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Biosynthesis of *Rhizobium trifolii* Capsular Polysaccharide: Enzymatic Transfer of Pyruvate Substitutions into Lipid-Bound Saccharide Intermediates†

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The activity of capsular polysaccharide pyruvyltransferase catalyzing the pyruvylation of acidic heteropolysaccharide was measured in *Rhizobium trifolii* 843 and 0403 *rif*. This enzyme activity was determined with EDTA-treated cells, uridine diphosphate-sugar precursors, and phosphoenol [1-^{14}C]pyruvate. Activity was measured by the incorporation of radioactivity into organic solvent-soluble glycoconjugates. Enzymatic pyruvylation of capsular polysaccharide occurred from phosphoenolpyruvate at the lipid-bound saccharide stage.

The biosynthesis of extracellular polysaccharide of *Klebsiella aerogenes* (18), *Xanthomonas campestris* (12), *Acetobacter xylinum* (5), *Aerobacter aerogenes* (22), and *Neisseria meningitidis* (15) involves lipid-bound intermediates (17). The lipid carrier is a C_{55} polyisoprenol (bactoprenol) linked to the growing oligosaccharide chain through an acid-labile phosphodiester bond (13). Synthesis of β-(1->2)-glucan from UDP-[^{14}C]glucose by *Rhizobium japonicum* (7) and *R. phaseoli* (2) and of lipid-bound saccharides by *R. meliloti* has been reported (19, 20, 23). Many bacterial polysaccharides are pyruvylated (17), including the acidic heteropolysaccharides of *Rhizobium* spp. (4, 10). However, there are few publications (3, 12) on the in vitro pyruvylation of polysaccharides. Just published recently was a report on the in vitro synthesis of a lipid-linked pyruvylated oligosaccharide of wild-type strain NA-30 of *R. trifolii* (3).

Age-dependent changes in noncarbohydrate substitutions (pyruvate, acetate, 3-hydroxybutanoate) of *R. trifolii* 0403 capsular polysaccharide (CPS) have been related to its transient lectin-binding ability (1, 16), and mutant strains with altered pyruvate levels in CPS have been described (8, 11). In this paper, we report (i) the optimal conditions for in vitro pyruvylation of CPS, using *R. trifolii* wild-type strains 843 and 0403 *rif*, and (ii) that enzymatic pyruvylation of CPS occurs from phosphoenolpyruvate at the lipid-bound saccharide stage.

Portions of our work have been described (A. E. Gardiol, Ph.D. dissertation, Michigan State University, East Lansing, 1985) and presented at the Sixth International Symposium on Nitrogen Fixation, Oregon State University, Corvallis, 4 to 9 Aug. 1985 (11).

R. trifolii ANU 843 was obtained from M. Djordjevic and B. Rolfe at the Australian National University (Canberra), and *R. trifolii* 0403 *rif* was from K. Nadler at Michigan State University.

Cells were grown in BIII broth (6) to stationary phase (100 Klett units in a Klett-Summerson colorimeter with a no. 66 filter), centrifuged at 12,000 × *g* for 30 min, and washed with BIII medium followed by 10 mM EDTA adjusted to pH 8.0 with 10 mM Tris base. Pelleted cells were suspended with 1 volume of buffer, frozen at −80°C, and thawed at room temperature (20). This freeze-thawing cycle was repeated eight times, yielding EDTA-treated cells that were permeable to sugar nucleotide diphosphates. Protein content was measured by the Folin phenol method (14), with a bovine serum albumin standard.

The in vitro activity of CPS pyruvyltransferase was measured by a modification of the method of Ielpi et al. (12). The standard incubation mixture (total volume, 50 μl) contained the following: Tris hydrochloride buffer (pH 8.2), 3.5 μmol; $MgCl_2$, 0.4 μmol; 2-mercaptoethanol, 2.0 μmol; EDTA-treated cells (0.4 to 1 mg of protein); UDP-glucose, 15 nmol; UDP-galactose, 8 nmol; UDP-glucuronic acid, 8 nmol; and phosphoenol [1-^{14}C]pyruvic acid (PEP) (15 mCi/mmol), 29 nmol as the cyclohexylammonium salt (Amersham Corp., Arlington Heights, Ill.). The reaction was performed for 20 min (unless otherwise indicated) at 15°C and stopped by adding 0.5 ml of 70 mM Tris hydrochloride buffer (pH 8.2) containing 5 mM EDTA. The mixtures were centrifuged at 12,000 × *g* and 4°C. The pellets were washed twice with buffer without EDTA, extracted twice with 0.2 ml of chloroform-methanol-water (1:2:0.3), and centrifuged. Samples of this extract (supernatant), containing lipid-bound oligosaccharides (LBO), were mixed in a liquid scintillation mixture (Scinti Verse II; Fisher Scientific Co., Pittsburgh, Pa.) and counted in an LS 7000 liquid scintillation counter (Beckman Instruments, Inc., Irvine, Calif.). CPS pyruvyltransferase activity was expressed as the radioactivity (counts per minute) incorporated into the LBO extract during the incubation period per indicated amount of protein. One unit of CPS pyruvyltransferase was defined as the amount of enzyme that incorporated 200 cpm (13 pmol) of labeled PEP into LBO in 20 min. Specific activity was expressed as units per milligram of protein.

The major acidic heteropolysaccharide of the wild-type *R. trifolii* strains contains glucose, galactose, and glucuronic acid. To determine if, in the conditions of this assay, the cells were able to incorporate these labeled glycosyl components into LBO from their respective UDP ^{14}C-labeled sugar

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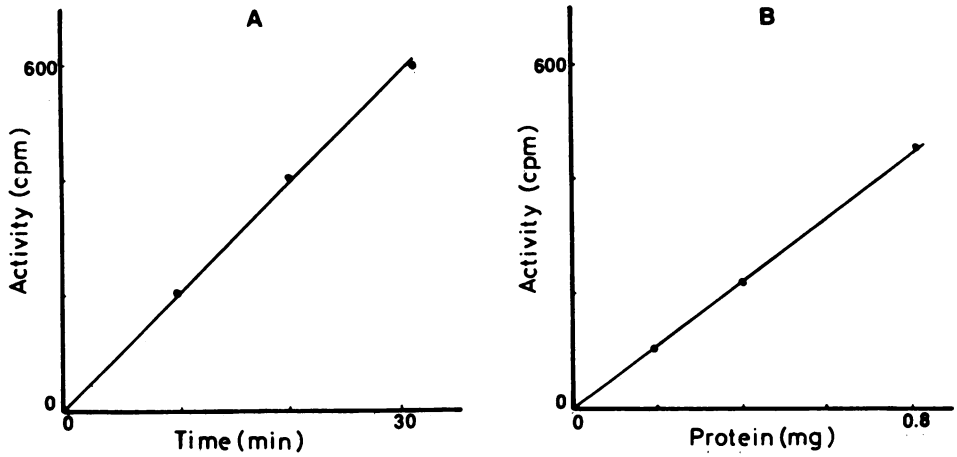

FIG. 1. CPS pyruvyltransferase activity in *R. trifolii* 843 as function of the incubation time (A) or the amount of protein (B). Enzyme assays were performed with the standard incubation mixture containing EDTA-treated cells, UDP-sugar donors, and [1-^{14}C]PEP. Activity was expressed as the radioactivity (counts per minute) incorporated from [1-^{14}C]PEP into lipid-bound saccharides soluble in organic solvent per 0.6 mg of protein at indicated times (A) or per indicated amount of protein in 20 min (B).

precursors, a modified standard incubation mixture was used. Individual UDP-U-^{14}C-labeled sugar precursors were added separately to the standard incubation mixture, and the corresponding unlabeled UDP-sugars as well as PEP were omitted. The incubation mixture contained UDP-[U-^{14}C]glucose (293 mCi/mmol, 0.85 nmol), UDP-[U-^{14}C] galactose (297 mCi/mmol, 0.85 nmol), or UDP-[U-^{14}C]glucuronic acid (269 mCi/mmol, 0.95 nmol) and the remaining two unlabeled UDP-sugars at the same concentration as in the standard incubation mixture. Control samples consisted of heat-inactivated cells.

The LBO extract from a standard incubation mixture (scaled up 30-fold) was dried under a stream of nitrogen at room temperature. Mild acid hydrolysis, designed to cleave the pyrophosphate bridge between the lipid and the saccharide, was performed in 0.01 N HCl for 10 min at 100°C, and the solution was partitioned in chloroform-methanol-water (3:2:1) (20). The aqueous (top) and organic (bottom) phases were separated, and radioactivities of aliquots were measured.

Gel filtration chromatography of the radioactive, acid-cleaved moiety that partitioned into the aqueous phase was performed at room temperature in a column (100 by 2 cm) of Bio-Gel P6 (100–200 mesh). The solvent was 0.1 M pyridine acetate (pH 5.0), and the flow rate was 8 ml/h. Fractions of 3 ml were collected, and aliquots of 0.5 ml were mixed with 3 ml of Scinti Verse II and counted. Blue dextran was used to determine the void volume. Standards consisted of lactose and the modified oligosaccharide fragment from *R. trifolii* 843 CPS (isolated after depolymerization, using a β-lyase which generates 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid in place of one glucuronic acid residue [1]), and their elution was detected by the phenol-sulfuric acid method (9). Solvent in radioactive fractions was evaporated under a stream of nitrogen at 30°C, and the saccharide residues were partially depyruvylated in 0.01 N HCl for 90 min at 100°C. Samples were then neutralized, evaporated under nitrogen, and redissolved in water. Components were separated by ascending paper chromotography on Whatman no. 1 paper, using *n*-butanol–propionic acid–water (21:11:14) as solvent and sodium pyruvate as standard. Radioactivity on the paper was detected with a radiochromatogram scanner, and unlabeled compounds were detected by the alkaline silver nitrate method (21).

The optimal conditons for assaying CPS pyruvyltransferase activity (under which the highest incorporation of radioactivity from [1-^{14}C]PEP into LBO was achieved) were first established for *R. trifolii* 843 and used in the standard assay. The optimum pH within the range of 7.0 to 9.5 was 8.2 at 15°C, and activity was unchanged at temperatures within the range of 0 to 15°C and then decreased at temperatures above 15°C at pH 8.2. The reaction was linear up to 30 min, and total activity was proportional to protein concentration added (Fig. 1A and B). Results presented in Fig. 1 were corrected for the low amount of radioactivity of control (boiled enzyme) samples, due to slight solubility of labeled PEP in the organic solvent. The concentration of the UDP-sugar substrates in the complete incubation mixture was not limiting since a further increase did not increase product formation (Table 1). The label incorporated from [1-^{14}C]PEP into product when unlabeled UDP-sugar donors were not exogenously added to the incubation mixture was threefold lower than when the standard incubation mixture was used (Table 1). This indicated that biosynthesis of the saccharide moiety of the product can be increased by exogenously supplementing the concentration (provided by the EDTA-treated cells used in the assay) of the necessary UDP-sugar precursors. The addition of unlabeled PEP (four-

TABLE 1. In vitro assay of CPS pyruvyltransferase activity in *R. trifolii* 843

Incubation mixture	Sp act (U/mg)
Complete[a]	3.73
3 × [UDP-sugars][b]	3.70
− [UDP-sugars][c]	1.24
+ 4 × cold [PEP][d]	0.56
Boiled enzyme (5 min)	0.59

[a] Standard incubation mixture containing EDTA-treated cells, exogenously added 8 nmol of UDP-galactose, 8 nmol of UDP-glucuronic acid, 15 nmol of UDP-glucose, 29 nmol of [1−^{14}C]PEP, and buffer salts, pH 8.2.

[b] Threefold-higher concentration of exogenously added UDP-sugars than in the standard incubation mixture.

[c] No exogenously added UDP-sugars.

[d] Fourfold-higher concentration of unlabeled PEP than that of labeled PEP.

TABLE 2. Incorporation of radioactivity from labeled UDP-sugars into lipid-bound saccharides in EDTA-treated cells of *R. trifolii* 843[a]

Labeled, UDP-substrate	Unlabeled, UDP-substrate	Glycosyltransferase activity[b]
UDP-[^{14}C]glucose	UDP-galactose + UDP-glucuronic acid	2,548
UDP-[^{14}C]galactose	UDP-glucose + UDP-glucuronic acid	5,020
UDP-[^{14}C]glucuronic acid	UDP-glucose + UDP-galactose	21,668

[a] Assay was performed with the standard incubation mixture to which the indicated labeled UDP-substrate was added (with the omission of the corresponding unlabeled UDP-substrate as well as PEP). The incubation mixture contained the other two indicated unlabeled UDP-substrates. Concentrations of the UDP-substrates were as follows: UDP-[^{14}C]glucose, 17 μM; UDP-[^{14}C]galactose, 17 μM; UDP-[^{14}C]glucuronic acid, 19 μM; UDP-glucose, 300 μM; UDP-galactose, 160 μM; UDP-glucuronic acid, 160 μM.

[b] Expressed as the radioactivity (counts per minute) incorporated into glycoconjugate product soluble in chloroform-methanol-water (1:2:0.3) in 20 min per milligram of cellular protein. The results shown are the average of three replicates.

fold more than labeled PEP) to the standard incubation mixture containing labeled PEP decreased the radioactivity incorporated into the product to the background level obtained with boiled control samples (Table 1). This indicated that CPS pyruvyltransferase catalyzed the incorporation of PEP into the lipid-bound product. The possibility of label incorporation into product from further metabolism of PEP (e.g., through an acetyl-coenzyme A intermediate) was eliminated because [1-^{14}C]PEP would be converted into unlabeled acetyl-coenzyme A.

There was incorporation of radioactivity into lipid-bound saccharides when UDP-[^{14}C]glucose, UDP-[^{14}C]galactose, or UDP-[^{14}C]glucuronic acid was used separately in the incubation mixture which contained the other two unlabeled UDP-substrates (Table 2). Thus, the cells used in this experiment are also capable of incorporating the glycosyl components of *R. trifolii* acidic CPS into lipid-bound saccharides. The greatest incorporation of label was obtained with the combination of UDP-[^{14}C]glucuronic acid plus UDP-glucose plus UDP-galactose in the incubation mixture (Table 2). Consistent with our results, similar studies with *R. trifolii* NA-30 (3) reported 3.5-fold-higher incorporation of radioactivity into product with UDP-[^{14}C]glucuronic acid (7.1 μM) plus UDP-glucose (178 μM) than with either UDP-[^{14}C]glucose (7.1 μM) plus UDP-glucuronic acid (178 μM), only UDP-[^{14}C]glucuronic acid (7.1 μM), or only UDP-[^{14}C]glucose (7.1 μM) in the incubation mixture.

Mild acid treatment of the labeled LBO product synthesized in vitro resulted in the transfer of all radioactivity from the LBO extract (37,000 cpm) to the aqueous phase when partitioned in chloroform-methanol-water (3:2:1). This indicated that the label was incorporated into the carbohydrate moiety of the LBO product and not the lipid itself. To determine if the labeled moiety of the aqueous soluble product was pyruvate, we fractionated this product by gel filtration chromatography. Analysis of the fractions showed that two major labeled products (peaks V_e/V_0 = 3.3, 3.5) eluted after the modified oligosaccharide fragment of *R. trifolii* 843 CPS. Depyruvylation of these isolated products followed by paper chromatography with standard pyruvate showed that the labeled moiety of these aqueous soluble products was indeed pyruvate.

The specific activities of CPS pyruvyltransferase from *R. trifolii* 843 and 0403 *rif* were very similar (3.79 and 3.73 U/mg of protein, respectively) in these two nodulating wild-type strains isolated from very different ecological environments.

We conclude that pyruvylation of CPS in *R. trifolii* 843 and 0403 occurs by enzymatic transfer from phosphoenolpyruvate to lipid-bound saccharide intermediates. Consistent with these results, the pyruvylation of a lipid-linked oligosaccharide of *R. trifolii* NA-30 from phosphoenolpyruvate has recently been described (3).

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LITERATURE CITED

1. **Abe, M., J. E. Sherwood, R. I. Hollingsworth, and F. B. Dazzo.** 1984. Stimulation of clover root hair infection by lectin-binding oligosaccharides from the capsular and extracellular polysaccharides of *Rhizobium trifolii*. J. Bacteriol. **160**:517–520.
2. **Amemura, A.** 1984. Synthesis of (1->2)-β-D-glucan by cell-free extracts of *Agrobacterium radiobacter* IFO 12665bl and *Rhizobium phaseoli* AHU 1133. Agric. Biol. Chem. **48**:1809–1817.
3. **Bossio, J. C., N. Inon de Ianino, and M. A. Dankert.** 1986. *In vitro* synthesis of a lipid-linked acetylated and pyruvylated oligosaccharide in *Rhizobium trifolii*. Biochem. Biophys. Res. Commun. **134**:205–211.
4. **Carlson, R. W.** 1982. Surface chemistry, p. 199–234. *In* W. Broughton (ed.), Nitrogen fixation, vol. 2. *Rhizobium*. Clarendon Press, Oxford.
5. **Couso, R. O., L. Ielpi, R. C. Garcia, and M. A. Dankert.** 1982. Synthesis of polysaccharides in *Acetobacter xylinum*. J. Biochem. **123**:617–627.
6. **Dazzo, F. B.** 1982. Leguminous root nodules, p. 431–446. *In* R. G. Burns and J. H. Slater (ed.), Experimental microbial ecology. Blackwell Scientific Publications, Oxford.
7. **Dedonder, R. A., and W. Z. Hassid.** 1964. The enzymatic synthesis of a (β-1,2-)-linked glucan by an extract of *Rhizobium japonicum*. Biochim. Biophys. Acta **90**:239–248.
8. **Djordjevic, M. A., P. R. Schofield, and B. G. Rolfe.** 1985. Tn5 mutagenesis of *Rhizobium trifolii* host-specific nodulation genes results in mutants with altered host-range ability. Mol. Gen. Genet. **200**:463–471.
9. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
10. **Dudman, W. F.** 1984. The polysaccharides and oligosaccharides of *Rhizobium* and their role in the infection process, p. 397–404. *In* C. Veeger and W. E. Newton (ed.), Advances in nitrogen fixation research. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague.
11. **Gardiol, A. E., R. I. Hollingsworth, F. B. Dazzo, M. A. Djordjevic, and B. G. Rolfe.** 1985. Alterations in capsular polysaccharide of Tn5 induced mutants in the *R. trifolii* nodulation region, p. 267. *In* H. J. Evans, P. J. Bottomley, and W. E. Newton (ed.), Nitrogen fixation research progress. Martinus Nijhoff, Dordrecht, The Netherlands.
12. **Ielpi, L., R. O. Couso, and M. A. Dankert.** 1981. Xanthan gum biosynthesis. Pyruvic acid acetal residues are transferred from phosphoenolpyruvate to the pentasaccharide-P-P-lipid. Biochem. Biophys. Res. Commun. **102**:1400–1408.
13. **Lennarz, W. J., and H. G. Scher.** 1972. Metabolism and function of polyisoprenol sugar intermediates in membrane-associated reactions. Biochim. Biophys. Acta **265**:417–441.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **Masson, L., and B. E. Holbein.** 1985. Role of lipid intermedi-

ate(s) in the synthesis of serogroup B *Neisseria meningitidis* capsular polysaccharide. J. Bacteriol. **161**:861–867.
16. **Sherwood, J. E., J. M. Vasse, F. B. Dazzo, and G. L. Truchet.** 1984. Development and trifoliin A-binding ability of the capsule of *Rhizobium trifolii*. J. Bacteriol. **159**:145–152.
17. **Sutherland, I. W.** 1985. Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. Annu. Rev. Microbiol. **39**:243–270.
18. **Sutherland, I. W., and M. Norval.** 1970. Synthesis of exopolysaccharide by *Klebsiella aerogenes* membrane preparations and the involvement of lipid intermediates. Biochem. J. **120**: 567–576.
19. **Tolmasky, M. E., R. J. Staneloni, and L. F. Leloir.** 1982. Lipid-bound saccharides in *Rhizobium meliloti*. J. Biol. Chem. **257**:6751–6757.
20. **Tolmasky, M. E., R. J. Staneloni, R. A. Ugalde, and L. F. Leloir.** 1980. Lipid-bound sugars in *Rhizobium meliloti*. Arch. Biochem. Biophys. **203**:358–364.
21. **Trevelyan W. E., D. P. Procter, and J. S. Harrison.** 1950. Detection of sugars on paper chromatograms. Nature (London) **166**:444–445.
22. **Troy, F. A., F. E. Frerman, and E. C. Heath.** 1971. The biosynthesis of capsular polysaccharide in *Aerobacter aerogenes*. J. Biol. Chem. **246**:118–133.
23. **Ugalde, R. A., J. Handelsman, and W. J. Brill.** 1986. Role of galactosyltransferase activity in phage sensitivity and nodulation competitiveness of *Rhizobium meliloti*. J. Bacteriol. **166**: 148–154.