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Nodulation protein NodL of *Rhizobium leguminosarum* O-acetylates lipo-oligosaccharides, chitin fragments and N-acetylglucosamine *in vitro*

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Summary

Upon induction of their nodulation genes, the root nodule-inducing *Rhizobium* bacteria produce lipo-oligosaccharide signal molecules. All lipo-oligosaccharides identified from *Rhizobium leguminosarum* bv. *viciae* carry an O-acetyl group at the C-6 position of the non-reducing terminal sugar, the presence of which is important for biological activity and host specificity. Previously we showed that a functional *nodL* gene product is required for the presence of this O-acetyl moiety. The production of polyclonal antibodies against isolated NodL protein, using a NodL-overproducing *Escherichia coli* strain is described. These antibodies were used (i) to elucidate the subcellular localization of the NodL protein, which appeared to be present in the cytosol, and (ii) for the purification of native NodL protein from *E. coli*. Here we provide biochemical proof that purified NodL protein has transacetylating activity *in vitro* with acetyl-CoA as the acetyl donor. NodL protein appeared to be able to acetylate various substrates, such as lipo-oligosaccharides, chitin fragments and N-acetylglucosamine. For chitinpentaoase as the substrate we have shown, using mass spectrometry and NMR spectroscopy, that NodL protein substitutes one O-acetyl group at the C-6 position of the non-reducing terminal sugar.

Introduction

Bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*, collectively referred to as rhizobia, are

able to induce nodules on the roots of leguminous plants (for a review see Brewin, 1991). The bacteria infect the roots where they induce the formation of nodules in which they differentiate into bacteroids. The plant supplies the bacteroids with a carbon source, whereas the bacteroids convert atmospheric nitrogen into ammonia, which in turn is used by the plant as a nitrogen source. This symbiosis is a host-specific process, in which each bacterial species or biovar nodulates a particular set of host plants (Fisher *et al.*, 1992).

The host specificity is determined by a two-way signal-exchange between plant and bacterium. In the first step the host plant excretes flavonoids which are sensed by the bacteria present at or near the root surface, thereby activating the constitutively formed *nodD* gene product (for a review see Schlaman *et al.*, 1992). This leads to the transcription of the other inducible *nod* genes. A specific recognition by NodD of the flavonoids secreted by the host plant appears to be a determinant of host specificity (Spaijk *et al.*, 1987; Horvath *et al.*, 1987). In the second step the bacterium, through its activated *nod* genes, produces Nod metabolites that have been characterized as lipo-oligosaccharides (for a review see Dénarié *et al.*, 1992). Purified lipo-oligosaccharides can induce several biological responses on the host plant root which are often indistinguishable from those occurring in the first stages of infection and nodule formation. Examples are pre-infection thread formation (Van Brussel *et al.*, 1992) and nodule primordium formation (Spaijk *et al.*, 1991; Truchet *et al.*, 1991).

Structural analysis of Nod metabolites from various rhizobial strains have shown that they have a basic structure consisting of a β -1,4 linked N-acetylglucosamine sugar backbone and an N-acyl group substituted on the non-reducing terminal sugar. The length of the sugar backbone varies from three to five residues. Structural variability is also present in the fatty acyl chain. For instance, *Rhizobium meliloti* Nod metabolites contain a C16 acyl group with two or three double bonds (Lerouge *et al.*, 1990; Schultze *et al.*, 1992), whereas lipo-oligosaccharides of *Rhizobium leguminosarum* bv. *viciae* contain a C18 chain with one or four double bonds (Spaijk *et al.*, 1991). Furthermore, additional groups can be attached to the sugar backbone. For example, an O-acetyl group can be attached to C-6 of the non-reducing terminal residue

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in *R. meliloti* and *R. l. bv. viciae* and a sulphate group is attached to the C-6 position of the reducing terminal residue in *R. meliloti* (Lerouge *et al.*, 1990; Spaink *et al.*, 1991; Roche *et al.*, 1991; Schultze *et al.*, 1992). Recently, other modifications have been reported to be present in the lipo-oligosaccharides of other rhizobia. For example, other additional sugar moieties can be present, like a 2-*O*-methylfucose in *Bradyrhizobium japonicum* and *Rhizobium* sp. strain NGR234 (Sanjuan *et al.*, 1992; Price *et al.*, 1992; Carlson *et al.*, 1993) and *D*-arabinose in *Azorhizobium caulinodans* (Mergaert *et al.*, 1993). Carbamoyl and *N*-methyl groups can be present in *Rhizobium* sp. strain NGR234 and *A. caulinodans* (Price *et al.*, 1992; Mergaert *et al.*, 1993). Some of these structural variations were shown to be determinants of the host specificity of nodulation (Spaink *et al.*, 1991; Truchet *et al.*, 1991).

Homologies at the amino acid level with other functionally known proteins and *nod* mutant studies have given indications about the functions of some Nod proteins (for a review see Spaink, 1992). *In vitro* studies have been scarce. For NodP and NodQ, it has been shown *in vitro* that they function together as an ATP-sulphurylase (Schwedock *et al.*, 1990). For NodB, *in vitro* studies have shown that it functions as a chito-deacetylase (John *et al.*, 1993).

In *R. l. bv. viciae*, all identified lipo-oligosaccharides carry an *O*-acetyl group, which is required for pre-infection

thread formation and nodule meristem formation by purified Nod metabolites (Spaink *et al.*, 1991; Van Brussel *et al.*, 1992). Analysis of *nodL* mutants has shown that the NodL protein is required for the presence of the *O*-acetyl group (Spaink *et al.*, 1991). Furthermore, the NodL protein shows homology with acetyltransferases LacA, CysE and NifP and acyltransferase LpxA (Downie, 1989; Evans *et al.*, 1991; Baev *et al.*, 1992).

In this paper we describe the purification of NodL protein and provide biochemical proof that it has transacetylating activity *in vitro* using acetyl-CoA as the acetyl donor. The various candidate acceptors tested, lipo-oligosaccharides, chitin fragments and *N*-acetylglucosamine appeared to be acetyl-accepting substrates.

Results

Overproduction of NodL protein

In order to overproduce NodL protein in *Escherichia coli*, the *nodL* gene of *R. l. bv. viciae* was cloned in expression vector pET9A, resulting in plasmid pMP3401 (Fig. 1). *E. coli* strain BL21(DE3) harbouring plasmid pMP3401 produced an IPTG-inducible protein, which migrated as a 20 kDa protein in SDS-PAGE (Fig. 2A, lane 4). This apparent molecular weight is in agreement with the predicted size of the NodL protein (Canter Cremers *et al.*,

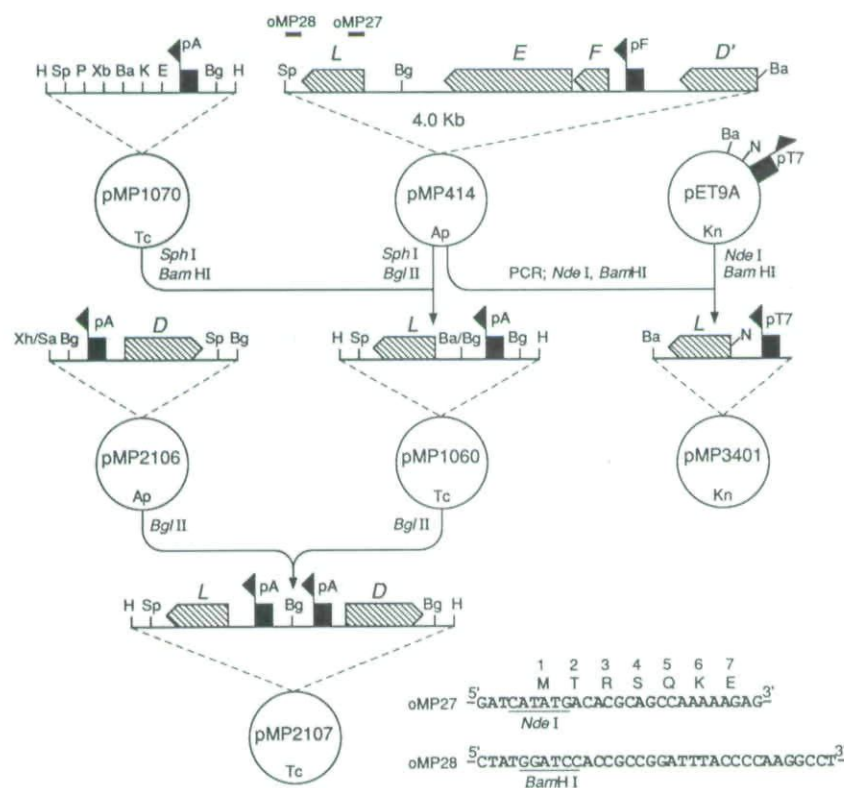


Fig. 1. Construction of plasmids. pMP414 contained a *R. l. bv. viciae nod* region of pRL1J1 (Canter Cremers *et al.*, 1989). pMP3401 and pMP2107 are expression plasmids for the overproduction of NodL protein in *E. coli* and *Rhizobium*, respectively. Both plasmids contain the entire *nodL* gene cloned behind the *nodA* and T7 promoters, respectively. pMP1070 is a derivative of pMP263 (Spaink *et al.*, 1987) and is used as an expression vector. pMP2106 contained a complete *nodD* gene from *R. l. bv. trifolii*, which was cloned into pMP1060 resulting in pMP2107. Suitable restriction sites for cloning the *nodL* gene in expression vector pET9A (Studier *et al.*, 1990) were introduced by PCR with primers oMP27 and oMP28. Hatched boxes indicate *nod* gene sequences; black boxes with an arrow on top represent promoters. Abbreviations: Tc, Ap and Kn, tetracycline, ampicillin and kanamycin-resistance genes, respectively. Restriction sites: H, HindIII; Sp, SphI; P, PstI; Xb, XbaI; Ba, BamHI; K, KpnI; E, EcoRI; Bg, BglII; N, NdeI; Xh, XhoI; Sa, SalI.

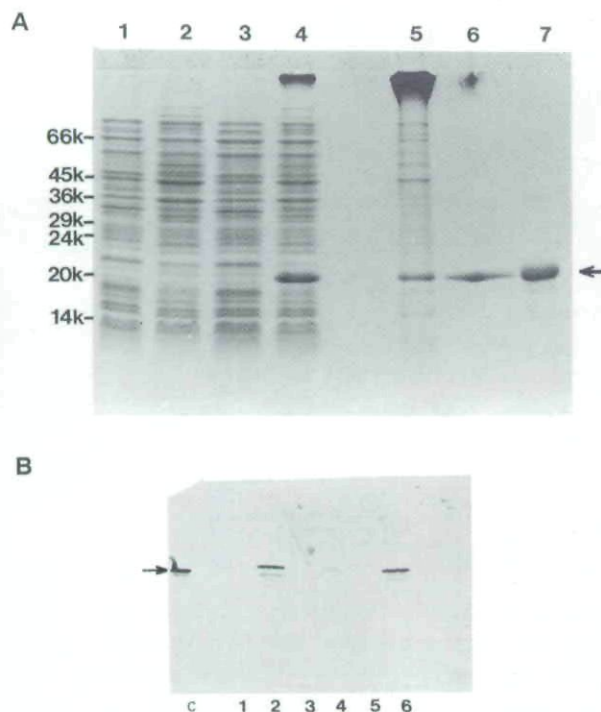


Fig. 2. A. SDS–polyacrylamide gel analysis of overproduction and purification of NodL protein from *E. coli*. Lanes 1 through to 4 show total cell protein profiles, whereas lanes 5 through to 7 show the subsequent purification steps. The position of NodL protein is indicated by an arrow. Lanes: 1 and 2, *E. coli* BL21(DE3) harbouring pET9A grown in the presence or absence of IPTG; 3 and 4 *E. coli* BL21(DE3) harbouring pMP3401 grown in the presence or absence of IPTG; 5, protein aggregates isolated by low-spin centrifugation; 6, denatured protein aggregates after isolation out of gel; 7, NodL purified out of gel.

B. Western blot analysis of total cell proteins of *E. coli* and *Rhizobium* using antibodies raised against NodL protein. Approximately 10^6 *E. coli* cells and 10^8 *Rhizobium* cells were used for analysis. Lanes: c, reference: purified NodL protein from *E. coli* harbouring pMP3401 used for raising antibodies; 1 and 2, cell proteins of *E. coli* BL21(DE3) harbouring pMP3401 grown in the absence or presence of IPTG; 3 and 4, cell proteins of *R. l. bv. viciae* strain 248 grown in the absence or presence of naringenin; 5 and 6, cell proteins of *Rhizobium* strain LPR5045 harbouring pMP2107 grown in the absence or presence of naringenin. The position of NodL protein is indicated by an arrow.

1989). After electrophoresis protein aggregates were observed in the border region of the stacking and separating gels (Fig. 2A, lane 4). Such protein aggregates could be isolated after lysis of the cells by low-spin centrifugation (Fig. 2A, lane 5). The aggregates, presumably inclusion bodies, were purified by excision out of the SDS–polyacrylamide gel. After denaturation in urea, it appeared that these aggregates mainly consisted of a 20 kDa protein (Fig. 2A, lane 6). This band was isolated from the gel and used for immunization of a rabbit. Collected serum reacted with the IPTG-inducible 20 kDa protein, as shown by Western blot analysis (Fig. 2B, lanes c, 1 and 2). The serum also reacted with a flavonoid-inducible 20 kDa

protein of wild-type strain *R. l. bv. viciae* strain 248 and the NodL-overproducing *Rhizobium* strain LPR5045 harbouring pMP2107 (Fig. 2B, lanes 3, 4, 5, and 6). In the induced situations of BL21(DE3) harbouring pMP3401, and LPR5045 harbouring pMP2107, the serum also reacted with a small band running slightly faster than the 20 kDa protein (Fig. 2B, lanes 2 and 6). Since this band is only observed in preparations from induced cells, it is likely to represent a specific degradation product of the NodL protein. From these results we conclude that the antiserum specifically reacts with NodL protein.

Subcellular localization of NodL protein

Cells of the wild-type *R. l. bv. viciae* strain 248 were grown in the absence or presence of the inducer naringenin. After fractionation into periplasmic, cytoplasmic and membrane components, immunoanalysis of these fractions showed that NodL protein was only detected in the cytoplasmic fraction (Fig. 3). The presence or absence of NodL protein in these fractions was not affected by omitting 1 M KCl during isolation of the membranes (data not shown). NodL was not detected in the spent growth medium (data not shown). These results show that the NodL protein can be isolated from the cytoplasmic fraction.

Purification of native NodL protein

After isolation of the soluble protein fraction from IPTG-induced BL21(DE3)(pMP3401) cells, NodL protein was purified to homogeneity by a three-step procedure, consisting of ammonium sulphate precipitation, BlueB affinity chromatography and ResourceQ anion exchange chromatography. The presence of NodL protein was monitored by immunoanalysis using the antibodies raised against NodL protein. SDS–PAGE of NodL-containing protein fractions at successive stages of purification is shown in Fig. 4. The results show that after the final purification step only one band is present as judged by SDS–PAGE followed by Fast Green staining (Fig. 4, lane 5).

Thin-layer chromatography analysis of *in vitro* acetylation products

In order to show *in vitro* trans-acetylating activity of the NodL protein isolated from *Rhizobium*, NodL had to be overproduced in *Rhizobium*, as it had been observed that NodL protein was produced in very small amounts in *R. l. bv. viciae* strain 248 (Fig. 2B, lane 4). The general expression vector pMP1070 was constructed, which can be used to express proteins under the control of the *nodA* promoter in *Rhizobium* strains that contain a

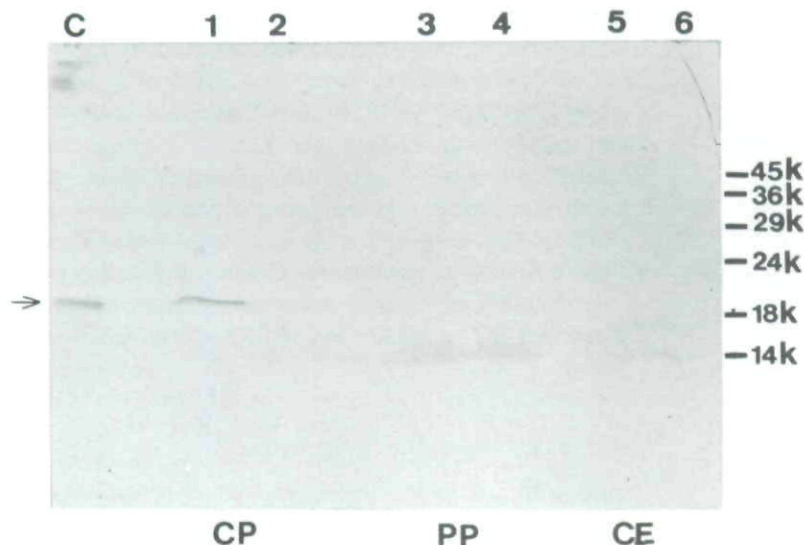


Fig. 3. Subcellular localization of NodL protein in *R. l. bv. viciae* wild-type strain 248. Cellular fractions obtained from equal amounts of cells grown in the presence (odd lanes) or absence (even lanes) of the inducer naringenin were analysed by immunoblotting using antibodies raised against NodL protein. Lanes: c, reference: purified NodL protein; 1 and 2, cytoplasmic fraction (CP); 3 and 4, periplasmic fraction (PP); 5 and 6, membrane fraction (CE). The position of NodL protein is indicated by an arrow.

functional *nodD* gene (Fig. 1). In order to overproduce NodL protein in a Sym plasmid-cured *Rhizobium* strain, the *nodL* and *nodD* genes were cloned into plasmid pMP1070 resulting in plasmid pMP2107 (Fig. 1). Induced cells of LPR5045 harbouring pMP2107 produced NodL in larger quantities than wild-type *R. l. bv. viciae* strain 248 cells, as was shown by immunoblotting (Fig. 2, lanes 4 and 6). Soluble protein fractions isolated from non-induced and induced cells of *Rhizobium* strain LPR5045 harbouring pMP2107 were incubated with [14 C]acetyl-CoA and lipo-oligosaccharides as candidate acetyl acceptor substrates. Only when cells had been induced with flavonoids, labelled reaction products were detected by thin-layer chromatography (TLC) (data not shown).

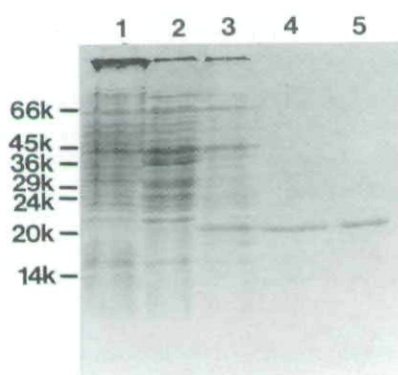


Fig. 4. Purification of native NodL protein from *E. coli* BI21(DE3) harbouring pMP3401 after induction with IPTG. Fractions of the subsequent purification steps were analysed by SDS-PAGE and stained with Fast Green. Lanes: 1, total cell proteins; 2, soluble protein fraction; 3, 30–50% ammonium sulphate precipitate; 4, purified fraction after BlueB dye chromatography; 5, protein fraction after Resource Q anion exchange chromatography. The position of NodL protein is indicated by an arrow.

These spots were not observed if strain LPR5045 was used. The same spots were also observed when a dissolved ammonium sulphate precipitate of soluble proteins isolated from induced cells of *E. coli* strain BI21(DE3) harbouring pMP3401 was used (data not shown). To show that the NodL protein was indeed responsible for the appearance of the reaction products, purified native NodL protein from *E. coli* was incubated with [14 C]acetyl-CoA and several candidate acetyl acceptor substrates. A radioactive reaction product was formed using NodRlv-V(C18:4) as a substrate (Fig. 5A, lane 2), which runs at the position of NodRlv-V(C18:4;Ac) (Fig. 5A, lane 1) (Spaink *et al.*, 1991). The reaction product was not detectable by TLC analysis after the reaction mixture had been treated with sodium methoxide, which leads to the hydrolysis of an *O*-acetyl group leaving a hydroxyl group (Fig. 5A, lane 3), confirming that the reaction product carries an *O*-acetyl group. These results suggest that the formed reaction product is NodRlv-V(C18:4;Ac). Also the use of NodRlv-IV(C18:4), NodRm-IV(C16:2;S) or NodRm-IV(C16:0;S) as substrates resulted in the formation of radioactive reaction products (Fig. 5A, lanes 4, 5 and 6), which migrate at the positions expected for their *O*-acetylated derivatives (Spaink *et al.*, 1991). Only in the case of NodRm-IV(C16:0;S) were two reaction products observed (Fig. 5A, lane 6), which might be due to a contamination of NodRm-IV(C16:1;S) in the sample. When purified NodL protein and [14 C]acetyl-CoA were incubated with *N*-acetylglucosamine or chitin fragments (di-, tri-, tetra- and pentameric forms of *N*-acetylglucosamine), radioactive reaction products were detected on TLC (Fig. 5B, lanes 2, 3, 4, 5 and 6), indicating that these are acetylated derivatives. A radioactive spot running at $R_f = 0.58$ was present in all cases, even when no saccharides were added (Fig. 5B, lane 1). This spot also appeared

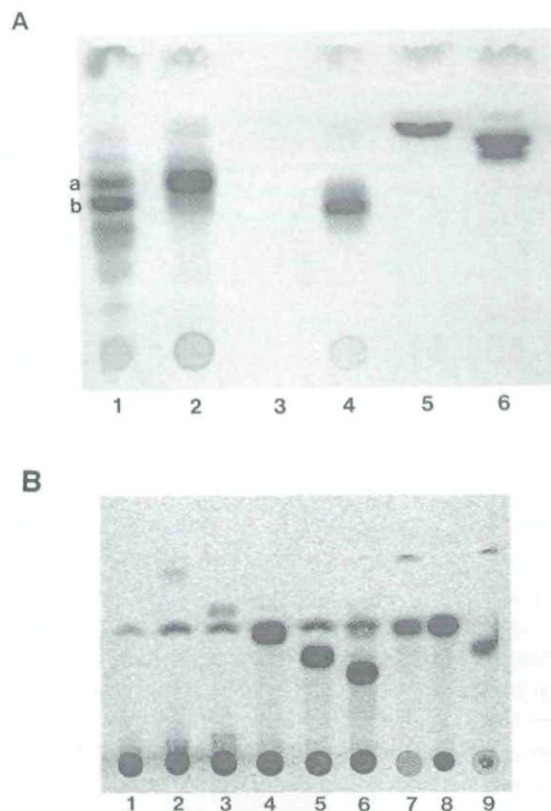


Fig. 5. Thin-layer chromatography (TLC) analysis of reaction products owing to the activity of NodL protein.

A. After incubation reaction mixtures, containing NodL protein, [$1\text{-}^{14}\text{C}$]acetyl-CoA and various lipo-oligosaccharides, were extracted with *n*-butanol and analysed using reverse-phase C18-coated silica plates. Lanes: 1, reference compounds: ^{14}C -labelled lipo-oligosaccharides isolated from *Rhizobium* strain RBL5560 (Spaink *et al.*, 1991), the positions of NodRlv-V(C18:4;Ac) and NodRlv-IV(C18:4;Ac) are indicated by a and b, respectively; 2, reaction mixture containing NodRlv-V(C18:4); 3, reaction mixture containing NodRlv-V(C18:4) treated afterwards with sodium methoxide; 4, reaction mixture containing NodRlv-IV(C18:4); 5, reaction mixture containing NodRm-IV(C16:2;S); 6, reaction mixture containing NodRm-IV(C16:0;S). B. TLC analysis of reaction mixture containing NodL protein, [$1\text{-}^{14}\text{C}$]acetyl CoA and various chitin fragments. Lanes: 1, no saccharides added to the reaction mixture; 2, *N*-acetylglucosamine; 3, dimer of *N*-acetylglucosamine; 4, trimer of *N*-acetylglucosamine; 5, tetramer of *N*-acetylglucosamine; 6, pentamer of *N*-acetylglucosamine; 7,8, reference compound (^{14}C -labelled *N*-acetylglucosamine); 9, reference compound (^{14}C -labelled dimer of *N*-acetylglucosamine obtained by chitinase degradation of ^{14}C -labelled lipo-oligosaccharides).

when only [$1\text{-}^{14}\text{C}$]acetyl-CoA was incubated in the buffer (data not shown), suggesting that this spot was a spontaneously formed acetyl-CoA derivative. The reaction products formed when using the monomers and dimers of *N*-acetylglucosamine as substrates (Fig. 5B, lanes 2 and 3), ran at different positions to the reference compounds (monomer and dimer of *N*-acetylglucosamine) (Fig. 5B, lanes 7, 8 and 9). This result also indicates that these reaction products contain an additional acetyl group. For *N*-acetylglucosamine we had to incubate the reaction

mixture six-times longer in comparison to the oligomeric forms to detect a reaction product. In all these cases only one reaction product was formed, suggesting that only one acetyl moiety was substituted per substrate molecule.

Complete chemical analysis of an in vitro reaction product

High-pressure liquid chromatography (HPLC) analysis of reaction mixtures consisting of NodL protein, *N,N',N'',N'''*-penta-acetylchitinopentaose (further on referred to as chitinpentaose) and acetyl-CoA showed that peak A (Fig. 6), representing chitinpentaose, decreased with time. One new peak, called peak B (Fig. 6) increased with time, suggesting that this is a modified form of chitinpentaose. After 24 h a more than 60% conversion of peak A into peak B had taken place. The compound represented by peak B and the untreated chitinpentaose were both analysed using FAB-MS (data not shown). The positive ion spectrum of peak B contained a single major $M+H^+$ pseudomolecular ion at m/z 1076, corresponding to a chitinpentaose bearing a single acetyl group, while the untreated sample gave an analogous pseudomolecular ion at m/z 1034 for the unacetylated chitinpentaose. The site of acetylation was determined by carrying out collision-induced dissociation tandem mass spectrometric analyses of peak B and the untreated species (Fig. 7). A^+ -type fragment ions were generated from both samples demonstrating that the sample in peak B contains a single species in which an additional acetyl group is attached to the non-reducing terminal GlcNAc (Fig. 7B).

Peak B and the untreated chitinpentaose were also examined using 1- and 2-D ^1H -NMR analyses (nuclear magnetic resonance) (Fig. 8). A singlet with a chemical shift of 2.17 p.p.m. in the 1-D ^1H -NMR spectrum of peak B showed that peak B contains an additional *O*-acetyl group which is absent from the untreated sample (data not shown). This *O*-acetyl group was shown to be attached to C-6 since in the 2-D spectrum of peak B two characteristic geminal protons, H6 and H6', were observed at δ 4.53 and δ 4.31 (Fig. 8B), which were not present in the spectrum of chitinpentaose (Fig. 8A). These data are in agreement with the NMR data obtained from the *O*-acetylated lipo-oligosaccharides (Spaink *et al.*, 1991).

In conclusion, the results of the NMR and mass spectrometric analyses show that the product formed by the action of NodL protein on chitinpentaose is a chitinpentaose derivative bearing a single *O*-acetyl group specifically substituted at C-6 of the non-reducing terminal residue.

Discussion

All structurally identified lipo-oligosaccharides of *R. l. bv. viciae* contain an *O*-acetyl group at the C-6 position of

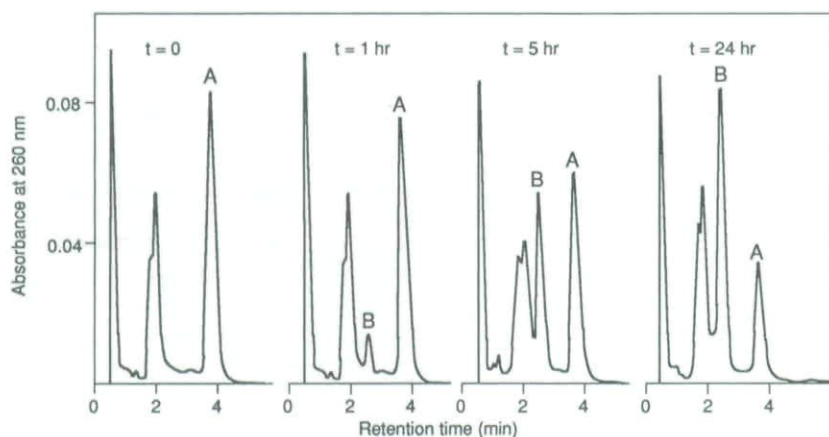


Fig. 6. HPLC analysis of reaction product(s) resulting from the activity of NodL. Samples from a reaction mixture containing NodL protein, chitinpentose and acetyl-CoA were taken at the following time points (t): t=0, t=1 hour (hr), t=5 hr and t=24 hr and analysed on an amine-HPLC column. Peak A represents chitinpentose and peak B the newly formed reaction product.

the non-reducing terminal sugar residue, the presence of which is important for biological activity (Spaiink *et al.*, 1991). Other rhizobial lipo-oligosaccharides can also contain such an *O*-acetyl group, like those isolated from *R. meliloti* (Truchet *et al.*, 1991), *Rhizobium leguminosarum* bv. *trifolii* (H. P. Spaiink and J. E. Thomas-Oates unpublished results) and the distantly related *B. japonicum* (Carlson *et al.*, 1993). From studies of *nodL* mutants it was concluded that NodL protein of *R. l. bv. viciae* is involved in the addition of the *O*-acetyl group (Spaiink *et al.*, 1991). The *nodL* gene products are homologous with *E. coli* LacA and CysE, which have acetyltransferase enzymatic activity (Downie *et al.*, 1989). The nucleotide sequence of a *nodL* gene from *R. meliloti* has recently been reported, the deduced gene product of which shows 83% homology with the deduced NodL protein of *R. l. bv. viciae* (Baev *et al.*, 1992). The aim of the present study was to test biochemically whether NodL protein indeed has *O*-acetyltransferase activity and to determine the possible substrate(s) for the NodL protein. Therefore we have set up an *in vitro* test system using purified NodL protein.

Mono-specific polyclonal antibodies towards NodL were obtained. These were used for subcellular localization studies and in the purification of NodL protein. From a theoretical evaluation of the tendency of the NodL protein to form trans membrane helices, using the method of Engelman *et al.* (1986), it was suggested that the *nodL* gene product is anchored in the cytoplasmic membrane (Canter Cremers *et al.*, 1989). In contrast, our results show that NodL protein is not an integral membrane protein. Even omitting the use of high salt in the fractionation procedure, which removes proteins bound to the membrane by electrostatic interactions, did not result in the co-purification of NodL protein with the membrane fraction, indicating that the NodL protein is not associated with the cytoplasmic membrane via electrostatic interactions. The soluble protein fraction was used to purify NodL protein for further studies.

In our *in vitro* test system acetyl-CoA was used as the acetyl donor, since it is the most general acetyl donor in nature and is also used by homologues of NodL such as LacA and CysE (Zabin, 1963; Kredich *et al.*, 1963). Various derivatives of *N*-acetylglucosamine were tested as acetyl acceptors. Crude cell lysates from a *nodL* mutant strain did not show transacetylating activity on any of the tested substrates, whereas crude cell lysates from a strain overproducing NodL did show activity. To prove that NodL was responsible for the observed transacetylating activity, we used a NodL protein preparation which was purified to homogeneity from *E. coli*. The key purification step was the use of BlueB dye-matrix chromatography, for which NodL protein appeared to have an affinity. Various lipo-oligosaccharides such as NodRlv-IV(C18:4), NodRlv-IV(C18:4), NodRm-IV(C16:2,S), NodRm-IV(C16:0,S) can be acetylated by NodL (Fig. 5). These results indicate that the absence or presence of a sulphate group at the reducing-terminal sugar residue is not essential for the function of NodL. Furthermore, they show that NodL protein can function with an acyl chain that can both vary in length as well as in the number of double bonds. The fact that lipo-oligosaccharides of *R. meliloti* can be acetylated by the NodL of *R. l. bv. viciae* gives support to the notion that *nodL* gene can be considered as a common nodulation gene (Canter Cremers *et al.*, 1989). The presence of the acyl chain is not required by acetyl-accepting substrates for NodL protein, because the mono-, di-, tri-, tetra- and pentamer of *N*-acetylglucosamine can also be acetylated. Since *O*-acetylated forms of chitin have not been reported previously and therefore no standards were available, we determined the chemical structure of the single reaction product formed when chitinpentose was added to our *in vitro* system. This reaction product contained one *O*-acetyl group at the C-6 of the non-reducing terminal sugar (Figs 7B and 8B).

The biochemical functions of the *nodB* and *nodH* gene products have also been studied *in vitro*. It has been

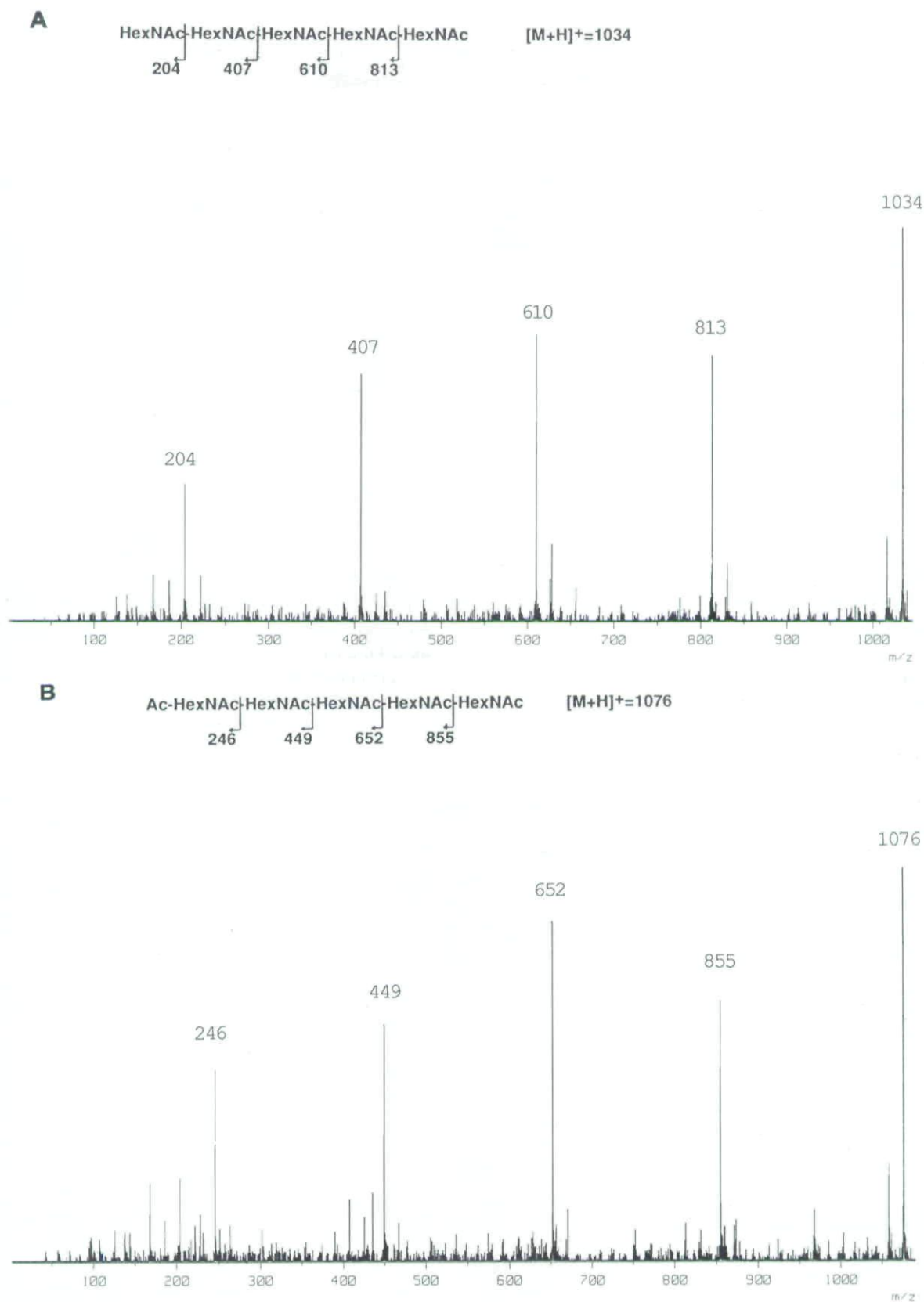


Fig. 7. Collision-induced dissociation mass spectrometry of untreated chitin (A) and peak B (B) from Fig. 6.

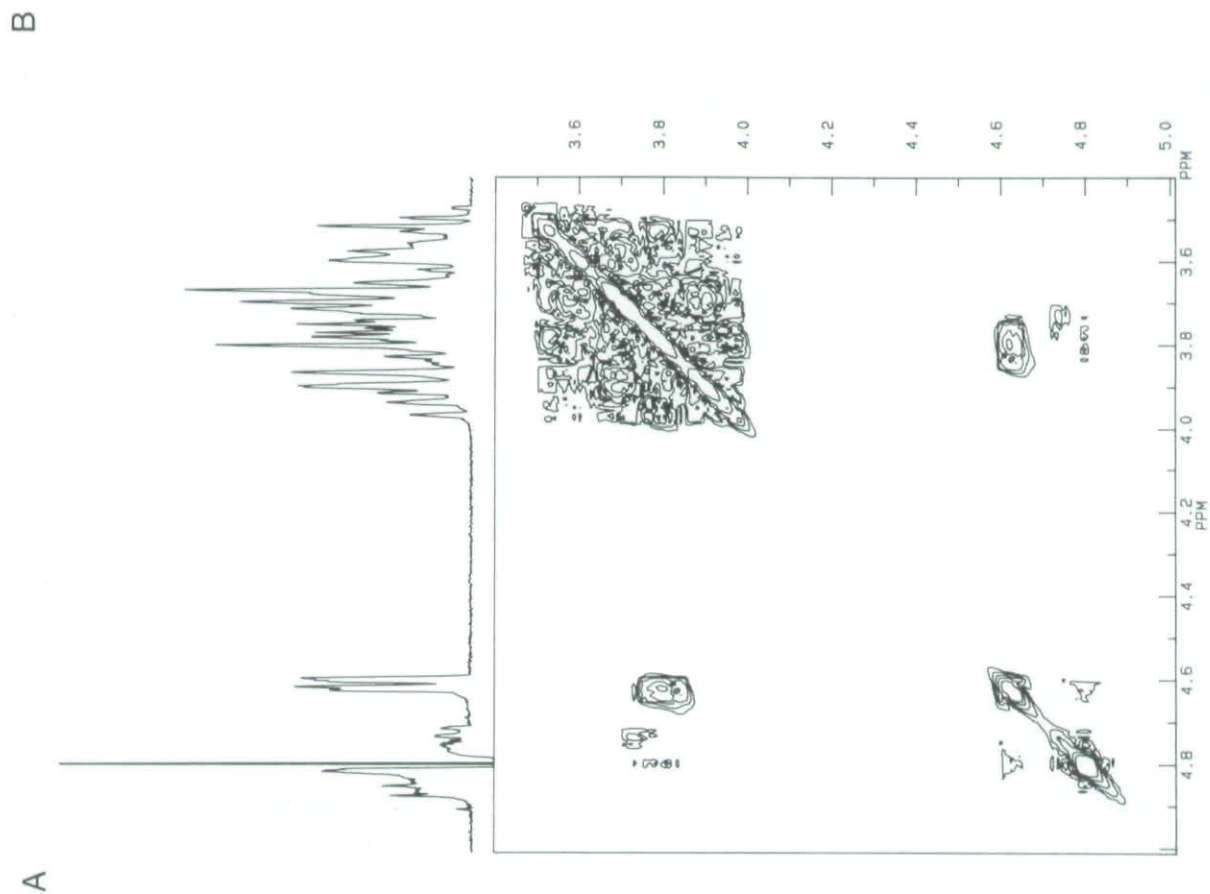


Fig. 8. Two-dimensional ^1H -NMR analysis of untreated chitinpentaose (A) and peak B (B) from Fig. 6. In the spectrum of peak B (B) two characteristic geminal protons H6 and H6' are observed at $\delta 4.53$ and $\delta 4.31$.

shown that both proteins can use chitin fragments as substrates. *In vitro* studies with NodB protein showed that it can remove the *N*-acetyl group from the non-reducing *N*-acetylglucosamine residue of chitinbiose, chitintriose and chitintetraose (John *et al.*, 1993). These chitin fragments, which are substrates for NodB, are also substrates for NodH, which sulphurylates the reducing *N*-acetylglucosamine residue of these compounds (E. M. Atkinson and S. R. Long, personal communication). NodB and NodH proteins differ from the NodL protein in that *N*-acetylglucosamine seems not to be a substrate.

NodC is predicted to have homology with chitin synthase (Bulawa and Wasco, 1991; Atkinson *et al.*, 1992; Debéllé *et al.*, 1992). Recently it was shown that NodC protein is able to produce chitin fragments in the absence of the other *nod* genes (Spaink *et al.*, 1993b). Together with the observation that NodB deacetylates chitin fragments *in vitro* (John *et al.*, 1993), this has led to a model for the biosynthesis of the lipo-oligosaccharides (Spaink *et al.*, 1993b). In this model deacetylated chitin fragments, produced by means of NodC and NodB, are acylated by an as yet unknown transacylase, which was speculated to be NodA protein (Spaink *et al.*, 1993b). Our observation that NodL protein is localized in the cytoplasmic fraction (Fig. 3) indicates that the natural substrate for NodL is also localized in the cytoplasm or on the inside of the cytoplasmic membrane. *N*-acetylglucosamine does not appear to be as good a substrate as the chitin fragments, indicating that NodL is active after synthesis of the chitin backbone. Therefore our results suggest that the biosynthesis of the chitin backbone of the lipo-oligosaccharides occurs in the cytoplasm or the inside of the cytoplasmic membrane. On the basis of our results we cannot define the *in vivo* substrate for NodL protein. Therefore K_m values for lipo-oligosaccharides and substrates which could be intermediates in their biosynthesis have to be determined and compared with estimates of the concentrations of such compounds in the cell. Furthermore, the presence of *O*-acetylated biosynthetic intermediates *in vivo* should be investigated.

Previously it has been shown that large chitin fragments are elicitors of the diterpene biosynthesis pathway in rice (Ren *et al.*, 1992). Chitin fragments containing up to four residues were also tested for elicitor activity, but were unable to induce the diterpene biosynthesis pathway. Chitin fragments have also been tested for their ability to induce transient membrane depolarization in suspension-cultured rice cells (Kuchitsu *et al.*, 1993). It was shown that the dimer of *N*-acetylglucosamine could not induce a depolarization, whereas the heptamer was able to induce a transient membrane depolarization. Chitinpentaoase has been tested for its activity on *Vicia* roots, but failed to elicit a biological response similar or related to one of the stages of infection or nodule formation (Spaink *et al.*, 1991). We are now able to produce chitin fragments which contain

an *O*-acetyl group at the C-6 position of the non-reducing terminal sugar. It will be of interest to test these novel derivatives in these test systems.

Lipo-oligosaccharides can elicit various biological responses on the plant root, but so far nothing is known about the fate of these signal compounds in the plant. Using our *in vitro* test system we are now able to produce radiolabelled derivatives of various (lipo-)chitin fragments, which can be either be acylated or sulphated. These specifically labelled derivatives could be useful for the study of the fate of Nod metabolites in the plant.

It has previously been hypothesized that rhizobial lipo-oligosaccharides mimic endogenous plant signal molecules (Truchet *et al.*, 1991; Bulawa *et al.*, 1992; Spaink *et al.*, 1993a). Support for this hypothesis has come from the discovery of chitinase-sensitive lipophilic molecules in *Lathyrus* plants (Spaink *et al.*, 1993a). As we have shown that the NodL protein acetylates various chitin derivatives, our *in vitro* acetylation system could also be helpful in the search for hypothetical chitin derivatives produced by plants.

Experimental procedures

Bacterial strains and growth conditions

The *Rhizobium* strains used were the wild-type *R. l. bv. viciae* strain 248 (Josey *et al.*, 1979) and the Sym plasmid-cured *R. l. bv. trifolii* strain LPR5045 (Hooykaas *et al.*, 1982). The cells were grown on TY-medium supplemented with 20% B⁻ medium (Spaink *et al.*, 1991). For induction of *nod* genes, naringenin was added to a final concentration of 3.75 µM. *E. coli* strain BL21(DE3) (Studier *et al.*, 1990) was grown in Luria-Bertani (LB) medium and the T7-polymerase gene was induced by adding IPTG to a final concentration of 2.5 µM. Strains harbouring plasmids were grown in the presence of the appropriate antibiotics (Spaink *et al.*, 1987).

Construction of plasmids

In order to clone the *nodL* gene in the expression vector pET9A (Studier *et al.*, 1990), PCR was performed with *Taq* polymerase (Biozym Nederland) with primers oMP27 and oMP28, and pMP414 as the template (Fig. 1), with standard procedures (Innis *et al.*, 1990). The PCR product was cloned as a *NdeI*-*Bam*HI fragment into pET9A, producing pMP3401 (Fig. 1). The *nodL* insert of pMP3401 was checked for mutations by nucleotide sequencing according to standard procedures (Sanger *et al.*, 1977), but no mutations affecting the amino acid sequence were found.

For expression of proteins in *Rhizobium*, a general expression vector pMP1070 (Fig. 1) was constructed by substitution of an *EcoRI*-*PstI* fragment in pMP263 (Spaink *et al.*, 1989) for an *EcoRI*-*PstI* polylinker fragment from pIC20R (Marsh *et al.*, 1984).

To construct a plasmid for overproduction of the NodL protein in a cured *Rhizobium* strain, the following strategy was

employed. A *Bgl*II–*Sph*I fragment from pMP414 (Fig. 1), containing the *nodL* gene, was cloned downstream of the *nodA* promoter in pMP1070, resulting in pMP1060 (Fig. 1). Subsequently, plasmid pMP2106 (Fig. 1) was treated with *Bgl*II and the isolated *nodD*-containing fragment was cloned into pMP1060, resulting in pMP2107 (Fig. 1).

Raising antibodies

Cells of *E. coli* BL21(DE3) harbouring pMP3401 were grown for 8 h in LC medium (Canter Cremers *et al.*, 1989) complemented with 1.0% glucose for catabolic repression. Subsequently, the culture was 1:100 diluted in 1 l LC medium supplemented with IPTG and grown for 16 h. To isolate aggregates of NodL protein, the following procedure was followed. After harvesting, the cells were resuspended in 25 ml ice-cold 50 mM Tris HCl, pH 8.5. Cells were broken by three passages through a French pressure cell and cell lysates were centrifuged for 30 min at $900 \times g$. The pellet was resuspended in 5 ml sample buffer and proteins were separated using a 15% SDS–polyacrylamide gel as described by Lugtenberg *et al.* (1975). Protein aggregates were retarded at the border of stacking and separating gels. This border region was excised and incubated for 1 h with shaking in 4 M urea and the mixture was separated using 15% SDS–polyacrylamide gel. After staining with Coomassie brilliant blue, the major protein band of approximately 20 kDa was isolated by electroelution. One-hundred micrograms of the isolated protein (in a volume of 0.5 ml) was suspended 1:1 (v/v) in Freund's complete adjuvant and injected subcutaneously into a New Zealand white rabbit. A booster injection containing 100 µg of the isolated protein was given after 14 d. Antiserum was collected 10 d after the booster injection. The titre of the NodL antiserum was determined by spotting 10 ng NodL protein on to nitrocellulose filters. Subsequently, immunoanalysis of the spots was performed with different dilutions of the antiserum, showing the titre of the antiserum to be 24 000.

Cell fractionation and protein analysis

Cells of *R. l. bv. viciae* strain 248 were grown for 16 h in the absence or presence of naringenin. Periplasmic and cytoplasmic proteins were isolated as described by de Maagd *et al.* (1986). Isolation of membrane proteins was achieved by adding KCl to lysed cells to a final concentration of 0.2 M and pelleting the total membranes by centrifugation at $120\,000 \times g$ for 16 h. In an equivalent experiment KCl was omitted. Isolation of secreted proteins from the culture medium was carried out as described previously (de Maagd *et al.*, 1986).

Proteins were separated using 15% SDS–polyacrylamide gels, which were stained using Fast Green. In case of immunoanalysis, proteins were transferred to nitrocellulose and detection carried out as described previously (de Maagd *et al.*, 1986). The blot was incubated with antiserum against NodL protein, diluted 3000-fold in 0.05% Tween 20 buffer. After washing, the nitrocellulose was incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Sigma) using methods recommended by the manufacturers.

Quantification of NodL protein was achieved by a modified Lowry procedure (Dulley *et al.*, 1975).

Purification of NodL protein

NodL was purified from *E. coli* strain BL21(DE3) harbouring pMP3401 for enzymatic studies. Induced cells from 5 l of culture medium were harvested by centrifugation and resuspended in 50 ml ice-cold 20 mM Tris/HCl, pH 7.5. The cells were broken by three passages through a French pressure cell. Unbroken cells and membranes were removed by centrifugation for 16 h at $120\,000 \times g$. Ammonium sulphate precipitations were performed on the soluble protein fraction at concentrations of 30% and 50%. The 50% precipitate was dissolved in 20 mM Tris/HCl, pH 7.5 (buffer A) and dialysed for 16 h against buffer A. Subsequently the protein sample was loaded on to a 2 ml Blue B dye matrix column (Amicon) equilibrated with buffer A. After washing with buffer A, NodL protein was eluted from the column in one step with 0.2 M KCl. The eluted protein sample, containing the NodL, was dialysed against 20 mM Tris/HCl pH=8.0 (buffer B) for 16 h. This sample was then loaded on to a Resource Q anion exchanger column (Pharmacia LKB) equilibrated with buffer B. Following washing with buffer B, proteins were eluted with a linear gradient of 0–1 M NaCl in buffer B. Fractions containing NodL protein were pooled, dialysed for 16 h against 20 mM phosphate buffer pH=7.3 and concentrated by ultrafiltration over a 10 kDa filter (Amicon corp.). Protein samples obtained during purification steps were analysed by SDS–PAGE and immunoanalysis.

In vitro transacetylating assay

In order to demonstrate transacetylating activity *in vitro*, 75 µl of purified NodL (5 µg) in 20 mM phosphate buffer pH=7.3 was incubated with 50 nCi (0.8 nmol) [14 C]acetyl-CoA (Amersham) and several candidate acetyl acceptors. These reaction mixtures were incubated for 4 h at 28°C. Purified lipo-oligosaccharide (20 µg) dissolved in 1% CHAPS (w/v) was added to the reaction mixtures, which contained a final concentration of 0.01% CHAPS (w/v). NodRlv-IV(C18:4,Ac) and NodRlv-V(C18:4,Ac) were purified as described previously (Spaijk *et al.*, 1991) and de-O-acetylated by incubation for 1 h at 40°C in 1 N sodium methoxide in methanol. After acidifying with acetic acid and evaporating to dryness, lipo-oligosaccharides were dissolved in water-saturated *n*-butanol. The *n*-butanol phase was washed three times with an equal volume of *n*-butanol saturated water to remove sodium acetate. Purified lipo-oligosaccharides from *R. meliloti* were kindly provided by Dr Michael Schulze (CSV, Gif Sur Yvette, France). The putative substrates *N*-acetylglucosamine (Sigma), *N,N'*-diacetylchitobiose (Sigma), *N,N',N''*-tri-acetylchitotriose (Sigma), *N,N',N'',N'''*-tetraacetylchitotetraose (Seika-gaku corp.) and *N,N',N'',N''',N''''*-pentaacetylchitopentaose (Seika-gaku, Corp.) (for brevity the latter compound is called chitinpentaose in this paper) were tested, they were added to the reaction mixture in amounts of 20, 40, 60, 80 and 100 µg respectively. In case of *N*-acetylglucosamine the reaction mixture was incubated for 18 h. For a quantitative isolation of the reaction product, 20 µg purified NodL protein was incubated with 1 mg acetyl-CoA (Sigma) and 1 mg chitinpentaose for 24 h at 28°C.

Analysis of in vitro reaction products

Lipo-oligosaccharides were isolated from the reaction mixtures by *n*-butanol extraction. Samples were concentrated by evaporation and chromatographed on reverse-phase C18-coated silica plates (Sigma) using a mobile phase of acetonitrile:water (1:1 v:v). In case of *N*-acetylglucosamine and the oligomers of *N*-acetylglucosamine, reaction mixtures were concentrated by evaporation and chromatographed on NH₂ TLC plates (Merck) using a mobile phase of acetonitrile:water (65:35 v:v).

Reaction mixtures containing chitinpentase and acetyl-CoA were separated on a nucleosil 120–7 NH₂ HPLC column obtained from Macherey Nagel. Before loading on to the column, acetonitrile was added to the reaction mixture to a final concentration of 75% and the sample was filtered over a 45 µm Spin × 8170 nylon membrane (Costar). Compounds were separated using an isocratic elution in 75% acetonitrile in water.

NMR-spectroscopy and mass spectrometry

Fast atom bombardment (FAB) mass spectra were obtained using MS 1 of a JEOL JMS-SX/SX 102 A tandem mass spectrometer operated at 10 kV accelerating voltage and using mono-thioglycerol as matrix. The FAB gun was operated at an emission current of 10 mA with xenon as the bombarding gas. Spectra were scanned at a speed of 30 sec for the full mass range specified by the accelerating voltage used, and were recorded and averaged using a Hewlett-Packard HP 9000 data system running JEOL COMPLEMENT software.

Collision-induced dissociation (CID) tandem mass spectra were obtained using all four sectors of the same instrument under similar conditions, and using helium as the collision gas at a pressure sufficient to reduce the parent ion to one third of its original intensity.

¹H NMR spectra were measured on a Bruker MSL-400 spectrometer (equipped with an aspect 3000 data system). The thoroughly dried samples were dissolved in D₂O. The description of the 2D-COSY experiment is given by Aue *et al.* (1976).

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