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NOTES

Mass Spectrometric Analysis of Chitin Oligosaccharides Produced by *Rhizobium* NodC Protein in *Escherichia coli*

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A system for studying the in vivo activity of *Rhizobium* NodC protein in *Escherichia coli* has been developed. Using thin-layer chromatography, high-performance liquid chromatography, and mass spectrometry, we show that in this system *R. leguminosarum* bv. viciae NodC protein directs the synthesis of chitinpentaose, chitintetraose, chitintriose, and two as yet unidentified modified chitin oligosaccharides.

Rhizobial lipochitin oligosaccharides are signal molecules which are essential for nodulation (5, 8). Several reports strongly suggest that NodC is an *N*-acetyl-glucosaminyltransferase which is involved in the synthesis of precursor chitin oligosaccharides (6, 12, 13). However, final proof is lacking since the chemical structures of the metabolites produced by NodC in the absence of the other *nod* genes have not been reported. In previous reports, the detection of *nodC*-dependent metabolites in a *Rhizobium leguminosarum* bv. viciae strain lacking functional *nodAB* genes has been described (12, 13), but the amounts produced were too low for chemical analysis. Here we report the production of *nodC*-dependent metabolites in *Escherichia coli*, their purification, and structural analysis.

Analysis of *nodC*-dependent metabolites in *E. coli* by TLC. To obtain expression of *nodC* in *E. coli*, we introduced plasmid pMP2065 (13), constructed by cloning the *R. leguminosarum* bv. viciae *nodC* gene under the control of the T7 promoter in the expression vector pET9a (14), into *E. coli* JM101. Strains carrying either pMP2065 or the vector pET9a without an insert were grown overnight at 37°C in LC medium in the presence of 50 μg of kanamycin per ml, diluted 1:100 in fresh medium, and grown to an A_{620} of 0.2. To 1 ml of these cultures, 0.2 μCi of D-[1-^{14}C]glucosamine (GlcN; 50 mCi mmol^{-1}; obtained from Amersham, International, Amersham, England) was added, and *nodC* expression was induced by infection with the T7 RNA polymerase-containing phage mGP1-2 (16). After various periods of incubation at 37°C, bacteria were collected by centrifugation and extracted by the method of Bligh and Dyer (1). The aqueous phase was dried, and the material was dissolved in 10 μl of water. A volume of 1 μl of this sample was applied to a silica 60-NH_2 thin-layer chromatography (NH_2-TLC) plate and developed by using acetonitrile-water (65:35, vol/vol) as the mobile phase. The results (Fig. 1) show that within 20 min after induction of *nodC*, four *nodC*-dependent radiolabelled spots can be detected. Since these spots were absent from extracts of the control strain, we conclude that they are due to the expression of *nodC*. Extracts from JM101/pMP2065 (*nodC*) cells that were induced overnight contained very minor amounts of *nodC*-dependent metabolites (Fig. 1).

HPLC analysis and purification of *nodC*-dependent metabolites from *E. coli*. For high-performance liquid chromatographic (HPLC) analysis, extracts from 5-ml cultures of JM101/pMP2065 and the control strain JM101/pET9a were prepared after 2 h of *nodC* induction in the presence of D-[1-^{14}C]GlcN (0.2 μCi ml^{-1}) as described above. Acetonitrile was added to a final concentration of 75%, after which the samples

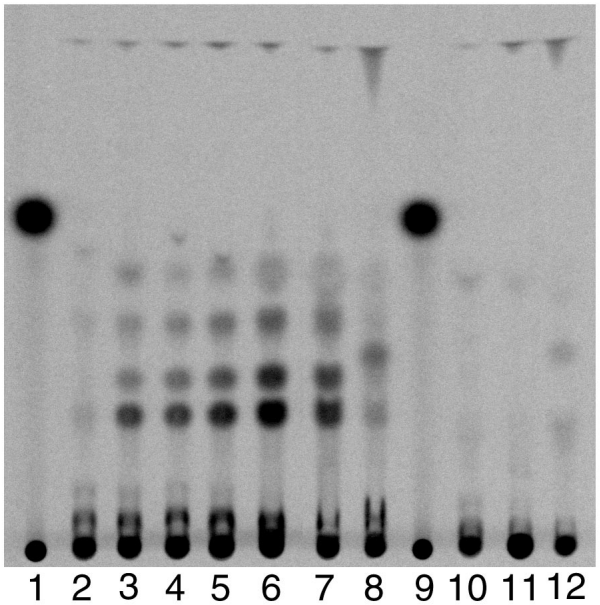

FIG. 1. TLC analysis of in vivo-radiolabelled NodC metabolites. After induction of *nodC* expression in the presence of D-[1-^{14}C]GlcN, cells were extracted and the resulting aqueous samples were analyzed on an NH_2-TLC plate (Merck, Darmstadt, Germany) as described in the text. Results were visualized with a PhosphorImager system (Molecular Dynamics, Sunnyvale, Calif.) in combination with ImageQuant software. Lanes: 1 and 9, *N*-acetyl-D-[1-^{14}C]GlcN; 2 to 8, JM101/pMP2065 (*nodC*), 10 min, 20 min, 40 min, 1 h, 2 h, 6 h, and 20 h after induction, respectively; 10 to 12, JM101/pET9a (control), 10 min, 4 h, and 20 h after induction, respectively.

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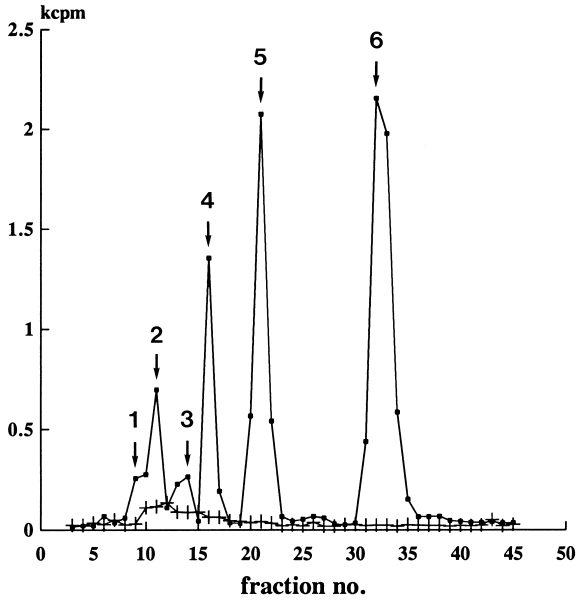

FIG. 2. NH_2-HPLC analysis of in vivo-radiolabelled *nodC*-dependent metabolites. Extracts from strain JM101/pMP2065 (*nodC*) (■) and JM101/pET9a (control; +), following D-[1-^{14}C]GlcN labelling as described in the text, were loaded onto a nucleosil 120-7 NH_2-HPLC column (Macherey-Nagel, Düren, Germany) and eluted with acetonitrile-water (75:25, vol/vol). Fractions (1 ml) were collected, and the radioactivity in 100 μl of each fraction was determined. Arrows indicate the fractions used for subsequent TLC analysis (see Fig. 3).

were loaded onto a nucleosil 120-7 NH_2-HPLC column, from which they were eluted isocratically in 75% acetonitrile in water at a flow rate of 1 ml min^{-1}. Fractions (1 ml) were collected, and 100 μl of each fraction was analyzed for the presence of radioactivity, using liquid scintillation counting. The elution profiles of extracts from the *nodC*-expressing strain and the control strain (Fig. 2) show that the two fastest-migrating *nodC*-dependent spots in the NH_2-TLC system (Fig. 1) are both separated into two peaks in the HPLC system. In a control experiment in which *N*-acetyl-D-glucosamine (GlcNAc) oligomer standards were chromatographed, HPLC peaks 1, 3, 5, and 6 (Fig. 2) were found to elute at the same position as the chitin oligosaccharides $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$ and $(GlcNAc)_5$, respectively (data not shown). Fractions corresponding to each peak top (as indicated in Fig. 2) were dried, taken up in a small volume of water, and analyzed on a silica 60 TLC plate, using 1-propanol–ammonium hydroxide (32% ammonia solution)–water (60:40:1.5, vol/vol/vol) as the mobile phase. The results (Fig. 3) of assays using this separating system also indicate that peaks 1, 3, 5, and 6 of Fig. 2 are chitin oligosaccharides and that peaks 2 and 4 are structurally different. The HPLC-purified radiolabelled compounds from peaks 2, 4, 5, and 6 (Fig. 2) were incubated with 0.002 U of *Streptomyces griseus* chitinase (Sigma) per ml for 1 h at pH 6.0. The compounds from peaks 5 and 6 were completely digested, yielding chitin disaccharides and a mixture of chitin di- and trisaccharides, respectively (Fig. 3B, lanes 6 to 9). This finding is in agreement with the HPLC results, which suggested the presence of chitin tetra- and pentasaccharides in peaks 5 and 6, respectively. The additional *nodC*-dependent compounds from peaks 2 and 4 (Fig. 2), which did not migrate as linear chitin oligosaccharides on HPLC, are also degraded by the chitinase (Fig. 3B, lanes 2 to 5). This result indicates that these are modified chitin oligosaccharides.

To purify the *nodC*-dependent metabolites for mass spectrometric analysis, aqueous extracts from 100-ml cultures of strains JM101/pMP2065 (*nodC*) and JM101/pET9a (control) were prepared as described above. To the concentrated extract of strain JM101/pMP2065, 10 nCi of a radiolabelled extract of

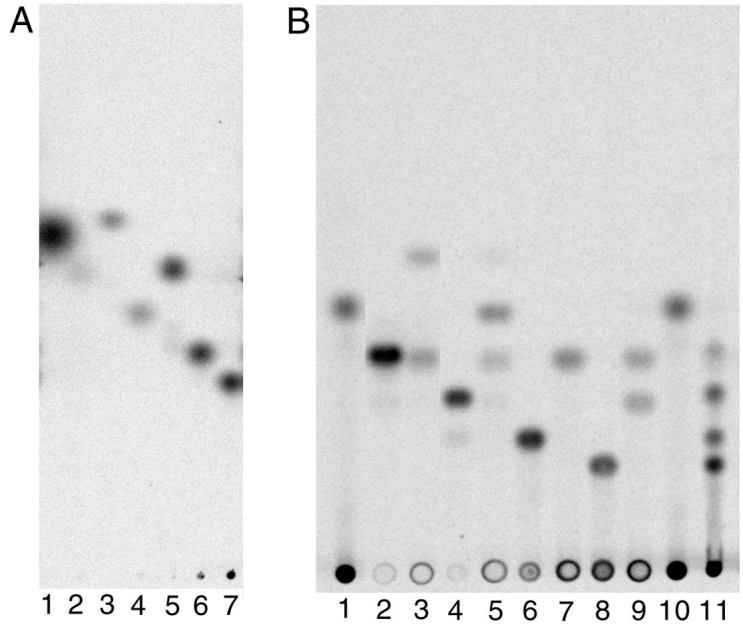

FIG. 3. TLC analysis of HPLC-purified, radiolabelled *nodC*-dependent metabolites. (A) D-[1-^{14}C]GlcN-labelled *nodC*-dependent metabolites, extracted from strain JM101/pMP2065 (*nodC*), were separated on an NH_2-HPLC column (Fig. 2), concentrated, and analyzed on a silica 60 TLC plate (Merck) as described in the text. Lanes: 1, *N*-acetyl-D-[1-^{14}C]GlcN; 2 to 7, HPLC peaks 1 to 6 of Fig. 2, respectively. (B) Chitinase treatment of HPLC-purified *nodC*-dependent metabolites. HPLC peaks 2, 4, 5, and 6 (Fig. 2) were incubated with chitinase as described in the text and subsequently analyzed on an NH_2-TLC plate (Merck). The even-numbered lanes from lanes 2 to 9 represent untreated samples, whereas the odd-numbered lanes show chitinase-treated samples. Lanes: 1 and 10, *N*-acetyl-D-[^{14}C]GlcN; 2 and 3, peak 2; 4 and 5, peak 4; 6 and 7, peak 5; 8 and 9, peak 6; 11, in vivo-radiolabelled NodC metabolites from strain JM101/pMP2065 (*nodC*).

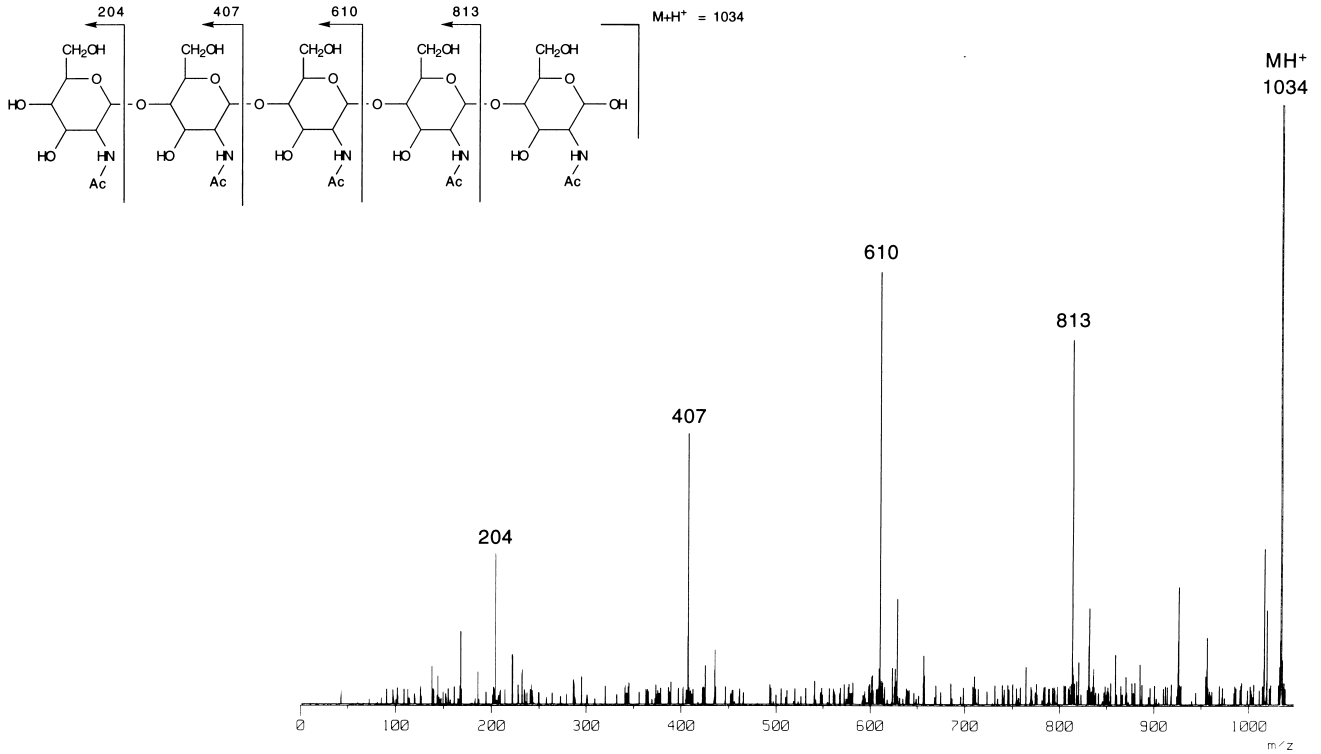

FIG. 4. Collision-induced dissociation tandem mass spectrometric analysis of the *nodC*-dependent $[M + H]^+$ ion at *m/z* 1034 in HPLC fraction 32 of Fig. 2 from strain JM101/pMP2065 (*nodC*).

the same strain (Fig. 1, lane 6) was added, and the mixture was separated by using NH_2-HPLC as indicated in Fig. 2. Of each fraction, 500 μl was assayed for the presence of radioactivity. The elution pattern observed was identical to that shown in Fig. 2 (data not shown).

Structural analysis of *nodC*-dependent metabolites. HPLC fractions obtained from strain JM101/pMP2065 (*nodC*) were analyzed by using fast atom bombardment (FAB) mass spectrometry. FAB mass spectra were obtained in the positive ion mode, using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer at 10- or 6-kV accelerating voltage. The FAB gun was operated at 6 kV with an emission current of 10 mA, using xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for *m/z* 100 to 1,500 (10 kV) or 1,500 to 4,000 (6 kV) and were recorded and processed on a Hewlett Packard HP9000 series data system, using the JEOL Complement software. Tandem mass spectra were obtained on the same instrument, using helium as the collision gas (in the third field free region) at a pressure sufficient to reduce the parent ion to one-third of its original intensity. The dried HPLC fractions were redissolved in 20 μl of dimethyl sulfoxide, and 2-μl aliquots of the sample solutions were loaded into a matrix of thioglycerol. The FAB mass spectrum of fraction 32 contains an $[M + H]^+$ pseudomolecular ion at *m/z* 1034, corresponding to an oligosaccharide consisting of five GlcNAc residues. The spectrum obtained on collision-induced dissociation of this ion contains A^+-type fragment ions formed by sequential cleavage of the glycosidic linkages with charge retention on the nonreducing terminal fragment (Fig. 4). These fragment ions, at *m/z* 813, 610, 407, and 204, correspond to a linear chitin pentasaccharide. No chitin oligomers were detectable in the other fractions. To improve sensitivity, samples were submitted to peracetylation (17). For peracetylation, 50% of the HPLC fractions were dried and 500 μl of trifluoroacetic anhydride-acetic anhydride (2:1, vol/vol) was added. After 30 min at room temperature, the samples were dried under vacuum, and the residues were redissolved in 20 μl of methanol prior to mass spectrometric analysis. The FAB mass spectrum of fraction 14 contains a minor $[M + H]^+$ pseudomolecular ion at *m/z* 964, corresponding to a peracetylated chitin trisaccharide, while that of fraction 21 contains an intense $[M + H]^+$ pseudomolecular ion at *m/z* 1251 for the corresponding tetrasaccharide. HPLC fractions from the control strain JM101/pET9a having equivalent retention times were also analyzed by FAB mass spectrometry. Ions for chitin oligomers were not observed in any of the fractions, confirming that the presence of *nodC* is necessary for the production of chitin oligosaccharides. In fractions 11 and 16, no molecular ions which could be linked to the presence of *nodC* were detected. The HPLC analysis shown in Fig. 2, however, clearly shows the presence of *nodC*-dependent compounds in these fractions (peaks 2 and 4). It will therefore be necessary to develop additional purification methods or use different chemical analysis strategies to determine the identities of these additional *nodC*-dependent compounds.

In conclusion, these results show that the *N*-acetyl-glucosaminyltransferase encoded by *nodC* directs the synthesis of (β1→4) *N*-acetyl-D-glucosamine oligomers and that none of the other *nod* gene products is needed for this activity. Although oligomers with a degree of polymerization of 2 to 5 have been detected, GlcNAc dimers and trimers seem to be minor products. The two modified chitin oligosaccharides detected could represent carrier-linked oligosaccharides, considering the fact that biosynthesis of many oligosaccharides is known to proceed through such intermediates (2, 7, 9). However, we cannot exclude the possibility that the appearance of modified chitin oligosaccharides is due to physiological differ-

ences between *E. coli* and *R. leguminosarum* bv. viciae and results from the presence in *E. coli* of acceptor molecules that do not exist in rhizobia. Our results show that an *E. coli* background can be used to study the biosynthesis of chitin oligosaccharides by NodC. Our system may also be useful in the study of proteins which are homologous to NodC, such as FbfA from *Stigmatella aurantiaca* (11) and DG42 from *Xenopus laevis* (3, 10), whose biochemical functions have not been reported.

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