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Down-Regulation of the *Rhizobium ntr* System in the Determinate Nodule of *Phaseolus vulgaris* Identifies a Specific Developmental Zone

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The *Rhizobium* NtrBC two-component system, although essential for the expression of genes involved in ammonium assimilation, is not required for nodule development and efficiency. Moreover, nitrogen assimilation was previously found to be absent in nitrogen-fixing bacteroids. We speculate that this switch-off in nitrogen assimilation may be mediated by the inactivation (dephosphorylation) of the NtrC protein or by its absence. We find that transcription of the *Rhizobium etli* ORF1-*ntrBC* operon is switched off during bacteroid differentiation in coincidence with the arrest of bacterial division occurring in the nodule. Free-living *R. etli* shows a similar behavior, since the ORF1-*ntrBC* promoter is active only in growing bacteria. The NtrC protein is expressed only in the young bacteroids located in the peripheral part of the nodule of *Phaseolus vulgaris* and this identifies the presence of a previously unrecognized zone of development in determinate nodules. The NtrC protein disappears from bacteroids within one cell layer, thus indicating protein turnover coincident with the arrest in bacterial division. In indeterminate type *Vicia hirsuta* nodules down-regulation of the ORF1-*ntrBC* promoter occurs when bacteroids stop dividing and before the developmental stage in which they express the *nifH* gene. We discuss the possibility that invagination in the peribacteroidal membrane of the bacteroids may inhibit their multiplication and, as a consequence, cause down-regulation of a set of genes as part of a developmental mechanism required to reduce bacteroid metabolic activities.

Additional keywords: histochemical localization, nitrogen metabolism.

The study of regulation of nitrogen metabolism in soil bacteria of the genus *Rhizobium* is of particular interest since their symbiotic interaction with legume plants is correlated with changes in nitrogen metabolism for both partners. During the development of the legume root nodules *Rhizobium* bacteria penetrate the curled root hairs, induce a nodule primordium containing new plant meristem and invade it through tubular structures called infection threads. Bacteria, surrounded by a plant-derived membrane, are released into the cytoplasm of plant cells, where they differentiate into morphologically distinct forms called bacteroids. Only mature differentiated bacteroids express the *nif* genes, responsible for the fixation of atmospheric nitrogen (Kondorosi and Kondorosi 1986; Downie and Johnston 1986; Long 1989; Nap and Bisseling 1990; Franssen et al. 1992). Early work on nitrogen metabolism of bacteroids suggested that ammonia produced through the activity of nitrogenase is exported into the plant cytoplasm (Bergersen and Turner 1967; Brown and Dilworth 1975; Glenn and Dilworth 1984). Therefore, unlike the situation observed in free-living diazotrophs in which fixed nitrogen is used for bacterial growth, uncoupling between nitrogen fixation and nitrogen assimilation in bacteroids is a prerequisite for the efficiency of the *Rhizobium*-legume symbiosis. Thus, the study of the mechanism(s) by which genes involved in nitrogen assimilation are switched off during the differentiation of bacteria into bacteroids is essential to understanding the symbiotic interaction.

The free-living form of *Rhizobium* in the soil assimilates nitrogen, obtained by direct uptake of ammonium salts or by nitrate reduction. When nitrogen sources become limiting, the efficiency of nitrogen assimilation is enhanced by the increased synthesis and activity of the ammonium carrier, of nitrate reductase, and of glutamine synthetase. In particular, transcription of genes coding for glutamine synthetase is controlled by the global nitrogen regulatory system (*ntr* system) including a two-component system, the protein kinase NtrB and the transcriptional regulator NtrC, and other gene products regulating the activity (namely phosphorylation) of the NtrC protein (Chiurazzi and Iaccarino 1990; Moreno et al. 1992; Patriarca et al. 1992; Patriarca et al. 1994). The published evidence suggests that ammonium sensing occurs through regulation of NtrC activity, as in free-living diazotrophs (Kennedy et al. 1994).

It is possible that the switch in nitrogen metabolism occurring during the differentiation of bacteria into bacteroids is mediated by the inactivation (dephosphorylation) of the NtrC protein, or by its absence. In fact, while nodule development occurs only under nitrogen limiting conditions, bacteroids in the cytoplasm of the nodular invaded cells do not appear to be nitrogen starved since *ntr*-regulated activities, such as those involved in ammonium assimilation, are switched off (Brown

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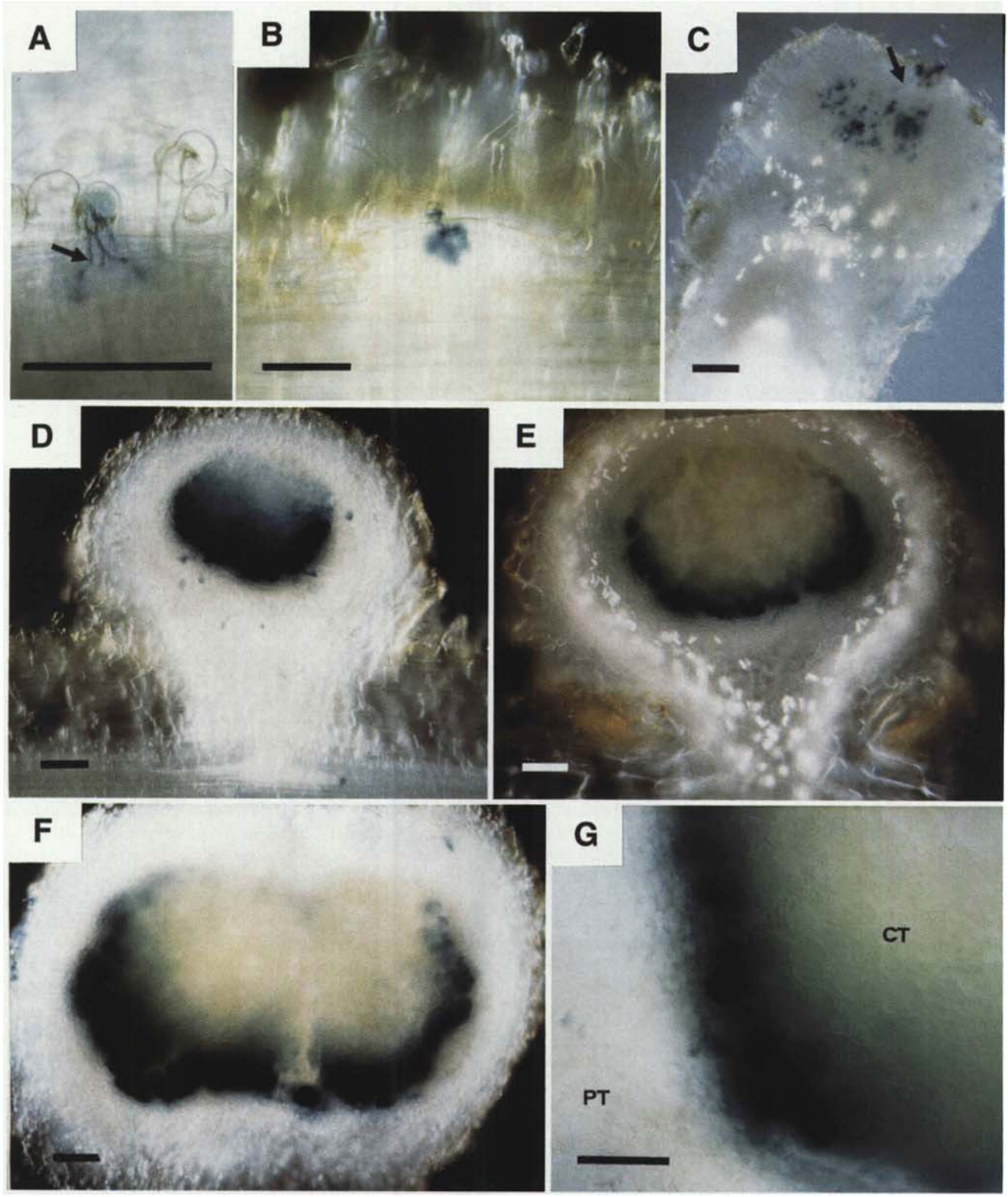

Fig. 1. Histochemical localization of β-galactosidase activity in different developmental stages of *Phaseolus vulgaris* nodules induced by *Rhizobium etli* carrying plasmid pAR66 (containing the ORF1-*ntrBC* promoter fused to *lacZ*). β-galactosidase activity is evident: **A** and **C** (arrows), in infection threads; **B,** in the first invaded cells of the outer cortex; and **C,** in invaded cells of a longitudinal section of an early emergent nodule. **D,** A longitudinal section of a 10-day-old nodule; β-galactosidase activity is evident in the invaded cells of the central tissue, but a decreasing proximal-distal gradient is observed. In longitudinal sections of (**E**) 15- and (**F**) 20-day-old nodules promoter activity is restricted to bacteroids of the most peripheral invaded cells. **G,** A magnification of the peripheral part of the nodule shown in **F**; peripheral tissues (PT) and central tissue (CT) are indicated. Bar = 100 μm. Nodules obtained from plantlets inoculated with *R. etli* carrying plasmid pMP220 (the promoterless vector) showed no activity.

and Dilworth 1975; Miflin and Lea 1976; Robertson and Farnden 1980; Cullimore et al. 1983; Howitt et al. 1986). Furthermore, genetic evidence indicates that the NtrC protein, although essential to enhance the expression of genes involved in ammonium assimilation, is not required for nodule development and efficiency (Szeto et al. 1987; Moreno et al. 1992; Udvardi et al. 1992). Therefore, the study of *ntrC* expression during nodule development is essential for a better understanding of the mechanisms leading to the changes in nitrogen metabolism occurring in the symbiosis.

The *ntrB* and *ntrC* genes of *Rhizobium etli* (formerly *R. leguminosarum* bv. *phaseoli*) are cotranscribed with an open reading frame (called ORF1) of unknown function (Patriarca et al. 1993). Two RNA 5′ ends, t_1 and t_2, each preceded by a sequence homologous to the –35/–10 (σ^{70}-dependent) promoter consensus sequence of *Escherichia coli*, were found upstream of ORF1. Transcription of the ORF1-*ntrBC* operon is two- to threefold higher when bacteria are grown in NH_4Cl compared with growth in KNO_3. A 20-fold-higher expression of this operon was found in a *R. etli ntrC*$^-$ mutant strain compared with the wild type, indicating negative autoregulation of ORF1-*ntrBC* expression by NtrC, probably due to the binding of this protein to a site overlapping the transcriptional start site t_1 (Patriarca et al. 1993; M. Martino and E. J. Patriarca, unpublished).

We show here, by using histochemical localization of β-galactosidase activity expressed from an ORF1-*ntrBC-lacZ* fusion and by immuno gold labeling of the NtrC protein, that the expression of the ORF1-*ntrBC* operon of *R. etli* decreases during development of *Phaseolus vulgaris* L. nodules and that this down-regulation correlates with bacteroid differentiation. Due to the absence of a persistent meristem these nodules (called determinate) have been described as globose structures in which all cells are progressing through the same stage of development (Newcomb et al. 1979). We find that in nitrogen-fixing (3-week-old) nodules the ORF1-*ntrBC* operon is expressed only in the young bacteroids present in a group of invaded cells located at the periphery of the central tissue. This pattern of expression shows the presence of a previously unrecognized developmental zone in the determinate type *Phaseolus vulgaris* nodules. This conclusion is supported by the analysis of the activity of the ORF1-*ntrBC* promoter in the indeterminate nodules of *Vicia hirsuta* (L.) S. F. Gray. Nodules of the indeterminate type are characterized by the presence of a persistent apical meristem from which the different tissues of the nodule develop. As a consequence, in a longitudinal section of a mature indeterminate nodule it is possible to recognize all the developmental zones. Down-regulation of ORF1-*ntrBC* expression occurs simultaneously with the arrest of division of the younger, undifferentiated bacteroids. Consistently, under free-living conditions we find that the *R. etli* ORF1-*ntrBC* promoter is active only in early growth phase.

RESULTS

Expression of the ORF1-*ntrBC* promoter in *Phaseolus vulgaris* nodules.

Phaseolus vulgaris plantlets were inoculated with wild-type *R. etli* (strain CE3) carrying plasmid pAR66 (containing the ORF1-*ntrBC* promoter fused to the *lacZ* gene). Roots were harvested at different times after inoculation and in situ expression of β-galactosidase was observed after staining with X-Gal. The ORF1-*ntrBC* promoter is active in bacteria growing inside the infection threads (Fig. 1A) and in the bacteroids released in the first invaded cells of the outer cortex during primordium formation (Fig. 1B). At a later stage β-galactosidase activity is evident in all invaded cells of an early emergent nodule (Fig. 1C). In a 10-day-old nodule, promoter activity is present in the invaded cells of the central tissue, but a decreasing proximal-distal gradient of activity begins to be observed in the longitudinal section shown in Figure 1D. In mature nodules (15- and 20-day-old, Fig. 1E and F, respectively) activity is restricted to bacteroids located in the most peripheral invaded cells. A higher magnification of the peripheral region of the central tissue (Fig. 1G) shows that expression of the ORF1-*ntrBC* promoter follows a peripheral-central decreasing gradient and that only two to three cell layers of invaded cells are stained. On the other hand, β-galactosidase activity from the *nifH* promoter (pRT124) is absent in infection threads and in the first invaded cells of the nodule primordium and it is evident in all invaded cells of a nitrogen-fixing (2- to 3-week-old) nodule (data not shown).

Activity of the ORF1-*ntrBC* promoter was also observed in *Phaseolus vulgaris* nodules induced by a *ntrC*$^-$ mutant of *R. etli* (strain CFN2012) carrying pAR66. The pattern observed was identical to that obtained with the wild-type strain (data not shown), the only difference being that incubation times required to detect the X-Gal staining were about 10-fold lower. This result demonstrates that the negative autoregulation of the ORF1-*ntrBC* promoter by NtrC acting in free-living *R. etli* (Patriarca et al. 1993) is operative in the nodule portion where NtrC is expressed and that down-regulation of the ORF1-*ntrBC* promoter is clearly a different phenomenon.

The NtrC protein in *Phaseolus vulgaris* nodules.

It has been previously proposed (Taté et al. 1994) that the youngest invaded cells are the most peripheral of the central tissue. It is therefore likely that the ORF1-*ntrBC* promoter is expressed only in bacteria that still have to differentiate into mature bacteroids. To test this hypothesis, immuno localization experiments were performed with a specific antiserum raised against the *R. etli* NtrC protein (Patriarca et al. 1993) on thin (70 to 100 nm) sections obtained from different regions of *Phaseolus vulgaris* nodules (Fig. 2). Gold particles showing the presence of the NtrC protein are observed in growing bacteria located inside the infection threads (Fig. 2A) and in undifferentiated bacteroids (Fig. 2B). NtrC-related gold particles are not observed in mature bacteroids (Fig. 2C), characterized by the presence of poly-β-hydroxybutyrate vesicles and by a surrounding zone of fibrous material (Cermola et al. 1994). To obtain a more global picture of the presence of NtrC protein, we performed experiments on semi-thin (1 to 2 μm) sections using silver enhancement in order to visualize the signal with a light microscope. The NtrC protein is present only in the most peripheral invaded cells of the central tissue (Fig. 2E), where the younger invaded cells are located (Fig. 2D).

Expression of the ORF1-*ntrBC* promoter in *Vicia hirsuta* nodules.

R. leguminosarum bv. *viciae* infecting *Vicia hirsuta* gives indeterminate nodules. We analyzed the expression of the

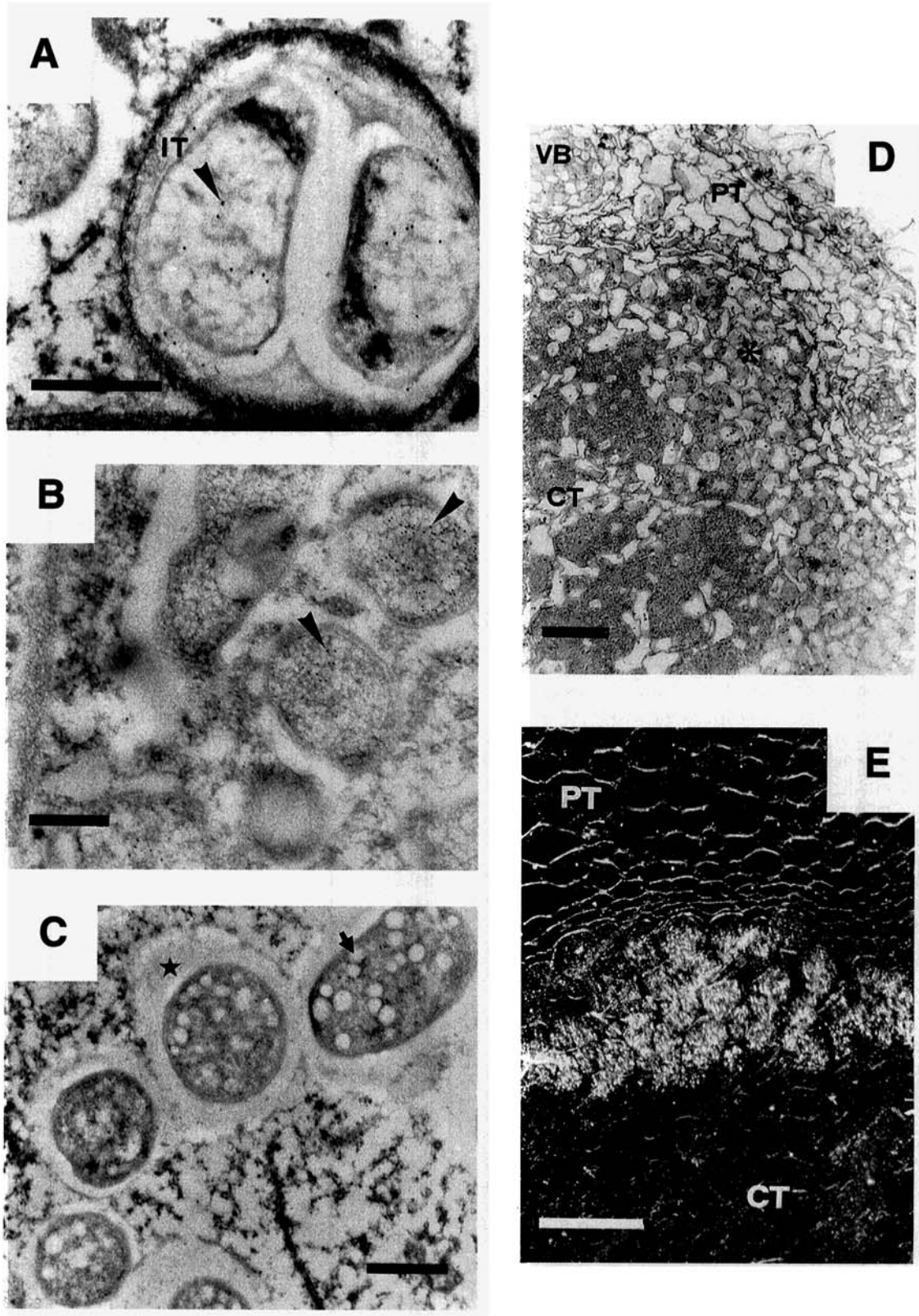

Fig. 2. Immuno localization of the NtrC protein on thin sections (70 to 100 nm. **A, B,** and **C,** Gold particles (arrowheads) are evident (**A**) in bacteria inside infection threads (IT) and (**B**) in young bacteroids. **C,** Background levels are observed in the bacteroids of mature symbiotic cells in the central part of the nodule. Fibrous material (star) and poly-hydroxybutyrate vesicles (arrow) are indicated. With a preimmune antiserum almost no gold granules were observed (data not shown). **D** and **E,** light microscopy of semi-thin (1 to 2 μm) sections of a 12-day-old bean nodule showing small invaded cells with few bacteroids at the periphery of the central tissue (asterisk in **D**) and immuno localization of the NtrC protein followed by silver enhancement to visualize the colloidal gold labeling (**E**). Peripheral tissues (PT), including vascular bundles (VB), and the central tissue (CT) are indicated. Bar equals 1 μm in **A–C**; 100 μm in **D–E.**

ORF1-*ntrBC* promoter in this system because in a longitudinal section of a mature indeterminate nodule it is possible to recognize all the developmental zones (Vasse et al. 1990). Proceeding from the apex of the nodule toward the root, the apical zone (zone I) corresponds to the nodule meristem, the invasion zone (zone II) is characterized by the presence of infection threads and of plant cells containing young bacteroids, and the interzone II-III shows a sharp developmental switch in starch accumulation (Vasse et al. 1990) and nitrogenase genes (*nif*) expression (Yang et al. 1991) continuing in the nitrogen-fixing zone (zone III). Young bacteroids, which occasionally divide inside the peribacteroidal vesicle, are observed in the root-distal portion of zone II, while only nondividing, elongated bacteroids are observed in the root-proximal portion.

Vicia hirsuta plantlets were inoculated with *R. leguminosarum* bv. *viciae* transconjugants containing either pAR66 or pRT124, carrying the ORF1-*ntrBC* or the *nifH* promoter fused to the *lacZ* gene, respectively. The ORF1-*ntrBC* promoter is active in growing bacteria inside the infection threads (Fig. 3A) and in bacteroids released in the first invaded cells of the inner cortex during primordium formation (Fig. 3B,C,D). In a more mature nodule (1 week old; Fig. 3E) a decreasing distal-proximal gradient begins to be observed. In a mature nodule (2 weeks old) the activity is restricted to bacteroids present in the younger invaded cells of zone II (Fig. 3G,H). On the other hand, the *nifH* promoter is switched on sharply in a single cell layer, the interzone II-III, where starch granules are also present (Fig. 3F) and it is expressed throughout zone III. Figure 3I shows that the ORF1-*ntrBC* promoter is active in bacteroids present in the plant cells of the root-distal part of zone II, decreases in the root-proximal part, and is not detectable in the bacteroids present in the last two to three cell layers just before the interzone II-III, where starch accumulation begins to be evident.

The ORF1-*ntrBC* promoter is growth-phase regulated.

Since the promoter of the ORF1-*ntrBC* operon is active only in bacteria multiplying inside infection threads or in dividing bacteroids the observed down-regulation of the ORF1-*ntrBC* mRNA might be due to growth-phase regulation. We tested this hypothesis by diluting late-exponential phase cells of *R. etli* into fresh rich medium and measuring ORF1-*ntrBC* mRNA levels by RNase protection analysis (Fig. 4). RNA purified from bacteria collected at different times during exponential growth was hybridized to an excess of ^{32}P-labeled antisense RNA generated from a DNA clone containing the *ntrBC* intergenic region and then digested with RNases A and T1. The experiment reported in Figure 4 shows that transcription from the ORF1-*ntrBC* promoter progressively increases after dilution of the culture. Upon further growth the intracellular level of ORF1-*ntrBC* mRNA diminishes and in late exponential cells almost no mRNA is detected.

Similar experiments were performed with RNA prepared from strain CFN2012, an *ntrC*$^-$ mutant of *R. etli*. The levels of the ORF1-*ntrBC* mRNA at different growth stages are higher than those obtained with the wild-type strain, but the expression pattern is the same (data not shown). Therefore, the autogenous regulation exerted by the NtrC protein on the ORF1-*ntrBC* promoter (Patriarca et al. 1993) is not required for the observed growth-phase regulation.

DISCUSSION

The experiments reported demonstrate that transcription of the ORF1-*ntrBC* operon in *R. etli* is switched off during development of *Phaseolus vulgaris* nodules and that this down-regulation correlates with bacteroid differentiation. β-galactosidase activity expressed from the ORF1-*ntrBC* promoter is detectable in infection threads and in all invaded cells of young nodules (Fig. 1). Later on, during nodule development, a decreasing root proximal-distal gradient is observed and in nitrogen-fixing nodules only bacteroids within the most peripheral invaded cell layers express the ORF1-*ntrBC* operon. In conclusion, during symbiotic interaction the ORF1-*ntrBC* promoter is active only in dividing bacterial cells (i) in bacteria growing inside the infection threads, and (ii) in young, undifferentiated bacteroids.

The analysis of expression of plant genes induced during nodule development has been used to suggest (Taté et al. 1994) that the youngest invaded plant cells are located at the periphery of the central tissue in nitrogen-fixing nodules of *Phaseolus vulgaris*. The experiments reported here show the presence of young (undifferentiated) bacteroids, expressing the ORF1-*ntrBC* operon into the same peripheral invaded cell layers. These results demonstrate that in the determinate nodules of *Phaseolus vulgaris* not all the invaded cells of the central tissue are progressing through the same stage of development. Thus, contrary to the conclusion drawn from morphological analysis on the determinate soybean nodules (Newcomb et al. 1979; Calvert et al. 1984; Selker 1988), the experiments reported here show the presence of developmental zones in the nodules of *Phaseolus vulgaris*. Moreover, while in indeterminate nodules the youngest invaded cells are distal to the root (Vasse et al. 1990), in the determinate nodule of *Phaseolus vulgaris* the youngest invaded cells are located at the periphery of the central tissue. These cells may be either the latest to be invaded or the latest to be differentiated. The presence of infection threads around the most peripheral invaded cells in mature nodules (R. Taté and E. J. Patriarca, unpublished results) supports the first hypothesis.

The analysis of the ORF1-*ntrBC-lacZ* expression in *Vicia hirsuta* indeterminate nodules (Fig. 3) permits a correlation between promoter activity and the physiology of the different developmental zones of this type of nodules. The signal causing down-regulation of the ORF1-*ntrBC* promoter is not the lack of oxygen, since it has been shown (Soupéne et al. 1995) that the O_2 concentration in the nodule changes only slightly in zone II, where the ORF1-*ntrBC* promoter is down-regulated. On the other hand, down-regulation of the ORF1-*ntrBC* promoter is not due to the appearance of ammonia synthesized through nitrogenase activity since (i) down-regulation takes place in the distal half of zone II, quite far from the interzone II-III where nitrogenase appears (Soupéne et al. 1995) and (ii) a Fix$^-$ mutant of *R. leguminosarum* bv. *viciae* carrying the ORF1-*ntrBC-lacZ* fusion shows the same pattern of expression (data not shown). A transcriptional down-regulation has also been reported in the case of the *nod* and *ropA* genes (Schlaman et al. 1991; Sharma and Signer 1990; de Maagd et al. 1994). The molecular mechanism causing down-regulation of these promoters in the developing nodule is presently unknown and certainly worth investigating. In free-living *R. etli* the ORF1-*ntrBC* promoter is more

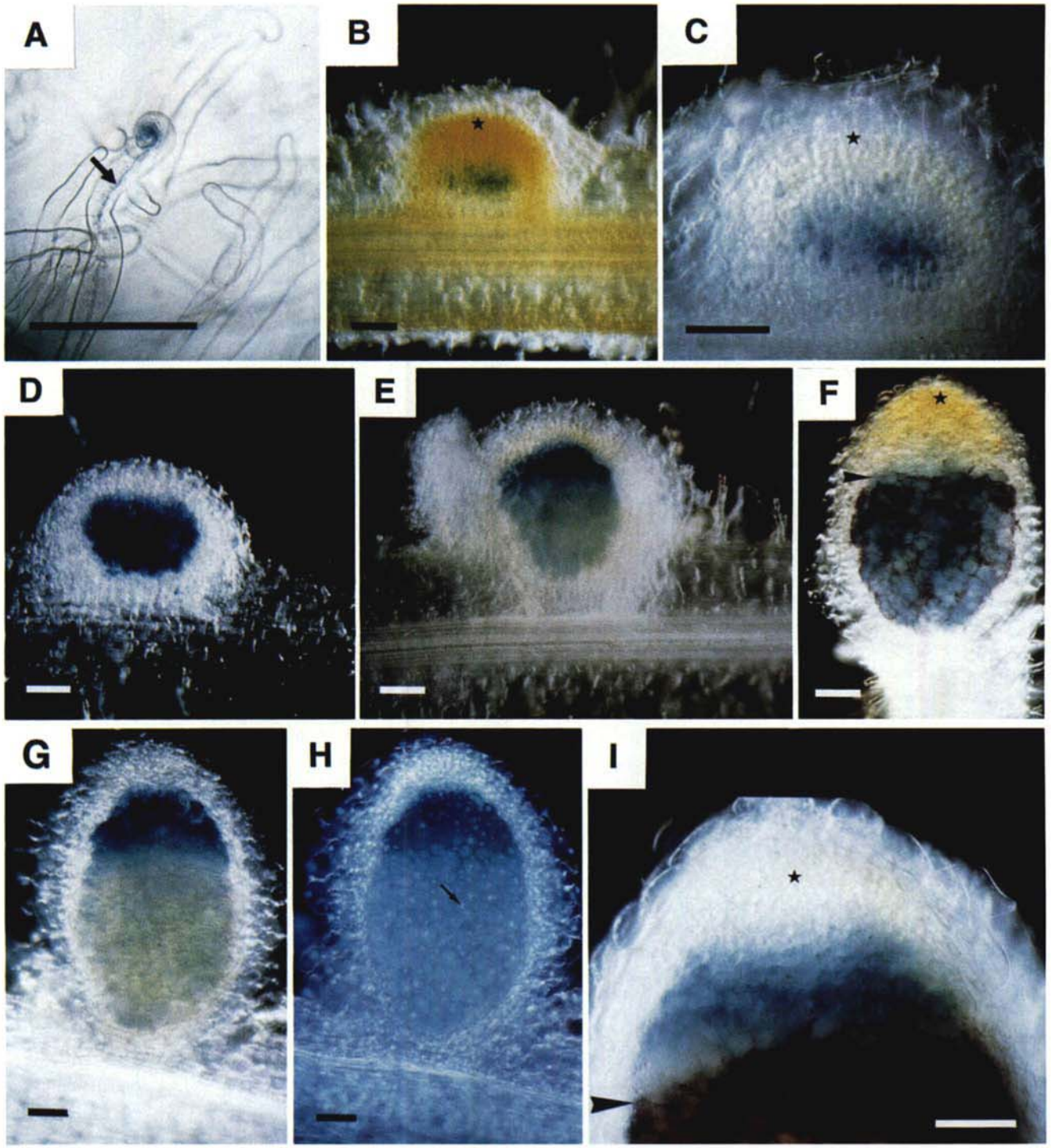

Fig. 3. Histochemical localization of β-galactosidase activity in different developmental stages of *Vicia hirsuta* nodules induced by *Rhizobium leguminosarum* bv. *viciae* carrying either pAR66 or pRT124. β-galactosidase activity expressed from the ORF1-*ntrBC* promoter (plasmid pAR66) is evident in infection threads (arrows in **A**) and in the first invaded cells of an early emergent nodule (**B, C,** and **D**). **E,** In a 10-day-old nodule a decreasing apical-proximal gradient of β-galactosidase activity is observed. In 20-day-old nodules β-galactosidase activity begins to be evident in the infection zone just behind the meristem (star) and it is not detectable in the last two to three cell layers of zone II (**G, H,** and **I**). **G,** The section shown is also stained with DAPI (4′,6-diamidino-2-phenylindole) to show nuclei of invaded cells. β-galactosidase activity from the *nifH* promoter (plasmid pRT124) begins to be evident in (**F**) interzone II-III and shows no activity in infection threads and in the first invaded cells of the nodule primordium (data not shown). **F** and **I,** Sections are also stained with I_2-KI showing starch granules in brown. Bar = 100 μm. Nodules obtained from plantlets inoculated with *R. leguminosarum* bv. *viciae* carrying plasmid pMP220 (the promoterless vector) showed no activity.

active in early logarithmic phase bacteria (Fig. 4) than at later stages. We have also found (M. Martino and E. J. Patriarca, unpublished experiments) that growth inhibition (induced by antibiotics or by amino acid starvation) causes a disappearance of the ORF1-*ntrBC* RNA. Therefore, it is possible that the transcriptional down-regulation of this promoter is a consequence of the observed arrest in bacterial division occurring in specific parts of determinate (this paper) and indeterminate (Vasse et al. 1990) nodules.

The immuno localization analysis shows that the NtrC protein abruptly disappears from bacteroids within one cell layer (Fig. 2). Gene products should not be diluted out after down-regulation of transcription because bacteroids only occasionally divide inside the peribacteroidal vesicle and therefore the sharp disappearance of NtrC suggests a mechanism for active removal of this protein from the bacteroids present within the younger invaded cells. Also the *ropA* gene product (OMPIII protein) was shown to disappear from bacteroids within a single cell layer of the invasion zone (de Maagd et al. 1994). It is therefore evident that in young bacteroids a developmental switch takes place: transcription of a set of genes is switched off and the gene products are removed, most likely through the action of proteases, possibly turned on because of nitrogen starvation (Goldberg and Dice 1974). This suggests a parallelism between the very early steps of sporulation of *Bacillus subtilis* and the onset of *Rhizobium* differentiation into bacteroids. Sporulation is induced either at the end of the logarithmic phase of growth, or by dilution in a nitrogen-free medium, and it is correlated to two events: transcription of a set of genes is down-regulated by modification of the vegetative sigma factor (Tijan and Losick 1974) and protein degradation is activated in the mother cell of bacteria (Kornberg et al. 1968).

Bacteroid development takes place in an invagination of the plasma membrane, the peribacteroid membrane. We assume that after invagination the transport systems do not reverse their polarity and therefore they will take up solutes from the membrane side, which was previously oriented toward the extracellular space and now is in the peribacteroidal space. This may be an efficient mechanism to starve bacteroids of all the substances that are normally taken up by the plant cell and feed them only with the few specific substances normally excreted by it. We speculate that the onset of bacteroid differentiation may be caused by a general starvation of the bacterium, mimicking a late exponential phase and therefore causing the activation of global regulatory mechanisms, as for example the down-regulation described in this paper, and activation of protein turnover. Later on, during the symbiotic interaction, the structure and function of the peribacteroid membrane gradually differentiates from that of the plasma membrane to incorporate functions needed in the symbiosis, as for example the dicarboxylic acids transport system (Ou Yang et al. 1990).

MATERIALS AND METHODS

Bacterial strains and plasmids.

Rhizobium leguminosarum bv. *viciae* LPR1105, a Rif^r derivative of RCR1001 (Hooykaas et al. 1977) and *Rhizobium etli* strains CE3 and CFN2012 (a *ntrC*::Tn*5* derivative of CE3) (Moreno et al. 1992), were grown at 30°C on TYR rich medium (Beringer 1974). Antibiotics used were as follows (μg ml^{-1}): tetracycline (5); nalidixic acid (20); kanamycin (30); and rifampicin (100).

Plasmid pAR66, containing the ORF1-*ntrBC* promoter region fused to the *lacZ* gene, was previously described (Patriarca et al. 1993). Plasmid pRT124, containing the *nifH* promoter (the *nifH* gene codes for a subunit of the nitrogenase complex) of *R. etli* fused to *lacZ*, was constructed by ligation of a 1.5-kb *Bgl*II DNA fragment from plasmid pCQ23 (Quinto et al. 1985) to *Bam*HI-digested pGEM3Z (Promega, Madison, WI), followed by digestion with *Eco*RI/*Xba*I and recloning into plasmid pMP220 (Spaink et al. 1987). pAR66 and pRT124 were conjugated from *E. coli* strain S17-1 to the above *Rhizobium* strains, as previously described (Amar et al. 1994).

RNase protection assays.

Plasmid pEP24a (containing a DNA fragment spanning 272 nucleotides of the *ntrBC* intergenic region; Patriarca et al. 1993) was used to synthesize ^{32}P-labeled antisense RNA with SP6 RNA polymerase (Promega Inc.) and [α-^{32}P]UTP (Amersham Corp., U.K.). RNA purification and RNase protection experiments were performed as previously described (Patriarca et al. 1993).

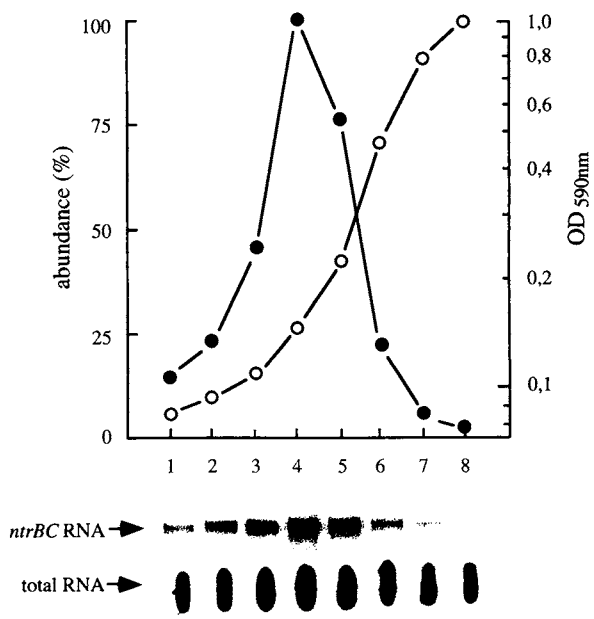

Fig. 4. ORF1-*ntrBC* mRNA levels during growth of *Rhizobium etli* in TYR medium at 30°C. A culture of bacteria grown to an OD_{590} of 1 was diluted into fresh medium and incubated at 30°C in a rotary shaker (Model G76, New Brunswick Scientific Co., Edison, NJ) at 200 rpm. RNase protection analysis was performed using RNA prepared from cells harvested at various times during growth (OD_{590} values, open circles). Aliquots were hybridized to an excess of ^{32}P-labeled antisense RNA probe generated from clone pEP24a, covering the *ntrBC* intergenic region (see Materials and Methods). In control experiments, not shown, the riboprobe annealed in the absence of RNA and treated with RNases A and T1 showed no protected fragment and the riboprobe alone showed, as expected, a band of 325 nucleotides. The radioactive signals were quantified (abundance percent, closed circles) with a PhosphorImager. Values were normalized to the signals obtained by a slot blot experiment (below the gel) using ^{32}P-labeled total DNA as a probe and to the value obtained at 0.14 O D_{590}, taken as 100.

Plant growth and histochemical localization.

Seeds were germinated and inoculated as previously described (Amar et al. 1994). Whole roots were collected at various times after inoculation and fixed, under a brief and gentle vacuum, with 2% glutaraldehyde, 0.3 M mannitol, and 10 mM 2-[N-morpholino]ethanesulfonic acid, pH 5.6 for 1 h at room temperature. This fixation treatment inactivates β-galactosidase activity of the plant tissue (Arsène et al. 1994). Whole root fragments and nodule sections were washed three times with 50 mM phosphate buffer pH 7.2 and immersed in a staining solution containing 10 mM phosphate buffer, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.03% Triton-X100 and 1 mM 5-bromo-4-chloro-3-indolyl β-galactopyranoside (X-Gal, INALCO SPA, Italy). The samples were incubated in the dark at 37°C for the required time, and then briefly cleared with sodium hypochlorite (Boivin et al. 1990), washed in phosphate buffer, rinsed in 70% ethanol, and then observed with a light microscope by means of dark- and bright-field optics. In more than 90% of the cases the X-Gal staining pattern was identical, indicating that the recombinant plasmids used are not lost from bacteria inside the plant, where there is no selective pressure. Nodules induced by *Rhizobium* strains carrying the pMP220 vector showed no background X-Gal stain. Starch granules were evidenced by staining sections in 0.1 M aqueous I_2-KI. The stained sections were photographed with a Nikon microscope in bright-field and epipolarization optics. To locate nuclei, nodule sections were incubated at 30°C for 15 min with 0.1 μg/ml DAPI (4′,6-diamidino-2-phenylindole) in 50 mM phosphate buffer pH 7.2, washed twice (10 min) with buffer, and observed under fluorescence microscopy.

Immuno localization.

Immuno gold labeling was performed using polyclonal rabbit antibodies raised against the *R. etli* NtrC protein. Root nodules were collected at 7, 12, or 18 days after inoculation and fixed for 40 min at 4°C with 4% paraformaldehyde, 0.1% glutaraldehyde, 0.05 M potassium phosphate buffer (pH 7.3), and 0.5 M sucrose. The fixed tissues were rinsed three times in the same solution for 30 min, dehydrated through a graded ethanol series, and infiltrated with LR white resin. Polymerization was performed in gelatin capsules under UV light at −10°C. Thin sections (70 to 100 nm) were obtained by means of an LKB ultratome-III equipped with a diamond knife and mounted on gold or nickel slotted grids, which were incubated in PBST (0.01 M phosphate buffer pH 7.4, 0.15 M NaCl, 0.5% Tween 20, 0.2% fat sterile milk) for 15 min, then treated with the first antibody in PBST without milk for 2.5 h at 4°C and washed six times (10 min) with PBST. Samples were exposed to 10 nm colloidal gold conjugated goat anti-rabbit IgG (British BioCell Inter., U.K.) diluted 1:40 in PBST for 2.5 h at 4°C, they were then washed six times (10 min) with 0.1 M Tris buffer, 0.5 M LiCl, and 0.05% DTT (dithiothreitol), and two times (5 min) with distilled water. Grids were dried and stained with 2% uranyl acetate for 20 min at 60°C, followed by Reynold's lead citrate for 1 min at 20°C, and then observed with a Siemens E102 electron microscope. Immuno localization was also performed with semi-thin (1 to 2 μm) sections using the same protocol. Silver enhancement (British BioCell Inter.) was applied to visualize the colloidal gold labeling with a light microscope.

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