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MINIREVIEW

Regulation of Nodulation Gene Expression by NodD in Rhizobia

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INTRODUCTION

Strains of the soil bacteria *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* spp. can infect plants, leading to a symbiotic interaction in which root nodules, and in the case of *Azorhizobium* spp. sometimes stem nodules, are formed. In these nodules the bacteria live in a differentiated form, the bacteroid, inside the cells of the host plant, and they fix nitrogen by reducing atmospheric nitrogen to ammonia. The ability of bacterial strains to form effective nodules is limited to certain host plants, usually restricted to plants belonging to the *Leguminoseae*. For instance, *Vicia* and *Pisum* spp. are host plants for *Rhizobium leguminosarum* biovar (bv.) viciae, *Trifolium* spp. are hosts for *R. leguminosarum* bv. trifolii, *Medicago* spp. are hosts for *Rhizobium meliloti*, *Glycine* spp. are hosts for *Bradyrhizobium japonicum*, and the tropical legume *Sesbania rostrata* is the host for *Azorhizobium caulinodans*.

A number of bacterial genes are important for the symbiosis. Among these are the nodulation genes, designated *nod* and *nol*. The organization of these genes in operons is very similar in *Rhizobium* and *Bradyrhizobium* spp. (Fig. 1). In the fast-growing species of *Rhizobium* the *nod* genes are localized on a large socalled Sym (symbiosis) plasmid, whereas in *Bradyrhizobium* and *Azorhizobium* spp. the *nod* genes are located on the chromosome. Initially, the *nod* genes were classified as common or host-specific nodulation (*hsn*) genes, which are, respectively, those interchangeable for nodulation function between different species or those involved in the host specificity of nodulation. This strict dichotomy is not clear for all *nod* and *nol* genes, however. The common *nod* genes comprise *nodA*, *-B*, *-C*, *-I*, and *-J*, all located in one operon, of which *nodABC* are essential for nodulation. Another essential gene is *nodD*, of which one or more alleles are present, depending on the rhizobial species (see below). The *nodD* gene behaves as a common *nod* gene for nodulation on some host plants, while in other cases it represents an important determinant of host specificity (18, 57). Several *hsn* genes are common to all *Rhizobium* spp., e.g., *nodFE*, *nodL*, and *nodM*. Many others, however, are present only in a particular set of rhizobial species or biovars, e.g., *nodO* in *R. leguminosarum* bv. viciae, *nodH* and *nodPQ* in *R. meliloti*, and *nodZ* in *B. japonicum*. In addition to these *nod* genes there are several recently characterized genes (designated *nod* or *nol*) which are regulated in the same way as *nod* genes but for which the effect on symbiosis is not yet clear.

The biochemical functions of only some of the Nod proteins are established. It is known that most of them are involved in the synthesis of extracellular bacterial signal compounds (31, 55). Apparently, more than one species of these factors are synthesized (55). These signal compounds have the general structure of a tetra- or pentamer of *N*-acetylglucosamines to which a variable acyl chain is linked (31, 55). The common *nod* genes are involved in the synthesis, and probably also the secretion, of the backbone structure. Several *hsn* genes are involved in the synthesis or addition of various extra moieties to this backbone (for a review, see reference 52).

THE *nodD* GENE

In *R. leguminosarum* bv. viciae and trifolii only one *nodD* gene is present, whereas other rhizobia carry more *nodD* alleles. Up to four *nodD* genes have been reported for *R. meliloti*; these are designated *nodD1*, *nodD2*, *nodD3*, and *syrM*. The *nodD* gene product is the transcriptional activator of the other *nod* genes (see below). However, it can also act as a repressor of transcription, as illustrated by the strong negative autoregulation observed in *R. leguminosarum* bv. viciae and trifolii (41, 53). Furthermore, the expression of *rhiA*, localized on the Sym plasmid of *R. leguminosarum* bv. viciae and coding for an abundant 24-kDa protein, is under negative control of NodD (11). On the basis of homology, NodD has been classified as a member of the LysR family of transcriptional regulators (19) (Fig. 2). Most of these act as transcriptional activators; some are repressors. All of these proteins require an inducing compound for activation. Although the cellular processes in which they act are very diverse, the proteins nevertheless share many common features. Their properties can be summarized as follows. (i) They are medium-sized proteins, 32 to 36 kDa. (ii) They have a helix-turn-helix DNA-binding motif in their N termini (19). The highest sequence conservation resides in this part of the proteins. (iii) They lack sequence homology in the C-terminal part. (iv) They are very often subject to negative autoregulation. (v) Their transcription frequently reads divergently from the genes which they control. (vi) Characteristics of in vitro binding to target DNA sequences are usually not changed by the presence or absence of inducers. (vii) For several of these proteins, mutants which activate transcription independent of inducing compounds have been described, suggesting a conformational change upon binding of inducers. (viii) They contain a common motif in their DNA target sites, designated the LysR motif (16).

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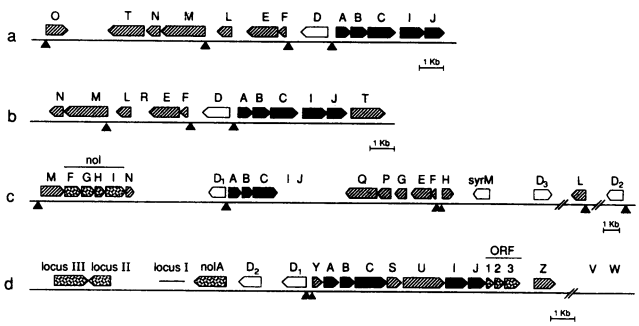

FIG. 1. Genetic organization of *nod* genes in *R. leguminosarum* bv. viciae (a), *R. leguminosarum* bv. trifolii (b), *R. meliloti* (c), and *B. japonicum* (d). The genes are presented as arrows which point according to the direction of their transcription. Common *nod* genes are indicated with black arrows, host-specific *nod* genes are indicated with shaded arrows, and the *nodD* genes are indicated with white arrows. *nol* genes, unknown open reading frames (ORF), and other *nod* loci are indicated with dotted arrows. Black triangles indicate the positions of *nod* boxes.

On the basis of sequence data, it is assumed that DNA binding occurs at the N termini of the proteins and that interaction with inducer molecules occurs at the C-terminal part. However, results with double mutants (4) and with hybrid *nodD* genes (56) demonstrated that the C-terminal part of NodD is also involved in DNA binding, suggesting that NodD does not consist of two separate functional domains. A comparable situation appears to exist for NahR (43), a LysR-type protein which shows strong sequence similarity to NodD (Fig. 2) (44).

TRANSCRIPTIONAL REGULATION OF *nod* GENES

Except for most *nodD* genes, the *nod* and *nol* genes are not transcribed in bacteria grown in the usual laboratory media. To induce their expression the following are required: (i) the NodD protein, the positive transcriptional regulator of the inducible *nod* genes; (ii) a *nod* box, a conserved DNA sequence upstream of the inducible *nod* genes which is essential for promoter function; and (iii) an inducer, usually a flavonoid from the root exudate of the host plant. Inducers for most fast-growing rhizobia usually are flavones and flavanones, whereas inducers for *Bradyrhizobium* spp. are often isoflavones. Plants also release flavonoids which can act as anti-inducers (9, 12). Interestingly, the *nodD1* genes of *B. japonicum* (61), *R. leguminosarum* bv. phaseoli (7), and *Rhizobium fredii* (1) are also preceded by a *nod* box sequence. For the former two species, the *nodD1* transcription levels are enhanced in the presence of NodD1 protein and certain flavonoids independently of other *nod* genes (8, 51). The expression of *nodD3* and *syrM* in *R. meliloti* is strongly interwoven in a complex way (28, 34, 42).

The expression of the inducible *nod* genes during symbiosis starts in the rhizosphere. The activity of the *nod* products leads to the production of extracellular bacterial signal compounds which in turn induce a wide range of plant responses, e.g., root hair deformation, meristematic activity in the cortex, and induction of some early nodulins (for a review, see references 37 and 52). When the bacteria have entered the host plant root, they multiply in the infection thread and are subsequently released into the cytoplasm of the newly formed meristematic cells, where they differentiate into bacteroids. Bacteroids are a differentiated form of

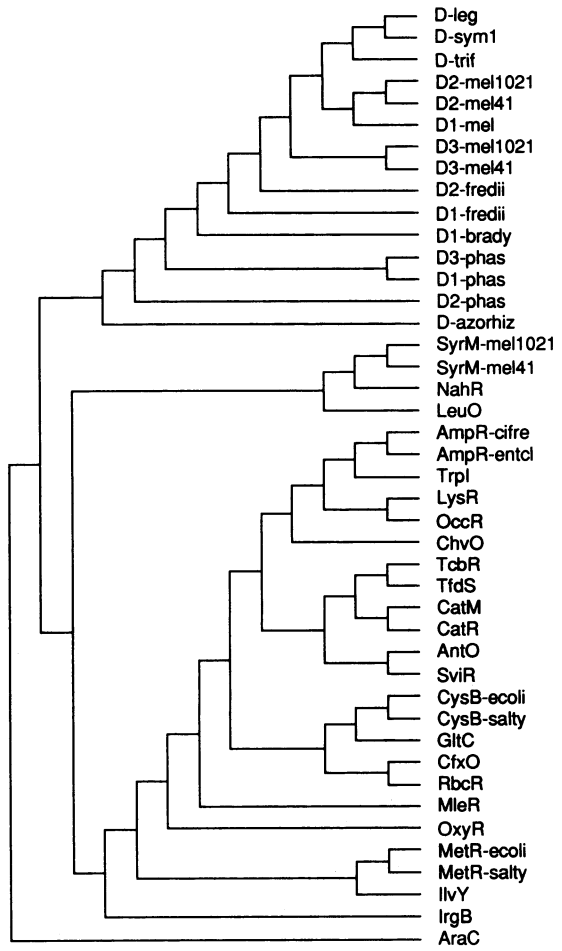

FIG. 2. Phylogenetic relationships among members of the LysR family of transcriptional regulator proteins as deduced by the program PAUP (59), version 3.0o for Macintosh. All of these sequences are available in the data bases and have been published. D, NodD sequences from *A. caulinodans* (azorhiz); *B. japonicum* (brady); *R. fredii* (fredii); *R. leguminosarum* bv. viciae (leg and sym1, two different strains), trifolii (trif), and phaseoli (phas); and *R. meliloti* (mel; 1021 and AK41 are two different strains). Other abbreviations: cifre, *Citrobacter freundii*; ecoli, *Escherichia coli*; entcl, *Enterobacter cloacae*; salty, *Salmonella typhimurium*. The sequence of AraC, to which NodD formerly was proposed to be homologous (50), was chosen as an outgroup (59). The position of AntO in this order of relationship is open to discussion since its function as an H^+/Na^+ antiport differs largely from that of the other proteins.

the bacteria which fix nitrogen and are unable to convert to bacteria. When the bacteria are released from the infection thread, the expression of the inducible *nod* genes stops and that of *nodD* decreases (46, 49).

Initially, the favored model of transcriptional activation of the inducible *nod* genes was one in which flavonoids enter the bacterial cytoplasm, where they bind to NodD protein and activate the protein through a conformational change. The activated NodD subsequently binds to the *nod* box, and because of this binding, the transcription of the respective genes is induced. The following observations made it necessary, however, to revise this model. (i) The NodD protein of *R. leguminosarum* bv. viciae is localized in the cytoplasmic membrane (48). (ii) Flavonoids are probably hardly present in the cytoplasm, but are thought to shuttle through the

cytoplasmic membrane since the molecules are alternately protonated and deprotonated (39, 40). (iii) In vitro, NodD can bind to the *nod* boxes also in the absence of flavonoids (13, 20, 29). (iv) Other proteins bind to the *nod* boxes as well, and they might be involved in the regulation of transcription from these promoters (16, 29). In the following paragraphs the various elements of the model of transcriptional activation of the *nod* genes are discussed; the NodD-mediated part of transcriptional activation will be discussed in more detail.

NodD as a membrane protein. In *R. leguminosarum* bv. viciae, NodD is an amphipathic cytoplasmic membrane protein, presumably inserted only in the inner monolayer (48). In *R. meliloti*, however, substantial amounts of NodD1 and NodD3 are present in the soluble fraction of a biochemical preparation (29, 32). By using computer analysis, a hydrophobic α-helix has been predicted for the presumed membrane-integrated part of NodD. This part contains three and four Pro residues for *R. leguminosarum* bv. viciae NodD and *R. meliloti* NodD1, respectively (48); Pro residues are known to break α-helices (6). It should be noted that Pro residues are found in membrane-located α-helices of many membrane proteins that function as receptor subunits or as transporters (for a review, see reference 62). For SyrM of *R. meliloti* a potential membrane-integrated helix domain also is predicted (28).

Binding of flavonoids to NodD and activation of NodD in the membrane. In vivo, the presumed interaction between NodD and flavonoids is likely to occur in the cytoplasmic membrane, since both partners are localized in this compartment (40, 48). This suggests that an analysis of the presumed binding is highly complicated. Indeed, a direct binding of flavonoids to NodD has not been shown, due to technical difficulties since flavonoids stick to all kinds of materials, including proteins (38). Nevertheless, results with mutant *nodD* genes (3, 22, 35, 56), analysis of inducible *nod* gene transcription in an isogenic background with *nodD* genes from various sources (18, 57), and an enhanced binding of *nod* box DNA by a 35-kDa protein in the presence of flavonoid inducers (16) together strongly suggest that NodD functions as a specific receptor for flavonoids. As stated above, NodD does not contain separate functional domains for DNA binding and flavonoid interaction. It was initially suggested from several studies with mutants that flavonoid binding occurs in the C-terminal part of the protein (3, 17, 22, 35), but this was not supported by the results of other NodD mutant studies (4, 56).

Since flavonoids are required for activation of the NodD protein, they presumably induce a conformational change in the protein. This notion is supported by the fact that it is possible to construct mutant and hybrid NodD proteins which activate the transcription of the inducible *nod* genes independent of flavonoids (3, 54).

Translocation of NodD from the membrane. We suggest that NodD is localized in the cytoplasmic membrane to facilitate binding of flavonoids. Consistent with this is the observation made with *R. meliloti*, in which NodD has been localized mainly in the cytosol, that migration to the cytoplasmic membrane occurs only when appropriate flavonoids are added to the cell (29). Binding of NodD to *nod* box DNA occurs by a soluble form of NodD in *R. meliloti* (13) and also in *R. leguminosarum* bv. viciae, although a minor fraction of cytoplasmic-membrane-located NodD can bind to *nod* boxes as well (45). Other proteins which have a reversible association with the membrane, similar to NodD, have been described. These are designated amphitropic proteins (5), and NodD presumably is such a membrane protein. In *R. meliloti*, a chaperonelike protein homologous to GroEL of *Escherichia coli* is necessary for the transcriptional activation by NodD (33). It is feasible that this protein is necessary for the translocation of NodD from the cytoplasmic membrane to keep it in a proper, soluble conformation. In this respect, it might be relevant that a 59-kDa protein was copurified with NodD1 from the cytosol of *R. meliloti*, since GroEL is a 60-kDa protein (13).

Binding of NodD to *nod* boxes. The specific binding of NodD to *nod* box DNA has been well established in vitro (13, 16, 20, 29). The *nod* box DNA region protected by NodD is identical in the presence and absence of flavonoids (14, 29). Comparable results are found for many proteins belonging to the LysR family. However, studies done with NahR demonstrate that differences in binding to the regulated promoter sequence are detectable only when the analyses are performed in vivo and not in vitro (23). For *R. meliloti* AK41 (29) and *A. caulinodans* (16) it has been reported that NodD has an higher affinity for the *nod* box in the presence of inducer than in its absence. An altered binding was not observed by others, however (13, 20).

In *Rhizobium* spp. the *nod* box is composed of three hyperconserved parts (53), whereas in *B. japonicum* the *nod* box sequence can be divided into four hyperconserved boxes (61). Recently, the presence of two inverted repeats with the sequence A-T-C-N_9-G-A-T within all known *nod* boxes was made evident (16). Such a structure favors the hypothesis that NodD binds as a tetramer to the *nod* box, as was also suggested by studies with *nod* box deletion mutants (61). Consistent with this are data from studies of *R. meliloti* in which one or more *nodD* genes were mutated and subsequently analyzed for inducing capacity, which revealed that NodD probably binds to the *nod* box as a dimer or a tetramer (21). This notion is further supported by the presence of a receiver module in the N-terminal half of NodD (35) which might be involved in multimerization of the protein (25). Two other members of the LysR family, CysB (36) and NahR (43), bind to their DNA-binding sites as tetrameric proteins.

Additional factors involved in expression of *nod* genes. A repressor of *nod* gene transcription, designated NolR, is present in many *R. meliloti* strains but not in the well-investigated strain 1021 (29, 30). NolR binds to the *nodD1* and *nodD2* promoter regions and not to any of the inducible *nod* promoters (29), and its major role is proposed to be in regulation of *nodD1*, *nodD2*, and *nodD3* transcription (30). Strong evidence for the presence of a repressor protein in *R. leguminosarum* bv. viciae is lacking (30), although *nolR*-homologous DNA can be detected on Southern blots under low-stringency conditions (27). In contrast, an additional protein which binds to the *nodF* box acts as an activator rather than as a repressor (45). This same protein or another one may also bind to *nod* box sequences of *nodA* and *nodM*, but not to those of *nodO*. In *A. caulinodans* at least three other proteins, smaller than NodD, were found to bind to *nod* box DNA, but their function is unknown (16).

Combined nitrogen represses *nodABC* transcription in both *R. meliloti* and *B. japonicum* (10, 60). The expression of *R. meliloti nodD3*, but not that of *nodD1* (10), and of *B. japonicum nodD1* (60) is under negative control of NH_4^+. In the latter case, neither NifA nor NtrC appears to be involved, but two binding sites for NtrC are found upstream of *nodD3* in *R. meliloti* (26). At a 10 mM concentration of NH_4^+, 40 and 20% inhibition of *nodD1* and *nodABC* expression, respectively, occurs in *B. japonicum* (60), whereas at least 30 mM NH_4^+ is required for measurable inhibitory effects in *R. meliloti* (10).

Transcriptional activation of inducible *nod* genes. The mechanism by which NodD induces transcription is still not understood. In *R. meliloti nod* promoter activity correlates with in vitro NodD DNA binding (15). RNA polymerase may be facilitated to bind to the promoter region which is located downstream from the binding site of NodD. Such a mechanism, e.g., by bending of the DNA helix, has been proposed for the members of the LysR family (19), although strong experimental data supporting this notion are still lacking. There is evidence, however, for bending of *nod* box DNA by NodD1 in *R. meliloti* (15). This problem will likely be resolved only when an in vitro system for transcriptional activation of the inducible *nod* genes is available. In vivo studies on NodD-*nod* box interaction should be undertaken in the near future.

Decrease of transcription of *nod* genes. In bacteroids, the inducible *nod* genes are not transcribed (46, 49), and their expression stops after the bacteria have been released from the infection thread into the plant cytoplasm (46). This phenomenon has been analyzed biochemically in *R. leguminosarum* bv. viciae and apparently is caused by ineffective binding of NodD in bacteroids to *nod* boxes, because of either a conformational change of the protein or its presence in another complex (47). Since high-level constitutive expression of the inducible *nod* genes in bacteroids results in Fix^- nodules (3, 24), the expression of these genes is undesirable in bacteroids. Moreover, the transcription of *nodD* is reduced in bacteroids (46, 49). In bacteroids of *R. leguminosarum* bv. viciae the level of *nodD* expression is around 35% of that of free-living cells, and this reduction may be caused by a bacteroid-specific repressor protein (47). In *R. meliloti* neither *nodD1* nor *nodD3* is transcribed, whereas the expression of *syrM* is enhanced in bacteroids (49, 58).

RELATIONSHIP BETWEEN NITROGEN FIXATION AND NodD PROTEIN

The role of NodD in bacteroids is poorly understood, since it appears not to be used for *nod* gene induction and thus flavonoid sensing. However, several relevant observations suggest that NodD is in some way linked to the process of nitrogen fixation. (i) When plants are infected with rhizobia containing the hybrid gene *nodD604*, which activates the transcription of *nod* genes independent from flavonoids, normal nodulation occurs but the levels of nitrogen fixation can be significantly higher (46, 54). This is not caused by a continuous expression of the inducible *nod* genes within the bacteroids (46). (ii) The *syrM* gene in *R. meliloti* is the least-conserved *nodD*-like gene known (Fig. 2) (2, 28), and it can therefore be assumed that the conformation of SyrM is different from that of the other NodD proteins. While the expression of the *nodD* genes is much lower in bacteroids than in free-living cells (46, 49), the reverse appears to be the case for the transcription of *syrM*: it is very low in free-living cells, grown aerobically or microaerobically, but high in nitrogen-fixing bacteroids (49, 58). (iii) In addition, the expression of *nodD3* of *R. meliloti* appears to be controlled by the general system for nitrogen-regulated gene expression NtrB-NtrC (26).

Despite these data, no molecular interaction of NodD with *nif* and/or *fix* genes is known, nor do we have any idea whether more proteins and/or factors are involved.

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