Molecular Microbiology (1997) **24**(6), 1119–1129

Sulphation of *Rhizobium* sp. NGR234 Nod factors is dependent on *noeE*, a new host-specificity gene

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Summary

Rhizobia secrete specific lipo-chitooligosaccharide signals (LCOs) called Nod factors that are required for infection and nodulation of legumes. In *Rhizobium* sp. NGR234, the reducing *N*-acetyl-D-glucosamine of LCOs is substituted at C_6 with 2-*O*-methyl-L-fucose which can be acetylated or sulphated. We identified a flavonoid-inducible locus on the symbiotic plasmid pNGR234*a* that contains a new nodulation gene, *noeE*, which is required for the sulphation of NGR234 Nod factors (NodNGR). *noeE* was identified by conjugation into the closely related *Rhizobium fredii* strain USDA257, which produces fucosylated but non-sulphated Nod factors (NodUSDA). *R. fredii* transconjugants producing sulphated LCOs acquire the capacity to nodulate *Calopogonium caeruleum*. Furthermore, mutation of *noeE* (NGRΔ*noeE*) abolishes the production of sulphated LCOs and prevents nodulation of *Pachyrhizus tuberosus*. The sulphotransferase activity linked to NoeE is specific for fucose. In contrast, the sulphotransferase NodH of *Rhizobium meliloti* seems to be less specific than NoeE, because its introduction into NGRΔ*noeE* leads to the production of a mixture of LCOs that are sulphated on C_6 of the reducing terminus and sulphated on the 2-*O*-methylfucose residue. Together, these findings show that *noeE* is a host-specificity gene which probably encodes a fucose-specific sulphotransferase.

Introduction

Establishment of symbioses between nitrogen-fixing rhizobia and legumes requires the expression of plant and bacterial genes (for reviews see Fellay *et al*., 1995a; van Rhijn and Vanderleyden, 1995). Symbiotic associations may be specific and result in the formation of specialized root organs called nodules, in which rhizobia reduce atmospheric dinitrogen to ammonia. In conjunction with flavonoids excreted by legume roots, the NodD proteins (members of the *lysR* family of transcriptional regulators) induce the expression of rhizobial nodulation genes (*nod*, *nol* and *noe*). Certain nodulation genes (e.g. *nodABC*) are present in all rhizobia, while others are host specific (*hsn*). Both are required for the synthesis of a family of lipo-chitooligosaccharides (LCOs) called Nod factors. Nod factors, which are β-1,4-linked tri-, tetra- or pentamers of *N*-acetyl-D-glucosamine, and are *N*-acylated at the non-reducing end, act as specific inducers of nodule formation (Lerouge *et al*., 1990; Truchet *et al*., 1991; van Brussel *et al*., 1992; Relič *et al*., 1993; Ehrhardt *et al*., 1996; Spaink, 1996). Production of the core oligosaccharide molecule is dependent on the activity of the *nodABC* genes. In turn, *hsn* genes direct the decoration of the core molecule with various adjuncts.

NGR234 is a fast-growing tropical *Rhizobium* species which nodulates more than 110 different genera of legumes (S. G. Pueppke and W. J. Broughton, unpublished), and produces over 80 different Nod factors (S. Jabbouri, unpublished). The non-reducing glucosamine of the basic NodNGR factor is *N*-methylated and possesses zero, one or two carbamoyl groups. At the reducing terminus, the *N*-acetyl-D-glucosamine (GlcNAc) is substituted on carbon position 6 with 2-*O*-methyl-L-fucose which may be acetylated, sulphated, or non-substituted (Price *et al*., 1992). Most of the genetic determinants needed for the establishment of a functional symbiosis are located on the 536 kb symbiotic plasmid pNGR234*a*. Three distinct host-specificity loci *hsnI*, *hsnII* and *hsnIII*, have been identified on pNGR234*a* (Broughton *et al*., 1996; Lewin *et al*., 1987). *hsnI* contains the regulatory *nodD1* gene and *nodZ* which is responsible for the transfer of the fucose group to the reducing *N*-acetyl glucosamine (Quesada-Vincens *et al*., 1997; Lopez-Lara *et al*., 1996; Mergaert *et al*., 1996). *hsnII* comprises two genes, *nodS* and *nodU*, which are essential for nodulation of tropical trees of the genus *Leucaena* (Lewin *et al*., 1990; Krishnan *et al*., 1992). *nodS* is involved in *N*-methylation and *nodU* in 6-*O*-carbamoylation of the non-reducing terminus of NodNGR factors

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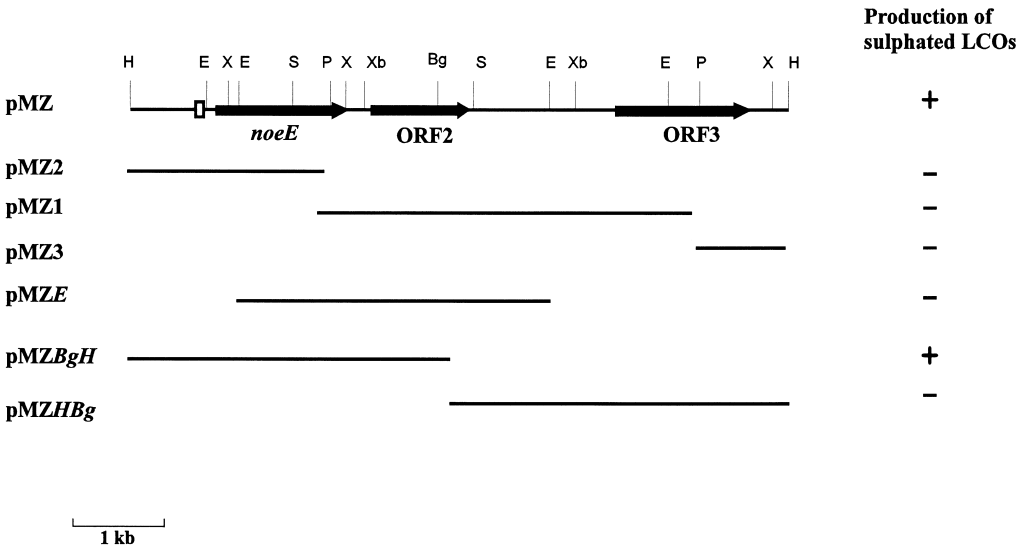


Fig. 1. Physical and genetic map of the NodNGR-sulphation locus. Subclones used to complement *R. fredii* USDA257 for the production of sulphated LCOs are also shown. Restriction sites are as follows: Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; X, *Xho*I; Xb, *Xba*I. Arrows indicate the direction of transcription of *noeE*, *orf2* and *orf3*. The *nod* box is indicated by a rectangle.

(Jabbouri *et al*., 1995). The *nodABC–nodIJ–nolO–noeI* operon makes up the *hsnIII* locus (Jabbouri *et al*., 1996; B. Relič, unpublished).

As far as is known, NGR234 is the only *Rhizobium* which sulphates the 2-*O*-methylfucose residue. However, Nod factors in which the methylfucose is 3-*O*-sulphated have been observed in different *Bradyrhizobium* strains (M. Ferro, personal communication). In *R. meliloti* strain RCR2011, three *hsn* genes, *nodPQ* and *nodH*, are required for 6-*O*-sulphation of the reducing GlcNAc and nodulation of the homologous host, *Medicago sativa* (Roche *et al*., 1991). *nodH* encodes a sulphotransferase which catalyses the transfer of sulphate from 3′-phosphoadenosine 5′-phosphosulphate (PAPS) to Nod factors (Schultze *et al*., 1995; Ehrhardt *et al*., 1995). PAPS, the activated sulphuryl donor, is synthesized by NodP and NodQ (Schwedock and Long, 1992).

Although two dispersed sequences homologous to *nodPQ* of *Rhizobium meliloti* were identified on the chromosome of NGR234 (Perret *et al*., 1991), homologues of *nodH* have not been found in NGR234. Here we show that transfer of a specific locus, *noeE* of pNGR234*a*, to *Rhizobium fredii* USDA257 extends the host range of the recipient to include *Calopogonium caeruleum* and results in the production of sulphated NodUSDA. *R. fredii* USDA257 was chosen as the recipient strain because it is genetically closely related to NGR234, and above all, produces fucosylated, but neither acetylated nor sulphated Nod factors (Bec-Ferté *et al*., 1994). Thus, *noeE* is a host-specificity gene, mutation of which abolishes sulphation of NGR234 Nod factors and nodulation of the edible yam *Pachyrhizus tuberosus*.

Results

Cloning of the NodNGR-sulphation locus

As sequences homologous to *nodH* of *R. meliloti* could not be detected in NGR234, we developed another approach to identify the sulphation locus. Assuming that the gene(s) is inducible by flavonoids (Fellay *et al*., 1995b) and is not shared by *R. fredii* USDA257 (Perret *et al*., 1994), a 7.2 kb *Hin*dIII DNA fragment of *hsnIII* downstream of the *nodABCIJ–nolO–noeI* operon was a likely candidate. This fragment was cloned into a broad-host-range plasmid to yield pMZ (Fig. 1) and transferred to USDA257. After induction with 10^{-6} M apigenin, Nod factors produced by the transconjugant USDA(pMZ) were labelled with [1-^{14}C]-D-glucosamine and analysed by reverse-phase thin-layer chromatography (TLC). Two major spots were detected in lipophilic extracts from the supernatant: spot A (retardation factor (Rf) ≅ 0.62) corresponds to Nod factors that are either acetylated or non-substituted on the 2-*O*-methylfucose; spot B (Rf ≅ 0.86) corresponds to sulphated Nod factors (Fig. 2a). Spot B is absent in extracts prepared from USDA257 cultures, showing that the appearance of the sulphated molecules is linked to pMZ. By subcloning increasingly smaller fragments, sulphation activity was restricted to a 3.4 kb *Bgl*II–*Hin*dIII fragment (pMZ*BgH*) (Fig. 1). Sequence analysis (EMBL Accession Number Y09415) revealed an open

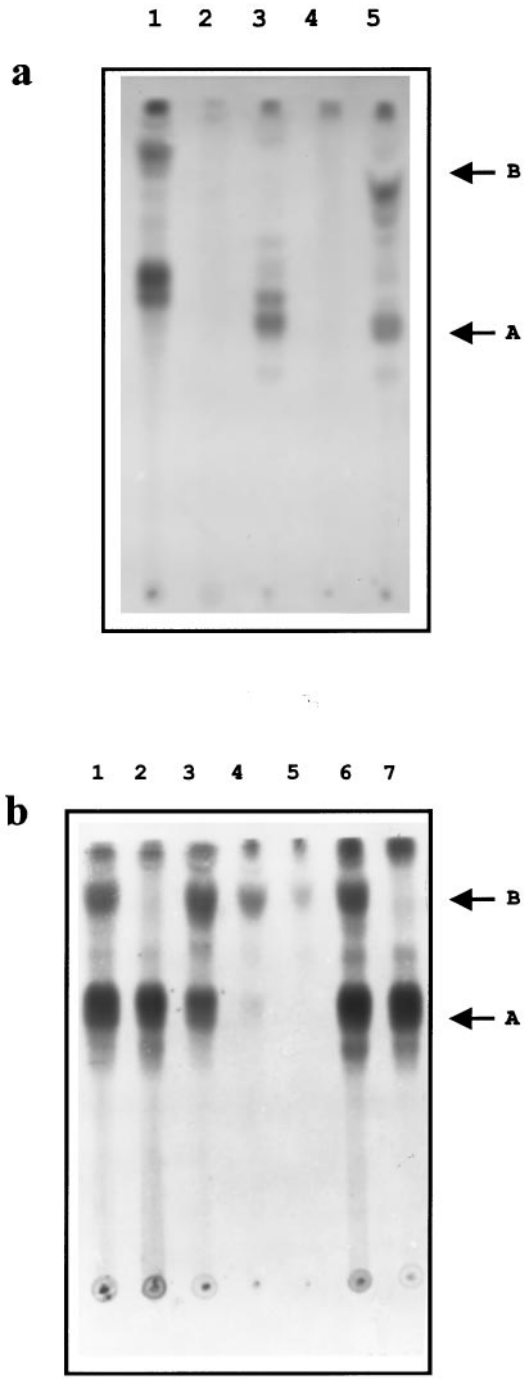


Fig. 2. Reverse-phase TLC analysis of radiolabelled LCOs. All samples except those indicated were labelled with [^{14}C]-glucosamine. Spots A and B indicate the position of non-sulphated and sulphated Nod factors, respectively.
A. Lane 1, apigenin-induced NGR234; lane 2, non-induced NGR234; lane 3, apigenin-induced USDA257; lane 4, non-induced USDA257; and lane 5, apigenin-induced USDA257(pMZ).
B. Lane 1, apigenin-induced NGR234; lane 2, apigenin-induced NGRΔ*noeE*; lane 3, apigenin-induced NGRΔ*noeE*(pMZ*BgH*); lane 4, apigenin-induced NGRΔ*nodZ*(pGMI449); lane 5, NGRΔ*nodZ*(pGMI449) induced by apigenin and labelled with [^{35}S]-Na_2SO_4; lane 6, apigenin-induced NGRΔ*noeE*(pGMI449); and lane 7, apigenin-induced NGRΔ*nodZ*.

reading frame (ORF) of 1278 bp (*noeE*) preceded by a well conserved *nod*-box consensus sequence 230 bp upstream of the probable translational start codon.

Structural analysis of the Nod factors produced by R. fredii *harbouring* noeE

Nod factors were purified from culture supernatants of USDA257(pMZ*BgH*) transconjugants by reverse-phase high-performance liquid chromatography (HPLC). As with NGR234, two major fractions were found (F1 and F2) which reacted positively with anthrone. Each fraction was analysed by fast-atom bombardment/mass spectrometry (FAB/MS) in the positive-ionization mode for non-sulphated molecules (F2) and both in the positive- and negative-ionization mode for sulphated products (F1). Molecular ions corresponding to non-sulphated LCOs (F2; a mixture of tri-, tetra-, and pentamers of GlcNAc with fucose or methylfucose on the reducing end and *N*-acylated on the non-reducing terminus by $C_{18:1}$, $C_{18:0}$ or $C_{16:1}$; see Fig. 4 later) are identical to those described for the wild-type strain USDA257 (Bec-Ferté *et al*., 1994; Jabbouri *et al*., 1995). Analysis of fraction F1 in the positive-ion mode indicated that the non-reducing glucosamine is identical to that of the wild type. On the other hand, pseudomolecular ions were shifted up by 80 Da to m/z 1482.7, 1279.7, and 1076.7, which correspond to $[M+H]^+$ ions having an additional sulphate group on penta-, tetra-, and trimeric Nod factors, respectively, acylated by $C_{18:1}$ (Fig. 3 shows the pentameric form). Ionization in the negative mode gave an identical series of $[M+H]^-$ ions (-2 Da) as shown in Fig. 3C (in which $C_{18:1}$ pentamers sulphated on the fucose or the methylfucose are indicated). The fragmentation pattern of the $[M+H]^+$ ions m/z 1482.7 to m/z 1402.7 shows that the sulphate group is located on the fucose residue (Fig. 3B). These results confirm the role of *noeE* in the sulphation of fucosylated LCOs. By studying the monosaccharide composition following acid hydrolysis and gas chromatography/mass spectrometry (GC/MS) analysis of the alditol acetate derivatives, a decrease in the ratio of moles of 2-*O*-methylfucose to moles of fucose (2.8 to 0.5) was observed after introduction of the *noeE* gene into wild-type USDA257.

*Nod factors produced by an NGR*Δnoe*E mutant are not sulphated*

To confirm that the sulphotransferase activity is dependent on the *noeE* gene, a NGR234 mutant was constructed by replacing the 320 bp *Eco*RI fragment containing the 5′ end of *noeE* with a spectinomycin-resistant (Sp^R) Omega interposon (see the *Experimental procedures*). RMM medium (Broughton *et al*., 1986) was supplemented with [1-^{14}C]-D-glucosamine, and lipophilic extracts were

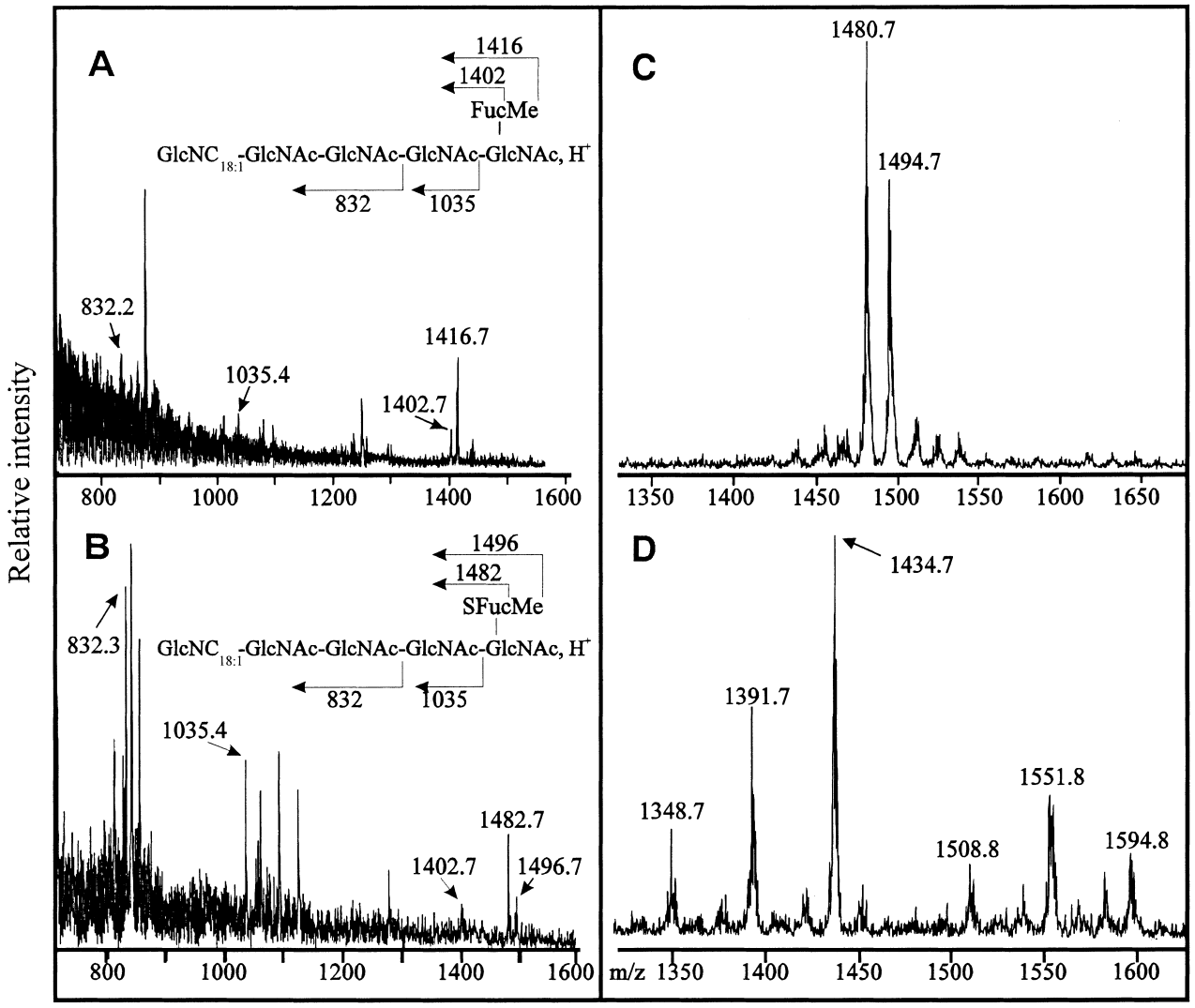
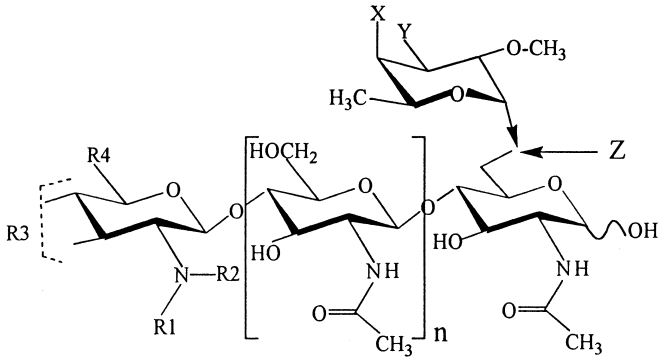


Fig. 3. A and B. FAB/MS spectra in the positive-ionization mode of pentameric LCOs acylated with vaccenic acid from wild-type USDA257 (A) and from the transconjugant USDA257(pMZ*BgH*) (B). The molecular ions $[M+H]^+$ at m/z 1402.7 of fucosylated molecules and 1416.7 of methylfucosylated molecules from spectrum A are shifted up by 80 Da in USDA257(pMZ*BgH*), and the fucosylated form is predominant in spectrum B.
C and D. FAB/MS spectra in the negative-ionization mode of sulphated LCOs from USDA257(pMZ*BgH*) (C) and from NGRΔ*noeE*(pGMI449) transconjugants (D). The major peaks of spectrum C correspond to $[M+H]^-$ ions of LCOs with sulphated fucose (m/z 1480.7) or sulphated methylfucose (m/z 1494.7) at the reducing terminus. Ions from spectrum D at m/z 1348.7, 1391.7 and 1434.7 correspond to sulphated but not fucosylated LCOs, as well as non-carbamoylated, or mono-carbamoylated or bis-carbamoylated LCOs, respectively. The $[M+H]^-$ pseudomolecular ions of m/z 1508.8, 1551.8 and 1594.8 are 160 Da heavier than those produced by NGRΔ*nodZ*$_1$(pGMI449) and correspond to molecules possessing an additional methylfucose group. Similar data was also obtained in the positive-ionization mode (data not shown).

prepared from the supernatants in which NGRΔ*noeE* or wild-type NGR234 had grown. Reverse-phase TLC analysis (Fig. 2b) showed that mutation of *noeE* resulted in the disappearance of spot B which corresponds to sulphated LCOs. Nod factors produced by the mutant were extracted, purified by HPLC and analysed by FAB/MS. In the negative-ionization mode, no significant ions corresponding to sulphated products were found from fractions collected at the expected HPLC retention time of 17.5 min (Price *et al*., 1992). In the positive-ionization mode, however, LCOs purified from a second major fraction (retention time = 22 min) yielded fragments and molecular ions $[M+H]^+$ identical to those of non-sulphated products produced by the wild-type NGR234. Confirmation that disruption of *noeE* is responsible for the disappearance of sulphated Nod factors was obtained by complementing the mutant NGRΔ*noeE* with pMZ*BgH*. Complementation restored spot B which corresponds to sulphated molecules (Fig. 2b). Nod factors produced by a mutant in which an Omega fragment was inserted in the *Bgl*II site downstream



	NGR234	USDA257	NGR234Δ*noeE*	USDA257 + *noeE*
R1	$C_{18:1}$, $C_{18:0}$, $C_{16:1}$	$C_{18:1}$, $C_{18:0}$, $C_{16:1}$	$C_{18:1}$, $C_{18:0}$, $C_{16:1}$	$C_{18:1}$, $C_{18:0}$, $C_{16:1}$
R2	CH_3	H	CH_3	H
R3	Carb or OH	OH	Carb or OH	OH
R4	Carb or OH	OH	Carb or OH	OH
n	3	1 or 2 or 3	3	1 or 2 or 3
X	Ac or OH	OH	Ac or OH	OH
Y	SO_4H or OH	OH	OH	SO_4H or OH
	NGR234Δ*nodZ*	**NGR234Δ*nodZ* + *nodH***	**NGR234Δ*noeE*+*nodH***	
R1	$C_{18:1}$, $C_{18:0}$, $C_{16:1}$	$C_{18:1}$, $C_{18:0}$, $C_{16:1}$	$C_{18:1}$, $C_{18:0}$, $C_{16:1}$	
R2	CH_3	CH_3	CH_3	
R3	Carb or OH	Carb or OH	Carb or OH	
R4	Carb or OH	Carb or OH	Carb or OH	
n	3	3	3	
X	----	-----	Ac or OH	
Y	----	-----	SO_4H or OH	
Z	OH	SO_4H or OH	SO_4H or OH	

Fig. 4. Structure of the major LCOs produced by *Rhizobium* sp. NGR234, *R. fredii* USDA257, and various derivatives thereof. n is the number of *N*-acetyl-D-glucosamine residues; R1 represents acyl chains, the number of double bonds and the length of which are indicated in the table; carb indicates carbamate group.

of *noeE* are identical to those produced by the wild-type bacterium, delimiting the sulphation locus to *noeE* (data not shown).

The fucose moiety is required for sulphation by NoeE and can be sulphated by NodH

The absence of sulphated NodNGR factors was also observed after disruption of the *nodZ* gene. This gene encodes a fucosyltransferase which is not linked to *noeE* (Quesada-Vincens *et al*., 1997). Nod factors produced by the *nodZ* mutant (NGRΔ$nodZ_1$) are neither fucosylated nor sulphated (Fig. 4), suggesting that the sulphation activity associated with NoeE is unable to act on sites other than the fucosyl moiety.

As mentioned above, transfer of sulphate from PAPS to *O*-6 of the reducing GlcNAc of NodRm factors is catalysed by NodH (Ehrhardt *et al*., 1995). The absence of fucose at position C_6 of the reducing GlcNAc of Nod factors produced by NGRΔ$nodZ_1$ should allow NodH to produce C_6-sulphated molecules. To test this theory, a broad-host-range plasmid containing *nodH* of *R. meliloti* (pGMI449) was transferred into NGRΔ$nodZ_1$. After labelling with [1-^{14}C]-D-glucosamine, the presence of sulphated Nod factors in the culture supernatant of the transconjugant was shown by TLC analysis. The ratio of sulphated (spot B) to non-sulphated (spot A) molecules was 5.8, as determined by densitometric scanning of the corresponding autoradiograph (Fig. 2b). To confirm the position of the sulphate group, LCOs from the transconjugant NGRΔ$nodZ_1$(pGMI449) were analysed by FAB/MS. In the positive-ion mode, fragment ions and pseudomolecular ions donated by the non-sulphated fraction were identical to those from NGRΔ$nodZ_1$. On the other hand, pseudomolecular ions from the sulphated fraction were shifted up by 80 $[M+H]^+$ or 102 $[M+Na]^+$ Da, demonstrating the presence of sulphated products. The sulphate group was assigned to the reducing GlcNAc by examining

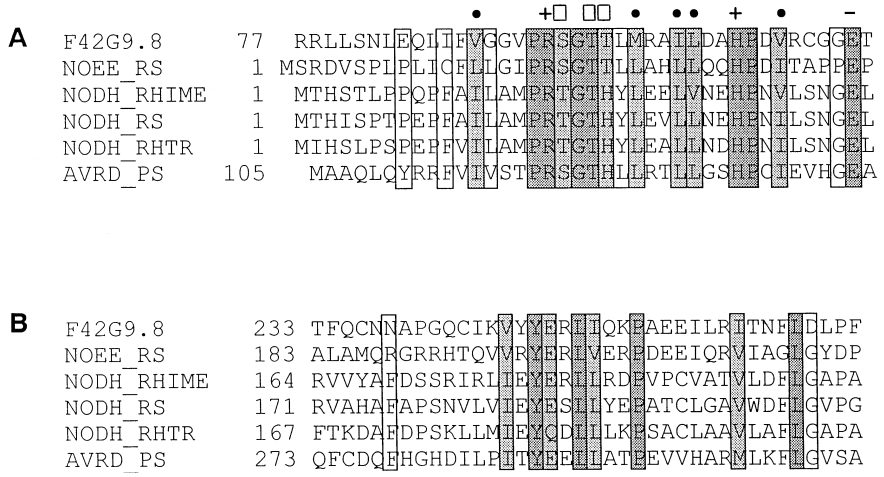


Fig. 5. Relationship of NoeE to other sulphotransferases. Partial alignments between NoeE from NGR234 (NOEE_RS; Accession No. Y09415) and NodH from *R. meliloti* (NODH_RHIME; SWISSPROT Accession No. P06236), *Rhizobium* sp. N33 (NODH_RS; GenBank/EMBL Accession No. U53327), and from *R. tropici* (NODH_RHTR; SWISSPROT Accession No. P52994) are shown. The predicted products of F42G9.8 from *C. elegans* (F42G9.8; GenBank Accession No. 1216308) and ORF4 of *P. syringae* pv. *tomato* (AVRD_PS; EMBL Accession No. J03681) are also included. Dark grey boxes represent identical residues of all six sequences, light grey boxes mark residues belonging to the same group, and white boxes highlight residues present in at least four sequences. Dots represent hydrophobic residues and rectangles represent polar residues, while the plus and minus signs indicate positively and negatively charged amino acids, respectively.

the fragmentation pattern of the $[M+H]^+$ ions (data not shown). GC/MS analysis of monosaccharides liberated by acid hydrolysis confirmed the absence of fucose or 2-*O*-methyl-L-fucose, showing that NodH sulphated the reducing GlcNAc at C_6.

To test if NodH is specific for the C_6 position of the reducing GlcNAc, pGMI449 was introduced into the *noeE* mutant, NGRΔ*noeE*. Production of sulphated LCOs was restored as shown by TLC analysis (Fig. 2b). In this case, the ratio between sulphated (spot B) and non-sulphated (spot A) molecules was 1.2. FAB/MS analysis of the non-sulphated products in the positive-ionization mode (spot A) revealed that they are identical to those produced by the wild-type strain. Two types of sulphated products were produced, however. Approximately 70% of these were identical to those produced by the transconjugant NGRΔ*nodZ*$_1$(pGMI449), i.e. they had no fucosyl moiety and a sulphate group on the reducing GlcNAc. An additional 30% of the sulphated molecules were 2-*O*-methyl-fucosylated. Fragmentation patterns located the sulphate group on the fucosyl moiety. In the negative-ionization mode, the presence of triplicate pseudomolecular ions corresponding to uncarbamoylated, mono- or bis-carbamoylated molecules was also confirmed (Fig. 3D).

Expression of the noeE *gene is controlled via a flavonoid-inducible* nod*-box promoter*

As mentioned above, the *noeE* gene is part of a flavonoid-inducible locus and is associated with an upstream *nod*-box promoter. Because production of sulphated Nod factors requires induction (Fig. 2), a 900 pb *Eco*RI–*Sph*I fragment containing the *noeE nod* box was cloned into pMP220, a broad-host-range β-galactosidase transcriptional fusion vector (Spaink *et al*., 1987). The resulting construct pNB*noeE* was then conjugated into NGR234. In non-induced cultures, the promoter was expressed at very low levels (less than 240 Miller units), and induction with apigenin increased the activity more than 14-fold (>3400 Miller units). Similar inducing activities were observed with daidzein and luteolin.

NGR234 possesses two copies of the transcriptional regulator *nodD*, one of which, *nodD1*, activates and the other, *nodD2*, represses expression of the *nodABC* genes (Fellay *et al*., in preparation). To test whether activity of the *noeE* gene is modulated by NodD proteins, pNB*noeE* was introduced into the *nodD* mutants. *noeE nod*-box activity was completely abolished in NGRΔ*nodD1* and unchanged in NGRΔ*nodD2*.

Structure of the noeE *gene*

Potentially, the *noeE* gene codes for a protein of 47 kDa with a calculated pI of 5.9. BLAST searches (Altschul *et al*., 1990) revealed weak and partial homology with F42G9.8, a gene product of *Caenorhabditis elegans* (GenBank Accession No. 1216308) of unknown function. As *noeE* probably codes for a sulphotransferase, comparisons were made between NoeE and NodH of *R. meliloti* (SWISSPROT Accession No. P06236), *Rhizobium tropici* (SWISSPROT Accession No. P52994), and *Rhizobium* sp. N33 (GenBank/EMBL Accession No. U53327), the F42G9.8 gene product from *C. elegans*, and ORF4 of the *avrD* locus of *Pseudomonas syringae* pv. *tomato* (EMBL

Table 1. Nodulation of *C. caeruleum*, *D. intortum*, *P. tuberosus*, *P. tetragonolobus*, and *V. unguiculata* by *Rhizobium* sp. NGR234 and NGRΔ*noeE*.

	Nodules[a] Per Plant Tested[b]				
Strain	*V. unguiculata*	*C. caeruleum*	*P. tuberosus*	*D. intortum*	*P. tetragonolobus*
NGR234	52 ± 0.7	23 ± 1.2	4 ± 1.1	20 ± 1.5	25 ± 1.1
NGRΔ*noeE*	50 ± 2.5	17 ± 1.9	0	9 ± 2.6	15 ± 1.8

a. Mean value for 16 to 30 plants. Standard deviations are included.
b. Plants were harvested 6 weeks (*V. unguiculata*) and 8 weeks (*C. caerulum*, *D. intortum*, *P. tuberosus*, and *P. tetragonolobus*) after inoculation with *Rhizobium* NGR234 or NGRΔ*noeE*.

Accession No. J03681) (which shows homology to NodH). Two regions contain identical as well as conserved residues in all six sequences (Fig. 5). The first region is close to the N-terminus, while the second corresponds, at least partially, to that described by Roche *et al*. (1991), which shares homology with mammalian sulphotransferases.

noeE *is a host specificity of nodulation (*hsn*) gene*

Several different hosts of NGR234 were inoculated with NGRΔ*noeE* and the efficiency of nodulation was compared with that of the wild-type strain. A functional *noeE* gene is required for nodulation of *P. tuberosus* (Table 1). Mutation of *noeE* reduces the number of nodules on *C. caeruleum*, *Desmodium intortum* and *Psophocarpus tetragonolobus*, whereas nodulation of *Vigna unguiculata* is not significantly affected. Furthermore, introduction of *noeE* into USDA257 extended its host range to include *C. caeruleum*, even though the nodules were unable to fix nitrogen. Thus, *noeE* has all the attributes of a true host specificity of nodulation (*hsn*) gene.

Discussion

Both gain- and loss-of-function techniques show that the new nodulation gene *noeE* is involved in sulphation of NodNGR factors. Introduction of *noeE* into USDA257 results in the production of sulphated Nod factors, while mutation of *noeE* in NGR234 abolishes synthesis of sulphated molecules. Three lines of evidence show that NoeE is probably a sulphotransferase. First, USDA257 does not contain sequences which hybridize to *noeE*. Thus, *noeE* could not drive production of sulphated Nod factors in USDA257 by recombination and so rescue a pre-existing, silent pathway. Second, inter-species complementation of the NGRΔ*noeE* mutant by *nodH* of *R. meliloti* restores sulphotransferase activity. Finally, NoeE and NodH share a highly conserved domain of approximately 30 N-terminal charged and polar amino acids. A second motif (Fig. 5B) is part of a domain that shares homology with NodH and mammalian sulphotransferases (Roche *et al*., 1991). This supports the hypothesis that domain A corresponds to the substrate-binding site and that domain B is involved in binding of activated sulphate (Roche *et al*., 1991). It is worth noting that two predicted hydrophobic regions (TM base software: Hofmann & Stoffel, 1993) that possess potential membrane-spanning segments are located in the vicinity of these two blocks of homologies. It is possible that these regions are hydrophobic pockets that are often found in the active sites of enzymes rather than in membrane-spanning segments.

Another interesting feature of NoeE is the predicted α-helix spanning the last 50 amino acid residues. The probability of this helix folding into a coiled coil is 0.9 using the method of Lupas *et al.* (1991) and 0.45 using Berger's PAIRCOIL programme (Berger *et al*., 1995). In coiled coils, α-helices of protein subunits wrap around each other to form multimers (Lupas, 1996). Deletion of the C-terminal domain (pMZ2; Fig. 1) abolishes sulphotransferase activity, suggesting that the active NoeE enzyme may be a multimer and that the predicted coiled-coil region is responsible for its oligomerization.

The sulphation activity dependent on *noeE* is specific for the fucose group of NGR234 Nod factors. This was first shown by the analysis of Nod factors produced by NGRΔ$nodZ_1$ which lack fucose (Quesada-Vincens *et al*., 1997) and which are not sulphated. This shows that the NoeE-sulphation activity is specific to position C_3 of the 2-*O*-methylfucose. When the *R. meliloti nodH* gene (on plasmid pGMI449) is introduced into NGRΔ$nodZ_1$, the C_6 position of the reducing GlcNAc is sulphated. Unlike LCOs from *R. meliloti*, those produced by NGRΔ$nodZ_1$ (pGMI449) are only partially sulphated. Perhaps NodH has a greater affinity for $C_{16:2}$ than $C_{18:1}$ acylated substrates (Schultze *et al*., 1995). The *nodH* gene of *R. meliloti* was also introduced into NGRΔ*noeE* resulting in the production of sulphated LCOs, of which 30% are sulphated on the 2-*O*-methylfucose and the remaining on the C_6 position of the reducing GlcNAc. NodH seems to be able to act both on fucose and GlcNAc moieties, the latter being the preferred substrate. This hypothesis is reinforced by the fact that the ratio of sulphated to non-sulphated molecules is greater in NGRΔ$nodZ_1$(pGMI449) as compared to NGRΔ*noeE*(pGMI449). In USDA257(*noeE*) transconjugants, sulphation and methylation of the fucose residue seem to be metabolically linked. Thus, the ratio of 2-*O*-methylfucose to fucose on Nod factors is lower when *noeE* is expressed. Similar results are obtained when the *nodPQH* genes of *R. meliloti* are introduced

Table 2. *Rhizobium* sp. and plasmids used in this study.

Strains/plasmids	Characteristics	Source/Reference
Strain		
NGR234	Rif^R derivative of wild-type strain NGR234 from *Lablab purpureus*	Lewin *et al.* (1990)
USDA257S1	Km^R derivative strain of wild-type USDA257, contains a silent Tn*5* insertion in the Sym plasmid	Heron *et al.* (1989)
NGRΔ*nodD1*	NGR234 derivative containing an Ω insertion in the *Bam*HI site of *nodD1*, Rif^R Sp^R	Relič *et al.* (1993)
NGRΔ*nodD2*	NGR234 derivative containing an Ω insertion in the *Bam*HI site of *nodD2*, Rif^R Km^R	R. Fellay *et al.* (in preparation)
NGRΔ*nodZ*$_1$	NGR234 carrying an Ω insertion in the *Eco*RV site of *nodZ*, Rif^R Sp^R	Quesada-Vincens *et al.* (1997)
NGRΔ*noeE*	*noeE*-deletion mutant of NGR234, Rif^R Sp^R	This work
Plasmid		
pBluescript-II KS+	Amp^R	Stratagene
pJQ200 SK	pACYC184-derived (p15A) suicide vector, Gm^R	Quandt and Hynes (1993)
pMP220	*IncP* expression vector containing a promoterless *lacZ* gene, Tc^R	Spaink *et al.* (1987)
pNB*noeE*	960 bp *Hin*dIII–*Sph*I fragment containing the *nod* box of *noeE* cloned in pMP220	This work
pRK2013	ColE1 replicon with RK2 *tra* genes, Nm^R Km^R	Figurski and Helsenki (1979)
pRK600	pRK2013 Nm^s::Tn*9*, Cm^R	Finan *et al.* (1986)
pBBR1MCS-5	Broad-host-range cloning vector, Gm^R	Kovach *et al.* (1994)
pRK7813	Broad-host-range *IncP1* vector, Tc^R	Jones and Gutterson (1987)
pMZ	7.2 kbp *Hin*dIII fragment containing *noeE* cloned in pRK7813	This work
pMZ1	3.9 kbp *Pst*I fragment cloned in pRK7813	This work
pMZ2	2.1 kbp *Pst*I–*Hin*dIII fragment cloned in pRK7813	This work
pMZ3	1.2 kbp *Hin*dIII–*Pst*I fragment cloned in pRK7813	This work
pMZ*BgH*	3.4 kbp *Bgl*II–*Hin*dIII fragment containing *noeE* cloned in pBBR1MCS-5	This work
pMZ*HBg*	3.8 kbp *Hin*dIII–*Bgl*II fragment cloned in pBBR1MCS-5	This work
pMZ*E*	3.3 kbp *Eco*RI fragment cloned in pBBR1MCS-5	This work
pGMI449	2.2 kbp *Bam*HI–*Bgl*II fragment carrying *nodH* of *R. meliloti* cloned in pLAFR3, Tc^R	Debellé and Sharma (1986)

Amp^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Gm^R, gentamicin resistance; Km^R, kanamycin resistance; Rif^R, rifampicin resistance; Sp^R, spectinomycin resistance; Tc^R, tetracycline resistance; Nm^s, neomycin-sensitivity gene.

into *R. tropici*. Only sulphated Nod factors are produced, approximately half of which are no longer *N*-methylated (Poupot *et al*., 1995). As *N*-methylation results from an *S*-adenosylmethionine-dependent pathway, it is possible that both sulphation and *N*-methylation use a limited sulphur pool. Unfortunately, the process of *O*-methylation of fucose is unknown.

noeE is essential for nodulation of the edible yam *P. tuberosus* by NGR234, and helps control the number of nodules on *C. caeruleum*, *D. intortum* and *P. tetragonolobus* but not *V. unguiculata*. Purified NodNGR(S) factors allow USDA257 to nodulate *C. caeruleum* (Relič *et al*., 1994), showing that the *noeE* gene helps extend the nodulation capacity of USDA257 to include *C. caeruleum*. Even although the reducing terminus of LCOs produced by NGRΔ*nodZ* (pGMI449) and wild-type *R. meliloti* are identical, this strain is not able to nodulate *M. sativa* (data not shown). This indicates that although the sulphate group is necessary for nodulation of *M. sativa*, the non-reducing end of Nod factors also contributes to the determination of host range (Debellé *et al*., 1996). In other words, the presence of substituents on the non-reducing terminus can have both positive and negative effects on nodulation.

Experimental procedures

Media, strains and bacterial crosses

Bacterial strains and plasmids used in this study are listed in Table 2. *Rhizobium* strains were grown at 28°C on RMM

medium (Broughton *et al*., 1986) containing succinate as the carbon source. Induction of *nod*-genes was performed by adding apigenin at 10 μM. Antibiotics were added at the following concentrations: rifampicin and spectinomycin, 100 μg ml^{-1}; tetracycline, 25 μg ml^{-1} and gentamicin, 15 μg ml^{-1}. Broad-host-range-plasmids were mobilized from *Escherichia coli* DH5α into *Rhizobium* by tri-parental matings using pRK2013 as the helper plasmid (Ditta *et al*., 1980).

*Molecular methods and construction of a NGRΔ*noeE *mutant*

Digestion with restriction endonucleases, bacterial transformation, cloning, and plasmid isolation were performed as described previously (Sambrook *et al*., 1989). A 2390 bp *Hin*dIII–*Xba*I fragment containing *noeE* was cloned into pBluescript-II KS+, and linearized using the same enzymes. The resulting plasmid was then digested by *Eco*RI, leading to an internal deletion of 230 bp in *noeE*. A Sp^R Omega interposon (Prentki and Krisch, 1984) was subsequently inserted into this site. Finally, the entire cassette was cloned into the *Spe*I–*Apa*I sites of the suicide vector pJQ200 SK. This vector carries the *sacB* gene of *Bacillus subtilis*, which is inducible by sucrose and lethal when expressed in Gram-negative bacteria. The recombinant plasmid was then mobilized into NGR234 by tri-parental mating using the helper plasmid pRK2013. Transconjugants were selected and purified on RMM plates containing 100 μg ml^{-1} rifampicin and 50 μg ml^{-1} spectinomycin. Single colonies were grown in liquid RMM and spread on plates containing both antibiotics and 5% (w/v) sucrose. Marker exchange by double cross-over in NGRΔ*noeE* was confirmed by Southern blot analysis.

Construction of gene fusions and monitoring of β-galactosidase activity

A 2.3 kb *Xba*I–*Hin*dIII fragment containing *nod* box–*noeE*, was cloned into pBluescript-II KS+. The resulting plasmid was digested with *Kpn*I–*Sph*I leading to excision of 900 bp of upstream sequences. This fragment was finally cloned into pMP220 (Spaink *et al*., 1987), generating pNB*noeE*. *Rhizobium* strains containing pNB*noeE* were grown in RMM media supplemented with 25 μg ml^{-1} tetracycline to an OD_{600} of 0.1. Apigenin was then added to the cultures to a final concentration of 10^{-6} M. At various times, the levels of β-galactosidase activity were determined according to Miller (1972).

Plant assays

Nodulation tests were performed in Magenta^{TM} jars (Lewin *et al*., 1990). All plants were grown at a day temperature of 28°C, a night temperature of 18°C, and a light phase of 16 h. To distinguish between Nod$^+$ and Nod$^-$ phenotypes, plants were harvested six to eight weeks after inoculation.

Purification of Nod factors

Rhizobia strains were grown at 27°C in 2 l Erlenmeyer flasks containing 1 l of RMM medium with or without 10^{-6} M apigenin (Price *et al*., 1992). Cells were grown to an OD_{600} of 1. After centrifugation (7000 × *g*, 30 min, 4°C), extracellular Nod factors were extracted from the supernatant as described previously (Price *et al*., 1992; Jabbouri *et al*., 1995). To label the Nod factors, RMM was supplemented with [^{14}C]-D-glucosamine (54 mCi mmol^{-1}) or [^{35}S]-Na_2SO_4 (500 Ci mmol^{-1}) (Amersham).

Analytical methods

For TLC analysis, radiolabelled *Rhizobium* cultures were grown to the exponential phase and the supernatant was applied to a C_{18} reverse-phase Sep-Pak (Millipore). After washing with 5 ml of distilled water, Nod factors were eluted with 5 ml of methanol. Following vacuum drying, samples were dissolved in 10 μl of methanol and applied to TLC plates (C_{18} reverse-phase high-performance thin-layer chromatography (RP–HPTLC); Aldrich Chemical Co.). Elution was performed with 9:1 (v/v) methanol/5.5 M ammonia. Separation of LCOs by HPLC was performed as described previously (Jabbouri *et al*., 1995). Mass spectra were recorded on an Autospec instrument (Fisons, VG-Analytical) fitted with a caesium-ion gun working at 20 kV. The acceleration voltage was 8 kV. A 1:1 mixture of meta-nitrobenzyl alcohol and glycerol was used as the matrix. Matrices were acidified with 0.1 M trifluoroacetic acid, or spiked with 10% sodium iodide for use in the positive-ionization mode. After alditol acetate derivatization, the monosaccharide analyses of LCOs were performed in the GC/MS mode by separation on a 15 cm Supelco SP fused silica column (Hewlet Packard) and fitted with an electron-impact ion source.

Acknowledgements

We thank Candy Monteiro and Myriam Ferro, for their help in mass spectrometry analysis, and Frédéric Debellé and Jean Dénarié for supplying *E. coli* (pGMI449). We are grateful to Hani Antoun for his critical review of the manuscript and to Dora Gerber for assistance with many aspects of this work. Financial support for this project was provided by the Fonds National Suisse de la Recherche Scientifique grants 31-30950.91f and 31-36454.92, and by the Université de Genève.

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