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Metabolic Engineering and Synthetic Biology–Original Paper

Development of modular expression across phylogenetically distinct diazotrophs

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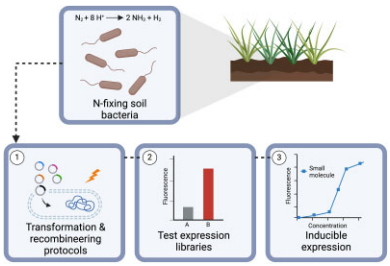
Abstract: Diazotrophic bacteria can reduce atmospheric nitrogen into ammonia enabling bioavailability of the essential element. Many diazotrophs closely associate with plant roots increasing nitrogen availability, acting as plant growth promoters. These associations have the potential to reduce the need for costly synthetic fertilizers if they could be engineered for agricultural applications. However, despite the importance of diazotrophic bacteria, genetic tools are poorly developed in a limited number of species, in turn narrowing the crops and root microbiomes that can be targeted. Here, we report optimized protocols and plasmids to manipulate phylogenetically diverse diazotrophs with the goal of enabling synthetic biology and genetic engineering. Three broad-host-range plasmids can be used across multiple diazotrophs, with the identification of one specific plasmid (containing origin of replication RK2 and a kanamycin resistance marker) showing the highest degree of compatibility across bacteria tested. We then demonstrated modular expression by testing seven promoters and eleven ribosomal binding sites using proxy fluorescent proteins. Finally, we tested four small molecule inducible systems to report expression in three diazotrophs and demonstrated genome editing in *Klebsiella michiganensis* M5al.

One-sentence summary: In this study, broad-host plasmids and synthetic genetic parts were leveraged to enable expression tools in a library of diazotrophic bacteria.

Keywords: diazotroph, biological nitrogen fixation, rhizosphere, synthetic biology

Graphical abstract



Introduction

Nitrogen is an established key nutrient for the development of plants and has a significant effect on crop yield (Lawlor et al., 2001). As a result, synthetic nitrogen sources are often added to improve crop performance. However, these synthetic fertilizers are generated using the energy intensive Haber–Bosch process which is estimated to use ∼2% of the world's energy output (Erisman et al., 2008). Synthetic fertilizers are also linked to negative environmental factors such as soil degradation and greenhouse gas emissions (Kongshaug, 1998; Sutton et al., 2011; Tripathi et al., 2020). Consequently, alternative nitrogen sources have become economically and environmentally attractive. Nitrogen-fixing bacteria, referred to as diazotrophs, possess the ability to perform biological nitrogen fixation (BNF), a potential route to lessen dependence on synthetic fertilizers. Diazotrophs can associate with crops and supply usable nitrogen (Fernandes & Rossiello, 1995; Robertson & Vitousek, 2009). Research in diazotrophs has aimed to increase the nitrogen supplied to crops and also to further understand mechanisms that lead to and sustain plant-diazotroph associations or microbe–microbe interactions in the rhizosphere to improve overall nitrogen release productivity (Pankievicz et al., 2019; Knights et al., 2021; Chakraborty et al., 2023). Despite the potential for engineering diazotrophs for improved BNF, only a limited set of tools have been developed in few diazotroph species (Venkataraman et al., 2023). Since diazotrophs are known to be highly diverse, exist in variable ecological niches, and vary in plant association, it is

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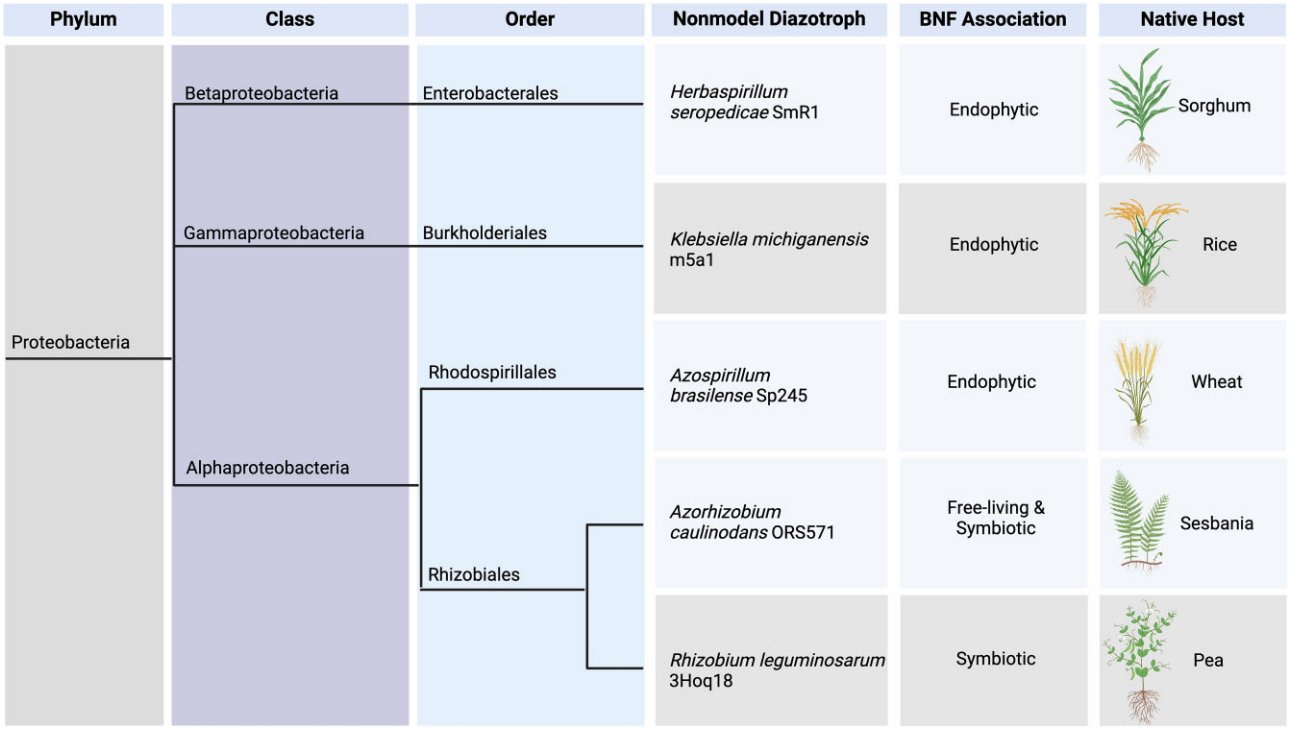

Fig. 1. Graphical representation of phylogenetic distribution. From left to right: the relative phylum, class, and order of each diazotroph (phylogenetic tree not to scale) followed by strain, nitrogen fixation modality (BNF association) as either endophytic (associated with roots), free-living (independent of plants), or symbiotic (root nodule symbiosis), and the native host from which the strain was isolated.

desirable to have genetic tools that can be applied to a wide range of diazotrophs (Pankievicz et al., 2019).

Our objective in this study was to build synthetic biology tools in a variety of diazotrophs across the proteobacterial clade to onboard potential model strains. We present here the development of plasmid-based expression systems in five diazotrophic bacterial species: *Klebsiella michiganensis* M5al (formerly referred to *K. oxytoca* M5al and *K. pneumonia* m5al) (Mahl et al., 1965), *Azospirillum brasilense* Sp245 (Baldani et al., 1983), *Herbaspirillum seropedicae* SmR1 (Pedrosa et al., 2011), *Azorhizobium caulinodans* ORS 571 (Dreyfus & Dommergues, 1981; Lee et al., 2008), and *Rhizobium leguminosarum* 3Hoq18 (Ramírez-Bahena et al., 2008). The relative phylogenetic relationships of our selected diazotrophs along with native host and their BNF modality are summarized in Fig. 1. Two of the selected diazotrophs (*R. leguminosarum,* and *Azor. caulinodans*) are of the order Rhizobiales and symbiotically form root nodules in legumes where high rates of BNF occur (Döbereiner, 1997). *Rhizobium leguminosarum* was originally isolated from pea nodules and has been shown to be as effective as fertilizer on sweet pea inoculants (Bedrous & Owais, 1982). *Azorhizobium caulinodans* is unusual in its ability to reduce nitrogen not only in tropical sesbania root nodules but also as a free-living soil microbe (Lee et al., 2008). Additionally, *Azor. caulinodans* has the ability to colonize wheat roots, increasing dry weight and nitrogen content, which makes it suitable for studying both legume and grass interactions (Sabry et al., 1997). More recently, *Azor. caulinodans* has been engineered to release extracellular ammonia (Haskett, Paramasivan et al., 2022) and express nitrogenase under the control of synthetic bacterial signals engineered into Medicago truncatula and barley (Haskett, Karunakaran et al., 2022).

The other three diazotrophs (*K. michiganensis, Azos. brasilense,* and *H. seropedicae*) are phylogenetically dispersed across alpha-, gamma-, and beta-proteobacteria and are known to form endophytic relationships with a variety of grasses; colonizing in and around root structures (Baldani et al., 1986; Orlandini et al., 2014; Yu et al., 2018). *Klebsiella michiganensis* strain m5al has long been a model for studying the molecular genetics of nitrogen fixation (Dixon & Postgate, 1971; Lin et al., 1993; Parejko & Wilson, 1970). *Klebsiella michiganensis* has also been shown to produce cell wall-degrading compounds and colonize rice seedling roots (Yu et al., 2018). *Azospirillum brasilense* natively associates with wheat and other grasses and has been shown to increase vegetative development of grape vines (Bartolini et al., 2017). *Azospirillum brasilense* has also been engineered to release ammonia through glutamine synthetase deactivation via multicopy genetic redundancy of adenylyltransferases (Schnabel & Sattely, 2021). Lastly, *H. seropedicae,* an endophyte of tropical grasses, is reported to increase crop biomass and a range of secretion systems suggesting high interaction with plants (Pedrosa et al., 2011; Alberton et al., 2013; Silveira Alves et al., 2019). Overall, endophytes make promising model organisms given their ability to associate in close proximity with crops to supply nitrogen.

Though all the selected diazotrophs have been subject to experimentation, they lack the support of tested genetic parts for stable, modular expression of exogenous DNA—an important yet time-consuming step for model strain onboarding due to the unbound experimental space needed to optimize parameters for growth and transformation. We focused on the transformation of broad-host plasmids using fluorescent markers as a proxy for heterologous gene expression. To modulate transcription and translation we explored alternative promoter sequences and ribosomal binding sites (RBSs). We show inducible gene expression with signaling molecules known to be present in root exudates. Finally, we demonstrated genome editing in *K. michiganensis* with a newly constructed recombineering system. Our results enabled the facile expression and manipulation of five diverse diazotrophs.

Table 1. Characterization of selected diazotrophs in this study

Strain	Media	Culture Temperature (°C)	Doubling time (min)	CFU/mL at 1 OD_{600}	RK2 Plasmid Kanamycin Concentration (μg/mL)	Transformation efficiency (CFUs/μg)
Azospirillum brasilsense Sp245	LB	30	85.2 ± 3.7	(1.18 ± 0.03) * 10^8	75	(2.3 ± 1.3) * 10^4
Klebsiella michiganensis m5al	LB	30	25.4 ± 2.6	(7.07 ± 1.0) * 10^7	75	(1.07 ± 0.3) * 10^4
Herbaspirillum seropedicae SmR1	LB	30	45.9 ± 8.0	(2.05 ± 0.8) * 10^8	250	(4.12 ± 0.9) * 10^3
Azorhiozbium caulinodans ORS 571	TY	28	96.6 ± 2.1	(2.32 ± 0.2) * 10^8	30	(6.45 ± 0.2) * 10^4
Rhizobium leguminosarum 3Hoq18	TY	28	127 ± 3.5	(1.67 ± 0.60) * 10^8	50	(7.33 ± 4.0) * 10^1

From left to right: diazotroph strain, growth media (LB or TY). Standard culture temperature. Doubling time in 96-well format (N.T.: not tested, strains failed to grow in 96-well plate format) (Supplementary Fig. S1). Colony formation units per 1 mL normalized to 1 OD_{600} in log phase (OD_{600} ~0.5–0.8). Kanamycin selection concentrations for each strain given as μg per mL for plasmids with origin of replication RK2. Transformation efficiencies (CFUs/μg) of each strain for plasmid TE554 harboring RK2 origin of replication (Supplementary Fig. S2). CFU/mL and transformation efficiency numbers represent three biological replicates and standard deviations are given as errors.

Our tools and methods can be leveraged for the rapid assessment of genetic components integral to sustained diazotroph-plant interactions and nitrogen release of diazotrophs.

Results and Discussion

Baseline Characterization of Diazotrophs for Lab Cultivation and Strain Engineering

We examined the literature and existing sequence databases to identify diazotrophs isolated from varied climates since the earliest reports in the 1940s (Wilson & Burris, 1947). From this information, we were able to down select several diazotrophs based on their availability in public repositories, existence of high-quality genomic sequencing information, and reports indicating cultivation under primarily aerobic laboratory conditions. Strain origins and accession numbers for American Type Culture Collection (ATCC), German Collection of Microorganisms and Cell Cultures (DSMZ), and National Center for Biotechnology Information (NCBI) accession numbers are summarized in Supplementary Table S1. Likely due to differences in strain collection standards over the past decades, exact geographical and climate isolation information is missing from several isolates. Additionally, laboratory cultivation of diazotrophs is often poorly characterized in the accompanying repository metadata, requiring further experimentation to onboard strains. More recent strain sequencing information indicates relatively large bacterial genome sizes (~6 Mb) but all have high quality scores as calculated by NCBI Prokaryotic Genome Annotation Pipeline (Parks et al., 2015) and fully assembled genomes. The GC content of the strains ranges from 56% to 68.5% in *K. michiganensis* and *Azos. brasilense*, respectively. All strains contain one chromosome but *Azos. brasilense* also maintains 6 plasmids ranging in size from 0.16 to 1.7 Mb (Orlandini et al., 2014). The strains designated as biosafety level (BSL) 1 except *K. michiganensis* m5al that harbors gene clusters for the transport of yersiniabactin, a critical virulence factor making it a potential pathogen and a BSL2 organism (Yu et al., 2018).

Next, we established baseline growth conditions for these non-model microbes for laboratory cultivation. The endophytic bacterial species (*K. michiganensis, Azos. brasilense,* and *H. seropedicae*) were cultured in standard Luria-Bertani (LB) media. In contrast, root nodule symbionts *R. leguminosarum,* and *Azor. caulinodans* showed more robust colony formation and higher revival rates from cryostorage in tryptone-yeast extract (TY) media (see Materials and Methods) suggesting the reduced salt concentration and ionic strength were beneficial for enhanced laboratory growth in several of these species. Growth assays were performed for the endophytic bacteria (*K. michiganensis, Azos. brasilense,* and *H. seropedicae*) as they readily grew in 96-well plate format. *Klebsiella michiganensis* was the fastest growing diazotroph we tested with a doubling time of 26.1 min. *Herbaspirillum seropedicae* and *Azos. brasilense* grew slower with a doubling time of 53 and 87.9 min, respectively (Table 1 and Supplementary Fig. S1).

Classical mutant analysis studies in diazotrophs rely on time-consuming homologous recombination-based methods for integrating plasmid vectors in species such as *Azotobacter vinelandii* (Dos Santos, 2019). Therefore, we wanted to express genes from stable, exogenously provided plasmid DNA as it enables the rapid characterization of genetic elements and expression of heterologous genes. We first identified the minimum inhibitory concentrations (MICs) for chloramphenicol (Supplementary Table S2) and kanamycin (Table 1) for each diazotroph with three broad host plasmids BBR1(Antoine & Locht, 1992), RSK1010 (Scherzinger et al., 1984), and RK2 (Thomas, 1981). Plasmids with origin of replication RK2 and kanamycin selection marker were stably replicated in five diazotrophic strains, facilitating the characterization of common genetic elements in parallel. Two additional diazotrophs (*Sinorhizobium meliloti 1021* and *Gluconacetobacter diazotrophicus Pal5*) were not readily transformed with any plasmids regardless of electroporation protocol implemented and as a result were not characterized further.

Many gene editing techniques require the introduction of linear DNA fragments or ssDNA (e.g., CRISPR or recombineering). This rules out the possibility of conjugative approaches with helper microbes using *tra*-family conjugation elements, which cannot target these DNA species for introduction into other microbes. Specifically, the ~90mer ssDNA oligonucleotides designed per targeted edit with recombineering cannot contain the *oriT* sequence required to initiate transconjugation since this additional DNA sequence would introduce a region of non-homology that blocks base-pairing with the targeted locus (Couturier et al., 2023). Therefore, we constructed small expression plasmids (~4 kb) each containing an origin of replication and antibiotic selection marker to determine transformation efficiency via electroporation (see Materials and Methods). Supplementary Table S3 summarizes the plasmids used in this study. Plasmids containing the origin of replication BBR1 or RSF1010 were not reliably transformed into *Azos. brasilense* and *R. leguminosarum* respectively. BBR1 and RSF1010 also yielded high day-to-day variation and were found to be low efficiency in some species (Supplementary Table S2, and additional data not shown). In contrast, plasmids containing the origin of replication RK2 and a kanamycin resistance marker were most widely successful across diazotroph strains. Of the strains that could be successfully transformed, the transformation

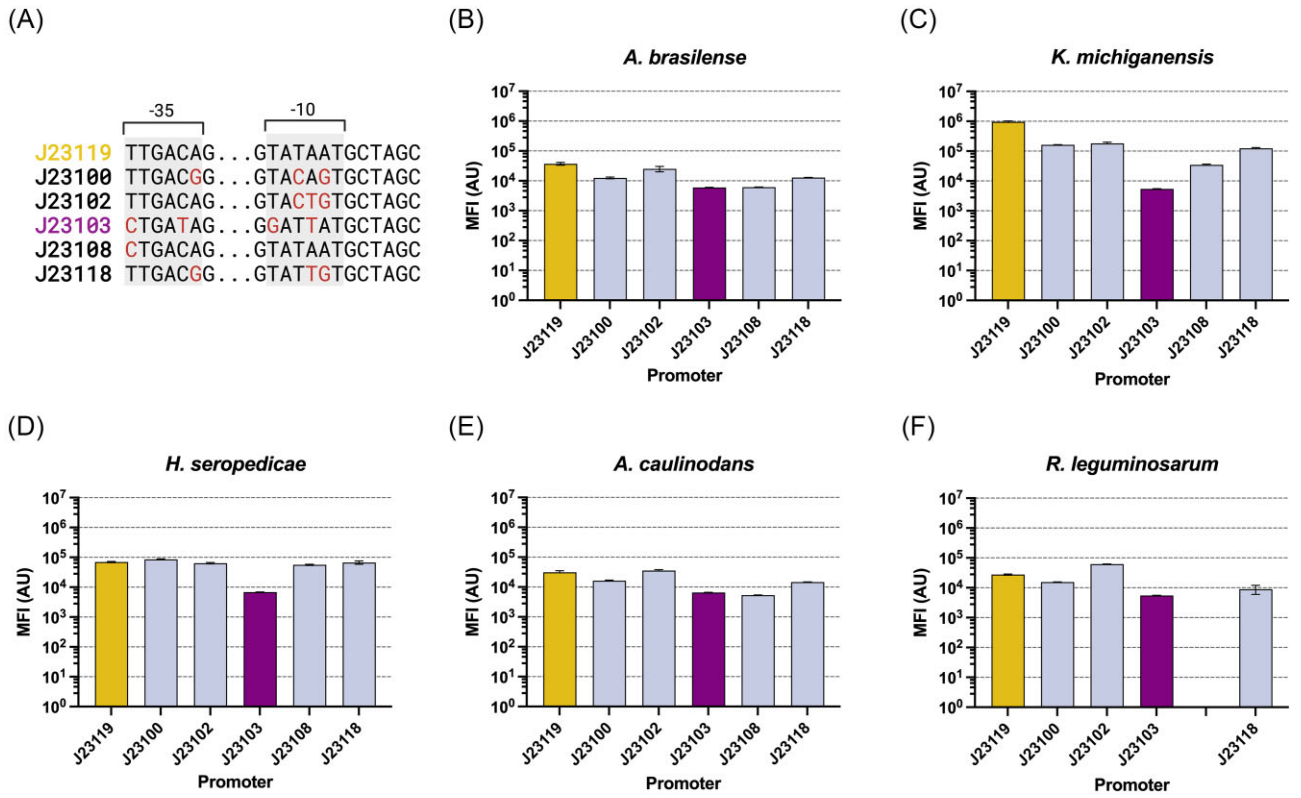

Fig. 2. Expression of sfGFP using Anderson Promoters. Schematic of the Anderson promoter library (A) highlighting nucleotide differences from wildtype promoter J23119 and the lowest expression promoter as assessed in *E. coli* J23103. Mean fluorescence intensity (MFI) in arbitrary units (AU) across selected diazotrophs in logarithmic scale (B–F). Promoters are organized from highest fluorescence to lowest (as reported in *E. coli*) for each diazotroph (left to right). Error bars represent standard deviation over three biological replicates. Full promoter sequences can be found in Supplementary Table S2.

efficiencies for plasmids containing the origin of replication RK2 were found to be consistent in these four diazotrophs (Table 1) exceeding ~4 × 10^3 colony formation units (CFUs) per µg DNA. Extraction and reintroduction of the same plasmid isolated from *Azos. brasilense* resulted in an increase of ~1 order of magnitude of the transformation efficiency, likely due to specific plasmid DNA methylation from replication in *Azos. brasilense*, preventing degradation as previously described (Mermelstein & Papoutsakis, 1993). While these transformation efficiencies are still several orders of magnitude lower than that of *Escherichia coli*, it is comparable in efficiency to other soil microbes like *Corynebacterium glutamicum* and *Bacillus subtilis* and sufficient for building mutant libraries (Inoue et al., 1990; Xue et al., 1999; Ruan et al., 2015). Despite the relatively lower efficiencies of the RK2 plasmids in *R. leguminosarum*, this electroporation protocol was reproducible with consistent transformation efficiency across several trials spanning several years and operators. Therefore, we were able to minimize the variety of plasmids used to enable transformation across multiple diazotroph species.

Genetic Elements for Constitutive Heterologous Protein Expression

Our understanding of gene expression in non-model bacteria remains incomplete; envisioned edits to cellular metabolism often have unpredicted phenotypic behavior (Wintermute & Silver, 2010; Rivier et al., 2023; Banerjee et al., 2024). As a first step in building tunable gene expression platforms, we needed to assess the activity of existing promoters and other genetic elements to determine if they were compatible with diazotrophs, which have divergent sigma factors and transcriptional machinery compared to *E. coli* (Zhan et al., 2016). To enable reliable dynamic expression, we leveraged the Anderson promoter library (https://parts.igem.org/Promoters/Catalog/Anderson). Initially created in *E. coli*, the Anderson promoter library has mutations to conserved regulatory motifs at the −35 and −10 upstream locations in a highly conserved short promoter sequence known to bind transcription factors with the general from 5′-NNNNNNGCTAGCTCAGTCCTAGGNNNNNNGCTAGC-3′. The result is a library of constitutive promoters with discrete transcription rates. We initially tested the consensus promoter from the Anderson collection (J23119) to confirm reliable constitutive expression of an sfGFP fluorescent reporter (Supplementary Fig. S3). We next constructed a plasmid library with a subset of Anderson promoters to modulate transcription (Fig. 2A), selecting promoters that exhibited a range of fluorescence expression in *E. coli* as a proxy for transcriptional induction and measured fluorescence of all samples 24 hr post induction. The representative library resulted in changes of expression of up to 100-fold in *K. michiganensis* and consistent expression changes of ~10-fold in the remaining diazotroph species compared to the consensus sequence (J23119) (Fig. 2B–F). At best, the consensus promoter J23119 had at least fivefold or higher expression than the J23103 promoter as observed in *E. coli*, but overall trends were more difficult to discern. One plasmid, containing promoter J23108, failed to transform into *R. leguminosarum* and we were unable to recover any colonies harboring the expression plasmid. When Anderson promoter variants were compared across diazotrophs,

the rank order of fluorescence expression was not conserved. Moreover, their ranked expression level was also different across each microbe, highlighting the necessity to test each expression element (Supplementary Fig. S4).

After modulating expression by varying promoter sequences, we next tested a library of RBSs to modulate translation. We built an RBS library using the De Novo DNA platform consisting of 11 variable RBSs predicted to yield a range of translation rates in *Azos. brasilense* (Farasat et al., 2014; Espah Borujeni et al., 2014, 2017; Ng et al., 2015; Reis & Salis, 2020). The resulting degenerate DNA RBS library genetic sequence (5′ GTGYASAGGCAARAARGASGTHTTTA 3′) was varied at six nucleotide locations and predicted to have a 1000-fold range of expression in *Azos. brasilense*. Full RBS sequences are summarized in Supplementary Table S4. The RBSs were then inserted into plasmids containing an mScarlet reporter along with the consensus Anderson promoter J23119 (Fig. 3A). The resulting *Azos. brasilense* optimized RBS-variant plasmids were examined for mScarlet fluorescence in all five microbes. We observed that the relative mScarlet fluorescence varied across all bacterial species, further enabling dynamic expression (Fig. 3B–F). The observed change in *Azos. brasilense* was ∼10-fold and followed the general trend as computationally predicted. Larger ranges of expression were noted for several species including a ∼100-fold range in *H. seropedicae* and *Azor. caulinodans*. However, the other four diazotrophs followed no correlation with the predicted values, underscoring the difference between bacterial strains (Fig. 3C–F). The predicted strongest (Fig. 3C–F, light blue) and lowest (Fig. 3B–F, pink) are not consistent across strains and do not constitute the experimentally determined strongest and weakest RBSs as predicted in *Azos. brasilense* (Fig. 3D). Moreover, the normalized relative fluorescence was not conserved across bacterial strains similar to the promoter sequences, again highlighting the need to test each individual component and tailor RBS libraries for each microbe (Fig. 3G). Several RBS plasmid variants failed transformation in several species, but the reason for this limitation is unknown. A second, smaller RBS library was built using the same predictive platform in *Azos. brasilense* to control expression of sfGFP offering a library of distinct RBS sequences (Supplementary Fig. S5A). The resulting library shows less dynamic range (∼10-fold) than the previous library but still could offer additional expression control given the variability of downstream applications or target genes (Supplementary Fig. S5B–F). Given the small library size relative to previous studies in *E. coli*, testing a larger RBS sample space (e.g., ∼500 RBS variants) could identify a broader expression range in diazotrophs (Espah Borujeni & Salis, 2016).

By testing a library of common genetic expression elements, we were able to test five strains in parallel. However, we found varying efficacies to modulate expression at the transcriptional and translational level. For example, the promoters tested for *K. michiganensis* result in a wider range of expression (∼100-fold) compared to the RBSs (<10-fold), but the opposite is true for *Azor. caulinodans* and *R. leguminosarum* (Fig. 4C, E, F). Nonetheless, our approach facilitated rapid parallel characterization of multiple strains and resulted in detectable fluorescence in all diazotrophs. The ability to modulate expression is crucial for the establishment of synthetic genetic networks, especially in diazotroph-plant or diazotroph-microbe interactions where signaling molecule interactions can vary across orders of magnitude (Cesco et al., 2010). Considerable effort has gone into further understanding how to more effectively create sustained microbe-diazotroph associations that result in higher amounts of ammonia for the plant. The ability to express exogenous DNA expands our capacity to investigate specific genes involved in these complex associations. Moreover, genetic circuits between plants and soil bacteria in the rhizosphere have been targets for monitoring and controlling plant physiology. The tools demonstrated here offer potential to leverage diazotrophic bacteria to modulate diazotroph strain behavior and affect their environment by the expression of heterologous genes, perhaps by changing the composition of the plant microbiome with new secreted metabolites or proteins.

Inducible Gene Expression Systems in Diazotrophs

In addition to the constitutive expression system generated above, conditional expression of heterologous genes is a desirable tool for the construction of synthetic circuits especially when potentially growth-inhibitory genes are introduced. Therefore, we tested several small molecule inducible systems previously optimized in the "Marionette" *E. coli* strain (Meyer et al., 2019). We specifically chose naringenin, arabinose, salicylic acid, and vanillic acid, which are inducer molecules that are also known to exist in root exudates (Vives-Peris et al., 2020). The promoter sequences for these inducible systems were subcloned into existing plasmid designs to drive expression of sfGFP. After construction, these plasmids were transformed into the selected diazotrophs and, as in *E. coli*, 0.05 μM–2000 μM inducer was added (Meyer et al., 2019). We found the most robust induction with salicylic acid, yielding over a 10 000× increase in expression compared to the uninduced control in *H. seropedicae* (Fig. 4A), *K. michiganensis* (Fig. 4B), and *R. leguminosarum* (Fig. 4C). In addition, arabinose induced expression in *K. michiganensis* and vanillic acid had limited effect in *R. leguminosarum* and only showed modest fold increases at millimolar concentrations. None of the tested promoter systems were portable into *Azos. brasilense* and *Azor. caulinodans* (Supplementary Fig. S6, refer to Figs. 3 and 4). It is possible that root exudate compounds may necessitate additional consideration as they may already exist in native diazotroph metabolism as suggested by the degradation of naringenin in *H. seropedicae* (Marin et al., 2013).

The expression systems in diazotrophs described here may be further leveraged to improve diazotroph ammonia release or plant associations. Genetic components of nitrogenase related genes could be expressed in a tailored fashion, potentially enabling scenarios where an increase in exogenous ammonia can be observed as has been previously reported in other diazotrophs (Mus et al., 2022). Moreover, plant associations could be engineered to increase diazotroph ability to establish in plant roots. Increased plant interaction could be fostered through the over-expression of genes known to be involved with endophytic relationships (Cole et al., 2017). Likewise, gene expression of biocontrol traits could offer methods for engineered strains to outcompete other bacterial species, including pathogens, and result in increased plant associations. Regardless of the downstream approach, coupling these expression-based tools with other methods such as adaptive evolution may be necessary given the complex nature of these biological systems (Nordgaard et al., 2022).

Demonstration of Genome Editing in *K. michiganensis*

Having developed a broad set of plasmids compatible with electroporation methods, we proceeded to test targeted genome editing using a plasmid-borne recombineering system in conjunction with a chemically synthesized oligonucleotide (Datta et al., 2008). By overexpressing components of the mismatch repair system containing a dominant negative MutL-E36K mutation, the

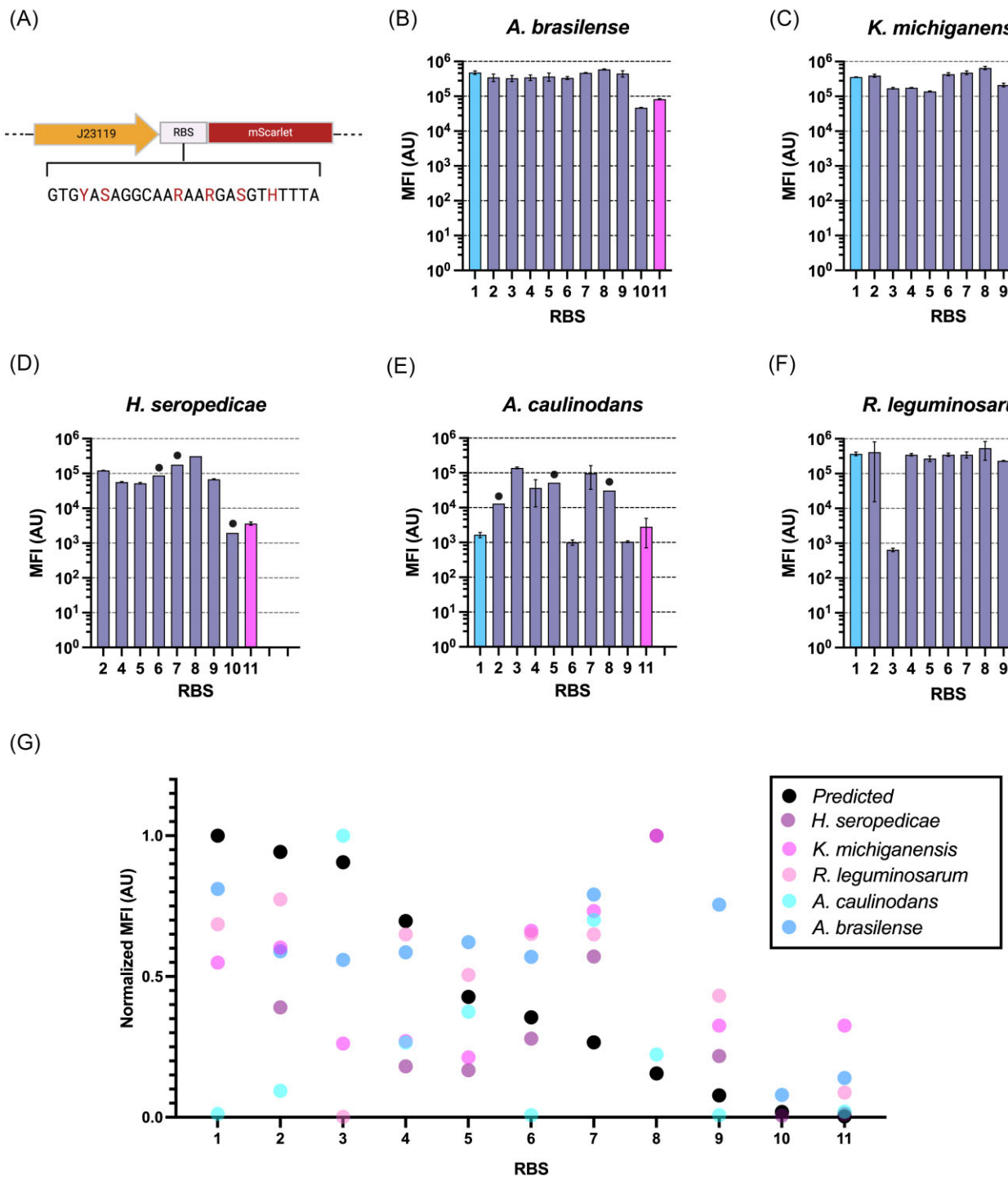

Fig. 3. Behavior of A. brasilense-optimized Ribosomal binding sites on gene expression across divergent diazotrophs. Representation of RBS plasmid library harboring mScarlet fluorescence protein (A) controlled by J23119 promoter and variable RBSs by altering nucleotide bases highlighted in red. Mean fluorescence intensity (MFI) in arbitrary units (AU) of mScarlet in logarithmic scale for selected diazotrophs (B–F). RBSs are organized by decreasing fluorescence in each diazotroph. Sequences 1 and 11 represent the RBS that have the highest and lowest predicted activity, respectively. Error bars represent standard deviation over three biological replicates. Dots denote one biological replicate. Full RBS sequences are available in Supplementary Table S2. Highest fluorescence normalized to a value of one reveals divergence of RBSs in different species (G).

provided oligo initiates lagging strand DNA replication, incorporating the desired genomic mutation (Fernández-Cabezón et al., 2021). To apply this method in a diazotroph, we used an oligo targeting the ribosomal 12S gene (*rpsL*) that confers streptomycin resistance by introducing a Lys to Arg mutation at amino acid residue 43 with three mutated nucleotides (Wannier et al., 2020). We picked *K. michiganensis* for this demonstration because it has the fastest doubling time and formed colonies after plasmid transformation within 2 days of plating. When this oligonucleotide is electroporated into cells expressing both *recT* and

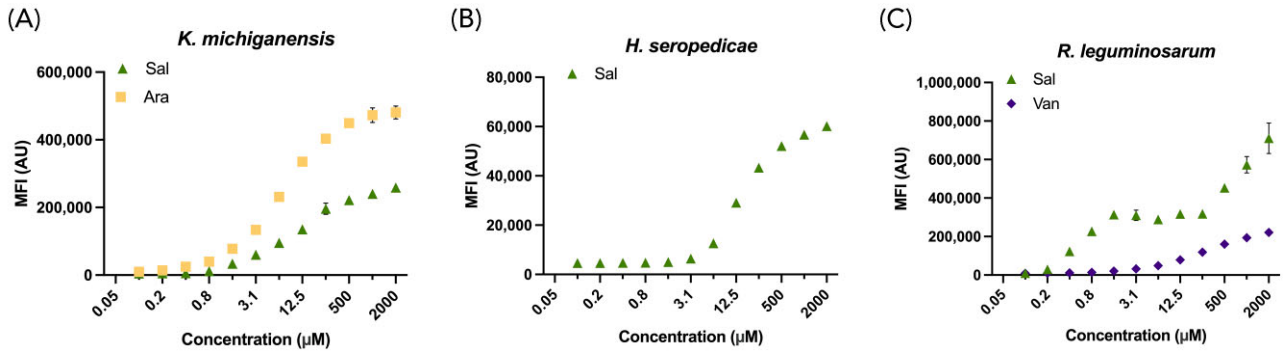

Fig. 4. Inducible promoter systems in diazotrophs. Mean fluorescence intensity (MFI) in arbitrary units (AU) of inducible promoter systems in H. seropedicae (A), K. michiganensis (B), and R. leguminosarum (C) plotted in linear scale. Small molecule inducers arabinose (Ara, squares), salicylic acid (Sal, triangles), and vanillic acid (Van, diamonds) were varied in concentration from 0.05 μm to 2000 μm. Error bars represent standard deviation over two biological replicates. Non-functional inducer systems are summarized in Supplementary Fig. S7.

mutL-E36K from *Pseudomonas aeruginosa*, we expect to see an increase in streptomycin resistant colonies compared to a control reaction. Consistent with this model, electroporation of *K. michiganensis* with this oligonucleotide yielded ~10 streptomycin resistant colonies with no streptomycin resistant clones without addition of the oligonucleotide. Several randomly chosen streptomycin resistant clones were sequenced at the *rpsL* locus and all were confirmed to harbor the expected K43R point mutation through Sanger sequencing where the identical mismatched nucleotides chemically synthesized in the oligonucleotide were incorporated into the *K. michiganensis* genome (Supplementary Fig. S7). These results demonstrate that genomic edits at different loci can be generated with other selection markers (e.g., auxotrophies or antibiotics). This strategy could be further extended with the use of Cas9 endonuclease counterselection, offering additional strategies that can be applied to diazotrophs. The ability to use the impaired mismatch repair system offers additional recombineering strategies in diazotrophs relying on lambda-red recombination (Venkataraman et al., 2023; Almási et al., 2024). Having additional recombineering tools as described here may be beneficial for the establishment of cutting-edge genetic engineering in a wider variety of diazotrophs.

Materials and Methods

Bacterial strains, Growth conditions, and Cell counts

Bacterial strains *H. seropedicae* SmR1, *K. michiganensis* M5al, and *Azos. brasilense* Sp245 were generously donated by Adam Deutschbauer. *Azorhizobium caulinodans* ORS571 and *R. leguminosarum* 3D1k2 were obtained from The ATCC and are described in the strain table (Table 1). All microbes used in this study were verified with 16 s rRNA sequencing using primers 27F (forward: 5′- AGAGTTTGATCMTGGCTCAG-3′) and 1492R (reverse: 5′-TACGGYTACCTTGTTACGACTT-3′) (Weisburg et al., 1991). All strains were stored in 10% glycerol at −80°C. Plasmid sequences are available at public-registry.jbei.org after generating a free user account and plasmids are available upon request to the corresponding author.

Azospirillum brasilense, *K. michiganensis*, and *H. seropedicae* were cultured in autoclaved LB media (Tryptone, 10.0 g/L; Yeast extract, 5.0 g/L; and NaCl, 10.0 g/L) at a temperature of 30°C. *Azorhizobium caulinodans* was cultured in autoclaved TY media (Tryptone 5.0 g/L, Yeast extract 3.0 g/L, and $CaCl_2$ x 2 H_2O 0.9 g/L) at 28°C. Tryptone, yeast extract, NaCl, and $CaCl_2$ were purchased from BD Biosciences (Milpitas, CA, USA).

Kinetic growth curves were generated by measuring optical density (OD) at a wavelength of 600 nm and pathlength of 1 cm every 10 min using a Molecular Devices Filtermax F5 plate reader (Molecular Devices LLC, San Jose, CA, USA). Microtiter plates were incubated at 30°C for the duration of the time course at "high" shake speed setting and linear shaking mode in a 96 well microtiter plate sealed with a Breathe-Easy® transparent membrane (Sigma–Aldrich). Three biological replicates were processed by growthcurver R package and doubling times were found for all strains (Sprouffske & Wagner, 2016).

Colony formation unit of bacterial strains were measured by growing each strain in their respective media (Table 1) for ~16–24 hr to logarithmic growth except for the fast-growing strain *K. michiganensis* that was grown for 16 hr then back diluted to an OD_{600} of 0.1 and grown for ~2 hr to logarithmic phase. Cells were then diluted in phosphate buffered saline (PBS, Thermofisher scientific, Waltham, MA, USA) to a range of dilutions (10 000×–2 000 000×) and plated onto LB agar or TY agar plates and incubated at 30°C for 2–5 days until colonies could be visualized. Dilution ranges were selected for calculating initial OD to CFU values where roughly 200 CFUs per plate (Tomasiewicz et al., 1980). Three biological replicates of CFUs were counted and back calculated to the original concentration and normalized to an OD_{600} of 1.

Plasmid Construction

All DNA constructs and oligos were designed using either Snapgene (BioMatters Ltd) or Primer3 (Koressaar & Remm, 2007) and plasmids were assembled using isothermal HiFi assembly (New England Biolabs (NEB), Ipswitch, MA, USA) following manufacturer's guidelines. Ribosomal binding site libraries were predicted using the De Novo DNA platform with either mScarlet or green fluorescent protein (GFP) as the protein coding sequence and *Azos. brasilense* as the host organism. DNA amplification was conducted following manufacturer's guidelines. Whole plasmid sequencing (Primordium Labs, Monrovia, CA, USA) was used to verify constructs as necessary. Standard *E. coli* bacterial transformation with Invitrogen XL-1 Blue competent cells (ThermoFisher, Carlsbad, CA, USA) was used to isolate new DNA constructs. Plasmid DNA was isolated using alkaline lysis and silica columns using Qiagen miniprep kits (Qiagen Inc, Germantown MD) following the manufacturer's guidelines.

Bacterial Transformations

Bacterial strains were grown in 5 mL of the appropriate media (Supplementary Table S1) for 24 hr at 30°C to reach saturation. Cultures were diluted to an OD_{600} of 0.1 in 25 mL of liquid media in a 250 mL flask and incubated until they reached an OD_{600} of ∼0.5 (approximately ∼2–6 hours). *Rhizobium leguminosarum* was notably slow growing and was diluted to an initial OD_{600} of 0.05 and incubated overnight to reach a comparable OD_{600} in logarithmic phase by the next morning. Cells were harvested by centrifugation at 5000 x *g* for 10 min and washed twice with ice-cold 10% (v/v) glycerol. The pellets were resuspended in 250 μL of 10% glycerol and 50 μL aliquots were prepared and stored at −80°C. Cell aliquots were thawed on ice for 5 min before adding 100 ng of plasmid DNA and electroporated on Bio-rad Micropulser (Bio-Rad, Hercules, CA, USA) using EC2 protocol (0.2 cm cuvette, 2.5 kV). This protocol was modified slightly for *R. leguminosarum* by resuspending 4 mL of culture media in 100 μL of 10% glycerol to increase the number of cells used per electroporation. Resulting transformants were then grown in liquid media for 5 hr and plated onto respective selection plates with appropriate antibiotic concentrations (Supplementary Table S1).

Transformation efficiency numbers were calculated by harvesting cell cultures (grown as described above) to logarithmic phase. Cells were diluted to an OD_{600} of 0.1 in PBS pH 7.4, then serial diluted to low concentrations (OD_{600} of 1.0×10^{-6}, 1.0×10^{-5}) and plated on respective agar plates. Colony formation units were counted and normalized to account for dilutions to determine equivalent CFUs for 1 unit of OD.

ssDNA Recombineering in *K. Michiganensis*

ssDNA recombineering was performed in *K. michiganensis* with slight modifications from our existing recombineering protocol used in *P. putida* (Czajka et al., 2022). The strain was transformed with a plasmid containing a 3-methyl benzoate (3 MB; m-Toluic Acid; Sigma–Aldrich Catalog No. T36609) inducible recT recombinase and $mutL^{E36K}$ (pTE583) was grown overnight, back diluted to a starting OD_{600} of 0.1, and allowed to grow for an additional 3 hr. The recombineering pathway genes were induced with 1 mM 3 MB for 4 hr before being electroporated as described with oligonucleotide containing a K43R triple point mutation (5′ GCAGAAACGTGGCGTATGTAC TCGTGTATATACCACCACTCCTCGTAAACCTAACTCCGCACTGCGTAAAGTTTGCCGTGTGCGTCTGAC 3′) (Integrated DNA Technologies, Redwood City, CA, USA). The oligo was ordered desalted and without further purification or base modifications. Resulting transformants were screened on solid LB agar media containing 100 µg/mL streptomycin (Sigma–Aldrich). Transformants were confirmed to incorporate the identical three nucleotide polymorphism by Sanger sequencing (Supplementary Fig. S7). No spontaneous streptomycin clones were recovered on the control transformation plates without electroporation with oligo.

Quantifying Fluorescent Expression

Flow cytometric analysis was conducted using an Accuri C6 flow cytometer equipped with an autosampler (BD Biosciences). Cells were cultivated in 1 mL media in 24 well deep well plates at 30°C and 250 rpm for 24 hr prior to being diluted to OD_{600} 0.1 in 500 μL of the respective medium in a 96 well microtiter plate for fluorescence measurements. Measurements were conducted in biological triplicate. A total of 30 000 events were recorded at the "low" flow rate setting with a core size of 22 μm. Green fluorescent protein was excited at 478 nm at 70 mW and emission detected at 530 nm.

Vanillic acid, arabinose, and salicylic acid were dissolved in sterile deionized water. Naringenin was dissolved in dimethylsulfoxide. Expression was induced via small molecules by incubating for 24 hr and induction of mScarlet was excited at 552 nm with default signal intensity and emission detected at 610 nm. For all fluorescent measurements, data acquisition was performed as described in the Accuri C6 Sampler User's Guide. The acquired data were analyzed in FlowJO (TreeStar Inc, San Carlos, CA, USA).

Conclusion

Here, we have outlined the growth and cultivation parameters for five diazotrophs in laboratory conditions and identified a suite of genetic elements. Specifically, we attempted to onboard seven phylogenetically distinct diazotrophs and found five suitable for exogenous DNA expression. We then tested eleven constitutive promoters and a total of 20 RBSs between two libraries. The tested genetic expression elements enable translation and transcription attenuation of up to ∼100-fold and ∼1000-fold, respectively. Additionally, four root exudate compounds were tested for the efficacy in induction, resulting in five systems of gene induction across three diazotrophs. A larger set of genetic tools will enable other researchers in the field to manipulate more components of rhizobial community members with the potential to improve bioenergy crop yields while reducing dependence on energetically expensive and environmentally detrimental fertilizers

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Supplementary Material

Supplementary material is available online at *JIMB* (www.academic.oup.com/jimb).

Author Contributions

Molecular biology and strain engineering: S.K., A.R., S.M., and T.E. Data interpretation and analysis: S.K., A.R., R.K., and T.E. Supervision: T.E. Drafted the manuscript: A.R., T.E. Acquisition of Funds: T.E. All authors have read, provided feedback, and approved the manuscript for publication.

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Conflict of Interest

None.

Data Availability Statement

The data underlying this article will be shared on reasonable request to the corresponding author.

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