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Novel lipochitin oligosaccharide structures produced by *Rhizobium etli* KIM5s

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Abstract

The novel lipochitin oligosaccharide (LCOs) structures produced by *Rhizobium etli* KIM5s were characterized using a nanoHPLC reverse-phase system coupled to an ion-trap mass spectrometer. This technique was shown to be more sensitive for structural elucidation of LCOs than previously used mass spectrometric methods. The structures of the LCOs of *R. etli* KIM5s, the majority containing six monosaccharide residues, differed from those synthesized by all other rhizobia analyzed to date. In addition, novel structures in which the chitin backbone was deacetylated at one or more GlcNAc moieties were found as minor compounds. The difference in host range of this strain compared to that of other known bean microsymbionts is discussed.

Keywords: NanoHPLC system; Ion-trap mass spectrometry; Lipochitin oligosaccharides (LCOs); Nodulation; *Rhizobium etli*

1. Introduction

Lipochitin oligosaccharides (LCOs) are signal molecules secreted by rhizobia (symbiotic nodule bacteria) in the presence of leguminous plant inducers.[1] A leguminous species can only engage in symbiosis with one or a few species of bacteria, and conversely, the microsymbionts exhibit a particular host range. This host range and the ability to colonize nodular tissues is mainly determined by the bacterial LCO structure together with the composition of the lipo- (LPS) and exopolysaccharides (EPS).[2] Because of their pivotal importance in nodulation, LCOs are also known as nodulation factors.[2,3] In the nodules, the bacteria can differentiate into nitrogen fixing bacteroids harboured intracellularly within a membrane-bound compartment, the symbiosome. In exchange for the fixation products, the plant supplies the bacteroids with di- and tri-carboxylic acids.[4] The most common LCO structure is built up by a chitin backbone. The number of GlcNAc residues generally varies from three to five units, depending on the species. The non-reducing terminus of this backbone is always acylated with a fatty acid that can be mono- or polyunsaturated. The length of the acyl group can vary from 16 to 26 carbon atoms depending on the rhizobial species, and its nature often reflects the composition of the bacterial membrane.[3,5] At both the reducing and non-reducing termini of the chitin chain, different substituents like acetyl, sulphatyl, carbamoyl, fucosyl and methyl groups may be present. The host specificity of the LCO produced by a rhizobial strain is defined by the number of GlcNAc monomers in the chitin backbone, the length and number of unsaturations of the acyl chain and the nature of the other substituents present in the molecule.[2,5] Few LCO

Abbreviations: LCOs: lipochitin oligosaccharides; nano-ESI, nano-electrospray ionization; MALDI TOF, nanoLC, nano liquid chromatography; MS, mass spectrometry; d.p.i., days post inoculation.

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structures differing from this general structure have been found. *Rhizobium* sp. GRH2 was reported to produce a LCO having an hexameric chitin backbone. This LCO species was found as a minor component of the total LCO pool synthesized by this strain.[6] In only two *Rhizobium* species a non-acetylated hexose forming part of the chitin polymer has been described. The LCOs produced by *Rhizobium tropici* strain CIAT899 contain a mannose moiety at the reducing terminal residue,[7] and *Sinorhizobium fredii* produces a minor LCO with a glucose substituting one of the GlcNAc moieties.[8] In several cases, it has been shown that a single substituent, the introduction of a new LCO decoration or a different chain length of the chitin-oligo, can drastically change the host range of the bacterium, whereas other LCO modifications have little or no effect on nodulation efficiency or host range.[2,3,5]

Different rhizobial species that are able to nodulate the same host plant often share a similar LCO structure. This is the case with *R. etli* CE3, which naturally infects common bean and with *Mesorhizobium loti*, which nodulates *Lotus* species. In both cases it was found that these bacteria produce LCOs that have as major component a chitin fragment that contains five GlcNAc moieties with a vaccenic (C18:1) fatty acid at the non reducing terminus and an acetylfucose attached to the C6 of the reducing terminal N-GlcNAc molecule. In some cases, a carbamoyl group is also present at the N-saccharide of the non-reducing terminus.[9,10] The *R. etli* strain CE3 can nodulate *Lotus japonicus*,[11] and *Lotus corniculatus*,[12] despite that these plants are not its natural host. *R. etli* KIM5s was isolated from nodulated *Phaseolus vulgaris* plants grown in Kimberly, ID, USA.[13] In several respects, KIM5s is a divergent or atypical *R. etli* strain. It has three *nifH* gene copies, two of them identical and one with a slightly different sequence. Both *R. etli* KIM5s *nifH* nucleotide sequences are distinct from that of the conserved CE3-type *nifH* sequence (Sepúlveda, E.; Romero, D. personal communication). Vinuesa et al. described a new and strain specific LPS O-antigen composition in *R. etli* KIM5s, as well as a distinct plasmid profile and gene organization in the plasmid-borne LPS O-antigen biosynthesis cluster.[14] However, PCR-RFLP analysis of the 16S rRNA and 23S rRNA loci, *nifH* copy number and several phenotypic traits (i.e., lack of growth on LB medium and intrinsic resistance to nalidixic acid) clearly indicate that KIM5s fits within *R. etli* (Vinuesa, P. unpublished). Strain KIM5s has been used in several earlier studies due to its high competitiveness for nodule occupancy and nitrogen fixation properties.[13,15] Nodulation experiments described in this work show that this strain is also able to nodulate the tropical legume *Macroptilium atropurpureum* (siratro), but fails to nodulate *L. japonicus*.

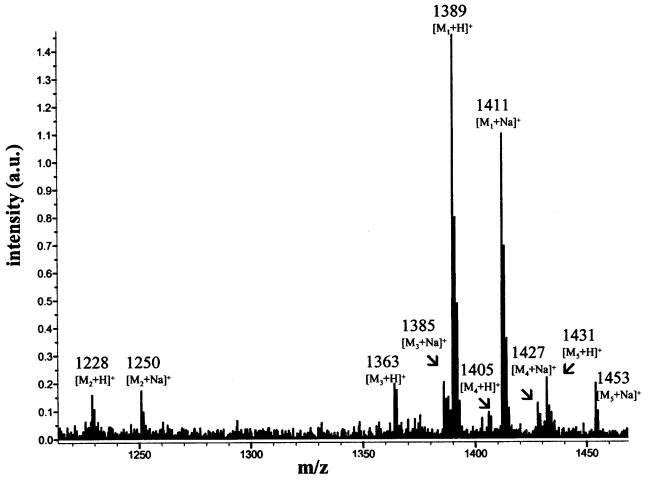


Fig. 1. MALDI TOF mass spectrum of HPLC fraction 2. HPLC fraction 2 of Kim5S LCOs was measured on a MALDI TOF mass spectrometer equipped with a nitrogen laser. The major peaks at m/z 1389 and 1411 correspond to a protonated ($[M_1 + H]^+$) and a sodiated ($[M_1 + Na]^+$) molecules, respectively, of a species with a molecular mass of 1388. Other species that show both molecules in the spectrum have molecular masses of 1227, 1362, 1404 and 1430. See species description in Table 1.

Here we isolated and elucidated the structure of the LCOs produced by this bacterial strain using a nano-HPLC reverse-phase system coupled to an ion-trap mass spectrometer. This technique, which had not been previously applied for LCO structural analysis, permitted fractionation of the LCOs molecules as a function of their hydrophobicity prior to mass spectrometric analysis. This study shows that *R. etli* KIM5s produces novel LCO structures that are clearly distinct from those synthesized by *R. etli* CFN42 or CE3.

2. Results

Mass spectrometry.—Three independent crude extracts were obtained after isolation of LCO mixtures from 1 L cultures of *R. etli* KIM5s (Section 3). One of these extracts was subjected to HPLC analysis, yielding two different LCO containing fractions. The HPLC-fractions obtained were subjected to MALDI TOF mass spectrometric analysis. Previously, this technique has been successfully used for mass determination of LCOs.[16] As an example, the mass spectrum of fraction 2 is shown in Fig. 1. Two major peaks are observed at m/z 1389 and 1411. These correspond with a protonated ($[M_1 + H]^+$) and a sodiated ($[M_1 + Na]^+$) molecule of a species with a molecular mass of 1388, respectively. Other species shown in the spectrum have molecular masses of 1227, 1362, 1404 and 1430. To further analyze the structures of the LCOs, all HPLC-fractions, as well as two crude extracts, were subjected to a nanoHPLC reverse-phase system. All eluting

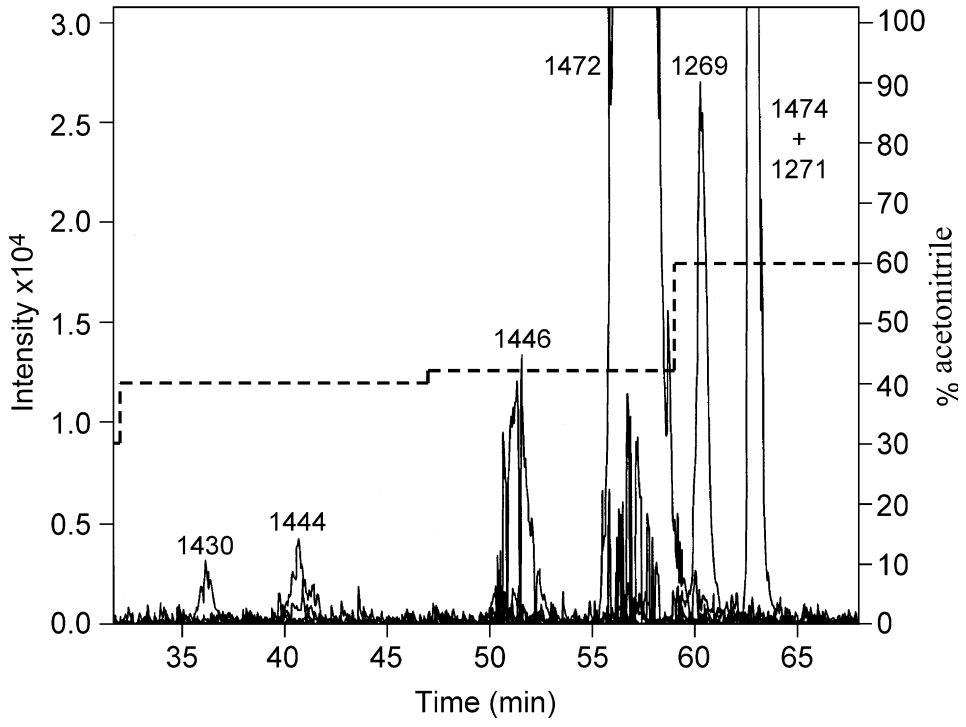


Fig. 2. NanoLC-chromatogram of the crude extract of the LCOs isolated from *R. etli* Kim 5S. LCOs from Kim5S were separated by using a nanoLC-system equipped with a reverse-phase capillary pre-column and an analytical column. A step gradient using two solvent-systems was applied for elution as described in the instrumental section. The numbers given above the peaks indicate the molecular masses of the eluting nanoLC-fractions.

nanoLC-fractions were measured online using an ion-trap mass spectrometer after nanoelectrospray ionization (nanoESI). As an example, the nanoLC-chromatogram (derived from the total ion current) of a crude extract is shown in Fig. 2.

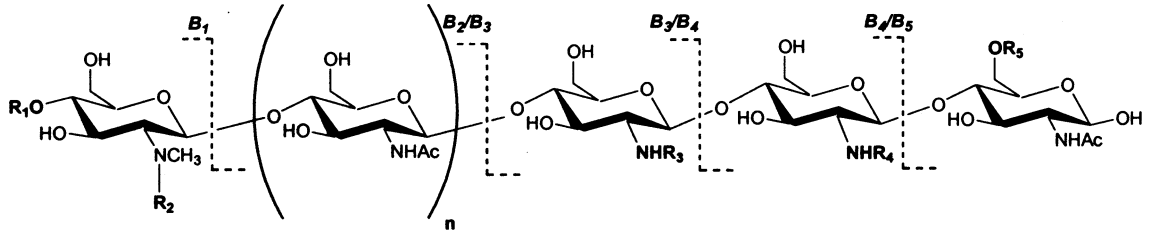
Of each LCO species that was present in a concentration suitable for MS/MS-experiments, the protonated molecule was fragmented by using helium as collision gas. Characteristic cleavages of the glycosidic bonds were observed, yielding mainly B_i-ions.[17] Because of the recently published reports on internal residue loss,[18] the precise location of the glucosamine residues could not be assigned with certainty. However, the results obtained after applying MS/MS to a reduced trisaccharide as a standard makes it unlikely that such internal residue loss is occurring in the combination of nanoESI with ion-trap fragmentation.[19,20] An overview of the complete analysis of LCO structures produced by strain KIM5s is shown in Table 1.

The major component of *R. etli* KIM5s has a molecular mass of 1472 and contains six GlcNAc residues. From the B_i-ions in the spectra, it is clear that the non-reducing terminus of this LCO species contains a vaccenic acid and a methyl group. No other substituents at either the reducing or the non-reducing terminus were found. This kind of LCO was published previously for *Rhizobium* sp. GRH2, isolated from *Acacia cyanophylla*. In contrast to *R. etli* KIM5s, in *Rhizobium* sp. GRH2 this LCO structure represented only a minor species of the total LCOs produced by the bacteria.[6]

Furthermore, two different LCO species were found in relatively smaller amounts, both in the HPLC-purified and in the crude extract. These species show one or two non-acetylated glucosamines as components of a hexameric chitin backbone. The glucosamines are most likely to occupy the fourth (M_w 1430), or both the fourth and fifth, positions of the chitin oligosaccharide (M_w 1388), counting from the non-reducing terminus. These LCOs both contain a C18:1 fatty acid on the non-reducing GlcNAc moiety. The presence of the glucosamine molecules was confirmed by MS/MS fragmentation, where a characteristic loss of 161 was observed instead of 203 for a GlcNAc residue. As an example, the MS/MS spectra of LCOs with molecular mass of 1430 and 1388, respectively, are shown in Fig. 3(A and B), respectively.

The loss of 161 was also detected in LCOs with molecular masses of 1227 and 1201, which were present in much lower amounts. Other minor components that could be identified by using a MS/MS procedure have molecular masses of 1474, 1404 and 1269. These LCOs all contain GlcNAc residues only, and differ from the major component (M_w 1472) in length and/or number of double bonds of the fatty acid and/or number of monosaccharides in the chitin backbone. The same

Table 1
LCO structures present in *R. etli* Kim5S and *R. etli* CE3



R. etli strain	M_w (MALDI TOF)	M_w (ion trap)	B_5	B_4	B_3	B_2	B_1	*n*	R_1	R_2	R_3	R_4	R_5	LCO structure [c]
Kim5S		1474	1254	1051	848	645	442	2	H	C18:0	Ac	Ac	H	NodKim5S-VI (C18:0, Me)
	1472	1472 [a]	1252	1049	846	643	440	2	H	C18:1	Ac	Ac	H	NodKim5S-VI (C18:1 Me)
		1458 [b]	n.d.	n.d.	n.d.	n.d.	n.d.	2	H	C17:1	Ac	Ac	H	NodKim5S-VI (C17:1 Me)
	1446	1446 [b]	n.d.	n.d.	n.d.	n.d.	n.d.	2	H	C16:0	Ac	Ac	H	NodKim5S-VI (C16:0 Me)
		1444	n.d.	n.d.	n.d.	n.d.	n.d.	2	H	C16:1	Ac	Ac	H	NodKim5S-VI (C16:1 Me)
	1430	1430 [a]	1210	1049	846	643	440	2	H	C18:1	Ac	H	H	NodKim5S-V,I N-Glc (C18:1 Me)
	1404	1404	1184	1023	820	617	414	2	H	C16:0	Ac	H	H	NodKim5S-V,I N-Glc (C16:0 Me)
	1388	1388 [a]	1168	1007	846	643	440	2	H	C18:1	H	H	H	NodKim5S-IV,II N-Glc (C18:1 Me)
	1362	n.d.						2	H	C16:0	H	H	H	NodKim5S-IV,II N-Glc (C16:0 Me)
		1271 [b]		n.d.	n.d.	n.d.	n.d.	1	H	C18:0	Ac	Ac	H	NodKim5S-V (C18:0 Me)
	1269	1269		1049	846	643	440	1	H	C18:1	Ac	Ac	H	NodKim5S-V (C18:1 Me)
	1227	1227		1007	846	643	440	1	H	C18:1	Ac	H	H	NodKim5S-IV,I N-Glc (C18:1 Me)
		1201		981	820	617	414	1	H	C16:0	Ac	H	H	NodKim5S-V (C16:0 Me)
CE3		1500 [a]	n.d.	1092	889	686	483	1	$C(O)NH_2$	C18:1	Ac	Ac	4-*O*-Ac-αFuc (1→	NodCE3-V (C18:1 Me,Ac,Fuc,Cb)
		1459 [b]	n.d.	n.d.	n.d.	n.d.	n.d.	1	H	C18:0	Ac	Ac	4-*O*-Ac-αFuc (1→	NodCE3-V (C18:0 Me,Ac,Fuc)
		1458 [b]	n.d.	n.d.	n.d.	n.d.	n.d.	1	$C(O)NH_2$	C18:1	Ac	Ac	αFuc (1→	NodCE3-V (C18:1 Me,Fuc,Cb)
		1457 [a]	n.d.	1049	846	643	n.d.	1	H	C18:1	Ac	Ac	4-*O*-Ac-αFuc (1→	NodCE3-V (C18:1 Me,Ac,Fuc)
		1415 [b]	n.d.	n.d.	n.d.	n.d.	n.d.	1	H	C18:1	Ac	Ac	αFuc (1→	NodCE3-V (C18:1 Me,Fuc)
		1255		1035	832	629	426	1	H	C17:1	Ac	Ac	H	NodCE3-V (C17:1 Me)

Schematic representation of the LCO structures analyzed by MALDI TOF and ion-trap mass spectrometry. When present in suitable amounts, MS/MS fragmentation was applied.

[a] Major compounds.

[b] Only present before HPLC. n.d., not determined.

[c] An alternative interpretation of the molecular masses can be done in which the *N*-methyl moiety is absent and the fatty acid chain presents one more carbon molecule.

holds for LCOs with molecular masses of 1458, 1446, 1444 and 1271. The low amounts of the compounds yielding these peaks made MS/MS-confirmation of the proposed structures impossible.

As a control, we isolated and analyzed the LCOs of *R. etli* CE3 and applied the same techniques as for *R. etli* KIM5s. In addition to all the LCO structures published for this strain,[9] a pentameric LCO carrying a C17:1 fatty acid was detected (see Table 1). The low amount of some of LCO compounds made further structural analysis by mass spectrometric fragmentation impossible. Therefore, these structures given in Table 1 should be considered as a proposal only. From our results it may be concluded that structural analysis using nanoLC-MS/MS is applicable for the identification of LCOs and is more sensitive than previously used methods. In addition, an approximate quantification of the different molecules present in the mixture is possible.

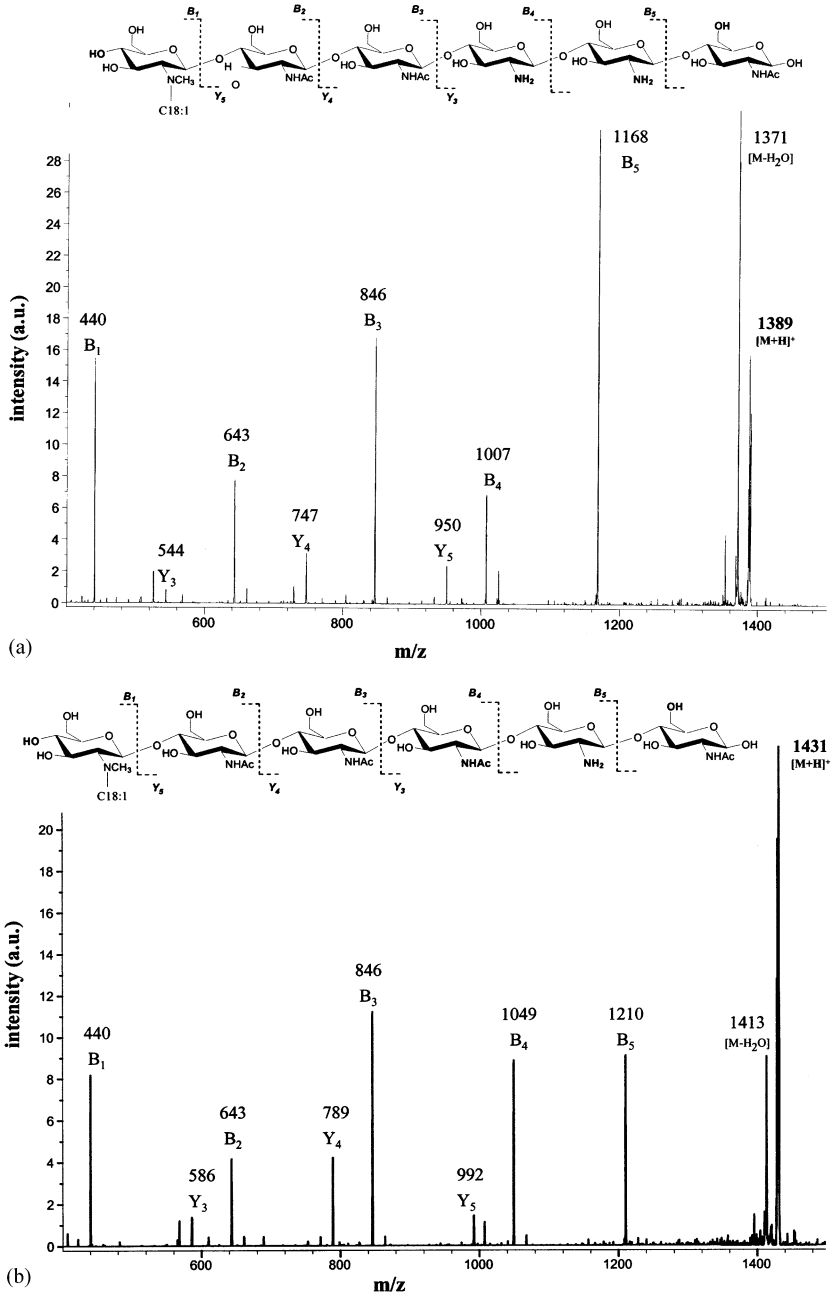


Fig. 3. NanoESI MS/MS mass spectrum of LCO with molecular mass 1430 and 1388. LCO species with an hexachitin backbone consisting of glucosamines non acetylated at one position (M_w 1430) (A) or at both the fourth and fifth positions (M_w 1388) (B) LCO species contain a C18:1 fatty acid at the non-reducing terminus. Molecular weights of the Y ions are depicted above their respective peaks.

Table 2
Bacterial strains

Rhizobial strain	Antibiotic resistance	Nodulation		Reference
		L. japonicus	Siratro	
R. etli CE3	Rif^r	+	n.d.	13
M. loti R7A	Rif^r	+	n.d.	36
R. etli KIM5s	Sp^r	−	+	37
S. fredii HH103	Str^r	n.d.	+	38

Rhizobial strains used during this work and nodulation tests performed on *L. japonicus* and *M. atropurpureum*. Rif^r, Rifampicine resistance; Spec^r, Spectinomycin resistance; Str^r, Streptomycine resistance. The antibiotics were added to the plates to the following final concentrations: Rif, 20 g/mL; Sp, 50 g/mL; Str, 250 g/mL. +, nodulation; −, lack of nodulation; n.d., not done.

Our results show that the major LCO synthesized by *R. etli* KIM5s consists of a hexa-chitin backbone with a methylated vaccenic acid at the non reducing terminus. No other substituents were present on its chitin backbone. Minor fractions of LCOs, where the fourth or both the fourth and the fifth glucosamine units were not acetylated, were also observed. To date, such LCO structures are not known to be produced by any other rhizobial strain.

Nodulation experiments.—Nodulation experiments showed that *L. japonicus* was nodulated by *R. etli* CE3, but not by strain KIM5s. The nodules formed by CE3 appeared later (25 d.p.i.) than those induced by the control strain *M. loti* R7A. They were also smaller and darker than the ones elicited by strain R7A (data not shown). Although *R. etli* KIM5s did not induce nodulation on *L. japonicus* (Fig. 4(B)), the roots inoculated presented black spots and slight thickening that were not observed in the inoculated control. These symptoms were present 11 d.p.i, coincident with the moment that young nodules induced by *M. loti* are visible (Fig. 4(A)). *R. etli* KIM5s (Fig. 4(C)), like other *R. etli* strains,[12] was able to nodulate siratro. These nodules were comparable in shape and colour to those formed by *S. fredii* on the same leguminous plant (Fig. 4(D)).

3. Experimental

Bacterial strains.—*R. etli* KIM5s and *R. etli* CE3 were incubated on YMB plates at 28 °C for 2–3 days with the proper antibiotics (Table 2). From these plates, 100 mL B$^-$ medium, supplemented with phosphate to a final concentration of 0.1 M, were initiated and grown under continuous agitation at 28 °C for 48 h. These pre-cultures were employed both for LCO isolation and for nodulation experiments with *L. japonicus*.

For LCO isolation, 1 L cultures were started with an OD_{660} of 0.1, grown overnight and subsequently extracted with 1-butanol as described by López-Lara et al.[10] Induction of LCO production was achieved by the addition of the flavanone naringenin to a final concentration of 1 mg/mL. For plant inoculation bacteria were brought to an OD_{660} of 0.1 either from the 100 mL-liquid pre-cultures or from plates without antibiotics.

LCO isolation and HPLC.—Bacterial cultures (1 L) were extracted with 1-butanol[10] and subsequently dried and dissolved overnight in 5 mL 60% acetonitrile (ACN)–water. Afterwards the solution was applied on an octadecyl extraction column (J.T. Baker, Philipsburg, USA) and directly collected, using the column as a rough filter rather than a selective separation of the LCOs. Using this method, we obtained three independent LCO crude extracts. For one of the experiments, the LCOs of *R. etli* KIM5s were submitted to HPLC before its mass spectrometric analysis. The crude extract was brought to a concentration of 30% ACN–water, and the equivalent to 312 mL culture was injected into the column. The HPLC protocol consisted of 30 min isocratic elution with 30% ACN–water followed by 20 min isocratic elution 40% ACN–water, 20 min isocratic elution with 42% ACN–water and 20 min isocratic elution with 60% ACN–water. The flow rate was 1 mL/min. Two different peaks were obtained at 30 and 60% ACN–water, respectively, when monitored at 206 nm (not shown). These fractions were collected, dried and redissolved in 1 mL 60% ACN–water before ion-trap analysis.

NanoHPLC and mass spectrometry.—HPLC fractions of *R. etli* KIM5s LCOs were measured on a Reflex III MALDI TOF mass spectrometer (Bruker Daltonics), equipped with a nitrogen laser. A sample (0.5 μL) was mixed in the sample well of a stainless steel target plate with α-cyano-4-hydroxy-cinnamic acid (10 mg/mL in 1:1 ACN–water, containing 0.2% trifluoroacetic acid) at a ratio of 1:1, and allowed to crystallize at rt. Positive-ion reflector mode spectra were obtained at an accelerating voltage of 20 kV and a reflector voltage of 23 kV, using a pulse delay of 20 μs. Data were processed using XMASS software and Biotools.

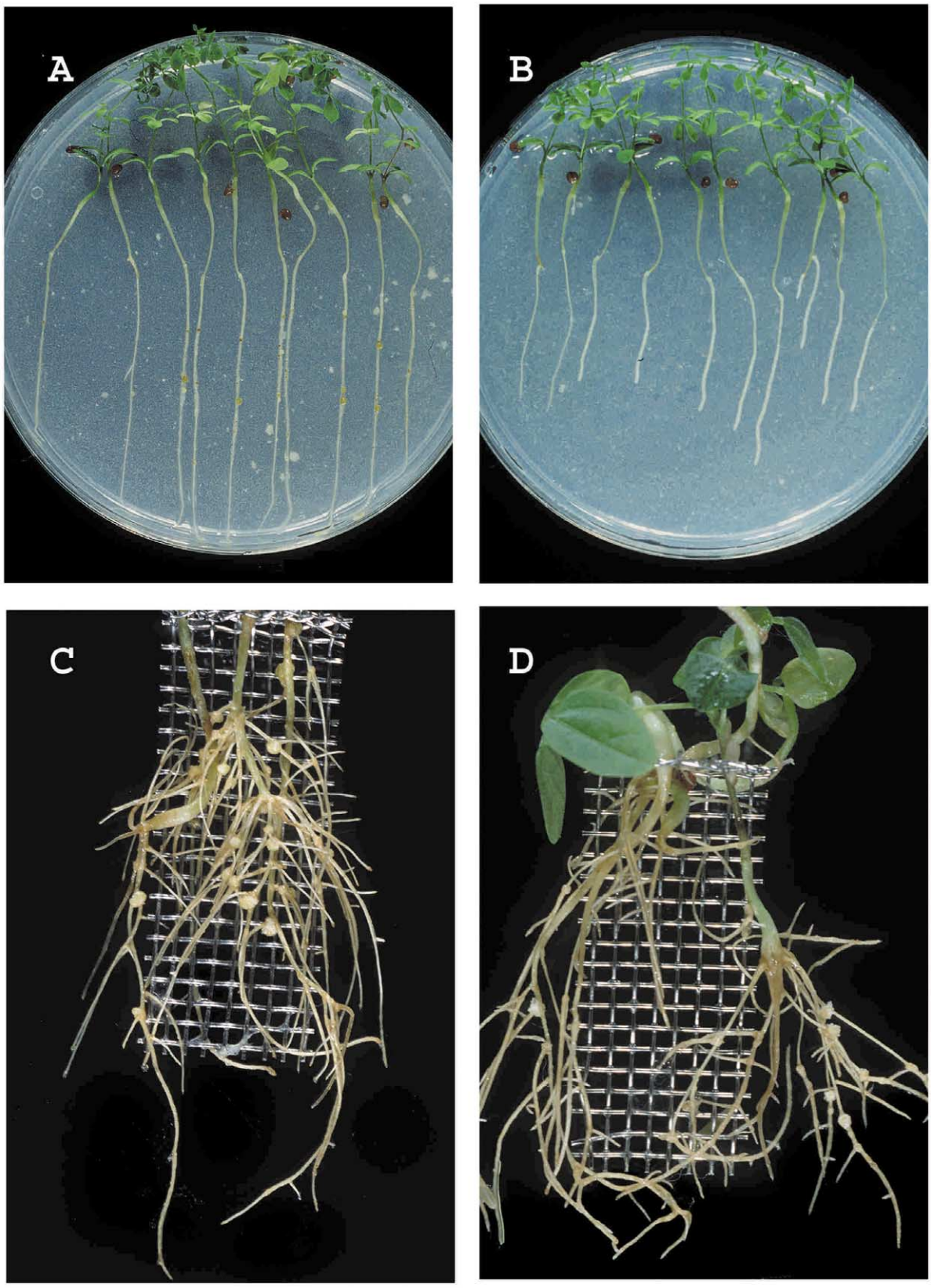


Fig. 4. Nodulation experiments on *L. japonicus* and *M. atropurpureum*. Nodulation phenotypes of *L. japonicus* (upper panel) after inoculation with *R. etli* CE3 (A) and *R. etli* Kim 5S (B), respectively, and *M. atropurpureum* (lower panel) after independent inoculations with *R. etli* Kim5S (C) and *S. fredii* HH103 (D). *R. etli* Kim5S fails to nodulate *L. japonicus* and nodulates *M. atropurpureum*.

For LC-MS/MS analysis, all LCOs were separated by using a nanoLC-system equipped with a reverse-phase capillary precolumn (internal diameter 0.3 mm, length 1 mm) and an analytical column [internal diameter 75 m, length 15 cm, pepmapC18™ (LCPackings, Amsterdam, The Netherlands)]. Typically, 1 μL of sample (from 1 mL 60% ACN–water, see "LCO isolation") was injected and concentrated on the precolumn. LCOs were eluted over the analytical column at a flowrate of 150 nL/min by applying step gradient using two solvent-systems (A, 1:19 ACN–water; and B, 9:1 ACN–water, both containing 1% AcOH indicated in Fig. 2). After online nanoESI, positive ions of each LCO were measured on an Esquire 3000 quadruple ion-trap mass spectrometer (Bruker Daltonics). Ions were scanned between 50 and 3000 Da with a scan speed of 13,000 Da/s at unit resolution. MS/MS experiments were performed by using the ion trap to select the precursor ion for fragmentation with helium collision gas. Recorded data were processed using ESQUIRE NT software and Biotools.

Plant growth and nodulation experiments.—*L. japonicus* seeds were surface sterilized, germinated, and grown on plates containing AVG (L-α-(aminoethoxyvinyl)glycine) as described.[21] Twenty plants distributed in two plates were inoculated with *R. etli* KIM5s or *R. etli* CE3. As a control, 10 plants were inoculated with the natural *Lotus* nodulating strain *M. loti* R7A. Each plate was inoculated with 200 μL of bacterial suspensions in an approximate concentration of 10^8 bacteria/mL (OD_{660} of 0.1).

Siratro seeds were treated in the same way as *L. japonicus* seeds, with the difference that the incubation time in sulphuric acid was brought up to 15 min. The plantlets were grown at 21 °C with an 8 h light/16 h dark period in transparent tubes containing 50 mL of Jensen medium.[22] Six plants were inoculated with *R. etli* KIM5s. As positive control, another six plants were inoculated with *S. fredii* strain HH103, which is known to nodulate siratro efficiently.[23]

Bacteria were added to the plants 3 days after being brought to the growth chamber. Nodulation was scored at 11 and 28 days post inoculation (d.p.i.) in the case of siratro, and 11, 25, 28 and 37 d.p.i. for *L. japonicus*.

4. Discussion

Small differences in the LCOs structures are often indispensable for specificity of the plant–bacteria interaction.[2,3,5] We describe the structure of a novel LCO molecule that is made by *R. etli* KIM5s. We also demonstrate that the application of a nanoLC system with reverse-phase capillary column directly coupled to an ion-trap spectrometer allows very fast and precise detection and analysis of LCOs, even in the presence of other molecules. The LCOs of *R. etli* KIM5s consist predominantly of hexameric chitin oligosaccharides and differ from each other in the length of their fatty acid and composition of their core oligosaccharide. Another characteristic of these hexameric LCOs is the absence of any other substituent than the methyl and acyl groups at the non-reducing GlcNAc moiety.

LCO structures that have one or two glucosamine molecules forming part of the chitin chain were found as minor components of the *R. etli* KIM5s LCO mixture. The rhizobial NodC protein is responsible for the synthesis and length of the chitin oligosaccharide backbone by the sequential addition of GlcNAc moieties to the elongating polymer.[24,25] The existence of LCOs containing glucosamine and hexameric molecules implicates the existence of an additional or different biosynthetic process to these previously described for LCO backbone synthesis, either by deacetylation of its LCOs after incorporation of GlcNAc into the chain or by the direct addition of glucosamine units into the chitin backbone. This function may be performed by NodC via an alternative mechanism, or by a different enzyme. The significance of this novel structure during plant–bacteria signalling is still unknown.

Common bean can be nodulated by a very heterologous group of rhizobia.[26–28] This implies that *P. vulgaris* is able to respond to a variety of LCOs, ranging from structurally simple ones like those produced by *Rhizobium* sp. GRH2[6] or those reported herein for *R. etli* KIM5s, to the structurally more complex LCOs synthesized by *R. etli* CFN42, *R. tropici* CIAT899 or CFN299.[7,9,29,30] It has been claimed that nodule induction in several *P. vulgaris* cultivars is achieved more efficiently by the more complex LCO species than by the less decorated ones.[6,31] Laeremans et al.[32] studied this issue in detail and reported that bean nodule induction efficiency, in decreasing order, is modulated by the LCO reducing end substitutions fucose, arabinose or sulfate and hydrogen. Interestingly, strain KIM5s, even though it produces nearly "naked" LCOs, has been reported to be a good competitor for nodule occupancy.[15,33] These data suggest that the structural complexity of LCOs per se does not necessarily affect nodulation efficiency and strain competitiveness for nodule occupancy. It should be tested, however, if a complex *nod* gene inducing cocktail, such as bean root exudate,[26] could induce the production of a different array of LCO species (more decorated ones) in *R. etli* KIM5s, than the observed after naringenin induction.

It is intriguing that strain KIM5s produces non-fucosylated LCOs despite the presence of a functional fucose biosynthesis locus,[14] and the presence of a naringenin-inducible *nodZ* (Nod-factor-specific fucosyltransferase) gene, as assessed by RT-PCR transcription analysis (Vinuesa, P. unpublished). Furthermore,

Corvera et al.[31] reported the presence of a *nolL* homologue in strain KIM5s based on high stringency southern hybridization experiments, and Quinto et al.[34] reported that by far, the most efficient substrates for NodZ-dependent transfucosylating activity are penta- and hexameric chitin fragments. The simplest working hypothesis we can think of to explain this observation would be that in *R. etli* KIM5s *nodZ* may be a pseudogene. We are currently investigating this issue. The use of a heterologous expression system for NodZ and NolL, such as that reported by Pacios-Bras et al.[21] seems to be a promising and suitable experimental system to test whether or not the hexameric LCOs produced by *R. etli* KIM5s can function as substrates for NodZ- and NolL-dependent fucosylation and acetylation of the reducing terminus GlcNAc moiety. Such a substitution may result in the extension or modification of the host range of strain KIM5s, which eventually would include the model legume *L. japonicus*.[5] Such an experimental system may aid in the dissection of the contributions of LCOs and other infection factors like surface polysaccharides in the infection process of *P. vulgaris* and *L. japonicus*.

The significance of the novel LCO structures for *R. etli* KIM5s is unknown, but we think that it has a direct relation with host specificity. This is supported by the observation that *R. etli* KIM5s does not succeed in nodulating *L. japonicus*, whereas this plant can be nodulated by *R. etli* CE3.[11] Furthermore, Stokkermans showed an equivalent effect between tetrameric non-decorated LCOs and pentameric methyl fucosylated ones on *Glycine soja* (soybean). Both LCO structures induced root hair deformations and nodule primordia formation on this legume. Tetrameric acetyl fucosylated factors did not induce plant responses and neither did pentameric non decorated.[35]

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