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Key Role of Bacterial NH_4^+ Metabolism in Rhizobium-Plant Symbiosis

Eduardo J. Patriarca,* Rosarita Tatè, and Maurizio Iaccarino

International Institute of Genetics and Biophysics, Consiglio Nazionale delle Ricerche, 80125 Naples, Italy

INTRODUCTION

Rhizobium-Plant Interaction

Soil bacteria of the family *Rhizobiaceae* are able to colonize the roots of compatible legume plants and to induce specialized organs therein, the N_2-fixing nodules, thus making the plant autotrophic for external N. Most of the literature deals with the properties of bacteria belonging to the genera *Rhizobium* and *Sinorhizobium* and, to a lesser extent, *Azorhizobium*, *Bradyrhizobium*, and *Mesorhizobium*. In this article we use the term rhizobia to include these five genera. From the point of view of the bacterial partner, nodule formation is a multistage process involving bacterial multiplication in the rhizosphere, recognition of the host plant, infection of the root hairs, and bacterial growth inside a network of tube-like structures made of plant cell wall-like material, called infection threads (ITs). When the ITs reach the center of a nodule primordium (formed by successive rounds of mitotic events), bacteria are released from the tip of ITs into the plant cells by endocytosis. Bacteria, at this stage called bacteroids (BTs), become enclosed in membrane vesicles arising from the plasma membrane, which act as a physical barrier between the symbiotic partners.

Once in the cytoplasm of plant cells, BTs divide a few times, arrest division, and differentiate morphologically and physiologically into a form able to reduce atmospheric N_2 and release the fixed N to the plant (for reviews, see references 19, 124, and 197). The organelle-like structure formed by the BT, together with the peribacteroid space and the surrounding plant membrane, is called the symbiosome (SB). After the onset of N_2 fixation, SBs continue to differentiate and eventually proceed towards lysis. It was recently shown that, in the symbiosis between *Sinorhizobium meliloti* and *Medicago sativa*, undifferentiated bacteria remaining in intracellular ramifications of the ITs infect the cytoplasm of senescent cells growing as free-living bacteria (186). This process occurs after the SBs are degraded. This observation may explain the benefit of the bacterial partner in the symbiosis.

Root Nodule

Fully developed nodules are vascularized plant organs formed by two main tissues, peripheral and central. The central tissue (CT) includes thousands of SB-containing cells, which in

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* Corresponding author. Mailing address: IIGB-CNR, Via G. Marconi 10, 80125 Naples, Italy. Phone: 0039081 725 7257. Fax: 0039081 725 7202. E-mail: patriarca@iigb.na.cnr.it.

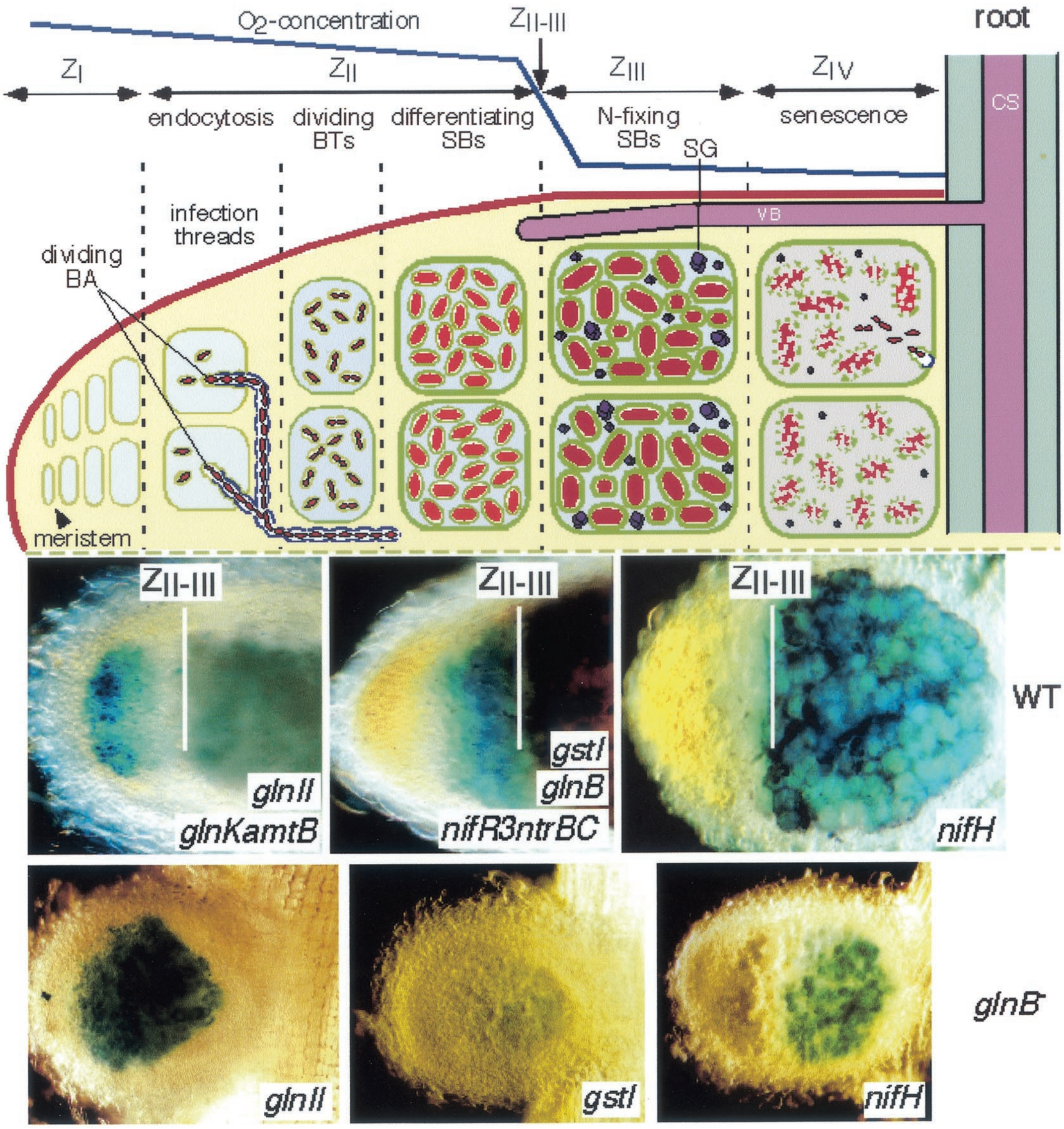

FIG. 1. (Top) Scheme of the indeterminate elongated nodule. BA, bacteria; BTs, bacteroids; SBs, symbiosomes; SG, starch grain; CS, central stele; VB, vascular bundle. The nodule zones (Z) are indicated. (Bottom) Histochemical localization of β-galactosidase activity in nodules of *V. hirsuta* induced by the wild-type (WT) and the *glnB* (P_{II}^-) strain of *R. leguminosarum* carrying *lacZ* transcriptional fusions to the genes indicated.

the literature are called either infected or invaded. Since SBs do not behave as pathogenic bacteria, we use the term invaded cells (ICs) for SB-containing cells. SBs are designed to accommodate the requirements for bacteroidal N_2 fixation and the metabolic exchanges occurring between the two symbiotic partners. Both partners genetically regulate nodule organogenesis and nitrogen fixation. The most studied nodules are of two types, indeterminate and elongated (e.g., from *Medicago sativa*, *Pisum sativum*, and *Vicia hirsuta*) and determinate and globose (e.g., from *Glycine max* and *Phaseolus vulgaris*). Indeterminate nodules are elongated because of the presence of a persistent meristem located at their apex (Fig. 1). As a consequence, in a longitudinal section they show different developmental zones containing ICs at different stages of differentiation, in which SBs also show a progressive differentiation (186, 199).

Determinate nodules are globose because they develop from a spherical meristem that is not evident in mature nodules (Fig. 2). They show a peripheral and a central tissue and, on the basis of histological observations (124, 125), it has been concluded that at each step of development all the ICs and the SBs of the CT are at a similar stage of differentiation. However, more recently it was shown that in developing determinate nodules of *P. vulgaris*, there is also a spatial component of development. In fact, during nodule organogenesis, it is possible to observe differentiating ICs containing only a few undifferentiated SBs and mature ICs filled with differentiated SBs formed by more than one BT (25). Moreover, some plant and bacterial genes are not expressed simultaneously in all cells of the CT (144, 176) (see below and Fig. 2) and signs of nodule senescence, such as the lysis of ICs, initiate their appearance specifically at its center (25).

Finally, the heterogeneity of the ICs is confirmed by the

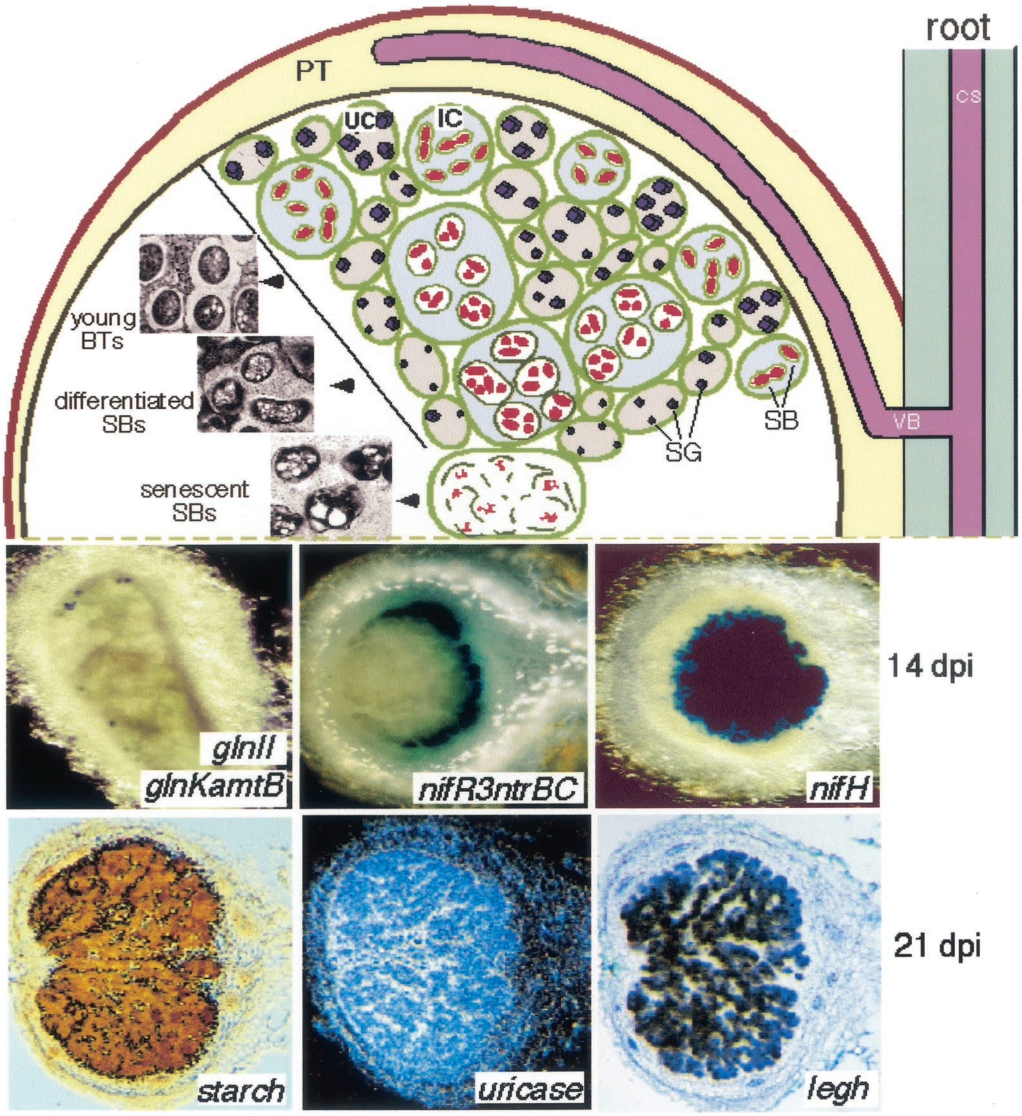

FIG. 2. (Top) Scheme of the determinate globose nodule. CS, central stele; VB, vascular bundle; PT, peripheral tissue; BTs, bacteroids; SBs, symbiosomes; SG, starch grain; IC, invaded cell; UC, uninvaded cell. (Middle) Histochemical localization of β-galactosidase activity in 14-day-postinoculation (dpi) nodules of *P. vulgaris* induced by *R. etli* carrying *lacZ* transcriptional fusions to the genes indicated. (Bottom) Sections of *P. vulgaris* nodules taken at 21 days postinoculation, showing amyloplast (starch) accumulation, and the in situ localization of uricase and leghemoglobin (legh) transcripts.

observation that in nodules elicited by bacterial mutants having delayed invasion ability, not all the meristematic cells are invaded simultaneously. This suggests either a difference in their susceptibility to invasion (181, 182) or that the first invaded meristematic cells divide several times before differentiation (149). Plant molecular markers are differentially expressed through the CT also in *G. max* nodules (83).

Nodule organogenesis, such as the formation of other plant organs (flowers, leaves, roots), proceeds through many temporally and spatially regulated developmental steps. It is induced by rhizobia, but the development of a mature and functional nodule requires the exchange of several signal molecules and metabolites between the prokaryotic and eukaryotic partners. Nodule organogenesis is studied with the help of bacterial and plant mutants, which reveal specific patterns of differentiation, and with the in situ study of molecular markers, which in this case are not only eukaryotic, but also prokaryotic. The combined efforts of physiologists, embryologists, molecular biologists, and cytologists are needed to make progress in this field.

Purpose of This Review

In the rhizobium-legume symbiosis, the bacterial partner differentiates into nondividing endocellular symbionts that, through the induction of the nitrogenase complex, convert atmospheric N_2 to NH_3/NH_4^+, which is eventually used by the plant. In contrast, most NH_4^+ produced through N_2 fixation in free-living diazotrophs is assimilated and used for bacterial growth. Null mutants of rhizobia (e.g., Tn*5* induced) (37, 174) affected in different genes involved in NH_4^+ assimilation were

TABLE 1. Genes for ammonium metabolism and their expression during symbiosis

Gene(s)	Function(s) of the product(s)	Mutant phenotype, free-living//symbiosis[a] (reference[s])	Promoter activity	
			Elongated/indeterminate	Globose/determinate
aapM	General amino acid permease	*Rl* unable to use glutamine-glutamate as C source//Nod$^+$ Fix$^+$ (204, 204a)	Unknown	Unknown
aatA	Aspartate aminotransferase	*Sm* unable to use aspartate//Nod$^+$ Fix$^-$ partially empty nodules (207)	High levels of enzyme activity in BTs (207)	Unknown
cysG	Syroheme synthetase	*Re* unable to grow on NO_3^-//Nod$^+$ Fix$^+$ (178)	Unknown	Unknown
glnA/*glnII*/*glnT*	Glutamine synthetase I, II, or III	Single mutants show no phenotype (54)//single mutants are Nod$^+$ Fix$^+$ (37); *Bj glnA glnII* only nodule primordia (22)	p*glnII* ON in ITs, OFF in BTs of ZI and later on (183)	p*glnII* ON in ITs, OFF in BTs after endocytosis (179)
glnB	P_{II} regulatory protein	*Rl* NtrC-activated promoters ON//Nod$^+$ Fix$^{+/-}$ (3, 183); *Sm* NtrC-activated promoters OFF//Nod$^{+/-}$ Ara$^+$ Fix$^-$, abnormal ITs (5)	ON in ITs and in BTs of ZI and ZII; OFF in BTs of ZIII and later on (52)	ON in younger BTs; OFF in differentiated BTs (R. Tatè, unpublished data)
glnD	Uridylyl removing/transferase of P_{II}	*Rl* constitutively deuridylylated P_{II}, NtrC-activated promoters OFF//Nod$^+$ Fix$^+$ (159)	ON at all stages of BTs differentiation (159)	Unknown
glnK	AmtB (NH_4^+ uptake) regulator	No mutants described	Same pattern as that of p*glnII* (179)	As p*glnII* (179)
gltB/*gltD*	Glutamate synthase subunits	Glutamate auxotrophs//*Sm* or *Re* Nod$^+$ Fix$^+$ (24, 95); *Bj* and *As* Nod$^+$ Fix$^-$ (71, 133)	High levels of enzyme activity in BTs (136)	High levels of enzyme activity in BTs (24)
gstI	*glnII* translation inhibitor	No mutants described	Same pattern as that of p*nifR3-ntrBC* (183)	As p*nifR3-ntrBC* (R. Tatè, unpublished)
nifA	Transcriptional activator	Early degeneration and lysis of BTs in both types of nodules (25, 58, 73, 209)	Same pattern as that of p*nifHDK* (212)	Unknown
nifHDK	Nitrogenase subunits	*Sm* Nod$^+$ Fix$^-$, ZIII not formed, early senescence (72, 73, 199); *Bj* and *Re* Nod$^+$ Fix$^-$, no early histological alterations, abnormal BT differentiation (8, 25, 58)	OFF in ITs and in BTs of ZI and ZII; ON in BTs of interzone II-III and in distal ZIII (144, 179, 180, 212)	OFF in ITs; ON in younger differentiating BTs (12, 180)
nifR3-ntrBC	Transcriptional regulator	Unable to use low concentration of NO_3^- or amino acids as N source//Nod$^+$ Fix$^+$ (108, 123, 174)	ON in BTs of ZI and ZII, OFF in N_2-fixing BTs of ZIII and ZIV (144)	ON in ITs and in younger BTs; OFF in differentiated BTs (144)
putA	Proline dehydrogenase	Unable to use proline//Nod$^{+/-}$ Fix$^+$ (80)	Same pattern as that of p*nifR3-ntrBC* (80)	Unknown

[a] *Rl, Rhizobium leguminosarum*; *Re, Rhizobium etli*; *Bj, Bradyrhizobium japonicum*; *Sm, Sinorhizobium meliloti*; *As, Azorhizobium sesbaniae*; Z, zone; ON, active; OFF, inactive.

found to be able to elicit the formation of efficient nodules (Nod$^+$/Fix$^+$ phenotype). Moreover, it has been shown that in isolated N_2-fixing BTs, NH_4^+ assimilation activities are either absent or expressed at very low levels (22, 37, 77, 162). These studies led to the conclusion that the bacterial ability to assimilate NH_4^+ is not needed for an efficient symbiosis (80a, 192a). However, they do not deal with the mechanism(s) of gene regulation acting during SB differentiation to uncouple N_2 fixation and NH_4^+ assimilation activities and do not exclude the possibility that the switching off of this pathway is essential for an efficient symbiosis.

The purpose of this review is to show that more detailed studies have revealed a very fine regulation of bacterial NH_4^+ metabolism during the establishment of the symbiosis. Down-regulation of structural and regulatory genes involved in NH_4^+ assimilation is a key and necessary step for the differentiation of efficient SBs and thus for the development of effective nodules. During SB differentiation, a conserved molecular mechanism acts selectively to switch off gene transcription and to remove the corresponding proteins, either before or when N_2 fixation is turned on.

NH_4^+ AS A REGULATORY SIGNAL IN SYMBIOSIS

Small supplements of combined N, such as NO_3^-, NH_4^+, and urea, added at sowing may benefit symbiosis by increasing seedling growth rate and the number, size, and efficiency of the resulting nodules. Excessive amounts of combined N depress nodulation by reducing the number of infective sites and/or the number of successful infections on the primary roots and by inhibiting nodule growth and functionality (for a review, see reference 213). NH_4^+ is at the same time the primary product and the main regulator of the symbiosis: either as such, or after conversion into organic compounds such as glutamate and glutamine, it regulates most stages of the symbiotic interaction. Table 1 lists some of the genes and phenotypes described below.

Action on Early Steps of Symbiosis

The production of flavonoids, such as luteolin and naringenin (146, 163), that are secreted by the root cells is induced by stress of many types, including N starvation (28, 33). Flavonoids induce transcription of the bacterial *nod* genes and thus the production of lipochitooligosaccharides called Nod factors. The Nod factors are in turn able to elicit the induction of a nodule primordium in the specific host plant and to stimulate the plant to produce flavonoids (16, 39, 97, 171). Except for the case of *Rhizobium leguminosarum* (9), the flavonoid-mediated induction of *nod* gene transcription is abolished in the presence of NH_4^+, which appears to act through its assimilation products (48, 114, 205).

NH_4NO_3 is known to be a potent inhibitor of root hair deformation, initial cortical cell division, and IT formation, but its effect on the Nod factor perception has been excluded (70). The presence of NH_4^+ in the medium reduces the ability of *Sinorhizobium meliloti* to produce exopolysaccharides, which play a critical role in the process of nodule invasion (44). Although bacterial lipopolysaccharides are necessary for competitiveness (93) and nodule invasion (23, 203), no reports on the regulation of their structure and synthesis by NH_4^+ have been reported. Their structure is modified in response to the pH of the medium (175), and the latter is strongly influenced by the N source. Thus, differences in N metabolism may indirectly provoke the structural alterations of the lipopolysaccharides observed in bacteria growing inside the ITs (164). The addition of N compounds, including NH_4^+, lowers the expression and/or the activity of some nodulins, plant gene products that are either specifically expressed or highly induced in the nodule (27; for a review, see reference 156).

Positive and Negative Regulator of Nodule Development

NH_4^+ acts as a negative signal when added from without. This effect has been mostly studied on the elongated nodules of *M. sativa* and *P. sativum*. When combined N is added to nodulated roots, nodule growth is arrested and nodule endodermis grows to progressively surround the apical meristem, which becomes inactive. When the N source is removed, the apical meristem is reactivated, and thus nodule growth resumes (189; G. Truchet, personal communication). Addition of NH_4^+ (and NO_3^-) also inhibits nitrogenase activity, possibly not directly but after conversion to glutamine, or through the regulation of a plant function (173), e.g., the reduction of the O_2 or C supply by the plant (15). In fact, it has been shown that isolated SBs do not take up NH_4^+ and that SBs in the nodule do not express the *amtB* gene, which is required for high-affinity NH_4^+ uptake activity (92, 179). Moreover, externally added NH_4^+ may never reach the SBs, since the NH_4^+-assimilating enzymes are highly expressed in the cytoplasm of ICs.

NH_4^+ produced through the action of the nitrogenase complex appears to be required as a positive signal necessary for normal development of indeterminate nodules (5, 199). Nodules induced by *S. meliloti* mutants unable to reduce N_2 because of a mutation in one of the structural genes for nitrogenase (*nifHDK*), and thus unable to produce NH_4^+, show normal early organogenesis, but later on the N_2-fixing zone (zone III) of the nodule is not formed, the apical meristem is inactivated, and early nodule senescence is observed (72, 73, 199). Therefore, completion of nodule development and sustained action of the meristem require nitrogenase activity. The regulatory signal for completion of nodule development may be either the NH_4^+ released by SBs or some other metabolic changes resulting from nitrogenase activity, such as enhanced consumption of O_2 and/or reducing power.

The presence of normal Fix^+ nodules inhibits the induction of further nodules on the root. In fact, plants inoculated with Fix^- rhizobia show a greater number of nodules than in normal nodulation due to reinfections taking place on secondary roots (72). In conclusion, NH_4^+ produced in the nodule appears to act locally as a positive regulatory signal for nodule and SB maintenance, whereas it acts systemically as a negative signal inhibiting the reinfection process. Moreover, NH_4^+ produced in the nodule may repress flavonoid and isoflavonoid production (the latter compounds being involved in the defense reaction [for a review, see reference 16]). In fact, the *chs* (chalcone synthase) gene, involved in flavonoids and isoflavonoid biosynthesis, is induced in mature and invaded (18 days postinoculation) nodules elicited by a Fix^- mutant of *S. meliloti* and not in nodules elicited by the wild-type strain (67). In nodules elicited by an *S. meliloti* strain mutated in the *nifA* regulatory gene, the SBs degenerate at an earlier stage of development than in nodules formed by a *nifH* mutant (73), indicating that NifA is involved in the expression of bacterial functions that are required for SB viability, in addition to those involving N_2 fixation.

A different behavior is observed in the case of determinate nodules. When *G. max* and *P. vulgaris* are inoculated with a *nifH* mutant strain and *Vigna unguiculata* is nodulated in the absence of N_2 (replaced by argon), the resulting nodules are similar (in shape, dimension, and histology) to normal nodules (8, 25, 58). This suggests that NH_4^+ is not a signal for nodule organogenesis and that younger BTs release NH_4^+ by a mechanism not involving nitrogenase action, such as the utilization of amino acids as a C source (see below). Instead, if these plants are infected with a *nifA* mutant strain, SBs are degraded at an early stage (25, 58, 209). This indicates that the *nifA* regulatory gene, as in the case of elongated nodules, is required for SB maintenance, while *nifH*, and thus nitrogenase activity, is not (60, 129).

Action on Symbiosomes

During development of both types of nodules, SBs undergo profound physiological and morphological changes. One of the most relevant is that, even though reached in different ways, their surface/volume ratio decreases during differentiation, reducing the capacity of SBs to exchange metabolites (and/or signals) with the plant.

SBs of elongated nodules during nodule development maintain a single BT, which increases up to 30-fold in size and shows polar swellings and Y shapes (199). Moreover, BTs undergo significant changes in DNA content and in the ratio of chromosomal to symbiotic plasmid DNA (14, 202, 210). The morphological changes of SBs become evident after the arrest of BT divisions occurring in the middle of zone II (Fig. 1) and thus before the onset of nitrogenase activity. The same shape modifications are observed in nodules elicited by Fix^- strains

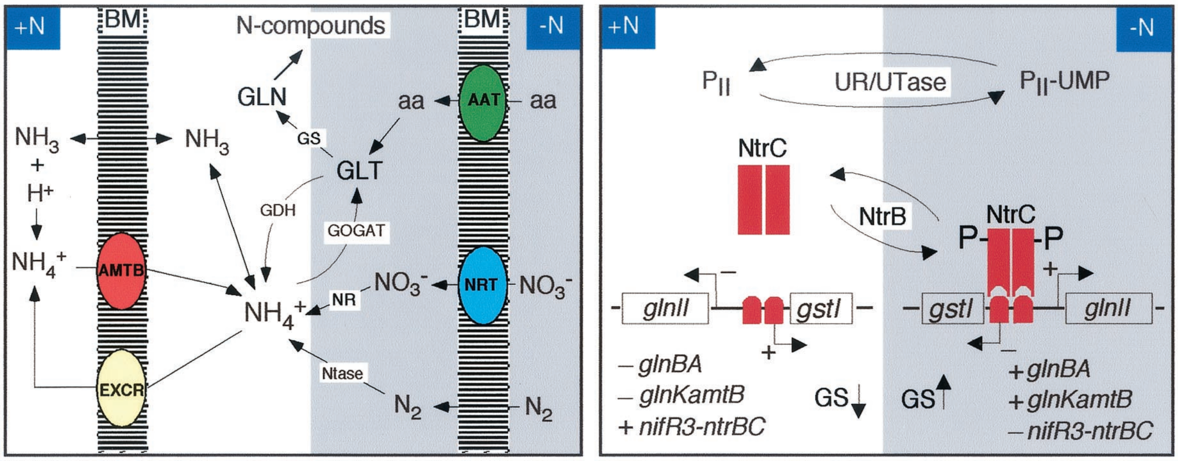

FIG. 3. NH_4^+ metabolism (left) and regulation of NH_4^+ assimilation (right), at high (left side of each panel) and low (right side) N concentrations. (Left panel) NH_3 is supposed to diffuse freely through the bacterial membrane (BM), while NH_4^+ requires a transporter (AmtB) for uptake and is supposed to need a different one for excretion (EXCR). N sources alternative to NH_4^+ are amino acids (aa), which require specific transport systems (AAT), and NO_3^-, requiring a transport system (NRT) (for reviews, see references 64, 82, and 121), and N_2. Ntase, nitrogenase; NR, nitrate reductase; GOGAT, glutamate synthase; GDH, catabolic glutamate dehydrogenase; GS, glutamine synthetase isozymes; GLT, glutamate; GLN, glutamine. (Right panel) Gene symbols are described in the text. A switch from high N (left side of the panel) to low N (right side) causes inactivation (uridylylation) of the P_{II} protein (P_{II}-UMP), phosphorylation of the NtrC protein (NtrC-P), and its binding to a sequence located at the *glnII-gstI* intergenic region, thus activating *glnII* and repressing *gstI* expression. The effect of NtrC-P on the transcription of other genes, such as *glnBA*, *glnK-amtB*, and *nifR3-ntrBC*, is indicated. P_{II}-UMP also mediates the posttranslational deadenylylation of GSI and thus its activation.

and when free-living bacteria are treated with cell cycle inhibitors (94) and C_4-dicarboxylic acids (193), excluding the involvement of NH_4^+ in this process. However, the appearance of C_4-dicarboxylic acids may provoke, at the corresponding stage of differentiation, severe changes in N metabolism, such as the inhibition of the utilization of amino acids as a C source and thus of NH_4^+ excretion (see below). Furthermore, the completion of SB differentiation takes place after nitrogenase activation (199).

Globose nodules develop SBs formed by multiple rod-shaped BTs, which do not increase in size (25, 34). The number of BTs per SB appears to increase more by fusion of younger SBs than by BT division inside a single membrane vesicle (25, 55, 127). Moreover, the merging of SBs proceeds through the fusion of their membranes. It increases during nodule senescence, when BT division stops, and appears to be related to the N status of the cell, since it is also accelerated when the plant is infected with Fix^- mutants (25). Lack of nitrogenase activity, and therefore of NH_4^+ production, might cause a change in pH at the cytoplasmic surface of SBs, thus activating a homotypic mechanism of membrane fusion that may involve SNARE proteins (49).

NH_4^+ METABOLISM

Rhizobia are able to take up and assimilate NH_4^+ from the external medium, to produce it intracellularly from N_2, nitrate, and amino acids, and to excrete it when in excess (Fig. 3).

NH_4^+ Uptake

NH_4^+ is in equilibrium with NH_3, the pKa being 9.25, and it is assumed that NH_3 crosses most but not all (86) biological membranes by unmediated diffusion and thus supports bacterial growth under certain conditions (see references 87 and 169 for discussion). Conversely, NH_4^+ crosses the membranes only through specific transporters. Evidence for a low-affinity NH_4^+ uptake system has been recently reported for *Corynebacterium glutamicum* (112). A high-affinity, energy-dependent transport system for NH_4^+, detected by uptake assays with radioactive methylammonium (an NH_4^+ analogue), has been described in many bacterial species, including rhizobia (77, 87, 134, 179). In rhizobia this system is activated under poor N conditions, such as the utilization of nitrate and glutamate as the sole N source, either to retrieve the NH_3/NH_4^+ lost to the outside or to sense their extracellular concentration (179, 191).

The *amtB* gene from *Azorhizobium caulinodans* and *Rhizobium etli* has been shown to be required for high-affinity NH_4^+ transport (117, 179). This gene codes for an integral membrane protein highly conserved in many organisms (184, 185) and it is cotranscribed with the *glnK* gene (coding for a P_{II}-like protein), forming a *glnK-amtB* operon. This operon is expressed from a promoter located upstream of *glnK*, whereas no promoter activity was detected in the intergenic region (179). The *glnK* promoter becomes inactive very early during symbiosis, and in correlation isolated BTs do not show high-affinity NH_4^+ uptake activity (92, 134, 179, 180). Downregulation of *amtB* expression is essential for the normal function of BTs (see below).

Nitrogen Fixation

The organization and regulation of the rhizobial genes coding for the nitrogenase complex have been reviewed (59). Their transcription is dependent on the NifA protein, which is expressed and/or activated under specific environmental conditions, including low O_2 tension (11). In *S. meliloti* the expression of the *nifA* gene under microaerobic free-living conditions requires the FixLJ two-component system and is negatively regulated by NH_4^+ through a process mediated by the FixL protein (35, 131). The posttranslational inactivation of nitrogenase mediated by the *draGT* genes and induced by NH_4^+, reported for other members of the diazotrophic α-proteobacteria (for a review, see reference 6), has not been identified in rhizobia.

In free-living diazotrophs such as *Klebsiella pneumoniae*, the NH_4^+ produced through the action of nitrogenase is assimilated for bacterial growth. The nitrogenase complex may be induced in free-living rhizobia but, with the exception of *A. caulinodans* and *Rhizobium trifolii* strain 0403 (40, 194), most of the NH_4^+ produced is excreted (132, 194). Therefore, in rhizobia, N_2 fixation and assimilation of the NH_4^+ produced are uncoupled, and this is essential for an effective symbiotic interaction. In fact, plants nodulated by strains unable to express an active nitrogenase complex and unable to release the NH_4^+ produced show signs of N deprivation, which are relieved by exogenously added combined N (5, 72, 73). However, it has recently been reported that an *ntrR* mutant strain of *S. meliloti* induces nodules with an enhanced N_2 fixation capacity due to the higher expression of *nif* genes (135). Moreover, this phenotype is not abolished by the exogenous addition of N, suggesting that in *S. meliloti* the expression of the *nif* genes is also N regulated during the symbiosis.

The symbiosis may be viewed as an exchange of photosynthate (in the form of C_4-dicarboxylic acids) for fixed N, produced by the SBs (80a, 147, 192a). During SB differentiation, BTs of globose nodules accumulate polyhydroxybutyrate and glycogen granules, perhaps because their capacity to fix N_2 decreases while C supply is still plentiful. In fact, ineffective nodules of *P. vulgaris* induced by a Fix$^-$ *R. etli* mutant strain show an earlier and higher accumulation of polyhydroxybutyrate compared to effective nodules (25). On the other hand, nodules induced by a mutant strain blocked in polyhydroxybutyrate biosynthesis show a higher and prolonged nitrogenase activity compared to nodules induced by the wild-type strain, indicating that nitrogenase and polyhydroxybutyrate biosyntheses compete for reducing power (26). *Rhizobium tropici* mutants unable to synthesize glycogen also show a better symbiotic performance (107a).

Internal Production from Nitrate and Amino Acids

NH_4^+ is produced inside rhizobia from nitrate (Fig. 3), which in the soil is formed by oxidation of NH_4^+ from residual fertilizer and mineralization of organic N and may reach concentrations up to 20 mM (for a review, see reference 213). Most species grow on NO_3^- as a sole N source and possess an assimilatory NO_3^- reductase, although some of them, including *R. etli*, do not express it in isolated SBs (104). A mutant strain of *R. etli* unable to reduce NO_2^- because of a mutation in the *cysG* gene and consequent lack of the siroheme cofactor does not grow on NO_3^-, but it induces effective nodules (178). Moreover, mutants of *Bradyrhizobium japonicum* lacking assimilatory NO_3^- reductase elicit the formation of normal nodules with somewhat better N_2 fixation characteristics compared to the parental strain (41). An *R. leguminosarum glnB* mutant strain lacking the regulatory P_{II} protein does not grow on NO_3^-, but it is Nod$^+$ and invades developing nodules (3). In conclusion, the rhizobial ability to assimilate NO_3^- is dispensable either for its growth during nodule invasion or for SB activity, even though NO_3^- may be the most abundant soil N source and at low levels stimulates nodulation (154, 213).

NH_4^+ is also produced from amino acids, which, like NO_3^-, behave as poor N sources in the presence of a rich C source. Unlike enteric bacteria, free-living rhizobia (and *Pseudomonas aeruginosa*) can use a single amino acid (such as glutamine, asparagine, or histidine) as the sole source of C and energy (99). Some mammalian cell lines also use glutamine as the principal energy source in the presence of hexoses (for a review, see reference 151). Amino acid utilization requires active uptake, deamination/deamidation, common metabolic pathways, such as the citric acid cycle and gluconeogenesis, and NH_4^+ excretion.

R. etli mutants showing very low levels of glutaminase, asparaginase, and aspartase activity are Nod$^+$/Fix$^+$ (46, 47, 50, 79). Enzymes involved in gluconeogenesis are expressed in BTs of *R. leguminosarum*, *S. meliloti*, and *Rhizobium* sp. strain NGR234 (57, 111, 137), and *R. etli* mutants impaired in this pathway induce only a few nodule primordia (R. Tatè, unpublished). Mutant strains altered in sugar catabolism are Nod$^+$/Fix$^+$ (for a review, see reference 204a). In the utilization of glutamine and histidine as a C source, glutamate is produced, which, for further catabolism, requires a dissimilatory glutamate dehydrogenase (GDH), distinct from the assimilatory one (99). Rhizobial mutants lacking this enzyme have not been described, but some mutants altered in glutamate catabolism show an altered symbiotic phenotype (see below). However, since *aap* (general amino acid permease) mutant strains of *R. leguminosarum* are unable to grow with glutamate as a carbon source but are Nod$^+$/Fix$^+$, it has been concluded that glutamate is not an important carbon and energy source in BTs of *R. leguminosarum* (204).

Assimilation

Assimilation of NH_4^+ in enteric bacteria takes place through the GS/GOGAT (glutamine synthetase/glutamate synthase) pathway and, at higher NH_4^+ concentrations, through GDH (for a review, see reference 152). As shown below, assimilatory GDH appears to be absent in rhizobia, while there are two GS isozymes, GSI and GSII. In some species a third GS has been identified (30, 53, 54, 162), which in free-living rhizobia is expressed only under special conditions. Why rhizobia express more than one GS isozyme remains an unresolved question. The present evidence is in agreement with the hypothesis that GSI is preferentially used for the assimilation of external NH_4^+, while GSII appears to be preferred for the assimilation of internal NH_4^+ when it is produced at low concentrations.

GSI, encoded by the *glnA* gene, is a dodecameric enzyme similar to the GS present in enteric bacteria. The intracellular concentration of GSI increases two- to fourfold when *R. leguminosarum* is grown on a poor N source compared to a rich one, while its activity is regulated by reversible adenylylation (153). When *R. leguminosarum* is switched from N-rich to N-poor conditions, GSI activity is essential to allow an efficient rate of GSII derepression (183). This suggests that under these conditions, GSI is required for a prompt synthesis of glutamine to be used for protein synthesis. Regulation of GSI concentration and activity (adenylylation) is similar in other rhizobial species (for a review, see reference 116).

GSII, encoded by the *glnII* gene, is a heat-sensitive (relative to GSI) octameric enzyme (103). The expression of the *glnII* gene is activated under N deficiency (for a review, see reference 54) and requires an upstream activating sequence (140). When *R. leguminosarum* is switched to an NH_4^+-containing medium, *glnII* transcription is 10-fold repressed, but GSII activity and the GSII protein become undetectable (103, 140). That this is due to a posttranscriptional control mechanism of *glnII* expression has recently been demonstrated (172, 183). The inhibition of *glnII* mRNA translation is mediated by the product of the *gstI* gene, located upstream and transcribed divergently of *glnII.* Under N-excess conditions, *glnII* transcription is low and *gstI* transcription is high, while the opposite is found under N-limiting conditions (Fig. 3, right panel).

The *gstI* gene codes for a small protein (63 amino acids) that inhibits translation of *glnII* mRNA, probably through direct binding to the 5′ untranslated region. In fact, in silico remote modeling predicts that the GstI protein may fold to match a domain of the *Escherichia coli* glutaminyl-tRNA synthetase. On the other hand, the 5′ untranslated region of *glnII* mRNA may adopt a secondary structure involving two loops, both of which contain the UUG anticodon sequence for glutamine (172, 183). The reason for the presence of such a sophisticated regulation of *glnII* expression is unknown. It is possible that GstI not only inhibits translation but at the same time stabilizes *glnII* mRNA for prompt expression when the intracellular concentration of glutamine becomes limiting. Under microaerobic free-living conditions as well as in isolated BTs of *B. japonicum*, *glnII* mRNA but neither GSII activity nor GSII protein was detected, and thus a posttranscriptional mechanism of regulation of *glnII* expression has been hypothesized (108). However, no evidence for the presence of the *gstI* gene in this bacterium has been reported. GSI but not GSII activity was detected in isolated BTs of *R. etli* (122).

Very low levels of GS activity are found in isolated N_2-fixing SBs, and genetic manipulation indicates that none of the GS isozymes is necessary for effective symbiosis (37, 54, 116, 192). However, a *glnA glnII* double mutant of *S. meliloti* induces normal invaded nodules (37), and GSIII activity was detected (162). A *glnA glnII* mutant of *B. japonicum* is severely affected in nodulation (22), and GSIII is absent in this species. These results apparently lead to contradictory conclusions that may be clarified if the symbiotic interaction is considered as a multistage process. In fact, while the ability of rhizobia to invade developing nodules may be dependent on the capacity to synthesize glutamine, GS activity appears to be dispensable for BT function.

Assimilatory GDH activity (Fig. 3, left panel) is absent in *R. leguminosarum*, and consequently this bacterium grows better on glutamine than on NH_4^+ as the sole N source (153). It is also absent in other rhizobial species (18, 76, 100, 114). This suggests that assimilatory GDH activity is not needed for efficient nodulation and function. In contrast, it has been shown that the expression of the *E. coli gdhA* gene, coding for an assimilatory GDH, reduces nodulation ability when expressed in *R. etli*, whereas if expressed in N_2-fixing BTs, it has detrimental effects on nodule efficiency (see below).

GOGAT activity is present in both free-living rhizobia and isolated SBs (20), and the structural genes, *gltB* and *gltD*, coding for GOGAT in *R. etli* have been isolated (24). Due to the absence of assimilatory GDH activity, mutants lacking GOGAT activity are glutamate auxotrophs (24, 45, 88, 95, 133, 136) (Fig. 3). *S. meliloti* and *R. etli* mutants lacking GOGAT are not altered in their symbiotic behavior (24, 95). Thus, during symbiosis, glutamate is provided in the nodule by the plant and is synthesized through a different pathway, such as amino acid degradation. In the case of *R. etli*, lack of GOGAT activity causes an enhancement of nodulation efficiency, probably correlated to an increased ability of BTs to release fixed N (24). In contrast, strains of *B. japonicum* and *A. caulinodans* lacking GOGAT are defective in symbiotic N_2 fixation (71, 133).

NH_4^+ Efflux

When rhizobia use either histidine or glutamine as the sole C source, due to the stoichiometry of the C and N requirements for bacterial growth, NH_4^+ is excreted and the extracellular concentration of NH_4^+ may become as high as 90 mM (42, 46). Since high intracellular concentrations of NH_4^+ are toxic, the extracellular levels are probably reached by means of an active mechanism of efflux (Fig. 3, left panel), but neither bacterial genes nor mutations involved in NH_4^+ efflux in rhizobia and other bacteria have been described. It has recently been proposed that the human *Rhesus*-associated RhAG proteins are involved in NH_4^+ efflux of mammalian cells. In fact, these proteins, showing similarity to members of the AMT/MEP NH_4^+ transporter family (105), facilitate the NH_4^+ efflux when expressed in *Saccharomyces cerevisiae* (106). Other members of the Rh superfamily are predominantly expressed in organs (liver, kidney, and skin) involved in ammonia genesis, excretion, and secretion (96). Glutamine (the most abundant amino acid in plasma) is the preferred oxidation substrate of erythrocytes (150) and of other mammalian cell lines (for a review, see reference 151), suggesting the necessity for NH_4^+ efflux.

In the rhizobium-legume symbiosis, most of the N_2 fixed in BTs is liberated to the host plant to satisfy its demand for N, and consequently the growth of nodulated plants becomes almost independent of alternative N sources (69). The form (e.g., NH_3/NH_4^+ and organic compounds, such as amino acids) and the mechanism (passive diffusion and transport) by which fixed N is exported are unclear. *B. japonicum* SBs isolated from crushed nodules have been shown to excrete alanine (206). *R. leguminosarum* SBs excrete different ratios of alanine and NH_4^+ according to the experimental conditions used (open or closed systems), but NH_4^+ excretion prevails in an open system where the supernatant is continuously removed (2, 147). It should be pointed out that in an intact nodule the N compounds excreted by SBs are promptly utilized by the plant, which is known to derepress NH_4^+ assimilation activities, as for example a nodule-specific GS (for a review, see reference 156). Therefore, the primary form excreted in the symbiosis by SBs is most likely NH_4^+, whereas other compounds like alanine are synthesized when NH_4^+ accumulates either in vitro

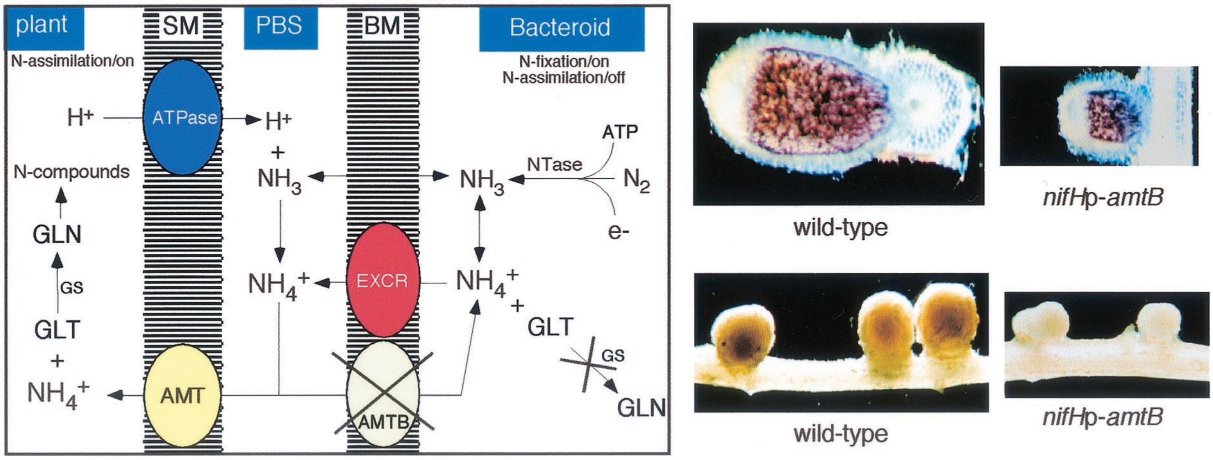

FIG. 4. NH$_4^+$ flux and assimilation inside invaded cells (left panel). NH$_3$ produced through nitrogenase (Ntase) action diffuses freely through the BT membrane (BM) and is converted into NH$_4^+$, which cannot be assimilated into glutamate (GLT) and glutamine (GLN) through glutamine synthetase (GS) action and is excreted, probably by an active mechanism (EXCR) into the peribacteroid space (PBS), where an ATPase present on the symbiosome membrane (SM) pumping protons keeps a high NH$_4^+$/NH$_3$ ratio, and a transport system for NH$_4^+$ uptake (AMT) moves NH$_4^+$ to the plant cytoplasm, where is assimilated. Note that, as described in the text, in BTs the NH$_4^+$ transporter AmtB is not expressed. (Right panel) Top: Longitudinal sections of *V. hirsuta* nodules elicited by the wild-type strain of *R. leguminosarum* and by the same strain carrying the *amtB* gene under the control of the *nifH* promoter. Bottom: Nodules of *Phaseolus vulgaris* induced by the wild-type strain of *R. etli* and by the same strain expressing *amtB* from the *nifH* promoter.

(closed chamber) or in vivo when the NH$_4^+$ assimilation capacity of the plant is reduced (2, 13).

It has been proposed (36, 192) that NH$_3$ might passively diffuse across the BT membrane into the relatively acidic peribacteroid space, where it is converted into NH$_4^+$. However, the present evidence does not exclude the existence of a channel for NH$_3$ or NH$_4^+$ efflux in free-living bacteria and in BTs during symbiosis (Fig. 4, left panel). The NH$_3$/NH$_4^+$ diffused or excreted outside the BTs reaches the plant cytosol through a channel located on the SB membrane and capable of transporting NH$_4^+$ (190) or through a channel mediating NH$_3$ flux (36). However, the plant gene(s) involved in this process remains unrecognized (81, 107). By complementation of a triple *MEP* mutant strain of *Saccharomyces cerevisiae*, genes coding for NH$_4^+$ transport systems have been identified in different plants (for a review, see reference 78) and recently in the legume *Lotus japonicus* (155). One of the *MEP* genes, coding for the high-affinity NH$_4^+$ uptake system of *S. cerevisiae*, may act as a sensor for extracellular NH$_4^+$ (98).

REGULATION OF NH$_4^+$ ASSIMILATION UNDER FREE-LIVING CONDITIONS

ntr Regulatory System

The regulation of N metabolism is best known in enteric bacteria, where the global N regulatory (*ntr*) system has been described (for reviews, see references 116 and 152). The *ntr* system senses the ratio of the intracellular concentration of glutamine to α-ketoglutarate and, through a cascade of regulatory proteins, regulates the activity of different promoters by means of a two-component system, comprising the protein kinase NtrB and the transcriptional regulator NtrC (Fig. 3, right panel). Most studies on the rhizobial *ntr* system have used the knowledge of the enteric one as a paradigm, even though differences in gene organization and regulation were found.

The *R. etli ntrBC* genes are not cotranscribed with *glnA* as in enteric bacteria, but with an open reading frame (ORF) that shows significant homology at the amino acid level with an ORF located upstream of the *fis* gene of *E. coli* and named *yhdG* (141). The YhdG protein belongs to the NifR3-SMM1 protein family of unknown function (Prosite PS01136). The same gene organization has been described for *Azospirillum brasilense*, *Mesorhizobium loti*, and *Rhodobacter capsulatus* (62, 82, 102). In the following description, the *ORF1-ntrBC* operon has been renamed *nifR3 ntrBC*. The *nifR3 ntrBC* operon of *R. etli* is transcribed from a promoter containing a sequence homologous to the −35/−10 consensus, identified by deletion analysis and runoff transcription experiments using specific oligonucleotides as DNA competitors (110). The switch from N-rich to N-poor conditions causes a 14-fold increase in NtrC concentration in enteric bacteria, but no increase (actually a 2-fold decrease) in *R. etli*. Under N deficiency, the DNA-binding activity of NtrC increases, and as a consequence some promoters (such as *glnII*) are activated, whereas others (such as *gstI*) are repressed (Fig. 3) (140–143, 183). This shows that, while in enteric bacteria N availability regulates both the intracellular concentration of NtrC and its activity, in *R. etli* only NtrC activity is regulated (110, 142). On the other hand, a mutant strain expressing a truncated form of the NtrC protein shows an intracellular concentration of *nifR3 ntrBC* mRNA at least 20-fold higher than that of the wild-type strain (141). This demonstrates the presence of a mechanism of autogenous regulation acting on either promoter activity or mRNA stability.

In *R. etli* the *ntrC* gene does not affect the utilization of either glutamine or histidine as the sole N source when added at 1 g/liter (123). Moreover, an *ntrC* mutant strain grows on 10 mM nitrate, but not on 3 mM (123). The same phenotype was observed in *ntrC* mutant strains of *B. japonicum* and *S. meliloti* (108, 174). It is possible that lack of growth at low nitrate concentrations is a consequence of a reduced bacterial capacity to maintain NH_4^+ inside the cells and/or to assimilate it. In fact, the genes involved in NH_4^+ uptake and assimilation (like *amtB* and *glnII*) are highly expressed in an NtrC-dependent mode in bacteria grown on nitrate (140, 143, 179). Moreover, it has been reported that the majority of the N limitation-induced genes are not regulated by the *ntr* system (119). It seems, therefore, that in rhizobia the main function of NtrC is the transcriptional regulation of genes involved in NH_4^+ metabolism.

In free-living diazotrophs, NtrC is necessary for expression of the *nifA* gene, which in turn activates expression of the other *nif* promoters (43), and as a consequence *ntrC* mutants are unable to grow when N_2 is the sole N source. By contrast, different rhizobia mutated in the *ntrC* gene are Nod$^+$ Fix$^+$ (108, 123, 174), showing that NtrC is not essential either for activation of *nif* and *fix* genes or for an efficient symbiosis. However, *P. vulgaris* plants nodulated with an *R. etli ntrC* mutant are invaded faster (R. Tatè, unpublished) and accumulate more N than plants nodulated with the wild-type strain (165). This suggests that NtrC exerts a negative control on other functions related to the symbiotic interaction. In fact, in *S. meliloti*, NtrC regulates *nod* gene expression as a function of the NH_4^+ concentration (48). In *R. leguminosarum*, NtrC represses the expression of the *aap* (general amino acid permease) operon (204), and in *R. etli* NtrC prevents the expression of the symbiotic terminal oxidase (167) and affects the degree of exopolysaccharide polymerization (C. Giordano and E. J. Patriarca, unpublished data). Moreover, NtrC is necessary for the alteration of nodulation exerted by the ectopic expression of the *amtB* and *gdhA* genes (114, 180).

P_{II} Regulatory Protein

In bacteria, the P_{II} protein, encoded by the *glnB* gene, has a key role in the regulation of NH_4^+ metabolism. In fact, P_{II} is involved in the covalent (and reversible) modification of both NtrC (through phosphorylation) and GSI (adenylylation), and thus in the regulation of their activity (5, 31, 32, 74, 75). In *S. meliloti*, *B. japonicum*, and *R. leguminosarum*, *glnB* is cotranscribed with *glnA* (GSI), forming a *glnBA* operon. The same gene organization is present in the genome of *M. loti* (82). An N-regulated promoter, activated by NtrC and located upstream of *glnB*, drives the generation of *glnBA* transcripts (29, 52, 109). Hence, *glnB* expression in *R. leguminosarum* is N regulated (fourfold lower in NH_4^+ compared to NO_3^-), while it is constitutive in enteric bacteria (for a review, see reference 84). The P_{II} proteins of *R. leguminosarum* and *S. meliloti*, as in enteric bacteria, are subject to covalent uridylylation, which modulates their activity (6, 32, 159). The degree of uridylylation is high under N limitation and undetectable under N excess (32). In bacteria growing in an NH_4^+-rich medium, unmodified P_{II} stimulates the adenylylation of GSI, inactivating the enzyme (4, 65). However, the influence of the different forms of P_{II} on the activity of NtrC varies among different rhizobia.

In *R. leguminosarum* the P_{II} protein is essential to inactivate (dephosphorylate) NtrC under conditions of N excess, but it is dispensable for the activation (phosphorylation) of NtrC, which, in the absence of P_{II}, becomes active independently of the N conditions (Fig. 3, right panel). In fact, transcription of the *glnII* gene (requiring NtrC for expression) is constitutive in a *glnB* mutant (3). In a *glnD* mutant lacking uridylyl removing/uridylyltransferase (UR/UTase), the enzyme catalyzing uridylylation/deuridylylation of P_{II}, the latter is constitutively deuridylylated, and therefore NtrC cannot be activated. As a consequence, the *glnII* promoter also remains silent under N-limiting conditions (159). An *R. leguminosarum glnB* mutant induces normal early organogenesis (3), but nitrogenase is only weakly expressed compared to the level in nodules induced by the wild-type strain (183). In contrast, a *glnD* mutant strain elicits the formation of normal, N_2-fixing nodules (159). Altogether, these data indicate that P_{II}, in its deuridylylated form, is essential for normal levels of nitrogenase expression, most probably due to the inactivation exerted by P_{II} on NtrC and/or GSI, ensuring a reduced NH_4^+ assimilation capacity of BTs (see below). This is supported by the observation that NtrC-activated promoters are switched off, in a P_{II}-dependent manner, very early during symbiosis (183).

In *S. meliloti* P_{II} is instead essential to activate (phosphorylate) NtrC under conditions of N starvation, and as a consequence, NtrC-dependent promoters are inactive in a *glnB* mutant (5). When *M. sativa* is infected with this mutant strain, most ITs abort early. Since strains lacking NtrC are able to induce efficient nodules, the abnormal phenotype of the *glnB* mutant cannot be explained by a requirement for NtrC phosphorylation. Therefore, P_{II} should be involved in the regulation of another bacterial function(s) essential at the onset of nodulation (growth in the ITs), such as the activation of genes needed for the synthesis of Nod factors, exopolysaccharides, and lipopolysaccharides. Otherwise, a constitutively dephosphorylated NtrC might repress and alter the expression of the above-mentioned genes. The phenotype of the *S. meliloti glnB* mutant strain cannot be explained by an action of the P_{II} protein on GSI adenylylation, since a *glnA* mutant strain impaired in adenylylation of GSI shows a normal symbiotic phenotype (4).

In the *R. leguminosarum glnB* mutant, the NtrC-activated genes (such as *glnII* and *glnK-amtB*) are highly expressed independently of the N source, but this strain does not grow on nitrate (3). It appears therefore that P_{II} activates another function, as yet unknown, required for nitrate utilization.

As mentioned above, a second *glnB*-like gene, *glnK*, located upstream of the *amtB* gene is present in different rhizobia (6, 82, 179). In *R. etli* the *glnK-amtB* operon is transcribed from a σ^{54}-dependent promoter requiring NtrC for activity (179). This promoter does not show a clear-cut sequence for NtrC binding, and therefore its activation by NtrC may be indirect. In some bacterial species, *glnB* and *glnK* are functionally equivalent (for a review, see reference 6). In rhizobia the function of GlnK is unknown, although it has recently been shown that in *E. coli* and *Azotobacter vinelandii*, GlnK binds to the membrane in an AmtB-dependent manner and that GlnK acts as a negative regulator of the transport activity of AmtB (33a). This interaction represents a novel form of signal transduction pathway sensing extracellular NH_4^+.

In *A. caulinodans*, a second two-component system (NtrY/NtrX) showing considerable homology to NtrBC has been identified (145) and found to regulate the expression of NtrC and NifA. *A. caulinodans* is a diazotrophic bacterium in which NtrC mediates the induction of *nifA* transcription under N limitation. Homologous genes are also present in *M. loti*, *R. etli*, and *S. meliloti* (64, 82; E. J. Patriarca and M. Merrick, unpublished data), but no evidence for their presence has been reported in the case of enteric bacteria. Sequence analysis predicts that NtrY (the homologue of the protein kinase NtrB) may be membrane associated through a transmembrane element. Therefore, NtrY may modulate the activity of NtrX (the NtrC equivalent) as a function of the extracellular N. If so, its function might be linked to that of the *glnK-amtB* operon.

REGULATION OF NH_4^+ ASSIMILATION IN DIFFERENTIATING SYMBIOSOMES

In Situ Detection

Most studies describing the presence of NH_4^+ assimilation enzymes in nodules use an in vitro biochemical approach. This means crushing nodules, purifying BTs and intact SBs, and assaying a specific gene product. Although direct and quantitative, it assumes reproducibility in the yield of isolation of SBs at different stages of development, the absence of contaminating plant products, and stability during the isolation procedure. The results obtained are the average values of a heterogeneous population of SBs and therefore are of limited usefulness for the understanding of in situ NH_4^+ metabolism in the nodule. Moreover, SBs analyzed in vitro are out of their natural environment, namely the cytoplasm of the plant cell, which may explain some of the contradictions that are found in the literature when comparing results obtained through biochemical and genetic methods.

Studies on the pattern of gene expression in developing nodules clarify the above contradictions. Although in situ localization of bacterial mRNA has been reported (158, 212), the histochemical localization of the product of a reporter gene (*gfp*, *gusA*, and *lacZ*) is more generally used because of the simplicity and efficacy of the method (7, 63, 161). Most studies make use of *lacZ* fusions because the protocol used to lower the background of β-galactosidase activity (7) and the use of stable plasmids (170, 208) permit clear establishment of when and where during nodulation a bacterial promoter is active.

As shown below, the in situ analysis of the activity of different promoters in the elongated nodule confirms and extends the conclusions reached by histological analysis (186, 199). In fact, with respect to the regulation of NH_4^+ metabolism in *V. hirsuta* nodules, this kind of analysis confirms the activation of *nif* gene expression in BTs of SBs located at the II-III interzone (144, 179) and allows the description of two previously unrecognized metabolic events (Fig. 1). The first one is the downregulation of genes required for NH_4^+ uptake and assimilation, observed when bacteria are released, by endocytosis, from the ITs into the meristematic host cells. The second one takes place later on, a few cell layers before the II-III interzone, where promoters of several N-regulatory genes (including *gstI*, *ntrC*, and *glnB*) are switched off.

In the globose nodule of *Proteus vulgaris*, the in situ analysis of bacterial promoters, together with earlier evidence of gradients of expression of eukaryotic gene products in this nodule (176), has been instrumental in revealing a spatial component of development. In particular, downregulation of the *nifR3-ntrBC* promoter clearly defines a previously unrecognized developmental zone (144). Furthermore, the analysis of the *nifHc* promoter indicates that *nif* genes are activated much earlier in the globose nodule than in the elongated nodule (180). Table 1 lists the topological expression of bacterial genes in nodules.

Expression of Bacterial Genes

The promoters of the *glnK-amtB* operon of *R. etli* and of the *glnII* gene of *R. leguminosarum* are N regulated and strictly dependent on NtrC for activation (see above) (140, 179). Their activity is lower in bacteria growing on glutamine as a sole C source (thus causing NH_4^+ efflux) than when growing on NH_4^+ (R. Tatè, unpublished). In the symbiosis, these promoters are switched off very early during nodule invasion (179, 183). In both elongated and globose nodules (Fig. 1 and 2), both promoters are active in bacteria growing inside the ITs but become inactive just when bacteria begin to invade nodule cells and thus are the earliest negatively regulated promoters so far recognized. In fact, transcription of *nod* genes, which mediates the nodule primordium induction, is switched off later during SB differentiation (161).

The *gstI* promoter in free-living *R. leguminosarum* is induced in an N-rich medium, while it is repressed under N-poor conditions (183). In developing nodules of *V. hirsuta*, the *gstI* promoter is active in SBs released in the first ICs of the inner cortex during primordium formation. In 1-week-old nodules, a decreasing distal-proximal gradient (with respect to the root) is observed, which becomes more evident later on, when the activity is restricted to the SBs present in the most distal cells of the invasion zone (zone II according to reference 199) (Fig. 1). The *gstI* promoter becomes inactive before transcription of the *nif* genes is activated (183). The pattern of *gstI* expression suggests that dividing BTs located in the distal part of zone II of elongated nodule sense a signal of N excess, whereas the most proximal ones are under severe N limitation and are unable to activate the *gstI* promoter. In the presence of GstI, *glnII* expression in BTs may be inhibited not only at the transcriptional level but also at the posttranscriptional level, as occurs under free-living conditions (183).

The *R. etli nifR3-ntrBC* promoter in *V. hirsuta* nodules is active in bacteria growing in ITs and in dividing BTs located in differentiating host cells of the distal zone II. It becomes inactive in the SBs of the last two or three cell layers before *nif* gene expression is switched on (Fig. 1) (144). The same pattern of expression is observed in Fix^- nodules, confirming that downregulation of the *nifR3-ntrBC* promoter is not due to the synthesis of NH_3 produced through nitrogenase activity. In globose nodules of *P. vulgaris*, the *nifR3-ntrBC* promoter is active in bacteria growing in the ITs, in the BTs released in the first ICs of the primordium, and in all ICs of early emergent nodules. In developed nodules, expression is limited to the BTs located in the two and three most peripheral layers of ICs (Fig. 2) (144). Immunolocalization of NtrC confirms this pattern of expression and shows that not only is transcription of the *nifR3-ntrBC* operon switched off but also that, since BTs at this stage do not divide, the NtrC protein is removed.

The *R. leguminosarum glnBA* promoter in *V. hirsuta* nodules shows a pattern of activity quite similar to that of the *nifR3-ntrBC* and *gstI* promoters, since it is active in bacteria growing inside the ITs and in BTs released in the first ICs of the inner cortex during primordium formation. In more mature nodules it is switched off, in correlation with the disappearance of NtrC, a few cell layers before *nif* gene expression (52).

In nodules of *P. vulgaris*, the *R. etli nifHc* promoter is induced at a very early stage of nodule invasion, namely, in BTs just released in the plant cells (180). This promoter is induced in a NifA-dependent manner both under free-living microaerobic conditions and during symbiosis (195). Instead, in elongated nodules the *R. etli nifHc* promoter is switched on abruptly in SBs located at interzone II-III (144), exactly where predicted by an in situ hybridization analysis revealing the endogenous *nifH* mRNA (212). In interzone II-III, the ICs are filled with nondividing, almost completely differentiated SBs (199), and a sharp drop in O_2 tension takes place (168) (Fig. 1). The earlier timing of NifA activation, and therefore of *nifHc* expression, in globose versus elongated nodules may be related to differences either in the organizational structure of the parenchyma (211) or in plant and/or bacterial metabolism, which generate a low O_2 tension environment (91, 120).

That the invasion of globose nodules takes place under microaerobic conditions is supported by the following data. First, in the youngest ICs of developing nodules of *P. vulgaris*, harboring only a few SBs formed by dividing BTs, a large number of condensed (crista-rich) mitochondria, which cluster, aggregate, and divide, are observed (25). Second, a terminal oxidase complex, which is expressed under microaerobic conditions in free-living bacteria, is required for the invasion process, since a cytochrome *c*-deficient mutant of *R. etli* induces empty nodules (166). Third, nitrogenase activity (requiring microaerobiosis) is detected in young soybean nodules, in which most of the BTs in the host cells are still actively dividing (12). These data indicate that the nitrogenase complex is induced in young dividing BTs during the invasion of globose nodules, even though the analysis of *nif* mutants indicates that nitrogenase activity is not essential for invasion.

ntr System

The *glnK-amtB* and *glnII* promoters become inactive in younger BTs when the *nifR3-ntrC* promoter is still active and the NtrC protein is present (see above). This suggests the inactivation of NtrC, because of a signal of N excess transmitted by P_{II}, and the activation of a different regulatory circuit involving a specific repressor(s) operating on both promoters. The availability of an *R. leguminosarum glnB* mutant and the in situ analysis of the *glnII* (NtrC-activated) and *gstI* (NtrC-repressed) promoters allowed us to test whether the *ntr* system senses the N status of SBs. In dividing BTs located in the ICs of the distal zone II of nodules induced by the wild-type strain, the NtrC-repressed *gstI* promoter is active, whereas the NtrC-activated *glnII* promoter is inactive (Fig. 1 and 2). In contrast, in nodules induced by the *glnB* mutant, the *gstI* promoter is switched off during endocytosis, whereas the *glnII* promoter remains active (Fig. 1) (183). The same reciprocal pattern of regulation occurs in wild-type and free-living *glnB* bacteria. This indicates that P_{II} is necessary for regulation of NtrC activity not only in free-living bacteria but also in BTs. The P_{II}-mediated inactivation of NtrC is the first recognized step in the molecular mechanism leading to the uncoupling of NH_4^+ assimilation and N_2 fixation in differentiated SBs. This event occurs early during host cell invasion, just when bacteria are released into the host cell, and therefore much earlier than when N_2 fixation begins to take place.

During symbiosis, the N status of rhizobia is determined by the composition of the medium in which they are immersed. The analysis of *ntr*-regulated promoters indicates that during endocytosis, the N metabolism of BTs changes from a condition of N deficiency to one of N richness (and C deficiency, which would result in a relative abundance of available N). The movement of the bacterium from a C-rich environment, the cell wall-like matrix of the ITs, into the intracellular environment of the BTs, which are surrounded only by the SB membrane, may reduce C availability. A condition of C limitation and N excess may also be generated by the utilization of amino acids as a C source (see below). A second factor causing a condition of C deficiency is the permeability of the SB membrane. The latter arises from an invagination of the plasma membrane, so that if it does not invert the polarity of its transport systems, the peribacteroid space is, at least initially, equivalent to the extracellular space. This implies that the IC may take up solutes, such as sugars, not only from the extracellular space but also from the peribacteroid space of the youngest BTs. However, later on during differentiation, the structure and function of the SB membrane as well as the composition of the peribacteroid space gradually change (200, 201). For instance, the induction of a C_4-dicarboxylate carrier on the SB membrane (138) may drastically change the physiology of the SBs.

N Sources

A successful symbiosis may take place in the absence of added N, and therefore nodule (and root) growth uses N sources provided by the seed. Bacteria grow extensively in the ITs, and BTs grow inside the ICs of the nodule (at least in elongated nodules) before nitrogenase begins to be expressed. An effective symbiosis is induced by a few bacteria, which multiply up to many millions. Sustained bacterial growth implies that the plant provides the necessary N compounds in return for a more abundant supply later on. The N source needed for growth is not NH_4^+ and NO_3^- (see above) and therefore should be a single or several N-containing organic compounds. Determining the symbiotic phenotypes (Nod and Fix) of auxotrophic mutants is a way to test the availability to rhizobia of a given substance in the developing nodule.

N-containing compounds such as purines and pyrimidines are not provided by the corresponding plant partners to *B. japonicum*, *R. etli*, *Rhizobium fredii*, and *R. leguminosarum*, even in the ITs, since auxotrophic strains induce only empty pseudonodules (128, 130). Mutants of *S. meliloti* induce Fix^- nodules, but a histological analysis was not performed (85).

R. etli strains defective in the biosynthesis of amino acids (such as aromatics, aspartate, cysteine, glutamate, glycine, histidine, isoleucine-valine, leucine, lysine, phenylalanine, and tryptophan) with the exception of an arginine and a methionine auxotroph, are able to induce nodules (24, 56, 178, 181, 182; S. Ferraioli, unpublished data). Analogous observations have been reported in the case of *S. meliloti*. In fact, except for an isoleucine-valine auxotroph (1), all other mutants analyzed (such as those requiring asparagine, leucine, methionine, ornithine, tryptophan, and tyrosine) are able to induce nodules on *M. sativa* (10, 85, 148, 188). With minor differences, the same conclusion was reached by analyzing amino acid-requiring mutants of *R. leguminosarum* (139). In most cases, however, the resulting nodules are partially or totally ineffective, but without histological observations, the nature of these alterations in most cases remains unrecognized.

Auxotrophic strains of *R. etli* incubated in minimal medium in the absence of their nutritional requirements are unable to produce Nod factors, but when inoculated on the roots of *P. vulgaris* without exogenously added amino acids, they are able to induce nodules (56, 178, 181, 182). Since Nod factors are essential for nodule induction, the plant must provide the necessary amino acids to the bacterium. The presence of amino acids in the root exudates of some legumes has been demonstrated (196), and it has been proposed that they are produced by lysis of the root cells detached from its cortex due to the outgrowth of developing lateral roots. However, it is not possible to exclude that root cells even under N starvation (a condition necessary for symbiosis) release amino acids to stimulate rhizobia both to divide and to produce Nod factors. Chemotaxis towards root exudates has been demonstrated (21).

The ability of most of the amino acid-auxotrophic strains to invade developing nodules indicates that the plant provides the amino acids necessary for bacterial growth occurring inside the ITs. The origin of the amino acids in the ITs is unknown, but, similar to what happens in the case of pollen tubes, membrane vesicles may fuse with the tip of developing ITs, allowing their elongation (16, 68). In this way they would provide nutrients in the form of amino acids and proteins. This may explain why bacteria grow only at the tip of ITs (63). After endocytosis, the composition of the peribacteroid space, as well as the composition of the SB membrane, is modified by fusion of the SB with secretory vesicles formed by budding of the Golgi cisternae and/or originated from the endoplasmic reticulum (201). A variety of hydrolytic enzymes, including proteases and protease inhibitors, have been identified in the peribacteroid space (113). Since BTs divide several times before differentiation and the SB membrane is relatively impermeable to amino acids (192), proteolytic activity may generate a mixture of free amino acids from proteins released through vesicle fusion.

Different lines of evidence indicate that undifferentiated BTs may use amino acids as a source of C and energy and that C_4-dicarboxylic acids are the C source utilized after SBs begin to differentiate (51, 198). An *S. meliloti* mutant strain altered in proline catabolism shows reduced nodulation efficiency and competitiveness, and the promoter for the *putA* (proline utilization) gene is active during root invasion and nodule formation, but not in N_2-fixing BTs (80). An *S. meliloti* mutant strain missing succinic semialdehyde dehydrogenase (involved in the γ-aminobutyrate bypass), and therefore impaired in glutamate catabolism, shows reduced nitrogenase activity and thus biomass-accumulating ability (61). The γ-aminobutyrate bypass is responsible, even though partly, for the catabolism of glutamate in BTs of *B. japonicum* (90). A mutant of *S. meliloti* missing aspartate aminotransferase (*aatA* gene) induces Fix^- nodules in which only a few BTs are present in the ICs (207). Since proline catabolism and aspartate aminotransferase activity generate glutamate, together these data suggest that glutamate catabolism plays an important role in the dividing BTs.

A very strong inactivation of the *glnII* promoter (NtrC activated) and derepression of the *gstI* promoter (NtrC repressed) are observed in free-living *R. leguminosarum* when an amino acid, such as glutamine and glutamate, is used as the sole C source (see above) (183). Therefore, the almost complete inactivation of NtrC taking place during endocytosis supports the notion that BTs use amino acids as a C source. Moreover, when rhizobia utilize an amino acid as a C source, they excrete NH_4^+, and if under these conditions *amtB* expression is forced by means of an inducible promoter, bacterial growth is inhibited (E. J. Patriarca, unpublished data). NH_4^+ excretion by young BTs may prepare the SBs and the cytoplasm of ICs, through the induction of specific plant genes, for the transport and assimilation of the NH_4^+ produced later by the nitrogenase.

Global Regulation

During SB differentiation, the transcription of genes coding for N-regulatory proteins (such as NtrC, GstI, and P_{II}) is switched off. It is clear, however, that transcriptional downregulation of these and other genes is not a consequence of the nitrogenase activity and of the NH_4^+ produced. In fact, many promoters are silenced at the same stage of differentiation in nodules induced by a Fix^- mutant (52, 144, 177). Moreover, some of these promoters, such as *gstI* and *nifR3-ntrBC*, are induced in free-living bacteria growing with NH_4^+ as an N source (183).

With almost the same pattern, the *lipA* (lipoic acid synthetase), *nod* (Nod factors), *putA* (proline utilization), and *ropA* (outer membrane protein III) genes are also downregulated (38, 80, 158, 161, 177). Over 350 proteins, including several proteins involved in N metabolism and the cell cycle, were found to be expressed at lower levels or undetectable in isolated SBs of *S. meliloti* compared to free-living bacteria (126). These include proteins encoded by genes that are either never expressed or inactivated very early (in the ITs) during symbiosis. This proteome analysis, however, reflects the pattern of expression obtained from a mixture of BTs at different stages of differentiation. Therefore, proteins encoded by genes that are downregulated (even abruptly) later on during BT differentiation, such as in interzone II-III (Fig. 1) could be considered as not altered. Therefore, the number of genes downregulated may be higher. Downregulation is extensive, involving genes related to different metabolic pathways, uncorrelated with respect to regulation and function. It seems, therefore, that in differentiating SBs there is a global mechanism for inactivation of gene expression.

It is known that in bacteria one of the mechanisms used to exert a global control of gene expression is the modification of the chromosome structure. DNA supercoiling varies as a function of the cellular energy status and plays an essential role in bacterial adaptation and survival to suboptimal growth conditions, such as stationary-phase growth, osmotic stress, and aerobic to anaerobic transition (for a review, see reference 157). It has been shown that before the sharp drop in O_2 concentration taking place at interzone II-III of nodules, a shallow gradient of decrease is observed in zone II (168) and, in addition, that the DNA superhelicity increases in BTs (66). The energy status (the ADP/ATP ratio) of BTs may change as a function of the decrease in the O_2 tension that is encountered inside the nodule, thus altering the superhelical density of the chromosome.

Another event taking place at this stage, in the middle of zone II, is the transition from dividing BTs (distal zone II) to nondividing differentiating BTs (proximal zone II). In bacteria sigma factors are considered global regulators of gene expression. The *sigA* gene, coding for the putative vegetative sigma factor of *R. etli*, has been identified (101). The intracellular levels of *sigA*, *lipA*, and *nifR3-ntrBC* transcripts are very high just after bacterial dilution in complete medium, decrease during bacterial divisions, and become very low, almost undetectable, as bacterial growth continues (101, 144, 177). In a similar manner, *sigA* may be downregulated during BT differentiation (144, 177) (Fig. 2), and as a consequence, a large set of genes necessary for bacterial growth and invasion but dispensable for the function of N_2-fixing BTs would be silenced.

A combination of immunolocalization and promoter analysis (and in situ mRNA hybridization) shows, in all cases analyzed, the colocalization of transcripts and proteins. Different proteins, including β-galactosidase, expressed from *lacZ* transcriptional fusions become undetectable in BTs located in proximal zone II. Since BTs in proximal zone II change their shape and enlarge but do not divide (199), protein disappearance is better explained by a mechanism of proteolysis than by dilution. Activation of proteolysis is observed in rhizobia incubated in minimal medium with glucose and mannitol but without added N (E. J. Patriarca, unpublished data). Changes in C and N supply, namely a switch from amino acids to C_4-dicarboxylic acids, which may result in severe N starvation, appear to take place in correlation with the beginning of BT differentiation. The removal of a large set of proteins, including the vegetative sigma factor, may explain the irreversibility of the process of BT differentiation. A parallel with the very early steps of the sporulation of *Bacillus subtilis* has been suggested (144). In fact, during the initial stage of sporulation induced by bacterial dilution in N-free medium, transcription of a set of genes is downregulated by inactivation of the vegetative sigma factor (187) and protein degradation is activated in the mother cell (89).

DOWNREGULATION OF NH_4^+ ASSIMILATION IS REQUIRED FOR EFFICIENT SYMBIOSIS

As described above, the promoters of genes for NH_4^+ uptake (*glnK-amtB*) and for its conversion into glutamine (*glnII*) are switched off very early during invasion of host cells, whereas those of genes involved in the regulation of NH_4^+ metabolism such as *ntrBC*, *glnBA*, and *gstI* are downregulated later on during SB differentiation. Accordingly, N_2-fixing BTs show very low levels of NH_4^+ assimilation activities, and mutant strains lacking genes required for NH_4^+ assimilation, such as *glnA*, *glnII*, and *gltB*, are able to induce normal N_2-fixing nodules. A key question is if this downregulation is necessary for the efficient functioning of the symbiosis. Of course, it is not possible to give an answer by using null mutants for these genes, and in fact, our present understanding of the necessity for downregulation comes from bacteria in which a specific function, NH_4^+ uptake and assimilation, is forced to be expressed in the symbiosis.

The expression of the *E. coli gdhA* gene (coding for an assimilatory GDH) was used to enhance the NH_4^+ assimilation capacity of *R. etli*, which does not possess an assimilatory GDH. Bacteria expressing *gdhA* show up to sixfold lower GOGAT activity, a sixfold-higher glutamate pool, and excrete some amino acids into the medium (114). The same alterations are observed when *gdhA* is expressed in an *ntrC* derivative. The ability of the wild-type strain of *R. etli* to induce nodules was completely inhibited when this strain carried *gdhA* (17, 18, 114). In contrast, the symbiotic behavior of the *ntrC* mutant strain was unaffected by GDH activity. Consistent with these results, *gdhA* expression reduced the capacity of the wild-type strain but not of the *ntrC* mutant to induce *nod* genes and thus to produce Nod factors in the presence of the appropriate flavonoid inducer (naringenin). These results indicate that bacteria must be N deprived to be able to nodulate and that NtrC is an essential component of the N-dependent regulatory circuit, which modulates the expression of *nod* genes as a function of the N status of the cells (114).

By driving the expression of *gdhA* from the *nifHc* promoter (which is under NifA control), it has been demonstrated that a high level of NH_4^+ assimilation in BTs is also detrimental at later steps of the symbiosis (115). The expression of GDH, and thus the enhancement of the NH_4^+ assimilation capacity of BTs, has a negative consequence on the ability for N translocation to the plant (115). In fact, GDH mediates an increase in the amino acid pool of BTs (including glutamate), whereas it reduces the ureide (the form of N transported to bean leaves) content in the xylem sap (115). Moreover, nitrogenase activity but not *nifHDK* expression is reduced in BTs expressing GDH, and premature senescence of some nodules is observed.

The effect of ectopic expression of the *amtB* gene allows investigation of whether BTs release NH_4^+. In the current model of NH_4^+ flux in the symbiosis (36, 192), the NH_3 produced in the BTs by the nitrogenase complex would diffuse to the peribacteroid space, where it is converted to NH_4^+. These NH_4^+ ions would reach the cytoplasm of the plant cell through an NH_4^+ channel located in the SB membrane and would then be fixed into amino acids. The requirements for this process are (i) a low level of GS activity (NH_4^+ assimilation) in BTs; (ii) the presence of NH_3/NH_4^+ in the peribacteroid space; (iii) the absence of an NH_4^+ uptake system in the bacteroid membrane; (iv) the presence of an NH_4^+ carrier in the SB membrane; and (v) high levels of assimilatory GS and GDH activities in the cytoplasm of the ICs.

The release of NH_3/NH_4^+ into the peribacteroid space was tested by forcing the expression of *amtB* in N_2-fixing BTs, in which this gene is normally not expressed (179, 180). It was predicted that the presence in BTs of an active mechanism for the uptake of NH_4^+ should retrieve the NH_4^+ present in the peribacteroid space, thus generating an energy-consuming futile cycle with negative consequence for the symbiotic interaction. The results obtained confirmed this prediction. In fact, driving the expression of *amtB* from the *nifHc* promoter (specifically activated by NifA under microaerobic conditions), the symbiosis is profoundly affected. Nodules (12 days old) are small, show abnormal pigmentation, a reduced number of ICs containing only a few undifferentiated SBs, and, in correlation, almost undetectable nitrogenase activity (measured as acetylene reduction). By forcing the expression of *amtB* in different genetic backgrounds, it was shown that the detrimental effect of *amtB* expression depends on the capacity of BTs to fix N and to efficiently assimilate NH_4^+ (180).

Surprisingly, the earliest effect of the expression of *amtB* was the arrest of bacterial division inside the host cells. Bacterial division, as well as nitrogenase activity, requires energy in the form of ATP and reducing equivalents. In *Azotobacter vinelandii*, it was observed that the addition of NH_4^+ to N_2-fixing cells rapidly inhibits nitrogenase activity by decreasing the flow of reducing equivalents, whereas at high concentrations it also reduces the intracellular ATP/ADP ratio (92). In contrast, addition of NH_4^+ does not affect nitrogenase activity of isolated SBs of *R. leguminosarum*, which are instead unable to take up NH_4^+ actively, and therefore it was concluded that NH_4^+ could affect the energized state of the membrane only when it is taken up (92). The *amtB*-mediated negative effect on BT growth supports the notion that, unlike free-living diazotrophs, BTs are programmed not only to fix N_2 but also to release most of the N fixed and that the N_2 fixation process in BTs is regulated to satisfy the plant requirement for N.

The ectopic expression of the *R. etli amtB* gene was also studied in *V. hirsuta* (R. Tatè, unpublished data) (Fig. 4, right panel). Nodules induced by an *R. leguminosarum* strain expressing *amtB* ectopically from the *nifH* promoter, and thus expressing it specifically in N_2-fixing BTs of zone III, are smaller than the control ones and their nitrogenase activity is strongly reduced. However, as predicted by the timing of NifA activation (Fig. 1), the detrimental effects mediated by *amtB* are evident after interzone II-III, similar to what was observed in nodules induced by Fix^- mutant strains.

In summary, the evidence obtained by driving the expression of *gdhA* and *amtB* in BTs permits us to conclude that the symbiotic association is based on the ability of rhizobia to uncouple N_2 fixation and NH_4^+ assimilation and that at least part of the NH_3/NH_4^+ produced by nitrogenase activity is directly released by the BTs.

CONCLUDING REMARKS

In the rhizobium-legume symbiosis, most of the fixed N is released to the plant and used by it (69). Nitrogenase expression is switched on, bacterial assimilation of the NH_4^+ produced is prevented, and fixed N is exported. The uncoupling of NH_4^+ production and assimilation is crucial in the symbiosis, but it has received relatively little attention (192a). Results obtained when analyzing free-living diazotrophs cannot be extrapolated to rhizobia. In fact, in *K. pneumoniae*, the NH_3 produced by N_2 fixation is assimilated for growth, whereas in free-living rhizobia it is not assimilated for growth but excreted.

Until now, the transition of rhizobial N metabolism that takes place when free-living bacteria differentiate into endosymbionts has been considered in terms of a single-step mechanism. Instead, it should be regarded as a multistep process requiring recognition of the plant, invasion of specific cortical cells, growth in the ITs, endocytosis, differentiation in the ICs into N_2-fixing BTs, and finally senescence. This implies several check points effected through the exchange of signal and metabolites, inside a plant that maintains a controlled internal environment through regulation and adaptation of its own N metabolism (for a review, see reference 118). During nodule development, the plant provides the necessary N compounds for bacterial growth and for BT differentiation in the ICs, while it simultaneously prepares itself to assimilate and transport the fixed N as glutamine in temperate legumes and as ureides in tropical legumes (see reference 160 for a review).

As emphasized in this review, the use of modern histochemical techniques (including in situ RNA hybridization, immunolocalization of proteins, and in situ promoter activity), combined with classical cytological and ultrastructural analyses, has made it possible to observe developmentally regulated changes (temporal and spatial) in bacterial and plant gene expression during the symbiotic interaction. The main conclusions reported in this review follow.

(i) For the growth of bacteria in the rhizosphere and inside the ITs, a mixture of amino acids is available, whereas for the differentiation of BTs inside the ICs, a smaller number of amino acids are available. This change in amino acid availability coincides with the internalization of BTs in the ICs and thus with the formation of the SB membrane.

(ii) Bacteria in the ITs are N starved, indicating that amino acids are used as an N source, whereas at the onset of endocytosis BTs are under conditions of N excess, as if from this stage amino acids are also used as a C source (resulting in an excess of NH_4^+).

(iii) Endocytosis is a key step in the symbiotic regulation of bacterial N metabolism in both elongated and globose nodules, since NtrC-activated promoters are switched off at this stage. The N-regulatory P_{II} protein plays a central role in the inactivation of NtrC, indicating that an intracellular signal of N excess occurs in BTs at endocytosis.

(iv) Downregulation of NH_4^+ assimilation begins with the early inactivation of the NtrC protein followed by downregulation of the *nifR3-ntrBC* promoter and is completed with the removal of NtrC. The absence of NtrC in BTs explains why nitrogenase expression and regulation of N metabolism are uncoupled. In fact, in free-living diazotrophs, NtrC activity is essential for the coordinated expression of genes involved in N_2 fixation and NH_4^+ assimilation.

(v) Nitrogenase expression takes place when gene products for NH_4^+ sensing (P_{II}), regulation (NtrC), and assimilation (AmtB and GS) are either inactivated or absent. As a consequence, most of the NH_3/NH_4^+ produced must be released to the plant to avoid detrimental effects on the SBs.

Symbiotic N_2 fixation provides N to cultivated plants, thus avoiding the negative environmental effects caused by chemical fertilizers. In the last two decades, the considerable interest in the rhizobium-legume symbiosis has produced scant results in the improvement of this process due to underestimation of its complexity and ignorance about the regulatory mechanisms involved. Knowledge of the regulation of N metabolism constitutes an important step towards correcting this situation. In fact, as described in this review, the efficiency of symbiotic N_2 fixation is improved by (i) increasing nitrogenase activity (overexpression of the *nif* genes and enhanced respiration); (ii) reducing the NH_4^+ assimilation capacity of the bacterial partner (NtrC- and GOGAT-deficient mutants); (iii) enhancing the NH_4^+ assimilation capacity of the plant (high levels of GS); and (iv) decreasing the sensitivity of the symbiotic interaction to the presence of inhibiting concentrations of NH_4^+ (NtrR). Vice versa, genetic manipulations that modify these processes in the opposite direction result in reduced N_2 fixation efficiency.

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