nolO and noel (HsnIII) of *Rhizobium* sp. NGR234 Are Involved in 3-O-Carbamoylation and 2-O-Methylation of Nod Factors*

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Loci unique to specific rhizobia direct the adunction of special groups to the core lipo-oligosaccharide Nod factors. Host-specificity of nodulation (Hsn) genes are thus essential for interaction with certain legumes. *Rhizobium* sp. NGR234, which can nodulate >110 genera of legumes, possesses three *hsn* loci and secretes a large family of Nod factors carrying specific substituents. Among them are 3-O (or 4-O)- and 6-O-carbamoyl groups, an N-acetyl group, and a 2-O-methylfucose residue which may bear either 3-O-sulfate or 4-O (and 3-O)-acetyl substituents. The *hsnIII* locus comprises a nod box promoter followed by the genes *nodABCJNolOnoei*. Complementation and mutation analyses show that the disruption of any one of *nodIJ*, *nolO*, or *noel* has no effect on nodulation. Conjugation of nolO into *Rhizobium fredii* extends the host range of the recipient to the non-hosts *Calopogonium caeruleum* and *Lablab purpureus*, however. Chemical analyses of the Nod factors produced by the NodI, NolO, and Noel mutants show that the *nolO* and *noel* gene products are required for 3 (or 4)-O-carbamoylation of the nonreducing terminus and for 2-O-methylation of the fucosyl group, respectively. Confirmation that NolO is a carbamoyltransferase was obtained from analysis of the Nod factors produced by *R. fredii* containing nolO; all are carbamoylated at O-3 (or O-4) on the nonreducing terminus. Since mutation of both *nolO* and *nodU* fails to completely abolish production of monocarbamoylated NodNGR factors, it is clear that a third carbamoyltransferase must exist. Nevertheless, the specificities of the two known enzymes are clearly different. NodU is only able to transfer carbamate to O-6 while NolO is specific for O-3 (or O-4) of NodNGR factors.

Nodulation genes (*nod*, *nol*, and *noe*) of the symbiotic soil bacteria *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* (collectively termed rhizobia), may be divided into two classes. One class comprises genes which, when mutated, completely abolish nodulation on all legumes. Since genes in this group share significant sequence homology and can be complemented between different rhizobial genera/species, they are often called “common.” *nodABCJ* and *nodD* are the best known examples. Genes of the second class are necessary for the interaction with certain, but not all, legumes. Expression of these genes permits nodulation of additional hosts, and for this reason, they have been termed host specificity of nodulation (= *hsn*) genes. By definition, they are unique to one or a few rhizobia.

In 1990, Lerouge *et al.* (1) showed that the products of the *nod* genes are N-acylated oligomers of N-acetyl-d-glucosamine. Numerous other investigators have confirmed these findings (for reviews see Refs. 2–5). Since these substances are the products of the *nod* genes, they are called Nod factors. Principal differences among the Nod factors of the various rhizobia concern the length of the core molecule as well as the substitutions to both the reducing and nonreducing residues. Presumably, the *hsn* genes are responsible for these substitutions.

With the discovery of Nod factors, it became possible to correlate *nod* gene expression with Nod factor structure. In their pioneering studies, Roche *et al.* (6) demonstrated that the *hsn loci*, *nodH* and *nodFQ*, are responsible for the 6-O-sulfation of the reducing N-acetyl-d-glucosamine of NodRM factors. Later work has shown that the first step in Nod factor assembly is performed by an N-acetylglucosaminyltransferase coded by *nodC* (7). Then, a deacetylase coded by *nodB* removes the N-acetyl moiety from the nonreducing end of the N-acetylgalcosamine oligosaccharides (8). Finally, an acyltransferase coded by *nodA*, links the acyl chain to the NH₂-free carbon C-2 of the nonreducing end of the oligosaccharide (9). *NodI* and *NodJ* are involved in the export of Nod factors (10, 11).

In *Rhizobium* sp. NGR234, three Hsn loci were discovered by transferring cosmids covering the symbiotic plasmid to heterologous rhizobia (i.e. rhizobia unable to nodulate NGR234 hosts), and asking if the transconjugants could form nodules on *Vigna unguiculata* (12, 13). In this way, we showed that HsnII, which is responsible for the host-specific nodulation of *Leucaena* species, contains *nodSU* (14), which are involved in N-methylation and 6-O-carbamoylation of NodNGR factors, respectively (15). HsnI contains five genes encoding a set of enzymes responsible for the synthesis GDP-fucose and its transfer by NodZ to NodNGR factors (16, 17).

Here we present a molecular analysis of the HsnIII locus (12, 13). In this, as in much of the work discussed above, we used the closely related *R. fredii* strain USDA257 because (a) it nodulates an exact subset of the NGR234 hosts; (b) many of the *nod* genes, and most of the essential, chromosomal genes, are extremely well conserved between the two strains (18–20); and (c) perhaps because of (b), transconjugants are more stable in the *R. fredii* background than in any other rhizobia. Combined, these properties facilitate extension of host-range studies in which NGR234 clones are introduced into USDA257 on broad host range, multiple copy plasmids. Since the nodulation re-
requirements of the two bacteria can be performed on legumes that are nodulated by NGR234 but not by USDA257.

These analyses show that HsmIII is downstream of nodABC, and includes the nodI/nolO Double Mutants—The 2.2-kb HindIII/BamHI fragment downstream of nodABCJII, which contains nolO, was cloned into the HindIII and BamHI sites of pBluescript SK (giving pSK2.2B/H). Digestion of pBluescript KS with BamHI and HindIII led to deletion of the EcoRI site in the multiple-cloning site. Consequently, an EcoRI fragment carrying the Km\(^\beta\) Omega interposon (25) could be cloned into the EcoRI site of nolO::Tn5 (26). Triparental matings (including the helper plasmid pRK2013) were used to mobilize nolO:Omega into NGR234. Selection of NGR110nolO was performed as described previously (27).

**Construction of nodU/nolO Double Mutants**—The 2.2-kb HindIII/BamHI fragment downstream of nodABCJII, which contains nolO, was cloned into the HindIII and BamHI sites of pBluescript SK (giving pSK2.2B/H). Digestion of pBluescript KS with BamHI and HindIII led to deletion of the EcoRI site in the multiple-cloning site. Consequently, an EcoRI fragment carrying the Km\(^\beta\) Omega interposon (25) could be cloned into the EcoRI site of nolO::Tn5 (26). Triparental matings (including the helper plasmid pRK2013) were used to mobilize nolO:Omega into NGR234. Selection of NGR110nolO was performed as described previously (27).

**Nodulation Tests**—Seeds of Calopogonium caeruleum (Benth.) Hemsl. were purchased from the Inland and Foreign Trading Co., Indus
O-Carbamoylation and O-Methylation of NodNGR Factors

Table II

| Phenotypes of Rhizobium sp. NGR234, R. fredii USDA257, and their various derivatives on nodulation and nitrogen fixation on different legumes |
|---------------------------------------------------------------|
| Calopogonium caeruleum | Glycine max | Lablab purpureus | Leuecaena leucocephala | Macroptilium atropurpureum | Vigna unguiculata |
|-------------------------|-------------|------------------|------------------------|-----------------------------|------------------|
| NGR234                  | Fix⁺        | Fix⁺             | Fix⁺                   | Fix⁺                        | Fix⁺             |
| NGRInolO                | Fix⁺        | n.t.+            | n.t.                   | n.t.                        | n.t.             |
| NGRIhoel                | Fix⁺        | n.t.+            | Fix⁺                   | Fix⁺                        | n.t.             |
| NGRIhoelU3InolO         | Fix⁺        | n.t.+            | n.t.                   | Fix⁺                        | n.t.             |
| USDA257                 | n.d.        | n.d.             | n.d.                   | n.d.                        | n.d.             |
| USDA257(p7.6E)          | Nod⁻        | Fix⁻             | n.d.                   | n.t.                        | Fix⁻             |
| USDA257(p6.8HR)         | Fix⁻        | Fix⁻             | n.d.                   | Fix⁻                        | n.d.             |
| USDA257(p6.8HRep)       | Nod⁻        | Fix⁻             | n.d.                   | Fix⁻                        | n.d.             |
| USDA257(p6.8HRep)       | Fix⁺        | n.t.             | Nod⁻                   | Fix⁻                        | n.d.             |
| USDA257(p2.2)           | Nod⁻        | Fix⁻             | n.d.                   | Fix⁻                        | Fix⁻             |
| USDA257(pNG77)          | Fix⁺        | Fix⁻             | Nod⁻                   | Fix⁻                        | Fix⁻             |
| USDA257(R3)             | Fix⁺        | Fix⁻             | n.t.                   | Fix⁻                        | Fix⁻             |
| USDA257(pA18)           | n.d.        | n.t.             | n.d.                   | Fix⁻                        | Fix⁻             |

n.t. = not tested.

Fig. 1. Genetic/physical map of the nodABCJIno1onoel locus. Sizes of the open reading frames are based on the complete sequence of the symbiotic plasmid pNGR234a (http://genome.imb-jena.de/archives). Restriction sites are marked by vertical lines as follows: B, BamHI; C, ClaI; E, EcoR1; H, HindIII; P, PstI; S, SstI; SaI, SpI; SpAl; and X, XhoI. Sites into which the spectinomycin resistant Omega cassette was inserted are marked by triangles. (+) and (−) correspond to the phenotypes Fix⁺ or Nod⁻ observed in USDA257 transconjugants containing different fragments of the HsnIII locus. nodI shows 65% identity (and 78% similarity) while nodJ shows 65% identity (and 77% similarity) to the same genes of R. leguminosarum (37).

RESULTS

Host Range Extension—At the outset, large fragments (≥40 kb) of the symbiotic plasmid of Rhizobium sp. NGR234 (pNGR234a) were cloned into the nontransmissible cosmid vector pJB8 (22, 30). Individual cosmids were mobilized into heterologous rhizobia by introducing the cis-acting DNA recognition site for conjugative DNA transfer (Mob site) of RP4 into the clones. This was accomplished by conjugating the Tn5-Mob vector pSUP5011 (31) into E. coli strains containing the pJB8 cosmids. Matings with another RP4 derivative permitted mobilization of the cosmids into various Agrobacterium/Rhizobium strains including Rhizobium loti strain NZP4010. R. loti (pWA54) transconjugants nodulated V. unguiculata at low frequency, and this locus was named HsnIII (12). Preliminary mapping showed that pWA54 partially overlaps with pWA46, but that the latter does not confer host-range extension on the transconjugants (13).

To search for a cosmid which would give a higher frequency of Nod⁺ transconjugants, a new clone bank was established in the broad host-range, transmissible, cosmid vector pRK7813 (32). Triparental matings were used to mobilize the individual pRK7813 cosmids into R. fredii USDA257. One clone
(pNG77) was identified that shared homology with pWA54 and permitted USDA257 to nodulate *C. caeruleum*. Among the legumes tested, pNG77 extended the host range to *Calopogonium* and *Lablab purpureus* but had no effect on nodulation of the other plants (Table II).

Examination of the nodulation capacity of *R. fredii* transconjugants harboring either HsnII (nodSU, contained on pA18) or HsnIII (carried on pNG77), clearly demonstrated that these are proper hsn loci. The nodSU genes permit nodulation of *Leucaena leucocephala* (but not *Calopogonium* and *Lablab*), while the reverse is true for HsnIII (Table II).

**Delimitation of the HsnIII Locus**—A series of subclones in pRK7813 were generated and mobilized into USDA257. The resulting transconjugants were used to inoculate *Calopogonium*, *Lablab*, and other legumes. Interestingly, the 7.6-kb EcoRI fragment of pNG77, which contains regions on either side of nodABC, was unable to extend the host range (Table II). On the other hand, clones containing the right most of two 6.8-kb HindIII fragments (p6.8HR) (Fig. 1), conferred the ability to nodulate both *C. caeruleum* and *L. purpureus* on the transconjugants. These data suggest that the HsnIII locus lies at the end of p6.8HR distal to nodABC.

Surprisingly, when the transconjugants were reisolated from *Calopogonium* nodules, they had lost resistance to tetracycline.

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**FIG. 2.** LSI-MS spectra in the positive ionization mode of the major LCOs (N-vaccenyl pentameric form) produced by derivatives of NGR234. A, NGRmnoLO possessing an N-methyl group and zero or one carbamoyl groups at the nonreducing terminus along with a partly acetylated fucose at the reducing end at m/z 1458 and 1501 for (MH)+, or their adducts +Na at m/z 1480 and 1523; nonacetylated form at m/z 1416, 1459 (MH)+, and 1458, 1481 for (MNa)+. B, NGRΔ(mnoSU) lacking an N-methyl but possessing zero or one carbamates at the nonreducing end in which the methylfucose is acetylated (m/z at 1501 and 1458 (MH)+, or their adducts +Na at m/z 1480 and 1523. C, NGRmnoI possessing an N-methyl group and one or two carbamates at the nonreducing terminus along with acetylated fucose (GMH)+ at m/z 1501 and 1544 or their adducts +Na at m/z 1523 and 1566. The structures of all LCOs are presented in Fig. 3.
(i.e., they were KmRcS), but retained their ability to nodulate Calopogonium. DNA isolated from nodules induced by USDA257(pNG77), and hybridized against an internal nodC probe of NGR234, showed that the restriction pattern of the USDA257 nodC locus in the re-isolates had changed to that of NGR234. Similar KmRcS reisolates were obtained from nodules induced by USDA257(p6.8H) transconjugants. Interestingly, the restriction pattern of the USDA257(p6.8H) nodC locus was identical to that obtained from USDA257(pNG77) transconjugants, suggesting that the common nod genes play a role in homologous recombination between the two species. R. fredii (pNG77) or R. fredii (p6.8H) reisolates from C. caeruleum nodules were stable as judged by their ability to nodulate both Calopogonium and Lablab when reinoculated onto them (Table II).

**Mutational and Sequence Analysis of HsnIII**—As R. fredii transconjugants harboring p7.6E and p2.2BH (Fig. 1) were unable to nodulate C. caeruleum while those containing p6.8HR were able to do so (Table II), the HsnIII locus is most probably located in the region of overlap between p7.6E and p2.2BH. To delimit possible genes in this region, we sequenced the plasmids represented by p7.6E and p2.2BH. As confirmed by complete sequencing of pNGR234α (33), seven open reading frames are contained in this locus (Fig. 1). A noncoding sequence of 173 bp separates nodABC from the 1,005-bp nodI. After a gap of only 3 bp, this is followed by another ORF of 789 bp that is highly similar to nodJ. nolO is downstream of nodJ and is followed by part of noeI. Insertion of an Omega cassette into either nodI or nolO or noeI had no effect on the capacity of the mutant to form nodules (Table II).

RNA competition/hybridization experiments were used to see whether the loci contained on pNG77 and p6.8HR were inducible by flavonoids. One h after exposure to apigenin, all of the fragments contained on p6.8HR (e.g., the 1.7- and the 3.6-kb BamHI fragments) as well as the 7.6-kb EcoRI fragment of pNG77 hybridized, showing that the entire locus is inducible.

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3 B. Relić, unpublished results.

| NGR234 | USDA257 | USDA257(p6.8HR) | NGRΩnodI | NGRΩnolO |
|--------|---------|-----------------|-----------|-----------|
| R1 CH₃ | H       | H               | CH₃       | CH₃       |
| R2 Carb or OH | OH | Carb or OH | OH       | OH       |
| R3 Carb or OH | OH | OH           | OH       | OH       |
| n 3 | 1 or 2 or 3 | 1 or 2 or 3 | 3 | 3 |
| X OCH₃ | OCH₃ or OH | OCH₃ or OH | OH | OH |
| Y SO₄H or OH | OH | SO₄H or OH | SO₄H or OH | SO₄H or OH |
| Z OAc or OH | OH | OAc or OH | OAc or OH | OAc or OH |

| NGRΩnolO | NGRΔ1 | NGRΔ1-nolO | NGRΩ26 | NGRΩ26-nolO |
|-----------|-------|------------|--------|------------|
| R1 CH₃ | H | H | CH₃ | CH₃ |
| R2 Carb or OH | Carb or OH | Carb or OH | OH | OH |
| R3 Carb or OH | OH | OH | OH | OH |
| n 3 | 1 or 2 or 3 | 1 or 2 or 3 | 3 | 3 |
| X OH | OCH₃ | OH | OCH₃ | OH |
| Y SO₄H or OH | SO₄H or OH | SO₄H or OH | SO₄H or OH | SO₄H or OH |
| Z OAc or OH | OAc or OH | OAc or OH | OAc or OH | OAc or OH |

**Fig. 3.** Structure of the major LCOs produced by NGR234, USDA257, and various derivatives thereof (n is the number of GlcNAc residues; Ac, acetyl; carb, carbamate).
Involvement of hsnIII Genes in 2-O-Methylation of the Fucose—Thin layer chromatographic analyses of the supernatants from ^14C]glucosamine-fed cultures of NGR\textit{nol}I, NGR\textit{nol}O, and NGR\textit{noeI} did not reveal differences from the wild-type bacterium (data not shown). We were thus able to exclude a role for these genes in fucosylation or sulfation of NodNGR factors. Other possible roles of the \textit{hsn}III genes were studied by subjecting partially purified (i.e. eluted from a \textit{C}\textsubscript{18} reverse-phase column) Nod factors to acidic methanolysis. Hydrophilic compounds were separated from the hydrophobic components by hexane extraction. GC/MS analyses of the methyl-esterified hexane-fraction did not reveal any changes in the N-linked acyl chain attached to the nonreducing terminus. This way, we were able to rule out any affect of the \textit{hsn}III genes on fatty acid synthesis.

In another approach (and after aldol-acetate derivatization), the monosaccharide compositions of the mutants were compared with those produced by the wild-type bacterium. Interestingly, the peak corresponding to 2-O-methylfucose was absent in Nod factors produced by all three mutants, while those corresponding to fucose, N-acetylglucosamine (GlcNAc), and N-methylglucosamine (MeGlcN) were present (see Fig. 3). This indicates that one or combinations of all three mutations in \textit{hsn}III prevent 2-O-methylation of the fucose. Since \textit{noda}B\textit{C}IJ\textit{nol}O\textit{noeI} probably form an operon (see above and Fig. 1), and each of the Omega insertions has a polar effect on expression of the down-stream genes, the simplest interpretation of the phenotype is that NoeI is the methyltransferase. To test this, supernatants of NGR\textit{noeI} cultures were purified by reverse phase (RP)-HPLC, and the appropriate fractions were analyzed by LSI-MS in the positive ionization mode. Fragment ions generated from the nonreducing terminus were unchanged in comparison to those given by wild-type extracts, indicating that the N-methyl and the set of carbamyl groups are present (Fig. 2C). In contrast, the mass of all pseudomolecular ions [M + H]\(^+\) was 14 Da less than those produced by the wild-type bacterium. To localize the origin of the 14-Da ion, metastable ion spectra (MS/MS) of the major Nod factors were taken. It is clear from these analyses that the fucose moiety lacks the methyl group since the first significant fragment ion corresponds to the loss of fucose (m/z 146) rather than methylfucose (m/z 160). Also, the \(^1H\) NMR spectrum lacks the signal of the 2-O-methyl at \(\delta = 3.35\) ppm. Thus mutation on \textit{noel} seems to suppress the production of NodNGR-factors containing 2-O-methylated fucose (Fig. 3).

\textit{Nod Factors Produced by NGR\textit{nol}O}—The LSI-MS spectra (positive ionization mode), of RP-HPLC-purified compounds extracted from the supernatants of NGR\textit{nol}O cultures revealed [M + H]\(^+\) molecular ions which correspond to sulfated or acetylated molecules. Fragment ions from the nonreducing terminus differ from analogous ions of Nod factors of the wild-type bacterium by the absence of his-carbamoylated products (absence of m/z 528, 526, and 498), which correspond to LCO species possessing two carabamates, acylated with C\textsubscript{18:0}, C\textsubscript{18:1}, and C\textsubscript{16:1} respectively, and N-methylated. Molecules in which the fucose is acetylated gave pseudomolecular ions that were shifted down by 57 Da in comparison to ions from NodNGR factors (Fig. 2A). This 57-Da difference corresponds to the sum of the mass of carabamoyl (43 Da) and methyl (14 Da) groups.
B/E spectra were performed on several molecular ions of LCOs extracted from the NGR\textsuperscript{nolO} mutant. Among them, the [M + H]\(^+\) ion at \(m/z\) 1459 revealed the following fragment ions: \(m/z\) 1313, 1092, 889, 686, and 483. Thus the ion \(m/z\) 1313 is derived from \(m/z\) 1459 by the loss of fucose (146 Da) rather than methylfucose (160 Da). Similarly, the loss of 43 Da must be from the nonreducing terminus, since no fragment ion corresponding to bis-carbamoylated ions (\(m/z\) 526) was observed. \(^{13}\text{C}\) NMR showed the absence of signals at 60, 156.1, and 155.7 ppm, confirming that no methyl group was attached to the carbamate groups in different LCOs produced by NGR234 and USDA257 and their derivatives.

In this way, we were able to provisionally assign the 2-O-methylation function to NoeI, and carbamoylation at the nonreducing terminus to NolO. Examination of Nod factors produced by NGR\textsuperscript{nodI} also showed the loss of one carbamoyl and the 2-O-methyl group, confirming the organization of nodIJ\textsuperscript{nolOnoeI} genes in the operon. Nevertheless, the height of the HPLC peaks of LCOs produced by the Nod\textsuperscript{I} mutant were only half those produced by loading extracts prepared from the same quantity of wild-type bacteria (data not shown).

\textbf{Fig. 5.} \(^{13}\text{C}\) NMR signals (Me\textsubscript{6}SO-d\textsubscript{4}/100 MHz) corresponding to the carbamate groups in different LCOs produced by NGR234 and USDA257 and their derivatives. \(A\), NGR234 (wild-type) (\(\delta = 156.5\) ppm corresponds to H\textsubscript{2}NCOO at O-6, and \(\delta = 156.1\) or 155.7 ppm to H\textsubscript{2}NCOO at O-3 or O-4). \(B\), NGR\textsuperscript{nodI}; \(C\), NGR\textsuperscript{nodO}; \(D\), USDA257\textsuperscript{nodsU}; \(E\), NGR\textsuperscript{A1nolO}; \(F\), NGR234 wild type after mild alkaline hydrolysis.

\textbf{Fig. 6.} Specific action of the carbamoyltransferases encoded by nod\textsuperscript{U} and nod\textsuperscript{O} of NGR234 in generating mono- and bis-carbamoylated Nod factors. Mutation in nod\textsuperscript{U} or nod\textsuperscript{O} gives only monocarbamoylated molecules. The absence of tris-carbamoylated forms is probably because NoO does not recognize position O-3 or O-4 when one of them is carbamoylated. Similarly, NoO is probably incapable of recognizing position O-3 (or O-4) when one of them is carbamoylated. Crosses on the arrows after the enzyme name show that the structure which follows is forbidden. Thus NoO is incapable of adding carbamate to 3-O (or 4-O) carbamoylated Nod factors. Although Nod factors carbamoylated at positions O-3 and O-4 do not normally exist, NodU would be incapable of 6-O carbamoylating them. In other words, native NodNGR factors can only be carbamoylated at two of the three possible positions; 3-O (or 4-O) and 6-O.
not yield fragment ions corresponding to N-methylated or bis-
carbamoylated Nod factors (Fig. 2B), but the monocarbamoy-
lated and monoacetamidated forms persisted (m/z 465, 469, and 471). Unexpectedly, the B1 oxonium ions of mass spectra generated from different fractions of the supernatants of NGR11nolO were similar to those from NGR1, showing that mutation of nodU and nolO were not sufficient to completely abolish carbamoylation (data not shown). The 2-O-methyl group was however absent in the supernatants of cultures from NGR1nolO (Fig. 2A) and NGR11nolO. Moreover the pseudomolecular ions from NGR11nolO or NGR1 revealed a short Nod factor backbone (3 or 4 GlcNAcs) confirming the role of nodS in ensuring pentameric NodNGR factors (15). In some cases, the molecular ions of the tri- and tetrameric LCOs were similar to fragment ions from the pentameric forms (see Fig. 2B, at 1299.7, 1256.7, 1293.7, 1096, and 1053 Da), but the relative abundances of the non-
carbamoylated and monocarbamoylated forms were reversed. MS and MS/MS analyses of pure HPLC fractions of these short LCOs clearly proved these structures. The monocarbamoylated forms were also observed in the LSI-MS spectra of LCOs puri-
fied from NGR11nodU1nolO.

Specific Carbamoylation by NolO—Further evidence that NolO specifies carbamoylation on positions other than O-6 was obtained by tandem mass spectrometry and by 15N or 13C NMR spectroscopy. Using model compounds, we showed that the metastable ion spectra of the oxonium ions from all isomers of mono-O-acetylated, N-acetylgalactosamine were different and that 3-O- and 6-O-carbamates behaved as the corresponding O-acetates (4-O-acetate was not used as a reference com-

Fig. 7. Relation of NoLO to other carbamoyltransferases. Partial sequence alignments between NodU (NODU-RHISN) and NolO (NOLO_ RHISS) of Rhizobium sp. NGR234, a carbamoyltransferase from Nocardia (NORF10A_1), NodU from A. caulinodans (NODU_AZOC), NodU (NODU_BRAJA) and NolO (NOLO_BRAJA) of B. japonicum. Boxes represent identical residues in all six sequences. nolO of NGR234 is 72% identical (and 82% similar) to nolO of B. japonicum, even though it is 211 amino acids longer (38). In the central domain, nolO of NGR234 shares 60% similarity and 40% identity with nodU of A. caulinodans (35).
tion termination signals of the bacteriophage T4D gene 32 (34), it is highly unlikely that any residual 3 (or 4)-O-carbamoyltransferase activity of NolO remains in NGRanoiO. It is thus likely that a third carbamoyltransferase specific for the O-6 of the nonreducing terminus exists. Since no other genes homologous to either nodU or nooO were found on pNGR234a (33), this suggests that the third carbamoyltransferase must be on another replicon.

**DISCUSSION**

Since mutation of noel has no effect on noduleation while host-range extension (complementation) studies with nooO render USDA257 Nod- on *C. caeruleum*, it seems likely that nooO is the principal host range determinant of the transferase activity of NolO. It is highly unlikely that any residual 3 (or 4)-O-carbamoyltransferase activity of the bacteriophage T4D gene 32 (34), this suggests that the third carbamoyltransferase must be on the nonreducing terminus exists. Since no other genes homologous to either nodU or nooO were found on pNGR234a (33), this suggests that the third carbamoyltransferase must be on another replicon. Since no other genes homologous to either nodU or nooO were found on pNGR234a (33), this suggests that the third carbamoyltransferase must be on another replicon.

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