Extracellular Polysaccharide Produced by a Virulent Strain (U-7) of *Pseudomonas solanacearum*

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An extracellular polysaccharide was isolated from the culture medium of a virulent strain (U-7) of *Pseudomonas solanacearum*. The polysaccharide caused tomato cuttings to wilt. It seemed to be composed mainly of *N*-acetylgalactosamine together with small amounts of rhamnose, glucose, and protein. This is, to our knowledge, the first example of homo-*N*-acetylgalactosaminoglycan in nature.

Pseudomonas solanacearum E. F. Smith is a well-known bacterium associated with wilt diseases of various plants.¹⁾ When grown in culture, most strains of this bacterium form two types of colonies, fluidal and afluidal. The former strains produce an extracellular polysaccharide (EPS) and are virulent; the latter, which lack the EPS, are all avirulent.²⁾ The EPS is probably the primary factor causing wilting by increasing the viscosity of the vascular stream.

Husain and Kelman³⁾ found that the EPS produced by a virulent strain of *P. solanacearum* obtained from naturally infected tomato is a complex polysaccharide of high molecular weight, with glucose as the main component. Dudman⁴⁾ investigated the chemical composition of EPS produced by strains of *P. solanacearum* infecting tomato and banana. These EPS are mixtures of polysaccharide, mainly composed of galactosamine (2-amino-2-deoxygalactose) and glucose, and certain simple peptides. Details of the chemical properties of the EPS itself are not known. In this report, we wish to describe the production and chemical composition of the EPS from a virulent strain (U-7) of *P. solanacearum*.

MATERIALS AND METHODS

General methods. Evaporation was under reduced pressure at a bath temperature not exceeding 45°C. Optical rotations were measured with a JASCO DIP-181 polarimeter using a 100-mm microcell. Zone electrophoresis was done on Whatman GF/A glass microfibre paper with 0.1 M sodium tetraborate buffer (pH 9.3)⁵⁾ at 1500 V for 30 min; detection was with 50% sulfuric acid. GLC was on a Shimadzu GC-7A instrument fitted with a flame ionization detector. Peak areas were measured with a Hewlett-Packard 3380A digital integrator. GC-MS (70 eV) were generated with a Hitachi M-80 mass spectrometer and M-003 data processor. The total sugar content was assayed by the phenol-sulfuric acid method.⁶⁾ Hexosamine was measured colorimetrically⁷⁾ after hydrolysis in hydrochloric acid. Sugar was analyzed by GLC of the alditol acetates on a Hewlett-Packard fused-silica capillary column (50 m × 0.31 mm).⁸⁾ The ¹³C-NMR (125 MHz) spectra for solution of EPS in D₂O were recorded on a Bruker AM-500 spectrometer at 30°C using a 5-mm tube and internal methanol (49.9 ppm from Me₄Si) as the standard. The infrared (IR) spectrum was obtained using a Nicolet 60SX. Total nitrogen was assayed by elemental analysis. Acetic acid was measured with GLC⁹⁾ using propionic acid as the internal standard. The GLC conditions were as follows: column, a glass column (100 × 0.3 cm) packed with Porapak QS (80~100 mesh); carrier gas, N₂ at 60 ml/min; injection temperature, 250°C; oven temperature, 150°C isothermal. Amino acids were analyzed in a JEOL JLC-6AH amino acid analyzer after hydrolysis of the sample in 6N hydrochloric acid at 110°C for 18 hr in a sealed glass

tube under nitrogen.

Bacterial cultures. The *P. solanacearum* strain U-7 was obtained from the culture collection maintained at the Utsunomiya Experiment Station, Japan Tobacco Inc. This strain is highly pathogenic to tobacco and tomato. The bacteria were grown at 27°C on an agar medium for 48 hr containing (per litre) 1 g of Casamino Acids, 10 g of Bacto-Peptone, 10 g of glucose, and 0.05 g of 2,3,5-triphenyl-tetrazolium chloride; then the bacteria were stored in sterilized distilled water in capped tubes at room temperature.¹⁰⁾ The liquid medium contained (per litre) dialyzed yeast extract and 15 g of glucose.

Extraction and purification of EPS. Bacteria were grown in a 50-ml Erlenmeyer flask containing 10 ml of liquid medium plus 0.2 ml of a sterilized aqueous suspension of about 1×10^9 cells/ml (optical density of 0.5 at 600 nm) on a reciprocal shaker at 28°C for 4 days. Cells were removed by centrifugation (15,000 g, 30 min) at 4°C. The supernatant was dialyzed against distilled water and freeze-dried to give crude EPS. For large-scale preparation, cultures containing 1 liter of liquid medium plus 10 ml of bacterial suspension in a 3-liters flask were incubated at 28°C on a rotary shaker for 4 days. After removal of cells by centrifugation, the supernatant was dialyzed against water and then poured into 4 volumes of ethanol with stirring in a cold room. After being kept in the cold overnight, the precipitate was collected by centrifugation, dissolved in water, centrifuged to remove insoluble materials, and then precipitated again by adding to 4 volumes of ethanol as before. The precipitate thus obtained was dissolved in water and freeze-dried to give purified EPS (yield, 1.5 g).

Wilt-inducing activity. Cuttings of 3-week-old tomato seedlings were placed in tap water for 1 hr before use. Five milliliters of an aqueous solution of the EPS was poured into a micro-vial, into which the tomato cuttings were inserted. After being left for 4 hr at room temperature, the cuttings were examined for symptoms of wilting, and rated on an arbitrary scale of 0~3 for the severity of wilting.

RESULTS

EPS Production

Tomato cuttings placed in the culture filtrate from *P. solanacearum* U-7 developed wilting symptoms reported by Husain and Kelman.³⁾ The culture filtrate of an avirulent strain (M4S) was less viscous than that of U-7 and did not cause wilt. The growth of the bacterium and the yield of EPS after various periods of incubation are shown in Fig. 1. After culture for 2 to 10 days, production of EPS

FIG. 1. Growth of *P. solanacearum* and the Yield of Crude EPS after Various Incubation Periods.

●—●, viable cells; ○—○, yield of EPS.

TABLE I. WILT-INDUCING ACTIVITY OF EPS

Concentration of EPS (mg/ml)	Wilting index ^a	
	Incubation period (hr)	
	1	4
5	2	3
2.5	2	3
1.25	2	3
0.625	1	2
0.3125	1	2
0.15625	0	0
0	0	0

^a 0, no wilting; 1~3, severity of wilting.

and bacterial growth were on about the same order: 12~13 mg of EPS/flask and $3 \sim 4 \times 10^{10}$ cells/ml, respectively. Addition of calcium carbonate (0.5%) increased the yield of the crude EPS by 10~20%. EPS production after 4 days of culture was influenced more by the amount of the dialyzable yeast extract (5 to 40 g/liter) than by that of glucose (10 to 25 g/liter); production was higher at the concentration of 10 g/liter glucose or 40 g/liter yeast extract.

Purification of the EPS

The EPS obtained from large-scale production seemed to be homogeneous from the results of zone electrophoresis and gel filtration on Sepharose 4B.

Wilt-inducing activity

The causing of wilt by the EPS was ex-

aminated using tomato cuttings. Table I shows the relationship between the concentration of the EPS and the symptoms of these cuttings after 1 or 4 hr incubation. The minimum concentration of the EPS that wilted the cuttings was 0.3 mg/ml.

Properties of the EPS

The EPS was water-soluble and its $[\alpha]_D^{23}$ -26.6° (*c* 0.1, water). Its molecular weight was about 600,000 by gel filtration. It gave approximately 3% sugar by the phenol-sulfuric acid method and 2% protein by the amino acid analysis. It was composed of galactosamine and small amounts of rhamnose and glucose, which were identified by GLC and GC-MS as the alditol acetates prepared after the hydrolysis. The major amino acids were alanine (21.3 mol%), aspartic acid (16.0), leucine (14.6), glutamic acid (13.1), serine (11.6), and threonine (10.1). Galactosamine was also detected by amino acid analysis. IR $\nu_{\max}$ cm^{-1} : 3291, 2977, 2932, 1730, 1650, 1550, 1379, 1315, 1247, 1125, 1060 and 949. Elemental analysis showed that C, H, and N were 38.92, 6.70, and 5.74%, respectively. Deamination of the EPS (sodium nitrite in aqueous acetic acid) did not make the molecule smaller since all of the materials were recovered from the inside of the dialysis tube after deamination reaction and successive dialysis. The EPS was not adsorbed on CM-Sephadex or AG 50W $\times 8$.

Galactosamine

The amount of galactosamine of the EPS was measured colorimetrically after hydrolysis with hydrochloric acid. Hydrolysis in 4N hydrochloric acid at 110°C for 4 hr, the usual conditions for hexosamine analysis, gave about 50% of galactosamine; weaker acidity yielded more galactosamine. The maximum yield of galactosamine (60%) was obtained when the EPS was hydrolyzed in 3N hydrochloric acid at 110°C for 30 min.

Acetyl groups

Acetyl groups were studied by GLC as acetic acid after hydrolysis with hydrochloric

acid. Hydrolysis in 2N hydrochloric acid at 100°C for 2 hr gave the maximum value of acetic acid (231 $\mu\text{g}/\text{mg}$ EPS). Without acid hydrolysis, the EPS did not give any acetic acid. Treatment of 1 mg of *N*-acetylgalactosamine in 2N hydrochloric acid at 100°C for 2 hr yielded 256 μg of acetic acid (theoretical value, 272 μg).

DISCUSSION

A virulent strain (U-7) of *P. solanacearum* produced EPS that wilted tomato cuttings. After 2 days of culture, the production of EPS reached a maximum (12~13 mg EPS/10 ml medium); this level did not much change when the culture period was longer. During culture, the cell numbers were unchanging ($3\sim 4 \times 10^{10}$ cells/ml), suggesting that the bacterium may not use the EPS as a carbon or nitrogen source.

Chemical analysis of the EPS by GLC of the alditol acetate derivatives after acid hydrolysis indicated that the major component of the EPS is galactosamine, which coincides with the results of Dudman⁴⁾ and Whatley *et al.*¹¹⁾ However, this analytical method cannot detect *N*-acetylhexosamine, since acid hydrolysis should cause de-*N*-acetylation of *N*-acetylhexosamine to the corresponding hexosamine. The possibility that the galactosamine residue is *N*-acetylated was considered from the basis of the following evidence. (a) The EPS molecule was not degraded under conditions that cause the deamination of galactosamine, and the material recovered from the inside of the dialysis tube still could cause wilt. (b) The EPS was not adsorbed on cation exchange resins (CM-Sephadex and AG 50W $\times 8$). (c) The IR spectrum had bands at 1550 and 1650 cm^{-1} , indicating the presence of an NH-CO linkage. (d) Acid hydrolysis of the EPS yielded 23% acetic acid, so the EPS was about 90% *N*-acetylgalactosamine, assuming that the acetic acid was exclusively derived from *N*-acetylgalactosamine. (e) The ¹³C-NMR spectrum (125-MHz) of the EPS showed signals of carbonyl (~ 175 ppm) and methyl groups (~ 23 ppm) of *N*-

FIG. 2. ^{13}C NMR Spectrum of EPS.

acetylgalactosamine (Fig. 2). (f) Elemental analysis showed that the N content of EPS was 5.74%, indicating that the EPS was about 85% *N*-acetylgalactosamine, assuming that all nitrogen was derived from the sugar residue. These observations strongly suggest that the main part of EPS is poly-*N*-acetylgalactosamine. The maximum value for the galactosamine content obtained colorimetrically was 60%, corresponding to 70% *N*-acetylgalactosamine. This underestimate may be due to the degradation of *N*-acetylgalactosamine residues during acid hydrolysis, suggesting that the EPS is not as acid-stable as other hexosamine polymers such as chitin and chitosan.

So far, five research groups have investigated the chemical compositions of EPS of *P. solanacearum*. Glucose was detected by Husain and Kelman,³⁾ galactosamine, glucose, and some amino acids by Dudman,⁴⁾ glucose, mannose, and various amino acids by Gowda and Rai,¹²⁾ galactosamine by Whatley *et al.*,¹¹⁾ and galactosamine, rhamnose, and glucose by Drigues *et al.*¹³⁾ Our finding that the main component of EPS is *N*-acetylgalactosamine is not in agreement with results of these groups. We do not know the reason for the discrepancy, but it might arise from differences in the strain or culture conditions used for the study, or both.

Galactosamine polymers (galactosaminoglycans) have been isolated from the culture fluid of *Aspergillus paraciticus*,¹⁴⁾ the spherule

wall of *Physarum polycepharum*,¹⁵⁾ the culture fluid of *Neurospora*,¹⁶⁾ and the culture fluid of *Cordyceps ophioglossoides*.¹⁷⁾ All these are fungi, whereas our study gives an example of galactosaminoglycan from bacteria. In the galactosaminoglycans reported so far, the galactosamine residues were partly acetylated or not at all. In *A. paraciticus*, an enzyme (deacetylase) is thought to participate in the synthesis of the polysaccharide.¹⁸⁾ In the EPS of *P. solanacearum*, almost all of the galactosamine residues were *N*-acetylated, so this bacterium does not have deacetylase.

The EPS preparation also contained neutral sugar and protein in very small amounts compared to *N*-acetylgalactosamine. Whether such minor components are part of the *N*-acetylgalactosaminoglycan or are contamination is not known.

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