Mutation or Increased Copy Number of nodE Has No Effect on the Spectrum of Chitolipooligosaccharide Nod Factors Made By Rhizobium leguminosarum bv. trifolii*

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The bacterial gene nodE is the key determinant of host specificity in the Rhizobium leguminosarum-legume symbiosis and has been proposed to determine unique polyunsaturated fatty acyl moieties in chitolipooligosaccharides (CLOSs) made by the bacterial symbiont. We evaluated nodE function by examining CLOS structures made by wild-type R. leguminosarum bv. trifolii ANU843, an isogenic nodE::Tn5 mutant, and a recombinant strain containing multiple copies of the pSym nod region of ANU843. 1H-NMR, electrospray ionization mass spectrometry, fast atom bombardment mass spectrometry, flame ionization detection-gas chromatography, gas chromatography/mass spectrometry, and high performance liquid chromatography/UV photodiode array analyses revealed that these bacterial strains made the same spectrum of CLOS species. We also found that ions in the mass spectra which were originally assigned to nodE-dependent CLOS species containing unique polyunsaturated fatty acids (Spaink, H. P., Bloemberg, G. V., van Brussel, A. A. N., Lugtenberg, B. J. J., van der Drift, K. M. G. M., Havercamp, J., and Thomas-Oates, J. E. (1995) Mol. Plant-Microbe Interact. 8, 155–164) were actually due to sodium adducts of the major nodE-independent CLOS species. No evidence for nodE-dependent CLOSs was found for these strains. These results indicate that the current model to explain how nodE determines host range in the R. leguminosarum-legume symbiosis.

Rhizobium, Bradyrhizobium, and Azorhizobium are bacterial genera that form nitrogen-fixing nodules on legume roots. In this symbiosis, the plant produces flavonoids that activate bacterial infection and nodulation of the corresponding host plant (1–4). These Nod factors are chitolipooligosaccharides (CLOSs) consisting of β-1,4-linked oligomers of N-acetylglucosamine bearing an amide-linked fatty acyl moiety at the nonreducing end and may contain other substituents (e.g. O-acetyl, sulfate, etc.) that make their biological activity host-specific (5). The current model for nod functions is that the common nod genes encode enzymes that synthesize the common backbone of CLOSs, and the host-specific nod genes encode enzymes that introduce these modifications in CLOS structures making them host-specific (6, 7).

Rhizobium leguminosarum bv. trifolii (hereafter called R. trifolii) is the bacterial symbiont of the legume host, clover (Trifolium spp.). In the most thoroughly studied wild-type strain (ANU843), the ability to nodulate white clover is controlled by regulatory (nodD), common (nodABCij), and host-specific (nodFERL, nodMN) nod genes residing within a 14-kb region on its resident symbiotic plasmid (pSym) (8, 9). Elegant studies have shown that NodE is the main determinant of nodulation host range for R. trifolii and its closest relative, the pea symbiont, R. leguminosarum bv. viciae (10, 11). Tn5 disruption of nodE (but not genes downstream of nodE) in ANU843 results in a unique dual phenotype, which is defective in nodulation of white clover and gain in the ability to nodulate a new host, peas (10). Evaluations of the nodE sequence and of CLOS structures made by certain recombinant nod-overexpressing strains have led to the current model proposing that NodE is a 3-ketoacyl synthase that controls the host range of R. leguminosarum bv. viciae and bv. trifolii by specifying the synthesis of unique conjugated tri- and tetraunsaturated fatty acid moieties with characteristic absorption maxima between 300 and 330 nm in CLOS species (4, 12).

It was originally thought that Rhizobium synthesizes only minute quantities of CLOSs and excretes them into the extracellular milieu where they can act on the host plant (3, 4, 13). However, we recently found that CLOS glycolipids can be purified in significantly higher yield (>1000-fold) from extracts of cell membranes of wild-type rhizobia than from culture supernatants (14, 15). In the present study, we have critically evaluated the proposed function of nodE by performing detailed structural analyses of CLOS species made by wild-type ANU843, an isogenic nodE::Tn5 mutant derivative ANU297, and a recombinant strain ANU845 pRTRF101 containing the cloned 14-kb HindIII pSym nod region of ANU843 on multiple copy plasmid pWBS5a introduced into the pSym-cured derivative ANU845. Our experiments reveal that ANU843 makes a large diversity of major and minor CLOS species, which does not change with impairment of nodE function or increased nod copy number. This spectrum of CLOSs does not, however, include molecules containing tri- or tetraunsaturated fatty acids.

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***The abbreviations used are: CLOS, chitolipooligosaccharide; kb, kilobase pair(s); FID-GC, flame ionization detection gas chromatography; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; GC/MS, gas chromatography/mass spectrometry; nod, nodulation; pSym, symbiotic plasmid; HPLC, high performance liquid chromatography.**
This necessitated a reevaluation of results of a recent report (12) in which six CLOS species from R. trifolii were proposed to contain nodE-independent Tri- or tetraunsaturated fatty acids. We show here that the mass spectral ions to which these latter structures were assigned are attributable to sodium adducts of the major nodE-independent CLOS species. Portions of this work were presented recently.²

MATERIALS AND METHODS

Bacterial Strains and Plasmids—R. trifolii wild-type strain ANU843, its isogenic nodE::Tn5 mutant derivative ANU297, and its pSym-cured derivative ANU845 were obtained from B. Rolfe, Australian National University (10, 16). ANU843 has only one copy of nodE and produces a flavonoid-inducible protein detected by immune blotting with polyclonal anti-NodE protein antiserum, whereas no flavonoid-inducible protein reactive with the same antiserum is detected in ANU297 (10, 11). Plasmid pRtRF101 contains the 14-kb HindIII pSym nod region of ANU843 (8) cloned on pWB5a (17), a derivative of the IncP-1 plasmid pRK290 that is maintained in five to eight copies/gene equivalent (18). Strain ANU845 pRtRF101 (from A. Squarini, Padova University) was constructed by triparental conjugation between the Escherichia coli donor strain HB101 pRtRF101 (from S. Long, Stanford University, (17)) and the pSym-cured recipient ANU845 (16), using as a helper plasmid pRK2013 hosted in E. coli JM109. E. coli strains were grown at 37°C in TY medium containing 10 μg/ml tetracycline for the donor and 30 μg/ml kanamycin for the helper. Rhizobium recipients were grown in TY medium at 28°C. 10 ml of mid-exponential phase cultures of each strain were pelleted. Cells were resuspended in 100 μl of saline solution, and 50–μl aliquots were mixed on nitrocellulose 0.22-μm filters on TY plates and incubated for 24 h. Cells on filters were resuspended in 2 ml of saline solution, and aliquots were plated on defined BIII medium (19) containing 2 μg/ml tetracycline. Transconjugants were purified by restreaking, and their identity was confirmed by plasmid profile analysis using a modified Eckhardt gel technique (20).

Plant Nodulation Tests—Axenic seedlings of Dutch white clover (Trifolium repens L.) were grown on slopes of nitrogen-free Fahraeus agar as described previously (19). Axenic seedlings of pea (Pisum sativum cv. Alcan) were grown in Erlenmeyer flasks containing 200 ml of Fahraeus agar (21). Inocula were grown at 30°C for 5 days on BIII agar (plus 30 μg/ml kanamycin for ANU297 and 2 μg/ml tetracycline for ANU845 pRtRF101). For nodulation tests, cells were diluted in Fahraeus medium and applied at a dose of 5 × 10⁶ cells/plant, with 12 plant replicates. Plants were incubated in a plant growth chamber (22) and examined periodically for emergence of root nodules with confirmation of their structure after root clearing (23). Uninoculated controls received sterile Fahraeus medium only.

Bacterial Cultures for CLOS Production—Cells were grown with constant shaking (150 rpm) at 30°C in 2-liter flasks containing 1 liter of BIII broth with 0.45 mM CaCl₂, (24) and 4 μM 7,7-dihydroxyflavone (2). BIII medium was further supplemented with 30 μg/ml kanamycin or 2 μg/ml tetracycline for ANU297 and ANU845 pRtRF101, respectively. Batch cultures were grown to post-exponential phase (9 × 10⁷ cells/ml) just preceding stationary phase (25, 26), harvested by centrifugation, and further washed with deionized water.

Extraction and Purification of Membrane Chitolipooligosaccharides—CLOSs were extracted from cells of 4 liters of broth culture using chloroform/1-propanol/methanol/water (1:2:2:3) and then fractionated by a series of reversed-phase chromatographies (14) to yield the isolated family of CLOSs.

Composition and Structural Analyses of the Isolated Chitolipooligosaccharides—200 MHz ¹H-NMR spectroscopy (14) was performed on 6-mg samples at 25°C in deuterated dimethyl sulfoxide with suppression of the water resonance. The chemical shifts were measured relative to an external trimethylsilylamine standard. UV absorption spectra were recorded in ethanol on a Varian DM5200 spectrophotometer. For compositional analyses, 3-mg samples were treated with 1 ml of 2% HCl in methanol at 80°C for 16 h and then evaporated to dryness and partitioned between 2 ml of water/chloroform (1:1). The fatty acid methyl esters extracted into the chloroform layer were identified by FID-GC and GC/MS analyses with comparison to authentic standards whenever possible. The aqueous layer containing the methyl glycosides was evaporated to dryness, peracetylated with acetic anhydride/pyridine (1:1) at 25°C for 18 h, and analyzed by GC/MS. FID-GC analyses of the fatty acid methyl esters were performed on a Varian 3740 gas chromatograph using a J & W Scientific DB-225 column (program: 150–230°C at 3°C/min with a 10-min hold at 300°C). Fatty acid methyl esters and peracetylated methyl glycosides were analyzed on a Hewlett-Packard 5995C GC/MS instrument using a J & W Scientific DB225 column (program: 150–230°C at 3°C/min with a 20-min hold at 230°C). ESI-MS analyses were performed on a Fisons Platform instrument using an electrospray inlet in either negative or positive ion mode, a mobile phase of acetonitrile/water (1:1), and a flow rate of approximately 10 μl/min. The instrument was tuned for unit resolution, and the capillary voltage was set at approximately 3 kV.

Screen for Extracellular Chitolipooligosaccharides—The supernatants from 2 liters of broth cultures of ANU843 and ANU297 were passed through C18 reverse phase silica beds (150 ml) followed by washing with 500 ml of water and then with 500 ml of 100% acetonitrile to release adsorbed lipophilic material. The acetonitrile eluate was concentrated to dryness and analyzed by HPLC using a Vydac C18 reverse phase column (linear gradient of 30–100% acetonitrile in water over 50 min, 1 ml/min, monitoring of the effluent at 303 nm).

Comparative Analysis of the Total Cellular Fatty Acids of ANU843 and ANU297—Methyl ester derivatives were prepared from equivalent cell pellets (27) and analyzed by GC/MS.

Reverse Phase HPLC Analysis of CLOSs with UV Photodiode Array Detection—CLOS samples were dissolved in 20% acetonitrile in water and injected onto a Vydac C18 reverse phase analytical HPLC column (linear gradient of 20–100% acetonitrile in water over 60 min, 20-min
hold at 100% acetonitrile, 0.6 ml/min). The effluent was monitored by a Waters 990 photodiode array detector, with acquisition of the full range UV spectrum of each 303-nm absorbing eluted peak.

RESULTS

Plant Nodulation Tests—Wild-type ANU843 efficiently nodulated all of the white clover plants but neither nodulated nor induced cortical cell divisions on pea roots. In contrast, ANU297 took 6 days longer to induce the first emerging nodules on white clover and incited 80% fewer nodules/plant by 1 month. ANU297 reisolated from surface-sterilized nodules of white clover retained the Kanr marker. In addition, ANU297 induced foci of cortical cell divisions resembling root nodule primordia and/or root nodules on 83% of the pea plants. These results were consistent with previously reported symbiotic phenotypes for these strains (10). In comparison with ANU843 on white clover, the recombinant strain ANU845 pRtRF101 was twice as efficient in early nodulation kinetics and induced 50% more nodules/plant by 1 month (figure not shown).3

Influence of nodE and nod Gene Dosage on Yield of Membrane CLOSs from ANU843—The final yields of membrane CLOSs from pelleted cells of ANU297 (2–3 mg/liter) and ANU845 pRtRF101 (2–19 mg/liter) were 5-fold lower and slightly higher, respectively, than the previously reported yield from an equivalent culture of ANU843 grown identically (14).

Structural Analyses of CLOSs from ANU843 and ANU297—Apart from resonances due to residual traces of 1-propanol, the 1H-NMR spectra of CLOSs from ANU843 and ANU297 were the same (Fig. 1). These spectra contained resonances of methylene and methyl protons from the fatty acyl chain (1.2 and 0.8 ppm, respectively), N-acetyl and O-acetyl groups (1.8–2.0 ppm), the methylene group adjacent to vinyl and carbonyl groups (2.0 and 2.1 ppm, respectively), the carbohydrate ring protons (3.0–4.5 ppm), and the vinyl protons of an isolated double bond (5.3 ppm). A set of common downfield signals between 7.0 and 7.8 ppm were attributed to a combination of amide and conjugated vinyl protons since the former protons exchanged upon treatment with deuterium oxide, whereas the latter remained intact (Fig. 1, insets).

GC/MS analyses of the peracetylated methyl glycosides indicated that glucosamine was the sole glycosyl component of CLOSs from ANU843 and ANU297 (figures not shown). The FID-GLC and GC/MS profiles of the fatty acid methyl esters from these same samples were also identical in every detail (Fig. 2). The major fatty acid component was cis-vaccenic acid (C18:1) with lesser amounts of C18:0, C16:0, and C16:1 fatty acids, consistent with previous results (14). Together these accounted for approximately 95% of the total fatty acids in the family of CLOSs from both strains. Additional fatty acids detected in both samples by FID-GC and GC/MS were C18:2 and C20:1. Selected ion chromatograms for the characteristic fragment at m/z 103 (28) also revealed 3-hydroxy-C14:0, 3-hydroxy-C16:0, and 3-hydroxy-C18:0 fatty acids (Fig. 2, C and D). Thus, nine different fatty acids are definitively identified in the family of CLOSs from ANU843 and ANU297.

Heterogeneity of CLOS species from ANU843 and ANU297 was further analyzed by both negative and positive mode ESI-MS. Like all soft ionization methods, ESI-MS is quite sensitive to the exact purity of the sample (concentrations of metal ions, anions, etc.) over which very little control can be exerted. Although the masses and relative abundance of ions are reproducible in multiple analyses of the same sample, the absolute values of ion intensities from different preparations usually vary to some extent and therefore should not be used in quantitation. Evaluation of high mass ions in several runs of posi-
tive and negative mode ESI-MS analyses showed that ANU843 produced a very diverse family of CLOSs (Fig. 3, A and C), and this same diversity was detected in CLOSs from ANU297 (Fig. 3, B and D), in agreement with the above 1H-NMR and GC/MS analyses. This diversity of CLOS species found by these methods consists of non-O-acetylated, mono-O-acetylated, and di-O-acetylated chitotri-, tetra-, and pentasaccharides bearing a large variety of amide-linked fatty acids (Table I). This list of
CLOS species from ANU843 includes the diversity of a previously published list (14) plus the di-O-acetylated CLOS species, III(C18:1, 2Ac) with M+ at m/z 933, IV(C16:0, 2Ac) with M+ at m/z 1110, and IV(C18:1, 2Ac) with M+ at m/z 1136 found by negative mode ESI-MS (Table I).

The occurrence of the six high mass ions previously assigned to unique nodE-dependent CLOS species of R. trifolii (12) was further investigated by positive mode ESI-MS. All CLOS species from ANU843 and ANU297 identified in negative mode ESI-MS spectra were also found in positive mode ESI-MS spectra (Fig. 3, C and D), either as pseudomolecular ions [M+H]+ (i.e. M + 1 u) or as their sodium adducts [M+Na]+ (i.e. M + 23 u). An important feature of the positive mode ESI-MS spectra of isolated CLOSs from ANU843 and ANU297 was the occurrence of major additional ions at m/z 1075, 1091, 1117, 1119, 1320, 1322 (Fig. 3, C and D). These ions were assigned to sodium adducts of the major CLOS species found in both strains because they are not detected in the negative mode ESI-MS spectra of the same samples, and their masses are fully consistent with the [M+Na]+ pseudomolecular ions of major CLOS species, namely IV(C18:1) at m/z 1075, IV(C16:0,Ac) at m/z 1091, IV(C18:1,Ac) at m/z 1117, IV(C18:0,Ac) at m/z 1119, V(C18:1,Ac) at m/z 1320, and V(C18:0,Ac) at m/z 1322. These same sodium adducts occur in variable intensities in the positive mode FAB mass spectra of these same samples (figures not shown).

A second major contribution of negative mode ESI-MS analyses of CLOS was the detection of fragment ions between m/z 220–360, which could be assigned to various long chain fatty acylamino groups. Each of the fatty acids identified above by GC/MS was also found by negative mode ESI-MS (Fig. 4, A and B). In addition, the ESI-MS analyses in the low mass range revealed several other ions tentatively assigned to saturated, unsaturated, and hydroxylated fatty acids ranging in chain length from C14 to C22 (Fig. 4, A and B). Once again, no qualitative differences were found in these diverse families of definitively and tentatively identified CLOS fatty acids from ANU843 and ANU297 (Table I). The possibility that nodE-dependent fatty acids containing conjugated polysaturations might be present in ANU843 CLOS was further addressed by UV absorption spectroscopy, since three or four double bonds in conjunction with the fatty acid carboxyl group would have characteristic absorption maxima with very high molar extinction coefficients (ε, 50,000–100,000) in the region of 300–330 nm (29). A comparison of the UV absorption spectra of the CLOSs from ANU843 and ANU297 showed no differences in the region of 300–330 nm, indicating that they had the same degree and types of unsaturation (Fig. 5, A and B).

The remote possibility that polysaturated nodE-dependent CLOSs might selectively accumulate in the extracellular milieu rather than in cell membranes was explored by reverse phase HPLC of the hydrophobic components isolated from the culture supernatants. These analyses indicated that every 303-nm absorbing peak in the profile of the ANU843 supernatant extract was also found in the profile of the ANU297 supernatant (figures not shown). No 303-nm absorbing peaks eluted between 20–35 min, the region where CLOSs typically elute under these chromatographic conditions.

We also evaluated the possibility that unique nodE-dependent fatty acids might have been excluded from our protocol to purify membrane CLOSs. GC/MS analyses detected no differ-

| Molecular mass | Positive mode FAB-MS or ESI-MS (M+H)+ or [M+Na]+ | Negative mode ESI-MS (M-) | Assignmenta | Assignment or alternate assignmentb |
|---------------|-----------------------------------------------|--------------------------|-------------|-----------------------------------|
| 847           | 848                                           | 847                      | NDc         | NodRlt-III(C18:2)                 |
| 849           | 850                                           | 849                      | ND          | NodRlt-III(C18:1)                 |
| 853           | 854                                           | 853                      | ND          | NodRlt-III(3OH-C14:Ac)            |
| 889           | 890                                           | 890                      | ND          | NodRlt-III(C18:2,Ac)              |
| 891           | 892                                           | 891                      | NodRlt-III(C18:1,Ac) | NodRlt-III(C18:0,Ac)              |
| 893           | 894                                           | 893                      | ND          | NodRlt-III(C18:0,Ac)              |
| 933           | 934                                           | 933                      | ND          | NodRlt-III(C18:2,Ac)              |
| 1042          | 1043                                          | 1042                     | ND          | NodRlt-IV(3OH-C16:0)              |
| 1050          | 1051                                          | 1050                     | ND          | NodRlt-IV(C18:2)                  |
| 1052          | 1053                                          | 1052                     | ND          | NodRlt-IV(C18:1)                  |
| 1054          | 1055                                          | 1054                     | ND          | NodRlt-IV(C18:0)                  |
| 1066          | 1067                                          | 1067                     | ND          | NodRlt-IV(C16:1,Ac)               |
| 1068          | 1069                                          | 1068                     | ND          | NodRlt-IV(C16:0,Ac)               |
| 1074          | 1075                                          | 1074                     | ND          | NodRlt-IV(C18:1)Na                 |
| 1076          | 1077                                          | 1076                     | ND          | NodRlt-IV(C18:0)Nad               |
| 1090          | 1091                                          | 1090                     | ND          | NodRlt-IV(C18:3,Ac)               |
| 1092          | 1093                                          | 1092                     | ND          | NodRlt-IV(C18:2,Ac)               |
| 1094          | 1095                                          | 1094                     | NodRlt-IV(C18:1,Ac) | NodRlt-IV(C18:0,Ac)              |
| 1096          | 1097                                          | 1096                     | NodRlt-IV(C18:0,Ac) | NodRlt-IV(C18:0,Ac)              |
| 1111          | 1111                                          | 1110                     | ND          | NodRlt-IV(C16:0,2Ac)              |
| 1112          | 1113                                          | 1112                     | NodRlt-IV(OH-C18:0,Ac) | NodRlt-IV(3OH-C18:0,Ac)          |
| 1116          | 1117                                          | 1116                     | NodRlt-IV(C20:4,Ac) | NodRlt-IV(C21:4,Ac)Na             |
| 1118          | 1119                                          | 1118                     | NodRlt-IV(C20:3,Ac) | NodRlt-IV(C20:3,Ac)Na             |
| 1120          | 1121                                          | 1120                     | ND          | NodRlt-IV(C20:2,Ac)               |
| 1122          | 1123                                          | 1122                     | ND          | NodRlt-IV(C20:1,Ac)               |
| 1136          | 1137                                          | 1136                     | ND          | NodRlt-IV(C18:1,2Ac)              |
| 1255          | 1256                                          | 1255                     | ND          | NodRlt-IV(C18:1)                  |
| 1259          | 1259                                          | 1259                     | ND          | NodRlt-IV(C18:0,Ac)               |
| 1319          | 1320                                          | 1319                     | ND          | NodRlt-IV(C20:4,Ac)               |
| 1321          | 1322                                          | 1321                     | ND          | NodRlt-IV(C20:3,Ac)               |

a From Spánek et al. (12). Boldface entries were reported to be nodE-dependent (12).
b From Orgambide et al. (14) and this study.
c ND, not detected.
d Assignment revised from Orgambide et al. (14) based on the absence of a corresponding molecular ion in negative mode ESI-MS analyses.
Fig. 4. Negative mode ESI-MS analyses of the low mass region of CLOs from ANU843 (A), ANU297 (B), and ANU845 prTRF101 (C). See Table II for ion assignments.
sequences in total cellular fatty acids from ANU843 and ANU297 grown identically to express their nod genes (Fig. 6, A and B). Some of these cellular fatty acids are found in both CLOSs and phospholipids of ANU843 (C16:0, C16:1, C18:0, C18:1, C18:2, and C20:1), some in both CLOSs and lipopolysaccharide (3OH-C14:0, 3OH-C16:0, and 3OH-C18:0), and others in phospholipids (C14:0, C17:0, and C19:0) (14, 30, 31). Other minor peaks were not characterized since they were found in samples from both ANU843 and ANU297 (Fig. 6, insets).

Next, we tested the possibility that production of nodE-dependent CLOSs from R. trifolii might be increased to detectable levels by using recombinant strain ANU845 pRtRF101 containing a higher gene dosage of the entire 14-kb pSym nod region from ANU843 in its pSym-cured background. Positive mode ESI-MS analysis of membrane CLOSs from this strain (Fig. 3E) revealed the same spectrum of CLOS species represented by their pseudomolecular ions [M+H]+ and/or sodium adducts [M+Na]+ as found in ANU843 (Fig. 3C) and ANU297 (Fig. 3D) (see Table I for ion assignments). Most importantly, no new molecular species of CLOS were found in this recombinant strain. Negative mode ESI-MS of the CLOS sample from the recombinant strain also did not reveal any new species in the high mass range (figure not shown). Furthermore, a careful examination of the fatty acyl amino ions in the low mass region of this negative mode ESI-MS spectrum indicated that its diversity of CLOS-associated fatty acids was identical to both ANU843 and ANU297 (compare Fig. 4C to Fig. 4, A and B).

Finally, we investigated the chemical nature of the 303 nm-absorbing components present in membrane CLOS fractions from ANU843, ANU297, and ANU845 pRtRF101 using reverse-phase HPLC with photodiode array detection (Fig. 7). The proportion of these components in the various samples was insignificant (less than one ten-thousandth of a percent of the total mixture), since the peaks were nearly nonexistent despite extremely high molar extinction coefficients (ε, 50,000–100,000) for tri- and tetraunsaturated conjugated esters (29). Even if CLOS species containing such fatty acids constituted 1% of the total mixture, the peaks corresponding to them on the HPLC profile would be at least 1000-fold higher than the regular saturated and monounsaturated fatty acylated species. The converse was observed. The intensities of the 303-absorbing peaks were only ~1% of the other peaks monitored at 220 nm (Fig. 7), indicating that the levels were below the parts per million range. In order for this vanishing small quantity of components to be significant, one would need to be able to reproducibly isolate these molecules to better than 99.9999% purity. In any event, the level of these trace species did not increase in the CLOS fraction from the recombinant strain carrying multiple copies of the 14-kb pSym nod region. Furthermore, none of the UV absorption spectra of the 303 nm-absorbing species could be attributed to conjugated polyunsaturated fatty acids since they all lacked the splitting due to vibrational coupling that is characteristic of such molecules (compare Fig. 5C to Fig. 7, B–D).

**DISCUSSION**

nodE is the key determinant of host-specificity in the R. trifolii clover symbiosis (10, 11). A recent study on CLOSs from R. trifolii has concluded that host specificity of this clover-nodulating rhizobia is determined by the hydrophobicity of six unique CLOS species containing nodE-dependent polyunsaturated fatty acyl moieties (12). However, in that study, the structures of the six CLOS species were only based on FAB-MS data, and insufficient quantities of CLOSs were available for other structural analyses.

Our recent development of protocols to isolate large quantities of CLOSs based on their physiological accumulation within rhizobial membranes (14, 15) has enabled us to critically reexamine the proposed function of nodE in wild-type R. trifolii. We compared the diversity of CLOS-associated fatty acids and native CLOS structures made by wild-type ANU843, an isogenic nodE::Tn5 derivative (ANU297), and a recombinant strain harboring multiple copies of pRtRF101, which contains the entire 14-kb pSym nod region from ANU843 (ANU845 pRtRF101). This is the same nod-encoded plasmid as the one used in the other study on nodE function (12), but it is intro-

| Fatty acyl amino ion (R-CO-NH₂) | Assignment |
|----------------------------------|------------|
| 225 C14:0                         |            |
| 227 C14:0                         |            |
| 243 3OH-C14:0                     |            |
| 253 C16:0                         |            |
| 255 C16:0                         |            |
| 257 OH-C15:0                      |            |
| 265 C17:2                       | OH-C16:3 |
| 271 3OH-C16:0                     |            |
| 281 C18:1                         |            |
| 283 C18:0                         |            |
| 293 C19:2                       | OH-C18:3 |
| 297 3OH-C19:0                      |            |
| 299 3OH-C20:2                     |            |
| 309 C20:1                         |            |
| 311 C20:0                         |            |
| 325 C21:0                         |            |
| 337 C22:1                       | OH-C22:0 |
| 339 C22:0                       | OH-C22:0 |
| 355 OH-C22:0                      |            |

* Tentative assignments inferred only from the mass of the acyl amino ion. These ions could also be fragments of larger ions.

**Fig. 5. UV absorption spectra of CLOSs from ANU843 (A), ANU297 (B), and trans-parinaric acid (C18:4) (C).** Note that the splitting due to vibrational coupling (270–320 nm) characteristic for trans-conjugated polyunsaturated fatty acids is absent in the spectra of CLOSs from ANU843 and ANU297. The small, sharp spikes in B are artifacts due to electronic perturbations of the instrument.
duced into the pSym-cured background of ANU843 itself rather than of wild-type strain RBL5020. A sequence of chemical and spectroscopic analyses showed that the families of CLOSs from ANU843, ANU297, and ANU845 pRtRF101 grown identically had the same structural diversity. Therefore, the diversity of CLOSs in the ANU843 background was not influenced by loss of nodE function or increased nod gene dosage. The only consistent alteration in CLOSs in the nodE::Tn5 mutant was a 5-fold decrease in relative quantity of CLOSs as compared with the ANU843 parent. Further studies will be necessary to determine if this reduced production without change in diversity of CLOSs may contribute to the delayed and decreased nodulation phenotype of the nodE mutant on white clover, and/or its acquired compatibility and suppression of host defense responses with peas (10, 22).

Despite major efforts, we found no conclusive evidence to support the existence of the six tentative "nodE-dependent" CLOS species from R. trifolii that had been reported as mono-O-acetylated chitotetra- and pentasaccharides, which bore the unique N-acyl polyunsaturated fatty acids C18:3, C20:2, C20:3, and C20:4 (12). The structures of these fatty acids had been deduced from positive mode FAB-MS analyses that indicated ions at m/z 1091 [IV(C18:3,Ac)], 1117 [IV(C20:4,Ac)], 1119 [IV(C20:3,Ac)], 1121 [IV(C20:2,Ac)], 1320 [V(C20:4,Ac)], and 1322 [V(C20:3,Ac)]. Indeed, we did find prominent ions at m/z 1091, 1117, 1119, 1320, and 1322 in positive mode ESI-MS analyses, but these ions were found in samples of CLOSs not only from ANU843 but also from ANU297 and ANU845 pRtRF101. Thus, these prominent ions are neither nodE-dependent nor require increased nod gene dosage to be detected. We reassigned these prominent ions in positive mode mass spectrometry to sodium adducts (M + Na)⁺ of major CLOS species with common N-acyl substituted (C16:0, C18:0, C18:1) (Table I). This reassignment is based on the facts that: 1) no corresponding molecular ions were found in negative mode ESI mass spectra of the same samples, 2) none of the proposed polyunsaturated fatty acids were found in the low mass range of the negative mode ESI-MS spectrum of the CLOSs, the FID-GC and GC/MS analyses of the CLOS-associated or total cellular fatty acids, or the UV absorption spectroscopy analyses of the CLOSs, and 3) the masses of these ions were fully consistent with those of the above sodium adducts. We therefore conclude that five of the six ions previously assigned to nodE-dependent CLOSs are not due to CLOSs containing polyunsaturated fatty acids, but rather to sodium adducts of the predominant, nodE-independent CLOS species, which contain one or no fatty acyl unsaturation (Table I). Sodium adducts of CLOSs commonly occur in positive mode mass spectra (3, 32, 33).

This reassignment of MS data is also consistent with the collision-induced dissociation fragmentation pattern for some of these ions (12), since the sodium ion is retained on all of the fragments derived from neutral losses from the metallated molecular ion species. For example, the collision-induced dissociation spectrum of the ion at m/z 1117 shows fragmentations at m/z 896, 693, and 490 (see Fig. 4A in Ref. 12). This result can be interpreted as sequential ruptures of interglycosidic linkages resulting in elimination from the tetrasaccharide adduct [IV(C18:1,Ac)]Na⁺ of a reducing GlcNac terminus to generate the fragment at m/z 896 [(M + Na)⁺ – 221], followed by subse-
quent eliminations of the two adjacent GlcNAc residues from the latter ion to generate fragments at m/z 693 ([M + Na]^+ - 221 - 203) and 490 ([M + Na]^+ - 221 - 2×203), respectively. Such neutral losses from the sodiated species of glycolipids are common (34–36).

In support of the proposal that one of the nodE-dependent CLOS species was IV(C20:4,Ac), a shift in mass from m/z 1117 to 1125 upon catalytic hydrogenation of a CLOS fraction was reported (12). However, since a mass spectrum of this CLOS fraction prior to reduction was not presented, alternate CLOS assignments of this m/z 1125 ion could be to the pseudomolecular ion [M + H]^+ of IV(C20:0, Ac) or the hydrogenated product of IV(C20:1, Ac). Although the high mass region of our ESI-MS analyses did not reveal these alternate CLOS species, other analyses did indicate C20:0 and C20:1 fatty acids in ANU843 CLOSs (see Figs. 4 and 6 and Table II).

The sixth CLOS ion reported as nodE-dependent (m/z 1121, IV(C20:2,Ac), [12]) was not detected in the high mass region of our positive mode or negative mode mass spectral analyses of ANU843, ANU297, or ANU845 pRtRF101, and could not be

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Fig. 7. HPLC profiles of the CLOS fractions with photodiode array UV detection. A, ANU843 at 220 nm; B, ANU843 at 303 nm; C, ANU297 at 303 nm; and D, ANU845 pRtRF101 at 303 nm.
assigned to a sodium adduct of any CLOS species found. In addition, the proposed C20:2 fatty acid moiety was not detected in the low mass range of the negative mode ESI-MS analysis of CLOSs, nor in the FID-GC and GC/MS analyses of CLOSs, nor in the GC/MS analyses of total cellular fatty acids from these strains. Thus, this unusual fatty acid in CLOSs of R. trifolii could not be confirmed and remains to be validated by alternate unambiguous methods. Even if the sixth “node-dependent” CLOS species is confirmed, its production would be restricted to only certain genetic backgrounds of R. trifolii and/or in vitro growth conditions, and therefore is unlikely to dictate host specificity for this entire biovar in nature. Although our study highlights the absence nodeE-dependent CLOS species in R. trifolii, it should be noted that convincing evidence has been presented for the existence of nodeE-independent CLOS species in the alfalfa symbiont, R. meliloti (37).

Several lines of evidence show that membrane CLOSs of ANU843 contain the total diversity of CLOSs, rather than the possibility that some nodeE-dependent CLOS species might be selectively exported from cells into the extracellular environment. First, all CLOS ions, but the one corresponding to the tentative IV(C20:2,Ac) species found in 1-butanol extracts of membranes, were also found in our membrane extracts of ANU843. Second, our analysis of culture supernatants of ANU843 and ANU297 showed no evidence of nodeE-dependent CLOS species bearing polynsaturated fatty acids that might have been absent in the membrane CLOS fraction. Third, the “specific” modifications of the fatty acids and carbohydrate groups of supposedly excreted CLOSs are also common to the other membrane phospholipid and glycolipid components (15). This again underlines the viewpoint that “excreted” CLOSs likely arise from “blebbing off” of membrane vesicles as a normal feature of bacterial cell division, and have no special structural features that distinguish them from membrane-derived CLOS species.

In summary, our studies show that wild-type R. leguminosarum bv. trifolii can produce a full spectrum of diverse chitooligosaccharide species independent of nodeE function, indicating a need to revise the current model explaining how this important gene determines host specificity in the R. leguminosarum-legume symbiosis.

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