

## Note

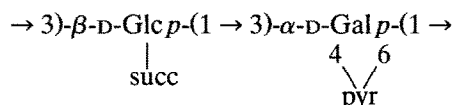
# Structure of the acidic exopolysaccharide of *Pseudomonas marginalis* Strain ATCC 10844 \*

Stanley F. Osman and William F. Fett

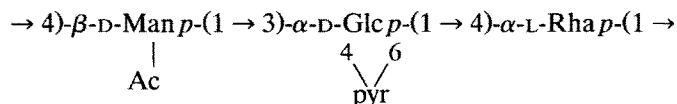
US Department of Agriculture, ARS, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, Pennsylvania 19118 (USA)

(Received June 9th, 1992; accepted in revised form October 20th, 1992)

*Pseudomonas marginalis* is a phytopathogenic bacterial species that is a causative agent of soft rot in stored fruits and vegetables. Work in our laboratory has indicated that strains of this species are heterogeneous with respect to the acidic exopolysaccharides (EPS) they produce. We have recently characterized<sup>1</sup> the acidic EPS from strain HT041B as:



and<sup>2</sup> the acidic EPS from strains PF-05-2 and PM-LB-1 as:



We now report the characterization of an additional acidic EPS isolated from the type stain (ATCC 10844) of *P. marginalis* as a (1 → 4)-β-D-glucan substituted alternately with fucose and an (*R*)-1-carboxyethyl substituent at the 3 position. The acidic nature of this polysaccharide is due to the presence of the 1-carboxyethyl substituent. This substituent has been found in the EPS of *Aerococcus viridans* var. *homari*<sup>3</sup>, *Klebsiella* type 66 (ref. 4) and *Mycobacterium lacticolum* strain 121 (ref. 5), none of which are plant pathogens. When grown on sucrose-containing agar medium, strain ATCC 10844 produces a neutral fructan (levan) as an EPS.

\* Mention of brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

## RESULTS AND DISCUSSION

Initial centrifugation of the cells plus EPS, obtained by washing of agar-plate cultures, resulted in a pellet of noncapsulated cells and a very turbid, viscous supernatant containing capsulated cells as determined by wet mounts using India ink. The capsular EPS could readily be removed by homogenization of cells left in the supernatant in a commercial blender. The resultant mixture of capsular and noncapsular EPS was then purified by dialysis, repeated acetone precipitation, ultracentrifugation, and anion-exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia, Inc.). From 30 agar plates, ~ 200 mg of partially purified EPS containing < 1% protein was obtained after the ultracentrifugation step. Elution of the EPS from the anion-exchange column required 0.2–0.4 M NaCl, indicating the polysaccharide to be acidic; however, tests for the presence of uronic acid, pyruvate, succinate, and phosphate were negative.  $^{13}\text{C}$  NMR analysis indicated the presence of a carboxyl carbon (182.2 ppm). Monosaccharide analysis of an  $\text{H}_2\text{SO}_4$  hydrolyzate by GLC–MS of the aldonitrile derivatives indicated that the EPS contained fucose, glucose and a late-eluting compound ( $t_R$  > galactose) in a molar ratio of 1:1:1. The late-eluting compound was not observed in the gas chromatograms of the alditol acetates. The  $^{13}\text{C}$  NMR spectrum showed 2 high field resonances at 16.0 and 18.2 ppm, one of which could be assigned to C-6 of fucose. The unknown sugar was isolated by preparative HPLC and treated with  $\text{BCl}_3$ . Glucose was identified as the sugar product of this reaction by GLC–MS of the aldonitrile derivative. The mass spectrum of the acetylated aldonitrile derivative of the carboxyl-reduced unknown was consistent with the identity of this compound as 3-*O*-(1-carboxyethyl)glucose in that ions arising from fragmentation on either side of the 3-substituted carbon, namely,  $m/z$  347 and 228, were observed. The *R* and *S* diastereomers of this compound were synthesized from 1,2,5,6-di-*O*-isopropylidene- $\alpha$ -D-glucofuranose and (*R*)- and (*S*)-2-chloropropanoic acid. The mass (aldonitrile derivative) and the  $^{13}\text{C}$  NMR (underivatized compounds) spectra were identical to those of the unknown compound. The aldonitrile derivatives of the diastereomers were separable by GLC. It was determined that the sugar was 3-*O*-[*R*-1-carboxyethyl]-D-glucose by co-chromatography of the synthetic compounds with the compound isolated from the EPS hydrolysate.

Permethylation analysis of the EPS indicated the presence of terminal fucose; 3,4-disubstituted glucose, and 4-substituted 3-*O*-[(*R*)-1-carboxyethyl]-glucose. Fucose was determined to be substituted at the 3-position of the 3,4 disubstituted glucose by permethylation analysis of the EPS in which the fucose had been removed by mild hydrolysis. The substitution of the carboxyethyl glucose was characterized on the basis of the MS fragmentation pattern of the methylated alditol acetate (Fig. 1). Determination of the anomeric configuration of the sugars was difficult because the polysaccharide solutions were extremely viscous at even 1 to 2 mg/mL. The  $^1\text{H}$  NMR spectrum (obtained at 80°C) showed broad anomeric resonances at 5.3 ppm and at ~ 4.4 and 4.5 ppm (the latter two were poorly

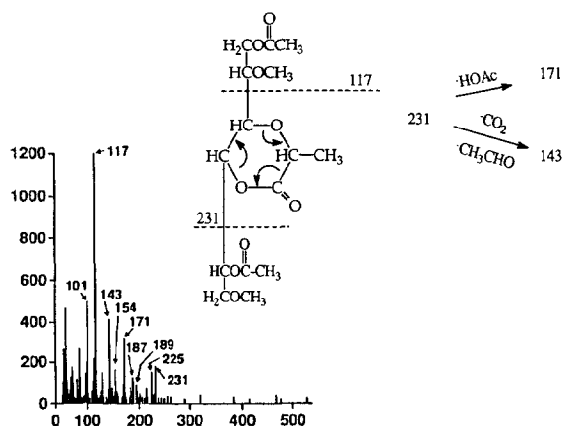
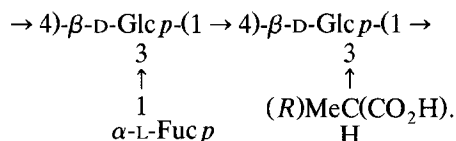


Fig. 1. Mass-spectral fragmentation pattern of the permethylated alditol acetate derivative of 3-O-[1-carboxyethyl]glucose.

resolved from the water peak). In the  $^{13}\text{C}$  NMR spectrum, anomeric signals were observed at 97.4, 102.8, and 103.6 ppm which suggested one  $\alpha$  and two  $\beta$  bonds, however it was not possible to obtain either H-1–H-2 couplings because of poor resolution in the proton spectrum or C-1–H-1 coupling because of the restriction on sample concentration due to the high viscosity of the solutions. Attempts to decrease the molecular weight of the polymer by treatment with cellulase and alpha-amylase were not successful.

In order to determine the anomeric configurations we resorted to the following procedure. The polysaccharide was subjected to mild hydrolytic conditions to selectively remove the terminal fucose (~85–90% of the fucose was removed). The resultant polymer, isolated by dialysis (12000 MW cut-off) and gel filtration, also yielded much less-viscous solutions probably due to limited backbone hydrolysis. The  $^1\text{H}$  NMR spectrum showed 2 doublets at 4.56 ( $J$  7.4 Hz) and 4.45 ppm ( $J$  8 Hz). A minor doublet for the fucose anomeric proton was observed at 5.4 ppm ( $J$  3.4 Hz).

The  $^{13}\text{C}$ - and  $^1\text{H}$  NMR shifts for the anomeric carbons and protons are consistent with pyranose ring structures. HPLC purified sugars from an acid hydrolyzate of the EPS were used for determining absolute configurations of glucose and fructose. The structure of the repeating unit of the EPS of *P. marginalis* strain ATCC 10844 is:



Interestingly most of the exopolysaccharides that we have isolated and characterized from different strains of *P. marginalis* are acidic; however the substituent

responsible for the acidic properties of the polysaccharide is variable. The composition of acidic EPS produced by strains of *P. marginalis* may be useful as a chemotaxonomic aid for the further differentiation of this species. The significance of EPS structure in host–pathogen interactions remains to be determined.

## EXPERIMENTAL

**Bacterial strain.**—ATCC (American Type Culture Collection) 10844 (type strain) of *P. marginalis* was obtained from Dr. C.-H. Liao (USDA, ERRC). The strain was stored on Difco *Pseudomonas* agar F (PAF) at 4°C.

**Preparation of EPS.**—Culture conditions, isolation and purification procedures were as previously described<sup>2</sup> except that bacteria were cultured for 1 week at 20 or 28 °C before harvesting EPS. The solid medium used (PAF) contained glycerol as the carbon source.

**General methods.**—Both GLC (Hewlett–Packard 5880A) and GLC–MS (Hewlett–Packard 5995B) analyses were carried out using an 15 M SP2230 capillary column (Supelco) or a 12 M Ultra capillary column (Hewlett–Packard). HPLC analyses were carried out on a Hewlett–Packard 1090 instrument equipped with a Hewlett–Packard 1037A refractive index detector. Optical rotations were obtained on a Perkin–Elmer 141 polarimeter. The <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained with a Jeol GX-400 spectrometer operated at 100 MHz for <sup>13</sup>C or 400 MHz for <sup>1</sup>H spectra. Spectra were run routinely at 60–75°C and Me<sub>2</sub>SO (undeuterated) was used as the internal standard in referencing both <sup>1</sup>H (2.7 ppm) and <sup>13</sup>C (40.15 ppm at 70°C) spectra.

Analytical methods for neutral sugars, glycuronans, pyruvate, acetate, and protein were as previously described<sup>6</sup>. Phosphate content was determined by the procedure of Barlett<sup>7</sup> with KH<sub>2</sub>PO<sub>4</sub> as the standard. The Taylor and Conrad procedure<sup>8</sup> was used for carboxyl reduction. The Hakomori procedure as described by Björndal, et al.<sup>9</sup> was used for permethylation analysis.

**Reduction of 3-O-(1-carboxyethyl)-glucose.**—HPLC-purified *O*-(carboxyethyl)glucose was treated with methanolic HCl followed by neutralization with AgCO<sub>3</sub>. The supernatant was concentrated, taken up in H<sub>2</sub>O and reduced with NaBH<sub>4</sub>. After neutralization with cation-exchange resin (Dowex 50W-X4), the methyl glycoside was hydrolyzed with 0.5 M trifluoroacetic acid.

**Hydrolysis of 3-O-[(*R*)-1-carboxyethyl]-glucose.**—(Carboxyethyl)glucose was isolated from an acid hydrolyzate of the EPS by preparative HPLC using an Aminex HPX-87C column (Bio-Rad) (column temperature, 65°C; eluent, H<sub>2</sub>O; flow rate, 0.4 mL/min). The retention time of the carboxyethyl glucose, under these conditions, was 10.3 min. Hydrolysis of *O*-(carboxyethyl)glucose was carried out using BCl<sub>3</sub> as previously described<sup>10</sup>.

**Preparation of 3-O-(1-carboxyethyl)-glucose.**—1;5,6-di-*O*-isopropylidene- $\alpha$ -D-glucopyranose was alkylated with either (*R*)- or (*S*)-2 chloropropanoic acid using the method described by Kochetkov et al.<sup>5</sup>. The crude product was taken up in

concd AcOH and H<sub>2</sub>O was added until the solution turned cloudy. The solution was then heated (95°C) and, after clarifying, more H<sub>2</sub>O was added until the solution became cloudy again. Heating was continued for another 90 min, the solution diluted with 2 vol of H<sub>2</sub>O and then extracted with CHCl<sub>3</sub>. The CHCl<sub>3</sub> extracts were evaporated to dryness under a stream of N<sub>2</sub>. Final purification was accomplished by preparative HPLC as already described.

*Partial hydrolysis of EPS.* — A solution of 50 mg of EPS in 0.05 M H<sub>2</sub>SO<sub>4</sub> was maintained at 70–75°C and aliquots removed periodically to monitor (by HPLC) the amount of fucose present in the solution. After the approximate theoretical amount of fucose was released from the EPS, the solution was cooled and adjusted to pH 3.5 with 1 N NaOH. The solution was then dialyzed (10 000 MW cut-off) and passed through a 10 DG desalting column (Bio-Rad). The polymer contained < 10% fucose as determined by GLC analysis of the aldononitrile derivatives of a hydrolyzate of the polymer.

#### ACKNOWLEDGMENTS

The authors thank Mr. Richard Boswell for his assistance in obtaining NMR spectra and Ms. Joanne O'Connor for her technical assistance.

#### REFERENCES

- 1 S.F. Osman, W.F. Fett, and M.L. Fishman, *J. Bacteriol.*, 166 (1986) 66–71.
- 2 S.F. Osman and W.F. Fett, *Carbohydr. Res.*, 199 (1990) 77–82.
- 3 L. Kenne, B. Lindberg, B. Lindqvist, and J. Lönnngren, *Carbohydr. Res.*, 51 (1976) 287–290.
- 4 P.-E. Jansson, B. Lindberg, J. Lönnngren and C. Ortega, *Carbohydr. Res.*, 132 (1984) 297–305.
- 5 N.K. Kochetkov, A.F. Sviridov, K.A. Arifhodzhaev, O.S. Chizhov, and A.S. Shashkov, *Carbohydr. Res.*, 71 (1979) 193–203.
- 6 S.F. Osman, W.F. Fett, and K.B. Hicks, *Carbohydr. Res.*, 176 (1988) 205–210.
- 7 G.R. Bartlett, *J. Biol. Chem.*, 234 (1959) 466–468.
- 8 R.L. Taylor and H.E. Conrad, *Biochemistry*, 11 (1972) 1383–1388.
- 9 H. Björndal, C.G. Hellerqvist, B. Lindberg, and S. Svensson, *Angew. Chem. Int. Ed. Engl.*, 9 (1970) 610–619.
- 10 T.G. Bonner and E.J. Bourne, *Methods Carbohydr. Chem.*, 2 (1963) 206–207.