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Reexamination of the Presence and Linkage of 3-Hydroxybutyryl Substituents in the Acidic Capsular Polysaccharide of *Rhizobium trifolii* 0403†

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We resolved previous conflicting results concerning the presence of 3-hydroxybutyryl substituents on the extracellular acidic polysaccharide from *Rhizobium trifolii* 0403. These substituents were indeed present in the polysaccharide and in the oligosaccharide fragments obtained by hydrogen fluoride solvolysis of the extracellular and capsular polysaccharides of the bacteria grown on plates. The 3-hydroxybutyrate substituent could be removed from the polysaccharide by 10 mM sodium deuteroxide without evidence of elimination, indicating that this substituent was ester linked.

Using ^{1}H nuclear magnetic resonance (NMR) spectroscopy, Hollingsworth et al. (4) discovered 3-hydroxybutyrate (3-HB) as a novel, covalently attached, noncarbohydrate substitution in the acidic capsular polysaccharide (CPS) and extracellular polysaccharide (EPS) of *Rhizobium trifolii* 0403 (*R. leguminosarum* biovar *trifolii* 0403). That study was done by analyzing isolated oligosaccharide fragments of unit size and uniform carbohydrate composition obtained after depolymerization of the CPS and EPS of this strain at near-neutral pH with either of two different bacteriophage-induced β-lyases, followed by P-10 gel filtration chromatography (4). These oligosaccharide fragments were also substituted with base-labile acetate groups and acid-labile pyruvyl acetals (1, 5). The 3-HB substituents were not removed by a mild base treatment, which was sufficient to remove the acetate esters from the oligosaccharide, suggesting that 3-HB was ether rather than ester linked (4). Quantitative ^{1}H NMR showed that the stoichiometry of 3-HB substitution per unit oligosaccharide fragment was greater for the CPS than for the EPS (hence, the CPS and EPS are not identical [5]) and increased with culture age (1).

More recently, Kuo and Mort (7) found no ^{1}H NMR signals for 3-HB in spectra of any of the oligosaccharide fragments obtained by HF solvolysis of the acidic EPS of *R. trifolii* 0403. On the basis of this result, they concluded that the acidic EPS of *R. trifolii* 0403 was not substituted with 3-HB. They did, however, find 3-HB substitution in HF-cleaved fragments of the EPSs from *R. trifolii* TA1 and *R. leguminosarum* 128C53 and 128C63 and showed that 3-HB was 3-O-acylated to the terminal galactose in the branch of the respective polysaccharide repeating unit.

The structures of the CPS and its unit oligosaccharide from *R. trifolii* 0403 are of interest because these molecules display an infection-related biological activity on white clover root hairs and bind with similar specific activity to the white clover lectin trifoliin A (1). Thus, it is especially important to clarify the discrepancies (4, 7) regarding the presence or absence of 3-HB and how it is linked to the acidic polysaccharide of *R. trifolii* 0403. Here we provide data that resolve these questions.

We first performed experiments to test the possibility that the *R. trifolii* 0403 cultures maintained in the two laboratories were not identical. For clarity, the cultures were designated 0403/FD (4) and 0403/AM (7) according to their source. Both cultures were streaked to check for culture purity on plates of defined BIII agar (3). Cells grown for 5 days at 30°C

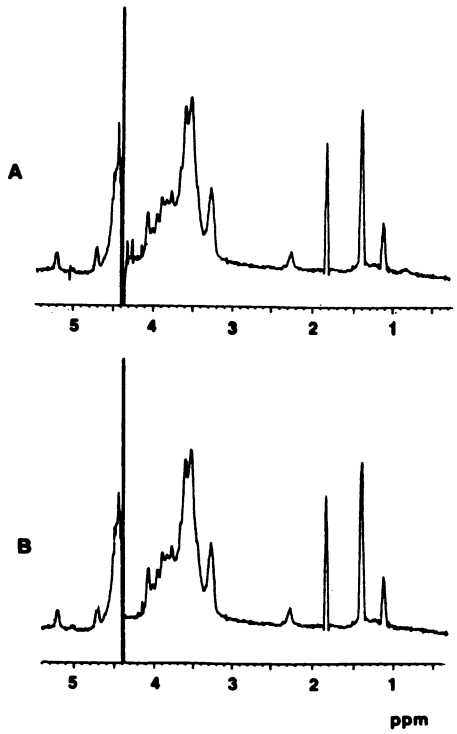

FIG. 1. ^{1}H NMR spectrum of deuteroxide-treated CPSs from *R. trifolii* 0403/FD (A) and 0403/AM (B).

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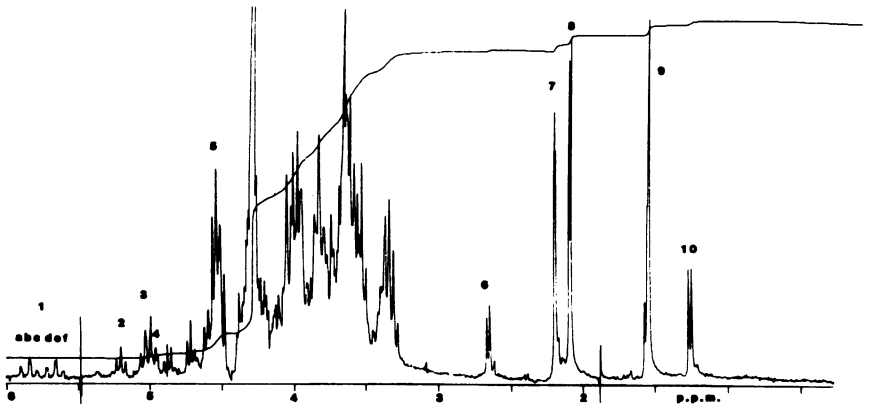

FIG. 2. ^{1}H NMR spectrum of an oligosaccharide fragment representing the repeating unit of the CPS from *R. trifolii* 0403/FD obtained after HF solvolysis. Peak identities: 1a to f, H-1 of glucosylfluoride residue split by anisotropic effects of substituents; 2, H-3 of 3-O-acetylated residue with gluco- configuration; 3, H-3 of 3-O-acetylated residue with gluco- configuration; 4, H-3 of galactose 3-O-acylated with 3-HB; 5, H-1 of β-linked sugars; 6, CH_2 of 3-HB; 7, CH_3 of acetate; 8, CH_3 of acetate; 9, CH_3 of pyruvate on galactose; 10, CH_3 of 3-HB. For explanation of assignments, see reference 7.

were analyzed for antigenic relatedness by indirect immunofluorescence microscopy with rabbit antisera against purified lipopolysaccharide or encapsulated cells of *R. trifolii* 0403/FD. Both cultures were also checked for nodulation ability by inoculation on roots of Louisiana Nolin white clover (*Trifolium repens* L.) seedlings grown axenically on enclosed −N agar plates (3).

Both the *R. trifolii* 0403/FD and 0403/AM cultures produced the same colonial morphology on BIII agar plates, showed positive immunofluorescence with immune antisera against the lipopolysaccharide and encapsulated cells of *R. trifolii* 0403/FD, and nodulated seedling roots of white clover. Thus, both cultures of *R. trifolii* 0403 displayed similar cultural, antigenic, and symbiotic properties.

Next, we isolated CPS by salt extraction of pelleted cells harvested from 5-day-old bacterial cultures grown as lawns on BIII agar plates as previously described (1, 5). The CPS was ethanol precipitated several times, dissolved in water, dialyzed, and lyophilized. (Poly-β-hydroxybutyrate, a cell storage product, is insoluble in aqueous systems and therefore would not be isolated with the CPS). A sample of the CPS was dissolved in 10 mM NaOD and briefly sonicated, and the ^{1}H NMR spectrum was obtained at 250 mHz (1, 4, 5) and 75°C. Another sample of the isolated CPS was depolymerized by HF solvolysis and fractionated by P-6 gel filtration (Bio-Rad Laboratories) (4). The carbohydrate fraction eluting at the same V_e/V_0 (1.8) as was previously observed (7) was deuterium exchanged and analyzed by ^{1}H NMR at 300 MHz and 70°C (7).

The ^{1}H NMR spectra of the CPSs from *R. trifolii* 0403/AM and 0403/FD were virtually the same (Fig. 1A and B). In both spectra, the resolved signals from 3-HB were readily assignable, in the same position, and in the same stoichiometry relative to acetate (at δ 1.83) and pyruvate (at δ 1.37 and δ 1.39). The methylene group of 3-HB appeared at δ 2.29, and the methyl group appeared at δ 1.13. In the fragment obtained by HF solvolysis (Fig. 2), the methylene and methyl group signals for 3-HB appeared at δ 2.67 and δ 1.27, respectively. In both cases, the methine group of 3-HB was obscured by the other carbohydrate signals. Thus, 3-HB substituents were present in the CPSs of both *R. trifolii* 0403 cultures.

In the deuteroxide-treated CPS, the methylene signals appeared further upfield than they did in the HF-generated fragments (Fig. 1A and B and Fig. 2). This meant that the carboxyl group of 3-HB was liberated by 10 mM base treatment. Also, when the spectrum was rerun after ethanol precipitation of the base-treated CPS, then both the acetate and 3-HB signals were absent (spectrum not shown). These results indicated that the 3-HB substituents were ester and not ether linked to the CPS. The acetate esters were presumably more base labile than were the 3-HB esters, since in an earlier study (4), the milder base treatment removed the acetate without affecting the 3-HB substituents.

The lack of 3-HB in the HF-cleaved products of the acidic EPS from *R. trifolii* 0403/AM, as reported in an earlier study (7), was probably a result of differences in growth conditions. Previous experience with rhizobia has shown that culture conditions (2) and culture age (1, 6, 8) can have a dramatic effect on their extracellular components.

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