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Review article

Function of Nodulation Genes of *Rhizobium*

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Abstract

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* induce nodules on the roots of leguminous plants in which the bacteria symbiotically fix nitrogen. Nodulation genes (*nod*) of the bacteria are essential in this process. The genetics of the *nod* genes of the intensively investigated species and the transcription activation of *nod* genes by plant-exuded flavonoïds are reviewed. The protein products of many *nod* genes are involved in the synthesis of low molecular weight compounds, designated Nod metabolites. Very recent data about the chemical structure of these Nod metabolites and their essential biological function in symbiosis are discussed.

Keywords: nodulation genes, *Rhizobium*, Nod metabolite

1. Introduction

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* are soil bacteria which are able to establish a symbiotic relationship with leguminous plants. This symbiosis is specific in that a certain species or biovar of bacteria forms a functional relationship with host plants of only one or a limited number of genera.

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The development of this symbiosis has been studied intensively and can now partly be described in molecular terms, especially as far as the bacterium is concerned. One of the first steps in symbiosis is the attachment of bacterial cells to the tip of root hair curling, and the formation of an infection thread. The infection thread, filled with bacteria, penetrates the cortex, while concomitantly meristems are induced at some distance from the infection thread. Bacteria are released in the newly formed meristematic cells and differentiate into bacteroids, which fix atmospheric nitrogen in the root nodules.

For root hair curling, infection thread formation and meristem induction, several bacterial genes are essential which are designated *nod* (for nodulation) genes. Bacterial genes which are not essential for nodulation but are located in the same regulon are also designated as *nod* genes or as *nol* genes. Besides these genes, other genes, which code for the synthesis of macromolecules at the surface of the bacteria, are important in the infection process, e.g. genes involved in exopolysaccharide synthesis.

This review deals with the genetics of the *nod* and *nol* genes and with their possible molecular functions of the rhizobia studied most intensively. These rhizobia include *Rhizobium leguminosarum* biovar *viceae*, which nodulates *Vicia, Lathyrus, Pisum* and *Lens*, *R. leguminosarum* bv. *trifolii* which nodulates *Trifolium* species and *R. meliloti* which is specific for plants like *Melilotus* and *Medicago*. In addition to these fast-growing strains we include some information on the slow-growing *Bradyrhizobium japonicum* which nodulates *Glycine* (soybean). Recently excellent reviews have been published by Long (1989) and by Nap and Bisseling (1991).

2. Nodulation genes and Their Regulation

In fast-growing rhizobia most *nod* genes are localized as a cluster on a large plasmid, designated Sym (for symbiosis) plasmid. In the slow-growing *Bradyrhizobium* no association of these genes with a plasmid has been reported. The genetic organization of *nod* genes is given in Fig. 1. The genes *NodA,B,C,I,J* are designated as common *nod* genes because they show cross-complementation in all *Rhizobium* species. In contrast, the other *nod* genes are designated as host-specific (*hsn*) *nod* genes. With the exception of most of the *nodD* genes in fast-growing rhizobia, the *nod* genes are not transcribed in cells cultured in the usual laboratory media. The NodD proteins act as positive regulators of the inducible *nod* operons upon activation by inducer molecules. These inducers have been identified as flavonoids (fast-growing *Rhizobium* species) or isoflavones (*Bradyrhizobium*) and are present in exudates of host plants (Innes et al., 1985; Mulligan and Long, 1985; Rossen et

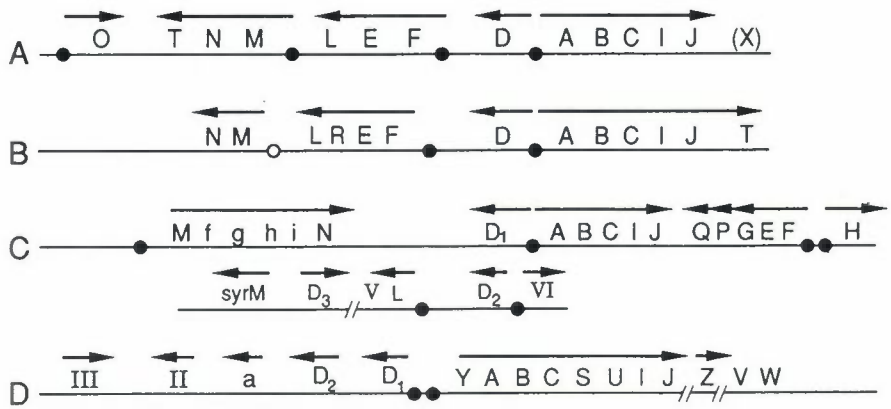

Figure 1. Nodulation of (A) *R. leguminosarum* bv. *viceae*, (B) *R. leguminosarum* bv. *trifolii*, (C) *R. meliloti* and (D) *B. japonicum*. *Nod* genes are indicated by capitals, *nol* genes with lower case and loci by roman characters. Operon structure and direction of transcription are indicated by arrows. Inducible promoters, containing a *nod*-box, are indicated by black dots, a supposed *nod*-box of *R. leguminosarum* bv. *trifolii* is indicated by an open dot. In *R. leguminosarum* bv. *viceae* some Sym plasmids, like in strain TOM, contain an additional gene *nodX* which confers extended host range to some *Pisum* strains (Canter Cremers et al., 1988; Davis et al., 1988). Data for *R. leguminosarum* bv *viceae* are taken from Downie et al., 1991, for *R. leguminosarum* bv. *trifolii* from Weinman et al., 1991, for *R. meliloti* from Kondorosi, 1991; Baev et al., 1991, for *R. meliloti* from Kondorosi, 1991; Baev et al., 1991 and for *B. japonicum* from Stacey et al., 1991.

al., 1985; Peters et al., 1986; Redmond et al., 1986; Firmin et al., 1986; Zaat et al., 1987; Banfalvi et al., 1988). Every legumenous species secretes its own, characteristic set of flavonoids (Zaat et al., 1988) and the NodD protein is adapted to a range of host plants in that the NodD protein of every species (biovar) is activated only by a limited range of these compounds. This selective activation is one of the determinants of host-specificity (Spaink et al., 1987b; Horvath et al., 1987). Other determinants of host specificity are discussed below. Besides inducing compounds also inhibitors of induction, e.g. umbelliferone and isoflavones are found in exudates (Djordjevic et al., 1987; Firmin et al., 1986). The transcription of inducible *nod* genes starts in the rhizosphere of the host root. Recently it has been shown that this expression is transient in that *nod* gene expression is turned off in bacteria which are to be released in the newly formed nodule cells (Sharma and Signer, 1990; Schlaman et al., 1991).

This mechanism of transcription activation of the inducible *nod* genes is known in outline. Each inducible operon is preceded by a highly conserved

DNA sequence, designated as *nod*-box, which is essential for promoter activity (Rostas et al., 1986; Spaink et al., 1987a). The inducing flavonoid binds probably directly to the NodD protein (Burn et al., 1987; Horvath et al., 1987; Spaink et al., 1987b) and activates this protein. However, the exact mechanism of transcription activation has not yet been elucidated. Inducing flavonoids accumulate in the cytoplasmic membrane (Recourt et al., 1989) and the NodD protein is located almost exclusively (Schlaman et al., 1989) or substantially (Kondorosi et al., 1989) in this membrane, strongly suggesting that NodD activation occurs in the cytoplasmic membrane. On the other hand, NodD binds to the *nod*-boxes and this binding is independent of induction (Hong et al., 1987; Fisher et al., 1988; Kondorosi et al., 1989). A model has been proposed (Schlaman et al., 1989; Schlaman et al., 1992) to reconciliate the different locations of NodD activation and NodD directed transcription activation.

Detailed studies of *nod* transcription activation show a complicated process, even more so because of differences between species: the biovars of *R. leguminosarum* contain one *nodD* gene, but *R. meliloti* contains 3 *nodD* genes and a *nodD* related gene *syrM*, each with different properties of activation and regulation (Göttfert et al., 1986; Honma and Ausubel, 1987; Györgypal et al., 1988; Mulligan and Long, 1987; Maillet et al., 1990; Honma et al. 1990). The relationships between these 4 genes are too complex to treat here (see Kondorosi et al., 1991a). The picture is also complicated by negative autoregulation of *nodD* transcription in *R. leguminosarum* biovars (Rossen et al., 1985) and the presence of a repressor of *nod* transcription, designated NolR, in some strains of *R. meliloti* (Kondorosi et al., 1991b). The different *Rhizobium* species evidently employ complicated systems to fine-tune the transcription of inducible *nod* genes. This subject is treated in more depth elsewhere (Schlaman et al., 1992).

3. Structure of Nod Metabolites

Most of the nod genes are involved in the synthesis and export of bacterial factors which induce symbiotic responses in the plant. The structures of a number of these Nod metabolites have been elucidated. They have a similar overall structure but show also significant differences (Lerouge et al., 1990; Truchet et al., 1991; Spaink et al., 1991b; Schultze et al., 1992) (Fig. 2). The Nod metabolites are designated according to the producing bacterial species (e.g. Rm and Rlv), the number of sugar moieties (e.g. IV), the length and the number of unsaturated bonds of the acyl chain (e.g. 16:2), and additional groups to the sugar backbone (e.g. S for sulphate and Ac for O-acetyl). For *R. meliloti* a family of Nod metabolites has been described (Schultze et al.,

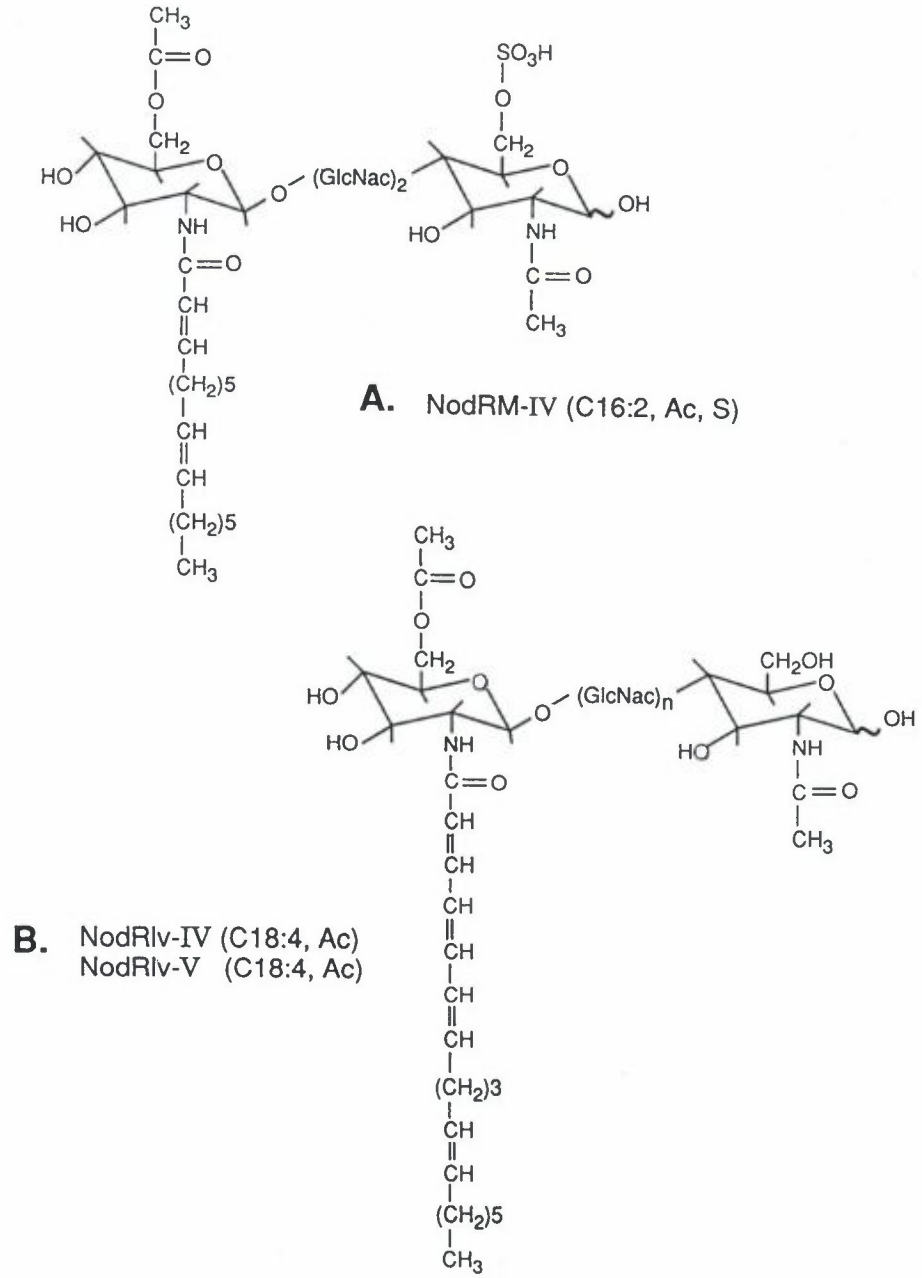

Figure 2. Chemical structure of bacterial factors with maximal biological activity. (A) Factors of *R. meliloti* (Lerouge et al., 1990; Truchet et al., 1991) and (B) Factors of *R. leguminosarum* bv. *viceae* (Spaink et al., 1991b. n is 2 or 3 in this factor. GlcNac, N-acetyl-glucosamine.

1992), containing containing three, four or five sugar moieties, C16:2 or C16:3 acyl chains, and a sulphate group at the reducing sugar moiety. The compound with the highest biological activity, Rm-IV(C16:2,S) is also found with O-acetylation at the non-reducing end of the N-acetyl glucosamine backbone. This acetylation improves the biological activity of the factor (Truchet et al., 1991). For *R. leguminosarum* bv. *viceae* two factors with complete biological activity have been described: NodRlv-IV(C18:4,Ac) and NodRlv-V(C18:4,Ac), differing in the number of N-acetyl-glucosamine (GlcNac) residues (Spaink et al., 1991b). The moieties involved in host-specificity are: (1) the sulphate group of the *R. meliloti* factors, (2) the highly unsaturated acyl chain of the *R. leguminosarum* bv. *viceae* factors, and (3) the O-acetyl group in the *R. leguminosarum* bv. *viceae* factors.

4. Biochemical Functions of Nod Genes

The *nodA,B,C* genes are essential for symbiosis, since mutants in *nodA nodB* or *nodC* are completely lacking in any plant response. They are presumably involved in the synthesis of the backbone structure of the Nod metabolite (Spaink et al., 1991a,b; Spaink et al., 1992), consisting of an oligosaccharide moiety and a C18:1 acyl chain, but their specific biochemical function is unknown at the moment. Gene *nodC* has homology with chitin synthase (Bulawa and Wasco, 1991) and cellulose synthase (M. Saxena, University of Texas, pers. comm.) and may therefore be responsible for the polymerization of the oligosaccharide backbone. The *nodA,B* genes have no known homologies. The genes *nodI,J* are less important in symbiosis. Mutants in these genes are only slightly impaired in some bacterium-plant combinations, but highly impaired in other combinations. Homology of *nodI* with ATP-dependent transport proteins (Higgins et al., 1986) and localization of NodI protein in the cytoplasmic membrane (Schlaman et al., 1990) suggest a role in export of Nod metabolites. This is supported by the observation that the INI response (see below) is delayed when using *nodI* and *nodJ* mutants (Van Brussel et al., 1990).

The host specific *nodF,E,L* genes of *R. leguminosarum* bv. *viceae* have an established role in the modification of the basic Nod metabolite into a biovar-specific compound. NodF and NodE have homology with acyl carrier protein (Shearman et al., 1986) and β-ketoacyl synthase (Bibb et al., 1989), respectively. NodE is the major determinant of host-specificity (Spaink et al., 1989) and both NodE and NodF proteins are involved in the synthesis of the specific (Spaink et al., 1991b) fatty acid moiety of the host specific *R. leguminosarum* bv. *viceae* Nod metabolite. NodL protein is responsible for the O-acetylation at the nonreducing end of the chitin backbone of the Nod metabolite (Spaink et

al., 1991a,b; Truchet et al., 1991). The *R. meliloti* NodP,Q proteins have ATP sulphurylase activity, which provides activated sulphate (Schwedock and Long, 1990; Roche et al., 1991a) and the NodH product is involved in the transfer of this activated sulphate group to the reducing end of the oligosaccharide (Roche et al., 1991a). It is remarkable that mutants in *nodE,F* in *R. leguminosarum* bv. *viceae* are not completely blocked in symbiosis with *Vicia sativa*, although they are less efficient in nodulation. This is explained by the observation that *nodO* can partially complement the loss of the *nodE,F* functions (Downie and Surin, 1990), although it codes for a completely unrelated protein which is exported from the cell, has homology to *E. coli* haemolysin and has Ca^{2+}-binding properties (Economou et al., 1990; de Maagd et al., 1989). The NodM protein has glucosamine synthetase activity (Baev et al., 1991; Downie et al., 1991) but is less important in symbiosis, since metants in *nodM* have a wild-type phenotype in *R. leguminosarum* bv. *viceae* (Canter Cremers et al., 1988) or show delayed nodulation in *R. meliloti* (Baev et al., 1991). The chromosome of *R. leguminosarum* bv. *viceae* has a functional *glmS* gene,providing the same function and *nodM, glmS* double mutants are understandably Nod^- (Marie et al., 1992). The other *nod* and *nol* genes are less well understood and contribute to a more efficient nodulation of a subgroup of host plants.

5. Biological Functions of Nod Metabolites

To test the biological activity of Nod metabolites several bioassays have been employed, based on plant responses in the early stages of symbiosis. The Nod metabolites NodRlv-IV(C18:4,Ac) and NodRlv-V(C18:4,Ac) elicit in the host plant *Vicia sativa* root hair deformation (Had), formation of short and thick roots, designated as Tsr (Van Brussel et al., 1986), *de novo* synthesis of flavonoïds, designated as INI (Van Brussel et al., 1990; Recourt et al., 1991) and induction of nodule meristems (Spaink et al., 1991b). The responses Had and Tsr are elicited at the surprisingly low concentration of 10^{-10}–10^{-11} M Nod metabolites, the other responses occur at $5 \cdot 10^{-8}$ M. INI and meristem induction are specific for these metabolites, since metabolites isolated from *R. leguminosarum* bv. *viceae* strains lacking the NodE or NodL proteins do not show these responses. In contrast, Had and Tsr are induced even by Nod metabolites produced by *Rhizobium* harbouring only *nodA,B,C,D*: NodRlv-IV(C18:1) and NodRlv-V(C18:1) (Spaink et al., 1991a,b). The sulphate group of the *R. meliloti* Nod metabolites NodRm-1 and Ac-NodRm-1 is essential for root hair deformation and meristem induction on the host *Medicago*, since stains mutated in *nodH*, which produce Nod metabolties without sulphate, are completely inactive on this host plant. Nod metabolites produced by a *nodH*

mutant elicit root hair deformation and Tsr on *Vicia sativa* instead (Roche et al., 1991a,b).

6. Prospects

Now that we are beginning to understand the structure of Nod metabolites and the biological processes they can bring about, it is clear that much of the future work will focus on the isolation of receptors for the Nod metabolites and on the elucidation of the pathways for the transduction of the signals leading to the biological responses. A promising approach to link Nod metabolites with changes in electric potentials of root hair cells has been reported recently (Ehrhardt et al., 1992).

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