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Rhizobium NodI and NodJ Proteins Play a Role in the Efficiency of Secretion of Lipochitin Oligosaccharides

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Thin-layer chromatographic analysis of extracts of D-[1-^{14}C]glucosamine-labelled rhizobia was used to analyze the effects of *nodI*, *nodJ*, and *nodT* on secretion of lipochitin oligosaccharide (LCO) signal molecules. Secretion was analyzed by comparing quantitities of radiolabelled LCOs present in the cellular and spent growth medium fractions. A second rapid and sensitive method was introduced to estimate the secreted LCO fractions by using D-[1-^{14}C]glucosamine-labelled cells grown in medium supplemented with chitinase. At various times after induction of LCO synthesis, the quantity of degradation products of LCOs was compared with the amount of nondegraded LCOs. In wild-type strains of *Rhizobium leguminosarum* biovars viciae and trifolii the *nodI* and *nodJ* genes (but not the *nodT* gene) strongly enhance the secretion of LCOs during the first 5 h after the induction of LCO synthesis. In LCO-overproducing strains the enhancement of secretion was observed only during the first 3 h after induction. At times later than 5 h after induction, a significant influence of the presence of the *nodI* and *nodJ* genes on LCO secretion was detectable neither in the wild type nor in LCO-overproducing strains. By using plasmids in which the *nodI* and *nodJ* genes are cloned separately under control of a flavonoid-inducible promoter, it was shown that both genes are needed for a wild-type level of LCO secretion. Therefore, these results demonstrate that *nodI* and *nodJ* play a role in determining the efficiency of LCO secretion.

Rhizobia, bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*, establish a nitrogen-fixing symbiosis in root nodules of leguminous plants. Lipochitin oligosaccharide (LCO) signal molecules produced by the rhizobia play a pivotal role in the infection process and the formation of root nodules. Various so-called *nod* (for nodulation) genes play an essential role in the biosynthesis of the LCOs (7). Common to all rhizobia are the genes *nodA*, *nodB*, *nodC*, *nodI*, and *nodJ*, which are usually organized in one transcriptional unit which is regulated by plant flavonoids (5, 12, 22). The first three of these genes were shown to be sufficient for the biosynthesis of LCO molecules (28). Evidence that NodC encodes a chitin oligosaccharide synthase, NodB encodes a chitin oligosaccharide deacetylase, and NodA encodes an acyltransferase is accumulating (1, 11, 15, 19, 29).

The *nodI* and *nodJ* genes are similar to genes involved in ATP-dependent polysaccharide secretion (32). NodI is homologous to KpsT from *Escherichia coli*, BexB from *Haemophilis influenzae*, and CtrD from *Neisseria meningitidis*, all of which contain an ATP-binding motif. In addition, the NodI protein was shown to be localized in the cytoplasmic membrane fraction and was shown to contain a leucine zipper motif indicative of the ability to form dimers (12, 21). NodJ is similar to KpsM, BexA, and CtrC, which, on basis of their genetic organization and their hydrophobicity patterns, are presumed to be cytoplasmic membrane proteins associated with KpsT, BexB, and CtrD, respectively (32).

Both on the basis of homology studies and on the basis of their genetic organization in one operon together with the *nodA*, *nodB*, and *nodC* genes, the NodI and NodJ proteins have been implicated in the secretion of LCO molecules (10, 32). Since NodT protein is similar to outer membrane proteins which form parts of secretion complexes, it was suggested to be involved in LCO secretion in a complex with NodI and NodJ (10). However, direct secretion studies of LCOs have yielded conflicting data. Spaink et al. (24) reported that inactivation of the *nodI*, *nodJ*, and *nodT* genes in a wild-type *Rhizobium leguminosarum* biovar viciae strain did not influence the amount of secreted LCOs after overnight induction of the *nod* genes. By using LCO-overproducing strains, a positive effect of the *nodI* and *nodJ* genes under the same conditions in the same chromosomal background was detected (23). In contrast, McKay and Djordjevic (16) reported that *nodI* and *nodJ* are essential for secretion of LCOs in an LCO-overproducing strain of *R. leguminosarum* biovar trifolii after overnight induction.

In this article we present data on the roles of *nodI* and *nodJ* in secretion of LCOs. Using D-[1-^{14}C]glucosamine labelling studies and a newly developed method for the rapid quantification of secreted LCOs, we have analyzed the influence of LCO overproduction, *nod* gene induction time, and chromosomal background on the LCO secretion process. These results show that *nodI* and *nodJ* play a role in the efficiency of LCO secretion.

MATERIALS AND METHODS

Plasmids and bacterial strains. The construction of plasmids is illustrated in Fig. 1. Plasmid pMP3510 was constructed by replacing the multicloning site of the IncP plasmid pMP1070 (3) by a synthetic oligonucleotide, producing the multiple cloning site indicated in Fig. 1. IncP and IncW plasmids were mobilized from *E. coli* to *Rhizobium* strains by using the method of Ditta et al. (8) and selection for resistance to rifampin (20 $\mu g \cdot ml^{-1}$), tetracycline (IncP plasmids, 2 $\mu g \cdot ml^{-1}$), and spectinomycin (IncW plasmids, 100 $\mu g \cdot ml^{-1}$) (29). *Rhizobium* strains are described in Table 1. Since the *nodL* gene, encoding an *O*-acetyltransferase (3), was shown to be important for the quantity of produced LCOs, we made use of derivatives of strain RBL5970 (which contains pMP2105) for the analysis of LCO (Table 1).

Analysis of LCOs. Cells were grown at 29°C in B$^-$ medium containing suitable antibiotics for plasmid selection as described previously (29). For secretion studies, cells were precultured overnight to an optical density at 620 nm of 0.3 and diluted to an optical density at 620 nm of 0.1 in 2.0 ml of B$^-$ medium. Sub-

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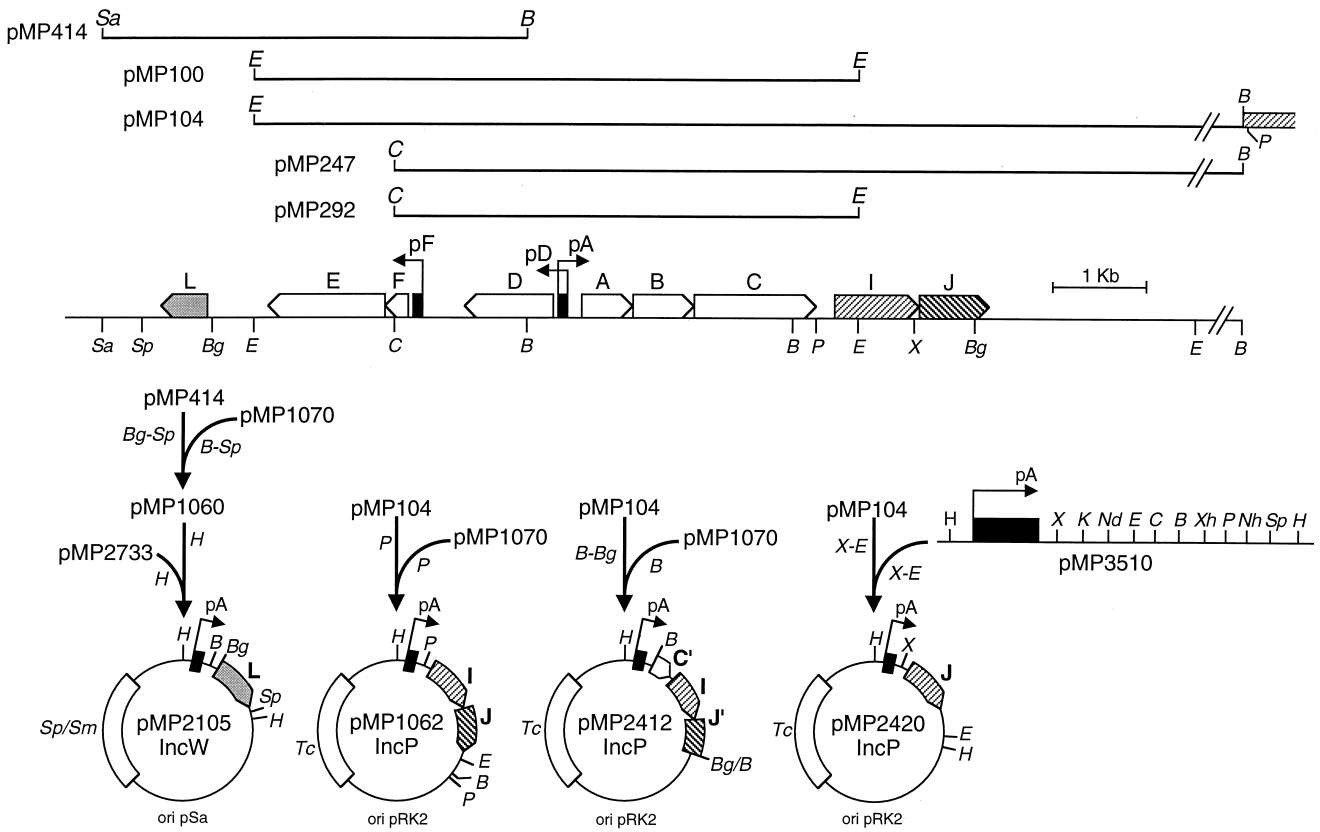

FIG. 1. Construction of plasmids. The genetic map and the encoded *nod* genes of part of the Sym plasmid pRL1JI of *R. leguminosarum* biovar viciae are shown. Restriction sites used for cloning: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; Nh, *Nhe*I; P, *Pst*I; Sp, *Sph*I; X, *Xba*I. Sp, Sm, and Tc, genes encoding resistance to spectinomycin, streptomycin, or tetracycline, respectively; ori, origin of replication; pA, promoter of *nodA*.

sequently, 0.4 μCi of D-[1-^{14}C]glucosamine (specific activity, 50 mCi · mmol^{-1}; Amersham, Buckinghamshire, United Kingdom) and a final concentration of 1.0 μM of the *nod* gene inducer naringenin was added. After the indicated period of time, the entire culture was centrifuged for 15 min at 18,000 rpm in Eppendorf tubes, and the spent growth medium fraction was extracted with 1.0 ml of water-saturated *n*-butanol as described previously (29). The pellet fraction obtained after centrifugation was resuspended in 0.5 ml of water and, following the addition of 0.5 ml of water-saturated *n*-butanol, was boiled for 15 min. As a control, the pellet fractions were extracted by the method of Bligh and Dyer (2) as described previously (29). Extracts were analyzed by thin-layer chromatography (TLC) as follows. Extracts were dried and dissolved in 20 μl of acetonitrile-water (1:1). A volume of 6 μl of the samples was loaded on Silica Gel 60 TLC plates (category HPTLC; Merck, Darmstadt, Germany) and developed by using *n*-butanol-ethanol-water (5:3:2) as the solvents (29). In this TLC system, LCOs of *R. leguminosarum* are separated according to differences in the lengths of the oligosaccharide backbone (13). The distribution of radioactivity on the TLC plates was analyzed by using a Phosphorimager and the ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.). Analysis of the uptake of added radiolabelled glucosamine showed that cells take up more than 50% of the label within 1 h.

Chitinase treatment of cultures. As an alternative strategy for analysis of LCO secretion, 0.01 U of chitinase (*Streptomyces griseus*, Sigma Chemical Co., St. Louis, Mo.) was added to the culture at the time of induction with naringenin. At the indicated time after induction, 1.0 ml of *n*-butanol was added to the entire culture, and the mixture was boiled for 15 min. *n*-Butanol extracts were analyzed as described above. Degradation products of LCOs were identified by comparisons with published standards of di-, tri-, and tetrasaccharidic derivatives of the LCO NodRlv-V ($C_{18:4}$, Ac) (13).

Test for cell lysis. Cell lysis was judged by the presence of cytoplasmic proteins in the spent growth medium and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6) and silver staining (31). Samples of the spent growth medium were prepared by the addition of 5% trichloracetic acid and centrifugation. The pellet fraction was subsequently washed three times with ether and dissolved in sample buffer (6).

RESULTS

Secretion of LCOs in *nod* gene-overproducing strains. In order to analyze LCO secretion shortly after induction of the *nod* genes, we constructed a series of *nod* gene-overproducing strains derived from strain RBL5970 (Table 1). After induction by naringenin, the production and secretion of LCOs by cells of these strains was tested by using radiolabelled D-[1-^{14}C]glucosamine. The results obtained by TLC analysis of ^{14}C-labelled LCOs present in the cell pellets and in the spent growth medium of strain RBL5970(pMP247) (*nodDABCIJ*) and of strain RBL5970(pMP292) (*nodDABC*) are shown in Fig. 2A. Radioactivity of the spots which were identified as LCOs consisting of five or four saccharide units (13, 28) is quantified in Fig. 3A for the strain containing plasmid pMP247 (*nodDABCIJ*) and in Fig. 3B for the strain containing pMP292 (*nodDABC*). The results of triplicate experiments showed that variation in the relative levels of LCOs was less than 15% at various times after induction (data not shown). Absolute counts could vary largely between independent experiments (e.g., see the legend to Fig. 2). We have also tested whether the presence of LCOs in the spent growth medium could be due to cell lysis. However, analysis of the spent growth medium for the presence of cytoplasmic proteins using polyacrylamide gel electrophoresis showed that this was not the case (data not shown). From these results we conclude that (i) the production of LCOs can be detected 40 min after induction, (ii) at 40, 60, and 80 min after induction the strain containing the *nodIJ* genes secretes signif-

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference
Strains		
LPR5045	*R. leguminosarum* biovar trifolii strain RCR5, cured from the Sym plasmid, Rif^r	14
RBL5560	LPR5045 with *R. leguminosarum* biovar viciae Sym plasmid pRL1JI *mep*::Tn*5*, wild-type nodulation on *Vicia* plants, Rif^r Km^r	26
RBL5729	LPR5045 Sm^r with pRL1JI *nodI*::Tn*5*, Rif^r Km^r	33
RBL5734	LPR5045 Sm^r with pRL1JI *nodJ*::Tn*5*, Rif^r Km^r	33
RBL5795	LPR5045 with pRL1JI *nodT*::Tn*5phoA*, Rif^r Km^r	4
RBL5970	LPR5045 with pMP2105, contains *nodL*^{b}, Rif^r Sp^r Sm^r	This study
ANU843	Wild-type *R. leguminosarum* biovar trifolii	20
ANU261	ANU843 *nodI*::Tn*5*, Km^r	9
Plasmids		
pMP100	IncP^b, contains *nodEFDABC*, Tc^r	This study
pMP104	IncP^b, contains *nodEFDABCIJ*, Tc^r	27
pMP247	IncP^b, contains *nodDABCIJ*, Tc^r	This study
pMP292	IncP^b, contains *nodDABC*, Tc^r	This study
pMP414	ColE1, contains *nodFEL*^{b}, Ap^r	This study
pMP1060	IncP, contains *nodL* under control of the promoter of *nodA*, Tc^r	3
pMP1062	IncP^b, contains *nodIJ* under control of the promoter of *nodA*, Tc^r	This study
pMP1070	IncP, expression vector containing the promoter of *nodA*, Tc^r	3
pMP2105	IncW^b, contains *nodL* under control of the promoter of *nodA*, Sp^r Sm^r	This study
pMP2412	IncP^b, contains *nodI* under control of the promoter of *nodA*, Tc^r	This study
pMP2420	IncP^b, contains *nodJ* under control of the promoter of *nodA*, Tc^r	This study
pMP2733	IncW, cloning vector, Sp^r Sm^r	29
pMP3510	IncP^b, expression vector containing the promoter of *nodA*, Tc^r	This study

[a] Ap^r, Km^r, Rif^r, Sp^r, Sm^r, and Tc^r, resistance to ampicillin, kanamycin, rifampin, spectinomycin, streptomycin, or tetracycline, respectively.
[b] See Fig. 1.

icantly higher levels of LCOs than the strain lacking the *nodIJ* genes, and (iii) 180 min after induction no significant difference in secretion of LCOs in the absence or presence of *nodIJ* was detected. The largest effect of *nodIJ* is found at 60 min after induction, when in the presence of *nodIJ* 67% of the LCOs are found in the medium fraction compared with 34% in the absence of *nodIJ* (Fig. 3A and B). The same conclu sions can be drawn by comparing the results obtained with the strains harboring pMP104 (*nodEFDABCIJ*) or pMP100 (*nodEFDABC*), as shown in Fig. 3C and D, respectively. The presence of the *nodFE* genes apparently does not influence LCO secretion.

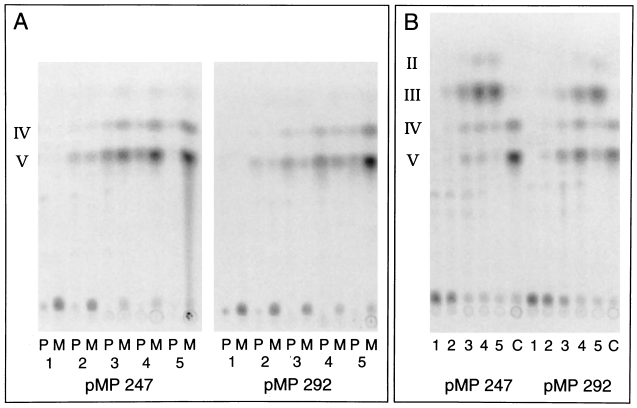

FIG. 2. TLC analysis of LCO production by the *nod* gene-overproducing strains RBL5970(pMP247) and RBL5970(pMP292). Extracts, equivalent to 0.6 ml of culture, were analyzed at various time points after induction with naringenin as follows: lanes 1, 20 min; lanes 2, 40 min; lanes 3, 60 min; lanes 4, 80 min; lanes 5, 180 min. (A) Analysis of *n*-butanol extracts of the pellet (P) and spent growth medium (M) fractions; (B) analysis of *n*-butanol extracts of the entire culture of bacteria grown in the presence of chitinase. Lanes C, extracts of cells grown 180 min after induction in the absence of chitinase. Standards: V, NodRlv-V ($C_{18:4}$, Ac); IV, NodRlv-IV ($C_{18:4}$, Ac); III, NodRlv-III ($C_{18:4}$, Ac); II, NodRlv-II ($C_{18:4}$, Ac). Total absolute counts (10^3 dpm) of LCOs for panel A (as an example): 2.4, 15.7, 21.1, and 24.1 for pMP247 and 1.4, 6.8, 12.6, and 31.2 for pMP292 at 40, 60, 80, and 240 min, respectively.

The presence of LCOs in the spent growth medium was also analyzed later than 180 min after induction. The results show that 6 h after induction, LCOs accumulate in the cell pellets in all tested *nod* gene-overproducing strains. Since the LCOs could be extracted by repeated washing, the accumulation of the LCOs in the cell pellets is presumably a result of the formation of insoluble complexes of the LCOs (data not shown) (25). The occurrence of this phenomenon makes secretion studies of LCOs at later times after induction very difficult.

We also tested the influence of the *nodI* and *nodJ* genes on LCO secretion by using a novel method which makes use of the sensitivity of LCOs to degradation by chitinase. The presence of chitinase in the suspension did not result in detectable cell lysis (results not shown), and, since chitinase cannot enter the bacterial cells, the quantity of degradation products of LCOs can be used as a measure of their secretion. The results obtained with strain RBL5970(pMP247) (*nodDABCIJ*) (Fig. 2B and Fig. 3A) show that the presence of chitinase in the growth medium leads to the digestion of the majority of the LCOs, resulting in the trisaccharide and disaccharide forms as early as 40 min after induction. In contrast, degradation of the LCOs produced by strain RBL5970(pMP292) (*nodDABC*) was minor during the first 80 min after induction (Fig. 2B and 3B). In conclusion, the results of the chitinase method correlate well with the direct secretion measurements presented in Fig. 2A. The same conclusions can be drawn with strains RBL5970 (pMP104) (*nodEFDABCIJ*) (Fig. 3C) and RBL5970(pMP100) (*nodEFDABC*) (Fig. 3D), confirming that the presence of the *nodFE* genes does not influence LCO secretion. These data also show that the newly developed method yields reproducible results.

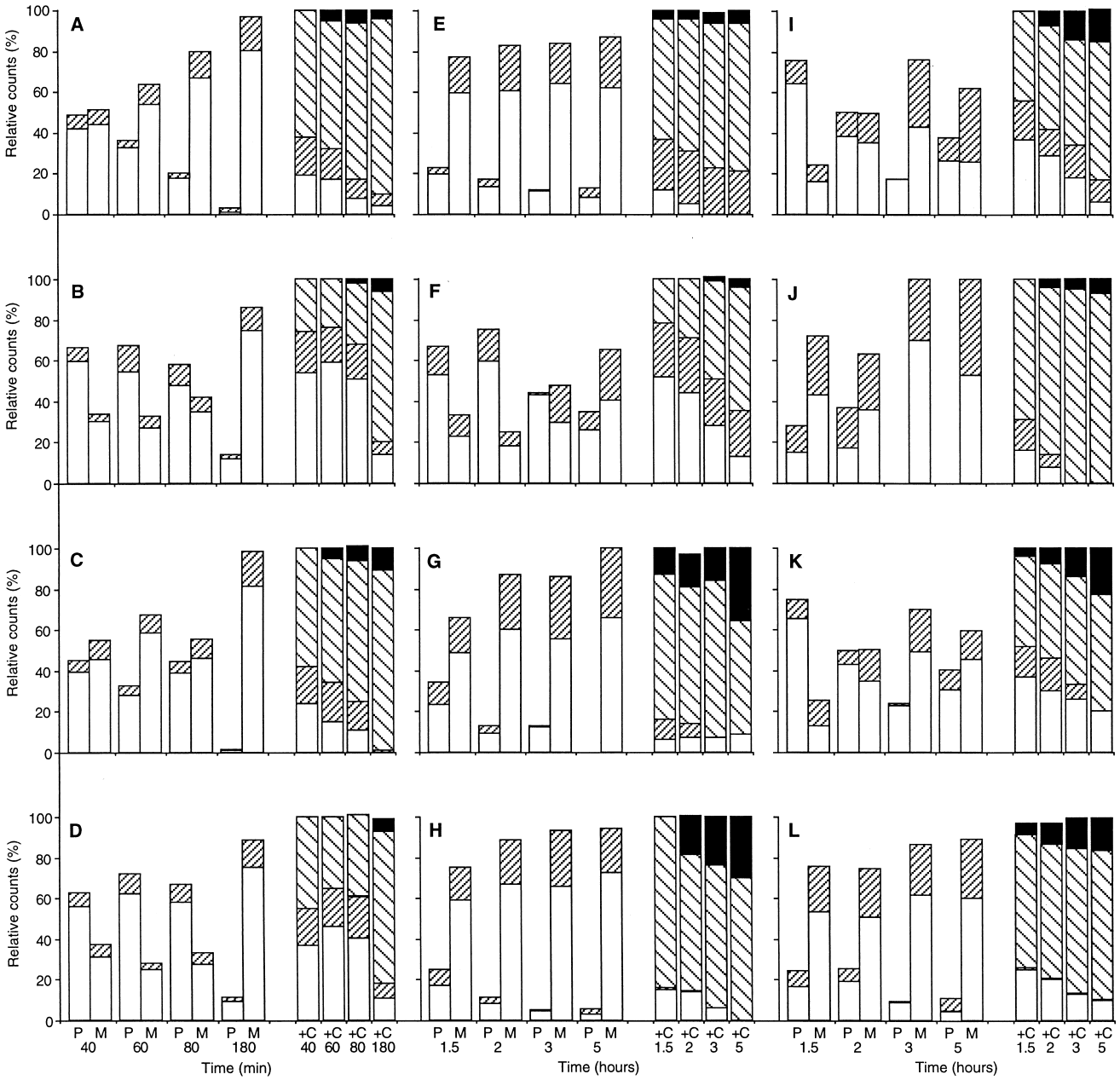

FIG. 3. Quantitative analysis of LCO secretion by various strains after *nod* gene induction. Radioactivity of TLC spots measured by the ImageQuant software is indicated as counts relative to the total count of radioactive LCOs produced at each time. LCOs: □, pentasaccharide; ▨, tetrasaccharide; ▧, trisaccharide; ■, disaccharide. (A) RBL5970(pMP247) (*nodDABCIJ*); (B) RBL5970(pMP292) (*nodDABC*); (C) RBL5970(pMP104) (*nodEFDABCIJ*); RBL5970(pMP100) (*nodEFDABC*); (E) ANU843 (wild type); (F) ANU261 (*nodI*::Tn*5*); (G) RBL5560 (wild type); (H) RBL5795 (*nodT*::Tn*5phoA*); (I) RBL5729 (*nodI*::Tn*5*); (J) RBL5729(pMP2412) (*nodI*); (K) RBL5734 (*nodI*::Tn*5*); (L) RBL5734(pMP2420) (*nodJ*). P, pellet fraction; M, spent growth medium fraction; +C, presence of chitinase in the growth medium.

Secretion of LCOs in a wild-type strain and a *nodI* mutant strain of *R. leguminosarum* biovar trifolii. The secretion of LCOs by wild-type strain ANU843 (20) and its *nodI*::Tn*5* derivative ANU261 (9) was analyzed at various times after induction. The results (Fig. 3E and 4A) show that the majority of the LCOs produced by the wild-type strain ANU843 are present in the spent growth medium as early as 1.5 h after induction by naringenin. Although the *nodI*::Tn*5* strain (Fig. 2A and 3F) also secretes LCOs, it does so much less efficiently than the wild-type strain. The role of the *nodI* gene seems more pronounced in the wild type than in the overproducing situation since even at 5 h after induction, the *nodI*::Tn*5* mutant secreted significantly smaller quantities of LCOs than the wild type. Similar results were obtained when LCOs were extracted from the cell pellets by the method of Bligh and Dyer (2) instead of *n*-butanol extraction (for an example, see Fig. 4C). LCO secretion was also analyzed by the chitinase method. The results with the wild-type strain ANU843 (Fig. 3E and 4B) show that the presence of chitinase in the growth medium leads to degradation of the majority of LCOs 1.5 h after in-

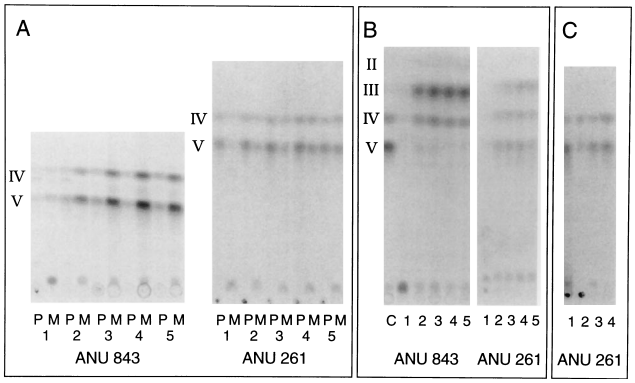

FIG. 4. TLC analysis of LCO secretion by *R. leguminosarum* biovar trifolii strain ANU843 and its *nodI*::Tn*5* derivative, ANU261. Cultures were analyzed at 1, 1.5, 2, 3, and 5 h after induction with naringenin (lanes 1 to 5, respectively). (A) Analysis of *n*-butanol extracts of the pellet (P) and spent growth medium (M) fractions; (B) analysis of *n*-butanol extracts of the entire culture of bacteria grown in the presence of chitinase. Lanes C, extracts of cells grown 180 min after induction in the absence of chitinase. (C) Comparison of different extraction methods using a culture of strain ANU261 grown for 5 h after induction with naringenin. Lanes: 1, water-methanol phase of a Bligh-Dyer (2) extract of the bacterial cell pellet; 2, chloroform phase of a Bligh-Dyer extract of the bacterial cell pellet; 3, *n*-butanol extract of the spent growth medium; 4, *n*-butanol extract of the bacterial cell pellet. See the legend to Fig. 2 for standards.

duction. In contrast, degradation of LCOs is much slower in strain ANU261 (Fig. 3F and 4B). At 5 h after induction, the difference in LCO degradation between the *nodI*::Tn*5* mutant and the wild type almost disappears.

Analysis of the role of the individual *nodI* and *nodJ* genes. The organization of the *nodI* and *nodJ* genes in one operon makes it difficult to study the function of the individual genes. To circumvent this problem, we have chosen an approach in which the *nodI* and *nodJ* genes were cloned separately under control of the *nodA* promoter (Fig. 1). The function of the cloned genes was analyzed in Tn*5* mutant derivatives of wild-type *R. leguminosarum* biovar viciae strain RBL5560. LCO secretion in the wild-type strain RBL5560 (Fig. 3G) was similar to that obtained with wild-type *R. leguminosarum* biovar trifolii strain ANU843 (Fig. 3E) in that LCOs were efficiently secreted into the growth medium within 1.5 h after induction by naringenin. This conclusion is supported by the experiment in which the chitinase method was used (Fig. 3G). Interestingly, a Tn*5phoA* insertion in the *nodT* gene (strain RBL5795), which on basis of sequence homology was suggested to play a role in LCO secretion (10), did not affect the efficiency of LCO secretion (Fig. 3H). In contrast, a Tn*5* insertion in *nodI* (strain RBL5729; Fig. 3I) strongly affected the efficiency of LCO secretion. The results obtained with this *nodI* mutant strain are therefore very similar to those obtained with the *R. leguminosarum* biovar trifolii *nodI* mutant strain ANU261. With the *nodJ*::Tn*5* mutant strain RBL5734 a phenotype similar to that of the *nodI*::Tn*5* mutant was observed (Fig. 3K). Introduction of plasmid pMP1062, containing the *nodIJ* genes, into the *nodI*::Tn*5* and *nodJ*::Tn*5* mutant strains restored the efficiency of LCO secretion to wild-type levels (data not shown). Surprisingly, LCO secretion of the *nodI*::Tn*5* mutant was also restored to wild-type levels by the introduction of plasmid pMP2412, which contains the *nodI* gene but lacks the *nodJ* gene (Fig. 3J). These results show that the Tn*5* insertion in *nodI* has no polar effect on the transcription of the *nodJ* gene located downstream. As expected, the *nodJ*::Tn*5* mutant was restored by the introduction of the *nodJ*-containing plasmid pMP2420 (Fig. 3L). In conclusion, both the *nodI* and *nodJ* genes are required for wild-type levels of LCO secretion.

DISCUSSION

The genetic organization of the *nodI* and *nodJ* genes together with the *nodA*, *nodB*, and *nodC* genes in one operon and the homologies of their gene products with various ATP-dependent secretion proteins have led to many speculations about their function (5, 10, 12, 22, 23, 32). However, direct proof for a role in the secretion of LCOs has not been reported. The study of the function of the NodI and NodJ proteins has been hampered because (i) LCOs are produced in very small quantities, making early detection after the start of their production difficult; (ii) LCOs are hydrophobic, and after secretion they may bind to various surfaces such as the growth containers and the bacterial cell surface (17, 24, 25); and (iii) in some strains the *nodI* and *nodJ* genes are present in one transcriptional unit, with other genes, such as *nodT* in *R. leguminosarum* biovar trifolii strain ANU843 (30), located downstream, and therefore polar effects of Tn*5* mutations cannot be excluded. In this work we have avoided these difficulties by constructing LCO-overproducing strains, developing a new method for studying LCO secretion, and making use of genetically well-defined systems in which the *nodI* and *nodJ* genes are cloned separately under control of an inducible promoter.

In our secretion studies, we used 1-^{14}C-labelled D-glucosamine, which allowed detection of LCOs early after induction. The TLC system used for analysis of LCOs has been described previously, and it has the advantage that it is suited for discrimination of oligosaccharide chain length (13). The use of a Phosphorimager provides a versatile method for direct quantification of produced radiolabelled LCOs. The presence of LCOs in the spent growth medium has been used as a measure for secretion of LCOs. It can be argued, however, that as a result of their hydrophobicity, LCOs can bind to various surfaces or can form insoluble complexes, and they therefore, even after secretion, can be present in the bacterial pellet fraction. It has been shown in this and other studies that LCOs can accumulate in the cell pellet fraction of wild-type bacterial cultures (16, 17, 25). We have noted that this occurs especially more than 6 h after induction of LCO production and in strains which overproduce LCOs as a result of the presence of cloned *nod* genes on high-copy-number plasmids. To circumvent these problems, we have developed a novel method which makes use of the sensitivity of extracellular LCOs for chitinase. Our results show that the conclusions obtained with the chitinase method are identical to those obtained with direct secretion studies shortly after induction of LCO production.

Both *nodI* and *nodJ* play a role in the efficiency of secretion of LCOs. This efficiency can be measured in terms of the ratios of secreted versus internal LCOs at fixed times after transcriptional induction of the *nod* genes by naringenin. Greater efficiency of LCO secretion by *nodIJ*-containing strains is apparent only shortly after induction. The time after induction after which an effect of the presence of *nodIJ* is no longer apparent is shorter in strains which contain *nod* genes cloned on multicopy plasmids than for wild-type strains. Control experiments show that the presence of LCOs in the spent growth medium of strains which do not harbor the *nodIJ* genes is not the result of cell lysis. Therefore, we can conclude that *nodI* and *nodJ* are not essential for secretion of LCOs. In contrast, McKay and Djordjevic (16) concluded that Tn*5* insertions in the *nodI* and *nodJ* genes prevented secretion of LCOs in the same chromosomal background as that used in our studies. It should be noted, however, that in their work LCO secretion was studied

after overnight induction of the *nod* genes in a *nod* gene-overproducing system and therefore the results are not comparable to those in our study. In order to explain the ability of *nodI* and *nodJ* mutants to secrete LCOs, one could assume the presence of chromosomal genes which are able to partially replace the function of *nodI* and *nodJ*. Such chromosomal genes could be homologs or even ancestors of the *nodIJ* genes or, alternatively, are unrelated genes involved in transport of molecules which are similar to LCOs. With respect to the *nodT* gene the nucleotide sequence of a chromosomal homolog with unknown function, which could explain the lack of a phenotype of *nodT* mutants, has been described (18). Our preliminary results obtained with the PCR technique also indicate the presence of *nodI* and *nodJ* homologs in various *Rhizobium* strains including the strains used in this study. If the regulation of such presumed homologs is growth condition dependent, this could explain anomalies found with the phenotypes of *nodI* and *nodJ* mutants as a result of the use of different test systems.

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