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Isolation, chemical structures and biological activity of the lipo-chitin oligosaccharide nodulation signals from *Rhizobium etli*

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Abstract

Rhizobium etli is a microsymbiont of plants of the genus *Phaseolus*. Using mass spectrometry we have identified the lipo-chitin oligosaccharides (LCOs) that are produced by *R. etli* strain CE3. They are *N*-acetylglucosamine pentasaccharides of which the non-reducing residue is *N*-methylated and *N*-acylated with *cis*-vaccenic acid (C18:1) or stearic acid (C18:0) and carries a carbamoyl group at C4. The reducing residue is substituted at the C6 position with *O*-acetylfucose. Analysis of their biological activity on the host plant *Phaseolus vulgaris* shows that these LCOs can elicit the formation of nodule primordia which develop to the stage where vascular bundles are formed. The formation of complete nodule structures, including an organized vascular tissue, is never observed. Considering the very close resemblance of the *R. etli* LCO structures to those of *R. loti* (I. M. López-Lara, J. D. J. van den Berg, J. E. Thomas Oates, J. Glushka, B. J. J. Lugtenberg, H. P. Spaink, Mol Microbiol 15: 627–638, 1995) we tested the ability of *R. etli* strains to nodulate various *Lotus* species and of *R. loti* to nodulate *P. vulgaris*. The results show that *R. etli* is indeed able to nodulate *Lotus* plants. However, several *Lotus* species are only nodulated when an additional flavonoid independent transcription activator (FITA) *nodD* gene is provided. *Phaseolus* plants can also be nodulated by *R. loti* bacteria, but only when the bacteria contain a FITA *nodD* gene. Apparently, the type of *nod* gene inducers secreted by the plants is the major basis for the separation of *Phaseolus* and *Lotus* into different cross inoculation groups.

Introduction

Soil bacteria of the genus *Rhizobium* are able to establish a symbiosis with specific leguminous plants by forming root nodules in which, after differentiation of the bacteria to bacteroids, atmospheric nitrogen is fixed. The shape and growth pattern of the nodule that is formed is determined by the plant. Determinate-type nodules develop from primordia in the outer cortex and indeterminate nodules develop from primordia in the inner cortex [7]. The specificities of various types

of rhizobia for host plants are very different. Some strains such as *Rhizobium* strain NGR234 [12] or strain GRH2 [14, accompanying paper] are able to nodulate a wide range of plant genera, whereas other strains, such as *R. etli*, only nodulate plant species belonging to a few genera (e.g. *Phaseolus*, a determinate nodule-forming plant) [19].

The *Rhizobium nod* (nodulation) genes, whose transcription is induced by flavonoids secreted by the plant, are involved in important stages of the nodule formation process and determine host specificity of the bacteria. These important roles of the *nod* genes are explained by the fact that they are required for the biosynthesis of lipo-chitin oligosaccharide (LCO) signal molecules. The LCO molecules from many rhizobial strains have been identified and they all appear to consist of an acylated chitin fragment which can contain strain-specific modifications [3, 9, 18, 20, 22]. Purified LCOs have been shown to induce several responses in the plant which are also induced by rhizobial infection. The responses that can be observed at the microscopic level include root hair deformation [10], root hair curling [17], formation of pre-infection threads [30], nodule primordia [2, 13, 24] and, in some cases, even complete nodule structures [25, 29].

In this paper we report our identification of the LCOs from *R. etli* strain CE3 and show that they are able to induce nodule primordia in the roots of the host plant *P. vulgaris*. Surprisingly, the structures of the LCOs are identical to those produced by various *R. loti* strains. We show that the barrier to nodulation of the non-host plant *Lotus* by *R. etli* is at the level of induction of the *nod* genes.

Materials and methods

Bacterial strains plasmids and growth conditions

R. etli strain CE3 [31] and *R. loti* strain E1R [13] were grown on TY medium (containing 16 g bactotryptone, 10 g bacto-yeast extract, and 5 g NaCl per litre). Strains overproducing *nod* metabolites were constructed by introducing *nodD* genes from different rhizobial species, by means of triparental mating and using pRK2013 as helper plasmid [4]. The use of plasmids pMP280 (*nodD* of *R. loti* biovar. *viciae*) and pMP604 (containing a hybrid *nodD* which confers flavonoid independent transcription activation) has been described previously [13]. Cells to be used for detection and purification of Nod factors were grown at 30 °C to an OD600 of 0.5–0.6 in liquid B^- medium [23]. Naringenin was added to the cultures when necessary for *nod* gene induction, to a final concentration of 1.5 μM. Strains containing plasmids were grown in the presence of 10 μg/ml tetracycline.

Detection of Nod metabolites using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC)

Bacteria were grown for 22 h in B^- medium and then diluted to an OD_{600} of 0.1. The cells were induced by the addition of naringenin and at the same time 0.5 μCi of *D*-[1-^{14}C]-glucosamine (50 mCi/mmol, Amersham) or 0.1 μCi *L*-[*methyl*-^{14}C]-methionine (55 mCi/mmol, Amersham) was added, and the culture was incubated at 29 °C for 18 h. Culture supernatants were extracted with a half volume of water-saturated *n*-butanol to yield a crude mixture of Nod metabolites. This fraction was evaporated to dryness, resuspended in 20 μl water-saturated *n*-butanol, and 1 μl of this solution was applied to octadecyl silica TLC plates (Sigma) as described [21]. Plates were developed in acetonitrile/water (1:1, v/v) and dried prior to detection of radiolabelled components using a Molecular Dynamics PhosphoImager equipped with Image Quant software. In order to obtain sufficient quantities of Nod metabolites for the structural determinations, cells were grown under the same conditions, but in the absence of radiolabelled glucosamine in 1 litre flasks. The culture supernatants were extracted with 0.2 volumes water-saturated *n*-butanol, and the extracts taken to dryness under vacuum. LCOs were redissolved in 20 ml 60 aqueous acetonitrile with vigorous shaking for 18 h, concentrated on an octadecyl

extraction column (J. T. Baker), and purified using reversed-phase HPLC, as described [13]. Quantities of the LCOs were estimated by comparison of HPLC peak intensities to a standard of the LCO NodRlv-V (C18;4, Ac) [24].

Bioassays

Seeds of *P. vulgaris* cv. Negro Jamapa were surface-sterilized with absolute ethanol for 1 min, and then with 20% (v:v) hypochlorite solution for 5 min, followed by three water washes to remove residual hypochlorite. Sterilized seeds were germinated aseptically on agar plates (0.5%). The seedlings were mounted in a curled wire in a tube (20 cm × 3 cm) with the roots in 25 ml of Fåhraeus medium [5]. The roots were inoculated at a point about 1 cm below the zone of emerging root hairs, with 1 μl of a solution containing a mixture of LCOs obtained after HPLC purification (the solution was prepared by pooling LCO-containing fractions 38–46 (peak I, Fig. 2), evaporating to dryness and redissolving in 1 ml 25% aqueous methanol). To each plant an amount of LCOs was added which is equivalent to that produced by 1 ml of naringenin-induced culture. The method of application of LCOs does not prevent their diffusion to other places of the root system. Roots were cleared for analysis according to the method of Truchet *et al.* [28]. Entire root systems, including inoculation sites, were bleached in 25% (v:v) hypochlorite solution for 20 min, and then stained in 0.01% methylene blue solution for 5 min and, when necessary, destained in 20% aqueous ethanol solution for 1 h. The frequency of nodule initiation was monitored under a stereoscopic microscope using magnifications of 30 × and 40 ×.

Chemical modifications of LCOs

Mild base de-esterification was carried out on 10% of peak I (Fig. 2), dried under vacuum, by redissolving the LCO in 250 μl of a 1:1 (v/v) mixture of 25% aqueous ammonia solution and methanol. The reaction was allowed to proceed at ambient temperature for 18 h when the volatile reagents were removed under vacuum. The product was redissolved in 10 μl DMSO and 1 μl used for MS analysis. Peracetylation was carried out on 10% of peak I, which was dried under vacuum and then treated with 250 μl of a mixture (2:1, v/v) of trifluoroacetic anhydride and glacial acetic acid. The reaction proceeded for 20 min at ambient temperature and the reagents were then removed under vacuum. The product was redissolved in 10 μl DMSO and 1 μl used for MS analysis. Permethylation was carried out using 10% of peak I, which was dried and redissolved in 250 μl anhydrous dimethyl sulphoxide. One or two pellets of NaOH were rapidly ground in a glass pestle and mortar and added to the sample solution. After 10 min at room temperature, 100 μl methyl iodide was added, and after a further 10 min, a second 100 μl aliquot was added. After 20 min, 300 μl methyl iodide was added and after a final 20 min the reaction was terminated by adding first 1 ml 10 mg/ml sodium thiosulphate solution, followed immediately by 1 ml dichloromethane. The mixture was shaken thoroughly, and after centrifugation, the aqueous layer was removed and discarded. The organic layer was washed 3 times with 1 ml water, and dried under a stream of nitrogen. In order to retain the carbamoyl group on permethylation this procedure was modified slightly, so that the first aliquot of methyl iodide was added immediately after the NaOH, instead of waiting for 10 min. All other amounts and times remained exactly the same. Permethylated LCO preparations were redissolved in 10 μl DMSO and 1 μl used for FAB-MS analysis. Conversion of the Permethylated LCOs to their partially methylated alditol acetates was achieved as described [11]. Monosaccharide composition analysis was carried out after conversion of 15% of the LCOs in peak I to their TMS methyl glycosides, as described [21].

Fast-atom bombardment mass spectrometry and collision-induced dissociation tandem mass spectrometry

Positive-ion fast-atom bombardment mass spectra were obtained using MS1 of a JEOL JMS-

SX/SX102A tandem mass spectrometer operated at 10 kV accelerating voltage. The FAB gun was operated at 6 kV accelerating voltage with an emission current of 10 mA and using 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 on a Hewlett Packard HP9000 data system running JEOL Complement software. Collision-induced dissociation mass spectra were recorded with the same machine, with nitrogen as the collision gas in the third-field free-region collision cell, at a pressure sufficient to reduce the parent ion to one third of its original intensity. In all experiments mono-thioglycerol was used as the matrix.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed using a Fisons MD800 mass spectrometer fitted with a Fisons GC8060 gas chromatograph, an on-column injector and helium as the carrier gas. Monosaccharide derivatives were separated on a DB-5MS column (0.32 mm × 30 m; J + W Scientific). TMS methyl glycosides were injected directly from solution in the TMS reagents (1 μl injected) and separated using the following temperature programme: 110 °C for 2 min, then ramping at 30 °C/min to 170 °C, holding for 2 min, then ramping at 4 °C/min to 240 °C, and holding for 10 min. Partially methylated alditol acetates (PMAAS) were injected in solution in dichloromethane (1 μl injected) and separated by using the following temperature programme: 50 °C for 2 min, then ramping at 40 °C/min to 130 °C, holding for 2 min, then ramping at 4 °C/min to 230 °C, and holding for 15 min. Mass spectra were recorded under conditions of electron impact in the positive ion mode with an electron energy of 70 eV, and were recorded using linear scanning from m/z 50–500 over 1 s.

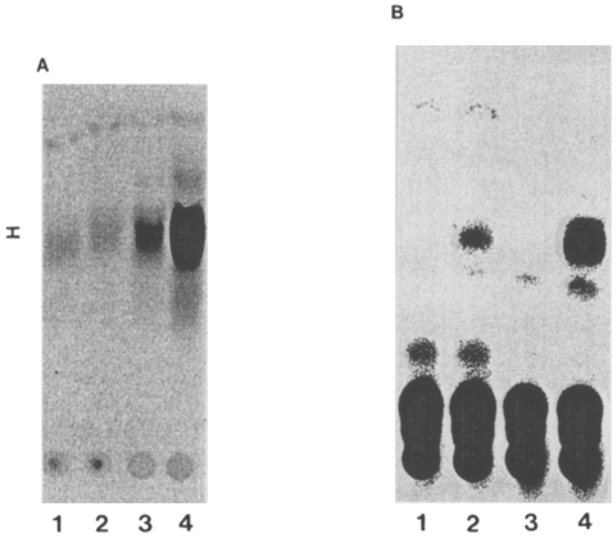

Fig. 1. Thin-layer chromatographic (TLC) analysis of radiolabelled metabolites of *R. etli*. A. *n*-butanol extracts of cells grown in the presence of [1-^{14}C]-glucosamine were analysed. B. *n*-butanol extracts of cells grown in the presence of *L*-[*methyl*-^{14}C]-methionine were analysed. Lanes: 1, strain CE3 without induction; 2, strain CE3 induced with naringenin; 3, strain CE3.pMP280 without induction; 4, strain CE3.pMP280 induced with naringenin.

Results

Production and purification of LCOs

The production of LCOs was assayed by *D*-[1-^{14}C]-glucosamine or *L*-[*methyl*-^{14}C]-methionine labelling studies as described in Materials and methods. In the presence of the flavonoid naringenin, *R. etli* strain CE3 produces radiolabelled metabolites which can be extracted with *n*-butanol and which behave like LCOs on TLC analysis (Figs. 1A and 1B). However, in the case of the labelling experiment with *D*-[1-^{14}C]-glucosamine, metabolites that behave similar in the TLC analyses were also produced in the absence of naringenin (Fig. 1A, lane 1).

In order to be able to purify sufficient quantities of LCOs to allow chemical analysis we constructed a series of *R. etli* LCO-overproducing strains by introducing an additional *nodD* gene. The best strain is obtained when the *nodD* gene of *R. leguminosarum* bv. *viciae* is used (Figs. 1A and 1B, lanes 3 and 4). The HPLC separation of the extracts obtained after the reversed-phase prepurification step yields, in addition to the peak

resulting from naringenin (at RT 18), two inducible broad peaks, the major one corresponding to HPLC fractions 38–45 (peak I) and the minor one to fractions 48–49 (peak II) (Fig. 2). Peaks I and II were dried and redissolved in 1 ml 25% aqueous acetonitrile and were used for further studies. It should be noted that in the absence of the inducer naringenin UV-absorbing peaks co-eluting with fractions 38–45 one also detected. This result is in agreement with the radiolabelling studies which indicate that in the absence of inducer the *R. etli* strain is also able to produce a minor quantity of compounds with similar chromatographic behaviour as LCOs (Fig. 1A).

Structural determination of purified LCOs

The two LCO-containing peaks, I and II, were analysed by FAB-MS in the positive ion mode. The FAB mass spectrum obtained from peak I (data not shown) contains signals corresponding to $[M + H]^+$ pseudomolecular ions at *m/z* 1501, 1459 and 1458, consistent with the presence of a LCO consisting of five GlcNAc residues and one deoxyhexose, a carbamoyl, an acetyl, a methyl and a C18:1 fatty acyl moiety and to LCOs lacking either the acetyl group or the carbamoyl group. The presence of signals at *m/z* 1609, 1567 and 1566, corresponding to thioglycerol adducts of the $[M + H]^+$ ions, is consistent with the presence of an unsaturated fatty acyl moiety.

The CID mass spectrum of the ion at *m/z* 1501 in peak I (Fig. 3), obtained on collision with nitrogen and recorded after scanning the second mass spectrometer for the fragment ions, contains signals at *m/z* 1483, 1092, 889, 686 and 483, corresponding to oxonium ions formed by cleavage of each successive glycosidic bond, with charge retention on the non-reducing terminus. These ions allow the sequence and substitution pattern of the LCO to be defined: the backbone

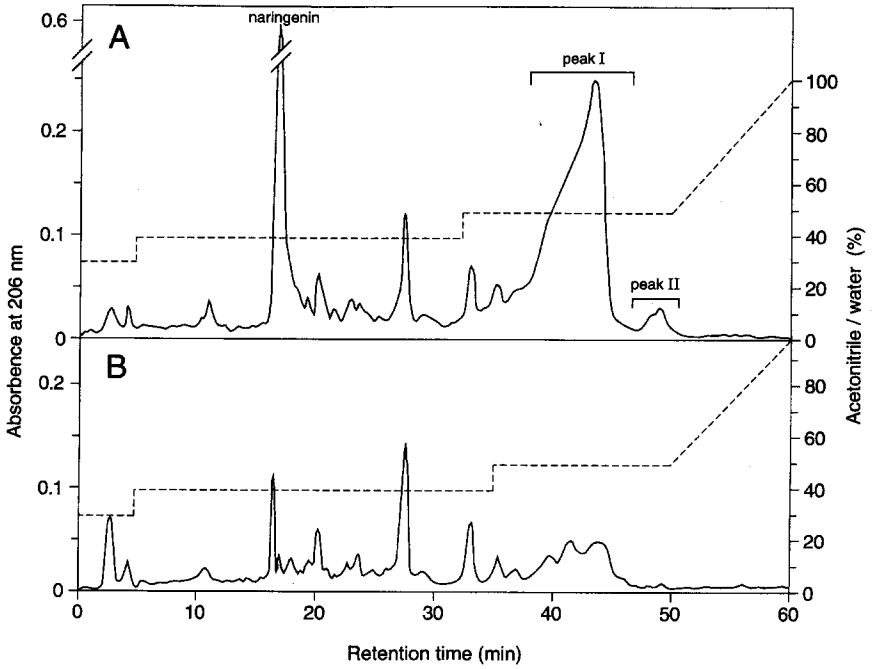

Fig. 2. High-performance liquid chromatography (HPLC) analysis of LCO extracts of *R. etli* strain CE3.pMP280. *n*-butanol extracts of 1 liter of bacteria grown in the presence (A) or absence (B) of naringenin and prepurified with reversed-phase extraction columns. The resulting samples were applied to a HPLC column using the shown acetonitrile/water gradient and fractions were collected each minute.

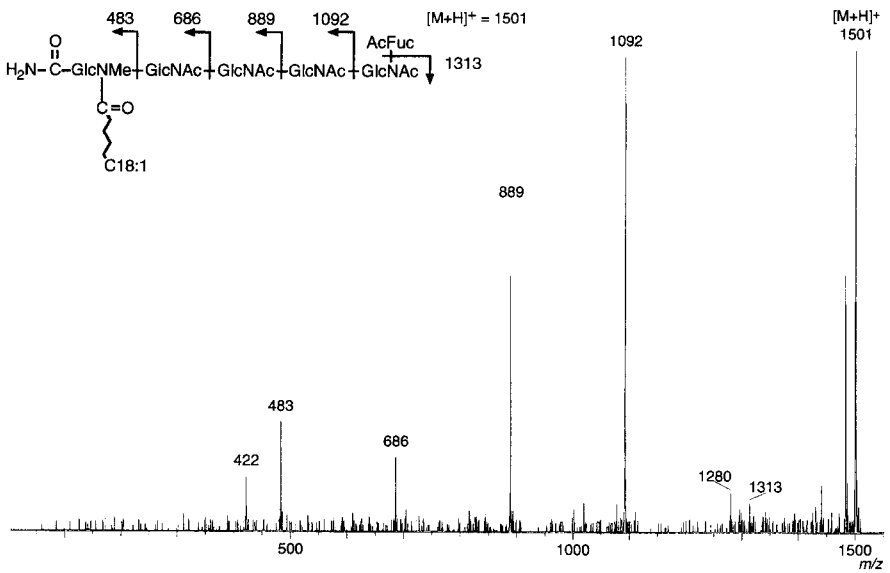

Fig. 3. CID mass spectrum and fragmentation scheme of the major LCO species in peak I of Fig. 2. The ion at *m/z* 422 arises by elimination of NH_2COOH from the oxonium ion at *m/z* 483.

is a linear GlcNAc5 oligosaccharide, bearing the C18:1 fatty acid, the methyl group and the carbamoyl group on the non-reducing terminal residue and an acetyl moiety and a deoxyhexose residue on the reducing terminal residue. The ion at *m/z* 1280 corresponds to an oxonium ion produced on loss of a single GlcNAc residue. This could arise either from the presence of a minor amount of an isomeric species in which the acetyl and deoxyhexose residue are not substituted on the reducing terminal residue, or alternatively could represent a low-abundance rearrangement ion analogous to that described by Ferro *et al.* [6]. The low-abundance ion at *m/z* 1313 arises on β-cleavage of an acetyl-deoxyhexose residue from the pseudomolecular ion, and is important in defining the attachment of the acetyl group to the deoxyhexose residue.

The FAB mass spectrum of peak II contains signals corresponding to $[M+H]^+$ ions at *m/z* 1503, 1461 and 1460, consistent with LCO species analogous to those found in peak I but bearing a C18:0 fatty acyl moiety instead of the C18:1 chains found in peak I. Ions corresponding to thioglycerol adducts are not observed, which is consistent with the presence of a saturated fatty acid. The CID mass spectrum of the pseudomolecular ion at *m/z* 1503 contains signals at *m/z* 1485, 1094, 891, 688, and 485, consistent with the presence of the saturated fatty acyl-containing analogue of the structure in peak I.

The nature of the linkages of the fatty acyl chain and the acetyl moiety to the LCO were established upon de-esterification of an aliquot of peak I in mild base. The FAB mass spectrum of the product contains a major $[M+H]^+$ pseudomolecular ion at *m/z* 1459 which represents a mass loss of 42 amu. This is consistent with cleavage of an acetyl ester under mildly basic conditions, with retention of the fatty acyl moiety, which is therefore concluded to be attached in amide linkage to the non-reducing terminal residue.

In order to determine whether the methyl group is attached to the nitrogen of the non-reducing terminal GlcNAc or to an oxygen, an aliquot of peak I was peracetylated. The FAB mass spectrum of the product contains a major $[M+H]^+$ pseudomolecular ion at *m/z* 2047. The mass shift with respect to the underivatised species corre-

sponds to the incorporation of 13 acetyl groups, which is consistent with the presence of 12 free hydroxyl groups, and the acetylation of the carbamoyl group in the native material. This indicates that the methyl group is attached to the nitrogen atom of the non-reducing terminal GlcNAc.

In order to preclude the theoretical possibility of the presence of a C19:1 fatty acyl group in place of a methyl group and a C18:1 fatty acyl chain, an aliquot of peak I was permethylated. The FAB mass spectrum of the product contains a signal corresponding to a $[M+H]^+$ pseudomolecular ion at m/z 1668. This mass corresponds to the loss of the acetyl and the carbamoyl groups with the incorporation of 18 (rather than 19) methyl groups, indicating that the nitrogen on the non-reducing terminal GlcNAc bears a C18:1 fatty acyl and a separate methyl group.

The identity of the deoxyhexose residue was determined after methanolysis of an aliquot of peak I, and GC-MS analysis of its TMS methyl glycosides. The peak pattern and retention times of the deoxyhexosyl derivative from peak I are identical to those obtained from an authentic fucose standard, and are quite distinct from those corresponding to the TMS methyl glycosides obtained from a rhamnose standard, allowing the deoxyhexose residue in the LCO to be assigned as fucose.

In order to establish the linkage between the monosaccharide residues, and the site of attachment of the fucose residue to the reducing terminal HexNAc, the remainder of permethylated peak I was hydrolysed, reduced and acetylated. The resulting PMAAs were analysed using GC-MS. Derivatives corresponding to terminal HexNAc, 1,4-disubstituted HexNAc, and 4,6-disubstituted HexNAc were identified. We thus conclude that the LCO has a linear 1,4-linked HexNAc5 backbone, to which the acetylated deoxyhexose residue is attached via C-6 of the reducing-terminal residue.

The C-atom to which the carbamoyl group is attached was determined following methylation of an aliquot of peak I under conditions where the carbamoyl group is retained [27]. Subsequently, this sample was hydrolysed, reduced and acetylated. The resulting PMAAs were analysed using GC-MS and the data from this experiment were compared with those obtained above. The intensity of the peak in the GC-chromatogram corresponding to the non-reducing terminal GlcNAc residue is greatly reduced, and derivatives corresponding to 3- and 6-substituted GlcNAcs are not observed, while the relative intensity of the peak corresponding to 4-substituted GlcNAc is more intense. These data are consistent with the conclusion that the carbamoyl group is located on C-4 of the non-reducing terminal GlcNAc.

Bioassays

The biological activity of the major LCO-containing fraction upon HPLC was assayed with an inoculation assay using *P. vulgaris* roots, and scored for induction of cortical cell division and production of nodule primordia. Nodule primordia are completely absent from uninoculated roots. An average of 90% of the roots inoculated with the LCO-containing fraction, or with a crude *n*-butanol extract, give a response in the formation of a discernible zone of cortical cell division or characteristic nodule primordia (Fig. 4A). Half of the primordia induced are observed at the site of inoculation, and the remainder are found elsewhere on the roots. Some of them eventually form nodule-like structures (Fig. 4B and 4C) with a frequency of three to five nodule-like structures per plant. The number of identifiable structures increases with increasing concentrations of LCOs applied (the maximum concentration tested was 10^{-7} M). They are observed as nodule primordia from the 5th day after inoculation (Fig. 4A), and as nodule-like structures from the 10th day after inoculation (Fig. 4B and 4C). These nodule-like structures are distinguishable from lateral roots since the focus of cell division in the nodule-like structures is located in the inner and outer cortex, while lateral root initiations contain a dense cortical primordium originating in the pericycle (Fig. 4D). When the purified LCO-containing fraction was used, there is a delay of about 6 days

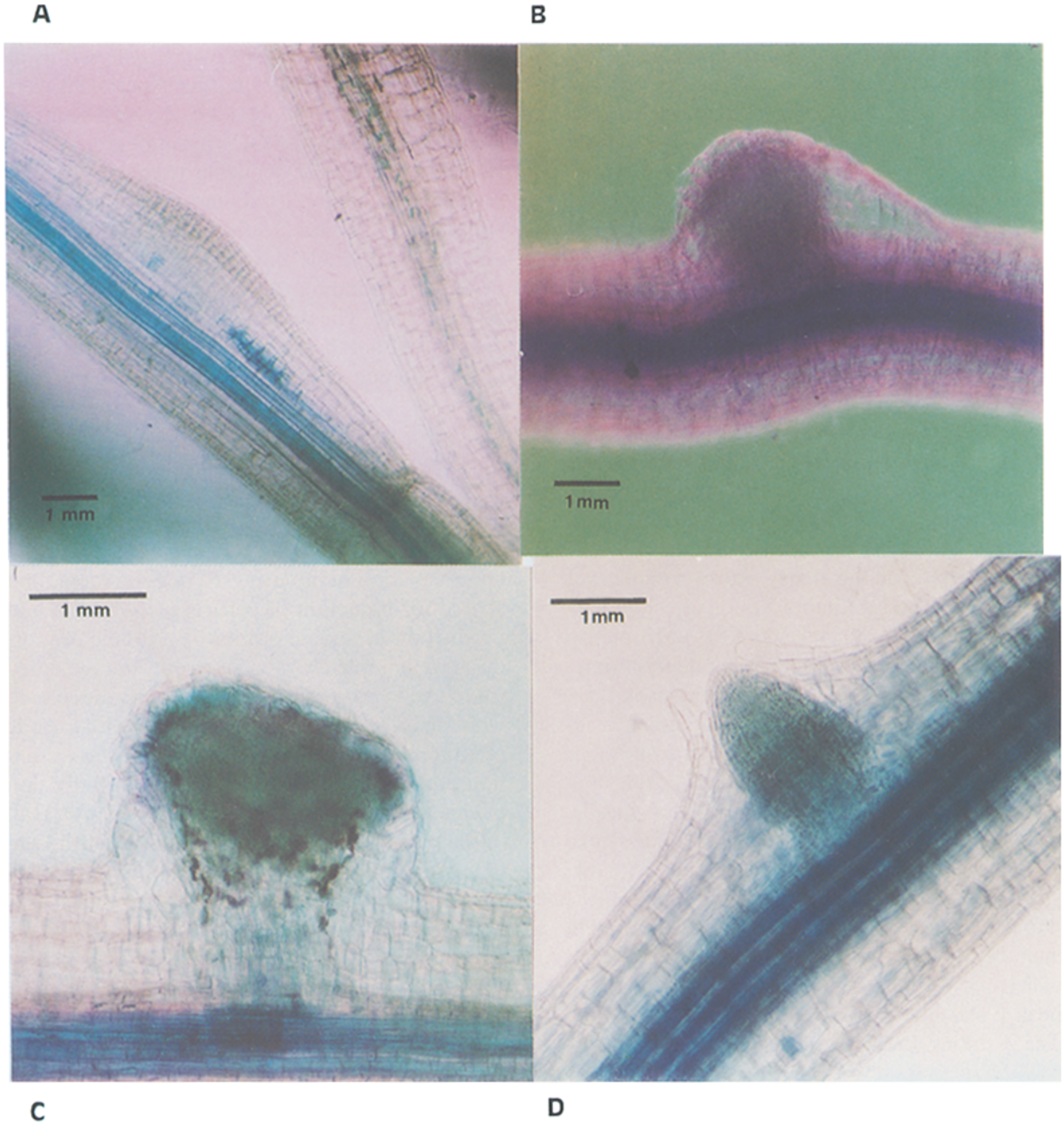

Fig. 4. Induction of nodule primordia on *Phaseolus vulgaris* by LCOs from *R. etli* (A–C) and comparison with a lateral root primordium (D). To each plant an amount of LCOs from peak I (Fig. 2) was added which is equivalent to that produced by 1 ml of naringenin-induced culture (approximate final concentration of LCOs: 10^{-7} M). Shown are examples of several stages of development observed 5 days (A) or 10 days (B and C) after inoculation.

in the development of the primordium and nodule-like structures compared to the crude extracts. The structures produced on inoculation with the LCO-containing fraction or crude extracts are smaller than the nodules produced by bacterial inoculation. In addition, although they

have the same bifurcated type of vascularization (Fig. 4C), it is not as structured as in the nodules induced by the bacteria [14, 26].

Nodulation experiments on Lotus *plants*

Considering the apparent identity of the structures of the LCOs isolated from *R. etli* with those identified from *R. loti* [13], we have investigated whether the former strain is able to nodulate on *Lotus* plants. The results (Table 1) show that *R. etli* is not able to induce the formation of nodules or nodular structure on *Lotus corniculatus* and *L. caucasicus*. However, a derivative of *R. etli* strain CE3, which contains plasmid pMP604, is able to induce nodules on the roots of these *Lotus* species. Since plasmid pMP604 harbours a *nodD* gene which confers flavonoid-independent transcription activation (FITA) of the *nod* genes, a host-specific barrier for *R. etli* to nodulate some *Lotus* plants is apparently the lack of suitable *nod* gene inducers excreted by these plants. *L. preslii* is nodulated by *R. etli* as efficiently as by *R. loti*. The latter finding shows that the identities in LCO structures produced by these rhizobia is also biologically relevant.

The reverse situation, i.e. when *P. vulgaris* is inoculated with *R. loti* strains, was also tested (Table 1). The results show that only the *R. loti* strain which contains the FITA *nodD* gene is able to nodulate *P. vulgaris*. We therefore conclude that the lack of *nod* gene inducers is apparently the major barrier of nodulation of bean plants by *R. loti* bacteria.

Discussion

In this paper we describe the structures and biological activities of *R. etli* strain CE3 which was isolated as an endosymbiont of *Phaseolus vulgaris*. The structures of the LCOs of various other rhizobia which nodulate *Phaseolus* have already been reported. The rhizobial strains concerned are *Rhizobium* strain NGR234 [16], strain GRH2 [14], *R. tropici* [15] and *R. fredii* [1] that all have broad host ranges. *R. etli* is different from these strains in that it has a more narrow host range [19]. It is therefore of interest to compare the structures of the LCOs from these various strains.

The LCOs of *R. etli* are β-1,4-linked pentasaccharides consisting of one *N*-acyl-*D*-glucosamine and four *N*-acetyl-D-glucosamines. The major compounds are substituted with a carbamoyl group at C4 and a methyl group at the amino group on the non-reducing-terminal residue, and an acetylfucose at C6 on the reducing-terminal residue. The position of the acetyl group on the fucose cannot be determined by mass spectrometric methods. For NMR analysis, sufficient quantities have not yet been isolated. The fatty acyl chain carried by the non-reducing terminal glucosamine is either vaccenic acid (C18:1) or stearic acid (C18:0). LCO spot I in the TLC system (Fig. 1B) and the corresponding HPLC peak I (Fig. 2) are broader than expected for a single compound. This is consistent with the results of the mass spectrometric studies, that show that peak I is a mixture of compounds. Some minor compounds, such as the de-*O*-acetylated LCO or that lacking the carbamoyl group could

Table 1. Frequency of nodulation[1] of *Lotus* and *Phaseolus* species by *R. etli* and *R. loti* strains.

Bacterial strains	Plant species			
	L. corniculatus	*L. preslii*	*L. caucasicus*	*P. vulgaris*
R. etli CE3	0	100	0	100
R. etli CE3pMP604	10	100	20	100
R. loti E1R	100	100	90	0
R. loti E1R.pMP604	nt	nt	nt	100

[1] The percentage of nodulated plants was scored 19 days after inoculation with rhizobia. For the *Lotus* species ten plants were used for each experiment. For *P. vulgaris* four plants were used for each experiment.

also have been generated during purification as a result of partial degradation.

The LCOs of *R. etli* are different from any of the LCOs produced by any of the broad-host-range strains mentioned above. The methyl group is found in the LCOs from all the broad-host-range strains except for *R. fredii* [1]. Fucosylated or 2-*O*-methylfucosylated LCO structures are found also in *B. japonicum*, *B. elkanii* and *R. fredii* although these are not *O*-acetylated [1, 2]. The LCOs of strain NGR234, having the broadest host range of all known rhizobia, can contain *O*-acetylated 2-*O*-methylfucose moieties [16]. Surprisingly, the LCOs of *R. etli* are indistinguishable from those of various *R. loti* strains [13]. These *R. loti* strains have a very narrow host range, mainly confined to species of the genus *Lotus*. This raised the question as to whether *R. etli* is able to nodulate *Lotus* species. The results show that, although *R. etli* itself is not able to nodulate *Lotus* plants, a derivative of *R. etli* harbouring a *nodD* gene which activates transcription in the absence of flavonoids efficiently nodulates *Lotus* plants. Apparently, the lack of inducers excreted by the *Lotus* plants is the major barrier to nodulation by *R. etli*.

We have tested the biological activity of the *R. etli* LCOs on the host plant *Phaseolus vulgaris*. The purified LCOs are able to induce cell divisions in the root, starting in the outer cortex and thus following a developmental pattern which is characteristic of determinate nodules. In the accompanying paper [14] it is shown that the purified LCOs from strain GRH2 are also able to induce nodule primordia on *P. vulgaris*. In that study it was shown that the nodule primordia induced by LCOs are not able to develop into complete nodule structures. Here we show that the nodule primordia induced by the purified LCOs of the homologous symbiont *R. etli* also do not develop into complete nodular structures including a well-organized vascular structure. Mixtures of the LCOs produced were more effective in inducing nodule primordia but they were also not able to induce complete nodule structures. The higher effectiveness of the mixtures might indicate synergistic effects or, alternatively, the loss of minor, biologically very active, LCO species during the purification. The combined results therefore suggest that an unknown rhizobial factor plays a role in the formation of full-grown nodules on *P. vulgaris*.

It has been shown that several *nod* genes encode enzymes involved in the processes of fatty acid biosynthesis, chitin synthesis and chitin modification [3, 20, 22]. Through their essential roles in the biosynthesis of the LCOs the *nod* genes are major mediators of the host specificity of nodulation. Geelen *et al.* [8] have provided evidence that *nodS* encodes a methyltransferase involved in the addition of the *N*-methyl substituent in *Azorhizobium*. Consistent with the presence of an *N*-methyl group in the LCOs of *R. etli* strain CE3 this strain also contains a *nodS* gene [32]. We are presently looking for the genes which are responsible for the addition of the acetylfucose and carbamoyl moieties.

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