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Original article

Involvement of indole-3-acetic acid produced by *Azospirillum brasilense* in accumulating intracellular ammonium in *Chlorella vulgaris*

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This study is dedicated to the memory of the Israeli soil microbiologist Prof. Yigal Henis (1926–2010) of the Faculty of Agriculture, The Hebrew University of Jerusalem in Rehovot, Israel, one of the pioneers in research on *Azospirillum*.

Abstract

Accumulation of intracellular ammonium and activities of the enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were measured when the microalgae *Chlorella vulgaris* was immobilized in alginate with either of two wild type strains of *Azospirillum brasilense* or their corresponding indole-3-acetic acid (IAA)-attenuated mutants. After 48 h of immobilization, both wild types induced higher levels of intracellular ammonium in the microalgae than their respective mutants; the more IAA produced, the higher the intracellular ammonium accumulated. Accumulation of intracellular ammonium in the cells of *C. vulgaris* followed application of four levels of exogenous IAA reported for *A. brasilense* and its IAA-attenuated mutants, which had a similar pattern for the first 24 h. This effect was transient and disappeared after 48 h of incubation. Immobilization of *C. vulgaris* with any bacteria strain induced higher GS activity. The bacterial strains also had GS activity, comparable to the activity detected in *C. vulgaris*, but weaker than when immobilized with the bacteria. When net activity was calculated, the wild type always induced higher GS activity than IAA-attenuated mutants. GDH activity in most microalgae/bacteria interactions resembled GS activity. When complementing IAA-attenuated mutants with exogenous IAA, GS activity in co-immobilized cultures matched those of the wild type *A. brasilense* immobilized with the microalga. Similarity occurred when the net GS activity was measured, and was higher with greater quantities of exogenous IAA. It is proposed that IAA produced by *A. brasilense* is involved in ammonium uptake and later assimilation by *C. vulgaris*.

Keywords: Ammonium uptake; *Azospirillum*; *Chlorella*; Indole-3-acetic acid

Note: The term ammonium is used to denote NH_3 and NH_4^+; chemical symbols are used when specificity is required.

1. Introduction

The genus *Chlorella* is a commonly studied unicellular, non-motile, green microalga inhabiting aquatic environments. Its main usefulness lies in numerous biotechnological applications, such as pigments for the food industry, products for the health food market, human and animal foodstuffs, applications for wastewater treatments and, potentially, as biofuels [1–3]. *Azospirillum* spp. are useful as plant growth-promoting bacteria that enhance the performance of many crops and environmental plants and algal species, including *Chlorella* spp [4,5]. This enhancement occurs via numerous simultaneously operating mechanisms, in tandem or cascading, in a process termed "Multiple Mechanisms Theory" [6]. One of the main mechanisms is production of large quantities of the phytohormone

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indole-3-acetic acid (IAA). IAA either directly affects the growth of both higher plants [7,8] and *Chlorella* spp [9]. or serves as a general signaling molecule in plant–bacteria interactions [10,11]. Green microalgae (like *Chlorella*) do not produce high levels of exogenous auxin-like compounds [12,13]. While the effect of the hormone brassinosteroid in *Chlorella vulgaris* is very well documented [14–17], only limited information is available on metabolic and growth processes induced by auxins in microalgae [4,18–20]. The synergetic relationships between brassinosteroid, IAA, and abscisic acid in the cell population, regulation of levels of phytohormones and accumulation of metabolites have also been demonstrated [21,22]).

Ammonium is one of the optimal nitrogen sources for *Chlorella* spp [23,24]. and requires less energy for assimilation because of the reduced state of the nitrogen [25]. Assimilation occurs via two metabolic pathways that involve the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT) (major route [26]) or via glutamate dehydrogenase (GDH, auxiliary route [27]). *Azospirillum brasilense* is also known to enhance ammonium uptake by the microalgae from a medium [28–30] and activities of GS and GDH in *C. vulgaris* [31].

Immobilization of microorganisms in polymers and beads prepared from polymers for production of various products and environmental and agricultural research is well known, and has given rise to increasing applications in the last two decades [1,32]. Immobilizing microalgae is also a common approach in several bioremediation applications [2]. Furthermore, immobilization of two microorganisms within the same matrix significantly reduces mobility [33,34], forces interactions and induces effects of one microorganism upon the other, as is the case for *A. brasilense* and *Chlorella* spp. [4,35–37] and *Chlorella* spp. with other bacteria [4,38]. The natural alginate polymer is the most commonly used for immobilizing microorganisms [39,40]. Immobilization of *Chlorella* spp. with *Azospirillum* spp. in alginate beads was also proposed as a model for studying plant–bacteria interactions [41] in a so-called "synthetic mutualism" or "synthetic symbiosis".

Considering all of the above, our hypothesis was that IAA produced by *A. brasilense* would enhance GS and GDH activity and increase ammonium uptake and assimilation in the microalgae. To test this hypothesis: (1) we measured the effect of two IAA-attenuated mutants of *A. brasilense* on intracellular ammonium accumulation in microalgal cells vs. induction of accumulation of intracellular ammonium by their intact parental wild-type strains; (2) the same was measured for GS and GDH activity; and (3) we complemented IAA-attenuated strains with exogenous IAA to reach the same level of IAA as in parental strains, and measured the effects of the combination (bacteria–exogenous IAA) on ammonium accumulation and the main enzymatic activity (GS) in comparison to the parental strains.

2. Materials and methods

2.1. Microorganisms and culture conditions

C. vulgaris Beijerinck (UTEX 2714, University of Texas, Austin, TX) and wild type *A. brasilense* Sp245 (EMBRAPA, Rio de Janeiro, Brazil) and *A. brasilense* Sp6 [42] and their IAA-attenuated mutants *A. brasilense* FAJ0009 [43] and *A. brasilense* SpM7918 [42] were used in all experiments. *C. vulgaris* was cultured for 6 days in mineral growth media (C30) [44] at 150 rpm in an orbital shaker, 28 ± 1 °C, and 80 μmoles photon m^{-2} s^{-1}. *A. brasilense* strains was cultured for 17 h in nutrient broth (NB; #N7519, Fluka, Sigma–Aldrich, St. Louis, MO, USA) at 35 ± 2 °C and 140 rpm. Cultures of the two mutants were supplemented with 25 μg L^{-1} kanamycin (#60615, Sigma–Aldrich).

2.2. Immobilization of microalgae and bacteria in alginate beads

Microorganisms were immobilized using the method described in de-Bashan et al. [29], where 40 mL of *C. vulgaris* culture (6.0×10^6 cells•mL^{-1}) was mixed with 160 mL of a sterile, 2000 cP 3% alginate solution (alginate mixed at 14,000 cP and 3500 cP), and stirred for 15 min. Using an automatic bead maker, this mixture was dropped into a 2% $CaCl_2$ solution under slow stirring [2]. The beads were stabilized for 1 h at 28 ± 1 °C and washed in sterile saline solution. *A. brasilense* (approximately 1.0×10^9 CFU mL^{-1}) was immobilized similarly. Immobilization normally reduces the number of organisms in the beads; therefore, a second incubation step was necessary (10% NB overnight) [45]. To combine both species in the same beads, a similar procedure was performed using 20 mL of each culture in a mixture (40 mL total). After the second incubation, the beads were rinsed three times in saline solution (0.85% NaCl) placed in 500 mL Erlenmeyer flasks (40 g of beads per flask) containing 200 mL of synthetic growth medium containing 25 mg L^{-1} NH_4Cl (SGM; described in de-Bashan et al. [37]). In treatments to induce IAA production, 200 μg L^{-1} tryptophan were added, an essential ingredient for IAA production in *Azospirillum* spp [6]. The flasks were placed on an orbital shaker for 2 days under the same conditions for culturing *Chlorella*.

2.3. Counting microorganisms after treatment

After each experiment, 48 h total incubation time, beads containing microorganisms were dissolved in 4% sodium bicarbonate solution at room temperature (~28 °C) for ~30 min. Then the microorganisms were counted. *C. vulgaris* was counted under a light microscope with a Neubauer hemocytometer [4] connected to an image analyzer (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD). *A. brasilense* was counted under a fluorescent microscope using fluorescein diacetate stain (#F7378, Sigma–Aldrich) [46].

Growth rate (μ) was defined by: $\mu = (\ln Nt_1 - \ln Nt_0)/(t_1 - t_0)$, where Nt_1 is the number of cells at sampling time and Nt_0 is the number of cells at the beginning of the experiment [47]. This was determined by taking 10 samples per treatment (5 replicates per treatment) after 24 and 48 h.

2.4. Application of exogenous IAA to cultures of C. vulgaris

Freshly prepared IAA (#I2886-25G, Sigma–Aldrich), dissolved in absolute ethanol and kept in the dark, was used. The concentration of exogenous IAA (ng μL^{-1}) corresponded to 44.53 for *A. brasilense* Sp245, 51.18 for *A. brasilense* Sp 6, 6.96 for *A. brasilense* FAJ0009 and 15.53 for *A. brasilense* SpM7918 [9]. The SGM medium used in this study has a strong buffer capacity. Additions of these quantities of IAA did not change the pH of the medium, which remained stable during the experiment.

2.5. Analytical methods

2.5.1. Determination of ammonium

In cultures, ammonium was analyzed colorimetrically by the phenate method [48] adapted to microplates [49].

For intracellular ammonium, we used the method described by Lin and Kao [50], with modifications: After dissolving the beads, the cells were rinsed 10 times (0.85% NaCl, 4500 × *g*) to eliminate traces of tryptophan that potentially interfere with detecting ammonium. The cells were stored at −80 °C. Frozen samples were macerated with pestle and mortar using acid-washed glass beads. To the macerate, 20 mL 0.03 mM H_2SO_4 (at pH 3.5) was added and the mixture was macerated again. The suspensions were centrifuged at 19,500× *g* for 15 min at 4 °C. Ammonium was measured in the supernatant, as described earlier. Since there is no practical way to measure the contribution of each microorganism partner in the immobilized assemblage, intracellular ammonium of microalgae immobilized with bacteria was calculated by subtracting the intracellular ammonium produced by the microalgae or a bacteria strain when cultivated alone from the intracellular ammonium measured in the mixed treatment. Based on preliminary tests, we assumed that the physiological behavior of free cells and immobilized cells of a single microorganism is similar. Calculation per cell was done by dividing these values by the number of developing cells at specific sampling times. In contrast to microelectrode measurements of ion transport in cells [51], this method cannot differentiate between vacuolar and cytoplasmatic ammonium. Reported ammonium levels are the sum of both.

2.5.2. Protein

Protein content of cell extracts was analyzed by dye-binding [52] using a protein analysis kit (BioRad Laboratories, Hercules, CA, USA). The reaction was quantified in a microplate reader at 595 nm (VersaMax, Molecular Devices, Sunnyvale, CA, USA). The net protein content of the microalgae during association is the total protein of the association minus the protein content of the bacteria when immobilized alone.

2.5.3. Enzymatic analysis

Extraction was as follows. Every 24 h, 13.3 g of beads were dissolved. Cells were harvested and washed twice with 0.85% saline solution at 4 °C at 6000 × *g* and frozen at −80 °C. Frozen samples were macerated with mortar and pestle in 1 mL cold 100 mM HEPES buffer (2-[4-(2-hydroxyethyl) piperazine-1-yl]ethanesulfonic acid) at pH 7.5 (#H3375, Sigma–Aldrich) and containing 10 mM DTT (dithiothreitol), using acid-washed glass beads. Extracts of the cells were prepared as described below for measuring intracellular ammonium. The suspensions were centrifuged at 10,500× *g* for 30 min at 4 °C [53] and the pellets were discarded. The supernatant was transferred to clean microtubes and served as the source of the enzyme.

Glutamate dehydrogenase (GDH) activity was measured in the aminating direction and determined by the decline in the rate of absorbance at 340 nm that results from oxidation of NADH in the presence of α-ketoglutarate and ammonium ions. This assay follows the method described by Frieden [54] modified for microplate analysis by de-Bashan et al. [31].

Glutamine synthetase (GS) activity was measured by the method described by Shapiro and Stadtman [55] and modified by de-Bashan et al. [31], as adapted for microplate analysis. Because there is no practical way to measure the contribution of the microalga and bacterium partners in an immobilized assemblage to GS and GDH enzymatic activity, as both have these enzymes, net activity was calculated by subtracting the activity detected in each control treatment (each partner alone) from the activity detected in the co-immobilized treatment.

2.5.4. Quantification of IAA-equivalent molecules

The IAA that was measured in our study is the exogenous IAA-equivalent molecules liberated by the microorganisms into the growth medium. For qualitative and quantitative determination of IAA-equivalent molecules, the colorimetric Salkowski's assay was used [56]. Salkowski's assay, a common assay for indole-type molecules including auxins [57], has been successfully used for *Azospirillum* [9,58] and IAA-equivalent molecular quantification in *Pseudomonas putida* [59]. Its limitations notwithstanding, Salkovsky's method can easily be performed under common laboratory conditions and is far handier for a routine quality check in experiments than the specific HPLC method recommended for absolute determination of IAA. This approach is especially useful when the absolute quantities of IAA production by these microorganisms had been previously quantified by the HPLC method [9], and the objectives of our study were to measure other growth parameters and concentrations of synthetic IAA that was added to the cultures.

2.6. Experimental design and statistical analysis

Several variants of microorganisms were used depending on the specific experiment. Each combination is listed in the respective Results subsections: (1) *Azospirillum* alone, (2) *Chlorella* alone, (3) immobilization of *Chlorella* with *Azospirillum*, (4) immobilization of each of the two mutants of *Azospirillum* and (5) immobilization of *C. vulgaris* with each of the mutants. Beads without microorganisms were used as a control. Because growth of *C. vulgaris* fluctuated when entire

experiments were repeated, as well as its protein content [30], *C. vulgaris* populations were counted in each experiment and protein content in every enzymatic experiment. The calculated values for growth and proteins were derived from specific experiments. Representative growth or protein quantity is presented here, but to save space, each population count or each protein determination in each experiment is not presented. Each experiment had five replicates where one Erlenmeyer flask served as a replicate. Each experiment was repeated at least three times. The average data of all experiments were used for statistical analysis by one-way ANOVA and Tukey's HSD post-hoc analysis at $p < 0.05$ or by Student's *t*-test at $p < 0.05$. Statistica 8.0 software was used (StatSoft, Tulsa, OK, USA).

3. Results

Comparing net accumulation of intracellular ammonium during the association induced by each wild-type *A. brasilense* and its IAA-attenuated mutants revealed that, after 24 h of incubation, only strain Sp6 induced a higher amount of intracellular ammonium than its respective mutant (SpM7918). The four IAA values that were evaluated correspond to the IAA produced by the mutant SpM7918 (15.53 ng μL^{-1}), and FAJ0009 (6.96 ng μL^{-1}), and wild type Sp6 (51.18 ng μL^{-1}), and Sp245 (44.53 ng μL^{-1}). However, after incubation for 48 h together, both wild types induced higher levels of intracellular ammonium than their respective mutants (Fig. 1a). When results of intracellular ammonium accumulation and potential IAA produced by each of these strains were compared, the more IAA that was produced, the higher the accumulation of intracellular ammonium (Fig. 2a). When intracellular accumulation of ammonium was calculated per cell, similar results were present (Fig. 1b).

The rate of growth of the microalgae at 48 h was higher for the wild types than their IAA-attenuated mutants, where variable growth effects were detected after 24 h. After initial vigorous growth (0–24 h), the growth rate significantly slowed down at 48 h, as was previously demonstrated for this microalga [60]. Yet the total population of cells at this time was greater. However, after 48 h, the microalgae co-immobilized with the wild-type strains had significantly higher growth rates than when co-immobilized with the corresponding IAA-attenuated mutants or when immobilized alone (Fig. 1c). The rate of growth of the microalgae was affected by the capacity of each strain of *A. brasilense* to produce IAA; the growth rate was faster when more IAA was produced (Fig. 2b).

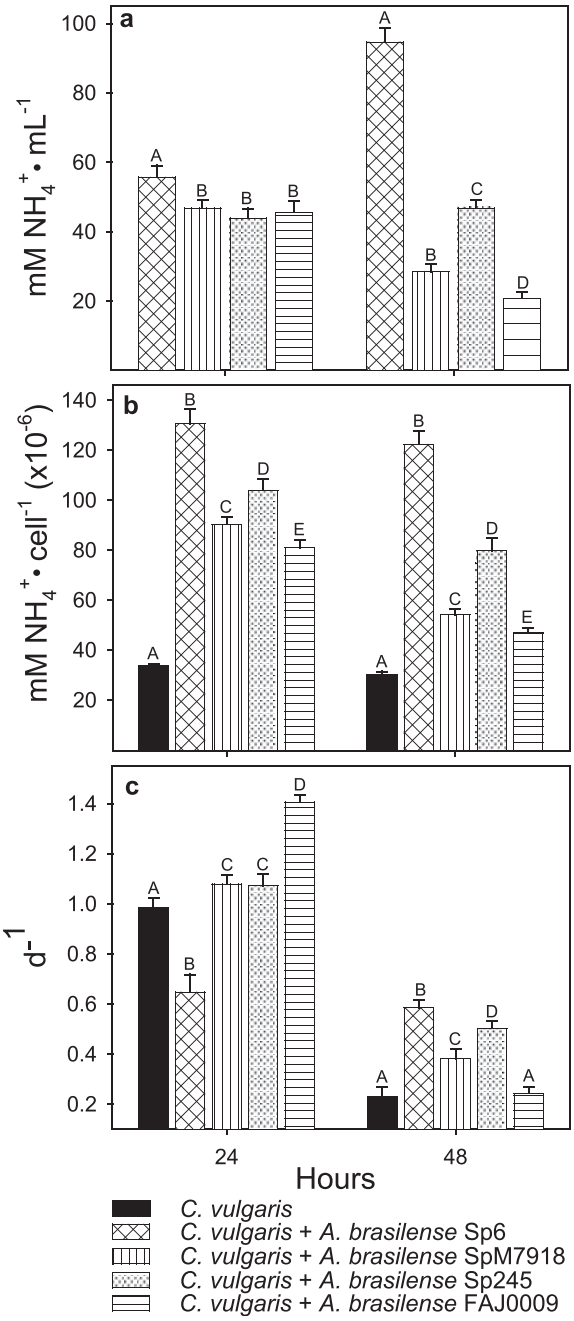

Fig. 1. Net accumulation of intracellular ammonium in *C. vulgaris* induced by *A. brasilense*. Accumulation per culture (a) or per cell (b). Rate of growth of the microalgae (c). Groups of 4 columns, in each subfigure separately, and denoted by a different letter, differ significantly by one-way ANOVA and Tukey's HSD post-hoc analysis at $p < 0.05$.

3.1. Effects of different levels of exogenous IAA on ammonium uptake and accumulation of intracellular ammonium by C. vulgaris

Application of four levels of exogenous IAA reported for *A. brasilense* and its IAA-attenuated mutants to cultures of *C. vulgaris* enhanced the growth of the population over 48 h (Fig. 3a), where enhanced growth induced by the lower levels of IAA (corresponding to the two mutants) was smaller than the wild type. Similarly, the growth rate of the microalga after 24 h increased when the IAA was increased. The growth rate after 48 h was lower, although the same pattern occurred (Fig. 3b). The level of ammonium uptake also increased compared to untreated *C. vulgaris* (Fig. 3c, d lower case letter analyses) and, after incubation for 24 h, was higher for levels of IAA corresponding to IAA production by the wild type *A. brasilense* (Fig. 3c, d, capital letter analyses).

Accumulation of intracellular ammonium in *C. vulgaris* cells followed a similar pattern for the first 24 h. The higher the level of applied exogenous IAA, the greater the amount of intracellular ammonium detected. This effect was transient and disappeared after incubation for 48 h (Fig. 3e). When accumulation of intracellular ammonium was calculated on a per-cell basis of the microalga after 24 h, intracellular ammonium was always higher when more IAA was applied. The quantity of IAA corresponded to the production of IAA by the wild type, yielding more intracellular ammonium than the production of IAA by the IAA-attenuated mutant (compare pairs in Fig. 3f). The effect significantly declined after 48 h.

3.2. Effects of different strains of A. brasilense on glutamine synthetase and glutamate dehydrogenase activity in C. vulgaris

In co-immobilized treatments containing both microorganisms at the culture level, GS activity varied according to the combination of the wild-type or mutant that was used. While GS activity induced by Sp6 vs. SpM was similar (Fig. 4a), in the other combination, Sp245 vs. FAJ0009, the wild type was always higher (Fig. 4b). In most treatments, inoculating *C. vulgaris* with a bacterium strain induced higher GS activity; the bacterial strains also had GS activity comparable to the activity detected in *C. vulgaris*, but lower than in co-immobilized treatments (Fig. 4a, b). When net activity was calculated, the wild type consistently induced higher GS activity in *Chlorella* than the IAA-attenuated mutants (Fig. 4c).

Under these conditions, GDH activity in most microalgae/bacteria combinations resembled those of GS (Fig. 5a, b), except after 24 h in Sp6 vs. SpM, where the mutant induced higher activity (Fig. 5a). Net GDH activity, similar to GS, was always higher in the wild type compared to its mutant (Fig. 5c). When enzymatic activities of GS and GDH were compared to the potential capacity of the strains to produce IAA, direct correlations occurred with both enzymes (Fig. 4d and 5d). In all cases, the concentration of protein in *Chlorella* immobilized with either wild-type bacteria was higher than with their respective mutants (Fig. S1).

3.3. Effect of complementing IAA-attenuated mutants with exogenous IAA on GS activity and accumulation of intracellular ammonium

In these complementation experiments, two approaches were used. One consisted of cultivating the microalgae immobilized with IAA-attenuated mutants of *A. brasilense* and supplementing the culture with exogenous IAA to reach the IAA quantity produced by the wild type. In the second approach, we cultivated the microalgae with exogenous IAA corresponding to the quantities of either the IAA produced by the wild type or by the corresponding mutant.

When complementing mutant FAJ0009 with exogenous IAA, GS activity in the microalgae immobilized with mutant bacteria matched those of wild type *A. brasilense* Sp245

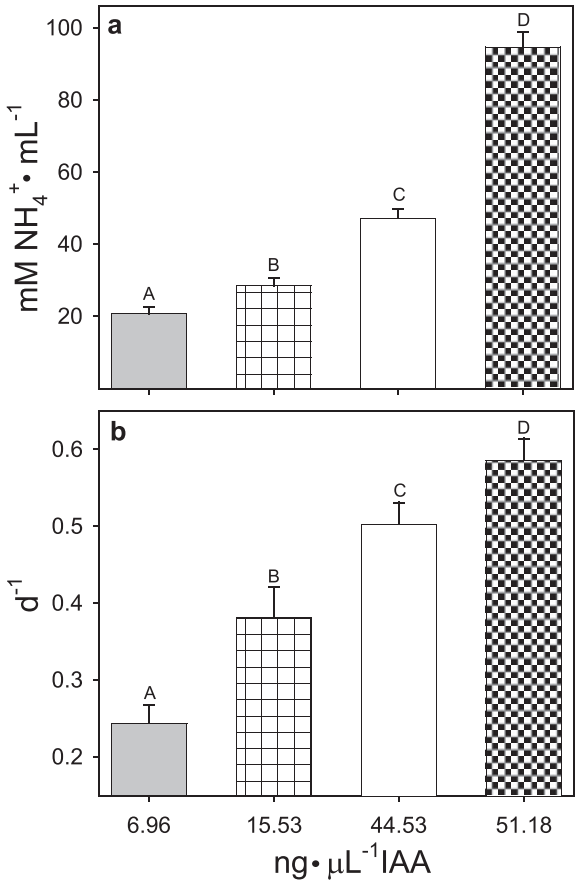

Fig. 2. Calculation comparing intracellular ammonium accumulation and the level of IAA produced by the four *Azospirillum brasilense* strains (a). Relationship between rate of growth and the level of IAA produced by each of the *A. brasilense* strains (b). The four IAA values evaluated correspond to IAA produced by mutant SpM7918 (15.53 ng μL^{-1}), and FAJ0009 (6.96 ng μL^{-1}), and wild type Sp6 (51.18 ng μL^{-1}) and Sp245 (44.53 ng μL^{-1}). Groups of 4 columns, in each subfigure separately, denoted by a different letter, differ significantly by one-way ANOVA and Tukey's HSD post-hoc analysis at $p < 0.05$.

immobilized with the microalgae (Fig. 6a). Similarly, adding exogenous IAA to cultures of *C. vulgaris* yielded the same pattern; the higher the amount of IAA supplied, the more the GS activity increased (Fig. 6b, c). The same pattern occurred when net GS activity was measured (Fig. 6d, e). Adding exogenous IAA to mutant FAJ0009 did not have an effect on intracellular ammonium accumulation after incubation for 48 h (Fig. 6f). GDH activities measured in complementation experiments were different in the two repetitions of the experiment, thus yielding less conclusive results than did GS activity (data not shown).

4. Discussion

Growth promotion of green microalgae *Chlorella* spp. induced by the microalgae growth-promoting bacteria (MGPB) *A. brasilense* was shown [4,41] and was attributed to formation of IAA by the bacteria [9]. Similarly, MGPB enhanced the capacity of the microalgae to uptake ammonium,

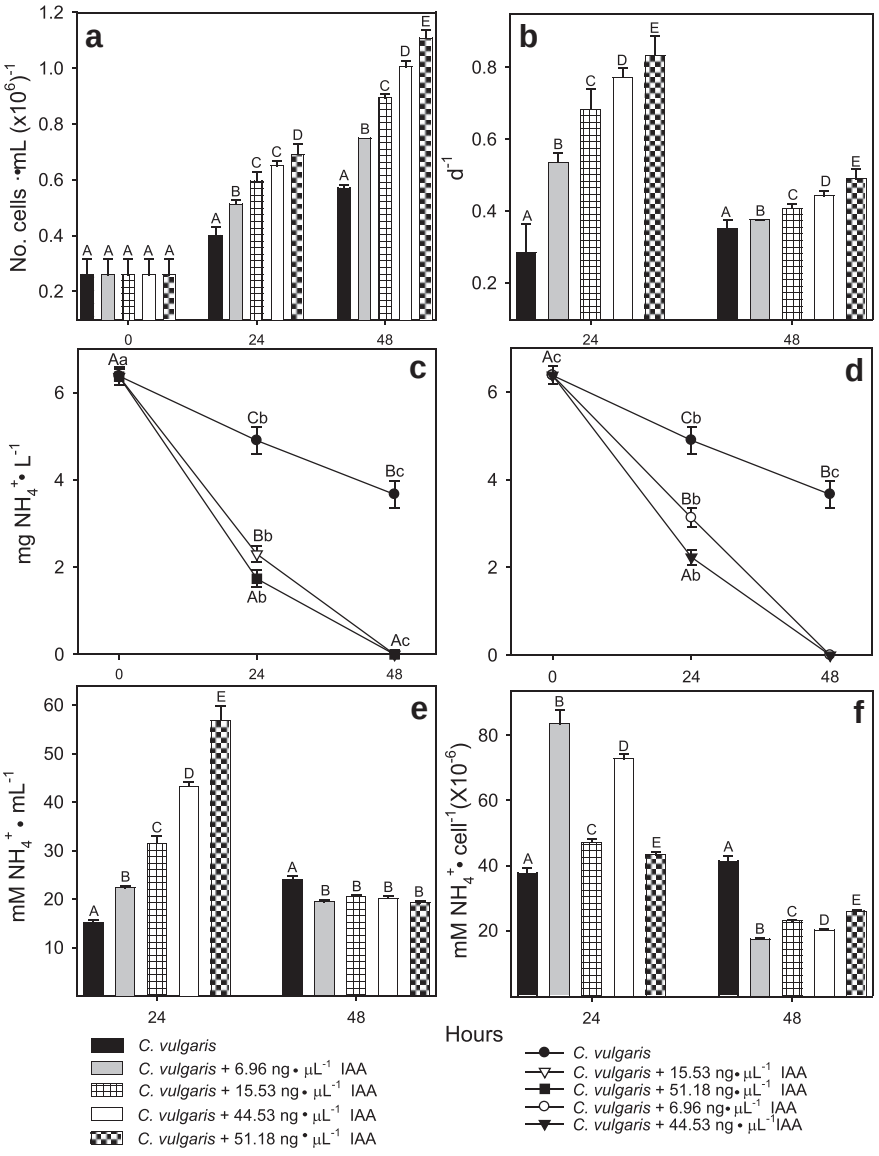

Fig. 3. Effects of different levels of exogenous IAA on ammonium uptake and accumulation of intracellular ammonium by *C. vulgaris*. Growth (a); growth rate (b); uptake of ammonium from the growth medium (c, d); accumulation of intracellular ammonium per culture (e) and per cell (f). Groups of 4 columns, in each subfigure separately, denoted by a different letter, differ significantly by one-way ANOVA and Tukey's HSD post-hoc analysis at $p < 0.05$. In subfigures c and d, different capital letters along a curve differ significantly, and different lower case letter at each time point differ significantly. Both analyses used one-way ANOVA and Tukey's HSD post-hoc analysis at $p < 0.05$.

either from sterile substrates [28,30,60,61] or from wastewater under a variety of conditions [29,62–64]. The specific topic addressed in this study is whether IAA produced in abundance by this genus of bacteria is involved in ammonium uptake and later assimilation in microalgal cells.

For uptake of NH_4^+, several ammonium transporters act as the major entry [25,65], similar to green macroalgae muskgrass *Chara* spp. and the floating plant crystalwort *Riccia fluitans* [66], and by direct diffusion of uncharged NH_3, which is highly permeable across biological membranes [66,67]. Ammonium fluxes in plant roots are difficult to control because ammonium establishes equilibrium between NH_4^+ and NH_3, each with a different membrane permeability. The equilibrium, at least for enteric bacteria, is sustained by the ammonium/methylammonium transport B (AmtB) protein [68]. In the microalgae *Chlorella* spp., ammonium transporters have not been specifically investigated. Assimilation of ammonium involves two enzymatic pathways, whereas there is no difference between ammonium assimilation pathways in algae and higher plants [69].

Similarly to uptake of phosphorus and iron by roots, based on transporters and the relatively simple proton efflux mechanism [70–72], ammonium assimilation pathways, with only a few enzymes involved, are some of the most complex biochemical mechanisms in microorganisms and plants. Numerous mechanisms, backup mechanisms and forms of enzymes are involved. To adjust cellular and ammonium levels that vary not only in response to uptake of external ammonium, but also to intracellular amino acid catabolism, ammonium transport processes need to be tightly regulated.

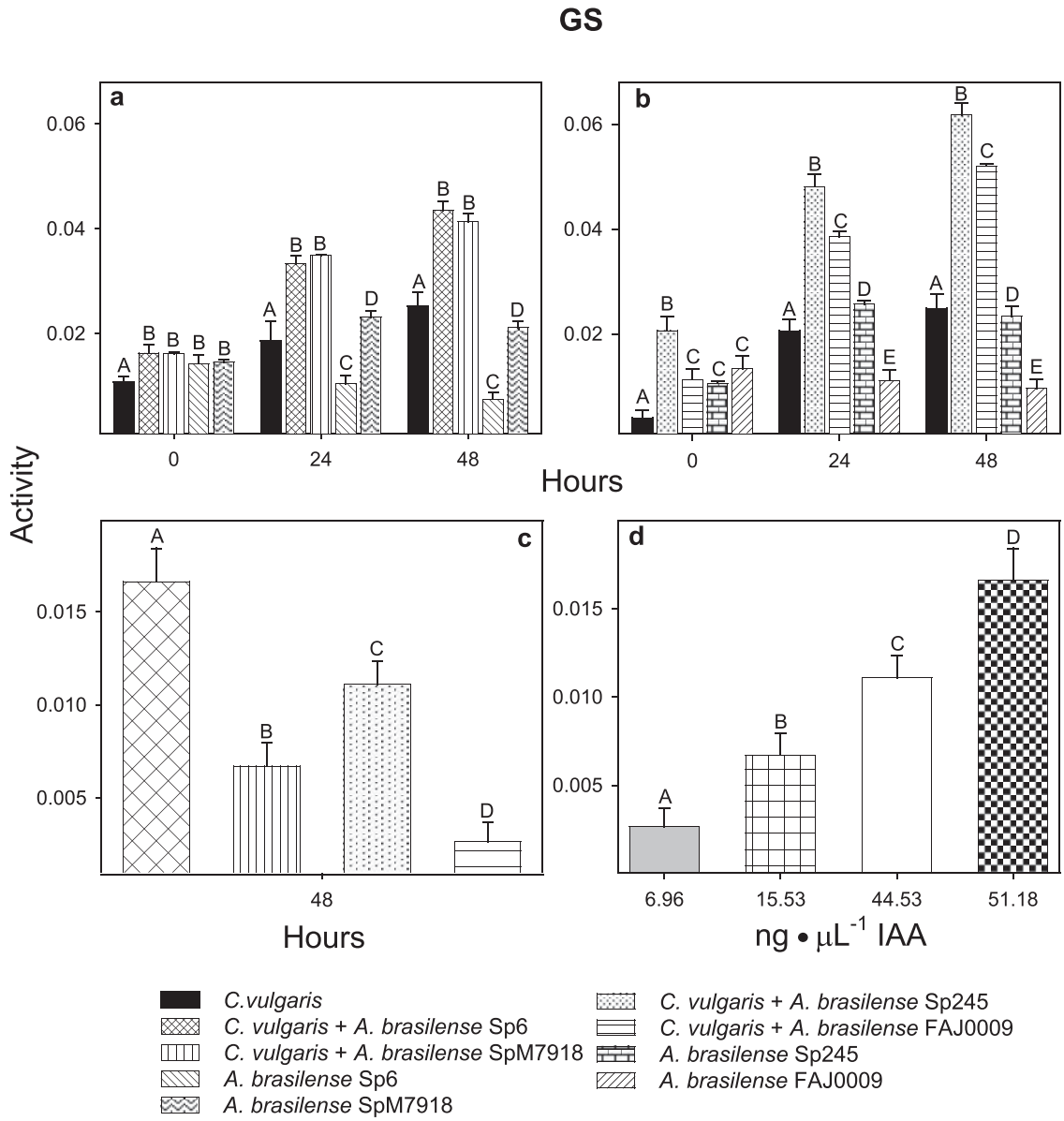

Fig. 4. Effects of different strains of *A. brasilense* on activity of glutamine synthetase (GS) in *C. vulgaris*. Activity of GS (a, b); net GS activity (c); comparison of GS vs. the capacity of strains to produce IAA (d). Groups of 4 or 5 columns, in each subfigure separately, denoted by a different letter differ significantly by one-way ANOVA and Tukey's HSD post-hoc analysis at $p < 0.05$. GS enzymatic activity was defined as: absorbance$_{660nm}$ 10 min^{-1} culture^{-1}.

Intracellular ammonium concentrations in non-stressed plants range between 2 and 45 mM [51], whereas concentrations of intracellular ammonium found in *C. vulgaris* are in agreement with higher plants. Ammonium nutrition leads to an immediate increase in cellular ammonium and amino acid pools [25]. In our study, it led to enhanced growth of the microalga population.

The most important pathway for ammonium assimilation involves the enzymes glutamine synthetase (GS) and glutamate synthase (GOGAT) [26]. Our study showed that the enhanced activity of GS corresponds to the quantity of IAA that a strain can produce. Activities of both GS and GDH are enhanced by immobilization of *Chlorella* spp. with *A. brasilense* [31]. GDH also participated in maintaining the balance of carbon and nitrogen within plant cells, which turns the carbon in amino acids back into reactions of carbon metabolism [27]. This happens because the balance between nitrogen and carbon metabolism is of major importance for the growth and well-being of all organisms. Similarly to GS, GDH-enhanced activity found in our study was related to the quantity of IAA available in the culture medium.

The effects of IAA have been far less thoroughly studied in microalgae than in higher plants, where IAA induces many significant effects [73]. The effect of auxins on *Chlorella* spp. has been rarely studied. Czerpak et al. [19] found strong stimulation by IAA of water-soluble protein, monosaccharides—aldohexoses and chlorophyll-*a* and -*b* content in *Chlorella pyrenoidosa*. Protein excretion by *C. pyrenoidosa* cells increased by applying several auxins, auxin precursors and chemical analogues of auxins [18,19]. Application of

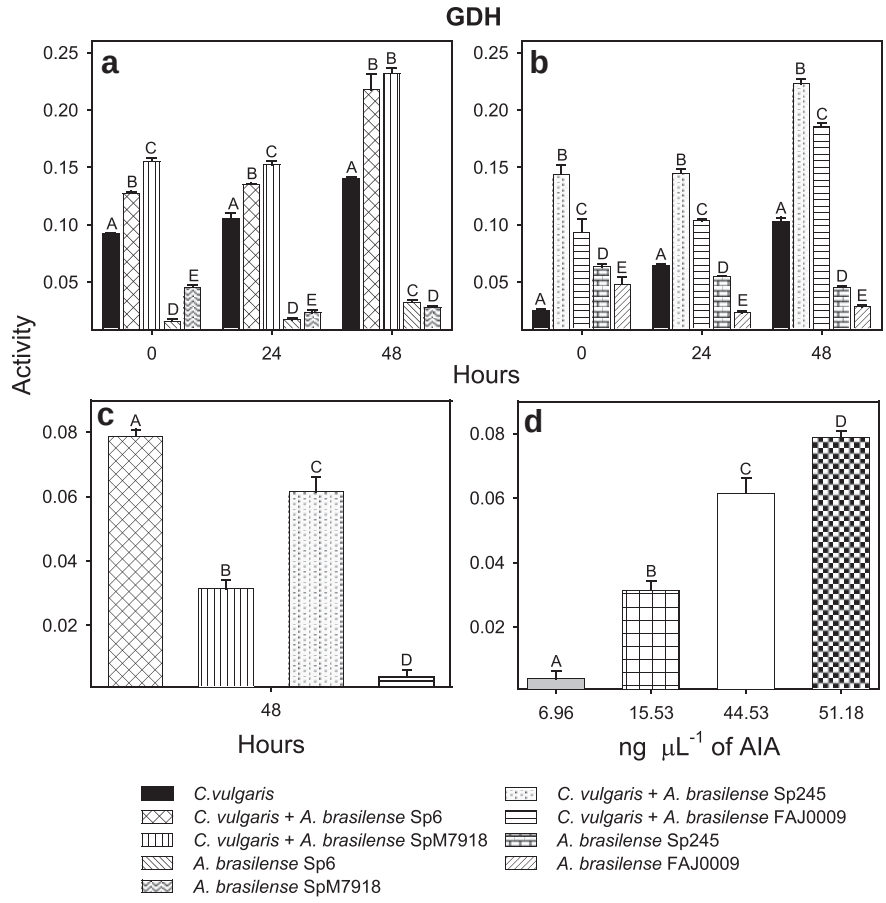

Fig. 5. Effects of different strains of *A. brasilense* on activity of glutamate dehydrogenase (GDH) in *C. vulgaris*. Activity of GDH (a, b); net GDH activity (c); comparison of GDH vs. the capacity of the strains to produce IAA (d). Groups of 4 or 5 columns, in each subfigure separately, denoted by a different letter, differ significantly by one-way ANOVA and Tukey's HSD post-hoc analysis at $p < 0.05$. GDH enzymatic activity was defined as Δ absorbance$_{340nm}$ 10 min^{-1} culture^{-1}.

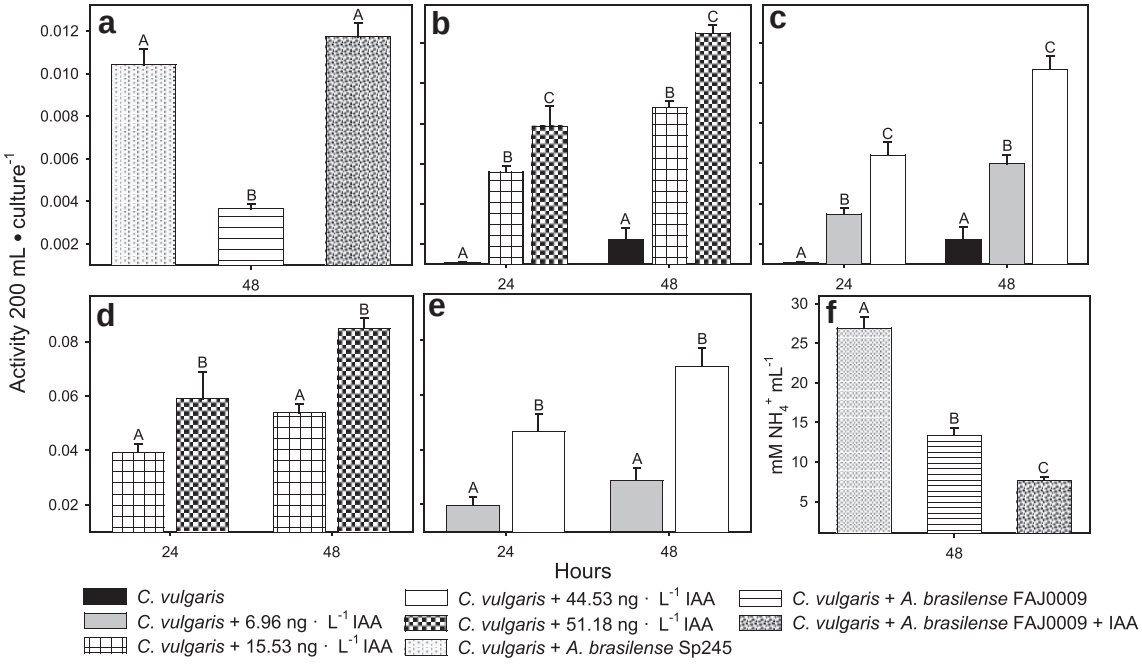

Fig. 6. The effect of complementation of IAA-attenuated mutants with exogenous IAA on activity of GS and accumulation of intracellular ammonium. Activity of GS after complementing the mutant FAJ0009 with exogenous IAA (a); activity of GS complementing *C. vulgaris* with exogenous IAA (b, c); net GS activity calculated after complementing with exogenous IAA (d, e); the effect on intracellular ammonium of addition of exogenous IAA to mutant FAJ0009 (f). Groups of 3 columns, in each subfigure separately, denoted by a different letter, differ significantly by one-way ANOVA and Tukey's HSD post-hoc analysis at $p < 0.05$ (in a,b,c,f). Pair of columns in subfigures d,e denoted by a different letter differ significantly by Student's *t*-test at $p < 0.05$.

exogenous IAA to *C. vulgaris* culture significantly increased the population of microalgae [4] and significantly increased GS and GDH activities [31]. This study has shown that there is a relationship between the capacity of a strain to produce certain quantities of IAA and the effect detected in GS and GDH enzymes; the higher the amount of IAA produced, the greater the enzymatic activity. IAA production and N_2-fixation in *A. brasilense* are linked in a yet-to-be-discovered way through a heat-shock protein codified by the *clpX* gene [74]. GS is the common user of nitrogen derived from N_2-fixation [27]. Therefore, IAA may be linked to greater activity of GS via enhanced biosynthesis of this enzyme. Alternatively, IAA may indirectly affect GS activity via the linkage with N_2-fixation of *A. brasilense*.

Thus far, it has not been possible to isolate *Azospirillum* mutants that are completely unable to synthesize IAA. Only a few IAA-attenuated strains are known and two were used in this study. These mutants produce 0.2%–5.0% of the level of IAA produced by the wild types [8,9]. Although the direct phytostimulation of the bacterial auxin on growth of microalgae is barely known [4], the effect of *Azospirillum* spp. on wheat was further enhanced by adding tryptophan, an essential precursor of IAA, and this could be mimicked by replacing *Azospirillum* spp. cells with IAA [75]. An *ipdC* knock-out mutant, the key enzyme for IAA biosynthesis in *A. brasilense*, which produces only 10% of wild-type IAA levels in *A. brasilense* Sp245 [43], had significantly less effect on wheat [76] and bean growth [77]. Using the same mutant in our study, it produced a lesser effect on intracellular ammonium and GS and GDH activity. Upon introduction of either a constitutive or a plant-inducible promoter into the wild-type strain, *ipdC* constructs further improved the plant growth-promoting effect of *A. brasilense* [76]. Similarly, use of several IAA-attenuated mutants of *A. brasilense* and *Azospirillum lipoferum* produced significantly less promotion of growth on multiplication of *C. vulgaris* compared with their respective wild types [9].

While external ammonium in plants may be very low, intracellular ammonium can be high, up to 40 times higher. The concentrations of intracellular ammonium reported in this study accords with the concentration reported in many plant species (Table 2 in Miller et al. [51]). Accumulation of intracellular ammonium in leaves resulted from water stress, exposure to excess Cu and senescence [78]. The close association of *C. vulgaris* and *A. brasilense* within the beads creates highly congested immobilizing conditions [35,63] that may provide physical stress for the microalgae, which may lead to increased intracellular ammonium.

Inorganic ions that accumulate in plant cells serve nutritional, osmotic, signaling and storage functions. Insufficient ion accumulation or excess accumulation may compromise these functions. While intracellular ammonium reserves may buffer the cytoplasm against short-term perturbations, when external sources of ions are removed, the reserves are usually exhausted within a few days [65]. Consumption of ammonium from a medium (or wastewater) by *Chlorella* spp. immobilized with *Azospirillum* spp. is very fast, normally within 24 h, and the medium is depleted of ammonium within 48 h. This makes the assemblage of microorganisms a formidable wastewater treatment tool [28,29]. Our study shows that, within 24 h, there is a consistent and significant accumulation of intracellular ammonium, possibly related to a yet-to-be-discovered process or to the quantity of IAA that the bacterial partner can excrete. This accumulation of intracellular ammonium is transient and significantly reduced after 48 h. A plausible theoretical explanation is that there is a difference in capacity between ammonium assimilation and ammonium uptake mechanisms by the microalgae. Both are enhanced when there is an interaction of the microalgae with *A. brasilense*. *A. brasilense* enhances the uptake of ammonium from the substrate to a greater extent than the enhancement it initiates in the enzymatic machinery of ammonium assimilation (GS and GDH) inside the cell. Consequently, the assimilation mechanism is unable to cope with the extra supply of ammonium. This resulted in some of the non-assimilated ammonium transiently accumulating as free ammonium inside the cell for about 24 h. The intracellular ammonium is later assimilated into glutamate when the substrate where the microalga is growing runs out of ammonium and the uptake mechanism stops. The internal supply of ammonium allows *C. vulgaris* to grow for a limited time without exogenous ammonium. Growth of *C. vulgaris* with limited or no ammonium has been previously demonstrated [30,36].

Considering these findings, a reservation should be added. The two enzyme activities and accumulation of intracellular ammonium in co-immobilization treatments cannot be separated to show the contribution of each of the two partners in this association. The microorganisms produced all these variables to a lesser extent when grown separately. This indicates that the association itself might have produced mutual stimulation in both microorganisms, and not simply in the microalga. This potential complication is currently under study.

In summary, this study shows that intracellular ammonium is accumulated in cells of *C. vulgaris* when interacting with the bacterium *A. brasilense*, and this is affected by the phytohormone IAA. The activity of two major enzymes of ammonium assimilation, GS and GDH, is also enhanced. The accumulation of intracellular ammonium is transient and allows the microalgae to grow when the external ammonium supply is depleted.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2014.12.010.

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