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Note

Structure of the exopolysaccharide of *Pseudomonas stutzeri* strain ATCC 17588

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The isolation and structural characterization of exopolysaccharides (EPSs) synthesized by *Pseudomonas* species has been an integral part of a program in this laboratory with the goal of possible commercialization of these polymers. The EPSs which we have characterized to date were all isolated from plant pathogenic pseudomonads belonging to rRNA-DNA homology group I [1]. Most of the EPSs were demonstrated to be acidic and included alginates as well as several novel polymers which lacked uronic acids but contained acidic substituents [2–5]. We now report on the characterization of the EPS of the human opportunistic pathogen, *Pseudomonas stutzeri*. This group I bacterium can be distinguished from most other members of the group by its ability to denitrify and its inability to produce a fluorescent pigment [1]. The structure of *P. stutzeri* EPS produced by two distinct colony types has been determined. The acidic nature is not due to the presence of uronic acids, but rather to a 1-carboxyethyl (lactic acid) substituent.

1. Results and discussion

By colorimetric assay, the EPS isolated from both colony types before column chromatography contained ~ 47% neutral sugar and no detectable uronic acid or amino sugar. GLC–MS analysis of the alditol acetate derivatives of a trifluoracetic acid hydrolyzate of EPS from both colony types of *P. stutzeri* indicated that they were

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identical in composition containing glucose, mannose, and an unknown compound in a ratio of 1:1:0.7. The ^{13}C NMR spectrum of the EPS showed a downfield resonance (182.2 ppm) and an upfield resonance (21.6 ppm) indicating the presence of an *O*-(1-carboxyethyl) substituent [5]. The mass spectrum of the alditol acetate was consistent with a lactic acid derivative of a deoxyhexose. The pure unknown was isolated by anion-exchange chromatography. GLC of the aldononitrile acetate of the sugar obtained from the acidic compound by BCl_3 hydrolysis indicated that the sugar component of the unknown was rhamnose.

The substitution pattern, as determined by permethylation analysis, indicated that the glucose was 4-*O*-substituted and the mannose was 3,4-di-*O*-substituted. The mass spectrum of the permethylated unknown was consistent with 3-*O*-(1-carboxyethyl)rhamnose. The ^{1}H NMR spectrum of the acetylated, lactonized, methyl glycoside of this compound was similar to the spectrum reported for methyl 3-*O*-(1-carboxyethyl) rhamnoside [6] and by duplication of the NOE experiment described in this reference it was determined that the configuration of the carboxyethyl substituent is *R*.

Partial hydrolysis of the EPS yielded a polysaccharide depleted in 3-*O*-(1-carboxyethyl)rhamnose and containing 3-substituted mannose (as determined by permethylation analysis) as well as 4-substituted glucose and 3,4-disubstituted mannose (the last being present in diminished amounts with respect to the sugar composition of the native EPS). The monosaccharide composition of the EPS is therefore 4-substituted glucose and 3-substituted mannose substituted in the 4 position with terminal 3-*O*-[(*R*)-1-carboxyethyl] rhamnose.

Signals for three anomeric protons were observed in the ^{1}H NMR of the native EPS at 4.45 ($J_{1,2}$ 7.6 Hz), 4.75 ($J_{1,2} < 2$ Hz), and 4.95 ppm ($J_{1,2} < 2$ Hz). The ^{1}H NMR spectrum of a partially hydrolyzed EPS, which was depleted in the rhamnose substituent as determined by GLC analysis of the sugar aldononitrile acetates, showed a significant decrease in the relative intensity (to the other anomeric resonances) of the resonance at 4.95 ppm; therefore this resonance was assigned to H-1 of the rhamnose derivative. Since the resonance at 4.45 ppm must be from the H-1 of β-glucose because of the large coupling constant, the resonance at 4.75 ppm was assigned to the mannose component. The coupled ^{13}C NMR spectrum indicated one α (99.5 ppm, $J_{C\text{-}1,H\text{-}1}$ 175 Hz) and two β (102.8 ppm, $J_{C\text{-}1,H\text{-}1}$ 161 Hz, and 104.2 ppm, $J_{C\text{-}1,H\text{-}1}$ 159 Hz) anomers. By selective decoupling it was determined that the substituted rhamnose anomeric proton (4.95 ppm) was bonded to the carbon resonating at 99.5 ppm; therefore the substituted rhamnose is in the α configuration (the configuration of the glucose had already been determined to be β from the H-1–H-2 couplings). The resonances at 102.8 ppm and 104.5 ppm were assigned to the C-1 of the mannose and glucose, respectively, using the same method. The complete structure of the repeating unit of *P. stutzeri* EPS is:

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→ 4)-β-D-Glcp-(1 → 3)-β-D-Manp-(1-
                              4
                              ↑
                              1
3-O-[(R)-carboxyethyl]-α-L-Rha
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The finding that *P. stutzeri* does not produce alginate as an acidic EPS is in agreement with two previous studies. Govan et al. [8] were unable to induce alginate

producing variants of a strain of *P. stutzeri* by selection for resistance to carbenicillin, in contrast to several other group I pseudomonads. In addition, molecular genetic studies by Fiahlo et al. [9] also indicated that a strain of *P. stutzeri* was devoid of homologues to *algD* and *algR1*, two genes required for alginate production by *P. aeruginosa*. However, Goldberg et al. [10] recently reported that a strain of *P. stutzeri* genetically manipulated to contain the regulatory gene *algT* on a plasmid did produce an alginate-like polymer. The conflicting nature of these results might be due to the use of different bacterial strains as this species is known to be very diverse [1].

2. Experimental

Bacterial strain.—The type strain (ATCC 17588) of *P. stutzeri* was obtained from the American Type Culture Collection, Rockville, MD. For short-term storage the bacterium was maintained on Difco *Pseudomonas* agar F at 4°C. Long-term storage was in trypticase soybroth (BBL) amended with 20% glycerol at −85°C. Upon initial culturing on PAF, two colony types (one flat and nonmucoid, the other raised and mucoid) were present. Both colony types were isolated and purified by single-colony cloning.

Preparation of EPS.—The two colony types gave mucoid growth on PAF when the glycerol content was raised from 1 to 5%. For EPS isolation both types were grown on this medium contained in Pyrex dishes (22 × 32 cm) (two dishes per strain) for 7 days at 20°C. The cells plus EPS were harvested using a bent glass rod and distilled water. After thorough mixing, the cells were removed by centrifugation (16300 *g*, 30 min). A concentrated aqueous solution of KCl was added to the supernatant to give a final concentration of 1% (w/v) and the EPS was precipitated by addition of 2-propanol (2 vol). After being kept at 4°C overnight, the precipitated EPS was collected by centrifugation (16300 *g*, 15 min), taken up in distilled water, and dialyzed against distilled water. After dialysis the samples were subjected to centrifugation (16300 *g*, 30 min) and freeze-dried. Yields for colony type I were ~ 800 mg and for colony type II ~ 500 mg.

Ion-exchange chromatography.—Anion-exchange chromatography was carried out on a DEAE-Sepharose CL-6B column. The column was eluted with a 0–1 M NaCl gradient prepared in 25 mM Tris-HCl, pH 7.2. Carbohydrate eluted between 0.40 and 0.60 M NaCl. The carbohydrate was not retained on CM-Sepharose at pH 5.0–8.0.

General methods.—Both GLC (Hewlett–Packard 5880A) and GLC–MS (Hewlett–Packard 5995B) analyses were carried out on a 15 m SP2330 capillary column (Supelco). Optical rotations were obtained on a Perkin–Elmer model 141 polarimeter. The ^{1}H and ^{13}C NMR spectra were obtained on a Jeol GX-400 spectrometer. Polysaccharide spectra were obtained at 60°C. Spectra were externally referenced to Me_4Si. The NOE difference spectra were obtained on a Varian Gemini-200 spectrometer using the pulse sequence of Kinns and Saunders [11]. Irradiation was carried out with a 3 Hz decoupling field.

Colorimetric assay methods for the determination of neutral, acidic, and amino sugars were as previously described [3]. Permethylation analysis was carried out by the

Hakomori procedure as described by Björndal et al. [12]. The absolute configuration of the sugars was determined by the method of Gerwig et al. [13].

Partial hydrolysis of EPS.—EPS (15 mg) was dissolved in 5 mL of 0.05 M H_2SO_4 and maintained for 2.5 h at 92°C. After neutralization with $BaCO_3$ the sample was filtered and chromatographed on a DG-10 (Bio-Rad) gel-filtration column. The carbohydrate fraction that eluted in the void volume was lyophilized (yield, 15 mg).

Isolation of rhamnose.—3-*O*-(1-carboxyethyl)rhamnose ($[\alpha]_D^{23} + 10°$) was isolated from a 2 M CF_3CO_2H hydrolyzate of *P. stutzeri* EPS by anion-exchange chromatography on DEAE-Sepharose CL-6B and then hydrolyzed with BCl_3 as previously described [14]. Pure rhamnose was isolated by anion-exchange chromatography as before.

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