Chapter - III

Nitrogen assimilation by *Azospirillum brasilense*
Azospirillum brasiliense is a nitrogen-fixing, gram-negative soil bacterium capable of growing in the presence of various nitrogen sources, both inorganic and organic. The regulation of nitrogen metabolism of this bacterium has been studied in this work. The amino acids glutamate and glutamine are formed in the bacterial cells as intermediates of the metabolism of all nitrogeneous compounds; thus for studying the nitrogen assimilation in this bacterium, it is necessary to have an understanding of the regulation of the enzymes responsible for the synthesis of glutamate and glutamine. Earlier work with this bacterium showed the presence of the enzymes glutamate dehydrogenase (GDH), glutamate synthase (GOGAT) and glutamine synthetase (GS) in the cells. The presence of a NADH-dependent GDH has been reported by Okon et al. (84) and Bani et al. (81), but their results showed different levels of this enzyme under similar conditions of growth; however, none of these workers found any change in the level of this enzyme in cells grown either with an excess or limiting amount of ammonia. In the case of GS the results of previous workers are not consistent with one another. In one report there was no significant increase in the level of the enzyme in the presence of limiting concentration of ammonia (84) and in another (81) there was about 1.6 fold increase in the level of GS under similar conditions of growth. These inconsistent results
obtained by the previous workers led us to estimate the levels of the enzymes GDH, GS and GOGAT in A. brasilense RG cells more carefully after growing the bacteria with different sources of nitrogen; this was done to find out whether the levels of these three enzymes are controlled by the nitrogen source in the growth medium.

The regulation of synthesis of GS, GOGAT and GDH has been further studied with a number of Nif$^-$ mutants. It was expected that some of the mutants that had been isolated in our laboratory would show altered regulation of ammonia assimilatory enzymes because of some defect in their general nitrogen regulation (Ntr).

Earlier work with Nif$^-$ mutants of A. brasilense has been done by several groups (81-83, 85). Jara et al. (82) have reported the isolation and genetic analysis of a few Nif$^-$ mutants; one of these mutants could be complemented by a plasmid containing the nifHDK sequence of A. brasilense. Bani et al. (81) have shown that in asm$^-$ mutants (mutants lacking the enzyme GOGAT) of A. brasilense, the synthesis of GS cannot be derepressed by ammonia limitation, which they think is because of the accumulation of high levels of glutamine in the cells. According to them the repression of GS in the asm$^-$ mutants is responsible for the Nif$^-$ phenotype. However, in their report on the Nif$^+$ revertants of these mutants (83) the same group of workers have shown that the glutamine levels in the original asm$^-$ mutant as well as the Nif$^+$ revertants were not significantly
different from the parental (wild type) strain. In their report on Nif" mutants of *A. brasilense*, Pedrosa and Yates (85) have suggested the presence of Ntr system and a cascade type regulatory mechanism for nif gene expression in *A. brasilense* as found in *K. pneumoniae* (45). However, they have not shown how the ammonia assimilatory enzymes are regulated in these mutants. The Nif" mutants isolated by them have defects in regulation of GS and utilization of nitrate but the utilization of histidine and proline was normal. Presence of ntrC like gene in *A. brasilense* was suggested because introduction of *K. pneumoniae* ntrC gene by conjugation into these *A. brasilense* Nif" mutants resulted in reversion to Nif" phenotype. The picture of the regulation of nitrogen assimilatory system that emerge from these reports is somewhat different from what is already known about Ntr of *E. coli* or *K. pneumoniae*. In the latter case, a defect in ntrC not only leads to Nif" phenotype but it also prevents the mutants from utilizing other nitrogen sources like arginine or proline.

3.1. Results

3.1.1. Growth patterns of *A. brasilense* RG in the presence of different N-sources

*Azospirillum brasilense* RG can use a variety of nitrogenous compounds as the sole source of nitrogen in the growth medium. In this work, the growth patterns of
Fig. 3.1. Growth pattern of *Azospirillum brasilense* RG in succinate salt (SS) medium containing various nitrogen sources. An overnight culture of cells grown in SS medium containing NH$_4$Cl (0.1%) and yeast extract (0.05%) was washed with and resuspended in normal saline; this cell suspension was used as the inoculum. The nitrogen sources were NH$_4$Cl (0.1% and 0.01%), KNO$_3$ (0.1%), glutamate (0.25%), glutamine (0.25%), proline (0.25%), arginine (0.25%), and casamino acids (0.5%).
the bacteria in various N-sources have been studied. The compounds used as the sole source of nitrogen were NH₄Cl, nitrate, glutamate, glutamine, proline, arginine and casamino acids (Fig. 3.1). The generation times of A. brasilense RG in different growth media had been calculated from plots on semi-logarithmic paper (plots not shown).

Table - 3.1: Generation times of A. brasilense RG in succinate salt medium supplemented with various N-sources.

<table>
<thead>
<tr>
<th>N-source</th>
<th>Generation times (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl (0.1%)</td>
<td>108</td>
</tr>
<tr>
<td>NH₄Cl (0.01%)</td>
<td>108</td>
</tr>
<tr>
<td>KNO₃ (0.1%)</td>
<td>108</td>
</tr>
<tr>
<td>Glutamate (0.25%)</td>
<td>110</td>
</tr>
<tr>
<td>Glutamine (0.25%)</td>
<td>108</td>
</tr>
<tr>
<td>Proline (0.25%)</td>
<td>115</td>
</tr>
<tr>
<td>Arginine (0.25%)</td>
<td>100</td>
</tr>
<tr>
<td>Casamino acids (0.5%)</td>
<td>105</td>
</tr>
</tbody>
</table>

*aConcentrations of the various nitrogen sources are given within parentheses.

Cells grown in the presence of NH₄Cl (0.1%) and yeast extract (0.05%) were used as the inoculum for the growth experiments. The doubling times of A. brasilense RG remained the same in the presence of different N-sources.
However, the growth patterns of the cells were different when different sources of nitrogen were present in the growth media. Cells showed a long period of lag (about 8 hours) in growth when nitrate, glutamate, proline or arginine were present in the medium as the only source of nitrogen. The initial lag in growth was considerably shorter (about 3 hours) when NH₄Cl, glutamine or casamino acids were used as N-source in the medium. The growth pattern of *A. brasilense* RG was also studied in the presence of limiting concentrations of NH₃ (0.01%). The growth of the cells stopped after about 6 hours probably due to the exhaustion of nitrogen source.

3.1.2. Regulation of ammonia assimilatory enzymes in *A. brasilense* RG

The ammonia assimilatory enzymes GDH, GOGAT and GS were measured according to the assay methods described in Section 2.2.4. The cells were grown in the presence of high and low concentrations of ammonia, glutamate, glutamine and casamino acids, and GDH and GOGAT were measured from the crude extracts of the cells (Section 2.2.5.1.). GS activity was measured in the whole cells (Section 2.2.5.2.).
Table 3.2: Specific activities of glutamate dehydrogenase, glutamate synthase and glutamine synthetase (γ-glutamyl transferase activity) in A. brasilense RG cells grown in succinate salt medium containing various N-sources.

<table>
<thead>
<tr>
<th>N-source</th>
<th>GDH</th>
<th>GOGAT</th>
<th>GS Mn²⁺</th>
<th>GS Mn²⁺Mg²⁺</th>
<th>Unadenylated form (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl (0.1%)</td>
<td>0.22</td>
<td>0.046</td>
<td>2.13</td>
<td>0.82</td>
<td>38</td>
</tr>
<tr>
<td>NH₄Cl (0.01%)</td>
<td>0.021</td>
<td>0.075</td>
<td>2.74</td>
<td>2.33</td>
<td>85</td>
</tr>
<tr>
<td>Glutamate (0.25%)</td>
<td>0.015</td>
<td>0.019</td>
<td>4.02</td>
<td>2.53</td>
<td>63</td>
</tr>
<tr>
<td>Glutamine (0.25%)</td>
<td>0.017</td>
<td>0.029</td>
<td>3.45</td>
<td>2.23</td>
<td>65</td>
</tr>
<tr>
<td>Casamino acids (0.5%)</td>
<td>0.009</td>
<td>0.019</td>
<td>2.20</td>
<td>1.40</td>
<td>63</td>
</tr>
</tbody>
</table>

GDH (NADPH-dependent), GOGAT and GS assays were carried out as described in Section 2.2.4. Specific activities are expressed as μmoles of product formed/min/mg protein. Specific activity values are the average of three experiments.

*aConcentrations of the various nitrogen sources are given in parentheses.

3.1.3. Regulation of the level of GDH in A. brasilense RG by the nitrogen source in the growth medium

GDH activity from Agospirillum brasilense had been measured by the earlier workers (81, 84) according to the assay procedure described by Meers et al. (2). The assay procedure of Meers et al. was found to be unsuitable...
for GDH of *A. brasilense* RG. When NADH-dependent GDH activity in the crude extracts was measured according to the method of Meers *et al.*, the results were erratic and irreproducible. A new modified assay method described in Section 2.2.4 was followed where NADPH was used as the cofactor for the reductive amination reaction. GDH of *A. brasilense* RG turned out to be a cold sensitive enzyme. The preparation of the crude extracts was carried out in cold (4°C) in the presence of 10% glycerol (v/v); glycerol was found to protect the enzyme against cold inactivation.

It is evident from Table - 3.2 that the level of GDH in the cells is strongly regulated by the nitrogen source of the growth medium. The specific activity of GDH was ten-fold higher in the extracts of cells grown with excess NH$_4$Cl (0.1%) as compared to the same from cells grown with limiting amount of NH$_4$Cl (0.01%). When glutamate was present in the medium as the only nitrogen source, the level of GDH in the crude extracts was further reduced. Glutamine or casamino acids also caused repression of synthesis of this enzyme. When excess ammonia was replaced by glutamine or casamino acids the enzyme levels were reduced by ten-fold and twenty-fold respectively.

Thus it can be concluded that the amino acids in general exert an inhibitory effect on the synthesis of GDH in *A. brasilense*. As the presence of glutamate in the medium repressed GDH, and high concentration of ammonium favoured its expression, one may conclude that the enzyme serves an
ammonia assimilatory function in _A. brasilense_ RG.

3.1.4. Regulation of the level of glutamate synthase by the N-source in the medium

GOGAT activity was measured according to the method of Meers et al. (2) except that potassium phosphate buffer was used instead of Tris-HCl. The activity of GOGAT was found to be higher in potassium phosphate buffer than in Tris-HCl buffer. It has been found that the specific activity of GOGAT in _A. brasilense_ RG grown in limiting NH$_4^+$ was only about 1.8 times higher than that in cells grown in high NH$_4^+$. It was also observed that glutamate and casamino acids could repress the synthesis of GOGAT by 2.4 fold when they replaced NH$_4^+$ as the N-source for growth; but GOGAT level did not alter appreciably when glutamine replaced NH$_4^+$.

3.1.5. Regulation of the level of GS by the N-source in the medium

The $\gamma$-glutamyl transferase activity of glutamine synthetase of _A. brasilense_ RG was assayed by the method of Miller et al. (89). Minor modifications in the concentrations of various substrates were made for the sake of convenience. In _A. brasilense_ RG, higher levels of glutamine synthetase were produced in the cells when the growth media contained glutamate or glutamine in the place of NH$_4^+$. Low concentration of NH$_4^+$ in the growth medium also favoured higher level of GS synthesis in the bacterium.
In the case of *A. brasilense* RG, adenylylation state of the enzyme (as measured by the γ-glutamyl transferase assay in the presence and absence of 60 mM MgCl₂) was nearly the same (about 40%) whether glutamate, glutamine or casamino acids was used as nitrogen source for growth by the bacterium. About 85% of the enzyme was in the unadenylylated form when the medium contained limiting amount of NH₃ whereas the enzyme remained mainly in the inactive or adenylylated form (38% of the enzyme was active) when excess amount of ammonia was present in the growth medium.

3.1.6. **Isolation of Nif⁻ mutant strains of**

*Azoospirillum brasilense* RG

*A. brasilense* RG cells were mutagenized by the chemical mutagen NMNG at a concentration (50 μg/ml) which produced about 95% killing of the cells. After mutagenesis the cells were grown overnight in SS-medium containing 0.1% NH₄Cl and 0.05% yeast extract. An aliquot of this culture was used to inoculate SS-medium containing 0.1% NH₄Cl - this step was necessary for enrichment of the cells deficient in nitrogenase over the auxotrophic mutants. Next step for enrichment of Nif⁻ mutant was carried out by growing the mutagenized cells in nitrogen-free SS semi-solid medium (i.e. under microaerophilic condition) in the presence of ampicillin and D-cycloserine. Since ampicillin and D-cycloserine cause lysis of dividing cells only, it was
expected that the cells which were able to fix nitrogen and grow in nitrogen-free semi-solid media would be killed by ampicillin and D-cycloserine, whereas those cells which were not able to fix nitrogen would be saved. D-cycloserine was used along with ampicillin because ampicillin is not a very potent bacteriolytic agent for A. brasilense (minimum inhibitory concentration of ampicillin was 500 \mu g/ml for A. brasilense RG). After ampicillin enrichment step, the cells were spread on YNS plates. The wild type nitrogen fixing cells grow into normal size colonies on YNS plates. The minute colonies were picked as presumptive Nif⁻ mutants and these were screened for Nif⁻ phenotype by spotting individual colonies on SS-agar plates one containing 0.1% NH₄Cl and another containing no nitrogen source. Colonies which showed normal growth on NH₄Cl containing plates and no growth on N-free plates were purified and assayed for the nitrogenase activity by the acetylene reduction method.

Table - 3.4: Levels of nitrogenase in the wild type and the Nif⁻ mutants of A. brasilense RG.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific activity of nitrogenase⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. brasilense RG</td>
<td>0.26</td>
</tr>
<tr>
<td>RG755</td>
<td>0.00</td>
</tr>
<tr>
<td>RG846</td>
<td>0.00</td>
</tr>
<tr>
<td>RG760</td>
<td>0.00</td>
</tr>
<tr>
<td>RG747</td>
<td>0.00</td>
</tr>
<tr>
<td>RG124</td>
<td>0.00</td>
</tr>
</tbody>
</table>

⁹ Specific activity of nitrogenase is expressed as \(\mu\)moles of \(\text{C}_2\text{H}_4\) formed/hr/mg protein.
Fig. 3.2. Growth patterns of the wild type and Nif\textsuperscript{-} strains of \textit{Azospirillum brasilense} RG in the presence of NH\textsubscript{4}Cl (0.1%) (A), NH\textsubscript{4}Cl (0.01%) (B), proline (0.25%) (C), arginine (0.25%) (D), glutamine (0.25%) (E), Glutamate (0.25%) (F), casamino acids (0.5%) (G). An overnight culture of cells grown in SS-medium containing NH\textsubscript{4}Cl (0.1%) and yeast extract (0.05%) was centrifuged, washed with and resuspended in normal saline; this cell suspension was used as the inoculum for the growth experiments.
3.1.7. Growth patterns of the Nif" mutants

The growth patterns of Nif" mutants in SS medium containing various nitrogen sources had been studied. The nitrogen sources used were NH₄Cl, nitrate, glutamate, glutamine, proline, arginine and casamino acids.

The growth patterns and the generation times of RG755, RG846, RG747 and RG760 were shown in Fig. 3.2(a–g) and Table 3.4 respectively. Growth pattern of the Nif" mutants in the presence of nitrate as the nitrogen source is not shown as the mutants showed absolutely no growth in this case. The growth patterns of the mutant RG124 in the presence of different N-sources were similar to those of RG747 — hence it was omitted from Fig. 3.2 and Table 3.4.

Table 3.4: Generation times of the Nif" mutants in SS medium supplemented with various nitrogen sources.

<table>
<thead>
<tr>
<th>N-source</th>
<th>Generation times of the mutant strains (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RG755</td>
</tr>
<tr>
<td>NH₄Cl (0.1%)</td>
<td>220</td>
</tr>
<tr>
<td>NH₄Cl (0.01%)</td>
<td>170</td>
</tr>
<tr>
<td>KNO₃ (0.1%)</td>
<td>No growth</td>
</tr>
<tr>
<td>Glutamate (0.25%)</td>
<td>No growth</td>
</tr>
<tr>
<td>Glutamine (0.25%)</td>
<td>162</td>
</tr>
<tr>
<td>Proline (0.25%)</td>
<td>No growth</td>
</tr>
<tr>
<td>Arginine (0.25%)</td>
<td>No growth</td>
</tr>
<tr>
<td>Casamino acids (0.5%)</td>
<td>57</td>
</tr>
</tbody>
</table>
The growth experiments for the Nif\(^-\) mutants were carried out under identical conditions as they were done for the wild type strain. The mutant RG747 was able to utilize various sources of combined nitrogen except nitrate for growth. The pattern of growth of RG747 in growth media containing glutamate, glutamine, casamino acids, arginine, proline and ammonium chloride were identical to that of the wild type strain. The mutant RG124 showed similar pattern of growth as the mutant RG747 (data not shown).

The mutant RG755 was unable to grow on nitrate, glutamate, proline or arginine when these compounds were present as sole sources of nitrogen in the growth medium. The growth rate of this mutant on NH\(_4\)Cl (0.1\%) and glutamine was slower and the same on casamino acids was faster in comparison with that of the wild type strain.

The mutant strain RG846 showed normal growth rates on NH\(_4\)Cl, glutamine and casamino acids. This strain was unable to grow on proline, arginine and nitrate while the presence of glutamate in the growth medium resulted in very slow growth.

The generation times of the mutant RG760 in NH\(_4\)Cl, glutamine and casamino acids were longer than the generation times of the wild type cells grown under similar conditions. RG760 was unable to grow on glutamate, proline, arginine and nitrate.
3.1.8. Levels of ammonia assimilatory enzymes in the Nif⁻ mutants

Table - 3.5: Specific activities of GDH in the Nif⁻ mutants grown in SS medium containing various N-sources.

<table>
<thead>
<tr>
<th>N-source</th>
<th>Specific activities of GDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RG755</td>
</tr>
<tr>
<td>NH₄Cl (0.1%)</td>
<td>0.9</td>
</tr>
<tr>
<td>NH₄Cl (0.01%)</td>
<td>1.1</td>
</tr>
<tr>
<td>Glutamate</td>
<td>ND</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.86</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>0.91</td>
</tr>
</tbody>
</table>

GDH (NADPH-dependent) assay was carried out as described in Section 2.2.4.1.
ND = Not determined.

Table - 3.6: Specific activities of GOGAT in the Nif⁻ mutants grown in SS medium containing various N-sources.

<table>
<thead>
<tr>
<th>N-source</th>
<th>Specific activities of GOGAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RG755</td>
</tr>
<tr>
<td>NH₄Cl (0.1%)</td>
<td>0.037</td>
</tr>
<tr>
<td>NH₄Cl (0.01%)</td>
<td>0.077</td>
</tr>
<tr>
<td>Glutamate (0.25%)</td>
<td>ND</td>
</tr>
<tr>
<td>Glutamine (0.25%)</td>
<td>0.12</td>
</tr>
<tr>
<td>Casamino acids (0.5%)</td>
<td>0.38</td>
</tr>
</tbody>
</table>

GOGAT assay was carried out as described in Section 2.2.4.2.
ND = Not determined.
Table 3.7: Levels of GS in the Nif\textsuperscript{−} mutants grown in SS medium containing various N-sources.

<table>
<thead>
<tr>
<th>N-source</th>
<th>Specific activities of GS</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RG755 (Mn\textsuperscript{++})</td>
<td>RG846 (Mn\textsuperscript{++})</td>
<td>RG124 (Mn\textsuperscript{++})</td>
<td>RG760 (Mn\textsuperscript{++})</td>
<td>RG747 (Mn\textsuperscript{++})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH\textsubscript{4}Cl (0.1%)</td>
<td>0.149</td>
<td>0.056</td>
<td>0.27</td>
<td>0.21</td>
<td>1.47</td>
<td>0.55</td>
<td>0.98</td>
</tr>
<tr>
<td>NH\textsubscript{4}Cl (0.01%)</td>
<td>0.137</td>
<td>0.155</td>
<td>0.23</td>
<td>0.66</td>
<td>2.1</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Glutamate (0.25%)</td>
<td>ND</td>
<td>ND</td>
<td>0.61</td>
<td>0.71</td>
<td>1.3</td>
<td>0.97</td>
<td>ND</td>
</tr>
<tr>
<td>Glutamine (0.25%)</td>
<td>0.405</td>
<td>0.17</td>
<td>0.71</td>
<td>0.87</td>
<td>1.6</td>
<td>1.1</td>
<td>ND</td>
</tr>
<tr>
<td>Casamino acids (0.5%)</td>
<td>0.167</td>
<td>0.29</td>
<td>0.46</td>
<td>1.0</td>
<td>1.23</td>
<td>0.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(\gamma\)-glutamyl transferase assay for GS was carried out as described in Section 2.2.4.3.

ND = Not determined.
3.1.9. Regulation of synthesis of the enzymes GDH, GOGAT and GS in the Nif<sup>−</sup> mutants

The Nif<sup>−</sup> mutants RG755 and RG846 showed almost similar patterns of regulation of the ammonia assimilatory enzymes. Both these mutants showed constitutive synthesis of high levels of GDH and low levels of GS. The GOGAT levels in the mutants were almost similar to those found in the wild type strain when excess or limiting concentrations of NH<sub>4</sub><sup>+</sup> was used as N-source for growth. However, in the presence of glutamine and casamino acids, GOGAT synthesis was not repressed as found in the case of wild type cells.

The mutants 747 and 124, showed normal regulation of synthesis of the enzymes GS, GOGAT and GDH in response to different N-sources in the growth medium. In the mutant 760, the enzyme levels were measured only in cells grown with the excess and limiting concentration of NH<sub>4</sub><sup>+</sup>. The mutant showed defects in the regulation of synthesis of GS and GDH. GS remained in adenylylated or inactive form even when the mutant cells were grown with limiting NH<sub>4</sub><sup>+</sup>. GDH level in this mutant, on the other hand, did not increase when the cells were grown with excess NH<sub>4</sub><sup>+</sup>. The GOGAT levels in RG760 grown in the presence of excess or limiting concentrations of NH<sub>4</sub><sup>+</sup> were the same as those found in the wild type cells.

It is evident that the mutants RG755, RG846 and RG760 have some defect in the regulation of synthesis of ammonia assimilatory enzymes GDH and GS, and in the utilization or
transport of the nitrate and amino acids, glutamate, proline and arginine. The mutant RG747 and RG124 do not show any defect in regulation of the enzymes GS or GDH or in the utilization of the amino acids, glutamate, proline and arginine.

On the basis of the pattern of regulation of the enzymes GS and GDH the Nif" mutants have been classified into three groups:

Table-3.8: Classification of Nif" mutants.

<table>
<thead>
<tr>
<th>Group</th>
<th>Nif&quot; mutants</th>
<th>Phenotype of the cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class-I</td>
<td>RG755 and RG846</td>
<td>Constitutive synthesis of high levels of GDH and low levels of GS. No growth on nitrate, proline, glutamate, arginine.</td>
</tr>
<tr>
<td>Class-II</td>
<td>RG747 and RG124</td>
<td>Normal regulation of synthesis GS and GDH. Normal growth on proline, glutamate, arginine. No growth on nitrate.</td>
</tr>
<tr>
<td>Class-III</td>
<td>RG760</td>
<td>No induction of GS and GDH synthesis by limiting ammonia and excess ammonia respectively in the media. No growth on proline, arginine, glutamate and nitrate.</td>
</tr>
</tbody>
</table>
3.2. Discussion

Synthesis of a large number of bacterial proteins such as the enzymes for ammonia assimilation, amino acid transport proteins and amino acid degradative enzymes and the enzymes and accessory components for nitrogen fixation is regulated by the availability of combined nitrogen in the growth medium. In enteric bacteria, extensive work has been done on the effect of nitrogen source on the induction or repression of several enzymes. In K. pneumoniae it has been found that the expression of hut (histidine utilization) operon, put (proline utilization) operon, ureA (urease) gene, nif (nitrogen fixation) regulon and glnA (glutamine synthetase) gene are repressed when the cells are grown in the presence of ammonia; the expression of these genes are induced when the ammonia concentration is limiting in the growth medium or when the cells were grown on a poor source of nitrogen (3). All the above genes are regulated by the general nitrogen regulatory or ntr system, the details of which has been extensively studied by genetic analyses. In the enteric bacteria K. pneumoniae and K. aerogenes, the synthesis of glutamate dehydrogenase is controlled in a way opposite to that of the enzymes belonging to the general nitrogen regulation system; mutants showing a Ntr⁻ phenotype show altered regulation of GDH.

In A. brasilense, although the presence of Ntr system has been demonstrated by Pedrosa and Yates (85), the
details of the Ntr system in this bacterium has not been elucidated. They have shown that some of their Nif\textsuperscript{−} mutants were unable to grow on nitrate and showed low levels of GS; these mutants were complemented for nitrogenase activity and growth on nitrate when K. pneumoniae nif\textsuperscript{A} or ntrC gene was introduced as a plasmid gene by conjugation. The authors suggested that the ntrC like gene product of A. brasilense was not required for the activation of N-assimilating system other than those required for the utilization of N\textsubscript{2} and NO\textsuperscript{−}.

Studies with A. brasilense RG shows that the activity of GS and the synthesis of GDH are very strongly regulated by the availability of NH\textsubscript{3} in the medium. The growth patterns of A. brasilense RG in different nitrogen sources are distinctly different. Cells previously grown in the presence of NH\textsubscript{4}Cl as the nitrogen source showed an initial lag of about 7.5 hours when grown in the presence of nitrate, glutamate, proline or arginine, and a lag of 3-4 hours in the presence of glutamine or casamino acids. The longer lag in growth could be because of the fact that in the cells, initially grown in the presence of ammonium, the transport or utilization systems for the nitrate and the amino acids glutamate, proline and arginine remained repressed. Thus it appears that the enzymes glutamine synthetase, glutamate dehydrogenase and the proline, arginine and nitrate utilization systems of A. brasilense RG are regulated by the nitrogen source in the growth medium.
A nitrogen regulatory or Ntr system similar to that reported in the case of *K. pneumoniae* and *K. aerogenes* may be present in *A. brasilense* too.

The presence of Ntr system in *A. brasilense* RG was further substantiated by studies with the Nif" mutants. The mutants that have been classified as regulatory mutants (Class-I and Class-III) showed an altered pattern of growth in different N-sources and altered pattern of induction of GDH and GS.

The mutants belonging to Class-I showed very low levels of total GS enzyme in the cells (10-20% of that found in the wild type). Unregulated synthesis of high levels of GDH occurs in the presence of various nitrogen sources in the growth medium. It has been already established that in the case of *A. brasilense*, the regulation of GS and GDH synthesis is almost similar to that in *K. aerogenes* and *K. pneumoniae*. Thus this group of mutants can be called Ntr" mutants although the ntr genes of *A. brasilense* still remain unknown. That these mutants are defective in general nitrogen regulation is also evident from the growth pattern of these mutants in various nitrogen sources. The cells showed poor growth in proline, arginine and nitrate. The transport and utilization of the amino acids proline and arginine have been found to be subject to Ntr in the case of *K. pneumoniae* and *K. aerogenes* (31). The growth characteristics of this class of mutants confirm that these amino acid utilization systems are subject to nitrogen
regulation (Ntr) also in *A. brasilense*. There have not been any earlier report on these types of mutants in *A. brasilense* or in any *N₂*-fixing bacteria. It appears that these mutants might have lesions in the *ntrC* or *ntrA* like genes which are responsible for the activation of Ntr system. In the mutant RG760, the enzyme GS showed little or no deadenylylation even in the presence of limiting concentration of NH₄⁺ in the growth medium. This type of mutant has not been reported earlier in the case of *A. brasilense*. This could be due to a defect in the *glnB* or *glnD* like gene whose product assesses the level of ammonia present in the cell. This mutant also showed inability of induction of GDH synthesis in the presence of excess ammonia. This mutant is essentially Ntr⁻ in phenotype as the cells were unable to utilize proline, arginine, glutamate or nitrate. Mutants having lesions in *glnB* or *glnD* in *K. aerogenes* and *K. pneumoniae* revert at a high frequency to give GlnC and NtrC phenotypes. Whether this also occurs in the case of *A. brasilense* has not been studied.

The mutants RG747 and RG124 showed normal growth on all nitrogen sources except nitrate. The levels of ammonia assimilatory enzymes in the presence of different nitrogen sources were similar to those found in wild type cells under similar conditions. Thus these cells show defects only in nitrogen fixation and utilization of nitrate. It has been suggested earlier by Brill (95) that defects in the transport of molybdenum into the cells may result in an
inability of the cells to fix nitrogen and to utilize nitrate as both nitrogenase and nitrate reductase are molybdenum co-factor containing enzymes. There are certain nitrate reductase deficient mutants of *E. coli* (NarD−) that are unable to fix N₂ when they carry a nif containing plasmid unless high levels of MoO₄²⁻ are added (96). Thus it may be suggested that the mutants RG747 and RG124 have defects in the molybdenum transport system.