

Structure of an Acidic Exopolysaccharide of *Pseudomonas marginalis* HT041B

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The exopolysaccharide of *Pseudomonas marginalis* HT041B has been characterized as a 1,3-linked galactoglucan in which galactose and glucose are in the α - and β -anomeric configurations, respectively. The polysaccharide is substituted with pyruvate at the 4 and 6 positions of galactose and with succinic acid at either the 2 or 4 position of glucose. This polysaccharide has been given the trivial name marginalan.

In a recent report, we characterized the structure of the acidic exopolysaccharide (EPS) of the phytopathogen *Pseudomonas syringae* pv. *glycinea* as an alginate similar in structure to alginates isolated from *P. aeruginosa*, *P. fluorescens*, *P. putida*, and *P. mendocina* (8). Subsequently, we examined the EPS of a wide range of plant pathogenic pseudomonads and found alginate production to be common among fluorescent types when glucose or gluconate was used as the carbon source in the culture medium; with sucrose as the carbon source, levan, levan and alginate, or alginate alone was produced, depending on the bacterial strain examined (3). On the basis of these results, we proposed that most, if not all, fluorescent pseudomonads are capable of synthesizing alginate. However, in recent experiments the EPS isolated from a strain of *P. marginalis* (an organism responsible for spoilage of fruits and vegetables in storage) grown on glycerol-containing medium was determined to be neither an alginate nor levan. As part of our investigation of bacterial EPS as a virulence factor of phytopathogenic bacteria, we undertook an examination of the structure of this polysaccharide.

General methods. Neutral sugar, uronic acid, and amino sugar analyses were done by colorimetric assays as previously described (9). Pyruvate concentration was determined by the method of Jeanes et al. (6), and succinate concentration was determined by the method of McComb and McCready (7). The method of permethylation analysis used in this laboratory has been described elsewhere (8). High-performance liquid chromatography (HPLC) analyses were carried out on an HP 1090 chromatograph (Hewlett-Packard Co.) fitted with a diode array detector and an HP 1037A refractive index detector, gas chromatography analyses were carried out on an HP 5880 chromatograph, and mass spectra were obtained on an HP 5990B GC-MS spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on a JEOL 400X.

Preparation of EPS. *P. marginalis* HT041B was obtained from C. Liao (this laboratory). The bacterium was grown on *Pseudomonas* agar F (Difco Laboratories) for 2 to 4 days at 20°C. Mucoid growth was removed from the agar surface by using water and a bent glass rod. Cells were removed by centrifugation and subsequent filtration (0.45- μ m filter). Crude EPS was dialyzed extensively against water at 4°C and was purified by the method of Sutherland (11). Protein content was further reduced to less than 1% by extraction

with cold buffered phenol (5). The partially purified polysaccharide was then chromatographed on a column packed with DEAE-Sepharose CL-6B (Pharmacia, Inc.), using a 0 to 1 M NaCl gradient in 0.05 M Tris hydrochloride (pH 7.2) for elution. The fraction eluting between 0.5 and 0.6 M NaCl was collected.

Constituent characterization. Sugars were identified by gas chromatography analysis of the aldonitrile derivatives. Both pyruvate and succinate were identified by ¹³C-NMR (Table 1) and HPLC and gas chromatography (methyl esters) co-chromatography with authentic samples.

Removal of substituents. Succinate ester was hydrolyzed at pH 11.5 (3 h, room temperature). The pyruvate ketal was hydrolyzed in 50 mM oxalic acid at 100°C for 90 min. In both reactions, the solutions were centrifuged, the supernatants were dialyzed, and the retained materials were lyophilized. Oxalic acid treatment also resulted in significant succinate hydrolysis.

Oligosaccharide preparation. EPS (50 mg) was hydrolyzed for 3 h at 95°C in 0.1 M H₂SO₄ (10 ml). After neutralization with BaCO₃ and deionization with Amberlite MB-3 resin (Sigma Chemical Co.), the sample was concentrated under a stream of N₂ to 1 ml. The sample was then fractionated by HPLC on an Aminex Q-15S Ca²⁺-form column (2 by 30 cm; Bio-Rad Laboratories), using deionized H₂O as the mobile phase. The fractions corresponding in retention volumes to the tetra-, tri-, and disaccharides and glucose and galactose (as determined by maltoligosaccharide and monosaccharide standards) were collected, neutralized with BaCO₃, filtered, and lyophilized. A sample of the disaccharide fraction was hydrolyzed to monosaccharides (1 M H₂SO₄); as expected, glucose and galactose were the only sugars present.

Glycosidase assay. To an aqueous solution of a monosaccharide-free disaccharide fraction (1 mg/ml), 1 mg of glycosidase was added, and the solution was incubated at 37°C for 3 h. The solution was then boiled for 1 min to denature the enzyme, filtered (0.45- μ m filter), and taken to dryness under a stream of N₂. The sample was divided into two fractions; one fraction was hydrolyzed with 1 M H₂SO₄, and both fractions were analyzed for monosaccharides in the usual manner.

NMR analysis. ¹³C-NMR spectra were obtained in D₂O at ambient pH (ca. 5) and also, for desuccinylated samples, at pH 12. At the latter pH, line broadening was greatly reduced. About 10,000 scans were accumulated for each spectrum, using a 5-s pulse delay.

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