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The structure of the acidic exopolysaccharide of *Pseudomonas marginalis* strains PF-05-2 And PM-LB-1

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ABSTRACT

The structure of an acidic exopolysaccharide of two strains of *Pseudomonas marginalis*, a bacterium which causes soft rots of various vegetables, has been determined to consist of a repeating unit of: →4)-β-D-Man*p*-(1→3)-α-D-Glc*p*-(1→4)-α-L-Rha*p*-(1-. The glucose is pyruvated at O-4 and O-6 and the mannose is acetylated at either O-2 or O-3.

INTRODUCTION

We have previously reported on the nature of the exopolysaccharide (EPS) produced by a number of phytopathogenic fluorescent pseudomonads in conjunction with our studies on plant–bacteria interactions[1,2]. Most pathovars of *Pseudomonas syringae* studied produce either the neutral fructan, levan, or the acidic glycuronan, alginate, as EPSs *in vitro*, depending upon the carbon source available to the bacterium[3]. However, we recently reported on the isolation and structural characterization of a unique acidic EPS from a strain of the soft-rotting bacterium *P. marginalis*, which has the disaccharide repeating unit[4] of →3)-β-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→). The galactose is pyruvated at O-4 and O-6 (acetal form) and the glucose is succinylated (either at O-2 or O-3). More recently, in examining the additional strains PF-05-2 and PM-LB-1 of *P. marginalis*, both of which cause soft-rot of bell peppers (capsicum) and produce levan as an EPS when grown on sucrose-containing agar media, we have isolated an additional, unique acidic EPS. The structural characterization of this EPS is the subject of this paper.

RESULTS AND DISCUSSION

From 40 agar plates, the strains yielded the following amounts of crude EPS: 76 and 173 mg of noncapsular and capsular EPS, respectively, for strain PF-05-2; and 329 and 57 mg of noncapsular and capsular EPS, respectively, for strain PM-LB-1. Each

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sample contained < 2% protein, < 5% uronic acid, and no hexosamine. Analysis by g.l.c. indicated that the crude capsular EPS of both strains contained glucose, mannose, and rhamnose (1:1:1) with a trace of galactose. The crude noncapsular EPS contained variable ratios of glucose (1.0), mannose (0.53 to 0.88), rhamnose (0.46 to 0.71), and galactose (0.41 to 0.68).

Anion-exchange chromatography of crude capsular EPS resulted in the elution of a single large peak between 0.3–0.5M NaCl. This material contained only glucose, mannose, and rhamnose (1:1:1). The noncapsular EPS also eluted as a single peak, primarily between 0.3–0.5M NaCl, but low amounts of hexose-containing material also eluted as a tail following the main peak. The composition of the material eluting as the main peak was glucose, mannose, and rhamnose (1:1:1) with only a trace of galactose. The later-eluting material consisted of mannose, xylose, rhamnose, glucose, and galactose (1:0.17:0.86:0.88:0.64). This material was not studied further. No neutral material containing sugar eluted with buffer alone for either sample.

The purified capsular and noncapsular EPS of *P. marginalis* strain PM-LB-1 was shown to be composed of rhamnose, glucose, mannose, pyruvate, and acetate in the approximate ratios of 1:1:1:1:1 by chemical and ^{13}C-n.m.r. analysis. Three anomeric carbons as well as the acetal carbon of the pyruvate were observed in the ^{13}C-n.m.r. spectrum (Fig. 1). The substitution pattern as determined by permethylation analysis was: 3,4,6-substituted glucose, 4-substituted mannose, and 4-substituted rhamnose. Permethylation analysis of the depyruvated polymer resulted in the derivative for 3-substituted rather than 3,4,6-substituted glucose.

Oxidation by periodic acid produced a non-dialyzable polymer that contained glucose and mannose. Since the mannose was not degraded, it must be acetylated at O-2

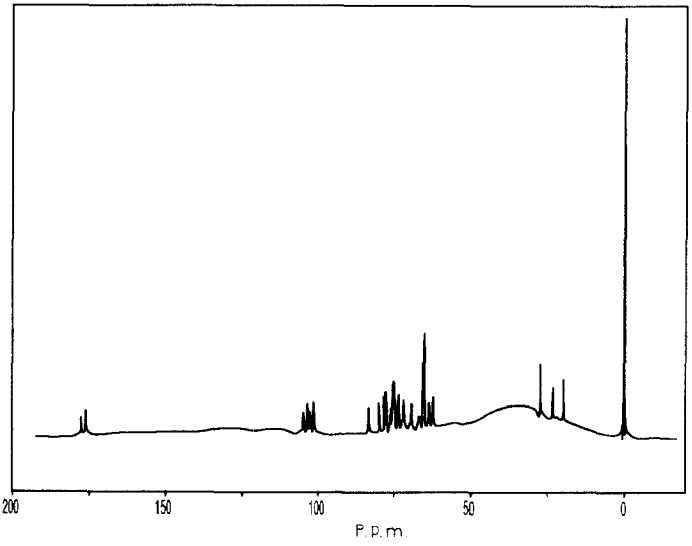


Fig. 1. ^{13}C-n.m.r. spectrum of *P. marginalis* PM-LB-1 EPS.

or O-3. Deacetylation followed by oxidation and hydrolysis yielded glucose as the only sugar. The sugar sequence of the acetylated polysaccharide was determined by permethylating the periodate-oxidation product, followed by partial hydrolysis, ethylation, and finally complete hydrolysis, reduction, and acetylation. The products of this sequence of reactions: 1,5-di-*O*-acetyl-4-*O*-ethyl-2,3,6-tri-*O*-methylhexitol; 1,3,5-tri-*O*-acetyl-4,6-di-*O*-ethyl-2-*O*-methylhexitol, and 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methylhexitol (derived from incomplete removal of the pyruvate substituent on mild acid hydrolysis) indicate that the sequence of sugars in the repeating unit is: mannose, glucose, and rhamnose.

Determination of the anomeric configuration was difficult, since n.m.r. data were ambiguous. Coupling constants of C-l–H-l indicated one α (168 Hz, 101.2 p.p.m.) and one β (159 Hz, 103.2 p.p.m.) linkage, however, the remaining coupling constant was 165 Hz (102.6 p.p.m.). The ^{1}H-n.m.r. spectrum of the intact polysaccharide was poorly resolved, even at 50° (higher temperature caused autohydrolysis of the pyruvate substituent). The ^{1}H-n.m.r. spectrum of the periodate-oxidation product (containing glucose and mannose as the only intact sugars) showed much better resolution and contained two major resonances (5.06 and 4.88 p.p.m.) neither of which had coupling constants greater than 3 Hz. Therefore, glucose must be α. The shifts also indicate the mannose was β. However, determination of the anomeric configuration of rhamnose required further experimentation. The polysaccharide was partially hydrolyzed in 0.05M H_2SO_4. Preferential hydrolysis of the rhamnosyl–mannose bond (as determined by $NaBH_4$ reduction, hydrolysis, and g.l.c. analysis of the sugars as the aldononitrile acetates and alditols as the alditol acetates) gave a mixture composed almost exclusively of rhamnose-terminated oligomers that were multiples of d.p. 3 (Fig. 2). The ^{1}H-n.m.r. spectrum of **1** (prepared by $NaBH_4$ reduction of d.p. 3) had two signals in the anomeric region 5.06 and 4.88 p.p.m. ($J_{1,2}$ 1.2 Hz), the n.m.r. spectrum of d.p. 6 (**2**) contained, in

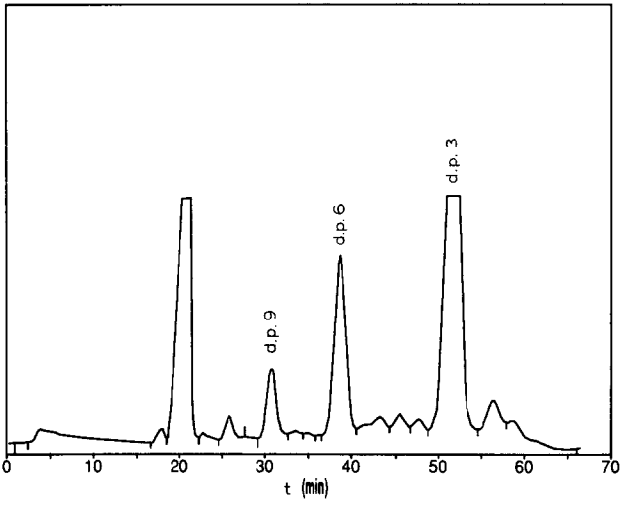


Fig. 2. L.c. of partially hydrolyzed *P. marginalis* PM-LB-1 EPS.

addition to these two resonances, a doublet ($J_{1,2} \sim 2$ Hz) centered at 5.16 p.p.m. From these results we conclude the rhamnose has the *α* configuration and mannose the *β*. These results are consistent with the interpretation of the n.m.r. spectrum of the Smith-degradation product of the polysaccharide.

The pyruvate C-2 carbon of the polysaccharide resonates, unexpectedly, at 104.6 p.p.m. This assignment was unambiguous from ^{13}C-n.m.r. DEPT and ADEPT spectra. Generally this carbon atom resonates further upfield[5] ($\sim$ 101–102 p.p.m.). Based on the shift of the pyruvate methyl group, 25.9 p.p.m. (assigned by comparison of ^{13}C-n.m.r. normal and DEPT spectra), C-2 of the pyruvate substituents has been assigned the *R* configuration[5]. The complete structure of the repeating unit of the acidic EPS of strain PM-LB-1 is therefore structure **3**. Acidic EPS of strain PF-05-2 was identical to that of strain PM-LB-1 as regards ^{13}C-n.m.r. analysis and was therefore considered structurally identical.

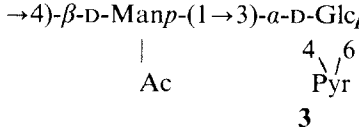
Initially, all strains of the soft-rotting bacterium *P. marginalis* were thought to have biochemical and physiological properties similar[6] to members of *P. fluorescens* biovar II. It is now clear, however, from the physiological and nutritional tests done by Liao and Wells on a number of *P. marginalis* strains[7] as well as by our studies on the nature of the EPS's produced by strains of *P. marginalis*, that this species is much more diverse than initially proposed[7].

Man-(1→3)-Glc-(1→4)-rhamnitol

1

Man-(1→3)-Glc-(1→4)-Rha-(1→4)-Man-(1→3)-Glc-(1→4)-rhamnitol

2



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EXPERIMENTAL

Bacterial strains. — *P. marginalis* strains PF-05-2 and PM-LB-1 were obtained from Dr. C.-H. Liao (USDA, ERRC). Both strains were originally isolated from rotted bell pepper. Strains were stored on Difco *Pseudomonas* agar F at 4°.

General methods. — Analytical methods for neutral sugars, glycuronans, pyruvate, acetate, hexosamines, and protein have been described[8]. The method of Bjørndal *et al.*[9] was used for permethylation and perethylation. Both g.l.c. (Hewlett–Packard 5880A) and g.l.c.–m.s. (Hewlett–Packard 5995B) analyses were carried out using an SP-2230 capillary (15 m) column (Supelco). Glucose and mannose enantiomers were determined by g.l.c. analysis of the sugar acetates using a Chirasil-Val (25 m) capillary column (Alltech). The rhamnose enantiomer was isolated by l.c. of an 0.5M H_2SO_4

hydrolyzate of the polysaccharide, using an Aminex HPX-87H column (Bio-Rad), 5mM H_2SO_4 eluent; $[\alpha]_D^{23}$ $-8°$.

The ^1H- and ^{13}C-n.m.r. spectra were obtained with a JEOL CX-400 spectrometer. The samples were dissolved in D_2O containing DSS as the internal standard. Spectra for the polysaccharide were obtained at 50° and spectra for the oligomers were obtained at both 35 and 70°.

Preparation of EPS. — Bacteria were grown on *Pseudomonas* agar F (Difco) for 3 d at 28°. Mucoid growth was scraped off using H_2O and a bent glass rod. After stirring vigorously, the suspension was centrifuged (90 min, 23 300*g*) resulting in a dense cell-pellet covered by a middle opaque layer and an upper clear supernatant. Light microscopy using wet mounts with India ink demonstrated that the opaque middle layer consisted of capsulated cells. The clear supernatant-fluid upper layer and the opaque layer were collected separately. The capsular EPS was removed from the cells by homogenization in a commercial blender. Re-centrifugation of this suspension as before resulted in a clear supernatant fluid overlaying a dense cell-pellet. Both the capsular and noncapsular EPS preparations were extensively dialyzed (12 000 mol wt. cutoff tubing) against H_2O at 4°. After concentration by lyophilization, the samples were subjected to precipitation with three volumes of ice-cold acetone after addition of $CaCl_2 \cdot 2H_2O$ (final concentration, 0.15%). Precipitated EPS was collected by centrifugation and the acetone precipitation step repeated once. EPS was then subjected to ultracentrifugation (100 000*g*, 4 h). The clear supernatant fluids were re-dialyzed as before, lyophilized, and designated as crude EPS.

EPS samples were further purified by anion-exchange chromatography on a column (1.5 × 46 cm) of DEAE-Sepharose CL-6B (Pharmacia). The column was equilibrated in 0.05M Tris-HCl buffer, pH 7.2. Equilibration buffer (400 mL), followed by a linear 0–1M NaCl gradient (400 mL) prepared in equilibration buffer, was used for elution at 0.4 mL.min^{-1}. Fractions (4 mL) were collected and tested for neutral sugar. Combined peak fractions were dialyzed against H_2O and lyophilized.

Oligomer preparation and analysis. — The polysaccharide (40 mg) was hydrolyzed with 0.05M H_2SO_4 (15 mL) for 4 h at 95°. After neutralization with $BaCO_3$ and concentration, d.p. 3 and d.p. 6 were isolated by h.p.l.c. on an Aminex HPX-22H column (Bio-Rad), with 5mM H_2SO_4 eluent. A portion of each fraction was rechromatographed to determine purity. The fractions were made neutral and evaporated; 2.1 mg of d.p. 6 and 4.3 mg of d.p. 3 were recovered. The fractions were reduced with $NaBH_4$ and after neutralization with Dowex H^+ resin and concentration (5X) from MeOH, a sample was hydrolyzed in 0.5M H_2SO_4 and the aldononitrile acetate derivatives prepared in the usual manner (the alditols, obviously, only forming their acetates). Two columns were used for g.l.c. analysis. Galactitol and glucitol acetates and the corresponding aldononitrile acetates were analyzed on an SP-2330 capillary column; rhamnitol acetate and the corresponding aldononitrile acetate, which were not resolved on the SP-2330 column, were analyzed on an HP-5 capillary (25 m) column (Hewlett–Packard).

Periodate oxidation. — Reaction conditions were similar to those described previously[8] with the exception that neutralization of the $NaBH_4$ reduction product was

monitored on a pH meter to prevent the pH of the solution dropping below 5; 1000 mol. wt. cutoff tubing was used for dialysis.

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