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IN VITRO SYNTHESIS OF A LIPID-LINKED ACETYLATED AND PYRUVILATED OLICOSACCHARIDE IN Rhizobium trifolii

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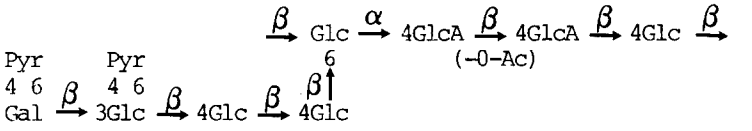
SUMMARY.- EDTA-treated Rhizobium trifolii cells (strain NA30) incorporate radioactivity from (^{14}C) labeled UDP-Glc, UDP-GlcA, Acetyl-Coa and/or phosphoenol pyruvate into chloroform:methanol:water (1:2:0.3) extracts. The incorporation products have properties of prenyl-phospho-sugars; mild alkaline hydrolysis of these extracts produce cyclic phosphate esters suggesting the presence of a diphosphate bridge, and mild acid or catalytic reduction-alkaline phosphatase treatments release four main componenets a, b, c and d, as judged by paper electrophoresis and chromatography and gel filtration studies. The four components can be obtained (^{14}C)acetyl-labeled, but only compound c and to a lesser degree compound b can be (^{14}C)pyruvate-labeled.

For the exopolysaccharide produced by this strain the following repeating unit has been proposed (Robertsen et al. (1981), Plant Physiol. 67, 389-400):



The results obtained suggest that the octasaccharide repeating unit (compound a) with one (compound b) or two (compound c) ketal pyruvate residues are assembled on a lipid acceptor. All these compounds are assumed to be intermediates in the biosynthesis of R. trifolii exopolysaccharide © 1986 Academic Press, Inc.

In the last few years a great deal of interest has been focused on the process of legume root infection by bacteria of the genus Rhizobium. The exopolysaccharides (EPS) that these microorganisms secrete seem to play a controversial role in the first stages of infection (1,2).

Less attention has been devoted to the biosynthesis of these EPS. The in vitro synthesis of a lipid-bound oligosaccharide repeating unit has been reported only for R. meliloti and Agrobacterium tumefaciens, a related species (3,4,5).

Dedicated to Dr. Luis F. Leloir on the occasion of his 80th birthday, September 6, 1986.

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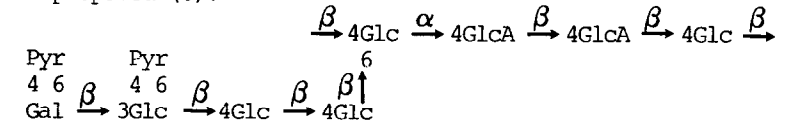
Rhizobium trifolii, strain NA30, which infects red clover, produces a fairly complex exopolysaccharide. For the repeating unit of these EPS, the following structure has been proposed (6):



An acetyl residue is also present in an unknown position.

In this communication, the in vitro synthesis from UDP-Glc and UDP-GlcA of a lipid-linked oligosaccharide with properties consistent with the above repeating unit, as well as the transfer of acetyl and ketal pyruvate residues from acetyl-Coa and phosphoenolpyruvate (PEP), respectively, is reported.

MATERIALS AND METHODS.- UDP-(^{14}C)Glucose (285.1 Ci/mol) and UDP-(^{14}C)glucuronic acid (285.1 Ci/mol) were prepared as previously described (7,8). Phosphoenol (1-^{14}C)pyruvic acid, cyclohexylammonium salt (12.4 Ci/mol) ((^{14}C)PEP) was purchased from the Radiochemical Centre, Amersham, England, and (1-^{14}C)acetyl-coenzyme A (56.6 Ci/mol) ((^{14}C)acetyl-CoA) from New England Nuclear. The heptasaccharide Rha →6Glc →6Glc →4GlcA →2Man →3Glc →4Glc and the hexasaccharide Glc →6Glc →4GlcA →2Man →3Glc →4Glc from Acetobacter xylinum, as well as the pentasaccharide Man →4GlcA →2Man →3Glc →4Glc from Xanthomonas campestris, all of them (^{14}C)glucuronic acid-labeled, were prepared as in previous work (9,10). Other chemicals were commercial samples.

Rhizobium trifolii, strain NA-30, a kind gift from Dr. William F. Dudman of the Division of Plant Industry of the Commonwealth Scientific and Industrial Research Organization (Canberra City, Australia), was grown and harvested as reported by Robertsen et al. (6). The enzyme preparations consisted of EDTA-treated cells obtained as in previous publications (8). Protein was determined by the method of Lowry, with a standard of bovine serum albumin (11).

Assay procedure: The standard mixture contained 70 mM Tris-HCl buffer, pH 8.2; 8 mM $MgCl_2$, EDTA-treated cells (1.05 mg protein) and the amounts of labeled or unlabeled sugar nucleotides, PEP and acetyl-CoA indicated in each case. The reactions were carried out in a total volume of 0.140 ml, at 10°C for 60 min and stopped by adding 1.0 ml of 70 mM Tris-HCl buffer, pH 8.2, containing 1.25 mM EDTA. The mixtures were centrifuged at 6,000 x g for 5 min and the pellets resuspended and washed three times with buffer without EDTA. The washed pellets were then extracted three times with (0.1 ml each) chloroform:methanol:water (1:2:0.3) (1203 solvent). This extract, which contains the lipid-linked oligosaccharides, will be referred to as "1203 extract".

Degradation techniques: The phosphate-sugar bond in the lipid-linked oligosaccharides was split by either mild acid hydrolysis, performed in 0.01 N HCl at 100°C for 10 min, or by catalytic reduction with Pt-H_2, followed by alkaline phosphatase treatment as reported previously (12). Cyclic phosphoric esters were opened by treating with 0.1 N HCl, at 100°C for 10 min and the phosphate residue was removed with alkaline phosphatase (from E. coli, Sigma) as in previous work (9). Total acid hydrolysis was carried out in 1 N, HCl at 100°C for 24 h in a sealed tube. Borohydride reduction was performed as in previous work (8)

Paper chromatography and electrophoresis were carried out in 2043 a Schleicher and Schull paper as previously reported (8). The following solvents were used:

(A) 1.2 M pyridinium acetate buffer, pH 6.5; (B) nitromethane·acetic acid:ethanol: boric acid saturated water (8:1:1:1); (C) 3 M ammonia in 70% (v/v) ethanol. Reducing substances were located with the alkaline silver reagent (13), UV absorbing compounds with a Mineralight lamp and radioactivity by scanning the paper strips on a Packard radiochromatogram scanner, model 7201.

RESULTS AND DISCUSSION

Incubations carried out with EDTA-treated cells in the presence of UDP-Glc and UDP-GlcA, either sugar nucleotide (^{14}C)labeled, lead to the formation of radioactive material extractable with 1203 solvent (Table 1, lines 1 to 4). The (^{14}C)GlcA incorporation from UDP-(^{14}C)GlcA was greatly increased in the presence of UDP-Glc (Table 1, line 2), but (^{14}C)glucose incorporation was not stimulated in the presence of UDP-GlcA (Table 1, line 4). These results have been justified considering that EDTA-treated cells are fairly crude enzyme preparations and that therefore contain endogenous acceptors and donors of the reactions studied in this work, as well as related enzymes (i.e. UDP-Gal-4-epimerase).

Aliquots of the 1203 extracts from experiments 1 or 2 were submitted without treatment to paper electrophoresis with buffer A: the radioactive material did not migrate, as expected for a lipid-linked sugar. But if the extracts were previously hydrolyzed with acid (pH 2, 100°C for 10 min), a procedure that splits the sugar-phosphate linkage, three major radioactive compounds, a (R_{UMP} = 0.73), b (R_{UMP} = 1.05) and c' (R_{UMP} = 1.29) were observed (Fig. 1,A).

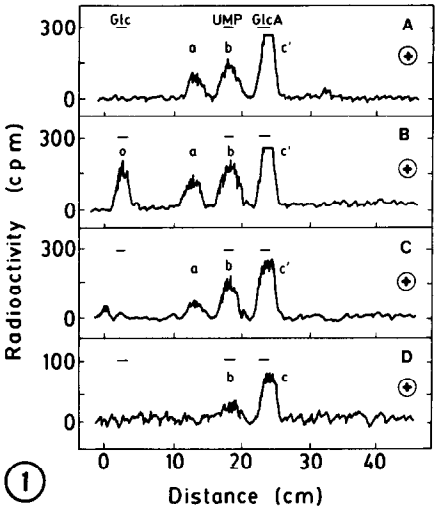
The paper electrophoresis profile obtained with (^{14}C)glucose-labeled material was similar, except for the presence of a new radioactive compound in the neutral area

TABLE 1

Incorporation of the different components of the lipid-linked saccharide into 1203 extract

Exp	Donor		Radioactive residue in 1203 extract (pmoles/mg protein)
	Labeled	Unlabeled	
1	UDP-(^{14}C)GlcA	----	21.4
2	UDP-(^{14}C)GlcA	UDP-Glc	65.6
3	UDP-(^{14}C)Glc	----	16.9
4	UDP-(^{14}C)Glc	UDP-GlcA	17.5
5	(^{14}C)Acetyl-CoA	----	11.5
6	(^{14}C)Acetyl-CoA	UDP-Glc + UDP-GlcA	18.4
7	(^{14}C)PEP	----	28.5
8	(^{14}C)PEP	UDP-Glc + UDP-GlcA	51.5

The incubations were carried out and processed as indicated in Materials and Methods. In each case, the concentration of the individual labeled and unlabeled sugar nucleotides was 7.1 uM and 178 uM, respectively. Experiments 5 and 6 were performed in the presence of 119 uM (^{14}C)Acetyl-CoA and experiments 7 and 8 in the presence of 414 uM (^{14}C)PEP. Aliquots of each 1203 extract were counted for radioactivity in Bray's solution with a Packard 2002 Tri-Carb Scintillation Counter.



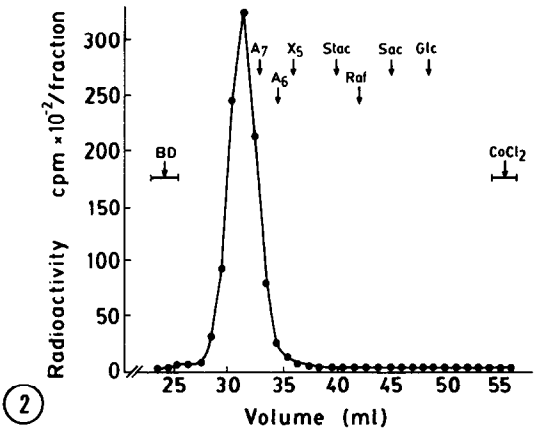


Fig. 1.- Analysis of the incorporation products
1203 extracts obtained as in legend to Table 1 were hydrolyzed in 0.01 M HCl, at 100°C for 10 min and submitted to paper electrophoresis with buffer A. The following combination of precursors was used: UDP-(^{14}C)GlcA and UDP-Glc (A); UDP-(^{14}C)Glc and UDP-Glc (B); (^{14}C)Acetyl-Co A and unlabeled sugar nucleotides (C). (^{14}C)Pyruvic acid-labeled oligosaccharides were obtained by catalytic reduction and alkaline phosphatase treatment of 1203 extracts obtained as in Table 1, experiment 8 (D).

Fig. 2.- Gel filtration
(^{14}C)Glucuronic acid-labeled compound a, obtained as in legend to Fig. 1, frame A, was sampled in a Bio Gel P2 (200-400 mesh) column (0.7 x 1.14 cm) in 0.1 M pyridinum acetate buffer pH 5.0. Fractions of 0.5 ml were collected at a rate of 0.1 ml/min and 0.025 ml aliquots were counted for radioactivity (-•-). The arrows indicate the elution volumes of the following standards: glucose (Glc), sucrose (Suc), raffinose (Raf), stachiose (Stac), pentasaccharide from X. campestris (X_5), hexasaccharide (A_6) and heptasaccharide (A_7) from A. xylinum. Blue Dextran and $CoCl_2$ were added as indicators of total exclusion and total inclusion volumes, respectively.

(compound o) (Fig. 1,B). With either label, the relative amounts of the different compounds varied according to the batch of enzyme being used.

Compound a, filtered through a Bio Gel P2 column, eluted before a heptasaccharide standard, in a position expected for an octasaccharide (Fig. 2). Compound b eluted slightly ahead of it, and compound c' produced two components: one in the octasaccharide area (compound c) and a trisaccharide (compound d) (not shown).

Submitted to acid conditions that release ketal pyruvate residues (pH 2, 100°C for 90 min) (14), compounds b and c turned out to migrate as compound a, upon paper electrophoresis with buffer A or Bio Gel P2 gel filtration. Compound a was not affected by this treatment. Acid hydrolysis of compound c for shorter times (20-30 min) produced a mixture of the three components.

Total acid hydrolysis of borohydride-reduced (^{14}C)glucose-labeled compounds a, b or c released labeled sorbitol, glucose and galactose, as judged by paper

chromatography with solvent B. Therefore the three compounds have glucose at the reducing end. On the other hand, and taking into consideration that GlcA is also incorporated, the presence of galactose is an indication that the whole repeating unit has been assembled since in the proposed structure glucose occupies the reducing end and galactose the non reducing end of the octasaccharide

All these results taken together indicate that very likely compound c is substituted with two ketal pyruvate residues, compound b with only one and compound a is the unsubstituted octasaccharide repeating unit (see below).

Acetylation: Incubations performed with unlabeled sugar nucleotides and (^{14}C)acetyl-CoA also incorporated radioactivity into 1203 extracts (Table 1, lines 5 and 6). Mild acid hydrolysis of this material produced a paper electrophoresis profile identical to the one observed with labeled sugar nucleotides, indicating that compounds a, b and c' could be acetylated (Fig. 1,C). The small stimulation observed in the presence of unlabeled sugar nucleotides suggests that endogenous acceptos and/or donors are present in the enzyme preparation (Table 1, line 6).

Incorporation of pyruvate residues: Similarly, incubations performed with unlabeled sugar nucleotides and (^{14}C)PEP incorporated radioactivity into 1203 extracts (Table 1, lines 7 and 8). Paper electrophoresis analysis of this fraction, after catalytic reduction and alkaline phosphatase degradation, produced mainly compound c and traces of compound b(Fig. 1,D). No compounds a or d were observed, as expected.

Diphosphate bridge: Mild alkaline treatment (chromatography with solvent C) of Glc or GlcA labeled 1203 extracts produced compounds with the properties of cyclic phosphate ester derivatives, which upon pH 1 hydrolysis and alkaline phosphatase treatment released radioactive material with the properties of compounds a, b, c and d. Without prior acid hydrolysis, the mild alkali products were insensitive to phosphatase degradation. The formation of cyclic phosphate esters under mild alkaline conditions is an indication that a diphosphate bridges the oligosaccharide to the lipid (8). In addition, 1203 extracts, Glc, GlcA or acetyl labeled, submitted to catalytic reduction, a procedure that cleaves the phosphate-prenol linkage releasing diphosphate saccharides, produced highly anionic compounds (as expected for the diphosphate derivatives). These substances, treated with alkaline phosphatase, released mainly compounds b, c and d and only traces of compound a. This result also suggests that the endogenous lipid acceptor is very likely a monophosphorylated allylic prenol (17), since dolichol derivatives are not degraded by this procedure (12). On the other hand, the above results also indicate that the acid conditions used to liberate the oligosaccharides release part of the ketal pyruvate residues present in them: catalytic reduction and alkaline phosphatase treatment, which avoid the acid hydrolysis step, produced mainly the fully pyruvilated compound c.

The participation of prenyl-phospho-sugars has been reported for the biosynthesis of two complex EPS: a capsular polysaccharide from Aerobacter aerogenes (15) and xanthan gum (16). In this communication, the in vitro accumulation of lipid-linked oligosaccharides with some of the properties expected for the repeating unit of the R. trifolii EPS is reported; it seems reasonable to assume that they constitute a stage in the pathway of this EPS biosynthesis. Furthermore, previous work from this laboratory has shown that in the biosynthesis of xanthan gum, the incoorporation of ketal-pyruvate residues from PEP, and acetylation, from acetyl CoA, occur at the prenyl phosphosugar stage (10,17). The same mechanism seems to be operating for the synthesis of R. trifolii EPS. The sequential assembly and the detailed structure of the octasaccharides a, b, and c is being investigated. Compound d has been characterized as a mixture of acetylated and non acetylated trisaccharide GlcA $\overset{\beta}{\rightarrow}$ 4GlcA $\overset{\beta}{\rightarrow}$ 4Glc (N. Iñon de Iannino, J.C. Bossio and M.A. Dankert, in preparation). For the EPS of other strains of R. trifolii, the presence of only one GlcA residue per repeating unit has been reported (18-19).

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