Auxotrophic Mutant Strains of *Rhizobium etli* Reveal New Nodule Development Phenotypes

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We report here the isolation and characterization of amino acid–requiring mutant strains of *Rhizobium etli*. We observe that the phenotype of most mutations, even when causing a strict auxotrophy, is overcome by cross-feeding from the host plant *Phaseolus vulgaris*, thereby allowing bacterial production of Nod factors and, consequently, nodule induction. Conversely, light and electron microscopy analysis reveals that the nodules induced by all mutants, including those with normal external morphology, are halted or strongly altered at intermediate or late stages of development. Moreover, some mutants induce nodules that display novel symbiotic phenotypes, such as specific alterations of the invaded cells or the presence of a reduced number of abnormally shaped uninvaded cells. Other mutants induce nodules showing an early and vast necrosis of the central tissue, a phenotype not previously observed in bean nodules, not even in nodules induced by a *Fix⁻* mutant. These observations indicate that amino acid auxotrophs represent a powerful tool to study the development of globose determinate-type nodules and emphasize the importance of establishing their histology and cytology before considerations of metabolic exchange are made.

Additional keywords: cell wall, root exudate, symbiosis, symbiosome differentiation.

Rhizobia are gram-negative bacteria that have the ability to interact, through the induction of N₂-fixing root nodules, with plants of the *Leguminosae* family. From the point of view of the bacterial partner, nodule formation involves infection of root hairs and bacterial growth inside a network of tubular structures called infection threads (ITs). When ITs reach cells of the nodule primordium (formed by successive rounds of mitosis), bacteria are released through endocytosis from the tip of IT ramifications into the cytoplasm of host cells. Bacteria become engulfed in a plant membrane, initially arising from the plasma membrane, which acts as a physical barrier between both partners. Intracellular bacteria, called bacteroids (BTs), divide few times and show physiological changes, morphological changes, or both, such as the activation of the nitrogenase complex that catalyzes the reduction of atmospheric N₂ into NH₃ and makes the plant autotrophic for external N (Mylona et al. 1995). The organellelike structure formed by the BT, together with the peribacteroid space and the surrounding plant membrane, is called a symbiosome (SB). Developed root nodules are vascularized organs formed by different tissues, with a

central tissue (CT) containing thousands of invaded plant cells (ICs) fully packed with differentiated SBs exchanging fixed N for C compounds (Van Rhijn and Vanderleyden 1995). Thus, at least two bursts of bacterial divisions are necessary to develop a nodule. The first one occurs inside the ITs, while the second takes place within the cytoplasm of the ICs (Turgeon and Bauer 1985). This implies that, during nodule organogenesis, *Rhizobium* metabolism should adapt to three different environments, namely rhizosphere, ITs, and host cell cytoplasm. In particular, during BT differentiation, a shift of bacterial metabolism from N assimilation to NH₄⁺ export is essential for an efficient symbiotic interaction (Arcondéguy et al. 1997; Mendoza et al. 1995; Patriarca et al. 1996; Tatè et al. 2001).

Nodule invasion occurs in the absence of exogenously added N, and it does not require bacterial genes involved in the assimilation of inorganic N compounds (such as NO₃⁻ or NH₄⁺) or in the biosynthesis of different amino acids (Castillo et al. 2000; Ferraioli et al. 2001; Kerppola and Kahn 1988; Pain 1979; Tatè et al. 1997, 1999a, 1999b; Truchet et al. 1980). The ability of most amino acid–requiring mutants to induce and invade developing nodules in the absence of their nutritional requirements indicates that, at least during early organogenesis, bacteria acquire amino acids from the plant. However, nodules elicited by amino acid–requiring mutants frequently show a reduced functionality, as indicated by low nitrogenase activity, measured as acetylene reduction activity (ARA), low N content of plants or signs of plant N starvation, or both (Kerppola and Kahn 1988; Pain 1979). The histology and cytology of these nodules, with only a few exceptions (Barsomian et al. 1992; Rossbach and Hennecke 1991; Tatè et al. 1999a, 1999b; Truchet et al. 1980), was not analyzed, and therefore, the intermediate (or late) step at which nodulation is altered remains unrecognized. In fact, a reduced level of ARA may be due to: (i) a reduced bacterial capacity to invade developing nodules, thus resulting in empty or partially empty nodules; (ii) the inability of SBs to induce normal levels of a functional nitrogenase complex, perhaps because of problems in C exchange and O₂ availability; (iii) a reduced or altered SB maintenance, or a premature nodule senescence, or both. On the other hand, a high level of ARA activity is not indicative of a correct nodule function, since it has been shown that ARA activity in nodules induced by some mutants is normal, while N fixed is not efficiently exported to the plant (Arcondéguy et al. 1997).

In this work, we report the isolation and characterization of several amino acid–requiring strains of *Rhizobium etli*. We analyzed the number, localization, shape, and ARA of nodules elicited by these strains on the roots of *Phaseolus vulgaris*. The nodules were examined at the histological, cytological, and ultrastructural level. The ability of the mutants to produce Nod factors was also analyzed. Three new abnormal nodulation

phenotypes are described, suggesting the presence of yet unrecognized metabolic signals required for specific steps of nodule development.

RESULTS

Isolation of amino acid–requiring mutants of *R. etli*.

To isolate amino acid–requiring mutant strains, the suicide plasmid pSUP1011 (Simon et al. 1983) was used to deliver Tn5 to *R. etli* wild-type strain CE3. A control experiment was performed with pSUP101 carrying the genetic markers of pSUP1011 except for Tn5 (Simon et al. 1983). Plasmid DNA was introduced into *R. etli* by electroporation, and kanamycin-resistant bacteria were obtained at high frequency only with pSUP1011.

By screening the Tn5 recipient strains on plates of NH_4^+ containing minimal medium (RMM) with or without supplemental casaminoacids ($5 \mu\text{g ml}^{-1}$), mutant strains showing amino acid–dependent growth were recovered at a frequency of about 0.5%. The presence of a single Tn5 copy *per* strain was confirmed by Southern blot analysis (discussed below). The nutritional requirement of each mutant strain was established by testing their growth phenotype on RMM plates supplemented with a single or a defined mixture of amino acids. Strain CTNUX1 is a slow grower on RMM, and it requires lysine for normal growth. Strain CTNUX10 requires asparagine, aspartate, or glutamate, but its growth defect is partially restored also by addition of leucine, valine, or histidine. Strain CTNUX36 requires leucine and strain CTNUX20 requires histidine, while CTNUX13 requires both isoleucine and valine. Strain CTNUX18 requires phenylalanine, while strain CTNUX26 requires all aromatic amino acids (i.e., tryptophan, phenylalanine, and tyrosine). Strain CTNUX27 requires glycine or serine.

The Tn5 flanking regions were cloned and sequenced as previously described (Tatè et al. 1997). In each mutant strain (Fig. 1A) the inferred amino acid sequence of the DNA region around the Tn5 insertion shares significant similarity to the sequence of a protein involved in the amino acid biosynthesis.

Strain CTNUX1 has a Tn5 insertion in a region (accession number AJ422134) that encodes a protein highly similar (up to 88% identity) to the diaminopimelate decarboxylase, the *lysA* gene product of *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*. LysA (EC 4.1.1.20) catalyzes the conversion of meso-2,6-diaminoheptanedioate to L-lysine. In strain CTNUX10, the open reading frame (ORF) interrupted by Tn5 (accession number AJ422137) is highly similar (up to 93% identity over 183 aa overlap) to the small subunit of glutamate synthase (GOGAT), the *gltD* gene product, of many bacteria. GOGAT (EC 1.4.1.13) catalyzes the conversion of 2-oxoglutarate and glutamine into glutamate. The lack of GOGAT in strain CTNUX10 can explain the rescue of the growth phenotype observed when the medium was supplemented with a compound that can be degraded to glutamate or a compound that can transaminate its nitrogen to 2-oxoglutarate, such as aspartate or asparagine. In strain CTNUX36, the ORF interrupted by Tn5 is highly similar (up to 93% identity over 174 aa overlap) to 3-isopropylmalate dehydratase (3-isopropylmalate isomerase), the *leuC* gene product of *S. meliloti*, *Mesorhizobium loti*, and *A. tumefaciens*. LeuC (EC 4.2.1.33) catalyzes the conversion of 3-isopropylmalate to 2-isopropylmaleate, the second step in leucine biosynthesis. Strain CTNUX20 has a Tn5 insertion in a locus (accession number AJ422139) encoding an ORF highly similar (up to 83%) to the C-terminal portion of phosphoribosylformimino-5 aminoimidazole carboxamide ribotide isomerase, the *hisA* gene product, of many bacteria. The reaction catalyzed by HisA (EC 5.3.1.16) is the fourth step in the pathway of histidine biosynthesis. Downstream of the ORF interrupted by Tn5, a second ORF (accession number AJ422139), highly similar to the imidazoleglycerol-phosphate synthase cyclase subunit (HisF), was identified. Strain CTNUX18 has a Tn5 insertion in a locus (accession number AJ422135) encoding an ORF highly similar (up to 87% over 176 aa overlap) to the chorismate mutase protein, the *pheA* gene product of *A. tumefaciens*, *S. meliloti*, and *M. loti*. PheA (EC 5.4.99.5) catalyzes the conversion of chorismate into prephenate, the first step in the pathway of phenylalanine biosynthesis. In strain CTNUX26, the ORF interrupted by Tn5 (accession number

A

Strain	Nodulation			Mutated gene
	nr ^a	mg ^b	ARA%	
CE3	57	1.15	100	none
CTNUX13	55	1.28	86.8	nd
CTNUX27	43	1.34	49.3	<i>serA</i>
CTNUX10	42	0.82	24.7	<i>gltD</i>
CTNUX20	25	0.75	38.1	<i>hisA</i>
CTNUX36	12	1.21	9.8	<i>leuC</i>
CTNUX18	11	1.50	ud	<i>pheA</i>
CTNUX26	4	2.30	ud	<i>aroK</i>
CTNUX1	-	--	--	<i>lysA</i>

^a number of nodules, ^b fresh weight of nodules
ud, undetectable

B

Fig. 1. A, Symbiotic features of auxotrophic mutant strains and of the wild-type strain CE3 of *Rhizobium etli*. The gene truncated by Tn5 in each mutant is also indicated. **B**, Nodules or nodulelike structures induced on the roots of *Phaseolus vulgaris* by the indicated strains.

AJ22136) is highly similar (up to 84% over 89 aa overlap) to shikimate kinase, the *aroK* gene product of many bacteria. AroK (EC 2.7.1.71) catalyzes the conversion of shikimate into shikimate-3-P, the fifth step in the biosynthesis of chorismate necessary for the biosynthesis of aromatic amino acids. Strain CTNUX27 has a Tn5 insertion in a locus (accession number AJ422140) encoding an ORF highly similar (82% identity over 125 aa overlap) to D-3-phosphoglycerate dehydrogenase (L-serine dehydratase), the *serA* gene product of *S. meliloti*. This enzyme (EC 1.1.1.95) catalyzes the conversion of 3-P-D-glycerate into 3-P-hydroxypyruvate, the first step in the 'phosphorylated' pathway of L-serine biosynthesis.

Symbiotic behavior of amino acid-requiring mutants.

To test the nodulation ability of the isolated amino acid-requiring mutants of *R. etli*, 5-day-old germinated seedlings of *P. vulgaris* were inoculated with 100 µl of a washed bacterial suspension containing about 10⁵ bacteria. At different times after inoculation, the number, fresh weight, and ARA of the resulting nodules were determined. Bacteria were isolated from the mutant-induced nodules (10 nodules, 21 days post inoculation [dpi]) and were shown to be kanamycin resistant and to maintain the original amino acid requirement. This indicates that the nodules were induced and invaded by the original

auxotrophs and not by prototrophics revertants. As in soybean (Rossbach and Hennecke 1991), we observe in beans a high variability in several parameters of nodulation, including the time of initiation, the number of nodules formed, and their localization (primary or secondary roots). In fact, at 14 dpi on bean roots inoculated with strain CE3 (wild type), it is possible to observe developed, spherical, dark red nodules as well as small, white (or pale red) developing nodules, indicating that the process of nodule formation is not synchronous (data not shown). Therefore, the parameters measured are the average of a heterogeneous population of small, middle, and fully developed nodules.

A typical experiment (Fig. 1) showed that all the mutant strains tested were able to induce nodules or nodulelike structures, although with a varied anatomy, size, and ARA. Strain CTNUX1 elicited the formation of few (1 or 2 per plant) small, white, tumorlike structures from which secondary roots developed (Fig. 1B). Conversely, strain CTNUX13 induced a normal number of nodules, and their size, fresh weight, color, and ARA were similar to that of nodules formed by the wild-type parent. Strains CTNUX27 and CTNUX10 elicited the formation of a slightly lower number of nodules than strain CE3. The shape and color of these nodules were normal (Fig. 1B), and the fresh weight of CTNUX27-induced nodules was comparable

Fig. 2. Semithin sections, observed by bright-field microscopy, of 21-days-post-inoculation (dpi) nodules elicited on the roots of *Phaseolus vulgaris* by strains **A**, CTNUX1; **B**, CTNUX26; **C**, CTNUX18 and, in the inset, an invaded cell of a nodule induced by the wild-type strain CE3. **D**, **E**, and **F**, Thin sections observed by transmission electron microscopy of 21-dpi nodules of *P. vulgaris* induced by strain CTNUX18. Bars = 10 µm, CW = cell wall, IC = invaded cell, RM = root meristem, SB = symbiosome, SG = starch granule, SM = symbiosome membrane, UC = unininvaded cell, VB = vascular bundle, and wt = wild type.

to that of wild-type nodules, whereas the fresh weight of CTNUX10-induced nodules was lower, due to the prevalence of small nodules (Fig. 1A). The ARA of these nodules (sampled at 14 dpi) was 50 and 25% of normal values, respectively, and normal values were not reached when measured at 21 dpi. Strains CTNUX20 and CTNUX36 induced fewer nodules than strain CE3; this alteration being more marked (up to 5-fold fewer nodules) in the case of strain CTNUX36 (Fig. 1A). The pink nodules induced by these strains also showed a delayed kinetics of ARA induction that was more pronounced in the case of strain CTNUX36. The ARA of nodules sampled at 14 dpi was 38 and 10% of the control value, respectively. At 21 dpi, ARA values increased up to 50 or 60% (data not shown). The last two mutants analyzed, CTNUX18 and CTNUX26, induced a very low number of nodules, up to 10-fold lower than strain CE3 (Fig. 1A). This effect was more remarkable in the case of strain CTNUX26. Strain CTNUX18 induced white or slightly pale nodules of normal size but without any detectable ARA (Fig. 1A and B). Strain CTNUX26 elicited the formation of few, white and ring-shaped nodules (Fig. 1B) with undetectable ARA (Fig. 1A). The mean fresh weight of these nodules was higher than in the case of nodules induced by the wild-type strain, because small nodules, due to later infections, were not present. Around the nodules elicited by both mutants, the external protuberances (swelling, lumps) observed in nodules induced by the wild-type strain were less or almost not organized (Fig. 1B). Their cellular nature was established by the observation of nodule sections. In normal nodules, these structures arise peripherally with respect to the nodule cortex but are not randomly distributed. In fact, their localization reflects the position of the internal vascular bundles developed in the nodule parenchyma.

The nodulation ability of some mutants was tested in the presence of the corresponding nutritional requirement. The amino acids were added (20 µg/ml) to the plant growth medium and were maintained for the first 7 dpi. Under these conditions, strains CTNUX1, CTNUX18, CTNUX20, and CTNUX27 were able to induce (14 dpi) a significant higher (between 2.5- and 7-fold) number of nodules than in the absence of the required amino acid. The nodule number also increased, even if slightly (up to 1.5-fold), when the nodulation ability of strain CE3 was assayed in the presence of the same amino acid added at the same concentration.

By thin layer chromatography analysis (Spaink et al. 1992), we observed that all the mutants, with the exception of strain CTNUX1 (which does not strictly depend on amino acids for growth), were unable to grow or to produce Nod factors when incubated in NO₃⁻ containing RMM. In contrast, all the mutants were able to grow and to produce Nod factors when incubated on the same minimal medium supplemented with one or more required amino acids (not shown).

Cytoarchitecture of nodules.

The effectiveness (ARA) of nodules elicited by most of the mutants was reduced, even when their size, shape, and color were unaffected or only slightly affected (Fig. 1A and B). For instance, nodules elicited by strains CTNUX10 and CTNUX27 were of normal shape and dimension, but the ARA was low and never reached normal values. This may be a consequence of a delayed nodule invasion or of either an altered process of SB differentiation, a reduced subsistence of BTs inside the host cells or both. To achieve a better definition of their alterations, root nodules or pseudonodules were examined by light and electron microscopy (Figs. 2 to 5). At least five nodules (the most developed ones harvested from different plants) induced by each mutant strain were analyzed, and essentially the same phenotype was observed.

Strain CTNUX1 elicited the formation of pseudonodules in which the ITs remained confined to the infected root hairs and in which a single vascular system developed in a central position (data not shown). These nodule primordia resulting from the outgrowth of the outer cortex did not show evident signs of infection (ITs or ICs) (Fig. 2A). It was possible to observe up to three groups of dividing cells, closely aggregated to form a conical structure similar to that of an apical root meristem (Fig. 2A), instead of the CT normally formed by ICs and UCs. In correlation, the formation of more than one secondary root from these pseudonodule structures was frequently observed (Fig. 1B).

Nodules induced by strains CTNUX18 and CTNUX26 show a similar, although not identical, tissue organization (Fig. 2B through F). Abnormally shaped ICs were observed in sections of 21-dpi nodules elicited by strain CTNUX18 (Fig. 2C; for comparison, the shape of an IC of a nodule induced by strain CE3 can be found in the inset). In these nodules, the ratio of ICs to UCs was lower than in normal nodules, and the ICs, even though harboring a few SBs (Fig. 2D), were larger than the surrounding UCs, showing a large nucleus with a prominent nucleolus and a cytoplasm rich with ribosomes (Fig. 2F) similar to what was observed in younger ICs of normal nodules (Cermola et al. 2000). In contrast with normal ICs, the cell wall of abnormal ICs was indented and irregular (Fig. 2F). Highly vacuolated UCs were interspersed among the ICs. UCs were of normal dimension and shape but contained a huge number of fully differentiated amyloplasts preferentially located at the cell periphery (Fig. 2C). While normal SBs contained many BTs, the SBs of strain CTNUX18 contained a single round- or Y-shaped BT with an electron-dense cytoplasm free of polyhydroxybutyrate inclusions (Fig. 2E). The same fibrous material present in the peribacteroid space of normal SBs (Cermola et al. 2000) surrounded these abnormally differentiated BTs, but their SB membrane was not clearly recognized, and it appeared to be fragmented. Similarly, strain CTNUX26 induced under-developed nodules but with a lower number of irregularly shaped ICs (indented cell wall) than in strain CTNUX18 (Fig. 2B).

Strain CTNUX27 induced nodules in which the peripheral vascular bundles and the nodule parenchyma were normally organized (data not shown). In contrast, the CT was strongly altered, with the ICs closely aggregated and welded and an abnormally low number of small, interspersed UCs (Fig. 3A and B). The ICs were polyhedral, whereas the strong staining observed suggests that their cell wall is thicker (or has a different composition) than that of normal ICs. Few ICs located at the center of the CT showed signs of cytoplasm degradation (Fig. 3B), although this alteration was less pronounced than in other mutant-induced nodules (see below). Inside the ICs, the density of SBs, the number of BTs per SB, and other morphological features, like the presence of polyhydroxybutyrate inclusions, was normal (Fig. 3C and E). Sometimes a heterogeneous staining of the cytoplasm of the ICs was observed in which the less-stained cell zone was always a peripheral part of the cytoplasm, closely related to at least one UC (Fig. 3D and F). Ultrastructurally, it was possible to observe that the UC shape (Fig. 3C and D) was strongly altered, with the cells adopting the form of the intercellular spaces left by the ICs. This suggests that these cells are unable to expand and, in correlation, that the vacuole occupies only part, and not all, of the cell volume, such as in normal UCs. Finally, these cells contained only one or two amyloplasts (Fig. 3C and D).

The developmental alterations found in nodules induced by strains CTNUX20, CTNUX10, and CTNUX36 were similar to each other. The early nodule organogenesis induced by these strains was not altered. In fact, the histology of CTNUX20-

induced nodules (Fig. 4B) was similar to that of normal nodules (Fig. 4A and A'), although the UCs, interconnected and interspersed between the ICs, were less evident than in normal nodules (Fig. 4B and B'). The vascular bundles (VB) were located at the nodule periphery and were surrounded by a differentiated nodule parenchyma formed by highly vacuolated cells that were interlocked (Fig. 4B'). Some cells of the nodule parenchyma (the most internal and related to the CT) contained many starch grains (Fig. 4B'). In most nodules, however, the central part of the CT showed signs of necrosis, appearing empty or containing only dispersed cell debris (Fig. 4B). This

effect was not evident in nodules induced by the Fix⁻ strain CFNX249(*nifH*₁-*nifH*₂) of *R. etli* (Fig. 4C and C'). The cell size and shape of some ICs located at the periphery of the CT was less regular than in normal nodules, with some ICs showing a clear-cut gradient of staining (Fig. 4B'). In thin sections of CTNUX20-induced nodules (21 dpi), these cells appeared to be normally differentiated (Fig. 5A). The SBs of the peripheral ICs were normally differentiated with a low number of BTs per SB (Fig. 5D). The peribacteroid space was rich with the fibrous material characteristic of normal SBs, and the host cell cytoplasm was rich with ribosomes (Fig. 5D). In the ICs

Fig. 3. Thin and semithin sections of 21-days-post-inoculation nodules of *Phaseolus vulgaris* induced by strain CTNUX27 and observed by **A and B**, bright-field microscopy and by **C, D, E, and F**, transmission electron microscopy. Bars = 10 μ m. BT = bacteroid, CT = central tissue, CW = cell wall, IC = invaded cell, PBS = peribacteroid space, SG = starch granule, SM = symbiosome membrane, and UC = uninvaded cell.

located at the middle of the CT (Fig. 5B), the number of BTs per SB increased. Since dividing BTs were not observed in these cells (Fig. 5B), the increased BT to SB ratio must be due to SB fusion. The extreme consequence of this process is that, in the central part of the CT, some ICs appear to harbor a unique SB containing thousands of BTs surrounded by the fibrous material characteristic of the peribacteroid space (Fig. 5C). The SB membranes were difficult to recognize, but sometimes membrane fragments were observed (data not shown). The BTs of this nodule zone were rich in polyhydroxybutyrate and other inclusions, as in wild-type BTs, but were irregularly shaped with the cell wall undergoing lysis (Fig. 5E). At the same time, the plant cell wall was altered as if lysis was beginning (Fig. 5C).

DISCUSSION

The symbiotic behavior of the bacterial mutants described in this paper provides new insight into bacterial traits essential for effective symbiotic interaction. The phenotypes observed deal with early, intermediate, or late nodule organogenesis.

Nodule induction.

In the absence of externally added N, all the mutant strains tested were able to induce at least a nodule primordium, or small swellings, on the roots of bean seedlings and, thus, to produce Nod factors, which are required to induce nodulation in *P. vulgaris* (Cardenas et al. 1995). We found that the mutants were able to grow and to produce Nod factors only after the required amino acid was added to the medium. Analogous results have been reported for other auxotrophs of *R. etli* (Ferraioli et al. 2001; Tatè et al. 1999a, 1999b). These observations led us to conclude that, to produce Nod factors, the mutants require amino acids that cannot be endogenously produced (by a proc-

ess of protein turnover). Some amino acids, such as serine, aspartate, glutamate, threonine, valine, glycine, isoleucine, and leucine were detected in root exudates of *P. vulgaris* (Ferraioli et al. 2001; S. Ferraioli, *unpublished data*). Amino acids may reach the rhizosphere either by the degradation of the root cortical cells (detached during the outgrowth of a secondary root) as previously speculated (van Egeraat 1975) or they may be actively released by N-starved root cells, a condition necessary for symbiosis. In the rhizosphere, amino acids may stimulate bacteria both to grow and to produce Nod factors. A correlation between bacterial division and production of Nod factors has not been demonstrated, but the promoters of *nod* genes are active in younger dividing bacteroids (invasion zone), whereas they are inactive in differentiated nondividing N₂-fixing bacteroids (Sharma and Signer 1990). Moreover, C16:1 and C18:1 fatty acids, such as those present in *Rhizobium* membranes, are precursors in the biosynthesis of some Nod factors, and specialized acyl carrier proteins are required for their acylation (Bladergroen and Spaink 1998; Ritsema et al. 1997). Since bacteria regulate membrane lipid synthesis as a function of the growth rate (Magnuson et al. 1993), a close coupling between Nod factors synthesis and bacterial growth is possible.

The number of nodules or nodulelike structures induced by a mutant strain appears to be determined by the availability of the amino acid required. In fact, strain CTNUX27 induces the formation of a relatively high number of nodules, and serine is one of the most abundant amino acids identified in the root bean exudate. On the other hand, low or undetectable levels of arginine, methionine, phenylalanine, and tryptophan were found in root exudates, and in correlation, the mutants requiring one of these compounds were either unable to induce nodules or were able to induce a small number of under-developed nodules (Fig. 1A) (Ferraioli et al. 2001; Tatè et al. 1999a, 1999b). Thus, the final number of nodules (or pseudonodules)

Fig. 4. Semithin sections of 21-days-post-inoculation nodules induced on *Phaseolus vulgaris* by different strains of *Rhizobium etli* and observed by bright-field microscopy. **A and A'**, Strain CE3; **B and B'**, CTNUX20; and **C and C'**, CFNX249 *nifH*⁻. BT = bacteroid, IC = invaded cell, NZ = necrotic zone, UC = uninvaded cell, and VB = vascular bundles.

induced by a strain may depend on its capacity to divide and, consequently, to reach one or more of the competent zones of the roots. This hypothesis is supported also by the observation that, due to reinfections, *nif* strains induce a greater number of nodules than the wild type (Hirsch et al. 1983), whereas strains CTN18 and CTN26 are *Fix*⁻ but form few nodules. Finally, we observed that all the mutants assayed were able to induce a significantly higher number of nodules (or nodulelike structures) when the plant growth minimal medium was supplemented with the required amino acid.

Amino acid availability inside the ITs.

R. etli invades a developing nodule through intercellular ITs (Cermola et al. 2000), and it has been proposed that ITs grow by incorporation of membrane vesicles at their tip, as in growing pollen tubes (Bladergroen and Spaik 1998; Gage et al. 2000; Gualtieri and Bisseling 2000). The evidence presented here indicates that bean cells provide various amino acids for bacterial divisions occurring inside the ITs. These amino acids may be present in the membrane vesicles as such or may be produced by degradation of plant proteins, in which case their relative abundance would be determined by the amino acid composition of the proteins. In contrast, the analysis of purine and pyrimidine auxotrophs of *R. etli* indicates that these compounds are not available to bacteria growing inside the ITs (Noel et al. 1988; Newman et al. 1994).

Induction of the nodule meristem.

Strain CTN1 induced only uninvaded root swellings formed by the cells located at the periphery of a normally developed nodule (parenchyma and vascular bundles). In contrast, the cells proper of a normal CT (ICs and UCs) were not evident. Moreover, lateral roots arose from these abortive pseudonodules (Figs. 1B and 2A). Similar results have been reported in the case of *lps* mutants of *R. etli* (Vandenbosch et al. 1985). Which cells of a pseudonodule differentiate into root meristematic cells is presently unknown, but it is worth noting that apical root meristems are located (Fig. 2A) far away from the central stele of the root. Similarly, meristematic cells of elongated nodules arise from the most distal cells of the nodule primordium (Timmers et al. 1998). Normal lateral roots derive from cells of the pericycle, and thus, the vascular bundle observed at the center of these pseudonodules may be involved in the induction of lateral roots. Otherwise, some cells of the nodule primordium could be reprogrammed (in the absence of bacterial invasion), to differentiate into a conical root-apical meristem rather than into a globose nodular meristem. The bacterial factor or factors necessary to induce the globose meristem that generates the CT of a bean nodule is presently unknown, but while *Rhizobium* invasion is required, Nod factors are not sufficient. In fact, externally added Nod factors purified from *R. etli* elicit the formation of pseudonodules (nodule primordia) that develop only to the stage in which a central vascular bundle is formed and never to a complete nodule structure (Cardenas et al. 1995).

Differentiation of ICs and nodule development.

Ineffective (*Fix*⁻) root nodules are usually of a smaller size than normal (*Fix*⁺) nodules. This has been correlated with the decline in mitotic activity of the nodule meristem from which the CT (ICs and UCs) is generated (Newcomb et al. 1979). However, strains CTN18 and CTN26 induce *Fix*⁻ nodules with approximately the same dimension and mass of wild-type nodules, even if they contain a low number of ICs. These results, as well as previous ones (Tatè et al. 1999a), show that meristem activity is not arrested in *Fix*⁻ *P. vulgaris* nodules. In CTN18- and CTN26-induced nodules, the cells of the

CT are well distinguished in ICs and UCs, even though the low number of morphologically differentiated ICs observed harbor only a few SBs. The absence of correlation between the degree of invasion and the cell volume reached was observed also in nodules elicited by a methionine-requiring mutant of *R. etli* (Tatè et al. 1999a). These results show that, while bacterial invasion is required to induce the process of cell differentiation, the cell dimension reached is not determined by the degree of invasion. Moreover, the observation that the process of lipid vesicles formation is strongly induced both in normal ICs and in cells with arrested or delayed invasion (Verma and Hong 1996) supports the hypothesis that these processes may be uncoupled.

One of the most remarkable phenotypes of CTN18- and CTN26-induced nodules is that the cell walls of the ICs are irregular (indented), due either to zones of fragility or to a generalized lack of rigidity. These abnormal indentations involve the cell wall of adjacent cells. In plants, adjacent cells are joined by a middle lamella that is mainly composed of pectins. In common beans, polygalacturonase inhibitor proteins regulate the activity of the extracellular polygalacturonase (Desiderio et al. 1997). Thus, in 21-dpi nodules induced by strains CTN18 and CTN26, either the expression or the activity of these inhibitors, or both, may be reduced. Why these bacterial strains cause plant cell wall softening is unknown, but the effect seems to be specific. In fact, while other mutants of *R. etli* induce similar abnormal nodules formed by a very low number of ICs harboring undifferentiated SBs, this kind of abnormal cell shape was never observed (Tatè et al. 1999a, 1999b).

In nodules induced by strain CTN27, the ICs are full of differentiated SBs, thus indicating that the bacterial requirement for serine, glycine, or both to divide inside the ICs is fulfilled by the plant. Conversely, only few abnormally shaped UCs are observed (Fig. 3C and D). UCs are dispersed among the ICs and are only rarely interconnected, such as in normal nodules (Fig. 4A and A'). The biosynthesis of serine and glycine is also the pathway for the biosynthesis of one-carbon (C₁) units and, in the ICs of determinate nodules, C₁ units are involved in the *de novo* synthesis of purines and, thus, in the assimilation of fixed N (Schubert 1986). It has been proposed that purines are released by the ICs to the UCs and that, in the latter, purines are converted into ureides (Schubert 1986). Accordingly, uricase is expressed only in the UCs of mature bean nodules (Tatè et al. 1994). The signal or signals triggering the differentiation of nodule meristematic cells into UCs is presently unknown. However, our observations suggest that glycine metabolism by the ICs may be directly or indirectly involved in this process.

In nodules elicited by strains CTN10, CTN20, and CTN36, the ICs are filled with differentiated SBs, thus indicating that the corresponding required amino acids are provided by the plant to dividing BTs, although the SB membrane is impermeable to many amino acids and sugars (Udvardi and Day 1997). Thus, BTs may acquire amino acids through the hydrolysis of plant proteins released into the peribacteroid space. The fusion of membrane vesicles with the SB membrane in developing bean nodules has been previously observed (Cermola et al. 2000). It has been postulated that SBs have properties of a lytic compartment, and some lytic enzymes and protease inhibitors were recognized in the peribacteroid space (Verma 1992).

Finally, in nodules elicited by strains CTN10 and CTN20, ICs and UCs located at the center of the CT undergo lysis, and sometimes, only cell debris are observed (Fig. 4B). These alterations appear more correlated with a necrotic lesion than with the normal senescence of nodules. In fact, it has been previously described (Cermola et al. 2000) that, dur-

ing nodule senescence (normally occurring or induced by dark stress), the morphological alterations involve, initially and mainly, the cytoplasm of ICs that become homogeneous. The reason why a process of premature cell death is induced in these nodules is presently unknown, but the lack or reduction of nitrogenase activity is excluded. In fact, in 21-dpi nodules induced by *Fix⁻* mutants, the necrosis of the CT is not observed (Fig. 4) (Cermola et al. 2000). Furthermore, due to the different nutritional requirements of these strains, the rationalization of a common signal is not obvious. Most likely the plant recognizes differentiated SBs of these strains as pathogens (Lam et al. 2001) or reacts to dead BTs inside the ICs. It is important to remark that the necrosis of the CT, although occurring early during nodule invasion, was observed in nodules elicited by a *nifA* mutant strain but not in *nifH* strains of *Bradyrhizobium japonicum* on soybean roots (Fischer et al. 1986).

MATERIALS AND METHODS

Growth conditions and bacterial strains.

R. etli strains were grown at 30°C in rich medium containing (g l⁻¹): tryptone (5), yeast extract (3) and CaCl₂·2H₂O (0.88). RMM was used as a chemically defined medium containing (g l⁻¹) MgSO₄·7H₂O (0.25), CaCl₂·2H₂O (0.1), K₂HPO₄ (1), and

KH₂PO₄ (1); and (μg l⁻¹) FeCl₃·6H₂O (10), MnSO₄·H₂O (20), ZnSO₄·7H₂O (20), CuSO₄·5H₂O (20), CoCl₂ (20), Na₂MoO₄ (20), biotin (20), calcium pantothenate (20), and thiamin (20). *E. coli* strains were grown at 37°C in tryptone-yeast extract medium containing (g l⁻¹) tryptone (10), yeast extract (5), and NaCl (10). Antibiotics (μg ml⁻¹) nalidixic acid (20), kanamycin (50), and ampicillin (100) were added to the medium as needed. All media were solidified with 1.5% agar (Difco Laboratories, Franklin Lakes, NJ, U.S.A.). RMM medium was supplemented with NH₄Cl (10 mM) or KNO₃ (10 mM) and glucose (1%) as N and C sources, respectively. Amino acids sterilized by filtration were added at a concentration of 20 μg ml⁻¹.

Tn5 mutagenesis.

R. etli was grown at 30°C with shaking (250 rpm, rotary shaker) in rich medium and cells competent for electroporation were prepared as previously described (Tatè et al. 1997). DNA of plasmid pSUP1011 (Simon et al. 1983) was purified using Qiagen columns (Promega, Madison, WI, U.S.A.). Electroporation was performed as previously described (Tatè et al. 1997). Electroporated cells were resuspended in 1 ml of medium containing (g l⁻¹): tryptone (20), yeast extract (3), KCl (0.19), CaCl₂ (2.1), MgSO₄ (2.5), and mannitol (10) and incubated with shaking for 4 h at 30°C. The cells were then plated on rich medium

Fig. 5. Thin sections of 21-days-post-inoculation nodules of *Phaseolus vulgaris* induced by strain CTNUX20 and observed by transmission electron microscopy. Bars = 10 μm, BT = bacteroid, IC = invaded cell, PBS = peribacteroid space, SG = starch granule, SM = symbiosome membrane, and UC = uninvaded cell.

agar containing kanamycin. Colonies appearing after 3 days of incubation at 30°C were streaked on TYR or RMM plates. Strains with growth defects on RMM were purified at least twice on nonselective medium and were retested extensively.

DNA manipulations.

Single digests of genomic DNA were performed using either *Bam*HI or *Eco*RI. For each digestion, 5 µg of genomic DNA from each strain were used, and the mixtures were incubated for 4 h at 37°C. DNA modifying enzymes (Promega) were used as specified by the manufacturer. Southern hybridization against a 5.5-kb *Hpa*I fragment obtained from pSUP1011 DNA and containing the Tn5 DNA was performed under conditions of high stringency (Sambrook et al. 1989). The Tn5 flanking regions were cloned and sequenced as previously described (Tatè et al. 1997).

Nodulation test and acetylene reduction activity.

Seeds of *P. vulgaris* cv. Negro Jamapa were surface sterilized with 5% hydrogen peroxide for 30 min, washed extensively with sterile water, and 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 bacteria resuspended in 50 mM phosphate buffer (pH 7.0) at 10⁶ ml⁻¹. Plants were cultured in a growth chamber maintained at 65 to 75% relative humidity, at 23°C in light and 19°C in dark, and with a 16-h-daylight period, using a N-free medium (Jensen 1942). For the measurement of ARA of nodulated roots, plants were harvested, and the root system was immediately detached and incubated in five replicates at 25°C in 15-ml rubber-cap tubes containing 1/10 (vol/vol) acetylene. The ethylene produced at different time points was quantified with a Sigma 3B gas chromatograph (Perkin-Elmer, Foster, CA, U.S.A.) equipped with a Porapak T column.

Optical and electron microscopy.

Nodules were excised from the primary root, sliced manually, and 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 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, dehydrated by passing through a series of graded ethanol, and embedded in Poly/Bed 812 resin (Polyscience, Warrington, PA, U.S.A.). Ultrathin sections were obtained with a Reichert OMU2 microtome equipped with a diamond knife (Diatome, Fort Washington, PA, U.S.A.), stained with uranyl acetate and lead citrate, and observed in a Philips EM208 electron microscope. Semithin sections (1 to 2 µm thick) of the same samples were used for optical observation after staining with toluidine blue (0.04% in distilled water).

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