Growth Temperature Regulation of Host-Specific Modifications of Rhizobial Lipo-Chitin Oligosaccharides: The Function of *nodX* Is Temperature Regulated

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Lipo-chitin oligosaccharides (LCOs) are usually produced and isolated for structural analysis from bacteria cultured under laboratory rather than field conditions. We have studied the influence of bacterial growth temperature on the LCO structures produced by different *Rhizobium leguminosarum* strains, using thin-layer chromatographic, high-performance liquid chromatographic, and mass spectrometric analyses. Wild-type *R. leguminosarum* bv. *viciae* A1 was shown to produce larger relative amounts of *nodX*-mediated, acetylated LCOs at 12°C than at 28°C, indicating that the activity of *nodX* (a gene encoding an LCO *O*-acetyl transferase) is temperature dependent. Interestingly, symbiotic resistance genes *sym1* and *sym2* found in primitive pea cultivars are also temperature sensitive, only being active at low temperatures, at which they block nodulation by *R. leguminosarum* bv. *viciae* strains lacking *nodX*. We therefore propose that the gene-for-gene relationship between plant and bacterium has a temperature-sensitive mechanism as an adaptation to environmental conditions. An *R. leguminosarum* bv. *trifolii* strain was also shown to produce larger relative amounts of *nodX*-mediated, acetylated LCOs at 12°C than at 28°C. The major components synthesized by the two strains are produced at both temperatures but in different relative amounts, while some minor components are only produced at one of the two temperatures.

Additional keywords: fast atom bombardment, matrix-assisted laser desorption/ionization, nano-electrospray, Q-Tof.

The symbiosis between rhizobial bacteria and leguminous plants is a species-specific process that results in the formation of nitrogen-fixing root nodules. Successful nodulation results from a series of complex plant-microbe interactions

that involve the production and mutual recognition of specific plant and microbial signal molecules. The plant signals are secreted flavonoids that induce the transcription of nodulation, or *nod*, genes in the bacterium. Many of these gene products are involved in the biosynthesis and secretion of lipo-chitin oligosaccharides (LCOs), or Nod factors, the signal molecules secreted by the bacterium. LCOs consist of an oligosaccharide backbone of two to six β -1 $\rightarrow$ 4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues. A fatty acid group, the structure of which can vary from species to species, is attached to the nitrogen of the nonreducing-terminal residue. The structures of the LCOs are further elaborated by the addition of one or more of a range of different possible groups whose biosynthesis and/or transfer are mediated by the *nod* genes. The *nodL* gene, for example, encodes an *O*-acetyl transferase that specifically transfers an acetyl group to C-6 of the nonreducing-terminal residue (Downie 1989; Spaink et al. 1991), and *nodX* is involved in the addition of an acetyl group to C-6 of the reducing-terminal GlcNAc residue of pentameric LCOs (Davis et al. 1988). The nature of the fatty acyl chain, the number of GlcNAc residues, and the combination of extra substituents, together determine the host specificity of the bacterium.

To date, three *nodX*-bearing *Rhizobium leguminosarum* biovars—*R. leguminosarum* bv. *viciae* TOM, *R. leguminosarum* bv. *viciae* A1, and *R. leguminosarum* bv. *trifolii* ANU843—have been shown to produce *nodX*-mediated, acetylated LCOs. In addition to the GlcNAc₅ LCOs bearing the specific *nodX*-mediated acetyl group on the reducing terminal residue, these strains all produce LCOs bearing the *nodL*-mediated acetyl group on the nonreducing terminal residue. LCOs consisting of a GlcNAc backbone of four to five residues are produced by strain TOM and of three to five residues are produced by strain A1, and they carry as the major fatty acyl components C_{18:1} and C_{18:4} (Firmin et al. 1993; Ovtsyna et al. 1999). *R. leguminosarum* bv. *trifolii* ANU843, or derivatives of this strain, produce LCOs composed of three to five GlcNAc residues that bear a range of different fatty acids (C_{14–20}, with zero to four double bonds, and with or without a

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hydroxyl group) (Bloemberg et al. 1995; Orgambide et al. 1995; Philip-Hollingsworth et al. 1995).

Poor nodulation, which can lead to substantial loss of legume yield due to nitrogen deficiency, has been attributed to a range of environmental conditions, including unfavorable soil pH, high salinity, drought, the presence of ions such as aluminum and nitrate, and deficiencies in essential elements including calcium and phosphorus (Bordeleau and Prévost 1994; Richardson et al. 1988; Sadowsky and Graham 1998; Trotman and Weaver 1995). Soil temperature is also an important environmental variable (Gibson 1971) that influences the growth and survival of rhizobia in the soil, their interaction with the legume host, and competition for nodulation. Low temperatures reduce nodule formation and nitrogen fixation in the rhizobial symbionts of temperate legumes; however, in the extreme environment of the high arctic, native legumes can be nodulated and the nodulating rhizobia can fix nitrogen at rates comparable to those observed in temperate climates, indicating that both plants and their rhizobia have successfully adapted to arctic conditions (Bordeleau and Prévost 1994; Ek-Jandér and Fåhræus 1971).

Most studies in which LCOs are produced and isolated for structural analysis are carried out with bacteria grown under laboratory rather than field conditions, typically with a growth temperature of 28°C, at which the bacteria are considered to grow best. In the soil, however, bacteria grow and reproduce at much lower temperatures. To study the effects of growing bacteria at temperatures very different from those under which they naturally grow, we have analyzed the profile of LCOs produced, at a range of growth temperatures, by *R. leguminosarum* bv. *viciae* TOM (from Turkey), *R. leguminosarum* bv. *viciae* A1 (from northwest Russia), *R. leguminosarum* bv. *trifolii* ANU843 (from Australia), and *R. leguminosarum* bv. *trifolii* Tromsø 20-15 (from northern Norway), with thin-layer chromatographic (TLC) screening, and, for some of the strains, additionally with high-performance liquid chromatography (HPLC) and mass spectrometry.

In addition to the general reduction in nodulation at temperature extremes, there are also specific legume-*Rhizobium* spp. combinations that demonstrate temperature sensitivity. For example, *R. leguminosarum* bv. *trifolii* TA1 forms nodules on *Trifolium subterraneum* cv. Woogenellup in the laboratory at growth temperatures above 25°C but not below 22°C, although it nodulates the majority of its host clover cultivars below 22°C (Lewis-Henderson and Djordjevic 1991a, 1991b). In the field, strain TA1 is renowned for its failure to nodulate cultivar Woogenellup (Gibson 1968). McKay and Djordjevic (1993) suggested that there might be a direct effect of environmental conditions on transcription, translation, or post-translation events that affect the production and secretion of LCOs. Host-controlled restriction of nodulation has also been reported for primitive pea cultivars from the Middle East that are able to form nodules with most European *R. leguminosarum* bv. *viciae* strains at 26°C but not at 18°C (Kozik et al. 1995; Lie 1978). Resistance to nodulation by these strains at low temperatures is conferred by *sym1* or *sym2* (Lie 1984), recessive, allelic plant genes that are responsible for strain-specific nodulation behavior. *R. leguminosarum* bv. *viciae* strains able to overcome this resistance carry an additional gene, *nodX*, first described in strain TOM (Davis et al. 1988). Introduction of this gene into European *R. leguminosarum* bv.

viciae strains overcomes the resistance, indicating a gene-for-gene relationship between *sym1* or *sym2* and *nodX*. It has been proposed that *sym2* is involved in the early stages of the infection process controlling LCO-induced infection thread formation and that it encodes an LCO receptor or part thereof that specifically recognizes the *nodX*-modified LCOs (Firmin et al. 1993; Geurts 1998; Geurts et al. 1997; Kozik et al. 1995).

Kozik et al. (1995) suggested that pea lines from the Middle East carrying the resistance genes *sym1* or *sym2* are temperature sensitive, since it is only at low temperatures that they block nodulation by *R. leguminosarum* bv. *viciae* strains lacking *nodX*. In the present study, we have tested whether the bacteria also demonstrate temperature-sensitive regulation, i.e., whether the activity of *nodX* is also temperature dependent. The *R. leguminosarum* bv. *viciae* strains TOM and A1, both of which are able to nodulate Afghan pea, were induced with the plant flavonoid naringenin to produce LCOs at 6, 12, 18, and 28°C to see whether, with TLC, there is a demonstrable difference in the relative amounts of *nodX*-mediated, acetylated LCOs produced at the different temperatures. Recently, it was shown that *nodX* from wild-type *R. leguminosarum* bv. *trifolii* ANU843 is very similar to the *nodX* genes from strains TOM and A1 (Ovtsyna et al. 1999). We have therefore additionally studied the influence of growth temperature after induction with naringenin on the LCO structures produced by two *R. leguminosarum* bv. *trifolii* ANU843 strains. The structures of the LCOs produced by strain A1 and one of the *R. leguminosarum* bv. *trifolii* ANU843 strains were identified following HPLC fractionation, with mass spectrometry.

RESULTS

TLC analysis.

Normal-phase TLC analysis with radioactive detection was carried out as an initial screen to study the influence of temperature on the resulting profile of LCO structures extracted from the bacteria. It was already known that reducing the growth temperature from 28 to 18°C resulted in an increase in the amount of radiolabeled LCOs produced by *R. leguminosarum* bv. *trifolii* (Spaink et al. 1995). Four *nodX*-containing strains, wild-type *R. leguminosarum* bv. *trifolii* ANU843^{RR}, *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003, wild-type *R. leguminosarum* bv. *viciae* A1, and wild-type *R. leguminosarum* bv. *viciae* TOM, were induced to produce LCOs at 6, 12, 18, and 28°C (Fig. 1). Clearly, for all strains, LCO spot intensities at different growth temperatures are different. The TLC profiles for strains A1 and TOM are similar, with the most intense spots produced at 6°C and, in contrast, no radiolabeled and therefore observable spots at 28°C. Strains ANU843^{RR} and ANU843^{RR}.pRI4003 give LCO profiles with the most intense LCO spots obtained at 6 and 12°C and at 12 and 18°C, respectively. The intensity ratios of the spots obtained at a particular temperature are different for these two strains, probably due to the fact that strain ANU843^{RR}.pRI4003 contains extra copies of its *nod* genes that may well result in the production of slightly different ratios of LCO structures. The absence of observable LCO spots from strains A1 and TOM on growth at 28°C, the commonly used growth temperature, shows that it is advisable to induce

uncharacterized strains to produce radiolabeled LCOs for TLC analysis at a range of different growth temperatures.

The chromatographic migration of LCOs from strain ANU843^{RR} produced at 29°C has been published (Spaink et al. 1995b) and was used to identify the spots containing monoacetylated GlcNAc₃, GlcNAc₄, and GlcNAc₅ LCOs (indicated with Roman numerals in Figure 1) in the profiles of the other strains. Surprisingly, at 6, 12, and 18°C, in addition to spots of minor intensity, an extra spot is present in between the two major spots corresponding to monoacetylated GlcNAc₄ and GlcNAc₅ LCOs in the profile of strain ANU843^{RR}. Repetition of these experiments resulted in the same profiles showing this extra spot. Since the introduction of an acetyl group increases the hydrophobicity of an LCO and thus increases its *R_f* value on normal-phase TLC, this extra spot might correspond to pentameric LCOs bearing not only the *nodL*-mediated acetyl substituent but also a *nodX*-mediated acetyl substituent. This extra spot was not detected in the TLC profiles (not shown) of LCOs obtained at different temperatures from a related *R. leguminosarum* strain, *R. leguminosarum* bv. *viciae* LPR5560 (Spaink et al. 1991), which does not contain *nodX* and which was used as a reference in the TLC experiments. From strain ANU843^{RR} grown at 6°C, this extra spot seems to be at its relatively most intense when compared with the two major spots. Similarly for strain ANU843^{RR}.pRI4003 grown at 28°C, this extra spot is weak in relation to the spots for the tetrameric and pentameric monoacetylated LCOs, while it is one of the major spots at the lower temperatures. In addition, this extra spot is the most intense spot produced at 6°C by strains A1 and TOM, while at 18°C it is the weakest spot produced by these two strains, indicating that for each strain the intensity of the extra spot, presumably corresponding to *nodX*-mediated, doubly acetylated, pentameric LCOs, relative to the monoacetylated GlcNAc₄ and GlcNAc₅ LCO spots increases with decreasing growth temperature. However, only radiolabeled LCOs are visualized with TLC with radioactive detection and the growth temperature might influence the efficiency of uptake or incorporation of the radiolabel. We therefore fractionated the LCOs with HPLC with photodiode array detection and identified the LCOs obtained with mass spectrometry to obtain more detailed structural information on the range and relative amounts of LCOs produced at the different temperatures. The large-scale isolation of LCOs was performed at 12 and 28°C. Strain A1 was used because it produces much larger amounts of LCOs and a wider spectrum of different structures than does strain TOM (Firmin et al. 1993; Ovtsyna et al. 1999). In addition to the Australian wild-type *R. leguminosarum* bv. *trifolii* ANU843^{RR} and ANU843^{RR}.pRI4003, wild-type *R. leguminosarum* bv. *trifolii* Tromsø 20-15 originating from the north of Norway was induced at 12 and 28°C for large-scale LCO production and isolation. The HPLC profiles obtained at the two different temperatures were compared in order to study the possible influence of the climatic origin of the bacteria on the amount of LCOs produced at high and low temperatures.

HPLC analysis.

The reverse-phase HPLC photodiode array profiles of the large-scale bacterial cultures of strains A1 (Fig. 2A,B), ANU843^{RR}.pRI4003 (Fig. 2C,D), ANU843^{RR} (not shown),

and Tromsø 20-15 (not shown) induced with naringenin to produce LCOs at 12 and 28°C show clear differences. The relative intensities of the HPLC peaks at the two temperatures are significantly different, indicating, as do the results of the TLC analysis, that the temperature affects the relative amounts of the different LCOs produced. Induction with naringenin of all strains except for ANU843^{RR}.pRI4003 at the two temperatures was initiated in cultures having comparable OD₆₂₀ values. Based on the absorption values at 206 nm for the different peaks in the HPLC profiles, strains A1 (from northwestern Russia) and Tromsø 20-15 (from northern Norway) seem to produce larger absolute amounts of LCOs at 12°C than at 28°C, while the opposite holds for strain ANU843^{RR} (from Australia). In the case of the arctic strain *R. leguminosarum* bv. *trifolii* Tromsø 20-15, it clearly seems to suggest that the best yield of LCOs is obtained at low growth temperatures, which are presumably closer to the natural soil temperature for this strain. Interestingly, incorporation of radiolabeled glucosamine is not necessarily most efficient at the temperature at which large amounts of LCOs are produced (see Figure 1), suggesting that incorporation of glucosamine into the LCOs is taking place in competition with normal 'household' glucosamine metabolism and stressing the desirability of carrying out radiolabeling experiments for initial screening with TLC analysis for uncharacterized strains at different temperatures. For example, the *R. leguminosarum* bv. *trifolii* ANU843^{RR} strains do not show an observable spot on a TLC plate from LCOs produced at 28°C in the presence of radiolabeled glucosamine, while the results obtained with HPLC analysis show that these strains produce relatively large amounts of (unlabeled) LCOs at this temperature.

Figure 2 shows that for both strains A1 and ANU843^{RR}.pRI4003 the most intense peak (recorded at 206 nm absorbance) elutes later in the HPLC profile of LCOs produced at 12°C than in that for those produced at 28°C. The presence of absorbance maxima at 260 nm (not shown) and 303 nm (Fig. 2A,B) in the HPLC profiles obtained from strain

Fig. 1. Normal phase thin-layer chromatography (TLC) profile of radiolabeled lipo-chitin oligosaccharides (LCOs) produced by (lanes 1–4) wild-type *Rhizobium leguminosarum* bv. *trifolii* ANU843^{RR}, (lanes 5–8) *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003, (lanes 9–12) *R. leguminosarum* bv. *viciae* A1, and (lanes 13–16) *R. leguminosarum* bv. *viciae* TOM, at (lanes 1, 5, 9, 13) 6°C, (lanes 2, 6, 10, 14) 12°C, (lanes 3, 7, 11, 15) 18°C, and (lanes 4, 8, 12, 16) 28°C. III, spots corresponding to GlcNAc₃-containing LCOs; IV, spots corresponding to GlcNAc₄-containing LCOs; V, spots corresponding to GlcNAc₅-containing LCOs. Lanes 1–4 originate from a different TLC plate than lanes 5–16. This explains the small difference in *R_f* values of the LCO spots on the two plates, e.g., the *R_f* values of the spots corresponding to the GlcNAc₃-containing LCOs.

A1 is consistent with the published structures (Ovtsyna et al. 1999; Spaink et al. 1991) and these probably correspond to LCOs having C₁₈ fatty acids with two and three double bonds in conjugation with the carbonyl group, respectively. In addition to these absorbance maxima, the HPLC profiles from strain ANU843^{RR}.pRI4003 also have peaks with 330 nm absorbance maxima (Fig. 2C,D) corresponding to LCOs bearing fatty acyl chains in which four double bonds are in conjugation with the carbonyl group as reported (Spaink et al. 1995a; van der Drift et al. 1996). The HPLC profiles at 303 and 330 nm (Fig. 2A and B, respectively) are different for the LCOs produced at the different growth temperatures.

To identify the LCO structures present in the HPLC fractions and to study the differences due to growth temperature in more detail, the HPLC fractions from *R. leguminosarum* bv. *viciae* A1 and *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003, which were chosen because of the large amount of LCOs they produce, were analyzed by fast atom bombardment mass spectrometry (FAB-MS) and nano-electrospray quadrupole time-of-flight mass spectrometry (nanoES-Q-TOF-MS). To obtain structural information on individual LCO components, collision-induced dissociation tandem mass spectrometry (CID-MS/MS) was carried out. Fragmentation of an LCO precursor ion is induced on collision with air, and the fragment ions formed are recorded in the tandem mass spectrum. The MS analysis of LCOs is reviewed by van der Drift et al. (1998).

MS analysis of LCOs from *R. leguminosarum* bv. *viciae* A1.

FAB-MS was performed on all HPLC fractions collected from strain A1 obtained at 12 and 28°C. FAB-MS is well suited for the rapid screening of LCOs, providing information on the molecular weight and on the presence of double bonds in the fatty acyl chain. "Thioglycerol adduct" ions are observed 108 mass units above the pseudomolecular ions in the FAB mass spectra when a double bond is present in the corresponding glycolipid and thioglycerol is used as the matrix (Fukuda et al. 1985). With FAB, LCO species ionize predominantly to give [M+H]⁺ pseudomolecular ions. The species identified from the FAB mass spectra, together with those assigned from the nanoES experiments (see below) are summarized in Table 1. If a peak could not be assigned without ambiguity, the relevant HPLC fraction was analyzed by FAB-MS with 3-nitrobenzyl alcohol as matrix, which contains a large amount of sodium. When the spectra run in 3-nitrobenzyl alcohol were compared with those run in thioglycerol, the relevant LCO signals were shifted up in mass by 22 mass units, due to the formation of [M+Na]⁺ rather than [M+H]⁺ pseudomolecular ions, thus facilitating the assignment of the structures. Additional structural information from LCOs in the 12°C fraction 4 and 28°C fraction 3 was obtained by mild base de-esterification (see Table 1), which selectively removes *O*-acetyl groups. The MS shifts resulting from this treatment were used to determine the number of *O*-acetyl groups present in the different LCOs and to confirm the pro-

Fig. 2. High-performance liquid chromatography (HPLC) profiles of *n*-butanol extracts from (A,B) *Rhizobium leguminosarum* bv. *viciae* A1 and (C,D) *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003 obtained at growth temperatures of (A,C) 12°C and (°) 28°C. Absorbance was recorded at 206 nm. Dotted lines correspond to (A,B) 303 nm and (C,D) 330 nm absorbance. Fractions were pooled as indicated.

posed structures. To verify the presence of some minor species giving ions that are difficult to distinguish from the background in the FAB mass spectra, nanoES-Q-TOF-MS was performed because of its higher sensitivity and lower background. In addition, to obtain detailed structural information on individual LCO components, different pseudomolecular ions present in the mass spectra were analyzed by CID-MS/MS, with nanoES-Q-TOF-MS (Table 1). A rough indication of the relative amounts of the various LCOs produced was obtained based on the relative intensities of the corresponding pseudomolecular ions in the mass spectra together with consideration of the corresponding peak intensities at 206 nm in the HPLC chromatogram. The LCO components, however, are generally spread over several HPLC fractions so that a direct comparison is not straightforward. To compare the LCOs produced at the two temperatures more directly, composite representative pooled fractions (see Materials and Methods) were generated for the LCOs

produced at each temperature and these were analyzed by MS. Different dilutions of the pooled fractions were prepared and analyzed with nanoES, FAB, and matrix-assisted laser desorption/ionization (MALDI) to obtain a series of spectra in which the relative intensities of the pseudomolecular ions for the different LCO components could be compared.

The results of the MS analysis of the individual HPLC fractions show that the major LCO components are produced at both temperatures, but some minor LCO components are produced at only one temperature (see Table 1). In the mass spectra of all HPLC fractions, ions corresponding to doubly acetylated, pentameric LCO species are present, but, importantly, with a significantly higher intensity in those fractions obtained from LCOs produced at 12°C than at 28°C. These observations are consistent with the indications obtained from TLC and HPLC analyses and support the hypothesis that more *nodX*-mediated, doubly acetylated LCOs are produced at 12°C

Table 1. Summary of lipo-chitin oligosaccharide (LCO) structures identified as being produced at 12 and 28°C by *Rhizobium leguminosarum* bv. *viciae* A1, analyzed by fast atom bombardment–mass spectrometry (FAB-MS) and nano-electrospray quadrupole time-of-flight mass spectrometry (nanoES-Q-TOF-MS) in combination with collision-induced dissociation tandem mass spectrometry (CID-MS/MS)

LCO structure ^a	[M+Na] ⁺ (<i>m/z</i>)	12°C FAB # ^b	ES ^c	28°C FAB # ^b	ES ^c	nanoES-MS/MS ^d	–Ac ^e
V(C _{18:0} ,Ac,Ac)	1364	10	+	10,11	+	12,28	–
V(C _{18:1} ,Ac,Ac)	1362	5-10	+	1-11	+	12,28	+
V(C _{18:3} ,Ac,Ac)	1358	5,6	+	5	+	12,28	–
V(C _{18:4} ,Ac,Ac)	1356	3-6	+	5	+	12,28	+
V(C _{16:0} ,Ac,Ac)	1336	3,4	+	5		12,28	+
V(C _{18:1} -OH,Ac)	1336					12,28	
V(C _{16:1} ,Ac,Ac)	1334	1-3	+	–	+	12,28	–
V(C _{18:2} -OH,Ac)	1334					12,28	
V(C _{18:0} ,Ac)	1322	10	–	9-11	–	–	–
V(C _{18:1} ,Ac)	1320	4-10	+	1-11	+	12	+
V(C _{18:1} ,redAc) ^f	1320					12	
V(C _{18:2} ,Ac)	1318	4	+	5	+	–	+
V(C _{18:3} ,Ac)	1316	4	+	5	+	12,28	+
V(C _{18:3} ,redAc) ^f	1316					12	
V(C _{18:4} ,Ac)	1314	3,4	+	3-5	+	12,28	+
V(C _{18:4} ,redAc) ^f	1314					12	
V(C _{16:0} ,Ac)	1294	3,4	+	5	+	12,28	+
V(C _{16:0} ,redAc) ^f	1294					12,28	
V(C _{16:1} ,Ac)	1292	1-4	+	1-5	+	–	+
V(C _{18:0})	1280	–	–	10	–	–	–
V(C _{18:1})	1278	4-8	+	5-10	+	12	+
IV(C _{18:1} -OH,Ac)	1133	4,5	+	5	+	12	+
IV(C _{18:0} ,Ac)	1119	10	+	10,11	+	12,28	
IV(C _{18:1} ,Ac)	1117	4-10	+	1-11	+	12,28	+
IV(C _{18:2} ,Ac)	1115	4,5	+	3	+	12,28	+
IV(C _{18:3} ,Ac)	1113	5	+	3	+	12,28	–
IV(C _{18:4} ,Ac)	1111	3-6	+	1-5	+	12,28	+
IV(C _{16:0} ,Ac)	1091	3-6	+	4-6	+	12	+
IV(C _{16:1} ,Ac)	1089	1-4	+	1-5	+	12	+
IV(C _{18:0})	1077	10	–	10,11	–	–	–
IV(C _{18:1})	1075	5-10	+	6-10	+	12	–
IV(C _{18:4})	1069	4	+	5	–	12	+
IV(C _{14:1} -OH)	1035	4	+	4,5	+	28	–
III(C _{18:1} ,Ac)	914	6-10	+	8-10	+	12,28	–
III(C _{18:2} ,Ac)	912	4	+	–	–	–	+
III(C _{18:1})	872	5-9	–	–	–	–	–

^a Nomenclature (substituents listed “clockwise” in the order in which they appear in a two-dimensional depiction of the structure drawn with Haworth projections of the monosaccharides and starting with the fatty acid; Spink 1992).

^b High-performance liquid chromatography (HPLC) fraction number; LCOs observed as [M+H]⁺ pseudomolecular ions with FAB-MS.

^c nanoES-Q-TOF-MS performed on representative, composite HPLC fractions; LCOs observed as [M+Na]⁺ pseudomolecular ions. +, pseudomolecular ion observed; –, pseudomolecular ion not observed.

^d nanoES-MS/MS performed on pseudomolecular ion from culture grown at 12 and/or 28 oC or not performed (–).

^e De-O-acetylation, mild base de-esterification carried out on HPLC fraction 4 obtained at 12 oC and fraction 3 obtained at 28°C (+).

^f redAc = *O*-acetyl group on reducing-terminal GlcNAc residue.

than at 28°C. Clearly, the relative amounts of the major LCOs produced are different at the two temperatures, indicating growth temperature dependence. For example, hardly any HexNAC₃-containing LCOs are produced at 28°C, while in HPLC fractions 8, 9, and 10 obtained at 12°C, HexNAC₃ LCOs are major components.

All LCO components that have recently been reported to be produced at 28°C (Ovtsyna et al. 1999) are observed. In addition, we have identified some extra, minor pseudomolecular ions corresponding to III(C_{18:1}), IV(C_{18:4}), IV(C_{18:0}), V(C_{18:0}), V(C_{16:2},Ac), V(C_{16:1},Ac), V(C_{18:0},Ac), and V(C_{16:1},Ac,Ac) produced at 12 and/or 28°C. These LCOs were probably not observed by Ovtsyna et al. (1999) since they only induced the bacteria to produce LCOs at 28°C and did not collect and analyze all HPLC fractions.

The nanoES-Q-TOF mass spectra of the representative pooled HPLC fractions derived from strain A1 at 12 and 28°C (Fig. 3A,B) obtained under the same instrumental conditions contain mainly [M+Na]⁺ ions and clearly show differences in relative ion intensities for LCOs produced at the different temperatures. Remarkably, the ion at *m/z* 1362.6 corresponding to V(C_{18:1},Ac,Ac) is the second most intense ion in the spectrum of LCOs produced at 12°C, while in the spectrum of LCOs produced at 28°C the same ion is present at much lower intensity, confirming that relatively more *nodX*-mediated, doubly acetylated, pentameric LCOs are produced at 12°C than at 28°C. In addition, other *nodX*-mediated, doubly acetylated LCOs (V(C_{16:0},Ac,Ac) at *m/z* 1336.7 and V(C_{18:4},Ac,Ac) at *m/z* 1356.7) also yield more intense pseudomolecular ions in the spectrum of the LCOs produced at 12°C. In keeping with what was observed through FAB analysis of the individual HPLC fractions, the pseudomolecular ion for the HexNAC₃-containing LCO (III(C_{18:1},Ac) at *m/z* 914.6) is more abundant in the nanoES spectra of the LCOs produced at 12°C than in that of those produced at 28°C.

The FAB and MALDI spectra of the pooled HPLC fractions (not shown) yield results that are directly comparable with those from the nanoES spectra. Instrumental conditions were varied and different dilutions were analyzed to study their influence on the relative intensities of the ions, and only small differences were observed.

In summary, *R. leguminosarum* bv. *viciae* A1 produces relatively larger amounts of *nodX*-mediated, doubly acetylated, pentameric LCOs at 12°C than at 28°C. To see whether this is observed with other *nodX*-containing bacteria, the HPLC fractions from the large-scale culture of *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003 that was induced to produce LCOs at 12°C and 28°C were also analyzed following the same procedures used for strain A1.

MS analysis of LCOs from *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003.

All individual HPLC fractions from strain ANU843^{RR}.pRI4003 obtained at 12 and 28°C were submitted to FAB-MS analysis and some were also analyzed by nanoES-Q-TOF-MS/MS. In addition, FAB-MS, MALDI-TOF-MS, and nanoES-Q-TOF-MS combined with CID-MS/MS were performed on the two composite, representative, pooled HPLC fractions of LCOs produced at 12 and 28°C. The species identified from the FAB mass spectra together with those

assigned from the nanoES experiments (see below) are summarized in Table 2.

Doubly acetylated, pentameric LCOs are observed in the HPLC fractions obtained at both temperatures. However, in the FAB mass spectra of HPLC fractions 6, 7, and 8 obtained

Fig. 3. Positive ion mode nano-electrospray quadrupole time-of-flight (nanoES-Q-TOF) mass spectra (*m/z* 900–1400) of the representative composite high-performance liquid chromatography (HPLC) fractions from (A,B) *Rhizobium leguminosarum* bv. *viciae* A1 and (C,D) *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003 produced at (A,C) 12°C and (B,D) 28°C. Instrumental settings: capillary voltage (A,B) 2500 V and (C,D) 1700 V, cone voltage 100 V. Summary of assignments is given in Table 1 for *R. leguminosarum* bv. *viciae* A1 and in Table 2 for *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003. Peak assignments based on MS/MS data: I, *m/z* 929.6 polyester HO-(CH₂-CH₂-CH₂-CO-O)₅-CH₂-CH₃+Na⁺; II, *m/z* 972.6 adduct of III(C_{18:1},Ac)+Na⁺ and 58 mass units (perhaps sodium chloride, although the low ion intensity makes it difficult to be certain); III, *m/z* 1448.8 unassigned contaminant (from its MS/MS spectrum, not a lipo-chitin oligosaccharide [LCO]); IV, *m/z* 1175.6 adduct of IV(C_{18:1},Ac)+Na⁺ and 58 mass units (perhaps sodium chloride, although the low ion intensity makes it difficult to be certain); V, *m/z* 1253.5 adduct of IV(C_{18:1},Ac)+Na⁺ and 136 mass units; VI, *m/z* 1336.7 V(C_{16:0},Ac,Ac); VII, *m/z* 1356.7 V(C_{18:4},Ac,Ac); VIII, ions not corresponding to LCOs that have also been observed in the late-eluting HPLC fractions 11–14 obtained at 28°C; IX, *m/z* 1175.6 adduct of IV(C_{18:1},Ac)+Na⁺ and sodium chloride; X, *m/z* 1378.7 adduct of V(C_{18:1},Ac)+Na⁺ and sodium chloride. *, also shown to correspond to potassium-cationized IV(C_{18:1},Ac). **, also shown to correspond to potassium-cationized V(C_{18:1},Ac,Ac).

Table 2. Summary of lipo-chitin oligosaccharide (LCO) structures identified as being produced at 12 and 28°C by *Rhizobium leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003, analyzed by fast atom bombardment–mass spectrometry (FAB-MS) and nano-electrospray quadrupole time-of-flight mass spectrometry (nanoES-Q-TOF-MS) in combination with collision-induced dissociation tandem mass spectrometry (CID-MS/MS)

LCO structure ^a	[M+Na] ⁺ (m/z)	12°C FAB # ^b	ES ^c	28°C FAB # ^b	ES ^c	nanoES- MS/MS ^d	LCO structure ^a	[M+Na] ⁺ (m/z)	12°C FAB # ^b	ES ^c	28°C FAB # ^b	ES ^c	nanoES- MS/MS ^d
V(C _{20:0} ,Ac,Ac)	1392	–	+	–	–	12	V(C _{16:3} -OH)	1262	4	+	3,4	–	–
V(C _{20:1} ,Ac,Ac)	1390	11	+	–	+	12	V(C _{16:1})	1250	2,3	+	2	–	–
V(C _{22:2} -OH,Ac)	1390					28	IV(C _{20:0} -OH,Ac)	1163	3	+	2	+	–
V(C _{20:2} ,Ac,Ac)	1388	11	+	–	+	12,28	IV(C _{20:1} -OH,Ac)	1161	–	+	–	+	–
V(C _{20:3} ,Ac,Ac)	1386	9-13	+	9-11	+	12,28	IV(C _{18:2} -OH,Ac,Et)	1159	11,12	+	13,14	+	28
V(C _{20:4} ,Ac,Ac)	1384	9-13	+	8-10	+	12,28	IV(C _{20:2} -OH,Ac)	1159					12,28
V(C _{20:5} ,Ac,Ac)	1382	12	+	–	+	12,28	IV(C _{20:3} -OH,Ac)	1157	–	+	13,14	+	28
V(C _{20:0} -OH,Ac)	1366	3	+	–	+	12,28	IV(C _{20:4} -OH,Ac)	1155	–	+	12,13	+	28
V(C _{18:0} ,Ac,Ac)	1364	9-13	+	9,13	+	12,28	IV(C _{18:0} ,Ac,Et)	1147	11-12	+	13	+	12,28
V(C _{20:1} -OH,Ac)	1364					28	IV(C _{20:0} ,Ac)	1147					12,28
V(C _{18:1} ,Ac,Ac)	1362	6-13	+	5-10,13	+	12,28	IV(C _{18:1} ,Ac,Et)	1145	11-12	+	13,14	+	12,28
V(C _{20:2} -OH,Ac)	1362					28	IV(C _{20:1} ,Ac)	1145					12,28
V(C _{18:2} ,Ac,Ac)	1360	11-13	+	13	+	12,28	IV(C _{20:2} ,Ac)	1143	12	+	12,13	+	12,28
V(C _{20:3} -OH,Ac)	1360					12,28	IV(C _{20:3} ,Ac)	1141	9-12	+	10-13	+	12,28
V(C _{18:3} ,Ac,Ac)	1358	11-13	+	–	+	12,28	IV(C _{20:4} ,Ac)	1139	9-12	+	7-10	+	12,28
V(C _{20:4} -OH,Ac)	1358					12,28	IV(C _{20:5} ,Ac)	1137	8-10	+	6,7	+	12,28
V(C _{16:1} -OH,Ac,Ac)	1350	–	+	–	+	12	IV(C _{18:0} -OH,Ac)	1135	1-3	+	5	+	12,28
V(C _{18:0} ,Ac,Et)	1350					12,28	IV(C _{18:1} -OH,Ac)	1133	1-4	+	3-7	+	12,28
V(C _{20:0} ,Ac)	1350					12,28	IV(C _{18:2} -OH,Ac)	1131	13	+	5-9,12-14	+	28
V(C _{16:2} -OH,Ac,Ac)	1348	7,8	+	5-7,9	+	12,28	IV(C _{22:1})	1131					28
V(C _{18:1} ,Ac,Et)	1348					12,28	IV(C _{22:2})	1129	13	+	13	+	–
V(C _{20:1} ,Ac)	1348					12,28	IV(C _{18:0} ,Ac)	1119	9-12	+	8-11	+	12
V(C _{20:2} ,Ac)	1346	8	–	–	+	28	IV(C _{18:1} ,Ac)	1117	5-12	+	4-13	+	28
V(C _{20:3} ,Ac)	1344	–	–	–	+	28	IV(C _{18:2} ,Ac)	1115	5-7	+	4,5	+	28
V(C _{20:4} ,Ac)	1342	5,11	+	–	+	28	IV(C _{18:3} ,Ac)	1113	4	+	3,4	+	28
V(C _{16:0} ,Ac,Ac)	1336	3-5	+	2-4	+	12	IV(C _{18:4} ,Ac)	1111	4	+	3	+	28
V(C _{18:1} -OH,Ac)	1336					12,28	IV(C _{16:0} -OH,Ac)	1107	3	+	–	+	12,28
V(C _{16:1} ,Ac,Ac)	1334	2-5	+	4	+	12	IV(C _{16:1} -OH,Ac)	1105	1,2	+	1	+	12,28
V(C _{18:2} -OH,Ac)	1334					12,28	IV(C _{20:1})	1103	9-12	+ ^f	8-13	+ ^{f,g}	12,28
V(C _{16:2} ,Ac,Ac)	1332	5	+	–	+	12	IV(C _{20:2})	1101	6-12	+ ^f	5-13	+ ^f	12,28
V(C _{18:3} -OH,Ac)	1332					12,28	IV(C _{20:3})	1099	6-10	– ^h	5-14	+ ^{f,g}	12,28
V(C _{18:0} ,Ac)	1322	8-12	+	13	+	–	IV(C _{16:0} ,Ac)	1091	4,5	+	3,4	+	–
V(C _{18:1} ,Ac)	1320	4-13	+	4-13	+	28	IV(C _{16:1} ,Ac)	1089	2-4,11	+	1-3,10,11	+	–
V(C _{18:2} ,Ac)	1318	4,5	+	3-5	+	–	IV(C _{16:2} ,Ac)	1087	2,3	+	–	+	–
V(C _{18:3} ,Ac)	1316	4	+	3	–	–	IV(C _{14:0} -OH,Ac)	1079	1	+	1	+	12,28
V(C _{18:4} ,Ac)	1314	2	–	1,2	–	–	IV(C _{18:0})	1077	8-11	+ ^f	8,9	+ ^f	28
V(C _{16:0} -OH,Ac)	1310	3	+	2,3	+	12,28	IV(C _{18:1})	1075	4-10	+ ^f	3-11	+ ^f	28
V(C _{14:0} ,Ac,Ac)	1308	2,3	+	–	+	12	IV(C _{18:2})	1073	2	+ ^f	4	+	–
V(C _{16:1} -OH,Ac)	1308					12,28	IV(C _{18:3})	1071	2,3	+ ^f	–	–	–
V(C _{14:1} -OH,redAc ^e ,Et)	1308					28	IV(C _{16:1} -OH)	1063	1-3	+	1	+	12
V(C _{14:1} ,Ac,Ac)	1306	1-3	+	–	+	12	IV(C _{16:2} -OH)	1061	1,2	+	1	+	12
V(C _{16:2} -OH,Ac)	1306					12,28	IV(C _{16:0})	1049	4,5	+ ^f	3,4	+ ^f	–
V(C _{20:2})	1304	5-7	+	4-6	+	28	IV(C _{16:1})	1047	2-5	+ ^f	1-3	+ ^f	–
V(C _{20:3})	1302	5,12	+	4	–	–	IV(C _{16:2})	1045	2	+ ^f	1-3	+ ^f	–
V(C _{16:0} ,Ac)	1294	4	+	3,4	+	12	III(C _{18:0} ,Ac)	916	9-12	+	9-13	+ ^g	12,28
V(C _{16:1} ,Ac)	1292	2-4	+	1-4	+	12	III(C _{18:1} ,Ac)	914	5-9	+	4-11	+	12,28
V(C _{16:2} ,Ac)	1290	2	+	–	–	12	III(C _{18:2} ,Ac)	912	5,6	+	3-5	+ ^g	12,28
V(C _{14:0} -OH,Ac)	1282	1	+	–	–	–	III(C _{18:1})	872	6,7	+ ^f	5-7	–	–
V(C _{18:0})	1280	8	+	–	+	–	III(C _{18:2})	870	4,6	+ ^f	4-7	–	–
V(C _{18:1})	1278	4-8	+	3-10	+	–	III(C _{18:3})	868	2	+ ^f	1	–	–
V(C _{18:2})	1276	2	+	2	+	–	III(C _{14:0} ,Ac)	860	–	+	2,3	–	12
V(C _{16:1} -OH)	1266	1	+	2	+	12	III(C _{14:1} ,Ac)	858	1	+	2,3	–	12
V(C _{16:2} -OH)	1264	1,2	+	2	+	12							

^a Nomenclature (substituents listed “clockwise” in the order in which they appear in a two-dimensional depiction of the structure drawn with Haworth projections of the monosaccharides and starting with the fatty acid; Spaink 1992).

^b High-performance liquid chromatography (HPLC) fraction number, LCOs observed as [M+H]⁺ pseudomolecular ions by FAB-MS.

^c nanoES-TOF-MS performed on representative composite samples, LCOs observed as [M+Na]⁺ pseudomolecular ions. +, pseudomolecular ion observed; –, pseudomolecular ion not observed.

^d NanoES-MS/MS performed on pseudomolecular ion from culture grown at 12 and/or 28 oC or not performed (-).

^e RedAc = *O*-acetyl group on reducing-terminal GlcNAc residue.

^f Ion observed at very low intensity in nanoES mass spectrum relative to ion intensity observed in FAB mass spectrum, indicating that this ion is the result of cleavage of an *O*-acetyl group during FAB ionization.

^g Ion also shown to correspond to a fragment ion (B₄).

^h Ion shown to correspond to a fragment ion (B₄).

at 12°C, a pseudomolecular ion at m/z 1340.7 corresponding to the major doubly acetylated LCO V(C_{18:1},Ac,Ac) is present as one of the major ions, while this ion is only observed as a minor ion in the spectra from LCOs obtained at 28°C, indicating, as do the results of the TLC analysis, that strain ANU843^{RR}.pRI4003 produces a larger relative amount of *nodX*-mediated, doubly acetylated LCOs at 12°C than at 28°C. In line with the results obtained from strains A1 and TOM, the *nodX*-mediated acetyl group is only attached to pentameric LCOs produced at either of the two temperatures. This acetyl group is attached to 18 different LCO species produced at 12°C and only to 10 produced at 28°C, while 28°C is the temperature at which more LCOs are produced, suggesting a higher activity of *nodX* at the lower temperature.

To compare the relative amounts of the LCO structures present more directly, aliquots of the HPLC fractions obtained at each temperature were pooled to yield representative composite fractions of LCOs produced at each of the two temperatures and were analyzed by different MS techniques. Additional CID-MS/MS analyses of many of the pseudomolecular ions in the composite fractions were performed to determine the structures of most of the individual LCO components (see Table 2). In the initial nanoES-Q-TOF mass spectra, abundant [M+K]⁺ ions were present in addition to [M+Na]⁺ ions, complicating the spectra. After addition of a small amount of sodium chloride to the fractions, the intensity of the [M+K]⁺ ions was reduced significantly and the spectra clearly show differences in relative ion intensities from LCOs produced at 12°C (Fig. 3C) and 28°C (Fig. 3D). The major LCO structures are produced at both temperatures but yield ions having different relative intensities, while some minor LCO components are only observed at one temperature. Clear differences in the relative ion intensities of the ion at m/z 1362.7 ([M+Na]⁺ for the major doubly acetylated LCO structure) due to the difference in growth temperature are observed. In addition, in the spectrum obtained from LCOs produced at 28°C this ion was shown by MS/MS to consist of a mixture of the doubly acetylated LCO V(C_{18:1},Ac,Ac) and the monoacetylated LCO V(C_{20:2-OH},Ac). The difference between the amounts of *nodX*-mediated, doubly acetylated LCOs produced by strain ANU843^{RR}.pRI4003 at 12 and 28°C is comparable with that observed for strain A1. However, the relative abundance of the ion at m/z 1362.7 is lower in the spectrum of LCOs produced at 12°C by strain ANU843^{RR}.pRI4003 than in that of those produced by strain A1 (Fig. 3A,B).

Both tetrameric and pentameric LCOs bearing C_{18:1} (at m/z 1075.6 and 1278.7, respectively) or C_{16:1} together with one acetyl substituent (at m/z 1089.6 and 1292.7, respectively) are clearly produced at 12°C, but are barely observable or are absent at 28°C (see Figure 3C,D). At the lower temperature, more LCOs bearing fatty acyl chains containing a hydroxyl group are produced, since pseudomolecular ions observed at m/z 1061.6 (IV(C_{16:2-OH})), 1063.6 (IV(C_{16:1-OH})), 1264.7 (IV(C_{16:2-OH})), and 1266.7 (IV(C_{16:1-OH})) are present at higher relative intensities in the spectrum obtained from LCOs produced at 12°C than in that from those produced at 28°C. Furthermore, the relative amount of HexNAc₃ LCOs produced is much higher at 12°C than at 28°C (cf. nanoES mass spectra from LCOs from strain A1; Fig. 3A,B). LCOs that bear highly unsaturated fatty acids containing up to five double bonds are produced as minor components at both temperatures. The

relative amounts do not seem to be influenced significantly by the different growth temperatures. No absorption maxima above 330 nm, corresponding to four double bonds in conjugation with the carbonyl group, are observable in the isograms obtained from LCOs produced at 12 and 28°C at the retention times corresponding to the fractions in which the C_{20:5} fatty acyl-bearing LCOs are observed, suggesting that not all five double bonds are in conjugation with the carbonyl group.

The FAB and MALDI mass spectra of the pooled HPLC fractions at different dilutions (not shown) are comparable with the nanoES mass spectra, with only some minor differences in relative ion intensities observed.

Figure 4 shows the nanoES tandem mass spectrum of the sodium-cationized species at m/z 1384.7 corresponding to V(C_{20:4},Ac,Ac) produced at 28°C. Although this precursor ion is of very low intensity (see Figure 3D), a good quality tandem mass spectrum was obtained. A range of different fragment ions is present, corresponding to A-, B-, C-, Y-, and BY-type ions (nomenclature after Domon and Costello 1988), allowing the structure to be identified (see fragmentation scheme in Figure 4). The series of B_n and Y_n ions observed at m/z 512, 715, 918, 1121, and m/z 489, 692, 895, respectively, shows that one acetyl group, that mediated by *nodL*, is located on the nonreducing-terminal HexNAc residue, while the second acetyl group, that mediated by *nodX*, is positioned on the reducing-terminal residue. In comparison to the high-energy tandem mass spectra obtained by FAB-MS of protonated precursor ions, Y_n ions and double cleavage ions (B_mY_n) are of higher relative abundance in the nanoES spectrum obtained from sodiated species obtained at relatively low collision energies. [M+Na]⁺ ions corresponding to hydroxylated fatty acyl-containing LCOs are isobaric with [M+K]⁺ ions corre-

Fig. 4. Nano-electrospray tandem mass spectrum of lipo-chitin oligosaccharide (LCO) component V(C_{20:4},Ac,Ac) produced at 28°C by *Rhizobium leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003 on collision of m/z 1384.7 from the pooled high-performance liquid chromatography (HPLC) fraction. Instrumental settings: capillary voltage 2800 V, cone voltage 140 V, collision energy 70–72 eV. The m/z values are quoted as nominal masses.

sponding to their nonhydroxylated analogs. Y_n and double cleavage ions, however, allow us to distinguish fragments derived from $[M+Na]^+$ and from $[M+K]^+$ precursors and so to determine the identity of the cationizing species and thus to reveal the presence of a mixture of isobaric $[M+Na]^+$ and $[M+K]^+$ precursor ions; e.g., B_3Y_4/B_4Y_3 ions originating from sodiated HexNAc₅-containing precursor ions are observed at m/z 429 (see Figure 4), while the potassium-cationized analogs are present at m/z 445. B ions alone do not allow such distinctions to be made as the B ion series from an $[M+Na]^+$ precursor bearing a hydroxylated fatty acid and those from an $[M+K]^+$ precursor bearing a nonhydroxylated fatty acid are isobaric. Using nanoES-MS/MS, we were able to distinguish between many of the isobaric LCO structures present in the pooled fractions obtained from *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003 (see Table 2).

In some tandem mass spectra a mass difference of 249 mass units was observed between the parent ion and the highest mass B ion corresponding to the reducing HexNAc residue and an additional group with a mass of 28 mass units. Bloemberg et al. (1995) described this additional group present in LCOs from *R. leguminosarum* bv. *viciae* RBL5900 (pMP2109,pMP2368,pMP3444) as being located on C-1 of the reducing-terminal HexNAc residue and suggested that it probably corresponds to an ethyl glycoside. This sort of glycosidic substitution has also been observed in LCOs from other related microorganisms, e.g., in *Bradyrhizobium japonicum* glycerol was reported to be glycosidically linked to the reducing-terminal GlcNAc residue (Carlson et al. 1993). We have been able to identify, by CID-MS/MS, five different LCOs containing such an ethyl glycoside (Et), i.e., IV(C_{18:1},Ac,Et), IV(C_{18:0},Ac,Et), V(C_{18:1},Ac,Et), and V(C_{18:0},Ac,Et) produced at both temperatures, and IV(C_{18:2-OH},Ac,Et) produced only at 28°C (see Table 2). The ethyl glycoside-containing LCOs elute later than their analogs lacking this substituent, consistent with the expectation that this group increases the overall hydrophobicity of the LCO molecules. The tandem mass spectrum obtained from the precursor ion at m/z 1348.8 (Fig. 5) nicely demonstrates the identification of a mixture of LCOs (V(C_{18:1},Ac,Et), V(C_{16:2-OH},Ac,Ac), and V(C_{20:1},Ac)) present in the pooled HPLC fraction obtained at 28°C that have the same molecular mass but different tandem mass spectra. The B_n and Y_n ions observed at m/z 490, 693, 896, 1099, and m/z 272, 475, 678, 881, respectively, allow the ethyl glycoside-containing LCO V(C_{18:1},Ac,Et) to be identified (see fragmentation scheme in Figure 5).

Interestingly, unusual fragment ions of low intensity are observed in tandem mass spectra of LCOs bearing hydroxy fatty acyl chains from both *R. leguminosarum* bv. *viciae* A1 and *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003. In the tandem mass spectrum of IV(C_{18:1-OH},Ac) present in HPLC fraction 4 produced at 12°C from strain A1 at m/z 1133.8 (Fig. 6) these unusual ions are clearly present at m/z 268, 471, 674, and 895. The latter ion corresponds in mass to a species consisting of four HexNAc residues and one acetyl group, indicating that the fatty acyl chain is cleaved in such a way that an amide-bound C₂H₃O group remains on the nonreducing-terminal residue (loss of 238 mass units from the precursor ion). This kind of fragment ion from amide-linked 3-hydroxy fatty acids was described for the first time in post-source decay spectra of different lipopolysaccharide-derived lipid A structures

(Lindner and Luethje 1998). We propose that the mechanism of this fragmentation corresponds to a McLafferty rearrangement of the fatty acyl chain following cleavage between C-2 and C-3 comparable with that occurring on fragmentation of hydroxy fatty acids under conditions of electron ionization (Murphy 1993). The ions in Figure 6 at m/z 268, 471, and 674 are the B₁, B₂, and B₃ analogs of this B₄ ion at m/z 895. In theory, these ions could also correspond to B_mY_n ions arising from an LCO bearing an acetyl group on the reducing-terminal residue (redAc) instead of the *nodL*-mediated acetyl group on the nonreducing-terminal residue, but there is no other evidence in the spectrum for the presence of such a *nodX*-mediated acetyl group on tetrameric LCOs, e.g., a B₃ ion at m/z 870 is not observed. The loss of 60 mass units from

Fig. 5. Nano-electrospray tandem mass spectrum obtained on collision of m/z 1348.8, which corresponds to a mixture of lipo-chitin oligosaccharide (LCO) components V(C_{18:1},Ac,Et), V(C_{16:2-OH},Ac,Ac), and V(C_{20:1},Ac) produced at 28°C by *Rhizobium leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003. Spectrum obtained from the representative composite high-performance liquid chromatography (HPLC) fraction. Instrumental settings: capillary voltage 2800 V, cone voltage 140 V, collision energy 70–72 eV. The m/z values are quoted as nominal masses. Fragment ions originating from V(C_{18:1},Ac,Et), V(C_{16:2-OH},Ac,Ac), and V(C_{20:1},Ac) are in bold, in italics, and underlined, respectively. Peak assignment: I, m/z 104 also observed in tandem mass spectra from ions not corresponding to LCOs; II, m/z 447 Y₂.

these unusual ions is observed at m/z 835, 614, and 411 and shows that the O-linked acetyl group is still present and is lost as acetic acid (CH_3COOH). The fragment ion at m/z 853 probably results from the loss of ketene (CH_2CO , 42 mass units). Loss of ketene is also observed in the tandem mass spectra of m/z 1384.7 and 1348.8 (Figs. 4 and 5) from the amide-bound acetyl group of the $\text{B}_2\text{Y}_4/\text{B}_3\text{Y}_3/\text{B}_4\text{Y}_2/(\text{B}_5\text{Y}_1)$ ions (which lack the O-acetyl group), resulting in the fragment ion at m/z 184.

DISCUSSION

In this study, TLC, HPLC, and MS analyses have shown that wild-type *R. leguminosarum* bv. *viciae* A1 and *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003 both produce larger relative amounts of *nodX*-mediated, doubly acetylated LCOs after induction with naringenin at 12°C than at 28°C. The second, *nodX*-mediated O-acetyl group is only transferred to pentameric LCOs, where it is attached to the reducing residue, with the major species being $\text{V}(\text{C}_{18:1}, \text{Ac}, \text{Ac})$. This species is the second most abundant component produced at 12°C by strain A1, while the most abundant component is $\text{IV}(\text{C}_{18:1}, \text{Ac})$. Interestingly, the total amount of LCOs produced was larger at a growth temperature of 12°C for strain A1 and at 28°C for strain ANU843^{RR}.pRI4003 while for both strains *nodX* shows

a higher activity at 12°C. The extra spot in between the two spots corresponding to GlcNAc_4 and GlcNAc_5 LCOs observed in the TLC profiles at the lower temperatures therefore presumably corresponds to the *nodX*-mediated, doubly acetylated, pentameric LCOs.

Our results indicate that the activity of the *nodX* gene in *R. leguminosarum* bv. *viciae* A1 and *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003 is temperature sensitive. This temperature sensitivity could operate at the transcriptional, translational, or post-translational level. Interestingly, resistance genes *sym1* and *sym2* from primitive pea cultivars are active at the lower temperature, blocking nodulation by *R. leguminosarum* bv. *viciae* lacking *nodX*. We therefore propose that the gene-for-gene relationship between plant and bacteria has a temperature-sensitive mechanism as an adaptation to environmental conditions.

As the *nodX* gene is incomplete on pRtRF101, a 14-kb *HindIII* fragment clone of the ANU843 Sym plasmid that confers very efficient nodulation ability for red or white clover, LCOs containing the *nodX*-mediated acetyl group were not observed from LPR5045.pRtRF101 (Spaink et al. 1995a). Ovtsyna et al. (1999) therefore reasoned that *nodX* is not required for nodulation of these hosts and that any role of *nodX* in nodulation of these hosts is therefore probably limited to one influencing the efficiency of nodulation rather than host range. We now suggest that *nodX* from *R. leguminosarum* bv. *trifolii* might be involved in the determination of host range on some clover cultivars as it is for *R. leguminosarum* bv. *viciae* strains on primitive pea cultivars. If this is the case, then it is possible that such clover cultivars might also possess analogs of the *sym1* and *sym2* resistance genes, which would act as an extra selection criterion for improving the efficiency of symbiosis under cold conditions for bacterial strains adapted to low temperatures. It would therefore be interesting to determine whether clover plants bear *sym1* or *sym2* analogs or other symbiosis genes having a similar function.

Our results also show additional interesting effects of growth temperature on LCO structures. The major components produced by the two strains are produced at both temperatures but in different relative amounts while some minor species are only produced at one of the two temperatures. At 12°C, relatively more GlcNAc_3 - and GlcNAc_5 -containing LCOs are produced by both strains. In addition, LCOs bearing hydroxy fatty acyl chains from strain ANU843^{RR}.pRI4003 are produced more abundantly at 12°C than at 28°C. In addition, the proportion of mono-unsaturated fatty acids, mainly $\text{C}_{16:1}$ and $\text{C}_{18:1}$, was relatively larger at 12°C consistent with the observation that the growth temperature markedly affects the fatty acid composition of free-living bacteria from *R. leguminosarum* bv. *viciae* strains CBh5 and CBp7 (Théberge et al. 1996); the proportion of unsaturated fatty acids increased significantly when the day/night temperature was lowered from 22/15°C to 15/10°C. The amount of highly unsaturated fatty acids from strain ANU843^{RR}.pRI4003, however, does not seem to be influenced significantly by growth temperature in our experiments.

HPLC analysis of the LCOs produced at 12°C and 28°C by three *R. leguminosarum* strains originating from different climatic regions—Australia (ANU843^{RR}), northwestern Russia (A1), and northern Norway (Tromsø 20-15)—shows how important the environmental condition of growth temperature is

Fig. 6. Nano-electrospray tandem mass spectrum of lipo-chitin oligosaccharide (LCO) component $\text{IV}(\text{C}_{18:1}\text{-OH}, \text{Ac})$ produced at 12°C by *Rhizobium leguminosarum* bv. *viciae* A1. Spectrum obtained on collision of m/z 1133.8 from h-performance liquid chromatography (HPLC) fraction 4. Instrumental settings: capillary voltage 1500 V, cone voltage 100 V, collision energy 60 eV. The m/z values are quoted as nominal masses. *, $\text{C}_{16}\text{H}_{30}\text{O}$ from fatty acyl chain; Peak assignment: I, m/z 242 $\text{B}_2\text{Y}_3/\text{B}_3\text{Y}_2/\text{B}_4\text{Y}_1$ from $[\text{IV}(\text{C}_{18:1}, \text{Ac})+\text{K}]^+$; II, m/z 346 $0.2\text{A}_4\text{Y}_2$; III, m/z 362 $0.2\text{A}_4\text{Y}_2$ from $[\text{IV}(\text{C}_{18:1}, \text{Ac})+\text{K}]^+$; IV, m/z 626 0.2A_2 ; V, m/z 632 B_4Y_3 ; VI, m/z 835 895-HOAc ; VII, m/z 853 $895\text{-CH}_2\text{CO}$.

for LCO yield. In the case of the arctic strain *R. leguminosarum* bv. *trifolii* Tromsø 20-15, it is clear that the lower laboratory growth temperature better reflects the bacterium's normal soil temperature than do the higher growth temperatures tested.

More than 100 different LCO components have been identified from *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003. These consist of trimeric, tetrameric, and pentameric GlcNAc backbones bearing C₁₄₋₂₂ fatty acids that contain zero to five double bonds and sometimes a hydroxyl group, zero to two acetyl substituents and occasionally an ethyl glycoside (see Table 2). Several species are minor components and only observable by nanoES-Q-TOF-MS. Importantly, structural information on these minor components can be obtained with CID-MS/MS because of the high sensitivity of the Q-ToF mass spectrometer in the MS/MS mode. van der Drift et al. (1998) showed that, to obtain CID mass spectra of comparable quality, one-thousandth of the amount of an LCO-containing HPLC fraction from *Mesorhizobium loti* needed to generate a spectrum with FAB-MS was sufficient to produce a spectrum of better quality with nanoES-Q-TOF-MS. nanoES-Q-TOF-MS is thus very well suited for use as an alternative to FAB-MS for the rapid screening of LCOs. However, the additional information on the presence of a double bond in the fatty acyl chain provided by the observation of a thioglycerol adduct in the FAB mass spectrum is sacrificed. CID-MS/MS of the pooled HPLC fractions is sometimes complicated when a precursor ion consists of both [M+Na]⁺ and [M+K]⁺ pseudomolecular ions for different molecular species and/or corresponds to a mixture of LCO structures with the same molecular mass and/or contains ¹³C isotopes of LCOs slightly lower in mass.

In the course of this study, we noticed that different strains of *R. leguminosarum* bv. *trifolii* produce LCOs bearing quite different fatty acyl chains; in particular, some strains appear to be much better able to produce the species-specific polyunsaturated fatty acyl chains (Spaink et al. 1995a; van der Drift et al. 1996) than others (Olsthoorn et al. 1997; Orgambide et al. 1995; Philip-Hollingsworth et al. 1995). Since making these observations we have focused a good deal of research effort in our laboratories on determining the reason for this observation. This has resulted in our now being able to identify the mechanism by which production of *R. leguminosarum* bv. *trifolii*-specific polyunsaturated fatty acylated LCOs is regulated (M. M. A. Olsthoorn, M. Harteveld, H. R. M. Schlaman, J. E. Thomas-Oates, and H. P. Spaink, *paper in preparation*).

MATERIALS AND METHODS

Bacterial strains.

R. leguminosarum bv. *trifolii* ANU843^{RR} is a spontaneous, rifampicin-resistant derivative of wild-type ANU843 (Djordjevic et al. 1985) that, after inoculation on red clover, was reisolated from nitrogen-fixing nodules. Plasmid pRI4003 contains all the known *nod* and *nif* genes from *R. leguminosarum* bv. *trifolii* ANU843 and is able to restore the ability to induce nitrogen-fixing nodulation on clover to pSym-cured strains (Innes et al. 1988). *R. leguminosarum* bv. *trifolii* Tromsø 20-15, which was isolated from white clover nodules obtained in the arctic zone, was a gift from M. Svenning (Tromsø Univer-

sity, Norway). *R. leguminosarum* bv. *viciae* A1 is a wild-type strain nodulating Afghan peas and isolated from soils of northwestern Russia (Chetkova and Tikhonovich 1986). *R. leguminosarum* bv. *viciae* TOM is a wild-type strain nodulating Afghan peas and isolated from Turkish soil (Lie 1978).

Chemical modification.

Mild base de-esterification of 5% of HPLC fraction 4 from strain A1 obtained at 12°C and 15% of fraction 3 from strain A1 obtained at 28°C was carried out in 200 µl of methanol and 200 µl of 25% aqueous ammonia for 18 h at ambient temperature followed by evaporation to dryness.

TLC analysis.

Bacteria were grown in B⁻ medium (Spaink et al. 1992) including 1 mM potassium phosphate buffer (pH 7.4) at 28°C and 200 rpm (Aqua Shaker; Adolf Kühner, Basel, Switzerland). Spectinomycin (10 µg/ml) was added to the ANU843^{RR}.pRI4003 culture. The cultures of strains ANU843^{RR}.pRI4003, A1, and TOM were diluted to an optical density at 620 nm (OD₆₂₀) of 0.05, while the ANU843^{RR} culture was diluted to an OD₆₂₀ of 0.09. LCOs were radiolabeled by growing 3 ml of bacterial cultures overnight in the presence of 0.4 µCi [1-¹⁴C]-D-glucosamine (specific activity 50 mCi/mmol; Amersham, Buckinghamshire, U.K.) and naringenin (1 µg/ml) as inducer at 6, 12, 18, and 28°C. After addition of 2 ml of water-saturated *n*-butanol and boiling for 10 min, LCOs were isolated from the cultures by extraction. The extracts were dried and redissolved in 30 µl of water-saturated *n*-butanol. TLC analyses were carried out with normal phase Silica 60 plates (Aldrich Chemical, Milwaukee, WI) developed with butanol:ethanol:water (5:3:2, vol/vol/vol). Five microliters of each extract was used for each TLC analysis. The LCOs from *R. leguminosarum* bv. *trifolii* ANU843^{RR}.pRI4003, *R. leguminosarum* bv. *viciae* A1, and *R. leguminosarum* bv. *viciae* TOM were analyzed on the same plate, while those from *R. leguminosarum* bv. *trifolii* ANU843^{RR} were analyzed on a separate one. Radioactivity was detected with a phosphor imaging system (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software.

HPLC analysis.

Bacteria were grown to an OD₆₂₀ of 0.3 for strain A1 for induction at both 12 and 28°C, of 1.2 and 1.0 for strain ANU843^{RR}.pRI4003 for induction at 12 and 28°C, respectively, of 1.0 and 0.8 for strain ANU843^{RR} for induction at 12 and 28°C, respectively, and of 0.72 and 0.73 for strain Tromsø 20-15 for induction at 12 and 28°C, respectively. Spectinomycin (10 µg/ml) was added to the ANU843^{RR}.pRI4003 cultures. The cultures were then diluted to 3 liters with B⁻ medium containing 1 µg of naringenin per ml as inducer and were incubated at 12 or 28°C overnight at 100 rpm (Air Cooled Shaker; Marius Instrumenten, Utrecht, NL) for LCO production. LCOs were extracted from the cultures with *n*-butanol (300 ml/liter) and dried. The samples were redissolved in acetonitrile/water (3:2, vol/vol) overnight, purified over an octadecyl silica column (J. T. Baker, Deventer, The Netherlands, 1 mg of sorbent), and subsequently submitted to reverse-phase HPLC on a Pep-S column (5 µm, 5 × 250 mm; Pharmacia, Uppsala, Sweden). Elution was performed with a stepwise gradient of mixtures of acetonitrile and water

(highest grade; J. T. Baker) at a flow of 1 ml/min and monitored with a Jasco MD-910 photodiode array detector for strains A1 and ANU843^{RR}.pRI4003 and a Pharmacia RSD2140 photodiode array detector for strains ANU843^{RR} and Tromsø 20-15. The gradient used for strain A1 was 20% acetonitrile for 10 min, 30% for 40 min, 40% for 30 min, and 80% for 10 min. The gradient used for strain ANU843^{RR}.pRI4003 was 20% acetonitrile for 10 min, 30% for 30 min, 40% for 20 min, 42% for 30 min, 50% for 10 min, 60% for 10 min, and 80% for 10 min. The gradient used for strains ANU843^{RR} and Tromsø 20-15 was 30% acetonitrile for 30 min, 40% for 20 min, 42% for 20 min, 50% for 20 min, 60% for 10 min, and 80% for 15 min. HPLC fractions of 1 ml were collected and dried. The resulting HPLC fractions were redissolved in 10 to 1,000 µl of dimethyl sulfoxide prior to FAB-MS analysis. To make a more direct comparison of the different LCOs produced at the two temperatures with MS analysis, aliquots from each HPLC fraction were taken so that the volume of the aliquot reflected the number of minutes over which the fraction had been collected and the volume of dimethyl sulfoxide in which it was dissolved. These aliquots were pooled to yield a composite representative fraction for each temperature and strain and the fractions were dried.

MS.

Positive ion mode FAB-MS was carried out with the first two sectors of a Jeol JMS-SX/SX 102A tandem mass spectrometer operated at 10 kV (mass range m/z 200 to 2000) accelerating voltage. The FAB gun was operated at 4 kV accelerating voltage with an emission current of 10 mA and 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 processed on a Hewlett-Packard HP 9000 data system with the Jeol Complement software. The probe was loaded with 1 µl of sample solution into a matrix of thioglycerol (2 to 3 µl). A matrix of 3-nitrobenzyl alcohol was used to generate $[M+Na]^+$ ions.

nanoES-Q-TOF-MS was performed with a Micromass Q-ToF hybrid tandem mass spectrometer equipped with a Z-spray sample introduction system and gold-coated glass capillaries in a nanospray ionization source. The capillary was loaded with 1 to 3 µl of an individual or pooled HPLC fraction in acetonitrile:water:formic acid (50:50:0.1, vol/vol/vol) and a potential of +1500 to 2800 V was applied to it. Sodium chloride (5 mM) was added to the two pooled HPLC fractions from ANU843^{RR}.pRI4003 to displace potassium. The source temperature was 80°C. The quadrupole was used in the R_f -only mode. TOF mass spectra were integrated every 2.3 s over the m/z 200 to 2000 range and recorded with a cone voltage varying between 25 and 140 V. The data were recorded and processed with the MassLynx software, version 3.0. Mass calibration was performed by multiple-ion monitoring of the singly charged $[M+Na]^+$ maltooligosaccharide signals derived from corn syrup (Sigma Chemicals, St. Louis, MO). CID-MS/MS was performed with the quadrupole to select the precursor ion for fragmentation in the hexapole collision cell. Argon was used as the collision gas and the collision energy varied between 40 and 80 eV. Product ions were analyzed with the orthogonal TOF analyzer.

MALDI-TOF-MS experiments were performed on a PerSeptive Biosystems Voyager-DE STR with a BioSpectrometry

Workstation with PerSeptive GRAMS/386 software. Positive ion mode spectra were recorded in the reflectron mode over the m/z 600m to 2000 mass range with an acceleration voltage of 20 kV, grid voltages of 15.4 and 14.6 kV for the pooled HPLC fractions from strains A1 and ANU843^{RR}.pRI4003, respectively, a guide wire voltage of 4 V, and a delay time of 150 ns. 2,5-Dihydroxybenzoic acid was used as the matrix, made up as a saturated solution (10 mg/ml) in acetonitrile:water (1:1, vol/vol) containing 10 mM sodium chloride that was mixed 1:1 (vol/vol) with the pooled HPLC fractions in acetonitrile:water:formic acid (50:50:0.1, vol/vol/vol). The preparations were irradiated at 337 nm with 3 ns pulses from a nitrogen laser.

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