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A CORE OLIGOSACCHARIDE COMPONENT FROM THE LIPOPOLYSACCHARIDE OF *Rhizobium trifolii* ANU843

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ABSTRACT

The major oligosaccharide from the core region of the lipopolysaccharide from *R. trifolii* ANU843 was isolated and its structure determined. It is a trisaccharide consisting of two galacturonic acid residues and one 3-deoxy-D-*manno*-2-octulosonic acid (KDO) residue. The two galacturonic acid residues are terminally linked alpha to the C-4 and C-7 atoms of KDO. This structure was determined through use of ^{1}H- and ^{13}C-n.m.r. spectroscopy, f.a.b.-m.s., and g.l.c.–m.s. techniques. This oligosaccharide had not previously been reported to be present in the lipopolysaccharides from Gram-negative bacteria.

INTRODUCTION

Rhizobia are Gram-negative bacteria that have a nitrogen-fixing symbiotic relationship with legume plants. As Gram-negative bacteria they have the usual surface polysaccharides consisting of lipopolysaccharides (LPSs), and extracellular (EPSs) and capsular polysaccharides (CPSs). All of these molecules have been hypothesized to play a role in the symbiotic infection process[1] and therefore have been the subject of much study.

The LPSs have been the least studied of all of the *Rhizobium* polysaccharides. The LPSs from *R. leguminosarum* are reported to specifically inhibit the binding of these bacteria to the host pea-root[2]. Lectin from the host plant reportedly binds specifically to LPSs from the symbiont bacteria[3–5]. There are also reported changes in the LPS compositions from mutant rhizobia that are defective in nodulation[6–8]. Some of these changes are qualitative[6,7] whereas others are quantitative[8]. More recently it has been shown that for some symbiotic mutants of *R. phaseoli* and *R. japonicum* the O-antigen portion of the LPS may be missing[9–11]. In the case of the *R. phaseoli* mutant it was demonstrated that the defect in nodulation, aborted

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infection threads, is linked to the defect in the LPS[9,11]. In order to assess the role(s) of *Rhizobium* LPSs in symbiosis it is important to characterize these molecules structurally.

Initial studies of *Rhizobium* LPSs revealed that they can vary greatly in composition among different species, as well as among strains of a single species[12,13]. More recently, it has been determined that the core regions of LPSs from *R. phaseoli, R. leguminosarum,* and *R. trifolii* all have molecular weights of ~600–700 and are composed largely of galacturonic acid[14]. The O-antigens of *Rhizobium* LPSs are highly variable in composition, and are likely to consist of a complex oligosaccharide rather than a repeating oligosaccharide[14]. These O-antigens commonly contain methylated sugars and methylated amino sugars, and when a heptose is present it is found in the O-antigen and not in the core[14,15]. A more detailed study of *R. trifoli* LPSs has shown that both the O-antigen and the core oligosaccharides contain 3-deoxy-D-*manno*-2-octulosonic acid (KDO) at their reducing end, indicating that both are linked to the rest of the LPS molecule *via* KDO[15]. These results show that *Rhizobium* LPSs are quite different from those from *Salmonella* and *E. coli.* However the LPSs from the *Rhodospirillaceae* family may be more similar to those of *Rhizobium* species. They are reported to have short O-antigens which also contain methylated sugars[16]. Also, the O-antigen from *Rhodopseudomonas sphaeroides* is an oligosaccharide that contains glucuronic acid and has KDO at its reducing end[17].

We now describe the structure of the major core fraction from *R. trifolii* ANU843. This structure has not been observed in LPSs from other Gram-negative bacteria.

EXPERIMENTAL

General. — *Rhizobium triolii* ANU843 was obtained from Dr. Barry Rolfe of The Australian National University, Canberra City, Australia. The bacteria were grown as previously described[14,15]. The LPSs and their O-antigens and core oligosaccharides were isolated as previously described[14,15].

Further purification of the core oligosaccharides. — The core oligosaccharides were initially isolated by mild, acid hydrolysis of the LPSs, followed by gel-filtration chromatography[14,15]. Further purification was achieved by chromatography on a column of Sephadex G-25, using an ammonium formate buffer[18], pH 3.3, prepared by adjusting the pH of a 10% formic acid solution to 3.3 with ammonium hydroxide. The peaks respectively containing uronic acid and hexose were collected, lyophilized, and desalted in a G-10 column. These fractions still contained small amounts of salts, and were further purified by gel filtration using Bio-Gel P-2 with de-ionized water as the eluant.

Analytical techniques. — Uronic acids were identified by reduction[19] followed by preparation of the alditol acetates[20] and analysis by gas–liquid chromatography (g.l.c.) using a 15-m column of Supelco SP2330 fused silica. Reduction of the uronic

acids with borodeuteride followed by g.l.c.–mass spectrometry (m.s.) analysis of the alditol acetates[21] was also used to identify the uronic acids. Uronic acids and KDO were quantitated by colorimetric assays using *m*-hydroxybiphenyl and 2-thiobarbituric acid, respectively[22,23]. In the KDO assay, the hydrolysis conditions were modified as previously described[15], in that the acid concentration was increased to 2M and the time of hydrolysis to 1 h.

Glycosyl linkages were determined by a modification of a previously described procedure[24]. The oligosaccharide (1 mg) was prereduced with $NaBH_4$. The sample was permethylated using potassium dimethyl sulfoxide anion and CH_3I. The carboxyl groups of the permethylated prereduced, oligosaccharide were converted into methyl esters by using methanolic HCl. The methyl esters were reduced with $LiAlD_4$. The resulting oligosaccharide was hydrolyzed, the sugars acetylated and the acetates subjected to g.l.c.–m.s. at 70 eV in a column packed with 3% of OV225 on 80/100 Supelcoport.

Peracetylation of the G25-2-P2 oligosaccharide was performed under acidic conditions, using the mixed anhydride of acetic acid and trifluoroacetic acid. The sample was dissolved in acetic acid (100 μL) and 2:1 acetic acid–trifluoroacetic anhydride (200 μL). The reaction was allowed to proceed for 3 h at room temperature. The excess of the reagents was removed by evaporation under a stream of N_2. The residue was mixed with chloroform, and partitioned between chloroform and M HCl saturated with NaCl. The chloroform layer was dried (sodium sulfate) and evaporated to dryness.

N.m.r. spectra (^1H and ^{13}C) for solutions in D_2O were recorded with a Bruker WM250 spectrometer operated at 250 MHz for protons, and 62.8 MHz for carbon atoms. Proton spectra were recorded at 80°. All chemical shifts are referenced to Me_4Si. Fast-atom-bombardment mass spectrometry (f.a.b.-m.s.) using a 2-thioglycerol matrix was performed with a JEOL HX-110 HF spectrometer.

RESULTS AND DISCUSSION

Purification and composition of core oligosaccharides. — Gel-filtration, using G-25, of ANU843 core oligosaccharide, obtained by mild acid hydrolysis of the LPS and isolated by gel-filtration chromatography on Sephadex G-50, gave two peaks, G25-1 and G25-2. These peaks yielded 4 and 40 mg, respectively, from 400 mg of LPS. Calibration of the G-25 column with stachyose, maltotriose, lactose, and D-glucose indicated that the major fraction, G25-2, was eluted with a molecular weight of ~700 as previously reported[15]. After desalting, the major fraction (G25-2) was further purified through Bio-Gel P-2. One major peak, G25-2-P2, which contained two sugars, galacturonic acid and KDO, resulted. Fraction G25-2-P2 was further analyzed by methylation, n.m.r.-spectral and f.a.b.-m.s. techniques as already described and later discussed.

Structural analysis of the core oligosaccharide (G25-2-P2) from R. trifolii *ANU843.* — Gas-liquid chromatography–m.s. analysis of the alditol acetates

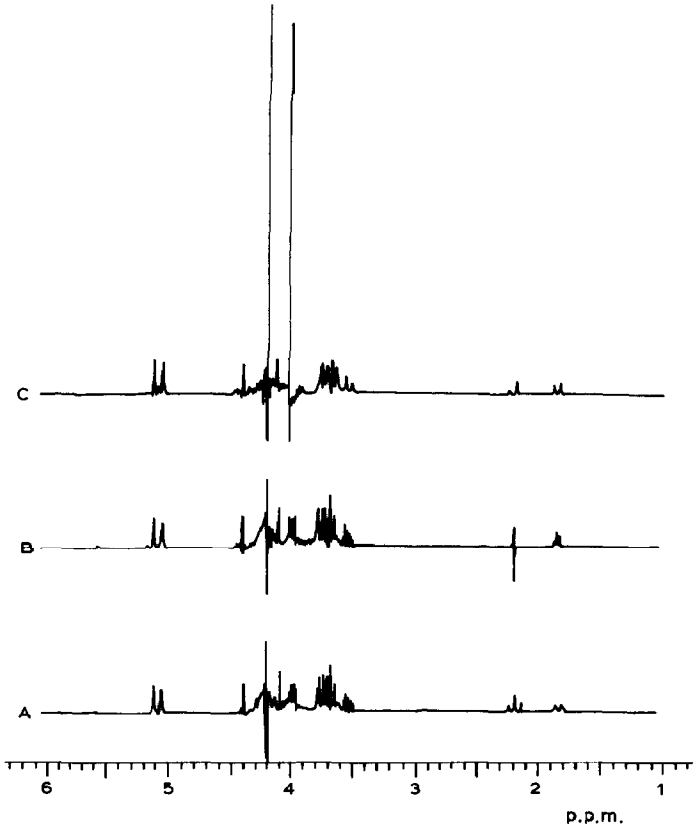

Fig. 1. (A) 250-MHz, ^{1}H-n.m.r. spectrum of the G25-2-P2 oligosaccharide. (B) Spectrum after irradiation of the triplet at δ 2.17. [Note the collapse of the doublet of doublets at δ 1.82 and the perturbation of the signals at δ 4.05.] (C) The spectrum after irradiating at δ 4.05. [Note the collapse of the triplet at δ 2.17 and the doublet of doublets at δ 1.82.]

derived from the purified core oligosaccharide (G25-2-P2) after deuterium labeling of the carboxyl carbon atoms indicated that galacturonic acid is the sole hexose component. The ^{1}H-n.m.r. spectrum of G25-2-P2 in deuterium oxide (see Fig. 1A) contains two signals which are attributable to α-anomeric protons on a pyranose ring. The first signal is a doublet (J 3.8 Hz) at δ 5.12. The second signal, also a doublet having the same splitting, appears at δ 5.04. In the ^{1}H-n.m.r. spectrum, there are no other signals which can be assigned to an anomeric proton.

The ^{13}C-n.m.r. spectrum (see Fig. 2) contains three signals that can be attributed to anomeric carbon atoms. These signals appear at δ 101.3, 100.3, and 97.1. A signal at δ 34.7 can be attributed to a methylene carbon atom in a ring. These resonances, along with the positive 2-thiobarbituric acid reaction, suggested that the third anomeric resonance in the ^{13}C-n.m.r. spectrum is due to C-2 of KDO. Thus, a composition of only galacturonic acid and KDO, and the presence of two

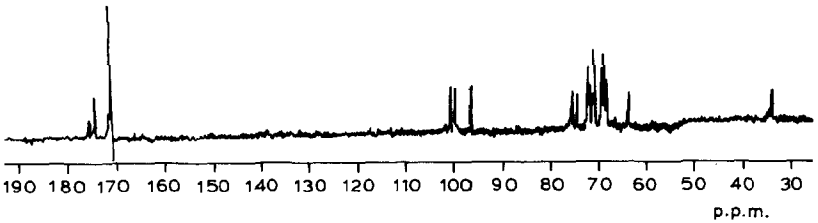

Fig. 2. 62.8-MHz, ^{13}C-n.m.r. spectrum of the G25-2-P2 oligosaccharide. [The intense peak at δ 171.4 is due to free formate. Note that only three signals appear in the anomeric-carbon region. Also note the signal at δ 34.7 which is due to C-3 of KDO.]

anomeric protons and three anomeric carbon atoms, one of which is due to a KDO residue, show that G25-2-P2 consists of one KDO and two galacturonic acid residues. The composition of G25-2-P2 was confirmed by f.a.b.-m.s. of the underivatized compound (see Fig. 3). The peak at *m/z* 613 can be assigned to the protonated, monosodium salt of G25-2-P2, and the ion at 635 to the disodium salt. Some scans (see Fig. 3, inset) contain another ion at *m/z* 657 which is due to the trisodium salt.

In an earlier report[15], it was shown that one of the D-glucuronic acid units of this oligosaccharide must be linked to O-4 or O-5, or both, of KDO, because the 2-thiobarbituric acid test was negative unless the trisaccharide was first hydrolyzed in relatively strong acid, *i.e.*, 2M. This 2-thiobarbituric acid test result can be obtained only if O-4, O-5, or both, are blocked, and therefore do not allow periodate rupture of the C-4 to C-5 bond of KDO[25]. The linkages of the sugar units in G25-2-P2 can be deduced from the n.m.r. spectra, f.a.b.-m.s., and methylation analysis.

The f.a.b.-m.s. spectrum (see Fig. 3) does not contain any fragments that could be due to a galacturonic acid disaccharide. Thus, both D-galacturonic acid

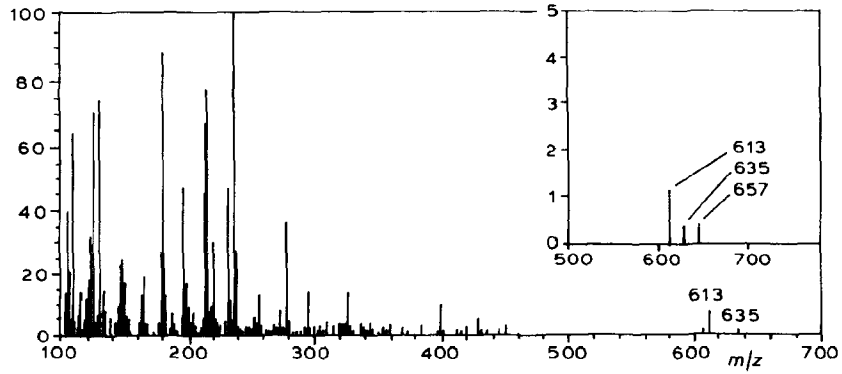

Fig. 3. F.a.b.-mass spectrum of the G25-2-P2 oligosaccharide. [The ion at *m/z* 613 is due to the protonated monosodium salt of the trisaccharide. The inset shows a single scan in which the qausimolecular ion of the di- and tri-sodium salts respectively appear at *m/z* 635 and 657.]

units must be substituted directly on the KDO ring. Methylation analysis confirmed the terminal location of the D-galactosyluronic acid groups. Gas-liquid chromatography of the methylation products of G25-2-P2 results in two peaks with relative areas of 1:2. The major product has fragments of *m/z* 235, 191, 162, and 118. These fragments are derived from a 2,3,4-tri-*O*-methylalditol acetate derivative of a hexose having two deuterium atoms on C-6 and one deuterium atom on C-1. This product arises from the terminally linked, D-galactosyluronic acid groups of G25-2-P2. The remaining methylation product, presumably due to KDO, because it is the only other component from G25-2-P2 (see the foregoing discussion), has fragments with *m/z* 45, 161, and 205. These fragments are consistent with a KDO residue linked at O-7, not at O-5 or O-8. Therefore, the data suggest that the remaining D-galactosyluronic acid group is joined to O-4 of KDO. The data for this KDO derivative were not completely satisfactory, because characteristic fragments from the reducing end of the molecule (*e.g.*, *m/z* 249) were not present. It is possible that the KDO residue, was incompletely methylated due to steric hindrance. Several variations of the methylation procedure were attempted to resolve this problem, but all our attempts gave similar results.

Both ^{13}C- and ^{1}H-n.m.r.-spectral analysis supported the conclusion that KDO is substituted at O-4 and O-7. Fig. 1 shows that the protons of the methylene group of the KDO appear in the proton spectrum as a doublet of doublets (J 12.5 and 2.9 Hz) at δ 1.82 and a triplet (J 12.5 Hz) at δ 2.17. The triplet can be assigned to the axial proton (H-3*a*) which should have a large splitting from the neighboring *trans*-vicinal proton and another large splitting from the other geminal proton. The equatorial proton (H-3*e*) should have a comparatively small vicinal coupling, as it is *cis* to the proton on C-4. The connectivities between the triplet at δ 2.17 and the doublet of doublets at δ 1.82 were confirmed by spin-decoupling. Irradiation of the triplet caused collapse of the doublet of doublets, and perturbation of the signal at δ 4.05 (see Fig. 1B). This defines the position of the H-4 resonance of KDO at δ 4.05. Irradiating the signals at δ 4.05 (see Fig. 1C) caused collapse of the triplet to a strongly coupled doublet, and removal of the smaller splitting on the doublet of doublets at δ 1.82. The chemical shifts of H-3*a* and H-3*e*, namely, δ 2.17 and 1.82 respectively, are reversed from their normal positions for KDO[25,26]. Normally, the methylene H-3*e* and H-3*a* for β-KDO have resonances of δ ~2.4 and ~1.8, respectively[25,26]. In the case of α-KDO, the chemical shift for the H-3*e* atom decreases to[25,26] ~2.0. Thus, the resonance for H-3*e* is normally downfield from that of H-3*a* by ~0.2 to 0.6 p.p.m. In our case, H-3*e* is upfield from H-3*a* by 0.35 p.p.m. A similar result has been reported[27] for an α-L-Rha-(1→5)-KDO disaccharide isolated from plant cell-walls, in the spectrum of which the H-3*e* signal is upfield from H-3*a* by ~0.30 p.p.m. It has been reported[25] that the methyl glycoside of the ammonium salt of KDO has the expected H-3*e* and H-3*a* resonances, whereas the ammonium salt of KDO (with a free reducing end) has the H-3*e* resonance upfield from H-3*a* by 0.10 p.p.m. Thus, a portion of the unusually small chemical shift of H-3*e* of G25-2-P2 can be accounted for by the fact that KDO is at

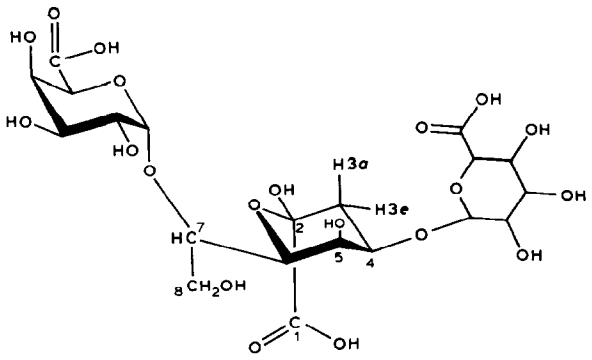

Fig. 4. The structure of the G25-2-P2 oligosaccharide, showing the carboxyl group of the α-D-galactosyluronic acid group on O-4 of KDO in close proximity to the axial H-3 atom of KDO.

the reducing end of this molecule. This is also probably true for the plant cell-wall disaccharide already mentioned. However, the remaining upfield shift of this proton must be caused by the substituent groups attached to the KDO residue. This unusually small chemical shift of H-3*e* indicates that this proton is in the shielding zone of a carbonyl group, because all other possible anisotropic effects should lead to a downfield shift. This shielding effect could be due to the carboxyl group of a uronic acid group substituted on O-4 of KDO. Examination of molecular models showed that the interactions between an α-linked D-galactosyluronic acid group on O-4 of KDO and the KDO ring are much less when the planes of the two rings are at right angles to each other. In this orientation, the carbonyl group of the D-galacturonic acid group is close to H-3*a* of KDO (see Fig. 4). The ^{1}H-n.m.r. spectrum also indicated that KDO is not substituted at O-5, as substitution at O-5 would be expected to destabilize the pyranose ring because of the severe 1,3 interaction with the axial proton on C-3. This would result in flattening of the KDO pyranose ring and a concomitant decrease in the *trans*-diaxial couplings from the maximum of ~12 Hz. It would also result in an increase in the *cis* couplings. The values observed for these splittings indicated that there is no ring distortion. Thus, it is probable that one of the galacturonic acid is linked to O-4 and not to O-5 of KDO. The ^{13}C-n.m.r. spectrum (Fig. 3) indicates that the remaining galacturonic acid residue is not linked to O-8 and therefore must be linked to O-7. At δ 63.8 is observed a carbon resonance which is assigned[28] to C-8 of KDO. The absence of any appreciable downfield shift in the position of the C-8 resonance from δ 63.8 to 68–72 indicates that there is no substituent on that carbon atom. Thus, the ^{13}C- and ^{1}H-n.m.r. spectra indicate that the D-galactosyluronic acid groups are not linked at either O-8 or O-5 of KDO, and therefore must be linked at O-4 and O-7.

In order to confirm that KDO is not linked at O-8 or O-5 (and therefore must be linked at O-4 and O-7), the G25-2-P2 oligosaccharide was peracetylated, and the acetate analyzed by ^{1}H-n.m.r. spectroscopy. The resonances of protons

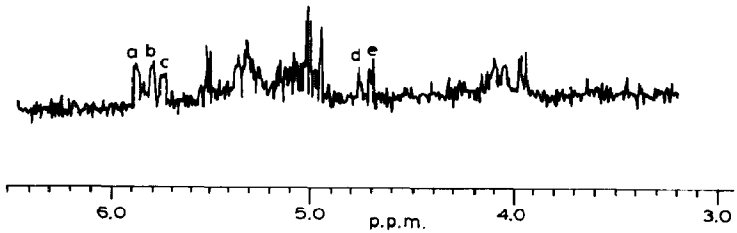

Fig. 5. Partial ^{1}H-n.m.r. spectrum of the peracetate of the oligosaccharide. [Resonances A, B, and C are due to H-4 of galacturonic acid and to H-5 of KDO. These three protons have an identical orientation relative to their two neighboring protons and display very small couplings, often <2 Hz, with these neighbors. Resonances D and E are due to H-5 atoms of the two D-galactosyluronic acid groups. In addition, the absence of signals between δ 3.0 and 4.0 indicates that the primary hydroxyl group of KDO is esterified.]

attached to carbon atoms bearing hydroxyl groups undergo a substantial, downfield shift on conversion of the hydroxyl group into an acetoxyl group, and can often be assigned by chemical shifts and coupling constants. The ^{1}H-n.m.r. spectrum of the peracetate (see Fig. 5) contains between δ 5.7 and 5.9 three resonances which are readily assignable to the protons on C-4 of the two D-galactosyluronic acid groups and on C-5 of the KDO residue. This assignment is possible because, in the entire molecule, these are the only three protons having two *cis*-neighbors, and hence they display very small spectral splitting. They are also the only three such equatorial protons, and therefore have the largest downfield resonance positions. Thus, this experiment showed that O-5 of KDO is not involved in a glycosyl linkage, as it was free for acetylation. The primary hydroxyl group of KDO, *i.e.*, HO-8, was also acylated, as evidence by the lack of resonances between δ 3.0 and 4.0. This shows that C-8 of KDO is not involved in a linkage. Thus, these data confirmed that KDO is substituted at O-4 and O-7, as was inferred from the ^{13}C- and ^{1}H-n.m.r. spectra already described.

Summary. — Herein, we have presented data which show that the major oligosaccharide component of the core from *R. trifolii* ANU843 LPS has the structure shown in Fig. 4, namely, 3-deoxy-4,7-di-*O*-(α-D-galactopyranosyluronic acid)-β-D-*manno*-2-octulopyranosic acid. This structure has not been reported to be present in the LPS from other Gram-negative bacteria. However, previous work had shown that core oligosaccharides from an *R. leguminosarum* LPS and from *R. phaseoli* LPS have compositions which are almost identical[14] to the one given herein.

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REFERENCES

1 R. W. CARLSON, in W. J. BROUGHTON (Ed.), *Nitrogen Fixation*, Vol. 2, *Rhizobium*, Clarendon Press, Oxford, 1982, pp. 199–234.
2 G. KATO, Y. MARUYAMA, AND M. NAKAMURA, *Agric. Biol. Chem.*, 44 (1980) 2843–2855.
3 G. KATO, Y. MARUYAMA, AND M. NAKAMURA, *Agric. Biol. Chem.*, 43 (1979) 1085–1092.
4 W. KAMBURGER, *Arch. Microbiol.*, 12 (1979) 83–90.
5 E. M. HRABAK, M. R. URBANO, AND F. B. DAZZO, *J. Bacteriol.*, 148 (1981) 691–711.
6 R. RUSSA, T. URBANIK, E. KOWALCZUK, AND Z. LORKIEWICZ, *FEMS Lett.*, 13 (1982) 161–165.
7 R. RUSSA, T. URBANIK, W. ZURKOWSKI, AND Z. LORKIEWICZ, *FEMS Lett.*, 13 (1981) 81–85.
8 R. W. CARLSON AND M. YADAV, *Appl. Environ. Microbiol.*, 50 (1985) 1219–1224.
9 K. D. NOEL, K. A. VANDEN BOSCH, AND B. KULPACA, *J. Bacteriol.*, in press.
10 G. STACEY, V. PUVANESARAJAH, F. M. SCHELL, AND D. GERHOLD, *J. Bacteriol.*, 169 (1988) 137–141.
11 R. CARLSON, S. KALEMBASA, D. TUROWSKI, P. PACHORI, AND K. D. NOEL, *J. Bacteriol.*, 169 (1988) 4923–4928.
12 R. W. CARLSON, R. E. SANDERS, C. NAPOLI, AND P. ALBERSHEIM, *Plant Physiol.*, 62 (1978) 912–917.
13 L. P. T. M. ZEVENHUIZEN, I. SCHOLTEN-KOERSELMAN, AND M. A. POSTHUMUS, *Arch. Microbiol.*, 125 (1980) 1–8.
14 R. W. CARLSON, *J. Bacteriol.*, 158 (1984) 1012–1017.
15 R. W. CARLSON, R. SHATTERS, J. DUH, E. TURNBULL, B. HANLEY, B. G. ROLFE, AND M. A. DJORDJEVIC, *Plant Physiol.*, 84 (1986) 421–427.
16 H. MAYER AND J. WECKESSER, in E. T. RIETSCHEL (Ed.), *Chemistry of Endotoxin*, Elsevier, Amsterdam, 1984, pp. 221–247.
17 P. V. SALIMATH, R. N. THARANATHAN, J. WECKESSER, AND H. MAYER, *Eur. J. Biochem.*, 144 (1984) 227–234.
18 S. P. DJORDJEVIC, M. BATLEY, AND J. W. REDMOND, *J. Chromatogr.*, 354 (1986) 507–510.
19 W. F. DUDMAN, L.-E. FRANZEN, J. E. DARVILL, M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 117 (1983) 141–156.
20 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345.
21 R. HOLLINGSWORTH, M. ABE, J. SHERWOOD, AND F. B. DAZZO, *J. Bacteriol.*, 160 (1984) 510–516.
22 N. J. BLUMENKRANTZ AND B. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
23 A. WIESSBACH AND J. HURWITZ, *J. Biol. Chem.*, 234 (1958) 705–709.
24 P. PREHM, S. STIRM, B. JANN, AND K. JANN, *Eur. J. Biochem.*, 56 (1975) 41–55.
25 F. M. UNGER, *Adv. Carbohydr. Chem. Biochem.*, 38 (1981) 323–388.
26 K. JANN, in L. ANDERSON AND F. M. UNGER (Eds.), *Bacterial Lipopolysaccharides*, American Chemical Society, Washington, D.C., 1983, pp. 171–191.
27 W. S. YORK, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 138 (1985) 109–126.
28 K. BOCK AND C. PEDERSEN, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–66.