Effect of glutamate transport and catabolism on symbiotic effectiveness in *Rhizobium leguminosarum* bv. *phaseoli*

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Abstract

Seven Tn*5* induced mutants unable to use glutamate as sole carbon and nitrogen source were isolated from the effective *Rhizobium leguminosarum* bv. *phaseoli* strain P121-R. As indicated by restriction and hybridisation analysis, all the mutants arose from a single Tn*5* insertion in the chromosome. The ^{14}C-glutamate uptake rate of the mutants was 76 to 88% lower than that of strain P121-R. Inoculation of *Phaseolus vulgaris* cv. Labrador with these mutants significantly decreased shoot dry matter yield and the total nitrogen content respectively, as compared to inoculation with the parental strain P121-R. All the mutants formed nodules, however they were smaller, white to greenish and approximately 30% less numerous than those formed by strain P121-R. These observations suggest that glutamate transport and catabolism in *R. leguminosarum* bv. *phaseoli* P121-R may play an important role in the establishment of an effective symbiosis in field bean. None of the mutants isolated was an auxotroph. All mutants were unable to grow on aspartate suggesting that glutamate and aspartate, probably have the same transporter as indicated in *Rhizobium meliloti* and in *Bacillus subtilis*. All mutants readily used glutamine, proline, arginine as sole carbon and nitrogen source, but grew more slowly than the wild type strain. On the other hand, all the mutants were impaired in growth on histidine and γ-aminobutyrate as sole carbon and nitrogen source. As the catabolism of these amino acids occurs predominantly through glutamate, our results indicate that mutants are also impaired in their ability to use histidine and γ-aminobutyrate as a nitrogen source. Our results also suggest that other amino acids catabolized through the glutamate pathways may be an additional important carbon source for bacteroids in nodules.

Introduction

Rhizobia and bradyrhizobia are gram-negative bacteria that can fix atmospheric nitrogen within the root nodules of their symbiotic legume partners. Many data are consistent with the hypothesis that N_2 fixation is not limited by photosynthate, but rather by utilization of carbon within the nodule (Vance and Heichel, 1991). Until now the exact nature of the carbon source supplied by the plant to the nitrogen fixing bacteroids in nodules remains an unresolved issue. Generally in rhizobia, extensive evidence indicates that the four-carbon dicarboxylic acids are a major source of

reducing equivalents to the nitrogen fixing bacteroids (Streeter, 1991; Werner, 1992). However, many recent reports suggest that other carbon sources may also play a major role in the nitrogen fixing process in nodules. For example, a mutant of *Rhizobium meliloti* lacking aspartate aminotransferase activity, required for aspartate catabolism, was found to be Fix⁻, indicating that aspartate might be an essential substrate for bacteria in nodules (Rastogi and Watson, 1991). Watson et al. (1993) showed that there was one transport system in *Rhizobium meliloti* for both aspartate and glutamate, regulated independently from the Dct system. Tolner et al. (1995) also demonstrated the existence of a similar transporter for both glutamate and aspartate in *Bacillus*

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subtilis. Kahn et al. (1985) suggested a model in which an amino acid such as glutamate carries carbon and energy into the bacteroids. This possibility was supported by Salminen and Streeter (1987) who showed uptake, incorporation and respiration of exogenous ^{14}C-glutamate by bacteroids from soybean nodules. By using a different experimental system, Bergersen and Turner (1988) also observed that both O_2 consumption and N_2 fixation by soybean bacteroids may be stimulated by exogenous glutamate. Glutamate catabolic activity was detected in both the free-living and bacteroid forms of *R. meliloti* indicating that this amino acid may be implicated as an additional carbon source for effective nitrogen fixation (Fitzmaurice and O'Gara, 1991). In fact a Fix^- mutant of *R. meliloti* defective in the degradation of glutamate was isolated (Fitzmaurice and O'Gara, 1993). This mutant lacked the functional γ-aminobutyrate (GABA) bypass, a glutamate degradation pathway possibly playing an important role in the symbiotic nitrogen fixation process. The present work presents results obtained with some Tn5 induced mutants of *R. leguminosarum* bv. *phaseoli* showing that glutamate or other amino acids using its catabolic pathways are probably major additional carbon sources for bacteroids in field bean (*Phaseolus vulgaris* L.) nodules.

Materials and methods

Bacterial strains and growth conditions

Strain P121-R is a stable derivative of the very effective strain of *Rhizobium leguminosarum* bv. *phaseoli* P121 (Lalande et al., 1986), resistant to 50 μg mL^{-1} of rifampicin and obtained as described by Chao et al. (1986). Rhizobia were cultured at 30°C on TY media (Beringer, 1974) or on BM_1 minimal media (Lafrenière et al., 1987) modified by using 200 μg L^{-1} of biotin and 200 μg L^{-1} of thiamine and containing 5 g L^{-1} of filter sterilized (0.22 μm pore size) carbohydrates or amino acids as sole carbon source. When an amino acid was tested as a sole carbon and nitrogen source, BM_1 media was prepared without nitrate. *Escherichia coli* S17.1 and SM10 were grown on LB media (Maniatis et al., 1982) at 37°C. Solid media were prepared by adding 15 g L^{-1} of agar or Noble agar (Difco) for nutritional studies. Antibiotics were used in solid media at the following final concentrations (μg mL^{-1}): chloramphenicol, 20; kanamycine, 100; neomycin, 100; rifampicine, 50.

Transposon mutagenesis and isolation of mutants

Tn5-derivatives were prepared by conjugal transfer of pSUP2021 to strain P121-R as described by Simon et al. (1983). Transconjugants were subjected to three carbenicillin enrichment cycles in BM_1-glutamate, (Lafontaine et al., 1989), and glutamate negative mutants were isolated by replica plating (accoutran replica plater, Schleicher and Schuell) of the transconjugants on BM_1-glucose-nitrate and BM_1-glutamate without nitrate and containing rifampicin and kanamycine. Presumptive glutamate negative mutants selected are transconjugants showing a good growth after seven days on BM_1-glucose-nitrate and no growth or a very weak growth on BM_1-glutamate medium.

Nutritional studies

To determine the utilization of different carbon sources by strain P121-R and by the isolated mutants, washed cells used as inocula were prepared as previously described (Antoun et al., 1984). Using a replica plater (8 × 6 array, Sigma) inocula were placed in alternate wells of sterile Elisa multiple well plates, and on BM_1 plates with different carbon sources supplemented with rifampicin and kanamycine. The plates were incubated at 30°C and growth was observed after 7 days. Some confirmatory tests were performed in 50 mL Erlenmeyer flasks containing 10 mL of BM_1-carbohydrate media with antibiotics. The flasks were incubated on a rotary shaker at 30°C and growth was monitored daily.

DNA isolation and Southern hybridization

Total DNAs from the mutants and wild type were isolated by standard procedures (Maniatis et al., 1982), digested with *Eco*R1, separated by electrophoresis on 0.6% agarose gel and then transferred and covalently linked to a nylon High-bond N membrane (Amersham Corp.) as previously described by Laberge et al. (1989). DNA probe labelled with ^{32}P for hybridization (internal *Hind*III fragment of Tn5 isolated from pSUP2021) was prepared with the nick translation system of Gibco BRL (Burlington, Ontario). To determine if the Tn5 insertion occurred in plasmids, Southern blot of an Eckhardt gel containing the lysate of all the mutants was also hybridized with the Tn5 probe.

Transport assays

For transport assays, cells were cultivated on BM_1-glucose-nitrate, washed twice in phosphate buffer saline and resuspended in BM_1 without carbon source as described by Bigwaneza et al. (1993). Transport assays were performed at 25 °C as described previously (Bigwaneza et al., 1991). The reaction mixture (1500 μL) contained 1350 μL of cells suspended in BM_1 without carbon source and 150 μL of L-[^{14}C(U)]-glutamic acid (NEN Research Products, DuPont Canada Inc., Mississauga, Ontario), or 150 μL L-[^{14}C(U))-aspartic acid (Amersham Canada), The final concentration of labelled glutamate or aspartate was 100 μM. The specific activity in the reaction mixture was 1 μCi $\mu mole^{-1}$ for glutamate and 0.2 μCi $\mu mole^{-1}$ for aspartate. The rates of transport were calculated from the amount of radioactivity accumulated by 250 μL of the cells at 2 minutes intervals during 10 minutes.

Plant tests

The symbiotic properties of strain P121-R and of the mutants were assessed as previously described (Lalande et al., 1986), using *Phaseolus vulgaris* cv. Labrador grown in nitrogen free nutrient solution. Seeds were surface sterilized by soaking for 30 seconds in 95% ethanol, and 8–10 minutes in 6% sodium hypochlorite, followed by 10 rinses in sterile distilled water. Plastic pots (11 cm diameter) were sterilized with a 1.4% dimethyl benzyl ammonium chloride solution (Sanitizer no. 1, Oakite Products of Canada, Bramalea, Ontario), and filled with an autoclaved mixture of 1 volume sand and 2 volumes vermiculite. Each pot received 5 seeds, and 200 mL of a nitrogen free Hoagland's nutrient solution (Bordeleau et al., 1977). Plants were thinned to 2 per pot one week after sowing and they were inoculated by adding 40 mL sterile nutrient solution containing approximately 10^8 mL^{-1} washed bacterial cells (initially grown on BM_1-glucose-NO_3). Uninoculated controls received 40 mL of sterile nutrient solution without bacteria. One week later, a second inoculation was performed. Plants were watered with sterile distilled water when required and twice a week received nutrient solution. Plants were harvested at anthesis at 42 days. Dry matter yields and plant total-N content were measured as previously described (Bordeleau et al., 1977). Plants were grown under a 12 h light period (150–250 μEm^{-2} s^{-1}) and a 12 h darkness at 18–22 °C. The experimental design was a randomized complete block with 4 replicates.

Results

Isolation of the glutamate mutants

After 2–3 carbenicillin enrichment cycles, the screening of 12,000 clones yielded some mutants unable to grow or showing very poor growth after 7 days on solid BM_1-glutamate minimal media containing glutamate as sole carbon and nitrogen sources while the wild type strain showed dense white colonies after only three days. Mutants G14, G20, G21, G22, G23, G24 and G25 were chosen for further studies, because they showed no growth on BM_1 glutamate liquid medium after 7 days incubation while the parent strain P121-R showed a dense growth.

Physical analysis of the Tn5 insertions in the mutants

Restriction and hybridization analysis (data not shown) indicated the presence of one *Eco*RI fragment hybridizing to the Tn5 probe in all mutants selected, indicating that they arose from a single transposition event. In addition, a Southern blot of an Eckhardt gel of all the glutamate mutants (data not shown) demonstrated that the Tn5 insertion was not located on any of the symbiotic plasmids, and the insertion was therefore assumed to be chromosomal.

Symbiotic properties of the mutants

After 6 weeks of growth under controlled conditions, all bean plants inoculated with the glutamate negative mutants formed ineffective fix⁻ associations (Figure 1). All the mutants formed nodules, however they were smaller, white to greenish and approximately 30% less numerous than those formed by strain P121-R (Figure 1). The shoot dry matter yield of bean inoculated with all these mutants was significantly lower than that obtained with strain P121-R (Figure 2A). For all the mutants the shoot dry matter yield is about half of that of plants inoculated with P121-R and was not significantly different from the uninoculated control. Similar results were observed with the plant total nitrogen content (Figure 2B).

Glutamate transport

As indicated by the transport rates, all the mutants were unable to take up ^{14}C-glutamate or ^{14}C-aspartate at the same rate as P121-R (Figure 3). In fact, the percentage of glutamate uptake of the mutants as compared
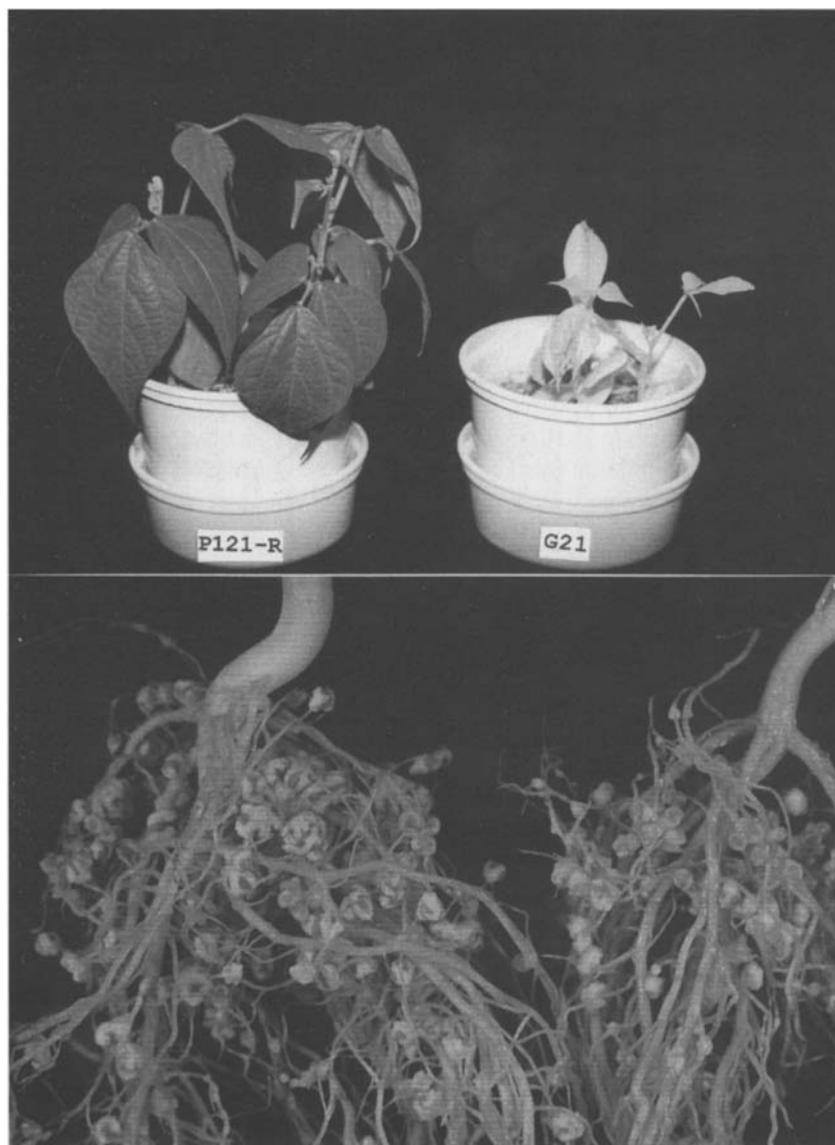
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Figure 1. On the left, Fix⁺ phenotype of *Phaseolus vulgaris* cv. Labrador plants inoculated with the effective wild type strain P121-R of *Rhizobium leguminosarum* bv *phaseoli*. Nodule formed (below) are big and pinkish. On the right, the Fix⁻ phenotype produced by the ineffective glutamate mutant G21. Plants are yellow and they form less nodules (below) that are smaller and whitish.

to strain P121-R during 10 minutes vary from 12% for G21 to 24% for G25. The aspartate uptake of the mutant G21 was also reduced at the same level as the glutamate uptake (10%).

Catabolism of different carbon sources

Like the parental strain P121-R in minimal agar media, all the mutants were able to use the following compounds as sole carbon source: arabinose, glucose, mannitol, pyruvate, fumarate, malate, succinate, and α-2-oxoglutarate.

The utilization of different amino acids as sole carbon and nitrogen sources by the mutants isolated is presented in Table 1. All the mutants were able to catabolize glutamine, proline and ornithine as sole carbon and nitrogen sources, but colonies were smaller than the wild type strain. All the mutants isolated were impaired in Gaba, aspartate, arginine, histidine, valine, phenylalanine and tryptophane utilization, but were not
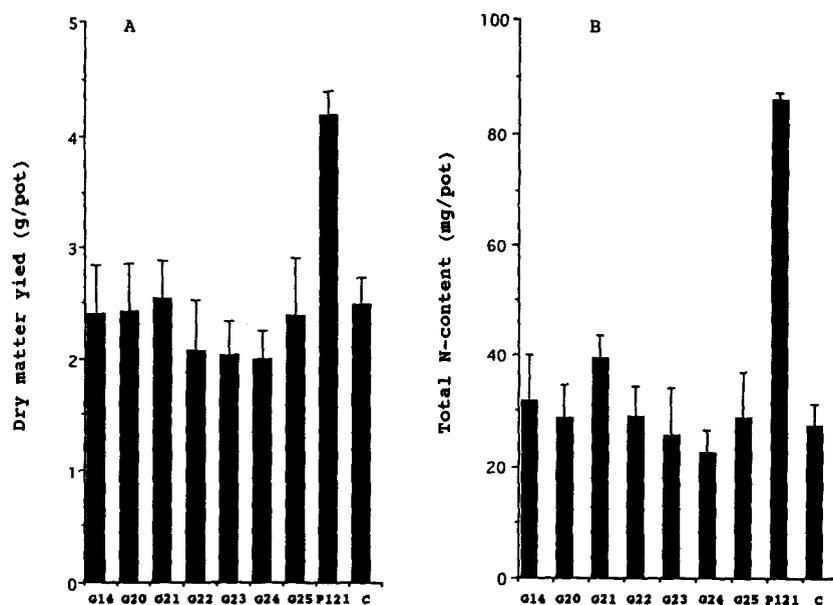
Figure 2. Variation in shoot dry matter yield (A) and total nitrogen content (B) of *Phaseolus vulgaris* L. inoculated with *Rhizobium leguminosarum* bv. *phaseoli* strain P121-R and its glutamate negative mutants G14, and G20 to G25. C is the uninoculated control. Values are means of 4 replications. Bars indicate the values of the standard deviation.
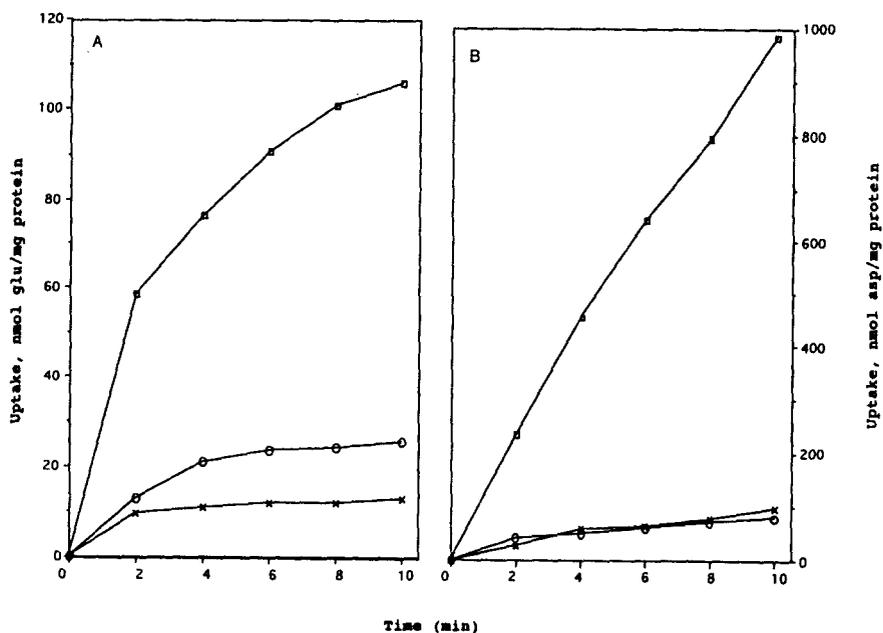

Figure 3. Variation in ^{14}C-glutamate (A) and ^{14}C-aspartate (B) uptake rates during 10 min. of *Rhizobium leguminosarum* bv. *phaseoli* strain P121-R (-□-) and its negative glutamate mutants G21 (-x-) and G25 (-○-). Strains were grown in minimal medium (Lafrenière et al., 1987) with glucose as the carbon source and nitrate as the nitrogen source. Uptake was assayed by using 100 μM aspartate or glutamate and ^{14}C-labelled substrates. Each value is the mean of at least 2 replications.

affected in the catabolism of alanine and asparagine except for G14 (Table 1). In liquid nitrate medium the mutants G20 and G14 were able to use Gaba as sole carbon source, while the mutants G21 and G22 showed very weak growth and mutants G23, G24 and G25 showed no growth.

Table 1. Utilization of some amino acids as sole carbon and nitrogen sources by the glutamate mutants of strain P121-R of *Rhizobium legumi-nosarum* bv *phaseoli*

Mutant	Gaba[a]	Asp	His	Ala	Val	Phe	Try	Asn
G14	-[b]	-	-	-	-	-	-	-
G20	-	-	-	+	-	-	-	+
G21	-	-	-	+	-	-	-	+
G22	-	-	-	+	-	-	-	+
G23	-	-	-	+	-	-	-	+
G24	-	-	-	+	-	-	-	+
G25	-	-	-	+	-	-	-	+
P121-R	+	+	+	+	+	+	-	+

[a] Asp-aspartate, His-histidine, Ala-alanine, Val-valine, Phe-phenylalanine, Try-tryptophane, Asn,-asparagine.
[b] -, no growth and +, growth after 7 days incubation. All the mutants were negative on glutamate, arginine and positive on glutamine, proline, ornithine.

Discussion

We have described the isolation of 7 mutants of *R. leguminosarum* bv. *phaseoli* unable to catabolize glutamate as sole carbon and nitrogen source on agar minimal medium. The fact that the Tn5 insertion was chromosomal and not located on any of the symbiotic plasmids indicates that the symbiotic genes were not affected and that the mutation is probably metabolic. It is important to notice here the difficulty to obtain glutamate catabolic mutants because of the many ways by which this amino acid is catabolized in rhizobia. In fact, the catabolism of glutamate in rhizobia could occur via the tricarboxylic acic cycle following conversion of glutamate to 2-oxoglutarate by glutamate dehydrogenase. Another potential route is the γ-aminobutyrate shunt, which leads to succinate formation. Glutamate catabolism to proline or via glutamic semialdehyde to arginine is also a possibility (Fitzmaurice and O'Gara, 1991; Salminen and Streeter, 1990). In liquid minimal medium with nitrate and glutamate as sole carbon source, all mutants showed no growth after 11 days incubation while the parental strain P121-R completed its growth in 7 days. Inoculation of *Phaseolus vulgaris* with these mutants significantly decreased the shoot dry matter yield and the total nitrogen content respectively, as compared to inoculation with the parental strain P121-R. With the exception of mutant G21, all plants inoculated with the mutants had a total N content similar to that of the uninoculated control (Figure 2B), confirming their Fix⁻ phenotype. Both experimental systems used by Salminen and Streeter (1987) and

Bergersen and Turner (1988) to elucidate the importance of glutamate in biological nitrogen fixation of the symbiosis between *Bradyrhizobium japonicum* and *Glycine max*, support the proposed mechanism of Khan et al. (1985), but do not prove that it operates in vivo in nodules. Bergersen and Turner (1988) indicated that a confirmatory study of the nitrogen fixing capacity of nodules produced by mutants of rhizobia which are unable to utilize glutamate is required to confirm their results. Our results present a contribution to elucidate the role of glutamate in biological nitrogen fixation. These observations also suggest that glutamate transport and catabolism in *R. leguminosarum* bv. *phaseoli* P121-R may play an important role in the establishment of an effective symbiosis in field bean.

All the mutants showed reduced glutamate and aspartate transport activities during 10 minutes as compared to the wild type strain and this reduction ranged from 76% (G25) to 88% (G21) for glutamate and about 90% (G21) for aspartate. The fact that the transport of both glutamate and aspartate were similarly reduced indicates the existence of the same transporter for both glutamate and aspartate in *Rhizobium leguminosarum* bv. *phaseoli* as was previously observed in *Rhizobium meliloti* (Watson et al., 1993) and *Bacillus subtilis* (Tolner et al., 1995). The use of the carbonyl cyanide m-chlorophenylhydrazone (CCCP) as an inhibitor to the glutamate transport showed that about 10% of the transport monitored occur passively (data not shown). These results indicate that G21 is totally blocked in the glutamate transport and that the other mutants are also severely altered in this activity. Our data also show, that

the glutamate transport is an active process requiring energy and confirm the result of Poole et al. (1985). Consequently, glutamate transport and its utilization by *Rhizobium leguminosarum* bv. *phaseoli* may be an important factor to support symbiotic nitrogen fixation in *Phaseolus vulgaris* L..

None of the mutants isolated were auxotrophs. All the mutants like their parent strain P121-R were able to grow on succinate, malate and fumarate as sole carbon source, therefore excluding the possibility of the loss of symbiotic effectiveness resulting from mutations in the *dct* genes (Lafontaine et al., 1989; Streeter, 1991; Werner, 1992).

All mutants readily used glutamine and proline as sole carbon and nitrogen source but slower than the wild type strain. However, under the same conditions all the mutants were impaired in growth on arginine, histidine, aspartate and Gaba. As the catabolism of these amino acids occurs predominantly through glutamate, our results indicate that the mutants are unable to use histidine or Gaba as sole nitrogen source, but are able to use them as sole carbon source. In fact, in minimal liquid media with nitrate, mutants G14 and G20 were able to catabolize Gaba; mutants G21 and G22 showed very weak growth and mutants G23, G24 and G25 were unable to grow. Our results indicate that in *R. leguminosarum* bv. *phaseoli*, functional catabolic enzymes of γ-aminobutyrate and succinic semialdehyde may be essential for effective symbiotic nitrogen fixation as was show for *R. meliloti* (Fitzmaurice and O' Gara, 1993)

The data presented in this work show that in *Rhizobium leguminosarum* bv. *phaseoli*, glutamate or other amino acids (i.e. aspartate, histidine, Gaba) implicated in the glutamate catabolic pathways could be important carbon sources for the bacteroids in nodules, in addition to organic acids. Our work also corroborate previous observations made with *Rhizobium meliloti* showing the importance of glutamate (Fitzmaurice and O'Gara, 1991,1993; Kahn et al., 1985 or aspartate (Rastogi and Watson, 1991) catabolism for the establishment of an effective symbiosis.

Acknowledgements

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