Glutamine Utilization by *Rhizobium etli*

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Submitted 20 October 2003. Accepted 2 February 2004.

We undertook the study of the use of glutamine (Gln) as the source of carbon and energy by *Rhizobium etli*. Tn5-induced mutagenesis allowed us to identify several genes required for Gln utilization, including those coding for two broad-range amino acid transporters and a glutamate dehydrogenase. The isolated mutants were characterized by the analysis of their capacity i) to grow on different media, ii) to transport Gln (uptake assays), and iii) to utilize Gln as the C energy source (CO₂ production from Gln). We show that Gln is degraded through the citric acid cycle and that its utilization as the sole C source is related to a change in the bacterial cell shape (from bacillary to coccoid form) and a high susceptibility to a thiol oxidative insult. Both these data and the analysis of *ntr*-dependent promoters suggested that Gln-grown bacteria are under a condition of C starvation and N sufficiency, and as expected, the addition of glucose counteracted the morphological change and increased both the bacterial growth rate and their resistance to oxidative stress. Finally, a nodulation analysis indicates that the genes involved in Gln transport and degradation are dispensable for the bacterial ability to induce and invade developing nodules, whereas those involved in gluconeogenesis and nucleotide biosynthesis are strictly required.

Rhizobia (including the bacterial genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*) are α -proteobacteria found as free-living cells in the soil or as intracellular symbionts in root nodules induced on legumes. Biochemical and genetic evidence (including the symbiotic phenotype of amino acid-requiring mutants), indicates that during the initial stages of the symbiotic interaction, the plant host supplies invading bacteria with amino acids that may be used not only as a source of N but also as a source of C and energy (Patriarca et al. 2002). At later stages of the symbiosis, the plant instead supplies nondividing (N₂-fixing) bacteroids (the intracellular form of rhizobia), mainly with intermediates of the TCA (tricarboxylic acid) cycle, such C4-dicarboxylic acids (malate, fumarate, or succinate) that are used as sources of energy (Dunn 1998; Poole and Allaway 2000), although the phenotype of bacterial mutants

altered in amino acid transport indicates that amino acid cycling is essential for N fixation in the *Rhizobium leguminosarum*-*Pisum sativum* symbiosis (Lodwig and Poole 2003; Lodwig et al. 2003).

Previous studies have shown that free-living rhizobia, including *Rhizobium etli*, are able to utilize amino acids, including asparagine (Asn), histidine, or glutamine (Gln), as a sole source of C and energy (Dilworth and Glenn 1982; Duran and Calderon 1995). Among these, Gln is of particular interest, since it is the final product of NH₄⁺ assimilation, and its intracellular level is a measure of the N status of the cells (Merrick and Edwards 1995; Patriarca et al. 2002). In fact, the expression of nitrogen assimilation and catabolism genes is regulated by the global nitrogen regulatory system as a function of the intracellular α -ketoglutarate/Gln ratio. The importance of Gln is indicated also by the presence of alternative pathways for both biosynthesis and degradation. For instance, in *R. etli*, Gln synthesis from NH₄⁺ and glutamate (Glt) may be catalyzed by three different Gln synthetase isoenzymes (Espin et al. 1994), while Gln degradation may occur either by hydrolytic deamidation (yielding NH₄⁺ plus Glt) or by the transaminase- ω -amidase pathway (yielding glycine, alanine, α -ketoglutarate, and NH₄⁺) (Duran and Calderon 1995). Moreover, Gln synthesis and degradation may operate simultaneously, thus generating a Gln cycle at the expense of ATP (Encarnacion et al. 1998; Mora 1990).

To date, only a few bacterial species other than rhizobia, for instance, *Bordetella bronchiseptica* (Plotkin and Bemis 1998), *Helicobacter pylori* (Stark et al. 1997), and *Pseudomonas aeruginosa* (Lu and Abdelal 2001), have been described as being able to grow using Gln as the sole C source. However, the genes required to make use of Gln, as well as the physiological consequence of its utilization, are largely unknown. Thus, with the aim of defining this process, we isolated mutant strains of *R. etli* defective in Gln utilization and identified the corresponding mutated genes. The isolated mutants were characterized by analyzing their capacity to grow on different media, to transport Gln (uptake assays), and to utilize Gln as C and energy source (CO₂ production). Moreover, the consequence of the use of Gln as the sole C source on bacterial growth rate, cellular morphology, and oxidative stress were analyzed. Furthermore, to gain insight into the C/N status of Gln-grown cells, the analysis of *ntr*-dependent promoters, performed in different genetic backgrounds and under different growth conditions, was included. Finally, the ability of the isolated mutants to induce and invade developing nodules on roots of *Phaseolus vulgaris* was assayed.

RESULTS

Isolation and characterization of Gln utilization mutants.

Preliminary observations showed that the wild-type strain CE3 of *R. etli* plated on a chemically defined minimal medium

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Nucleotide sequence data are available in the EMBL database under accession numbers AJ18827, AJ18828, AJ18829, AJ18830, AJ18831, AJ580303, AJ580304, AJ580305, AJ580306, AJ580307, and AJ580308.

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(RMM; Tatè et al. 1997) supplemented with Gln as the sole source of C and N and containing bromocresol purple (BCP) as a pH indicator, induced the alcalinization (BCP shifted to violet) of the surrounding medium (Fig. 1). Thus, to recognize bacterial strains with an altered capacity to utilize Gln, both the growth phenotype (on liquid or solid medium) and the pH or color variations of the growth medium were exploited. A group of 11 Tn5-induced mutants named CTGUT (for CE3 Tn5 Gln utilization) were identified, isolated, and subjected to further characterization.

Probing the total genomic DNA of CTGUT strains (digested either with *EcoRI* or *BamHI*) with a labeled Tn5 DNA fragment established that each strain carried only one Tn5 (data not shown). The transposon DNA flanking regions were cloned, and the corresponding nucleotide sequence, including that of the insertion point, was established (Table 1). In strain CTGUT14, the open reading frame (ORF) truncated by Tn5 is highly similar to AapM, a component of the general L-amino acid transport Aap system previously identified in *R. leguminosarum* (Walshaw and Poole 1996). Upstream to *aapM*, a second ORF highly similar to the C-terminal domain of AapQ was identified, thus suggesting that, in *R. etli* as in

R. leguminosarum, an *aapJQMP* operon is present. In strains CTGUT27 and CTGUT40, the Tn5-disrupted ORFs code for proteins highly similar to BraD and BraE, respectively, which are components of the branched-chain amino acid ABC transporter (Bra system), the second general amino acid permease system of *R. leguminosarum* (Hosie et al. 2002). Strain CTGUT3 carries a Tn5 insertion in an ORF coding for a protein highly similar to the NADH-dependent Glt dehydrogenase (the *gdhB* gene product) of different bacteria, including that of *P. aeruginosa* (Lu and Abdelal 2001). In strain CTGUT6, the Tn5 insertion interrupted an ORF coding for a protein similar to different aldehyde dehydrogenase transmembrane proteins and, among these, to the NAD-dependent succinate semialdehyde dehydrogenase, the *gabD* gene product (Lutke-Eversloh and Steinbuchel 1999) that is involved in the GABA (γ -aminobutyric acid) shunt pathway for Glt deamination and decarboxylation. In strain CTGUT19, the Tn5-disrupted ORF codes for a protein highly similar to ketopantoate hydroxymethyl transferase (the *panB* gene product) of different bacteria. PanB catalyzes a step in the biosynthesis of coenzyme A (CoA), which is required in different anabolic pathways and for the function of the TCA

Fig. 1. Growth ability and pH variations induced by a group of mutants of *Rhizobium etli*. Bacteria (10^4 in 5 μ l) were spotted on plates of a chemically defined minimal medium (RMM; Tatè et al. 1997) (initial pH 5.63) supplemented with glucose (Glc), succinate (Suc), NH_4Cl (Am), glutamine (Gln), or asparagine (Asn) at the concentrations (mM) indicated. As a pH indicator, 0.04% bromocresol purple (violet-purple at pH values higher than 6.8, yellow at pH values lower than 5.2) was added. After 96 h of incubation at 30°C, the resulting colonies were photographed (Stemi SV11 Zeiss microscope). The strains are indicated at the top.

Table 1. Mutant strains of *Rhizobium etli* isolated and characterized in this study

Strain	Accession number	Gene product	Sequence similarity ^a
CTGUT3	AJ518827	Glutamate dehydrogenase (<i>gdhB</i>)	<i>Sm</i> , CAC47889 (81%/179 aa) E = 1e-79
CTGUT5	AJ518828	Fructose bisphosphate aldolase (<i>fbaB</i>)	<i>At</i> , AAK89666 (76%/174 aa) E = 1e-71
CTGUT6	AJ580303	Aldehyde dehydrogenase	<i>MI</i> , BAB49892 (78%/169 aa) E = 7e-72
CTGUT14	AJ518831	General L-aa transport system permease (<i>aapM</i>)	<i>RI</i> , CAA57935 (75%/104 aa) E = 8e-42
CTGUT15	AJ518829	Glutathione reductase (<i>gor</i>)	<i>At</i> , AAK87390 (74%/168 aa) E = 9e-67
CTGUT16	AJ580304	5'-P-ribosyl-5-amino-imidazole synthetase (<i>purM</i>)	<i>At</i> , AAK86944 (89%/189 aa) E = 2e-88
CTGUT19	AJ580305	Ketopantoate hydroxymethyl-transferase (<i>panB</i>)	<i>Rp</i> , CAD27226 (95%/82 aa) E = 5e-37
CTGUT27	AJ518830	Branched-chain aa permease (<i>braD</i>)	<i>Sm</i> , CAC49879 (70%/134 aa) E = 2e-35
CTGUT28	AJ580306	Aspartate carbamoyltransferase catalytic chain (<i>pyrB</i>)	<i>Sm</i> , CAC45883 (91%/158 aa) E = 4e-72
CTGUT40	AJ580307	Branched-chain aa permease (<i>braE</i>)	<i>Sm</i> , CAC49878 (56%/89 aa) E = 3e-18
CTGUT42	AJ580308	Phosphoenolpyruvate carboxy-kinase (<i>pckA</i>)	<i>At</i> , AAL41066 (88%/151 aa) E = 7e-78

^a Organism, Genbank accession number, (identity/amino acid overlap), significance; *At* = *Agrobacterium tumefaciens*; *MI* = *Mesorhizobium loti*; *RI* = *Rhizobium leguminosarum*; *Rp* = *Rhizobium phaseoli*; *Rs* = *Ralstonia solanacearum*; and *Sm* = *Sinorhizobium meliloti*.

cycle (Kurtov et al. 1999). A group of strains was mutated in genes involved in biosynthetic pathways, such as gluconeogenesis, expected to be active when Gln is utilized as the sole C source. For instance, in strain CTGUT5, the Tn5-truncated ORF is highly similar to the fructose-bisphosphate aldolases (the *fbaB* gene product) of class I that are more related to some eukaryotic aldolases rather than to glycolytic bacterial aldolases. Aldolase catalyzes one of the two irreversible steps of gluconeogenesis, and accordingly, strain CTGUT5 could not grow on RMM containing Gln, Asn, or succinate (Suc) as the unique source of C but grew on glucose (Glc). In strain CTGUT42, the Tn5-truncated ORF is highly similar to the phosphoenolpyruvate carboxykinase (the *pckA* gene product) that catalyzes the conversion of oxaloacetate into phosphoenolpyruvate. In two strains, CTGUT16 and CTGUT28, the mutated gene (*purM* and *pyrB*, respectively) is involved in the synthesis of nucleotides, and as expected, they grew when supplemented with their nutritional requirements (data not shown).

Gln as C or N source and pH variation.

All the mutants analyzed showed defects of growth in liquid RMM-Gln (5 mM), thus with Gln as sole source of N and C energy. Strain CTGUT15(*gor*) grew more slowly than did its wild-type parent strain CE3 (doubling time 9.5 and 5.3 h, respectively). Some strains, including CTGUT19(*panB*), CTGUT40(*braE*), CTGUT42(*pckA*) (doubling time between 35 and 45 h), CTGUT6(*ald*), and CTGUT27(*braD*) (doubling time >50 h) grew at very low rates. Finally, strains CTGUT3(*gdhB*), CTGUT5(*fbaB*), and CTGUT14(*aapM*) showed no detectable growth after 72 h of incubation. On solid RMM-Gln (5 mM), the mutant strains showed a variable behavior (Fig. 1). Remarkably, it was observed that strains CTGUT6(*ald*), CTGUT19(*panB*), CTGUT27(*braD*), and CTGUT40(*braE*) grew as well as strain CE3. Moreover, with the exception of CTGUT5(*fbaB*) and CTGUT42(*pckA*), all the strains tested were able to utilize Suc or Asn as a sole

C source. To test their ability to utilize Gln as a sole source of N as well as a putative Gln growth-inhibitor effect, the CTGUT mutants were spotted on solid RMM-Glc supplemented with different concentrations of Gln. On RMM-Glc (5 mM) supplemented with either 0.1 mM or 5 mM Gln (Fig. 1), all the mutants grew as well as strain CE3, thus indicating that they are able to utilize Gln as sole N source.

As shown in Figure 1, when plated on RMM-Gln (5 mM) the growth of *R. etli* caused the alkalization of the surrounding medium (BCP shifted to violet). On the contrary, on RMM-Glc/Gln (5:0.1 mM) or RMM-Glc/NH₄⁺ (5 mM each), the mutants as well as strain CE3 (wild type) caused acidification of the medium (BCP shifted to yellow). On RMM-Glc/Gln (5 mM each), the pH variations induced were variable (Fig. 1). Some strains, including CE3, CTGUT5(*fbaB*), CTGUT6(*ald*), and CTGUT42(*pckA*), induced a weak alkalization of the medium (BCP shifted to pink). Strains CTGUT19(*panB*), CTGUT27(*braD*), and CTGUT40(*braE*) shifted the color of the medium to pale pink, while CTGUT3(*gdhB*) and CTGUT14(*aapM*) shifted it to yellow. In addition, on RMM-Suc/NH₄⁺ (5 mM each) or RMM-Asn (5 mM), and with the exception of CTGUT5(*fbaB*) and CTGUT42(*pckA*) (defective in gluconeogenesis and thus requiring Glc to grow), all the mutants as well as strain CE3 induced strong alkalization (BCP violet). Considering the acidification related to Glc utilization and the alkalization related to Gln (Suc or Asn) utilization, these data suggest that *R. etli* strains use Gln as C source even in the presence of Glc. Furthermore, the pH variations induced by strains CTGUT5(*fbaB*) and CTGUT42(*pckA*) suggest that Gln is used even when bacteria are unable to utilize Gln as sole C source.

Uptake and intracellular fate of Gln.

Since they are unable to grow efficiently on RMM-Gln, the CTGUT mutants were grown on RMM-Gln/Glc (5 mM each). In the presence of an equal concentration of Glc and Gln, the capacity of *R. etli* to take up Gln was reduced but not abolished. In fact, the rate of Gln uptake measured in cells of strain CE3 (wild type) grown either on RMM-Gln/Glc (5 mM each) or RMM-Gln (5 mM) was 28.5 ± 3.2 nmol mg⁻¹ min⁻¹ and 40.5 ± 2.7 nmol mg⁻¹ min⁻¹, respectively.

As shown in Figure 2, the CTGUT mutants showed a variable behavior, even if, the ability to transport Gln was not abolished in any of them. Strains CTGUT5(*fbaB*), CTGUT6(*ald*), CTGUT19(*panB*), and CTGUT42(*pckA*) transported Gln at almost the same rate as did strain CE3. Strain CTGUT3(*gdhB*) showed a Gln uptake capacity 30% lower than that of strain CE3. Instead, strains CTGUT14(*aapM*), CTGUT27(*braD*), and CTGUT40(*braE*) showed a significantly reduced capacity (60 to 70% lower than strain CE3), thus indicating that *R. etli* may transport Gln by more than one permease and that the general amino acid transport systems Aap and Bra are directly or indirectly, involved in Gln uptake by *R. etli*.

The production of CO₂ from Gln gives an estimate of the flow of Gln through the citric acid cycle to produce energy (Duran et al. 1995; Reitzer et al. 1979). Therefore, *R. etli* bacteria were grown in RMM-Glc/Gln (5 mM each), were harvested, washed, and incubated (15 min) in RMM-Gln (40 μM) supplemented with [¹⁴C]-Gln, and the production of ¹⁴CO₂ was measured (Fig. 2). The rate of Gln degradation by strain CE3, calculated on the assumption that each Gln molecule is completely degraded, was 23.3 ± 3.8 nmol mg⁻¹ min⁻¹, implying that about 80% of the Gln molecules transported inside the cells is degraded to CO₂. The production of CO₂ from Gln was also measured in the presence of fluorocitrate or malonate that in-

Fig. 2. Glutamine (Gln) uptake and respiration of different CTGUT mutants of *Rhizobium etli*. The strains were grown in a chemically defined minimal medium (Tatè et al. 1997) (RMM) with Gln and glucose (5 mM each) up to exponential phase (optical density at 590 values of 0.6 to 0.8), were harvested, and were resuspended in RMM supplemented with [¹⁴C]-Gln. The rates of Gln uptake (white bars) and of Gln respiration (¹⁴CO₂ production) (black bars) were determined for strain CE3 (wild type) and a number of mutant strains (indicated at left). Data are means of three independent assays, and the variations of experimental values are indicated (error bars).

hibit two enzymes of the citric acid cycle, namely aconitase and succinate dehydrogenase, respectively (Gardner and Fridovich 1991). Fluorocitrate (100 μ M) and malonate (2.5 mM) inhibited the growth of *R. etli* on RMM-Gln (5 mM) and the $^{14}\text{CO}_2$ production by 97 and 99%, respectively. These data indicate that Gln is mainly degraded through the citric acid cycle and that, at least during the first 15 min of incubation on RMM-Gln, more than 95% of the $^{14}\text{CO}_2$ produced came from Gln respiration.

The ability of the mutants to respire Gln was variable. The $^{14}\text{CO}_2$ production by strains CTGUT5(*fbaB*), CTGUT6(*ald*), and CTGUT42(*pckA*) was slightly reduced (20 to 35% lower than in CE3). Strains CTGUT3(*gdhB*) and CTGUT19(*panB*) produced CO_2 from Gln but at a reduced rate (65 to 70% lower than CE3). This defect is most remarkable, considering that these strains showed a normal (or slightly reduced) ability to transport Gln. Finally, in correlation with their reduced capacity to transport Gln, strains CTGUT14(*aapM*), CTGUT27(*braD*), and CTGUT40(*braE*) produced CO_2 from Gln at very low rates (75 to 80% lower than CE3).

The incorporation of the C skeleton of Gln into macromolecules was measured. To this purpose, *R. etli* bacteria were incubated with [^{14}C]-Gln, and the radioactivity precipitated by trichloroacetic acid was measured as previously described (Thorne and Williams 1997). The rate of Gln incorporation into macromolecules by strain CE3 was 6.4 ± 0.5 nmol mg^{-1} min^{-1} , implying that about 20% of the Gln uptake by the cells is incorporated into cellular components. This value was reduced by about 80% in the presence of a growth-inhibiting concentration (300 $\mu\text{g ml}^{-1}$) of chloramphenicol, thus indicating that, during the incubation time (15 min, 40 μM Gln), proteins are the cellular components labeled by ^{14}C from [^{14}C]-Gln.

Gln utilization and oxidative stress.

The mutation of strain CTGUT15 appeared to be unrelated to Gln utilization, since the Tn5-disrupted ORF codes for a protein highly similar to the glutathione reductase (the *gor* gene product) of different bacteria (Table 1). As expected for

a *gor* mutation, strain CTGUT15 (inoculated in complete TYR [tryptone/yeast extract/Rhizobium] medium) was more susceptible than was strain CE3 (the wild-type parent) to treatment with diamide (Fig. 3A), a reagent that specifically oxidizes thiol groups, thus depleting the cells of reduced glutathione (GSH) (Kosower and Kosower 1995). On the other hand, strains CTGUT15 and CE3 showed almost the same degree of sensitivity to H_2O_2 (Fig. 3B), which mediates damage to DNA, proteins, and lipids. These results indicate that *gor* is essential to defend *R. etli* cells against an externally added thiol oxidative stressor. Moreover, as shown in Figure 3C, strain CE3 of *R. etli* showed a higher (at least 10-fold) susceptibility to diamide when incubated in RMM-Gln than when incubated in RMM-Glc/Gln or RMM-Glc/ NH_4^+ . Conversely, strain CTGUT15 was highly susceptible to diamide, even in the presence of Glc (Fig. 3D). In RMM-Glc/Gln or RMM-Glc/ NH_4^+ , the cultures of strains CTGUT15 and CE3 reached almost the same optical density at 590 nm values ($\text{OD}_{590} = 1$) after 20 h of incubation in the absence of diamide. Thus, the different susceptibility observed is not a consequence of different growth ability.

Gln utilization and cellular shape.

The maximal value of turbidity reached by a batch culture of strain CE3 in liquid RMM-Gln was dependent, even if not as a strict linear function, on the initial concentration of Gln used (Fig. 4). Unexpectedly, after reaching its maximal value, independently of the initial concentration of Gln used, the turbidity of the cultures decreased (Fig. 4A), albeit, as established by counting the colony-forming units, most bacterial cells were viable (data not shown). Thus, the morphology of *R. etli* grown on RMM-Gln was examined (Fig. 4B). Initially (OD_{590} value ranging between 0.25 and 0.3), *R. etli* cells were rod-shaped; later on (OD_{590} values of 0.7), the cells became shorter in length, while after the drop in turbidity (0.65 OD_{590}), most bacteria appeared spherical (coccoid), and the remaining rod-shaped cells were concatenates of short unit-length cells that eventually may develop coccoid bacteria. In contrast, the cells harvested from RMM-Gln/Glc (5

Fig. 3. Effect of oxidative stressors on the growth of *Rhizobium etli*. Strains CE3 (wild type, open circles) and CTGUT15 (*gor::Tn5*, solid circles) were inoculated (initial optical density at 590 nm [OD_{590}] = 0.05) in TYR supplemented with **A**, diamide or **B**, H_2O_2 at the indicated concentrations. After incubation (20 h, 30°C) the OD_{590} reached by the cultures was measured and expressed as a percentage of the OD_{590} value reached (100%) by the culture in the absence of stressor. In TYR, 100% was an OD_{590} equal to 1.2 and 1.5 for strains CE3 and CTGUT5, respectively. The effect of diamide on strains **C**, CE3 and **D**, CTGUT15 was assayed in chemically defined minimal medium (RMM)-glutamine (Gln) (open triangles), RMM-glucose (Glc)/Gln (open rhomb), or RMM-Glc/ NH_4^+ (solid triangles). In **C**, 100% was equal to 0.48, 1.05, and 0.97 in RMM-Gln, RMM-Glc/ NH_4^+ , and RMM-Glc/Gln, respectively. In **D**, 100% was equal to 0.85 and 1.01 in RMM-Glc/ NH_4^+ and RMM-Glc/Gln, respectively.

mM each) maintained their initial rod-shaped morphology, and only few cells appeared to be shorter than normal. These data indicate that, in cells growing with Gln as sole C source, the growth cell cycle becomes perturbed, more likely due to abnormalities in the establishment of their midpoint to form the septum.

N and C status of Gln-grown cells.

It has previously been established that the activity of NtrC-dependent promoters depends on the intracellular α -ketoglutarate/Gln ratio (C/N status) (Merrick and Edwards 1995; Patriarca et al. 2002). Therefore, the activity of two well-characterized NtrC-regulated promoters, namely, *glnIIp* (*glnII* codes for Gln synthetase II) and *gstIp* (*gstI* codes for the Gln synthetase translational inhibitor) was measured in *R. etli* bacteria grown under different conditions. It is important to remark that under conditions of C excess and N starvation, NtrC activates *glnIIp* and simultaneously represses *gstIp* (Patriarca et al. 1993; Spinosa et al. 2000; Tatè et al. 2001). As shown in Figure 5A, *glnIIp* activity was 10- to 17-fold higher in cells grown on RMM-Glc/Gln (1:1) or RMM-Glc/Gln (50:1) than in cells grown on RMM-Gln. Conversely, the *gstIp* activity was lower (3- to 6-fold) in cells grown on RMM-Glc/Gln (1:1) or RMM-Glc/Gln (50:1) than in cells grown on RMM-Gln. These data indicate that the intracellular α -ketoglutarate/Gln ratio of Gln-grown cells (*glnIIp* repressed, *gstIp* induced) is sufficiently low to inactivate NtrC and, consequently, that they are under condition of C starvation and N excess. In correlation with a condition of N sufficiency, it has previously been shown that Gln-grown cells of *R. etli* excrete ammonium (Duran et al. 1995). Hence, to avoid a futile cycle of glutamine degradation- NH_4^+ excretion and NH_4^+ uptake-organization), the genes *amtB* (NH_4^+ transport) and *glnII* (NH_4^+ assimilation) should be repressed in Gln-grown bacteria. In *glnB* (coding for nitrogen regulatory P_{II}) mutants of rhizobia, these genes are constitutively expressed, unrelated to the N status of the cells (Amar et al. 1994; Tatè et al. 2001), and as shown in Figure 5B,

strain BS11 (P_{II}^- mutant) of *R. leguminosarum*, unlike its wild-type parent LPR1105, was unable to grow on RMM-Gln. Conversely, strain BS11 grew, as well as strain LPR1105, on either complete TYR medium or RMM-Glc/ NH_4^+ .

Symbiotic behavior of CTGUT mutants.

In order to assess the nodulation capacity of CTGUT mutants, roots of *Phaseolus vulgaris* seedlings were inoculated and incubated for two to three weeks, and the resulting nodules or nodule-like structures were examined. Most of the mutants analyzed, including CTGUT3(*gdhB*), CTGUT6(*ald*), CTGUT14(*aapM*), CTGUT15(*gor*), CTGUT19(*panB*), CTGUT27(*braD*), and CTGUT40(*braE*) were able to induce and to invade developing nodules that, when developed, showed the anatomy of wild-type nodules (Fig. 6A) and normally organized central and peripheral tissues. For instance, a section of a 3-week-old nodule induced by strain CTGUT3(*gdhB*) is shown in Figure 6C. Moreover, by measuring the acetylene reduction activity, we found that the nitrogenase complex was normally induced in the resulting nodules (data not shown). In contrast, strains CTGUT5(*fbaB*), CTGUT16(*purM*), CTGUT28(*pyrB*), and CTGUT42(*pckA*) induced only few nodule-like structures in which the infection threads, when observed, appeared to be confined to the invaded root hair and were never observed in the central region of developing nodule primordia (data not shown). Frequently, lateral roots developing from these undeveloped nodule-like structures were observed (Fig. 6B).

DISCUSSION

The genetic analysis presented here indicates that *R. etli* bacteria require two broad-range amino acid transporters, namely, Aap and Bra, to use Gln efficiently as the sole source of C and energy. These transporters were extensively characterized in *R. leguminosarum*, although their involvement in Gln uptake and utilization was not directly assayed (Hosie et

Fig. 4. A, Effect of glutamine (Gln) concentration on the growth of *Rhizobium etli*. Strain CE3 was inoculated (optical density at 590 nm (OD_{590}) = 0.05) in chemically defined minimal medium (RMM; Tatè et al. 1997) supplemented with 5 (open squares), 10 (open triangles), 25 (open rhomb), or 50 mM (open circles) Gln as the sole source of C and N (initial pH 5.02). The OD_{590} reached at different times, as well as the final pH value of the cultures, are indicated. **B,** Cellular morphology of strain CE3 grown in RMM-Gln (10 mM). Bacteria were immobilized with agarose (3%) and photographed. Strain CE3 grown in RMM-glucose/Gln (5:1) was used as a control.

al. 2002; Lodwig et al. 2003; Walshaw and Poole 1996). It was observed, however, that the Bra system of *R. leguminosarum* is required to use Glt as the sole C source on liquid but not in solid medium (Hosie et al. 2002); the same growth differences were observed when the ability of *R. etli* bra mutants (strains CTGUT27 and CTGUT40) to utilize Gln as the sole C source was analyzed. Why the Bra system is essential for Gln and Glt utilization on liquid medium remains an unanswered question, although the bra mutants showed a significantly reduced Gln uptake and respiration capacity (Fig. 2). Furthermore, two other, apparently unrelated mutant strains, namely, CTGUT6 and CTGUT19, showed the same growth phenotype (Fig. 1).

Once inside the cells, Gln may be converted into Glt by deamidation, and it has previously been shown that *R. etli* requires glutaminase activity to utilize Gln as a sole C source (Duran et al. 1995). Glt may be channelled into the TCA cycle through different ways, namely the GABA shunt, Glt dehydrogenase (GDH), or transaminases (Dunn 1998; Poole and Allaway 2000). The growth phenotype of strain CTGUT3(*gdhB*) suggests that, in *R. etli*, the reaction catalyzed by GDH is a major route of Glt catabolism. The residual Gln degradation capacity (CO_2 production) of strain CTGUT3 (Fig. 3) may be a consequence of the glutaramate ω -amidase pathway that, in *R. etli*, produces mainly glycine, alanine, and α -ketoglutarate (Duran and Calderon 1995). Moreover, the GDH of *R. etli* appears to be a catabolic enzyme devoid of Glt biosynthetic activity. In fact, mutant strains of *R. etli* lacking GOGAT activity required Glt to grow even when incubated at high NH_4^+ concentrations (Bravo and Mora 1988; Ferraioli et al. 2002), whereas strain CTGUT3(*gdhB*) grew as well as strain CE3 on RMM-Glc/ NH_4^+ (Fig. 1). Furthermore, in order to avoid a futile cycle of Glt degradation and synthesis when growing with Gln as the sole C source, a GDH enzyme with assimilatory activity must be absent or inactive. In correlation, it has been shown that, unlike its wild-type parent, a mutant strain of *Neurospora crassa* lacking assimilatory GDH activity and partially lacking glutamine synthetase (GS) activity (GDH: GS^{+/−}) is able to grow on Gln as its sole C source (Calderon and Mora 1989).

One remarkable physiological consequence of the utilization of Gln as a sole C source is a difficulty of the cells to

remove oxidative stressors such as diamide (Fig. 3). The intracellular level of GSH depends on the level of reduced nicotinamide adenine dinucleotide phosphate (NADPH), which is mainly produced by the oxidative arm of the pentose phosphate pathway (PPP). Since PPP activity depends on Glc availability, it is reasonable to speculate that, using Gln as the sole C source and thus making Glc by gluconeogenesis, *Rhizobium* bacteria are less capable to respond to oxidative stressors by channeling glucose through the PPP. This would explain why the addition of Glc to Gln-grown bacteria reduces the bacterial susceptibility to diamide.

Pleomorphism in rhizobia has been observed by inhibiting septation (Latch and Margolin 1997), under microaerobic (N_2 -fixing) conditions (Kaneshiro et al. 1983), or in the presence of Suc (Urban and Dazzo 1982). In all these cases, however, the cell dimension increases up to 30-fold and this change is accompanied by swelling and branching, similar to what happens during symbiosome differentiation. Conversely, we observed that Gln-grown bacteria undergo a reductive cell division process (bacillary to coccoid), with a consequent decrement of the cell volume and mass (Fig. 4). It has previously been observed that C-starved cells of *R. phaseoli* become coccoid (Thorne and Williams 1997), and our data (Fig. 5) indicate that Gln-grown cells of *R. etli* are C-starved. The mechanism by which C-starvation induces a morphological transition remains unknown, but it has been proposed that oxidative stress may play a role in the bacillary-coccoid transformation of *Helicobacter pylori* (Nakamura et al. 2000), and we found that Gln-grown cells of *R. etli* are highly sensitive to oxidative stress (Fig. 3).

Fast-growing rhizobia, including *R. etli*, are highly sensitive to acid pH, and this is relevant under some environmental conditions such as the symbiosis (Zahran 1999). Our data suggest that bacteria may control pH levels (i. e., keep the external pH at values near neutrality) by regulating the use of amino acids as a C source (i. e., varying the proportion of sugars and amino acids used). Furthermore, using one or more amino acids as an energy source (even partially), the sugars available in the

Fig. 5. A, Activity of NtrC-regulated promoters in glutamine (Gln)-grown bacteria. Strain CE3 was transformed with plasmids pAR212a (*gstIP-lacZ*) or pAR35 (*glnIIP-lacZ*). The resulting strains were grown in chemically defined minimal medium (RMM; Tatè et al. 1997) supplemented with Gln or with Gln plus glucose (Glc), and the β -galactosidase activity was measured. Data (Miller units) are means of three independent assays, and errors are indicated. **B,** Ability of strain BS11 (*glnB*[−]) of *Rhizobium leguminosarum* to use Gln. Bacteria (10^4 in 5 μ l) in complete medium (TYR) or in minimal medium RMM supplemented with Gln or with Glc plus NH_4^+ (Am) and with bromocresol purple (0.04%) as a pH indicator. After 96 h of incubation at 30°C, the resulting colonies were photographed. The wild-type strain LPR1105 was used as a control.

Fig. 6. Nodules induced by *Rhizobium etli* strains. A, Nodule (21-day-old) induced by *R. etli* CE3 on the roots of *Phaseolus vulgaris*. **B,** Abortive nodule (AN) (21-day-old) induced by strain CTGUT5(*fbaB*) and developing numerous lateral roots (LR). **C,** Thin section of a 21-day-old nodule induced by strain CTGUT3(*gdhB*). The peripheral tissues (PT) including the vascular bundles (VB) and the central tissue (CT) formed by invaded (IC) and uninvaded cells (UC) are indicated.

rhizosphere may be channeled into the production of exopolysaccharides and lipopolysaccharides that are determinants of the capacity of rhizobia to establish an effective symbiotic interaction with their host plant (Patriarca et al. 2002).

The nodulation phenotype of CTGUT mutants (Fig. 6) indicates that bacterial Gln catabolism is dispensable for the symbiotic interaction and that glucose and nucleotides are not available for *R. etli* during nodule invasion. It has previously been shown that strains affected in gluconeogenesis, including *pckA* mutants of *R. leguminosarum*, *S. meliloti*, and *Rhizobium* NGR234, may induce the formation of root nodules, even though, in some rhizobia-legume pairs, the resulting nodules contain few or not-invaded host cells (Finan et al. 1991; McKay et al. 1985; Osteras et al. 1991). As previously suggested (Finan et al. 1991), these different symbiotic behaviors may reflect differences in the composition or amount, or both, of the metabolites made available by each legume to their symbiotic bacteria. Gluconeogenesis is strictly required for growth when using amino acids or C₄-dicarboxylates as the sole C source. However, rhizobia mutants unable to transport (uptake) C₄-dicarboxylates, including those of *R. etli*, are able to invade developing nodules (Lodwig and Poole 2003). In conclusion, even though the identity of C sources used by *R. etli* to invade developing nodules on the root of *Phaseolus vulgaris* remains unknown, the availability of a complex mixture of amino acids cannot be excluded. This is based on the following observations: i) with the exception of strains CTGUT5(*fbaB*), CTGUT16(*purM*), CTGUT28(*pyrB*), and CTGUT42(*pckA*), all the strains analyzed could grow using amino acids other than Gln, including Asn (Fig. 1), as their sole C source; and ii) most of the amino acids auxotrophs of *R. etli* so far analyzed were found to be able to invade developing nodules (Patriarca et al. 2002).

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The wild-type strain CE3 of *R. etli* (Noel et al. 1984) was grown at 30°C with shaking (200 rpm, G67 rotary shaker; New Brunswick, NY, U.S.A.) in TYR complete medium or in RMM, a chemically defined minimal medium (Tatè et al. 1997), and the N and C sources used were indicated for each experiment. Antibiotic (Sigma Chemical Co., St Louis) concentrations were as follows: tetracycline, 5 µg ml⁻¹; nalidixic acid, 20 µg ml⁻¹; and kanamycin, 30 µg ml⁻¹. All media were solidified with 1.5% agar (Difco Laboratories, Detroit).

Tn5 mutagenesis and cloning of the flanking regions.

A general Tn5 mutagenesis was performed, as previously described (Tatè et al. 1997). Mutant strains were isolated on TYR medium supplemented with kanamycin. Tn5-induced mutants (3,000 strains) were inoculated in 96-well microtiter plates containing 100 µl of complete medium (TYR) per well. Plates were incubated at 30°C for 48 h (allowing bacterial growth) and then were replicated in 96-well microtiter plates (liquid medium) or onto petri plates (solid medium), both containing RMM-Gln (initial pH 5.05). After two to three days of incubation at 30°C, 178 strains (5.6%) showing growth defects on solid medium (including colony dimension, morphology, etc.) or that were unable to increase the pH of the liquid medium (i.e., maintaining the initial yellow color or shifting only to a pale pink color), or both, were chosen for further characterization. Amino acid auxotrophs (about 90% of the mutants) were recognized by plating on RMM-Glc supplemented with casaminoacid (5 mg ml⁻¹) plus tryptophan (50 µg ml⁻¹) and excluded from further analysis.

The growth phenotype was tested also in RMM containing Glc plus Gln or NH₄Cl as C and N sources, respectively.

Digestions of total DNA were performed with either *Bam*HI or *Eco*RI. Restriction enzymes were used according to the directions of the manufacturers (New England Biolabs, Inc., Milan). For each digestion, 5 µg of total DNA was used, and the reaction mixtures were incubated for 4 h at 37°C. Southern hybridization against a 5.5-kb *Hpa*I fragment obtained from pSUP1011 and containing the Tn5 DNA was performed under high stringency conditions. To clone the Tn5 flanking regions, single and double digestions of total DNA (0.5 µg) were performed with *Bam*HI or *Bam*HI/*Cla*I or *Bam*HI/*Xba*I followed by ligation to pGEM7Zf+ (Promega Corp., Milan) digested with the same restriction enzymes. After transformation into *Escherichia coli* DH5α, the strains resistant to kanamycin were isolated and plasmid DNA was purified using the Qiaprep Kit (Qiagen, Milan).

DNA sequencing and sequence analysis.

DNA sequence was determined at the Automatic Sequencing Service, Institute of Genetics and Biophysics, CNR-Area di Ricerca, Naples, Italy. A standard Tn5 outward-reading primer, Tn5out (5'-GAAAGGTTCCGTTTCAGGACGCTAC-3') was used. The DNA and predicted protein sequences were analyzed with the University of Wisconsin Genetics Computer Group (Madison, WI, U.S.A.) software. Database searches for similarity were performed with BLAST software (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, U.S.A.). The DNA sequences were deposited in the EMBL database, and the accession numbers are indicated in Table 1.

Gln transport assays.

Uptake assays were performed by rapid-filtration method as described elsewhere (Tatè et al. 1998). Bacteria were grown at 30°C under aeration (rotating shaker, 200 rpm) up to an OD₅₉₀ value ranging between 0.6 and 0.8 and were centrifuged (4,000 × g, 10 min) at 4°C, and the bacterial pellet was washed twice with sterile water and was resuspended in RMM-Gln (40 µM final concentration). L-[U-¹⁴C]Gln (50 µCi ml⁻¹, 244 µCi µmol⁻¹) purchased from Amersham-Pharmacia Biotech (Milan) was used. The protein content of the bacterial suspension was determined with the Bio-Rad kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Measurement of CO₂ production from Gln.

Bacteria were grown as described above (Gln transport assays) and were resuspended in 0.2 ml (OD₅₉₀ = 3.0) of RMM-Gln (40 µM final concentration). After the addition of 10 µl (2 nmoles) of a labeled L-[U-¹⁴C]Gln solution (50 µCi ml⁻¹, 244 µCi µmol⁻¹) (Amersham-Pharmacia Biotech), the mixture was incubated (15 min, 30°C) and the reaction was stopped by addition of cold Gln (50 mM final concentration) plus 1 ml of 0.9 M HClO₄. The ¹⁴CO₂ liberated from this acid mixture was captured in a CO₂ trap (appended from the cap) containing 1 M benzethonium hydroxide (Sigma Chemical Co., St. Louis) in CH₃OH. After 3 h of incubation, the trap was removed and its contents were diluted in 50% scintillation mix (Ultima Gold, Packard; Cambera Company, Meriden, CT, U.S.A.) and were counted (β-Counter; Beckman Coulter, Inc. Fullerton, CA, U.S.A.). The protein content of the bacterial suspension was determined with the Bio-Rad kit.

Nodulation tests and optical microscopy.

Seeds of *Phaseolus vulgaris* cv. Negro Jamapa were surface-sterilized with 5% hydrogen peroxide for 30 min, were

washed extensively with sterile water, and were germinated at 23°C for 72 h on a petri dish containing water-agar (1%). The principal root of germinated seeds was inoculated with 100 µl of a bacterial suspension (10^6 ml⁻¹) in 50 mM phosphate buffer (pH 7.0). Plants were cultured in a growth chamber maintained at 65 to 75% relative humidity, 23°C in light, 19°C in dark, and with a 16-h-daylight period, using a N-free medium (Tatè et al. 1999). For optical microscopy, nodules were excised from the primary root, were sliced manually, and were fixed in 50 mM potassium phosphate buffer (pH 7.4) containing 4% paraformaldehyde, 3% glutaraldehyde, and 4% sucrose. Nodule samples were subjected to short pulses of gentle vacuum until they sank, and after 3 h, they were rinsed in phosphate buffer and were postfixed for 1 h in 50 mM potassium phosphate buffer (pH 7.4) containing 1% osmium tetroxide. After fixation, the samples were rinsed three times in the same buffer, were dehydrated by passing through a series of graded ethanol, and were embedded in Poly/Bed 812 resin (Polyscience, Warrington, PA, U.S.A.). Semithin sections (2 µm thick) were obtained with a Leica microtome equipped with a diamond knife (Diatome, Biel, Switzerland), were stained with toluidine blue (0.04% in distilled water), and were observed in a Zeiss Axiophot microscope.

ACKNOWLEDGMENTS

We thank P. Poole, M. Merrick, and J. Guardiola for useful discussion, C. Rallo and M. Valenzi for computer analysis, C. Sole and R. Vito for technical assistance, and A. Secondulfo for preparing the manuscript. This work was partially supported by Ministero Università Ricerca Scientifica e Tecnologica-CNR L. 488/92 (cluster 02), Ministero Politiche Agricole e Forestali and Ministero Istruzione Università e Ricerca-Fondi Investimenti Ricerca di Base (RBNE01KZE7).

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