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Gene Deletions Resulting in Increased Nitrogen Release by *Azotobacter vinelandii*: Application of a Novel Nitrogen Biosensor

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Azotobacter vinelandii **is a widely studied model diazotrophic (nitrogen-fixing) bacterium and also an obligate aerobe, differentiating it from many other diazotrophs that require environments low in oxygen for the function of the nitrogenase. As a free-living bacterium, *A. vinelandii* has evolved enzymes and transporters to minimize the loss of fixed nitrogen to the surrounding environment. In this study, we pursued efforts to target specific enzymes and further developed screens to identify individual colonies of *A. vinelandii* producing elevated levels of extracellular nitrogen. Targeted deletions were done to convert urea into a terminal product by disrupting the urease genes that influence the ability of *A. vinelandii* to recycle the urea nitrogen within the cell. Construction of a nitrogen biosensor strain was done to rapidly screen several thousand colonies disrupted by transposon insertional mutagenesis to identify strains with increased extracellular nitrogen production. Several disruptions were identified in the ammonium transporter gene *amtB* that resulted in the production of sufficient levels of extracellular nitrogen to support the growth of the biosensor strain. Further studies substituting the biosensor strain with the green alga *Chlorella sorokiniana* confirmed that levels of nitrogen produced were sufficient to support the growth of this organism when the medium was supplemented with sufficient sucrose to support the growth of the *A. vinelandii* in coculture. The nature and quantities of nitrogen released by urease and *amtB* disruptions were further compared to strains reported in previous efforts that altered the *nifLA* regulatory system to produce elevated levels of ammonium. These results reveal alternative approaches that can be used in various combinations to yield new strains that might have further application in biofertilizer schemes.**

Nutrient requirements are directly linked to biomass production, and any potential increased improvement in the scale of biomass yield will necessitate a proportional increase in the demand for essential nutrients. For all photosynthetic organisms (photoautotrophs such as land plants, algae, and cyanobacteria) with requisite light energy and water, nitrogen is the most limiting and expensive nutrient input for aquaculture and agricultural production alike (1). A majority of our current nitrogen fertilizer production is tied to the burning of fossil fuels to generate ammonia from molecular nitrogen (N_2 gas) through the Haber-Bosch process, which accounts for 3 to 5% of world natural gas consumption, or about 1 to 2% of total worldwide energy expenditures (1–3). In developed countries, industrial nitrogen production is accompanied by a huge economic and energetic cost overall (2), while this key nutrient limits agricultural productivity in developing countries, where energy and infrastructure costs prohibit the utilization of the Haber-Bosch process to produce ammonia from atmospheric nitrogen on a large scale.

The development of biological approaches to improve biofertilizers represents a unique opportunity for future generations to lower the potential economic costs and environmental impacts of current fossil-fuel-dependent industrial methods for the production of ammonia-derived fertilizers (4–6). Various laboratories have been investigating the potential of *Azotobacter* species to provide nitrogen to specific crops for many years, with various degrees of success (5–11). Although many nitrogen-fixing bacteria produce nitrogen in environments requiring very low oxygen, *Azotobacter vinelandii* has evolved the capability of fixing nitrogen as a free-living aerobe, despite the fact that nitrogenase is inherently sensitive to oxygen. This makes it an ideal organism for coculture with a broader range of plants, as growth in microaerobic or anaerobic environments is not required. The ultimate long-term goal of biofertilizers would be to develop potential alternatives to the energy intensive Haber-Bosch process.

Biofertilizers provide a range of potential benefits versus current industrial nitrogen production routes. *In situ* biofertilizer production would circumvent and displace transportation costs and associated environmental impacts related to production and distribution of Haber-Bosch-derived industrial fertilizers. Biological assimilation of nutrients in addition to timed-release of nitrogen compounds would mitigate issues associated with agricultural residue runoff from excessive application of industrial fertilizers, leading to eutrophication of nearby water supplies and streams. Both higher land plants and microalgae are known to produce extracellular carbon as a potential source of fixed carbon to support beneficial heterotrophs that make up part of the rhizosphere (5, 12–15).

Current biofuel feedstock crops such as corn for ethanol require substantial amounts of nitrogen inputs. Potential future production of biomass using next-generation feedstocks such as algae promise significant improvements in overall yield that could be orders of magnitude higher than current conventional land

Received 16 February 2015 **Accepted** 13 April 2015

Accepted manuscript posted online 17 April 2015

Citation Barney BM, Eberhart LJ, Ohlert JM, Knutson CM, Plunkett MH. 2015. Gene deletions resulting in increased nitrogen release by *Azotobacter vinelandii*: application of a novel nitrogen biosensor. Appl Environ Microbiol 81:4316–4328. doi:10.1128/AEM.00554-15.

Editor: H. L. Drake

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doi:10.1128/AEM.00554-15

TABLE 1 Mutant strains constructed and/or used in this study

A. vinelandii strain	Plasmid utilized	Genetic features[a]	Parent strain
DJ[b]	None	Wild type	
AZBB010	pPCRNH3-15	Δ*nifLA*::Str^r	DJ
AZBB020	pPCRNH3-14	Δ*nifLA*::*nifA*	AZBB010
AZBB023	pPCRSCRK31	Δ*scrX*::*lacZ*-Kan^r	DJ
AZBB030	pPCRURE3	Δ*ureABC*::Str^r	DJ
AZBB035	pPCRURE3	Δ*ureABC*::Str^r and Δ*nifLA*::*nifA*	AZBB020
AZBB063	pPCRNH3-21	Δ*nifLA*::Tet^r and Δ*scrX*::*lacZ*-Kan^r	AZBB023
AZBB085	pEB001	*amtB*::Kan^r-transposon and Δ*ureABC*::Str^r and Δ*nifLA*::*nifA*	AZBB035
AZBB086	pEB001	*amtB*::Kan^r-transposon and Δ*ureABC*::Str^r and Δ*nifLA*::*nifA*	AZBB035
AZBB088	pEB001	*amtB*::Kan^r-transposon and Δ*ureABC*::Str^r	AZBB030
AZBB091	pEB001	*amtB*::Kan^r-transposon and Δ*ureABC*::Str^r	AZBB030
AZBB093	pEB001	*amtB*::Kan^r-transposon and Δ*ureABC*::Str^r and Δ*nifLA*::*nifA*	AZBB035
AZBB094	pEB001	*amtB*::Kan^r-transposon and Δ*ureABC*::Str^r and Δ*nifLA*::*nifA*	AZBB035
AZBB102	pPCRAMTBK3	Δ*amtB*::Kan^r	DJ
AZBB103	pPCRAMTBK3	Δ*amtB*::Kan^r and Δ*ureABC*::Str^r	AZBB030
AZBB148	pPCRNH3-42	*nifL*::Kan^r similar to MV376[c]	AZBB010
AZBB150	pPCRNH3-43	*nifL*::Kan^r incorporated slightly upstream of SmaI site	AZBB010
AZBB158	None	Spontaneous mutation of AZBB148 resulting in Nif$^+$ phenotype	AZBB148
AZBB163	None	Spontaneous mutation of AZBB150 resulting in Nif$^+$ phenotype	AZBB150

[a] Tet^r, tetracycline resistance; Str^r, streptomycin resistance; Kan^r, kanamycin resistance.
[b] Setubal et al. (35).
[c] Brewin et al. (8).

plant crops (16, 17). Since a majority of the current nitrogen requirements for the growth of biofuel crops will likely be derived from energy intensive industrial processes such as Haber-Bosch, the energy use efficiency of current biofuel crops has been questioned (18). A significant amount of the energy acquired from corn ethanol and soybean biodiesel or next-generation biofuel crops such as algae may actually need to be diverted back to these industrial processes to supply the energy required for additional industrial nitrogen fixation. Proposed improvements in final biomass yield will require concomitant increases in macronutrient inputs such as nitrogen. In a world where societies will require more of our agricultural resources to feed expanding populations, and significant portions of our agricultural lands are currently being converted from food to fuel crop production, the impacts and requirements of current methods to provide nitrogen for present and future crops will only increase in importance (18–20).

The long-term aim of biofertilizers is to circumvent the energy cost and the associated greenhouse gas emissions tied to the production and distribution of conventional Haber-Bosch derived nitrogen fertilizers. The potential of biofertilizers can be enhanced by identifying alternative methodologies of nitrogen delivery using diazotrophic bacteria to provide a renewable source of nitrogen to meet the growth requirements of the associated photosynthetic species. Although model symbiotic systems between specific plants and nitrogen fixing bacteria are well established (21, 22), these are limited to a small number of commodity crops. This approach is directed toward expanding similar symbiotic relationships to a broader range of crops or next-generation biomass sources.

In the present study, we use various approaches to increase the production of extracellular nitrogen by *A. vinelandii*. These approaches include rational manipulations designed to convert urea from a common metabolite into a terminal product. Further application of transposon mutagenesis combined with a nitrogen biosensor reveals a specific gene deletion resulting in increased nitrogen release by *A. vinelandii*. These alterations are compared to results obtained following the reconstruction of strains reported previously to elevate production of ammonium by *A. vinelandii* following disruption of the regulatory gene product of *nifL*. These reconstructed strains were used to make comparisons between the results and reported phenotypes of previous constructs, as well as the strains constructed here, to highlight the potential to produce fixed nitrogen species that might be applied to various biofertilizer schemes.

MATERIALS AND METHODS

Bacterial culture. *A. vinelandii* DJ was obtained from Dennis Dean (Virginia Tech) and grown on standard B plates (23) unless otherwise specified. *Escherichia coli* WM3064 was used for conjugation (24, 25) and grown on lysogeny broth (LB) or on BYE medium (B plates containing 5 g of yeast extract/liter).

Algal culture and cell counts. Cultures of *Chlorella sorokiniana* UTEX 1602 were obtained from the UTEX culture collection of algae (Austin, TX) and have been maintained for several years by subculturing on solid media (11). Algae strains were cultured in a freshwater medium as described previously (26). Algal cells in solution were measured using a hemocytometer following the directions of the manufacturer (Hausser Scientific, Horsham, PA).

Genetic constructs of *Azotobacter vinelandii*. *Azotobacter vinelandii* AZBB030 and AZBB035 used as initial target strains, and AZBB063 used as a biosensor strain, were constructed as detailed in Table 1 by transforming *A. vinelandii* DJ with the various plasmids listed in Table 2. The primers used to clone genes or genome segments are listed in Table 3. A graphical representation of the three strains compared to the wild-type *A. vinelandii* strain is shown at the top of Fig. 2. Following the transposon experiments described below, *A. vinelandii* AZBB102, AZBB103, AZBB148, and AZBB150 were constructed by using a similar approach to confirm the phenotype found during the transposon experiments or construct strains used as controls. Methods for the manipulation of *A. vinelandii* have been described previously (23, 27, 28).

Urea quantitation. To measure extracellular urea concentrations from cultures of *A. vinelandii*, 1-ml samples were first spun at maximum

TABLE 2 Key plasmids and relevant derivatives of these plasmids used for the construction of *A. vinelandii* manipulated strains

Plasmid[a]	Relevant gene(s) cloned or plasmid manipulation[b]	Parent vector	Source or reference
pEB001	Plasmid containing Mariner transposon and transposase		24
pBB052	pUC19 with Kan^r from pUC4K in place of Amp^r	pUC19	50
pBB053	Removed NdeI site from pUC19 by silent mutation	pUC19	25
pBB073	Moved spectinomycin/streptomycin resistance cassette from pHP45Ω into EcoRI site of pBB052	pBB052	51
pBBTET3	pUC19 with Tet^r in place of Amp^r	pUC19	25
pPCRKAN4	Cloned Kan cassette from pBBR1MCS-2 into pBBTET3	pBBTET3	25
pLACZF12	Cloned *lacZ* gene from *E. coli* into pBBTET3 and removed various restriction sites by silent mutation with site-specific mutagenesis	pBBTET3	This study
pPCRSCRK2	Cloned *scrX* gene and flanking regions from *A. vinelandii* into pBB053	pBB053	This study
pPCRSCRK5	Removed restriction sites from pPCRSCRK2 by blunt fill-in; performed PCR to remove *scrX* gene from plasmid	pBB053	This study
pPCRSCRK7	Performed additional blunt fill-in and site-specific mutagenesis to remove additional restriction sites	pBB053	This study
pPCRSCRK28	Moved Kan cassette from pPCRKAN4 into pPCRSCRK7, then removed restriction sites by site-specific mutagenesis with silent mutations	pBB053	This study
pPCRSCRK31*	Moved *lacZ* gene from pLACZF12 into pPCRSCRK28	pBB053	This study
pPCRNH3-10	Cloned *nifA* gene from *A. vinelandii* into pBB114	pBB114	This study
pPCRNH3-11	Cloned *nifLA* genes and flanking regions from *A. vinelandii* into pBB053	pBB053	This study
pPCRNH3-12	Removed restriction site from pPCRNH3-11 by blunt fill-in	pBB053	This study
pPCRNH3-13	Performed PCR to remove *nifLA* genes from pPCRNH3-12 and add XbaI and BamHI sites	pBB053	This study
pPCRNH3-14*	Moved *nifA* gene from pPCRNH3-10 into pPCRNH3-13	pBB053	This study
pPCRNH3-15*	Moved Str cassette from pBB073 into pPCRNH3-13	pBB053	This study
pPCRNH3-21*	Moved Tet cassette pBBTET3 into pPCRNH3-13	pBB053	This study
pPCRNH3-42*	Removed segment of *nifL* gene from pPCRNH3-11 and inserted Kan cassette from pPCRKAN4 similar to approach taken to construct pBB369	pBB053	8; this study
pPCRNH3-43*	Removed larger segment of *nifL* gene from pPCRNH3-11 than was done in pPCRNH3-42 and inserted Kan cassette from pPCRKAN4 slightly further upstream of *nifA* as shown in Fig. 7	pBB053	This study
pPCRURE1	Cloned *ureABC* genes and flanking regions from *A. vinelandii* into pUC19	pUC19	This study
pPCRURE2	Performed PCR to remove *ureABC* genes from pPCRURE1	pUC19	This study
pPCRURE3*	Moved Str cassette pBB073 into pPCRURE2	pUC19	This study
pPCRAMTBK1	Cloned gene *amtB* and flanking regions from *A. vinelandii* into pBB053	pBB053	This study
pPCRAMTBK2	Performed PCR to remove gene *amtB* from pPCRAMTBK1	pBB053	This study
pPCRAMTBK3*	Moved Kan cassette from pPCRKAN4 into pPCRAMTBK2	pBB053	This study

[a] The sequences of all plasmids in this study are available upon request. Plasmids indicated by an asterisk (*) are completed vectors that were used to transform *A. vinelandii*.
[b] Tet^r, tetracycline resistance; Amp^r, ampicillin resistance; Str^r, streptomycin resistance; Kan^r, kanamycin resistance.

speed on a microcentrifuge for 1 min (~21,000 × *g*), and then the supernatant was removed and stored at −80°C or used immediately. Urea was quantified using the method of Mather and Roland (29) with slight alterations. The reagent was prepared fresh immediately before use by dissolving 10 mg of $FeCl_3 \cdot 6H_2O$ in 50 ml of acid solution (8% H_2SO_4 and 1% H_3PO_4). Next, 75 mg of diacetyl monoxime was dissolved in 5 ml of H_2O and combined with the acid solution. Then, 5 mg of thiosemicarbazide was dissolved in 5 ml of H_2O and combined with the acid solution. A standard curve was prepared with a maximum concentration of 1 mM urea. To run the assay, 300 μl of sample or standard was combined with 1 ml of the reagent and mixed thoroughly in a 1.5-ml microcentrifuge tube (polypropylene). Samples were incubated for 20 min at 90°C and then cooled on ice and allowed to sit for 20 min before measuring on a UV-visible spectrophotometer at 520 nm.

Ammonium quantitation. Ammonium was quantified by several different methods that have been described previously (7, 9, 30, 31) using either fluorescent or colorimetric approaches. For low levels of ammonium obtained from specific culture supernatants, a derivation of the phthalaldehyde method described previously (32, 33) was utilized. In these assays, 500 μl of assay reagent (270 mg of phthalic dicarboxaldehyde dissolved in 5 ml of ethanol and then added to 100 ml of 0.2 M phosphate buffer [pH 7.3] and 50 μl of β-mercaptoethanol) was combined with up to 500 μl of culture supernatant and allowed to react at room temperature for 30 min. Samples were analyzed on a Varian Cary Eclipse fluorescence spectrophotometer using an excitation wavelength of 410 nm and an emission wavelength of 472 nm.

Elemental analysis of cells and supernatants. Cells were collected by centrifugation at ~12,000 × *g* and frozen. Supernatants were separated from the cells for analysis of the remaining solids. Samples were lyophilized and thoroughly mixed using a spatula to ensure a homogeneous mixture. Samples of cells and/or supernatants were analyzed for the percent carbon, hydrogen, and nitrogen at the Stable Isotope Lab in the Geology Department at the University of Minnesota.

Random transposon mutagenesis. *A. vinelandii* strains AZBB030 and AZBB035 were transformed by a transposon insertion methodology using *Escherichia coli* WM3064 and the mariner transposon from plasmid pEB001 (24, 34). Briefly, ~50 μl of *A. vinelandii* cells was scraped from a fresh agar plate of cells with a sterile loop and resuspended in 500 μl of sterile phosphate buffer. Separately, ~50 μl of *E. coli* WM3064 cells containing the pEB001 plasmid were resuspended in 1 ml of sterile LB broth. Next, 100 μl of the suspended *A. vinelandii* cells and 20 μl of the suspended *E. coli* WM3064 cells were combined and mixed with a pipettor and then spotted onto BYE plates supplemented with 100 μM 2,6-diaminopimelic acid (DAP; 50 μl of a 10-mg/ml stock). These cells were incubated overnight at 30°C, transferred with a sterile loop to 100 ml of B medium, and grown overnight at 30°C in a shaker table at 180 rpm. After growth in B medium, 1 ml of culture was removed and pelleted in a microcentrifuge at 12,000 × *g*, and then all but 100 μl of supernatant was removed. The cells were resuspended in the 100 μl of remaining media, plated onto B plates supplemented with kanamycin (Kan; 3 μg/ml) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 80 μg/ml), and pretreated with a lawn of *A. vinelandii* AZBB063. Plates were then

TABLE 3 Primers used in this study

Primer	Sequence (5′–3′)[a]	Purpose
BBP919	GACTAGAA TTCGTGCAGA AACATCTCTA CCCGGAAG	*ureABC* gene and flanking region cloning
BBP920	GACTAAAG CTTGGAACAG AAGACGATGA GGATGC	*ureABC* gene and flanking region cloning
BBP938	GACTAGGA TCCGTGAAGA TCAGCAGCTT GTCTTTCTC	*ureABC* gene deletion
BBP939	GACTAGGA TCCAAGAAGG ACCTGATCCA CAACG	*ureABC* gene deletion
BBP1147	GAGCAAGC TTCATGGTCA GGTCGTGGCC TTC	*nifLA* gene and flanking region cloning
BBP1148	GACAGGAT CCGGTCAGCA CTTCGGACAC CAC	*nifLA* gene and flanking region cloning
BBP1209	GACATCTA GACTCTCATA TGGTGCCTCG TCTATCCAAG AAAACC	*nifLA* gene deletion
BBP1210	GACATCTA GAGGATCCGA CCCTCCGGCA ATGGATG	*nifLA* gene deletion
BBP1149	GACAGGAT CCATATGAAT GCAACCATCC CTCAGCGCT C	*nifA* gene cloning
BBP1150	GACATCTA GACTAGATCT TGCGCATGTG GATGTTGAG	*nifA* gene cloning
BBP2099	NNNGATAT CGGGCATTCC GCCCGACCTG GTGCTG	*nifL* gene segment deletion 1
BBP2100	NNNGATAT CGACGACGAT GCCCTGGGCC AGCAACTG	*nifL* gene segment deletion 1
BBP2101	NNNGATAT CCCGGAGAAG GCGCTGCCCT G	*nifL* gene segment deletion 2
BBP2102	NNNGATAT CCATCAGCAC CCGCGTGGAG AAC	*nifL* gene segment deletion 2
BBP1674	NNNTCTAG AGGAAGCTAT CCGACGAGGA CAGCCGAG	*scrX* gene and flanking region cloning
BBP1675	NNNGAATT CCTGAGCGCA GAATTTAGAT ATTGATACTC ATAGTC	*scrX* gene and flanking region cloning
BBP1710	NNNATGCA TATGGACTTC CTATTGTTGA CATTATTGGT GG	*scrX* gene deletion
BBP1711	NNNATGCA TGACAGAATT CAGATCTCAC GCCATAAGCT GTTAGCATTT TTCTTG	*scrX* gene deletion
BBP1669	NNNTCTAG AGGATCCCAT ATGCATACCA TGATTACGGA TTCACTGGCC GTCG	*lacZ* gene cloning
BBP1671	NNNAAGCT TGGATCCTAT TTTTGACAC CAGACCAACT GGTAATGGTA G	*lacZ* gene cloning
BBP1967	NNNAAGCT TCGAAGACAT GGCACTCCGA GGCGTTGGCC AGAC	*amtB* gene and flanking region cloning
BBP1968	NNNTCTAG AGATAGATTC CCTGCCAGGT CCCCAG	*amtB* gene and flanking region cloning
BBP1969	NNNAGATC TGGTTACAAC CTCTGAGTGT CGGGAG	*amtB* gene deletion
BBP1970	NNNAGATC TCAGCGTCAT TGATTATTCT CCTGGGGCG	*amtB* gene deletion
BBP982	GAAGGGCA GCAGCAGGTA GAGG	*ureABC* gene deletion confirmation
BBP983	CAGCAGTT CGCGAAGACT GTCGAAG	*ureABC* gene deletion confirmation
BBP1836	CTCAACGT TCGCCAGGTA TATGCCGAAC TC	*scrX* gene deletion confirmation
BBP1837	CACATAGG ATGAAACGTC ACCGAGCTTG TTCG	*scrX* gene deletion confirmation
BBP950	GAGCACAC CCATCACGGT CAGAG	*nifLA* gene deletion confirmation
BBP1322	GATCTCCA TCGACTCGAT CTTGTCCAGG GTGAAC	*nifLA* gene deletion confirmation
BBP2006	CACGTGCC AGGAATTCCT CCATG	*amtB* gene deletion confirmation
BBP2007	CTGTGGAC GATGGCCAGG GACATGGATC	*amtB* gene deletion confirmation

[a] Specific restriction enzyme sites added to primers are underlined for clarity.

incubated at 30°C for several days until colonies formed. Colonies were selected that revealed the blue phenotype, indicating that the integrated transposon had altered the *A. vinelandii* AZBB030 or AZBB035 target strains so they could support the growth of the nitrogen-dependent (Nif^-) phenotype of the *A. vinelandii* AZBB063 biosensor strain. Colonies that turned blue were transferred to a B plate containing streptomycin (Str; 0.5 μg/ml) to confirm and isolate only the transposon-modified strain and then again checked for the proper blue phenotype by streaking clean isolates onto an X-Gal- and kanamycin-supplemented plate with a lawn of *A. vinelandii* AZBB063. False-positive colonies were further tested by streaking onto B plates containing streptomycin and X-Gal.

Transposon insertion analysis. Strains exhibiting the desired phenotype were first purified by streaking individual colonies several times on a B plate supplemented with streptomycin. Cells were then scraped from a clean plate (~100 μl of cells), and genomic DNA was isolated using a ZR Fungal/Bacterial DNA miniprep kit (Zymo Research, Irvine, CA). Genomic DNA was digested with PstI, purified using a DNA Clean and Concentrator-25 kit (Zymo Research), and ligated using T4 DNA ligase (New England BioLabs, Ipswich, MA). PCR was then run on the ligated DNA using the primer BBP1241 (5′-GACCGCTATCAGGACATAGCGT TG-3′), which aligns near the end of the *kan* gene used as the selectable marker indicating transposon insertion. This approach relies on the potential of two similar DNA fragments containing the insert and sequence downstream the point of insertion to ligate and orient directionally toward one another. Although this is a rare event, PCR can then be utilized to amplify these segments for sequencing. The PCRs were analyzed by gel electrophoresis to confirm amplification, cleaned using the DNA Clean and Concentrator-25 kit, and sent for Sanger sequencing with the same primer BBP1241. All sequencing products for positive phenotypes identified a segment matching between 200 and 700 bp of genomic sequence from *A. vinelandii* DJ (35).

Coculture of *Azotobacter vinelandii* and *Chlorella sorokiniana*. A minimal amount of cells from strains of *A. vinelandii* and *C. sorokiniana* were spotted onto B plates substituting plant cell culture tested agar (Sigma, P/N A7921) for Bacto agar and grown on a custom light table for several days to make a qualitative assessment of the potential of various strains to support the growth of the algae. Equivalent starting quantities of cells were spotted to the plates. For liquid culture experiments, equivalent quantities of cells were inoculated into 60 ml of B medium in a 125-ml Erlenmeyer flask and grown under a bank of fluorescent lights with 14:10 light-dark cycles, while monitoring numbers of cells/ml daily. The light intensity for both experiments was ~200 μmol min^{-1} m^2. Liquid cultures were mixed on a shaker table with constant shaking at 160 rpm.

RESULTS

Urea as a terminal nitrogen compound. An initial aim pursued in this effort was to modify *A. vinelandii* metabolism to convert a common nitrogen metabolite into a terminal nitrogen product. Urea was selected as an initial target based on a careful analysis of *A. vinelandii* metabolic pathways and known enzymes. *A. vinelandii* contains genes for a known urease enzyme system (*ureABC*) (35). Substitution of the *ureABC* operon with a streptomycin antibiotic marker resulted in *A. vinelandii* AZBB030 that was unable to metabolize extraneously provided urea, even after several days, whereas the wild-type *A. vinelandii* strain was able to clear as

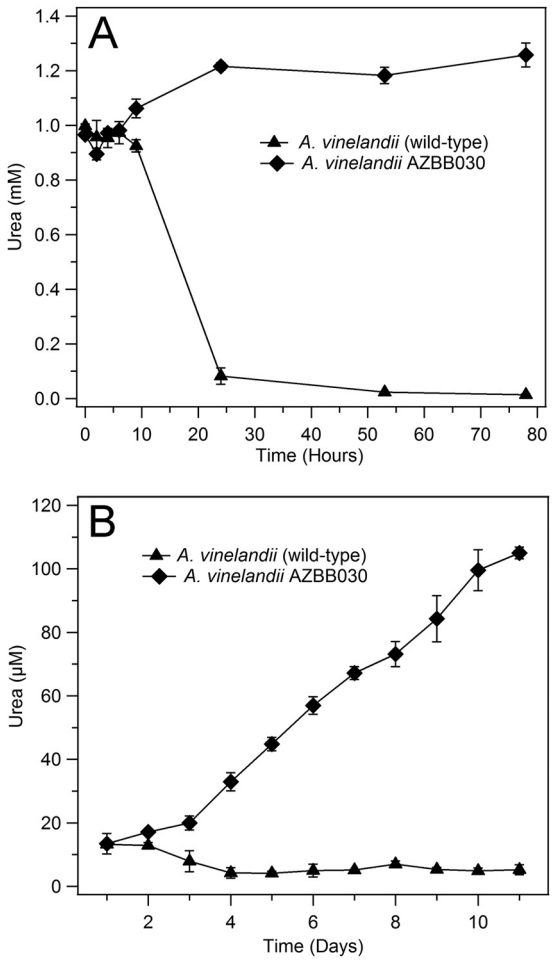

FIG 1 Urea analysis of key *A. vinelandii* strains. Shown above are the results of urea analysis of the *A. vinelandii* wild-type strain and the Δ*ureABC*::Str^r strain *A. vinelandii* AZBB030. (A) Results of an experiment where both strains were provided ~1.2 mM urea at the time of inoculation, illustrating that *A. vinelandii* AZBB030 does not metabolize urea to an appreciable amount, while the wild-type strain depletes the urea from the culture during the first 24 h of growth. (B) The urea levels in *A. vinelandii* AZBB030 increase over time versus the wild-type strain, reaching levels in excess of 100 μM. All results and statistics are calculated based on triplicate samples.

much as 2 to 3 mM urea within 24 h after the start of exponential growth (Fig. 1A), demonstrating that urea had become a terminal product.

Based on the genome annotation (35), *A. vinelandii* lacks a known arginase gene but contains all of the remaining genes constituting a functional urea cycle. Further analysis of urea levels in control samples revealed that the Δ*ureABC*::Str^r *A. vinelandii* AZBB030 strain was accumulating urea (Fig. 1B), either through the action of a yet-to-be-characterized arginase enzyme system or through alternative metabolic pathways present in *A. vinelandii* that yield urea as a by-product. This accumulation of urea in the μM range (~100 μM after about 10 days of culture under the conditions used here) resulted in only slight improvements supporting the growth of nondiazotrophic strains in coculture but confirmed the potential to increase urea levels using strategies planned for later experiments. The possibility of identifying a potential novel arginase or urea cycle alternatives in *A. vinelandii* remain points of interest for future studies.

Construction of a nitrogen biosensor strain. The second goal in this effort was to modify or identify a bacterial strain that could serve as an indicator of extracellular nitrogen production for use in applications to screen transposon mutagenesis experiments. Initially, several strains of bacteria were considered possible candidates for this role, based on physical characteristics such as color that would make them easy to differentiate on plates. However, each of the potential biosensor strains would require incorporation of antibiotic selection markers to be grown together with transposon treated *A. vinelandii* and did not guarantee that these strains would be able to transport or metabolize all of the potential extracellular forms of nitrogen produced by *A. vinelandii*. Alternatively, we elected to construct a strain of *A. vinelandii* that could fulfill this biosensor purpose. First, borrowing from a technique described previously, *lacZ* from *Escherichia coli* MG1655 was incorporated into *A. vinelandii* directly downstream of the *scrX* promoter (23, 36), resulting in a blue colored phenotype when grown in the presence of X-Gal. Next, we replaced the nitrogenase regulatory genes *nifLA* with a tetracycline antibiotic marker. These genes regulate expression of nitrogenase when molybdenum is available (8). This modification resulted in a strain that grew extremely slowly in the absence of extraneously provided nitrogen sources such as ammonium or urea (Nif$^-$ phenotype) but grew well when a nitrogen source was provided as a component of the medium. Combining these two modifications, this new *A. vinelandii* AZBB063 met all of the desired requirements of our nitrogen biosensor; requiring extraneous nitrogen for growth, appearing blue when grown on plates containing X-Gal, and having a strong potential to metabolize any nitrogen containing metabolites that might be excreted by a modified or wild-type *A. vinelandii* strain. A diagram of the alterations made to the *A. vinelandii* AZBB063 nitrogen biosensor strain versus wild-type *A. vinelandii* is shown in Fig. 2.

Construction of a Δ*nifL* strain. In addition to the Δ*ureABC*::Str^r *A. vinelandii* AZBB030 that accumulates low levels of urea, it was also initially of interest to construct a deregulated nitrogenase strain. Reports more than 2 decades ago by Bali et al. (7) described the construction of an *A. vinelandii* strain yielding copious quantities of ammonia (as high as 35 mM in later reports [8]) by disrupting the *nifL* gene but leaving the *nifA* gene intact behind various promoters. Based on this previous report, we hypothesized that transforming the Δ*nifLA*::Str^r substituted *A. vinelandii* strain with a plasmid that would incorporate *nifA* behind the promoter for *nifL* (Δ*nifLA*::*nifA*), should result in a similar phenotype that is deregulated for nitrogenase production. Isolation of *A. vinelandii* AZBB035 containing *nifA* in the place of *nifLA*, along with Δ*ureABC*::Str^r, did rescue the nitrogen-fixing phenotype (Nif$^+$) capable of growth on standard B plates but did not result in copious quantities of ammonium being released into the supernatant. Ortiz-Marquez et al. recently constructed a strain using a similar approach to that taken here based on the same concepts from the report by Bali et al. and reported an increase in ammonia production, although not to the same levels as reported by Bali et al. or Brewin et al. (7–9). In each of these cases, the approaches taken to increase ammonia production were slightly different from what was pursued here (see the more detailed analysis of these differences below). When either *A. vinelandii* AZBB030 or AZBB035 were grown on a plate in the presence of the nitrogen biosensor *A. vinelandii* AZBB063, extracellular nitrogen produced by either strain (in the form of either urea or ammonium) was not sufficient

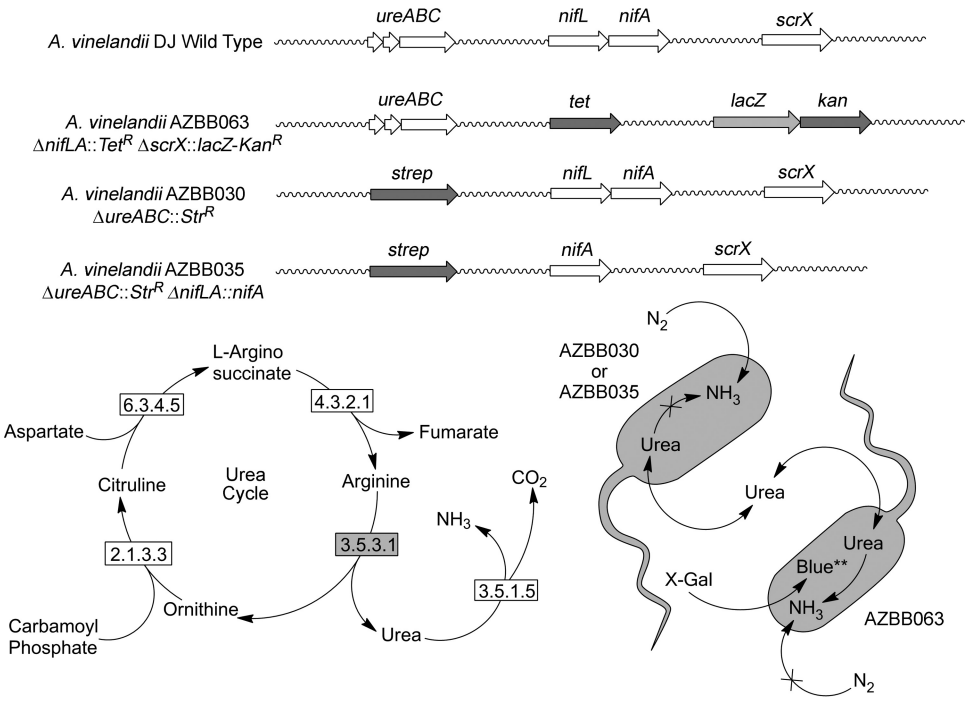

FIG 2 Descriptions of various strains constructed for this work. Shown above are simple representations of modifications made to various regions of the *A. vinelandii* genome to construct the nitrogen biosensor *A. vinelandii* AZBB063, which results in minimal growth in the absence of extraneously provided nitrogen and yields a blue phenotype when grown in the presence of X-Gal. Additional constructs *A. vinelandii* AZBB030 and AZBB035 contain Δ*ureABC*::Str^r, resulting in the accumulation of minor amounts of urea (Fig. 1). *A. vinelandii* AZBB035 also contains a deregulated nitrogenase by first removing the *nifLA* genes and then replacing them with *nifA* behind the *nifL* promoter, resulting in a rescued nitrogen fixing phenotype. The bottom left shows the urea cycle and urease enzyme (3.5.1.5) as it has been annotated in *A. vinelandii*. A known gene for arginase (3.5.3.1) has not been identified in *A. vinelandii*. The bottom right illustration indicates how urea as a terminal product could potentially support the growth of the nitrogen biosensor *A. vinelandii* AZBB063 if quantities of urea increased to a level sufficient to meet the nitrogen requirements of this strain. Additional metabolites produced by *A. vinelandii* AZBB030 and AZBB035 following transposon mutagenesis experiments could also substitute for the urea in this representation, as was found in these studies.

to support significant growth of the *A. vinelandii* AZBB063 biosensor strain, in contrast to the results found in experiments described below for other strains.

Transposon mutagenesis experiments to increase nitrogen output. Transposon mutagenesis can be used to deliver random gene disruptions. This approach was utilized here based on the assumption that disrupting specific pathways might result in the accumulation of nitrogen containing metabolites (including urea) that would escape the cell and become available to other organisms in coculture. Alternative disruptions might lead to the loss of metabolites through various transport phenomena. With the target *A. vinelandii* AZBB030 and AZBB035 and the nitrogen biosensor *A. vinelandii* AZBB063 strains in hand, the next goal was to use transposon mutagenesis to introduce random gene disruptions and look for a phenotype that results in the production of increased levels of extracellular nitrogen products. Using the techniques described in Materials and Methods to incorporate transposons through random insertions, conjugated cells of *A. vinelandii* AZBB030 or AZBB035 were spread over a plate containing a lawn of *A. vinelandii* AZBB063, along with X-Gal and kanamycin, and then grown for several days until colonies developed, indicating insertion of the transposon within the genome. Important to this approach, the initial plate used to select colonies is devoid of added nitrogen compounds, such that any insertions resulting in an undesirable disruption to nitrogen fixation or essential genes would be lost. After several additional days, numerous colonies turned blue, indicating potential extracellular nitrogen production (Fig. 3A). Approximately 15 colonies were isolated (from roughly 3,000 screened) by careful transfer, and the phenotypes were confirmed and tested more thoroughly. After genomic DNA isolation, PCR was performed to amplify the transposon and genomic region where the transposon was incorporated.

Approximately half of the *A. vinelandii* colonies isolated contained the blue phenotype when grown in the presence of X-Gal, even in the absence of the nitrogen biosensor *A. vinelandii* AZBB063 (Fig. 3C). Sequencing revealed that *lacZ* and the downstream kanamycin cassette from the *A. vinelandii* AZBB063 (Fig. 2) had been transferred to either *A. vinelandii* AZBB030 or AZBB035. This false-positive result is likely due to the survival of a small amount of the *E. coli* WM3064 conjugation strain that introduced *lacZ* from the *A. vinelandii* AZBB063 to the nitrogen-fixing target strains following plating on solid medium. The false-positive phenotype was easily differentiated from strains with a true-positive result, which only revealed the blue phenotype when grown together with the *A. vinelandii* AZBB063 biosensor strain. An alternative construction of the indicator strain separating the kanamycin antibiotic selection marker from the *lacZ* gene should minimize the potential of these false-positive strains in future experiments. Although this false-positive result is an unfortunate occurrence, the percentage of these strains overall is quite small (<10 total colonies out of several thousand screened) and still

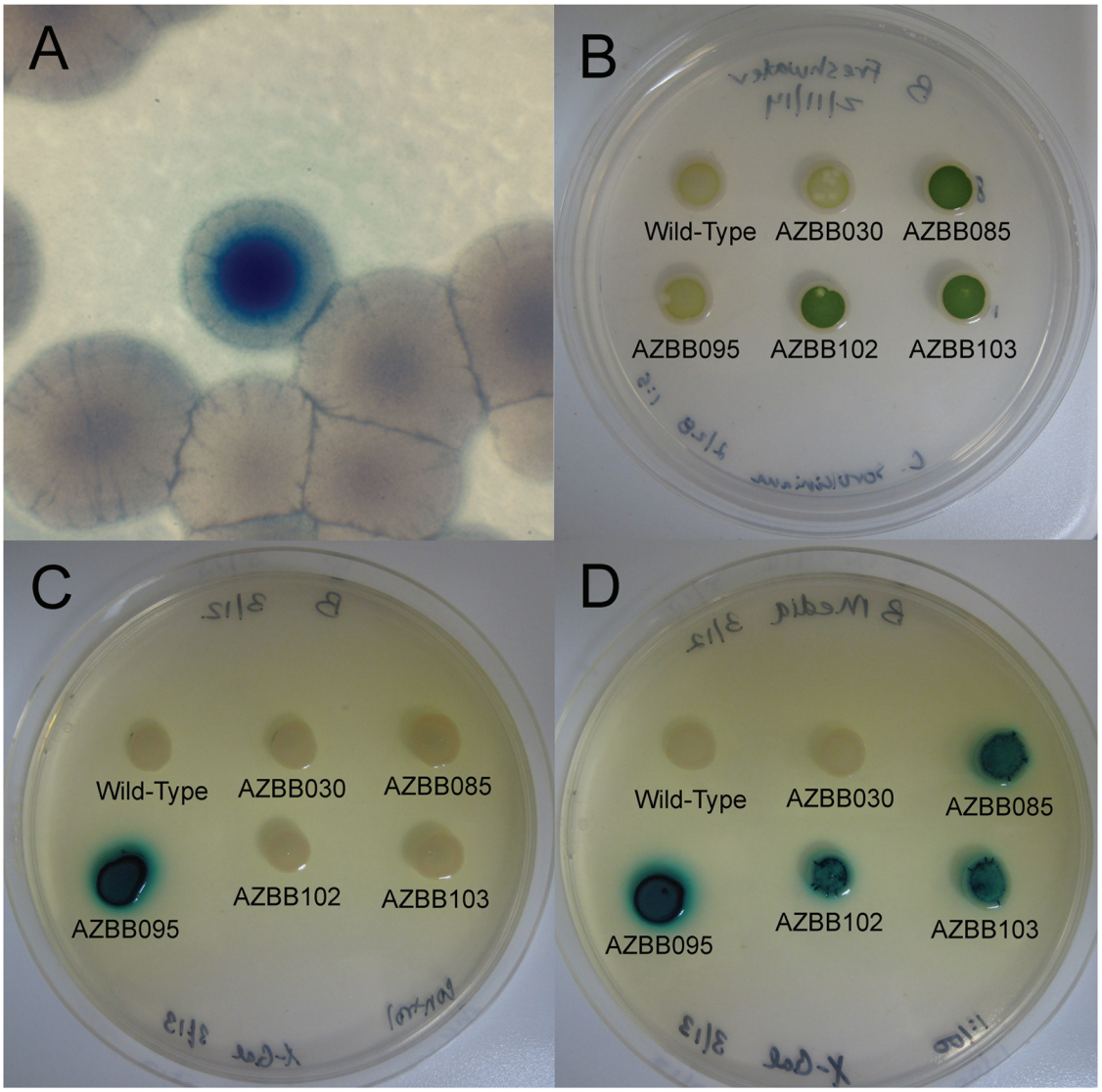

FIG 3 Phenotypes of various *A. vinelandii* strains constructed or obtained in these studies. (A) Example of the blue phenotype found for a specific colony using the screening technique with the nitrogen biosensor *A. vinelandii* AZBB063 during the transposon mutagenesis experiment. (B) Various strains of *A. vinelandii* constructed for the present study and grown as spots on a derivation of B medium with *C. sorokiniana* to determine whether the strain could provide sufficient nitrogen to support the growth of the green alga. (C) Example of a screen for false-positive strains that had unintentionally obtained the *lacZ* gene from *A. vinelandii* AZBB063, resulting in a false-positive phenotype when screened together with *A. vinelandii* AZBB063. In this experiment, pure strains were grown alone on B medium supplemented with X-Gal. (D) Various strains spotted onto a B plate together with a small amount of *A. vinelandii* AZBB063 and X-Gal. These results indicate that *A. vinelandii* AZBB085, AZBB102, and AZBB103 are all able to support the growth of a second strain (either *A. vinelandii* AZBB063 or *C. sorokiniana*) that is unable to obtain nitrogen through the action of a functioning internal nitrogenase.

allowed for the identification of true-positive colonies, as described below.

The remaining strains yielding the correct blue phenotype when cocultured with the *A. vinelandii* AZBB063 biosensor strain were also tested by spotting a small amount of each strain together with the green algae *Chlorella sorokiniana* onto a B plate (devoid of added nitrogen compounds) to determine whether the strain could provide sufficient extracellular fixed nitrogen to support the growth of this algal strain. Figure 3B shows an example where strain *A. vinelandii* AZBB085 supported the growth of *C. sorokiniana* while wild-type and the initial target *A. vinelandii* AZBB030 or AZBB035 did not. *A. vinelandii* AZBB095, a false-positive strain, was also unable to support the growth of *C. sorokiniana* (Fig. 3B and C). Testing the potential to support an algal strain is a nice secondary screen, although whether a specific strain of algae can utilize the various extracellular nitrogen compounds produced is likely strain dependent.

Identification of gene deletions resulting in increased extracellular nitrogen. *A. vinelandii* AZBB085 and five additional strains showed the correct blue phenotype only when spotted on plates with *A. vinelandii* AZBB063. Each of these strains had the kanamycin cassette transposon inserted into the gene *amtB*, and this occurred in both *A. vinelandii* AZBB030 and AZBB035 derived colonies. Although it was possible that some of the colonies could be replicates of the same transposon insertion event, only one of the six cases isolated here contained the same insertion and

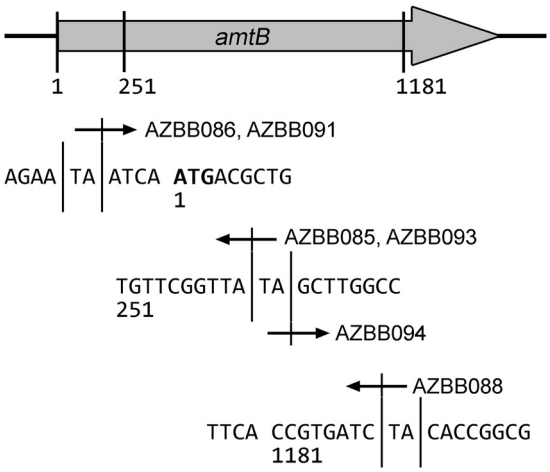

FIG 4 Insertions into *amtB* resulting in nitrogen production phenotypes. At the top of the figure is a drawing of the *amtB* gene region of *A. vinelandii*. The three different regions where insertions were found in the present study during the nitrogen production screen are marked on the diagram, and the sequence of each region is shown below. Specific TA base regions where insertions were made are marked with dividers on either side, and arrows are included depicting the region that was sequenced immediately outside the transposon inverted repeat when sequenced with a primer that complemented a segment within to the kanamycin selection marker from within the transposon. Specific strain numbers of *A. vinelandii* from Table 1 are labeled beside each arrow.

orientation for the same conjugation experiment (into either *A. vinelandii* AZBB030 or AZBB035). In all other cases, the insert was either in a different location, or it was inserted in a different direction (Fig. 4). This result indicated a clear potential for developing this phenotype through a targeted substitution of the entire *amtB* gene. Since it was also feared that the phenotype might potentially be the result of multiple insertions which were not obvious, we developed a strategy to substitute *amtB* using standard genetic techniques (a double-homologous-recombination approach) without the use of a transposon. The *amtB* gene has been proposed to play a role in the transport of ammonia or ammonium, although a specific role for *amtB* in *A. vinelandii* is still unclear (37–41). Based on this finding and on what is already known about these various genes and the nature of the nitrogen screen, three potential explanations were considered to explain the nitrogen secreting phenotype following *amtB* disruption. First, deleting *amtB* might result in increased extracellular ammonium if loss of this gene hindered the ability of the strain to recover ammonia or ammonium that leaks from the cell by natural processes, as has been described in other strains (42). Second, *amtB* might play a role in the transport of an alternative nitrogen compound, which requires the *amtB* still present in the *A. vinelandii* AZBB063 biosensor strain to transport this compound back into the cell. Finally, there was the remote possibility that *amtB* could also play a role in urea uptake in *A. vinelandii*, although the likelihood of this was considered low. Any of these explanations would still support a successful application of this approach to yield a strain of *A. vinelandii* with increased biofertilizer potential. To rule out the last option, *amtB* gene deletion/replacements were developed for both the Δ*ureABC*::Str^r strain and also wild-type *A. vinelandii*.

Construction of an *amtB* deletion/replacement *A. vinelandii* strain. Using the same approach that was taken to generate *A. vinelandii* strains AZBB030, AZBB035, and AZBB063, we further constructed *A. vinelandii* strains AZBB102 and AZBB103. *A. vinelandii* strain AZBB102 contains only Δ*amtB*::Kan^r, whereas AZBB103 contains Δ*amtB*::Kan^r along with Δ*ureABC*::Str^r. Both strains resulted in the blue phenotype when grown together with *A. vinelandii* AZBB063 and also supported the growth of *C. sorokiniana* when a nitrogen source was not supplemented in the medium (Fig. 3B and D). This indicates that urea accumulation is not required for the observed phenotype, which was further confirmed by determining that urea production and release had not increased versus *A. vinelandii* AZBB030 and AZBB035 urea levels in these strains (results similar to Fig. 1B).

Analysis of ammonium production by strains AZBB102 and AZBB103. Since *amtB* is proposed to be involved in the transport of ammonia or ammonium (38–41), this seemed to be the most probable explanation for supporting the growth of either the algae or the *A. vinelandii* biosensor strain AZBB063. Cells were grown for as long as a week, while supernatant was removed and ammonium was quantified using several different techniques that have been used previously by ourselves (27, 30) or reported by others (9, 31) to identify ammonium. Previous reports of modifications to the *nifLA* operon have reported the production of copious amounts of ammonium (7, 8). Our analysis of the supernatant from strain AZBB102 versus *A. vinelandii* wild type did find elevated levels of ammonium in the media, but levels were in the low micromolar range (Fig. 5A), which is in contrast to the dramatic increases reported for *nifLA* modifications (7–9).

These results indicate that deletion or disruption of the *amtB* gene in *A. vinelandii* results in a slow release of ammonium into the media. Although the levels of ammonium detected in *A. vinelandii* strain AZBB102 were low, these were consistently and significantly higher than what was found in the wild-type strain control (Fig. 5A). A further elemental analysis of carbon, hydrogen, and nitrogen (CHN analysis) of supernatants that were collected and lyophilized did not find significant amounts of nitrogen accumulating in the supernatants, although an analysis of the dry cell materials did find elevated nitrogen levels (3.63% ± 0.11% nitrogen for strain AZBB102 versus 2.36% ± 0.05% for the wild type, an increase of ca. 50%) under the growth conditions tested here. This indicates that the low level of ammonium found from the analysis described in Fig. 5A is the most likely source of the nitrogen produced by *A. vinelandii* AZBB102 and not an alternative form of nitrogen.

Potential of *A. vinelandii* AZBB103 to support the growth of the green alga *C. sorokiniana*. The approach using coculture on solid media (Fig. 3B) is a simple screen that provides a qualitative assessment of the ability of *A. vinelandii* strains to support algae or other organisms. In the absence of specific methods of quantitation (or if identification of the nitrogen compound responsible for supporting algal growth were unclear), cell growth of a nondiazotroph in coculture can be utilized to estimate the potential of the strain as a biofertilizer. Cocultures of *A. vinelandii* AZBB103 and *C. sorokiniana* were grown in a simple B medium devoid of added nitrogen under a bank of fluorescent lights along with a control containing the algae and *A. vinelandii* wild type to determine levels of algal cells that might be supported by the extracellular nitrogen being released when sufficient sugar is provided in the medium. Figure 6 shows the results of the coculture of the Δ*amtB*::Kan^r and Δ*ureABC*::Str^r strain *A. vinelandii* AZBB103 with *C. sorokiniana*. As can be seen, the levels of algae cells in-

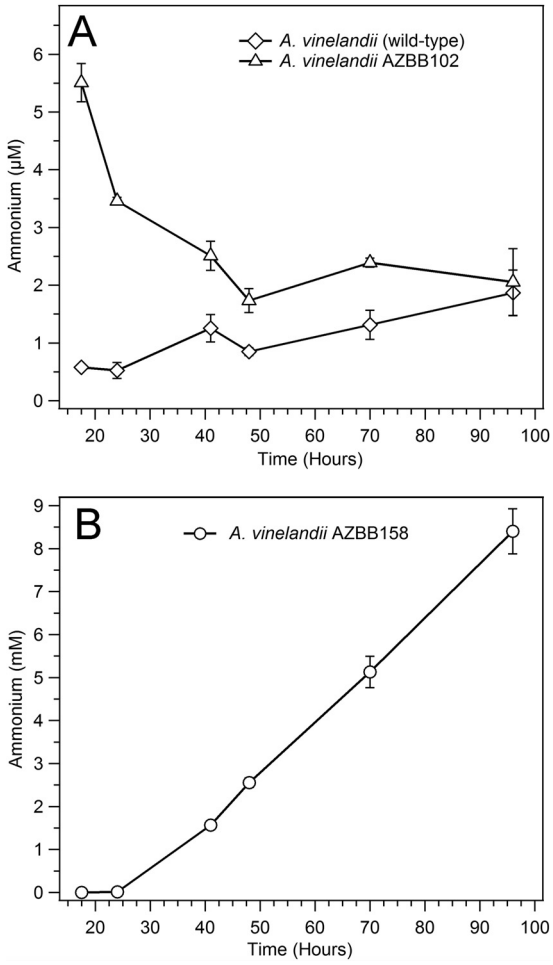

FIG 5 Ammonium levels accumulating in *A. vinelandii* strain supernatants. (A) Results of an analysis of *A. vinelandii* strain supernatants for the wild type and the Δ*amtB*::Kan^r AZBB102 strain. The levels of ammonium are consistently higher for *A. vinelandii* AZBB102, although the levels decrease over time and were not found to increase above 10 μM during the entire experiment. The highest levels of ammonium were found early during the growth, while the strain was still in the exponential stage of growth. (B) The levels of ammonium found in the *nifLA* disruption based on the previously described construction (8) resulted in a phenotype that accumulated significant amounts of ammonium, although these levels showed a lag following inoculation before increasing after about 24 h of growth. The averages and standard deviations shown are the results for triplicate samples. All cultures were grown at 22°C while shaking at 160 rpm.

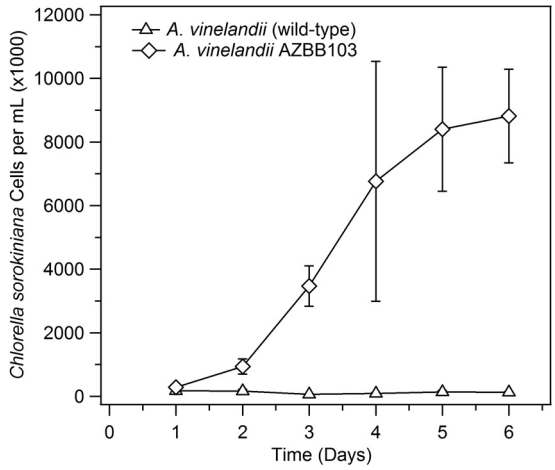

FIG 6 Coculture of *A. vinelandii* AZBB103 and *C. sorokiniana*. Shown above are the results obtained when *A. vinelandii* AZBB103 (Δ*amtB*::Kan^r and Δ*ureABC*::Str^r) was grown as a coculture with *C. sorokiniana* cells over a period of 6 days. Cells were counted by using a hemocytometer. A control containing *A. vinelandii* wild-type grown in coculture with *C. sorokiniana* was also included for comparison. The large variance seen at day 4 for *A. vinelandii* AZBB103 is potentially related to cell clumping. After day 4, cell clumps were broken up by repeatedly passage through a pipette tip prior to counting cells. All results and statistics are calculated based on triplicate samples.

creased dramatically when cultured with *A. vinelandii* AZBB103 versus the *A. vinelandii* wild-type strain (a similar result was found with *A. vinelandii* AZBB102). The improvement in the yield was >50-fold after only a few days of growth.

Reconstruction of a high ammonium production phenotype. Since the Δ*amtB*::Kan^r ammonium release phenotype differs from what has been reported by others for disruptions to the *nifLA* operon (7–9), and since our initial efforts to construct a high-ammonium-excreting strain were only partially successful (Nif$^+$ phenotype, but no dramatic release of ammonium to the medium for *A. vinelandii* AZBB020), we finally pursued efforts to reconstruct an *A. vinelandii* strain equivalent to what was previously reported by Brewin et al. (8). An attempt to obtain the exact strain reported by Brewin et al. (8) from Martin Drummond was unsuccessful, since the strain was likely lost following his retirement (personal communication from Martin Drummond). Using the approach detailed by Brewin et al., we constructed a plasmid similar to pBB369 (our pPCRNH3-42) and transformed *A. vinelandii* AZBB010 with this plasmid, isolating kanamycin-resistant colonies (*A. vinelandii* AZBB148). *A. vinelandii* AZBB148 was confirmed to have incorporated the kanamycin resistance cassette in the proper location and direction in the genome, but did not yield a Nif$^+$ phenotype. A slightly different strain transformed with plasmid pPCRNH3-43 (*A. vinelandii* AZBB150) also did not yield a Nif$^+$ phenotype. However, when each of these strains were transferred to B medium devoid of an added nitrogen source and grown as liquid cultures, spontaneous mutants did arise after several days that were Nif$^+$ and accumulated high levels of ammonium in the medium (Fig. 5B). These evolved strains were isolated on solid medium and designated *A. vinelandii* AZBB158 and AZBB163, respectively. *A. vinelandii* AZBB158 was grown in B medium similar to what was done for *A. vinelandii* AZBB102 and *A. vinelandii* wild-type and was found to yield ammonium at levels approaching 10 mM after 4 days of growth at 22°C, differentiating this with the phenotype found with Δ*amtB*::Kan^r (Fig. 5A). The levels of ammonium measured following depletion of the sucrose from the media reached >20 mM after more than a week of culture at 22°C (~35 mM at 30°C for *A. vinelandii* AZBB163). The *nifLA* regions of both *A. vinelandii* strains AZBB158 and AZBB163 were amplified, sequenced, and found to contain a point mutation to each residing in the segment of DNA upstream of the kanamycin promoter (Fig. 7). This mutation resided in the same location within the kanamycin cassette in both of the evolved Nif$^+$ strains (AZBB158 and AZBB163), and lies in close proximity to the BamHI restriction site that is used to shuttle the kanamycin cassette between vectors. Both Brewin et al. and Bali et al. used the kanamycin cassette from plasmid pUC4-KIXX (7, 8), which is to the best of our knowledge practically identical to the kanamycin

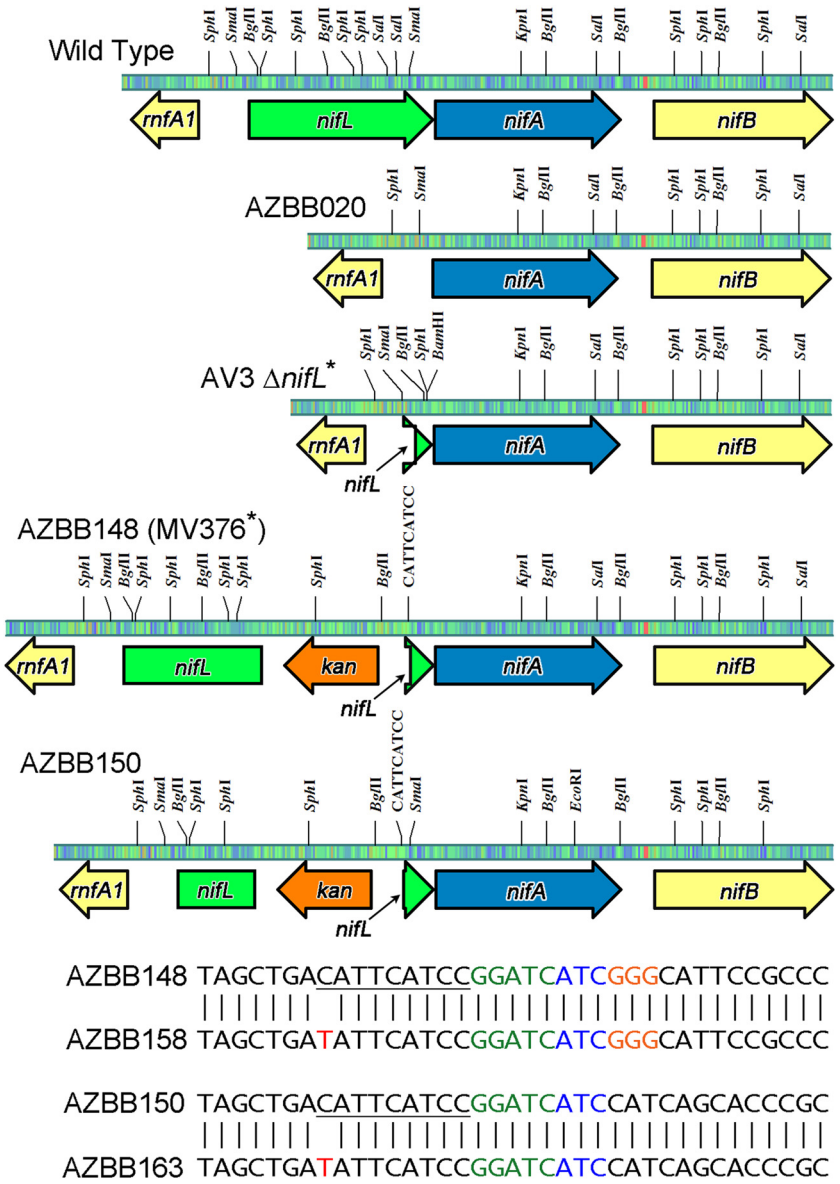

FIG 7 Illustration of different approaches to deregulate nitrogen fixation and excrete ammonium through manipulation of the *nifLA* operon. Shown is an illustration of the *nifLA* genome region from *A. vinelandii* (wild type) and various final constructs developed here or reported by others (7–9). The Δ*nifLA*::*nifA* construct *A. vinelandii* AZBB020 resulted in a nitrogen-fixing (Nif$^+$) phenotype but did not increase levels of ammonium in the extracellular space, as initially anticipated. The strain *A. vinelandii* AV3 Δ*nifL* constructed by Ortiz-Marquez et al. (9) took a similar approach but left a small segment of *nifL* intact and resulted in μM levels of ammonium and a Nif$^+$ phenotype (drawn as described previously [9]). Strain *A. vinelandii* AZBB148 was constructed similar to the approach to construct *A. vinelandii* strains MV376 and MD367 (7, 8), except that pPCRKAN4 was used as the source of the kanamycin cassette instead of pUC4-KIXX. *A. vinelandii* AZBB150 was constructed so that this same pPCRKAN4 derived kanamycin cassette would be inserted into *nifL* slightly further upstream while removing a larger section of the *nifL* gene. Both *A. vinelandii* AZBB148 and AZBB150 were found to be Nif$^-$ but became Nif$^+$ following a spontaneous mutation to yield *A. vinelandii* AZBB158 and AZBB163. The location of this mutation (underlined region) is shown in the alignments and is also marked on the illustration for the *nifLA* region of each of these strains. The sequence shown in green represents the remainder of the polished BamHI site, while the sequence drawn in blue is the remnant of the EcoRV site. The remainder of the SmaI site used here and previously to construct strains MV376 and MD367 (7, 8) is shown in orange for *A. vinelandii* AZBB148 and AZBB158. The C-to-T mutations for *A. vinelandii* AZBB158 and AZBB163 are shown in red. The figure was constructed using the program pDRAW32 (AcaClone Software).

cassette of pPCRKAN4, except for a small segment of DNA just prior to the restriction sites that flank the cassette in each case. As shown in Fig. 7, the kanamycin cassette from pPCRKAN4 contains the sequence (CCCAGTAGCT, where the underlined TAGCT is shown at the beginning of the alignments in Fig. 7) that is derived from Tn*5*. This CCCAGTAGCT sequence is the same in both the pPCRKAN4 kanamycin cassette and the pUC4-KIXX cassette (pUC4-KIXX cassette sequence kindly provided by Haruyasu Kinashi [43, 44]). Following the CCCAGTAGCT sequence, the pPCRKAN4 contains GACATTCATCC, which is also derived from Tn*5*, and GGATCATC, which is the product of the filled-in BamHI and EcoRV site ligation product used to incorporate the

cassette into the *nifL* region of both pPCRNH3-42 and pPCRNH3-43. This GACATTCATCC GGATCATC sequence is what differentiates the pPCRKAN4 from the pUC4-KIXX sequence, which contains CGAGAAGCTTCCC downstream of the CCCAGTAGCT sequence and is the remnant of the XhoI, HindIII, and SmaI sites (XhoI also utilizes the CT from the CCCAGT AGCT upstream sequence). Following this sequence, both strain *A. vinelandii* AZBB148 and MV376 (8) are then followed by GGG CATTCCGCCCG, which is the remainder of the SmaI site and *A. vinelandii* sequence lying upstream of the nifA gene. Thus, the only difference between our *A. vinelandii* AZBB148 and *A. vinelandii* MV376 in this region is the GACATTCATCC GGAT CATC sequence that is in place of CGAGAAGCTTCCC, respectively. The modification to *A. vinelandii* AZBB148 and AZBB150 that resulted in the Nif$^+$ phenotype was GATATTCATCC GGAT CATC (where C mutated to the underlined T). We assume that the difference in this short region of sequence is responsible for why we were unable to obtain the high ammonium producing phenotype using our pPCRKAN4 derived kanamycin cassette instead of the cassette obtained from pUC4-KIXX. Further features of this region will be investigated in the future. This highlights the unanticipated importance of the source and size of the kanamycin cassette when attempting to incorporate this cassette. As pointed out previously (8), obtaining the phenotype is directionally dependent on the kanamycin cassette in order to achieve the proper Nif$^+$ and high-ammonium-yielding phenotype and is further evidence that high ammonium production is dependent on a component of the kanamycin cassette that enhances *nifA* expression.

DISCUSSION

The efforts pursued in this work highlight three different approaches to increasing extracellular nitrogen production to enhance the biofertilizer potential of *A. vinelandii*. Efforts to convert urea into a terminal nitrogen product by deleting a key enzyme involved in nitrogen recycling resulted in μM levels of urea accumulating in the medium and further demonstrated successful limitation of reuptake of the urea nitrogen by *A. vinelandii* (Fig. 1). The levels of urea nitrogen obtained by deleting the urease are similar to the total nitrogen produced by strain *A. vinelandii* AV3 (Δ*nifL*) constructed by Ortiz-Marquez et al. (9) using their alternative approach to produce elevated levels of ammonium.

In addition to new approaches described here, we also reevaluated the potential of *nifL* disruptions through two additional approaches in this work. Previous reports have described several methods to increase levels of ammonium excreted by *A. vinelandii* through disruption of the *nifL* gene from the *nifLA* operon (7–9). An illustration of various modifications made to the *nifLA* region here and previously is shown in Fig. 7. Ortiz-Marquez et al. (9) recently published a report applying a derivation of the approach first described by Bali et al. in 1992 (7) that disrupts and deletes a section of the *nifL* gene while leaving *nifA* intact. Ortiz-Marquez et al. constructed an almost complete deletion of *nifL* (9), yielding a strain that also accumulated ammonium in the medium, although Ortiz-Marquez et al. reported ammonium levels of ~250 μM after 48 h (9), whereas Bali et al. and Brewin et al. had reported levels of ~10 mM and >25 mM, respectively, over somewhat longer time periods (7, 8). As pointed out by Ortiz-Marquez et al. (9), direct comparisons with the strains obtained by Bali et al. and Brewin et al. are difficult to make since reconstructions of certain strains were unobtainable even by the original authors. Brewin et al. did report confirmation of the original *A. vinelandii* strain MV376 phenotype described by Bali et al. but reported that other strains were difficult to rescue from their stocks (7, 8, 45). Our initial approach here using a complete deletion of the *nifL* gene resulted in a Nif$^+$ phenotype but no significant accumulation of ammonium in the growth medium versus the wild type. After applying a directed evolution approach, we were able to isolate a similar phenotype to what was reported using the approach reported by Brewin et al. (8). This required first isolating our initial *A. vinelandii* strains in the presence of a fixed nitrogen source in the medium and then isolation of a spontaneous Nif$^+$ mutation. These evolved strains resulted in the production of >10 mM ammonium after 4 days of culture (Fig. 5B), similar to the initial reports by Bali et al. (43). A more recent report by Ortiz-Marquez et al. (46) coupled their *A. vinelandii* Δ*nifL* strain previously described (9) with point mutations to glutamine synthase to yield ammonium levels up to 7 mM (46), although the long-term stability of their strain may be problematic based upon the potential for recombination events within the cell. The *A. vinelandii* AZBB158 and AZBB163 are also potentially problematic, since they are prone to contamination by spontaneous cheaters (cells of *A. vinelandii* that evolve to quit fixing nitrogen and then prey upon the ammonium released by the remaining ammonium excreting cells). However, these results indicate a potential to further improve nitrogen output through further modification of the *nifLA* operon, even without having to modify glutamine synthase.

Disruption or deletion of the *amtB* gene identified using the screening technique described here resulted in a low level of ammonium accumulation in the extracellular space (Fig. 5A). This was capable of supporting the growth of nondiazotrophs in coculture when sucrose was provided in the medium to support the growth of the *A. vinelandii*. Although the overall levels of ammonium quantified in the supernatants were low, they were significantly higher than what was found for the wild-type strain (Fig. 5A). We hypothesize that inclusion of a strain that can utilize the low levels of ammonium may rapidly deplete this nitrogen reservoir, driving additional ammonium across the membrane by diffusion to maintain a constant, but low level of ammonium flux across the membrane.

There is an active debate, as well as contrasting results, concerning the role that *amtB* plays in the transport of ammonia or ammonium in various bacteria (38–41), including reports of increasing extracellular ammonium when *amtB* is deleted while also demonstrating that cells can obtain ammonium from the extracellular space even in the absence of *amtB* (42, 47). The *amtB* gene has been implicated in the release of ammonium previously in other bacteria. Castorph and Kleiner reported that ammonium transport mutants (Amt$^-$) excreted ammonium in *Klebsiella pneumonia* (48). Meletzus et al. isolated *amtB* mutants from *A. vinelandii* and showed that *amtB* mutants could grow on limiting ammonium but were unable to transport methylammonium (37). Experiments testing for the loss of ammonium were not described in the Meletzus et al. study, although it was concluded that *amtB* has no function that is of vital importance in *A. vinelandii* (37). Our results confirm that deletion of *amtB* in *A. vinelandii* did not dramatically affect the growth rate or health of the *A. vinelandii* but indicate that *amtB* may be important for minimizing the loss of ammonium to the environment by this species. Another recent report found that ammonium was excreted in an *amtB* mutant strain of *Pseudomonas stutzeri* yielding extracellular ammonium

levels of ~6 μM (42), which is very similar to the levels obtained here (Fig. 5A), while a separate report linked upregulation of *amtB* to the ability of *Rhodococcus erythropolis* to utilize atmospheric ammonia, with disruption of *amtB* resulting in a growth defect under nitrogen-limiting conditions (47).

The identification of the *amtB* disruption demonstrates how the screening approach using a nitrogen biosensor strain can yield new strains producing elevated extracellular nitrogen products regardless of whether the specific nitrogen compound produced was targeted by the approach. This broad screening potential could have further benefits in identifying additional potential "nitrogen shuttle" compounds that might have further utility for providing nitrogen in coculture. *A. vinelandii* also produces additional nitrogen shuttle compounds such as siderophores that are suitable for supporting the growth of algae in coculture (11), as well as additional extracellular proteins (49). In these studies, the levels of nitrogen released to the culture medium by the *amtB* disruption were very low (i.e., a low micromolar range) and might be missed by less sensitive assays.

A. vinelandii strains AZBB030, AZBB102, and AZBB103 did not require the introduction of any foreign genes (though antibiotic markers were used under this approach). These disruptions that were shown to increase extracellular nitrogen levels when sufficient sugar is provided to the media by deleting/replacing *ureABC* and/or *amtB* could also be constructed as gene deletions using markerless techniques developed in our laboratory (to be published) or through congression approaches (23), resulting in true gene deletions that would not be transgenic but simply gene deficient. Each of these modifications could be coupled to one another or combined with approaches that alter the *nifLA* operon to enhance total nitrogen output further, similar to the approach taken by Ortiz-Marquez et al. with *nifLA* alterations coupled to *glnA* mutations (46). The development of stable genetic constructs that lack foreign genes would yield biofertilizer strains with a greater potential for utilization in algal culture and that could be applied to current conventional agricultural crops as an enhanced biofertilizer as well.

ACKNOWLEDGMENTS

This study is supported by grants (RC-0007-12) from the Initiative for Renewable Energy and the Environment (Institute on the Environment), the MnDRIVE transdisciplinary research initiative through the University of Minnesota based on funding from the state of Minnesota to B.M.B, and the Biotechnology Institute at the University of Minnesota for fellowship funding to L.J.E.

We thank Juan Torrecilla and Peter Nixon for assistance in preliminary urea production assays. We thank Eric Lenneman for assistance in obtaining specific photographs. We thank Haruyasu Kinashi for providing a sequence of the pUC4-KIXX cassette region. We thank the Stable Isotope Laboratory at the University of Minnesota for assistance with CHN analysis.

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