Research Note

The *Rhizobium etli* *trpB* Gene Is Essential for an Effective Symbiotic Interaction with *Phaseolus vulgaris*

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A mutant strain (CTNUX4) of *Rhizobium etli* carrying Tn5 unable to grow with ammonium as the sole nitrogen source was isolated and characterized. Sequence analysis showed that Tn5 is inserted into a *trpB* (tryptophan synthase)-homologous gene. When tested on the roots of *Phaseolus vulgaris*, strain CTNUX4 was able to induce only small, slightly pink, ineffective (Fix⁻) nodules. However, under free-living conditions, strain CTNUX4 was unable to produce flavonoid-inducible lipo-chitin oligosaccharides (Nod factors) unless tryptophan was added to the growth medium. These data and histological observations indicate that the lack of tryptophan biosynthesis affects the symbiotic behavior of *R. etli*.

Additional keywords: *trpFBA* operon.

The soil bacterium *Rhizobium etli* has the ability to form nitrogen-fixing nodules on the roots of common bean (*Phaseolus vulgaris*). From the point of view of the bacterial partner, the nodule formation involves bacterial multiplication in the rhizosphere, the recognition and infection of the root hairs, bacterial growth inside a network of tubular structures called infection threads, and the release of bacteria from the threads into the cytoplasm of host cells. Within the younger infected plant cells, the bacteria (called bacteroids), surrounded by a peribacteroid membrane of plant origin, differentiate into nondividing symbiosomes that are organelle-like structures able to reduce atmospheric dinitrogen into ammonia (for reviews, see Verma 1992; Panagiota et al. 1995; van Rhijn and Vanderleyden 1995).

It has been established that symbiosomes depend on their host plant for carbon and energy, but it is largely unclear which source(s) (such as carbon, sulfur, and nitrogen) the bacteria require, or have available, during their growth in the rhizosphere and inside the infection threads, and during symbiosome differentiation inside the infected plant cells. By analysis of the symbiotic behavior of auxotrophic mutants, it

has been demonstrated that various metabolic pathways of *R. etli* bacteria, although they may not pertain exclusively to symbiosis, are nevertheless important for an efficient symbiotic interaction (Noel et al. 1988; Taté et al. 1997b, 1999).

In this paper we report the isolation and characterization of a tryptophan auxotrophic mutant, strain CTNUX4, of *R. etli*. To isolate mutants, the suicide plasmid pSUP1011 (Simon et al. 1983) was used to deliver Tn5 to the *R. etli* wild-type strain, CE3. Mutants showing tryptophan-dependent growth were identified by screening simultaneously on plates of ammonium-containing minimal medium (RMM; Hooykaas et al. 1977) with or without supplemental tryptophan (20 µg ml⁻¹). In this form, strain CTNUX4, able to grow only in the presence of tryptophan, was isolated.

Probing of the total genomic DNA (5 µg) of strain CTNUX4, with a labeled Tn5 DNA fragment (5.5-kb *HpaI* fragment) established that, in this mutant, the insertion was located on a single 8.1-kb *EcoRI* fragment. Moreover, when the genomic DNA was digested with *BamHI*, two fragments of the size of 6.6 and 6.7 kb hybridized (data not shown). Therefore, strain CTNUX4 carried only one Tn5. By selecting transformants of *Escherichia coli* DH5α that were kanamycin resistant, the plasmid pAR173, carrying a portion of Tn5 (including the kanamycin resistance cassette) plus the adjacent genomic DNA, was isolated from strain CTNUX4 (Fig. 1A). Southern hybridization of various restriction digests of DNA obtained from the wild-type strain of *R. etli*, CE3, probed against a DNA fragment (1.6-kb *HpaI/HindIII* fragment) from pAR190 (Fig. 1A), yielded at least one strongly hybridizing band for each digest. Of the hybridizing bands, one (an approximately 7.5-kb *BamHI* fragment) was chosen to construct an enrichment library with plasmid pTR101 (which specifies tetracycline resistance) as a vector (Weinstein et al. 1992). After library was screened with the same probe, a plasmid called pAR201 carrying a 7.3-kb *BamHI* DNA fragment was isolated and a restriction map obtained (Fig. 1B). From pAR201, several overlapping DNA fragments were identified, cloned, and sequenced (Fig. 1C). The sequence was confirmed with synthetic oligonucleotides. A sequence analysis revealed three major open reading frames (ORFs). The deduced protein of the first ORF showed significant homology to that of the C-terminal portion of the *trpF* gene product (phosphoribosyl anthranilate isomerase) of various organisms. The deduced

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the *Pseudomonas aeruginosa* *trpB* gene (SwissProt accession no. P07345), and 65% (over 391-aa overlap) with the product of the *Acinetobacter calcoaceticus* *trpB* gene (SwissProt accession no. P16706). The predicted protein of the third ORF,

Fig. 1. A, Strategy to clone the Tn5 DNA flanking region from strain CTNUX4 of *Rhizobium etli*. A partial restriction map of the Tn5 DNA (hatched box) is shown. Black bar indicates the DNA fragment used as a probe to clone the corresponding DNA region from the wild-type strain CE3. Arrowhead indicates Tn5-outward reading primer. **B,** Physical map of the *R. etli* DNA cloned into pAR201 with a partial restriction map. Position of Tn5 insertion in the mutant strain CTNUX4 of *R. etli* is indicated. Insert of plasmids pRT153a and pRT153b and translated sequence of *trpB* are indicated. Restriction site positions: B, *Bam*HI; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; Ha, *Hpa*I; S, *Sac*I; X, *Xho*I. **C,** Nucleotide and derived amino acid (single-letter code) sequences of the *R. etli* *trpB* gene. Stop codon (stop), exact position of Tn5 insertion in strain CTNUX4, and restriction enzyme recognition sites are indicated. Data will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number AJ245564.

located in the 3'-flanking region of *trpB*, shares a high degree of identity only with the *trpA* gene product of various organisms. In bacteria, the *trpA* gene codes for the α subunit of the tryptophan synthase complex. Thus, the sequence analysis strongly suggests that a *trpFBA* operon is present in *R. etli* and that the gene interrupted by Tn5 in strain CTNEX4 encodes the β subunit (the *trpB* gene product) of the tryptophan synthase complex (EC 4.2.1.20).

To test the nutritional requirements of *R. etli* strains, a chemically defined RMM, containing 5 mM NH_4Cl and 1% of glucose as nitrogen and carbon sources, respectively, was used. Strain CTNEX4 could grow well in a tryptophan-containing medium but could not grow in a medium supplemented with any other amino acid, including aromatic amino acids such as phenylalanine or tyrosine. Unlike strain CE3, mutant CTNEX4 could not grow in minimal medium in the presence of any of the compounds known to be intermediates of tryptophan biosynthesis in bacteria, such as chorismate, anthranilic acid, and indole. Therefore, in strain CTNEX4 the terminal pathway of tryptophan biosynthesis is not functional.

To test the ability of the wild-type locus to complement the growth phenotype, either pTR101 (vector alone) or pAR201 (see Figure 1) was introduced into *R. etli* CTNEX4 by electroporation. Transformants bearing pAR201 (CTNEX4-pAR201) could grow in the absence of tryptophan, whereas those bearing pTR101 (CTNEX4-pTR101) could not. Therefore, the DNA fragment carried by pAR201 contains the information necessary to complement the Tn5 mutation.

To confirm that the cloned sequence encodes a protein functionally homologous to the TrpB protein, its ability to suppress the tryptophan auxotrophy caused in *E. coli* by a *trpB* mutation was tested. For this purpose, derivatives of strain JMB9 carrying either a *trpB*9578 or a *trpA*33 mutation were transformed with plasmid pTR101 (vector alone) or with plasmid pAR201(*trpB*) (see Figure 1). After the four transformants were plated on minimal medium, it was observed that none could grow in the absence of tryptophan. Since the *R. etli* promoters, which are recognized by the vegetative sigma factor, the *sigA* gene product, are not recognized efficiently in enteric bacteria (Luka et al. 1996; Taté et al. 1997a),

Fig. 2. Root nodules (2 weeks old) of *Phaseolus vulgaris* induced after inoculation with strain (A) CE3 or (B) CTNEX4 of *Rhizobium etli*. C, Root of *P. vulgaris* grown in the absence (-TRP) or presence of $20 \mu\text{g ml}^{-1}$ of tryptophan (+TRP). Root hairs (RH) are indicated. Bars indicate a length of $500 \mu\text{m}$.

we speculate that the lack of complementation observed might be simply due to the lack of expression of the *R. etli trpB* gene in *E. coli*. To test this hypothesis, a 2.6-kb *Hind*III DNA fragment (carrying the *trpB* gene of *R. etli*) from pAR201 was cloned into *Hind*III-digested pGEM7Zf⁺ in both orientations (Fig. 1B). The resulting plasmids pRT153a and pRT153b were used to transform the *trp* mutants of *E. coli* and the resulting strains were plated on minimal medium. Colonies of strain JMB9-*trpB*9578 carrying plasmid pRT153a were evident 48 h after plating, while colonies of strain JMB9-*trpB*9578 carrying pRT153b were never observed. Moreover, neither plasmid

pRT153a nor pRT153b relieved the tryptophan auxotrophy of strain JMB9-*trpA*33. On the other hand, all the transformed strains could grow when plated on medium supplemented with tryptophan (20 $\mu\text{g ml}^{-1}$). Therefore, the *trpB* gene of *R. etli* relieved, when expressed from the *lac* promoter present in the pGEM7Zf⁺ vector, the tryptophan auxotrophy of a *trpB* mutant (but not a *trpA* mutant) strain of *E. coli*. In enteric bacteria, the *trpB* gene product (the β subunit) together with the *trpA* gene product (the α subunit) form the tryptophan synthase complex. This multimer complex enzyme, which has an $\alpha_2\beta_2$ structure, catalyzes the conversion of indoleglycerol

Fig. 3. A and C, Sections of 3-week-old root nodules of *Phaseolus vulgaris* induced after inoculation with strain (A) CE3 or (C) CTNUX4 of *Rhizobium etli*. Nodules were harvested, fixed, and stained with methylene blue. Hand-cut sections (made with a razor blade) were obtained, and then viewed by bright-field microscopy. The central tissue (CT), invaded cells (IC), uninvaded cells (UC), and vascular bundles (VB) are indicated. Bars indicate a length of 100 μm . B and D, Ultrastructural differentiation of symbiosomes of nodules (15 days old) elicited on roots of *P. vulgaris* by strain (B) CE3 or (D) CTNUX4 of *R. etli*. The symbiosomes (SB) and starch grains (SG) are indicated. Bars indicate a length of 10 μm .

phosphate and serine to tryptophan and glyceraldehyde 3-phosphate (Crawford 1989; Pittard 1996), the last step of tryptophan biosynthesis. Our data indicate that the tryptophan synthase β subunit of *R. etli* (the *trpB* gene product) is able to form a functionally effective multimer with the α subunit of *E. coli*. This can be explained by considering that the degree of similarity between the primary structure of the TrpB proteins of both species (55% identical residues over 384-aa overlap) is sufficient not only to maintain the catalytic activity, but also to confer an interchanged three-dimensional (3D) structure. Accordingly, the 3D structure of the TrpB protein from *R. etli* (predicted by the SwissModel program) appears to be almost identical to the 3D structure of the TrpB protein of *Salmonella typhimurium*, previously established by X-ray crystallographic analysis (Hyde et al. 1988).

To obtain more information about the function of *R. etli* TrpB, measurements of tryptophan synthase activity were performed in different strains of *R. etli*. Bacteria grown in TYR rich medium (Taté et al. 1999) were harvested by low speed ($2,500 \times g$) centrifugation, washed, and resuspended in RMM. Cells were maintained for 3 h with shaking at 30°C, harvested by high speed ($8,000 \times g$) centrifugation, and disrupted by sonic oscillation. With the resulting crude extracts used as a source of tryptophan synthase, the disappearance of indole was measured spectrophotometrically, as previously described (Miles 1970). Taking the tryptophan synthase activity measured with the wild-type strain CE3 ($0.41 \text{ nmol min}^{-1} \text{ mg}^{-1}$) as 100%, the activity of strain CTNUX4-pAR201 was about 117% ($0.48 \text{ nmol min}^{-1} \text{ mg}^{-1}$), while the activity of the CTNUX4 mutant was less than 1% (about $0.0026 \text{ nmol min}^{-1} \text{ mg}^{-1}$). Therefore, in *R. etli* the *trpB* gene product is essential for the conversion of indoleglycerol phosphate and serine to tryptophan and glyceraldehyde 3-phosphate. Strain CTNUX4-pAR201 showed a higher tryptophan synthase activity than strain CE3, but this effect may be mediated by the copy number of the *trpFBA* operon.

To test the capacity of strain CTNUX4 (*trpB::Tn5*) of *R. etli* to nodulate *P. vulgaris*, roots (about 5 to 7 cm long) of germinated seeds were inoculated with either strain CTNUX4 or wild-type strain CE3, used as a control. Two weeks after inoculation the number, localization, shape, and acetylene reduction activity of nodules induced by both strains were not comparable (Fig. 2). The total number of nodules induced by the mutant strain, CTNUX4, was lower (about 10 to 15%) than the number induced by the reference strain. Moreover, nodules induced by strain CTNUX4 were smaller in size than nodules elicited by strain CE3, white or pale pink, and located preferentially on the secondary roots (Fig. 2B). To test the possibility that the original inoculum of the mutant strain had been contaminated with prototrophic revertants, bacteria were reisolated from nodules (five nodules per plant, 10 plants) induced by strain CTNUX4 and tested for the auxotrophic marker. The nodules were harvested, surface sterilized by sodium hypochlorite treatment, and crushed one at a time. The bacteria were then diluted and plated on complete medium with the addition of either nalidixic acid ($20 \mu\text{g ml}^{-1}$) or nalidixic acid plus kanamycin ($50 \mu\text{g ml}^{-1}$) to determine the number of CFU present. The same number of colonies were observed in both types of plates. Moreover, aliquots of cells were also streaked on plates of minimal medium with or without supplemental tryptophan ($20 \mu\text{g ml}^{-1}$). After 3 days of in-

cubation, colonies could be observed only on tryptophan-containing plates. The absence of nodulation in uninoculated controls and the presence of Trp⁻ kanamycin-resistant bacteria in all nodules tested indicate that nodules were formed by auxotrophic bacteria and not by prototrophic revertants. In conclusion, strain CTNUX4 of *R. etli* was able to induce nodulation on common beans. To test the effect of the addition of tryptophan on the nodulation ability of the *trpB* mutant strain, CTNUX4, nodulation experiments were performed with a plant food minimal medium (Jensen 1942) supplemented with tryptophan. However, it was observed that the addition of tryptophan (from 5 to $20 \mu\text{g ml}^{-1}$) affects the root growth of bean and alters the distribution of root hair lengths (Fig. 2C). Therefore, we could not determine if the addition of tryptophan restores the invasion ability of strain CTNUX4.

The nitrogen fixation capacity of the nodulated roots was established by measuring the acetylene reduction activity (ARA), as previously described (Taté et al. 1997b). Taking the ARA of nodules (15 days old) induced by strain CE3 as 100% (between 15 and $17 \mu\text{mol}$ of ethylene $\text{h}^{-1} \text{ g}$ of fresh nodules), the activity of nodules induced by strain CTNUX4 was less than 4%, and it was about 60% when nodules induced by the transformed strain CTNUX4-pAR201 were assayed. Normal values were obtained with nodules elicited by strains CE3-pTR101. Moreover, from nodules elicited by strain CTNUX4-pAR201, only prototrophic bacteria resistant to kanamycin ($50 \mu\text{g ml}^{-1}$) and tetracycline ($5 \mu\text{g ml}^{-1}$) were isolated, thus indicating that plasmid pRT101 does not affect the nodulation of *R. etli* strains and that it is stable during the growth of bacteria inside the nodules. How nodules elicited by strain CTNUX4-pAR201 have a low level of nitrogenase activity is currently unknown. However, nodules (15 days old) induced by strain CTNUX4-pAR201 showed a normal histological organization but, as judged by their size, are delayed with respect to the control nodules (data not shown). Since strain CTNUX4-pAR201 showed a higher level of tryptophan synthase activity, it is possible to speculate that alteration of the intracellular level of tryptophan may have a detrimental effect on the symbiotic interaction (see below).

To allow observation of the histological organization of nodules induced by the *trpB* mutant strain of *R. etli*, nodules were harvested at different times after inoculation, and fixed. After fixation, some nodules were sectioned with a razor blade, stained with methylene blue, and viewed by bright-field microscopy (Fig. 3A, C) while others were resin-embedded, sectioned, and viewed by electron microscopy (Fig. 3B, D). Nodules (12 days old) elicited by strain CTNUX4 showed the presence of a central tissue with invaded and uninvaded cells, a nodule parenchyma, and the vascular bundles located at the periphery (data not shown). However, despite this normal histological organization, a heterogeneous population of invaded plant cells was observed. In contrast, in nodules (12 days old) elicited by the wild-type strain, CE3, the population of invaded cells appeared almost homogeneous and the younger invaded cells constituted a small fraction (located at the periphery of the central tissue) of the total number of invaded cells (Taté et al. 1994; Patriarca et al. 1996). Histological examination of 21-day-old nodules induced by strain CTNUX4 (Fig. 3C) showed that they contain a smaller central zone than the control nodules (Fig. 3A) with masses of cells having no visible infection. In the invaded cells of nodules

induced by strain CTNEX4, most bacteroids were like rod-shaped dividing bacteria, with a higher density of ribosomes and a lower accumulation of poly- β -hydroxybutyrate vesicles (Fig. 3B, D). Symbiosomes expressing various stages of early development are present but with the bacteroids never reaching the maturity seen in the control nodules (Fig. 3B), thus suggesting that the bacteroid differentiation process is delayed or arrested. Moreover, some bacteroids located in young invaded cells appear irregular in shape, are thinner than the wild-type bacteroids, and apparently undergo premature senescence (Fig. 3D). These observations indicate that even though the *trpB* mutant of *R. etli* is able to induce the formation of a nodule primordium (i.e., able to produce Nod factors) both its ability to invade a nodule and its capacity to differentiate in nitrogen fixing symbiosomes are strongly altered.

Fig. 4. A, Thin-layer chromatographic (TLC) analysis of radiolabeled compounds produced by *Rhizobium etli*. Bacteria were grown overnight at 30°C (from an initial OD_{590} value of 0.05) in ammonium-containing minimal medium (RMM) containing D-[1- ^{14}C]glucosamine (1 μCi , 50 mCi mmol^{-1}). Nitrate and mannitol were used as the nitrogen and carbon source, respectively. The culture medium (1 ml) was extracted with water-saturated *n*-butanol (0.5 ml). The *n*-butanol was removed by evaporation and the dry pellet was resuspended in 20 μl of *n*-butanol. Samples (3 μl) were loaded on a reverse-phase C_{18} -coated silica ODS (100% octadecyl silanization) plate (Sigma, Milan, Italy) with acetonitrile/water (1:1, vol/vol) as a solvent. The plate was dried and the radiolabeled compounds were visualized with a PhosphorImager gel scanner (Molecular Dynamics, Sunnyvale, CA) equipped with Image Quant software, after 24 h of exposure. Samples: lanes 1–4, strain CE3 (wild-type); lanes 5–8, strain CTNEX4 (*trpB* mutant). Bacteria were incubated in the presence of 2 μM naringenin (lanes 2, 4, 6, and 8), in the absence of the inducer (lanes 1, 3, 5, and 7), or in the presence of 20 $\mu\text{g ml}^{-1}$ of tryptophan (lanes 3, 4, 7, and 8). **B,** TLC analysis of radiolabeled compounds produced by strain CTNEX4 of *R. etli* in the presence of growth-limiting concentrations of tryptophan. Bacteria were grown in RMM supplemented with D-[1- ^{14}C]glucosamine (1 μCi , 50 mCi mmol^{-1}) and tryptophan (added at the concentrations indicated). The initial optical density (OD_{590}) value was 0.05 (in all cases), while the final OD_{590} value of each culture is indicated. Samples (3 μl) were prepared, loaded, and visualized as described in **A**.

To test the ability of strain CTNEX4 to produce Nod factors, its growth medium was investigated for the presence of flavonoid-inducible lipo-chitin oligosaccharides by radiolabeling the cells with D-[1- ^{14}C]glucosamine (Fig. 4). The radiolabeled compounds were extracted and analyzed by thin-layer chromatography (TLC), essentially as previously described (Spaink et al. 1992). In the presence of the inducer, naringenin, the wild-type strain, CE3, used as a control, produced two main radiolabeled spots (Fig. 4A, lane 2). As previously observed (Cardenas et al. 1995), metabolites that show similar chromatographic behavior were also detected, although at lower levels, in the uninduced culture (Fig. 4A, lane 1). Moreover, the addition of tryptophan (20 $\mu\text{g ml}^{-1}$) to the growth medium of strain CE3 showed no effect on the production of flavonoid-inducible compounds. In contrast, strain CTNEX4 was unable to produce radiolabeled compounds either in the presence or in the absence of naringenin (Fig. 4A, lanes 5 and 6) unless tryptophan (its nutritional requirement) was added to the growth medium (Fig. 4A, lanes 7 and 8). The production of flavonoid-inducible compounds was determined also with cells of strain CTNEX4 maintained for 16 h with shaking at 30°C (from an initial OD_{590} of 0.1) in RMM, but supplemented with different concentrations of tryptophan (from 0.5 to 15 $\mu\text{g ml}^{-1}$). Increasing the amount of tryptophan added to the culture increases both the bacterial growth and the amount of radiolabeled compounds released (Fig. 4B, lanes 2 to 6). However, up to a concentration of 5 $\mu\text{g ml}^{-1}$ of tryptophan, the ratio between the intensity of the specific radioactive spots and the number of bacteria (expressed as the optical density value) was almost the same. Therefore, it is possible to conclude that strain CTNEX4 of *R. etli* is able to produce Nod factors also in the presence of growth-rate-limiting concentrations of tryptophan.

The Nod factors (lipo-chitin oligosaccharides produced by the bacterium) act as morphogenetic signals able to elicit host-specific plant responses such as the induction of cortical cell division (Spaink and Lugtenberg 1994; Long 1996). It has previously been shown that Nod^- strains of *R. etli* are unable to induce nodules or nodulelike structures on the roots of common bean, and that purified Nod factors of *R. etli* are able to elicit the formation of nodule primordia that develop to the stage at which vascular bundles are formed (Cardenas et al. 1995). Strain CTNEX4 (*trpB*) of *R. etli* is able, in the absence of externally added tryptophan, to induce nodules (Nod^+) on the roots of *P. vulgaris*. However, the nodule invasion ability of the mutant appears to be strongly altered, thus suggesting that the root cells of *P. vulgaris* are able to provide their symbiotic bacteria with an amount of tryptophan sufficient for the bacterial production of Nod factors (which trigger the program of nodule development) but not sufficient to allow a successful nodule invasion. Accordingly, we show that strain CTNEX4 produced flavonoid-inducible compounds in the presence of growth-limiting concentrations of tryptophan but not in the absence of tryptophan (Fig. 4). Without enough tryptophan the bacteria should not be able to divide at normal rates inside the infection threads or just after being released into the cytoplasm of invaded cells. Consequently, the low number of infected cells observed in nodules elicited by strain CTNEX4 should simply be due to the uncoupling between the timing of nodule development and the rate of bacterial division inside the nodule.

After germination (i.e., during plant development) and in the absence of alternative nutrients, the seed storage reserves are continuously consumed because they are used to build the nascent plant. Accordingly, it has been reported (Bialek et al. 1992) that in *P. vulgaris* the concentration of free tryptophan (from the proteolysis of storage proteins) decreases during plant development (the tryptophan level is about 10-fold higher in the axes of young seedlings than the level measured for older seedlings). Therefore, we speculate that the nodule invasion ability of strain CTNEX4 could be influenced by the age/dimension of developing roots. To test this hypothesis, roots of *P. vulgaris* were inoculated with strain CTNEX4 at 2, 5, or 7 days after germination. It was observed that the nodules reached later stages of development (based on their dimension and pigmentation) only after inoculation of young (with a 1- to 2-cm-long principal root) germinated seeds (data not shown). Moreover, it was observed that mutant strains of *R. etli* requiring phenylalanine (strain CTNEX18, Phe⁻) or requiring all three aromatic amino acids (strain CTNEX26, Aro⁻) display the same symbiotic phenotype of strain CTNEX4 (Trp⁻), i.e., small, white nodules with a very low nitrogenase activity (R. Taté and E. J. Patriarca, *unpublished data*). These data strongly suggest that the abnormal symbiotic phenotype of these auxotrophic mutants of *R. etli* is due to a shortage of aromatic amino acids during nodule invasion.

It has been observed that auxotrophic mutants of *Rhizobium meliloti* blocked at the later steps of the pathway of tryptophan biosynthesis (such as *trpA* or *trpB* mutants) are effective symbionts, i.e., are able to elicit Fix⁺ nodules (Barsomian et al. 1992). These data indicate that, unlike what is observed in *P. vulgaris*, the root cells of *Medicago sativa* supply the bacteria with enough tryptophan not only to produce Nod factors but also to invade at a normal rate the nodule primordium and to differentiate in nitrogen-fixing symbiosomes. These observations indicate that the bacterial nutrient(s) are specific for each symbiotic relationship, as previously postulated (Schubert 1986).

Finally, when we investigated the effect of the addition of tryptophan on the symbiotic phenotype of strain CTNEX4, we observed that, in the presence of tryptophan (added to the plant growth medium), bean roots showed an increased number of secondary roots and an abnormal elongation of the root hairs (Fig. 2C). This phenotype was not observed when germinated seedlings were grown in the presence of other amino acids such as cysteine, methionine, or arginine (Taté et al. 1997b, 1999; and data not shown). Several genetic and physiological studies indicate that the phytohormone auxin (indole acetic acid) is a regulator of root development, stimulating both the formation of lateral root primordia and the root hair elongation (for a review, see Schiefelbein et al. 1997). It has also been determined that tryptophan is the biosynthetic precursor of auxin in the axes of germinating seedlings of *P. vulgaris* (Bialek et al. 1992). Therefore, the addition of tryptophan might alter the auxin homeostasis (biosynthesis/degradation) of growing bean roots, thus provoking the phenotype observed.

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