ACS Synthetic Biology

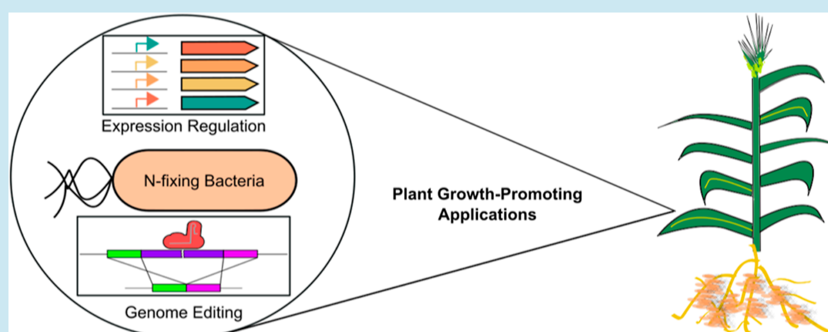
pubs.acs.org/synthbio Research Article

Synthetic Biology Toolbox for Nitrogen-Fixing Soil Microbes

Maya Venkataraman, Audrey Yñigez-Gutierrez, Valentina Infante, April MacIntyre, Paulo Ivan Fernandes-Júnior, Jean-Michel Ané, and Brian Pfleger*

Cite This: ACS Synth. Biol. 2023, 12, 3623−3634 Read Online

ACCESS Metrics & More Article Recommendations Supporting Information



ABSTRACT: The soil environment adjacent to plant roots, termed the rhizosphere, is home to a wide variety of microorganisms that can significantly affect the physiology of nearby plants. Microbes in the rhizosphere can provide nutrients, secrete signaling compounds, and inhibit pathogens. These processes could be manipulated with synthetic biology to enhance the agricultural performance of crops grown for food, energy, or environmental remediation, if methods can be implemented in these nonmodel microbes. A common first step for domesticating nonmodel organisms is the development of a set of genetic engineering tools, termed a synthetic biology toolbox. A toolbox comprises transformation protocols, replicating vectors, genome engineering (e.g., CRISPR/Cas9), constitutive and inducible promoter systems, and other gene expression control elements. This work validated synthetic biology toolboxes in three nitrogen-fixing soil bacteria: *Azotobacter vinelandii*, *Stutzerimonas stutzeri* (*Pseudomonas stutzeri*), and a new isolate of *Klebsiella variicola*. All three organisms were amenable to transformation and reporter protein expression, with several functional inducible systems available for each organism. *S. stutzeri* and *K. variicola* showed more reliable plasmid-based expression, resulting in successful Cas9 recombineering to create scarless deletions and insertions. Using these tools, we generated mutants with inducible nitrogenase activity and introduced heterologous genes to produce resorcinol products with relevant biological activity in the rhizosphere.

KEYWORDS: *diazotrophs, CRISPR, Cas9, synthetic biology toolbox, genome editing, nitrogenase*

Downloaded via CO BRASILEIRA PESQUISA AGROPECUARIA on December 18, 2023 at 12:33:47 (UTC). See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

■ INTRODUCTION

Plant growth-promoting bacteria can be used for developing beneficial microbial communities and improving agricultural yields. In particular, associative nitrogen-fixing bacteria (diazotrophs) have the potential to offset fertilizer usage and provide fixed nitrogen to fertilizer-intensive cereal crops such as wheat, corn, or sorghum, making them appealing targets for developing improved microbial inoculants.[1,2] However, diazotrophs remain poorly characterized with few tools for robust genetic engineering essential to meet applied goals. Well-characterized genetic engineering toolboxes can accelerate the pace of trait enhancement in nonmodel microbes.[3,4] Therefore, in this study, we investigated three diazotrophs for tool development and production of relevant products for the rhizosphere: *Stutzerimonas stutzeri* A1501 (formerly known as *Pseudomonas stutzeri*), *Azotobacter vinelandii* DJ, and a new isolate of *Klebsiella variicola*.

S. stutzeri A1501 has previously been studied extensively for its ability to fix nitrogen, and the regulation of this fixation has been studied in great detail.[5−7] Despite these studies, synthetic biology tools remain limited. Previous work achieved genetic modifications via a triparental conjugation strategy, with deletions achieved via homologous recombination of antibiotic selection markers.[8,9] Similarly, *A. vinelandii* DJ is a very well-studied obligate aerobe with an unusual metabolism that allows nitrogen fixation at atmospheric conditions.[10] It is an exceptional model due to its natural competency under metal-starved conditions[11] as well as expression of three different nitrogenase

Received: July 10, 2023
Revised: October 28, 2023
Accepted: October 31, 2023
Published: November 21, 2023

© 2023 American Chemical Society

https://doi.org/10.1021/acssynbio.3c00414
ACS Synth. Biol. 2023, 12, 3623−3634

enzymatic complexes (MoFe, VFe, and FeFe cores).[12−14] It was used previously to elucidate the structure[15,16] of the nitrogenase enzyme complex. Using homologous recombination with antibiotic selection, as well as *sacB* counterselection, *A. vinelandii* mutants that secrete high amounts of ammonia when grown in batch conditions have been produced.[17−19] Finally, while *Klebsiella pneumoniae* has been a model for studying biological nitrogen fixation for decades,[20,21] safety concerns over the use of these strains prevented agricultural use. However, more recently, many *K. variicola* isolated from maize and sorghum have been sequenced and found to lack human/animal virulence genes, opening the path for using such *K. variicola* for genetic engineering and agricultural applications.[22,23] For example, KV37-1036 was used as a nitrogen-fixing bacterial supplement in field studies.[24] Genetic edits of this strain relied on screening for insertions using the integration of an antibiotic resistance marker using a single crossover, followed by a *sacB* or other counterselection strategy to remove the plasmid backbone.[25] With these preliminary studies, *K. variicola* shows promise as a malleable chassis organism and an effective nitrogen-fixing symbiont for fertilizer-intensive crops.

While these three bacterial species have all been studied in some capacity, the ability to rapidly modulate expression in these diazotrophs more quickly and reliably would allow for further investigation of plant−microbe interactions and improve nutrient exchange. This study aimed to validate tools for advanced genetic engineering in diazotrophs. First, we characterized synthetic biology tools in these organisms using fluorescent reporters, focusing on broad-host plasmids, constitutive promoters, and inducible systems. Once we validated robust protein expression using these tools, we developed a genome-editing workflow for these diazotrophs. Using a Cas9/λRed-mediated recombineering strategy, we demonstrated two modes of action for plant growth promotion. In the first mode, we created strains with changes in nitrogenase expression that could be used as tools to investigate the mechanisms of nitrogenase regulation. In the second mode, we demonstrated the production of alkylresorcinol compounds using the *sbARS2* type III polyketide synthase (PKS) gene from sorghum.[26] Production of alkylresorcinols is an important precursor step to the production of sorgoleone, which has antifungal, antimicrobial, and herbicidal activities that could lend benefit to the rhizospheres of a variety of crop species.[27−31]

■ RESULTS AND DISCUSSION

Isolation of Nitrogen-Fixing Bacteria from Sorghum Roots. Mechan-Llontop et al. (2023) recently reported the isolation of diazotrophs from the epicuticular wax and the aerial root mucilage of sorghum.[22] Similarly, we isolated diazotrophs from the aerial root mucilage of sorghum (Supporting Information Table S1), including two *K. pneumoniae* strains, two strains in the genus *Paenibacillus*, one *Erwinia endophytica* strain, and an isolate of *K. variicola* (isolate A3). *K. variicola* A3 was isolated on nitrogen-free, solid artificial mucilage media.[32] Molecular confirmation of *nifH* was performed as described for all organisms[33] and initial acetylene reduction assays (ARAs) were performed in a semisolid nitrogen-free medium (0.2% agar) to confirm nitrogenase presence and activity. All strains were assayed for the following plant growth-promoting traits: auxin production using Salkowski's reagent,[34] organic and inorganic phosphate solubilization,[35] siderophore production,[36] and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity.[37] The *K. variicola* strain produced ∼43 μg/mL indole-3-acetic acid *in vitro* and solubilized both organic and inorganic phosphate. The strain did not produce siderophores or display ACC deaminase activity on plates. The plant growth-promoting activity of all isolated strains is summarized in Supporting Information Table S1. Because the strain was an excellent nitrogen fixer and displayed strong plant growth-promoting traits, the genome of *K. variicola* A3 was sequenced (NCBI Accession PRJNA793885). Since this isolate has no virulence gene for plants or animals, it was used as one of the chassis for this study along with the well-characterized *S. stutzeri* A1501 and *A. vinelandii* DJ. A summary of this isolate is shown in Table 1.

Table 1. Characteristics of the *Klebsiella variicola* Strain Used in This Study[a]

classification	bacteria; Pseudomonadota; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; <> *Klebsiella/Raoultella*<> group; *Klebsiella*
species	*Klebsiella variicola* subs. *variicola*
taxonomy ID	244366
virulence score	no virulence loci
genome length	5,365,114 bp
protein-coding genes	5019
plasmids	1
GC content	57.6%
*t*RNAs	67
antibiotic resistance	ampicillin 100 μg/mL, chloramphenicol 12.5 μg/mL, streptomycin 50 μg/mL
host	aerial root mucilage of *Sorghum bicolor*
place of collection	Live Oak, FL, USA
year of collection	2020
auxin production	43.45 μg/mL
siderophore production	−
organic phosphate solubilization	+
inorganic phosphate solubilization	+
ACC deaminase activity	−

[a]Data obtained from this isolation work as well as the Pathogenwatch platform for virulence loci (https://pathogen.watch).

Plasmid and Promoter Characterization in Nitrogen-Fixing Bacteria. The most critical element of a synthetic biology toolbox is the ability to introduce and stably express protein from heterologous DNA. Therefore, we first aimed to characterize the compatibility and relative expression of broad-host plasmids in these nonmodel organisms. We used four broad-host plasmids previously identified in the literature: RK2,[38] BBR1,[39] RSF1010,[40] and pRO1600,[41,42] as well as two plasmids generally more specific to *Enterobacteriaceae*, p15A[43] and ColE1.[44] The transformation efficiencies of different plasmid backbones are summarized in Table 2. All six plasmid backbones were transformed consistently in *K. variicola* A3, indicating its compatibility with these *Enterobacteriaceae* plasmids. While all plasmids generated antibiotic-resistant colonies of *S. stutzeri*, only the BBR1 and RSF1010 backbones were transformed reliably. Vectors containing RK2, pRO1600, p15A, and ColE1 origins were transformed into *S. stutzeri* with very low efficiencies with no plasmid backbone detectable by PCR, suggesting that surviving colonies may be the result of

Table 2. Transformation Efficiencies of Different Plasmid Backbones in Three Diazotrophs[a]

transformation efficiencies (CFU/μg DNA)	*K. variicola*	*S. stutzeri*	*A. vinelandii*
RK2	**$(2.0 \pm 0.4) \times 10^3$**	$(2 \pm 2) \times 10^1$	**$(4 \pm 1) \times 10^4$**
BBR1	**$(2 \pm 1) \times 10^3$**	**$(1.3 \pm 0.1) \times 10^4$**	**$(5.0 \pm 0.5) \times 10^4$**
RSF1010	**$(1.3 \pm 0.7) \times 10^3$**	**$(4 \pm 1) \times 10^4$**	$(3 \pm 2) \times 10^1$
pRO1600	**$(1.3 \pm 0.1) \times 10^4$**	$(3 \pm 2) \times 10^0$	**$(7 \pm 0.5) \times 10^3$**
p15A	**$(1.20 \pm 0.01) \times 10^4$**	$(2 \pm 1) \times 10^1$	0
ColE1	**$(7 \pm 2) \times 10^3$**	$(3 \pm 0.4) \times 10^1$	0

[a]Bolded values indicate plasmids that transform and express reliably. Standard deviations are from biological duplicates.

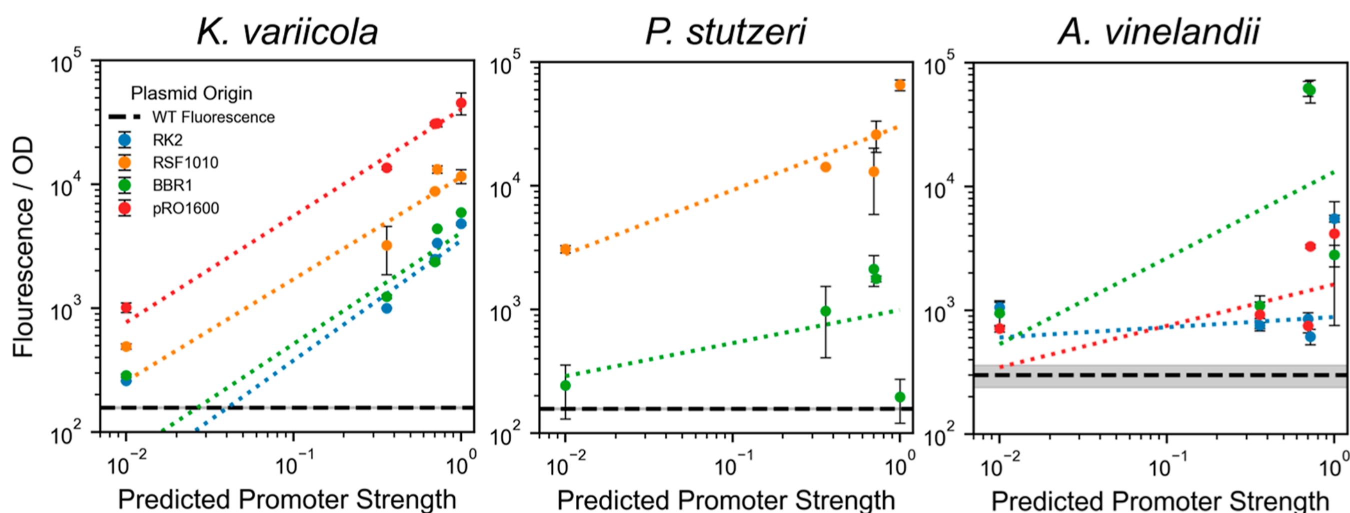


Figure 1. Constitutive promoter fluorescence in different broad-host backbones in *K. variicola*, *S. stutzeri*, and *A. vinelandii*. Predicted promoter strengths are from original work in *E. coli*.[46] Error bars show the standard deviation from biological triplicates.

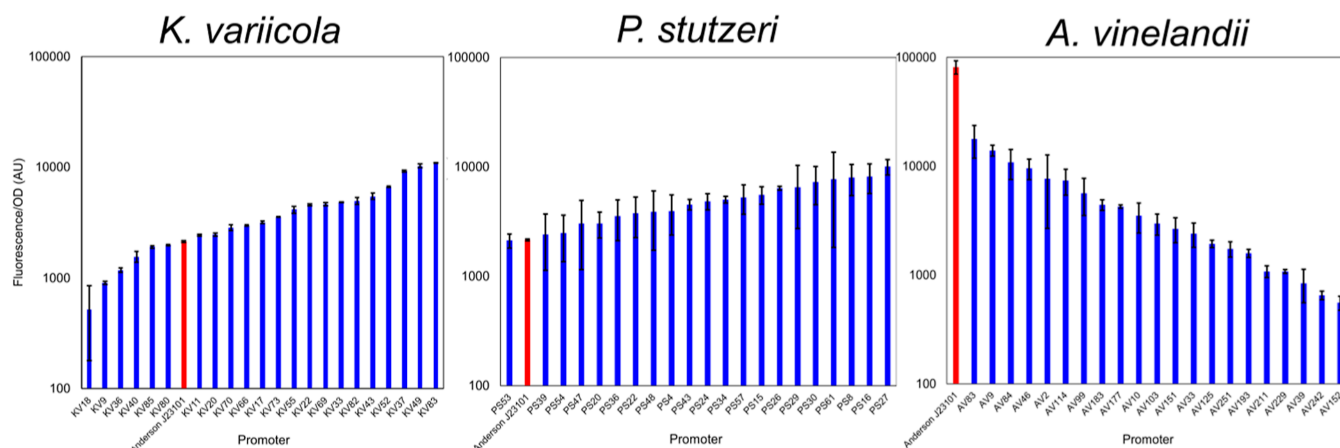


Figure 2. Promoter modulation in *K. variicola*, *S. stutzeri,* and *A. vinelandii* using degenerate oligos results in dynamic ranges in expression. A subset of promoters from each organism was taken to capture the full range of expression from the generated library. The red bar in each plot indicates the original fluorescence values from the starting promoter (Anderson Promoter J23101). Error bars show standard deviation from biological triplicates.

spontaneous mutation or uncommon ectopic integration events of the antibiotic resistance marker. The original cosmid from which the RK2 origin is derived can replicate in *S. stutzeri* A1501,[6,45] so additional replication machinery from the cosmid may be necessary to restore functionality. Finally, *A. vinelandii* was able to maintain RK2, BBR1, and pRO1600 backbone plasmids, albeit with slower growth in minimal media (Figure S1).

After validating stable vector backbones, we also verified the function of five constitutive promoters using fluorescent reporters. Using a subset of the Anderson promoter library,[46] we confirmed that we could get a range of constitutive expression levels. Reporter expression in *K. variicola* on broad-host plasmid origins was consistent with values reported in *Escherichia coli*. In contrast, expression in *A. vinelandii* and *S. stutzeri* was more inconsistent with the values reported in *E. coli* (Figure 1). These results indicated that we could successfully achieve constitutive expression in all three organisms with reliably maintained plasmids. We sought additional promoters from a degenerate promoter library for further granularity in transcriptional control. The BBR1 backbone was functional in all three organisms; therefore, all subsequent promoter testing

https://doi.org/10.1021/acssynbio.3c00414
ACS Synth. Biol. 2023, 12, 3623−3634

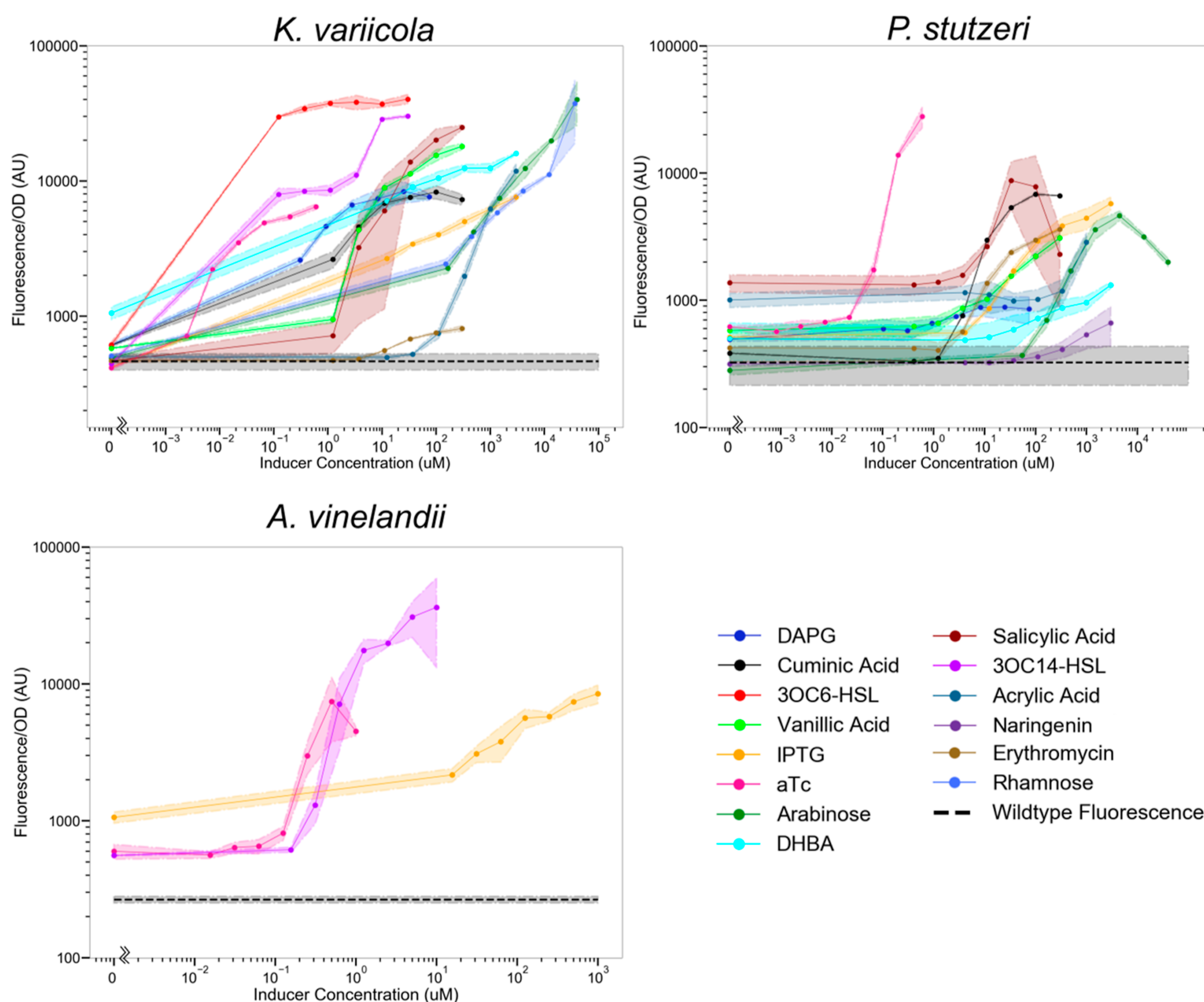


Figure 3. Induction plots of inducer systems in *K. variicola*, *S. stutzeri*, and *A. vinelandii*. The same set of 15 inducers was tried for all three organisms on a BBR1 backbone: DAPG, cuminic acid, 3OC6-HSL, vanillic acid, IPTG, anhydrotetracycline, arabinose, choline, naringenin, DHBA, salicylic acid, 3OC14-HSL, acrylic acid, erythromycin, and rhamnose. The shaded region represents the standard deviation from the biological triplicates. Only induction systems that were transformable and functional on plasmids in each organism are shown (nonfunctional inducer data in Figure S3).

used this backbone. Since the J23101 promoter from the Anderson library showed reliable constitutive expression in all three organisms, we mutagenized this promoter by PCR using degenerate oligos (5′ttacaNNNNNNNNNNNNNNNNNtattatNNNNNN′3) to generate a library for cloning. The oligo design kept the −35 and −10 regions of the J23101 promoter intact to maximize the number of clones with functional constitutive expression based on our initial results. While we could not capture the full diversity of this library through screening using 96-well plates, we could demonstrate ranges of expression between the original strong promoter and empty vector. A subset of isolates that capture the range of the fluorescence of sequenced promoters is shown in Figure 2. From these promoter sequences, we demonstrate that the interspatial region between the −35 and −10 regions can significantly impact expression significantly. We also analyzed a set of 11 different ribosome binding sites (RBSs) after the J23101 promoter to find additional ways to modulate protein expression. The strength of these RBSs was predicted by using the Salis lab calculator[47] and resulted in a dynamic range of over 1.5 orders of magnitude in fluorescence, thereby demonstrating the ability to modulate both transcription and translation in the three species (Figure S2).

Inducible Promoter Systems Are Variable Across Different Bacteria. Inducible promoters allow for conditional expression of heterologous proteins—a critical capability for designing synthetic circuits and tailored metabolism. Therefore, we validated the function of the 12 inducible systems previously developed in the "Marionette" strain of *E. coli*.[48] We also validated two additional plasmid-based systems from the same paper (acrylic acid and erythromycin biosensors) and a rhamnose-inducible cassette from previous work in soil bacteria, bringing the total number of tested induction systems to 15.[49] As the regulatory proteins responsive to the Marionette inducers were previously evolved to limit crosstalk, they offered a good starting set of inducible systems to onboard into new organisms using a broad host backbone. The results for the tests of these 15 induction systems are listed in Figure 3. Almost all of these inducible systems worked comparably in *K. variicola* as *E. coli*, with the exception of the naringenin and choline induction

https://doi.org/10.1021/acssynbio.3c00414
ACS Synth. Biol. 2023, 12, 3623−3634

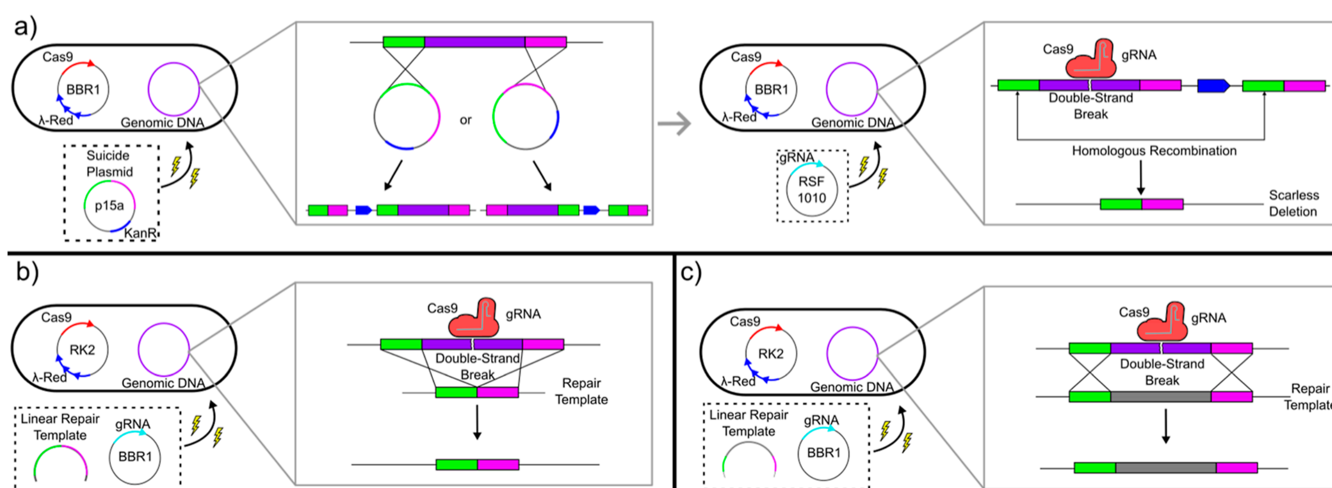


Figure 4. Schematic for Cas9/λ-Red recombineering in *S. stutzeri* (a) and *K. variicola* (b,c). For *S. stutzeri*, the repair template was first integrated into the genome using antibiotic selection for a single crossover event. In a second step, the gRNA plasmid was introduced via electroporation to induce positive selection for clean knockouts in cells. In contrast, genome editing in *K. variicola* utilized a one-step protocol for simultaneous introduction of the gRNA plasmid as well as the repair template, with the deletion workflow in (b) and the insertion workflow in (c). In all cases, electroporation was used to introduce DNA into each organism.

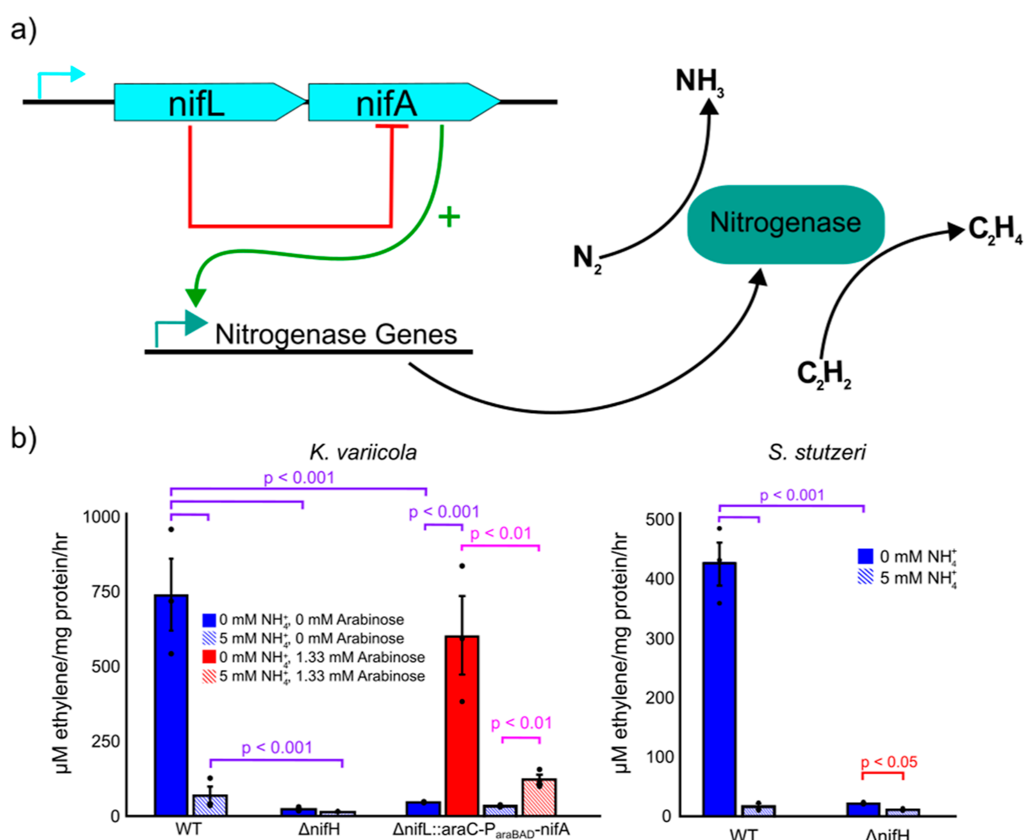


Figure 5. Nitrogenase activity can be induced in *K. variicola* with arabinose and abolished in both *K. variicola* and *S. stutzeri*. (a) Schematic of nitrogenase regulation through the *nifLA* operon in *K. variicola* and *S. stutzeri*. Acetylene reduction is used to approximate nitrogen fixation activity. (b) Δ*nifL::araC-araBAD-nifA* mutant changes the nitrogenase system to arabinose-inducible in Burk's media. In both *K. variicola* and *P. stutzeri*, Δ*nifH* strains show dysregulation of nitrogen fixation. *P*-Values indicate the results of a two-way ANOVA on log-transformed data with the use of the Bonferroni correction ($P < 0.05$ threshold). Error bars show the standard error from biological triplicates.

systems (Figure S3). Again, results were more variable in *S. stutzeri* and *A. vinelandii* with fewer induction systems transformable and functional in these organisms. Many induction systems in *A. vinelandii* showed nonfunctional induction on plasmids, with only three successful cassettes. However, some of the induction systems for *A. vinelandii* were functional when the regulators and responsive expression cassette were integrated into the genome of the organism (Figure S4). This suggests that plasmid systems are nonideal for *A. vinelandii* and may not yield an accurate representation of

https://doi.org/10.1021/acssynbio.3c00414
ACS Synth. Biol. 2023, 12, 3623−3634

possible expression from the chromosome. Additionally, in both *S. stutzeri* and *A. vinelandii*, the fold-change with induction tended to be smaller than that in *K. variicola* A3, indicating more significant differences in regulation from *E. coli*, which is to be expected given their larger evolutionary distances from *E. coli*. By starting from a large pool of initial inducers, we could find functional inducer systems for all three diazotrophs for further design work, even when many cassettes were nonresponsive to induction.

Developing Gene-Editing Systems Using Synthetic Biology Parts. With these characterized expression systems for three diazotrophs, we next aimed to develop robust genome-editing tools, with an outline of our gene-editing approach shown in Figure 4. Using the design from previous work in *E. coli* and *Pseudomonas putida*,[50] we developed a λRed/Cas9 recombineering protocol for scarless genome editing with positive counterselection against the original wild-type DNA for these organisms. Based on our broad-host plasmid compatibility, we used a two-plasmid system using RK2/BBR1 backbones for *K. variicola* and *A. vinelandii* and BBR1/RSF1010 backbones for *S. stutzeri*. One plasmid contained constitutively expressed Cas9 and arabinose-inducible λRed genes. The other plasmid contained the gRNA for the given target site, allowing for many different guides to be introduced sequentially to make subsequent edits by cycling of the gRNA plasmid. Using these methods and recombineering conditions, we generated scarless mutants in *S. stutzeri* and *K. variicola*. However, we could not successfully create genome edits in *A. vinelandii* using a Cas9-mediated system, likely due to a lack of protein production in our plasmid-based systems (Figure S5). Therefore, we continued with our genome-editing pipeline with only *S. stutzeri* and *K. variicola*.

First, we aimed to create mutants of nitrogen-fixing organisms that abolish nitrogen fixation activity. We deleted *nifH*, a critical subunit in the nitrogenase enzyme complex, in *K. variicola* and *S. stutzeri* to create a negative control, nonfixing strain. This strategy has successfully been used previously to abolish fixation in many other diazotrophs.[51,52] Using our Cas9-editing strategy, we were able to generate strains of both organisms that were unable to fix nitrogen (Figure 5). This initial mutation demonstrated the success of our genome-editing tools, so we next moved to test other systems for deregulated nitrogen fixation.

Previous work in *A. vinelandii*, *K. variicola* strain KV37-1036, and other diazotrophs show that replacement of *nifL* with a constitutive promoter results in increased expression of *nifA*, which in turn increases expression of the nitrogenase complex by activating transcription of the *nif* genes required for nitrogenase synthesis.[24,53] These modifications have resulted in an over-production of ammonia, which can increase nitrogen availability for symbiont crops. Using our Cas9-editing strategy, we created an Δ*nifL*::J23101 promoter mutant in both organisms to recapitulate these findings. While this change resulted in a significant difference in nitrogenase activity, we did not observe the deregulation from extracellular ammonia concentrations as expected. Rather, complete removal of the *nifL* CDS resulted in abolishment of nitrogen fixation activity, with nitrogenase activity comparable to a Δ*nifH* strain (Figure S6). Interestingly, fixation did not occur even under conditions where extracellular ammonia was absent, indicating that these strains did not fix nitrogen even when it was essential. Through the construction of these initial mutants, we observed that *K. variicola* was more responsive to our genetic engineering tools, with a higher rate of transformation success. Since *K. variicola* was most amenable to Cas9-mediated editing, we decided to further explore other gene expression mutations for deregulating ammonia production. In particular, we cleanly knocked out *nifL* with no promoter as well as made a knockout of the ammonia importer (Δ*amtB*) in a *nifL* knockout strain. However, none of the genomic knockouts fixed nitrogen more robustly than the wild-type strain, even in the absence of a fixed nitrogen source. Other previous work with nitrogenase deregulation in *K. variicola* has left parts of the N and C terminus of the protein CDS intact to minimize operon disruption,[24] which may explain the differences in fixation between our observations and prior literature.

With our *K. variicola* isolate, we instead aimed to have nitrogenase respond to plant-relevant signals. Arabinose is a carbohydrate source found in both root exudates and mucilage of aerial roots,[54,55] making it a useful metabolite to use as a proof-of-concept for plant-inducible controls. We knocked out *nifL* in *K. variicola* and replaced it with an arabinose promoter cassette. This strain did not fix nitrogen when arabinose was absent, even under nitrogen-free conditions, but showed nitrogenase activity at levels comparable to wild type upon arabinose induction (Figure 5). When ammonia is present in the media, this strain still sees some inducible activity, albeit with a weaker expression upon the same level of arabinose induction. This fixation occurs even at a low arabinose concentration relative to the maximum induction seen with sfGFP (1.33 vs 40 mM), indicating that using these signals to induce gene expression could be promising even in suboptimal conditions.

In both *K. variicola* and *S. stutzeri*, our Cas9 knockout strategies had high rates of success. The one-step method for *K. variicola* demonstrated 93% efficiency for the creation of Δ*nifL::araC-P_{araBAD}-nifA* and 100% efficiency for creating the Δ*nifH* strains. Similarly, the two-step method showed 67% deletion efficiency for creating a Δ*nifL::J*23101 strain in *S. stutzeri* and a 100% efficiency for creating a Δ*nifH* strain, indicating that our λRed/Cas9 two-plasmid system is usable in several diazotrophs for reliable editing. The editing efficiencies in *S. stutzeri* are comparable, though slightly lower on average, to the editing efficiencies seen in the two-step protocol previously in *P. putida*, where editing efficiencies were approximately 93% across four deletions. *K. variicola* seems to show more robust editing efficiencies using the one-step protocol compared to *P. putida*, as the one-step protocol only had an editing efficiency of approximately 5% when used prior.[50]

Heterologous Production of Alkylresorcinols in *K. variicola*. With *K. variicola* as our most promising chassis organism for rapid genome editing and reliable expression, we demonstrated gain-of-function in this organism using genomic insertion of the *sbARS2* gene from *Sorghum bicolor* as a first step toward generating relevant bioactive rhizosphere compounds. The *sbARS2* gene encodes a type III PKS that generates alkylresorcinol products from the condensation of long-chain CoA species with malonyl CoA. In *S. bicolor*, generating this alkylresorcinol is a key step in the pathway to generate sorgoleone, a desirable bioactive end product.[56] In the rhizosphere, sorgoleone acts as both an herbicide as well as a nitrification inhibitor, making it a desirable product to improve fertilization efficiency for crops.[28,29] By integrating *sbARS2* into the genome of *K. variicola* under arabinose induction (genotype *yciA-araC-P_{araBAD}-sbARS2*), we demonstrated gas chromatography−mass spectrometry (GC−MS) peaks consistent with the production of resorcinol products that are absent in the wild-type and uninduced strains (Figure 6). These alkylresorcinols

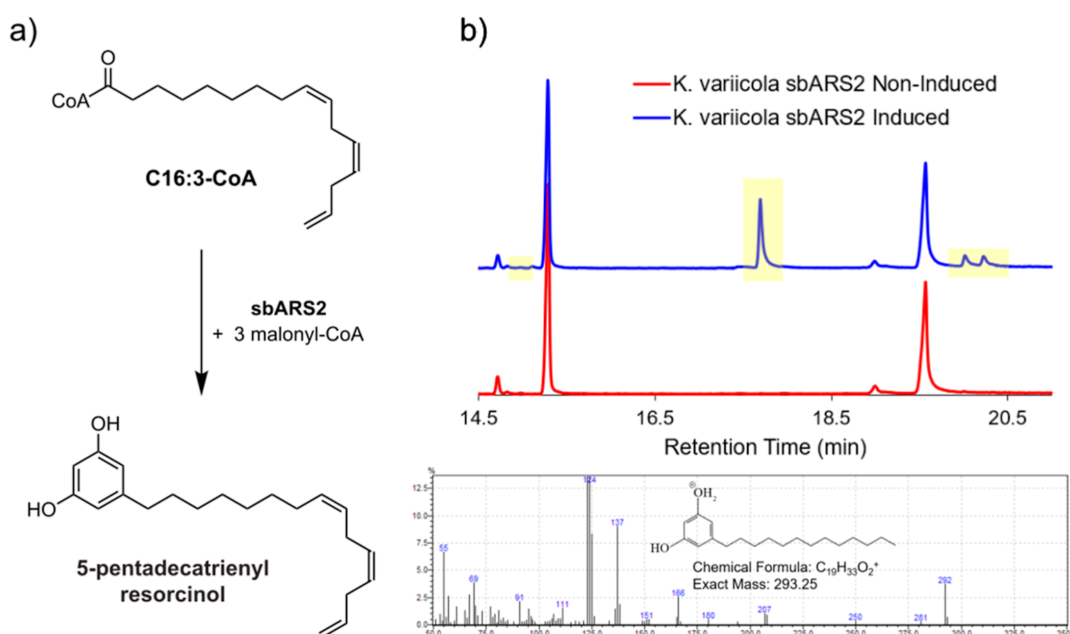


Figure 6. Alkylresorcinol generated in *K. variicola* eluted between 14 and 21 min with *sbARS*2 integrated into the genome. (a) Native alkylresorcinol synthesis reaction catalyzed by *sbARS*2 in *S. bicolor*. (b) In the supernatant extract of *K. variicola*, the MS fragmentation pattern shown was not seen in uninduced samples and indicates production of C14 alkylresorcinols as the dominant product, with a small amount of C12 and C16 products also produced (smaller peaks highlighted in yellow). The chemical structure from a similarity search of the NIST database is shown.

are also secreted into the supernatant, indicating that they would be released into the rhizosphere environment by our engineered *K. variicola* strain. Due to the promiscuity of *sbARS*2 for different CoA species, we saw a variety of alkylresorcinol chain lengths at different retention times (Figure S7). The heterologous production of alkylresorcinol products is the first major committed step to generate sorgoleone as well as other metabolites in *K. variicola*, which could be beneficial in the rhizosphere environment for engineering novel plant−microbe interactions. The lack of an antibiotic marker also facilitates repeated integrations, allowing for several engineering strategies to be employed at once for maximizing the benefits of engineered bacteria. Through this proof-of-concept integration, we observed a 29% efficiency for the integration of *sbARS*2.

Discussion. Working with nonmodel microbes often requires several rounds of trial and error to find systems that are robust for different organisms, and diazotrophs are no exception. The variability of expression from broad-host plasmids, even when taken from close relatives, makes it difficult to predict which plasmids will yield the best expression in different organisms. This inconsistency slows progress when evaluating multiple organisms simultaneously, as many parts need to be redesigned at each step; for example, BBR1 was the only reliable plasmid origin for all three organisms in our hands. Even with transformable plasmids, the expression strength remains varied, making transient plasmid systems difficult to tune for many different cases or organisms. Despite starting with a highly expressed promoter in all three organisms, as was the case with the promoter library, there was a wide range of responses to promoter modulation even with a strong initial promoter. For example, the original sequence was the strongest library member in *A. vinelandii*, but middling in *K. variicola*. Evaluating modular synthetic biology parts in several organisms eases the onboarding of heterologous expression cassettes for future work; the efficacy of these initial tools in an organism is often a harbinger of future expression, allowing for the selection of an ideal chassis.

Genome integration remains a reliable strategy for ensuring stability, and the Cas9/λ-Red strategy presented here is a reliable counterselection to avoid background wild-type cells without the use of antibiotic markers. Cas9 positive selection can also be propagated over several passages for complete removal of wild-type cells or heterozygous colonies, which allows for more robust genomic engineering and iterative knockouts that can recycle antibiotic cassettes. However, since Cas9 is constitutively expressed in our plasmids, these systems may not be reliable for organisms with higher basal expression, as Cas9 expression can be toxic at high levels even without a gRNA.[61,62] Having a dual-inducible system may allow for this system to be portable to other organisms, streamlining onboarding of new strains. While we attempted to create one-plasmid, inducible systems, we were ultimately unable to provide a strong enough selective pressure to create homozygous knockouts, and heterozygous colonies reverted to the wild type when patch-plated over several generations. Successful transformation of *S. stutzeri* with the Cas9- and pgRNA-editing plasmids was also less reliable, and transformations often needed to be repeated to yield edited colonies. The one-step protocol for Cas9/λ-Red recombineering in *S. stutzeri* yielded no colonies when attempted.

Nonetheless, using previously studied broad-host plasmids and promoters, we were able to successfully transform and evaluate the performance of several constitutive and inducible expression systems in three different soil microbes. From these initial tests, we were able to construct systems for scarless genome integration in two of these nitrogen-fixing organisms. Additionally, we demonstrated the validity of these knockouts by modulating the nitrogen fixation activity of these strains. Finally, as a proof-of-concept for functional gene insertions, we produced alkylresorcinol metabolites as potential sorgoleone precursors in *K. variicola* through scarless genome integration,

https://doi.org/10.1021/acssynbio.3c00414
ACS Synth. Biol. 2023, 12, 3623−3634

which may be useful for slowing denitrification or deterring growth of fungal pathogens in the soil environment. Future work could involve developing chain-length specificity and quantifying release of these alkylresorcinols into the media as well as developing bacteria that respond to relevant root exudates for robust fixation and association. Synthetic biology tools for diazotrophs will be essential for modulating their nitrogenase activity and secondary metabolite production in engineered rhizospheres.

■ METHODS

Plasmids, Bacterial Strains, and Growth Conditions. The bacterial strains and plasmids used in this study are shown in Tables S1 and S2, respectively. *E. coli* DH5*α*, *K. variicola* A3, and *S. stutzeri* A1501 were grown in LB medium at 37 °C. For antibiotic selection, LB medium was supplemented with kanamycin (50 *μ*g/mL, Kan50), gentamicin (30 *μ*g/mL, Gent30), carbenicillin (34 *μ*g/mL, Cm34), or tetracycline (10 *μ*g/mL, Tet10) for plasmid maintenance. *A. vinelandii* was grown in Burk's medium (0.89 g/L K_2HPO_4, 0.22 g/L KH_2PO_4, 20 g/L sucrose, 0.2 g/L $MgSO_4·7H_2O$, 0.119 g/L $CaCl_2$, 7.35 mg/L $CuSO_4$, 69.4 mg/L $Na_2B_4O_7·10H_2O$, 48.4 mg/L $MnSO_4·2H_2O$, 2 mg/L $Na_2MoO_4·2H_2O$, 29.9 mg/L $ZnSO_4·7H_2O$, 14.4 mg/L $FeSO_4·7H_2O$, and 19.3 mg/L $Na_2EDTA·2H_2O$) at 30 °C. Burk's medium was supplemented with kanamycin (0.5 *μ*g/mL, Kan0.5) and carbenicillin (50 *μ*g/mL, Carb50) for plasmid maintenance in *A. vinelandii*, as well as with NH_4OAc (15 mM) for testing constitutive and inducible expression from plasmids in *A. vinelandii*. Burk's semisolid media (Burk's + 1% agar) was used for ARAs for *K. variicola* and *S. stutzeri.*

Isolation of Sorghum Diazotrophs and Plant Growth-Promoting Trait Characterization. Wild-type diazotrophs were isolated on nitrogen-free, solid artificial mucilage media (Fahraeus medium supplemented with 113.7 *μ*g/mL arabinose, 354.4 *μ*g/mL glucose, and 377 *μ*g/mL fucose) by streaking mucilage onto plates, incubating at 28 °C for 2−4 days, and using 16S primers to identify individual colonies (forward primer 5′-ACTCCTACGGGAGGCAGCAGT, reverse primer 5′-TACGGTTACCTTGTTACGACTT). Molecular confirmation of *nifH* (IGK3-GCIWTHTAYGGIAARGGIGGIATHG-GIAA, DVV-ATIGCRAAICCICCRCAIACIACRTC) was performed as described for all organisms.[33]

All strains were assayed for the following plant growth-promoting traits: auxin production using Salkowski's reagent,[34] organic and inorganic phosphate solubilization,[35] siderophore production,[36] and ACC deaminase activity.[37] For auxin production, strains were incubated overnight at 28 °C in TY broth supplemented with additional tryptophan before being incubated with Salkowski reagent and quantifying indole on a Biotek plate reader. For phosphate solubilization, strains were incubated on plates at 28 °C of 10 g/L glucose, 0.5 g/L $(NH_4)_2SO_4$, 0.5 g/L yeast extract, 0.3 g/L NaCl, 0.3 g/L KCl, 0.03 g/L $FeSO_4·7\,H_2O$, 0.3 g/L $MgSO_4·7\,H_2O$, and 0.03 g/L $MnSO_4·4H_2O$ for 2−4 days supplemented with organic or inorganic sources of phosphate (0.2 g/L lecithin with 1 g/L $CaCO_3$ or 5 g/L $Ca_3(PO_4)_2$, respectively). Production of a cleared plate halo (solubilization positive) was recorded. For siderophore production, strains were incubated on TY-CAS/HDTMA plates for 48 h at 28 °C, and production of orange halos (siderophore positive) was recorded. For ACC deaminase activity, strains were incubated at 28 °C for 2−4 days on plates with ACC as the sole nitrogen source [0.1% sucrose, 0.1% 1-aminocyclopropane-1-carboxylic acid (MP Biomedicals), 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.013% $CaCl_2$, and 0.013% $FeSO_4$], and growth was recorded.

Molecular Techniques. For cloning, plasmids were isolated by using a QIAprep Spin Miniprep Kit, and oligonucleotides were purchased from IDT. Q5 High-Fidelity 2× Master Mix (NEB) and GoTaq Green (Promega) were used for PCR amplification and colony PCRs, respectively. Gibson Assembly[57] was used to create knockout and insertion plasmids for genomic modifications, and these plasmids were subsequently used as PCR templates to create linear repair templates for genome editing. Blunt end ligation (T4 PNK/T4 DNA Ligase, NEB) was performed to swap out the guide sequences in the gRNA plasmids. The *sbARS*2 coding sequence was codon-optimized from the *Sorghum bicolor* sequence (accession number XM_002441794) using an online codon optimization tool (Integrated DNA Technologies). The optimized sequence was synthesized with an N-terminal histidine tag (Twist Biosciences). Plasmids and colony PCRs were sequenced either through Sanger Sequencing (Functional Biosciences) or through next-generation sequencing (Plasmidsaurus).

Transformation. All transformation methods were adapted from Choi and Schweizer.[58] For the transformation of *K. variicola*, *A. vinelandii*, and *S. stutzeri*, 3 mL of stationary phase cultures were spun down at 7000*g* for 2 min. Cells were washed twice with either ice-cold 300 mM sucrose (*A. vinelandii* and *S. stutzeri*) or 10% glycerol (*K. variicola*) and then resuspended in 300 *μ*L of their respective wash solutions. Fifty microliters of washed cells were mixed with 1−5 *μ*L of DNA (100 ng/*μ*L to 1 *μ*g/mL). The mixture was transferred to 1 mm electroporation cuvettes and electroporated using a Bio-Rad Micropulser set to 1.8 kV (Ec1 preset). Cells were recovered in 1 mL of LB media (*K. variicola* or *S. stutzeri*) or 1 mL of Burk's medium (*A. vinelandii*) for 1 h following electroporation, then 100−200 *μ*L of cells were spread on Burk's media plates with the appropriate antibiotics. Transformation efficiencies for different plasmid backbones were calculated by dividing the average number of CFUs in the outgrowth by the amount of DNA added to the cuvette.

Additionally, triparental mating was used to transform *S. stutzeri* A1501 with an integrated suicide vector for Cas9 editing. *S. stutzeri* harboring pMVV205 (pBBR1-*λ*Red/Cas9) were grown overnight in 5 mL LB-Gent30, along with *E. coli* cells harboring the suicide vector (a ColE1 backbone grown in LB-Kan50) and *E. coli* cells harboring conjugation helper plasmid pRK600 (LB-Cm34).[43] The following morning, 100 *μ*L was taken from each stationary phase culture and mixed with 700 *μ*L of LB. Cells were spun down at 7000*g* for 2 min and resuspended in 1 mL of fresh LB twice. Finally, the pellet was resuspended in 50 *μ*L of LB and placed on a 13 mm mixed cellulose ester membrane on an LB plate. Cells were grown at 37 °C for 8 h, resuspended in 200 *μ*L sterile phosphate-buffered saline (PBS), and plated on LB Gent30/Kan50 to select for *S. stutzeri* cells with a successful single-crossover event of the antibiotic marker.

Quantifying Promoter Expression. Strains containing sfGFP expression plasmids were grown overnight in LB Kan50 for *S. stutzeri* and *K. variicola* or for 72 h in Burk's medium supplemented with Kan0.5 for *A. vinelandii*. After outgrowth, stationary phase cells were inoculated at an OD_{600} of ∼0.1 in 200 *μ*L of fresh LB or Burk's + NH_4OAc with appropriate antibiotics in a Corning 96-well plate with black walls and flat clear bottom. Samples were incubated on a shaking platform for 24 h (*K. variicola* and *S. stutzeri*), or for 60 h (*A. vinelandii*), then OD_{600} and fluorescence measurements were taken using an M1000

https://doi.org/10.1021/acssynbio.3c00414
ACS Synth. Biol. 2023, 12, 3623−3634

Tecan plate reader, using excitation/emission values of 485/510 nm for samples expressing sfGFP at a manual instrument gain of 90. Fluorescence/OD_{600} values are reported at 24 h for *K. variicola* and *S. stutzeri* and 60 h for *A. vinelandii*.

Genome Editing in *K. variicola* and *S. stutzeri*. For genome editing of *K. variicola*, cells were transformed by using a one-step λRed/Cas9 recombineering protocol. *K. variicola* cells containing pJMR52 (pRK2-λRed/Cas9) were grown overnight in 5 mL LB-Gent30. The following day, the expression of the λRed genes was induced with 0.2% L-arabinose, and cells were incubated for 45 min at 37 °C. These cultures were used to prepare electrocompetent cells, as described above. Cells were transformed with ∼500 ng pgRNA plasmid and ∼500 ng linear repair template by electroporation and allowed to recover in 1 mL of LB for 3 h at 37 °C. The recovered cells were selected on LB Gent30/Tet10. Transformants were screened for the desired knockout or insertion using colony PCR with primers flanking the gene of interest. After verifying the presence of the mutation, cells were passaged with antibiotics for the gRNA and Cas9 plasmids for 48 h to remove the remaining wild-type DNA. Following passaging, all positive hits were screened for the desired knockout or insertion. Colonies were also screened with a secondary colony PCR to check for the complete removal of wild-type DNA. Plasmids were cured from *K. variicola* by growing the cells overnight for 48 h in LB media without antibiotics and plating on LB agar. Single colonies were screened for loss of plasmid-based antibiotic resistance by patch plating.

For genome editing in *S. stutzeri*, cells were edited with a two-step recombineering protocol. Cells containing pMVV205 (pBBR1-λRed/Cas9) were grown overnight in LB-Gent30. In the first step, *S. stutzeri* cells were then transformed with a suicide plasmid containing the repair template of interest using conjugation, as described above. These colonies were subsequently grown overnight in 5 mL of LB-Gent30, Kan50, then induced with 0.2% L-arabinose and incubated for 1 h at 37 °C. Cells were transformed with 1 μg of pgRNA plasmid, then recovered for 4 h in 1 mL of LB at 37 °C before plating on LB Gent30, Tet10. Transformants were subsequently screened and cured of plasmids identically to *K. variicola*. Guide sequences for Cas9 gRNAs in both strains were chosen with CasOT to minimize off-target activity.[59]

Acetylene Reduction Assay (ARA). Nitrogenase activity was estimated by ARA gas chromatography using a GC-2010 instrument (Shimadzu) equipped with a 60 m Rt-Alumina BOND/KCL column (Restek). *K. variicola* and *S. stutzeri* were grown in LB medium overnight. Bacteria were collected by centrifugation (5 min, 3000*g* at 4 °C) and suspended in 1 X PBS buffer, then resuspended to 0.1 at 600 nm (OD_{600} = 0.1). An aliquot of 3 mL of Burk's semisolid medium (Burk's + 1% agar) was added to 10 mL vials (Supelco-27303U). The medium was inoculated with 30 μL of the cells in PBS. Inoculated and control vials were incubated at 30 °C for 72 h. From each vial, 1 mL of air in the headspace was removed with a syringe, 1 mL of acetylene (99% v v^{-1}) was injected, and the vials were incubated at 30 °C for 16 h. The ethylene produced by nitrogenase activity was quantified using a standard curve with known ethylene concentrations. The protein content in Burk's semisolid medium was determined using Bradford's reagent and quantified using a standard curve of albumin.[60]

Extraction of Alkylresorcinols. To produce alkylresorcinols, the *K. variicola* strain was grown overnight. The following morning, 5 mL of LB media were inoculated to an OD_{600} of ∼0.05 and induced at an OD_{600} of 0.7 with 0.6% w/v L-arabinose. Twenty-four hours after inoculation, cells from the 5 mL culture were collected via centrifugation and separated from the supernatant. The metabolites from the cell-free supernatant were extracted into 4 mL ethyl acetate. After vortexing the ethyl-acetate/supernatant mixture vigorously for 3 min, the mixture was placed on a shaking platform for 2 h, then centrifuged at 1000*g* for 10 min. The top layer of ethyl acetate was removed and evaporated under reduced pressure. The product was resuspended in 200 μL of fresh ethyl acetate for GC−MS analysis.

GC/MS Analysis of Alkylresorcinols. GC−MS was performed on a coupled Shimadzu GCMS-QP2010S. One microliter of alkylresorcinol extracts was injected into an HP-5 ms Ultra Inert column (5% phenyl-methylpolysiloxane; 30 m length; 0.25 mm inner diameter; and 0.25 μm film thickness; Agilent) with a split ratio of 1:10. The extracts were separated using the following temperature program: 120 °C (2 min), 16 °C min^{-1} to 200 °C, 6 °C min^{-1} to 320, and 320 °C (15 min). The column flow rate was 1.50 mL min^{-1} with an injection temperature of 320 °C. Helium was used as the carrier gas, with an inlet pressure of 1.2 bar. The mass spectra were recorded at an ion source and interface temperature of 230 and 275 °C, respectively. The spectra were collected from the *m*/*z* range of 50−500.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.3c00414.

Additional isolated diazotrophs, list of strains and plasmids used in this study, additional data for induction system functionality, and additional MS data (PDF)

DNA sequences for all sequenced constitutive promoters from the library (XLSX)

GenBank (.gb) files for all plasmids constructed for this study (ZIP)

Sequencing for all edited loci (ZIP)

AUTHOR INFORMATION

Corresponding Author

Brian Pfleger − *Department of Chemical and Biological Engineering, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States; Microbiology Doctoral Training Program, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States;* orcid.org/0000-0002-9232-9959; Email: brian.pfleger@wisc.edu

Authors

Maya Venkataraman − *Department of Chemical and Biological Engineering, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States*

Audrey Yñigez-Gutierrez − *Department of Chemical and Biological Engineering, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States*

Valentina Infante − *Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States*

April MacIntyre − *Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States; Valent BioSciences, Libertyville, Illinois 60048, United States;* orcid.org/0000-0002-1867-6661

https://doi.org/10.1021/acssynbio.3c00414
ACS Synth. Biol. 2023, 12, 3623−3634

Paulo Ivan Fernandes-Júnior − *Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States; Brazilian Agricultural Research Corporation (Embrapa), Tropical Semi-Arid Research Center (Embrapa Semiárido), Petrolina, Pernambuco 56302-970, Brazil*

Jean-Michel Ané − *Department of Bacteriology and Microbiology Doctoral Training Program, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States*

Complete contact information is available at:
https://pubs.acs.org/10.1021/acssynbio.3c00414

Author Contributions

MV, AYG, VI, and PIF designed and executed the experiments. AMM isolated, characterized the plant growth-promoting traits, and sequenced the *K. variicola* A3 strain. MV wrote the manuscript and created all figures with critical feedback and help from VI, JMA, and BFP.

Funding

This work was partly supported by a US Department of Energy grant #DE-SC0021052 and a US Department of Agriculture grant #2020-67013-32675 to JMA. This material is based upon work supported in part by the Great Lakes Bioenergy Research Center, US Department of Energy, Office of Science, Office of Biological and Environmental Research under award numbers DE-SC0018409 and DE-FC02-07ER64494 to BFP and JMA. MV is the recipient of a Graduate Research Fellowship from the National Science Foundation and a traineeship through the NIH Biotechnology Training Program at UW-Madison (NIGMS T32 GM135066).

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Pankievicz, V. C. S.; Irving, T. B.; Maia, L. G. S.; Ané, J. M. Are We There yet? The Long Walk towards the Development of Efficient Symbiotic Associations between Nitrogen-Fixing Bacteria and Non-Leguminous Crops. *BMC Biol.* **2019**, *17*, 99.

(2) Pankievicz, V. C. S.; do Amaral, F. P.; Ané, J. M.; Stacey, G. Diazotrophic Bacteria and Their Mechanisms to Interact and Benefit Cereals. *Mol. Plant-Microbe Interact.* **2021**, *34*, 491−498.

(3) Teh, M. Y.; Ooi, K. H.; Teo, S. X. D.; Mansoor, M. E. B.; Lim, W. Z. S.; Tan, M. H. An Expanded Synthetic Biology Toolkit for Gene Expression Control in Acetobacteraceae. *ACS Synth. Biol.* **2019**, *8* (4), 708−723.

(4) Markley, A. L.; Begemann, M. B.; Clarke, R. E.; Gordon, G. C.; Pfleger, B. F. Synthetic Biology Toolbox for Controlling Gene Expression in the Cyanobacterium Synechococcus Sp. Strain PCC 7002. *ACS Synth. Biol.* **2015**, *4* (5), 595−603.

(5) Desnoues, N.; Lin, M.; Guo, X.; Ma, L.; Carreño-Lopez, R.; Elmerich, C. Nitrogen Fixation Genetics and Regulation in a Pseudomonas stutzeri Strain Associated with Rice. *Microbiology (N. Y.)* **2003**, *149* (8), 2251−2262.

(6) Xie, Z.; Dou, Y.; Ping, S.; Chen, M.; Wang, G.; Elmerich, C.; Lin, M. Interaction between nifL and nifA in the Nitrogen-Fixing Pseudomonas stutzeri A1501. *Microbiology* **2006**, *152* (12), 3535−3542.

(7) Zhan, Y.; Yan, Y.; Deng, Z.; Chen, M.; Lu, W.; Lu, C.; Shang, L.; Yang, Z.; Zhang, W.; Wang, W.; Li, Y.; Ke, Q.; Lu, J.; Xu, Y.; Zhang, L.; Xie, Z.; Cheng, Q.; Elmerich, C.; Lin, M. The Novel Regulatory NcRNA, NfiS, Optimizes Nitrogen Fixation via Base Pairing with the Nitrogenase Gene nifK MRNA in Pseudomonas stutzeri A1501. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113* (30), E4348−E4356.

(8) Zhan, Y.; Deng, Z.; Yan, Y.; Zhang, H.; Lu, C.; Yang, Z.; Shang, L.; Huang, Y.; Lv, F.; Liu, Y.; Liu, Y.; Wang, S.; Chen, S.; Zhang, X.-X.; Cheng, Q.; Lin, M. NfiR, a New Regulatory Noncoding RNA (NcRNA), Is Required in Concert with the NfiS NcRNA for Optimal Expression of Nitrogenase Genes in Pseudomonas stutzeri A1501. *Appl. Environ. Microbiol.* **2019**, *85*, July.

(9) Shang, L.; Yan, Y.; Zhan, Y.; Ke, X.; Shao, Y.; Liu, Y.; Yang, H.; Wang, S.; Dai, S.; Lu, J.; Yan, N.; Yang, Z.; Lu, W.; Liu, Z.; Chen, S.; Elmerich, C.; Lin, M. A Regulatory Network Involving Rpo, Gac and Rsm for Nitrogen-Fixing Biofilm Formation by Pseudomonas stutzeri. *npj Biofilms Microbiomes* **2021**, *7* (1), 54.

(10) Carruthers, B. M.; Garcia, A. K.; Rivier, A.; Kacar, B. Automated Laboratory Growth Assessment and Maintenance of Azotobacter vinelandii. *Curr. Protoc.* **2021**, *1* (3), No. e57.

(11) Eberhart, L. J.; Knutson, C. M.; Barney, B. M. A Methodology for Markerless Genetic Modifications in Azotobacter vinelandii. *J. Appl. Microbiol.* **2016**, *120* (6), 1595−1604.

(12) Pérez-González, A.; Jimenez-Vicente, E.; Gies-Elterlein, J.; Salinero-Lanzarote, A.; Yang, Z. Y.; Einsle, O.; Seefeldt, L. C.; Dean, D. R. Specificity of NifEN and VnfEN for the Assembly of Nitrogenase Active Site Cofactors in Azotobacter vinelandii. *mBio* **2021**, *12* (4), 10.

(13) Chisnell, J. R.; Premakumar, R.; Bishop, P. E. Purification of a Second Alternative Nitrogenase from a nifHDK Deletion Strain of Azotobacter vinelandii. *J. Bacteriol.* **1988**, *170* (1), 27−33.

(14) Yang, J.; Xie, X.; Wang, X.; Dixon, R.; Wang, Y. P. Reconstruction and Minimal Gene Requirements for the Alternative Iron-Only Nitrogenase in Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111* (35), No. E3718.

(15) Keable, S. M.; Vertemara, J.; Zadvornyy, O. A.; Eilers, B. J.; Danyal, K.; Rasmussen, A. J.; De Gioia, L.; Zampella, G.; Seefeldt, L. C.; Peters, J. W. Structural Characterization of the Nitrogenase Molybdenum-Iron Protein with the Substrate Acetylene Trapped near the Active Site. *J. Inorg. Biochem.* **2018**, *180*, 129−134.

(16) Georgiadis, M. M.; Komiya, H.; Chakrabarti, P.; Woo, D.; Kornuc, J. J.; Rees, D. C. Crystallographic Structure of the Nitrogenase Iron Protein from Azotobacter vinelandii. *Science* **1992**, *257* (5077), 1653−1659.

(17) Ortiz-Marquez, J. C. F.; Do Nascimento, M.; Curatti, L. Metabolic Engineering of Ammonium Release for Nitrogen-Fixing Multispecies Microbial Cell-Factories. *Metab. Eng.* **2014**, *23*, 154−164.

(18) Barney, B. M.; Eberhart, L. J.; Ohlert, J. M.; Knutson, C. M.; Plunkett, M. H. Gene Deletions Resulting in Increased Nitrogen Release by Azotobacter vinelandii: Application of a Novel Nitrogen Biosensor. *Appl. Environ. Microbiol.* **2015**, *81* (13), 4316−4328.

(19) Ambrosio, R.; Ortiz-Marquez, J. C. F.; Curatti, L. Metabolic Engineering of a Diazotrophic Bacterium Improves Ammonium Release and Biofertilization of Plants and Microalgae. *Metab. Eng.* **2017**, *40* (January), 59−68.

(20) Milenkov, M.; Thummer, R.; Glöer, J.; Grötzinger, J.; Jung, S.; Schmitz, R. A. Insights into Membrane Association of Klebsiella pneumoniae nifL under Nitrogen-Fixing Conditions from Mutational Analysis. *J. Bacteriol.* **2011**, *193* (3), 695−705.

(21) Dixon, R. A.; Buck, M.; Drummond, M.; Hawkes, T.; Khan, H.; Macfarlane, S.; Merrick, M.; Postgate, J. R. Regulation of the Nitrogen Fixation Genes in Klebsiella pneumoniae: Implications for Genetic Manipulation. *Dev. Plant Soil Sci.* **1986**, *90*, 225−233.

(22) Mechan-Llontop, M. E.; Mullet, J.; Shade, A. Phyllosphere Exudates Select for Distinct Microbiome Members in Sorghum Epicuticular Wax and Aerial Root Mucilage. *Phytobiomes J.* **2023**, *7* (2), 184−197.

(23) Zhang, L.; Zhang, M.; Huang, S.; Li, L.; Gao, Q.; Wang, Y.; Zhang, S.; Huang, S.; Yuan, L.; Wen, Y.; Liu, K.; Yu, X.; Li, D.; Zhang, L.; Xu, X.; Wei, H.; He, P.; Zhou, W.; Philippot, L.; Ai, C. A Highly Conserved Core Bacterial Microbiota with Nitrogen-Fixation Capacity Inhabits the Xylem Sap in Maize Plants. *Nat. Commun.* **2022**, *13* (1), 3361.

(24) Wen, A.; Havens, K. L.; Bloch, S. E.; Shah, N.; Higgins, D. A.; Davis-Richardson, A. G.; Sharon, J.; Rezaei, F.; Mohiti-Asli, M.; Johnson, A.; Abud, G.; Ane, J. M.; Maeda, J.; Infante, V.; Gottlieb, S. S.;

Lorigan, J. G.; Williams, L.; Horton, A.; McKellar, M.; Soriano, D.; Caron, Z.; Elzinga, H.; Graham, A.; Clark, R.; Mak, S. M.; Stupin, L.; Robinson, A.; Hubbard, N.; Broglie, R.; Tamsir, A.; Temme, K. Enabling Biological Nitrogen Fixation for Cereal Crops in Fertilized Fields. *ACS Synth. Biol.* **2021**, *10* (12), 3264−3277.

(25) Bloch, S. E.; Temme, K.; Tamsir, A.; Higgins, D.; Davis-Richardson, A.; Clark, R.; Gottlieb, S. Guided microbial remodeling, a platform for the rational improvement of microbial species for agriculture. US20220127627A1, 2020.

(26) Baerson, S. R.; Rimando, A. M.; Pan, Z. Probing Allelochemical Biosynthesis in Sorghum Root Hairs. *Plant Signal. Behav.* **2008**, *3* (9), 667−670.

(27) Tesfamariam, T.; Yoshinaga, H.; Deshpande, S. P.; Srinivasa Rao, P.; Sahrawat, K. L.; Ando, Y.; Nakahara, K.; Hash, C. T.; Subbarao, G. V. Biological Nitrification Inhibition in Sorghum: The Role of Sorgoleone Production. *Plant Soil* **2014**, *379* (1−2), 325−335.

(28) Sarr, P. S.; Ando, Y.; Nakamura, S.; Deshpande, S.; Subbarao, G. V. Sorgoleone Release from Sorghum Roots Shapes the Composition of Nitrifying Populations, Total Bacteria, and Archaea and Determines the Level of Nitrification. *Biol. Fertil. Soils* **2020**, *56* (2), 145−166.

(29) Uddin, M. R.; Park, S. U.; Dayan, F. E.; Pyon, J. Y. Herbicidal Activity of Formulated Sorgoleone, a Natural Product of Sorghum Root Exudate. *Pest Manag. Sci.* **2014**, *70* (2), 252−257.

(30) Dayan, F. E.; Howell, J.; Weidenhamer, J. D. Dynamic Root Exudation of Sorgoleone and Its in Planta Mechanism of Action. *J. Exp. Bot.* **2009**, *60* (7), 2107−2117.

(31) Suzuki, Y.; Kono, Y.; Inoue, T.; Sakurai, A. A Potent Antifungal Benzoquinone in Etiolated Sorghum Seedlings and Its Metabolites. *Phytochemistry* **1998**, *47* (6), 997−1001.

(32) Van Deynze, A.; Zamora, P.; Delaux, P. M.; Heitmann, C.; Jayaraman, D.; Rajasekar, S.; Graham, D.; Maeda, J.; Gibson, D.; Schwartz, K. D.; Berry, A. M.; Bhatnagar, S.; Jospin, G.; Darling, A.; Jeannotte, R.; Lopez, J.; Weimer, B. C.; Eisen, J. A.; Shapiro, H. Y.; Ané, J. M.; Bennett, A. B. Nitrogen Fixation in a Landrace of Maize Is Supported by a Mucilage-Associated Diazotrophic Microbiota. *PLoS Biol.* **2018**, *16* (8), No. e2006352.

(33) Gaby, J. C.; Rishishwar, L.; Valderrama-Aguirre, L. C.; Green, S. J.; Valderrama-Aguirre, A.; Jordan, I. K.; Kostka, J. E. Diazotroph Community Characterization via a High-Throughput nifH Amplicon Sequencing and Analysis Pipeline. *Appl. Environ. Microbiol.* **2018**, *84* (4), No. e01512.

(34) Gang, S.; Sharma, S.; Saraf, M.; Buck, M.; Schumacher, J. Analysis of Indole-3-Acetic Acid (IAA) Production in Klebsiella by LC-MS/MS and the Salkowski Method. *Bio-Protocol* **2019**, *9* (9), No. e3230.

(35) Chen, Q.; Liu, S. Identification and Characterization of the Phosphate-Solubilizing Bacterium Pantoea Sp. S32 in Reclamation Soil in Shanxi, China. *Front. Microbiol.* **2019**, *10*, 2171.

(36) Schwyn, B.; Neilands, J. B. Universal Chemical Assay for the Detection and Determination of Siderophores. *Anal. Biochem.* **1987**, *160*, 47−56.

(37) Honma, M.; Shimomura, T. Metabolism of 1-Aminocyclopropane-1-Carboxylic Acid. *Agric. Biol. Chem.* **1978**, *42* (10), 1825−1831.

(38) Thomas, C. M. Molecular Genetics of Broad Host Range Plasmid RK2. *Plasmid* **1981**, *5* (1), 10−19.

(39) Antoine, R.; Locht, C. Isolation and Molecular Characterization of a Novel Broad-host-range Plasmid from Bordetella Bronchiseptica with Sequence Similarities to Plasmids from Gram-positive Organisms. *Mol. Microbiol.* **1992**, *6* (13), 1785−1799.

(40) Scholz, P.; Haring, V.; Wittmann-Liebold, B.; Ashman, K.; Bagdasarian, M.; Scherzinger, E. Complete Nucleotide Sequence and Gene Organization of the Broad-Host-Range Plasmid RSF1010. *Gene* **1989**, *75* (2), 271−288.

(41) Olsen, R. H.; DeBusscher, G.; McCombie, W. R. Development of Broad-Host-Range Vectors and Gene Banks: Self-Cloning of the Pseudomonas aeruginosa PAO Chromosome. *J. Bacteriol.* **1982**, *150* (1), 60−69.

(42) Silva-Rocha, R.; Martínez-García, E.; Calles, B.; Chavarría, M.; Arce-Rodríguez, A.; De Las Heras, A.; Páez-Espino, A. D.; Durante-Rodríguez, G.; Kim, J.; Nikel, P. I.; Platero, R.; De Lorenzo, V. The Standard European Vector Architecture (SEVA): A Coherent Platform for the Analysis and Deployment of Complex Prokaryotic Phenotypes. *Nucleic Acids Res.* **2013**, *41* (D1), 666−675.

(43) Chang, A. C. Y.; Cohen, S. N. Construction and Characterization of Amplifiable Multicopy DNA Cloning Vehicles Derived from the P15A Cryptic Miniplasmid. *J. Bacteriol.* **1978**, *134* (3), 1141−1156.

(44) Hershfield, V.; Boyer, H. W.; Yanofsky, C.; Lovett, M. A.; Helinski, D. R. Plasmid ColE1 as a Molecular Vehicle for Cloning and Amplification of DNA. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71* (9), 3455−3459.

(45) Shao, Y.; Yin, C.; Lv, F.; Jiang, S.; Wu, S.; Han, Y.; Xue, W.; Ma, Y.; Zheng, J.; Zhan, Y.; Ke, X.; Lu, W.; Lin, M.; Shang, L.; Yan, Y. The Sigma Factor AlgU Regulates Exopolysaccharide Production and Nitrogen-Fixing Biofilm Formation by Directly Activating the Transcription of pslA in Pseudomonas Stutzeri A1501. *Genes* **2022**, *13* (5), 867.

(46) *Anderson Promoter Collection. iGEM, Registry of Standard Biological Parts*; University of São Paulo, 2018.

(47) Salis, H. M. The Ribosome Binding Site Calculator. *Methods Enzymol.* **2011**, *438*, 19−42.

(48) Meyer, A. J.; Segall-Shapiro, T. H.; Glassey, E.; Zhang, J.; Voigt, C. A. Escherichia Coli "Marionette" Strains with 12 Highly Optimized Small-Molecule Sensors. *Nat. Chem. Biol.* **2019**, *15* (2), 196−204.

(49) Cook, T. B.; Jacobson, T. B.; Venkataraman, M. V.; Hofstetter, H.; Amador-Noguez, D.; Thomas, M. G.; Pfleger, B. F. Stepwise Genetic Engineering of Pseudomonas Putida Enables Robust Heterologous Production of Prodigiosin and Glidobactin A. *Metab. Eng.* **2021**, *67*, 112−124.

(50) 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* (7), 517−527.

(51) Ribbe, M. W.; Hu, Y.; Guo, M.; Schmid, B.; Burgess, B. K. The FeMoco-Deficient MoFe Protein Produced by a nifH Deletion Strain of Azotobacter vinelandii Shows Unusual P-Cluster Features. *J. Biol. Chem.* **2002**, *277* (26), 23469−23476.

(52) Bloch, S. E.; Clark, R.; Gottlieb, S. S.; Wood, L. K.; Shah, N.; Mak, S. M.; Lorigan, J. G.; Johnson, J.; Davis-Richardson, A. G.; Williams, L.; McKellar, M.; Soriano, D.; Petersen, M.; Horton, A.; Smith, O.; Wu, L.; Tung, E.; Broglie, R.; Tamsir, A.; Temme, K. Biological Nitrogen Fixation in Maize: Optimizing Nitrogenase Expression in a Root-Associated Diazotroph. *J. Exp. Bot.* **2020**, *71* (15), 4591−4603.

(53) Ortiz-Marquez, J. C. F.; Do Nascimento, M.; Dublan, M. d. l. A.; Curatti, L. Association with an Ammonium-Excreting Bacterium Allows Diazotrophic Culture of Oil-Rich Eukaryotic Microalgae. *Appl. Environ. Microbiol.* **2012**, *78* (7), 2345−2352.

(54) Kraffczyk, I.; Trolldenier, G.; Beringer, H. Soluble Root Exudates of Maize: Influence of Potassium Supply and Rhizosphere Microorganisms. *Soil Biol. Biochem.* **1984**, *16* (4), 315−322.

(55) Van Deynze, A.; Zamora, P.; Delaux, P.-M.; Heitmann, C.; Jayaraman, D.; Rajasekar, S.; Graham, D.; Maeda, J.; Gibson, D.; Schwartz, K. D.; Berry, A. M.; Bhatnagar, S.; Jospin, G.; Darling, A.; Jeannotte, R.; Lopez, J.; Weimer, B. C.; Eisen, J. A.; Shapiro, H.-Y.; Ané, J. M.; Bennett, A. B. Nitrogen Fixation in a Landrace of Maize Is Supported by a Mucilage-Associated Diazotrophic Microbiota. *PLoS Biol.* **2018**, *16* (8), No. e2006352.

(56) Cook, D.; Rimando, A. M.; Clemente, T. E.; Schröder, J.; Dayan, F. E.; Nanayakkara, N. D.; Pan, Z.; Noonan, B. P.; Fishbein, M.; Abe, I.; Duke, S. O.; Baerson, S. R. Alkylresorcinol Synthases Expressed in Sorghum bicolor Root Hairs Play an Essential Role in the Biosynthesis of the Allelopathic Benzoquinone Sorgoleone. *Plant Cell* **2010**, *22* (3), 867−887.

(57) Gibson, D. G.; Young, L.; Chuang, R. Y.; Venter, J. C.; Hutchison, C. A.; Smith, H. O. Enzymatic Assembly of DNA Molecules up to Several Hundred Kilobases. *Nat. Methods* **2009**, *6* (5), 343−345.

(58) Choi, K. H.; Schweizer, H. P. Mini-Tn7 Insertion in Bacteria with Single AttTn7 Sites: Example Pseudomonas aeruginosa. *Nat. Protoc.* **2006**, *1* (1), 153−161.

(59) Xiao, A.; Cheng, Z.; Kong, L.; Zhu, Z.; Lin, S.; Gao, G.; Zhang, B. CasOT: A Genome-Wide Cas9/GRNA off-Target Searching Tool. *Bioinformatics* **2014**, *30* (8), 1180−1182.
(60) Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(61) Cho, S.; Choe, D.; Lee, E.; Kim, S. C.; Palsson, B.; Cho, B. K. High-Level dCas9 Expression Induces Abnormal Cell Morphology in Escherichia coli. *ACS Synth. Biol.* **2018**, *7* (4), 1085−1094.
(62) Wendt, K. E.; Ungerer, J.; Cobb, R. E.; Zhao, H.; Pakrasi, H. B. CRISPR/Cas9 Mediated Targeted Mutagenesis of the Fast Growing Cyanobacterium Synechococcus elongatus UTEX 2973. *Microb. Cell Factories* **2016**, *15* (1), 1−8.

https://doi.org/10.1021/acssynbio.3c00414
ACS Synth. Biol. 2023, 12, 3623−3634