Acta Biochimica Polonica

Vol. 44 No. 1/1997

1–12

QUARTERLY

Review

The paper was presented at the XXXII Meeting of the Polish Biochemical Society

Nodulation genes in the *Rhizobium* — plant signal exchange

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Received: 27 November, 1996

Key words: *Rhizobium* — legume symbiosis, plant flavonoids, nodulation genes, synthesis and modifications of Nod factors, biochemical functions of *nod* gene products, plant root cell depolarization

The process of the host-plant recognition by rhizobia is complex and multi-step. The interaction between legumes and microorganisms results in the induction of the root nodule. This symbiotic interaction is highly host-specific. Bacteria within nodules fix atmospheric nitrogen. This process is of immense ecological and economic significance. The subject of this presentation is the molecular mechanism by which the bacterium determines its host-specific characteristics. First flavonoids secreted by the plant roots induce the transcription of bacterial genes involved in nodulation, the so-called *nod* genes. This leads to the next step of the signalling system, i.e. the production and secretion of lipo-oligosaccharide molecules by rhizobia. These signal molecules have various discernible effects on the roots of the host leguminous plants. The bacterial nodulation factors were isolated and structurally identified as substituted and N-acylated chitin oligosaccharides. These prokaryotic signals play a key role in the symbiosis by controlling the host specificity of the bacteria. They constitute a new class of signalling molecules able to elicit nodule organogenesis in leguminous plants in the absence of bacteria. More recent studies implicate involvement of root cell membrane depolarization and ion selective channels in the communication processes that initiate nodule formation.

Bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, collectively called rhizobia, are able to invade the roots of their leguminous host plants where they trigger formation of a new organ called the root nodule. In these root nodules a differentiated form of rhizobia, the bacteroid, is able to fix atmospheric nitrogen and deliver it efficiently to legumes, among them to agriculturally significant crops. The amount of nitrogen biologically fixed surpasses the quantity of nitrogen applied in agriculture in mineral fertilizers. In nodules the bacteria are surrounded by a plant-de-

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Abbreviations: GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; UDP-GlcNAc, uridine diphosphate *N*-acetylglucosamine; LCO, lipochitooligosaccharide; Fuc, fucose; Me-Fuc, methylfucose; Ara, D-arabinosyl residue.

rived membrane. This membrane regulates the exchange of soluble molecules between the plant cytoplasm and the bacteria. Nitrogen-fixing symbioses between legumes and rhizobia may be highly specific. This specificity is determined by several steps of chemical signals between the symbiotic partners [1]. Recent studies implicate also electrical signalling and ion channels in the communication processes that initiate nodule formation [2]. The nodulation process commences when the symbiotic partners encounter one another in the rhizosphere. Communication is mediated by exchange of diffusible chemical signals [3], foremost among which are flavonoids from the plant and Nod factors from the bacteria [4, 5]. Flavonoids exuded from the plant roots serve as chemoattractants and growth enhancers of particular bacterial strains [6]. In many rhizobial species flavonoids also enable activation of *nod* genes by a transcription factor encoded by *nod D* [1, 7]. The flavonoid requirement is not universal, so, flavonoids are viewed as only broad range determinants of host specificity. The ultimate determination of host specificity resides in bacterial compounds synthesized by the *nod* gene encoded enzymes secreted into the rhizosphere. Once the recognition process between legume and bacteria has been completed, a series of developmental changes start with root hair curling and initiation of cell division in the inner cortical layer of the root where the nodule will form. Then an invagination called the infection thread is produced and through this passageway bacteria enter the plant [8].

Rhizobial genes controlling infection, nodulation and host-range

The *Rhizobium* genes essential for infection can be divided into two classes. One class includes several sets of genes involved in formation of the bacterial cell surface, such as the genes determining the synthesis of exopolysaccharides (*exo* genes) [9–17] lipopolysaccharides (*lps* genes) [18–20] and β-1,2 glucans (*ndv* genes) [21]. A possible role of *exo* and *lps* genes in the determination of host specificity has been suggested, but no clear genetic evidence has yet been given that *Rhizobium* surface components are determinants of the host-range specificity [22–24]. The second class consists of the nodulation (*nod, nol*) genes [25, 26]. Some of the *nod* genes appear to be interchangeable in their nodulation function between different species and are designated as common *nod* genes [27, 28]. On the other hand, some *nod* genes are involved in the nodulation of a particular host and are called host-specific *nod* genes (*hsn* genes) [29]. In most *Rhizobium* species the *nod* genes reside on large symbiotic plasmids (pSym) that also carry the *nif* and *fix* nitrogen-fixing genes [30–34]. In genera *Bradyrhizobium* and *Azorhizobium* as well as *Rhizobium loti*, the symbiosis related genes are localized on the chromosome [34, 35].

Nod factors

A major function of the *nod* genes is to ensure signal exchange between the two symbiotic partners. In the first step, flavonoids excreted by the plant induce, in conjunction with the NodD protein, the transcription of bacterial *nod* genes [36]. In a second step the bacterium, by means of structural *nod* genes produces lipooligosaccharide signals (LCOs) called Nod factors [37] that induce various root responses [38]. The mechanisms underlying host specificity depend on both the regulatory and the structural *nod* genes. Induction of nodulation genes requires flavonoids excreted by the host plant root, the transcriptional activators NodD protein and the NodD binding *cis* regulatory element, the nod box. The structure of a given NodD protein determines which flavonoids act as *nod* gene inducers. Therefore, the flavonoid NodD interactions represent the first major host-specific step in the establishment of symbiosis [39]. In several cases flavonoids inhibit *nod* gene activation by effective inducers [40, 41]. The antiinducers usually have similar structures to those of inducers, and inhibition can be overcome by increasing the concentration of the inducers [42]. The *nod* gene functions are required for nodule induction and probably for the maintenance of nodule development in the case of indeterminate nodules formed on temperate

legumes such as pea, alfalfa or vetch. The number of *nodD* gene copies varies from one to five in different rhizobia. *Rhizobium tropici* strains contain 4–5 *nodD* copies, but only one allele seems to activate *nod* gene expression [43]. However, elaboration of flavonoid induced proteins by *Rhizobium fredii* is regulated by both *nodD1* and *nodD2* genes [44]. The interaction of NodD proteins with flavonoids differs not only from species to species or from strain to strain but also within a single strain among individual NodD alleles. The fact that individual NodD proteins differ in their flavonoid specificity suggests a direct contact between NodD and the inducer [45]. NodD belongs to the LysR family of the bacterial regulatory proteins [46]. Regulation of the *nod* genes is under a dual control involving activators and a repressor which contribute to optimal nodulation [47–49]. In *Bradyrhizobium japonicum* there is a NodD-independent control of nodulation with involvement of the *nodVW* genes [50]. Most *Rhizobium nod* genes are not expressed in cultured cells but are induced in the presence of the host plant [51, 52]. The *nod* genes for which a role has been demonstrated can be divided into four classes on the basis of the following steps in Nod factor production: (1) synthesis of a precursor molecule (*nodM*); (2) synthesis of a common lipooligosaccharide structure (*nodABC*); (3) host specific modifications of the basic structure (*nod E, F, G, H, L, P, Q, S, X, Z,* and *nolK, nolO);* (4) accessory functions like Nod signal excretion (*nod I, J,* and *nol F, G, H,* and *I*) [1]. UDP-GlcNAc is one of the earliest precursors of Nod factors. This molecule is also a precursor for the biosynthesis of lipid A and peptidoglycan. There is now evidence that the products of the common *nod* genes, *nodABC,* are enzymes that assemble the GlcNAc core of the molecule [53]. The common *nodABC* genes have been identified in all rhizobial strains. They are structurally conserved and functionally interchangeable between *Rhizobium, Bradyrhizobium* and *Azorhizobium* species without the host range being altered [33]. In all species studied except *Rhizobium etli, R. loti* and *Rhizobium* sp. the *nodABC* genes are part of a single operon [53–55]. Inactivation of the *nodABC* genes abolishes the ability of bacteria to elicit any symbiotic reaction in the plant [56]. The product of the *nodM* gene has been shown to be a glucosamine synthase. *NodC* gene has a significant sequence similarity to chitin synthase, so it has been suggested that it plays a role in polymerization of GlcNAc residues [56, 57]. It has been shown that NodB is an N-deacetylase that removes the acetyl group from the terminal non-reducing sugar residue of the LCO. The deacetylated molecule then serves as an acceptor for a transacylation reaction catalyzed by NodA which might preferably incorporate a specific unsaturated fatty acid characteristic of some *Rhizobium* species [55]. It has been proposed that Nod factor synthesis may take place in a multienzyme compartment. It is also reasonable to expect that N-acetylation of GlcN and activation to UDP-GlcNAc is localized in this complex compartment [3, 58]. The induction of the root nodule is triggered by the presence of specific substituted LCOs produced by the bacteria. The application of such signal mole-

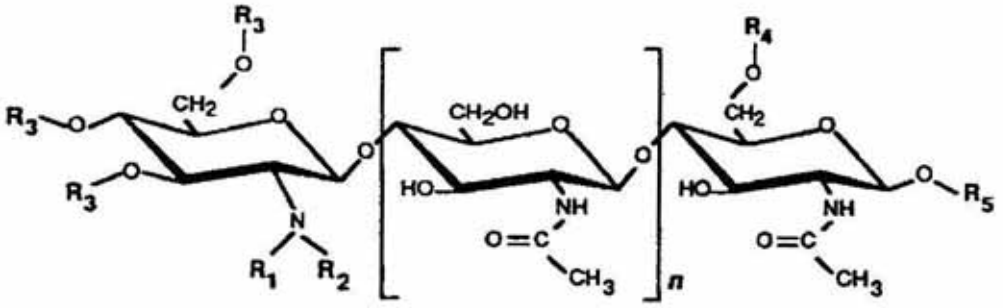

Figure 1. Structure of Nod factors from rhizobia.

R_1, H or methyl; R_2, long chain fatty acid; R_3, H or carbamoyl or acetyl; R_4, H or acetyl or sulfuryl or additional sugar (arabinosyl or fucosyl); R_5, H or glycerol group; n, 1, 2 or 3

cules to the roots of a compatible host induces root hair curling and cortical cell division. The structures of the compounds produced by *R. meliloti* were the first to be determined [59, 60]. A key function is fulfilled by the rhizobial LCOs, which in purified form, even at nanomolar concentration, are sufficient to induce the formation of root-nodule primordia and often complete nodule-like structures on host plants [1, 61, 62].

Modifications at the terminal reducing and non-reducing end of the Nod factor

Conversion of the basic structure into a host-specific Nod factor takes place by addition of various substituents [57, 63]. Little is known about the time when these modifications take place during Nod factor synthesis. Modifications at the non-reducing end of the Nod factor include acetylation, carbamoylation, N-methylation and N-acylation with a multiply unsaturated fatty acid. Carbamoyl groups have been reported on Nod factors from the broad host-range *Rhizobium* NGR234 [64], *Bradyrhizobium elkanii* [65], *Azorhizobium caulinodans*, *R. loti* and *R. etli* [66]. These species also produce LCOs which have no carbamoyl groups [65]. When they are present there can be one or two carbamoyl groups located on O-3 or O-4 of the N-acyl GlcN of Nod factors from *Rhizobium* NGR234 strain [64] or on O-6 in the case of *A. caulinodans* [66]. The genes responsible for carbamoylation have not yet been identified. The *nodL* gene is responsible for O-acetylated product. A 6-O-acetyl group on the non-reducing terminal N-acyl GlcN has been reported in LCOs from *Rhizobium meliloti*, *Rhizobium leguminosarum* bv. *viciae* [67, 68], *B. japonicum* and *B. elkanii* [65]. This modification appears to be crucial to host-range determination only in the case of *R. leguminosarum* bv. *viciae* [69]. NodE and NodF are unique to those *Rhizobium* species which contain multiply unsaturated fatty acids in their LCOs, i.e. *R. meliloti* and *R. leguminosarum*. In other rhizobia, in which *nodEF* have not been identified, the fatty acyl substituant is usually C18:1 with some C:16. The N-methyl group has been reported in LCOs from *Rhizobium* NGR234 [64], *B. elkanii* [65], *A. caulinodans* [66] and *R. tropici* [70]. NodS is responsible for the addition of this methyl group [66]. Since LCOs with and without modified groups can be synthesized by a single strain, it is difficult to speculate on the step and cellular location at which such groups are added during Nod factor synthesis. The modifications of the NodL, NodF and NodE products occur probably prior to their transport across the cell membrane [71, 72]. The localization of NodA (Nod factor acylation) in the cytosol supports the concept that fatty acids acylation occurs on the cytosolic side of the bacterial cell membrane [72]. The Nod factor reducing end can be modified by sulfation, O-acetylation, 2-*O*-methylfucosylation as well as by D-arabinosylation [73]. Interestingly, most *Rhizobium* species produce several different LCOs. The variation, which may allow a *Rhizobium* strain to nodulate different host plants, must depend on the specificity and efficiency of the respective biosynthetic steps [74, 75]. A sulfate at O-6 of the reducing end GlcNAc residue has been reported for *R. meliloti* and *R. tropici* [67, 70]. The products of *nodPQ* and *nodH* genes are responsible for sulfation of the *R. meliloti* Nod factor. An acetyl group at O-6 of the reducing GlcNAc residue is found in LCOs from *R. leguminosarum* bv. *viciae* strain TOM [49]. This strain has an additional *nod* gene, *nodX*, which permits nodulation of Afghanistan peas. Nod factors from *Rhizobium* NGR234, *B. japonicum*, *B. elkanii* and *R. fredii* contain MeFuc at O-6 of the reducing GlcNAc residue [64, 65, 76, 77]. Since both *R. fredii* and *B. elkanii* also have LCOs modified by Fuc it is possible that Fuc is transferred to the Nod factor prior to methylation at O-2. It is thought that NodZ may be a Fuc transferase. Perhaps these modifications at the reducing end take place in the periplasm at the time the molecule is exported out of the bacterium.

The relationship of host-range to Nod factor modifications

Some of the Nod factor modifications restrict while others extend the symbiotic host-range. Modifications which restrict the host-range include the sulfation at O-6 of the

reducing GlcNAc residue and the presence of a multiply unsaturated fatty N-acyl substituted C16:2, in the case of *R. meliloti* and a C18:4 N-acyl substituent in *R. leguminosarum* bv. *viciae* [69]. The D-Ara residue at O-6 of the reducing GlcNAc in the LCO from *A. caulinodans* is another example which suggests that such a substitution is required for specific interaction with *Sesbania*, a unique host to *A. caulinodans* [66]. *B. japonicum nodZ* mutants produce Nod factors that lack MeFuc and are defective in their ability to nodulate siratro, but still nodulate soybean. So, MeFuc appears to be necessary for *B. japonicum* to include siratro in its host-range. The *N*-methyl group on the terminal N-acyl GlcN of LCO from *Rhizobium* NGR234, *R. tropici, R. loti, R. etli, A. caulinodans* and *B. elkanii* may extend their host-range since transfer of *nodS*, the gene for the methyl transferase, to *R. fredii*, whose Nod factors do not normally have the N-methyl group, results in the extension of its host-range to include *Leucaena* [78]. Specific fatty acid structures determined by NodE have also been shown to be important for Nod factor activity towards vetch and clover. NodE dependent factors towards clover carry polyunsaturated acyl chains lacking the *cis* double bond that is present in the Nod factors of *R. leguminosarum* bv. *viciae*. However, recent results indicate [79] that the difference in the host-range between the *R. leguminosarum* bv. *viciae* and *trifolii* results from the overall hydrophobicity of the highly unsaturated fatty acyl moieties of LCOs rather than from a specific structural feature. Further evidence that the receptor-ligand interaction is not the only parameter that determines host-specific Nod factor activity is given by the increasing number of reports showing that non-legumes respond to LCOs [1]. The latest observation shows that Nod factors and cytokinins induce similar cortical cell division and amyloplast deposition in alfalfa roots [3]. Thus, Nod factor molecules might act as endogenous plant growth regulators. Similar compounds could be involved in yeast and even in vertebrate development [80]. The host-specific activity of Nod factors can be explained most easily by the presence of specific receptors that recognize molecules with different modifications. However, the remarkable variation of the terminal substituents on the Nod factors of the rhizobia that nodulate *Phaseolus* suggests that these modifications may also have another function than to contribute to receptor-ligand affinity [74]. The activity of LCOs can also be determined by the presence of plant enzymes involved in the metabolism of the Nod factor. It has been shown that the Nod factors of *R. meliloti* are rapidly inactivated in the rhizosphere of alfalfa by the action of chitinases and that the rate of degradation depends on a structural modification of the Nod factor [81]. Therefore, different structural moieties of *R. meliloti* LCOs has been investigated in a rapid assay that avoids degradation of the Nod factor. A few seconds after the addition of Nod factors the membrane was depolarized and ion channels have been formed. These changes were found to depend on the sulfate modification, whereas they were not influenced by *O*-acetyl group on the non-reducing residue. These data suggests that a sulfate group is involved in a receptor-ligand interaction, whereas O-acetylation may be involved either in recognition by a distinct class of receptors or in protecting the molecule against degradation [2, 74]. It has been speculated that the symbiotic relationship evolved from one that was initially pathogenic. In plants pathogenic bacteria may produce or elicit signals of an electrical nature [80]. Further studies provided evidence that electrical signalling is important in the *Rhizobium*-legume interaction [81]. Mechanisms of membrane depolarization include cation uptake, anion efflux and inhibition of the hyperpolarizing H^+-ATPase found on the plant plasma membrane. Previous assays of nodulation capacity by *Rhizobium* mutants set the stage for the next experiments [82]. A deletion mutant of *R. leguminosarum* bv. *viciae* lacking seven host-specifying *nod* genes (*nodEFLMNTO*) produces a Nod factor that has a C18:1 fatty acid side chain rather than the C18:4 fatty acid that is normally synthesized. This mutant is unable to nodulate [83]. Nodulation is partially restored by introduction of *nodEF* genes involved in biosynthesis of the C18:4 fatty acid [69]. Surprisingly, the nodulation

capacity is also partially restored to the deletion mutant by a plasmid carrying *nodO*. The *nodO* gene does not appear to be involved in the synthesis of the Nod factor. This gene encodes a secreted Ca^{2+}-binding protein with homology to the bacterial pore-forming hemolysins [84]. It has been found then that the purified product of *nodO* gene inserted into an artificial bilayer formed a cation-selective ion channel. It is suggested that the channel formed by NodO permits cation uptake *in vivo*, producing or enhancing the membrane depolarization. If NodO does trigger membrane depolarization, this effect may increase the efficacy of, or increase the plant's sensitivity to an abnormally weak signal caused by abberant Nod factors.

Strain variability and gene redundancy

Comparing *R. leguminosarum* bv. *trifolii* strains ANU843 and TA1 it was found that in the latter strain *nodT* is absent and a second copy of *nodD* is present. *NodT* is a positively acting cultivar specificity determinant controlling nodulation of *Trifolium subterraneum* by *R. leguminosarum* bv. *trifolii* [83]. Gene redundancy is another problem, particularly with *nodD* and other *nod* genes as well. *R. leguminosarum* has two glucosamine synthases, required for nodulation and development of nitrogen-fixing nodules [85].

Lectins and intracellular alkalinization

Nod factors appear not to be the only factors involved in determination of host–plant specificity during the nodulation process. In addition, the recognition mechanism of the symbionts has been reported to include legume lectins. Lectins are sugar-binding (glyco)proteins usually harbouring at least two sugar-binding sites per molecule. Upon the introduction of the pea lectin gene into clover, the transgenic plant became infected by *R. leguminosarum* bv. *viciae* [86]. It has been shown recently that alfalfa root hair cells respond to *R. meliloti* LCOs by rapid intracellular alkalinization. The response was most sensitive to the sulfated LCO with concomitant depolarization of the plasma membrane potential. Non-sulfated LCO elicited a pH change only at elevated concentrations without membrane depolarization. These results indicate that sulfated and non-sulfated LCOs act independently and suggest the existence of two Nod signal perception systems [87].

Nodulins

The nodule is a new organ of the plant with a defined structure [88–90]. During its differentiation a set of specific genes called nodulin genes are activated in the root or in the developing nodule [91]. In roots or root hairs, a set of plant genes is specifically activated by the presence of symbiotic bacteria shortly after infection [92]. Some of them can also be induced by a Nod factor such as Enod12, whereas others are activated at later stages. Many of the early nodulins characterized so far seem to be structural cell wall proteins. Putative nodulins participating in a signalling cascade are most likely of low abundance and therefore more difficult to identify [1]. It is worth to note that nodulin 26 is also an ion channel [6]. Purified nodulin 26 incorporated into a lipid bilayer formed a channel through which both cations and anions could permeate.

CONCLUSION

The current data suggest that the host specificity of Nod factor activity is determined at multiple levels. While the existence of specific plant receptors (yet to be identified) must be postulated, some of the structural features of Nod factors may be involved in increasing the stability of the signal molecules or in the transport processes. Very little is known about how the Nod signals are specifically perceived by host-plant cells. Nod factors act at extremely low concentrations which suggests the involvement of high-affinity receptors and signal amplification *via* a transduction cascade. Rapid, on a time scale of milliseconds, and transient depolarization of alfalfa root hair membranes in response to Nod factors has been reported [6]. This response shows a high degree of speci-

ficity towards cognate Nod signals indicating its possible involvement in Nod signal transduction [93, 94]. However, it remains unknown how plasma membrane depolarization would trigger the symbiotic programme. Therefore, should changes in plasma membrane potential play a role in Nod signal transduction? They are unlikely to be sufficient by themselves, but probably would require additional events.

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