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# LOCATION AND IDENTITY OF THE ACYL SUBSTITUENTS ON THE EXTRACELLULAR POLYSACCHARIDES OF *Rhizobium trifolii* AND *Rhizobium leguminosarum**

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ABSTRACT

The basic structures of the extracellular polysaccharides of *Rhizobium leguminosarum* and *Rhizobium trifolii* were found to be identical, but their acylation patterns differ. Liquid hydrogen fluoride at −40° degrades the two polysaccharides to a series of oligosaccharides representing the repeating units of the polysaccharides and their higher homologs. At −23°, it degrades the polymers to a mixture of oligosaccharides from which a tetrasaccharide constituting a unit of the backbone of the polysaccharide, and a trisaccharide representing all but the non-reducing terminus of the side chain, could be readily purified. The location and identity of the acyl substituents were determined by $^1$H-n.m.r. spectroscopy, methylation analysis, and f.a.b. mass spectrometry. The unusual substituent D-3-hydroxybutanoate was found esterified to O-3 of a terminal 4,6-*O*-pyruvic acetalated D-galactose in both strains of *R. leguminosarum*, and in one of the three strains of *R. trifolii* tested. All of the strains tested contained a 3-*O*-acetyl substituent on the (1→4)-β-D-glucopyranosyl residues in the backbone of the polysaccharide. Only the *R. leguminosarum* polysaccharides contained a combination of 2- and 3-*O*-acetyl groups on the branching sugar of the backbone of the polymer.

## INTRODUCTION

Various authors have suggested that the extracellular polysaccharides of Rhizobia may play a role in the specificity of the infection of legumes by Rhizobia[1]. However, the finding that the extracellular polysaccharides of *R. leguminosarum* and *R. trifolii* are seemingly identical[2] cast doubt on this hypothesis. However, the identity and specific locations of the acyl substituents shown[2] to be present were not determined, leaving the possibility that the acyl-substitution patterns could differ.

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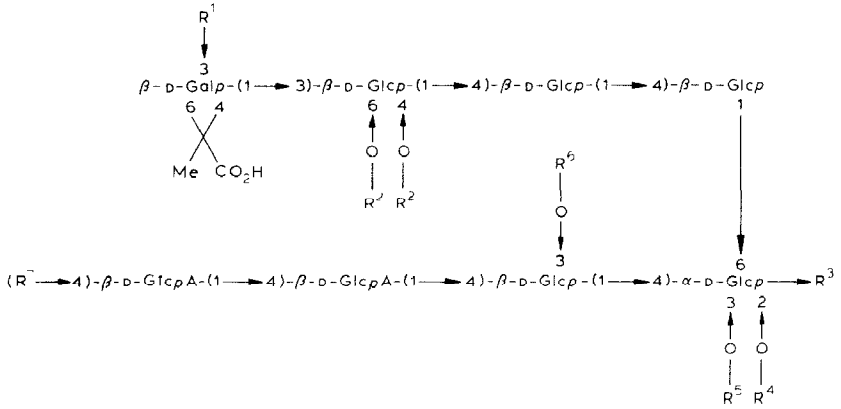

| Saccharide | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | $R^6$ | $R^7$ |
|---|---|---|---|---|---|---|---|
| Intact polysaccharide *R. leguminosarum* 128c 53 | OH or 3-hydroxybutanoate | pyruvic acetal | more repeats | H or Ac | H or Ac | Ac | more repeats |
| ≧4 Repeating units[a] oligosaccharides *R. leguminosarum* 128c 53 | OH or 3-hydroxybutanoate | H or ? | more repeats | H or Ac | H or Ac | Ac | more repeats |
| ≧4 Repeating units[a] deacylated oligosaccharides *R. leguminosarum* 128c 53 | OH | H or ? | more repeats | H | H | H | more repeats |
| Repeating unit[a] *R. leguminosarum* 128c 53 and 128c 63 | OH or 3-hydroxybutanoate | H or ? | F | H or Ac | H or Ac | Ac | OH |
| Repeating unit[a] *R. trifolii* TA1 | OH or 3-hydroxybutanoate | H or ? | F | H | H | Ac | OH |
| Repeating unit[a] *R. trifolii* NA30 and 0403 | OH | H or ? | F | H | H | Ac | OH |

[a]Generated with HF at −40°.

Fig. 1 Structure of the polysaccharides and oligosaccharides described

Acyl substituents are sometimes immunodominant[3], and could thus be very important in recognition processes.

From work with other acylated polysaccharides[4–6], we expected that solvolysis of the bacterial polysaccharides with liquid HF would produce acylated oligosaccharides useful in locating and identifying the acyl substituents.

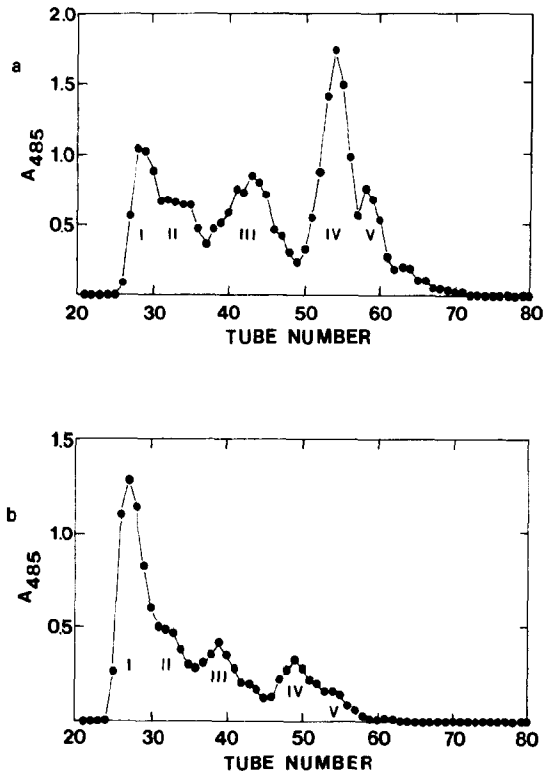


Fig. 2. Chromatography, on Bio-Gel P6, of the oligosaccharides produced from (a) *R. trifolii* NA30 and (b) *R. leguminosarum* 128c 53 by treatment with liquid HF for 15 min at −40°.

## RESULTS AND DISCUSSION

From the basic structure of the polysaccharides (see Fig. 1), and the behavior of various polysaccharides in HF, we considered that, at −40°, only a single cleavage should occur in the polysaccharide, at the α-D-glucosyl linkage, yielding the octasaccharide repeating units with all of the acyl substituents attached, but could not predict the behavior of the pyruvic acetal group.

Solvolysis of the *R. trifolii* and *R. leguminosarum* polysaccharides with HF for 15 min at −40° degraded them to a series of fragments that were eluted from a column of Bio-Gel P-6 as four major fractions. Fig. 2 shows the elution pattern for the polysaccharides of *R. trifolii* NA30 and *R. leguminosarum* 128c 53. The elution pattern of fragments obtained was always consistent with the cleavage of the polysaccharides at the α-D-glucosyl linkage, giving rise to a series containing different numbers of the repeating octasaccharide unit. Table I shows the relative amounts of material contained in the four major peaks for the five bacterial isolates tested. For the *R. trifolii* strains and *R. leguminosarum* 128c 63, the fraction corresponding to a single repeating unit preponderated, whereas, for *R. leguminosarum* strain

TABLE I

RELATIVE PERCENTAGE OF MATERIAL IN EACH OF THE FOUR MAJOR PEAKS IN FIG. 2, FOR THE FIVE BACTERIAL ISOLATES TESTED

| *Bacterial isolate* | *I* | *II* | *III* | *IV* |
|---|---|---|---|---|
| *R. leguminosarum* 128c 53 | 49.5 | 17.6 | 16.7 | 16.2 |
| *R. leguminosarum* 128c 63 | 17.5 | 13.7 | 25.5 | 43.3 |
| *R. trifolii* NA30 | 16.0 | 16.3 | 31.9 | 35.8 |
| *R. trifolii* 0403 | 10.9 | 10.8 | 32.8 | 45.5 |
| *R. trifolii* TA1 | 25.5 | 13.5 | 34.2 | 26.8 |

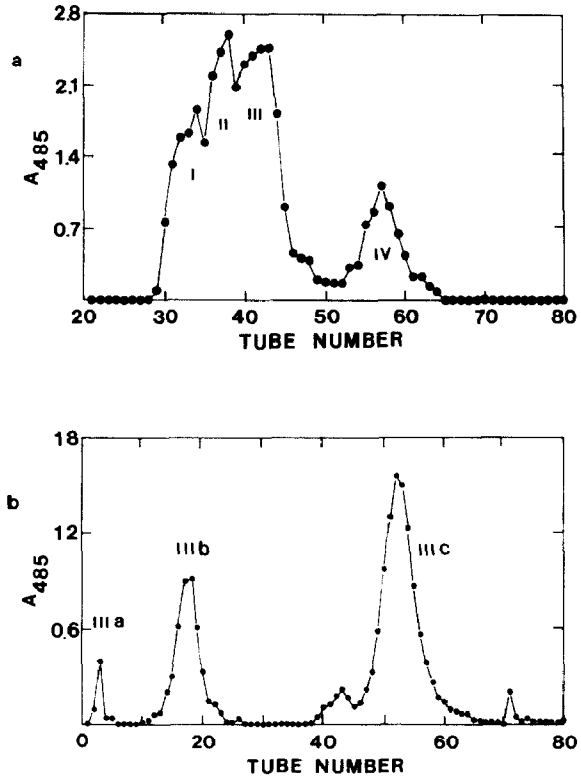


Fig. 3. (a) Chromatography, on Bio-Gel P2, of the oligosaccharides produced from *R. leguminosarum* 128c 53 by treatment with liquid HF for 15 min at −23° (b) Chromatography of fraction III [Fig. 3(a)] on a column of QAE Sephadex anion-exchanger, using a linear gradient of 0.05 to 0.50M imidazole buffer, pH 7.

128c 53, multimers of the repeating unit were abundant. *R. leguminosarum* 128c 53 seemed to have a structural feature hindering cleavage at the α-D-glucosyl linkage. Longer treatment (1 h) produced a greater proportion of the single repeating unit, but increased undesired cleavage at the terminal D-galactosyl group.

More severe treatment with HF, for 15 min at −23°, degraded all of the

TABLE II

ASSIGNMENTS FOR $^1$H-N M R SPECTRA IN FIG 4

| *Spectrum No.* | *Signal No.* | *P.p.m.* | *Spacing in Hz (F,H) or (H,H)* | *Relative integration (No. of H)*[a] | *Proton* | *Assignment* |
|---|---|---|---|---|---|---|
| 4a | 1c, 1f | 5.70 | 52.8 (F,1); 3.6(1,2) | 0.6 | H-1 | α-4,6-Glc(F) |
| | 3 | 5.21 | 3.6(1,2) | 0.15 | H-1 | α-4,6-Glc |
| | 5 | 5.03 | 8.9(2,3); 8.9(3,4) | 0.9 | H-3 | β-4Glc-3Ac |
| | 9 | 4.71 | doublet, doublet | 1.7 | H-1 | unidentified |
| | 10 | 4.5 | 8.9(1,2) | 5.5 | H-1 | β-anomeric pyranoside |
| | 13 | 2.10 | singlet | 2.7 | OAc | β-4Glc-3Ac |
| | 14 | 1.55 | singlet | 3.0 | $CH_3$ of pyruvic acetal | β-4,6-Gal and β-4,6-Glc |
| 4b | 1a,1d | 5.79 | 53.6 (F,1); 3.6(1,2) | 0.3 | H-1 | α-4,6-Glc(F)-2Ac |
| | 1b,1e | 5.73 | 51.4 (F,1); 3.6(1,3) | 0.2 | H-1 | α-4,6-Glc(F)-3Ac |
| | 4 | 5.21 | 10(1,2); 10(2,3) | 0.2 | H-3 | α-4,6-Glc-3Ac |
| | 6 | 4.96 | 9.3(2,3); 3.6(3,4) | 0.3 | H-3 | β-4,6-Gal-3 (3-H.B.)[b] |
| | 7 | 4.86 | d,d,d (F,2)(1,2)(2,3) | 0.7 | H-2 | α-4,6-Glc (F) |
| | 8 | 4.8 | | | H-1 | β-pyranose |
| | 11 | 2.67 | 6.4 (doublet) | 0.9 | $CH_2$ | 3-H.B.[b] on β-4,6-Gal-3 (3-H.B.)[b] |
| | 12 | 2.20 | singlet | 1.8 | OAc | |
| | | 2.21 | singlet | 0.4 | OAc | |
| | | 2.22 | singlet | 0.4 | OAc | |
| | 13 | 2.11 | singlet | 1.4 | OAc | |
| | | 2.10 | singlet | 1.4 | OAc | |
| | 15 | 1.27 | 7.1 (doublet) | 1.4 | $CH_3$ | 3-H.B.[b] on β-4,6-Gal-3 (3-H.B.)[b] |
| 4c | 2a | 5.52 | 3.6(1,2) | 0.3 | H-1 | α-4,6-Glc-2Ac |
| | 2b | 5.45 | 3.6(1,2) | 0.2 | H-1 | α-4,6-Glc-3Ac |
| | 2c | 5.39 | 3.6(1,2) | 0.1 | H-1 | α-4,6-Glc |

[a]Number of protons is integrated based on Me of pyruvic acetal, which is assumed to be 3.0. The integration is not exact, because of the complexity of the spectrum. As there are at least three components present in the spectrum, unit quantities were not expected. [b]3-H.B. is 3-hydroxybutanoate.

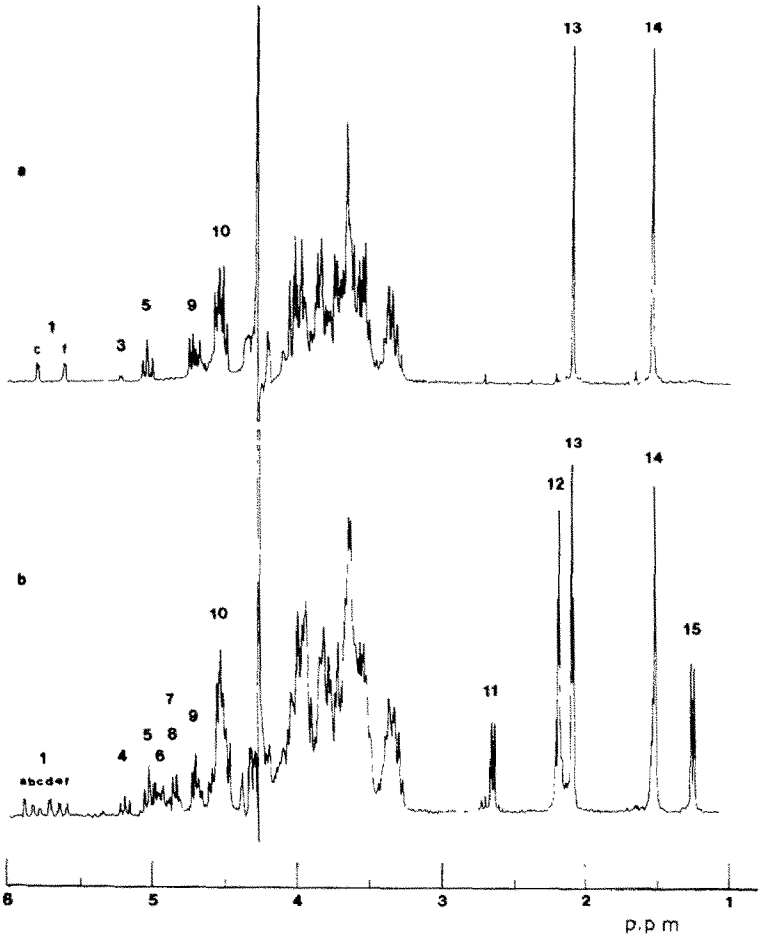


Fig. 4a,b. $^1$H-N.m.r. spectra of (a) repeating unit of *R. trifolii* NA30, and (b) repeating unit of *R. leguminosarum* 128c 53.

polysaccharides tested into smaller fragments that could be purified by Bio-Gel P-2 and anion-exchange chromatography. Fig. 3a shows the elution profile of the *R. leguminosarum* 128c 53 polysaccharide on Bio-Gel P-2 after HF treatment at −23°. Peak IV was shown, by its composition, methylation analysis, and $^1$H-n.m.r. spectrum, to be cellotriose, arising from the side chain. Peak III was further purified by chromatography on QAE Sephadex (see Fig. 3b). Peak IIIb from the QAE Sephadex chromatography was a tetrasaccharide from the side chain of the polysaccharide, and (the more tightly bound) peak IIIc was the tetrasaccharide unit from the backbone of the polysaccharide (see later). Peaks I and II from the P-2 column were not further fractionated.

*N.m.r. spectroscopy.* — As would be expected, the $^1$H-n.m.r. spectra of the intact polysaccharides were not well resolved, and could not be used for detailed structural analysis. In contrast, the fragments gave relatively well resolved spectra that provided useful structural information.

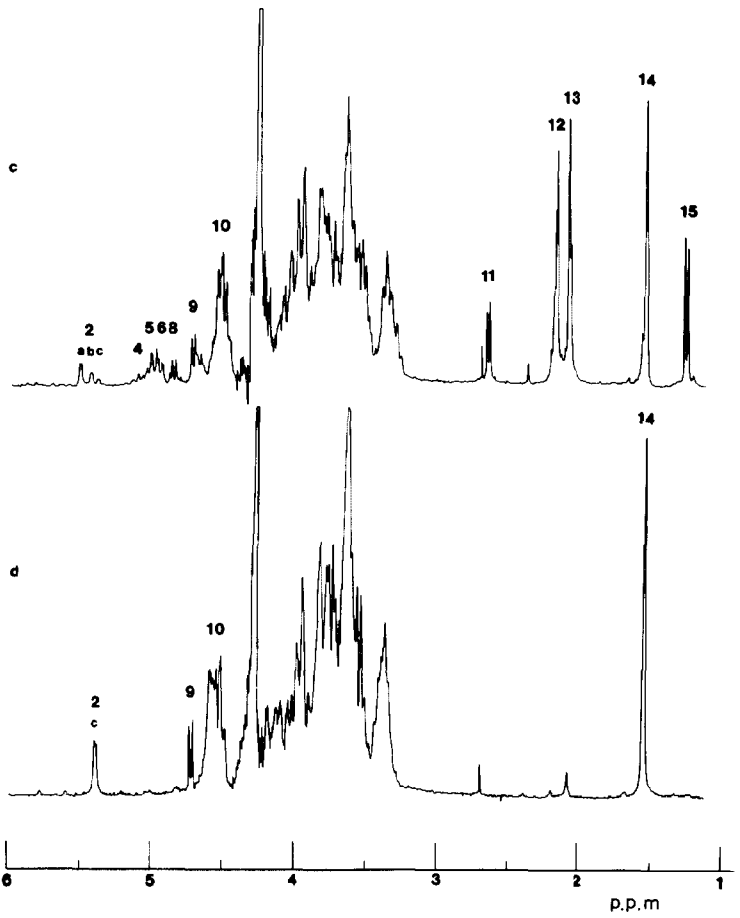


Fig. 4c,d. $^1$H-N.m.r. spectra of (c) multiple repeating units of *R. leguminosarum* 128c 53, and (d) the sample in 4(c) deacylated treatment with 10 mM KOH.

Fig. 4 shows the $^1$H-n.m.r. spectra (of the fragments) that yielded the most information; the assignments are summarized in Table II. Fig. 4a is for *R. trifolii* NA30 polysaccharide following treatment with HF at −40° and fractionation on a Bio-Gel P-6 column; the sample was drawn from the fourth peak. The spectrum corresponds well with that reported by Jansson *et al.*[7] for the intact polysaccharide of *R. trifolii* U226, but lacks a signal at 5.38 p.p.m. and has three additional signals, at 5.70, 5.03, and 4.7 p.p.m. Signals 13 and 14 (at 2.1 and 1.55 p.p.m.) respectively correspond to acetate and pyruvic acetal. The complex signal at ~4.5 p.p.m. corresponds to the anomeric protons of the β-linked residues. Jansson *et al.*[7] ascribed a signal at 5.38 p.p.m. to H-1 of an α-linked D-glucosyl residue. The disappearance of this signal, and the appearance of a doublet of doublets centered at 5.70 p.p.m. is consistent with conversion of the α-linked sugar into α-D-glucosyl fluoride[8] ($J_{1,2}$ 3.4, $J_{1,F}$ 51.8 Hz). The generation of oligosaccharide fluorides under similar condi-

tions of HF solvolysis had been reported[5]. The small signal (No. 3) at 5.21 p.p.m. is probably that of H-1 in a free, reducing α-D-glucose. Peaks centered at 5.03 p.p.m. correspond with H-3 of 3-*O*-acetylated β-(1→4)-linked D-glucose[9]. This assignment is confirmed by the mass-spectral and methylation data presented. The signals at ~4.7 p.p.m. appear to be those for anomeric protons; they were not resolved in the spectrum of the intact polysaccharide.

Integration of the signals indicated one pyruvic acetal and one acetate per repeating unit. Thus, one of the pyruvic acetal groups must have been removed by the HF treatment. Because of the presence of only one acetate per repeating unit, the location of only one acetate needed to be determined in order to characterize the polysaccharide of *R. trifolii* NA30 completely.

The spectra of corresponding fractions from *R. trifolii* TA1 and from *R. trifolii* 0403 were very similar to those spectra of fractions from NA30, with two additional peaks (11 and 15) appearing in the TA1 spectrum. Signals 11 and 15 arose from a 3-hydroxybutanoic ester, as explained later.

Fig. 4b shows the $^1$H-n.m.r. spectrum of the corresponding oligosaccharide fraction from the polysaccharide of *R. leguminosarum* 128c 53 treated in the same way as the *R. trifolii* NA30 polysaccharide. The anomeric region is much more complicated in spectrum 4b than in spectrum 4a, although the basic structural unit is the same for the two samples, and consequently, the differences must be due to acyl substituents. Upfield, 4b indicates the presence of more acyl substituents than in 4a. The signal for acetate is more complex, in that there are now two signals in the region of signal 13 in spectrum 4a, and another pair of signals, No. 12, is present slightly downfield. Two other signals, Nos. 11 and 15, both doublets, are also present in the spectrum 4b. From the chemical shifts, coupling constants, intensity ratios, enzymic determination, and co-chromatography with standards (see later), these two signals, Nos. 11 and 15, were assigned to a 3-hydroxybutanoic ester. From the total signal integration, it appears that there are ~2 acetyl groups, 0.5 3-hydroxybutanoyl group, and a little more than one pyruvic acetal per molecule. The chemical shifts of signals 13 and 5 from the acetyl methyl and H-3 of the acylated D-glucose are almost identical in spectra 4a and 4b, and, as found by methylation analysis, are due to an acetyl group at the same position in each repeating unit.

The signal for H-1 of the α-D-glucosyl fluoride is very different in spectra 4a and 4b. Three sets of signals (1a, d; 1b, e; and 1c, f) in spectrum 4b take the place of the one set (1c, f) in spectrum 4a. This indicates three different environments around H-1 in the material affording spectrum 4b. We explain this by proposing that, in some cases, O-2 of the D-glucosyl fluoride is acetylated, thus pulling the signal for H-1 downfield to 1a and 1d, and, in other cases, O-3 of the D-glucosyl fluoride is acetylated, shifting the signal for H-1 downfield to 1b and 1e. The third environment would arise when the D-glucosyl fluoride is not acetylated, and then the signal would be that seen in spectrum 4a. The first part of this proposition was substantiated by the finding that peaks 1a and 1d could be collapsed into singlets by proton decoupling at 4.86 p.p.m.

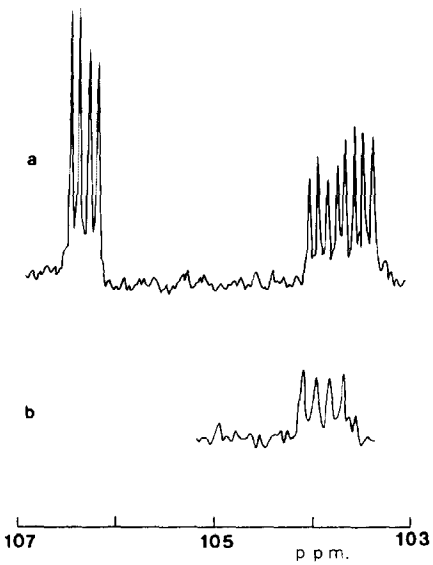


Fig. 5. $^{19}$F-N.m.r. spectra of one repeating unit for (a) *R. leguminosarum* 128c 53 and (b) *R. trifolii* 04030, using trifluoroacetic acid as an external standard.

Atom H-2 of a D-glucosyl fluoride acetylated at O-2 should give a complex signal[8], split by H-1 (3.6 Hz), F-1 (22.8 Hz), and H-3 (7.9 Hz) and be considerably downfield because of the acetyl group. Unfortunately, a doublet (signal No. 8), probably from an anomeric proton, is superposed on the complex signal (signal No. 7). The signal for H-3 of an $\alpha$-D-glucosyl fluoride acetylated on O-3 would be a triplet only very slightly split by the fluorine[8], probably a little farther downfield than that on an $\alpha$-linked D-glucose. Signal No. 4 fits this situation.

$^{19}$F-N.m.r. spectroscopy confirmed that there are three different environments around C-1 of the D-glucosyl fluoride. Three sets of quadruplets were found between 103 and 106 p.p.m., downfield from trifluoroacetic acid. Only one quadruplet, at 103.83 p.p.m., was found for *R. trifolii* 0403 (see Fig. 5).

Signal No. 6 is assigned to H-3 of D-galactose esterified with 3-hydroxybutanoic acid. Such a signal should have splitting from both an equatorial and an axial hydrogen atom, and be drawn downfield by the adjacent ester group. Evidence to support the assignment of the acylation of the D-galactose in this way was educed both from methylation analysis and enzymic determination.

The spectrum 4c is that of the larger fragments (Peak I on BioGel P-6) obtained from treatment of the *R. leguminosarum* 128c 53 polysaccharide at $-40°$. Spectrum 4d is from the same material after deacylation with 10mM KOH. These two spectra should reflect the intact polysaccharide without the influence of a fluoride atom on each repeating unit. Spectrum 4c is similar to 4b, except that there is only a trace of the signals for H-1 of D-glucosyl fluoride, and, instead, there are three signals for H-1 of $\alpha$-D-glucose in the region where Jansson *et al.*[7] observed the

signal for α-linked D-glucose, the smallest one being almost exactly at the chemical shift that they reported. Signal No. 4, from H-3 of the 3-*O*-acetylated α-D-glucose, is now a little farther upfield, probably because it is not adjacent to a fluorine atom, and the signal from H-2 of the 2-*O*-acetylated α-D-glucose is probably obscured upfield. Deacylation greatly simplified the spectrum. The three signals for H-1 of α-D-glucose collapsed to one, and all of the complex signals in the anomeric region disappeared. Only one of the signals, from a β-linked glycosyl unit (signal No. 9) remained separated from the cluster at ~4.5 p.p.m.

The $^1$H-n.m.r. spectra of oligosaccharides from *R. leguminosarum* 128c 63 showed the same features as those of strain 128c 53, but less signal density in the region which corresponds to acetylation at O-2 and O-3 of the branching α-D-glucose.

The $^1$H-n.m.r. spectrum of the intact polysaccharide of *R. leguminosarum* 128c 53 showed molar ratios of acetate:pyruvic acetal:3-hydroxybutanoate of ~3:4:1, showing that, apart from the loss of almost one pyruvic acetal group per repeating unit, the substituents on the oligosaccharides accounted for those on the polysaccharides.

A problem often encountered in locating acyl substituents is that acyl migration occurs under a variety of relatively mild conditions[7]. The possibility that the acetyl groups on the branching D-glucose were initially on one position, but migrated during the HF solvolysis, cannot be excluded. However, this is considered unlikely, because there was no detectable migration of the acetyl group on the adjacent D-glucose residue.

*F.a.b. mass spectrometry.* — A very useful technique for studying complex oligosaccharides is fast-atom-bombardment (f.a.b.) mass spectrometry, which provides[10] the molecular weight of the oligosaccharides and, sometimes, fragmentation data.

The f.a.b. mass spectrum of the putative repeating-unit generated by treatment of the polysaccharide of *R. leguminosarum* 128c 53 with HF at −40° showed peaks at *m/z* 1585 and 1607, corresponding to the octasaccharide fluoride + 2 acetate + pyruvic acetal + 3-hydroxybutanoate + $H^+$ or $Na^+$. One of the two pyruvic acetal groups must have been hydrolyzed off during preparation of the oligosaccharides, as indicated by the n.m.r. data. There were peaks of similar intensity at *m/z* 1499 and 1521, corresponding to the repeating unit without the 3-hydroxybutanoate, again + $H^+$ or $Na^+$, respectively. The only other peaks of note were at *m/z* 1233 and 1255, corresponding to a heptasaccharide formed by loss of the pyruvic acetalated, 3-hydroxybutanoylated D-galactose and 34 additional mass units. Because the fragment is present as both the protonated and sodium ion form, it is unlikely that it is generated as a fragment in the mass spectrometer, but rather, it may be generated during the reaction in liquid HF. More information about the reactions of pyruvic acetalated sugars in HF is needed before the apparent loss of the two acetal ring-oxygen atoms, along with the loss of pyruvic acetal from the D-glucose, can be explained.

Fraction IIIc from *R. leguminosarum* 128c 53 (see Fig. 3b) was purified by gel filtration and ion-exchange chromatography, and was expected to be the tetrasaccharide backbone of the polysaccharide. It gave an f.a.b. mass spectrum that supported this proposed structure. The highest-molecular-weight ions, 781 and 803, correspond to a tetrasaccharide fluoride containing two uronic acid residues and two acetates, confirming the presence of two acetyl groups in the backbone tetrasaccharides of *R. leguminosarum*. The lack of a significant ion at 599 (loss of D-glucosyl fluoride), and the presence of a major ion at 557 (due to loss of the acetylated sugar fluoride) indicated that the trisaccharide GlcA-GlcA-Glc contains only one acetyl group. Therefore, the other acetyl group is present on the $\alpha$-linked D-glucose in the original polysaccharide.

Fraction II from the Bio Gel P-2 column (see Fig. 3a) contained a mixture of slightly larger oligosaccharides and the intact side-chain of the polysaccharide. Ions at *m/z* 1225 and 1267 correspond to a heptasaccharide fluoride respectively containing one or two acetyl groups and an ion at *m/z* 1063 corresponds to a hexasaccharide fluoride containing one acetyl group. A peak of a di-*O*-acetylated hexasaccharide would coincide with a peak from the glycerol matrix. No indication of corresponding hexa- or hepta-saccharides containing 3-hydroxybutanoate or pyruvic acetal was seen, although all of the high-mass ions were of low intensity. An intense ion at *m/z* 825 corresponds to the tetrasaccharide fluoride of the side chain plus a 3-hydroxybutanoate and a pyruvic acetal group. A weaker ion, at *m/z* 867 may indicate that some of the side chain is also acetylated. An intense ion at *m/z* 481 can be explained by loss of two D-glucose residues from the side chain, and an even stronger one, at *m/z* 319, by a D-galactosyl residue carrying a 3-hydroxybutanoyl and a pyruvic acetal group, formed by loss of three D-glucose residues.

Overall, the mass spectra are consistent with a structure in which there is an acetyl group on both of the D-glucose residues in the backbone of the polymer, and a 3-hydroxybutanoic acid esterified to the terminal pyruvic acetalated D-galactose of the side chain.

*Identification of* D-*3-hydroxybutanoate.* — $^1$H-N.m.r. spectroscopy of some of the oligosaccharides showed doublets at 2.67 and 1.27 p.p.m. having a ratio of 2:3. No acyl substituents on polysaccharides have previously been reported that exhibit such n.m.r. signals. On the assumption that both signals were caused by the same substituent, the splittings and chemicals shifts of the two signals were used to predict the identity of the substituent. Both of the protons must be adjacent to a single proton in order to give rise to doublets. The signal at 1.27 p.p.m. is situated where slightly deshielded methyl protons would be expected to resonate, and that at 2.67 p.p.m. is very similar in shift to that for succinate methylene protons. The signal for the single proton (-C*H*OH-) splitting these two signals was not visible, and so it must lie among the signals for the sugar protons.

Treatment of the oligosaccharides with alkali removed the signals (at 2.67 and 1.27 p.p.m.) for the substituent, indicating that it was esterified to the oligosaccharides. The simplest small residue, commonly occurring as a metabolite, that fits these data is that of 3-hydroxybutanoic acid.

Published spectra[11] for the ethyl ester and the dimer of 3-hydroxybutanoic acid showed doublets at 1.28 and 2.5 p.p.m. The signal for H-3 of the dimer was at 4.21 p.p.m., and would thus be obscured in our oligosaccharide spectra.

The identity of the substituent was confirmed by co-chromatography as the trimethylsilyl derivative, using gas–liquid chromatography[12]. It was also substantiated by finding ions of the expected molecular weight in the f.a.b. mass spectra of oligosaccharides containing the substituent. 3-Hydroxybutanoic acid has one optically active center. Because it is a metabolite of the Rhizobia, it is probable that a single optical isomer is present. The enzyme 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) is only active on the D, that is (*R*), isomer of 3-hydroxybutanoate. When the 3-hydroxybutanoate was hydrolyzed from the oligosaccharide with 2M trifluoroacetic acid, and then exposed to 3-hydroxybutyrate dehydrogenase under the conditions used for assaying D-3-hydroxybutanoate[13], it was completely degraded, whereas only 50% of the commercially available DL-3-hydroxybutanoate was degraded under these conditions; this confirmed the presence of D-3-hydroxybutanoate.

While this manuscript was in preparation, Hollingsworth *et al.*[14], in a preliminary communication, reported finding 3-hydroxybutonate bound in an ether linkage to an oligosaccharide generated from *R. trifolii* 0403 by using an enzyme from a bacteriophage. We found no 3-hydroxybutanoate in the oligosaccharides isolated from our *R. trifolii* isolate that had the same strain designation. Also, in all of the cases in which we found it, 3-hydroxybutanoic acid was ester-linked. The chemical shifts of the proton resonances for the 3-hydroxybutanoate are all ~0.2 p.p.m. upfield in the $^1$H-n.m.r. spectra of the phage enzyme-produced oligosaccharides compared with the ones produced by using HF. An explanation of the discrepancy may be available when more of the structure of the oligosaccharide produced by the bacteriophage enzyme is known.

In another preliminary communication, Knirel *et al.*[15] reported finding 3-hydroxybutanoic acid in amide linkage in an unusual, sialic acid-like sugar [5,7-di(acylamido)-3,5,7,9-tetradeoxynonulosonic acid] present in the lipopolysaccharides of two strains of *Pseudomonas aeruginosa* and one of *Shigella boydii*.

*Methylation analysis.* — One of the most difficult structural features of a polysaccharide to determine is the stoichiometry and exact location of acyl substituents. A procedure to mask free hydroxyl groups with methyl vinyl ether, and then to methylate acylated oxygen atoms (methyl replacement) has been used[16], but, in our hands and those of others[7], the method has given unsatisfactory results.

The method devised by Prehm[17], using methyl trifluoromethanesulfonate as a methylating agent with a hindered base in trimethyl phosphate, appeared promising to us as a method for methylating free hydroxyl groups while retaining acyl substituents. Even Prehm's method did not prove to be ideal, as our attempts to use this reagent on intact polysaccharides failed, probably because of the low solubility of the polysaccharides in trimethyl phosphate. However, all of the fragments generated by the HF treatments could be effectively methylated. Table

TABLE III

RELATIVE MOLAR RATIO[a] OF DEDUCED SUGAR LINKAGES FROM FRACTION IV OF VARIOUS BACTERIAL ISOLATES

| *Glycosyl residue* | *Positions of O-methyl groups* | R. leguminosarum *128c 53* | | R. leguminosarum *128c 63* | | R. trifolii *NA30* | | R. trifolii *0403*[b] | | R. trifolii *TA1* | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *LiBH*$_4$ | | *LiBH*$_4$ | | *LiBH*$_4$ | | *LiBH*$_4$ | | *LiBH*$_4$ | |
| | | *before* | *after* | *before* | *after* | *before* | *after* | *before* | *after* | *before* | *after* |
| Glucosyl | 2,3,4,6 | 0.13 | 0.13 | 0.71 | 0.40 | 0.28 | 0.27 | 0.74 | 0.79 | 1.14 | 0.29 |
| Glucosyl | 3,4,6 | 0.30 | 0.18 | 0.23 | | 0.09 | 0.07 | | | | 0.18 |
| Glucosyl | 2,4,6 | 0.45 | 0.37 | 0.25 | 0.38 | 0.58 | 0.57 | 0.16 | 0.22 | 1.22 | 0.61 |
| Glucosyl | 2,3,6 | 1.90 | 2.26 | 1.44 | 2.29 | 1.88 | 2.56 | 1.30 | 1.46 | 1.22 | 2.60 |
| Glucosyl | 2,3,4 | 0.05 | 0.96 | 0.04 | 0.82 | 0.11 | 0.89 | 0.15 | 0.85 | | 0.67 |
| Glucosyl | 2,6 | 0.84 | 0.76 | 0.90 | 0.80 | 0.94 | 0.80 | 0.95 | 1.09 | 0.93 | 0.69 |
| Glucosyl | 3,6 | 0.10 | 0.13 | | | | | 0.21 | 0.22 | | 0.16 |
| Glucosyl | 2,3 | 0.40 | 1.44 | 1.11 | 2.21 | 1.29 | 2.01 | 1.30 | 2.07 | 0.35 | 1.67 |
| Galactosyl | 2,3 | 0.26 | 0.29 | 0.54 | 0.51 | 0.67 | 0.70 | 0.22 | 0.32 | 0.33 | 0.47 |
| Glucosyl | 2 | 0.82 | 0.77 | 0.32 | 0.25 | 0.16 | 0.14 | 0.14 | 0.19 | 0.16 | 0.13 |
| Galactosyl | 2 | 0.50 | 0.56 | 0.46 | 0.35 | | | | | 0.64 | 0.38 |
| Glucosyl | 3 | 0.25 | 0.16 | | | | | 0.06 | 0.11 | | 0.15 |
| Total | | 6.0 | 8.0 | 6.0 | 8.0 | 6.0 | 8.0 | 5.23 | 7.32 | 6.0 | 8.0 |

[a]Normalized to 6 and 8 residues for the nonreduced and reduced samples. [b]Normalized to take into account the major loss of D-galactose that occurred with this sample.

III shows the results obtained for peak IV isolated from the P-6 columns for all of the polysaccharides tested. The results before and after reduction of the methylated oligosaccharides with[18] $LiBH_4$ in oxolane (tetrahydrofuran) showed increases in 2,3,4-tri-*O*-methylglucose and 2,3-di-*O*-methylglucose, because of reduction of the terminal and 4-linked D-glucuronic acid residues to afford D-glucose derivatives.

The results of these methylation experiments cannot be explained by any combination of stoichiometric acylations of the repeating units of the polysaccharides. We propose that they were caused by a combination of naturally occurring, non-stoichiometric acylations, slight under-methylation, partial solvolysis of the terminal D-galactosyl group from the oligosaccharides, and an artifact produced by the HF treatment.

One feature common to all of the oligosaccharides is the presence of an almost stoichiometric amount of 3,4-disubstituted D-glucose. This unit is not seen on using the Hakomori method of methylation[19] and, thus, this shows that one of the 4-linked D-glucose residues is 3-*O*-acylated. $^1$H-N.m.r. spectroscopy also showed an almost stoichiometric acylation of one sugar residue in all of the oligosaccharides. Methylation analysis of fraction IIIc (see Fig. 3b), the backbone tetrasaccharide, from *R. leguminosarum* 128c 53 and 128c 63, and from *R. trifolii* TA1, also showed the presence of almost stoichiometric amounts of 3,4-disubstituted D-glucose. $^1$H-N.m.r. spectroscopy of this fraction confirmed the presence of the 3-*O*-acetyl group in the tetrasaccharide. This indicated that the β-linked D-glucosyl residue in the backbone is the one that is 3-*O*-acetylated in all cases.

Oligosaccharides from *R. leguminosarum* 128c 53 and 128c 63, and from *R. trifolii* TA1, contain tri-*O*-substituted D-galactose, accounting for ~50% of the D-galactose present (see Table III). As the D-galactose is at a nonreducing terminus and is 4,6-*O*-pyruvic acetalated, one in two residues must also be acylated. The signals at 4.96 p.p.m. in the $^1$H-n.m.r. spectra substantiated such an acylation. Identification of the acyl substituent came from analysis of HF–ether-soluble sugars. As part of the workup procedure from the HF treatment of the polysaccharides, the oligosaccharides were precipitated from the HF with ether, and the HF–ether solution was made neutral with calcium carbonate. After evaporation of the ether, any sugars which had been in solution in the HF–ether mixture could be extracted from the calcium carbonate, fluoride residue with water. Sugars extracted from the calcium carbonate, fluoride, in experiments in which polysaccharides from *R. leguminosarum* 128c 53 and 128c 63, and *R. trifolii* TA1, were treated in HF at −40°, were found by methylation analysis to be substituted, giving 2,3-di-*O*- and 2-*O*-methyl-galactose in almost equal proportions. G.l.c. analysis showed the presence of 3-hydroxybutanoate and pyruvate. These two HF–ether-soluble sugars must therefore be 4,6-*O*-pyruvic acetalated galactose and 4,6-*O*-pyruvic acetalated, 3-*O*-(3-hydroxybutanoyl)ated D-galactose.

Assignment of additional acylations from methylation data is complicated by the presence of di- and tri-substituted D-glucose in the intact polysaccharides. However, acylation of a portion of the branched D-glucose in the backbone of the poly-

saccharide at O-2 was suggested by the presence of 2,4,6-trisubstituted D-glucose in the oligosaccharide from *R. leguminosarum* 128c 53.

According to the methylation data (see Table III), some linkages that should not be present in a repeating unit of these polysaccharides are present, and one that should be present is not well represented. The presence of terminal D-glucose indicates that, to some extent, the HF cleaved the polysaccharide at a position other than the α-D-glucosyl linkage. However, both of the sugars that should be the non-reducing termini (pyruvic acetalated D-galactose and glucuronic acid) are present in almost stoichiometric amounts. The sugar not well represented is the 4,6-*O*-pyruvic acetalated, 3-*O*-β-D-galactosylated D-glucose. The presence of a small proportion of D-galactose in the HF–ether-soluble portion shows that some of the terminal sugars were cleaved, but the presence of an almost stoichiometric amount of D-galactose shows that most of it remained. The $^1$H-n.m.r. and the f.a.b. mass spectra showed that most of the oligosaccharides contain only one pyruvic acetal group. Thus, the pyruvic acetal group on the D-glucose is mostly removed by the HF. If the HF simply hydrolyzed off the pyruvic acid, the resulting D-glucose would be 3-linked. Some 3-linked glucose is present, but in varying proportions.

## CONCLUSION

It was concluded that, at −40°, liquid HF selectively cleaves the α-glycosidic linkage of the backbone of these polysaccharides, leaving the acyl substituents attached. However, the presence of a pyruvic acetal group on the penultimate D-glucosyl residue of the side chain leads to a variety of results, including hydrolysis of the pyruvic acetal group, loss of the D-galactosyl residue, or conversion of the D-glucosyl residue into a form labile to the reagents used in the methylation analysis employed.

The oligosaccharides produced by using HF allowed us to locate the acyl substituents of all five polysaccharides tested (see Fig. 1). All of the polysaccharides are 3-*O*-acetylated on the unbranched D-glucose of the backbone. *R. trifolii* TA1 and both strains of *R. leguminosarum* contain D-3-hydroxybutanoic acid esterified to O-3 of ~50% of the D-galactosyl residues. *R. leguminosarum* strain 128c 53 and, to a lesser extent, strain 128c 63 are also acetylated at a combination of O-2 or O-3 of the branching D-glucose of the backbone of the polysaccharide. The presence or absence of acetyl groups on the branching D-glucose of the polysaccharide correlated with the species of *Rhizobium* from which each polysaccharide was obtained.

## EXPERIMENTAL

Rhizobial strains *R. leguminosarum* 128c 53, 128c 63, *R. trifolii* 0403, and NA30 were gifts from Janet Darvill. *R. trifolii* TA1 was a gift from T. V. Bhuvaneswari. Polysaccharides were collected from cultures grown on defined medium as previously described[20].

*Partial degradation of polysaccharides with hydrogen fluoride.* — The poly-

saccharides were partially degraded by treatment with liquid hydrogen fluoride for 15 min at −40° or for 15 min at −23° as described previously, using the configuration for the apparatus in Fig. 2 of ref. 21.

*Liquid chromatography.* — Treated polysaccharide samples were fractionated on a column (2.5 × 50 cm) of Bio-Gel P-6 (Bio-Rad Laboratories, Richmond, California), and eluted with 0.05M sodium acetate buffer (pH 5.2). When the polysaccharide was treated with HF at −23° for 15 min, the product was fractionated on a Bio-Gel P-2 column (2.5 × 55 cm), and eluted with 0.05M sodium acetate buffer (pH 5.2). Portions of the effluent were then placed on a column (0.8 × 16 cm) of QAE-Sephadex anion-exchanger (40–120 μm; Sigma Chemical Co., St. Louis, Missouri), and eluted with imidazole acetate (Sigma Chemical Co.) buffer, pH 7.0, using a linear gradient between 0.05 and 0.50M (total volume, 160 mL).

Aliquots (25 μL) were taken from each 2-mL fraction, and tested by using the phenol–sulfuric acid assay[22]. All volumes were half those given in the reference.

*Methylation.* — The methylation method devised by Prehm[17] was used, as this procedure does not remove acetic esters during the methylation reactions. A small amount (0.6 mg) of oligosaccharide, dried over phosphorus pentaoxide, was suspended in trimethyl phosphate (100 μL, Aldrich Chemical Co., Milwaukee, WI) by sonication. 2,6-Di-(*tert*-butyl)pyridine (15 μL; Aldrich Chemical Co.) and methyl trifluoromethanesulfonate (10 μL; Aldrich Chemical Co.) were added to the suspension under nitrogen, and the mixture was allowed to react for 2 h at 50°. The methylated oligosaccharide was purified by adding water, and loading on a Sep-Pak $C_{18}$ cartridge (Waters Associates, Inc., Milford, MA). The methylated sugars were eluted with methanol as described[23], and divided into two equal portions; one was directly converted into partially methylated alditol acetates, and the other was reduced in order to convert uronic acids into hexoses.

*Reduction of uronic esters to alcohols.* — A procedure of Brown *et al.*[18] was simplified. After a methylated oligosaccharide (0.3 mg) had been dried over phosphorus pentaoxide in a vial, tetrahydrofuran (100 μL; Pierce Chemical Co., Rockford, IL) and a small pinch (~20 grains) of lithium borohydride (Aldrich Chemical Co.) were added, and the mixture was allowed to react for 2 h at 50°. When the reaction was over, the excess of $LiBH_4$ was decomposed by addition of glacial acetic acid (10 μL). The mixture was diluted with water and purified by passage through a Sep-Pak $C_{18}$ cartridge[23].

*Hydrolysis of methylated oligosaccharides.* — Hydrolysis was conducted in 88% formic acid (100 μL) for 1 h at 100°. After evaporation of the acid, 2M trifluoroacetic acid (100 μL) was added, and the hydrolysis was continued for 1.5 h at 121°.

*Reduction of monosaccharides.* — A procedure devised by Blakeney *et al.*[24] was used. After the methylated, hydrolyzed sample (0.3 mg) had been dried over phosphorus pentaoxide, M ammonia (10 μL) and 0.3M potassium borohydride in dimethyl sulfoxide (100 μL; Aldrich Chemical Co.) were added and allowed to react for 1.5 h at 40°. After the reduction, the excess of potassium borohydride was decomposed by addition of glacial acetic acid (10 μL).

*Acetylation.* — The procedure of Blakeney *et al.*[24] was used. To each sample of reduced material were added 1-methylimidazole (20 μL) and acetic anhydride (200 μL) and the reaction allowed to proceed for 10 min at room temperature. The excess of acetic anhydride was decomposed by addition of distilled water (5 mL). The alditol acetates were purified by loading on a stainless-steel guard-column packed with Bondapak™ $C_{18}$–porasil B (37–75 μm; Waters, Inc.) by using a reciprocating pump, and then eluting with methanol[23]. Water in the effluent was absorbed by addition of anhydrous sodium sulfate. After evaporating the methanol, ethyl acetate (25 μL) was added, to dissolve the alditol acetate derivatives. A 3-μL aliquot was then transferred to another vial, and diluted with 2,2,4-trimethylpentane (80 μL).

*Gas–liquid chromatography.* — The alditol acetate derivatives were separated on a DB-1 fused silica column (30 m × 0.25 mm, i.d.) (J & W Scientific Inc., CA) fitted to a Tracor 560 Gas Chromatograph (Austin, TX) equipped with a flame-ionization detector, and a capillary injection-system (J & W Scientific Inc.) was used for on-column injection, with helium as the carrier gas. Routinely, a 1-μL sample was injected. The oven-temperature program was as follows: injection at 105°, hold 4 min at 160°, then raise the temperature at 2°/min to 220°.

*$^1$H-N.m.r. spectroscopy.* — $^1$H-N.m.r. spectra were recorded with a Varian (Palo Alto, CA) XL-300 n.m.r. spectrometer (300 MHz) at both 25 and 70°. Polysaccharide samples (4 mg) were three times dissolved in $D_2O$ (Sigma Chemical Co.) and freeze-dried, to exchange the hydrogen for deuterium, and then dissolved in $D_2O$ (480 μL). Sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate (TSP) was used as the internal standard (0.00 p.p.m.). The data were recorded with 9k of memory and 400 transients. $^{19}$F-N.m.r. spectra were recorded at 25°, with trifluoroacetic acid as the external standard (0.00 p.p.m.). The data were recorded with 10k of memory and 1500 transients.

*Deacylation of oligosaccharides.* — The oligosaccharide (15 mg) was added to 10mM KOH (15 mL) and allowed to react for 5 h at room temperature under nitrogen[25]. The solution was then made neutral with M formic acid, and freeze-dried. The deacylated oligosaccharide was desalted by using a column of Bio-Gel P-2 in 0.05M sodium acetate buffer.

*F.a.b. mass spectrometry.* — Samples were suspended in glycerol, and analyzed with a Finnigan-Mat (San Jose, CA) high-resolution mass spectrometer at the NIH mass spectrometry facility of the Massachusetts Institute of Technology.

*Determination of 3-hydroxybutanoate; chemical method.* — A procedure of Horning *et al.*[12] was used. A suitable amount (100 μg) of sugar sample was hydrolyzed with 2M trifluoroacetic acid (100 μL) for 1 h at 100°. After cooling, the excess of acid was evaporated with a stream of nitrogen, and then sodium malate (5 μg; Sigma Chemical Co.) was added as an internal standard. After the mixture had been dried over phosphorus pentaoxide, 2% methoxyamine hydrochloride (20 μL; Pierce Chemical Co.) in pyridine (Aldrich Chemical Co.) was added, and allowed to react for 3 h at room temperature. The pyridine was evaporated. *N,O*-bis(tri-

methylsilyl)trifluoroacetamide (15 μL; Pierce Chemical Co.) was added, and allowed to react for 2 h at room temperature. The trimethylsilylated sample was diluted with dichloromethane (100 μL) and 1 μL was injected on-column. The temperature program was: inject at 40° and hold for 5 min at 60°, then increase at 4°/min to 180°. The peak corresponding to 3-hydroxybutanoate was identified by comparison with authentic sodium DL-3-hydroxybutanoate.

*Determination of 3-hydroxybutanoate by an enzymic method*[13]. — A suitable amount (100 μg) of the repeating unit of *R. leguminosarum* 128c 53 was dried, 2M trifluoroacetic acid (100 μL) was added and the mixture was heated for 1 h at 100° to hydrolyze 3-hydroxybutanoic acid from the oligosaccharide. After cooling to room temperature, the acid was evaporated under $N_2$. To the treated sample, were added water (0.67 mL), 0.1M hydrazine–Tris buffer, pH 8.5 (0.33 mL), and 14mM β-nicotinamide-adenine dinucleotide (33 μL) and mixed well. The absorbance was read at 340 nm against a blank solution of water (0.67 mL) and hydrazine–Tris buffer (0.33 mL).

After the addition of 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30; 15.6 units/mL of Tris buffer; 3.3 μL), the absorbance of the mixture in the sample cuvet was read and recorded until the reaction was complete (~60 min). The amount of 3-hydroxybutanoate was determined by use of a calibration curve (0–10 μg of DL-3-hydroxybutanoate).

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