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ROLE OF GLUTAMATE DEHYDROGENASE AND GLUTAMINE SYNTHETASE IN *CHLORELLA VULGARIS* DURING ASSIMILATION OF AMMONIUM WHEN JOINTLY IMMOBILIZED WITH THE MICROALGAE-GROWTH-PROMOTING BACTERIUM *AZOSPIRILLUM BRASILENSE*[1]

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Enzymatic activities of glutamate dehydrogenase (GDH) and glutamine synthetase (GS) participating in the nitrogen metabolism and related ammonium absorption were assayed after the microalga *Chlorella vulgaris* Beij. was jointly immobilized with the microalgae-growth-promoting bacterium *Azospirillum brasilense*. At initial concentrations of 3, 6, and 10 mg · L^{-1} NH_4^+, joint immobilization enhances growth of *C. vulgaris* but does not affect ammonium absorption capacity of the microalga. However, at 8 mg · L^{-1} NH_4^+, joint immobilization enhanced ammonium absorption by the microalga without affecting the growth of the microalgal population. Correlations between absorption of ammonium per cell and per culture showed direct (negative and positive) linear correlations between these parameters and microalga populations at 3, 6, and 10 mg · L^{-1} NH_4^+, but not at 8 mg · L^{-1} NH_4^+, where the highest absorption of ammonium occurred. In all cultures, immobilized and jointly immobilized, having the four initial ammonium concentrations, enzymatic activities of *Chlorella* are affected by *A. brasilense*. Regardless of the initial concentration of ammonium, GS activity in *C. vulgaris* was always higher when jointly immobilized and determined on a per-cell basis. When jointly immobilized, only at an initial concentration of 8 mg · L^{-1} NH_4^+ was GDH activity per cell higher.

Key index words: Azospirillum; Chlorella; **enzymes; glutamate dehydrogenase; glutamine synthetase; microalgae; nitrogen metabolism; PGPB; PGPR; plant-growth-promoting bacteria**

Abbreviations: **GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; SGM, synthetic growth medium**

Assimilation of ammonium is a critical biochemical process for plant growth and development (Lu et al. 2005), and it involves two enzymatic pathways. The most important is the GS and glutamate synthase (GOGAT or GltS) pathway (Vanoni and Curti 2005). The other one is the possible reversible reductive amination of α-oxoglutarate, catalyzed by GDH, which functions as an auxiliary pathway (Zaliha et al. 1997, Miflin and Habash 2002, Lu et al. 2005). Essentially, there is no difference between ammonium assimilation pathways in algae and higher plants (Huppe and Turpin 1994, Inokuchi et al. 2002). GS functions as the major assimilatory enzyme for ammonia derived from nitrogen fixation and nitrate and ammonia nutrition. One specific important feature of GS is its high affinity for ammonia and, consequently, its ability to incorporate ammonia efficiently into several organic configurations. It also assimilates ammonia released from photorespiration and breakdown of proteins. Prokaryotes and eukaryotes were

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once thought to express different forms of GS: prokaryotes express GS type I, while eukaryotes express GS type II (Eisenberg et al. 2000). Recently, GS type II was detected in symbiotic plant bacteria (Yuan et al. 2001), but GS type I has not been detected in eukaryotes, so far. Type I and type II forms have some, but not all, of their effectors (parameters that regulate their activity) in common. The expression of GS in bacteria is highly regulated by nitrogen starvation. Full expression requires growth in a nitrogen-limited environment. In eukaryotic GS, this phenomenon has not been observed. It has been reported that hormones can induce changes in the rate of GS type II biosynthesis (Eisenberg et al. 2000).

Together with GS, a number of other enzymes play key roles in maintaining the balance of carbon and nitrogen within plant cells. One of them is GDH, which returns the carbon in amino acids back into reactions of carbon metabolism (Miflin and Habash 2002). Higher plants and microalgae contain two isoenzymes of GDH that differ in physical, chemical, and immunological properties. These isoenzymes are regulated differently during the cell cycle: (i) a constitutive NAD-GDH isoenzyme that is located in mitochondria, and (ii) a chloroplastic, light-dependent, ammonium-inducible NADP-GDH (Prunkard et al. 1986). Bacteria have only NADP-dependent GDH.

Earlier studies show that joint immobilization of the microalgae-growth-promoting bacterium *Azospirillum* with *Chlorella* affects absorption (uptake and assimilation) of ammonium in *Chlorella* (de-Bashan et al. 2002a, 2004, 2005, 2008a, Hernandez et al. 2006). Our hypothesis was that joint immobilization of *A. brasilense* Cd and *C. vulgaris* would have an effect on GS and GDH activity in *C. vulgaris*.

MATERIALS AND METHODS

Microorganisms and initial growth conditions. Prior to immobilization in beads, 10 mL of axenic *C. vulgaris* UTEX 2714 was inoculated into 100 mL sterile mineral medium C30 and incubated at 22°C ± 2°C and stirred at 140 rpm under light intensity of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 7 d (Gonzalez et al. 1997).

A. brasilense Cd (DSM 7030, Brunschweig, Germany) was used in these experiments, and *A. brasilense* Sp6 (Katholieke Universiteit Leuven, Belgium) was used as the complementary strain. The bacteria were stored in liquid nitrogen and, for daily use, were kept on nutrient agar slants (Sigma, St. Louis, MO, USA). Two days before immobilization, a loop of *Azospirillum* was transferred to 25 mL liquid nutrient broth (Sigma) and incubated overnight at 37°C ± 2°C at 120 rpm. The day before immobilization, 3–4 mL preinoculum was introduced into 50 mL fresh nutrient broth and incubated at 37°C for 17 h (Bashan et al. 1993).

Immobilization of C. vulgaris *and* A. brasilense *in alginate beads.* Microorganisms were immobilized according to the method described in de-Bashan et al. (2004). Briefly, axenic cultures (either *C. vulgaris* or the microalgae-growth-promoting bacterium *A. brasilense*) were mixed with 2% alginate solution. The solution was dripped from a sterile syringe into 2% $CaCl_2$ solution, with periodic mixing of the solution. For joint immobilization of the two microorganisms in the same bead, after washing the cultures, each of them was resuspended in 10 mL 0.85% saline solution and then mixed in the alginate before forming the beads. Because immobilization normally reduces the number of *Azospirillum* cells in the beads, a second overnight incubation in diluted nutrient broth was necessary.

Culturing conditions for joint immobilization of microorganisms, solubilization of beads, and cell counts. Four concentrations of ammonium (10, 25, 27, and 30 $mg \cdot mL^{-1}$ NH_4Cl, which correspond to 3, 6, 8, and 10 $mg \cdot mL^{-1}$ NH_4^+) were tested. Although these levels are somewhat smaller than those common in highly contaminated wastewater reservoirs (Abeliovich 1982), they present the concentrations of ammonium occurring in domestic Mexican wastewater with which we have worked (de-Bashan et al. 2002a, 2004) and are in agreement with studies of growth of *Chlorella* spp., of which this study is a continuation (Gonzalez and Bashan 2000, de-Bashan et al. 2005, 2008b). Experiments were carried out in synthetic growth medium (SGM; Gonzalez and Bashan 2000). After secondary multiplication of the microorganisms inside the beads, the beads were washed twice with saline solution (0.85% NaCl), and beads weighing 40 g were added to 200 mL SGM. Batch cultures were incubated in Erlenmeyer flasks at 28°C with continuous stirring at 140 rpm under 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity for 48 h. Cells of the microorganisms released from the beads and were counted, using five beads solubilized by immersion in 5 mL 4% sodium bicarbonate for 30 min at room temperature (24°C–26°C). *A. brasilense* was counted by plating a series of dilutions in 0.85% saline solution on nutrient agar plates (Sigma), and *C. vulgaris* was counted with a Neubauer hemocytometer (Bright line counting chamber, Hausser Scientific Company, Harsham, PA, USA).

Ammonium analysis. Ammonium ions were measured by the salicylate method, a standard water analysis technique using a special kit (Hach, Loveland, CO, USA) and a spectrophotometer (Hach DR 4000).

Effect of joint immobilization of C. vulgaris *with* A. brasilense *on GS and GDH enzyme pathways. Extraction:* After incubating for 48 h, the beads dissolved. Cells were harvested and washed twice with 0.85% saline solution at 4°C at 6,000*g* and resuspended in 3 mL 100 mM HEPES (4-[2-hydroxyethyl]-1-piperazineethane sulfonic acid) buffered at pH 7.5 and containing 10 mM DTT (dithiothreitol). Extracts of the cells were prepared by sonicating the cells for 1 min and repeating this five times, with a cooling period in ice between each sonication. The suspensions were centrifuged at 10,500*g* for 30 min (Hartmann et al. 1988), and the pellet was discarded. The supernatant was transferred to clean microtubes and served as the source of the enzyme.

Protein: Protein content of cell extracts was analyzed by dye binding (Bradford 1976), using a protein analysis kit (Bio-Rad, Hercules, CA, USA). The reaction was quantified in a microplate reader at 595 nm (VersaMax, Molecular Devices, Sunnyvale, CA, USA).

Enzyme analyses—glutamate dehydrogenase (GDH, EC 1.4.1.3): GDH activity was measured in the aminating direction and determined by the rate of decrease in absorbance at 340 nm, which results from oxidation of NADH in the presence of α-ketoglutarate and ammonium ions (Frieden 1963). This assay was modified for microplate analysis, as follows:

Reagents—0.1 M sodium phosphate buffer at pH 7.5; 0.005 M α-ketoglutarate monosodium salt, freshly prepared in phosphate buffer; 0.004 M NADH disodium salt, freshly prepared in 0.001 M sodium carbonate; 3.0 M ammonium chloride in phosphate buffer; 0.0025 M EDTA disodium salt in distilled water.

Procedure—The reaction mixture for the microplate contains 5.4 mL phosphate buffer; 200 μL α-ketoglutarate; 200 μL NADH; 400 μL ammonium chloride; 200 μL EDTA. The reaction mixture (300 μL) was mixed with 10 μL enzyme extract and preincubated in the microplate reader at 27°C for 10 min. The reaction was monitored in a microplate reader at 340 nm at 27°C for 10 min. The slope was calculated (vmax · min^{-1}) using SoftMax Pro software (Molecular Devices). To validate the method, and as an additional control, a parallel assay with a standard enzyme was performed. For this, a commercial GDH (from bovine liver, Calzyme, San Luis Obispo, CA, USA) was prepared in phosphate buffer at a final concentration of 0.11 units · mL^{-1} and then assayed similarly to the extracts from the microalga. Enzyme activity was defined as (i) enzyme unit, absorbance · min^{-1} · mg $protein^{-1}$ and (ii) absorbance · min^{-1} · $cell^{-1}$.

Glutamine synthetase (GS: EC 6.3.1.2): In a preliminary experiment performed to ensure that the extraction procedure did not affect GS activity because this enzyme is highly labile and numerous proteases affect its activity, a mixture of protease inhibitors (0.49 mg polyvinylpolypyrrolidone [PPVP]; 220 μL phenylmethanesulfonyl fluoride [PMSF; stock solution 1.5 μM]; and 5 μL [stock solution 2 μM] *trans*-epoxysuccinyl-L-leucylamide [4-guanidine] butane [E-64] [all from Sigma]) was added to the extraction buffer before sonication.

This experiment was done in a suspension culture of the microalga, where 10 mL *C. vulgaris* was inoculated into 90 mL C30 medium (Gonzalez et al. 1997) with either of two sources of nitrogen, NH_4 (25 mg · L^{-1} NH_4Cl) or KNO_3 (5 mg · L^{-1}). The cultures were incubated for 48 h at 28°C and stirred at 130 rpm and exposed to 60 μmol · m^{-2} · s^{-1} light. At the end of the incubation, the cells were harvested and washed twice with 0.85% saline solution. The final pellets were resuspended in 4 mL HEPES plus DTT buffer. The mixture of inhibitors was added to half of the cultures grown with ammonium or nitrate. The other half of the cultures was sonicated without inhibitors. Extraction was carried out as explained earlier.

GS activity was measured (Shapiro and Stadtman 1972) with the following reaction mixture:

Reagents (all analytical grade)—1 M imidazole–HCl buffer at pH 7.0; 0.12 M ATP solution at pH 7.0; 2 M L-glutamate solution at pH 7.0; 1 M NH_4Cl solution; 1.67 M $MgCl_2$ solution; 29 mM $FeSO_4$ solution dissolved in 0.3 N H_2SO_4 (freshly prepared); 53 mM ammonium molybdate dissolved in 7.5 N H_2SO_4.

Procedure—The reaction mixture contained 1 mL imidazole HCL buffer; 2.5 mL ATP solution; 2.0 mL L-glutamate solution; 1.0 mL NH_4Cl solution; 0.6 mL $MgCl_2$ solution; 2.9 mL deionized water. The reaction mixture (0.2 mL) and 0.1 mL deionized water were extracted by pipette into a test tube previously washed with phosphorus-free detergent and rinsed with H_2SO_4 and deionized water to avoid phosphorus residues in the tubes. The mixture was equilibrated (preincubated) at 37°C for 5 min. A 0.1 mL sample was added to the mixture and incubated at 37°C for 10 min. $FeSO_4$ solution (1.8 mL) was added to stop the reaction; 0.15 mL ammonium molybdate reagent was added. Absorbance was read at 660 nm in a spectrophotometer (Hach 4000, Hach Co., Loveland, CO, USA).

Each sample had its own blank that was prepared as follows: to a 0.1 mL sample, 0.8 mL $FeSO_4$ solution was added, and the mixture was submerged in a hot bath (100°C) for 10 min. Then all the procedures were followed as outlined above. To validate the method and as an additional control, the activity of commercial GS from *E. coli* (Sigma) was determined parallel to the enzyme from *Chlorella* extracts. This enzyme was diluted in distilled water to a final concentration of 1.3 mg · mL^{-1}.

Activity was calculated with the following formula: volume activity (U · mL^{-1}) = absorbance × 0.719 × dilution factor where 1 unit of enzyme (U) is defined as the amount of enzyme that produces 1 μmol phosphate released from ATP per minute at 37°C at pH 7.0. Enzyme activity was defined as: (i) enzyme unit, U · mL^{-1} · mg $protein^{-1}$, and (ii) enzyme activity, U · mL^{-1} · $cell^{-1}$.

Experimental design and statistical analysis. We used two joint immobilization systems of *C. vulgaris,* one with *A. brasilense* Cd and the other with *A. brasilense* Sp6. As the results are very similar, for clarity and space saving, only results with strain *A. brasilense* Cd are presented. Each treatment was done in triplicate using one Erlenmeyer flask as a replicate. Each experiment was repeated in full three or four times. Blank beads (without microorganisms) were prepared similarly to serve as controls. Reaction mixtures without enzymes or boiled enzymes served as additional controls. Results of all experiments were analyzed by one-way analysis of variance (ANOVA) and then by Tukey's HSD post-hoc analysis at the significance level of $P \leq 0.05$. STATISTICA software was used (Statsoft, Tulsa, OK, USA). Comparisons between immobilization and joint immobilization treatments within each ammonium concentration were done by Student's *t*-test at $P \leq 0.05$. Linear regressions were performs with Sigma Plot v. 8 software (SPSS, Chicago, IL, USA) and statistically analyzed with STATISTICA at various *P* levels.

RESULTS

Growth and protein concentration in joint immobilization of C. vulgaris *and* A. brasilense *under different initial ammonium concentrations.* As shown in Figure 1A, at initial ammonium concentration of 3 or 6 mg · L^{-1}, combined immobilization enhanced the growth of *C. vulgaris* with *A. brasilense* Cd. However, the larger population did not yield higher protein content in the system (Fig. 1C) or in greater absorption of ammonium per cell (Table 1). The population of *A. brasilense* when jointly immobilized is lower than the population of *A. brasilense* immobilized alone (Fig. 1B). The total protein content of *A. brasilense* Cd cultures immobilized alone is higher than in the other cultures.

Under the initial 8 mg · L^{-1} of ammonium concentration, growth of *C. vulgaris* jointly immobilized with *A. brasilense* is not enhanced, compared to *C. vulgaris* immobilized alone (Fig. 1A), although ammonium absorption per cell (but not per culture) significantly increased in *C. vulgaris* that was jointly immobilized with *A. brasilense* Cd (Table 1). Populations of *A. brasilense* Cd, whether immobilized alone or jointly immobilized, are not statistically different (Fig. 1B), but total protein content under combined immobilization significantly increased (Fig. 1C).

Under an initial 10 mg · L^{-1} ammonium concentration, the results are somewhat similar to those obtained at initial concentrations of 3 mg · L^{-1} ammonium. Growth of *C. vulgaris* was enhanced by joint immobilization with *A. brasilense* (Fig. 1A), total protein content of the jointly immobilized systems is lower (Fig. 1C), and ammonium absorption per cell is similar, regardless of whether measurement is per culture or single cell (Table 1). Populations of *A. brasilense* Cd are similar to populations grown

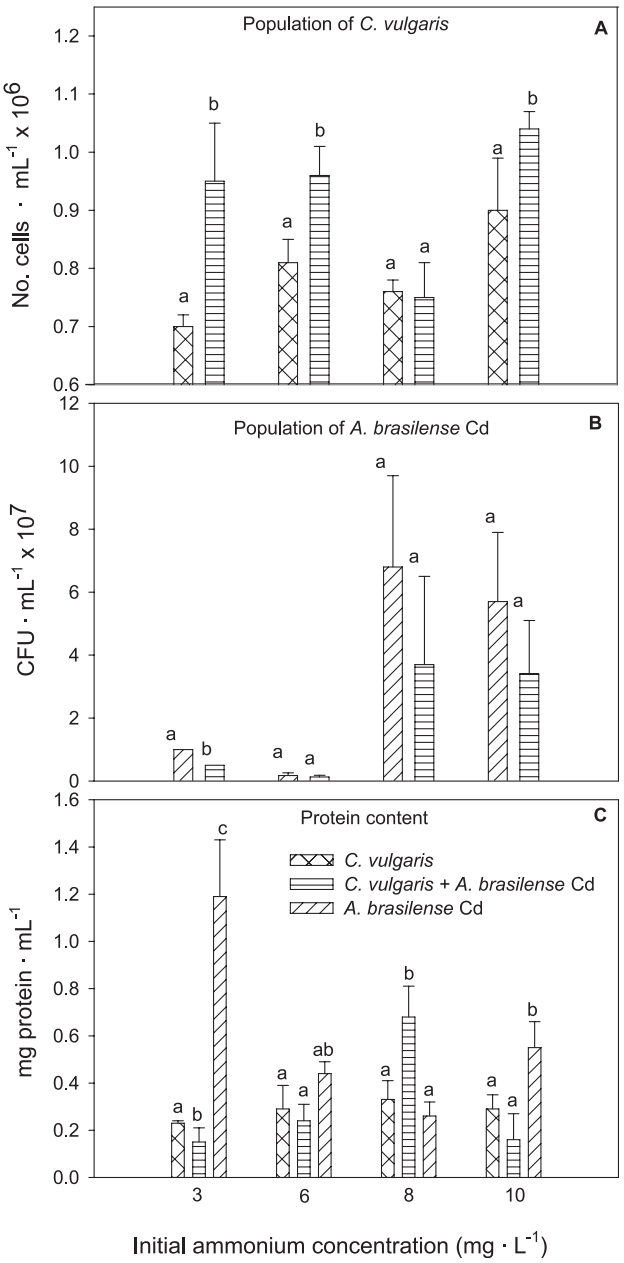

FIG. 1. Populations of (A) *Chorella vulgaris*, (B) *Azospirillum brasilense* Cd, and (C) protein content of the systems in experiments with an initial concentration of 3, 6, 8, or 10 mg · L^{-1} NH_4^+. Columns in each panel, for each ammonium concentration denoted with different letters, differ significantly at $P \leq 0.05$ using Student's *t*-test (A, B) or one-way ANOVA in groups of three columns (C). Whisker bars represent the standard error; absence indicates that the standard error is negligible.

under an initial 8 mg · L^{-1} ammonium concentration (Fig. 1B), yet they maintain the same trend—the bacterial populations are always smaller in jointly immobilized systems, but results with 10 mg · L^{-1} ammonium concentration differ from the 8 mg · L^{-1} ammonium concentration because cells have higher protein content when grown alone (Fig. 1C). The most significant result of the ammonium absorption experiments is absorption per cell at 8 mg · L^{-1} NH_4^+, where joint immobilization caused higher absorption than absorption by the microalga alone. In all other combinations, the absorption was similar (Table 1). Similar results were obtained with *A. brasilense* Sp6 (data not shown).

Possible correlations between absorption of ammonium per cell, removal of ammonium by the entire culture, and the development of the microalgal populations under the four ammonium concentrations were evaluated separately. At each of the three repetitions of each concentration, a slightly different population was developed. When evaluated at the single-cell level, and when the microalga was immobilized alone, there were negative, significant direct linear correlations at concentrations of 3, 6, and 10 mg · L^{-1} NH_4^+, but not at 8 mg · L^{-1} NH_4^+ (Fig. 2A). The same phenomena occurred during joint immobilization with *A. brasilense* Cd (Fig. 2B). The concentration of 8 mg · L^{-1} NH_4^+ stood out as an exception. Joint immobilization at that concentration, however, presented a higher correlation than that of immobilization alone, but it was low and not statistically significant (Fig. 2, A and B).

Evaluating possible correlations between the microalga population and the level of removal of ammonium from the medium showed that these two parameters, in general, are less correlated. When immobilized alone, a significant negative correlation was found only for the 10 mg · L^{-1} NH_4^+ concentration ($r^2 = 0.95$, $P \leq 0.05$). However, when the two microorganisms were jointly immobilized, the linear correlations were positive for 3 ($r^2 = 0.97$, $P \leq 0.05$) and 10 ($r^2 = 0.97$, $P \leq 0.05$) mg · L^{-1} NH_4^+. Again, at the initial ammonium concentration of 8 mg · L^{-1}, the population was not correlated with the removal of ammonium by the culture. In all experiments, the following enzymatic activities were monitored with special attention to the concentration of 8 mg · L^{-1} NH_4^+.

Activity of GS in jointly immobilized systems. To evaluate the contribution of protease inhibitors of GS activity, three inhibitors were tested: PMSF, an irreversible inhibitor of serine proteases; E-64, an inhibitor of cysteine proteases that does not affect cysteine residues of other enzymes; and PVPP, which improves stability of enzymes. Exploratory experiments with these protease inhibitors showed no positive effect of the inhibitors on GS enzymatic activity. On the contrary, extracts lacking inhibitors had higher activity (activity with inhibitors: 1.46×10^{-4} U · mL^{-1} · $cell^{-1}$; activity without inhibitors: 2.39×10^{-4} U · mL^{-1} · $cell^{-1}$). Therefore, proteins were extracted without inhibitors.

Glutamine synthetase activity in extracts from *A. brasilense* was negligible when immobilized alone (Fig. 3, A–H). Two ways of expressing the results (per unit of enzyme calculated from total

TABLE 1. Final concentration of ammonium in synthetic growth medium (SGM) and ammonium absorption per cell after incubation for 48 h in three immobilization systems at three initial concentrations of ammonium.

Initial concentration of ammonium (mg · L^{-1})	Final concentration of ammonium (mg · L^{-1} NH_4^+)*		Absorption of ammonium per cell (ng · $cell^{-1}$)**	
	Cv	Cv + Cd	Cv	Cv + Cd
3	0.23 ± 0.04a	0.1 ± 0.04a	4.28 ± 0.2a	3.47 ± 0.4a
6	2.9 ± 0.01a	3.0 ± 0.01a	4.42 ± 0.01a	3.66 ± 0.01a
8	5.60 ± 0.8a	4.5 ± 0.6a	4.10 ± 0.4a	6.00 ± 0.1b
10	1.14 ± 0.16a	0.0 ± 0.4a	8.90 ± 0.1a	8.00 ± 0.1a

Cv, *Chlorella vulgaris*; Cd, *Azospirillum brasilense* Cd.
For each pair of values, different lowercase letters in each row differ significantly at $P \leq 0.05$ using Student's *t*-test.
*After 48 h incubation.
**Calculated value.

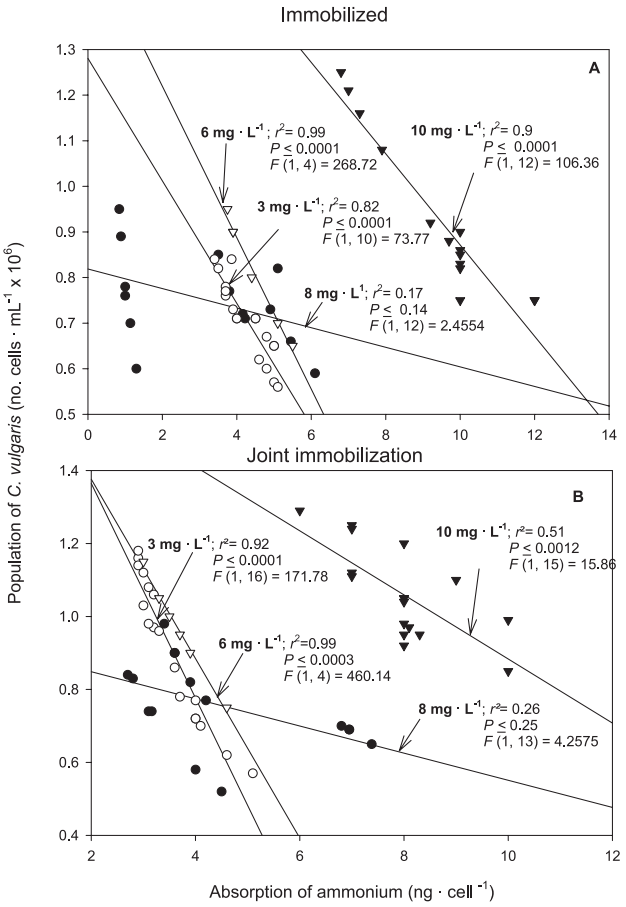

FIG. 2. Linear correlations between developing populations of *C. vulgaris* and absorption of ammonium per cell in immobilized (A) and jointly immobilized with *Azospirillum brasilense* (B) systems at four ammonium concentrations (3, 6, 8, or 10 mg · L^{-1}).

protein or enzymatic activity per cell) are presented in Figure 3.

GS activity differed slightly, depending on how the results are expressed. Regardless of the initial ammonium concentration, GS activity was always higher when *C. vulgaris* was jointly immobilized with *A. brasilense* Cd and activity was measured as per cell (Fig. 3, A–D). This relationship remained when activity was measured as per unit of enzyme for initial ammonium levels of 3, 6, and 10 mg · L^{-1} NH_4^+ (Fig. 3, E, F, and H). However, this pattern was reversed when the initial ammonium level was 8 mg · L^{-1} NH_4^+ (Fig. 3G).

Activity of GDH in jointly immobilized systems. Activity of GDH was measured in the same manner as GS activity. GDH in cultures of *A. brasilense* Cd alone is low or very low (Fig. 4, A–E, G, H). This level happened regardless of the initial ammonium concentration in the culture medium and the two ways to calculate enzyme activity. The sole exception was activity at 6 mg · L^{-1} NH_4^+, when measured as total protein content (Fig. 4F). Therefore, GDH of *A. brasilense* Cd can be ignored in the overall analysis of GDH activity in jointly immobilized systems.

When GDH activity is calculated per cell at the initial concentrations of 6 and 8 mg · L^{-1} NH_4^+, GDH activity is higher in jointly immobilized systems (Fig. 3, B and C) and correspondingly lower at 3 or 10 mg · L^{-1} NH_4^+ (Figs. 4, A and D). However, when GDH activity was calculated per enzyme unit, including the protein content of the cells in the equation, the results were reversed [higher GDH activity in jointly immobilized system at 3, 6, and 10 mg · L^{-1} NH_4^+ (Fig. 4, E, F, and H) and lower activity at 8 mg · L^{-1} NH_4^+ (Fig. 4G)].

DISCUSSION

Nitrogen and carbon metabolisms and the balance between the two are of major importance for the growth and well-being of all organisms. Since these are some of the most complex biochemical mechanisms in organisms, numerous mechanisms, backup mechanisms, and enzymes and forms of enzymes are involved. In plants, GS plays a major role because it is located in and functions in the center of the complex matrix of plant nitrogen metabolism. GS functions as the major assimilatory enzyme for exogenous ammonia, but also for reassimilating ammonia resulting from breakdown of proteins (Miflin and Habash 2002). The GS/GOGAT pathway is the major route for assimilation of ammonia when ammonia is in short supply

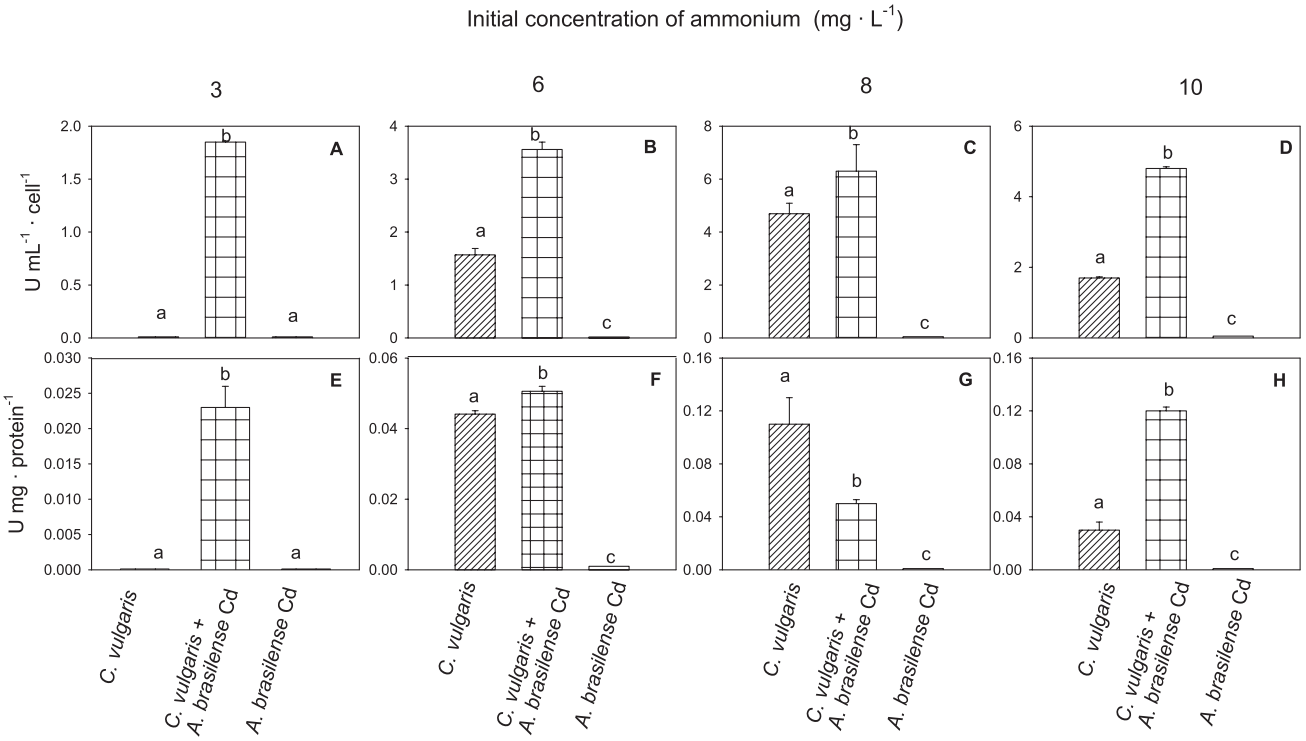

FIG. 3. Activity of glutamine synthetase (GS) in cultures of *Chlorella vulgaris* immobilized alone or with *Azospirillum brasilense* Cd. Units of activity per cell (A–D) and units of activity per mg of protein (E–H) at the four different initial concentrations of ammonium (3, 8, 6, or 10 mg · L^{-1}). In each panel, where a column is denoted with a different lowercase letter, differences are significant at $P \leq 0.05$, using one-way ANOVA. Bars represent the standard error; absence indicates that the standard error is negligible.

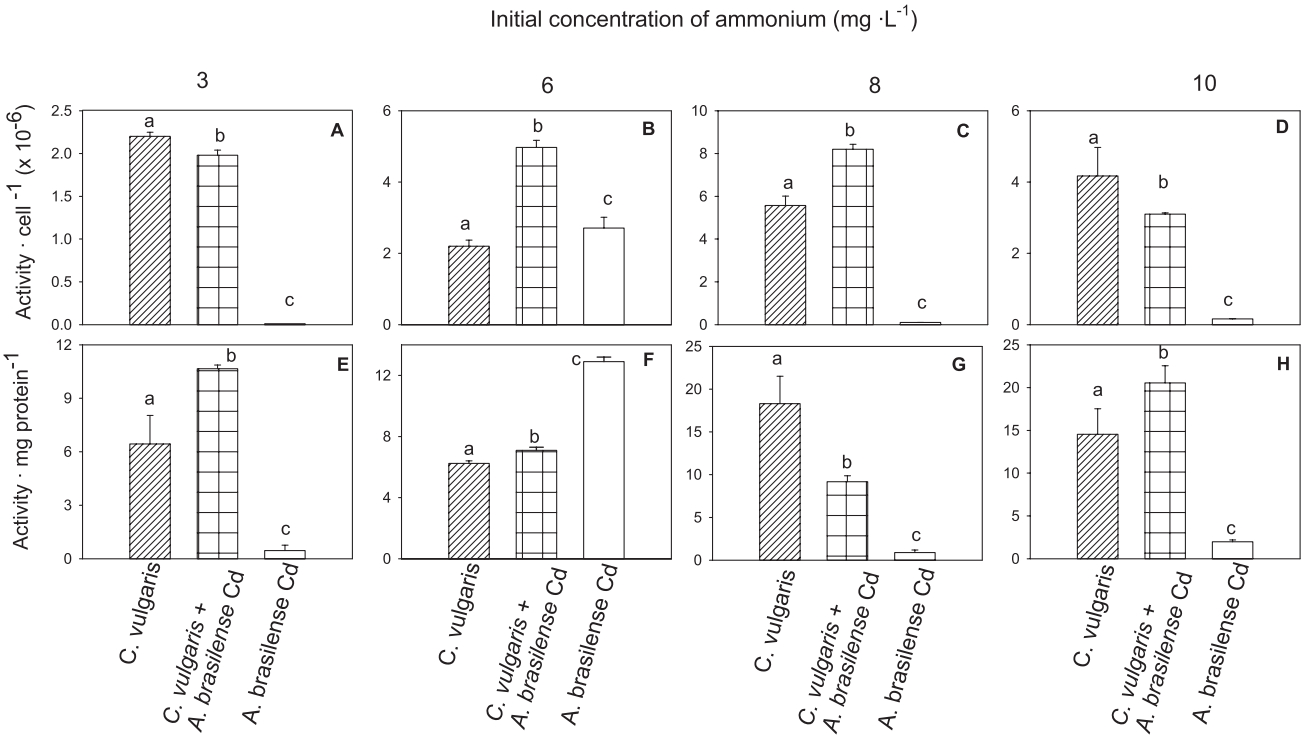

FIG. 4. Activity of glutamate dehydrogenase (GDH) in *Chlorella vulgaris* immobilized alone or with *Azospirillum brasilense* Cd. Activity per cell (A–D) and activity of enzyme per mg of protein (E–H) at different initial concentrations of ammonium (3, 8, 6, or 10 mg · L^{-1}). In each panel, where a column is denoted with a different lowercase letter, differences are significant at $P \leq 0.05$ using one-way ANOVA. Whisker bars represent the standard error; absence indicates that the standard error is negligible.

(Zaliha et al. 1997). Its expression is affected by biotic and abiotic factors, such as drought, nitrogen deficiency, and microbial infections (Santos et al. 2004). In an auxiliary role, GDH participates in a less-known role in maintaining the balance between carbon and nitrogen in the cell (Miflin and Habash 2002). These authors stated that ''GS and plant nitrogen metabolism is best viewed as a complex matrix continually changing during the development cycle of plants'' (Miflin and Habash 2002, p. 979).

Nitrogen metabolism of *Chlorella* is no different from other photosynthetic eukaryotes (Huppe and Turpin 1994). The effect of bacteria associated with microalgae on nitrogen metabolism of microalgae is unknown except for a few of our studies related to *A. brasilense* (de-Bashan et al. 2002a, 2004, 2005) and for *Phyllobacterium myrsinacearum,* which occurs naturally with *Chlorella* sp. and affects nitrate absorption of *C. vulgaris* (Gonzalez-Bashan et al. 2000).

Absorption of ammonia from the growth medium by *Chlorella* sp. is a process involving three enzymes, GS, GDH, and GOGAT. In reality, these enzymes, especially GS, are some of the most complicated in plant life, and the complex environment in which they operate in any plant is under continuous investigation, yet still without a definite answer about their exact operation, if there is an exact operation. For example, GS is a product of multiple genes with complex promoters that ensure the expression of the genes in response to a number of environmental variables affecting the nutritional status of the cell (Miflin and Habash 2002). The role of GDH in assimilating ammonia in higher plants has not been firmly established. The results presented in this study show that *A. brasilense* has an effect on GS and GDH activity in *C. vulgaris*. When jointly immobilized at 8 $mg \cdot L^{-1}$ NH_4^+, *C. vulgaris* absorbs more ammonium than *C. vulgaris* alone, for the activity of GS and GDH per cell is higher. However, in cases when only the activity of GS increases (in the experiments with final concentrations of ammonium of 3, 6, and 10 $mg \cdot L^{-1}$ NH_4^+), this higher activity did not yield a higher absorption of ammonium per cell, as a whole. Only at initial 8 $mg \cdot L^{-1}$ NH_4^+ did absorption of ammonium increase. This is the only concentration of ammonium in which absorption per cell or per culture and the population of the microalga was not correlated. Although in the majority of microalgal species, as in other plants, assimilation of ammonium is via the GS/GOGAT pathway, *Chlorella* is known to favor activation of the GDH pathway to ensure ammonium assimilation (Tischner and Lorenzen 1980).

In the system with jointly immobilized microorganisms, GS and GDH were present and able to function. However, only in cases where they function simultaneously did absorption of ammonium from the medium increase, and this was concentration dependent. There are at least four plausible explanations for the importance of GDH activity in the jointly immobilized system for enhancing ammonium absorption in *C. vulgaris,* even though GS activity is higher in all cases.

1. The ''GDH shunt,'' as named by Miflin and Habash (2002) acts when carbon is limited and nitrogen is abundant (Lu et al. 2005). The plant can give priority to carbon metabolism and keto-acid production over nitrogen metabolism. Carbon was somewhat limited for *C. vulgaris* in our experiments, notwithstanding the presence of arabinose, which is a source of carbon for *A. brasilense*, but not for *C. vulgaris* (Myers and Hubbell 1987), and the only source of carbon for growth was by photosynthesis. Only at 8 $mg \cdot L^{-1}$ NH_4^+ was the activity of GDH per cell higher, reflecting the complexity of the regulation and the possibility that it is a concentration-dependent activity.
2. The GDH shunt increases under various stress conditions (Srivastava and Singh 1987). The presence of many *A. brasilense* cells, in close proximity in the bead (Lebsky et al. 2001), can be considered a stressful condition for the microalga. In an earlier study, *C. vulgaris* responded to joint immobilization with *A. brasilense* by producing more lipids and fatty acids in the same manner as when it was under nitrogen stress (de-Bashan et al. 2002b).
3. Our results with the assay of GDH are in the aminating direction, but not in the deaminating direction. This finding suggests that at high availability of nitrogen, GDH is acting in the assimilatory mode, converting ammonium to glutamate. However, this may reflect a laboratory artifact. According to Miflin and Habash (2002), the aminating direction is the easier way to measure GDH activity. Therefore, the presence of the enzyme suggests that it has a role, but not what the role is. In this study, the results suggest an assimilatory role for the enzyme because the high activity leads to higher ammonium absorption.
4. We may assume an auxiliary role of GDH in this system. Only when GS and GDH are functioning together is there higher assimilation of ammonium per cell in the jointly immobilized system.

As stated earlier, there are no reports on the effect of the interaction between bacteria and microalgae on the activity of any of these enzymes. In higher plants, Ribaudo et al. (2001) reported that maize inoculated with *Azospirillum* showed increased GDH and GS activity in root cell free extracts over controls that were not inoculated. In peanuts inoculated with *Bradyrhizobium* sp., GS activity greatly increased in inoculated roots, compared to peanuts that were not inoculated (Terzo et al. 2005).

There are two patterns of ammonium absorption in *C. vulgaris*: (i) Joint immobilization with

A. brasilense enhanced growth of *Chlorella vulgaris* but did not affect absorption of ammonium of the latter. (ii) Joint immobilization enhanced absorption of ammonium by the microalga without affecting the growth of the *Chlorella* population (de-Bashan et al. 2005). With the complexity of these enzymes, it is not yet clear what factor(s) regulate the enzymatic activity. However, three possible factors can be considered: (i) Carbon can act as a regulator of the enzymatic activity. In cases when carbon is limited and nitrogen is in excess, the imbalance in the C/N ratio requires the operation of the GDH shunt because there may be a strong demand to obtain carbon from amino acids. GDH is one of the few enzymes capable of releasing amino nitrogen from amino acids to give keto-acid (oxoglutarate) and NH_3^+ that can be separately recycled for use in respiration and amide formation (Miflin and Habash 2002). In *Arabidopsis* for example, NADH-GDH activity and its gene expression increase with limited carbon (Turano et al. 1997). In any case, the two pathways, acting concomitantly, could provide a mechanism that responds to the different needs of cells for nitrogen and carbon compounds (Miflin and Habash 2002). (ii) It is possible that *C. vulgaris* uses all of the ammonium present in the bead and releases oxygen to the medium. At the same time, the interior of these large beads may become oxygen deficient (O'Reilly and Scott 1995). In these micro-aerophyllic spaces, *A. brasilense* can be fixing nitrogen and releasing it into the bead where *C. vulgaris* resides. As GS is the main enzyme in the assimilation of ammonia derived from nitrogen fixation, it is clear that *A. brasilense* induces higher GS activity. (iii) Indole-3-acetic acid (IAA) production and N_2-fixation in *A. brasilense* are linked in an unknown way through a heat-shock protein codified by the *clpX* gene (Rodriguez et al. 2006). Furthermore, hormones can induce changes in the rate of GS biosynthesis in eukaryotes (Eisenberg et al. 2000). There are indications that IAA is involved in promoting growth of *C. vulgaris* by *A. brasilense* (Gonzalez and Bashan 2000). 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 nitrogen fixation of *A. brasilense*; GS (in this case, of the microalga) is the common user of nitrogen derived from N_2 fixation (Miflin and Habash 2002).

Our data on enzymatic activities are presented in two ways: in traditional enzyme units (containing the total protein content in the equation) and enzyme activity per cell, a common way to express enzyme activity in microalgae (Ahmad and Hellebust 1990, Rees et al. 1995). Presenting data in the traditional enzyme units created confusing conclusions. The total concentration of proteins is proportionally inverted to the total population of the microalga and, furthermore, is independent of the activity of the enzymes. Therefore, presenting data as traditional enzyme units can lead to overestimates or underestimates of the real activity of these enzymes.

In summary, joint immobilization with *A. brasilense* affects the specific activity of GS and GDH in *C. vulgaris,* which affects ammonium assimilation by the microalga, and both are concentration dependent and optimum at 8 $mg \cdot L^{-1}$ ammonium. The results indicate that greater activity of GS and GDH increases absorption of ammonium by *C. vulgaris.*

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