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Metabolic engineering of ammonium release for nitrogen-fixing multispecies microbial cell-factories

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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. In this study we compared the effect of controlling the maximum activation state of the *Azotobacter vinelandii* glutamine synthase by a point mutation at the active site (D49S mutation) and impairing the ammonium-dependent homeostatic control of nitrogen-fixation genes expression by the *ΔnifL* mutation on ammonium release by the cells. Strains bearing the single D49S mutation were more efficient ammonium producers under carbon/energy limiting conditions and sustained microalgae growth at the expense of atmospheric N_2 in synthetic microalgae–bacteria consortia. Ammonium delivery by the different strains had implications for the microalga's cell-size distribution. It was uncovered an extensive cross regulation between nitrogen fixation and assimilation that extends current knowledge on this key metabolic pathway and might represent valuable hints for further improvements of versatile N_2-fixing microbial-cell factories.

1. Introduction

Concerns on energy depletion and environmental decay highlight a need for the development of alternative and sustainable sources of energy, food, feed and biomaterials. Since agriculture has a limited capacity to satisfy the current demand of all these services, over-exploitation according to available technology may incur severe environment deterioration (Sayer and Cassman, 2013). Microalgae-based bioprocesses represent a very promising set of alternatives to plant-based biofuels, feed and bulk chemicals (Wijffels et al., 2013). These bioprocesses might alleviate pressure on agriculture for food supply and extensive land-use change (Brennan and Owende, 2010). Currently, microalgal biomass is produced commercially mostly as high value products such as human food supplements, animal feed, cosmetics and pharmaceuticals (Gong et al., 2011). However, biofuels from microalgae are not commercial yet mostly because production of algae biomass is currently too expensive and too energy intensive for low commercial-value products (Chisti, 2013).

Similar to intensive agriculture, one of the drawbacks of implementing massive cultivation of microalgae is the unsustainable requirements of fertilizers, especially N. Microalgae have an average composition of $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$, with N accounting for 4–8% on a dry biomass basis making the bioprocess considerably more N-intensive than traditional agriculture. For example, it has been estimated that for the production of 1 kg of triacylglycerol (the principal feedstock of biodiesel) from microalgae biomass it would be needed 0.36 kg of N (0.46 kg NH_4^+ or 1.6 kg NO_3^-) (Peccia et al., 2013). This situation may not only negatively impact on production costs, but also represents a significant share of total energy inputs, may promote competence with agriculture and also direct and/or indirect detrimental effects on the environment (Crutzen et al., 2007; Miller, 2010).

Thus several alternatives have been proposed such as the use of waste water as a source of inexpensive nutrients (Olguín, 2012), N-recycling from biomass after separation of the energy carrier (Peccia et al., 2013; Wernick and Liao, 2013) and the use of N from the air by biological N_2 fixation (BNF) (Ortiz-Marquez et al., 2013, 2012).

All known N_2-fixing organisms (diazotrophs) correspond to the bacterial or archaeal domains of life (Raymond et al., 2004). Nonetheless, some eukaryotes (including plants and algae) engage in N_2-fixing symbioses with bacteria that allow them to take N, although indirectly, from the air (Zehr, 2013).

BNF is catalyzed by nitrogenases in a high energy-demanding reaction requiring at least 16 ATP to fix 1 N_2. The molybdenum nitrogenase is an oxygen sensitive complex of dinitrogenase (NifDK heterotetramer) and dinitrogenase reductase (NifH homodimer).

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The dinitrogenase contains at its active site for N_2 reduction, the iron and molybdenum cofactor (FeMo-co) which biosynthesis comprises dozens of gene products, among which NifB catalyzes the first committed step in the pathway (Rubio and Ludden, 2008). 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 works as a regulatory switch that 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). NifL senses autonomously the oxygen status by its FAD-containing PAS domain and the N status by interacting and receiving signals from GlnK (Dixon and Kahn, 2004). In *A. vinelandii* ammonium is incorporated into amino acids by a "low ammonia pathway" catalized by 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 protein 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).

Symbiotic bacteria normally execute a sophisticated interplay of signals with their partners to assure the specificity of the interaction before starting to cross-feed N-fixation products to their hosts (Charpentier and Oldroyd, 2010). On the other hand, free-living diazotrophs fix sufficient N_2 for their own needs and typically do not excrete significant amounts of N_2-fixation products into their environment (Colnaghi et al., 1997). However, two different kinds of mutations have been associated with an enhanced capacity of ammonium excretion in diverse diazotrophic bacteria: disruption of the NifA/NifL-dependent ammonium control of *nif* genes expression or partial inhibition of GS or GOGAT for deficient ammonium assimilation (Colnaghi et al., 1997). The first aspect has been addressed quite successfully in *A. vinelandii* by either overexpression of NifA or deletion of NifL (Bali et al., 1992; Brewin et al., 1999; Colnaghi et al., 1997; Ortiz-Marquez et al., 2012).

It was recently shown that conversely to the *A. vinelandii* wild type strain a *ΔnifL* mutant engaged in an artificial C for N mutualistic relationship with eukaryotic oleaginous microalgae. This genetically-engineered multispecies platform produced oil up to 30% on a dry biomass basis at the expense of C and N from the air (Ortiz-Marquez et al., 2012).

On the other hand, the ammonium assimilation by the GS–GOGAT pathway has remained difficult to modify in *A. vinelandii* probably because this bacterium relies exclusively on this pathway for N-assimilation and/or its inability to transport glutamine has precluded the isolation of conditional mutant strains as in other bacteria (Colnaghi et al., 2001, 1997).

Thus understanding the regulation of N_2 fixation and assimilation and especially ammonium release by aerotolerant non-symbiotic bacteria is of prime interest for the rational design of versatile biofertilizers for sustainable agriculture and/or 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).

In this work we show the ammonium excretion properties of *A. vinelandii* mutant strains impaired in the regulation of N_2 fixation and/or ammonium assimilation. Glutamine synthetase-deficient strains presented a moderate diazotrophic growth defect, excreted significant amounts of ammonium into the medium and show a higher ratio of excreted ammonium to sugar spent than mutant strains impaired in the regulation of N_2 fixation. Accordingly, these strains improved the growth of microalgae at the expense of atmospheric N_2 in a synthetic microbial consortium.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A. vinelandii strain DJ was the *wt* strain used in this study. DJ and DJ33 (*ΔnifDK*) were kindly provided by Dennis Dean (Virginia Tech). *A. vinelandii* strains AV2 (*ΔnifA::sp*), and AV3 (*ΔnifL*) isolation has been described previously (Ortiz-Marquez et al., 2012). *A. vinelandii* strains AV4 (DJ, *glnA* D49), AV5 (*ΔnifL*, *glnA* D49), AV6 (DJ, *glnA* D49S) and AV7 (*ΔnifL*, *glnA* D49S) were isolated in this work (Table 1). All *A. vinelandii* strains were maintained in Burk's modified medium (Strandberg and Wilson, 1968), incubated at 29 ± 1 °C with shaking at 200 rpm. When required the media were supplemented with 20 μg ml^{-1} spectinomycin 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 derepression of BNF and/or diazotrophic growth analysis cells were collected by centrifugation for 3 min at 1136*g* and then transferred to ammonium-free medium.

For ammonium or glucose consumption experiments, cells were transferred to fresh Burk's modified medium containing 5.6 mM ammonium acetate or 55.5 mM glucose substituting for sucrose, respectively.

Chlorella sorokiniana strain RP maintenance and assays were conducted in nitrogen-free BG11 medium (Rippka et al., 1979), supplemented with 2 mM ammonium or inoculated with *A. vinelandii* strains at initial cell-ratio of 1:1 in 500 ml sterile-air bubbled-bottles at 30 °C and 50 μmol photons m^{-2} s^{-1} continuous white light, as reported before (Ortiz-Marquez et al., 2012) with modifications. For ammonium-limiting conditions *C. sorokiniana* cells were cultivated for 24 h at the expense of 0.5 mM ammonium until the nitrogen source was completely exhausted from the medium and then supplemented with ammonium or inoculated with *A. vinelandii* strains. For extreme ammonium-limiting conditions ammonium-cultivated cells were collected, rinsed with ammonium-free medium and incubated under nitrogen-deprivation condition for 24 h until ammonium addition or inoculation with bacterial cells.

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.

2.2. Isolation of A. vinelandii site-directed mutants of glnA

For site-directed mutagenesis of *A. vinelandii* GS encoded by the *glnA* gene, sequence alignments of GSs were carried out to identify a candidate amino acid in *A. vinelandii* GS that might display the critical function of D50 or D51 in ammonium binding and deprotonation as in *E. coli* (Liaw et al., 1995; Liaw and Eisenberg, 1994) or *Anabaena azollae* (Crespo et al., 1999) GSs, respectively. Thus the candidate D49 was identified for *A. vinelandii* GS. To construct a D49S mutant a 831-bp DNA fragment was amplified by PCR, corresponding to the promoter region of *glnA* and the region comprising the codon for amino acid D49, using the primers *glnA*-F (5′-CAG GCA AAA GAG GGG GCG GGA TTA TAG C-3′) and *glnA*-R (5′-TGGCGGTGCGGATTTCGTGGTCGTGGT-3′) and ligated into the pGEM-T Easy vector (Promega). After sequence confirmation, the D49S mutation was introduced by PCR amplification with the mutagenic primers D49S-F (5′-CGG CAA GAT GTT CTC CGG CTC

Table 1
Bacterial strains, plasmids and primers.

Bacterial strains	Characteristics	Source
Azotobacter vinelandii		
DJ	*wild type*, Nif$^+$	D. Dean
DJ33	*ΔnifDK;* Nif$^-$	D. Dean
AV2	*ΔnifA::sp;* Nif$^-$; Sp^R	Ortiz-Marquez et al. (2012)
AV3	*ΔnifL;* Nif$^+$	Ortiz-Marquez et al. (2012)
AV4	DJ × pAGS1; Nif$^+$; Ap^R. Merodiploid strain containing a truncated and a complete copy of *glnA*	This study
AV5	AV3 × pAGS1; Nif$^+$; Ap^R. Merodiploid strain containing a truncated and a complete copy of *glnA*	This study
AV6	DJ × pAGS2; Nif[a]; Ap^R. Merodiploid strain containing a truncated copy of *wt glnA* and a complete copy of the mutant allele of *glnA*, D49S	This study
AV7	AV3 × pAGS2; Nif[b]; Ap^R. Merodiploid strain containing a truncated copy of *wt glnA* and a complete copy of the mutant allele of *glnA*, D49S	This study
Escherichia coli DH5α	F'*endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17* (rK$^-$ mK$^+$) λ$^-$	Laboratory collection
Plasmids		
pAGS1	pGEMT-Easy vector carrying a truncated copy of *wt glnA* of 831 bp	This study
pAGS2	Similar to pAGS1 but containing a truncated copy of mutant allele of *glnA* D49S	This study
Primers		
glnA-F	CAG GCA AAA GAG GGG GCG GGA TTA TAG C	This study
glnA-R	TGGCGGTGCGGATTTCGTGGTCGTGGT	This study
D49S-F	CGG CAA GAT GTT CTC CGG CTC CTC CAT C	This study
D49S-R	GAT GGA GGA GCC GGA GAA CAT CTT GCC G	This study
FwD49	GAC TTC TTC GAA TAC GGC AAG ATG TTC GAC	This study
FwD49S	GAC TTC TTC GAA TAC GGC AAG ATG TTC TC	This study
RvD49	AGA GCC AGC AGC AGG AGC AGG CTG GGC AAT	This study

[a] Slow diazotrophic growth.
[b] Very slow diazotrophic growth.

CTC CAT C-3′) and D49S-R (5′-GAT GGA GGA GCC GGA GAA CAT CTT GCC G-3′), where the modified nucleotides carrying the desired mutations have been underlined. The PCR reactions were incubated in the presence of the methylation-sensitive restriction enzyme *DpnI* to remove the template and used to transform *E. coli* cells. After sequence confirmation of the mutation the resulting plasmid was used to transform competent cells of *A. vinelandii* strains DJ or AV3. Originally, the merodiploid strains were identified by incorporation of the complete vector conferring resistance to ampicillin by single recombination at the *glnA* locus of the *A. vinelandii* chromosome resulting in a tandem of a complete and a truncated copy of *glnA* (Fig. 3). The mutations were first confirmed by PCR amplification with an allele-specific PCR primer (5′-GAC TTC TTC GAA TAC GGC AAG ATG TTC TC-3′) and then by PCR amplification of the region flacking the mutation and further sequencing of the amplicon.

Control strains (AV4 and AV5) have been constructed by transforming *A. vinelandii* strain DJ or AV3 with the non-mutagenized plasmid to evaluate whether GS function was perturbed in the merodiploids.

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

2.3. GS activity assays

2.3.1. Cells permeabilization

A. vinelandii cells were cultivated in Burk's modified medium containing ammonium overnight and then refreshed in the same medium for an additional 3-4 h. Cells were collected by centrifugation at 1136*g* for 3 min, and transferred into either ammonium-containing or ammonium-free medium (for nitrogenase derepression and/or GS activation) and incubated for 5 h. Cells were collected and rinsed with permeabilization buffer containing 50 mM imidazole, pH 7.5; 5 mM β-mercaptoethanol; 10% (v/v) chloroform; 0.005% (w/v) SDS. Finally cells were resuspended in permeabilization buffer at OD_{600}∼5 and kept on ice until GS activity determination (about 5 min).

2.3.2. GS biosynthetic activity-assay

The assays were run as reported (Shapiro and Stadtman, 1970) with modifications. The biosynthetic reaction mixtures (230 μl) containing 43 mM imidazole-HCl, pH 7.0, 43 mM $MgCl_2$, 86 mM Na-glutamate, pH 7.0; and 125 mM or 0.1 mM NH_4Cl for substrate saturating or substrate limiting assays, respectively; were blended with 30 μl of permeabilized cells and preincubated for 5 min at 29 °C ± 1 °C with shaking at 120 rpm. Reactions were allowed to proceed by addition of 7.5 mM NaATP, pH 7.0 and incubated for 15 min until addition of 1.8 ml of 28.8 mM $FeSO_4.7H_2O$ 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.

2.3.3. GS γ-glutamyl transferase activity-assay

Assays were conducted as reported (Stadtman et al., 1979) with modifications. The γ-glutamyl transferase reaction-mixtures (430 μl) containing 88 mM imidazole-HEPES, pH 7.0; 4.4 mM glutamine, pH 7.0; 0.7 mM $MnCl_2$; and 46 mM $NaH_2AsO_4.H_2O$ were homogenized with 300 μl of permeabilized cells and pre-incubated for 5 min at 29 °C ± 1 °C with shaking at 120 rpm. Reactions were allowed to proceed for 15 min after addition of 30 μl of hydroxylamine-HCl, pH 7.0, and 0.475 mM NaADP, pH 7.0 until addition of 2 ml of a 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. Immnublot analysis

Immunoblotting for NifDK, and NifB using specific immune sera was described previously (Curatti et al., 2005). In brief, total proteins from boiling 0.04 OD_{600} of cells in SDS-PAGE sample buffer (10 μl of a preparation obtained by mixing one volume of cells resuspended in deionized H_2O at OD_{600} of 8.0 and one

volume of two-fold concentrated SDS-PAGE sample buffer) were separated onto 10% polyacrylamide-SDS denaturing gels. Proteins were blotted onto nitrocellulose filters by using a semidry trans-blot device (Bio-Rad), and anti-NifDK or anti-NifB specific polyclonal antibodies raised in rabbits were used at a 1:10,000 dilution. Secondary anti-rabbit-IgG antibodies coupled to alkaline phosphatase were used for the nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate colorimetric development of the signals.

2.5. Analytical methods

Ammonium determinations were carried out by the indophenol method essentially as reported previously using a standard of NH_4Cl (Ortiz-Marquez et al., 2012). Glucose was determined by the Somoyi–Nelson method using a standard curve of glucose (Nelson, 1944). Lipids were isolated and gravimetrically determined essentially as already described (Ortiz-Marquez et al., 2012).

3. Results

3.1. Effect of MSX on growth, GS activity and ammonium release of A. vinelandii

To the best of our knowledge attempts to redirect to flux of N towards ammonium excretion by genetic modification of diazotrophic bacteria at more than one level have not been reported. Thus as a first step to address this possibility we made use of the GS inhibitor methionine sulfoximine (MSX) (Liaw and Eisenberg, 1994) on *A. vinelandii* cells. We observed that growth of the wild-type strain was moderately sensitive when using ammonium as the N source but very sensitive during diazotrophic growth (Fig. 1A). On the other hand, the ammonium-excreting *ΔnifL* strain (Ortiz-Marquez et al., 2012) presented no major difference in sensitivity to MSX according to the N source. In this case the growth defect was apparently of the same magnitude to that of the wild-type strain growing at the expense of ammonium (Fig. 1B).

Under these conditions ammonium excretion by either strain was enhanced and cells started to release ammonium sooner. While MSX-treated wild-type cells accumulated ammonium up to 1.5 mM and maintained that concentration, the *ΔnifL* strain accumulated comparatively very high concentrations of ammonium in the medium up to 7 mM only transiently a few hours after the treatment (Fig. 1C).

GS activity of permeabilized cells was more severely reduced in the wild-type strain growing diazotrophically than at the expense of ammonium, especially at a moderate concentration of the inhibitor. In agreement with the growth performance, GS activity remained less sensitive to MSX in the *ΔnifL* strain, especially when cells used N_2 as the sole source of N (Fig. 2).

3.2. Site directed mutagenesis of A. vinelandii GS

The previous results suggested the possibility of increasing the release of ammonium by *A. vinelandii* cells by a combination of disruption of the feedback control of ammonium cell status on nitrogenase expression/activity and the flux of ammonium towards amino acids synthesis.

Previous work in *E. coli* and *Salmonella typhimurium* (Liaw et al., 1994), and *Anabaena azollae* (Crespo et al., 1999) GS, showed that residues D50 or D51, respectively, are involved in ammonium binding and deprotonation at the active site of GS in these bacteria. Thus, by multiple sequence alignments we identified D49 in *A. vinelandii* GS as a candidate amino acid for a site-directed mutagenesis approach and obtained the *A. vinelandii* single mutant strain D49S, AV6 and double mutant strain *ΔnifL*-D49S, AV7 (Fig. 3).

Diazotrophically-cultivated D49S cells presented a residual GS activity (biosynthetic assay) of 25% but no observable change when cells were cultivated in the presence of ammonium. GS activity could be efficiently abolished in every case by addition of MSX (Fig. 2). On the other hand, we confirmed that the incorporation of non-replicating plasmids in the genome of *A. vinelandii* at the GS locus, although adding a truncated copy of GS did not

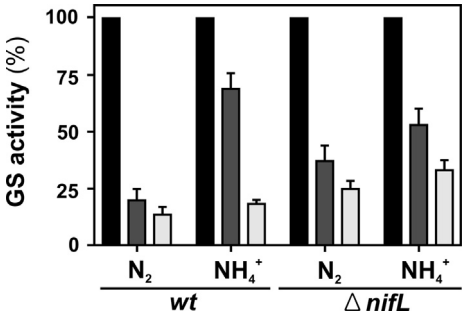

Fig. 2. Effect of L-methionine sulfoximine on GS activity in *A. vinelandii*. GS activity (biosynthetic reaction) in wild-type or *ΔnifL* strains incubated with 0 μM (■); 100 μM (■); or 500 μM (■) MSX under diazotrophic or non-diazotrophic growth conditons. Activities were expressed as a percentage of that in the absence of MSX which was 11.09 ± 2.72 μmol PO_4^{3-} OD^{-1} min^{-1} or 3.47 ± 0.85 μmol PO_4^{3-} OD^{-1} min^{-1} for the wild-type strain cultivated, diazotrophically or in the presence of ammonium, respectively or 2.59 ± 0.51 μmol PO_4^{3-} OD^{-1} min^{-1} or 2.76 ± 0.65 μmol PO_4^{3-} OD^{-1} min^{-1} for the *ΔnifL* strain cultivated, diazotrophically or in the presence of ammonium, respectively. Data represents the mean and standard deviation of 3 independent experiments.

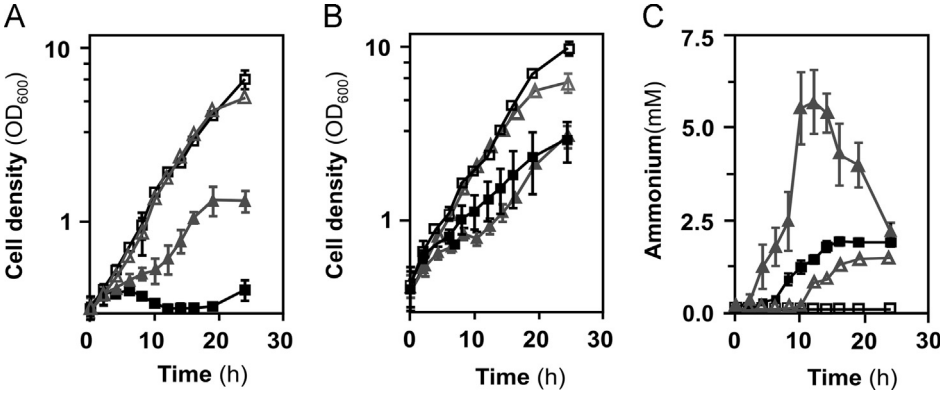

Fig. 1. Effect of L-methionine sulfoximine on growth and ammonium excretion in *A. vinelandii*. (A) Diazotrophic and (B) non-diazotrophic growth curves of *A. vinelandii* wild-type (■, □) or *ΔnifL* (▲, △) strains in the presence (■, ▲) or absence (□, △) of 500 μM MSX. (C) Ammonium accumulation in the spent medium of *A. vinelandii* strains at different time points under diazotrophic growth conditions. Symbols are as in (A) and (B). Each data point in (A–C) represents the mean and standard deviation of four independent experiments.

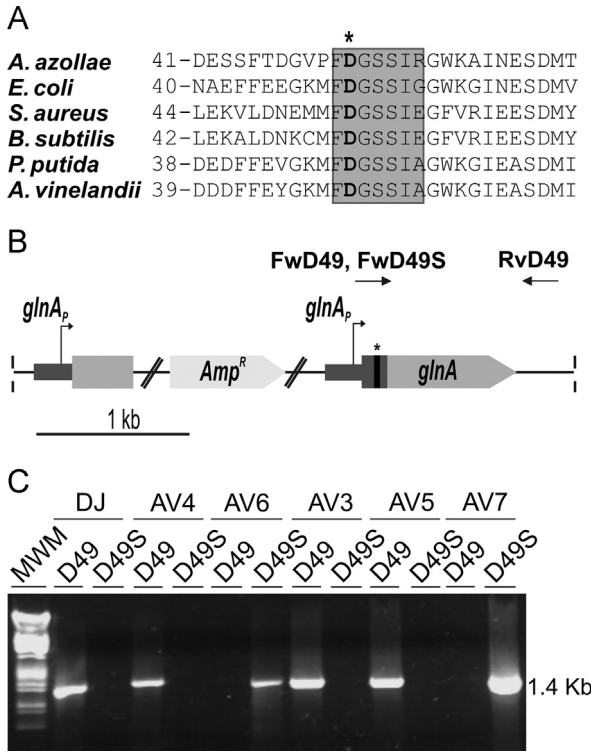

Fig. 3. Isolation of *A. vinelandii* GS D49S mutant strains. (A) Partial amino acids alignment of bacterial GSs showing the highly conserved region comprising the aspartic acid (D49 in *A. vinelandii*) presumably involved ammonium binding. (B) Genetic map of *A. vinelandii glnA* (encoding GS) locus in the D49S mutant strains (* indicates the relative position of D49). (C) PCR analysis of the *glnA* locus in mutant strains using the primers FwD49 or FwD49S and RvD49 as shown in (B).

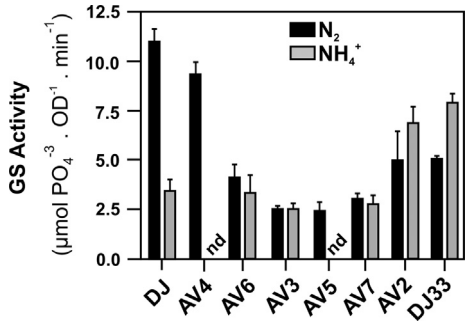

Fig. 4. GS activity of *A. vinelandii* mutant strains impaired in N_2 fixation and/or N assimilation. Strains were cultivated in the presence of ammonium and then transferred to medium containing (NH_4^+) or lacking (N_2) ammonium for 5 h before GS activity determination according to the biosynthetic assay. AV2, *ΔnifA::sp*; AV3, *ΔnifL*; AV4, D49; AV5, *ΔnifL*, D49; AV6, D49S; AV7, *ΔnifL*, D49S; DJ, wild-type; and DJ33, *ΔnifDK*. Data represent the mean and standard deviation of 4 independent experiments for AV2, AV4, AV5 and DJ33; 6 for AV6 and AV7; and 10 for DJ and AV3 in the presence of ammonium; and 19 or 12 for DJ or AV3 in the absence of ammonium, respectively. nd, not determined.

produce any noticeable effect on GS activity (Fig. 4) or *A. vinelandii* growth. In the last case, after subjected to the induction protocol (see Materials and methods) *wt* or merodiploid cells reached OD_{600} of 4.8 ± 0.9 or 5.3 ± 1.0, respectively, while *ΔnifL* or the isogenic merodiploid cells reached an OD_{600} of 4.8 ± 0.8 or 4.3 ± 0.8, respectively.

Because of the predicted function of D49 *A. vinelandii* GS in ammonium binding, we compared GS activity of the mutant strains at ammonium limiting concentrations according to the reported $Km_{NH_4^+}$ for *A. vinelandii* GS of 87 µM (Table 2) (Barnes et al., 1983). Under this condition GS activity was 37% or 25% of that of the substrate-saturating assay for the wild type or D49S strain, respectively. This result would be consistent with a role of *A. vinelandii* D49 in ammonium binding at the active site of GS.

3.3. GS activity in the *A. vinelandii ΔnifL* strain

Conversely to the wild-type, the *ΔnifL* strain presented no increase in GS activity when ammonium cultivated cells were transferred to diazotrophic growth conditions for 5 h. However, the *ΔnifL*-D49S double mutant strain did not present any additive effect on the inhibition of GS activity (Fig. 4).

Either the wild-type or the mutant GS activity was less sensitive to ammonium-limiting conditions in the *ΔnifL* genetic background resulting in 50% or 41% residual activity, respectively, in comparison with that of the corresponding saturating-substrate assays (Table 2).

We investigated if the lack of response of GS activity to the nitrogen source in the *ΔnifL* mutant strains was related to an ammonium-status-related inactivation by adenylylation. Thus we determined the Mn^{2+}-dependent γ-glutamyl transferase activity of GS that has been previously shown to be higher in the adenylylated enzyme as a result of ammonium loading (Colnaghi et al., 2001). As shown in Table 3, this activity was about 3-fold higher in the *ΔnifL* mutant strain than in wild-type cells regardless the N source used. These results suggested that GS might be constitutively adenylylated in the mutant strain irrespective the exogenous supply of ammonium. This lack of regulation was also observed in the double-mutant strain, although total activity was lower (Table 3). Thus, the *ΔnifL* mutation appeared to produce a status of N sufficiency in the cells that triggers a similar response of GS inactivation that that produced by exogenous loading of ammonium. However, experimental enhancement of nitrogen deficiency by exposing either *ΔnifA* or *ΔnifDK* mutant strains, that are unable to fix N_2, to 5 h of ammonium deprivation did not result in GS activation. Unexpectedly, GS activity in these mutant strains

Table 2
GS activity of *A. vinelandii* mutant strains under ammonium-limiting conditions.

Strains	GS Biosynthetic activity[a]	
	Saturating NH_4^+ [b]	Limiting NH_4^+ [c]
DJ	9.23 ± 1.50	3.43 ± 0.84
AV3	2.56 ± 0.53	1.29 ± 0.17
AV6	3.81 ± 0.83	0.96 ± 0.06
AV7	3.06 ± 0.49	1.28 ± 0.40

[a] µmol PO_4^{3-} OD^{-1} min^{-1}.
[b] 125.0 mM.
[c] 0.1 mM. Data correspond to the mean and standard deviation of 4 independent experiments.

Table 3
GS activities in *A. vinelandii* wild-type and mutant strains.

Strains	GS Biosynthetic activity[a]		GS transferase activity[b]		Activities ratio[c]	
	N_2	NH_4^+	N_2	NH_4^+	N_2	NH_4^+
DJ	11.09 ± 2.72	3.47 ± 0.85	1.09 ± 0.2	3.24 ± 0.32	0.12	0.94
AV3	2.59 ± 0.51	2.76 ± 0.65	4.42 ± 0.62	10.18 ± 2.50	1.71	3.69
AV6	4.15 ± 0.95	3.25 ± 0.75	1.89 ± 0.60	2.67 ± 0.19	0.46	0.82
AV7	3.06 ± 0.40	2.78 ± 0.65	3.87 ± 0.38	5.05 ± 0.07	1.26	1.81

[a] µmol PO_4^{3-} OD^{-1} min^{-1}.
[b] µmol γ-glutamyl hydroxamate OD^{-1} min^{-1}.
[c] Mn^{2+}-dependent transferase/Mg^{2+}-dependent biosynthetic activities. Each data point represents the mean and standard deviation of at least 4 independent experiments.

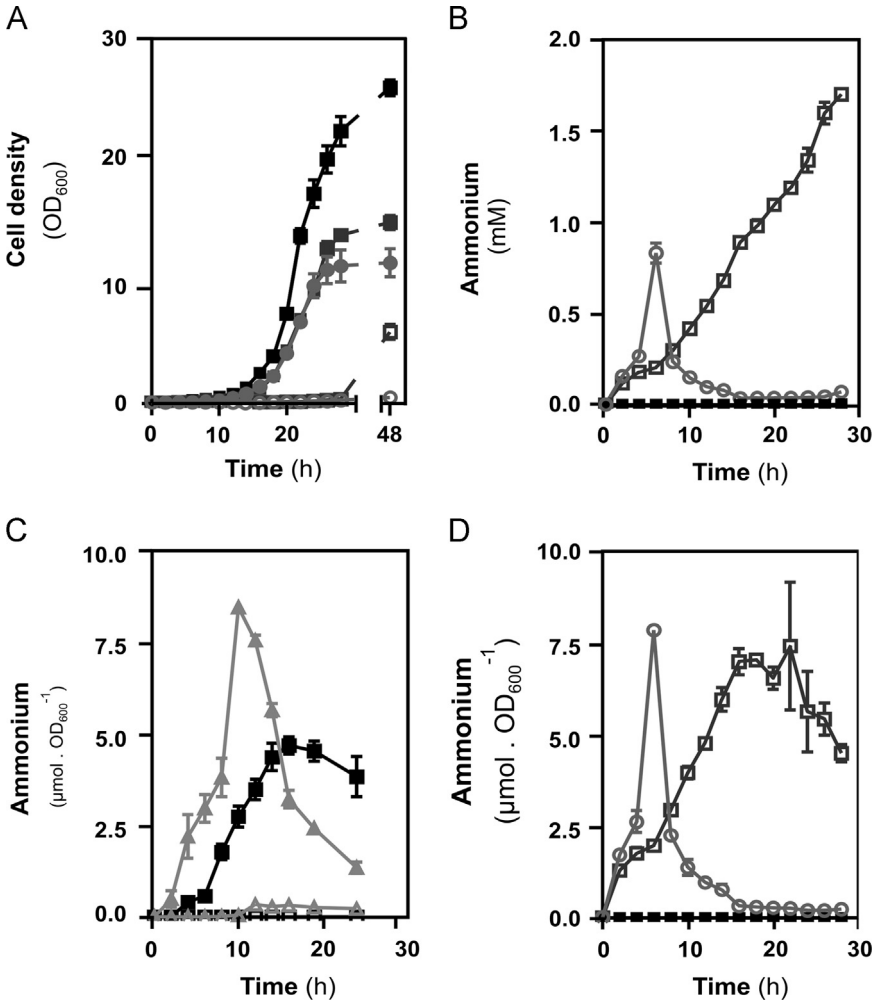

Fig. 5. Effect of the D49S mutation on growth (A) or ammonium excretion (B) of *A. vinelandii* mutant strains. Diazotrophically cultivated DJ (■); AV6 (▫); or AV7 (○); or AV6 (■); or AV7 (●) cells in the presence of ammonium. (C and D) comparison of the time course of ammonium accumulation as a ratio to the biomass production (OD_{600}) of MSX treated cells (C) or strains bearing the D49S mutation (D). In (C), DJ (■); or AV3 (▲) in the presence or DJ (▫); or AV3 (△) in the absence of MSX. In (D) symbols are as in (A and B). Each data point represents the mean and standard deviation of 4 independent experiments.

cultivated under ammonium sufficiency, condition under which they do not present any observable growth defect (not shown), was higher than that of the wild-type or *ΔnifL* strains (Fig. 4).

3.4. Effect of the D49S mutation on growth and ammonium release of A. vinelandii

Both D49S strains grew at the expense of ammonium moderately slower and reached the stationary phase prematurely at lower cell densities. However, they showed a strong diazotrophic growth defect, especially the double-mutant strain, when cells were shift from ammonium replete medium to medium lacking any other source of N than air (Fig. 5A). Conversely to the *ΔnifL* strain that started to accumulate ammonium into the medium at stationary phase of growth (Fig. 1), strain D49S started to release ammonium from its long lag phase for at least 20 h, in the absence of any noticeable growth (Fig. 5B and D). When the ammonium accumulation in the medium was standardized by the biomass produced (OD_{600}), the accumulation profile was very similar to that obtained after pharmacological inhibition of GS with MSX (Fig. 5C and D). Furthermore, the rapid increase and decrease of ammonium accumulation of the *ΔnifL* strain when GS was inactivated by MSX was confirmed by the D49S mutation on the *ΔnifL* genetic background.

Also an unintentionally-created D49A, A54I mutant allele of GS (A54 is not as phylogenetically conserved than D49, Fig. 3A) resulted in a mutant strain that excreted ammonium when transferred to the wild-type genetic background but decreased the ammonium accumulation of the *ΔnifL* mutant strain (not shown).

Growth and ammonium concentration in the medium was also analyzed when cells were allowed to grow and consume a limiting amount of ammonium. Under these conditions the wild-type strain exhausted all the ammonium from the medium in 10 h and continued growing diazotrophically. As a reference, a *ΔnifA* strain consumed ammonium at the wild-type rate and failed to grow diazotrophically. The strains bearing the *ΔnifL* and/or the D49S mutation presented growth profiles between the limits set by the wild-type and the *ΔnifA* strains (Fig. 6A) and left a significant amount of ammonium in the medium (Fig. 6B).

3.5. Effect of carbon limitation on growth and ammonium release of A. vinelandii mutant strains

A. vinelandii has a slight preference for sucrose than glucose (Wong, 1988). Thus, we substituted 1% glucose for 2% sucrose in the Burk's medium to induce a minor to moderate carbon limitation in *A. vinelandii*. We confirmed a delay to reach the logarithmic phase of growth, a slightly lower diazotrophic growth rate and

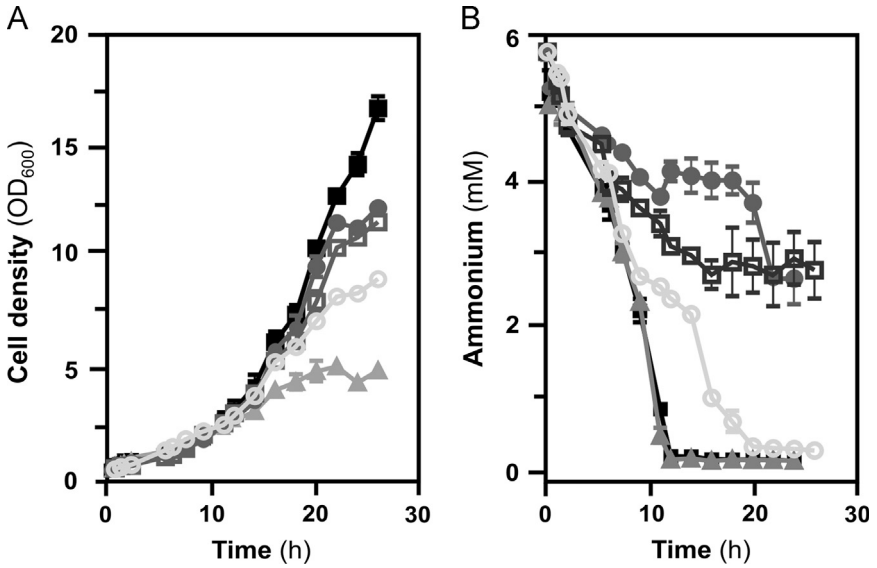

Fig. 6. Growth (A) and time course of ammonium concentration in the medium (B) when cells were allowed to growth and consume a limiting amount of ammonium. DJ (■); AV2 (▲); AV3 (●); AV6 (□); or AV7 (○). Each data point represents the mean and standard deviation of 6 independent experiments for DJ, 4 for AV2 and AV3 and 2 for AV6 and AV7.

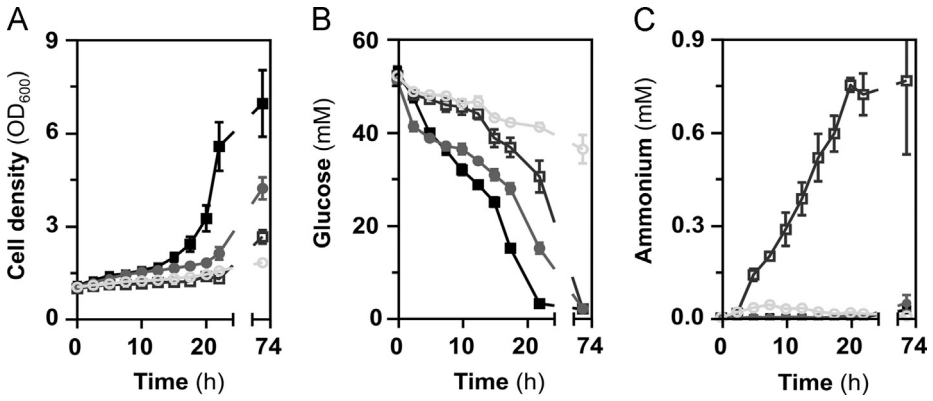

Fig. 7. Diazotrophic growth (A) and time course of glucose consumption (B) or ammonium excretion (C) of *A. vinelandii* mutant strains. C. DJ (■); AV3 (●); AV6 (□); or AV7 (○). Each data point represents the mean and standard deviation of 4 independent experiments.

a stationary phase at a lower cell density for the wild-type strain (Figs. 1 and 7A). This condition exacerbated the diazotrophic growth defect of all the mutant strains. This was particularly noticeable in the *ΔnifL* strain, which grows at nearly wild-type rates when is not carbon constrained (Fig. 1A and B). The strains consumed glucose at rates proportional to their growth rates (Fig. 7B). Under this condition only the single mutant strain D49S excreted significant amounts of ammonium into the medium (Fig. 7C).

3.6. Effect of GS inhibition on nitrogenase expression in A. vinelandii

Conversely to the wild-type strain, either in the *ΔnifL* or D49S strain, the dinitrogenase NifDK was expressed even in cells cultivated in the presence of an excess of ammonium. However, in the double-mutant strain NifDK accumulation was mostly undetectable under the experimental conditions used even after a very long derepression time of 20 h. Lower accumulation of NifDK in the *ΔnifL* strain was also observed after treating the cells with MSX for 5 h concomitantly with the transition from ammonium-replete medium to medium lacking that N source. Since it is known that NifDK polypeptides have a slow turn-over rate in *A. vinelandii* (Martínez-Noël et al., 2011) we analyzed the effect of the D49S mutation or MSX on the accumulation of the fast turning-over polypeptides of NifB. Similarly to the accumulation of NifDK, NifB did not accumulate in the *ΔnifL*, D49S strain and presented a more prominent decrease after inhibition of GS by MSX (Fig. 8).

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

Similarly to the *ΔnifL* mutant strain, the D49S and also the double-mutant strain allowed the diazothophic culture of the eukaryotic microalgae *C. sorokiniana* in artificial consortia in either nitrogen-limiting or extreme nitrogen-limiting assays (Fig. 9). In the absence of supplementation with an additional source of carbon and energy than the photosynthetic exudates from the microalgae, the D49S strain consistently supported a slightly higher population of *C. sorokiniana* cells than the *ΔnifL* strain (Fig. 9A and B). On the other hand, in cultures supplemented with 3 mM sucrose, the *ΔnifL* strain consistently performed better at nitrogen-fertilization of microalgae cells, while the double-mutant strain showed an intermediate response between that of the single mutant strains. These results are consistent with the observation that the D49S strain produced more ammonium than the *ΔnifL*

strain under carbon-limiting conditions as those expected in the artificial consortia.

According to the availability of bio-active N microalgae accumulate biomass and lipids as a direct or inverse relationship, respectively (Do Nascimento et al., 2012). Consequently, inoculation with each of the bacterial mutant strains resulted in an increase in microalgae biomass with a lower lipids content (Fig. 10A). Unexpectedly, when inoculated with the double-mutant strain, the microalgae compensated the low final cell-number (in comparison to that produced by either single mutant strain) by increasing the average cell-volume (Fig. 10B; Supplemental Fig. S1). In this case, the microalgae were on average about 50% or 250% larger than the control cells supplemented with 0.5 mM ammonium or no bioavailable N-source, respectively, regardless if calculated from the mean or median cell-volume. Thus the combination of data of cell-number and cell-volume fit reasonably well those of biomass determination.

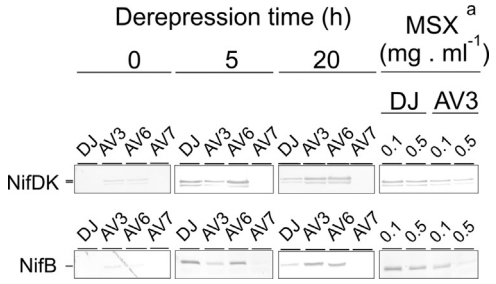

Fig. 8. Accumulation of Nif proteins in *A. vinelandii* mutant strains impaired in N_2 fixation and/or N assimilation. Time course of NifDK or NifB proteins accumulation and comparison to MSX treated cells concomitantly to ammonium removal for 5 h. A representative experiment is shown out of 2 independent assays with consistent results.

4. Discussion

In addition to being an essential part of the nitrogen cycle on Earth, BNF has a remarkable importance for current agriculture and a great potential as an alternative to help meeting the reactive nitrogen demand in a sustainable way for the generations to come.

However, the great majority of organisms of industrial relevance are not N_2-fixers *per se*. Thus the most prevailing alternatives comprise selection and optimization of either symbiotic or associative N_2-fixing bacteria to be used as biofertlilizers in agriculture or introducing the complete pathway for BNF into non-diazotrophic hosts (Beatty and Good, 2011; Oldroyd and Dixon, 2014).

The development of versatile ammonium excreting strains has been lately extended from plant crops for traditional agriculture to the biofertilization of eukaryotic microalgae and/or probably non-diazotrophic cyanobacteria as promising feedstocks for the future production of food, feed, biofuels and biomaterials. The proposal takes advantage of the fact that in natural systems, microalgae release dissolved organic carbon from 0% to 80% of photosynthates and around 6–16% in photobioreactors (Hulatt and Thomas, 2010). An analysis of *C. sorokiniana* exudates showed that it accumulates in the culture medium 122 μM sucrose as the major soluble carbohydrate and most readily usable C source for accompanying bacteria (Watanabe et al., 2008). Thus this strategy might turn "wasted" energy into N-fertilizer for sustainable production of algal biomass in an integrated bioprocess. In addition, this strategy would bypass most of the constraints associated with introducing

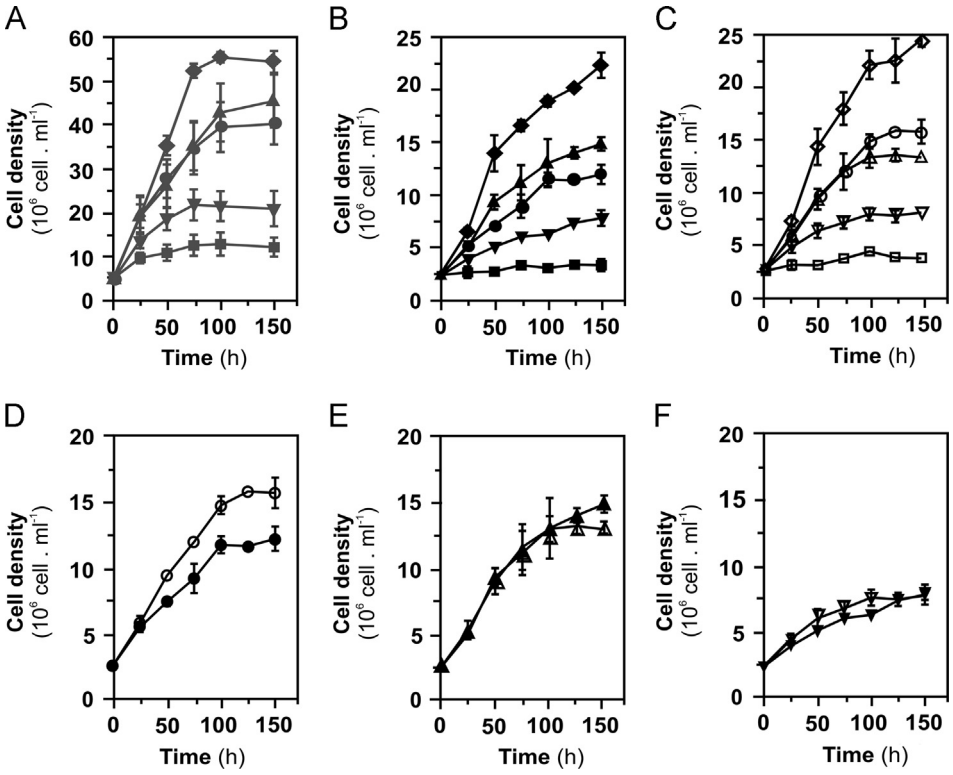

Fig. 9. N-biofertilization properties of genetically modified *A. vinelandii* mutant strains in artificial symbiotic association with eukaryotic microalgae. Microalgae growth curves under ammonium-limiting conditions (A), or extreme ammonium-limiting conditions (B and C), without (B) or with (C) the addition of 0.1% sucrose. (♦) Supplemenattion with 0.5 mM ammonium chloride; (■) no addition of a bioavailable N-source; and inoculation with *A. vinelandii* strains (●) AV3; (▲) AV6; or (▼) AV7. Empty symbols represent runs supplemented with 0.1% sucrose. (D–F) represent a replotting of data in (B and C) to highlight the effect of C-limitation on the strains performance. Each data point represents the mean and standard deviation of four independent experiments.

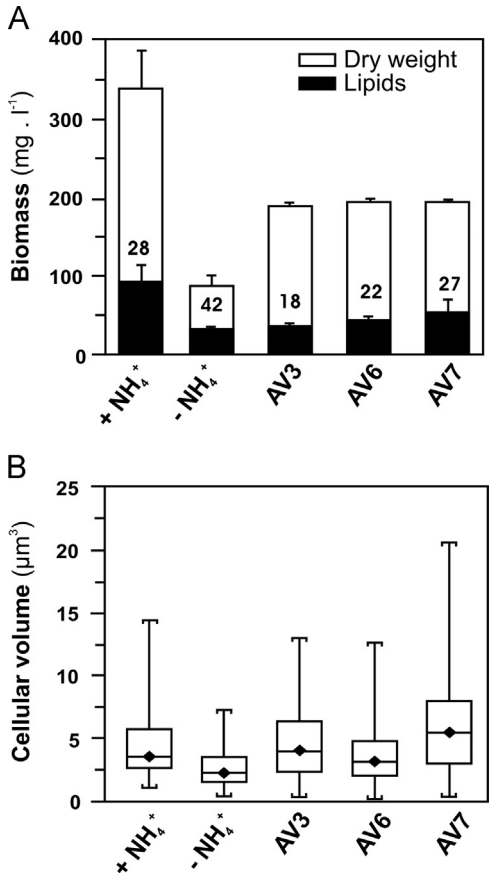

Fig. 10. Effect of inoculation with *A. vienlandii* strains on microalgal biomass, lipid content and cell size. (A) Biomass and lipids yield of artificial microalgae–*Azotobacter* consortia under extreme ammonium-limiting conditions at 150 h. Numbers within the boxes represent the lipids content as a percentage of dry biomass. Each data point represents the mean and error bars represent the range of two independent experiments. (B) Box and whisker plot of the microalgae cell-volume when cultivated in artificial microalgae–*Azotobacter* consortia under extreme ammonium-limiting conditions at 150 h. Boxes indicate the lower quartile, median (horizontal line within the box), and upper quartile values. Whiskers are located at extreme values within 1.5 times the inter-quartile range from the ends of each box. Population sizes were: 179; 113; 216; 268; and 250 cells from two independent experiments of cultures in the presence of 0.5 mM ammonium, no addition of a bioavailable N-source or inoculated with *A. vinelandii* strains AV3, AV6, or AV7 strains, respectively.

complete recombinant pathways in host for which genetic compatibility and/or metabolic coupling is poor (Ortiz-Marquez et al., 2013, 2012).

The aim of this work was to further optimize N_2-fixing bacteria with respect to ammonium release to be used as N_2-fixing parts in multispecies microbial cell-factories comprising photosynthetic (microalgae) and N_2-fixing (bacteria) synthetic partners. We report the isolation and characterization of the *A. vinelandii* GS-D49S strain with remarkably different ammonium-excretion properties than previously isolated *A. vinelandii* Δ*nifL* strains impaired in the N-related homeostatic control of nitrogenase expression (Bali et al., 1992; Brewin et al., 1999; Colnaghi et al., 1997; Ortiz-Marquez et al., 2012). *A. vinelandii* GS-deficient strains performed consistently better than the previously characterized Δ*nifL* strains (Ortiz-Marquez et al., 2012) in terms of both ammonium release into the medium of axenic cultures or microalgae growth-promotion at the expense of atmospheric N_2 in synthetic microalgae–bacteria consortia. This improvement might be related to the fact that this strain is a more efficient ammonium producer under carbon/energy limiting situations, as those expected in microalgae–bacteria consortia using air as sole sources of C and N. Since BNF is an extremely energy demanding processes, the demonstration that restricting amino acids synthesis and bacterial biomass production resulted in a more energy efficient strategy for biological production of ammonium than deregulating nitrogenase might be helpful for future directions in genetic engineering of BNF.

The expectations of sustained hyperproduction of ammonium in hypothetical strains with increased synthesis and reduced allocation to other pathways (biosynthesis of amino acids) was partially met. Although the double mutant strain Δ*nifL*, D49S has a considerably improved initial rate of ammonium release, it fails to maintain that status for more than 5 h and allowed less microalgal cells proliferation than either single mutant strain using N_2 as the sole source of N. Nevertheless, inoculation with this strain resulted in as much microalgae biomass from atmospheric N_2 than either single mutant strain because of a compensation in the alga's biovolume that tended to be higher. Although beyond the scope of this study, this unexpected result might be significant for microalgae biotechnology, since difficulty of cells harvesting, largely dependent on cell size and/or density, is one of the drawbacks for most microalgae biomass applications (Chisti, 2013). Although the mechanism underlying this effect is not understood at this moment, it is tempting to speculate that it could be related to differences in the time course of ammonium delivery among the different modes of N supply to the algae cells.

It has been recently proposed that for a rational design of exchangeable N_2-fixing synthetic circuits for future applications of BNF it would be necessary to override the extensive metabolic regulation that underlies N_2 fixation (Temme et al., 2012; Wang et al., 2013). Furthermore, results presented herein, especially the characterization of the double mutant strain Δ*nifL*, D49S, allowed us to uncover a variety of features and/or regulatory aspects interconnecting both N-fixation and assimilation.

In the strains bearing the D49S mutation the increase in metabolic flux towards the release of ammonium was met at the expense of the biosynthesis of amino acids (and probably of general proteins biosynthesis). This situation might result in a generalized pleiotropic effect on cell physiology, cell integrity and catalytic capacity. Thus it is anticipated that optimization of the level of residual GS activity might increase further the release of ammonium out of the cells. MSX has been extensively used to experimentally enhanced ammonium release in bacteria and/or screening for GS deficient strains (Colnaghi et al., 1997). We demonstrated that partial inhibition of GS by MSX phenocopied the growth performance and ammonium excretion of the D49S mutant strain to a reasonably high level of detail as that observed for the fast but transient accumulation of ammonium in the Δ*nifL* genetic background. This result highlights the convenience of using this inhibitor to experimentally model the effect of different levels and/or modes of inhibition of GS on ammonium release to further optimize strains for this trait.

Either the Δ*nifL* or the D49S mutant strains accumulated up to 2 mM ammonium in the culture medium. However, while the Δ*nifL* strain started to accumulate ammonium towards its stationary phase of growth, mostly in the absence of biomass production, the D49S strain accumulated ammonium during lag and logarithmic phases of growth. It is intriguing that although the Δ*nifL* strain presented low levels of GS activity at lag/early logarithmic phase of growth, similar to those of the D49S strain, that fact did not result in ammonium accumulation before the stationary phase of growth. Inhibition of GS in the Δ*nifL* strain appears to be exerted mostly at the level of GS adenylylation, confirming that this regulatory mechanism might operate in a similar way irrespective of the source of ammonium: external ammonium loading or N_2 fixation. However, it is not currently understood why mutations in *nifA* or *nifDK* did not produce the opposite effect.

Although somehow predictably, we uncovered not previously demonstrated mutant phenotypes of *ΔnifL* strains: a diazothophic growth defect under suboptimal supply of C and especially, the cellular stress imposed by defective ammonium assimilation and synthesis of amino acids as that of the D49S strain. Both situations might be the result of the inability of the mutant strains to restrict excessive synthesis of Nif proteins when C, amino acids and/or energy are in limiting supply.

Additionally, NifD, NifK and NifB accumulation was only partially repressed in the presence of ammonium in both single mutant strains and increased after ammonium removal of the medium. In the case of the single D49S mutation, this indicates that regardless the presumed shortage in amino acids supply a signal transduction cascade senses the metabolic imbalance, triggers *nif* genes activation and keeps forcing the accumulation of Nif proteins. However, the fact that Nif proteins accumulation was almost completely abolished in the *ΔnifL*, D49S strain is not completely understood at this moment. Nevertheless, it appears promising that the double mutant cells have a transient capacity to release very high amounts of ammonium soon after switching the cells from medium containing ammonium to medium lacking that N source even at very low levels of NifDK and NifB. Thus it remains to be identified the mechanism by which this engineered ammonium-excretion pathway switches off suddenly and cells resume growth, although slowly, apparently at the expense of the previously released ammonium.

5. Conclusion

To the best of our knowledge this is the first study that compared experimentally the ammonium excretion properties of the same bacterium impaired at the levels of N-fixation and/or assimilation. Progress has been made in the optimization of the ammonium excretion properties of bacteria to be used as N-biofertilizer. Future work should be directed to advance in the understanding of the underlying regulatory aspects to further increase the flux of N into N-fertilizers for improved multispecies microbial cell-factories taking C and N from the air.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.03.002.

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