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Research Article

Engineering the Maize Root Microbiome: A Rapid MoClo Toolkit and Identification of Potential Bacterial Chassis for Studying Plant−Microbe Interactions

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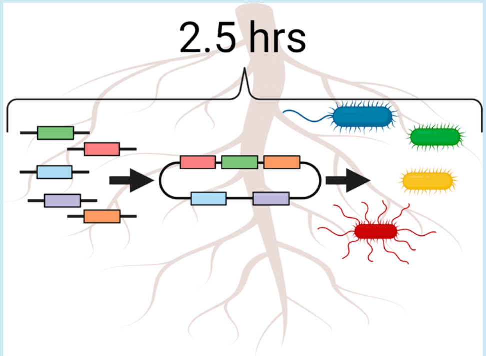

ABSTRACT: Sustainably enhancing crop production is a global necessity to meet the escalating demand for staple crops while sustainably managing their associated carbon/nitrogen inputs. Leveraging plant-associated microbiomes is a promising avenue for addressing this demand. However, studying these communities and engineering them for sustainable enhancement of crop production have remained a challenge due to limited genetic tools and methods. In this work, we detail the development of the Maize Root Microbiome ToolKit (MRMTK), a rapid Modular Cloning (MoClo) toolkit that only takes 2.5 h to generate desired constructs (5400 potential plasmids) that replicate and express heterologous genes in *Enterobacter ludwigii* strain AA4 (*Elu*), *Pseudomonas putida* strain AA7 (*Ppu*), *Herbaspirillum robiniae* strain AA6 (*Hro*), *Stenotrophomonas maltophilia* strain AA1 (*Sma*), and *Brucella pituitosa* strain AA2 (*Bpi*), which comprise a model maize root synthetic community (SynCom). In addition to these genetic tools, we describe a highly efficient transformation protocol (10^7−10^9 transformants/μg of DNA) 1 for each of these strains. Utilizing this highly efficient transformation protocol, we identified endogenous Expression Sequences (ES; promoter and ribosomal binding sites) for each strain via genomic promoter trapping. Overall, MRMTK is a scalable and adaptable platform that expands the genetic engineering toolbox while providing a standardized, high-efficiency transformation method across a diverse group of root commensals. These results unlock the ability to elucidate and engineer plant−microbe interactions promoting plant growth for each of the 5 bacterial strains in this study.

KEYWORDS: *modular cloning, synthetic community, maize, promoter trapping, electroporation, toolkit*

1. INTRODUCTION

Microbiomes have diverse impacts on plant health, including nutrient acquisition,[1−3] stress response,[1,4,5] and disease resistance.[1,6−8] Determining the mechanisms behind these plant growth-promoting phenotypes has been largely due to advancements in 'omics technologies and in high-throughput phenotypic measurements. For example, 16S amplicon sequencing has greatly expanded our understanding of which microbes associate with plant hosts and which are correlated with growth promotion, while high-throughput assays such as nutrient profiling,[2,4] functional genomics/metagenomics,[3,8] and transposon mutant libraries[5−7] have helped uncover the genetic bases of these properties. Many of these studies are facilitated by the use of simplified systems, such as synthetic communities or single microbes inoculated with their plant host,[2−4,7] which greatly reduces system complexity and allows individual interactions to be studied. However, the study of plant−microbe interactions is still limited by a lack of genetic tools and methods for most members of the plant microbiome.[9,10]

To bridge this gap, genetic toolkits have been developed with a variety of applications for a variety of microorganisms. We can broadly classify these toolkits into two groups: basic expression and advanced applications toolkits. Toolkits in the basic expression group generally provide a set of plasmids or promoter sequences that provide a range of expression levels for the selected microorganism. Toolkits in the advanced application group are diverse but enable researchers to perform more complex tasks than expression of recombinant proteins such as genome editing, genome integration, creation of gene circuits, directed evolution, etc. For model organisms (e.g., *Escherichia coli*,[11−15] *Pseudomonas putida*,[16−19] *Saccharomyces cerevisiae*,[20−23] etc.), a plethora of toolkits in the advanced application group exist that standardize and streamline more complex operations like the ones mentioned earlier. A subset of these are Modular Cloning (MoClo) toolkits that utilize the

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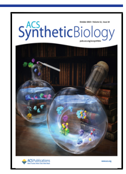

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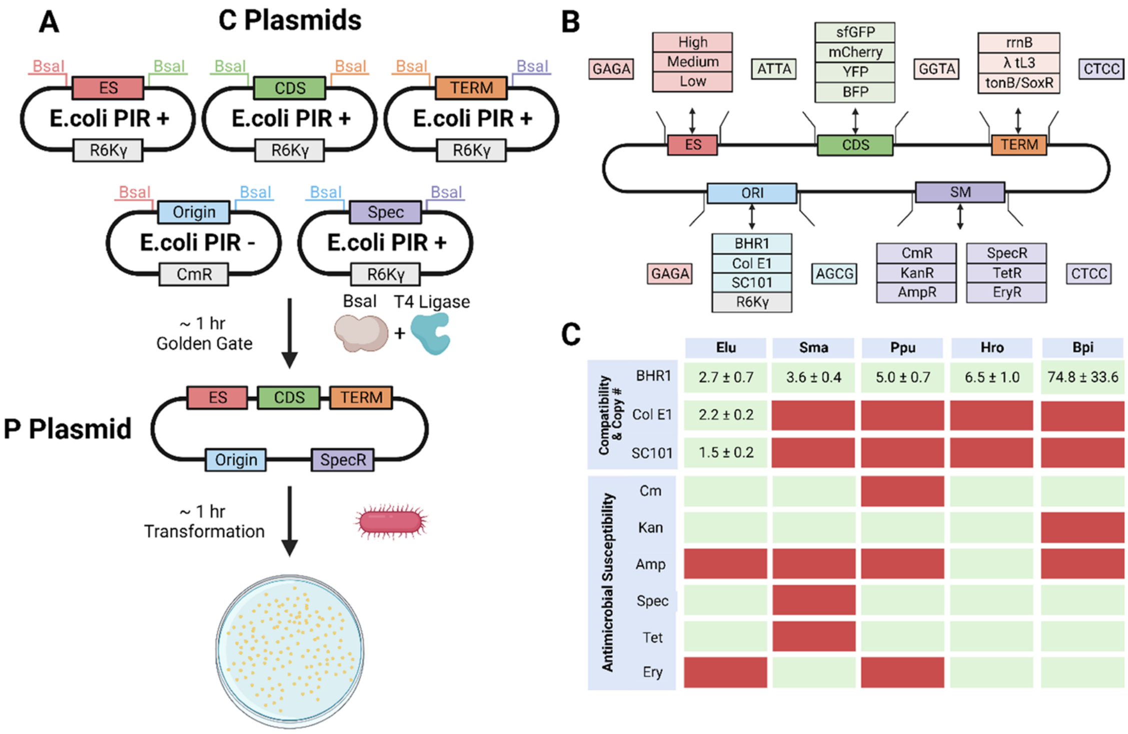

Figure 1. Maize Root Microbiome ToolKit (MRMTK): An overview of the MRMTK cloning process, parts within the toolkit, origin compatibility, origin copy number, and antimicrobial susceptibility for each strain. (A) Cloning plasmids (C plasmids) are used as starting material with BsaI restriction enzyme and NEBridge MasterMix to clone the product plasmid (P plasmid). P plasmids can then be transformed into the desired SynCom strain of interest with a time of ~2.5 h from plasmid to plate. (B) Overview of all 45 parts included in the MRMTK and their 4 bp golden gate overhang sequences. (C) Table showing the compatibility and copy number of the origins of the MRMTK for each strain as well as their antimicrobial susceptibility. Green boxes indicate that the origin is replicated in the strain and that the strain is susceptible to the antimicrobial agent. Red boxes indicate that the origin is not replicated in the strain and that the strain will grow on that antimicrobial agent.

golden gate cloning technique, such as the Yeast Toolkit (YTK),[20] EcoFlex,[11] CIDAR,[15] etc. MoClo toolkits provide standardized parts and efficient cloning strategies that make it straightforward to combinatorially assemble parts for the expression of proteins and/or the more advanced applications listed above. Genetic toolkits are also emerging for nonmodel microorganisms.[24−29] These are usually specific to a single species and define various regulatory elements (e.g., promoters, ribosomal binding sites (RBSs), and terminators) to enable a range of expression levels and can include some advanced applications like genome editing or gene circuit design. There have also been efforts to create genetic toolkits/collections that are generalizable to larger groups of organisms like Gram-negative bacteria, proteobacteria, and both prokaryotes and eukaryotes.[30−32] In the context of plant-associated microbes, the well-studied *Bacillus subtilis*[33−35] and *Pseudomonas* species[16−19] have genetic toolkits that allow for tunable and inducible expression, genome engineering and protein secretion. While genetic tools are being adapted and developed in more nonmodel, plant-associated microbes,[36,37] single microbiome toolkits and high transformation efficiencies (a prerequisite for high-throughput genetics) remain an elusive bottleneck.

To start defining genetic tools for plant microbiomes, we focused on the 7-member synthetic maize root microbiome (SynCom) developed by Niu et al.[38] This SynCom was chosen for its simplicity, its representation of the complex and diverse maize root microbiome, and its ability to model disease resistance and community assembly. Currently, there are 6 studies[7,39−43] that have utilized this SynCom, indicating that it is an emerging standard for plant microbiome research. This SynCom has enabled studies of community stability/dynamics,[39,40] microbe-dependent heterosis in maize,[41] development of protein extraction protocols for meta-proteomics in maize,[42] identification of biocontrol strains against nematodes,[7] and metabolic analysis of the SynCom strains.[43] Genetic tools are available for two SynCom species: *Enterobacter ludwigii* strain AA4 (*Elu*, formerly *E. cloacae*) and *P. putida* strain AA7 (*Ppu*) where genetic tools/methods developed in *E. coli* have been shown to be adaptable for *Enterobacter* strains[44−46] and a plethora of tools developed in other *P. putida* strains that may be adapted to *Ppu*.[16−19] The rest of the SynCom, *Stenotrophomonas maltophilia* strain AA1 (*Sma*), *Brucella pituitosa* strain AA2 (*Bpi*, formerly *Ochrobactrum pituitosum*), *Curtobacterium pusillum* strain AA3 (*CPU*), *Chryseobacterium indologenes* strain AA5 (*Cin*), and *Herbaspirillum robiniae* strain AA6 (*Hro*, formerly *Herbaspirillum frisingense*), vary in the availability of genetic tools. Transposon mutants have been generated in *Herbaspirillum*[47,48] and *Stenotrophomonas* species,[49,50] expression vectors have been constructed for *Brucella* species,[51,52] but no tools are available for *Chryseobacterium* and *Curtobacterium* species to the author's knowledge. Furthermore, transformation methods have yet to be established for *Chryseobacterium* species, but either electroporation and/or conjugation has been used to transform species in the same genera for the rest of the SynCom.[53−58]

In the work presented here, we outline a set of genetic tools and a universal, highly efficient transformation method for 5 of the SynCom members (*Elu*, *Ppu*, *Hro*, *Sma*, and *Bpi*) to act as a

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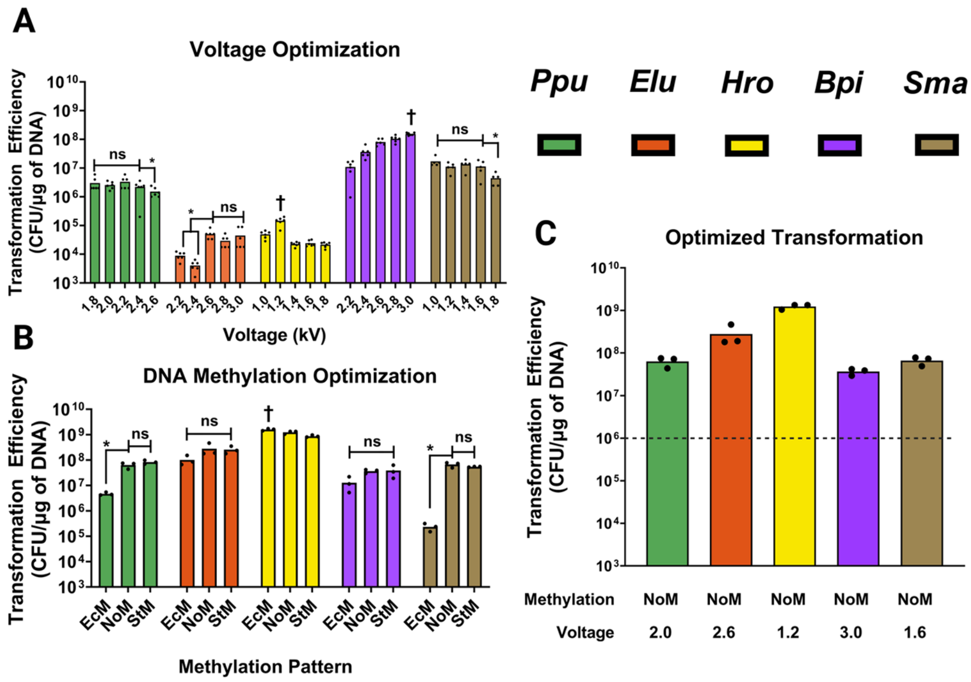

Figure 2. High-efficiency transformation of SynCom strains: (A) Electroporation voltages for each strain. Each dot represents a technical replicate (n = 5) Note: The optimal range of voltages shown for Ppu and Sma were determined from the extended range of voltages in Figures S1 and S2. (B) Methylation pattern of transformed plasmids optimized for each strain. *E. coli* methylation pattern (EcM), no methylation pattern (NoM), and strain methylation pattern (StM). Each dot represents a biological replicate (n = 3). (C) The highest-efficiency transformation protocol for each strain based on the methylation pattern of the transformed DNA and the voltage. Each dot represents a biological replicate (n = 3). † denotes the optimal voltage/methylation pattern, and * denotes significant differences between the averages. ns denotes no significant differences between the averages. For 1A and 1B, optimal conditions were determined by one-way ANOVA with multiple comparison using Tukey–Kramer and HSU's MCB in JUMP Pro17.

foundation for future high-throughput characterization and interrogation of plant–microbe colonization plus interactions in the maize root microbiome. Specifically, we developed a standardized MoClo plasmid toolkit for the strains *Elu*, *Ppu*, *Hro*, *Sma*, and *Bpi* that enables rapid cloning directly into these SynCom members in about 2.5 h. Additionally, we have developed a high-efficiency transformation method and used it as a proof of concept to identify expression sequences (ESs; specifically promoters and RBSs) via promoter-trapping screening, enabling tuning of protein expression in each of the strains.

2. RESULTS AND DISCUSSION

2.1. Maize Root Microbiome ToolKit (MRMTK). The maize root microbiome toolkit (MRMTK) is a MoClo toolkit that allows users to create their desired product plasmids (P plasmids) with the MRMTK cloning plasmids (C plasmids) and transform their P plasmid into their desired organism within 2.5 h (Figure 1A). Specifically, the MRMTK consists of 45 C plasmids (Table S1) that follow the common PCR-free format of MoClo toolkits[20,59] and that can be used to create 5400 total P plasmids. The MRMTK C plasmids contain one of 5 different part types (origin of replication, selection marker, terminator, expression sequence, or fluorescent protein). All C plasmids, annotated as Clo##, contain the R6kγ origin of replication (with the exception of Clo1-Clo3 and Clo1′-3′) and are maintained in the Invitrogen *E. coli* PIR1 cell line. The R6kγ origin of replication requires the PIR gene for replication, and since *Elu*, *Ppu*, *Hro*, *Sma*, and *Bpi* do not contain the PIR gene, plasmids containing this origin are not maintained in these strains. This allows for the direct use of the C plasmids as starting materials for cloning without the risk of them being replicated in the desired SynCom strain. Clo1-3 and Clo1′-3′ contain the Broad Host Range origin (BHR1), a temperature-sensitive origin (SC101 + RepA protein), or the ColE1 origin with the chloramphenicol (Clo1-3) or ampicillin (Clo1′-3′) selective markers. These two sets of plasmids were generated to allow users to have a selection marker for their P plasmid that is different from the origin-containing C plasmids used for cloning. Multiple origins also allow for the creation of dual-plasmid systems often seen in genome editing systems.[12,46]

All C plasmids can be cloned into a P plasmid using golden gate cloning with the restriction enzyme BsaI. While standard golden gate methods work with the MRMTK, we have found that using the NEBridge Master Mix enables a plasmid-to-plate time of 2.5 h. If users wish to utilize parts that are not provided with the kit (Figure 1B), then we suggest manual PCR with the appropriate golden gate overhangs. The golden gate overhangs for the MRMTK were selected based on the high-fidelity overhangs listed in Supplementary Table S8 in Potapov et al.[60] The selective marker compatibility, copy number, and origin compatibility were determined for each of the 5 strains (Figure 1C). We found that all origins are maintained at low copy numbers in each of the strains and that all strains are sensitive to the antibiotic combinations of spectinomycin or tetracycline combined with kanamycin, chloramphenicol, or erythromycin, making these combinations good selective markers for future microbiome experiments using the SynCom.

The MRMTK has several features that enable further expansion. Specifically, Clo2 and Clo3 contain the SC101 and ColE1 origins, respectively, that can replicate in Elu. These origins are present in an inducible CRISPR-Cas toolkit that has

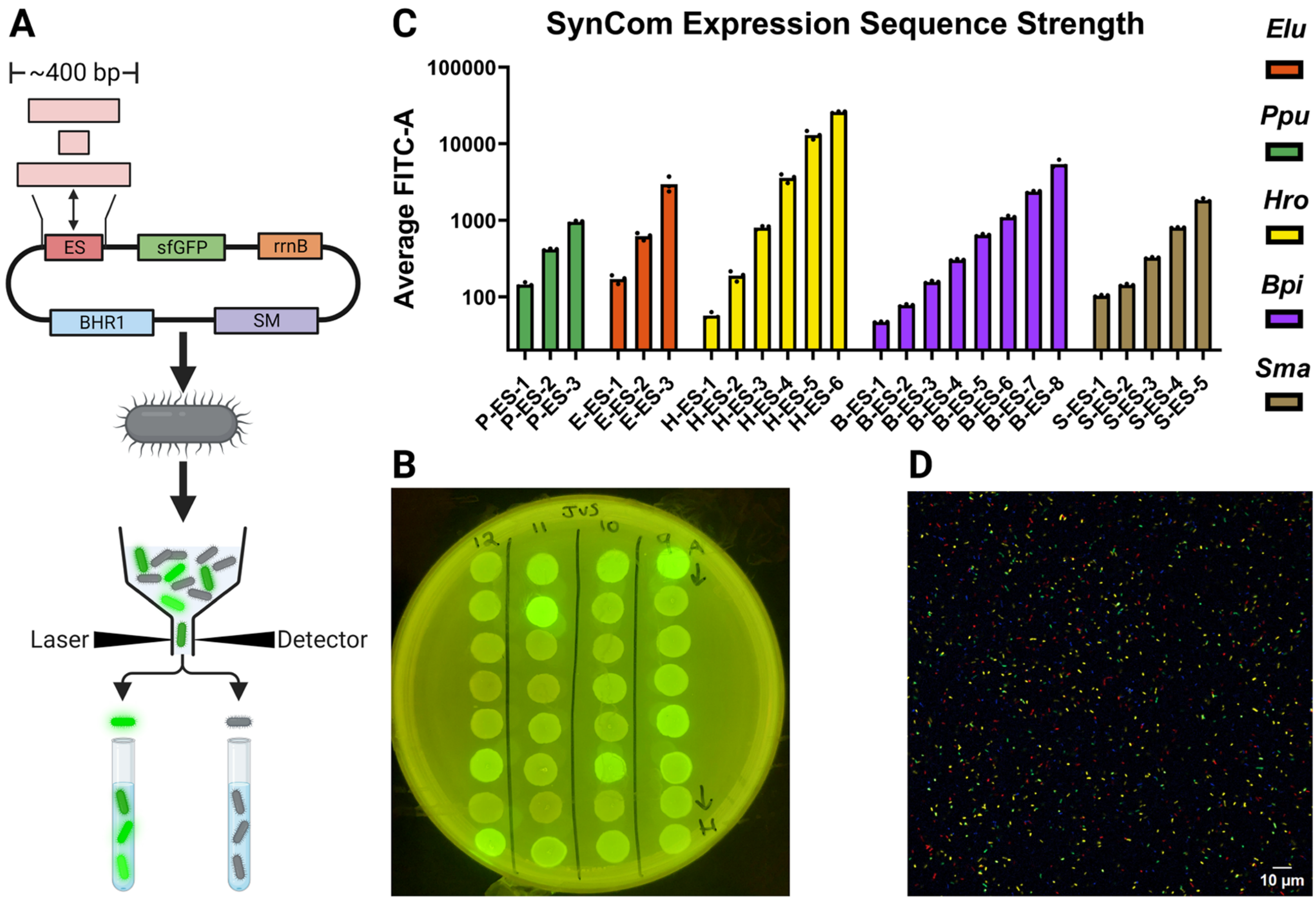

Figure 3. High-throughput expression sequence determination: (A) Overview of the promoter trapping method coupled with FACS used to screen for variable expression sequences within the 5 SynCom strains. Genomic DNA fragments from 200 to 600 bp in length were ligated into a vector containing sfGFP and transformed into the strain of interest. Transformants were then sorted for varying degrees of fluorescence by FACS. (B) Image of 24 Hro isolates after FACS sorting. (C) Average FITC-A values of sfGFP expressed with each ES in their respective strains. All ESs were run in biological triplicate in their respective strain. (D) Confocal image of Hro expressing each of the fluorescent proteins sfGFP, mCherry, BFP, and YFP.

been used for genome editing in *Enterobacter cloacae* FRM.[46] While not included in this set of vectors, MRMTK is designed to enable the implementation of these promoters and CDSs through PCR with proper golden gate overhangs. Furthermore, incorporation of other CRISPR tools such as the dual-plasmid, CRISPR-prime editing system developed by Tong et al. could also be incorporated through similar means.[12] Additionally, Clo4 contains the nonreplicative origin R6Kγ plus an origin of transfer (oriT) sequence. While the nonreplicative origin enables the rapid cloning protocol, the oriT sequence on Clo4 enables its use for conjugation and has been employed in genomic editing methods such as transposon mutant libraries.[7] These are important functionalities for plasmid systems as a deep understanding of plant−microbe interactions can come from genetic knockouts/knock-in experiments.[61,62]

2.2. Standardized Transformation Protocols for High-Efficiency Transformation. To enable more advanced experimentation, such as genome editing and high-throughput screening, high-efficiency DNA transformation is required. Transformation efficiency varies drastically between microbes and based on the transformation method employed; thus, optimization of a DNA transformation protocol is typically developed separately for each strain.[54−56] To streamline experimentation within SynCom, we aimed to develop a standardized transformation protocol that works for each strain of SynCom to enable high-throughput experimentation. Electroporation was chosen over other methods of DNA transformation as it has offered the highest transformation efficiency for the majority of SynCom relatives in prior work.[54−56,63] We first tested different electroporation voltages for each strain (Figure 2A). The strains *Bpi* and *Hro* had clear optimal voltages of 3.0 and 1.2 kV, respectively, with the rest of the strains having a larger range of voltages being optimal. Specifically, *Sma*, *Ppu*, and *Elu* had a range of voltages (1.0−1.6, 1.8−2.4, and 2.6−3.0 kV, respectively) that were significantly higher than the rest of the tested voltages (Figure 2A). Additional voltages tested in *Ppu* and *Sma* are shown in Figures S1 and S2.

We next asked whether each strain's endogenous restriction-modification (RM) systems significantly impact the transformation efficiency. Briefly, RM systems are native defense systems that protect the host from foreign DNA (identified via its methylation pattern) and have been shown to have significant effects on transformation efficiency.[64−67] The impact of DNA methylation on the transformation efficiency was tested using DNA extracted from NEB's *E. coli* DH5α strain (containing the Dam and Dcm methyltransferases, EcM), *E. coli* 135 (an *E. coli* mutant without any restriction-

Table 1. Expression Sequence Library Statistics[a]

	genome size (Mbp)	total number of transformants (CFU)	library coverage	total CFUs sorted	number of sfGFP cells sorted	colonies sequenced	repaired ES
Elu	4.80	7.30×10^4	6.08×	1.41×10^{10}	7076	20	3
Sma	4.66	5.46×10^5	46.83×	3.71×10^9	2011	17	5
Ppu	6.14	7.13×10^4	4.65×	6.13×10^7	1982	20	3
Hro	5.45	5.13×10^5	37.68×	1.17×10^8	4326	7	6
Bpi	5.47	1.47×10^6	107.55×	3.94×10^9	910	15	8

[a]Library coverage is based on the total number of transformants, the average size of the insets (400 bp), and the genome size of the strain. Colonies sequenced represent the colonies selected from the sorting plates that were sequenced.

modification systems, NoM), and the SynCom strain of interest (StM). The impact of methylation varied across each strain with a significant decrease in efficiency occurring for EcM in *Sma* and *Ppu* (Figure 2B). Interestingly, none of the transformation efficiencies were enhanced by the endogenous methylation patterns of each strain versus NoM, despite the existence of multiple RM systems in each strain (Table S4). Overall, the most efficiently transformable strain was *Hro* with an efficiency >10^9 CFU/μg of DNA. This rivals the efficiency of commercially available electrocompetent *E. coli* cells, which often have transformation efficiencies of 10^9−10^{10} CFU/μg of DNA. Furthermore, *Hro* lacks endogenous RM systems, making it a prime candidate for shuttling DNA libraries to other strains, especially since we found the transformation efficiency of *E. coli* 135 to be relatively low (<10^3 CFU/μg). Taken together, these results provide protocols to deliver DNA into SynCom at a high efficiency, which is a prerequisite for utilizing advanced genetic tools such as genome editing or variant screens.

2.3. High-Throughput Functional Screening for Endogenous Expression Sequences. Next, we leveraged the highly efficient transformation protocol to curate a library of expression sequences for each of the 5 SynCom strains. To do this, we used a promoter trapping method in which randomly generated genomic fragments from each SynCom strain were ligated upstream of sfGFP, generating a library that was directly transformed without intermediate passage through *E. coli*. The transformants were then pooled and sorted using fluorescence-activated cell sorting (FACS) (Figure 3A). Sorted cells were plated, and 96 colonies over a large range of fluorescent intensity were isolated for further processing. Isolated colonies were grown for verification through flow cytometry, plated, and sequenced (Figure 3B). Repair of the resulting sequences was often necessary, as the ligated fragments would sometimes contain part of the downstream gene they transcribed. In these instances, the sequences were "repaired" by deleting the downstream gene fragment, and the fluorescence intensities of the repaired expression sequences were remeasured using flow cytometry.

Collectively, we found 3−8 unique expression sequences (ES; 25 in total) for each of the 5 transformable strains that covered a 5- to 300-fold range of expression levels, without the need to obtain transcriptomic or proteomic data (Figure 3B). The most common downstream genes for the collection of ESs across the SynCom strains were ribosomal proteins, transporter proteins, and proteins with no known function (Table S5). The transformation protocols we identified enabled us to obtain complete coverage of each strain's genome in these screens with over 100-fold coverage of *Bpi* (Table 1). It is important to note that direct transformation of the cloning reactions into *Sma* or *Bpi* yielded very poor results, with 10 and 40% coverage of their genomes, respectively (Figure S1). To overcome this, we transformed the cloning reactions directly into *Hro* and then transformed the extracted DNA to the respective strain for sorting. This boosted the coverages of each genome over 200-fold, enabling us to screen for more expression sequences and providing more evidence that *Hro* is very useful for shuttling experiments (Figure 3C). ESs defined for *Bpi* were then utilized to clone *Bpi* with the expression plasmid for all of the fluorescent proteins available in the MRMTK (Figure 3D). Additional confocal images were taken with other SynCom strains where their ESs were utilized and cloned into their respective strain with each of the fluorescent proteins (Figure S5).

2.4. Discussion. This report presents a rapid, MoClo system and a highly efficient transformation method for 5 bacterial strains within the maize root microbiome. The MRMTK provides an array of fluorescent proteins and the ability to tune expression levels over a wide range for each strain. This modular format allows users to effortlessly integrate any sequence of interest not already within the toolkit. Utilization of the high-transformation-efficiency protocol allowed us to find a range of expression sequences for each strain, thereby demonstrating its potential for high-throughput applications such as mutant-library generation, functional metagenomics/genomic screening, genome editing, directed evolution, etc.

While our focus centered around establishing a standardized platform for engineering multiple strains in the SynCom, the potential applications of our work may extend beyond the five strains we studied. Proteobacteria, one of the most dominant bacterial phyla of the Maize root microbiome,[38,68,69] is represented well by these five strains. It is reasonable to hypothesize that our method and part toolkit would also efficiently transform and function with other soil bacteria. Many members of microbial communities have proven to be transformable by either electroporation or conjugation protocols,[50,54,55,70,71] even if the conditions require optimization for high efficiency in some members. Further optimization of other transformation variables not addressed here (final pellet optical density, time of recovery, growth media, etc.) and incorporation of conjugation would greatly aid in enhancing and potentially enabling the transformation of other members of the maize microbiome not studied here. Additionally, further expansion of the toolkit to include parts such as inducible promoters and other origins of replication would enable a more complex engineering study of microbe−plant interactions within the maize SynCom as well as other rhizosphere bacteria.

RM systems often hinder or prevent high-throughput screening within nonmodel organisms, which we observed in our methylation experiments. In this study, we demonstrated

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the ability to overcome these difficulties by shuttling DNA through *Hro*. The high efficiency of transformation into *Hro*, coupled with nondigesting RM systems (Table S4), makes this strain an ideal candidate for shuttling DNA to nonmodel bacteria. Existing functional screening studies often utilize commercially available *E. coli* to generate and shuttle DNA libraries due to its high transformation efficiency.[6,72,73] However, the total number of transformants screened may drop drastically when shuttled to a screening strain that is sensitive to *E. coli*'s methylation pattern (*Ppu* or *Sma*). For screens of plant microbiome metagenomes, *Hro* could address these concerns as it is a native member of the maize root microbiome, does not have digesting RM systems that would inhibit transformation into other strains, and has the potential to express genes not actively expressed in competent *E. coli* strains.[74] Furthermore, *Herbaspirillum* strains themselves have been shown to be important members of microbiomes. Specifically, *Herbaspirillum* strains promote plant growth and fix nitrogen for not only maize roots but other plant hosts.[75−80] Taken together, *Hro* has great potential for engineering the microbiome and acting as a potential chassis for understanding plant−microbe interactions.

The ESs defined in this study showed stable expression under the culture conditions that we employed. While many of the ESs are upstream of housekeeping genes (ribosomal proteins), there are a significant number that are not, and their strengths may be context-dependent. For example, the ES with the highest fluorescence intensity we observed was H-ES6 from *Hro*'s genome. This ES lies 1.3 kb upstream of a predicted gene of unknown function. Furthermore, this gene was located in a 35.5 kb cluster of genes with unknown functions in *Hro*'s genome. While this result is intriguing, this indicates that the activity of all ESs should be further validated under a variety of culture conditions and, perhaps more importantly, in planta. Additionally, ES variation in each strain could be further expanded through ribosomal binding site (RBS) libraries in each strain using tools such as the RBS Calculator DNA from the Salis lab. Since RBS libraries have been vital for understanding translation and enabling variable expression in bacterial strains, leveraging them would greatly enable genetic engineering and aid in understanding the factors involved in expression within each of the SynCom strains.[81,82]

Collectively, we see the MRMTK as greatly facilitating engineering of the maize microbiome and experimentally querying microbe−microbe and plant−microbe interactions. This toolkit expands the taxonomic range of microbe engineering within the plant−microbiome space, where tools and methods are currently sparse, and provides a basis for incorporation of advanced genetic engineering and control methods in the plant−microbiome space.

3. EXPERIMENTAL SECTION

3.1. Bacterial Strains, Media, and Growth Conditions. The SynCom strains used in this study were *S. maltophilia* strain AA1 (Sma), *B. pituitosa* strain AA2 (Bpi), *E. ludwigii* strain AA4 (Elu), *Herbaspirillum robineae* strain AA6 (Hro), and *P. putida* strain AA7 (Ppu), all of which were obtained as a generous gift from Dr. Manuel Kleiner. *E. coli* strains used were DH5α (NEB cat C2987H), *E. coli* PIR1 (Invitrogen cat C101010), and *E. coli* 135 (kind gift of Dr. Chase Beisel). All *E. coli* strains were grown in lysogeny broth medium (LB) from BD Scientific (BD 240210) and incubated at 37 °C and 250 rpm. Antibiotic-containing media were made with the following concentrations: chloramphenicol (*Cm*; 34 μg/mL), kanamycin (Kan; 50 μg/mL), ampicillin (Amp; 100 μg/mL), spectinomycin (Spec; 150 μg/mL), tetracycline (Tet; 10 μg/mL), or erythromycin (Ery; 250 μg/mL). All SynCom strains were grown in tryptic soy broth without dextrose (TSB; BD 286220) and incubated at 30 °C and 250 rpm. All plates were made according to manufacturer recommendations with 1.5% agar. The SynCom strains *Elu*, *Sma*, and *Ppu* grown on TSB plates were incubated overnight at 30 °C, while *Bpi* and *Hro* were incubated for 2 days at 30 °C. All SynCom strains were plated from −80 °C stocks. Transformation recovery media (SOC Media) was made by autoclaving 28 g of SOB Broth powder in 1 L of dH_2O and, once cooled, 20 mL of sterilized 1 M $MgSO_4$ and 20 mL of sterilized 1 M glucose were added.

3.2. Plasmids and Primers. All primers and plasmids used for the construction of the C plasmids, transformation optimization experiments, and expression sequence experiments are listed in Tables S2 and S3, respectively. C plasmids can be found in Table S1. All C plasmids can be found on Addgene.

3.3. Antibiotic Susceptibility Assays. Full-strength TSB agar plates with corresponding antibiotics were used to determine the susceptibility of each strain. Specifically, each strain was cultured overnight in TSB, struck out onto each antibiotic plate, and incubated at 30 °C for 48−72 h. Any colonies appearing on the plates after the incubation time were considered as not susceptible toward that antibiotic.

3.4. Molecular Cloning. Colony PCR reactions for sequence verification were performed with NEB OneTaq HotStart 2x MasterMix (M0484S) and PCR reactions for molecular cloning were performed with a Q5 HotStart 2x MasterMix (M0494S). All PCR reactions were performed according to the manufacturer's protocol. All plasmids were verified using a combination of whole-plasmid sequencing from Plasmidsaurus and Sanger sequencing from Genewiz (Azenta Life Sciences). Molecular cloning reactions were performed with a NEB Q5 mutagenesis kit (E0552S), NEB HiFi Master Mix (E2621S), or NEBridge MasterMix (M1100S) according to the manufacturer's protocol for each kit/master mix. Molecular cloning for creating the toolkit C plasmids was performed with the NEB HiFi master mix. Molecular cloning utilizing the C plasmids was performed with NEBridge Mastermix and equimolar amounts of each C plasmid. For most of the cloning plasmids maintained in Invitrogen's *E. coli* PIR1 cells, plasmid dimers formed and were accounted for when equal molar amounts of C plasmids were added during cloning. The length of each C plasmid is included in Table S1. The plasmids that were used to clone the plasmids created in this study can be found in Table S3.[20,57,83−86]

The empty vectors used for optimizing the transformation protocol were cloned using the NEB Q5 mutagenesis kit and Gibson cloning using the NEB HiFi master mix starting from the plasmid pBBR122. Annotation of pBBR122 using plannotate[87] revealed that the Kan gene split pBBR122's origin of replication into two parts (Figure S4). In order to generate the empty vectors used during transformation (only the origin of replication and a single selection marker), a series of 3 cloning reactions were conducted. The first reaction removed the MobA gene sequence to generate pBBR122-ΔMobA. The second cloning reaction inserted the rrnB and T7Te terminators from pBbB1c-GFP between the origin of replication and the *Cm* resistance gene to generate pBBR122-

DT-ΔMobA. The third cloning reaction removed the KanR gene to generate pBBR122-CmR. A set of cloning reactions (reaction 4 in Figure S4) swapped out the CmR gene via gibson cloning with the antibiotic resistance genes for Kan, Amp, Spec, Tet, and Ery. A total of eight plasmids were generated from pBBR122 with pBBR122-CmR and pBBR122-KanR used for optimizing the transformation protocol.

In order to screen the genome of each strain for functional ESs, the base vectors pBBR122- CmR-sfGFP and pBBR122-KanR-sfGFP were created from pBBR122-CmR, pBBR122-KanR, and pYTK0047 using Gibson assembly using NEB HiFi Master Mix. Specifically, the promoter sequence, ribosome binding site, and sfGFP coding sequence from pYTK047 were cloned in between the T7Te and rrnB terminators in pBBR122-CmR and pBBR122-KanR using Gibson assembly according to the manufacturer's protocol.

Two sets of plasmids were created for the plasmid copy number assay. The first set of plasmids that were generated (pBBR122-CmR-sfGFP', pBBR122-KanR-sfGFP', pSC101-CmR-sfGFP', and pColE1-CmR-sfGFP') were transformed into each strain and cloned with 16 Gibson assembly using NEB HiFi Master Mix. The exception was pBBR122-CmR-sfGFP' as it was generated while screening for ESs in Elu. The plasmid pBBR122-KanR-sfGFP' replaced the promoter and ribosome binding site of pBBR122-KanR-sfGFP with the Elu ES3 from pBBR122-CmR-sfGFP'. The plasmids pSC101-CmR-sfGFP' and pColE1-CmR-sfGFP' replaced the BHR1 origin with the SC101 and ColE1 origins from Clo3 and Clo2, respectively. The second set of reference plasmids were generated (pZL266-Elu-DAHP-sfGFP, pZL296- Hro-rpoS-sfGFP, pZL300-Bpi-rpoN-sfGFP, pZL293-Sma-rpoD-sfGFP, pZL299-Ppu-rpoDsfGFP) to create a standard curve for qPCR. This second set used pBBR122-KanR-sfGFP as a backbone and contained the qPCR reference genes (Table S6) via gibson cloning using NEB HiFi MasterMix.

Cloning for confocal imaging at North Carolina State University's Cellular and Molecular Imaging Facility (CMIF) was done following the toolkit format. The C plasmids Clo1, Clo5, Clo8, Clo13, Clo16, Clo 19, Clo25, Clo33, Clo38, and Clo39−42 were mixed at a concentration of 50 μM and cloned with BsaI-HF-V2 restriction enzyme and NEBridge Master Mix according to the manufacturer's protocol. Reactions for each construct were purified using a Zymo Clean and Concentrator-5 kit (D4004) according to the manufacturer's protocol. Purified DNA was then transformed directly into Bpi according to the optimized electroporation protocol below.

3.5. Optimized Electroporation Protocol. Single colonies of each strain were grown in 3 mL of TSB media overnight (12−18 h) and sub-inoculated at a 1:40 ratio into 25 mL of TSB media the next day. 1 mL of each overnight culture was aliquoted for genomic DNA extraction using Zymo's Quick-DNA Fungal/Bacterial Miniprep Kit (D6005). Extracted genomic DNA was used for 16S PCR with standard 27 forward and 1492 reverse primers to verify the purity of the culture to be transformed. PCR conditions followed the standard protocol outlined for NEB OneTaq HotStart 2x Master Mix with an annealing temperature of 49 °C, a cycle extension time of 1 min 30 s, and a final extension time of 2 min. Cultures were harvested at an OD_{600} of 0.4−0.6 by centrifugation at 5000*g* for 5 min at 4 °C. The supernatant of harvested cells was decanted, and the pellet was resuspended gently with a pipet using 25 mL of ice-cold 10% glycerol. The cells were washed by this process three times. The cells were finally resuspended with 250 μL of ice-cold 10% glycerol and normalized to an OD_{600} of 25. 50 μL of the normalized cell suspensions was electroporated with 2 μL of plasmid DNA (up to a maximum of 5 μL) at the respective strain's optimal voltage in 1 mm gap electroporation cuvettes (USA Scientific, Inc. 91041050) with a Biorad MicroPulser electroporator. Electroporated samples were recovered at 30 °C and 250 rpm for 1 h in 950 μL of SOC media. After recovery, serial dilutions of cell recovery solutions were made in a 96-well plate with total volumes of 250 μL. 10 μL of each serial dilution was then spot-plated onto a single TSB plate containing either *Cm* or Kan. Once dried, plates were then incubated overnight or for 2 days depending on the strain being tested. While this protocol was being optimized, the plasmids pBBR122-CmR and pBBR122-KanR were used for 100 ng. Furthermore, while the electroporation voltages were optimized, the OD of the final cell suspension was consistent across replicates in each strain but was not normalized to an OD of 25 for each strain.

3.6. Plasmid Copy Number Assay. Plasmid copy number was quantified by using quantitative PCR (qPCR). Each strain was transformed with either pBBR122-CmR-sfGFP′ or pBBR122-KanR-sfGFP′. Elu was additionally transformed with pSC101-CmR-sfGFP′ and pColE1-CmR-sfGFP′. Three individual colonies for each strain were inoculated into liquid TSB from a freshly streaked TSB plate with the appropriate antibiotic and grown at 30 °C overnight. Cultures were sub-inoculated to an initial OD_{600} = 0.01 in 25 mL of TSB with *Cm* or Kan and grown to late log phase for genomic DNA extraction. Ppu was the only strain transformed with pBBR122-KanR-sfGFP' and grown in TSB with Kan. Total DNA was isolated from 1 mL of late log phase cell cultures using the Zymo Quick-DNA Miniprep Kit (D3025) according to the manufacturer's protocol. The reference plasmids, containing sfGFP and each strain's reference gene, were grown overnight in LB media and extracted using a Zyppy Plasmid Miniprep Kit (D4020).

Since the sfGFP gene is present in all plasmids, a section of the gene was used to determine the plasmid copy number. For generating the standard curve, a single copy gene in each strain's genome was selected as the reference gene (Table S6). Primers for each strain's reference gene were designed using Primer-Blast with default settings for annealing temperatures of 60 °C (Table S2). Serial dilutions of the plasmids used for standard curve generation were made with concentrations ranging from 10^4 to 10^9 copies/μL.

Quantitative PCR was performed on a Biorad FX384 Touch Real-Time PCR Detection System. A qPCR mixture of 6 μL was prepared using SsoAdvanced Universal SYBR Green Supermix (Cat #1725270): 0.7 μL of PCR grade water, 0.15 μL of each primer (final concentration 0.375 μM), 3 μL of Supermix, and 2 μL of template DNA. All analyses of the qPCR curves were performed using Microsoft Excel.

3.7. High-Throughput Expression Sequence Determination. Genomic DNA (gDNA) for each strain was extracted and verified using 16S PCR as described above. 90 μL of gDNA for each strain was then sonicated with a Covaris S220 ultrasonicator at a duty factor of 5 and 200 cycles per burst for 80 s. Fragmented gDNA was extracted at a size range of 200−600 bp (average of 400 bp) from a 1% agarose gel run at 125 V for 20 min using Zymo's gel extraction kit (D4001) according to the manufacturer's protocol. The gDNA fragments were then end-repaired using the LGC Biosearch Technologies End-It DNA End-Repair Kit (ER81050) and subsequently purified using a Zymo Clean and Concentrator-5 kit (D4004) according to the manufacturer's instructions. The ES library vectors were prepared by inverse-PCR from the starting plasmids pBBR122-CmR-sfGFP or pBBR122-KanR-sfGFP. 1 μL DpnI (R0176L) was added to the PCR reaction mixture and incubated at 37 °C for 3 h. After digestion, the backbone was purified using a Zymo Clean and Concentrator-5 kit.

Ligation of the MRMTK vector and gDNA fragments was performed using the LGC Biosearch Technologies Fast-Link DNA Ligation Kit (LK6201H) overnight at room temperature. The overnight ligation reaction was deactivated at 70 °C for 10 min and cleaned with Zymo Clean and Concentrator-5 kit. The ligation reaction was eluted twice with 6 μL of nuclease-free water preheated to 55 °C. 5 μL of ligation reactions was transformed into their respective strains at their optimized voltages. 25 μL of the recovery solution was used to make serial dilutions to count the number of total transformants, and the remaining volume was inoculated into 50 mL of TSB with either *Cm* or Kan and incubated overnight at 26 °C. The total number of transformants for each library was then used to calculate the library coverage of each strain's genome using the following formula:

$$\text{library coverage} = \frac{\text{number of total transformants} \times \text{average insert size (400 bp)}}{\text{genome size of the strain}}$$

The next day, any libraries in the stationary phase were sub-inoculated at a ratio of 40:1 in 10 mL of TSB with antibiotics and incubated at 30 °C for 2 h to reach log phase. Cells that were ready for sorting were pooled and harvested by centrifugation at 5000*g* for 5 min. The harvested ES libraries were then resuspended in 1× PBS and sorted.

Harvested ES libraries in 1× PBS were sorted for green fluorescence using a BD FACSmelody. Sorting gates, voltages, and thresholds were determined using the WT strain and strains expressing GFP as a positive control for each sorting experiment. Specifically, a threshold for FITC-A was set for each strain just above the WT strain's signal such that nonfluorescent cells were not sorted. Plasmid pBBR122-CmR-sfGFP was used as a positive control for sorting Elu cells, pBBR122-KanR-sfGFP' was used as a positive control for sorting Ppu cells, and pBBR122-CmR-sfGFP' was used as a positive control for sorting Hro, Bpi, and Sma cells. After sorting the libraries through the BD FACSmelody, the sorted cells were plated across multiple TSB agar plates with either *Cm* or Kan (Table 1). Up to 96 fluorescent colonies were selected visually with a UV flashlight for additional screening and sequencing. Selected colonies were incubated in 2 mL of TSB+*Cm* overnight at 30 °C and at 250 rpm. The next morning, the cells were sub-inoculated into 2 mL of fresh media at a 1:40 volume ratio and grown for 2−3 h in the same conditions. The fluorescence of each culture was determined using a BD Accuri C6 Plus Flow Cytometer. A subset of these colonies with the largest range of fluorescence and without overlapping fluorescent strength was then sent for Sanger sequencing.

Plasmid DNA was extracted from cultures using Zymo's Zyppy miniprep kit (D4020). DNA extraction followed the manufacturer's protocol, with the exception of adding 240 μL of the 7× Lysis buffer and 450 μL of neutralization buffer. The promoter region of the plasmid was amplified using NEB Hot Start 2x Master Mix and sequenced via Sanger sequencing. Based on the Sanger sequencing results, ESs containing part of the downstream gene were repaired via the NEB Q5 mutagenesis kit according to the manufacturer's instructions. The resulting repaired ESs were then revalidated in biological triplicate in the same fashion as the initial selection of sorted sfGFP cells.

3.8. Cell Preparation and Confocal Imaging. WT and fluorescent protein expressing Bpi, Sma, Elu, Ppu & Hro and Bpi engineered to express each fluorescent protein were cultured in TSB and the appropriate antibiotic to an OD_{600} between 0.1 and 0.2. 5 μL of each strain culture was combined in a single microcentrifuge tube and mixed. 5 μL of this homogeneous mixture was placed on a glass microscope slide and covered with a glass coverslip for imaging. The slide was imaged on the Zeiss Axio Observer.Z1/7 confocal microscope at 40× magnification (Figure 3D). A closeup image of the fluorescent strains (Figure 3E) was taken via 4× scan zoom using Zeiss Zen Microscopy software. The excitation/emission spectra for each fluorescent protein were as follows: sfGFP (488/509); mCherry (587/610); BFP (384/448); YFP (508/524).

3.9. Statistical Analyses. All statistical analyses were performed using JMP Pro 17. One-way ANOVA, Tukey−Kramer, and HSU's MCB were used to determine the best voltage and methylation pattern for each strain's transformation protocol. Voltage optimization was performed with technical replicates of $n = 5$ or 6 where each replicate was an independent transformation from the same cell culture. The rest of the experiments were performed with biological replicates of $n = 3$ where each replicate was a culture isolated from a single colony.

■ ASSOCIATED CONTENT

Data Availability Statement

The Maize Root Toolkit can be found on Addgene.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.3c00371.

MRMTK plasmids, plasmids used, primers used, SynCom RM systems, ES information, and qPCR reference gene information (Tables S1−S6) (XLSX)

Extended voltage optimization for *Ppu*, *Sma*, ES library sizes, deconstruction of pBBR122, and confocal images of fluorescent SynCom members (Figures S1−S5) (PDF)

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Author Contributions

J.v.S. and J.C. completed the molecular cloning of the cloning plasmids for the MRMTK. J.v.S. designed and completed all plasmid compatibility and antibiotic susceptibility experiments. J.v.S. and Z.L. designed the designed the plasmid copy number experiments. Z.L. completed the cloning and plasmid copy number experiments. N.C. and J.v.S. designed the experiments for transformation optimization and ES library generation and identification. J.v.S. completed all experimentation related to transformation optimization and ES library generation and identification. J.C., J.v.S., and N.C. designed the experiments for imaging fluorescent protein-expressing strains. J.C. and J.v.S. completed the experiments for imaging fluorescent protein-expressing strains. J.v.S. completed the statistical analysis and created the figures. J.v.S., Z.L., J.C., and N.C. all contributed to writing the manuscript.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Trivedi, P.; Leach, J. E.; Tringe, S. G.; Sa, T.; Singh, B. K. Plant—microbiome interactions: from community assembly to plant health. *Nat. Rev. Microbiol.* **2020**, *18*, 607−621.

(2) Zhang, J.; Liu, Y.-X.; Zhang, N.; Hu, B.; Jin, T.; Xu, H.; Qin, Y.; Yan, P.; Zhang, X.; Guo, X.; et al. NRT1. 1B is associated with root microbiota composition and nitrogen use in field-grown rice. *Nat. Biotechnol.* **2019**, *37*, 676−684.

(3) Finkel, O. M.; Salas-Gonz′alez, I.; Castrillo, G.; Conway, J. M.; Law, T. F.; Teixeira, P. J. P. L.; Wilson, E. D.; Fitzpatrick, C. R.; Jones, C. D.; Dangl, J. L. A single bacterial genus maintains root growth in a complex microbiome. *Nature* **2020**, *587*, 103−108.

(4) Eida, A. A.; Ziegler, M.; Lafi, F. F.; Michell, C. T.; Voolstra, C. R.; Hirt, H.; Saad, M. M. Desert plant bacteria reveal host influence and beneficial plant growth properties. *PLoS One* **2018**, *13*, No. e0208223.

(5) Thorgersen, M. P.; Xue, J.; Majumder, E. L.; Trotter, V. V.; Ge, X.; Poole, F. L.; Owens, T. K.; Lui, L. M.; Nielsen, T. N.; Arkin, A. P.; et al. Deciphering microbial metal toxicity responses via random bar code transposon site sequencing and activity-based metabolomics. *Appl. Environ. Microbiol.* **2021**, *87*, No. e01037-21, DOI: 10.1128/AEM.01037-21.

(6) Mendes, R.; Kruijt, M.; de Bruijn, I.; De Bruijn, I.; Dekkers, E.; van der Voort, M.; Van Der Voort, M.; Schneider, J. H.; Piceno, Y. M.; DeSantis, T. Z.; Andersen, G. L.; Bakker, P. A. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* **2011**, *332*, 1097−1100.

(7) Zhao, Y.; Yuan, Z.; Wang, S.; Wang, H.; Chao, Y.; Sederoff, R. R.; Sederoff, H.; Yan, H.; Pan, J.; Peng, M.; et al. Gene sdaB Is Involved in the Nematocidal Activity of *Enterobacter ludwigii AA4* Against the Pine Wood Nematode *Bursaphelenchus xylophilus*. *Front. Microbiol.* **2022**, *13*, No. 870519, DOI: 10.3389/fmicb.2022.870519.

(8) Hjort, K.; Presti, I.; Elväng, A.; Marinelli, F.; Sjoling, S. Bacterial Chitinase with phytopathogen control capacity from suppressive soil revealed by functional metagenomics. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 2819−2828.

(9) Martínez-García, E.; de Lorenzo, V. Molecular tools and emerging strategies for deep genetic/genomic refactoring of *Pseudomonas*. *Curr. Opin. Biotechnol.* **2017**, *47*, 120−132.

(10) Yan, Q.; Fong, S. S. Challenges and advances for genetic engineering of non-model bacteria and uses in consolidated bioprocessing. *Front. Microbiol.* **2017**, *8*, 2060.

(11) Moore, S. J.; Lai, H.-E.; Kelwick, R. J.; Chee, S. M.; Bell, D. J.; Polizzi, K. M.; Freemont, P. S. EcoFlex: a multifunctional MoClo kit for *E. coli* synthetic biology. *ACS Synth. Biol.* **2016**, *5*, 1059−1069.

(12) Tong, Y.; Jørgensen, T. S.; Whitford, C. M.; Weber, T.; Lee, S. Y. A versatile genetic engineering toolkit for *E. coli* based on CRISPR-prime editing. *Nat. Commun.* **2021**, *12*, No. 5206.

(13) Yang, S.; Liu, Q.; Zhang, Y.; Du, G.; Chen, J.; Kang, Z. Construction and characterization of broad-spectrum promoters for synthetic biology. *ACS Synth. Biol.* **2018**, *7*, 287−291.

(14) Shin, J.; Noireaux, V. An *E. coli* cell-free expression toolbox: application to synthetic gene circuits and artificial cells. *ACS Synth. Biol.* **2012**, *1*, 29−41.

(15) Iverson, S. V.; Haddock, T. L.; Beal, J.; Densmore, D. M. CIDAR MoClo: improved MoClo assembly standard and new *E. coli* part library enable rapid combinatorial design for synthetic and traditional biology. *ACS Synth. Biol.* **2016**, *5*, 99−103.

(16) Calero, P.; Jensen, S. I.; Nielsen, A. T. Broad-host-range ProUSER vectors enable fast characterization of inducible promoters and optimization of p-coumaric acid production in *Pseudomonas putida KT2440*. *ACS Synth. Biol.* **2016**, *5*, 741−753.

(17) Sathesh-Prabu, C.; Tiwari, R.; Kim, D.; Lee, S. K. Inducible and tunable gene expression systems for *Pseudomonas putida KT2440*. *Sci. Rep.* **2021**, *11*, No. 18079.

(18) Cook, T. B.; Rand, J. M.; Nurani, W.; Courtney, D. K.; Liu, S. A.; Pfleger, B. F. Genetic tools for reliable gene expression and recombineering in *Pseudomonas putida*. *J. Ind. Microbiol. Biotechnol.* **2018**, *45*, 517−527.

(19) Wirth, N. T.; Kozaeva, E.; Nikel, P. I. Accelerated genome engineering of *Pseudomonas putida* by I-SceI—mediated recombination and CRISPR-Cas9 counterselection. *Microb. Biotechnol.* **2020**, *13*, 233−249.

(20) Lee, M. E.; DeLoache, W. C.; Cervantes, B.; Dueber, J. E. A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synth. Biol.* **2015**, *4*, 975−986.

(21) An-adirekkun, J. M.; Stewart, C. J.; Geller, S. H.; Patel, M. T.; Melendez, J.; Oakes, B. L.; Noyes, M. B.; McClean, M. N. A yeast optogenetic toolkit (yOTK) for gene expression control in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **2020**, *117*, 886−893.

(22) Malcı, K.; Watts, E.; Roberts, T. M.; Auxillos, J. Y.; Nowrouzi, B.; Boll, H. O.; Nascimento, C. Z. S.; Andreou, A.; Vegh, P.; Donovan, S.; et al. Standardization of Synthetic Biology Tools and Assembly Methods for *Saccharomyces cerevisiae* and Emerging Yeast Species. *ACS Synth. Biol.* **2022**, *11*, 2527−2547.

(23) Simakin, P.; Koch, C.; Herrmann, J. M. A modular cloning (MoClo) toolkit for reliable intracellular protein targeting in the yeast *Saccharomyces cerevisiae*. *Microb. Cell* **2023**, *10*, 78.

(24) DeLorenzo, D. M.; Rottinghaus, A. G.; Henson, W. R.; Moon, T. S. Molecular toolkit for gene expression control and genome modification in *Rhodococcus opacus PD630*. *ACS Synth. Biol.* **2018**, *7*, 727−738.

(25) Xia, P.-F.; Ling, H.; Foo, J. L.; Chang, M. W. Synthetic biology toolkits for metabolic engineering of *cyanobacteria*. *Biotechnol. J.* **2019**, *14*, No. 1800496.

(26) Mózsik, L.; Pohl, C.; Meyer, V.; Bovenberg, R. A.; Nygård, Y.; Driessen, A. J. Modular synthetic biology toolkit for filamentous fungi. *ACS Synth. Biol.* **2021**, *10*, 2850−2861.

(27) Larroude, M.; Park, Y.-K.; Soudier, P.; Kubiak, M.; Nicaud, J.-M.; Rossignol, T. A modular Golden Gate toolkit for *Yarrowia lipolytica* synthetic biology. *Microb. Biotechnol.* **2019**, *12*, 1249−1259.

(28) Crozet, P.; Navarro, F. J.; Willmund, F.; Mehrshahi, P.; Bakowski, K.; Lauersen, K. J.; P′erez-P′erez, M.-E.; Auroy, P.; Gorchs Rovira, A.; Sauret-Gueto, S.; et al. Birth of a photosynthetic chassis: a MoClo toolkit enabling synthetic biology in the microalga *Chlamydomonas reinhardtii*. *ACS Synth. Biol.* **2018**, *7*, 2074−2086.

(29) Chai, M.; Deng, C.; Chen, Q.; Lu, W.; Liu, Y.; Li, J.; Du, G.; Lv, X.; Liu, L. Synthetic biology toolkits and metabolic engineering applied in *Corynebacterium glutamicum* for biomanufacturing. *ACS Synth. Biol.* **2021**, *10*, 3237−3250.

(30) Schuster, L. A.; Reisch, C. R. A plasmid toolbox for controlled gene expression across the Proteobacteria. *Nucleic Acids Res.* **2021**, *49*, 7189−7202.

(31) Prior, J. E.; Lynch, M. D.; Gill, R. T. Broad-host-range vectors for protein expression across gram negative hosts. *Biotechnol. Bioeng.* **2010**, *106*, 326−332.

(32) Martínez-García, E.; Fraile, S.; Algar, E.; Aparicio, T.; Vel′azquez, E.; Calles, B.; Tas, H.; Bl′azquez, B.; Mart′ın, B.; Prieto, C.; et al. SEVA 4.0: an update of the Standard European Vector Architecture database for advanced analysis and programming of bacterial phenotypes. *Nucleic Acids Res.* **2023**, *51*, D1558−D1567.

(33) Fu, G.; Yue, J.; Li, D.; Li, Y.; Lee, S. Y.; Zhang, D. An operator-based expression toolkit for *Bacillus subtilis* enables fine-tuning of gene expression and biosynthetic pathway regulation. *Proc. Natl. Acad. Sci. U.S.A.* **2022**, *119*, No. e2119980119.

(34) Radeck, J.; Kraft, K.; Bartels, J.; Cikovic, T.; D̈urr, F.; Emenegger, J.; Kelterborn, S.; Sauer, C.; Fritz, G.; Gebhard, S.; Mascher, T. The *Bacillus* BioBrick Box: generation and evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis*. *J. Biol. Eng.* **2013**, *7*, 29.

(35) Li, Y.; Wu, Y.; Liu, Y.; Li, J.; Du, G.; Lv, X.; Liu, L. A genetic toolkit for efficient production of secretory protein in *Bacillus subtilis*. *Bioresour. Technol.* **2022**, *363*, No. 127885.

(36) Wang, Z.; Fang, Y.; Shi, Y.; Xin, Y.; Gu, Z.; Yang, T.; Li, Y.; Ding, Z.; Shi, G.; Zhang, L. Analysis of Xylose Operon from *Paenibacillus polymyxa ATCC842* and Development of Tools for Gene Expression. *Int. J. Mol. Sci.* **2022**, *23*, 5024.

(37) Li, R.; Shi, H.; Zhao, X.; Liu, X.; Duan, Q.; Song, C.; Chen, H.; Zheng, W.; Shen, Q.; Wang, M.; et al. Development and application of an efficient recombineering system for *Burkholderia glumae* and *Burkholderia plantarii*. *Microb. Biotechnol.* **2021**, *14*, 1809−1826.

(38) Niu, B.; Paulson, J. N.; Zheng, X.; Kolter, R. Simplified and representative bacterial community of maize roots. *Proc. Natl. Acad. Sci. U.S.A.* **2017**, *114*, E2450−E2459.

(39) Xun, W.; Liu, Y.; Li, W.; Ren, Y.; Xiong, W.; Xu, Z.; Zhang, N.; Miao, Y.; Shen, Q.; Zhang, R. Specialized metabolic functions of keystone taxa sustain soil microbiome stability. *Microbiome* **2021**, *9*, 35.

(40) Xiao, Y.; Angulo, M. T.; Friedman, J.; Waldor, M. K.; Weiss, S. T.; Liu, Y.-Y. Mapping the ecological networks of microbial communities. *Nat. Commun.* **2017**, *8*, No. 2042.

(41) Wagner, M. R.; Tang, C.; Salvato, F.; Clouse, K. M.; Bartlett, A.; Vintila, S.; Phillips, L.; Sermons, S.; Hoffmann, M.; Balint-Kurti, P. J.; Kleiner, M. Microbe-dependent heterosis in maize. *Proc. Natl. Acad. Sci. U.S.A.* **2021**, *118*, No. e2021965118.

(42) Salvato, F.; Vintila, S.; Finkel, O. M.; Dangl, J. L.; Kleiner, M. Evaluation of protein extraction methods for metaproteomic analyses of root-associated microbes. *Mol. Plant−Microbe Interact.* **2022**, *35*, 977−988.

(43) Krumbach, J.; Kroll, P.; Wewer, V.; Metzger, S.; Ischebeck, T.; Jacoby, R. P.Metabolic analysis of a bacterial synthetic community from maize roots provides new mechanistic insights into microbiome stability, 2021. https://doi.org/10.1101/2021.11.28.470254.

(44) Casavant, N. C.; Beattie, G. A.; Phillips, G. J.; Halverson, L. J. Site-specific recombination-based genetic system for reporting transient or low-level gene expression. *Appl. Environ. Microbiol.* **2002**, *68*, 3588−3596.

(45) Bai, R.; Chu, W.; Qiao, Z.; Lu, P.; Jiang, K.; Xu, Y.; Yang, J.; Gao, T.; Xu, F.; Zhao, H. Metabolic regulation of NADH supply and hydrogen production in *Enterobacter aerogenes* by multi-gene engineering. *Int. J. Hydrogen Energy* **2023**, *48*, 909−920.

(46) Liu, X.; Tian, J.; Liu, L.; Zhu, T.; Yu, X.; Chu, X.; Yao, B.; Wu, N.; Fan, Y. Identification of an operon involved in fluoride resistance in *Enterobacter cloacae FRM*. *Sci. Rep.* **2017**, *7*, No. 6786.

(47) Rothballer, M.; Eckert, B.; Schmid, M.; Fekete, A.; Schloter, M.; Lehner, A.; Pollmann, S.; Hartmann, A. Endophytic root colonization of gramineous plants by *Herbaspirillum frisingense*. *FEMS Microbiol. Ecol.* **2008**, *66*, 85−95.

(48) Wagh, J.; Chanchal, K.; Sonal, S.; Praveena, B.; Archana, G.; Kumar, G. N. Inoculation of genetically modified endophytic Herbaspirillum seropedicae Z67 *Herbaspirillum seropedicae Z67* endowed with gluconic and 2-ketogluconic acid secretion, confers beneficial effects on rice (*Oriza sativa*) plants. *Plant Soil* **2016**, *409*, 51−64.

(49) Meng, W.; Qiao, K.; Zhang, W.; Liu, F.; Gao, X.; Hu, X.; Zhu, J. Development and Application of a New Arabinose-Inducible Vector in High-Attachment Strain *Stenotrophomonas AGS-1* from Aerobic Granular Sludge. *ACS Synth. Biol.* **2022**, *11*, 69−76.

(50) Huang, T.-P.; Somers, E. B.; Wong, A. C. L. Differential biofilm formation and motility associated with lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes in *Stenotrophomonas maltophilia*. *J. Bacteriol.* **2006**, *188*, 3116−3120.

(51) Tian, M.; Qu, J.; Bao, Y.; Gao, J.; Liu, J.; Wang, S.; Sun, Y.; Ding, C.; Yu, S. Construction of pTM series plasmids for gene expression in *Brucella* species. *J. Microbiol. Methods* **2016**, *123*, 18−23.

(52) Seleem, M. N.; Jain, N.; Alqublan, H.; Vemulapalli, R.; Boyle, S. M.; Sriranganathan, N. Activity of native vs. synthetic promoters in *Brucella*. *FEMS Microbiol. Lett.* **2008**, *288*, 211−215.

(53) Miteva, V.; Lantz, S.; Brenchley, J. Characterization of a cryptic plasmid from a Greenland ice core *Arthrobacter* isolate and construction of a shuttle vector that replicates in psychrophilic high G+ C Gram-positive recipients. *Extremophiles* **2008**, *12*, 441−449.

(54) Ye, X.; Dong, H.; Huang, Y.-P. Highly efficient transformation of *Stenotrophomonas maltophilia S21*, an environmental isolate from soil, by electroporation. *J. Microbiol. Methods* **2014**, *107*, 92−97.

(55) Cho, J.-H.; Kim, E.-K.; So, J.-S. Improved transformation of *Pseudomonas putida KT2440* by electroporation. *Biotechnol. Tech.* **1995**, *9*, 41−44.

(56) Khorramizadeh, M. R.; Gheibi, A.; Khanahmad, H.; Kardar, G. A.; Boshtam, M.; Rezaie, S.; Kazemi, B. Optimization and Comparison of Different Methods and Factors for Efficient Transformation of *Brucella abortus RB51 strain*. *Adv. Biomed. Res.* **2019**, *8*, No. 37, DOI: 10.4103/abr.abr_14_19.

(57) Perry, B. J.; Yost, C. K. Construction of a mariner-based transposon vector for use in insertion sequence mutagenesis in selected members of the *Rhizobiaceae*. *BMC Microbiol.* **2014**, *14*, 298.

(58) Andreote, F. D.; Mortatti, M.-J.; de Souza, A.-O.; Walter, M.; Joao, L.; Welington, L. Impact of genetically modified *Enterobacter cloacae* on indigenous endophytic community of Citrus sinensis seedlings. *J. Microbiol.* **2004**, *42*, 169−173.

(59) Hahn, F.; Korolev, A.; Sanjurjo Loures, L.; Nekrasov, V. A modular cloning toolkit for genome editing in plants. *BMC Plant Biol.* **2020**, *20*, 179.

(60) Potapov, V.; Ong, J. L.; Kucera, R. B.; Langhorst, B. W.; Bilotti, K.; Pryor, J. M.; Cantor, E. J.; Canton, B.; Knight, T. F.; Evans, T. C., Jr.; Lohman, G. J. S. Comprehensive profiling of four base overhang ligation fidelity by T4 DNA ligase and application to DNA assembly. *ACS Synth. Biol.* **2018**, *7*, 2665−2674.

(61) Arnold, M. F. F.; Shabab, M.; Penterman, J.; Boehme, K. L.; Griffitts, J. S.; Walker, G. C. Genome-wide sensitivity analysis of the

microsymbiont *Sinorhizobium meliloti* to symbiotically important, defensin-like host peptides. *mBio* **2017**, *8*, No. e01060-17, DOI: 10.1128/mBio.01060-17.
(62) Budiharjo, A.; Chowdhury, S. P.; Dietel, K.; Beator, B.; Dolgova, O.; Fan, B.; Bleiss, W.; Ziegler, J.; Schmid, M.; Hartmann, A.; Borriss, R. Transposon mutagenesis of the plant-associated *Bacillus amyloliquefaciens ssp. plantarum FZB42* revealed that the nfrA and RBAM17410 genes are involved in plant-microbe-interactions. *PLoS One* **2014**, *9*, No. e98267.
(63) Madhaiyan, M.; Peng, N.; Te, N. S.; Hsin I, C.; Lin, C.; Lin, F.; Reddy, C.; Yan, H.; Ji, L.; et al. Improvement of plant growth and seed yield in Jatropha curcas by a novel nitrogen-fixing root associated *Enterobacter* species. *Biotechnol. Biofuels* **2013**, *6*, 140.
(64) Riley, L. A.; Guss, A. M. Approaches to genetic tool development for rapid domestication of non-model microorganisms. *Biotechnol. Biofuels* **2021**, *14*, 30.
(65) Zhang, G.; Wang, W.; Deng, A.; Sun, Z.; Zhang, Y.; Liang, Y.; Che, Y.; Wen, T. A mimicking-of-DNA-methylation-patterns pipeline for overcoming the restriction barrier of bacteria. *PLoS Genet.* **2012**, *8*, No. e1002987, DOI: 10.1371/journal.pgen.1002987.
(66) Johnston, C. D.; Cotton, S. L.; Rittling, S. R.; Starr, J. R.; Borisy, G. G.; Dewhirst, F. E.; Lemon, K. P. Systematic evasion of the restriction-modification barrier in bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116*, 11454−11459.
(67) Lee, J. Y. H.; Carter, G. P.; Pidot, S. J.; Gu′erillot, R.; Seemann, T.; Goņcalves da Silva, A.; Foster, T. J.; Howden, B. P.; Stinear, T. P.; Monk, I. R. Mining the methylome reveals extensive diversity in *Staphylococcus epidermidis* restriction modification. *mBio* **2019**, *10*, No. e02451-19, DOI: 10.1128/mBio.02451-19.
(68) Chen, L.; Xiuli, X.; Zhang, J.; Redmile-Gordon, M.; Guangsen, N.; Qingyun, W. Soil characteristics overwhelm cultivar effects on the structure and assembly of root associated microbiomes of modern maize. *Pedosphere* **2019**, *29*, 360−373.
(69) Xiong, C.; Zhu, Y.-G.; Wang, J.-T.; Singh, B.; Han, L.-L.; Shen, J.-P.; Li, P.-P.; Wang, G.-B.; Wu, C.-F.; Ge, A.-H.; et al. Host selection shapes crop microbiome assembly and network complexity. *New Phytol.* **2021**, *229*, 1091−1104.
(70) Rubin, B. E.; Diamond, S.; Cress, B. F.; Crits-Christoph, A.; Lou, Y. C.; Borges, A. L.; Shivram, H.; He, C.; Xu, M.; Zhou, Z.; et al. Species-and site-specific genome editing in complex bacterial communities. *Nat. Microbiol.* **2022**, *7*, 34−47.
(71) Klümper, U.; Riber, L.; Dechesne, A.; Sannazzarro, A.; Hansen, L. H.; Sørensen, S. J.; Smets, B. F. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *ISME J.* **2015**, *9*, 934−945.
(72) Crook, N.; Ferreiro, A.; Gasparrini, A. J.; Pesesky, M. W.; Gibson, M. K.; Wang, B.; Sun, X.; Condiotte, Z.; Dobrowolski, S.; Peterson, D.; Dantas, G. Adaptive strategies of the candidate probiotic *E. coli* Nissle. *Cell Host Microbe* **2019**, *25*, 499−512.
(73) Crofts, T. S.; McFarland, A. G.; Hartmann, E. M. Mosaic Ends Tagmentation (METa) Assembly for Highly Efficient Construction of Functional Metagenomic Libraries. *mSystems* **2021**, *6*, No. e00524-21, DOI: 10.1128/mSystems.00524-21.
(74) Uchiyama, T.; Miyazaki, K. Functional metagenomics for enzyme discovery: challenges to efficient screening. *Curr. Opin. Biotechnol.* **2009**, *20*, 616−622.
(75) da Cunha, E. T.; Pedrolo, A. M.; Bueno, J. C. F.; Pereira, T. P.; Soares, C. R. F. S.; Arisi, A. C. M. Inoculation of *Herbaspirillum seropedicae strain SmR1* increases biomass in maize roots DKB 390 variety in the early stages of plant development. *Arch. Microbiol.* **2022**, *204*, 373.
(76) Alves, G. C.; Videira, S. S.; Urquiaga, S.; Reis, V. M. Differential plant growth promotion and nitrogen fixation in two genotypes of maize by several *Herbaspirillum* inoculants. *Plant Soil* **2015**, *387*, 307−321.
(77) Brusamarello-Santos, L. C.; Gilard, F.; Brul′e, L.; Quiller′e, I.; Gourion, B.; Ratet, P.; Maltempi de Souza, E.; Lea, P. J.; Hirel, B. Metabolic profiling of two maize (*Zea mays L.*) inbred lines inoculated with the nitrogen fixing plant-interacting bacteria *Herbaspirillum seropedicae* and *Azospirillum brasilense*. *PLoS One* **2017**, *12*, No. e0174576.
(78) Neiverth, A.; Delai, S.; Garcia, D. M.; Saatkamp, K.; de Souza, E. M.; de Oliveira Pedrosa, F.; Guimarães, V. F.; dos Santos, M. F.; Vendruscolo, E. C. G.; da Costa, A. C. T. Performance of different wheat genotypes inoculated with the plant growth promoting bacterium *Herbaspirillum seropedicae*. *Eur. J. Soil Biol.* **2014**, *64*, 1−5.
(79) Baldani, J. I.; Baldani, V.; Seldin, L.; D̈obereiner, J. Characterization of Herbaspirillum seropedicae gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium. *Int. J. Syst. Bacteriol.* **1986**, *36*, 86−93.
(80) Elbeltagy, A.; Nishioka, K.; Sato, T.; Suzuki, H.; Ye, B.; Hamada, T.; Isawa, T.; Mitsui, H.; Minamisawa, K. Endophytic colonization and in planta nitrogen fixation by a *Herbaspirillum sp.* isolated from wild rice species. *Appl. Environ. Microbiol.* **2001**, *67*, 5285−5293.
(81) Jeschek, M.; Gerngross, D.; Panke, S. Combinatorial pathway optimization for stream- lined metabolic engineering. *Curr. Opin. Biotechnol.* **2017**, *47*, 142−151.
(82) Wang, H. H.; Isaacs, F. J.; Carr, P. A.; Sun, Z. Z.; Xu, G.; Forest, C. R.; Church, G. M. Programming cells by multiplex genome engineering and accelerated evolution. *Nature* **2009**, *460*, 894−898.
(83) Cubitt, A. B.; Heim, R.; Adams, S. R.; Boyd, A. E.; Gross, L. A.; Tsien, R. Y. Understanding, improving and using green fluorescent proteins. *Trends Biochem. Sci.* **1995**, *20*, 448−455.
(84) Lynch, M. D.; Gill, R. T. Broad host range vectors for stable genomic library construction. *Biotechnol. Bioeng.* **2006**, *94*, 151−158.
(85) Lee, T. S.; Krupa, R. A.; Zhang, F.; Hajimorad, M.; Holtz, W. J.; Prasad, N.; Lee, S. K.; Keasling, J. D. BglBrick vectors and datasheets: a synthetic biology platform for gene expression. *J. Biol. Eng.* **2011**, *5*, 12.
(86) Jiang, Y.; Chen, B.; Duan, C.; Sun, B.; Yang, J.; Yang, S. Multigene editing in the *Escherichia coli* genome via the CRISPR-Cas9 system. *Appl. Environ. Microbiol.* **2015**, *81*, 2506−2514.
(87) McGuffie, M. J.; Barrick, J. E. pLannotate: engineered plasmid annotation. *Nucleic Acids Res.* **2021**, *49*, W516−W522.