In Vitro Sulfotransferase Activity of NoeE, a Nodulation Protein of *Rhizobium* sp. NGR234

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Soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium* liberate morphogenetic lipochitin-oligosaccharides (Nod factors) into legume rhizospheres. Nod factors, which are synthesized by the products of rhizobial nodulation (*nod*) genes, vary in core length as well as in the number and type of substitutions. In *Rhizobium* sp. NGR234, the N-acylated pentamers of N-acetyl-D-glucosamine carry an O-methylfucose group on the reducing terminus that is substituted, on a mutually exclusive basis, with either an acetyl or a sulfuryl group. A sulfotransferase encoded by *noeE* is required for adjunction of activated sulfate donated by 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Here we show that when expressed in NGR234 cured of its symbiotic plasmid (= ANU265) or when purified as a fusion protein (MBP-NoeE), NoeE transfers sulfate from PAPS to fucosylated lipochitin-oligosaccharides. Enzyme assays showed that sulfotransferase activity is dependent on the presence of an acyl group (stearic and vaccenic acids were tested) since no activity was detected when fucosylated oligochitins (oligomers of two to six N-acetyl-D-glucosamine units) were used as substrates. Thus, NoeE is unique in that it is the only characterized sulfotransferase that is specific for fucosylated Nod factors. It probably acts after NodA, which acylates the amino-sugar backbone.

Symbiotic interactions between rhizobia and legumes are based on the exchange of specific signals. In response to flavonoids released by the plant, rhizobia synthesize and excrete lipochitin-oligosaccharides (LCOs), called Nod factors (for recent reviews, see Hanin et al., *in press*; Mergaert et al. 1997; Schultze and Kondorosi 1996). Nod factors are mitogens (Truchet et al. 1991) that also induce membrane depolarization (Ehrhardt et al. 1992), rapid elevations in intracellular free calcium (Ehrhardt et al. 1996; Gehring et al. 1997), root-hair deformation (Heidstra et al. 1994; Price et al. 1992), and entry of bacteria into their hosts (Relić et al. 1994). Together, these responses lead to the formation of root nodules in which differentiated rhizobia reduce atmospheric nitrogen to ammonia.

Many *nod* (or *nol* and *noe*) gene products are involved in the synthesis of Nod factors. NodA, NodB, and NodC are required for the synthesis of the Nod-factor "core" (Atkinson et al. 1994; Geremia et al. 1994; Spaink et al. 1994), which consists of oligomers of three to six N-acetyl-D-glucosamine residues (GlcNAc) that are N-acylated at the nonreducing terminus. Specific *nod* genes are for the most part responsible for the decoration of the Nod-factor backbone in which various adjuncts are added to both termini. These include O-acetyl, O-carbamoyl, and N-methyl groups as well as various fatty acids at the nonreducing end. Acetyl, arabinosyl, fucosyl, or sulfuryl, and, less commonly, glyceryl or mannosyl groups are present on the reducing sugar (Dénarié et al. 1996). Such substitutions vary from one *Rhizobium* sp. to another and play an important role in the control of the host range. As an example, mutation of *nodH* of *R. meliloti* abolishes the production of sulfated Nod factors as well as the ability to nodulate the homologous host *Medicago sativa*. On the other hand, NodH mutants are able to nodulate the heterologous legume *Vicia sativa* (Faucher et al. 1988; Roche et al. 1991). *nodS* encodes an N-methyltransferase that is specific for the nonmethylated products of the *nodBC* genes (Jabbouri et al. 1995; Geelen et al. 1995; Waelkens et al. 1995). A functional *nodS* gene is required for nodulation of *Leucaena leucocephala* (Lewin et al. 1990; Krishnan et al. 1992).

Sulfated Nod factors are found in *Rhizobium* sp. NGR234 (Price et al. 1992), *Rhizobium* sp. N33 (Cloutier et al. 1996), *R. tropici* (Poupot et al. 1993; Laeremans et al. 1996; Folch-Mallol et al. 1996), and *Sinorhizobium teranga* bv. *acaciae* strains ORS1602 and ORS1645 (Lorquin et al. 1997). The *nodPQ* genes of *R. meliloti* are required for sulfation of NodRm factors (Roche et al. 1991). NodP and NodQ form a multi-functional complex that possesses both ATP sulfurylase and adenosine 5'-phosphosulfate (APS) kinase activities. Activities of both enzymes are necessary for the synthesis of 3'-phosphoadenosine 5'-phosphosulfate (PAPS), the sulfate donor (Schwedock et al. 1994). In vitro assays show that NodH catalyzes the transfer of sulfate from PAPS to C-6 of the reducing GlcNAc of NodRm factors (Schultze et al. 1995; Ehrhardt et al. 1995). Recent genetic and chemical data suggest that sulfation of NodNGR factors is dependent on *noeE* (Hanin et al. 1997). In continuation of this work, we show here that NoeE purified as a fusion protein acts only on entire, fucosylated Nod factors.

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RESULTS

In vitro activity of NoeE.

noeE was placed under the control of the transcriptional activator *nodD1* (Hanin et al. 1997) by cloning it and its associated *nod* box into a broad-host-range vector (giving pMZBgH). To enable induction of *noeE* expression by flavonoids, it was necessary, however, to conjugate this plasmid into ANU265 (= NGR234 cured of its symbiotic plasmid) along with *nodD1*, which is carried on the plasmid pA28 (Price et al. 1992). Crude extracts of this construct, ANU265(pA28,pMZBgH), and ANU265(pA28) (negative control) were tested for their capacity to transfer sulfate from PAPS to the fucose moiety of Nod factors.

First, sulfotransferase activity was assayed with USDA257 Nod factors (NodRf factors) as substrates. NodRf factors are mainly mixtures of trimers, tetramers, and pentamers of GlcNAc acylated with vaccenic acid. Unlike the LCOs of NGR234, the 2-*O*-methylfucose (or fucose) is not substituted (Bec-Ferté et al. 1994), and for this reason they are particu-

larly convenient substrates for NoeE. Cell-free extracts of ANU265 (pA28,pMZBgH) and ANU265(pA28) were incubated at 27°C for 2 h with [³⁵S]PAPS. The reaction products were subjected to C₁₈ reverse-phase thin-layer chromatography (TLC) (Fig. 1). Controls consisted of NodNGR factors labeled in vivo with D-[¹⁴C]glucosamine. The major spot at Rf (retardation factor) $\cong$ 0.62 corresponds to NodNGR factors that are either acetylated or nonsubstituted on the 2-*O*-methylfucose (Fig. 1A, lane 1, spot a) while spot b (Rf $\cong$ 0.86) corresponds to sulfated Nod factors (Fig. 1A, lane 1, spot b) (Price et al. 1992). Interestingly, extracts prepared from apigenin-induced ANU265(pA28,pMZBgH) cultures also possess a compound that migrates to about the same position as spot b (Fig. 1A, lane 2). This spot was absent when extracts from ANU265(pA28) were used (Fig. 1A, lane 3). Thus, NoeE is responsible for the in vitro transfer of sulfate from PAPS to USDA257 NodRf. The slightly lower migration rate of sulfated NodRf factors is probably due to fact that they are less oligomerized than those of NGR234, containing three and four GlcNAc's, which makes them more hydrophobic than those with five.

Fig. 1. Silica-gel, thin-layer chromatography (TLC) analysis of *noeE*-dependent activity. Cell-free extracts of (A, lane 2; B, lanes 3 and 4; C, lane 2) ANU265(pA28,pMZBgH) or (A, lane 3; B, lane 2) ANU265(pA28) were incubated with [³⁵S]3'-phosphoadenosine 5'-phosphosulfate (PAPS) and various substrates. After incubation, Nod factors were recovered on C₁₈-SepPack cartridges and eluted with methanol. Portions of the methanolic fraction were spotted onto C₁₈-coated silica plates. Nod factors of NGR234 labeled with D-[¹⁴C]glucosamine produced in vivo by whole cells of NGR234 were loaded as a standard onto lane 1. A, B, and C, Spot a corresponds to Nod factors that are either acetylated or nonsubstituted on the 2-*O*-methylfucose; spot b corresponds to sulfated Nod factors (Hanin et al. 1997). A mixture of Nod factors taken from *Rhizobium fredii* strain USDA257 was used as substrate and incubated with protein extracts of (A, lane 2) ANU265(pA28,pMZBgH) or (A, lane 3; B, lane 2) ANU265(pA28). Purified, deacetylated NodNGR factors V (*N*-Me; MeFuc) acylated by (B, lane 3) C_{18:1} or (B, lane 4) C_{18:0} were used as substrates. C, Comparison of NoeE and NodH sulfotransferase activities with nonfucosylated Nod factors of NGR Ω nodZ₁ (NodNGR Ω nodZ₁-V(*N*-Me,C_{18:1})) with extracts of (lane 2) ANU265(pA28,pMZBgH) and (lane 3) NGR Ω noeE(pGMI449). The spot that migrates close to solvent front in lanes A2 and C3 originates from radioactive PAPS. (s = start point of migration; f = solvent front).

Whereas sulfated NodNGR factors are acylated with C_{16:1} or C_{18:1}, (Hanin et al. 1998), the nonsulfated forms may also possess a C_{18:0} fatty acid (S. Berck, *unpublished*). To test whether modification of the nonreducing terminus prevents recognition of the substrate by NoeE, NodNGR-V(N-Me, MeFuc)(C_{18:0}) or NodNGR-V(N-Me, MeFuc)(C_{18:1}) factors were prepared from those of the wild-type bacterium by mild alkaline hydrolysis. Absence of the acetyl and carbamoyl groups was verified by ¹³C- and ¹H-NMR (Jabbouri et al., *in press*). When added to the *in vitro* system described above, NoeE sulfated both NodNGR-V(N-MeFuc)(C_{18:0}) and NodNGR-V(N-MeFuc)(C_{18:1}) factors (Fig. 1B, lanes 3 and 4). Due to their increased hydrophobicity, C_{18:0} N-acylated LCOs migrate at a slightly slower R_f than the corresponding C_{18:1} acylated ones.

In contrast to NodH, which is able to sulfate fucose and the reducing GlcNAc of LCOs, NoeE is unable to sulfate non-fucosylated Nod factors *in vivo* (Hanin et al. 1997). To see if this is also true *in vitro*, Nod factors produced by

NGRΔ*nodZ*₁, which completely lack fucose (Quesada-Vincens et al. 1997), were incubated with cell-free extracts of ANU265(pA28,pMZBgH) and [³⁵S]PAPS. TLC analyses of the reaction products revealed traces of radioactivity in non-fucosylated Nod factors (Fig. 1C, lane 2), suggesting that they are not good substrates for NoeE. Substrate specificities of NodH and NoeE were compared by incubating cell-free extracts of NGRΔ*noeE*(pGMI449)(*nodH*) with fucosylated NodNGR-V(N-Me,MeFuc,C_{18:1}) factors. In NGRΔ*noeE*(pGMI449), transcriptional activation of *nodH* occurs via its own promoter and *noeE* is inactivated by an interposon (Hanin et al. 1997). Incorporation of labeled sulfate into fucosylated NodNGR factors (Fig. 1C, lane 3) thus confirms earlier suggestions that, while NodH is able to sulfate either the terminal reducing GlcNAc (Ehrhardt et al. 1995) or fucose, NoeE is specific for the latter.

Intracellular localization of sulfotransferase activity.

Membrane and cytosolic fractions of induced and noninduced cell extracts of ANU265(pA28,pMZBgH) were fractionated by ultra-centrifugation (100,000 × *g*, 1 h, 4°C). Sulfotransferase activity of each fraction was assayed by incubating 200 μg of the extract with [³⁵S]PAPS as well as both de-acetylated and de-carbamoylated NodNGR factors. TLC analysis of the methanolic fraction showed that the major sulfotransferase activity was located in the cytosolic fraction (Fig. 2).

Expression of sulfotransferase activity in *Escherichia coli* and purification of NoeE.

To purify NoeE, the gene was cloned into the expression vector pMAL-c2 (see Materials and Methods). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of total protein extracts of *E. coli* DH5α containing pMAL-*noeE* showed that the in-frame insertion of *noeE* downstream of *malE* (Fig. 3) resulted in the expression of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible MBP-NoeE fusion protein with a predicted mass of 90 kDa (Fig. 4, lane 3). This fusion protein was eluted from an affinity column by maltose (Fig. 4, lane 4) (yield ≅ 7 mg of MBP-NoeE per liter of DH5α [pMAL-*noeE*] culture). To further purify NoeE, the fusion protein was fractionated by ion-exchange chromatography on a DEAE Sepharose CL-6B column (Amersham Pharmacia Biotech, Uppsala, Sweden). Sulfotransferase activity was found in several fractions that were pooled and analyzed by SDS-PAGE (Fig. 4, lane 5). NoeE was then released from the appropriate fraction by cleavage with Xa factor, which recognizes the IEGR located between MBP and NoeE. Cleavage produced three major proteins of approximately 47, 43, and 32 kDa (Fig. 4, lane 6). Since NoeE has a predicted molecular mass of 47 kDa, the bands at 32 and 43 kDa probably correspond to the Xa factor and MBP, respectively (Fig. 4, lanes 7 and 8). Surprisingly, the band at 47 kDa (NoeE) was much less intense than the MBP band at 43 kDa. Similar results were obtained when the gels were stained with silver. Nevertheless, only the MPB-NoeE fusion protein possessed sulfotransferase activity (Fig. 5). Perhaps this suggests that NoeE is relatively unstable, a view that is supported by a protein Param stability index of 46. It is also possible that the purified enzyme is rapidly denatured. This is suggested by the loss of sulfotransferase activity after cleavage by factor Xa (Fig. 4, lane 6, asterisk, and Fig. 5, lane 3).

Fig. 2. Thin-layer chromatography (TLC) analysis (with C₁₈-coated silica) of the intracellular location of NoeE activity. The (lane 2) cytosolic and (lane 3) membrane fractions of ANU265(pA28,pMZBgH) were incubated with [³⁵S]3'-phosphoadenosine 5'-phosphosulfate (PAPS) and deacetylated NodNGR factors [NodNGR-V(N-Me, C_{18:1}, MeFuc)]. After 2 h of incubation, Nod factors were recovered with a C₁₈-SepPack. Lane 1, NodNGR factors labeled *in vivo* with D-[¹⁴C]glucosamine (control): spot a corresponds to Nod factors that are either acetylated or nonsubstituted on the methylfucose; spot b represents sulfated Nod factors (Hanin et al. 1997). As the spot, which migrates with the solvent front, only appeared sporadically, it is considered nonsignificant. Lane 4, NodNGR-V(N-Me, C_{18:1}, MeFuc) factors were incubated with ANU265(pA28). (s = start point of migration; f = solvent front).

Comparisons of the sulfotransferase activity of MBP-NoeE and cell-free extracts of ANU265(pA28,pMZBgH) to the Nod factors described above did not reveal any differences in substrate specificity (data not shown), suggesting that fusion to MALE has no effect on activity of the enzyme.

Must fucosylated oligochitins be acylated to serve as substrates for NoeE?

In reactions catalyzed by the NGR234 NodZ protein (see Materials and Methods), unlabeled, fucosylated oligochitins (FOCs) were synthesized with β -GDP-fucose and dimers to

Fig. 3. Construction of pMAL-*noeE*. A DNA fragment containing the entire *noeE* open reading frame was amplified by polymerase chain reaction with PR1/PR2 primers and the cosmid pXB285 as template (Perret et al. 1991). Primer PR1 carries a *Bam*HI site that was used for cloning of *noeE* in frame with *malE*. Restriction sites: B, *Bam*HI; Xb, *Xba*I. The bases marked in bold in PR1 represent the *Bam*HI site, and the letters above correspond to amino acids.

hexamers of GlcNAc: [(GlcNAc)₂ to (GlcNAc)₆]. Structures of the products were confirmed by electro-spray mass spectrometric analysis, which gave the expected molecular ions [M+Na⁺] at m/z = 593, m/z = 796, m/z = 999, and m/z = 1,202 (data not shown).

Sulfotransferase activity was assayed as described above, except that the concentrations of [³⁵S]PAPS and protein extracts were increased two- to 10-fold, and unreacted [³⁵S]PAPS was removed by anion-exchange chromatography. Cell-free extracts of induced and noninduced *E. coli* DH5α(pMAL-c2-*noeE*) or ANU265(pA28,pMZBgH) were unable to transfer sulfate to any of the fucosylated oligochitins. To check that the absence of spots corresponding to sulfated FOCs was not due to the resolving power of the TLC system used, other TLC plates (cellulose-coated and PEI-cellulose plates) were also employed without result (data not shown).

Another potential source of error lies in the difficulty of separating the hydrophilic FOCs, unreacted PAPS, and their derivatives from the sulfated products. To circumvent this problem, we prepared ¹⁴C-labeled FOCs and used them as substrates in sulfation assays with unlabeled PAPS (see Materials and Methods). Silica-gel TLC of the reaction products did not reveal spots corresponding to sulfated oligochitins, even when the reaction products were concentrated by DEAE anion-exchange chromatography (Fig. 6). Nevertheless, when nonfucosylated Nod factors [NodNGR-V(*N*-Me, C_{18:1} or C_{18:0})] were labeled with [¹⁴C]-GDP-fucose by NodZ of NGR234 and used as substrates in sulfotransferase assays, sulfated molecules were detected with reverse-phase TLC (data not shown).

Finally, we tested whether methylation was the missing condition for sulfation of the FOCs. To do this, 2-*O*-methylfucosyl-chitobiose was synthesized chemically (V. Tor-

gov, *unpublished*), and used in standard assays, but the combination 2-*O*-methylfucosyl-chitobiose and MBP-NoeE did not lead to sulfated products (data not shown). Taken together, these data suggest that only acylated and fucosylated LCOs can serve as substrates for the sulfotransferase encoded by *noeE*.

DISCUSSION

Included among the Nod factors secreted by NGR234 are those that are sulfated on C3 of the fucose group (Price et al. 1992). Here we present further evidence that the sulfotransferase required for sulfation of position C3 is encoded by *noeE*. Mutation of *noeE* abolishes synthesis of sulfated LCOs, while introduction of *noeE* into the closely related strain USDA257 permits production of sulfated NodRf factors (Hanin et al. 1997).

Although there is surprisingly little homology between the two proteins, NodH thus fulfills a role similar to that of NoeE of NGR234 (Hanin et al. 1997). Nevertheless, NodH is able to confer sulfotransferase activity on an NGRΩ*noE* mutant. The

Fig. 4. Coomassie blue-stained sodium dodecyl sulfate–polyacrylamide electrophoresis gel showing purification of NoeE. Lane 1, molecular mass markers (Amersham Pharmacia Biotech, Uppsala, Sweden). Lanes 2 and 3, total proteins of un-induced and isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *Escherichia coli* pMAL-*noeE* cells. Lane 4, MBP-NoeE fusion protein eluted from an amylose affinity column. Lane 5, MBP-NoeE fusion eluted from DEAE-Sepharose (arrow). Lane 6, MBP-NoeE fusion digested with factor Xa (NoeE indicated by asterisk). Lanes 7 and 8, reference compounds (Bio-Rad Laboratories, Hercules, CA): maltose binding protein (MBP) and factor Xa, respectively.

Fig. 5. Reverse-phase thin-layer chromatography (TLC) analysis of products from reactions with the purified NoeE sulfotransferase. Lane 1, NodNGR factors labeled with ¹⁴C in vivo. (reference compound, see Figure 1A, lane 1). Lanes 2 and 3, respectively: reaction products formed after incubation with the MBP-NoeE fusion protein (fraction from Figure 4, lane 5) and MBP-NoeE digested with factor Xa (fraction from Figure 4, lane 6). Reaction mixtures also contained [³⁵S]3'-phosphoadenosine 5'-phosphosulfate (PAPS) and deacetylated NodNGR factors (NodNGR-V [*N*-Me, C_{18:1}, MeFuc]). Nod factors were recovered on C₁₈-SepPacks after incubation. (s = start point of migration; f = solvent front).

major difference between these two proteins appears to be that NodH is able to sulfate both nonfucosylated and fucosylated Nod factors, whereas NoeE is specific for fucosylated Nod factors (Hanin et al. 1997). The in vitro assays reported here confirm this observation.

Biochemical proof that NoeE has sulfotransferase activity in vitro was provided by showing that (i) cell-free extracts of ANU265(pA28,pMZBgH) were able to transfer sulfate from PAPS to fucosylated Nod factors, and (ii) a fraction enriched in NoeE as a fusion protein (MBP-NoeE) has the same capacity (Fig. 4, lane 5). Assays with different substrates showed that neither an *N*-methyl group, nor saturated or unsaturated fatty acids on the nonreducing terminus, nor 2-*O*-methyl fucose had any effect on NoeE activity. On the other hand, NoeE is apparently incapable of transferring sulfate to fucosylated oligosaccharides that lack an acyl chain. This contrasts with NodH, which is able to sulfate both acylated and non-acylated oligochitins (Ehrhardt et al. 1995; Schultze et al. 1995). This apparently absolute requirement of NoeE for a fatty acid on the oligochitins strongly suggests that it must act after the basic Nod factors have been synthesized (i.e., after NodA, which acylates the oligomers). Obviously, NoeE must also act after NodZ has fucosylated the molecules.

Fractionation by high-speed centrifugation showed that, like NodA, NodB, NodL, NodS, and NodZ (Carlson et al. 1994), NoeE is cytosolic. Only NodC is located in the inner cytoplasmic membrane (Barny et al. 1996). It thus seems likely that Nod-factor biosynthesis occurs on the cytosolic side of the cytoplasmic membrane.

A number of sulfotransferases have been isolated from various bacterial, plant, and mammalian species. These include phenol sulfotransferases, heparan sulfotransferases, alcohol sulfotransferases, and amine sulfotransferases (Yamazoe et al. 1994). All are distinct, but so far NoeE is the only known enzyme that is specific for fucosylated LCOs.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and cell-free protein extracts.

Bacterial strains and plasmids used in this study are listed in Table 1. *Rhizobium* strains were grown at 27°C in/on RMM3 media (Broughton et al. 1986), using 10⁻⁶ M apigenin to induce *nod* genes. *E. coli* strains were grown in Luria broth supplemented with IPTG (0.3 mM final concentration) and ampicillin (100 µg/ml). Cell-free extracts of ANU265(pA28), ANU265(pA28,pMZBgH), and *E. coli* (pMAL-*noeE*) were prepared by suspending the cells in 10 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.4) supplemented with 1 mM of the protease inhibitor PMSF (phenyl methan-sulfonyl fluoride) and passed through a "French" press. Afterward, the extracts were separated into membrane and cytosolic fractions (Quesada-Vincens et al. 1997).

Purification and labeling of Nod factors.

NodNGR factors were isolated as described previously (Price et al. 1992; Jabbouri et al. 1995). ¹⁴C-labeled NodNGR factors were prepared from overnight cultures of NGR234

Fig. 6. Silica-gel, thin-layer chromatography (TLC) analysis of sulfotransferase activity with fucosylated oligochitins as substrates. ¹⁴C-labeled, fucosylated oligochitins were incubated with 20 µg of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) and 300 µg of protein extract of DH5α pMAL-*noeE*. Lanes 1, 3, 5, 7, and 9: position of the (GlcNAc)₂Fuc, (GlcNAc)₃Fuc, (GlcNAc)₄Fuc, (GlcNAc)₅Fuc, and (GlcNAc)₆Fuc standards. Lanes 2, 4, 6, 8, and 10: reaction mixtures containing ¹⁴C-labeled (GlcNAc)₂Fuc, (GlcNAc)₃Fuc, (GlcNAc)₄Fuc, (GlcNAc)₅Fuc, or (GlcNAc)₆Fuc, respectively. (s = start point of migration; f = solvent front).

grown in the presence of 10^{-6} M apigenin and 1 μ Ci of D- $[^{14}\text{C}]$ glucosamine (specific activity, 54 Ci/mol; Amersham Pharmacia Biotech) (Quesada-Vincens et al. 1997).

Biosynthesis of fucosylated oligochitins.

Cell-free extracts of *E. coli* DH5 α (pPROEX-1Z) were the source of fucosyltransferase activity used to synthesize both radiolabeled and unlabeled fucosylated oligochitins. Labeling reactions in which 100 to 150 μ g of oligochitins [(GlcNAc) $_2$ to (GlcNAc) $_6$] were mixed with 0.1 μ Ci of GDP- $[^{14}\text{C}]$ fucose (50 mCi/mmol; DuPont NEN, Boston) and 400 to 600 μ g of protein extract were performed according to Quesada-Vincens et al. (1997). To synthesize unlabeled, fucosylated oligochitins, 1 mg of di-*N*-diacetylchitobiose (Sigma Chemical, St. Louis, MO), 1 mg of tri-*N*-triacylchitotriose (Sigma), 2 mg of tetra-*N*-tetraacylchitotetraose (Sigma), and 5 mg of penta-*N*-pentaacylchito-pentaose (Seika-gaku, Chuo-ku, Tokyo) were added separately to 500 μ g of β -GDP-fucose. After the addition of 3 to 4 mg of cell-free protein extract, the mixture was incubated overnight at 27°C. Unreacted GDP-fucose was removed by anion-exchange chromatography on DEAE-Sephadex A25 (Amersham Pharmacia Biotech) and the reaction products analyzed by electro-spray-mass spectrometry.

Enzymatic assay of sulfotransferase activity with Nod factors as substrates.

In 100 μ l of buffer A (40 mM KCl, 5 mM MgCl $_2$, 50 mM Tris-HCl, pH7.5), the reaction mixture contained 1 μ Ci $[^{35}\text{S}]$ PAPS (1.6 Ci/mM; DuPont NEN, Boston), 40 nM candidate sulfate acceptors (Nod factors), and 200 μ g of cell-free protein extract. Reactions were incubated at 27°C for 2 h and stopped by boiling for 3 min; the nonsoluble material was removed by centrifugation at $10,000 \times g$ (30 min, 4°C). Then,

the supernatant was loaded onto C $_{18}$ SepPack cartridges (Millipore, Milford, MA) and, after washing with water, the Nod factors were eluted with methanol. The methanolic fraction was concentrated by evaporation and separated on C $_{18}$ reverse-phase TLC plates with 9:1 (vol/vol) methanol/ 5.5 M ammonia as the mobile phase. Autoradiographs were prepared by overlaying the dried TLC plates with Fuji RX film (Fuji Photo Film, Tokyo).

Enzymatic assay of sulfotransferase activity with oligochitins as substrates.

One sixth of the volume of fucosylated oligochitins, synthesized as described above, was incubated overnight (at 27°C) with 2 to 10 μ Ci $[^{35}\text{S}]$ PAPS in buffer A. Afterward, the reaction mixture was applied to a DEAE-Sephadex A25 (Amersham Pharmacia Biotech) anion-exchange column equilibrated with water. Step-wise elution with ammonium acetate (10 ml of 0, 50, 250, 750, and 2 M of NH $_4$ CH $_3$ COOH) removed the majority of the unreacted $[^{35}\text{S}]$ PAPS at a concentration equivalent to 750 mM NH $_4$ CH $_3$ COOH. Sulfated lipooligochitins were eluted at an ammonium acetate concentration of 250 mM. Sulfation of labeled, fucosylated oligochitins was assayed with 20 or 150 μ g of unlabeled PAPS in a reaction mixture of 100 μ l. Two microliters of each reaction mixture was spotted onto Silicagel-60 plates. Chromatography-autoradiography with Silicagel-60 HPTLC plates (Merck, Darmstadt, Germany) and butanol/ethanol/H $_2$ O (45/30/25) as the solvent was used to monitor incorporation of $[^{35}\text{S}]$ sulfate into fucosylated oligochitins.

Candidate sulfate acceptors and their de-acetylation.

Potential substrates for the sulfotransferase encoded by *noeE* included the Nod factors of *R. fredii* strain USDA257 (=

Table 1. Bacterial strains and plasmids^a

Strain/plasmid	Characteristics	Source/reference
<i>Escherichia coli</i> DH5 α	SupE44DlacU169 (F80 <i>lacZ</i> DM15) <i>hsdR17recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan 1983
<i>Rhizobium</i> spp.		
NGR234	Rif ^r derivative of wild-type strain NGR234, from <i>Lablab purpureus</i>	Trinick 1980
ANU265	Heat-cured derivative of NGR234 (Sp ^r) (lacks pNGR234a)	Morrison et al. 1983
ANU265(pA28,pMZBgH)	ANU265 containing pA28, (Tc ^r) and pMZBgH, (Gm ^r)	This work
NGR Ω <i>noeE</i>	Insertion mutant of <i>noeE</i> of NGR234 (Rif ^r ,Sp ^r)	Hanin et al. 1997
NGR Ω <i>nodZ</i> ₁	NGR234 carrying an Ω insertion in the <i>EcoRV</i> site of <i>nodZ</i> (Rif ^r , Sp ^r)	Quesada-Vincens et al. 1997
Plasmids		
pBluescriptKS ⁺	phage f1, <i>lacaZ</i> ⁺ (Ap ^r)	Stratagene, La Jolla, CA
Lorist 2	5.6-kbp cosmid, Km ^r	Gibson et al. 1987
pRK2013	Tra ⁺ helper plasmid for mobilization, (Km ^r)	Figurski and Helinski 1979
pBBR1MCS-5	Broad-host-range cloning vector, Gm ^r	Kovach et al. 1994
pMZBgH	3.4-kbp <i>Bgl</i> III- <i>Hind</i> III fragment containing <i>noeE</i> cloned into pBBR1MCS-5, (Gm ^r)	Hanin et al. 1997
pMAL-c2	Protein expression vector, (Ap ^r)	New England Biolabs, Beverly, MA
pMAL- <i>noeE</i>	1.4-kbp polymerase chain reaction (PCR) fragment containing <i>noeE</i> cloned into pMAL-c2, (Ap ^r)	This work
pProEX-1Z	1.2-kbp PCR fragment containing <i>nodZ</i> cloned into pProEX-1, (Ap ^r Tc ^r)	Quesada-Vincens et al. 1997
pProEX-1	Protein expression vector, (Ap ^r)	Life Technologies, Gaithersburg, MD
pGMI449	2.2-kbp <i>Bam</i> HI- <i>Bgl</i> II fragment carrying <i>nodH</i> of <i>Rhizobium meliloti</i> cloned in pLAFR3, (Tc ^r)	Debellé and Sharma 1986
pA28	2.2-kbp <i>Eco</i> RI- <i>Pst</i> I fragment containing <i>nodD1</i> of NGR234 cloned in pRK7813, (Tc ^r)	Price et al. 1992
pXB285	A Lorist 2 clone from pNGR234a, Km ^r	Perret et al. 1991
pKSB- <i>noeE</i>	1.4-kbp PCR fragment containing <i>noeE</i> cloned into pBluescriptK ⁺ , Ap ^r	This work

^a The following abbreviations are used: ampicillin (Ap), gentamycin (Gm), kanamycin (Km), spectinomycin (Sp), and tetracycline (Tc).

NodRf), de-acetylated and de-carbamoylated NodNGR factors [NGR-V (N-Me, C_{18:1} or C_{18:0}, MeFuc)], as well as the fucosylated oligochitins described above. The lipooligosaccharides of NGR Δ noeE are oligomers of five GlcNAc residues carrying N-linked acyl (C_{18:1} or C_{18:0}) and N-methyl groups and 2-O-linked methyl-fucose [= NGR-V (N-Me, MeFuc, C_{18:1}, or C_{18:0})]. De-acetylation was performed in 0.2 M NaOH (37°C, 1h). Afterward, the reaction mixture was neutralized with 0.2 M HCl, and applied to a C₁₈ reverse-phase SepPack column, washed with water, and eluted with methanol.

Purification of NoeE.

noeE was cloned downstream of the malE gene, which encodes a maltose-binding protein. To do this, polymerase chain reaction with the primers pR₁ and pR₂ was used to amplify noeE and to introduce a BamHI site upstream of the first ATG (see Figure 3). The amplified fragment was then digested with XbaI and cloned into the EcoRV and XbaI sites of pBluescript KS, giving pKS-BnoeE. Finally, pKS-BnoeE was digested with BamHI and XbaI and the 1.4-kb fragment containing noeE was cloned into the protein expression vector, pMAL-c2 (which in turn, was digested by the same enzymes). Then, this recombinant plasmid, pMAL-noeE, was transformed into E. coli DH5 α and the transconjugants screened as described by the manufacturer (New England Biolabs, Beverly, MA). Two hours after induction at 37°C, the fusion protein, called MBP-NoeE, was purified from 1 liter of culture. To do this, the bacteria were recovered by centrifugation, re-suspended in buffer B (20 mM Tris-HCl pH7.4, 200 mM NaCl, 1 mM EDTA), containing 1 mM dithiothreitol (DTT) and 1 mM phenylmethane sulfonyl fluoride, and disrupted in a "French" pressure cell. After centrifugation at 10,000 $\times$ g, the soluble fraction was loaded onto an amylose affinity column (New England Biolabs) pre-equilibrated with buffer B. MBP-NoeE was eluted from this column in buffer B containing 10 mM maltose. MBP-NoeE was then loaded onto a DEAE-Sephacrose (Amersham Pharmacia Biotech) column and eluted with a gradient of zero to 500 mM NaCl in 20 mM Tris-HCl (pH 8.0). Fractions containing MBP-NoeE were concentrated and desalted with Microcon-10 micro-concentrators (Amicon, Beverly, MA). Finally, 10 mg of this fusion protein was digested with 0.2 μ g of Xa factor for 18 h at 4°C. After each purification step, the degree of purity was checked by SDS-PAGE on 12% polyacrylamide gels that were stained with Coomassie blue.

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