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# Metabolic engineering of a diazotrophic bacterium improves ammonium release and biofertilization of plants and microalgae

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ABSTRACT

The biological nitrogen fixation carried out by some Bacteria and Archaea is one of the most attractive alternatives to synthetic nitrogen fertilizers. However, with the exception of the symbiotic rhizobia-legumes system, progress towards a more extensive realization of this goal has been slow. In this study we manipulated the endogenous regulation of both nitrogen fixation and assimilation in the aerobic bacterium *Azotobacter vinelandii*. Substituting an exogenously inducible promoter for the native promoter of glutamine synthetase produced conditional lethal mutant strains unable to grow diazotrophically in the absence of the inducer. This mutant phenotype could be reverted in a double mutant strain bearing a deletion in the *nifL* gene that resulted in constitutive expression of *nif* genes and increased production of ammonium. Under GS non-inducing conditions both the single and the double mutant strains consistently released very high levels of ammonium ( > 20 mM) into the growth medium. The double mutant strain grew and excreted high levels of ammonium under a wider range of concentrations of the inducer than the single mutant strain. Induced mutant cells could be loaded with glutamine synthetase at different levels, which resulted in different patterns of extracellular ammonium accumulation afterwards. Inoculation of the engineered bacteria into a microalgal culture in the absence of sources of C and N other than $N_2$ and $CO_2$ from the air, resulted in a strong proliferation of microalgae that was suppressed upon addition of the inducer. Both single and double mutant strains also promoted growth of cucumber plants in the absence of added N-fertilizer, while this property was only marginal in the parental strain. This study provides a simple synthetic genetic circuit that might inspire engineering of optimized inoculants that efficiently channel $N_2$ from the air into crops.

## 1. Introduction

It is anticipated that the increase in the human population and greater per capita incomes of 2.3 billion people towards 2050 would demand a nearly 100% increase in agricultural crop production (Tilman et al., 2011). The current trends for increasing agricultural production imply intensification with increased productivities in the same land area or extensification with greater land clearing in developed or developing regions of the world, respectively (Tilman et al., 2011).

The use of fertilizers, especially nitrogen, will be crucial to increase productivity since up to 90% (typically 40–60%) of crop yield can be attributed to commercial fertilizer inputs (Stewart and Roberts, 2012). Despite the welfare that the industrial production of N fertilizers by the Haber–Bosch process has signified, and still represents, to food security, both its production and use bring an injurious footprint to the environment (Erisman et al., 2008). Paradoxically, while crop productivity is chronically low due to limited access to N-fertilizers in some regions of the undeveloped world, in other more developed regions, mainly due to wrong application time and dose, the N use efficiency by cereal crops has dropped to nearly 30%. This results in a major loss of the N-fertilizer into the atmospheric, terrestrial and aquatic environments producing a variety of adverse side effects (Tilman et al., 2002; Canfield et al., 2010).

Additionally, concerns on depletion of fossil fuels and environmental decay have precipitated the need to develop alternative and sustainable sources of energy, such as biofuels from crops. Biofuels have been classified as of first, second and third generation according to the nature of its feedstocks as mainly intended for food, agricultural waste (mostly lignocellulose) and microalgae, respectively (Wijffels

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et al., 2013). Thus biofuels technology would pose an additional constraint towards food security, more prominently in the case of first generation biofuels but also indirectly in those cases that may dispute the use of arable land and agrochemicals, especially N-fertilizers (Ortiz-Marquez et al., 2014).

One of the most promising alternatives for affordable and environmentally benign N-fertilizers is promoting the agricultural use of biological nitrogen fixation (BNF), consisting of the biological production of ammonia from atmospheric $N_2$ (Canfield et al., 2010). BNF is an essential part of the geochemical cycle of N that accounts for about two-thirds of the total fixed $N_2$ while most of the remainder is due to the Haber–Bosch process. While the only known strict diazotrophs belong to the Domains Bacteria or Archaea, some eukaryotes (mainly plants and algae) engage in trophic associations with $N_2$-fixing bacteria that allow them to gain access to the atmospheric $N_2$. The legume–rhizobium symbiosis has been successfully used in agriculture for decades and also inoculation of crop plants with different $N_2$-fixing bacteria has been proved satisfactory towards the reduction of synthetic N-fertilizer requirements (Curatti and Rubio, 2014).

More recently, it has been proposed that metabolic engineering/synthetic biology has potential to take agricultural exploitation of BNF for crops production to a next level by means of (1) engineering new symbioses between plants and $N_2$-fixing bacteria (Oldroyd and Dixon, 2014; Mus et al., 2016); (2) enhancing of $N_2$-fixing bacterial endophytes or free living diazotrophs (Geddes et al., 2015); and (3) directly transferring of bacterial *nif* genes into crops (Curatti and Rubio, 2014; López-Torrejón et al., 2016; Ivleva et al., 2016).

BNF is catalyzed by oxygen sensitive nitrogenases in a high energy-demanding reaction requiring at least 16 ATP to fix 1 $N_2$. The molybdenum nitrogenase is a complex of dinitrogenase (NifDK hetero-tetramer) and dinitrogenase reductase (NifH homodimer) that requires dozens of additional genes to assemble an active enzyme. The γ-proteobacterium *Azotobacter vinelandii* is a free-living diazotroph that is exquisitely adapted to carry out aerobic BNF among other anaerobic metabolic pathways (Setubal et al., 2009). In this bacterium, the $N_2$ fixation genes for the molybdenum nitrogenase (*nif* genes) are activated by NifA-RpoN, while the antiactivator NifL interacts with and inhibits NifA to prevent *nif* genes expression when ammonium is available and/or in response to elevated concentrations of oxygen (Dixon and Kahn, 2004) (Fig. 1). In *A. vinelandii,* ammonium is incorporated into amino acids by a cyclic pathway comprising glutamine synthetase and glutamate synthase (GS-GOGAT pathway) (Kleinschmidt and Kleiner, 1978). In bacteria, GS is tightly regulated to attain cellular N homeostasis; under N-limiting conditions GlnD uridylylates the signal transduction proteins PII and GlnK increasing their rate of deadenylylation of GS (activation) by stimulating the adenylyl-removing activity of GlnE. Conversely, under N-sufficiency GlnD deuridylylates PII and GlnK to revert the GlnE-dependent activation of GS (Colnaghi et al., 2001) (Fig. 1).

Thus, *A. vinelandii*, as most free-living diazotrophs, makes use of concerted mechanisms for cellular N homeostasis and typically does not fix $N_2$ in excess nor excretes significant amounts of $N_2$-fixation products (Ortiz-Marquez et al., 2012). Three different kinds of mutations have been associated with an enhanced capacity of ammonium excretion in *A. vinelandii* and other diazotrophic bacteria: 1) disruption of the NifA/NifL-dependent ammonium control of *nif* genes expression; 2) partial inhibition of GS or GOGAT for deficient ammonium assimilation (Ortiz-Marquez et al., 2014); and 3) disruption of the ammonium/methylammonium transporter AmtB, although to lower relative level (Barney et al., 2015) (Fig. 1). *A. vinelandii* cells bearing a point mutation at the active site of GS (*glnA* D49S) displayed a moderate mutant phenotype under diazotrophyc growth conditions and excreted ammonium into the medium up to 1.7 mM. Conversely, double mutant strains (*ΔnifL*, *glnA* D49S) presented a stronger diazotrophic growth defect and although presented an increased initial rate of ammonium release into the medium, the maximum concentration remained lower than the single mutant cells at 1.0 mM, presumably as a consequence of the severe growth defect imposed by the combination of the mutations. Inoculation of either single or double mutant strains into a microalgal culture sustained microalgae growth at the expense of atmospheric $CO_2$ and $N_2$ in a synthetic microalgae-bacteria consortium (Ortiz-Marquez et al., 2014).

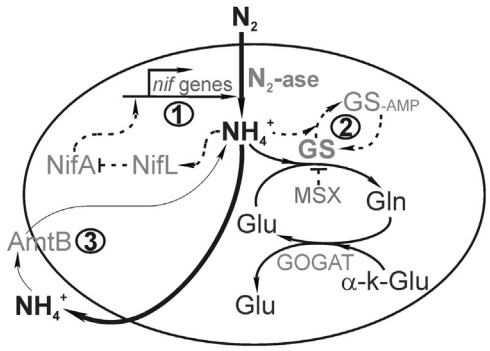


**Fig. 1.** Simplified schematic of $N_2$ fixation, ammonium assimilation and control of nitrogen homeostasis in *A. vinelandii*. Nitrogenase catalyses the conversion of atmospheric $N_2$ into ammonium, which is further assimilated into glutamate by the GS-GOGAT cycle. When cellular ammonium sufficiency is attained by supplementation of a suitable source of nitrogen or as a result of $N_2$ fixation, increased cellular level of ammonium down-regulates *nif* genes expression by a NifLA-dependent pathway (1) and promotes adenylylation of GS (inhibition of the Gln biosynthetic reaction and activation of the non-physiological γ-glutamyl transferase activity) (2). While ammonium diffuses in and out of the cell, the high-affinity ammonium-methylammonium transporter AmtB scavenges and uptakes ammonium at μM concentrations (3). It has been shown that modification of either of these three pathways promotes ammonium accumulation in the growth medium of *A. vinelandii* cells (see main text for details). $N_2$-ase, Fe-Mo nitrogenase; GS, glutamine synthetase; GS-AMP, adenylylated GS; GOGAT, glutamine-2-oxoglutarate aminotransferase; Glu, glutamate; Gln, glutamine; α-k-Glu, α-2-oxoglutarate; MSX, methionine sulfoximine. Solid lines indicate metabolic flux and dash lines regulatory circuits.

Thus, genetic engineering of ammonium release by aerotolerant non-symbiotic bacteria would be of prime interest for: 1) the development of versatile N-biofertilizers for sustainable agriculture (Geddes et al., 2015; Mus et al., 2016) and/or 2) the construction of exchangeable $N_2$-fixing parts for synthetic biology approaches for the development of multispecies microbial cell-factories comprising $CO_2$-fixing and $O_2$ evolving microalgae or cyanobacteria (Ortiz-Marquez et al., 2013; Smith and Francis, 2016).

This report shows high levels of ammonium accumulation (up to 20 mM) in the growth medium of *A. vinelandii* conditionally-lethal mutant strains that express *glnA* under an exogenously inducible promoter (*trc$_P$-glnA*). Single mutant strains (*trc$_P$-glnA*) excreted ammonium at very low concentrations of the inducer, which corresponds with extremely slow growth. On the other hand double mutant strains (*trc$_P$-glnA; ΔnifL)* grew and excreted high levels of ammonium under a wider range of concentrations of the inducer. Mutant cells could be loaded with GS activity at different levels allowing an additional level of control of cells population and ammonium release. Inoculation of the engineered bacteria proved to be a good replacement of N-fertilizers for growth of both microalgae and cucumber plants.

## 2. Materials and methods

### 2.1. Organisms and culture conditions

*A. vinelandii* strain DJ was the *wt* strain used in this study and was kindly provided by Dennis Dean (Virginia Tech). *A. vinelandii* strain AV3 (*ΔnifL*) isolation has been described previously (Ortiz-Marquez et al., 2012). *A. vinelandii* strains DJ, AV3, AV11 (*trc$_P$-glnA*), AV12 (*ΔnifL, trc$_P$-glnA*), and AV13 (*trc$_P$-glnA; ΔnifA::sp*) were maintained in Burk's modified medium (Strandberg and Wilson, 1968), incubated at 29 ± 1 °C with shaking at 200 rpm. When required media were

supplemented with 20 μg.ml$^{-1}$ spectinomycin, 1.5 μg.ml$^{-1}$ kanamycin or 50 μg.ml$^{-1}$ ampicillin. Inocula were routinely prepared from cells cultivated in the presence of 29 mM $NH_4Cl$ or ammonium acetate for solid or liquid medium, respectively, and represented the ammonium-replete condition. For the derepression of BNF and diazotrophic growth analysis, cells were collected by centrifugation at 1,136×*g* for 3 min and then transferred to ammonium-free medium.

*Escherichia coli* strain DH5α was used for molecular cloning purposes and was cultured in Luria-Bertani medium, supplemented with appropriate antibiotics at 37 °C with shaking at 150 rpm.

*Scenedesmus obliquus* strain C1S was cultivated in nitrogen-free BG11 medium (Rippka et al., 1979), BG11 medium supplemented with 2 mM ammonium chloride or nitrogen-free BG11 medium inoculated with *A. vinelandii* strains at an initial algal-bacterial cell-ratio of 1:1. The mixed cultures were cultivated in 500 ml culture bottles bubbled with sterile air at 30 °C and 50 μmol photons.m$^{-2}$.s$^{-1}$ continuous white light, as reported before (Ortiz-Marquez et al., 2014).

Cucumber (*Cucumis sativus*) seeds were surface sterilized by rinsing with $H_2O$, incubated in 5% (w/v) sodium hypochlorite for 2 min and then rinsed with several changes of sterile $H_2O$. Seeds were placed onto humidified vermiculite and lightly covered with a thin layer of the substrate. Three to five plantlets were allowed to grow hydroponically by feeding sterile INTA13 medium (0.88 mM $CaCl_2{\cdot}2H_2O$; 1.014 mM $MgSO_4$; 0.99 mM $Na_2HPO_4$; 0.73 mM $KH_2PO_4$; 4.13 μM $Na_2MoO_4{\cdot}2H_2O$; 3.15 μM $MnSO_4{\cdot}H_2O$; 4.01 μM $CuSO_4$; 3.48 μM $ZnSO_4$; 16.17 μM $H_3BO_3$ and 14.8 μM $FeCl_3{\cdot}6H_2O$; pH 6.5) from the tray that held the 1-l pots. When indicated, 3 mM $NaNO_3$ was included in the medium as the N-fertilizer. Illumination was set at 125 μmol photons.m$^{-2}$.s$^{-1}$ with a photoperiod of 16 h light/8 h darkness. Temperature was kept constant at 23 °C.

For inoculation with *A. vinelandii* strains the DJ strain was cultivated in Burk's liquid medium containing ammonium, whereas the same medium supplemented with 50 μg.ml$^{-1}$ ampicillin, 1.5 μg.ml$^{-1}$ kanamycin and 25 μM IPTG was used for strains AV11 and AV12. Bacteria were subsequently subcultivated in N-free Burk's medium for 4 h. Cells were collected by centrifugation at 2000×*g* for 3 min and rinsed with Burk's phosphates buffer to remove the inducer and the antibiotic. Cells were mixed with extensively washed and sterilized vermiculite at 1×10$^7$ cells g$^{-1}$ of substrate. Data were collected 25 days after cucumber seedlings germination.

### 2.2. Isolation of A. vinelandii mutant strains

For the construction of the *trc$_P$-glnA* allele a 1.4-kpb DNA fragment was PCR amplified using the oligonucleotides 5'- GGA CCA TAT GTC GAA GTC GTC TCA ACT G and 5'-CCG TCT AGA TCA GAC GCT GTA GTA CAG GTC GTA TTC, and *A. vinelandii* strain DJ total DNA as template. Using the restriction enzyme sites placed in the oligonucleotides, the 1.4-kpb amplicon was ligated into the *NdeI* and *XbaI* sites of pRHB169 (a pTrc99 derivative bearing a kanamycin resistance gene, kindly provided by Jose Angel Hernandez) to generate pRHB169-*glnA*(1). Then a 0.98-kpb DNA fragment immediately upstream of the *glnA* promoter was PCR amplified using the oligonucleotides 5′- GGG GTA CCG TCG ATC TTC AGG CGC TC and 5′- CCC AAG CTT CTG CTC TGT TTA TGT GC, and *A. vinelandii* strain DJ total DNA as template. The resulting amplicon was ligated into pTZ57R/T (Thermo Fischer) and released with the restriction enzyme *HindIII* using one restriction site placed in one of the oligonucleotides and a second restriction site located in the multiple cloning site of pTZ57R/T. The 0.98-kpb *HindIII-HindIII* DNA was ligated into the *HindIII* site of pRHB169-*glnA*(1) and clones with the desired direction were named pRHB169-*glnA*(2). Linearizing pRHB169-*glnA*(2) with *KpnI* results in a *glnA* allele in which pRHB169 substitutes for the *glnA* promoter (*glnA$_P$*) and places the gene under the transcriptional control of the *trc$_P$* promoter and the *E. coli lacI$^Q$* repressor (Fig. 3). The identity of the construct was confirmed by sequencing in a commercial facility (Macrogen, Korea). The linearized vector was transferred into the *A. vinelandii* DJ genome by double recombination to generate the mutant strain AV11. The correct genotype of this strain was confirmed by PCR amplification of the recombinant locus using the oligonucleotides 5′- GCT GGC ACG ACA GGT TTC CC and 5′- ATG CTG GGG GTC GGC AGC and oligonucleotides 5′- ATG CGC GTC GCG GTG TCG and 5′- TTC TTC GCC GGT CGG GCC TGC C for the *wt* locus to ascertain full segregation of the mutation (Supplementary Fig. 1A). For the isolation of *A. vinelandii* strain AV12 (*ΔnifL, trc$_P$-glnA*), strain AV11 (*trc$_P$-glnA*) was transformed with plasmid pRHB138 (Curatti et al., 2005) to create strain AV13 (*trc$_P$-glnA; ΔnifA::sp*) that was resistant to spectinomycin and unable to grow diazotrophically. Strain AV13 was then transformed with plasmid pRHB143 (Ortiz-Marquez et al., 2012) that bears the *A. vinelandii nifLA* locus with an almost complete in-frame deletion of *nifL* and an intact copy of *nifA* to generate strain AV12 that recovered the ability to grow diazotrophically and became spectinomycin sensitive (Fig. 3). Methods for *A. vinelandii* transformation have been described before (Jacobson et al., 1989; Curatti et al., 2005). The incorporation of the *ΔnifL* mutation in the genome of strain AV13 was confirmed by PCR amplification of the mutant locus using oligonucleotides 5′- CGG GAT CCA TAA GCG ACC TCA CCT GCT CGG CTA AAC and 5′- TGA GGG ATC CTT GCA TTC ATG GGC ATT CCC TGT as shown before (Ortiz-Marquez et al., 2012).

Nucleic acid extraction and manipulation were conducted using conventional protocols (Sambrook et al., 2001).

### 2.3. GS activity assays

*A. vinelandii* cells were collected by centrifugation at 1,136×*g* for 3 min, and resuspended in a buffer containing 50 mM imidazole pH 7.5; 5 mM β-mercaptoethanol; 10% (v/v) glycerol; 0.5 mM phenylmethanesulfonyl fluoride; 0.3 μg.μl$^{-1}$ lysozyme; 5 U DNAse I and 5 U RNAse I and incubated at room temperature for 15 min. Cells were disrupted in a sonicator at 40% maximum power and 3 s on – 3 s off pulses for one min twice while kept in a water-ice bath. Samples were clarified by centrifugation at 14,000×*g* a 4 °C during 30 min and desalted through a BioGel P10 (Biorad®) resin previously equilibrated in 50 mM imidazole, pH 7.5; 10% (v/v) glycerol.

GS glutamine biosynthetic activity assays were run as reported before (Shapiro and Stadtman, 1970) with modifications. The reaction mixtures (200 μl) containing protein extracts (about 100 μg protein); 50 mM imidazole-HCl, pH 7.0; 50 mM $MgCl_2$; 100 mM Na-glutamate, pH 7.0; 125 mM $NH_4Cl$ were preincubated at 28 °C for 5 min. Then reactions were allowed to proceed by addition of 7.5 mM NaATP, pH 7.0 and incubated at 28 °C for 15 min until addition of 1.8 ml of 28.8 mM $FeSO_4{\cdot}7H_2O$ in 0.015 N $H_2SO_4$ to stop the reactions. The assays were developed by adding 0.15 ml of 6.6% (w/v) $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ in 7.5 N $H_2SO_4$ and comparing the absorbance at 660 nm against a standard curve made with $Na_2HPO_4$.

To assay GS deadenylylation, protein crude extracts were desalted through a BioGel P10 (Biorad®) resin previously equilibrated in 0.1 M Tris-HCl, pH 9.0; 0.5 mM $MgCl_2$; and 10% (v/v) glycerol. Preparations were incubated in the presence of 15% (w/w) of their protein content of snake venom phosphodiesterase (Sigma) and incubated at 28 °C for 6 h. Then the reactions were desalted again as indicated previously before proceeding to assay the glutamine biosynthetic-activity of GS. Control reactions of as-extracted GS crude extracts were processed the same way but omitting the addition of phosphodiesterase.

To assay the γ-glutamyl transferase activity of GS, protein crude extracts were desalted through a BioGel P10 (Biorad®) resin previously equilibrated in 50 mM imidazole, pH 7.5% and 10% (v/v) glycerol. Assays were conducted as reported (Stadtman et al., 1979) with modifications. The γ-glutamyl transferase reaction-mixtures (700 μl) containing protein extracts (about 200 μg protein); 54.21 mM imidazole-Hepes, pH 7.5; 27.39 mM glutamine, pH 7.0; 0.43 mM $MnCl_2$; and 28.53 mM $NaH_2AsO_4{\cdot}H_2O$ were preincubated for 5 min at 28 °C

with shaking at 120 rpm. After the addition of 30 µl of a solution containing 2 M hydroxylamine-HCl, pH 7.0; and 0.54 mM NaADP, pH 7.0, the reactions were allowed to proceed for 15 min at 28 °C until addition of 2 ml of a reaction stop mixture containing 0.20 M $FeCl_3$; and 1% (v/v) trichloroacetic acid in 0.25 N HCl. Activity was calculated from the spectrophotometric readings at 540 nm using a standard curve made with γ-glutamyl hydroxamate.

### 2.4. Analytical methods

Total proteins were determined using the Bradford's assay (Bradford, 1976) using bovine serum albumin as a standard. Ammonium determinations were carried out by the indophenol method as reported previously using a standard of $NH_4Cl$ (Ortiz-Marquez et al., 2012).

## 3. Results and discussion

### 3.1. Effect of MSX on growth and ammonium release of A. vinelandii

In a previous report (Ortiz-Marquez et al., 2014) we showed that *A. vinelandii* mutant cells bearing a point mutation at the active site of GS (*glnA* D49S) displayed a similar phenotype as the parental *wild-type* strain in the presence of 0.5 mM of the GS inhibitor methionine sulfoximine (MSX) (Liaw et al., 1994) regarding diazotrophic growth and ammonium release. We had further showed that supplementation of an ammonium-excreting *ΔnifL* strain with MSX resulted in an additive effect on ammonium accumulation in the growth medium. However, the double mutant strain (*ΔnifL, glnA* D49S) presented a strong mutant phenotype under diazotrophic conditions and modest release of ammonium into the medium (Ortiz-Marquez et al., 2014).

To analyse in more detail the relationship among inhibition of GS, growth rate and ammonium release in *A. vinelandii*, we analyzed the effect of MSX-dependent partial inhibition of growth on ammonium release. Fig. 2 shows that at concentrations of MSX that only partially compromised diazotrophic growth, the level of ammonium release into the medium was higher but tended to be transient. More pronounced inhibition of GS and growth at higher concentrations of MSX resulted in lower levels of ammonium accumulation in the medium. In this case the ammonium concentration in the medium tended to remain more persistent in the absence of bacterial growth (Fig. 2).

Thus, previous results (Ortiz-Marquez et al., 2014) and new evidence (Fig. 2) led us to infer at least three hypothesis: 1) enhancing ammonium production and diminishing its incorporation into amino acids by metabolic engineering have great potential to improve the ammonium excreting properties of *A. vinelandii*; 2) chronically low activity of GS, as that produced by the *glnA* D49S allele, and thus sub-optimal synthesis of amino acids could be incompatible with the deregulation (enhancement) of Nif protein expression in a *ΔnifL* genetic background (Ortiz-Marquez et al., 2014); and 3) the additive effect on ammonium release by treating *ΔnifL* strains (AV3) with MSX (Ortiz-Marquez et al., 2014) might be better mimicked by a conditional mutation allowing the control of GS activity exogenously for fine tuning GS activity and/or uncoupling growth and ammonium release.

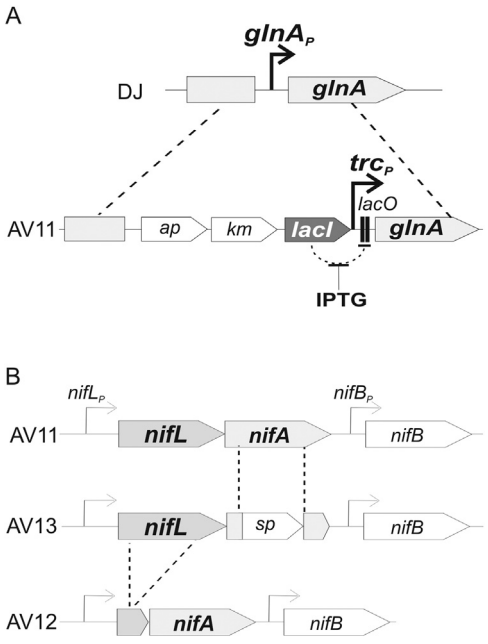


**Fig. 3.** Genetic constructs for the modification of $N_2$ fixation and ammonium assimilation in *A. vinelandii*. (A) Strain AV11 (*trc$_P$-glnA*) was generated by replacement of the *wt glnA* promoter (*glnA$_P$*) with an IPTG inducible promoter (*trc$_P$*) from *E. coli* by double homologous recombination at the *A. vinelandii glnA* locus. (B) Strain AV12 (*ΔnifL; trc$_P$-glnA*) was obtained by inactivating *nifA* with a Sp resistance gene in strain AV11 to generate the Nif$^-$ strain AV13. Then AV13 was transformed with a DNA fragment containing an in-frame deletion of *nifL* and an intact copy of *nifA* that reverted the Nif$^-$ into a Nif$^+$ phenotype.

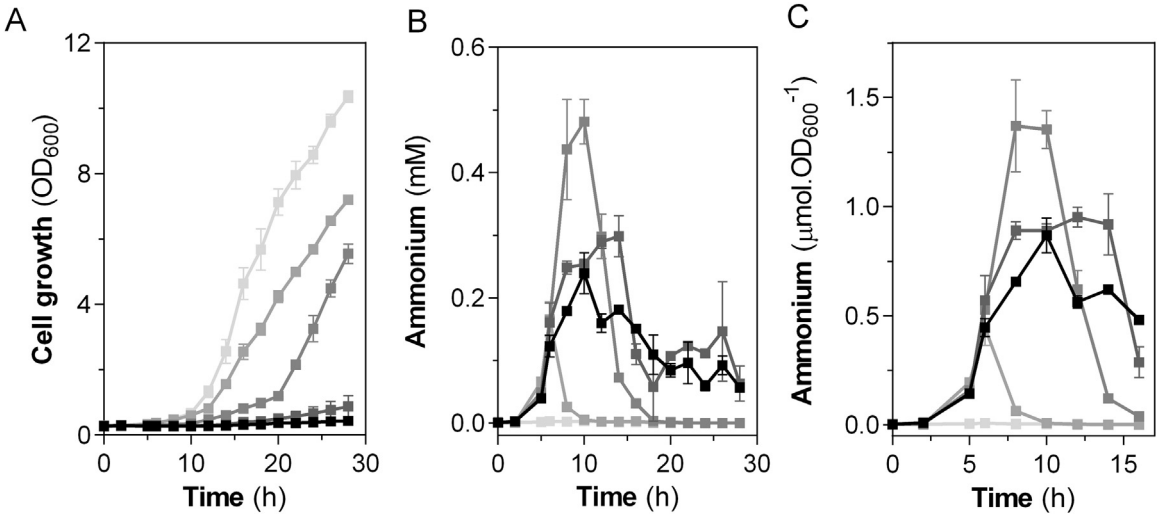


**Fig. 2.** Effect of MSX on cell growth and ammonium release in *A. vinelandii*. Cells of the *A. vinelandii* strain DJ were cultivated diazotrophically in the presence of 0 µM (□); 125 µM (▒); 250 µM (▒); 500 µM (■); or 750 µM (■) MSX. (A) Cells growth; (B) ammonium accumulated in the medium; (C) ratio ammonium accumulation:$OD_{600}$. Each data point represents the media and standard error of two independent assays.

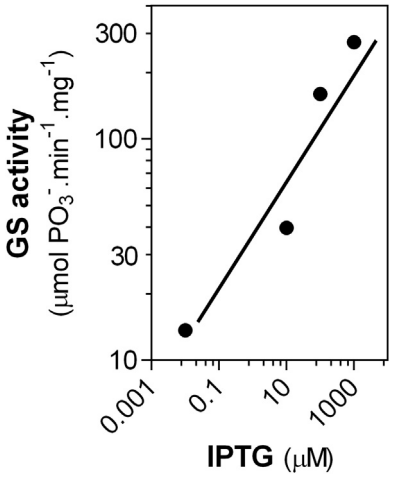


**Fig. 4.** Effect of the concentration of IPTG on GS total activity. Cells were deprived of ammonium and IPTG for 48 h and then incubated in the presence of 0 μM; 10 μM; 100 μM; or 1000 μM for 9 h. Protein extracts were incubated in the presence of snake venom phosphodiesterase for the determination of glutamine biosynthesis activity of GS. Results correspond to a representative experiment.

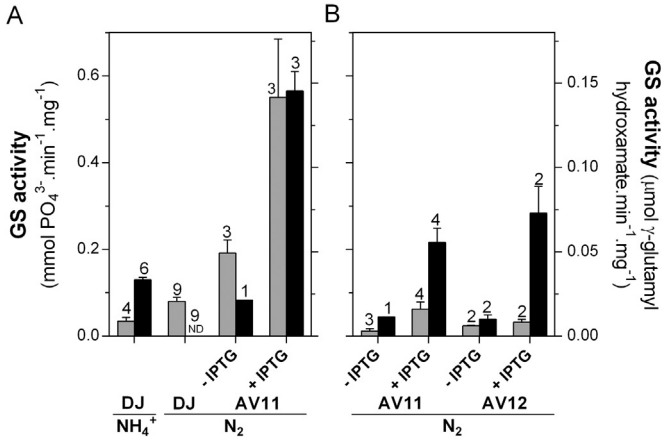


**Fig. 5.** GS activity of *A. vinelandii* $trc_P$-*glnA* mutant strains. (A) GS glutamine biosynthesis-activity (gray bars) or γ-glutamil transferase activity (black bars) of AV11 ($trc_P$-*glnA*) cells incubated for 6 h in culture medium with atmospheric $N_2$ as the sole source of N in the absence (-IPTG) or presence of 0.1 mM IPTG (+ IPTG). As a reference, activities are shown for *A. vinelandii* strain DJ cells cultivated under the same growth conditions or in the presence of 29 mM ammonium. (B) GS glutamine biosynthesis activity (gray bars) or γ-glutamil transferase activity (black bars) of AV11 ($trc_P$-*glnA*) or AV12 (*ΔnifL*; $trc_P$-*glnA*) cells incubated for 24 h in otherwise the same culture conditions as in (A). Digits above each bar represent the number of independent experiments for each data point. ND, not detected.

### 3.2. Isolation of *A. vinelandii* $trc_p$-*glnA* mutant strains for tuneable GS activity

We constructed *A. vinelandii* mutant strains in which the *wt glnA* promoter was replaced by the *E. coli* $trc_P$ promoter and the $lacI^Q$ repressor in the *wt* (AV11) or the *ΔnifL* (AV12) genetic backgrounds (Fig. 3). These mutant strains were routinely maintained in culture medium containing ammonium and 25 μM IPTG as the *glnA* inducer.

It is known that while the deadenylylated form of GS that is characteristic of cells deprived of ammonium has a more prominent glutamine biosynthetic activity, the adenylylated form that accumulates in ammonium cultivated cells presents an enhanced γ-glutamyl transferase activity (Kleinschmidt and Kleiner, 1978) and Fig. 1. Thus, treatment of protein extracts with snake venom phosphodiesterase converts GS into its deadenylylated form (Kleinschmidt and Kleiner, 1978) facilitating the analysis of total GS activity.

*A. vinelandii* strain AV11 cells were incubated in medium lacking ammonium and IPTG for 48 h and then transferred to medium containing different concentrations IPTG for 9 h. Protein extracts of these samples were incubated in the presence of snake venom phosphodiesterase for the determination of total GS activity (Fig. 4). An almost linear relationship was observed ($R^2$=0.97) for concentrations of IPTG from 0 to 1 mM, indicating that in this strain GS activity could be exogenously controlled within a reasonably high dynamic range.

We observed that soon after ammonium step-down (6 h) AV11 ($trc_P$-*glnA*) cells presented higher GS activities (glutamine biosynthetic activity and γ-glutamyl transferase activity) than the *wt* strain, especially under IPTG inducing conditions (Fig. 5A). Although both GS activities dropped markedly at longer times (24 h), the effect was more pronounced for the glutamine biosynthetic activity in the absence of IPTG (Fig. 5B). A similar pattern was observed for AV11 ($trc_P$-*glnA*) and AV12 (*ΔnifL;* $trc_P$-*glnA*) cells (Fig. 5B).

These results suggested that a large pool of adenylylated (inactive) GS might accumulate in the mutant cells during cells maintenance and/or inoculum preparation, and that this GS pool could be activated by deadenylylation under non-inducing conditions. Thus, AV11 cells were loaded with GS activity by cultivating them in the presence of ammonium and 10 or 25 μM IPTG overnight, which resulted in cells exhibiting different levels of GS γ-glutamyl transferase activity (Fig. 6A). Upon transfer to medium lacking IPTG and ammonium, a rapid decrease of γ-glutamyl transferase activity down non-detectable levels was observed at 12 or 36 h for cells loaded with GS with 10 or 25 μM IPTG, respectively (Fig. 6A). A concomitant increase in the glutamine biosynthetic activity of GS was also apparent (Fig. 6B). This increase reached a maximum at 6 or 12 h for cells loaded with GS at 10 or 25 μM IPTG, respectively, and then started to decline.

These results were further confirmed by converting the GS activity of protein extracts of AV11 cells loaded with γ-glutamyl transferase activity (Fig. 6A, 10 μm IPTG) into the glutamine biosynthetic activity by *in vitro* deadenylylation assays using snake venom phosphodiesterase (Fig. 6C).

In contrast to the previous assays (Fig. 5), the glutamine biosynthetic activity of GS only decreased to about one third of its maximum level by the end of the sampling time. A possible explanation for this would be that while the previous assays started at a lower cell density, for technical reasons the second set of experiments was conducted at considerably higher cell density, only allowing one round of cell division by the end of the experiments, at the time cells reached stationary phase of growth. Thus, cell division would promote dilution of GS in daughter cells under non-inducing conditions. Nevertheless these experiments could not rule out leakage from the $trc_P$ promoter under non-inducing conditions and slow *de novo* synthesis of GS in AV11 cells.

Taken together, these results suggested that mutant cells can accumulate a large pool of adenylylated/inactive GS under IPTG-inducing growth conditions. GS can be then deadenylylated/activated *in vivo* by transferring the cells into growth medium lacking IPTG to produce a slowly-decaying active form of GS.

### 3.3. Effect of tuning GS activity on *A. vinelandii* growth and ammonium release

As a consequence of the previous analysis we expected the ammonium excretion profile of the AV11 strain in non-inducing medium to show some differences according to the previous induction trajectory of the cells. Thus, we cultivated AV11 cells in medium containing ammonium and 25 or 100 μM IPTG overnight and transferred them into diazotrophic growth conditions in the absence of IPTG. While cells previously incubated in the presence of 100 μM IPTG did not accumulate ammonium in the culture medium, cells induced with a lower concentration of the inducer (25 μM) accumulated ammonium up to 9 mM (Fig. 7).

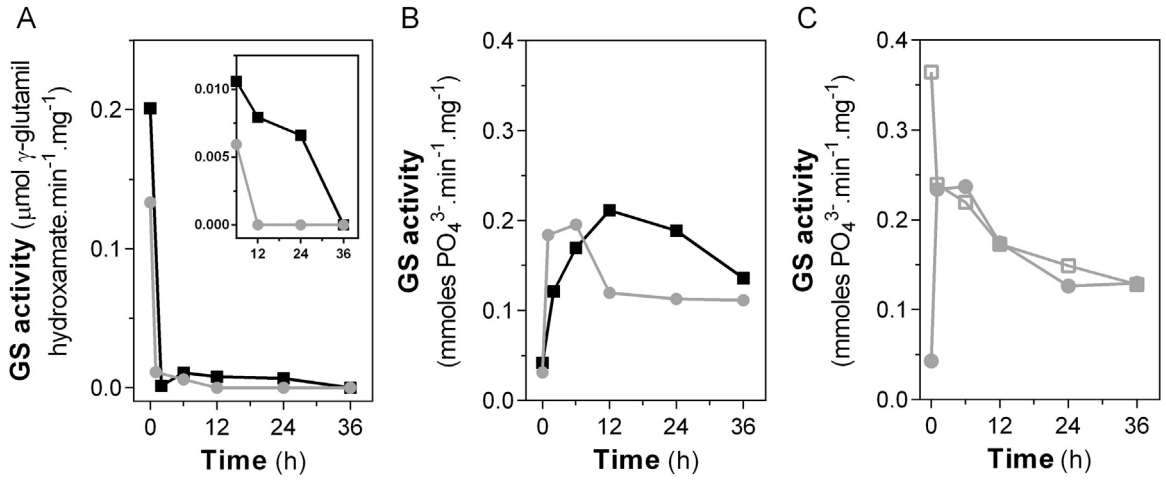


**Fig. 6.** Time course of *in vivo* changes in GS activities in *A. vinelandii* AV11 (*trc$_P$-glnA*) strains. Time course of (A) GS γ-glutamyl transferase activity and (B) GS glutamine biosynthesis activity of AV11 cells that had been cultivated in the presence of 29 mM ammonium and 10 μM (●) or 25 μM (■) IPTG for 16 h and then washed and transferred into fresh medium lacking IPTG and ammonium (data point 0 h). The inset in (A) represents a re-scaling of the plot to highlight GS activity declination at longer times. (C) GS glutamine biosynthesis activity of AV11 cells loaded at 10 μM IPTG as in (A and B). Cell extracts were incubated for 6 h in the presence (□) or absence (●) of snake venom phosphodiesterase before determination of GS glutamine biosynthesis activity. The data represent a single representative experiment.

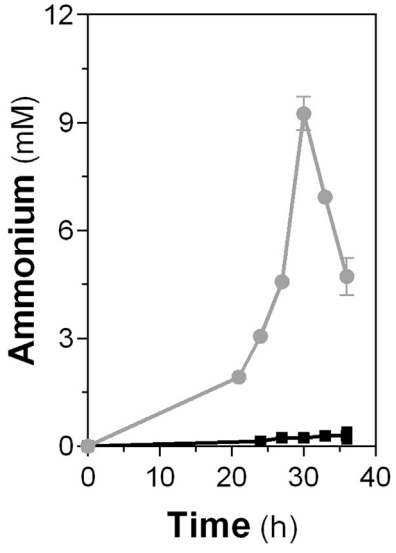


**Fig. 7.** Effect of previous induction of *glnA* on ammonium release of *A. vinelandii* AV11 (*trc$_P$-glnA*) cells. AV11 cells were cultivated for 16 h in the presence of 29 mM ammonium and (●) 25 μM or (■) 100 μM IPTG and then washed and incubated in medium lacking ammonium and IPTG (data point 0 h) before ammonium determination in the spent medium. The data represent the mean and standard deviation of two independent assays. When not visible, error bars fell behind the symbols.

Thus, for a more detailed analysis of the effect of inducing GS activity at different levels on *A. vinelandii* growth and ammonium accumulation we set up standard conditions for inducing the cells as follows: 1) cultivation onto Burk's solid plates at 0.3 mM IPTG; 2) culture overnight in liquid medium containing ammonium and 25 μM IPTG; 3) transfer into medium lacking ammonium and IPTG for 6 h; and 4) finally transfer into the desired inducing condition. After applying this protocol to AV11 cells we observed a strong diazotrophic growth defect under non-inducing conditions and accumulation of ammonium up to 7 mM ( Fig. 8 A). Cells improved their growth proportionally to the increment of the concentration of IPTG. The maximum ammonium accumulation (15 mM) was observed at 40 h in the presence of 1 μM IPTG, which produced a moderate to severe diazotrophic growth defect. No ammonium was detected in the culture medium when cells were induced with 100 μM IPTG (Fig. 8A). On the other hand, and conversely to AV7 (*ΔnifL, glnA* D49S) (Ortiz-Marquez et al., 2014) no diazotrophic growth defect was observed for the double mutant strain AV12 (*ΔnifL, trc$_P$-glnA*) regardless the *glnA* gene induction condition since cells apparently grew at the expense of the ammonium previously accumulated in the growth medium. Interestingly, AV12 cells still accumulated substantial levels of ammonium in the growth medium (around 5 mM) at the same time that growth was robust and cell density very high ($OD_{600}$ > 10). These cells excreted ammonium under every tested condition with a maximum level over 22 mM at an induction level of 0.1–1 μM IPTG, and produced characteristic peaks of ammonium excretion at an induction time of 24 h, especially at higher GS induction levels (Fig. 8B). This ammonium release profile was similar to that of AV7 (*ΔnifL, glnA* D49S) or MSX-treated AV3 (*ΔnifL*) cells (Ortiz-Marquez et al., 2014), although at a 5-fold higher level.

High ammonium excretion by other *nifL* mutants of *A. vinelandii* such as MV376 has been reported before (Bali et al. 1992; Blanco et al., 1993; Barney et al. 2015). However, at least part of the high ammonium release and growth instability of strain MV376 appeared to be due to an additional modification of *nifA* expression by a polar effect (Brewin et al., 1999). This was further confirmed more recently by re-constructing an MV376-like *A. vinelandii* strain (Barney et al., 2015). The fact that *A. vinelandii* strains bearing a *nifL* mutation tend to display a nearly wild-type phenotype under standard ("optimal") growth conditions, but a growth defect under suboptimal conditions (Ortiz-Marquez et al., 2014) leads us to speculate that even a more severe perturbation of N-homeostasis by deleting *nifL* and enhancing NifA activity might help to explain the observed instability of strain MV376 and highlights the convenience of maintaining modification of this trait by metabolic engineering within certain limits in order not to compromise cell integrity and physiology beyond a level that optimizes ammonium excretion.

### 3.4. Growth and ammonium release of mixed populations of AV11 cells loaded with different levels of GS activity

We reasoned that the possibility of loading *A. vinelandii* cells at different levels of GS activity would allow to prepare heterogeneous populations of cells. For example, mixing induced and non-induced cells would result in altered patterns of bacterial population growth and the amount and/or time course of ammonium release into the growth medium.

To challenge the hypothesis, two bacterial populations were prepared by cultivating AV11 cells, for 4 h either in the absence (non-induced cells) or in the presence of 50 μM IPTG (induced cells). Then,

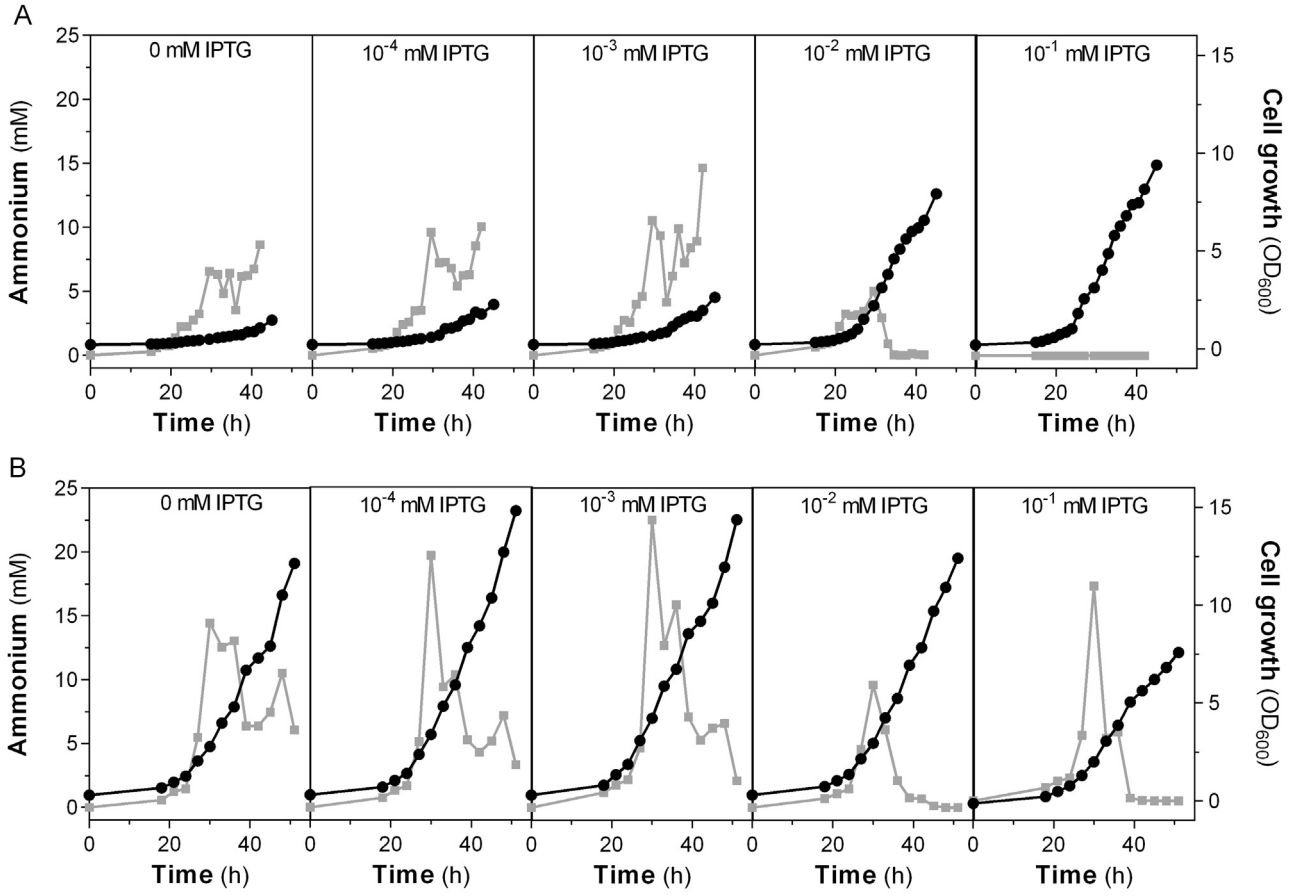


**Fig. 8.** Effect of *glnA* induction on growth and ammonium release of *A. vinelandii* $trc_P$-*glnA* cells. Growth (•) and ammonium accumulation in the spent medium (■) of *A. vinelandii* (A) AV11 ($trc_P$-*glnA*) or (B) AV12 ($trc_P$-*glnA; ΔnifL*). Data shows the mean of four independent experiments and error bars were omitted for clarity.

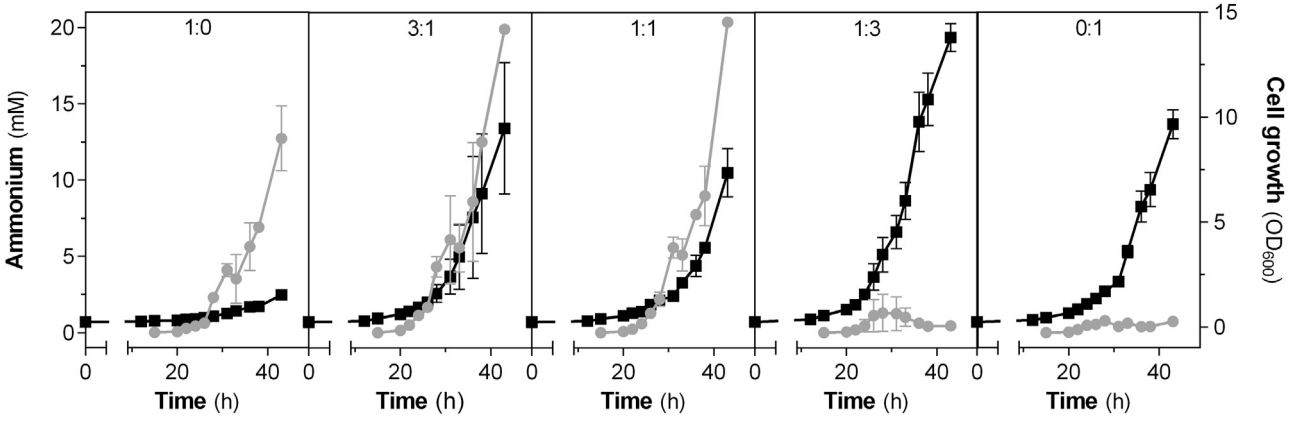


**Fig. 9.** Growth and ammonium release of heterogeneous populations of *A. vinelandii* AV11 ($trc_P$-*glnA*) cells. Growth (■) and ammonium accumulation in the spent medium (●). Two bacterial populations of *A. vinelandii* AV11 ($trc_P$-*glnA*) were cultivated for 16 h in the presence of 29 mM ammonium and 25 μM IPTG, washed and then transferred to medium either lacking IPTG (non-induced) or containing 50 μM IPTG (induced) for 4 h, and washed again. Then mixtures were set at 1:0; 3:1; 1:1; 1:3; or 0:1 ratios of non-induced to induced cells, respectively (indicated at the top of each panel). Finally the cell mixtures were incubated in N-free medium lacking IPTG (data point 0 h). Each data point represents the mean and standard error of two independent experiments.

mixtures of non-induced and induced cells were set at 1:0; 3:1; 1:1; 1:3; or 0:1 ratios, respectively. Mixed populations were then incubated in N-free medium lacking IPTG. The expected growth and ammonium release of cultures at 1:0 and 0:1 initial ratios was confirmed (Fig. 9). At 3:1 and 1:1 ratios, mixed populations attained levels of ammonium of up to 20 mM and high density of cells. Interestingly, ammonium accumulation exceeded the expected values for cells loaded at different levels of GS activity (non-induced and induced cells) acting independently from each other. At an initial ratio of 1:3 the mixed populations accumulated ammonium at very low levels and cells proliferated more than in the other combinations, presumably because of an excess of GS-loaded cells (induced) acting as commensals at the expense of ammonium released by GS-depleted cells (non-induced). The details of the complex population dynamics of these synthetic heterogeneous populations are not completely understood and remain beyond the scope of the present study. Nevertheless, these results provide proof-of-concept for the possibility of loading bacterial cells at different levels of GS activity to modulate the levels of cell density and ammonium delivery. Longer term experiments under a different experimental design would be needed to investigate whether this strategy can extend

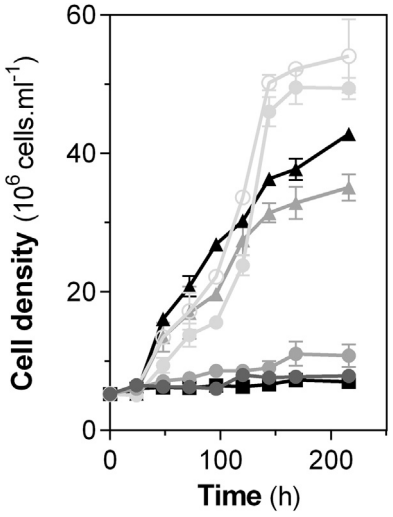


**Fig. 10.** N-fertilization of microalgal cultures by inoculation with *A. vinelandii* AV11 (*trc$_P$-glnA*) cells. Growth curves of *S. obliquus* C1S cells cultivated in BG11 medium using air as the sole source of carbon and nitrogen and inoculated with *A. vinelandii* AV11 cells in the absence (○) or the presence of 1 μM (●), 100 μM (●) or 10 mM IPTG (•). As a reference, microalgal axenic cultures without any source of N (other than air) (■), ammonium (▲) or ammonium and 10 mM IPTG (▲), are shown. Each data point represents the mean and standard error of two independent experiments.

the ammonium excretion phase of metabolically-engineered heterogeneous bacterial populations and to better understand the population dynamics.

### 3.5. Co-culture of A. vinelandii ammonium-excreting strains with microalgae

Comparable to intensive agriculture, one of the drawbacks of implementing massive cultivation of microalgae is the unsustainable requirements of fertilizers, especially N (Ortiz-Marquez et al., 2014).

Similarly to *A. vinelandii* AV3 (*ΔnifL*) (Ortiz-Marque et al., 2012), AV6 (*glnA* D49S), or AV7 (*ΔnifL, glnA* D49S) (Ortiz-Marquez et al., 2014), non-induced or 1 μM IPTG-induced AV11 cells promoted robust growth of the eukaryotic microalgae *S. obliquus* in a mutualistic artificial consortium taking $CO_2$ and $N_2$ from the air and apparently exchanging products of fixed C and N. Conversely, the N-fertilizing effect was completely lost when the medium was supplemented with 0.1 or 10 mM IPTG (Fig. 10).

Mutation of the ammonium/methylamonium transporter gene *amtB* of *A. vinelnadii* also resulted in ammonium excreting strains, although to a much lower extent than strains bearing mutations in *nifL* or *glnA*. AmtB-deficient strains also allowed cultivation of microalgae at the expense of $N_2$ from the air in a culture medium supplemented with sucrose (Barney et al., 2015).

More recently, Smith and Francis assembled a co-culture of an engineered strain (*cscB*) of the cyanobacterium *Synechococcus elongatus* that excretes sucrose and an *A. vinelandii* strain that excretes ammonium (AV3, *ΔnifL*). The co-culture produced polyhydroxybutyrate from $CO_2$ and $N_2$ from the air (Smith and Francis, 2016).

Altogether these synthetic $N_2$-fixing microalgal-bacterial consortia represent proof-of-principle for the idea that engineering communication and/or cross-feeding between selected partners is an alternative to genetic engineering of complex traits into industrial host that might be hard or impossible to achieve due to metabolic incompatibilities and/or other technical issues.

### 3.6. A. vinelandii ammonium-excreting strains as N-biofertilizer of cucumber plantlets

Genetic engineering of $N_2$-fixing endophytes or rhizospheric bacteria is probably the most straightforward alternative to N-fertilization of crop plants by means of BNF (Geddes et al., 2015). As a proof-of-concept for the potential of the bacteria genetically optimized in this work as N-biofertilizers for plants, hydroponic cultures of cucumber plants (*C. sativus*) were set using extensively water-washed vermiculite as an inert substrate irrigated with nutrients solution lacking N and selected ammonium-excreting bacteria. *A. vinelandii* strains DJ (*wt*), AV11 (*trc$_P$-glnA*), or AV12 (*ΔnifL, trc$_P$-glnA*) were inoculated into the substrate at $1\times10^7$ cells. g substrate$^{-1}$. As a reference control, non-inoculated pots were irrigated with nutrients solution containing 3 mM $NaNO_3$. Inoculation with AV11 or AV12 promoted weight increments of leaves and roots after 25 days in comparison with non-inoculated or DJ inoculated-plants (Fig. 11). Inoculation at higher bacterial density of $2.5\times10^8$ cells. g substrate$^{-1}$ resulted in a more prominent response for plantlets inoculated with AV11 (*trc$_P$-glnA*) cells but also a delay in the development of plants inoculated with AV12 (*ΔnifL, trc$_P$-glnA*),

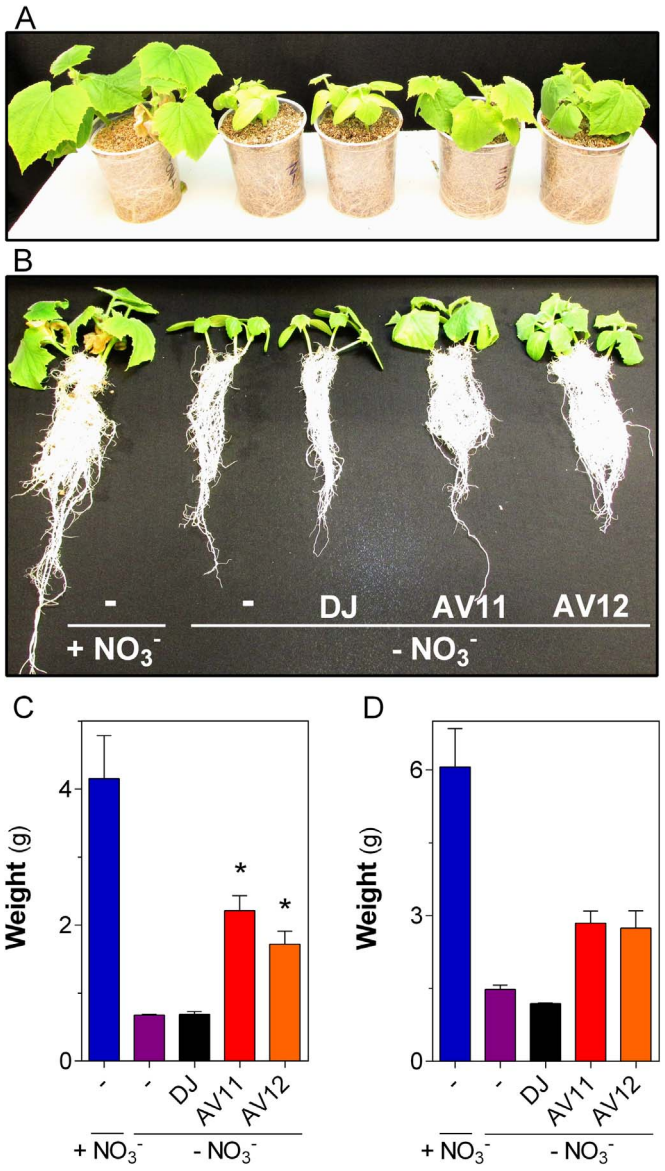


**Fig. 11.** N-fertilization of cucumber plantlets by inoculation with *A. vinelandii* strains. (A and B) Representative assays of cucumber plantlets fertilized, from left to right, with 3 mM $NO_3^-$, no-N and no-N but inoculated with *A. vinelandii* strains DJ (*wt*), AV11 (*trc$_P$-glnA*), or AV12 (*ΔnifL, trc$_P$-glnA*) which were previously cultivated in the absence of IPTG. Detailed analysis of leaves (C) and roots (D) weight at 25 days of germination. Each data point represents the mean and standard deviation of six plantlets. *Means were statistically different (P≤0.005) in a Kruskal Wallis statistical test.

possibly due to ammonium toxicity (Roosta et al., 2009), although this possibility was not confirmed in this study. This observation was coincidental with the determination of up to 50 μM ammonium in the hydroponic chamber of pots inoculated with AV12 cells but no ammonium in pots inoculated with AV11 ($trc_P$-*glnA*) cells.

During the last 40 years, there has been a rapid expansion in genetic engineering and synthetic biology tools (precision expression control, multi-gene DNA synthesis and assembly, synthetic regulation, simplifying design, etc.) that allowed engineering and transfer of nitrogenase activity into a variety of non-diazotrophic bacteria (Cannon et al., 1974; Temme et al., 2012; Smanski et al., 2014; Yang et al., 2014; Zhang et al., 2015; Li et al., 2016). Since the factors that are required for nitrogen fixation are to date more defined than those that govern colonization of plants, it has been proposed that engineering robust colonizers that fix $N_2$ could be of more practical use in the near future than alternative approaches (Geddes et al., 2015). In fact, it has been recently shown that inoculation of maize and wheat with the robust rhizosphere colonizer *Pseudomonas protegens* Pf-5×940 that had been engineered to express *P. stutzeri* A1501 *nif* genes (Setten et al., 2013), largely improved N content and biomass accumulation in both vegetative and reproductive tissues. These demonstrations represented a breakthrough for robust BNF in major cereal crops (Fox et al., 2016).

Besides optimizing and transferring the ability to fix $N_2$ to selected hosts by genetic engineering/synthetic biology approaches, it is clear that delivery of BNF products to the target host is also another challenging trait that should be optimized in coordination with the pathway for $N_2$-fixation (Curatti and Rubio, 2014; Mus et al., 2016). Mus and colleagues recently revised the biochemical mechanisms by which the diazotrophic partners of naturally occurring $N_2$-fixing symbiotic or mutualistic interactions regulate their metabolism to release fixed-N in exchange for fixed-C (Mus et al., 2016). Although it was evident that there is not a universal mechanism, it became apparent that down-regulation of ammonium assimilation, mostly exerted at the level of GS activity, and the concomitant ammonium release is a widely distributed mechanism in nature (Mus et al., 2016).

We provided here proof-of-principle of a simple synthetic genetic-circuit that allowed tuning GS activity of the model diazotroph *A. vinelandii* in such a way that ammonium release and cell proliferation could be adjusted within a reasonably high dynamic-range. These results might inspire the construction of optimized inoculants that efficiently channel N from the air into crops. For example, diazotrophic bacteria such as *A. vinelandii*, *P. protegens* Pf-5×940, or any other robust rhizosphere or endophytic colonizers could be genetically modified with synthetic constructs for fixed optimized levels of GS activity and/or conditionally-lethal alleles of the GS gene under the control of an inducible promoter sensing an inexpensive inducer that is not present or is not abundant in the rhizosphere or plant tissue. These strategies would be further powered by the combinatorial effect of heterogeneous bacterial populations for an optimized balance between cells proliferation and ammonium release. Control of cells permanence in the environment could be a desirable trait for precision agriculture using genetically modified bacteria.

## 4. Conclusion

There is increasing interest in promoting the use of BNF as an alternative to help meeting the fixed nitrogen demand in a sustainable way for the generations to come.

This report provides proof-of-principle for the use of genetically modified bacteria as an alternative N-fertilizer for plants and micro-algae. We show that the combination of mutations on *nifL* for constitutive *nif*-genes expression and attenuation of ammonium assimilation into amino acids by modification of GS activity in *A. vinelandii* resulted in extremely high levels of ammonium release into the growth medium in excess of 20 mM.

Uncoupling growth (GS sufficiency) and ammonium release (GS deficiency) in conditional lethal strains resulted in a significant improvement of ammonium release and some control of the bacterial population. The possibility of loading cells at different levels of GS activity resulted in a novel level of control of both cell proliferation and ammonium release in “heterogeneous” bacterial populations.

These results might inspire further developments of metabolic engineering/synthetic biology towards advanced biofertilizers for channelling $N_2$ from the air into selected crops for sustainable agriculture.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2017.01.002.

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