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Association with an Ammonium-Excreting Bacterium Allows Diazotrophic Culture of Oil-Rich Eukaryotic Microalgae

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Concerns regarding the depletion of the world's reserves of oil and global climate change have promoted an intensification of research and development toward the production of biofuels and other alternative sources of energy during the last years. There is currently much interest in developing the technology for third-generation biofuels from microalgal biomass mainly because of its potential for high yields and reduced land use changes in comparison with biofuels derived from plant feedstocks. Regardless of the nature of the feedstock, the use of fertilizers, especially nitrogen, entails a potential economic and environmental drawback for the sustainability of biofuel production. In this work, we have studied the possibility of nitrogen biofertilization by diazotrophic bacteria applied to cultured microalgae as a promising feedstock for next-generation biofuels. We have obtained an *Azotobacter vinelandii* mutant strain that accumulates several times more ammonium in culture medium than wild-type cells. The ammonium excreted by the mutant cells is bioavailable to promote the growth of nondiazotrophic microalgae. Moreover, this synthetic symbiosis was able to produce an oil-rich microalgal biomass using both carbon and nitrogen from the air. This work provides a proof of concept that artificial symbiosis may be considered an alternative strategy for the low-N-intensive cultivation of microalgae for the sustainable production of next-generation biofuels and other bioproducts.

At present, two of the main challenges of humankind are the sustainable production of food and the identification and development of alternative sources of energy in the framework of environmental protection and profitability (30). The depletion of the world's reserves of oil and the general concern regarding global climate change, presumably due to the increase in the levels of atmospheric CO_2, have promoted an intensification of research and development toward the production of biofuels, among other alternative sources of energy, during the last years. Biofuels are produced from biomass, in this way being derivatives of solar energy through the photosynthetic process. There are different types of biofuels according to the source of materials used as feedstocks. First-generation of biofuels are based on production from sugar, starch, or oils from different plant crops, while second-generation production uses agricultural and forest lignocellulosic residues or nonedible energetic crops. The main advantages of biofuels are their character of being renewable and neutral regarding CO_2 emissions into the atmosphere (2, 22, 34). However, life cycle analyses of these approaches have raised concerns related to land use changes; the impact on food production; and carbon, water, and nitrogen footprints (13, 21, 38).

On the other hand, third-generation or next-generation biofuels make use of eukaryotic microalgae or cyanobacterial biomass as a feedstock for a diversity of biofuels, especially biodiesel from eukaryotic microalgal lipids. Some of the main advantages of microalga-derived biofuels are (i) a high oil yield per unit of surface and time, (ii) the possibility for the use of marginal lands, (iii) the possibility for a better economy of water and nutrients, and (iv) the possibility of using residues from industries and other sources as inexpensive nutrients. With regard to the latter aspect, it is expected that the coupling of large-scale culturing of microalgae to CO_2-generating power plants will be an interesting strategy for the mitigation of the greenhouse effect (10, 25, 40, 45).

Regardless of the nature of the feedstock (plants or microalgae), fertilizers are required for sustained high-yield biomass production. Fertilizers can constitute a significant share of the total energy inputs in agriculture for food, feed, or energy production, particularly for nitrogen-intensive crops (11, 40, 41, 42, 44).

Microalgae have an average composition of $CH_{1.7}O_{0.4}N_{0.15}P_{0.0094}$, with N and P accounting for 4 to 8% and 0.1% on a dry biomass basis, respectively. These nutrients are in a limiting supply for microalga proliferation in both marine and freshwater environments (22). Thus, fertilizers, especially N, may constitute a significant input of energy if wastewater is not used as a source of inexpensive nutrients. It has been calculated that with the demand for N fertilizer for the complete replacement of European Union (EU) fossil fuel oil use by renewable sources, even accounting for the total N content of the EU waste, there would still be a deficit of ca. 10^5 kg of N · year^{-1} (22). Other calculations estimated that, without nutrient recycling, nearly 45% of all the fossil energy input for large-scale microalga cultivation is linked to fertilizers, while more than 90% of it corresponds to N fertilizer (11).

Additionally, the production and use of N fertilizers have raised concerns related to the mitigation of global warming. For nitrogen fertilizers, greenhouse gas (GHG) emissions arise both as a result of the fossil energy inputs needed to capture and process atmospheric nitrogen and also from complex processes that result in the production and release of nitrous oxide (N_2O) into the atmosphere as a potent GHG. N_2O accounts for 0.6% of the mass of the GHG released but 65% of CO_2 equivalents of the global warming potential (8, 13, 45).

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An alternative, natural way of converting the gaseous nitrogen from the air into biologically active nitrogen is called biological nitrogen fixation (BNF). All known nitrogen-fixing organisms (diazotrophs) are prokaryotes corresponding to the bacterial and archaeal domains of life (35).

The interaction of plants with diazotrophic bacteria in the soil is a widespread means by which plants obtain part of the nitrogen that they need (12). BNF takes place by means of complex enzymes known as nitrogenases. The molybdenum nitrogenase is an ATP-hydrolyzing, redox-active, and oxygen-sensitive complex of two component proteins: the dinitrogenase (NifDK heterotetramer) and the dinitrogenase reductase (NifH homodimer). The dinitrogenase contains the iron and molybdenum cofactor (FeMo-co) at its active site for N_2 reduction. Additionally, some organisms contain alternative nitrogenases, where Mo is replaced by either V or Fe (Vnf or Anf nitrogenase, respectively), which are hierarchically derepressed when Mo or V is not available (18). Dozens of gene products are required for the assembly and function of nitrogenases. Among them, NifB is critical because it catalyzes the first committed step in the biogenesis of FeMo-co and the cofactors of the alternative nitrogenases (36).

In the *Proteobacteria*, nitrogen fixation genes for the molybdenum nitrogenase (*nif* genes) are subjected to transcriptional activation by NifA (a member of the enhancer-binding protein family), together with the RNA polymerase RpoN (sigma factor σ^{54}). Although the regulatory cascades differ among even related diazotrophs, each regulatory circuit ultimately results in the regulation of NifA expression or the modulation of its activity in response to oxygen and/or fixed nitrogen. In the *Gammaproteobacteria*, *nifA* is cotranscribed in an operon with *nifL*, which encodes an antiactivator protein that regulates NifA activity in response to oxygen and fixed nitrogen by interacting with NifA to form an inhibitory complex under conditions that are unsuitable for nitrogen fixation (17).

While symbiotic bacteria tend to excrete assimilated N during specific bacterium-host interactions, free-living diazotrophs fix sufficient nitrogen for their own needs and do not generally excrete significant amounts of nitrogen fixation products into their environment (3). However, two different kinds of mutations have been associated with an enhanced capacity for ammonium excretion in diverse diazotrophic bacteria: those resulting in the partial inhibition of ammonium assimilation or interference with the mechanism by which ammonium inhibits either the synthesis or activity of the nitrogenase (12).

Despite it being appreciated for decades that microalgae normally co-occurred in nature with consortiums of associated heterotrophic bacteria, much less is known about these interactions than about plant-bacterium interactions in the rhizosphere. The isolation of bacteria from microalgal cultures has allowed the identification of many bacterial groups that produce either positive or negative effects on microalga growth (20, 43). However, relatively few studies have been conducted with model organisms in artificial symbiotic communities toward understanding the basis of these interactions and further improving their robustness by genetic engineering. Two of the few exceptions are studies conducted with *Chlorella* spp. and *Azospirillum brasilense* (16) and with *Chlamydomonas* sp. and *Azotobacter* sp. (29).

In this work, we have isolated a novel and stable *Azotobacter vinelandii* mutant strain with an almost complete deletion of the *nifL* gene that expresses nitrogenase constitutively and excretes ammonium into the surrounding medium. This strain considerably improves the growth promotion by *A. vinelandii* of nondiazotrophic microorganisms, including oleaginous microalgae, when no source of nitrogen other than air was supplemented into the medium. These results constitute a proof of concept for the development of N biofertilizers for clean and inexpensive biomass for next-generation biofuels and other bioproducts.

MATERIALS AND METHODS

Strains and culture conditions. *Chlorella sorokiniana* strain RP, *Pseukirchneriella* sp. strain C1D, and *Scenedesmus obliquus* strain C1S were isolated form freshwater ponds in Buenos Aires, Argentina, in 2009 and were identified by traditional and molecular methods (Table 1). These microalgae were routinely maintained in BG11 medium supplemented with either 2 mM NH_4^+, NO_3^-, or urea. Two different culture settings were used: one consisting of 25-ml cultures in 100-ml Erlenmeyer-type flasks with manual shaking twice a day and the other consisting of 150-ml cultures in homemade air-lift-type photobioreactors continuously bubbled with sterile air from the bottom. In both cases, cultures were incubated at 29°C ± 1°C and under continuous light at 50 μmol photons · $m^{-2} \cdot s^{-1}$.

A. vinelandii strain DJ was the wild-type (wt) strain used in this study. *A. vinelandii* strains DJ, AV2 (Δ*nifA*::Sp), AV3 (Δ*nifL*), UW211 (*nif*H_p-*lacZ*), UW217 (Δ*nifA*::Sp *nif*H_p-*lacZ*), and UW226 (Δ*nifL nif*H_p-*lacZ*) were maintained in Burk's modified medium containing 5 mM NH_4^+, supplemented with spectinomycin (Sp) in the case of Δ*nifA*::Sp strains. Cultures were incubated at 29°C ± 1°C with shaking at 200 rpm. For nitrogenase derepression, *A. vinelandii* cultures were cultivated in the presence of 5 mM NH_4^+, collected by centrifugation at 3,000 rpm for 3 min, and then transferred into NH_4^+-free medium.

When either bacteria or microalgae were cultured onto solid medium, water-washed agar was used at 1% (wt/vol).

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

Isolation of *Azotobacter vinelandii* mutant strains. The general strategy for DNA manipulation and *A. vinelandii* transformation was previously reported (14). Basically, competent *A. vinelandii* cells were prepared by culturing the strains in medium lacking Mo and Fe overnight, and competent cells were transformed with 0.5 to 1 μg linearized plasmids in half-strength Burk's modified medium containing 10 mM MOPS (morpholinepropanesulfonic acid) buffer (pH 7.4) and 8 mM $MgCl_2$. Cells were allowed to recover overnight in Burk's modified medium and then subjected to the appropriate selection on solidified medium (14). To obtain the Δ*nifA*::Sp strain, plasmid pRHB138 bearing an almost complete deletion of the *nifA* open reading frame (14) was introduced into *A. vinelandii* DJ by transformation and selection for spectinomycin-resistant extransformants. This strain was unable to grow diazotrophically. For the isolation of the Δ*nifL* strain, an in-frame *nifL* deletion was introduced into the *A. vinelandii* chromosome. To generate a Δ*nifL* mutant allele, a 1,408-bp DNA fragment from the *rnf-1* gene region upstream of *nifL* was generated by PCR using the oligonucleotides 5′-GCCGGATCCTCGTCGAGCTGCCCTTCTCG GCTC and 5′-TGG GGA TCC GCG CAT GCT CCA CCG TCT GGC GAA and ligated into pUK21 between the EcoRI and BamHI sites, as the corresponding sequences were included in the primers (underlined) to facilitate cloning for the construction of plasmid pRHB142. A 2,003-bp DNA fragment from the *nifA* region downstream of *nifL* was then generated by PCR using the oligonucleotides 5′-GCCGGATCCTCGTCGAGC TGCCCTTCTCGGCTC and 5′-AGGTTCTAGAGCTGATCGTGCGTG CTGCCGCAG, containing convenient restriction enzyme sites (underlined), and ligated into the BamHI and XbaI sites of pRHB142 to generate pRHB143, containing an in-frame deletion of *nifL* from amino acids Ile36 to Ile508 and a complete copy of *nifA* (Fig. 1).

Linearized plasmid pRHB143 was used to transform nondiazotrophic

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Microalgae		
Chorella sorokiniana strain RP	Isolated from freshwater ponds in Buenos Aires, Argentina, in 2009	Laboratory collection
Scenedesmus obliquus strain C1S	Isolated from freshwater ponds in Buenos Aires, Argentina, in 2009	Laboratory collection
Pseukirchneriella sp. strain C1D	Isolated from freshwater ponds in Buenos Aires, Argentina, in 2009	Laboratory collection
Azotobacter vinelandii		
DJ	Wild type	D. Dean
AV2	Δ*nifA*::Sp	This study
AV3	Δ*nifL*	This study
UW211	$nifH_p$-*lacZ*	14
UW218	Δ*nifA*::Sp $nifH_p$-*lacZ*	14
UW226	Δ*nifL* $nifH_p$-*lacZ*	This study
Escherichia coli DH5α	F′ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17*(r_K^- m_K^+) λ^-	34
Plasmids		
pUK21	Cloning vector	37
pRHB138	Source of *A. vinelandii* Δ*nifA*::Sp allele	14
pRHB142	Partial construction of *A. vinelandii* Δ*nifL* allele	This study
pRHB143	Source of *A. vinelandii* Δ*nifL* allele	This study

strain AV2 (Δ*nifA*::Sp), and diazotrophic extransformants were recovered and further analyzed for the introduction of the allele containing an intact copy of *nifA*, the in-frame deletion of *nifL*, and the release of the Sp marker of the Δ*nifA*::Sp mutant allele. The genotypes of the strains were confirmed by PCR amplification of the mutated locus.

For an analysis of the effect of *nifA* and *nifL* mutations on *nif* gene expression, reporter strains bearing a transcriptional fusion between the *nifHDK* promoter, the *E. coli lacZ* gene, and the Δ*nifA*::Sp (14) or the Δ*nifL* mutation were obtained. For the latter one, the nondiazotrophic Δ*nifA*::Sp $nifH_p$-*lacZ* strain (14) was reverted back to a diazotrophic strain

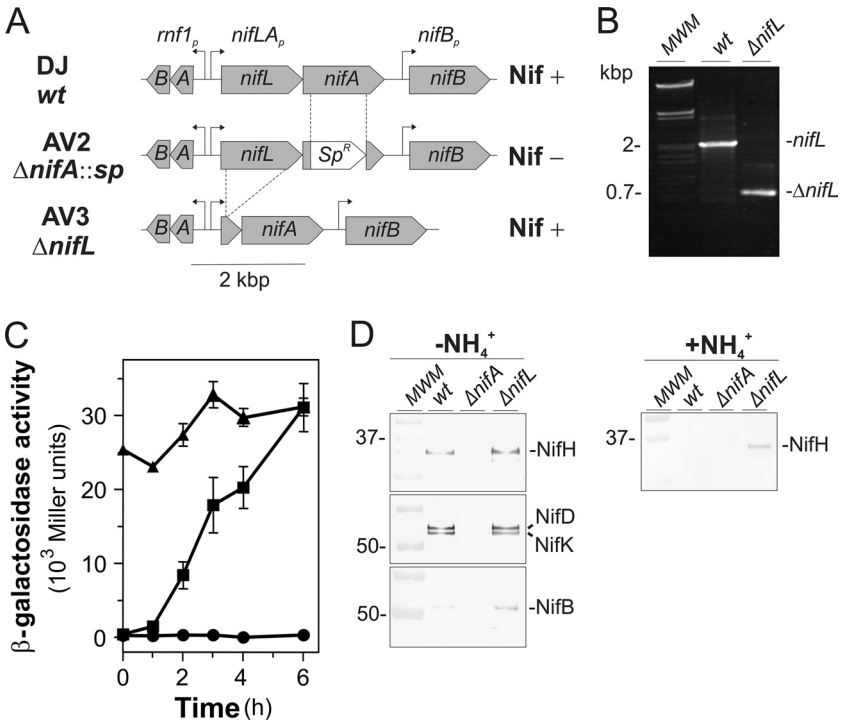

FIG 1 Isolation of *Azotobacter vinelandii* mutant strains with unregulated *nif* gene expression. (A) Physical map of the genetic loci of *A. vinelandii* and derivative *nifLA* strains. (B) PCR analysis of the *nifL* locus. (C) β-Galactosidase activity of *A. vinelandii* reporter strains bearing a transcriptional fusion between the *nifHDK* promoter and the *Escherichia coli lacZ* gene in the wt (squares), Δ*nifA*::Sp (circles), or Δ*nifL* (triangles) genetic background. Each data point represents the mean and standard deviation (SD) from two independent experiments. (D) Immunoblots of Nif proteins from the indicated *A. vinelandii* strains cultivated in the presence of ammonium or transferred into ammonium-free medium for 4 h. MWM, molecular weight marker (in thousands).

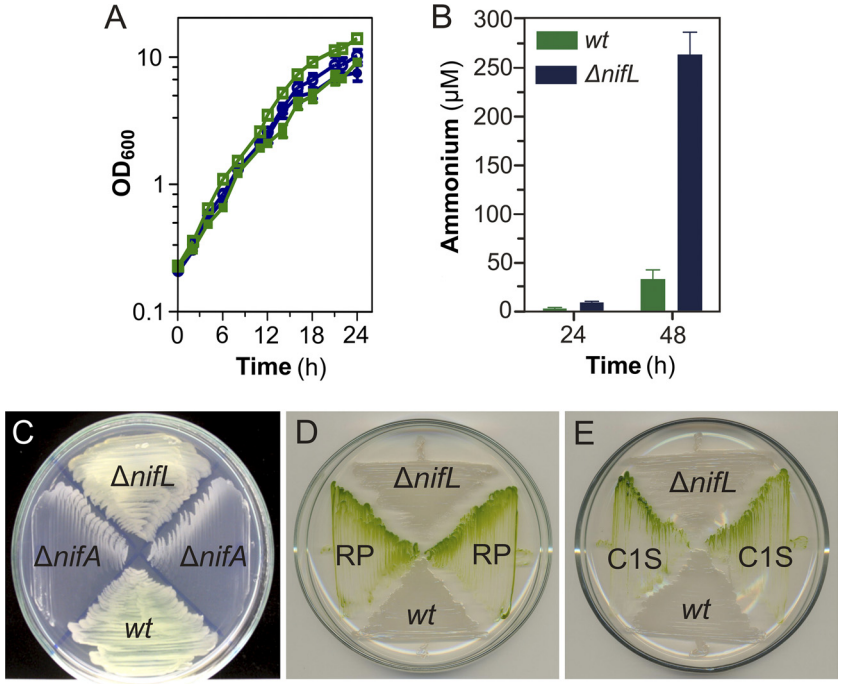

FIG 2 Ammonium excretion properties of an *Azotobacter vinelandii* Δ*nifL* mutant strain. (A) Growth curves of the *A. vinelandii* wt (green squares) and Δ*nifL* (blue circles) strains under diazotrophic (full symbols) or nondiazotrophic (open symbols) growth conditions. OD_{600}, optical density at 600 nm. (B) Ammonium accumulation in the spent medium of *A. vinelandii* strains at different time points. (C to E) Coculture in proximity of *A. vinelandii* wt and Δ*nifL* strains on solid Burk's modified medium lacking a source of nitrogen other than air with the nondiazotrophic *A. vinelandii* Δ*nifA* strain (C) or the eukaryotic microalga *Chlorella sorokiniana* strain RP (D) or *Scenedesmus obliquus* strain C1S (E). Each data point represents the mean and SD from two (A) or six (B) independent experiments. When not visible, error bars fall within the symbols or bars.

by transformation with linearized plasmid pRHB143, and further confirmation of the genotype was done by PCR analysis.

Lipid methods. Microalgal cells were induced for neutral lipid accumulation by N deprivation. Lipids were basically extracted according methods described previously by Bligh and Dyer (6), by vigorously vortexing the cells in a 1:2:2 mixture of water (containing 1 M NaCl)-methanol-chloroform and glass beads. Extraction from each cell pellet was repeated three times, and the pellet was evaporated as a single sample. Nile red staining of neutral lipid droplets (9) was used to monitor neutral lipid accumulation by fluorescence microscopy (Nikon Eclipse E600) after incubation in 20% dimethyl sulfoxide for 30 min in the dark.

Miscellaneous methods. β-Galactosidase assays were conducted as described previously (14), using SDS-chloroform-permeabilized bacterial cells and *o*-nitrophenyl-β-D-galactopyranoside as a substrate. The amount of released *o*-nitrophenol was determined spectrophotometrically at 420 nm. Immunoblotting for NifH, NifDK, and NifB using specific immune sera was also described previously (15). In brief, for NifDK and NifB, 10 μg total proteins was separated on 10% polyacrylamide–SDS-containing denaturing gels, while 12% polyacrylamide gels were used for NifH. Proteins were blotted onto nitrocellulose filters by using a semidry transblot device (Bio-Rad), and specific polypeptides were exposed with rabbit polyclonal antibodies raised against highly purified proteins from *A. vinelandii* and secondary anti-IgG antibodies coupled to alkaline phosphatase for the nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate colorimetric development of the signals.

Determinations of ammonium concentrations were carried out by use of an indophenol method essentially as reported previously (4). Forty microliters of phenol solution (100 g $liter^{-1}$ in 95% ethanol), 40 μl of sodium nitroprusside (5 g $liter^{-1}$), and 100 μl sodium hypochlorite (0.1 M in 200 g $liter^{-1}$ sodium citrate and 10 $liter^{-1}$ sodium hydroxide) were added to 1-ml ammonium-containing samples. The mixtures were incubated at room temperature for 30 min, and the ammonium concentration was determined spectrophotometrically at 660 nm using a standard curve of ammonium chloride as a reference.

Nucleic acid extraction and manipulation were conducted by using conventional protocols (37).

RESULTS

Isolation of an ammonium-excreting *Azotobacter vinelandii* mutant strain. With the double purpose of confirming that the contribution of *Azotobacter* spp. in natural or artificial associations with nondiazotrophic organisms is linked to the excretion of N fixation products and further improving that property by metabolic engineering, we have isolated a new *A. vinelandii* mutant strain with an almost complete deletion of the *nifL* gene (Fig. 1A and B). Unlike the wt strain, *A. vinelandii* Δ*nifL* cells expressed the structural genes for nitrogenase, *nifHDK*, constitutively, regardless of the availability of ammonium in the cultured medium (Fig. 1C). Also, the expressions of other *nif* genes were upregulated in Δ*nifL* strain AV3, as shown for the critical gene for FeMo-co synthesis, *nifB* (Fig. 1D). The parental strain of AV3, AV2 (Δ*nifA*::Sp), showed no derepression of *nif* genes upon the removal of ammonium from the medium (Fig. 1C and D). Similar results regarding *nif* gene expression were obtained previously by other researchers for different mutant alleles of either *nifL* or *nifA* (3). However, although a mutant strain with a similar Δ*nifL* mutant allele was obtained previously, it was not further analyzed because the strain was instable (5, 7). The *A. vinelandii* Δ*nifL* strain obtained in this work grew only slightly slower diazotrophically and excreted about 10-fold more ammonium than the wild-type strain (Fig. 2A and B). The pH remained practically unchanged during growth,

suggesting that it is unlikely that a rise in pH may have limited further growth and/or ammonium release.

***Azotobacter vinelandii* ammonium-excreting cells allowed diazotrophic cultures of nondiazotrophic microorganisms.** As a preliminary approach to study the properties of *A. vinelandii* and especially those of the ammonium-excreting derivative strain, a simple assay was used to visually score the growth of nondiazotrophic organisms at the expense of diffusible N-containing compounds produced by *A. vinelandii* or derivative strains on solidified Burk's modified medium containing sucrose as a source of carbon and energy for *A. vinelandii*. As shown in Fig. 2C to E, neither an *A. vinelandii* Δ*nifA*::Sp strain nor oleaginous eukaryotic microalgae of the genera *Chlorella* and *Scenedesmus* were able to grow on solid medium not supplemented with ammonium unless they were streaked in proximity to the *A. vinelandii* ammonium-excreting strain. In comparison, the wild-type bacterial strain was unable to sustain any noticeable growth of any nondiazotrophic microorganism analyzed in this work.

To analyze the effects of different initial bacterium-to-microalga ratios on the diazotrophic growth of the microalga *Pseudokirchneriella* sp., mixtures of serial dilutions of both microorganisms were spotted onto solidified Burk's modified medium containing sucrose.

We observed a more vigorous growth of the microalgae at higher dilutions of the bacterium (see Fig. S1 in the supplemental material), suggesting that initial bacterium-to-microalga ratios equal to or less than 1 were enough to sustain a considerable diazotrophic growth of the microalgae. On the other hand, for higher initial bacterium-to-microalga ratios in a medium optimized for bacterium proliferation, the bacteria outcompeted the microalgae, possibly by exhausting one or more nutrients before the onset of microalgal colonies. However, other allelopathic effects could not be ruled out.

Artificial symbiosis between photosynthetic microalgae and ammonium-excreting *Azotobacter*. Since N_2 fixation (either industrial or biological) is a high-energy-demand process (12, 17, 18, 23, 27), the main challenge for the use of an N_2-fixing bacterium as an N biofertilizer for the culturing of microalgae is whether microalgal photosynthetic exudates can drive N_2 fixation and ammonium excretion by the bacterium in either a natural or an artificial symbiosis.

As shown in Fig. 3, when inoculated at an arbitrary initial ratio of microalgal to bacterial cells of 1:1, the *A. vinelandii* wt strain had a very limited capacity to sustain microalgal growth in the absence of supplemented ammonium. Conversely, the ammonium-excreting strain did it much better and mimicked ammonium amendment up to a comparable concentration of 0.5 mM (Fig. 3A to C), especially when the microalga partner was *Chlorella sorokiniana* (Fig. 3B and C). Microalgal growth was N limited under these conditions and attained maximal neutral or total lipid accumulation in 4 to 5 days (not shown). This effect was observed by using two different culturing techniques: Erlenmeyer-type flasks with manual shaking twice a day to allow cyclic cell sedimentation and homemade air-lift-type photobioreactors. This beneficial effect of the *A. vinelandii* mutant strain as a sole source of nitrogen was observed for the eukaryotic microalgae *C. sorokiniana* strain RP, *Pseudokirchneriella* sp. strain C1D, and *S. obliquus* C1S, all isolated from freshwater ponds in Buenos Aires, Argentina (Fig. 2 and 3, and see Fig. S1 in the supplemental material). These results suggest that ammonium-excreting *A. vinelandii* strains may be regarded as generally beneficial for microalgae under conditions of an otherwise limiting supply of nitrogen.

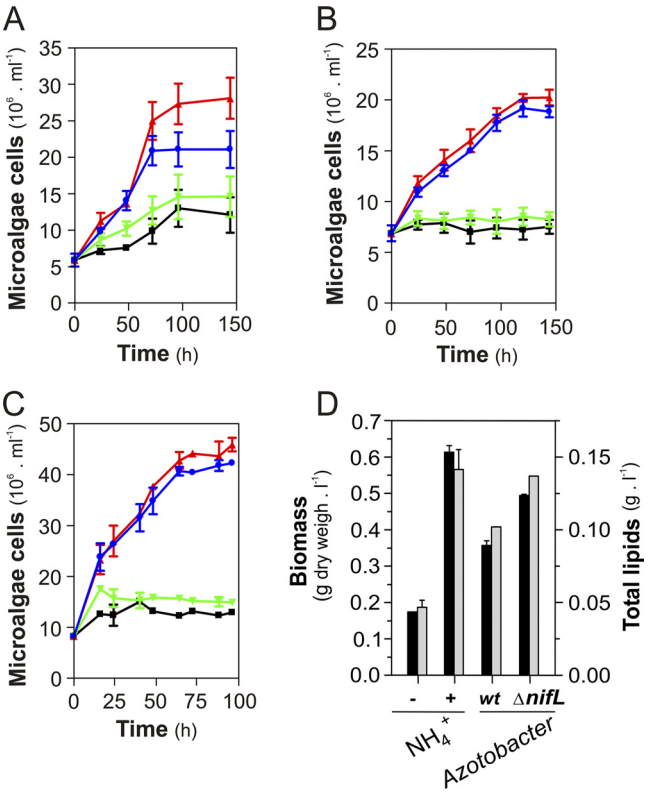

FIG 3 Growth of microalgae in coculture with *Azotobacter vinelandii* strains using air as the sole source of carbon and nitrogen. (A to C) The microalgae used were *Pseudokirchneriella* sp. strain C1D (A) and *C. sorokiniana* strain RP (B and C) in BG11 medium lacking any source of nitrogen other than air (black circles), with 0.5 mM ammonium chloride (red triangles), with the *A. vinelandii* wt strain (green triangles), or with the *A. vinelandii* Δ*nifL* strain (blue circles). Cultures were incubated in Erlenmeyer-type flasks with manual shaking (about 10 strokes each time) twice a day (A and B) or in homemade air-lift-type photobioreactors bubbled with air (C). Each data point represents the mean and SD from four independent experiments. (D) Microalgal biomass and lipid yields after coculture with *A. vinelandii* strains. Cultures were set as described above for panel C, using the microalga *C. sorokiniana* strain RP. The amounts of biomass (black bars) or lipids (gray bars) were determined after 4 days of culture. Each data point represents the mean and SD from two independent experiments. When not visible, error bars fall within the bars.

Both biomass yield and total lipid accumulation on the fifth day of coculture, when *C. sorokiniana* tends to reach the stationary phase of growth, roughly mirrored changes in the number of microalgal cells (Fig. 3D). At this time, the oil content of the biomass approached 25 to 30% of the dry biomass (wt/wt) under every culture condition analyzed. At earlier stages of growth, the addition of the ammonium-excreting bacterium exerted a an effect similar to that of ammonium in delaying the accumulation of neutral lipids, as analyzed by Nile red fluorescence (not shown).

Under the experimental conditions used, the *Pseudokirchneriella* strain C1D growth-promoting activity of the *A. vinelandii* ammonium-excreting strain appeared not to be limited by energy, since the addition of glucose did not result in a further stimulation of alga growth (Fig. 4). Conversely, alga proliferation remained limited by nitrogen, since ammonium concentrations higher than 0.5 mM decreased the doubling time and produced a higher biomass yield for the strains analyzed in axenic cultures (not shown).

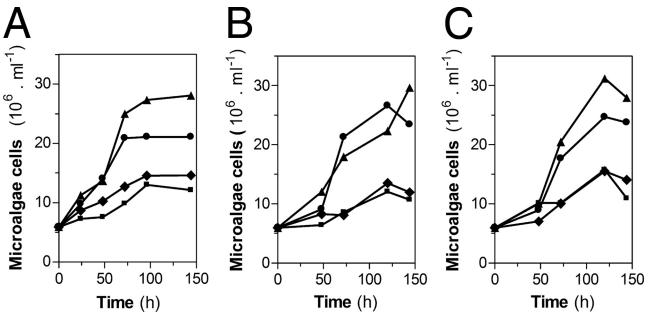

FIG 4 Growth curves of microalgae in coculture with *Azotobacter vinelandii* strains using glucose as a carbon source and air as the sole source of nitrogen. (A to C) The microalga used was *Pseudokirchneriella* sp. strain C1D in the absence (A) or presence of 0.1 mM (B) or 0.5 mM (C) glucose in BG11 medium lacking any source of nitrogen other than air (squares), with 0.5 mM ammonium chloride (triangles), with the *A. vinelandii* wt strain (diamonds), or with the *A. vinelandii* Δ*nifL* strain (circles). Cultures were incubated in Erlenmeyer-type flasks with manual shaking (about 10 strokes each time) twice a day. Data in panel A are the same as those in Fig. 3A to facilitate direct comparisons and represent the means of data from four independent experiments. Data from panels B and C correspond to single determinations of representative experiments.

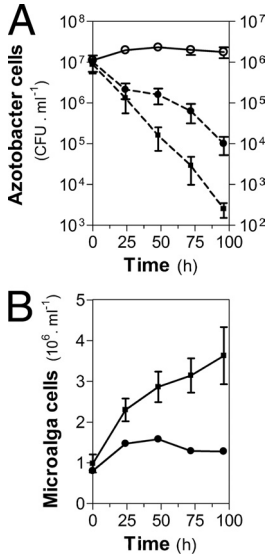

FIG 5 *Azotobacter vinelandii* and microalga growth curves for the third consecutive coculture using air as the sole source of carbon and nitrogen. Cultures were performed as described in the legend of Fig. 3C, using the microalga *C. sorokiniana* strain RP. (A) Viable cells (CFU) of the *A. vinelandii* wt (dashed line and full squares) or Δ*nifL* (dashed line and full circles) strain during the first coculture (left axis) or the Δ*nifL* strain (full line and open circles) after the third consecutive coculture (right axis). (B) Microalga cell counts during the third consecutive coculture with the *A. vinelandii* Δ*nifL* strain (full squares) or axenically (full circles). See the text for details. Each data point represents the mean and SD from four independent experiments, except for a representative single determination for the axenic culture of the microalgae shown in panel B.

Successive cocultivation with microalgae ameliorates loss of viability of *Azotobacter* in mineral medium. Unlike the microalgae, neither the wt nor the ammonium-excreting bacterial strain increased its cell numbers during coculture. Actually, numbers of bacterial cells counted by an optical microscope tended to decrease slowly (not shown). However, while the number of viable cells determined by plate counting decreased dramatically for the wt strain, it remained more stable for the ammonium-excreting strain (Fig. 5A), suggesting that it is engaged in a more robust symbiotic relationship with microalgae than the wt strain when air is the sole source of carbon and nitrogen.

To further investigate if the stability of the symbiotic relationship could be improved, we set symbiotic cultures onto solid medium containing carbon but lacking nitrogen and then transferred an inoculum from these cultures into liquid medium lacking both carbon and nitrogen for 7 days and subcultivated an aliquot of this inoculum in fresh medium lacking both nutrients to analyze the growths of both types of cells. It was observed that only a few generations of coculturing were enough to obtain an improved symbiotic inoculum in relation to cells that met each other for the first time during artificial symbiosis (Fig. 5B).

On the other hand, since mostly microalgal cells proliferated during coculture, and considering the large difference in the cell volume (ca. 100-fold, modeled for spherical cells), the resulting biomass resembles that of an axenic culture of microalgae.

DISCUSSION

The large-scale production of biofuels from microalgae or other feedstock may demand unsustainable inputs of nitrogen fertilizers, requiring huge inputs of fossil energy to be produced, in addition to a secondary effect on GHG emission after application (11, 41, 44). Thus, among other alternatives, many researchers have proposed an intensification of the exploitation of biological nitrogen fixation for the low-input cultivation of either plants or microalgae, including the use of metabolic engineering to introduce the nitrogen fixation pathway into crops or for the addition of valuable metabolic pathways to nitrogen-fixing cyanobacteria or other selected hosts (11, 19, 22, 24).

The coupling of wastewater treatment to the large-scale cultivation of microalgae for biofuel production and/or other purposes has been repeatedly proposed during the last decades (32), and its relevance has been highlighted by a more recent life cycle analysis (28).

In this work, an alternative and/or complementary approach was considered for the diazotrophic cultivation of oleaginous microalgae based on the assembling of microbial synthetic communities. Basically, the approach makes use of organisms specialized for aerobic nitrogen fixation (*A. vinelandii*) and photosynthesis-derived oil accumulation (microalgae), under the assumption that an enhancement of already efficient complex pathways by genetic engineering could be simpler than introducing whole pathways (including the extended regulatory networks) into suboptimal hosts (selected for other outstanding capabilities). As a proof of concept, this work demonstrates the feasibility of the approach by the assembly of an artificial symbiosis between an efficient nitrogen fixer (even at supra-ambient O_2 concentrations) genetically manipulated for high-level ammonium excretion and a fast-growing oleaginous microalga. Results presented in this work together with data from previous reports (29, 33) suggest that *Azotobacter* spp. might be a robust platform for this strategy using a variety of microalgae.

An important aspect of the approach is that the energy required for N_2 fixation appears to be provided by microalgal exudates. As recently shown for *Chlorella* sp. cells cultivated in photobioreactors, exudates may represent up to 17% of the total

assimilated CO_2 (depending on the culture conditions) (26). If photosynthetic oil is to be collected form cells (as most reports have proposed), microalgal exudates can constitute an energy loss from the system and a rich medium for the proliferation of undesirable organisms (26). Moreover, from an energy-saving perspective, it is also noteworthy, as suggested by results shown in Fig. 5, that the potential outcome of a more stable and robust symbiosis may also contribute to significantly reduce (or eliminate) the need for N fertilizers for microalgae and sugars for bacterial inoculum preparations. Similar results for the stabilization of the artificial symbiosis between *Azotobacter* sp. and *Chlamydomonas* sp. for an extended period of time have been reported, where the internalization of *Azotobacter* sp. into microalgal cells was described (33). Although the growths of both types of cells, especially that of the microalgae, were less robust than when cells were previously cultivated in specific carbon- or nitrogen-containing media, this work suggests that it may be possible to developed more stable symbiotic relationships after accelerated coevolution triggered by a metabolic engineering intervention.

Despite the artificial nature of the symbiotic relationship between microalgae and *A. vinelandii*, the use of this bacterium appears to be advantageous as a working model for basic research, with some potential for biotechnological applications as well. Apart from the exceptional ability of this genus for efficient biological nitrogen fixation at supra-ambient concentrations of oxygen, a vast amount of genetic and biochemical information is available for this bacterium (23, 39). This fact is expected to promote further research into genetic improvements of different aspects of *A. vinelandii* metabolism toward the promotion of growth of plants and microalgae.

The phenotype of *nifL* mutations in *A. vinelandii*, especially the trait of ammonium excretion, has been intriguing. Higher-level ammonium excretion by other *nifL* mutants of *A. vinelandii*, such as MV376 and others, was reported previously (3). Although the potential usefulness of these mutants as substitutes for nitrogen fertilizers for crop plants was proposed previously, a direct demonstration of such an application is lacking, to the best of our knowledge. Part of the bulk ammonium released by strain MV376 appeared to be due to a modification of *nifA* expression by a polar effect (3, 5, 7). Strains bearing an apparently nonpolar partial deletion, such as MV440 (5), could not be recovered from storage for further research, nor could they be isolated again from the same vectors and parental strains. Attempts to isolate a more complete deletion (Phe27 to Ile413) were equally unsuccessful, for unknown reasons (7). The identification of a positive transcription-regulatory element within the coding region of *nifL* that is likely to be required for the full activity of the *nifLA* promoter of *A. vinelandii* (31) might add a level of complexity to the regulation of *nif* gene expression comprising NifLA activity. While the parental strain of all these *nifL* mutants was strain UW, mutant strain AV3 isolated in this work is a derivative of strain DJ, a high-frequency-transforming variant of UW, the complete genome sequence of which has been determined (39). Whether subtle differences in genetic background also contribute to the level of ammonium released is currently unknown. Nevertheless, although beyond the scope of the present study, the isolation of an apparently stable mutant strain with an even more complete in-frame deletion of *nifL* from amino acids Ile36 to Ile508, presenting a level of NifA activity high enough to sustain higher levels of *nifHDK* expression and NifH, NifD, NifK, and NifB polypeptides than the wild-type strain (Fig. 1), may contribute to future research aimed at either understanding the structure and regulation of NifL or improving the production of ammonium by biological nitrogen fixation.

In addition, genetic and biochemical information on *A. vinelandii* is available to address other complementary mutations to boost ammonium excretion by altering ammonium assimilation into amino acids (12) and the production of hormone-like substances, antimicrobials, and phosphate volatilization (1). Furthermore, the *Azotobacter* platform might also allow the ectopic expression of genes for selected traits from less characterized bacteria naturally associated with microalgae (43).

Biofertilization as a nitrogen source might provide an alternative to the use of wastewater or residues from different industries as sources of nitrogen. This alternative might be especially interesting in those cases where the geographical (or temporal) segregation of points of production and use of wastewater may constrain the sustainability of the process or when the presence of toxic contaminants, normally occurring in domestic or industrial residues, limits the choice of microalgal species according to tolerance issues.

This work provides a proof of concept that artificial symbiosis may be considered an alternative strategy for the low-N-intensive cultivation of microalgae for the sustainable production of next-generation biofuels and other bioproducts.

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