Among these, microbial chain elongation produces C_6–C_8 medium chain fatty acids (MCFAs) from diverse waste streams—including agricultural residues, organic waste, and industrial syngas.[3–7] In microbial chain elongation, chain elongating bacteria (CEB) use the reverse β-oxidation (RBO) cycle, a growth-coupled anaerobic metabolism, to produce MCFAs using waste-derived electron donors.[3,8,9] While MCFAs have broad applications in agriculture[10], food and cosmetic products[11–13], and specialty chemicals and fuels[14], current chain elongation systems face limitations in product yield, selectivity, and versatility.[11,15,16] Improving the technology's productivity and expanding its product range, particularly towards higher-chain MCFAs (C_9-C_{12}) and their derivatives, necessitates the development of genetic tools for CEB to manipulate native metabolic pathways and introduce heterologous functions.

Isolated in the 1940s, *Clostridium kluyveri* is the model CEB for ethanol-based chain elongation.[17] It has been extensively studied to elucidate chain elongation physiology[3,18–20] and applied in pure culture for the bioproduction of C_6-C_8 MCFAs[3,5,21] and in microbial consortia for C_4-C_6 alcohols[13,22–24]. However, a lack of tools to genetically manipulate *C. kluyveri* limits deeper understanding of its metabolism and prevents metabolic engineering efforts to improve productivity and expand product range. Efforts to genetically manipulate *C. kluyveri* are impeded by its native restriction-modification (RM) systems[25], which are bacterial defense mechanisms that protect cells from exogenous DNA (e.g., bacteriophage genomes and plasmids) by recognizing and cleaving sequences lacking host-specific methylation patterns.[26] Type I, II, and III RM systems encode DNA methyltransferases that methylate host DNA at specific recognition sites, along with restriction endonucleases that cleave unmodified DNA; in contrast, Type IV systems encode only restriction endonucleases that target DNA with non-native methylation patterns.[27] In effect, improperly methylated DNA is degraded by RM systems upon introduction into a host cell, which poses a critical barrier to DNA delivery for genetic manipulation.[28]

Overcoming RM systems using targeted DNA methylation is a demonstrated strategy for enabling plasmid delivery via conjugation and electro-transformation in other *Clostridium* species.[29–34] In contrast to electro-transformation, conjugation may confer some partial evasion from RM systems by delivering single-stranded DNA, which restriction endonucleases cannot cleave.[35,36] Concurrent work has combined these strategies and demonstrated a triparental conjugation system for *C. kluyveri*, which relies on a methylating *Escherichia coli* strain and a conjugative *E. coli* helper strain for plasmid mobilization into *C. kluyveri*.[37] A drawback of triparental conjugation systems is the additional complexity of maintaining two separate *E. coli* strains and the reliance on two separate conjugation events, which may prolong mating times, increase mutations in the mobilized plasmids, and lead to variable transconjugant recovery.[37,38] To complement this recent development, we report a streamlined biparental methylation-conjugation system for *C. kluyveri* DSM555^T. Our approach uses a single *E. coli* donor strain engineered for both methylation and conjugation, following a workflow established for other *Clostridium* species.[39] Using this system, we

demonstrate plasmid transfer and functional expression of the Fluorescence-Activated absorption-Shifting Tag (FAST)[40] anaerobic fluorescent reporter in *C. kluyveri,* an essential tool for quantifying protein expression dynamics and characterizing regulatory elements. This system opens the doors for genetic techniques to knockout/knock-in genes that could enable better control of MCFA production and access to other product classes[41], expanding the potential applications of *C. kluyveri* beyond what is possible without metabolic engineering.

Results

To enable exogenous plasmid delivery into *C. kluyveri* DSM555^T, we first characterized its native RM systems via analysis of genomic methylation patterns (methylome) determined using long-read nanopore sequencing. The *C. kluyveri* methylome contains five significant motifs, which were assigned with a putative *C. kluyveri* RM system, by comparison to the entry for *C. kluyveri* on the Restriction Enzyme Database (REBASE)[42] and all other characterized systems. This allowed us to devise a strategy to protect against the predicted RM systems, summarized in Table 1. Only motifs linked to restriction endonuclease domains required pre-methylation for effective plasmid transfer. Among the observed motifs, only the CCGG motif was associated with a Type II RM system containing a discrete restriction-endonuclease (CKL_2670) and a corresponding DNA methyltransferase (CKL_2671) amenable to pre-methylating exogenous plasmid DNA. The remaining RM systems predicted by REBASE (S1) are unlikely to pose significant barriers for plasmid transfer. Specifically, the CKL_2595 restriction endonuclease domain lacks an associated methylase gene required for its activity[43,44] and has no unaccounted motif in the methylome, suggesting it is inactive. The other systems encode fused (CKL_2694 & CKL_2332) or multimeric (CKL_3239) methylase-restriction domains with competing activities that preclude effective restriction.

Table 1: Significant methylation motifs in *C. kluyveri* DSM555^T, their associated RM systems, and strategy for the methylation-conjugation system

Methylation Motif*	Fraction Methylated	Associated Methylase Domain	Associated Restriction Domain	RM System Type	Comments	Action
CCA**A**G (6mA)	0.9998	CKL_3239	CKL_3240	III	R_2M_2 multimer required for restriction activity; competing restriction and methylation activity[44,45]	Simultaneous plasmid delivery and knockout if plasmid transfer is hindered
G**A**TC (6mA)	0.9957	CKL_2314	None	II	Missing R domain; no restriction activity of concern	No action required
GWTA**A**T (6mA)	0.9933	CKL_2694 & CKL_2332	Fused to Methylase Domain	IIG	Fused RM domains; competing restriction and methylation activity[46]	Simultaneous plasmid delivery and knockout if plasmid transfer is hindered
CCGG (5mC)	0.9903	CKL_2671	CKL_2670	II	Restriction activity of concern	Express CKL_2671 in *E. coli* sExpress to protect transferred plasmids
CAAAA**A**R (6mA)	0.9749	CKL_3158	None	II	Missing R domain; no restriction activity of concern	No action required

*The boldfaced, underlined base denotes the methylated base in the motif. 6mA and 5mC denote N6-methyldeoxyadenosine and 5-Methylcytosine base modifications, respectively.

To simultaneously protect plasmids from the CCGG-targeting RM system in *C. kluyveri* and enable exogenous plasmid delivery, we integrated the CKL_2671 methylase into the conjugal donor strain *E. coli* sExpress, previously shown to be an effective donor strain for other *Clostridium* species.[47] Figure 1 outlines the strain construction workflow and functional validation of the methylating conjugal donor. The CKL_2671 methylase gene was PCR-amplified from the *C. kluyveri* DSM555^{T} genome and integrated into the IncPβ conjugative plasmid R702 via λ-Red homologous recombination (S1) and transformed into *E. coli* Express to generate the methylating conjugal donor, *E. coli* sExpress CKL_2671. To assess whether *E. coli* sExpress CKL_2671 could protect plasmids from CCGG-targeting restriction endonucleases, we co-transformed it with pMTL82151, from the pMTL80000 *Clostridium* shuttle plasmid system[48], and subjected it to restriction digestion assays. pMTL82151 carries a Gram-positive replicon from pBP1[49] (*Clostridium botulinum*), an antibiotic resistance marker for thiamphenicol (*catP*), an IncPβ-compatible origin of transfer (*oriT)*, and 14 CCGG sites (Figure 1D). To validate *in vivo* methylation of pMTL82151 using *E. coli* sExpress CKL_2671, plasmid DNA was extracted from the strain and digested with MspI, which cleaves at CCGG sites only if the first cytosine is unmethylated.[50] Both R702 and pMTL82151 were protected from complete digestion compared to the unmethylated control, confirming that the methylase is functional in *E. coli* (Figure 1D) and is responsible for methylating **C**CGG motifs in *C. kluyveri*.

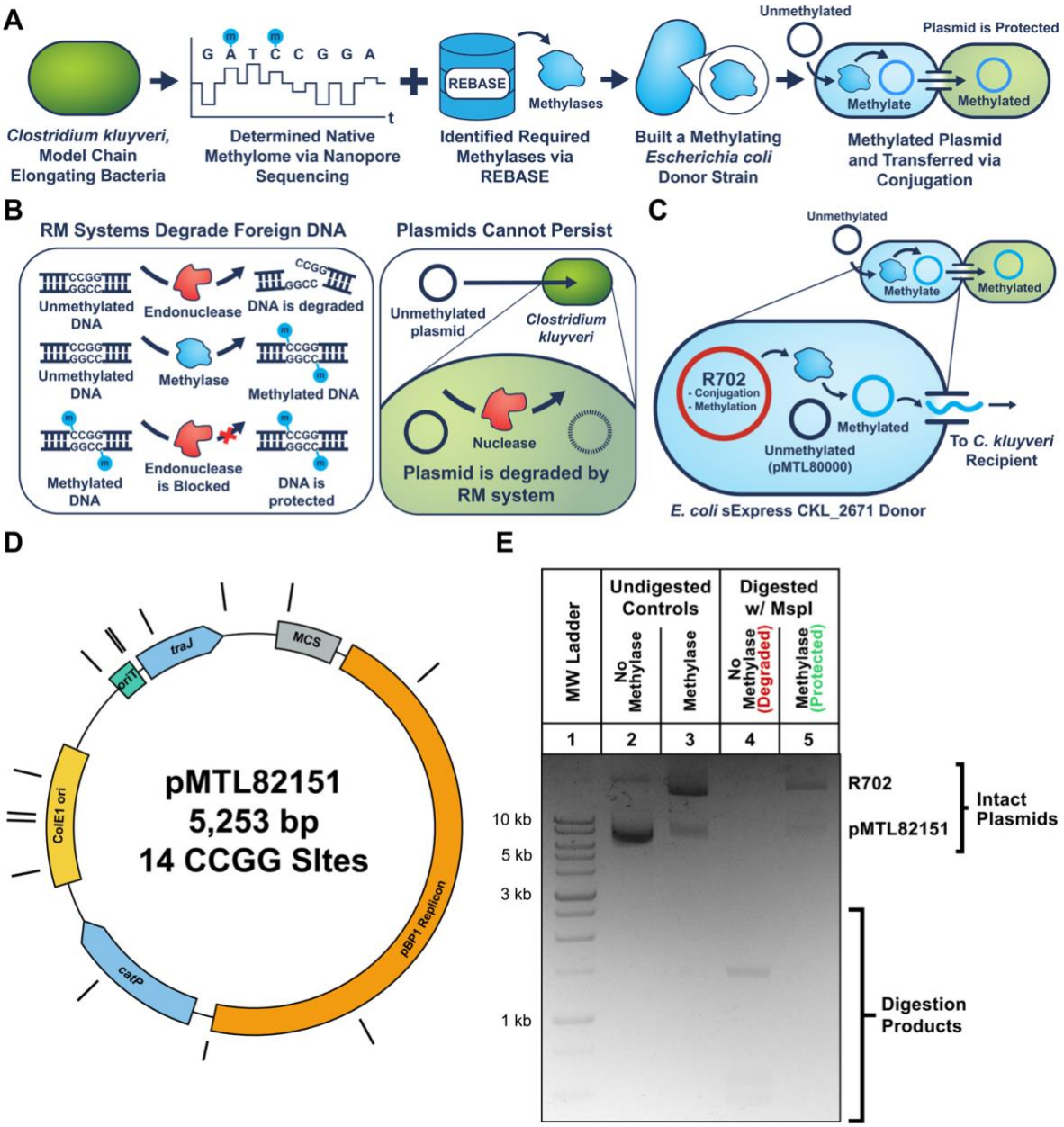


Figure 1: Development and functional validation of an *E. coli* methylation-conjugation strain. (A) Workflow for identifying and constructing *E. coli* donor strains expressing DNA methylases. Candidate methylases were selected based on methylome data and REBASE-annotated RM systems. (B) Conceptual overview of host RM systems, which degrade exogenous plasmid DNA lacking appropriate methylation. Plasmids propagated in standard *E. coli* are unmethylated at CCGG sites and are degraded upon entry into *C. kluyveri*. (C) Functional mechanism of the engineered *E. coli* sExpress CKL_2671 strain, which expresses the CKL_2671 methylase to methylate plasmids before conjugation. (D) Annotated plasmid map of pMTL82151, including features relevant for conjugation and selection: Multiple cloning site (MCS), pBP1 replicon, *catP*, ColE1 origin, *oriT*, and *traJ*. CCGG restriction sites targeted by MspI are highlighted as black bars. (E) MspI digestion products of plasmids extracted from *E. coli* strains with or without CKL_2671. Lane 1: Molecular weight marker (FroggaBio), Lanes 2-3: undigested controls; Lanes 4-5: plasmids

digested with MspI. Intact plasmid in Lane 5 confirms functional expression of CKL_2671 *in vivo*.

Following *in vivo* methylation, we tested whether *E. coli* sExpress CKL_2671 carrying pMTL82151 could transfer thiamphenicol resistance to *C. kluyveri*. Figure 2 summarizes the results of conjugation and phenotypic characterization of the resulting *C. kluyveri* transconjugants. Using *E. coli* sExpress CKL_2671 as the donor, we successfully transferred pMTL82151 to *C. kluyveri*, isolating transconjugants under thiamphenicol selection and D-cycloserine donor counterselection (Figure 2A). Wild-type *C. kluyveri* has no intrinsic resistance to thiamphenicol, predicted using the Comprehensive Antibiotic Resistance Database[51] and confirmed with controls. This validates the successful transfer of pMTL82151 and stable expression of the *catP* resistance gene. No colonies were obtained in the absence of methylation, demonstrating the necessity of pre-methylation for effective conjugation. Next, we used the strain to deliver protein expression plasmids and evaluated the expression of the anaerobic fluorescent reporter FAST in *C. kluyveri*. Two expression plasmids driven by the *Clostridium pasteurianum* ferredoxin promoter (P_{fdx}) were constructed and separately co-transformed into *E. coli* sExpress CKL_2671: pMTL82153 harbouring the *lacZ α*-subunit gene (as a non-fluorescent control), and pMTL82153-FAST encoding FAST. Upon conjugation into *C. kluyveri* (Figure 2B), the identities of the *C. kluyveri* transconjugants were confirmed by 16S rRNA gene amplicon sequencing and whole plasmid sequencing. The transconjugants were cultured and incubated with the required fluorophore for FAST fluorescence, 4-hydroxy-3-methylbenzylidene-rhodanine (HMBR).[40] Cultures expressing FAST exhibited visible yellow-green fluorescence under blue light excitation. In contrast, the wild-type and the non-fluorescent control cultures exhibited no visible fluorescence aside from red autofluorescence (Figure 2C). Quantitative fluorescence measurements further confirmed that FAST-expressing *C. kluyveri* cultures displayed significantly higher fluorescence compared to controls, with a peak emission at 542 nm (Figure 2D-E).

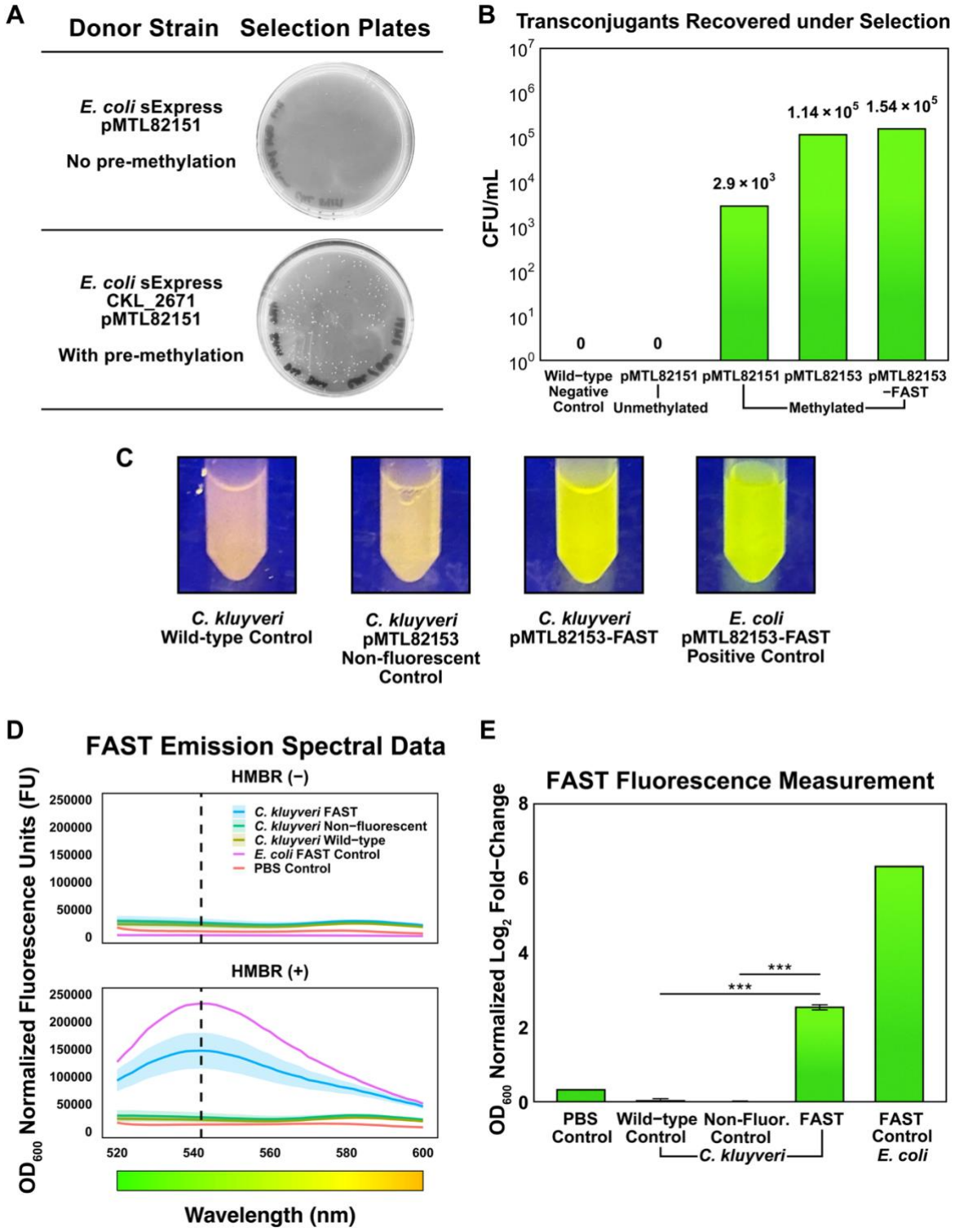


Figure 2: Demonstrating plasmid delivery and functional reporter expression in *C. kluyveri* DSM555^T. (A) Representative petri plate images of *C. kluyveri* DSM555^T transconjugants recovered under thiamphenicol selection, indicating successful plasmid transfer. (B) Quantification of transconjugant recovery demonstrates that methylation with CKL_2671 enables efficient DNA delivery, achieving >10³ CFU/mL. (C) *C. kluyveri* cultures imaged under blue light illumination exhibit visible fluorescence from strains expressing FAST. (D) Whole cell emission spectra (Ex. 485 nm) for

cultures with and without FAST expression and HMBR (shaded regions denote standard deviations). The peak wavelength for whole cells expressing FAST was measured at 542 nm. (E) Quantitative fluorescence measurements (Ex. 485 nm, Em. 542 nm) from plate reader assays show significant fluorescence in strains expressing FAST. One-way ANOVA ($p = 8.42 \times 10^{-8}$, $\alpha = 0.05$) and Tukey's HSD test ($\alpha = 0.05$, p-values < 0.001). Plasmids: pMTL82151 (base plasmid), pMTL82153 (non-fluorescent control plasmid expressing *lacZ α*-subunit), and pMTL82153-FAST (reporter plasmid expressing FAST). Wild-type: no plasmid control.

Discussion

This study establishes a foundational tool for the genetic engineering of the model chain elongating bacterium *C. kluyveri* DSM555^{T}. By characterizing its methylome using long-read nanopore sequencing and linking key motifs to predicted RM systems, we identified and validated the activity of the CKL_2671 methylase and used it to construct a methylating conjugal donor strain, *E. coli* sExpress CKL_2671. Using this strain, we demonstrated a streamlined biparental conjugation system for *C. kluyveri*, enabling stable plasmid transfer and heterologous gene expression. Our approach achieved efficient recovery of transconjugants and exhibits several key distinctions from other systems. Unlike triparental conjugation systems[34,37,38] that require separate *E. coli* donor and helper strains, our single-donor system simplifies the workflow and reduces the complexity of maintaining multiple strains. Additionally, we demonstrated that the pBP1 Gram-positive replicon is compatible for stable plasmid maintenance in *C. kluyveri,* expanding the plasmid toolkit for *C. kluyveri*. Importantly, we validated the functional expression of the anaerobic fluorescent reporter FAST, with engineered *C. kluyveri* strains exhibiting significantly higher fluorescence than control cultures. This marks an important step toward tunable heterologous protein expression in this organism and provides a tool for *in vivo* gene expression monitoring. To support broader adoption, the methylating donor strains developed in this study are available upon request (Associated Content).

While conjugation proved effective in our system, future efforts may benefit from developing an electroporation protocol for *C. kluyveri* to reduce the time-intensive nature of conjugation-based workflows. Although not tested here, the use of the commercially available MspI methyltransferase to pre-methylate plasmid DNA or the extraction of methylated plasmids from *E. coli* expressing CKL_2671 may circumvent RM barriers and enable electro-transformation. If successful, electroporation could offer a faster, more scalable alternative for DNA delivery into *C. kluyveri*, particularly for applications requiring iterative engineering or high-throughput library screening. Deactivating the RM systems in *C. kluyveri* via targeted knockouts could further simplify plasmid transfer into the strain, although such strategies are not applicable for engineering *C. kluyveri* within microbial consortia. Methylation-guided plasmid delivery remains critical for *in situ* engineering native microbial consortia[52], where preserving wild-type genotypes is needed for community stability and function.

Further, the workflow for creating the methylating donor strain can be extended to other CEB once methylomes for these organisms are determined. Future efforts will focus on expanding the toolkit for *C. kluyveri*, including tunable and inducible expression systems, using emerging fluorescent reporters with different emission wavelengths (e.g., evolved flavin-based fluorescent proteins (FbFPs)[53] and anaerobic,

221 blue fluorescent proteins[54]), which will be critical for high-throughput screening, multi-
222 gene expression tracking, and protein-protein interaction techniques like Fluorescence
223 resonance energy transfer (FRET)[55] which has been inaccessible in anaerobic
224 conditions.

225 Developing programmable genome editing tools for *C. kluyveri* is another
226 important next step. Potential strategies include adapting heterologous systems for
227 *Clostridium,* such as RiboCas[56], leveraging *C. kluyveri*'s endogenous CRISPR-Cas
228 system for native interference or editing capabilities[57], and applying transposon
229 mutagenesis libraries for high-throughput genotype-phenotype mapping.[58]
230 Establishing robust genome editing workflows will deepen our mechanistic
231 understanding of chain elongation and enable metabolic engineering of *C. kluyveri*.
232 Such tools could facilitate targeted improvements in chain elongation yield, chain
233 length selectivity, product tolerance, and expand the product spectrum of *C.* kluyveri
234 to other oleochemicals, increasing its utility as a chassis for sustainable industrial
235 biomanufacturing.

Methods

Bacterial Strains and Culture Conditions

E. coli strains were grown aerobically at 37 °C in LB medium or plates with appropriate antibiotics for each strain (S1). *C. kluyveri* DSM555^T was cultured anaerobically in Hungate tubes containing ATCC 1120 liquid medium at 37 °C and plated on Reinforced Clostridial Medium (RCM) agar supplemented with 100 µL of ethanol spread-plated before inoculation in an anaerobic chamber (COY, $N_2/CO_2/H_2$ atmosphere) and incubated at 37 °C in anaerobic containers (BD GasPak EZ Container Systems).

C. kluyveri Methylome and RM Systems Analysis

Genomic DNA from *C. kluyveri* DSM555^T was extracted using the Zymo Quick-DNA HMW MagBead Kit with modifications: bead beating (0.1 mm glass beads) and mutanolysin (50 U) were added to improve lysis. DNA was further purified with TotalPure NGS Beads (0.7× ratio) to remove low-molecular-weight fragments. Sequencing libraries were prepared with the ONT Rapid Barcoding Kit V14 and run on a MinION device (R10.4.1). Basecalling was done with dorado (v0.9.5), and an assembly generated using Flye[59] (v2.9.5), assessed with CheckM2[60] (v1.0.2), and taxonomically validated with GTDBtk[61] (v2.4.1). 6mA and 5mC modifications were detected via dorado's methylation model and mapped with minimap2[62] (-ax map-ont). Genome-wide patterns and high-confidence motifs were analyzed using modkit[63] (v0.4.4). Motifs associated with a putative *C. kluyveri* RM system based on REBASE. For RM systems lacking known recognition motifs, we predicted likely targets based on homology to characterized RM systems in REBASE. More details can be found in the Supporting Information (S1).

Construction of *Clostridium* Expression Plasmids

Clostridium shuttle plasmids for *E. coli* and *C. kluyveri* were assembled using modular parts from the pMTL80000 plasmid series from the Synthetic Biology Research Centre, University of Nottingham (SBRC). Restriction-digested or PCR-amplified fragments were assembled with NEBuilder HiFi Assembly and transformed into *E. coli* NEB5a (NEB). Final plasmids were sequence-verified by long-read sequencing (Plasmidsaurus).

Construction of the Methylating Conjugal Donor Strain

The *E. coli* sExpress CKL_2671 methylating conjugal donor was constructed based on a novel workflow leveraged for other *Clostridium* species from collaborators at the SBRC. The workflow included amplifying the *C. kluyveri* CKL_2671 methylase gene from its genome and cloning it into the arabinose-inducible RBV-1 vector via NEBuilder HiFi assembly and transformed into *E. coli* TOP10. The CKL_2671 expression cassette in RBV-1 includes homology arms for integration into the R702 plasmid. The CKL_2671 expression cassette was PCR-amplified and integrated into R702 via λ-Red recombineering in *E. coli* RE-InterStellar (harbouring R702 and pKD46cm). Recombinants were selected on apramycin and kanamycin, verified by PCR across the insertion site, and cured of pKD46cm. The recombinant R702 (containing CKL_2671 expression cassette) was extracted and transformed into *E. coli* Express (NEB) to obtain the final methylating conjugal donor, *E. coli* sExpress CKL_2671. *E.*

279 *coli* sExpress CKL_2671 was co-transformed with the assembled *Clostridium* shuttle
280 plasmids via heat-shock transformation. Functional methylation of these conjugal
281 donors was confirmed by protection against MspI digestion (methylation-sensitive).

282 **Anaerobic conjugation and validation of transconjugants**

283 *E. coli* sExpress CKL_2671 conjugal donors strains carrying the *Clostridium* shuttle
284 plasmids were mated with *C. kluyveri* under anaerobic conditions. *E. coli* and *C.*
285 *kluyveri* were mixed at a donor:recipient ratio of 1:1, spotted on RCM agar
286 supplemented with ethanol, and incubated for 48 h. Total biomass of the conjugal
287 mixture was harvested and plated at several serial dilutions on RCM agar
288 supplemented with ethanol along with thiamphenicol [7.5 μg/mL] to select for *C.*
289 *kluyveri* transconjugants and D-cycloserine [250 μg/mL] to select against the *E. coli*
290 donor strain. Transconjugants colonies were screened after 5 d by colony morphology,
291 inoculated into Hungate tubes containing ATCC1120 supplemented with
292 thiamphenicol and D-cycloserine at the same concentrations above and incubated at
293 37 °C for 3 d. Transconjugant cultures were passaged once, and nucleic acids were
294 extracted for 16S rRNA gene sequencing to confirm strain identity, and long-read
295 sequencing to confirm plasmid identity.

296 **FAST fluorescence measurement**

297 *E. coli* and *C. kluyveri* cultures were washed with PBS and resuspended to OD_{600} =
298 1.0 in PBS ± 20 μM 4-hydroxy-3-methylbenzylidene-rhodanine (HMBR, stock
299 prepared in DMSO). Fluorescence was measured in flat-bottom, black 96-well plates
300 (Greiner, 200 μL/well) using a plate reader and normalized to the measured OD_{600}
301 (Tecan Infinite M Plex, Ex: 485 nm, Em: 542 nm).

Associated Content

The Supporting Information is available free of charge at [insert publication link]. Supporting Information: Additional experimental details, materials, and methods (PDF). The conjugal donor strains developed as part of this work are available upon request at cost (repository@forgegenetics.com).

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Author Contributions

E.A. conceived, designed, and performed all experiments, analyzed the data, and drafted the manuscript. A.A.B., B.G.L., and I.M.G. assisted with methylome analysis and interpretation. A.W.D. provided training and guidance for methylation strain construction and recombineering techniques. N.P.M., R.M., and C.E.L. supervised the project and contributed to experimental design, data interpretation, and manuscript review. All authors reviewed and approved the final manuscript.

336 **Notes**

337 A.W.D. is an employee of Forge Genetics Inc., which offers the methylating *E. coli*
338 strains described in this work at cost for academic and non-profit research. All other
339 authors declare no competing financial interests.

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Supporting Information (S1)

Restriction-Modification Systems Analysis for *Clostridium kluyveri*

The REBASE Database (New England Biolabs) was used to both bioinformatically predict the presence of restriction modification systems (types I-IV) and the highest probability recognition motif of each annotated methyltransferase and endonuclease within *C. kluyveri*. Predicted enzymes were automatically assigned locus tags and sorted based on RM type, methyltransferase (M) or endonuclease activity (R), and their homology predicted recognition motif. Independently collected methylome data was further used to narrow down motifs of interest by identifying discrete genomic motifs that had a high incidence of methylation (5mC, 4mC, and 6mA).

Using both data sets, annotated REBASE motifs for *C. kluyveri* were first cross referenced with the genomic motifs and methylation type when available. For all remaining enzyme sets the motif and methylation data of closest annotated relative was used. From this comparison six potential candidates were identified with matching motif data, indicating these systems were actively being express. Of these, only four targets (CKL_2332, CKL2670/2671, CKL_2694, and CKL_3239/3240) had an annotated endonuclease that is required for DNA cleavage and degradation, indicating that they are likely barriers to conjugation and thus targets for evasion or removal. With a list of four targets, the RM type between each system was then compared to assign a rank to each system based on both likelihood of plasmid cleavage and ease of targeting.

CKL 3239/3240 was the only annotated type III system, which requires the formation of a heteromeric protein complex that facilitates both cleavage and modification of DNA motifs. Due to both the stability and dual function of the complex, and the lack of suitable commercial isozymes, isolation of methylation activity is not always possible. Thus, these systems are challenging to evade through DNA pre-methylation and require RNA interference strategies or gene knockouts to remove. Similarly, CKL_2332 and CKL_2694 are type IIG systems, consisting of a single protein with a fused restriction and modification domain, making the isolation impossible due to the significant overlap of the domains. CKL2670/2671 however, is a canonical type II system, consisting of a separated methylase and endonuclease pair with isolated modification and restriction activity. This enabled the selective pre-methylation of plasmid DNA using CKL_2671 to evade the restriction of CKL_2670 and confer a layer of epigenetic protection to plasmids pre-conjugation.

Table S1: REBASE Predicted RM systems for *C. kluyveri* and recognition sequence assignment

Source	Locus Tag	Type	System Name	Domain Activity	REBASE Predicted Rec. Seq.	Homology Predicted Rec. Seq.	Predicted Rec. Seq.	Methylation Type	Motif Present in Methylome?	Closest Characterized Homologous Enzyme	AA Identity (%)
Clostridium kluyveri DSM555 Genome	CKL_2596	I	2595	R		CAGNNNNWTCT	CAGNNNNWTCT		N	Cbo13037IVP	91
	CKL_1889	II	1889	M			Unassigned				
	CKL_1987	II	1987	M			Unassigned				
	CKL_2314	II	2314	M	G**A**TC		G**A**TC	6mA	Y		
	CKL_2332	II	2332	RM		GTTA**A**T	GTTA**A**T	6mA	Y	Cau10061II	56
	CKL_2649	II	2649	M		AB	AB	6mA	N	M.BceSVII	96
	CKL_2670	II	2671	R	CCGG		CCGG		Y	CceI	99
	CKL_2671	II		M	CCGG		CCGG	5mC (base undetermined)		M.CceI	99
	CKL_2694	II	2694	RM		GATA**A**T	GATA**A**T	6mA	Y	RM.CcaP7IV	47
	CKL_2938	II	2938	M	**A**CNNNNNC**T**CC		ACNNNNNCTCC	6mA	N		
	CKL_3158	II	3158	M		CAAAA**A**R	CAAAA**A**R	6mA	Y	M.CpaAIII	50
	CKL_3292	II	3292	M			Unassigned				
	CKL_3294	II	3292	M			Unassigned				
	CKL_3897	II	3897	M			Unassigned				
	CKL_3239	III	3239	M		CCA**A**G / TAAATC	CCA**A**G / TAAATC	6mA	Y (CCA**A**G)	M.Fla104114I	56
	CKL_3240	III		R		TAAATC	TAAATC	6mA	N	BcoSlacIP	56
pCKL555 (Endogenous Plasmid)	CKL_4008	II	4008	M	TGGCCA		TGGCCA	Modification undetermined	N		
	CKL_4048	II	4048	M			Unassigned				

618 **Methylome Analysis of *Clostridium kluyveri***

619 Genomic DNA was extracted from *Clostridium kluyveri* DSM 555 cultures harvested
620 at late exponential phase using the **Zymo Quick-DNA HMW MagBead Kit** (Zymo
621 Research), following the manufacturer's protocol optimized for high-molecular-weight
622 DNA suitable for long-read sequencing. Two modifications to the recommended
623 protocol were made to optimize genomic DNA yield and purity. First, cell pellets were
624 subjected to bead beating with sterile, acid-washed 0.1mm glass beads. Second, a
625 total of 50U mutanolysin (Sigma-Aldrich, M9901) was added to the enzymatic lysis
626 buffer. Additionally, the resulting high molecular weight gDNA was furthered purified
627 via TotalPure NGS Beads (Omega Bio-tek) using a 0.7X ratio of magnetic beads to
628 sample volume to deplete low molecular weight DNA fragments before library
629 preparation.

630 Purified gDNA was then prepared for sequencing on a minION device (Oxford
631 Nanopore Technologies) using Rapid Barcoding Kit V14 (SQK-RBK114) library
632 preparation followed by loading and sequencing on a minION flow cell (R10.4.1)
633 following manufacturer's protocols. The resulting raw data was basecalled with dorado
634 (v0.9.5) using the relevant high-accuracy model with 400bps translocation speed
635 (model v5.0.0). Reference genome assembly was performed with flye (v2.9.5) using
636 default settings. Thorough quality assessment of the resulting assembly was
637 completed with CheckM2 (v1.0.2) and confirmation of taxonomic assignment to *C.*
638 *kluyveri* DSM 555 was verified with GTDBtk (v2.4.1) against GTDB release 226.

639 Methylation of adenine (6mA) and cytosine (4mC and 5mC) bases were sensed with
640 dorado using appropriate modification-calling extensions of the canonical model
641 mentioned above (dna_r10.4.1_e8.2_400bps_hac). The resulting methylated
642 sequences were mapped to the reference genome assembly produced herein with
643 minimap2 (-ax map-ont; v2.28). Genome-wide methylation patterns were then
644 analyzed using modkit (v0.4.4); the proportion of mapped reads carrying modifications
645 at each position was summarized with modkit pileup (--filter-threshold 0.7) and motifs
646 with high proportions of modification were deduced by modkit motif search. Finally, the
647 log2 odds of the reported high confidence motifs were calculated with modkit motif
648 evaluate.

649 Table S3: Significant methylation motifs in *C. kluyveri*.

Motif[1]	Methylation Type	Fraction Methylated	High Count	Low Count	Mid Count	Log2 Odds
CCA**A**G	6mA	0.9998	4569	1	146	19.18
G**A**TC	6mA	0.9957	9070	39	1844	15.25
GWTA**A**T	6mA	0.9933	7904	53	381	14.50
CCGG	5mC	0.9903	6196	61	1169	19.96
CAAAA**A**R	6mA	0.9749	2835	73	875	12.19

[1]The boldface, underlined base denotes the methylated base in the motif

650

651 To associate detected methylation motifs with putative restriction-modification (RM)
652 systems, motif data were cross-referenced with REBASE, enabling annotation of *C.*
653 *kluyveri* methyltransferases and endonucleases based on homology to previously
654 characterized systems.

655 **Integration of CKL_2671 into R702**

656 The workflow conducted has been recently demonstrated in other *Clostridium* species
657 but had not been applied to *Clostridium kluyveri.* To construct the methylating conjugal
658 donor strain, the DNA methyltransferase (methylase) gene of interest (CKL_2671) was
659 amplified by PCR from the *C. kluyveri* genome, including the native ribosome binding
660 site and appropriate 5' overhangs for HiFi DNA assembly. The CKL_2671 gene was
661 then assembled into the *E. coli*-based recombineering vector RBV-1, which contains
662 an arabinose-inducible *pBAD* promoter controlling the methylase, a ColE1 origin of
663 replication for plasmid maintenance in *E. coli*, homology arms for R702, and dual
664 antibiotic markers (erythromycin and apramycin resistance).

665 RBV-1 was linearized by digestion with EcoRI and BsaI (NEB), and the gel-purified
666 PCR products were inserted into the linearized vector using NEBuilder HiFi DNA
667 assembly. The assembled plasmid was transformed into *E. coli* TOP10 cells, as other
668 strains may harbor active Type IV restriction systems that are incompatible with
669 methylase expression. Transformants were screened by colony PCR and confirmed
670 by sequencing. Functional validation of methylase activity was performed using
671 restriction digest assays with isoschizomers for the CCGG recognition sequence (MspI
672 and HpaII, NEB) corresponding to the predicted methylation motif of CKL_2671.

673 Once methylation activity was confirmed, the assembled methylase integration
674 cassette—including homology arms for R702 flanking the insertion site—were
675 amplified and inserted into the *E. coli* R702 conjugal plasmid using lambda Red
676 recombineering. This step employed an electro-transformation of the CKL_2671
677 integration cassette PCR product into *E. coli* RE-InterStellar, which harbours the R702
678 plasmid and pKD46cm, which carries the genes for the lambda Red system. At this
679 stage, culturing was performed at 30°C as pKD46cm contains a temperature sensitive
680 replicon. Recombinants were selected on LB agar containing apramycin and
681 kanamycin, pKD46cm was cured with subsequent culturing at 37°C, and correct
682 insertion into R702 was verified by PCR spanning the homology arm junctions and by
683 confirming the loss of spectinomycin resistance, which serves as a negative selection
684 marker.

685 Following this, the recombinant R702-CKL_2671 plasmid was extracted from *E. coli*
686 InterStellar and electro-transformed into E. coli Express to yield the base methylating
687 strain, E. coli sExpress CKL_2671. To create the conjugal donors with mobilizable
688 plasmids for *Clostridium, E. coli* sExpress CKL_2671 was transformed via heat-shock
689 with plasmids from the pMTL80000 *Clostridium* shuttle plasmid series. Again,
690 functional validation of methylase activity was performed using restriction digest
691 assays on plasmid extracts from the final conjugal donors. The system demonstrated
692 here supports foundational advancements for the genetic manipulation of *C. kluyveri*
693 DSM 555^{T} with potential for translation to *C. kluyveri* NBRC 12016, which has similar
694 RM systems based on REBASE predictions.

695

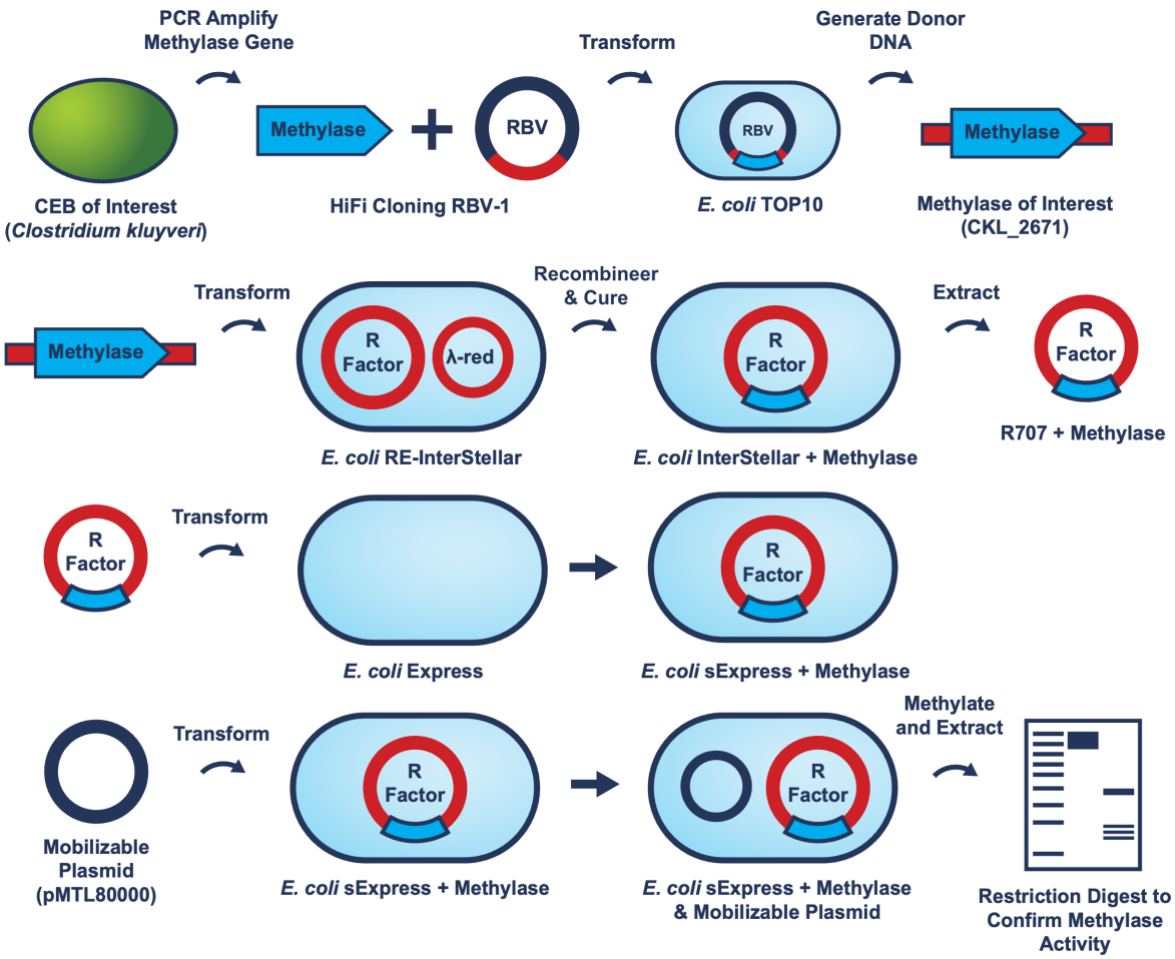


Figure S1: Schematic of the molecular cloning workflow for constructing the methylating conjugal donor strain, *E. coli* sExpress CKL_2671

699 **Bacterial Strains, Media, and Growth Conditions**

700 Table S2: A list of bacterial strains and their growth conditions used for this study.

Strain	Description	Antibiotics	Temp.	Growth Media	Source
Clostridium kluyveri DSM555	Type strain used for conjugation and wild-type controls		37°C	Hungate tubes: ATCC1120; Solid Media: Reinforced Clostridial Agar supplemented with ethanol	DSMZ
Escherichia coli NEB5a	Cloning strain		37°C	LB Miller broth and plates	NEB
Escherichia coli Express	Expression strain				
Escherichia coli NEB5a pMTL82151	Cloning strain carrying pMTL82151	chloramphenicol	37°C	LB Miller broth and plates	SBRC
Escherichia coli TOP10 RBV-1	Cloning strain carrying RBV-1	erythromycin			
Escherichia coli sExpress	Conjugal donor strain (dam+, dcm-) carrying R702	kanamycin			
Escherichia coli sExpress pMTL82151	Conjugal donor strain (dam+, dcm-) carrying R702 and pMTL82151	kanamycin; chloramphenicol			
Escherichia coli RE-InterStellar	λ-red recombination strain carrying R702 and pKD46-cam; derivative of E. coli Stellar (Takara Bio)	kanamycin; chloramphenicol	30°C		
Escherichia coli TOP10 RBV-1-CKL_2671	Cloning strain carrying RBV-1-CKL_2671 and used for recombineering into R702 and in vivo methylation assays	erythromycin	37°C	LB Miller broth and plates	This work
Escherichia coli RE-InterStellar CKL_2671	Intermediate strain carrying R702-CKL_2671 and pKD46-cam	apramycin; chloramphenicol	30°C		
Escherichia coli InterStellar CKL_2671	Intermediate strain carrying R702-CKL_2671 and cured of pKD46-cam	apramycin	37°C		

Escherichia coli NEB5a pMTL82153	Cloning strain carrying pMTL82153 and used for FAST fluorescence assays a non-fluorescent control	chloramphenicol	37°C	LB Miller broth and plates	This work
Escherichia coli NEB5a pMTL82153-FAST	Cloning strain carrying pMTL82153-FAST and used for FAST fluorescence assays as a positive control				
Escherichai coli sExpress CKL_2671	Methylating conjugal donor strain (dam+, dcm-) carrying R702-CKL_2671	apramycin	37°C	LB Miller broth and plates	This work
Escherichia coli sExpress CKL_2671 pMTL82151	Methylating conjugal donor strain (dam+, dcm-) carrying R702_CKL_2671 and pMTL82151	apramycin; chloramphenicol	37°C	LB Miller broth and plates	This work
Escherichia coli sExpress CKL_2671 pMTL82153	Methylating conjugal donor strain (dam+, dcm-) carrying R702_CKL_2671 and pMTL82153				
Escherichia coli sExpress CKL_2671 pMTL82153-FAST	Methylating conjugal donor strain (dam+, dcm-) carrying R702_CKL_2671 and pMTL82153-FAST				
Clostridium kluyveri DSM555 pMTL82151	Transconjugant carrying pMTL82151 and used to validate negative selection using thiamphenicol	thiamphenicol; D-cycloserine	37°C	Hungate tubes: ATCC1120; Solid Media: Reinforced Clostridial Agar supplemented with ethanol	This work
Clostridium kluyveri DSM555 pMTL82153	Transconjugant carrying pMTL82153 used for FAST fluorescence assays as a non-fluorescent control				
Clostridium kluyveri DSM555 pMTL82153-FAST	Transconjugant carrying pMTL82153-FAST used for FAST fluorescence assays				

701

702 **Primers**

703 Table S4: A list of primers used in this study.

Name	Sequence	Template	Function
CKL_2671_F	GTTTCTCCATACCCGTTTTTTTGGGTTAAGATAAAAGGTGATTGCAATGCGTGG	*Clostridium kluyveri* DSM555 genome	Assembly of RBV-1-CKL_2671
CKL_2671_R	CCGGAATCATATGACTCGAGTTTATAAAGCTATAGTTTTTATTTTTTCAATATCCTGCATTTCAGC		
LHA_F	TTCTACGGCACGTTTGAAGGCG	RBV-1-CKL_2671	Donor DNA PCR for CKL_2671 recombination into R702
RHA_R	GCAGCGACTTCCACGATGGG		
Int_scr_F	CCGAGCAGATCCTGCACGG	R702-CKL_2671	Screening for R702-CKL_2671
Int_scr_R	GGGAGTATGAAAAGTATGGCTGAAGCG		
82153_INS_F	GCCCCCTGCAGGATAAAAAAATT	pMTL83353	Assembly of pMTL82153
82153_INS_R	GGATTCAGAACGGCGCGCCATAAAAAT		
82153_VEC_F	ACAATTTTTTTATCCTGCAGGGGGCC	pMTL82151	
82153_VEC_R	ATTTTTATGGCGCGCCGTTCTGAATCC		
82153-FAST_INS_F	TTTTAAGGAGGTGTGTTACATATGGAACACGTAGCATTTGGAAGTG	pMTL82255	Assembly of pMTL82153-FAST
82153-FAST_INS_R	TTCTTATTTTTATGCTAGCGTCATACCCTCTTAACGAAAACCCAGTAG		
82153-FAST_VEC_F	AATGGCGCTAGCATAAAAATAAGAA	pMTL82153	
82153-FAST_VEC_R	ATCATGGTCATATGTAACACACCTCCTTAAAAA		
catP_scr_F	TTTGAGAGGGAACTTAGATGGTATTTGAAAAAATTG	pMTL80000 Clostridium shuttle plasmids	pMTL80000 colony PCR screening primers
catP_scr_R	TAAACTTAGGGTAACAAAAAACACCGTATTTCTACG		
27F	AGAGTTTGATCCTGGCTCAG	16S rRNA gene	16S rRNA gene amplification for strain identity verification
1492R	TACCTTGTTACGACTT		

704

705 **Plasmids**

706 Table S5: A list of plasmids used in this study.

Name	Description	Selection Markers	Source
RBV-1	Base recombineering plasmid	Erm	SBRC
R702	Base conjugative transfer plasmid	Kan	
pKD46-cm	Temperature sensitive λ-red recombination helper plasmid	Cam	
RBV-1-CKL_2671	Recombineering plasmid with CKL_2671	Erm, Apr	This work
R702-CKL_2671	Methylating, conjugative transfer plasmid	Kan, Apr	
pMTL82151	Base pMTL82151 plasmid	Cam	
pMTL82153	Non-fluorescent protein expression plasmid	Cam	
pMTL82153-FAST	Fluorescent protein expression plasmid	Cam	

707

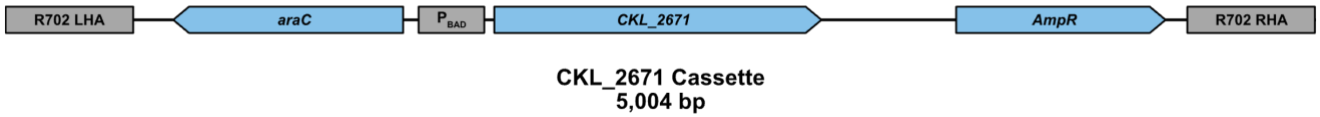


Figure S2: A schematic of the CKL_2671 Expression Cassette integrated into the R702 plasmid. R702 LHA: Left-hand homology arm (LHA) for the R702. araC: coding sequence for the P_{BAD} repressor protein. P_{BAD}: arabinose-inducible promoter controlling CKL_2671 expression. AmpR: resistance marker for apramycin negative selection. CKL_2671: a 5mC DNA methyltransferase from the *C. kluyveri* genome, methylating **C**CGG sites at the first cytosine. R702 RHA: right-hand homology arm (RHA) for the R702.

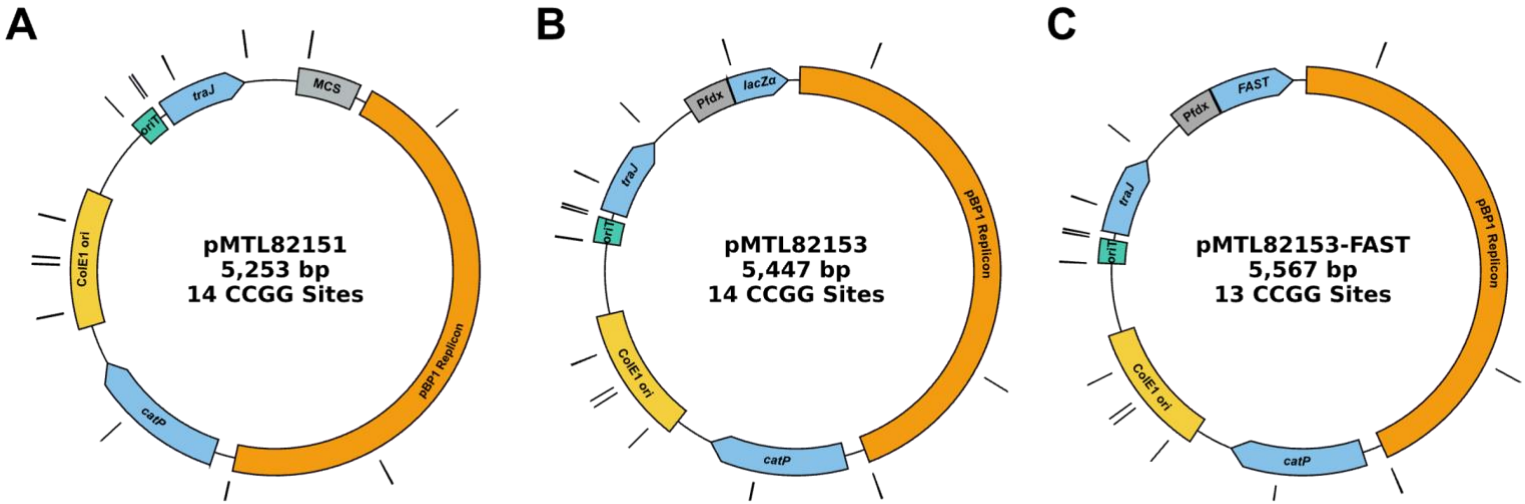


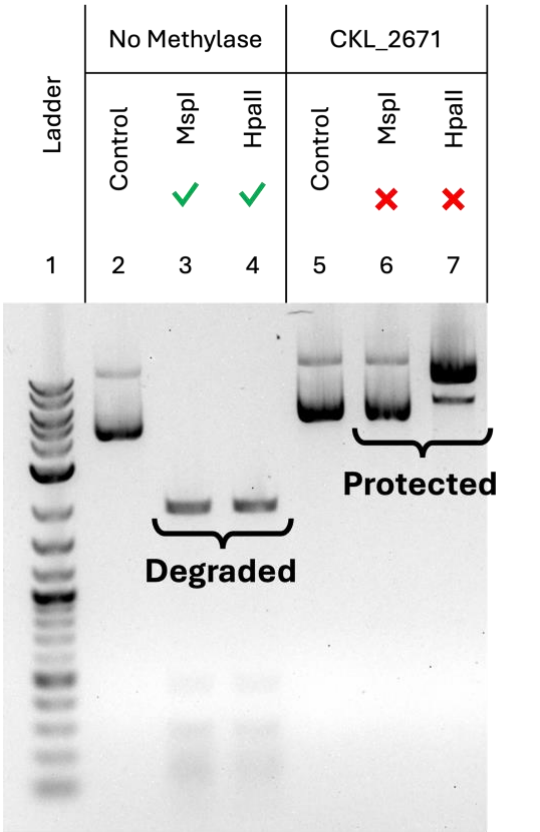
Figure S3: Plasmid maps of the pMTL80000 plasmids used in this study with annotated CCGG sites. pBP1 Replicon: Gram-positive compatible replicon from pBP1. *catP*: an antibiotic resistance marker for thiamphenicol. ColE1 ori: Gram-negative compatible origin of replication. *oriT:* an IncPβ-compatible origin of transfer. *traJ*: a coding sequence for the transcriptional activator for conjugative functions of R702. The plasmids differ at the insert region: (A) pMTL82151 contains a multiple-cloning site (MCS). (B) pMTL82153 contains an expression cassette for the lacZ α-subunit controlled by P_{fdx}. (C) pMTL82152-FAST contains an expression cassette for FAST controlled by P_{fdx}.

720 **Characterization of the CKL_2671 DNA Methyltransferase Activity**

721 Prior to constructing the methylation strain, we confirmed the *in vivo* activity and
722 modification specificity of CKL_2671 in *E. coli*. CKL_2671 was cloned into the
723 intermediate vector RBV-1 using HiFi Assembly. Expression of CKL_2671 in *E. coli*
724 RBV-1 was induced using 10 mM arabinose, and plasmid DNA was extracted and
725 subjected to MspI and HpaII digestion.

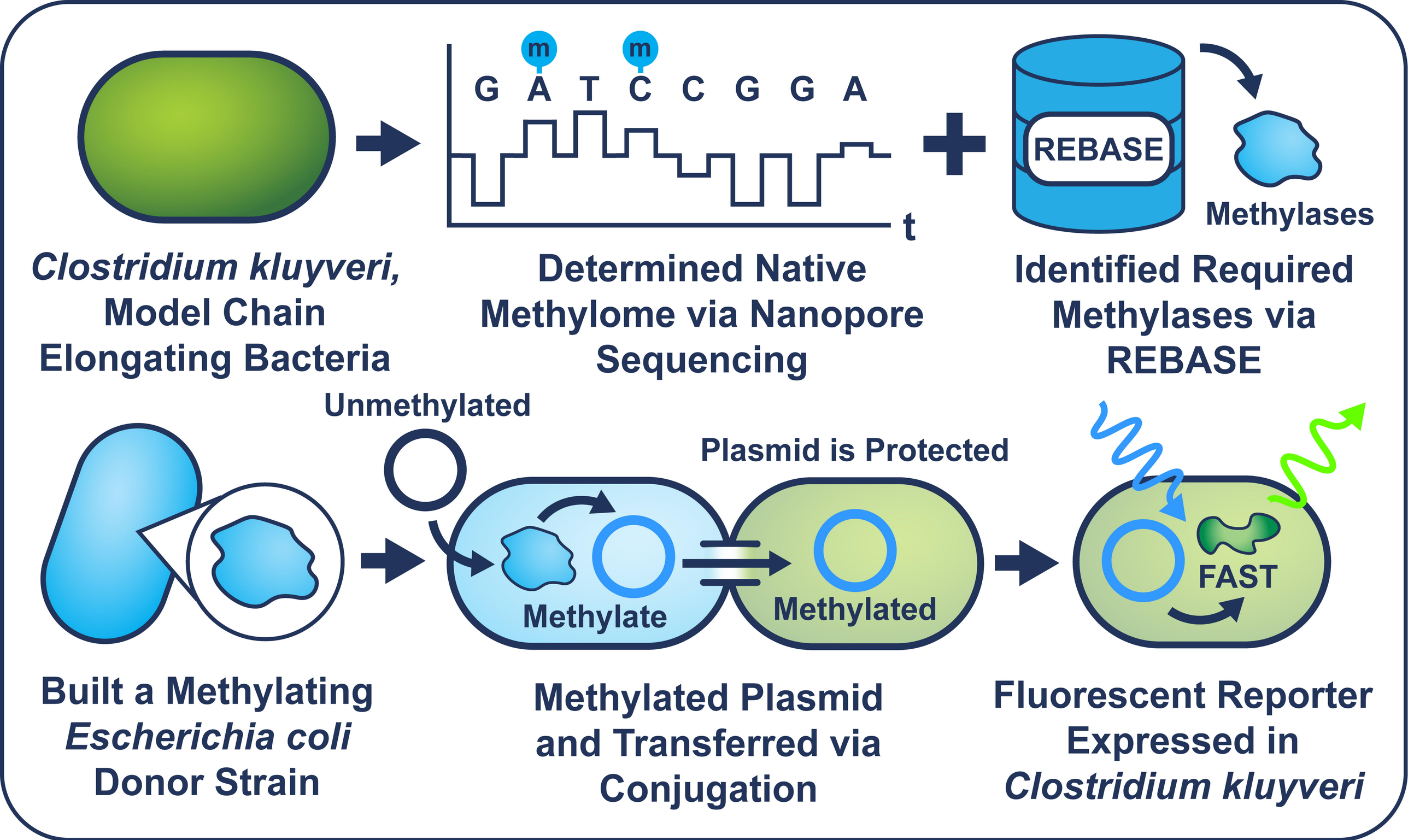
726 RBV-1-CKL_2671 was extracted and subjected to restriction digestion with MspI and
727 HpaII, which both recognize the CCGG sequence targeted by the CKL_2671,
728 methylase. These enzymes differ in their sensitivity to cytosine methylation: MspI is
729 blocked by methylation of the external cytosine (**C**CGG), while HpaII is blocked by
730 methylation of the internal cytosine (C**C**GG).

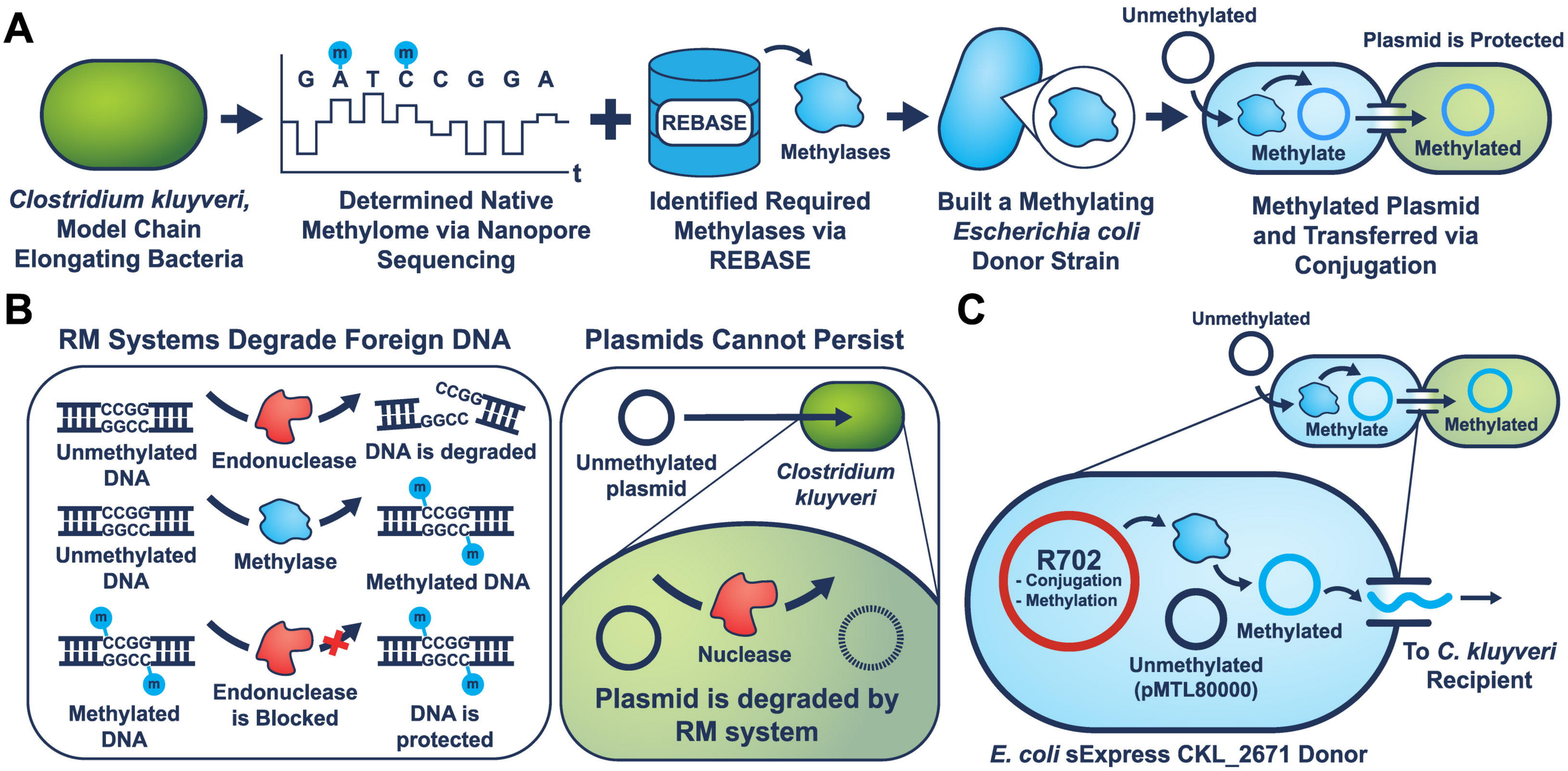
731 RBV-1-CKL_2671 was protected from cleavage by both enzymes, confirming *in vivo*
732 activity of CKL_2671 and confirming methylation at the external cytosine (**C**CGG). The
733 observed protection confirmed the role of CKL_2671 in the *C. kluyveri* methylome and
734 RM system and validated it as a viable candidate for methylation strain construction.

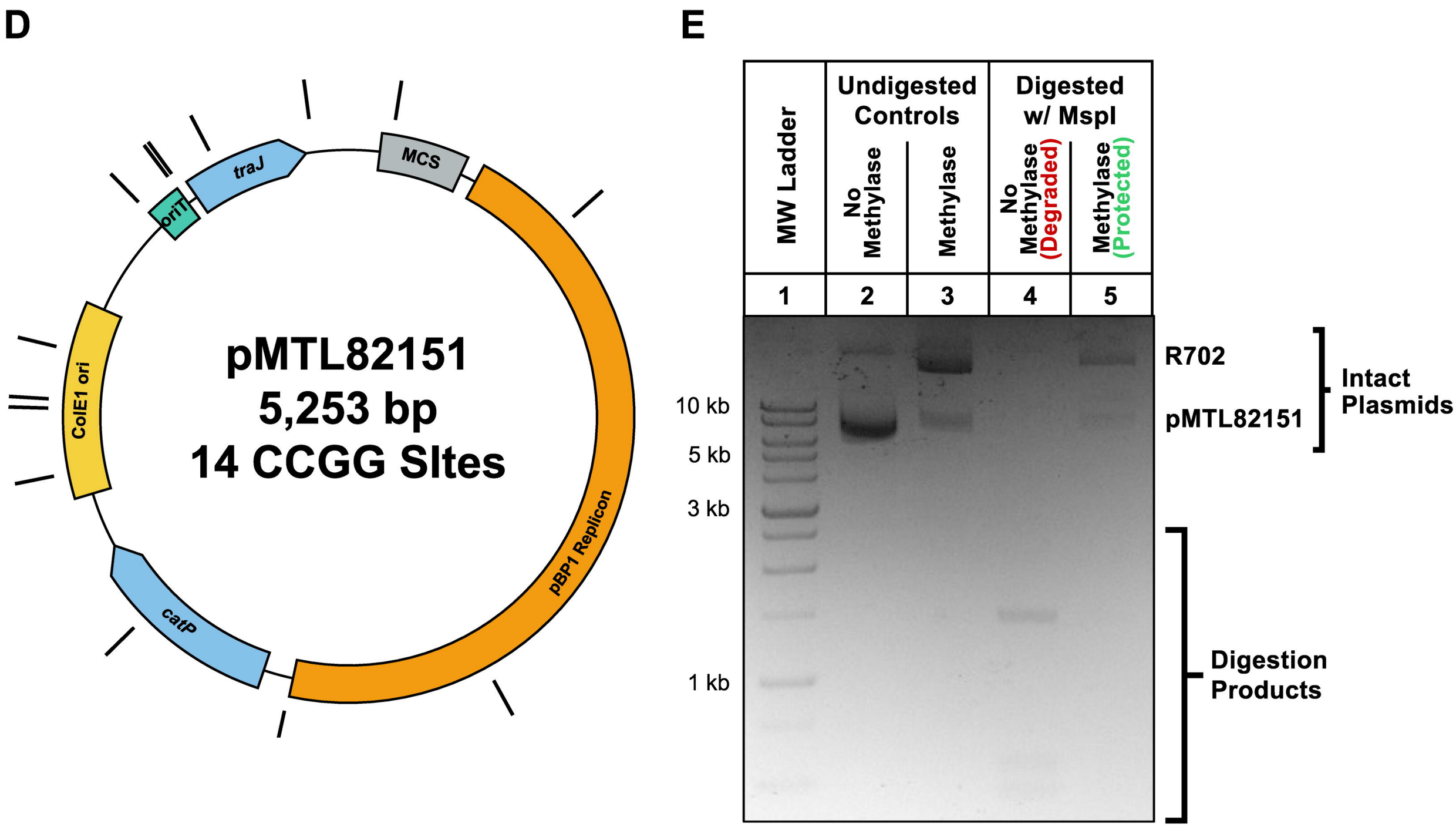


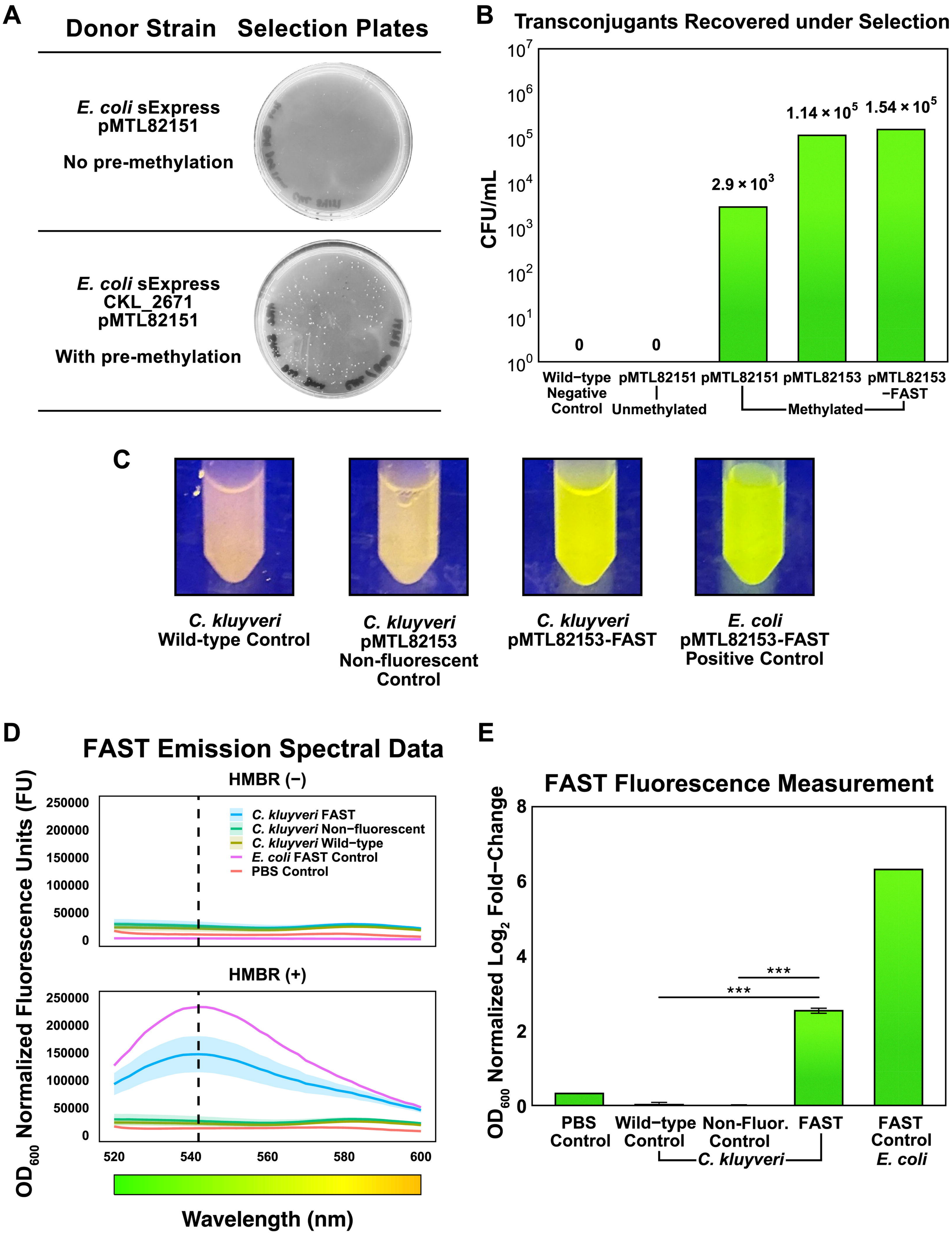
735

736 Figure S4: **3**The methylation sensitivity of the commercial restriction enzymes used in
737 the digest. MspI and HpaII both recognize the sequence CCGG, but their activity is
738 both blocked by methylation of the external cytosine. DNA gel electrophoresis image
739 of plasmid digests (1% agarose). Lanes 2 and 5 are undigested controls. Lanes 3-4
740 and 6-7 are plasmids digested with MspI and HpaII with (RBV-1-CKL_2671) or without
741 the methylase (RBV-1).


m
m
G A T C C G G A
t
REBASE
Methylases
Clostridium kluyveri, Model Chain Elongating Bacteria
Determined Native Methylome via Nanopore Sequencing
Identified Required Methylases via REBASE
Unmethylated
Plasmid is Protected
Methylate
Methylated
FAST
Built a Methylating *Escherichia coli* Donor Strain
Methylated Plasmid and Transferred via Conjugation
Fluorescent Reporter Expressed in *Clostridium kluyveri*


A
G A T C C G G A
m
m
t
REBASE
Methylases
Unmethylated
Plasmid is Protected
Methylate
Methylated
Clostridium kluyveri, Model Chain Elongating Bacteria
Determined Native Methylome via Nanopore Sequencing
Identified Required Methylases via REBASE
Built a Methylating Escherichia coli Donor Strain
Methylated Plasmid and Transferred via Conjugation
B
RM Systems Degrade Foreign DNA
Plasmids Cannot Persist
CCGG GGCC
Unmethylated DNA
Endonuclease
DNA is degraded
Methylase
Methylated DNA
Methylated DNA
Endonuclease is Blocked
DNA is protected
Unmethylated plasmid
Clostridium kluyveri
Nuclease
Plasmid is degraded by RM system
C
Unmethylated
Methylate
Methylated
R702
- Conjugation
- Methylation
Methylated
Unmethylated (pMTL80000)
To C. kluyveri Recipient
E. coli sExpress CKL_2671 Donor


D
traJ
MCS
oriT
ColE1 ori
pMTL82151
5,253 bp
14 CCGG Sites
catP
pBP1 Replicon
E
MW Ladder
Undigested Controls
No Methylase
Methylase
Digested w/ MspI
No Methylase (Degraded)
Methylase (Protected)
1
2
3
4
5
10 kb
5 kb
3 kb
1 kb
R702
pMTL82151
Intact Plasmids
Digestion Products


A
Donor Strain
Selection Plates
E. coli sExpress pMTL82151
No pre-methylation
E. coli sExpress CKL_2671 pMTL82151
With pre-methylation
B
Transconjugants Recovered under Selection
CFU/mL
0
0
2.9 × 10^3
1.14 × 10^5
1.54 × 10^5
Wild−type Negative Control
pMTL82151
pMTL82151
pMTL82153
pMTL82153 −FAST
Unmethylated
Methylated
C
C. kluyveri Wild-type Control
C. kluyveri pMTL82153 Non-fluorescent Control
C. kluyveri pMTL82153-FAST
E. coli pMTL82153-FAST Positive Control
D
FAST Emission Spectral Data
HMBR (−)
HMBR (+)
C. kluyveri FAST
C. kluyveri Non−fluorescent
C. kluyveri Wild−type
E. coli FAST Control
PBS Control
OD600 Normalized Fluorescence Units (FU)
Wavelength (nm)
E
FAST Fluorescence Measurement
OD600 Normalized Log2 Fold−Change
***
***
PBS Control
Wild−type Control
Non-Fluor. Control
FAST
FAST Control
C. kluyveri
E. coli