RESEARCH PAPER

Biological nitrogen fixation in maize: optimizing nitrogenase expression in a root-associated diazotroph

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Abstract

Plants depend upon beneficial interactions between roots and root-associated microorganisms for growth promotion, disease suppression, and nutrient availability. This includes the ability of free-living diazotrophic bacteria to supply nitrogen, an ecological role that has been long underappreciated in modern agriculture for efficient crop production systems. Long-term ecological studies in legume–rhizobia interactions have shown that elevated nitrogen inputs can lead to the evolution of less cooperative nitrogen-fixing mutualists. Here we describe how reprogramming the genetic regulation of nitrogen fixation and assimilation in a novel root-associated diazotroph can restore ammonium production in the presence of exogenous nitrogen inputs. We isolated a strain of the plant-associated proteobacterium *Kosakonia sacchari* from corn roots, characterized its nitrogen regulatory network, and targeted key nodes for gene editing to optimize nitrogen fixation in corn. While the wild-type strain exhibits repression of nitrogen fixation in conditions replete with bioavailable nitrogen, such as fertilized greenhouse and field experiments, remodeled strains show elevated levels in the rhizosphere of corn in the greenhouse and field even in the presence of exogenous nitrogen. Such strains could be used in commercial applications to supply fixed nitrogen to cereal crops.

Keywords: Ammonium production, biological nitrogen fixation, corn rhizosphere, diazotroph, *Kosakonia sacchari*, nitrogenase expression, regulatory nodes.

Introduction

One of the most significant agricultural inputs is nitrogen fertilizer, especially in the production of cereal crops. In the last 40 years, grain yield in corn (*Zea mays*) in the USA has increased linearly at a relative gain of 1.2% per year (Cassman and Liska, 2007). This increase in genetic gain is supported by technological advances in hybrid breeding, biotechnology, irrigation, conservation tillage, and fertilization practices. One of the earliest innovations contributing to increased cereal crop yields was the availability of synthetic nitrogen fertilizer via the Haber–Bosch process, which converts relatively non-reactive nitrogen gas to biologically available ammonia using high temperature and pressure (Erisman *et al.*, 2008). Today, commercial fertilizer is responsible for 40–60% of the world's food production, and global demand for nitrogen fertilizer remains strong (Roberts, 2009). While inorganic fertilizers have become fundamental to ensuring global food supplies through the 21st century, future increases in sustainable food production must be accomplished in an environmentally safe manner through ecological intensification (Roberts, 2009).

Increases in cereal crop yields in the second half of the 20th century necessitated a massive increase in the production and application of synthetic nitrogen fertilizer, of which about half is lost to the environment as aquatic or atmospheric pollutants (Lassaletta *et al.*, 2014; Zhang *et al.*, 2015). Since the 1960s, human use of synthetic nitrogen fertilizers has increased 9-fold, with further substantial increases of ~40–60% expected over the next 40 years (Sutton *et al.*, 2013)). The manufacture of synthetic nitrogen fertilizer consumes ~2% of the world's energy production (Glendining *et al.*, 2009), and its intensified use has disrupted the world's biogeochemical nitrogen cycle, causing major environmental, health, and economic problems (Rockstrom *et al.*, 2009; Kanter, 2018). The European Nitrogen Assessment, a comprehensive assessment of the massive anthropogenic increase in reactive nitrogen in the environment, has identified five key societal threats: water quality, air quality, greenhouse gas balance, ecosystems, and biodiversity and soil quality (Sutton *et al.*, 2011).

Crop-associated microbiota represent an attractive alternative to deliver nitrogen to cereal crops in an efficient and environmentally friendly manner. Microbes within the rhizosphere, endosphere, and phyllosphere of plants interact with crops through numerous mechanisms including nitrogen fixation, nutrient solubilization, metabolism of plant growth hormones, secretion of biocontrol metabolites, and stimulation of plant systemic defenses (Compant *et al.*, 2019). In the past two decades, tools for probing crop–microbe interactions have become more sophisticated (Cardinale, 2014; Haichar *et al.*, 2016; Kaul *et al.*, 2016), and the diversity of microbial species that can be domesticated and commercially formulated has expanded. Numerous efforts are underway to develop novel strains that confer useful agronomic properties and improve the efficiency and reliability of microbial-based products for large-scale farming operations (Kaminsky *et al.*, 2019). Realizing the broad potential of crop-associated microbes would be accelerated by leveraging crop–microbe interaction data to rationally and rapidly improve microbial genotypes and phenotypes. We have recently described a guided microbial remodeling platform by which microbes with plant-beneficial traits can be isolated, optimized, and developed for use as inoculants in agriculture (Bloch *et al.*, 2019b).

A small group of bacteria and archaea, known as diazotrophs, are capable of reducing dinitrogen to ammonium using nitrogenase enzymes (de Bruijn, 2015). This process of biological nitrogen fixation (BNF) is the major contributor to the nitrogen economy of the biosphere, accounting for 30–50% of the total nitrogen in crop fields (Ormeno-Orrillo *et al.*, 2013). While legumes (Fabaceae) form specialized symbiotic associations with diazotrophic rhizobia in root nodules, non-legume crops associate with diazotrophic bacteria which reside in the rhizosphere at the surface or interior of the root (de Bruijn, 2015). A root-associated, nitrogen-fixing bacterium could theoretically provide significant nitrogen to a cereal crop given adequate abundance and nitrogen-fixing activity in the rhizosphere, reducing producers' dependence on synthetic nitrogen. Several recent reviews discuss strategies and challenges associated with the use of diazotrophic bacteria for agricultural benefit in non-legumes (Mus *et al.*, 2016; Batista and Dixon,

2019; Bloch *et al.*, 2019a; Pankievicz *et al.*, 2019). However, due to the high energetic cost of BNF, requiring 16 ATPs per molecule of N_2 reduced, the abundance of synthetic nitrogen selects against and represses BNF in modern agricultural settings, preventing significant contributions of nitrogen by natural soil bacteria (Chapman *et al.*, 1949; Stewart, 1969; Dixon and Kahn, 2004; Weese *et al.*, 2015; Smercina *et al.*, 2019). Additionally, nitrogen-fixing bacteria efficiently assimilate fixed nitrogen into microbial biomass, preventing it from being released to the rhizosphere (Batista and Dixon, 2019). While several plant-associated diazotrophs with altered ammonium assimilation pathways have been shown to support the growth of algae (Ortiz-Marquez *et al.*, 2014; Barney *et al.*, 2015) and plants (Pankievicz *et al.*, 2015; Ambrosio *et al.*, 2017) in lab and greenhouse settings, few have been applied in the field (Bageshwar *et al.*, 2017), probably due to a lack of fitness in the rhizosphere (Colnaghi *et al.*, 1997). Recently, synthetic biology has been applied to refactor the regulation of genes encoding the nitrogenase enzyme complex (known as the *nif* cluster), enabling more control over nitrogenase expression in a range of species (Temme *et al.*, 2012; Wang *et al.*, 2013; Smanski *et al.*, 2014). In one instance, the transgenic nitrogen-fixing endophyte *Pseudomonas protogens* Pf-5 X940 led to improved nitrogen and biomass accumulation in inoculated corn and wheat (*Triticum aestivum*) plants in greenhouse conditions without nitrogen fertilizer (Fox *et al.*, 2016). Despite these advances, reports of diazotrophs that can overcome repression by exogenous fertilizer and express nitrogenase genes in fertilized field conditions are lacking.

As a step toward overcoming this challenge, we used our guided microbial remodeling platform (Bloch *et al.*, 2019b) to optimize the nitrogen fixation and ammonium assimilation regulatory networks in a corn root-associated bacterium. A strain of *Kosakonia sacchari* was isolated from corn roots and rewired to express nitrogenase and fix and release nitrogen in the presence of exogenous sources of nitrogen. The resulting strains are capable of expressing nitrogenase genes in the rhizosphere of corn in fertilized conditions, both in the greenhouse and in the field. Additionally, they are non-transgenic, potentially leading to accelerated regulatory approvals for commercialization. This work constitutes a critical proof of concept that rewiring the regulatory networks of microbes has the potential to up-regulate plant-beneficial phenotypes in the root microbiome.

Materials and methods

Media

Minimal medium contains (per liter) 25 g of Na_2HPO_4, 0.1 g of $CaCl_2\cdot2H_2O$, 3 g of KH_2PO_4, 0.25 g of $MgSO_4\cdot7H_2O$, 1 g of NaCl, 2.9 mg of $FeCl_3$, 0.25 mg of $Na_2MoO_4\cdot2H_2O$, and 20 g of sucrose. Growth medium is defined as minimal medium supplemented with glutamine to a final concentration of 10 mM. Super Optimal Broth (SOB) contains (per liter) 20 g of casein hydrolysate, 5 g of yeast extract, 0.5 g of NaCl, 2.4 g of $MgSO_4$, and 0.186 g of KCl (RPI, P/N S25040-1000).

Isolation of Kosakonia sacchari PBC6.1

Corn seedlings were grown from seed (DKC 66-40, DeKalb, IL, USA) for 2 weeks in a greenhouse environment controlled from 22 °C (night)

to 26 °C (day) and exposed to 16 h light cycles in agricultural soil collected from San Joaquin County, CA, USA. Roots were harvested and washed with sterile deionized water to remove bulk soil. Root tissues were homogenized, and the samples were centrifuged for 1 min at 13 000 rpm to separate tissue from root-associated bacteria. Supernatants were diluted and plated on NfB medium supplemented with 1.5% agar (Baldani *et al.*, 2014). Plates were incubated at 30 °C for 5–7 d. Colonies that emerged were tested for the presence of the *nifH* gene by colony PCR with primers Ueda19f and Ueda407r (Gaby and Buckley, 2012). Genomic DNA from strains with a positive *nifH* colony PCR was isolated (QIAamp DNA Mini Kit, Cat No. 51306, QIAGEN, Germany) and sequenced (Illumina MiSeq v3, SeqMatic, Fremont, CA, USA). Reads were assembled and annotated using a5 (https://academic.oup.com/bio-informatics/article/31/4/587/2748163) and Prokka (https://academic.oup.com/bioinformatics/article/30/14/2068/2390517), respectively, and the isolates containing nitrogen fixation gene clusters were utilized in downstream research.

Fluorescence microscopy

The *K. sacchari* PBC6.1 strain was selected for further study and gene editing. For fluorescence microscopy, corn seedlings were cultivated on unsterilized 1% Murashige and Skoog agar. PBC6.1 was transformed with plasmid PB114-RFP, a plasmid containing the pSC101 origin of replication, chloramphenicol resistance cassette, and a gene encoding red fluorescent protein (RFP) under the control of a strong constitutive promoter. Transformed cells were cultured overnight in SOB with 50 µg ml^{-1} chloramphenicol at 30 °C with shaking, and 1 ml of cell suspension was inoculated directly on the seed at the time of seeding. Seven days after germination, roots were removed from agar, gently washed, and cut into sections using a sterile razor blade. Root sections were mounted on microscope slides and imaged on a 6D Widefield Nike TI inverted fluorescence microscope located at the Nikon Imaging Center at the University of California, San Francisco.

Initial field trial with isolated diazotrophs

Of the first 49 strains isolated and genome sequenced, six were selected for a field study of corn root colonization, which was carried out near San Luis Obispo, CA, USA in the summer of 2015. Corn seed was coated with a culture suspension of each isolated diazotroph, resulting in ~10^7 viable bacterial cells per seed. Treated and untreated control (UTC) seeds of two maize genotypes (BR56M30 and P0876R) were planted on 24 June 2015. Root samples from three replicate plants per treatment per genotype were collected at 12 weeks after planting. Root samples were processed and assayed for colonization of the inoculant diazotrophs as described below.

Extraction of the root-associated microbiome

Roots were shaken gently to remove loose particles, and root systems were separated and soaked in an RNA stabilization solution (Thermo Fisher P/N AM7021) for 30 min. The roots were then briefly rinsed with sterile deionized water. Samples were homogenized using bead beating with 1/2 inch stainless steel ball bearings in a tissue lyser (TissueLyser II, Qiagen P/N 85300) in 2 ml of lysis buffer (Qiagen P/N 79216). Genomic DNA (gDNA) extraction was performed with the ZR-96 Quick-gDNA kit (Zymo Research P/N D3010), and RNA extraction was performed using the RNeasy kit (Qiagen P/N 74104).

Root colonization assay

Genomic DNA extracted from root samples was used to quantify root colonization using quantitative PCR (PCR) with primers designed by Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify unique regions of the wild-type (WT) genome. For the initial field colonization experiment, qPCR was carried out using the SYBR GreenER qPCR SuperMix Universal (Thermo Fisher P/N 11762100) kit, using only a forward and reverse amplification primer;

for all other assays, the Kapa Probe Force kit (Kapa Biosystems P/N KK4301) was used with amplification primers and a TaqMan probe containing a FAM dye label at the 5' end, an internal ZEN quencher, and a minor groove binder and fluorescent quencher at the 3' end (Integrated DNA Technologies). Primer and probe sequences used for qPCR are listed in Supplementary Table S1 at *JXB* online. qPCR efficiency was measured using a standard curve generated from a known quantity of gDNA from the target genome. Data were normalized to genome copies per g FW using the tissue weight and extraction volume. For each experiment, the colonization numbers were compared with UTC seedlings.

Acetylene reduction assay (ARA)

A modified version of the ARA (Temme *et al.*, 2012) was used to measure nitrogenase activity in pure culture conditions. Strains were propagated from a single colony in 3 ml of SOB for 24 h (30 °C, 200 rpm) and then subcultured 1:25 into growth medium and grown aerobically for 24 h (30 °C, 200 rpm). A 1 ml aliquot of the growth medium culture was then added to 4 ml of minimal medium supplemented with 0–10 mM glutamine in airtight Hungate tubes and grown anaerobically for 4 h (30 °C, 200 rpm). Headspace (10%) was removed then replaced by an equal volume of acetylene by injection, and incubation continued for 1 h. Subsequently, 2 ml of headspace was removed via a gas-tight syringe for quantification of ethylene production using an Agilent 6850 gas chromatograph equipped with a flame ionization detector (FID).

Ammonium excretion assay

Excretion of fixed nitrogen in the form of ammonium was measured using batch fermentation in anaerobic bioreactors. Strains were propagated from a single colony in 1 ml per well of SOB in a 96-well DeepWell plate. The plate was incubated for 24 h (30 °C, 200 rpm) and then diluted 1:25 into a fresh plate containing 1 ml per well of growth medium. Cells were incubated for 24 h (30 °C, 200 rpm) and then diluted 1:10 into a fresh plate containing minimal medium. The plate was transferred to an anaerobic chamber (Coy) with a gas mixture of >98.5% nitrogen, 1.2–1.5% hydrogen, and <30 ppm oxygen, and incubated at 1350 rpm, at room temperature for 66–70 h. Initial culture biomass was compared with end biomass by measuring the optical density (OD) at 590 nm. Cells were then separated by centrifugation, and supernatant from the reactor broth was assayed for free ammonium using the Megazyme Ammonia Assay kit (P/N K-AMIAR) normalized to biomass at each time point.

Gene editing

The genome modifications described in Fig. 2 were generated using genome editing methods described in a recently published patent application on our guided microbial remodeling platform (Bloch *et al.*, 2019b). Genome-edited strains were cured of all plasmids used to carry out genome editing by repeated subculturing followed by sequence verification of the desired edits. Primers used to verify the edits are listed in Supplementary Table S2.

Greenhouse assays to measure nifH transcription in PBC6.29, PBC6.99, PBC6.38, and PBC6.94

A planting medium with minimal background nitrogen was prepared using either pure sand (Fig. 5A–C) or a mixture of vermiculite and washed sand (Fig. 5D, E). The sand mixture was autoclaved for 1 h at 122 °C and ~600 g was measured out into a D40 Deepot (Stuewe and Sons) before planting corn seeds (DKC 66-40) at a depth of ~1 cm. For the 2 week assays, each seed was inoculated with either sterile phosphate-buffered saline (PBS; UTC controls) or an equal volume of microbial suspension using cells diluted to a set OD. For the 4 week assay, seedlings were inoculated with a suspension of cells drenched directly over the emerging coleoptile at 5 d after planting. Inoculum was prepared from 5 ml of overnight cultures in SOB, which was spun down and resuspended twice in 5 ml of PBS to remove residual SOB before final dilution to an OD
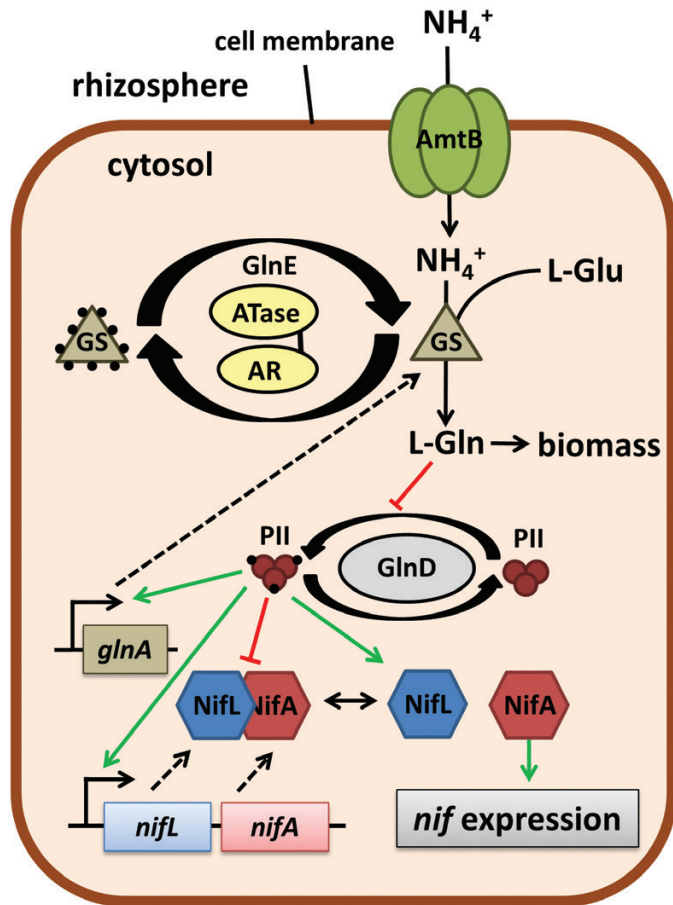
Fig. 1. *Kosakonia sacchari* PBC6.1 contains well-characterized nitrogen regulatory pathways. The regulatory network controlling nitrogen fixation and assimilation in PBC6.1 is shown, including the key nodes via AmtB, glutamine synthetase (GS; encoded by the *glnA* gene), GlnE depicted as the two-domain ATase-AR enzyme, GlnD, and PII proteins depicted as small trimeric proteins, NifL and NifA.

Fig. 2. Mutated sequences of the key genes of the nitrogen fixation and assimilation regulatory network of PBC6.1. (a) Representation of the Δ*nifL*::Prm mutations. The majority of the *nifL* gene was disrupted, leaving some nucleotides at the 5' and 3' ends. The start codon of *nifA*, which overlaps with the stop codon of *nifL*, was left intact. Promoter (Prm) sequences from two different loci in the PBC6.1 genome were inserted into the disrupted portion of the *nifL* gene to drive *nifA* transcription. (b) Representation of the Δ*glnE*$_{AR}$ mutations. The 5' region of the *glnE* gene was deleted, to remove the N-terminal adenylyl-removing (AR) domain of the resulting protein. A start codon was re-inserted at the 5' end of the truncated sequences to allow protein translation. (c) Representation of the Δ*amtB* mutation. The majority of the *amtB* gene was deleted, leaving some nucleotides at the 5' and 3' end. The deletion resulted in a frameshift, causing a stop codon just after the point of deletion. (d) Representation of the Δ*glnD* mutation. The entire coding region of the *glnD* gene was deleted, starting at the A of the ATG start codon and ending immediately after the gene's stop codon. No flanking regions were deleted. (e) Representation of the *nifHDK* operon and the Δ*nifH* mutation. The entire *nifH* coding region was deleted starting at the A of the ATG start codon and ending 9 bp downstream of the stop codon.

of 1.0. The plants were maintained under standard growth room conditions using fluorescent lamps and a 16 h daylength with a 26 °C day temperature and 22 °C night temperature. Plants were fertilized twice per week with a modified Hoagland's fertilizer solution containing 2 mM KNO$_3$. Additional watering during the assay was performed using either deionized water (Fig. 5A–C) or the modified Hoagland's solution with no KNO$_3$ The fertilizer solution contained (per liter) 3 mmol of CaCl$_2$, 0.5 mmol of KH$_2$PO$_4$, 2 mmol of MgSO$_4$, 17.9 μmol of FeSO$_4$, 2.86 mg of H$_3$BO$_3$, 1.81 mg of MnCl$_2$·4H$_2$O, 0.22 mg of ZnSO$_4$·7H$_2$O, 51 μg of CuSO$_4$·5H$_2$O, 0.12 mg of Na$_2$MoO$_4$·2H$_2$O, and 0.14 nmol of NiCl$_2$. All pots were watered with sterile deionized H$_2$O as needed to maintain consistent soil moisture. At 2 or 4 weeks after planting, plants were harvested, and nucleic acids were extracted from root tissue. Colonization of each strain was measured as described in the 'Extraction of the root-associated microbiome' and 'Root colonization assay' sections above. For microbial RNA quantification, roots were chopped into ~1 cm sections, and 5 g of root tissues was placed in conical tubes containing 10 ml of RNAlater solution (Invitrogen). Samples were vortexed for 10 min at 4 °C, and the solution containing the microbial cells was separated from root tissue using a 100 μm strainer. Microbial cells were then pelleted by centrifugation and resuspended in PBS. After digestion with lysozyme and proteinase K, microbial RNA was extracted using the RNeasy Kit (Qiagen) according to the standard protocol. The extracted RNA was used for NanoString analysis of *nifA*, *nifH*, and *rpoB* microbial transcript on an nCounter Sprint (Core Diagnostics, Hayward, CA, USA).
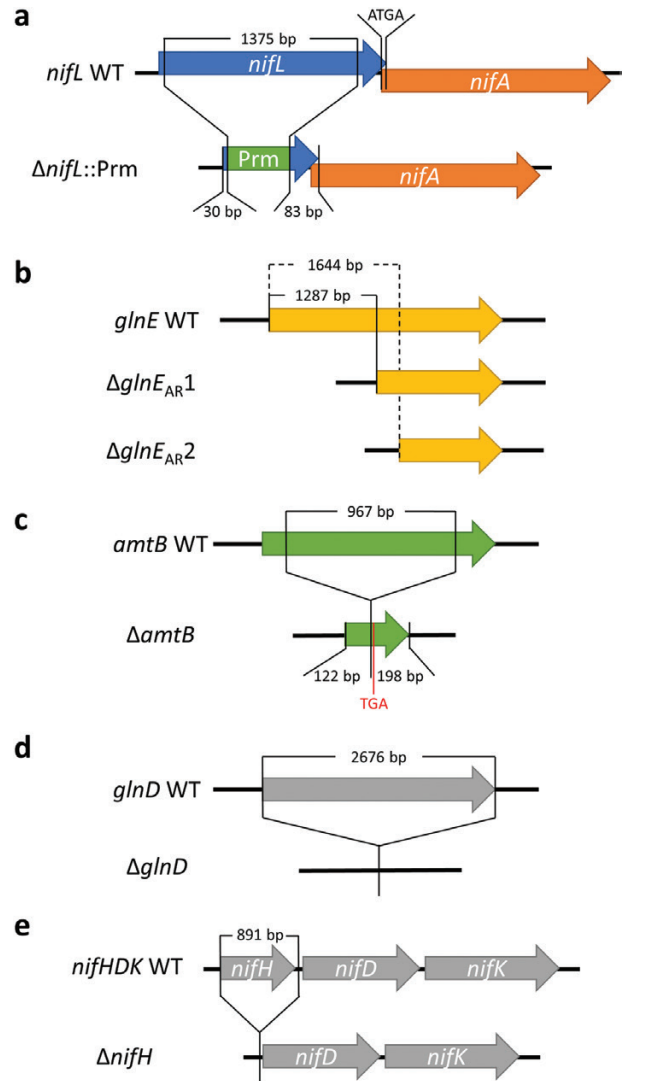
Field trials to measure colonization and nifH transcription

California

Two small-scale field trials were carried out in the late summer season in southern California. For the 2017 field trial, plots were prepared by fertilization to a level of 123 kg N ha^{-1}, and treated and UTC seeds

were planted in individual plots. Root samples from 12 replicate plants were collected at 13 d after planting for colonization analysis; at the same time, root samples were collected from 108 replicate plants to measure *nifH* transcript. Colonization samples were chilled to 4 °C and processed within 3 d of collection as described above. From each root sample, a 0.25 g tissue sample was collected from inner nodal and seminal roots, gDNA was prepared using the standard protocol described above, and the resulting sample was subjected to a colonization qPCR assay as described above. Root samples to be processed for RNA extraction were immediately packed in RNAlater at the field and frozen on arrival at the laboratory. Tissue samples of 1 g each were then homogenized, RNA was prepared using our standard protocol described above, and samples from three replicate plants were pooled to increase signal for downstream analysis, generating a total of 36 transcript samples per strain. The resulting RNA samples were analyzed via Nanostring for *nifH* and housekeeping genes. The 2018 field trial was carried out as above, except that the root samples were collected 6 weeks after planting, RNA was extracted from root samples immediately after sampling without RNAlater treatment or freezing, and RNA samples were not pooled but analyzed as individual plant samples. These trials were designed as small-scale trials to measure colonization and *nifH* transcript only; therefore, yield data were not collected.

Puerto Rico

Field trials were carried out during the winter season of Puerto Rico, USA. Treated and UTC seeds were planted in four sets of four replicate plots, each set fertilized with a different level of nitrogen (0, 67.25, 134.5, or 201.75 kg N ha^{-1} urea application 14 d after planting) for a total of 16 plots per microbial treatment. Root samples from 24 replicate plants were collected at 30 d after planting. Colonization samples were chilled to 4 °C and processed within 3 d of collection as described above. From each root sample, a 0.25 g tissue sample was collected from inner nodal and seminal roots and gDNA was prepared using the standard protocol described above. All root samples were subjected to a colonization qPCR assay, and a subset of these samples were subjected to microbiome profiling as described above. Root samples to be processed for RNA extraction were immediately packed in RNAlater at the field and frozen on arrival at the laboratory. Tissue samples of 1 g each were then homogenized, and RNA was prepared using our standard protocol described above. The resulting RNA samples were analyzed via Nanostring for *nifH* and housekeeping genes. All plots were measured for yield at harvest 118 d after planting. Though the nitrogen fertilizer level is expected to be correlated with yield, no such correlation was observed (R^2=0.00336, P=0.4319). However, yield was significantly correlated with plot row and range (R^2=0.147, P=6.4×10^{-8}, ANOVA), suggesting that nitrogen mobilization occurred during the growing season. We therefore averaged colonization, transcript, and yield data across all 16 plots for each treatment.

Illinois

Field trials were carried out during the standard corn-growing season in southern Illinois. Treated seeds and UTC seeds were planted in six replicate unfertilized plots. Root samples from one plant per plot were collected at 21 d after planting for colonization analysis; at the same time, root samples were collected from two replicate plants per plot to measure *nifH* transcript. Colonization samples were chilled to 4 °C and processed within 3 d of collection as described above. From each root sample, a 0.25 g tissue sample was collected from inner nodal and seminal roots, gDNA was prepared using the standard protocol described above, and the resulting sample was subjected to a colonization qPCR assay as described above. Root samples to be processed for RNA extraction were immediately packed in RNAlater at the field and frozen on arrival at the laboratory. Tissue samples of 1 g each were then homogenized, RNA was prepared using our standard protocol described above, and the resulting samples were analyzed via Nanostring for *nifH* and housekeeping genes.

Re-isolation of edited strains from field corn root samples

Roots from field samples taken 41 d after plating were homogenized as described above and dilution-plated to single colonies, and colonies were selected for screening based on morphotypic similarity to the parent inoculum. Isolates were screened using PCR primers specific to the mutations, and those with the correct band sizes were further verified by Sanger sequencing of the 16S rRNA region. Between two and 20 clones of each re-isolated strain were then purified and assessed for ARA activity relative to the original inoculant strain.

Results and discussion

We hypothesized that we could improve nitrogen contributions by BNF through identification and isolation of diazotrophs that closely associate with key crops, followed by gene editing to disrupt regulatory networks linking nitrogen sensing, fixation, and assimilation. To this end, corn plants were grown in agricultural soils, roots were collected, and microbial populations were extracted from the rhizosphere and endosphere. Isolates capable of growth on nitrogen-free media were purified, their genomes were sequenced, and the presence of the *nif* cluster was confirmed. Because the success of a microbial inoculant product depends on the ability of the strain to establish and persist in field conditions, selected strains were inoculated onto corn seeds which were grown in a field trial to identify strains capable of highly colonizing the root system. Genomic DNA from root samples taken 12 weeks after planting was extracted and subjected to a qPCR assay to quantify the colonization of inoculant strains. One of the most robust colonizers in the field study was a bacterium we classified through 16S rRNA and whole-genome sequencing as *Kosakonia sacchari* (strain PBC6.1), which was detected in 60% of inoculated plants and was not detected in any UTC plants. When detected, PBC6.1 colonized to ~2×10^5 CFU g^{-1} of root FW. *Kosakonia sacchari* is known to be associated with sugarcane (*Saccharum officinarum*) (Chen *et al.*, 2014; Gu *et al.*, 2014) and sweet potato (*Ipomoea batatas*) (Shinjo *et al.*, 2016), while other members of the genus *Kosakonia* (formerly part of *Enterobacter*) are beneficially associated with cereal crops including wheat (Kämpfer *et al.*, 2005), sugarcane (Taule *et al.*, 2016; Beracochea *et al.*, 2019; Taule *et al.*, 2019), rice (*Oryza sativa*) (Li *et al.*, 2016; Li *et al.*, 2017), and corn (Kämpfer *et al.*, 2016). Using fluorescence microscopy of roots inoculated with a strain of PBC6.1 expressing RFP, we observed colonization of the corn root surface along the perimeters of cells (Supplementary Fig. S1).

Genome sequencing revealed that PBC6.1 has a genome of at least 5.4 Mbp, and a *nif* gene cluster and a nitrogen metabolic regulatory network similar to those described in the literature (Fig. 1). The functions and regulation of the *nif* genes have been well studied, and nitrogen metabolism pathways in PBC6.1 are similar to those of the model organism for non-symbiotic nitrogen fixation, *Klebsiella oxytoca* M5A1 (Batista and Dixon, 2019). The *nifLA* operon directly regulates the rest of the *nif* cluster through transcriptional activation by NifA and nitrogen- and oxygen-dependent repression of NifA by NifL (Dixon and Kahn, 2004). Glutamine synthetase (GS) is responsible for rapid assimilation of newly fixed nitrogen in nitrogen-limiting conditions (van Heeswijk *et al.*, 2013). GS is reversibly

regulated by the two–domain adenylyltransferase (ATase) enzyme GlnE through the adenylylation and deadenylylation of GS to attenuate and restore activity, respectively (Jiang and Ninfa, 2009). The *nifLA* operon and GS (encoded by the *glnA* gene) are regulated at the transcriptional level by the PII protein regulatory cascade, which originates with the glutamine-sensing protein GlnD (van Heeswijk *et al.*, 2013; Batista and Dixon, 2019). In nitrogen starvation conditions, GlnD covalently modifies PII proteins, which leads to the up-regulation of nitrogen fixation and assimilation pathways. In the presence of exogenous nitrogen, GlnD removes the covalent modification from the PII proteins, leading to the repression of nitrogen fixation and assimilation genes (Batista and Dixon, 2019).

To assess the sensitivity of PBC6.1 to exogenous nitrogen and to predict activity in a fertilized field, nitrogenase activity in pure culture was measured with the classical ARA (Temme *et al.*, 2012) in the presence and absence of fixed nitrogen. PBC6.1 exhibited a high level of nitrogenase activity in nitrogen-free media, and exogenous fixed nitrogen in the form of 5 mM glutamine or ammonium repressed acetylene reduction (strain PBC6.1, Fig. 3). To evaluate how well PBC6.1 might transfer nitrogen to the roots of a host plant, we measured the release of newly fixed nitrogen into the media in an ammonium excretion assay. Strains were cultured in anaerobic conditions in nitrogen-free media, and the concentration of ammonium in the media was quantified after 3 d. Minimal ammonium from PBC6.1 could be detected (Fig. 4), suggesting that transfer of fixed nitrogen from the strain to a host crop might be limited by both *nif* repression by fertilizer and the microbe's rapid assimilation of newly fixed nitrogen.

Because of its robust colonization of corn roots, its genome containing the *nif* cluster, and its demonstrated nitrogenase activity in ARA, PBC6.1 was selected for further study and optimization for nitrogen fixation. We hypothesized that we could remodel the regulatory networks of PBC6.1 through intragenomic gene editing to enable nitrogen fixation and excretion of ammonium in high exogenous concentrations of fixed nitrogen. We targeted several critical nodes within the nitrogen fixation and assimilation gene regulatory network to increase nitrogen fixation and ammonium excretion in PBC6.1 (Fig. 2). First, disruption of *nifL* can abolish inhibition of NifA and improve *nif* expression in the presence of both oxygen and exogenous fixed nitrogen (Brewin *et al.*, 1999; Ortiz-Marquez *et al.*, 2014). Furthermore, expressing *nifA* under the control of a nitrogen–independent promoter could decouple nitrogenase biosynthesis from the PII protein regulatory cascade (Fig. 2a) (Bageshwar *et al.*, 2017). Secondly, truncation of the GlnE protein to delete its adenylyl-removing (AR) domain would lead to constitutively adenylylated GS, limiting ammonium assimilation by the microbe and leading to ammonium build up and release (Fig. 2b) (Clancy *et al.*, 2007). Thirdly, abolishing expression of AmtB, the transporter responsible for uptake of ammonium, could lead to greater extracellular ammonium by preventing reuptake of excreted ammonium (Fig. 2c) (Zhang *et al.*, 2012; Barney *et al.*, 2015). Finally, deletion of the GlnD protein would lead to a constant nitrogen sufficiency signal by eliminating the ability of the cells to covalently modify PII proteins (Fig. 2d). In combination with a *nifA* gene whose

transcription is decoupled from the PII regulatory cascade, this genotype should lead to a cell with constitutively up-regulated nitrogen fixation and down-regulated nitrogen assimilation, causing a net excess of ammonium and thereby ammonium excretion. To generate rationally designed microbial phenotypes without the use of transgenes, we employed two approaches: creating markerless and scarless deletions of genomic sequences encoding protein domains or whole genes, and rewiring regulatory networks by intragenomic promoter (Prm) rearrangement (Fig. 2). Through an iterative gene editing process, we generated several non-transgenic variant strains of PBC6.1 (Table 1), including a strain with the *nifH* gene deleted to serve as a negative control for nitrogenase expression (Fig. 2e).

Several *in vitro* assays were performed to characterize the edited strains. While PBC6.1 exhibited repression of nitrogenase activity at high exogenous glutamine concentrations, the edited strains reduced acetylene robustly at 5 mM glutamine and ammonium (Fig. 3). Strains containing the *ΔnifL*::Prm5 mutation show partial restoration of nitrogenase activity in the presence of 5 mM N, while strains containing the *ΔnifL*::Prm1 mutation show full restoration of nitrogenase activity in the presence of 5 mM N, reflecting the relative strength of the promoters inserted upstream of *nifA*. Additionally, some strains with the *ΔnifL*::Prm mutations show a decrease in growth rate in the presence of nitrogen (Fig. 3), suggesting that high levels of nitrogen fixation in nitrogen-replete conditions may carry a fitness cost. PBC6.90, the *ΔnifH* strain, exhibits no nitrogen fixation in any condition tested, as expected. We next measured whether edited strains excreted ammonium into the culture medium under nitrogen-fixing conditions (Fig. 4). While the *ΔnifL*::Prm5 mutation alone (PBC6.15) was insufficient to confer an ammonium excretion phenotype, the *ΔnifL*::Prm1 mutation alone (PBC6.14) led to significant excretion of ammonium. This suggests that an increase in nitrogenase expression and activity may be sufficient to generate an excess of ammonium ions within the cell which are not assimilated by GS, leading to passive excretion across the cell membrane, as has previously been observed in *Azotobacter vinelandii* (Bali *et al.*, 1992; Brewin *et al.*, 1999; Ortiz-Marquez *et al.*, 2014; Barney *et al.*, 2015). The *ΔglnE_{AR}* mutations led to an increase in ammonium excretion when stacked with the *ΔnifL*::Prm mutations, supporting our hypothesis that down-regulation of GS activity would lead to ammonium excretion. Furthermore, the *ΔglnD* mutation led to an additional increase in ammonium excretion, probably by causing a decrease in *glnA* expression. Notably, the ammonium excretion phenotype conferred by the *ΔglnE_{AR}* and *ΔglnD* mutations came with a corresponding decrease in growth rates, similar to what was observed in the acetylene reduction assay (Fig. 4). The *ΔamtB* mutations had no apparent effect on ammonium excretion or growth rate when stacked with the *ΔnifL*::Prm and *ΔglnE_{AR}* mutations. These results suggest that the edited strains may be able to fix nitrogen and transfer it to the crop in fertilized field conditions.

To determine whether the edited microbes were able to express nitrogenase in the rhizosphere of fertilized plants, we inoculated corn plants in greenhouse assays with PBC6.1 and a subset of edited strains to measure colonization of the corn
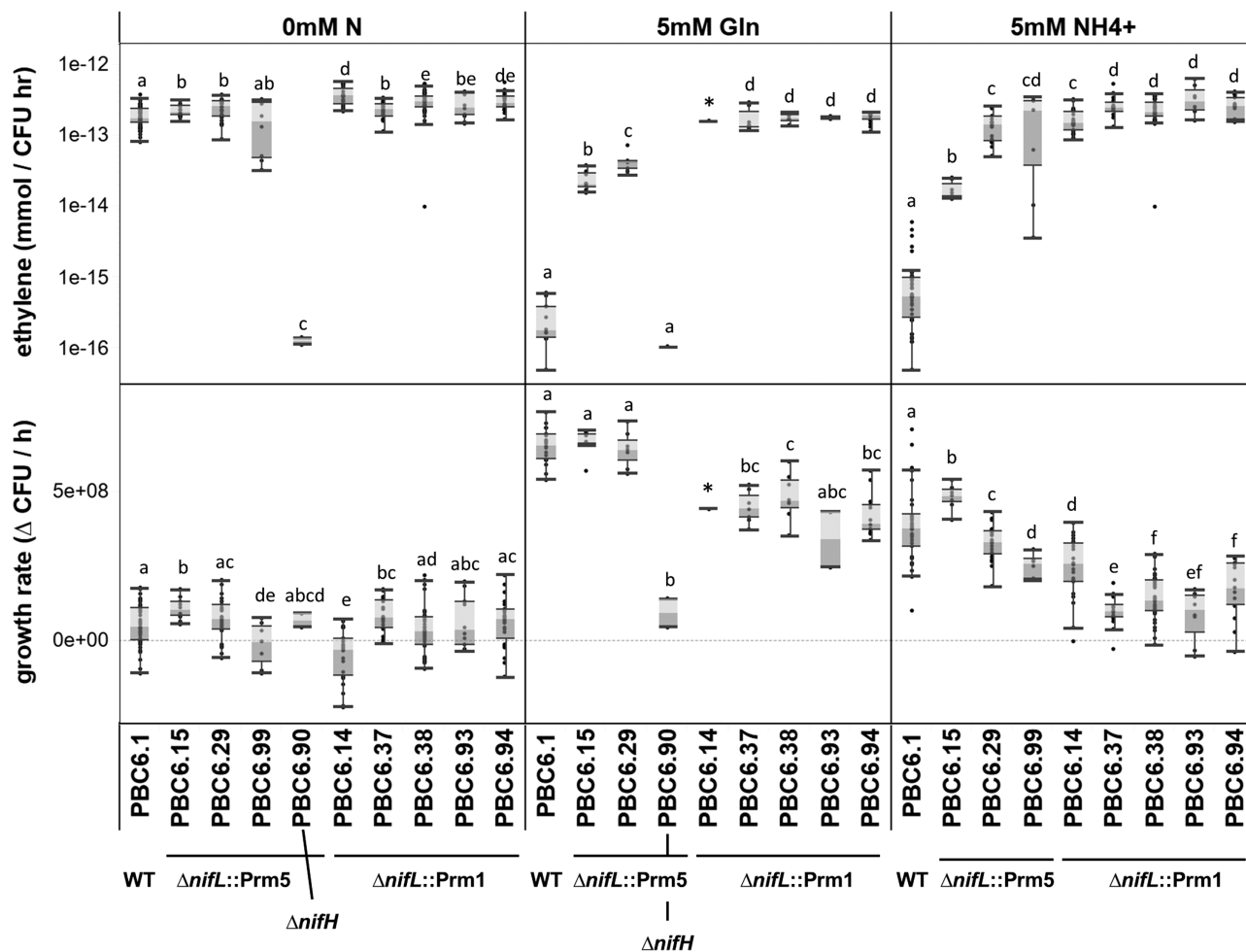
Fig. 3. Edited strains of PBC6.1 fix nitrogen independently of nitrogen status. Nitrogenase activity under varying concentrations and forms of exogenous nitrogen was measured with the acetylene reduction assay. The wild-type strain exhibits repression of nitrogenase activity as glutamine concentrations increase, while remodeled strains show nitrogenase activity in the presence of 5 mM glutamine (Gln) or NH_4^+. Each dot represents a single biological replicate; shaded boxes and whiskers represent the quartiles and range of the data, respectively; dots outside of whiskers are statistical outliers. Within each panel, letters indicate groups for which P was <0.05 as determined by a two-tailed, two-sample unequal variance t-test. An asterisk indicates where only one sample was analyzed. PBC6.90 was not tested in 5 mM NH_4^+; PBC6.99 was not tested in 5 mM glutamine.

rhizosphere and *nifH* transcription therein. In a first experiment, corn plants fertilized with 2 mM nitrate were harvested after the emergence of the second leaf collar, and the root metagenome and metatranscriptome were isolated. A probe-based method was used to measure the presence of PBC6.1 *nifA* and *nifH* transcripts relative to the housekeeping gene *rpoB*. The transcription of *nifA* was significantly up-regulated compared with the WT in all strains tested, all of which contained the *ΔnifL*::Prm5 mutation. Interestingly, PBC6.99 showed a significantly increased level of *nifA* (Fig. 5A) and *nifH* (Fig. 5B) transcript compared with its direct parent PBC6.29, suggesting that the *ΔglnD* mutation has an impact on *nifA* transcription. This is unexpected in the context of the *ΔnifL*::Prm5 mutation, which was hypothesized to confer a consistent level of *nifA* transcript in a variety of backgrounds. This suggests that the deletion of *glnD* has activated an unknown pathway which regulates *nifA* transcription, at least in the context of the inserted promoter. PBC6.29 showed no significant increase in *nifH* transcript in the corn rhizosphere, despite the significant increase in *nifA* transcription—this may be due to a basal level of *nifH* transcription in the WT strain

in the relatively low nitrogen conditions of the assay. As expected, *nifH* transcript was not detected in the *nifH* knockout strain (PBC6.90). Overall colonization was significantly higher than observed in the initial field study, probably reflecting both the earlier growth stage of the plant and the more controlled conditions of the greenhouse assay (Fig. 5C). All remodeled strains exhibited a decrease in colonization compared with the WT, with the greatest decrease observed in PBC6.99, suggesting that the high expression of *nifA* and *nifH* in that strain led to a fitness cost. However, the decrease in colonization of the *nifH* knockout strain (PBC6.90) suggests that this fitness cost is not entirely due to nitrogenase expression and may be a function of the nitrogen assimilation edits present in the strain.

In another study, we measured microbial colonization of root tissues and expression of *nifH* in strains containing the *ΔnifL*::Prm1 (PBC6.38 and PBC6.94) mutation 4 weeks after inoculation of the corn rhizosphere. Measurement of microbial transcription within root tissues showed that *nifH* expression was significantly increased in both edited strains compared with the WT (Fig. 5D), suggesting that the *ΔnifL*::Prm1 mutation
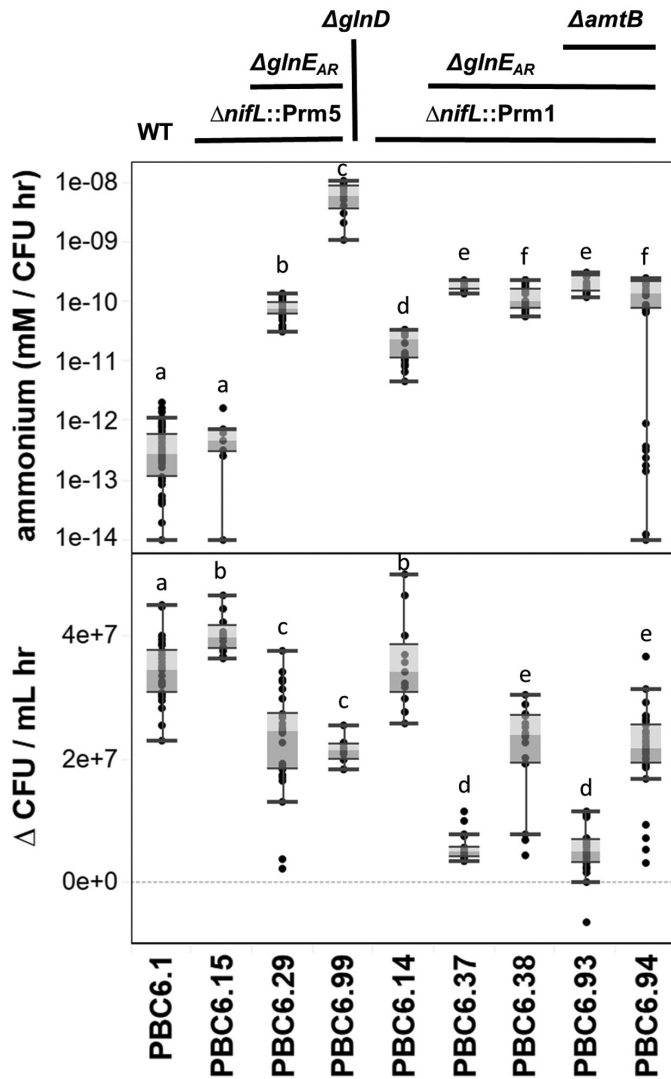
Fig. 4. Edited strains of PBC6.1 excrete fixed nitrogen into their environment. PBC6.1 and derived strains were grown in minimal nitrogen-free media in anaerobic conditions, and the resulting supernatant was cleared of cells and assayed for the presence of ammonium. While the WT strain cultured had little to no ammonium present, several gene-edited strains exhibited excretion of ammonium into the culture medium. Each dot represents a single biological replicate; shaded boxes and whiskers represent the quartiles and range of the data, respectively; dots outside of whiskers are statistical outliers. Data from multiple replicated experiments were combined. Within each panel, letters indicate groups for which P was <0.05 according to a two-tailed, two-sample unequal variance *t*-test.

Table 1. List of isolated and remodeled *K. sacchari* strains used in this work

Strain	Genotype
PBC6.1	WT
PBC6.15	$\Delta nifL$::Prm5
PBC6.29	$\Delta nifL$::Prm5 $\Delta glnE_{AR}$1
PBC6.99	$\Delta nifL$::Prm5 $\Delta glnE_{AR}$1 $\Delta glnD$
PBC6.90	$\Delta nifL$::Prm5 $\Delta glnE_{AR}$1 $\Delta nifH$
PBC6.14	$\Delta nifL$::Prm1
PBC6.37	$\Delta nifL$::Prm1 $\Delta glnE_{AR}$2
PBC6.38	$\Delta nifL$::Prm1 $\Delta glnE_{AR}$1
PBC6.93	$\Delta nifL$::Prm1 $\Delta glnE_{AR}$2 $\Delta amtB$
PBC6.94	$\Delta nifL$::Prm1 $\Delta glnE_{AR}$1 $\Delta amtB$

Prm, promoter sequence derived from the PBC6.1 genome; $\Delta glnE_{AR}$1 and $\Delta glnE_{AR}$2, different truncated versions of the *glnE* gene removing the adenylyl-removing domain sequence.

plot field trials using seeds coated with selected edited strains at three locations with a wide range of climates, soil types, and soil nitrogen levels. The Illinois trials represent typical growing conditions in a major corn–producing region of the USA, while California and Puerto Rico trials represent arid and tropical corn–producing regions, respectively. Because these trials were designed to study the persistence and nitrogenase expression of the bacteria in the rhizosphere, yield data were not collected, except for the Puerto Rico trial (Fig. 7). Root samples were collected between 2 and 5 weeks after planting for nucleic acid extraction to verify the presence of the strain and expression of the *nifH* gene (Fig. 6). PBC6.1 and edited strains colonize consistently across most of the locations tested, at levels of ~10^5 CFU g^{-1} of root FW (Fig. 6b, d, h). One exception was the Illinois location, at which the WT PBC6.1 strain colonized ~10^8 CFU g^{-1} of root FW, though this was not statistically significantly higher than the mutant strains (Fig. 6f). While this shows robust colonization by the strain in the field, these levels are significantly lower than values observed in the greenhouse (Fig. 5), possibly due to the combination of harsher abiotic conditions or the presence of a robust competing microbiome in the field. We observed consistently low normalized transcript levels of *nifH* in the WT parent strain PBC6.1 in all conditions tested, which ranged from unfertilized with residual nitrates measured at 16 kg nitrate N ha^{-1} to a field fertilized to a level of 123 kg N ha^{-1}. All edited strains show 5- to 10-fold increased transcription of *nifH* compared with the WT, though transcript results varied between strains and locations. PBC6.38 and PBC6.93 showed significantly increased *nifH* transcript at both locations where they were tested (Fig. 6e, g). While PBC6.29 did not show a significant increase in *nifH* transcript over PBC6.1 in the Illinois trial (Fig. 6e), it did show a significantly increased *nifH* transcript level in the 2018 California trial (Fig. 6d) and in the Puerto Rico trial (Fig. 6g), which is in contrast to the greenhouse results (Fig. 6b). The results correlate with differences in soil nitrogen levels—the greenhouse study was fertilized at low levels, and the Illinois trial was unfertilized, while the California and Puerto Rico trials were fertilized at levels consistent with farmer practice in the region. Strains with the $\Delta nifL$::Prm1 mutation showed significant increases in *nifH* transcript compared with PBC6.1 at all
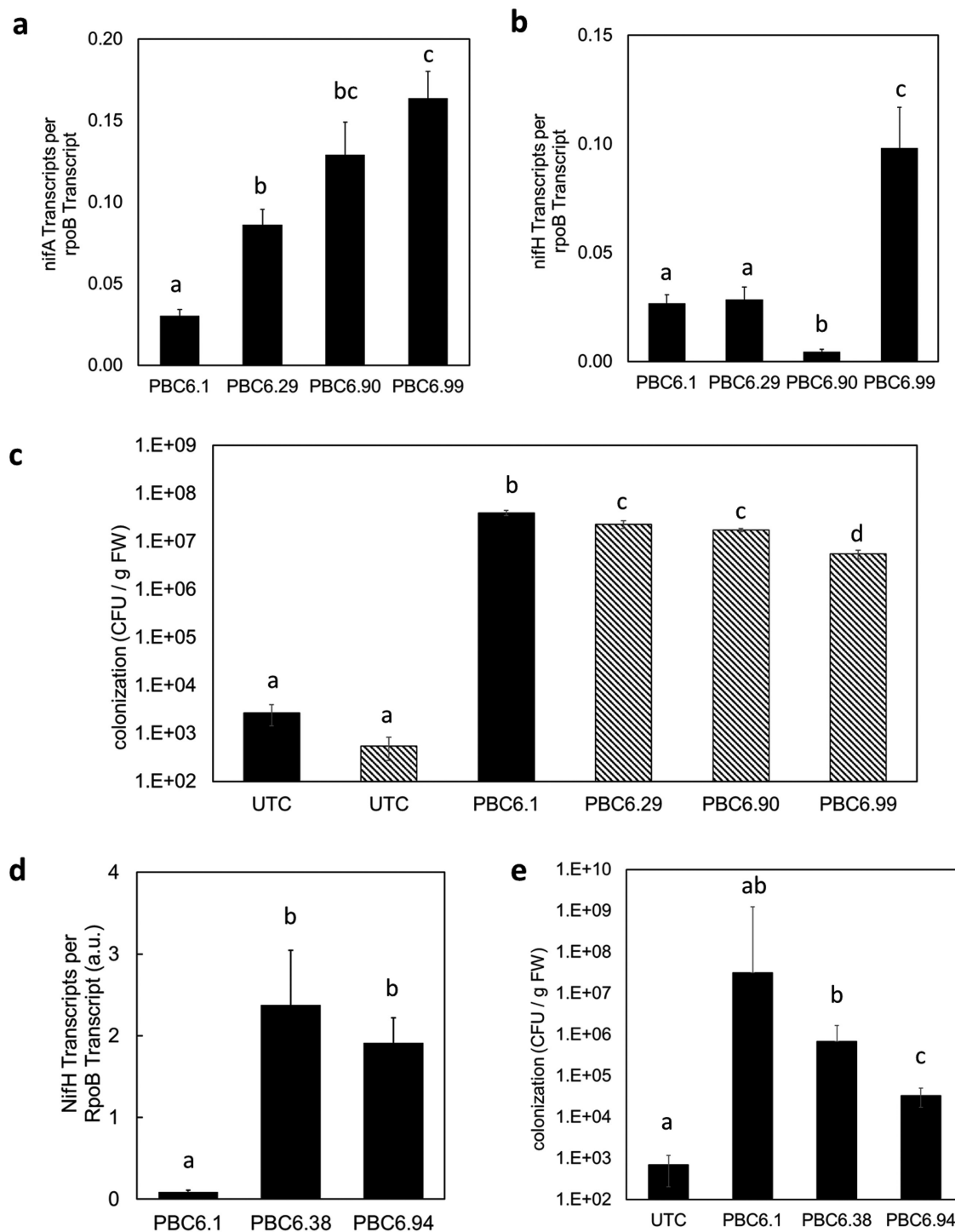
(in the absence of the $\Delta glnD$ mutation) confers a stronger nitrogenase up-regulation phenotype than the $\Delta nifL$::Prm5 mutation in the rhizosphere. Both edited strains showed a decrease in colonization compared with the WT, with PBC6.94 exhibiting significantly reduced abundance (Fig. 5E), again suggesting a fitness cost to high levels of nitrogenase expression and/or disruption of nitrogen assimilation.

In the past, translating microbial inoculants from the greenhouse to field conditions has posed significant challenges and, in some cases, has thwarted successful implementation of beneficial microbes in large-scale agriculture. Therefore, to measure the presence and nitrogenase expression of PBC6.1 and its derivatives in a variety of field conditions, we conducted replicated

Fig. 5. Greenhouse experiments demonstrate rhizosphere *nifH* transcription in fertilized corn. (A–C) Corn was planted in a controlled-environment growth chamber and inoculated with PBC6.1, PBC6.29, or PBC6.99. Upon the emergence of the second leaf collar, plants were harvested, and nucleic acids were extracted from the rhizosphere to quantify *nifA* (A) and *nifH* (B) transcript levels and colonization (C) of each strain. Data are presented as the mean of five root samples pooled from five plants each. (D and E) In a second experiment, 4 weeks after inoculation with PBC6.1 derivative strains, the corn root-associated microbiome was extracted. (D) *In planta* transcription of *nifH* was measured. The remodeled strains showed higher *nifH* transcription than PBC6.1 in the corn root environment. Error bars show the SE of the mean of at least three biological replicates. (E) Colonization is reported as the geometric means of at least eight biological replicates. In (C) and (E), black bars represent samples analyzed using primers targeting the *nifH–por2* intergenic region, and hatched bars represent samples analyzed using primers targeting the *ΔnifL::Prm5* genotype. Error bars show the SE of the mean. Within each panel, letters indicate groups for which P was <0.05 according to a two-tailed, two-sample unequal variance t-test.

locations, except PBC6.94, which showed high variability in the Illinois and Puerto Rico trials (Fig. 6a, e, g). In the Puerto Rico trial, yield data were collected. Because yield correlated

with plot location rather than fertilizer treatment, we assumed significant nitrogen mobilization across the field and therefore averaged the yield data across all plots and nitrogen treatments
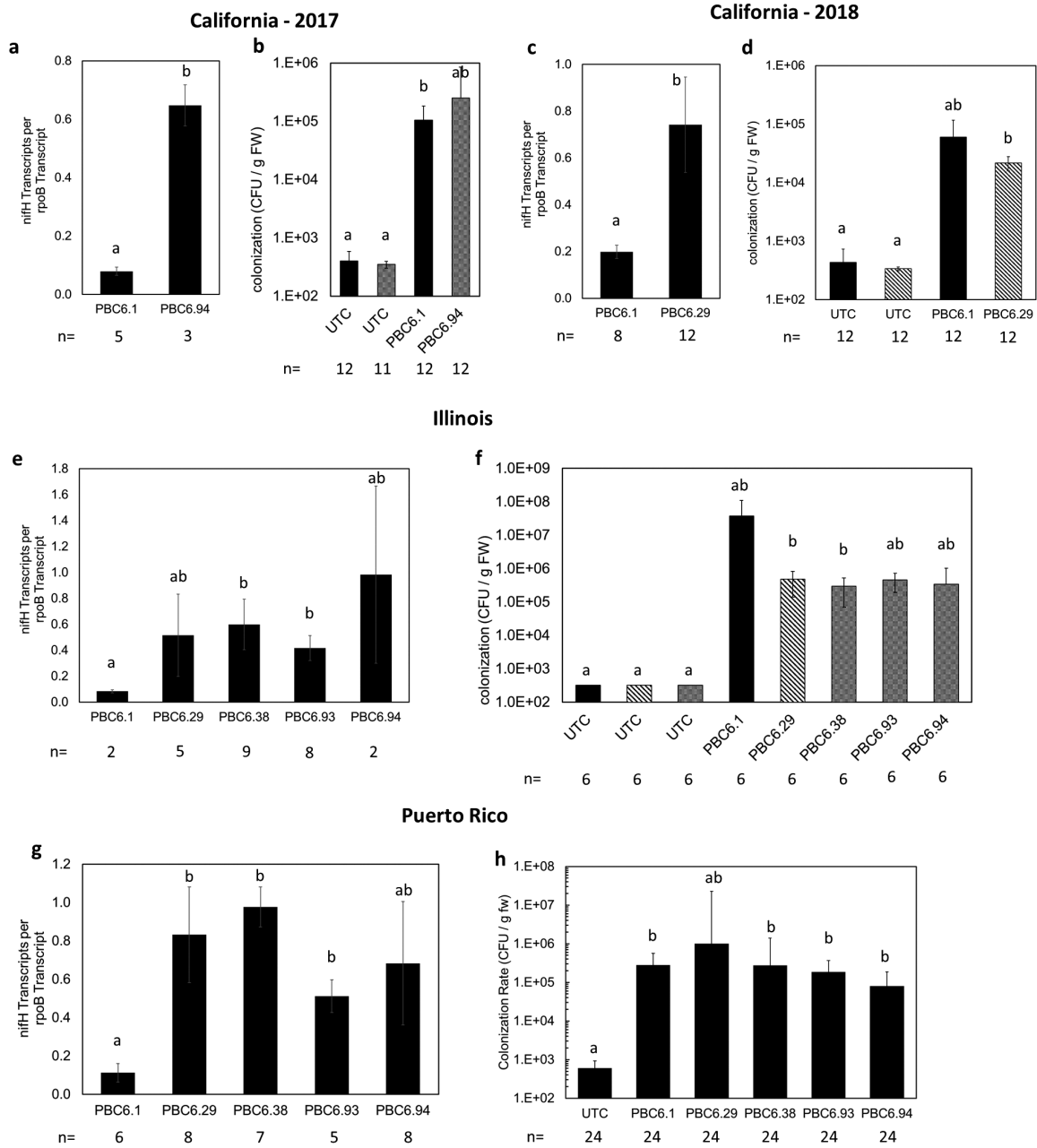
Fig. 6. Remodeled strains colonize corn roots and express nitrogenase in diverse locations, soil types, and nitrogen levels. Three replicated plot field trials were conducted by planting corn seed coated with PBC6.1 and derived strains. Root samples were collected, the root-associated microbiome was extracted, and *nifH* transcription (a, c, e, g) and colonization (b, d, f, h) were quantified. The California 2017 trial (a, b) was fertilized to a level of 123 kg N ha^{-1} prior to planting, and root samples were collected 13 d after planting; the California 2018 trial (c, d) was planted at the same location and fertilizer level as the previous year, and root samples were collected 6 weeks after planting. In Illinois (e, f), an unfertilized trial was planted in a field found to have 16 kg ha^{-1} residual nitrates, and root samples were collected 21 d after planting. In Puerto Rico (g, h), a fertilized trial was planted in a field with pre-plant nitrate N levels measured at 89 kg ha^{-1}, and root samples were collected 5 weeks after planting; because of heavy rains causing apparent leaching and pooling of fertilizer in fields, the samples were averaged across all fertilizer treatments. At all locations, remodeled strains show increased normalized *nifH* transcript levels and similar colonization when compared with PBC6.1. In (b, d, f, and h), black bars represent samples analyzed using primers targeting the *nifH–por2* intergenic region; shaded and hatched bars represent samples analyzed using primers targeting the *ΔnifL::Prm1* and *ΔnifL::Prm5* genotypes, respectively. Colonization values (b, d, and f) are reported as geometric means, and error bars on all panels represent the SE of the mean. Within each panel, *n* indicates the number of replicate plants; letters indicate groups for which *P* was <0.05 according to a two-tailed, two-sample unequal variance *t*-test.

(see the Materials and methods). The UTC plots produced a yield of 6.3 t ha^{-1}, and PBC6.1 and some of the edited strains showed significant increases in yield compared with the UTC (Fig. 7). While these data are highly variable and only represent a single field season, they suggest that these strains may have potential to improve the growth of corn. Additionally, clones of

edited strains were re-isolated from the roots of several plants in the Puerto Rico trial and found to be genetically consistent and to exhibit similar *in vitro* nitrogenase activity levels to the original inoculant strains (Fig. 8). These data confirm a true association between PBC6.1 derivatives and corn plants and that the strains retained their nitrogen-fixing capability in the
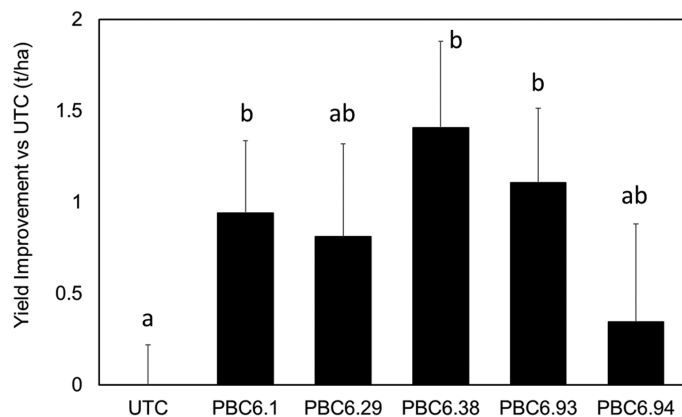
Fig. 7. Corn inoculated with PBC6.1 and its derivatives exhibited an increase in grain yield above UTC in the Puerto Rico field trial. Error bars represent the SE of the mean across 16 plots. Letters indicate groups for which *P* was <0.05 according to a two-tailed, two-sample unequal variance *t*-test.
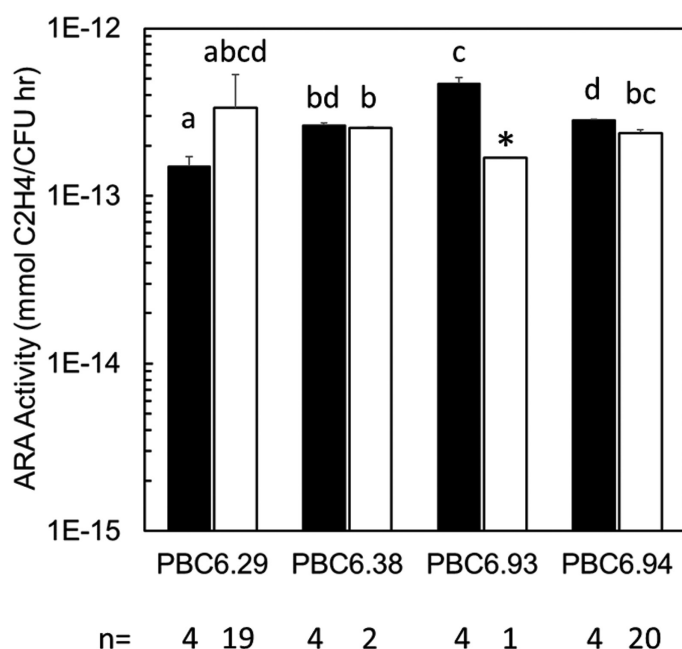

Fig. 8. Nitrogenase activity is observed in clones re-isolated from field-grown root samples. White bars represent the input strains used to inoculate the field trial; black bars represent clones reisolated from root tissues. Re-isolated clones show activity similar to the original inoculant strains. Error bars show the SE of the mean of at least two clones, except for PBC6.93, for which only one clone was re-isolated and analyzed. Letters indicate groups for which *P* was <0.05 as determined by a two-tailed, two-sample unequal variance *t*-test. Asterisk indicates the data where only one sample was analyzed.

field. Taken together, these data show that PBC6.1 and its derivatives colonize corn plants and survive during the growing season in diverse environments, and that nitrogenase expression is de-repressed in edited strains in a broad range of soil nitrogen levels.

Conclusions

Long-term ecological studies in legume–rhizobia interactions have shown that elevated nitrogen inputs can lead to the evolution of less cooperative nitrogen-fixing mutualists (Weese *et al.,*

2015). In this study, we have shown that a non-transgenic microbe can express nitrogenase in close association with corn roots in the presence of nitrogen fertilizer and can thrive in typical field conditions. Through gene editing, we generated strains in which nitrogenase biosynthesis is decoupled from the regulatory networks that sense and respond to cellular nitrogen status. These strains fix and excrete significant quantities of nitrogen into their environment at various levels of exogenous nitrogen (Figs 3, 4). By utilizing plant-by-plant measurements of colonization and transcription, we were able to study plant association and nitrogenase expression in these microbes on a molecular level (Figs 5, 6). We observed robust colonization and expression of nitrogenase in diverse field environments and soil types, and that functional clones can be re-isolated from field-grown roots 12 weeks after planting (Fig. 8). The persistence of gene-edited, ammonium-excreting strains in a field trial is a promising finding because translation to field conditions has been a challenge for the development of microbial products for production-scale agriculture, especially for ammonium-excreting diazotrophs in cereal cropping systems. Designing bacteria that fix nitrogen in the presence of exogenously fertilizer is a first step toward developing strains that can replace synthetic fertilizers in cereal crop production.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Fluorescence micrograph of an RFP-expressing PBC6.1 strain colonizing the surface of corn roots.

Table S1. Primers used in this study for genotypic detection.

Table S2. Primers used in this study for colonization assays.

Acknowledgements

Conflict of interest

The authors declare a competing financial interest: Pivot Bio has filed patent applications and is developing products based on the microbial strains in this study.

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