Current Review

The Biosynthesis of Rhizobial Lipo-Oligosaccharide Nodulation Signal Molecules

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Nitrogen-fixing soil bacteria of the family Rhizobiaceae (hereafter collectively called rhizobia), *Bradyrhizobium*, *Rhizobium*, and *Azorhizobium* species, produce a bewildering array of cell-surface and secreted carbohydrate structures, many of which are involved in the symbiotic association with leguminous plants. Most of these, for example, exopolysaccharides, lipopolysaccharides, and Kdo-rich polysaccharides (Carlson 1982; Carlson *et al.* 1992; Noel 1992; Gray *et al.* 1990; Gray *et al.* 1992; Leigh *et al.* 1992; Leigh and Walker 1994), have analogous structures in other Gram-negative bacteria. However, a specific characteristic of rhizobia is the ability to cause root nodule formation on legumes. This ability is, at least in part, determined by a family of unique lipo-oligosaccharides called Nod factors. Nod factors are chitin-like oligosaccharides of three to five 1,4-β-linked *N*-acetylglucosaminosyl (GlcNAc) residues, which are *N*-acylated on the terminal non-reducing glucosaminosyl (GlcN) residue, and may be further modified by the addition of another glycosyl residue, or other modifying groups such as sulfate, carbamyl, acetyl, and/or glycerol substituents. The expression of the bacterial genes responsible for the synthesis of these Nod factors (*nod* genes) is induced by flavonoid molecules excreted from the legume root. The "backbone" structure, i.e., the *N*-acylated chitin oligomer, is apparently synthesized by the protein products of the *nodABC* genes which are common to all rhizobia. Thus, we will refer to this structure as the "common" Nod factor. Gene products involved in determining host specific interactions, encoded by numerous other *nod* genes, are responsible for the various modifications. The types of Nod factors with their differing modifying groups are shown in Figure 1.

The proposed functions of the *nod* genes required for the biosynthesis and transport Nod factors are summarized in Table 1. The majority of these functions have been hypothesized from protein sequence comparisons. Enzyme activity has been demonstrated for NodB (John *et al.* 1993), NodL (Bloemberg *et al.* 1994), and NodPQ (Schwedock *et al.* 1990). In addition, specific *nodA*- and *nodC*-dependent enzyme activities have been demonstrated (Geremia *et al.* 1994; Röhrig *et al.* 1994). In spite of all the data summarized in Table 1, there are still many unanswered questions concerning the biosynthetic pathway of Nod factors. It is the intention of this brief review to address these questions, and, therefore, the following discussion is, by design, somewhat speculative.

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Synthesis of the "Common" Nod Factor.

The "common" Nod factor refers to the *N*-acylated chitin oligosaccharide that is synthesized by all rhizobia. Its synthesis depends on the "common" *nod* genes, i.e., *nodABC*. Present data show that the production and excretion of Nod factors does not depend on structural modifications dictated by the protein products of the host-specific *nod* genes, since rhizobia with mutations in these genes still excrete Nod factors (Lerouge *et al.* 1990; Roche *et al.* 1991b; Schultze *et al.* 1992; Spaink *et al.* 1991; Spaink *et al.* 1993a; Luka *et al.* 1993; Stacey *et al.* 1994).

UDP-GlcNAc synthesis.

UDP-GlcNAc is one of the earliest precursors to Nod factors. This molecule is also a precursor for the biosynthesis of lipid-A and peptidoglycan. These latter molecules are essential for viability and mutations affecting their synthesis are lethal. One of the early enzymes in the synthesis of *E. coli* lipid-A, UDP-GlcNAc 3-*O*-acyl transferase, has a Km for UDP-GlcNAc of 99 μM, while the intracellular concentration of this substrate is 100 μM in *E. coli* (Anderson *et al.* 1993; Mengin-Lecreu *et al.* 1989) and 164 μM in *Salmonella typhmurium* (Bochner *et al.* 1982). Since peptidoglycan synthesis also draws on the UDP-GlcNAc pool, it seems reasonable that rhizobia must have some means of increasing the concentrations of this substrate for Nod factor biosynthesis. Such increases in UDP-GlcNAc concentrations are probably met by the *nodM* and *nodN* gene products. These genes are located on the same operon in *R. meliloti* (Baev *et al.* 1992b), and in *R. leguminosarum* bv. *viciae* (Marie *et al.* 1992). It has been reported that the *nodM* encodes a fructose-1,6-diphosphate glutamine amidotransferase (GlcN synthase) (Baev *et al.* 1991), and shows sequence similarity to *glmS* from *E. coli* (Marie *et al.* 1992; Baev *et al.* 1992b). Thus, the increased concentrations of UDP-GlcNAc that may be required for Nod factor biosynthesis could be met, in part, by

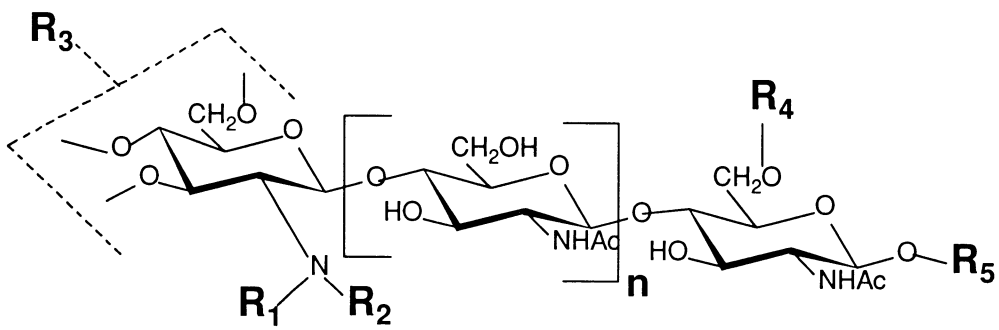
induction of *nodM*. The product of *nodN* has not been reported, however, one might propose that, since it is encoded on the same operon as *nodM*, it has the same function as the *glmU* gene in *E. coli* which is on the same operon as *glmS* (Walker *et al.* 1984). The sequence of *glmU* is similar to *gcaD* from *Bacillus subtilis* which encodes the enzyme, GlcNAc-1-phosphate uridyl transferase (Hove-Jensen 1992). However, the *nodN* sequence is not similar to *gcaD* (Price and Carlson, unpublished), and a role for *nodN* remains to be determined.

The biosynthesis of UDP-GlcNAc requires, in addition to GlcN synthase (the function of NodM), a mutase which converts GlcN-6-phosphate to GlcN-1-phosphate, a GlcN-1-phosphate *N*-acetyltransferase, and the GlcNAc-1-phosphate uridyl transferase. Thus, in addition to NodM, it seems likely that inducible genes which encode the mutase, *N*-acetyltransferase, and uridyl transferase may also be present if rhizobia require an increased pool of UDP-GlcNAc for Nod factor biosynthesis.

The Functions of NodA, NodB, and NodC.

Recent reports have indicated that the NodA, NodB, and NodC proteins are oligochitin GlcN *N*-acyltransferase, oligochitin GlcNAc de-*N*-acetylase and UDP-GlcNAc transferase enzymes, respectively (John *et al.* 1993; Geremia *et al.* 1994; Röhrig *et al.* 1994).

The NodB protein is able to de-acetylate the terminal GlcNAc residue of chitobiose, chitotriose, and chitotetrose, but has no activity on monomeric GlcNAc (John *et al.* 1993). The sequence of NodB is similar to chitin de-acetylase from the fungus *Mucor rouxii* which requires a substrate consisting of at least four GlcNAc residues (chitotetraose) for activity (Kafetzopoulos *et al.* 1993). NodB also has sequence similarity to an open reading frame (ORF) from *Bacillus stearothermophilus* (Kafetzopoulos *et al.* 1993). It was suggested that this ORF may encode an enzyme that de-*N*-acetylates peptidoglycan since such an enzyme has been isolated from a lysozyme resistant strain of *Bacillus cereus* (Kafetzopoulos *et al.* 1993). NodB does not show sequence similarity to at least two other bacterial de-*N*-acetylases (Price 1994); namely, LpxC, which converts UDP-3-*O*-acylGlcNAc to UDP-3-*O*-acylGlcN during the synthesis of lipid-A (Beall *et al.* 1987; Young *et al.* 1993), or NagA which converts GlcNAc-6-P to GlcN-6-P (Plumbridge *et al.* 1993). Thus, it is likely that Nod factor biosynthesis involves a novel pathway which does not rely on enzymes that are required for the synthesis of other essential molecules. The implication of these reports is that NodB de-*N*-acetylates a terminal GlcNAc residue on a chitinlike oligomer during the synthesis of the Nod factor. While the actual *in vivo* substrate for NodB remains to be determined, thin-layer chromatography (TLC) analysis of water soluble GlcN metabolites produced by a *R. leguminosarum* bv. *viciae*



Species	R1	R2	R3	R4	R5	n	Ref
R. meliloti	H	C16:2 C16:3	Ac(O-6) H	Sulfate	H	1,2,3	Lerouge *et al.* 1990 Roche *et al.* 1991 Schultze *et al.* 1992
R. leg. bv. viciae	H	C18:4 C18:1	Ac(O-6)	H Ac(O-6)	H	2,3	Spaink *et al.* 1991
B. japonicum	H	C18:1 C16:0 C16:1	Ac(O-6) H	MeFuc	H	3	Sanjuan *et al.* 1992 Carlson *et al.* 1993
B. elkanii	H Me	C18:1	Ac(O-6) H Cb	MeFuc Fuc	H Gro	2,3	Carlson *et al.* 1993
NGR 234	Me	C18:1 C16:0	Ac(O-6) H Cb (1,2)	MeFuc AcMeFuc MeFucS	H	3	Price *et al.* 1992
A. caulinodans	Me	C18:1 C18:0	Ac(O-6) Cb	D-Ara H	H	2,3	Mergaert *et al.* 1993
R. tropici	Me	C18:1	H	Sulfate	H	3	Poupot *et al.* 1993
R. fredii	H	C18:1	H	MeFuc Fuc	H	1,2,3	Bec-Ferte *et al.* 1993

Fig. 1. The types of substituent groups that have been reported for various rhizobial Nod factors. Me = methyl; C16:0 = palmitic acid, C18:1 = vaccenic acid, etc.; Ac = acetyl; Fuc = fucose; MeFuc = 2-*O*-methylfucose; Ara = arabinose, Gro = glycerol; Cb = carbamyl; S = sulfate.

nodB⁻ mutant indicates the presence of chitotriose to chitopentose oligomers (Spaink *et al.* 1993a).

It has been hypothesized that NodA catalyzes the transfer of the fatty acyl group from an acyl carrier protein (ACP) to the de-*N*-acetylated terminal GlcN residue on the chitin oligomer (John *et al.* 1993). As with NodB, NodA does not show sequence similarity to analogous enzymes involved in lipid-A synthesis (Röhrig *et al.* 1994; Price *et al.* 1994); namely, it does not show significant sequence similarity to LpxA or LpxD, the 3-*O*- and *N*-acyltransferases involved in the synthesis of UDP-2,3-diacyl-GlcN. Recently, it has been reported (Röhrig *et al.* 1994) that cell extracts of *R. meliloti* expressing *nodA* are able to acylate β-GlcN-(1→4)-[β-GlcNAc]$_2$-(1→4)–β-GlcNAc, while extracts of *nodA*⁻ mutants were inactive. Similarly, it has been shown that permeabilized cells of *E. coli* expressing *nodA* were able to acylate synthetic β-GlcN-(1→4)-[β-GlcNAc]$_2$-(1→4)–β-GlcNAc (S. Long, personal communication). These results show that *N*-acylation is dependent on *nodA* expression and support the hypothesis that NodA is an *N*-acyl transferase. However, it is important to purify the enzyme responsible for this activity to verify that it is NodA. That purified NodA has *N*-acylation activity has not been reported.

Several early reports suggested that NodC is involved in

Table 1. Summary of the genes required for Nod factor synthesis and transport

Component	Nod gene	Species	Gene product	Evidence	Function	Reference for gene product function
GlcNAc	*nodM*	Reported in *R. meliloti* and *R. leguminosarum*	GlcN synthase	Sequence similarity	GlcN synthesis	Baev *et al.* 1991 Marie *et al.* 1992
?	*nodN*	Reported in *R. meliloti* and *R. leguminosarum*	?	?	?	
GlcNAc	*nodC*	All	UDP-GlcNAc transferase	Sequence similarity to chitin synthase and enzyme activity	Required for nodulation	Atkinson *et al.* 1992 Debelle *et al.* 1992 Bulawa *et al.* 1992 Geremia *et al.* 1994
Fatty acids (R1)	*nodA*	All	*N*-Acyltransferase	Enzyme activity	Required for nodulation	Röhrig *et al.* 1994
Terminal GlcN	*nodB*	All	De-*N*-acetylase	Enzyme activity	Required for nodulation	John *et al.* 1993
Multiply unsaturated fatty acids (R1)	*nodE*	*R. meliloti*, *R. leguminosarum*	Condensing enzyme	Sequence similarity	Host-specificity determinant	Spaink *et al.* 1989
Multiply unsaturated fatty acids (R1)	*nodF*	*R. meliloti*, *R. leguminosarum*	Acyl carrier protein	Sequence similarity and has pantetheinic acid	Host-specificity determinant	Geiger *et al.* 1991
GlcN-methyl (R2)	*nodS*	NGR234, *R. tropici*, *A. caulinodans*, *R. fredii*, *B. japonicum*	*S*-Adenosyl methionine methyl transferase	Sequence similarity	Possible host-range determinant	Mergaert *et al.* 1993
GlcN *O*-acetyl (R3)	*nodL*	*R. melioti*, *R. leguminosarum*, *B. japonicum*, *B. elkanii*	*O*-acetyl transferase	Enzyme activity	Host-specificity determinant for *R. leguminosarum* only	Baev *et al.* 1992a Bloemberg *et al.* 1994
GlcN *O*-carbamyl (R3)	?	NGR234, *B. elkanii*, *R. fredii*, *A. caulinodans*	?	?	Possible host-range determinant	
Sulfate (R4)	*nodP*,*nodQ*	*R. meliloti*	ATP sulfurylase, APS kinase	Sequence similarity and enzyme activity	Host-specific determinant	Schwedock *et al.* 1990
Sulfate (R4)	*nodH*	*R. meliloti*	Sulfotransferase	Sequence similarity	Host-specific determinant	Roche *et al.* 1991a.
GlcNAc *O*-6 acetyl (R4)	*nodX*	*R. leguminosarum* bv. *viciae* TOM	Acetyl transferease	Sequence similarity, transfer of *nodX* results in factor with acetyl group	Host-range determinant	Firmin *et al.* 1993
Methylfucose, Fucose (R4)	*nodZ*	NGR234, *R. fredii*, *R. loti*, *B. elkanii*, *B. japonicum*	Fucosyl transferase (?)	Mutant factor lacks fucosyl residue	Host-range determinant	Stacey *et al.* 1994
Methyl group on fucosyl residue	?	NGR234, *R. fredii*, *R. loti*, *B. elkanii*, *B. japonicum*	?	?	?	
O-Acetyl on methylfucosyl residue	?	NGR234	?	?	?	
Sulfate on methylfucosyl residue	?	NGR234	?	?	Possible host-range determinant	
Glycerol (R5)	?	*B. japonicum*, *B. elkanii*	?	?	?	
None	*nodI*, *nodJ*	Reported in many species of rhizobia	ABC cassette transport proteins	Sequence similarity	Transport of Nod factor across the CM	Vazquez *et al.* 1993 McKay *et al.* 1993

the export of Nod factors that are synthesized by the action of NodA and NodB (Schmidt *et al.* 1988; John *et al.* 1988; Johnson *et al.* 1989). The data in those reports indicate that 1) *R. meliloti* cells expressing only *nodA* and *nodB* (i.e., *nodC* expression was not required) produce a factor that stimulates mitotic activity in tobacco tissue culture cells (Schmidt *et al.* 1988), and 2) that the NodC protein is located in the outer membrane (John *et al.* 1988; Johnson *et al.* 1989). However, the enzymatic activities of NodA and NodB, and their cytosolic location are more consistent with the recent reports suggesting that NodC is a UDP-GlcNAc transferase responsible for the synthesis of the chitin oligomer intermediate. Although, NodC has sequence similarity to chitin synthases (Bulawa 1992; Atkinson *et al.* 1992; Debellé *et al.* 1992), the order of similarity of NodC to other proteins is: DG42 > cellulose synthase > chitin synthase III > chitin synthase I and II (Bulawa 1992; Atkinson *et al.* 1992; Debellé *et al.* 1992; Spaink *et al.* 1993b). Recently, the sequence of NodC has been reported to be similar to ExoO, a β-1,4-glucosyl transferase required for the biosynthesis of the succinoglycan repeating unit from *R. meliloti* (Glucksmann *et al.* 1993b). DG42 is a protein found in *Xenopus laevis* whose function is not known. The similarity of NodC to the chitin synthases and DG42 resides in two regions near the carboxyl terminus. These two regions are separated by a short peptide, 20–30 amino acids. The function of the first region is unknown. However the second appears to encode a membrane spanning region (Bulawa 1992). This, and hydropathy plots, suggest that NodC is located in the membrane. A fitting location for NodC, if it is a UDP-GlcNAc transferase, would be the cytoplasmic membrane (CM). While earlier reports suggest that NodC may be located in the OM (John *et al.* 1988; Johnson *et al.* 1989) and, indeed, exposed on the surface of the cell (Johnson *et al.* 1989), a more recent report suggests that it is a CM protein (Barny *et al.* 1993). A CM location for NodC would allow the synthesis of the chitin oligomer on the inner face of the CM, presumably anchored to the CM via an aglycone moiety (e.g., undecaprenol pyrophosphate, see the discussion below). This would allow both NodA and NodB, located in the cytosol, access to the terminal end of the chitin oligomer. A diagram of the possible biosynthetic pathway of the "common" Nod factor is depicted in Figure 2. As mentioned above, it has recently been reported (Geremia *et al.* 1994) that cell extracts from *R. meliloti*, or *E. coli*, expressing *nodC* are able to incorporate GlcNAc from UDP-GlcNAc into chitin oligomers. These data demonstrate a *nodC*-dependent UDP-GlcNAc transferase activity and support the hypothesis that NodC is such a transferase. However, it has not been shown that purified NodC has such a transferase activity, and, therefore, it will be necessary to isolate this enzyme and show that it is, in fact, NodC.

Does Nod Factor Synthesis Involve a Lipid-Anchor?

As shown in Figure 2, *de novo* synthesis of the chitin oligomer via the UDP-GlcNAc transferase activity of NodC presumes the presence of an aglycone carrier/primer. However, the majority of Nod factors reported thus far have a free reducing GlcNAc residue. Although chitin oligomers can be obtained *in vitro* by incubating UDP-GlcNAc with *nodC*-expressing cell extracts, it is unlikely that the *in vivo* synthesis of Nod factors takes place with successive transfers of GlcNAc residues from UDP-GlcNAc to a free reducing GlcNAc residue. The synthesis of oligosaccharides and polysaccharides normally occurs by transfer to a sugar residue that is glycosidically linked to a lipid carrier, e.g., pyrophosphoryl undecaprenol (Whitfield *et al.* 1993). In certain instances the aglycone anchor can be a protein (Whitfield *et al.* 1993) (e.g., the NdvB protein in the synthesis of β-2-linked

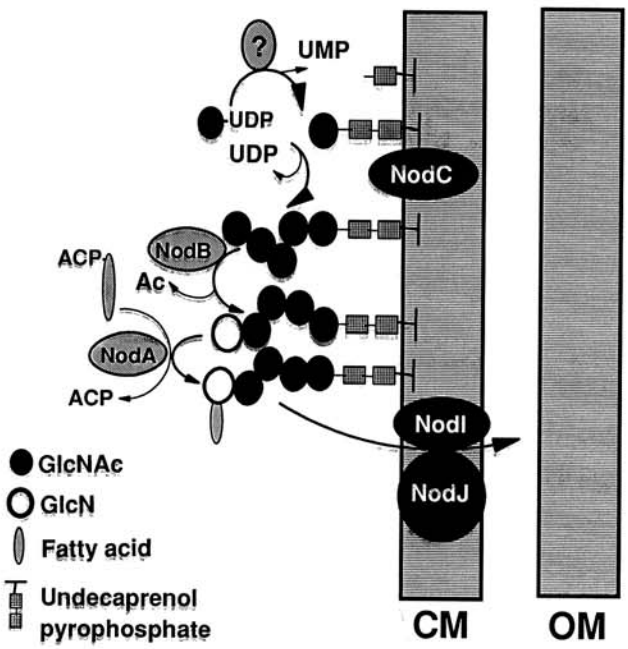


Fig. 2. A schematic diagram showing a possible route for the biosynthesis of the acylated chitin oligomer, i.e., the "common" Nod factor. In this schematic the initial reaction consists of the transfer of GlcNAc from UDP-GlcNAc to undecaprenol phosphate. This reaction could be catalyzed by NodC or by an, as yet, unidentified protein.

cyclic glucans [Ielpi *et al.* 1990]). The synthesis and polymerization of oligosaccharide repeating units of bacterial *O*-antigen polysaccharides, extracellular, and capsular polysaccharides, for example, occurs on an undecaprenol phosphate lipid carrier (Whitfield *et al.* 1993). Interestingly, for certain bacterial capsular antigens, the lipid anchor of the mature polysaccharide can consist either of lipid-A (normally the anchor for lipopolysaccharides) or phosphatidic acid (Whitfield *et al.* 1993; Jann *et al.* 1990, 1991), even though the repeating oligosaccharide unit of the antigen may be synthesized on an undecaprenol lipid carrier (Whitfield *et al.* 1993; Jann *et al.* 1990, 1991). Thus, for these polysaccharides, it is presumed (Whitfield *et al.* 1993) that a "trans-lipidation" occurs in which the poly- or oligosaccharide is transferred from the aglycone carrier to the lipid anchor found in the mature molecule, i.e., lipid-A or phosphatidic acid for LPSs or group II capsular polysaccharides, respectively. The biosynthesis of peptidoglycan, which is the bacterial polysaccharide most analogous to chitin, is also initiated on an undecaprenol phosphate carrier lipid.

In other organisms, such as crustacea, the synthesis of lipid-linked chitin oligosaccharide occurs on a polyprenol intermediate. This oligosaccharide is then transferred to a protein acceptor before extension by the sequential addition of more GlcNAc residues (Horst 1985). In the case of zoospores of *Blastocladiella emersonii*, a monoglycosylated diacylglycerolipid is thought to function as the lipid primer for chitin synthesis (Mills *et al.* 1980), with GlcNAc-Glc-diacylglycerol as an intermediate. Although glycosyldiacylglycerol glycolipids have been reported in certain photosynthetic bacteria, and in species of *Spirochaetes*, *Pseudomonads*, *Bacteroides,* and *Mycoplasma* (Kates. 1990), they are relatively uncommon in Gram-negative bacteria. However, a glycosyl diacylglycerol molecule has been found in *R. leguminosarum* bv. *trifolii* (Orgambide *et al.* 1992), and is reported to have biological activity similar to that of Nod factors (Orgambide *et al.* 1992). At the present time it is not understood why such structurally diverse molecules as glycosyl diacylglycerol and Nod factor would have similar biological activities.

Certain Nod factors from *B. elkanii* have been reported in which the "reducing-end" GlcNAc residue is glycosidically linked to glycerol (Carlson *et al.* 1993). As with *B. japonicum*, *B. elkanii* produces NodBj-V(C18:1,MeFuc) and NodBj-V(C16:0,MeFuc) Nod factors (Fig. 1) but, unlike *B. japonicum*, *B. elkanii* produces additional glycerol-containing Nod factors. In all of the glycerol-containing molecules from *B. elkanii*, the "reducing-end" GlcNAc residue contains fucose (Fuc), not 2-*O*-methylfucose (MeFuc), at *O*-6, e.g., NodBj-IV(C18:1,Fuc,Gro). Additionally, the majority of the glycerol-containing molecules have a chitin tetrasaccharide rather than a pentasaccharide as the backbone structure. Interestingly, a *nolO*$^-$ mutant *B. japonicum* strain USDA110 produces a C18:1 acylated chitin tetrasaccharide in which the "reducing"-end GlcNAc is glycosidically linked to glycerol, but does not contain either Fuc or 2-*O*-MeFuc, i.e., NodBj-IV(C18:1,Gro) (Luka *et al.* 1993). This *nolO*$^-$ mutant also produces the parental Nod factor, NodBj-V(C18:1,MeFuc) as well as its *O*-acetylated version (not normally produced by USDA110). The function of the *nolO* gene of *B. japonicum* is not known; however, these results suggest that its function(s)

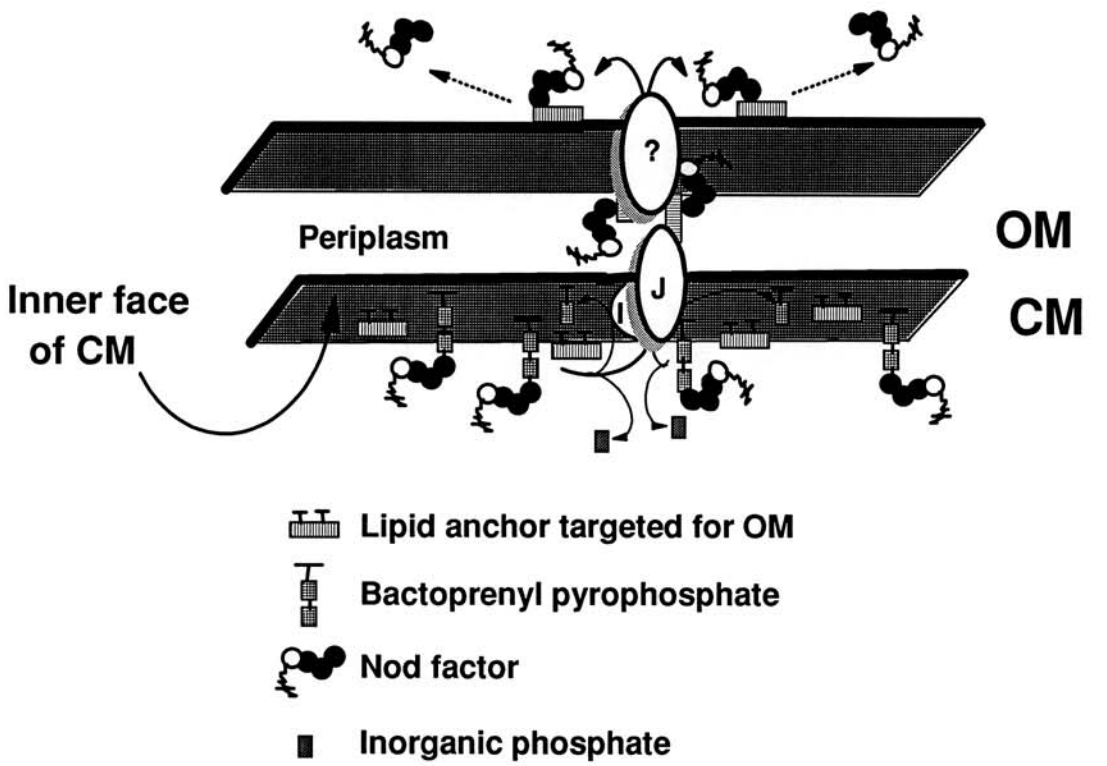


Fig. 3. A schematic diagram showing a possible mechanism by which Nod factors are exported out of the bacterium. During export the Nod factor is transferred from the lipid carrier (undecaprenol pyrophosphate) to a lipid anchor (e.g., diacylglycerol) which is ultimately removed by, as yet unidentified, hydrolases. The protein(s) involved in the exporting the Nod factor out of the cell has not been identfified.

may be important in regulating the size of the chitin oligosaccharide backbone, fucosylation of the Nod factor, and its *O*-acetylation. The fact that this glycerol-containing Nod factor is produced by the *nolO*⁻ mutant (Luka *et al.* 1993) also suggests that it may be a biosynthetic intermediate.

The presence of glycosyldiacylglycerol molecules in *Rhizobium*, together with the fact that glycerol has been found as the aglycone moiety of certain Nod factors, supports the possibility that (glycosyl)diacylglycerol molecules may serve as the lipid anchor in the synthesis of Nod factors. If true, then glycerol, as an aglycone substituent in certain Nod factors, implies that there is an acyl hydrolase(s) which removes the fatty acyl residues from the diacylglycerol lipid anchor. Such an enzyme has not been described in bacteria; however, there are such enzymes in plants. For example, all species of *Phaseolus* examined have acyl hydrolyases which remove the fatty acyl groups from glycosyldiacylglycerol glycolipids (Kates 1990). The lack of the glycerol moiety in the majority of Nod factors would suggest the presence of an additional hydrolase that removes the diacylglycerol anchor, or glycerol from the Nod factor during the production of the mature molecule. Both acyl and glycerol hydrolase activities could be involved in releasing the Nod factor from the OM. Mutants defective in these hypothetical activities may still be effective nodulators since Nod factors may be present on the outer surface of the bacteria and could be active, or released by host plant hydrolases. This may explain why such mutants have not been isolated. Very little, if any, extracellular Nod factor would be expected in such mutants, thus suggesting a screening method to isolate the mutants necessary to test this hypothesis.

As described above, it is also possible that the aglycone lipid anchor is pyrophosphoryl undecaprenol. In that case, as for the above glycolipid anchor, there would have to be an enzyme(s) that cleaves the lipid anchor, thus, producing the extracellular Nod factor. Thus far the glycerol aglycone substituent has only been found in *B. japonicum* and *B. elkanii* Nod factor preparations (Carlson *et al.* 1993; Luka *et al.* 1993) and, in the case of *B. japonicum,* only in the *nolO*⁻ mutant (Luka *et al.* 1993). It is conceivable that different species of the Rhizobiaceae have different lipid anchors; e.g., pyrophosphoryl undecaprenol in *Rhizobium*, and diacylglycerol in *Bradyrhizobium.*

Export of the Nod factor.

The export of the Nod factor out of the bacterium is thought to be mediated by NodI and NodJ. This role is supported by data that show that either *nodI*⁻ or *nodJ*⁻ mutants of *R. leguminosarum* bv. *trifolii* still synthesize, but fail to export, Nod factor (McKay and Djordjevic 1993). That report also shows that Nod factor export is, in part, mediated by environmental conditions such as pH and temperature (McKay and Djordjevic 1993). Additionally, the NodIJ proteins have been localized to the CM (Schlaman *et al.* 1990) which would be consistent with their function in transporting the Nod factor across the CM. However, another paper reports that nodI⁻ and nodJ⁻ mutants of *R. leguminosarum* continue to export Nod factor (Spaink *et al.* 1992). The latter evidence suggests that there may be secondary proteins which export Nod factor. Both NodI and NodJ show sequence similarity to KpsT and KpsM from *E. coli* (Vazquez *et al.* 1993), which, data indicate, comprise a transport system for the export of Kdo-

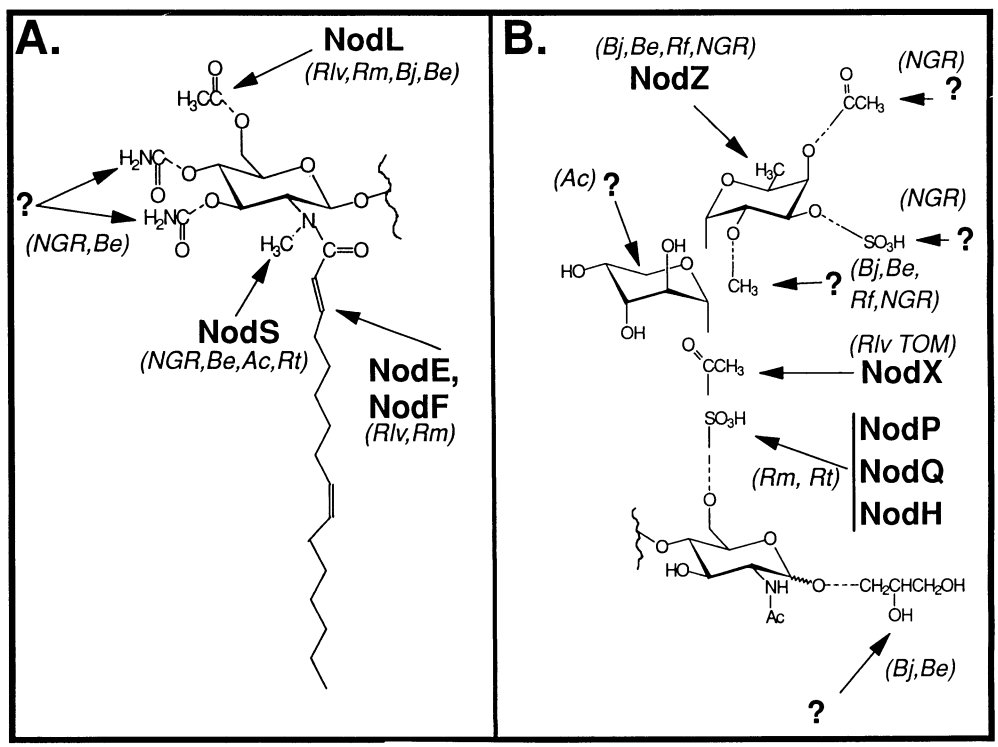


Fig. 4. Various modifications that occur on the non-reducing terminal GlcN residue **(A),** and on the "reducing" end **(B)** of the Nod factor. The various gene products involved and the rhizobial species are as indicated. NGR = *Rhizobium* sp. NGR234; Be = *B. elkanii*; Bj = *B. japonicum*; Ac = *Azorhizobium caulinodans*; Rf = *R. fredii*; Rm = *R. meliloti*; Rlv = *R. leguminosarum* bv. *viciae*; Rt = *R. tropici.*

containing capsular antigen across the CM (Smith *et al.* 1990; Pavelka, Jr. *et al.* 1991; Whitfield *et al.* 1993; Higgins *et al.* 1990). In fact, KpsT and KpsM comprise an ATP bindng cassette (ABC) export system in which KpsT contains the ATP-binding site and KpsM the membrane spanning domains (Fath *et al.* 1993). The KpsM protein is thought to extend into the periplasm (Fath *et al.* 1993; Pavelka Jr *et al.* 1991). Once the polysaccharide is transported into the periplasm, it has been suggested that periplasmic proteins, such as KpsD, act to secrete it out of the bacterium (Smith *et al.* 1990; Silver *et al.* 1993; Fath *et al.* 1993). This capsular polysaccharide export system apparently can transport a variety of capsular polyaccharides since it has been found in a various organisms which have different capsular antigens (Pavelka Jr *et al.* 1991; Smith *et al.* 1990). Interestingly, analogous Kdo-rich capsular antigens have been isolated from *R. fredii* and *R. meliloti*, and can functionally substitute for *R. meliloti* extracellular polysaccharides in the symbiotic infection of alfalfa (Reuhs *et al.* 1993; Petrovics *et al.* 1993). Preliminary data indicate that analogous capsular antigens are present in many species of the Rhizobiaceae (Reuhs and Carlson, unpublished data). The *nodI*$^-$ and/or *nodJ*$^-$ mutants have not been examined for defects in the export of capsular antigen. There are reports of secondary polysaccharide export genes similar to *kpsT* and *kpsM* in rhizobia; *ndvA* which may be involved in export of the β-1,2-glucan (Stanfield *et al.* 1988), and recently, the *nolFGHI* genes in *R. meliloti* (Saier *et al.* 1994). Perhaps these secondary transport genes account for the observation that *nodI*$^-$ and *nodJ*$^-$ mutants are not completely defective in nodulation but show delay in nodule formation; e.g., the capsular polysaccharide transport system may export sufficient Nod factor to enable delayed nodulation of the host.

The mechanism by which NodI and NodJ export Nod factors out of the bacteria is largely unknown. However, since NodI and NodJ are similar to KpsM and KpsT, it is possible to make some speculations by analogy to the hypothesized transport process for capsular antigens. As mentioned above, many capsular antigens are prepared on the inner face of the CM using undecaprenol phosphate as the lipid carrier (Whitfield *et al.* 1993; Jann *et al.* 1990, 1991). The lipid carrier for the synthesis of the group II capsular antigens which are transported by KpsT and KpsM has not yet been identified, however, it is hypothesized to be undecaprenol phosphate (Whitfield *et al.* 1993). In the case of capsular polysaccharides, after transport the lipid anchor is either lipid-A or phosphatidic acid (Jann *et al.* 1990; Jann *et al.* 1991; Whitfield *et al.* 1993). Therefore, it was suggested (Whitfield *et al.* 1993; Jann *et al.* 1990) that transport may involve a "trans-lipidation" from the lipid carrier on the CM (i.e., undecaprenol phosphate) to the lipid anchor (e.g., diacylglycerol) found in the mature capsular antigen. Mutants in a third gene, *kpsD*, fail to export CPS which then accumulates in the periplasm (Silver *et al.* 1987). This periplasmic form of the CPS was found to be substituted with phosphatidic acid (Kroncke, -D. *et al.* 1990), supporting the suggestion (Whitfield *et al.* 1993; Jann *et al.* 1990; Boulnois *et al.* 1987) that transfer across the CM via KpsT and KpsM is accompanied by "trans-lipidation" from undecaprenol phosphate to diacylglycerol. It has also been suggested (Whitfield *et al.* 1993) that, since *kpsM* has a dolichol-binding consensus sequence (Troy 1992), KpsM may comprise part of a transferase complex (i.e., catalyzing "trans-lipidation") in addition to being involved in the transport of the CPS across the CM. Furthermore, by using temperature-sensitive mutants for the expression of capsular antigens and by using capsule-specific antibodies, it was reported (Jann *et al.* 1990; Bronner *et al.* 1993; Boulnois *et al.* 1987) that capsule export occurs at discrete points in the OM since patches of capsule antigen were observed at early time points after shifting to permissive temperatures. At later time points, these capsular patches spread out over the entire surface of the bacterium. Additionally, there was an indication that these patches of capsular antigen appeared at sites where the CM and OM were in close proximity to one another (Jann *et al.* 1990; Bronner *et al.* 1993; Boulnois *et al.* 1987).

Based on these observations and hypotheses for capsular antigen, one could also speculate a similar mechanism for transport of the Nod factor as shown in Figure 3. In this mechanism, the Nod factor is synthesized by the action of NodABC on the inner face of the CM using an aglycone carrier, e.g., undecaprenol phosphate, see Figure 2. It is then transported through the CM and into the periplasm via the action of NodIJ, and secreted to the surface via an unidentified protein, perhaps a protein analogous to KpsD. As described above KpsD is a periplasmic protein and *kpsD*$^-$ mutants accumulate CPS in the periplasm. Additionally, KpsD has sequence similarity to ExoF, a protein from *R. meliloti* involved in EPS export (Muller *et al.* 1993; Reuber *et al.* 1993; Glucksmann *et al.* 1993a). During transport across the CM there may also be a transfer of the Nod factor from the undecaprenol phosphate carrier lipid to a lipid anchor which is targeted for the OM. This latter lipid anchor could be diacylglycerol. In the case of *Bradyrhizobium*, transfer would result in the release of undecaprenol pyrophosphate which is hydrolyzed to inorganic phosphate and undecaprenol phosphate. The Nod factors on the OM would then be released from the lipid anchor via putative hydrolases. An alternative possibility would be that the Nod factors are simply hydrolyzed from the undecaprenol pyrophosphate carrier (or other carrier) during transport and are released extracellularly. One possibility is that the putative hydrolase activity which removes the lipid anchor could reside in the KpsD-analogous protein. It has been reported that KpsD has sequence homology to PgpB, a phosphatidylglycerophosphate B phosphatase (Icho 1988a; Icho 1988b).

Present evidence indicates that, at least in some instances, the *N*-acyl substituent plays a role in determining host-specificity (discussed further below); however, in addition it may also be possible that this fatty acyl moiety causes the Nod factor to be associated with the membrane and is important for the Nod factor to be transported through the CM and/or OM. Such a function should be testable since it would imply that a mutation in *nodA* would result in the accumulation of de-*N*-acetylated chitin oligomers inside the bacterial cell.

Modification of the "common" Nod factor into a "host-specific" Nod factor.

Conversion of the "common" into a "host-specific" Nod factor takes place by the addition of various substituents. Such host-specific modifications occur on either the terminal *N*-acyl GlcN residue, or on the reducing-end GlcNAc. Little is known about when these modifications take place during Nod factor synthesis.

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

Host-specific alterations on the terminal *N*-acyl GlcN include acetylation, carbamylation, *N*-methylation, and *N*-acylation with a multiply unsaturated fatty acid, see Figure 4A.

Carbamyl groups have been reported on Nod factors from the broad host-range *Rhizobium* NGR234 (Price *et al.* 1992), *Azorhizobium caulinodans* (Mergaert *et al.* 1993) and *B. elkanii* (Carlson *et al.* 1993). These species also produce Nod factors without carbamyl groups (Price *et al.* 1992; Carlson *et al.* 1993). When present, there can be one to two carbamyl groups, and they are located on *O*-3 or *O*-4 of the *N*-acyl GlcN of Nod factors from NGR234 (Price *et al.* 1992), or on *O*-6 in the case of *A. caulinodans* (Mergaert *et al.* 1993). The genes responsible for carbamylation have not been identified.

The addition of the *O*-acetyl group appears to be a function of *nodL* since the NodL protein has sequence similarity to LacA, a transacetylase from *E. coli* (Baev *et al.* 1992a); and, more recently, since NodL has been shown to catalyze the transfer of *O*-acetyl groups from acetyl-CoA to chitin fragments (Bloemberg *et al.* 1994). A 6-*O*-acetyl group on the non-reducing terminal *N*-acyl GlcN has been reported in Nod factors from *R. meliloti*, *R. leguminosarum* bv. *viciae* (Spaink *et al.* 1991; Lerouge *et al.* 1990; Roche *et al.* 1991b), *B. japonicum* (Carlson *et al.* 1993), and *B. elkanii* (Carlson *et al.* 1993). This modification appears to be crucial to host-range determination only in the case of *R. leguminosarum* bv. *viciae* (Spaink *et al.* 1991).

NodE and NodF are required for the presence of multiply unsaturated fatty *N*-acyl substituents (Spaink *et al.* 1989; Demont *et al.* 1993; Geiger *et al.* 1991; Spaink *et al.* 1993a). The sequence of NodE is similar to β-ketoacyl synthase and NodF contains pantetheinic acid indicating that it is an acyl carrier protein (Spaink *et al.* 1989; Geiger *et al.* 1991; Spaink *et al.* 1993a); therefore, these proteins are proposed to be involved in the synthesis and transfer, via NodA, of the multiply unsaturated fatty acid to the terminal GlcN residue (Spaink *et al.* 1989; Geiger *et al.* 1991; Spaink *et al.* 1993a; Demont *et al.* 1993). NodE and NodF are unique to those *Rhizobium* species which contain multiply unsaturated fatty acids in their Nod factors, i.e., *R. leguminosarum* and *R. meliloti*. In other rhizobia, in which *nodEF* have not been identified, the fatty acyl substituent is usually C18:1, with some C16:0. This is also the case for *R. meliloti nodEF*$^-$ mutants (Demont *et al.* 1993). In addition, Nod factor preparations from these mutants are reported to contain long-chain ω-1 hydroxy fatty acids (Demont *et al.* 1993). Subsequently, it has been reported (Demont *et al.* 1994) that the presence of the ω-1 hydroxy fatty acids is regulated by the *nodD3* or *syrM* genes of *R. meliloti*. The variety of fatty acyl substituents shows that there is a lack of specificity of the *N*-acyl transferase. However, in the presence of NodEF, there apparently is a preference for the transfer of the multiply unsaturated fatty acids, presumably from NodF which acts as a specific acyl carrier protein (Geiger *et al.* 1991).

The *N*-methyl group is reported in Nod factors from *Rhizobium* NGR234 (Price *et al.* 1992), *B. elkanii* (Carlson *et al.* 1993), *A. caulinodans* (Mergaert *et al.* 1993), and *R. tropici* (Poupot *et al.* 1993). NodS is reported to be similar in sequence to *S*-adenosylmethionine-dependent methyl transferases and, therefore, is thought to be the enzyme responsible for the addition of this methyl group (Mergaert *et al.* 1993).

Since Nod factors with and without various modifying groups can be produced by a single strain, it is difficult to speculate on the time and cellular location at which such groups are added during Nod factor synthesis. The NodL, NodE, and NodF proteins have been localized to the CM (Spaink *et al.* 1989; Geiger *et al.* 1991; Spaink *et al.* 1993a; Bloemberg *et al.* 1994), and, thus, the modifications dictated by these proteins probably occur prior to transport across the CM. The localization of NodA, hypothesized to be the *N*-acyltransferase (see discussion above), in the cytosol also supports the concept that fatty acylation occurs on the cytosolic side of the CM (Johnson *et al.* 1989; Schmidt *et al.* 1986; Spaink *et al.* 1993a). The location of NodS has not been reported, and the carbamylation genes have not been identified.

Modifications at the Nod factor reducing end.

A summary of all the known modifications to the reducing end of various Nod factors, and the Nod proteins involved, are shown in Figure 4B.

Sulfate at *O*-6 of the reducing GlcNAc residue has been reported for the *R. meliloti* and *R. tropici* Nod factors (Poupot *et al.* 1993; Lerouge *et al.* 1990; Roche *et al.* 1991b). The sulfate group is required for biological activity on alfalfa (Lerouge *et al.* 1990; Truchet *et al.* 1991; Roche *et al.* 1991a). The NodPQ proteins are enzymes involved in the biosynthesis of 3′-phosphoadenosine-5′-phosphosulfate (PAPS), the activated form of sulfate (Roche *et al.* 1991a; Schwedock *et al.* 1990), and NodH is reported to be a sulfotransferase (Roche *et al.* 1991a). *R. meliloti* mutants defective in *nodPQ* still produce some sulfated Nod factor due to a secondary set of these genes (Schwedock *et al.* 1992). A *nodH*$^-$ mutant is defective in Nod factor sulfation (Roche *et al.* 1991a). Thus, NodPQ and NodH are proteins responsible for the sulfation of *R. meliloti* Nod factor. The location of these proteins within the bacterium has not been reported.

An acetyl group at *O*-6 of the reducing GlcNAc residue is found in Nod factors from *R. leguminosarum* bv. *viciae* TOM (Firmin *et al.* 1993). This strain has an additional *nod* gene, *nodX*, which permits nodulation of Afghanistan peas (Firmin *et al.* 1993; Davis *et al.* 1988). NodX has homology to transacetylases (Firmin *et al.* 1993), and transfer of this gene to strains in which it is normally absent results in the production of Nod factor with this acetyl group (Firmin *et al.* 1993). The structural modification mediated by NodX also extends the host-range of the symbiont to include Afghanistan peas. The location of NodX within the bacterium has not been reported.

A D-arabinsoyl (Ara) residue is found at *O*-6 of the reducing GlcNAc residue in Nod factors from *A. caulinodans* (Mergaert *et al.* 1993). The gene responsible for the addition of this residue has not been reported. Presumably, Ara is required for nodulation of *Sesbania*, the host for *A. caulinodans*.

Nod factors from *Rhizobium* NGR234, *B. japonicum*, *B. elkanii*, and *R. fredii* contain MeFuc at *O*-6 of the reducing GlcNAc residue (Price *et al.* 1992; Carlson *et al.* 1993; Sanjuan *et al.* 1992; Bec-Ferte *et al.* 1993). Since both *R. fredii* and *B. elkanii* also have Nod factors with Fuc (i.e., without the 2-*O*-methyl group) 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 since a *B. japonicum nodZ*$^-$

mutant produces Nod factor without MeFuc (Stacey *et al.* 1994). The putative gene encoding for the enzyme which catalyzes the 2-*O*-methylation of Fuc has not been reported. *Rhizobium* NGR234 produces Nod factors in which MeFuc can be modified by sulfation at *O*-3, or by acetylation at *O*-4. The genes responsible for these modifications have not been reported.

Little is known about when, or where, these host-specific modifications take place during Nod factor biosynthesis. Perhaps these modifications at the reducing end take place in the periplasm as the molecule is exported out of the bacterium. In the case of *B. elkanii,* the presence of Fuc is found only on those Nod factors in which the fucosylated GlcNAc residue is glycosidically linked to glycerol. If the glycerol substituent is derived from the diacylglycerol lipid anchor, this would imply that fucosylation occurs prior to hydrolysis of the lipid anchor, and that methylation of Fuc occurs after transfer to the Nod factor chitin backbone. Furthermore methylation of this Fuc may provide a better substrate for hydrolysis of the diacylglycerol anchor; while without methylation of Fuc, the Nod factor is the preferred substrate for an acyl hydrolase which removes the acyl substituents and leaves the glycerol moiety.

The relationship of host-range to Nod factor modifications.

Some of the Nod factor modifications appear to restrict while others extend symbiotic host-range. Modifications which restrict host-range include the sulfation at *O*-6 of the reducing GlcNAc residue, and the presence of a multiply unsaturated fatty *N*-acyl substituent, C16:2, in the case of *R. meliloti* (Lerouge *et al.* 1990; Roche *et al.* 1991b), and an *O*-acetyl and C18:4 *N*-acyl substituent in *R. leguminosarum* bv. *viciae* (Spaink *et al.* 1991). The D-Ara residue at *O*-6 of the reducing GlcNAc in the Nod factor from *A. caulinodans* is another example which suggests that it is required for specific interaction with *Sesbania* (Mergaert *et al.* 1993), a host unique to *A. caulinodans*.

Modifications which may play a role in extending host-range include MeFuc at *O*-6 of the reducing GlcNAc in Nod factors from *Rhizobium* NGR234, *B. japonicum*, and *R. fredii*. All of these strains have broad host-ranges relative to *R. leguminosarum* and *R. meliloti*, particularly *Rhizobium* sp. NGR234. *Bradyrhizobium japonicum nodZ*$^-$ mutants, which produce Nod factors that lack MeFuc, are defective in their ability to nodulate siratro, but still nodulate soybean. Thus, 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 Nod factors from *Rhizobium* NGR234, *R. tropici*, *A. caulinodans*, and *B. elkanii* (see Figure 1) is another example of a modification which may extend host-range since transfer of *nodS*, the putative 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* (Krishnan *et al.* 1992). A final example is the 6-*O*-acetyl group on the reducing GlcNAc residue in a Nod factor from *R. leguminosarum* bv. *viciae* TOM. The presence of this group requires NodX and results in the addition of Afghanistan pea to the host-range of strain TOM (Firmin *et al.* 1993; Davis *et al.* 1988). Thus, it is apparent that host-specific modifications include those which result in host-range extension as well as those which restrict host-range.

Two recent reports (Staehelin *et al.* 1994a; Staehelin *et al.* 1994b), suggest a possible mechanism for the host-specific effects of the various Nod factors. In the case of the Nod factor from *R. meliloti*, it was found that endo-chitinases from the host, alfalfa, rapidly degraded several Nod factors examined except NodRm-IV(C16:2,S) (Staehelin *et al.* 1994b), the alfalfa-specific molecule; i.e., the alfalfa chitinases rapidly degraded unsulfated, and pentasaccharide Nod factors. In the case of Nod factors from *Rhizobium* NGR234 and *B. japonicum*, it was reported that various hosts produced chitinases which differed in their ability to degrade the Nod factors produced by these rhizobia (Staehelin *et al.* 1994a). These reports suggest that host specific structural modifications of the various Nod factors function, in part, to protect them against rapid degradation by host chitinases.

Recently, using a variety of chemically synthesized *Bradrhizobium*-like Nod factors, it has been found that structural modifications, such as MeFuc, are not responsible for their biological activity on *Glycine soja,* and that nodules are initiated by a molecule consisting of an unmodified *N*-acyl chitin tetrasaccharide (Stokkermans *et al.*, submitted). However, addition of another GlcNAc residue, or MeFuc to the reducing terminus of this molecule resulted in a loss of biological activity; i.e., NodBj-V(C18:1,MeFuc) and Nod-IV(C18:1) were active, while NodBj-IV(C18:1,MeFuc) or NodBj-V(C18:1) were inactive. Thus, although unmodified chitotetramers are biologically active, the chitopentamers require modification by the addition of MeFuc. Perhaps MeFuc protects the pentameric Nod factors from degradation by host chitinases, or enables these chitinases to produce a product from NodBj-V(C18:1,MeFuc) which is active on soybean.

Summary.

While a great deal has been learned concerning the biosynthesis of Nod factors, there is much that remains to be determined. The functions of many Nod proteins involved in adding the host-specific modifications to the Nod factors remain to be unequivocally identified. Some of the genes required for these modifications have not yet been isolated, e.g., those involved in carbamylation, or addition of D-Ara. Additionally the cellular location of most of the Nod proteins and, concomitantly, the modifications they determine are not known.

The actual *in vivo* substrates for the NodABC proteins have not been identified, and the enzyme activities of purified NodA and NodC have not been demonstrated. The synthesis and export of the Nod factors most probably involves some type of carrier/anchor which remains unidentified. Analysis of GlcNAc metabolites from various mutants, e.g., *nodA*$^-$, *nodB*$^-$, or *nodC*$^-$ mutants, should facilitate the identification of the *in vivo* substrates involved in the synthesis of the "common" Nod factor and, thereby, lead to a greater understanding of Nod factor biosynthesis and transport. Finally, comparison of Nod factor biosynthesis to other examples of polysaccharide or glycolipid biosynthetic pathways suggest that several key enzymes remain to be identified. It is hoped that this discussion will be helpful in designing strategies for the detection and isolation of such novel enzymes.

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