Involvement of nodS in N-Methylation and nodU in 6-O-Carbamoylation of Rhizobium sp. NGR234 Nod Factors*

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Although Rhizobium sp. NGR234 and Rhizobium fredii USDA257 share many traits, dysfunctional nodSU genes in the latter prohibit nodulation of Leucaena species. Accordingly, we used R. fredii transconjugants harboring the nodS and nodU genes of NGR234 to study their role in the structural modification of the lipo-oligosaccharide Nod factors. Differences between the Nod factors mainly concern the length of the oligomer (three to five glucosamine residues in USDA257 and five residues only in NGR234) and the presence of additional substituents in NGR234 (N-linked methyl, one or two carbamoyl groups on the non-reducing moiety, acetyl or sulfogroups on the fucose). R. fredii (nodS) transconjugants produce chitopentamer Nod factors with a N-linked methyl group on the glucosaminyl terminus. Introduction of nodU into USDA257 results in the formation of 6-O-carbamoylated factors. Co-transfer of nodSU directs N-methylation, mono-6-O-carbamoylation, and production of pentameric Nod factors. Mutation of nodU in NGR234 suppresses the formation of bis-carbamoylated species. Insertional mutagenesis of nodSU drastically decreases Nod factor production, but with the exception of sulfated factors (which are partially N-methylated and mono-carbamoylated), they are identical to those of the wild-type strain. Thus, Nod factor levels, their degree of oligomerization, and N-methylation are linked to the activity encoded by nodS.

Symbiotic soil bacteria of the genera Azorhizobium, Bradyrhizobium, and Rhizobium (collectively termed rhizobia) interact with the roots of legumes to form nodules in which atmospheric nitrogen is reduced to ammonia. Signal exchange between the symbionts regulates the expression of both bacterial and plant genes involved in nodule development (1, 2). Flavonoids excreted by the legume roots activate both common and host-specific nod genes which direct the synthesis of lipo-oligosaccharide Nod factors. Secretion of these hormone-like substances into the plant rhizosphere induces root hair deformation and curling (3, 4), the formation of preinfection threads (5), and nodule-like structures (6). On a number of legumes, nod factors permit various natural or engineered Nod− mutants to enter the legume roots and to form nitrogen-fixing nodules (4, 7). All Nod factors so far identified are β-1,4-linked tri- or tetra- or pentamers of N-acetyl-D-glucosamine, N-acetylated at the non-reducing end, and N-acetylated on the other residues (1, 8). Essential differences among the Nod factors of the various species concern the substituents linked to both ends of the chitinic backbone. Among the substitutions found on the terminal non-reducing N-acetyl-D-glucosamine are an N-methyl group, carbamoyl groups, acetyl groups, and various fatty acids. Similarly, the reducing N-acetyl-D-glucosamine residue may be substituted with a sulfate group or with δ-arabinose, l-fucose, or 2-O-methylfucose. Furthermore, this additional saccharide may be acetylated or sulfated (1, 9).

Mutations in the nodABC genes, which are common to all rhizobia, abolish Nod factor production. nodC shares homology with chitin synthases (10), an observation which has been supported by in vitro studies (11). It thus seems likely that the first step in Nod factor synthesis involves the assembly of N-acetyl-D-glucosamine subunits by the N-acetylglucosaminyltransferase coded by nodC (12). When the growing chain reaches three to five residues, NodB probably removes the N-acetyl moiety of the non-reducing end (11). nodA is an N-acetyltransferase which links the acyl chain to the free NH₂ group on the oligomers synthesized by the NodC and NodB proteins (13, 14).

Host-specific nod genes are involved in the addition of extra groups to the core lipo-oligosaccharides. Modified factors permit nodulation of specific plants (16). For example, the nodH and nodPQ genes of R. meliloti are involved in the sulfation of the oligosaccharide signals (16). NodL shares homology with O-acetyltransferases; in vitro studies showed that NodL O-acetylates the non-reducing glucosaminyl residue (17). nodE and nodF are involved in synthesis of the lipid chain (18, 19), while nodZ and nodO play a role in fucosylation of Bradyrhizobium japonicum nodulation signals (20, 21). The nodSU genes of Rhizobium sp. NGR234 form an operon, the activity of which is required for nodulation of Leucaena leucocephala (22). nodSU are also present in R. fredii USDA257 (23), B. japonicum (24), and Azorhizobium caulindans (25), but no correlation exists between the ability to nodulate Leucaena and the presence of nodSU in these organisms (23). Moreover the nodSU genes from B. japonicum are unable to complement a nodS− mutant of NGR234 (24). On the other hand the transfer of nodSU from NGR234 to R. fredii USDA257 confers on the latter the ability to nodulate Leucaena (23). nodS shares similarities with S-adenosylmethyl transferases (25, 26). Support for this latter function has been obtained by in vitro labeling studies. The role of nodU remains to be elucidated.

Functions of nodS and nodU of NGR234 were determined by
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EXPERIMENTAL PROCEDURES

Bacteriological and Molecular Methods—Rhizobia, along with their relevant characteristics, are listed in Table I. Microbiological techniques were performed as described in Lewin et al. (22). Nodulation tests were conducted in Magenta™-type Leonard jars (22). DNA sequences were determined using the dideoxy chain termination procedure using double-stranded templates and the Sequenase™ II kit (U.S. Biochemical Corp.).

Placement of nodU Under Control of the nodSU nod Box—The cohesive ends of the 250-base pair Pvul-EcoRI fragment containing the nod box of the nodSU operon were filled in and ligated into the Smal site of pBS(+) +. Orientation of the insert in the resulting plasmid pNBSU was checked by sequencing. Concomitantly, the nodB gene (as well as the accompanying upstream sequences) was deleted from pA18 by double digestion with BamHI and EcoRI and replaced by the nod box of pNBSU (Fig. 1). The resulting plasmid pRAF25 was introduced into R. fredii USDA257 containing the helper plasmid pRK2013.

Purification of Nod Factors—Nod factors were isolated from a pigean-induced (10° C) culture media. Solid-phase extraction onto large scale C18 reverse-phase columns, followed by washing with ethyl acetate and preparative HPLC chromatography was performed essentially as described previously (27). Fractions reacting positively with anthrone were repurified on a C18 reverse-phase analytical column. A 20–90% methanol gradient was used for elution. Further purification was achieved on a propylamine-linked analytical column using a linear gradient of 100% acetonitrile/water/acetonitrile (80:20:20, v/v).

RESULTS

Nod Factors Produced by nodU− and nodSU− Mutants—Reverse-phase HPLC analysis of the supernatants from NGR(pA28) (nodU− nodSU−) (see Fig. 2) cultures revealed the same two nod factor peaks as in the overproducing strain. The first peak corresponds to a mixture of sulfated compounds, the second to non-sulfated Nod factors. FAB-MS spectra of NodNGR factors of the mutant strains were compared with those previously published (27). In the positive-ion mode, the spectra of compounds from peak A gave [M + Na]+ ions by sodium attachment of m/z 1597, 1569, 1554, and 1526 which are characteristic of sulfated Nod factors. Fragmentation of the non-reducing sugar yielded similar molecular ions which correspond to the glucosaminyl residue at the non-reducing end. They differ from analogous fragments of Nod factors originating from the over-producing strain NGR(pA28) by the absence of bis-carbamoylated ion products (lacking m/z 526 and 498 which correspond to species with two carbamates and C18:1 or C16:1, respectively).

Peak B contains Nod factors with an acetylated or non-substituted 2-O-methylfucosyl. As with the sulfated molecules, they differ from those of the NGR(pA28) by the presence of only one carbamoyl group (Fig. 2). Again, this is clearly seen on the introducing nodSU, nodS, and nodU into R. fredii USDA257 and by analyzing the Nod factors produced by the transconjugants. Analysis of the nod factors produced by nodSU− and nodU− mutants of NGR234 confirmed that the product of the nodS gene is involved in N-methyltransferase activity and that the 6-O-carbamoyltransferase activity is dependent upon expression of the nodU gene.

Table I

| Strain/plasmid | Characteristics | Source/refs. |
|---------------|-----------------|-------------|
| E. coli pBS(+) | pMB1, phage f1, lacZ′, Ap′ | Stratagene, La Jolla, CA |
| pRK2013 | Tra+ helper plasmid for mobilization (KmR) | 33 |
| pRK7813 Broad host range, IncP1 cosmid (TcR) | 34 |
| Rhizobium sp. NGR234 | RifR derivative of Rhizobium sp. NGR234 | 22 |
| NGR125 | NGR234 carrying an Ω insertion in nodS (RifR KmR) | 22 |
| NGR126 | NGR234 carrying an Ω insertion in nodU (RifR SpR) | 22 |
| pA28 2.2 kilobase EcoRI-PstI fragment containing nodD1 of NGR234 in pRK7813 (TcR) | 27 |
| pA18 EcoRI-HindII fragment containing nodSU of NGR234 cloned in pRK7813 (TcR) | 22 |
| pA16 EcoRI-PstI fragment containing nodS of NGR234 cloned in pRK7813 (TcR) | 22 |
| pA25 pA18 containing an Ω insertion in the BamHI site of nodS (KmR TcR) | 22 |
| pA26 pA18 containing an Ω insertion in the Hpal site of nodU (TcR SpR) | 22 |
| pRAF25 nod box::nodU of NGR234 cloned in pRK7813 (TcR) | This work |
| R. fredii USDA257 | R. fredii USDA2575. KmR derivative of wild-type strain USDA257. Contains a silent Tn5 insertion in the pSym. | 35 |

The abbreviations used are as follows: R signifies resistance against ampicillin (Ap), kanamycin (Km), rifampicin (Rif), spectinomycin (Sp), and tetracycline (Tc).
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Using the tomato cell suspension assay, we found that R. fredii (pA16) transconjugants produce 20 times more Nod factors than wild-type USDA257. HPLC analysis of the supernatants revealed three peaks (A-C) in the ratios 0:35:10:6, respectively. FAB spectra measured from the peaks showed a fragmentation sequence separated by 203 mass units which is characteristic of N-acetyl-o-glucosamine oligomers. Peak A gave [M + Na]⁺ at m/z 1424 and an associated peak (5%) yielded [M + Na]⁺ at m/z 1410. The former corresponds to nod factors containing five N-acetyl-o-glucosamine residues, a C₁₆:₁ acyl chain, 2-O-methylfucose, and an additional methyl group. The smaller peak (5%) lacks the 2-O-methyl substituent on the fucose seen in wild-type factors. [M + Na]⁺ ions of peak B arose at m/z 1452. Again, this mass is 14 Da more than that of the chitopentameric Nod factors of wild-type USDA257 possessing a C₁₆:₁ acyl chain. Sodium attached molecular ions from peak C ([M + Na]⁺) arise at m/z 1454, 2 Da higher than those of peak B which correspond to an acyl chain of C₁₈:₀ rather than C₁₆:₁. To locate the additional methyl group (14 Da), all products were studied in the presence of an acetylated matrix to enhance the formation of MH⁺ ions and their fragmentation. It is clear that the methyl group is borne on the ion fragment corresponding to the non-reducing sugar (oxonium B₁ ion). This result was confirmed by the metastable decay of MH⁺ ions (B/E scans) (Fig. 3B). The ion fragment at m/z 440 arises from decomposition of the m/z 1430 (peak B) corresponds to the non-reducing o-glucosamine end substituted by a C₁₆:₁ acyl chain and an additional methyl group (14 Da).

Localization of the methyl group was performed by (a) ¹H NMR analysis, (b) ¹³C NMR analysis, and (c) using gas chromatography of the hydrolysis products from peak B. The ¹³C NMR spectra gave a signal at δ = 27.3 ppm which corresponds to N-CH₃. In ¹H NMR (Fig. 4B), the singlet at δ = 2.7 ppm corresponds to the chemical shift of a methyl group bound to an amide nitrogen as in NodNGR and other N-methylated nod factors (26, 27). This ¹H NMR singlet is absent from the Nod factors of R. fredii USDA257 (30). Moreover, comparison of the gas chromatograph retention times of peracetyl derivatives of authentic N-methyl-D-glucosamine and of the acid hydrolysates of peak B, confirmed the presence of an N-methyl group on the glucosaminyl end.

Another observation of relevance to the role of nodS concerns the fact that wild-type USDA257 produces a majority of pentamers with relatively small amounts of tetramers and trimers (30). Introduction of nodS into R. fredii results not only in N-methylation of the Nod factors, but also in the complete disappearance of products containing three or four glucosamine residues (Fig. 2). Even though pA16 lacks only the carboxyl terminus of nodU, and the Omega (Ω) insertion in pA26 is very close to the N terminus, USDA257 transconjugants harboring either pA16 or pA26 have the same phenotype (22). Given these data, it is not surprising that the Nod factors produced by R. fredii (pA16) and R. fredii (pA26) are identical.

To discriminate between the roles played by nodS and nodU, the nod box of the nodSU operon was fused to the nodU gene. This way, the nodU gene could be expressed independently of nodS but under control of the same promoter (Fig. 1). The resulting plasmid pRAF25 confers the ability on USDA257 to nodulate Leucaena leucocephala (data not shown). In contrast to USDA257(nodS) transconjugants, R. fredii containing nodU (pRAF25) produces comparable amounts of Nod factors to those made by the wild-type USDA257 (tomato cell suspension culture assay). The HPLC profile of USDA257(pRAF25) transconjugants shows three peaks, D-F, which are present in the proportions 1:0:4:0:2, respectively. Using similar techniques to those described above, components with three, four, and five
N-acetyl-\(\beta\)-glucosamine residues were found with a predominance of the pentamer as in wild-type USDA257. Mass spectrometry showed that the methylfucose was still present. Similarly, fraction D gave \(B1\) ions at \(m/z\) 441 and 398. The latter corresponds to glucosamine bearing a C\(_{16:1}\) acyl chain as in Nod factors of wild-type USDA257. In the former, the shift up of 43 mass units is indicative of the presence an additional carbamoyl group. Similarly, fractions E and F contain mixtures of molecules that are either mono- or non-carbamoylated and possess either C\(_{18:1}\) or C\(_{18:0}\) acyl chains and three, four, or five N-acetyl-\(\beta\)-glucosamine residues (Fig. 2). B/E spectra performed on the different \([M+H]^+\) ions (Fig. 3A) confirmed the presence of an additional 43 mass units on the non-reducing sugar. Thus, introduction of nodU into R. fredii induces a partial mono-carnbamoylation at the non-reducing end.

Conjugation of both nodS and nodU (pA18) into R. fredii produced Nod factors than can be separated into three HPLC peaks which gave \([M+H]^+\) ions at \(m/z\) 1445, 1473, and 1475, indicating a chitotetrameric backbone (data not shown). \(B1\) ions of these peaks at \(m/z\) 455, 483, and 485 correspond to N-acetyl-\(\beta\)-glucosamine possessing acyl chains of C\(_{16:1}\) or C\(_{18:1}\) or C\(_{18:0}\), respectively, that are shifted up by 57 mass units in comparison with ions from USDA257 Nod factors. This 57 Da difference corresponds to the sum of the mass of two groups: 43 Da for carbamate and 14 Da for the N-methyl group. B/E spectra performed on the \([M+H]^+\) ion at \(m/z\) 1473 confirmed that these groups are attached to the non-reducing end (Fig. 3C). A \(^1H\) NMR signal at \(\delta = 2.7\) ppm corroborated the presence of an N-methyl group which also gave a signal at \(\delta = 27.2\) ppm in \(^13C\) NMR (Fig. 4A). The same spectrum confirmed the presence of a 6-O-carbamoyl group at \(\delta = 156.5\) ppm (Fig. 4A). The minor products (5% of the total) detected by FAB-MS also correspond to products containing five N-acetyl-\(\beta\)-glucosamine residues which are partially carbamoylated but not 2-O-methylated (data not shown).

Location of the Carbamoyl Groups—\(^13C\) NMR analysis in...
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We previously noted the striking nucleotide and amino acid sequence homologies between nodS of NGR234 and USDA257 (22, 23). Similarly, the nodU gene of NGR234 (EMBL, GenBank, and DDBJ accession number X89965) shows significant similarities to the amino acid sequences of NodU of R. fredii USDA257 (23), B. japonicum USDA110 (24), A. caulinodans (25), and ORF10 of Nocardia lactamurans (31) (data not shown). ORF10 of Nocardia is implicated in the biosynthesis of the cephamycin family of carbamoylated β-lactam antibiotics. Nevertheless, the fact that such well conserved and apparently functional genes behave differently in Rhizobium sp. NGR234 and B. japonicum USDA110 suggest that the NodU protein also may act on other substrates such as lipopolysaccharides as has recently been reported (32). We are currently investigating this possibility.

Our physical/chemical data suggest that the nodS gene product is involved in N-linked methyltransferase activity, while
that of the nodU gene is probably a 6-O-carbamoyltransferase.

An impediment to elucidating the role of nodSU in NGR234 is the probable existence of other genes encoding similar functions. Mutations in nodS drastically reduce nod factor production, but suppression of nodU has no effect on nod factor levels (28). Thus, definitive analysis of nodSU could only be obtained by introducing it into USDA257. Although both nodSU exist in USDA257, a deletion in the promoter region drastically reduces transcription of the operon (23). In spite of the extraordinary similarities between the two genomes, USDA257 produces nod factors that differ from those of NGR234 by the absence of acetyl and sulfated groups on the 2-O-methylfucose moiety and which lack both the N-linked methyl group as well as carboxyl groups on the non-reducing sugar. In R. fredii harboring nodS of NGR234, tri- and tetrameric nod factors are no longer produced, while N-methylation of the acyl chain of the pentameric species occurs. NodU causes partial 6-O-mono-carbamoylation of the non-reducing sugar but does not control the length of the oligomer. Together, nodSU yield pentamers that are N-methylated and mono-carbamoylated on C-6. Surprisingly, the activity of either gene is sufficient to confer nodulation of Leucaena species. Indeed, there is no apparent combination of N-linked methyl and carbamoyl groups with the number of saccharide repeating units which permits nodulation of Leucaena. This contrasts starkly with the requirement of sulfated nod factors of R. meliloti for nodulation of Medicago sativa (16). Nevertheless, mutations in nodS prevent NGR234 from nodulating Leucaena (22. 23). It should be noted, however, that nodSU of both B. japonicum and R. fredii are unable to complement nodSU− mutants of other (brady)rhizobia, including NGR234 (24).

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