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Molecular Microbiology (1994) 13(5), 821–831

Structural identification of metabolites produced by the NodB and NodC proteins of *Rhizobium leguminosarum*

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Summary

The *Rhizobium* nodulation genes *nodABC* are involved in the synthesis of lipo-chitin oligosaccharides. We have analysed the metabolites which are produced *in vivo* and *in vitro* by *Rhizobium* strains which express the single *nodA*, *nodB* and *nodC* genes or combinations of the three. *In vivo* radioactive labelling experiments, in which D-[1-^{14}C]-glucosamine was used as a precursor, followed by mass spectrometric analysis of the purified radiolabelled metabolic products, showed that *Rhizobium* strains that only express the combination of the *nodB* and *nodC* genes do not produce lipo-chitin oligosaccharides but instead produce chitin oligomers (mainly pentamers) which are devoid of the *N*-acetyl group on the non-reducing terminal sugar residue (designated NodBC metabolites). Using the same procedure we have shown that when the *nodL* gene is expressed in addition to the *nodBC* genes the majority of metabolites contain an additional *O*-acetyl substituent on the non-reducing terminal sugar residue (designated NodBCL metabolites). The NodBC and NodBCL metabolites purified after *in vivo* labelling were compared with the radiolabelled metabolites produced *in vitro* by *Rhizobium* bacterial cell lysates to which UDP-*N*-acetyl-D-[U-^{14}C]-glucosamine was added using thin-layer chromatography. The results show that the lysates of strains which expressed the *nodBC* or *nodBCL* genes can also produce NodBC and NodBCL metabolites. The same results were obtained when the NodB and NodC proteins were produced separately in two different strains. On the basis of these and other recent results, we propose that NodB is a chitin oligosaccharide deacetylase, NodC an *N*-acetylglucosaminyltransferase and, by default, NodA is involved in lipid attachment.

Received 11 March, 1994; revised 23 May, 1994; accepted 26 May, 1994. *For correspondence. Tel. (71) 275055; Fax (71) 275088.

Introduction

The interaction between bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* and their leguminous host plants results in the formation of nitrogen-fixing root nodules (see Brewin, 1991). Recently it has become apparent that the process of nodule formation is based on a mutual recognition of signal molecules produced by plant and bacteria. Flavonoid signal molecules produced by the plant are recognized by the guest bacteria, a process which is mediated by the regulatory NodD protein (see Schlaman *et al.*, 1992; Fisher and Long, 1992). As a result of this recognition, the NodD protein activates the transcription of the *nod* and *nol* genes and subsequently the bacteria produce signal molecules which are specifically recognized by the host plant (see Dénarié and Cullimore, 1993). The signal molecules (also called Nod metabolites) from many rhizobial strains have been identified and they all appear to consist of an acylated chitin oligosaccharide which can contain strain-specific modifications (see Spaink *et al.*, 1993c).

Several *nod* genes have been shown to be involved in the biosynthesis of the lipo-chitin oligosaccharide (LCO) signal molecules. The *nodA*, *nodB* and *nodC* genes, which are genetically organized in the *nodABCIJ* operon, are the only *nod* genes that are essential for the production of LCO. Moreover, *Rhizobium* strains which express the *nodABC* genes in the absence of the other *nod* genes can produce a basic LCO molecule which does not contain any host-specific substituents (Spaink *et al.*, 1991).

Recently, John *et al.* (1993) reported that the NodB protein functions *in vitro* as a deacetylase which can remove the *N*-acetyl group from the non-reducing terminal sugar residue of chitin oligomers. This function is in agreement with the observed homology of the NodB protein with a chitin deacetylase of the fungus *Mucor rouxi* (Kafetzopoulos *et al.*, 1993). A part of the NodC protein has been shown to be homologous to the presumed catalytic domain of various chitin synthases, suggesting a function as an *N*-acetylglucosaminyltransferase (Bulawa and Wasco, 1991; Atkinson and Long, 1992; Debellé *et al.*,1992; Bulawa, 1993). The localization of the NodC protein in the cytoplasmic membrane is consistent with such a

function (Barny and Downie, 1993). In a preliminary report, data were presented indicating that NodC could synthesize chitin oligomers in the absence of the other Nod proteins (Spaink *et al.*, 1993b). In the present study we have analysed the structures of glucosamine-derived or UDP-*N*-acetylglucosamine-derived metabolites which are produced *in vivo* and *in vitro* by strains which express the *nodA*, *nodB*, *nodC* and *nodL* genes or various combinations of these genes, in order to obtain a better insight into the function of the encoded proteins.

Results

Development of a test system for the analysis of Nod metabolites

The organization of the *nodABC* genes in one operon makes it difficult to study the function of the individual Nod products. To circumvent this problem we have chosen an approach in which the *nodB* and *nodC* genes are cloned separately using polymerase chain reaction (PCR) technology. In Fig. 1A the construction of plasmids is outlined. In the constructs pMP2150 and pMP2704, translation and transcription of the *nodB* and *nodC* genes is directed by the Shine–Dalgarno sequence derived from vector pET9a (Studier *et al.*, 1990) and the promoter of the *nodABCIJ* operon derived from vector pMP3402. In plasmid pMP2734, the separated *nodAB* genes are under the control of the original upstream sequences. Use was made of plasmid replicons of the incompatibility groups IncW (with *nodB*), IncP (with *nodC*) and IncQ (with *nodAB*) which, in the presence of appropriate antibiotics, appear to be stably maintained in the presence of each other. Inducible expression of these cloned *nod* genes by flavonoids in the absence of the other inducible *nod* genes is possible by using *Rhizobium* strain RBL5957 which harbours a transposon derivative in which the *nodD* gene is cloned (Tn*5*–*nodD*) (Fig. 1B).

In vivo *labelling studies using* D-[1-^{14}C]-glucosamine

R. leguminosarum biovar *viciae* strain RBL5562 (*nodA*::Tn*5*) (Table 1) harbouring various combinations

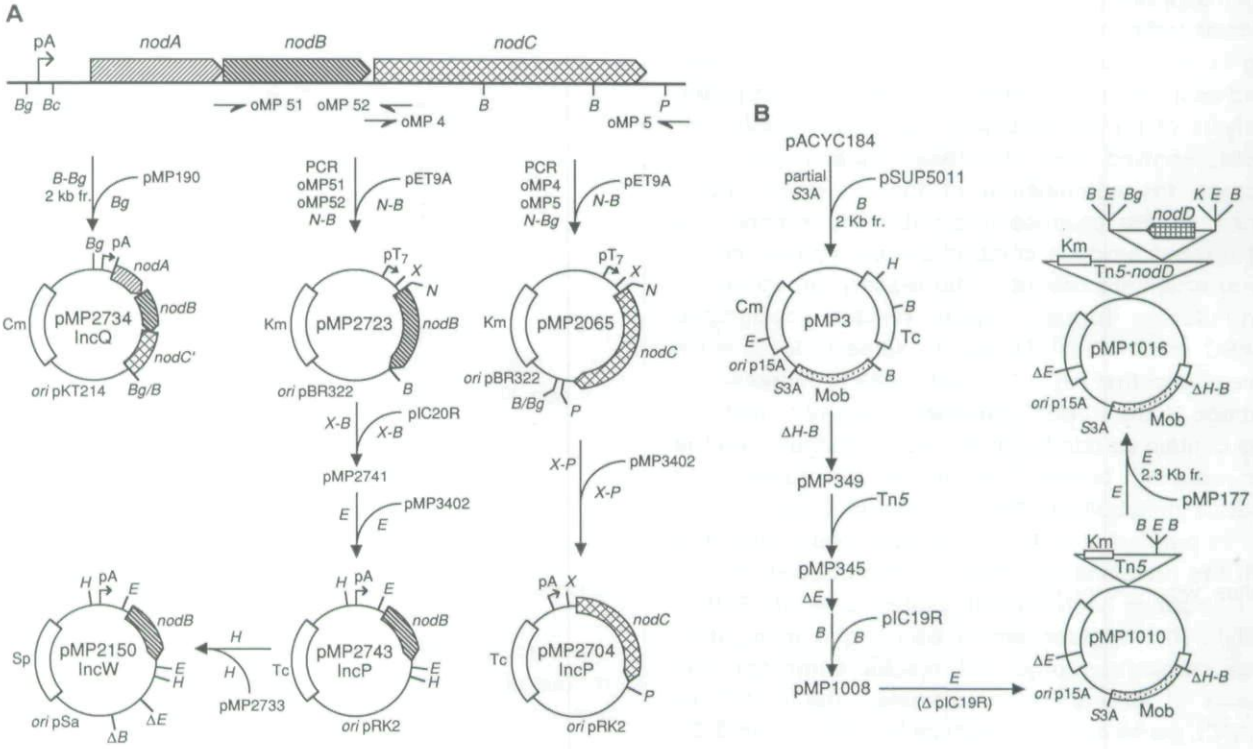

Fig. 1. Construction of plasmids and transposons.
A. Construction of pMP2734, pMP2150 and pMP2704. Shown on top is part of the *nod* region present in plasmid pMP292 and the positions of oligonucleotide primers oMP51, oMP52, oMP4 and oMP5, which were used for PCR.
B. Construction of general cloning vector pMP1010 and its derivative pMP1016. pMP1010 and its derivatives are mobilizable to *Rhizobium* but cannot replicate in this bacterium. Therefore, the plasmid can be used for general delivery of target genes which can be cloned into the Tn*5*-derivative (designated Tn*5*-1010). Cm, Km, Sp and Tc, chloramphenicol, kanamycin, spectinomycin and tetracycline-resistance genes, respectively; Fr., DNA fragment; p, promoter; Mob, plasmid mobilization region; △, removal of restriction site or DNA fragment. Restriction sites: *B*, *Bam*HI; *Bc*, *Bcl*I; *Bg*, *Bgl*II; *E*, *Eco*RI; *H*, *Hin*dIII; *K*, *Kpn*I; *N*, *Nde*I; *P*, *Pst*I; *S*3A, *Sau*3A; *X*, *Xba*I.

Table 1. Strains and plasmids used.

Description		Source/Reference
Strain		
LPR5045	Sym plasmid-cured derivative of strain RCR5, Rif^R	Hooykaas *et al.* (1982)
RBL5560	LPR5045 harbouring Sym plasmid pJB5JI (=pRL1JI *mep*::Tn*5*)	Spaink *et al.* (1987)
RBL5562	LPR5045 (pRL1JI) *nodA*::Tn*5*	Wijffelman *et al.* (1985)
RBL5957	LPR5045::Tn*5*–*nodD1016* (Fig. 1B)	This work
KMBL1164	*E. coli* K-12 *del(lac–proAB) thi*, F^-	Spaink *et al.* (1987)
Plasmid		
pACYC184	Inc15A, Cm^R, Tc^R	Chang and Cohen (1978)
pET9A	IncColE1, expression vector based on T7 promoter, Km^R	Studier *et al.* (1990)
pIC19/20R	IncColE1, cloning vectors, Amp^R	Marsh *et al.* (1984)
pJB5JI	IncpRL1JI, Sym plasmid containing Tn*5* insertion in the gene for medium bacteriocin production	Johnston *et al.* (1978)
pMP3	Inc15A, Cm^R, Tc^R (Fig. 1B)	This work
pMP177	pRt308-derived, contains *nodD* of *R. leguminosarum* bv. *trifolii*	This work
pMP190	IncQ, Sm^R, Cm^R	Spaink *et al.* (1987)
pMP292	IncP, Tc^R, contains *nodDABC* of *R. leguminosarum* bv. *viciae*	Spaink *et al.* (1993a)
pMP345	Inc15A, Cm^R, Km^R (Fig. 1B)	This work
pMP349	Inc15A, Cm^R (Fig. 1B)	This work
pMP1008	Inc15A/colE1, Km^R, Ap^R (Fig. 1B)	This work
pMP1010	Inc15A, Km^R, contains Tn*5*-1010, general cloning vector (Fig. 1B)	This work
pMP1016	Inc15A, Km^R, contains Tn*5*–*nodD1016*	This work
pMP1060	IncP, Tc^R, contains pA-*nodL*	Bloemberg *et al.* (1994)
pMP2065	pET9A-derived, Km^R (Fig. 1A)	H. R. M. Schlaman
pMP2410	IncQ, Cm^R, Sm^R, contains pA-*nodL* derived from pMP1060	This work
pMP2704	IncP, Tc^R, contains pA-*nodC* (Fig. 1A)	This work
pMP2723	pET9A-derived, Km^R (Fig. 1A)	This work
pMP2733	IncW, Spc^R, Sm^R, derived of pRI40 by removal of *Eco*RI and *Bam*HI sites	This work
pMP2734	IncQ, Cm^R, Sm^R, contains *nodAB* including the promoter region (Fig. 1A)	This work
pMP2743	IncP, Tc^R, contains pA–*nodB* (Fig. 1A)	This work
pMP2150	IncW, Spc^R, Sm^R, contains pA–*nodB* (Fig. 1A)	This work
pMP3402	IncP, Tc^R, derived of pMP1070 contains pA and multicloning site	This work
pRI40	IncW, Spc^R,Sm^R	Innes *et al.* (1988)
pRK2013	IncColE1, Tra^+, Km^R	Ditta *et al.* (1980)
pRL1JI	IncpRL1JI, wild-type Sym plasmid, conferring nodulation for *Vicia* plants	Johnston *et al.* (1978)
pSUP5011	Source of Mob fragment	Simon *et al.* (1986)

Cm^R, Km^R, Sp^R, Tc^R, Rif^R and Amp^R: chloramphenicol, kanamycin, spectinomycin, tetracycline, rifampicin and ampicillin(carbenicillin) resistance, respectively; pA, promoter of *nodA*; Mob, plasmid mobilization region; Inc, plasmid incompatibility group.

of the single *nod* gene-containing plasmids were grown in the presence of D-[1-^{14}C]-glucosamine and naringenin and tested for the production of LCO. The results of the thin-layer chromatography (TLC) analysis show that the absence of any of the NodA, NodB or NodC proteins prevents the production of LCO (Fig. 2A and data not shown). To be able to detect intermediates in the biosynthesis of the LCO, not only *n*-butanol extracts of the growth medium were analysed but also cellular metabolites which were extracted with chloroform/methanol/water (Bligh and Dyer, 1959). Both the aqueous (fraction A) and chloroform (fraction C) phases of the final Bligh–Dyer partition system were analysed using silica-TLC. The results of analysis of fraction A of strain RBL5562 (pMP2150(*nodB*)) (pMP2704(*nodC*)) show the presence of two inducible spots close to the application spot (Fig. 2A, lanes 11 and 12). However, these spots were not observed in the strains which lack either the *nodB* or *nodC* gene (Fig. 2A, lanes 3–10). These spots were also observed with strain RBL5562 (pMP2734(*nodAB*) (pMP2704(*nodC*)) and, surprisingly, even in the wild-type strain RBL5560 in addition to the two inducible spots which represent the LCO (Fig. 2A, lanes 1 and 2 and Fig. 2B, lanes 1 and 2). The production of LCO only in the presence of the *nodA* gene is an indication that the NodA protein is essential for the addition of the acyl group to the sugar backbone.

In the case of fraction A of strain RBL5562 (pMP2704(*nodC*)), very weak inducible spots were observed when more sample was applied and the TLC plates were exposed for four times as long to the phosphoimager screens (Fig. 2B, lanes 3, 4). The migration rate of these spots is similar to that of the reference *O*-acetylated chitin tri-, tetra- and pentasaccharides (Fig. 2B, lanes I(Ac) to V(Ac)). In fraction C of strain RBL5562 (pMP2704(*nodC*)) at least one weak inducible spot was also observed (Fig. 1C) which migrates faster than the LCO. The spots observed in Figs 2B and 2C were not observed for strains which in addition to pMP2704(*nodC*) also harboured pMP2150(*nodB*) or pMP2734(*nodAB*) (data not shown).

^{14}C-labelled metabolites were also analysed using an NH_2-TLC system. In fraction A of strain RBL5562 (pMP2150(*nodB*)) (pMP2704(*nodC*)) three inducible

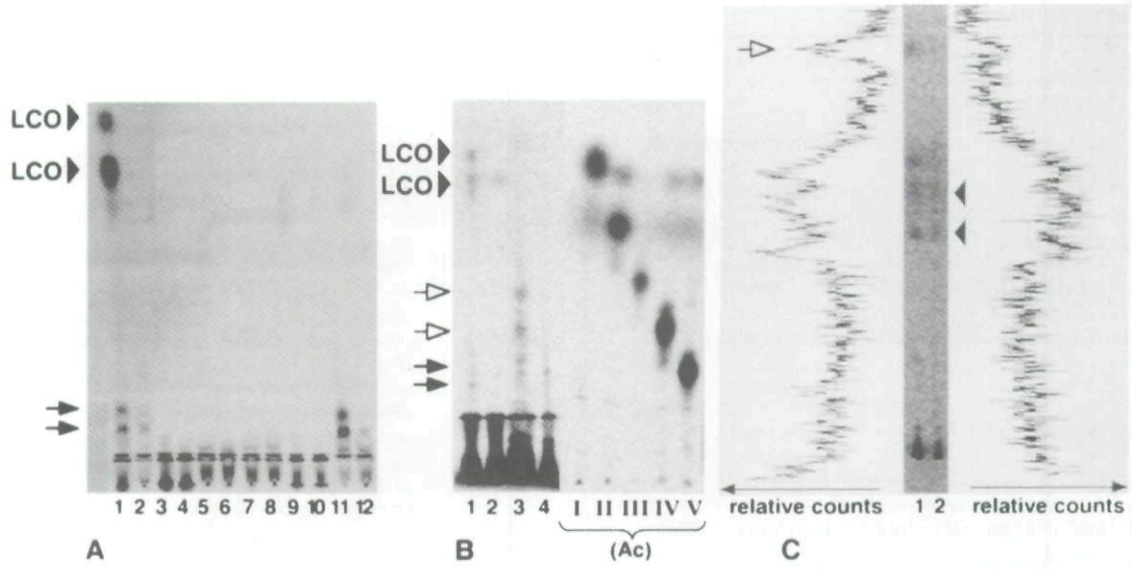

Fig. 2. Analysis of *in vivo* ^{14}C-labelled Nod metabolites by silica-TLC. Cells of strain RBL5562(*nodA*::Tn*5*) containing various plasmids or combinations of plasmids were grown in the presence of D-[1-^{14}C]-glucosamine and in the presence (odd numbers) or absence (even numbers) of the inducer naringenin. Cells were extracted using the method of Bligh and Dyer (1959) and after phase separation the chloroform/methanol phase (fraction C) and aqueous phase (fraction A) were collected and applied to TLC plates.
A. Fraction A of the following strains: lanes 1 and 2, RBL5562(pMP2734(*nodAB*), pMP2704(*nodC*)); lanes 3 and 4, RBL5562; lanes 5 and 6, RBL5562(pMP2150(*nodB*)); lanes 7 and 8, RBL5562(pMP2734(*nodAB*)); lanes 9 and 10, RBL5562(pMP2704(*nodC*)); lanes 11 and 12, RBL5562(pMP2150(*nodB*), pMP2704(*nodC*)).
B. Fraction A from the following strains (five times more sample was applied than in (A)): lanes 1 and 2, RBL5560; lanes 3 and 4, RBL5562(pMP2704(*nodC*)). Reference samples: I(Ac) to V(Ac) refer to *O*-acetylated *N*-acetylglucosamine and the chitin oligosaccharides chitinbiose, chitintriose, chitintetraose and chitinpentaose, respectively (see the *Experimental procedures*).
C. Lanes 1 and 2, fraction C of strain RBL5562(pMP2704(*nodC*)). In the graphs bordering the two lanes is plotted the quantitative output for the two lanes as generated by the Image Quant™ software. The position of the LCOs are indicated by arrowheads (not shown in Fig. 2C). The open arrows show the positions of the putative NodCL metabolites; the solid arrows are the positions of the NodBCL metabolites.

spots were observed (Fig. 3, lanes 4 and 5). These same inducible spots were observed in the concentrated spent growth medium (Fig. 3, lanes 8 and 9). In the corresponding fractions from strain RBL5957(pMP2150(*nodB*)) (pMP2704(*nodC*)), which does not contain a Sym plasmid, only a single inducible spot was observed (Fig. 3, lanes 6, 7, 10 and 11). In conclusion, the results shown in Figs 2 and 3 indicate that the *nodB* and *nodC* genes are involved in the production of metabolites which are different from LCO. Since apparently no other *nod* genes are needed for their production, these products are referred to as NodBC metabolites.

To test whether the NodL protein, which has been shown to be a transacetylase (Bloemberg *et al.*, 1994), determines the difference between the derivatives of strains RBL5562 and RBL5957, we have constructed strain RBL5957 (pMP2150(*nodB*), pMP2704(*nodC*), pMP2410(*nodL*)). The results of the glucosamine labelling experiments show that this strain produces a pattern of metabolites which is indistinguishable from that of strain RBL5562 (pMP2150(*nodB*), pMP2704(*nodC*)), showing that the *nodL* gene is indeed responsible for the difference in the profile of NodBC metabolites produced (data not shown). The metabolites produced by the strains RBL5562 (pMP2150(*nodB*), pMP2704(*nodC*)) and RBL5957 (pMP2150(*nodB*), pMP2704(*nodC*), pMP2410(*nodL*)) can therefore be referred to as NodBCL metabolites.

Purification of NodBC and NodBCL metabolites

For purification of NodBCL and NodBC metabolites, one-litre cultures of the strains RBL5562 and RBL5957, harbouring both the plasmids pMP2150(*nodB*) and pMP2704(*nodC*), were grown for 16 h in B$^-$ medium (Spaink *et al.*, 1991) in the presence of the inducer naringenin. The cells were centrifuged and the spent growth medium was evaporated, taken up in water and mixed with the radiolabelled fractions from the corresponding strain (Fig. 3, lanes 8 and 10). The samples were applied to a Sephadex G10 gel permeation column and fractions of the eluent were analysed quantitatively for the presence of the NodBC and NodBCL metabolites using TLC and subsequent scanning of the chromatograms using a phosphorimager. In a control experiment, radioactive samples were mixed with a reference sample which contained *O*-acetylated chitin oligosaccharides. The results (Fig. 4A) show that the NodBCL and NodBC metabolites elute at the

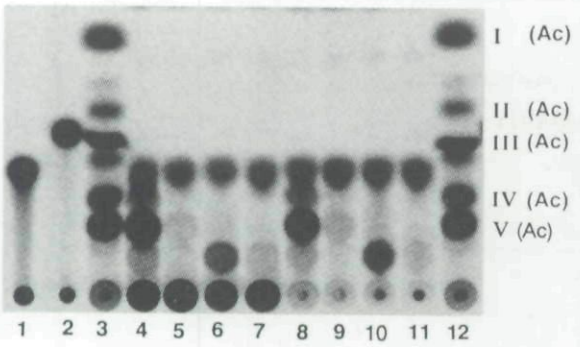

Fig. 3. Analysis of *in vivo* radiolabelled Nod metabolites using NH_2-TLC. Cells were grown in the presence of D-[1-^{14}C]-glucosamine and in the presence or absence of the inducer naringenin. The concentrated spent growth media (lanes 8–11) and the aqueous fractions of Bligh–Dyer extracts of cell pellets (lanes 4–7) were applied to TLC plates. Lane 1, D-[1-^{14}C]-glucosamine; lane 2, *N*-acetyl-D-[1-^{14}C]-glucosamine; lanes 3 and 12, mixed reference samples I(Ac) to V(Ac) as in Fig. 2B, lanes 4, 5, 8 and 9, strain RBL5562 (pMP2150(*nodB*), pMP2704(*nodC*)) grown in the presence (even numbers) or absence (odd numbers) of inducer; lanes 6, 7, 10 and 11, strain RBL5957(pMP2150(*nodB*), pMP2704(*nodC*)) grown in the presence (even numbers) or absence (odd numbers) of inducer.

position of the *O*-acetylated chitin pentasaccharide. Fractions 35 to 40 (Fig. 4A) were pooled and further purified using high-pressure liquid chromatography (HPLC). The radioactive profiles of the HPLC eluents were compared with the absorption profiles of the reference sample (the mixture of *O*-acetylated chitin oligosaccharides). The results (Fig. 4B) show that radioactive peaks elute at the positions expected for the NodBCL and NodBC metabolites on the basis of the results obtained from the TLC analysis (Fig. 3). Peak fractions were also analysed using TLC, showing that fractions 10 and 14 contain the major NodBCL and NodBC metabolites of the strain RBL5562 and RBL5957 derivatives, respectively (Fig. 5, lanes 7 and 9). These fractions were treated with the enzyme chitinase and subsequently analysed using TLC. The results show that the metabolites can be degraded by chitinase (Fig. 5, lanes 6 and 8).

Structural analysis of NodBC metabolites

Fractions generated in the final HPLC purification step (Fig. 4B) were analysed using both fast atom bombardment and collision-induced dissociation mass spectrometry (FAB-MS and CID-MS) in the positive-ion mode. In fraction 10 of strain RBL5562 (pMP2150(*nodB*), pMP2704(*nodC*)) an $[M+H]^+$ pseudomolecular ion was observed at *m/z* 1034 and A^+-type fragment ions formed by sequential cleavage of each glycosidic linkage with charge retention on the non-reducing portion of the molecule (oxonium ions) were observed at *m/z* 204, 407, 610 and 813 (Fig. 6A). These ions suggest a linear backbone of five *N*-acetyl-2-amino-2-deoxyglucose (GlcNAc) residues. CID-MS analysis of the product of de-esterification, however, revealed a reduction of 42 amu in the masses of the pseudomolecular ion (*m/z* 992) and all the oxonium fragment ions (*m/z* 162, 365, 568 and 771), indicating removal of an ester-linked acetyl moiety from the non-reducing terminal residue (Fig. 6B). This indicates the presence of a primary amine group on the non-reducing terminal residue instead of a secondary amine to which an acetyl moiety is bound. Minor fragment ions formed by glycosidic cleavage with hydrogen transfer and charge retention on the reducing terminus (β-cleavage) were observed at *m/z* 628 and 831. The fragment ion at *m/z* 204 is a double-cleavage ion, arising from a combination of A^+-type fragmentation and β-cleavage. After *N*-acetylation of the molecule using acetic anhydride, an $[M+H]^+$ pseudomolecular ion at *m/z* 1034 and A^+-type fragment ions (at *m/z* 204, 407, 610 and 813) were observed which are identical to those observed from the reference chitin pentasaccharide (Bloemberg *et al.*, 1994). These data demonstrate that the analysed NodBCL metabolite is a linear pentasaccharide $GlcNH_2GlcNAc_4$ bearing an acetyl ester on the $GlcNH_2$ residue.

The NodBC metabolite of strain RBL5957 (pMP2150(*nodB*), pMP2704(*nodC*)) (Fig. 4B, fraction 14) was also analysed using mass spectrometry. The CID mass spectrum obtained was identical to that from the de-*O*-acetylated product shown in Fig. 6B. It contained an $[M+H]^+$ pseudomolecular ion at *m/z* 992, A^+-type ions at *m/z* 162, 365, 568 and 771, β-cleavage ions at *m/z* 628 and 831 and a double-cleavage ion at *m/z* 204. These data indicate that the structure of this NodBC metabolite is $GlcNH_2GlcNAc_4$.

In vitro *labelling studies using UDP-*N*-acetyl-*D*-[U-^{14}C]-glucosamine*

To test whether NodBCL and NodBC metabolites can also be produced *in vitro*, lysates of cells grown in the presence of the inducer naringenin were incubated with ^{14}C-labelled UDP-*N*-acetyl-D-glucosamine. Cell lysates were subsequently extracted using the method of Bligh and Dyer (1959) and the aqueous fraction of the extracts was purified using HPLC as described in Fig. 4B. Radioactive peak fractions were analysed using TLC. The results from the derivatives of strains RBL5562 and RBL5957 containing both plasmids pMP2150(*nodB*) and pMP2704 (*nodC*) show that radiolabelled NodBCL and NodBC metabolites were also produced *in vitro* (Fig. 5, lanes 3 and 5). These metabolites have the same chromatographic characteristics as the NodBCL and NodBC metabolites observed for these strains after the *in vivo* radiolabelling (Fig. 5, lanes 9 and 7). The *in vitro* production of these metabolites was not observed when lysates from strains

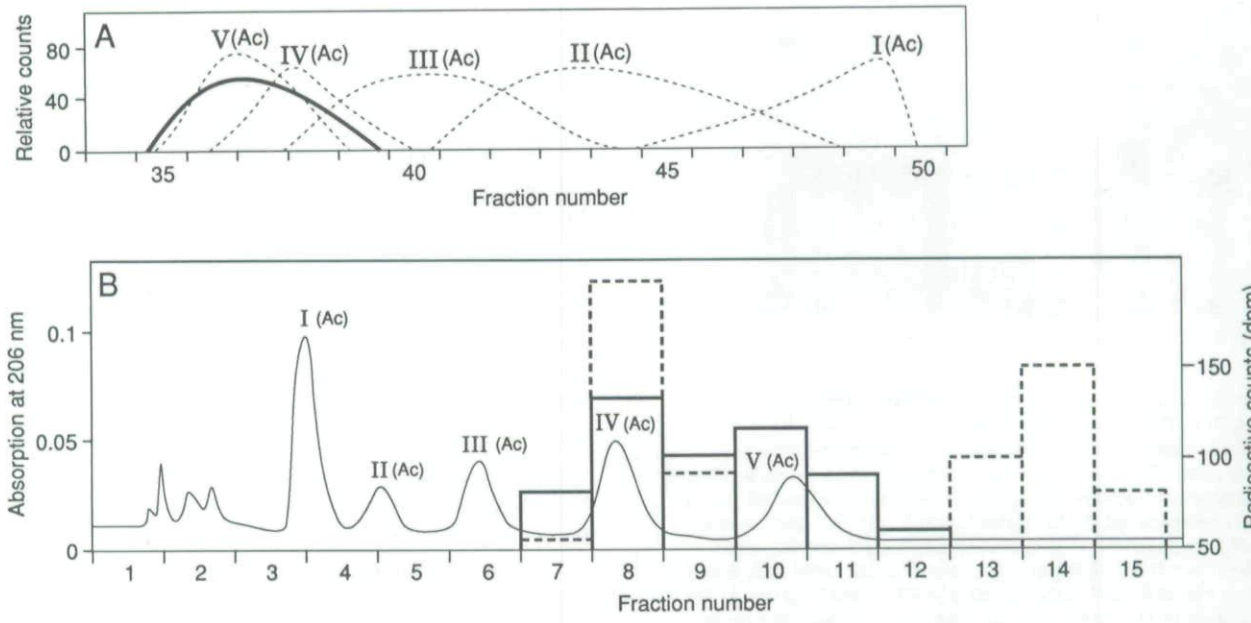

Fig. 4. Purification of NodBC metabolites from strains RBL5562 and RBL5957 harbouring plasmids pMP2150(*nodB*) and pMP2704(*nodC*).
A. Gel permeation chromatography on a Sephadex G10 column (1 × 75 cm, fraction volumes of 1 ml). The elution profile of radioactivity is indicated using a solid line for the bacterial extract of strain RBL5562(pMP2150, pMP2704). The elution profile for strain RBL5957 (pMP2150, pMP2704) was identical (data not shown). Also shown (broken lines) is a co-elution profile of a mixture of the radiolabelled *O*-acetylated reference compounds I(Ac) to V(Ac) (see Fig. 2). Quantification was achieved using phosphorimage analysis of TLC chromatograms of part of the eluted fractions. The original bar graph outputs of the Image Quant™ software are changed to smoothed lines.
B. NH_2-HPLC at a flow rate of 1 ml min^{-1}. The pooled fractions 35–40 (from A) from the strains RBL5562(pMP2150, pMP2704) (solid line bars) and RBL5957(pMP2150, pMP2704) (broken line bars) were applied and fractions of 1 ml were analysed for radioactivity as determined by liquid scintillation counting. Also indicated (thin line) is an independent HPLC profile (A_{206}) of the reference compounds I(Ac) through to V(Ac) (Fig. 2).

RBL5562 and RBL5957 that only contained plasmids pMP2150(*nodB*) or pMP2704(*nodC*) were used (data not shown). However, when the cell lysate of strain RBL5562(pMP2150(*nodB*)) was mixed with that of strain RBL5562(pMP2704(*nodC*)), the results were identical to those obtained with the strain which harboured both plasmids (data not shown).

For further analysis the NodBCL and NodBC metabolites produced *in vitro* were treated with the enzyme chitinase. The results show that chitinase treatment results in complete degradation (Fig. 5, lanes 2 and 4). The profiles of the breakdown products are indistinguishable from those of the metabolites produced *in vivo* (Fig. 5, lanes 6 and 8). The breakdown products migrate at the positions of glucosamine, *N*-acetylglucosamine and chitinbiose. Further analysis of the breakdown products using silica-TLC indicated that, as expected, other breakdown products were also produced which comigrated at the above mentioned positions in the NH_2-TLC (data not shown). These other breakdown products probably correspond to $GlcNH_2GlcNAc$ and the *O*-acetylated derivative of this compound. These results strongly suggest that the products of the *in vitro* system are identical to the NodBCL and NodBC metabolites which were subjected to structural analysis.

Discussion

In *R. leguminosarum* bv. *viciae*, the induction of the *nodABC* and *nodFEL* operons, in the absence of the other *nod* genes, leads to the production of the wild-type set of lipo-chitin oligosaccharides (Spaink *et al.*, 1993a). In this paper we show that the expression of only the *nodB* and *nodC* genes in *R. leguminosarum* is sufficient for the production of chitin oligosaccharides *in vivo* (designated NodBC metabolites). The major products were structurally identified as chitin pentasaccharides which lack an *N*-acetyl group on the non-reducing terminal sugar residue. One compound contains an additional *O*-acetyl substituent on this residue. The additional *O*-acetyl group was found in the strain RBL5562 that expresses the *nodL* gene. We assume that the presence of the additional *O*-acetyl substituent is the result of activity of the NodL protein since it is consistent with the function of this protein as a transacetylase that transfers an acetyl group onto the oxygen on C-6 of the non-reducing terminal residue of chitin oligosaccharides (Bloemberg *et al.*, 1994). The *O*-acetylated NodBC metabolite can, therefore, also be referred to as a NodBCL metabolite. The results of the TLC analysis (Fig. 3) suggest that, in addition to the NodBCL pentamer which was

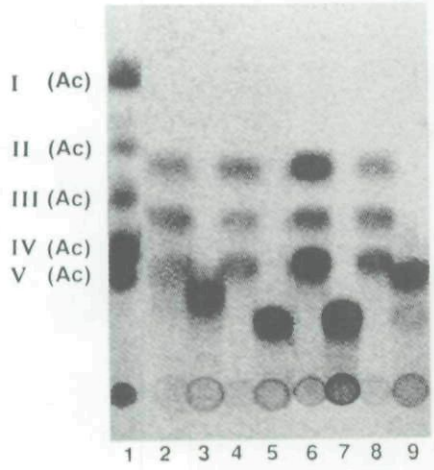

Fig. 5. Comparison of *in vitro* and *in vivo* radiolabelled NodBC metabolites by NH_2-TLC. HPLC-purified samples obtained from *in vitro* (lanes 2–5) and *in vivo* (lanes 6–9) labelling experiments were used. Samples were derived from strains RBL5562 (pMP2150, pMP2704) (lanes 2, 3, 8 and 9) and RBL5957 (pMP2150, pMP2704) (lanes 4–7). Samples of the even-numbered lanes were treated with chitinase. In lane 1 the reference sample containing the compounds I(Ac) through V(Ac) was applied (see Fig. 2).

structurally identified, a tetramer could also be produced, albeit in lower quantities. Therefore, the length of the NodBC(L) oligosaccharides seems to be confined to four or five sugar residues. *In vitro*, in the presence of UDP-*N*-acetyl-D-[U-^{14}C]-glucosamine, the same type of oligosaccharide is also produced by cell lysates of rhizobial strains in which at least the *nodB* and *nodC* genes were expressed.

These results are consistent with previous indications as to the functions of the NodB and NodC proteins. The NodB protein has been shown to have non-reducing-terminal de-*N*-acetylase activity on chitin oligomers ranging in size from two to five *N*-acetylglucosamine residues (John *et al.*, 1993). However, these authors had not shown that such substrates are produced by the rhizobial cells. For NodC, the homology with the various known classes of chitin synthases suggests that it acts as an *N*-acetyl-glucosaminyltransferase (Bulawa and Wasco, 1991; Atkinson and Long, 1992; Debellé *et al.*, 1992; Spaink *et al.*, 1993c). Geremia and co-workers (1994), who have performed radiolabelling studies using ^{14}C-labelled UDP-*N*-acetylglucosamine as a precursor, have obtained results which are strongly supportive for the presumption that the NodC protein of *Azorhizobium caulinodans* functions as an *N*-acetyl-glucosaminyltransferase. Our results from studies of strains which express the NodC protein in the absence of the NodB protein indicate that compounds which migrate as chitin oligomers on TLC are produced by means of NodC. However, the quantities of such metabolites produced is extremely low. In addition, there is also a *nodC*-dependent production of another type of compound which partitions into the chloroform phase of the Bligh–Dyer extract. The latter compound could perhaps be an oligosaccharide which is linked to a hydrophobic carrier such as bactoprenyl. In contrast, in the presence of NodB, a relatively large amount of radiolabelled glucosamine was incorporated into oligosaccharides, which allowed the isolation and identification of the reaction products. A possible explanation for these observations is that NodB, in addition to its role as a deacetylase, also has an additional function in the biosynthesis of oligosaccharides. Some possibilities are that (i) NodB enhances the enzymatic activity of NodC protein; (ii) NodB, by releasing a chitin oligomer from a bactoprenyl carrier, makes the limited amount of prenyl groups available for new rounds of synthesis; or (iii) in the absence of the *nodB* gene, NodC mainly directs the synthesis of long-chain chitin polymers which we have not detected chromatographically. In contrast, Geremia and co-workers (1994) have found high-level production of NodC-determined metabolites in the absence of the *nodB* gene. In their system the presence of the *nodB* gene did not lead to an increased production of Nod metabolites (D. Geelen, personal communication). The differences between the two systems might be the result of differences in the biochemical function of the NodC proteins of *R.l.* biovar *viciae* and the distantly related *A. caulinodans*.

Little is known about the biosynthesis of chitin in other organisms which could give clues to the biochemical function(s) of NodB and NodC. In brine shrimp, which produce the typical matrix glycoprotein that is often referred to as chitin, *in vitro* studies have yielded some information on the biosynthesis of the chitin glycoprotein (Horst, 1983). It has been suggested that, in this case, the biosynthesis involves an intermediate which consists of an oligomer of chitin ranging from two to approximately eight *N*-acetylglucosamine residues which is linked at the reducing end via a pyrophosphate linkage to a prenyl chain (Horst, 1983). However, it is not known which enzyme, perhaps analogous to NodC, is involved in this presumed step of the biosynthesis of the chitin glycoprotein.

Our identification of a NodBC metabolite as a terminally deacetylated chitin pentamer gives strong support for the hypothesis that the biosynthesis of lipo-chitin oligosaccharide molecules proceeds via such intermediates (John *et al.*, 1993; Spaink *et al.*, 1993b). If this is true, the biosynthesis of lipo-chitin oligosaccharides would be analogous to the biosynthesis of lipid A, which also proceeds via a deacetylated intermediate (Kelly *et al.*, 1993). There is a slight, but significant, similarity between the NodB protein and the EnvA protein, which is presumably involved in the deacetylation of lipid A precursors

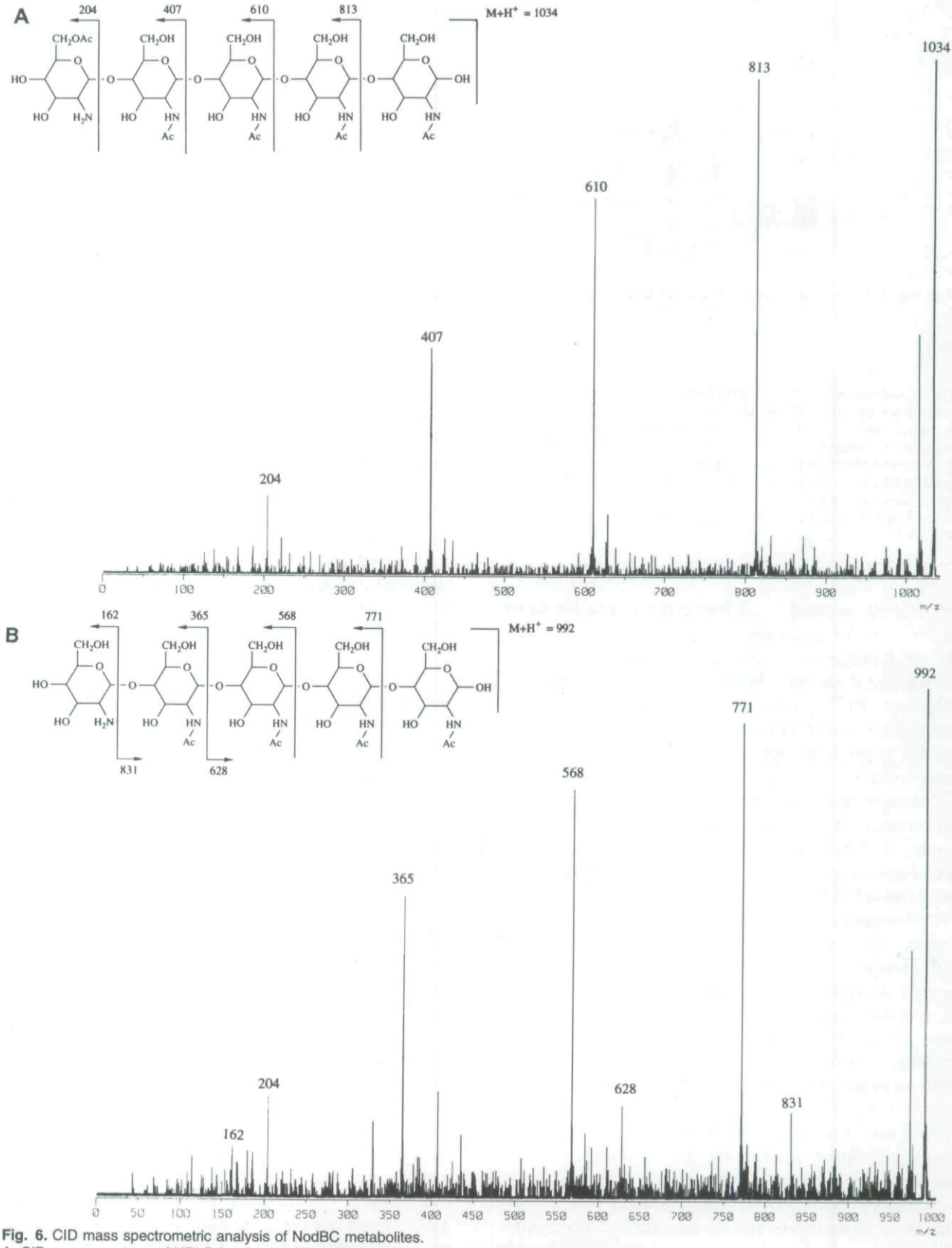

Fig. 6. CID mass spectrometric analysis of NodBC metabolites.
A. CID mass spectrum of HPLC fraction 10 (Fig. 4B) from strain RBL5562(pMP2150, pMP2704).
B. CID mass spectrum of the non-*O*-acetylated derivative. *m/z* values are quoted as nominal masses.

(Kelly *et al.*, 1993). This similarity (Spaink *et al.*, 1993b) could prove to be important if the structures of more deacetylating enzymes become available.

An important question remains as to which enzyme is responsible for the acylation of the chitin backbone. It would be very surprising if such an essential function in the biosynthesis of LCO were not encoded by a nodulation gene. Since the expression of the *nodABC* genes is sufficient for the production of lipo-chitin oligosaccharides and *Rhizobium* strains which only express the *nodBC* genes are unable to produce lipo-chitin oligosaccharides, the NodA protein is the most likely candidate for the function of transacylase. Indeed recent results demonstrate that the NodA protein is involved in the acylation of the NodBC metabolites leading to LCO in an *in vitro* system (T. Ritsema, personal communication).

Finally it should be noted that the NodBC and NodBCL metabolites could also have a biological function of their own. This is suggested by the observation that when the *nodA* gene is present the NodBCL product could also be observed (e.g. Fig. 2B, lane 1) and that these metabolites are also present in relatively large amounts in the growth medium (Fig. 3). It is remarkable that in *R. etli* strain CE3 the *nodA* and *nodBC* genes are situated in two different operons (Vásquez *et al.*, 1991) also suggesting a separate function for the lipo-chitin oligosaccharides and the NodBC metabolites. The availability of strains which only produce the NodBC(L) metabolites and not the lipo-chitin oligosaccharides will facilitate investigation of this possibility.

Experimental procedures

Bacterial strains and growth conditions

The strains and plasmids used are listed in Table 1. All *Rhizobium* strains were derived from the Sym plasmid-cured *R. leguminosarum* biovar *trifolii* strain LPR5045 (Hooykaas *et al.*, 1982). Plasmids were mobilized from *Escherichia coli* to *Rhizobium* as described previously using plasmid pRK2013 as a helper plasmid (Ditta *et al.*, 1980). Strains harbouring plasmids were grown on agar plates based on B^- medium in the presence of the appropriate antibiotics, listed in Table 1 (Spaink *et al.*, 1987). Concentrations of antibiotics used were chloramphenicol 10 mg l^{-1} and streptomycin 0.5 g l^{-1} (IncQ vectors), tetracycline 2 mg l^{-1} (IncP vectors) and spectinomycin 0.1 g l^{-1} (IncW vectors) for plasmid selection, and 20 mg l^{-1} rifampicin to select against *E. coli* after conjugation. Under these selective conditions, plasmids of the IncQ, IncP and IncW groups were stably maintained in each other's presence for at least 75 generations. This was confirmed by the observation that the strains stably maintained their capacity to produce Nod metabolites. For Nod metabolite production in liquid cultures, strains were always inoculated from bacterial cultures which were freshly grown on agar plates. For *nod* gene induction, naringenin was added to a final concentration of 4 μM. Strain RBL5957 was constructed by conjugal transfer of plasmid pMP1016 into strain LPR5045 resulting in kanamycin-resistant derivatives of the acceptor strain at a frequency of 10^{-4}. The DNA derived from one non-auxotrophic colony was tested with Southern hybridization for the presence of the intact *nodD* gene on the chromosome.

Molecular genetic techniques and construction of plasmids

Recombinant DNA techniques were carried out as described by Sambrook *et al.* (1989) and Innis *et al.* (1990). DNA restriction and polymerase enzymes were obtained from Pharmacia LKB and *Pfu* DNA polymerase used for PCR was obtained from Stratagene (USA). Restriction sites were removed using the Klenow fragment of DNA polymerase I as described by Sambrook *et al.* (1989). The construction of the plasmid is outlined in Fig. 1. Oligonucleotide primers used for PCR were oMP4 (5′-GAGAACATATGACCCTGCTCGCAACAAACCAG-C-3′), oMP5 (5′-TCCCAAGATCTGAGCTGCAGTGACGCG-TTCATCAC-3′), oMP51 (5′-GGACATATGAAGCGGCCCGC-ATATATGAGCGAAG-3′) and oMP52 (5′-TTTGGATCCT-CAGGGAAGTGAACGAATCTCGAATC-3′). The nucleotide sequence of the *nodB*-containing fragment of pMP2723 was checked and appeared not to contain any unwanted mutations. pMP2733 is an IncW cloning vector which is derived from pRI40 (Innes *et al.*, 1988) by removal of the *Eco*RI and *Bam*HI sites. pMP177 is a derivative of pIC20H which contains a *Kpn*I–*Bgl*II fragment derived from plasmid pRt308 (Djordjevic *et al.*, 1986). pMP2410 was constructed by inserting the *Hin*dIII fragment from pMP1060 containing the *nodL* gene under the control of the *nodA* promoter (Bloemberg *et al.*, 1994) into plasmid pMP190. pMP3402 is derived from pMP1070 (Bloemberg *et al.*, 1994) by exchange of the multicloning site for a synthetic DNA fragment generating the following sequence of restriction sites (in order of appearance behind the *nodA* promoter): *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Kpn*I, *Bam*HI, *Pst*I, *Nhe*I, *Sph*I and *Hin*dIII.

Reference compounds

The radiolabelled reference compounds 6-*O*-acetyl-*N*-acetylglucosamine, 6-*O*′-acetyl-*N*,*N*′-acetylchitobiose, 6-*O*″-acetyl-*N*,*N*′,*N*″-acetylchitotriose, 6-*O*‴-acetyl-*N*,*N*′,*N*″,*N*‴-acetylchitotetraose and 6-*O*⁗-acetyl-*N*,*N*′,*N*″,*N*‴,*N*⁗-acetylchitopentaose were prepared using purified NodL protein as described previously (Bloemberg *et al.*, 1994). These compounds are referred to as I(Ac) through to V(Ac) in Figs 2, 3 and 4. *N*-acetyl-D-[1-^{14}C]-glucosamine was obtained from Amersham (UK).

Radiolabelling

D-[1-^{14}C]-glucosamine (GlcN, 50 mCi $mmol^{-1}$) and uridine diphospho-*N*-acetyl-[U-^{14}C]-glucosamine (UDP-GlcNAc, 200 mCi $mmol^{-1}$) were obtained from Amersham (UK). For *in vivo* labelling, cells were grown in 1 ml cultures for 12 h in the presence of 0.2 μCi of GlcN. Cultures were inoculated at an initial optical density (260 nm) of 0.05. For *in vitro* labelling, 250 ml cultures were grown in the presence of naringenin to

OD_{260} = 0.5 and centrifuged. The cell pellets were resuspended in 25 ml buffer (50 mM Tris-HCl pH 7.4; 50 μM β-mercaptoethanol) and lysed by three passages through a French pressure cell. To 2 ml of cell lysate 0.22 ml $MgCl_2$ (0.1 M) and 0.2 μCi of labelled UDP-GlcNAc were added and the mixture was incubated for 14 h at 22°C.

Thin-layer chromatography

Cells or cell lysates were extracted according to Bligh and Dyer (1959). The aqueous (upper) and chloroform/methanol (lower) phases of the two-phase system were evaporated and taken up in 100 μl of acetonitrile/water (30:70, v/v) or chloroform, respectively. Unless indicated otherwise, 1/10 of the samples was applied to TLC plates. Samples were analysed on silica-60 TLC plates with concentration zone (Merck) using *n*-butanol/ethanol/water (50:30:20, v/v/v) as the mobile phase. Samples were chromatographed on NH_2-TLC plates (Merck) using a mobile phase of acetonitrile/water (70:30, v/v). Radioactivity on TLC plates was analysed with a PhosphorImaging system from Molecular Dynamics, using the Image Quant™ software.

Metabolite purification

For gel-permeation chromatography, a Sephadex G10 column (1 × 75 cm) was used which was equilibrated in buffer (0.15 M NH_4Ac, 7% propanol). The column was eluted with buffer at a flow rate of 0.5 ml min^{-1}. Fractions were evaporated and taken up in water. HPLC separation was performed on a nucleosil 120–7 NH_2-HPLC column obtained from Macherey Nagel. Acetonitrile was added to the reaction mixture to a final concentration of 70% and the sample was filtered over a 45 μm Spin X 8170 nylon membrane (Costar) before the mixture was loaded onto the column. Compounds were separated using an isocratic elution in 70% acetonitrile in water.

Mass spectrometry

FAB mass spectra were obtained using MS 1 of a JEOL JMS-SX/SX 102A tandem mass spectrometer operated at 10 kV accelerating voltage. 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 s 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. 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. Samples of 1–3 μl of the solutions were loaded into a matrix of monothioglycerol.

Derivitization of oligosaccharides

For de-*O*-acetylation, samples were taken up in 100 μl 1:1 methanol/concentrated NH_4OH (18 h, room temperature) and subsequently the mixture was dried under nitrogen. The residue was redissolved in 8 μl water prior to FAB mass spectrometric analysis.

For *N*-acetylation, samples were dissolved in 10 μl water and 0.5 ml of methanol/acetic anhydride (3:1, v/v) was added. The mixture was incubated for 2 h at room temperature and subsequently dried under vacuum.

Chitinase from *Streptomyces griseus* was obtained from Sigma and applied at 0.001 U ml^{-1} at pH 6.0. Oligosaccharides were digested for 6 h.

Acknowledgements

We are very grateful to H. R. M. Schlaman for making available to us plasmid pMP2065, and to E. Groot-Scheeren and P. M. Nelisse for their help with part of the experiments. We thank D. Geelen, M. Holsters (Ghent University) and T. Ritsema (Leiden University) for communicating results prior to publication. This work was supported by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organization for Scientific Research and by Grant BIO2-CT93-0400 from the European Community (DG12 SSMA). H.P.S. was supported by the Royal Netherlands Academy of Arts and Sciences.

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