

PURIFICATION AND CHARACTERIZATION OF *XANTHOMONAS CAMPESTRIS* pv. *GLYCINES* EXOPOLYSACCHARIDE

The exopolysaccharides (EPS) of virulent and avirulent strains of *Xanthomonas campestris* pv. *glycines*, causal agent of bacterial pustule disease of soybean, and one strain of the soybean non-pathogen *X. c.* pv. *campestris* were isolated, purified, and their compositions compared. EPS produced by *X. c.* pv. *glycines* in a completely defined medium appears to be identical to the well-characterized EPS produced by *X. c.* pv. *campestris* (commonly referred to as xanthan gum). The EPS of all strains was composed of the carbohydrates glucose, mannose and glucuronic acid with acetyl and pyruvyl substituents present. Permethylated analyses indicated EPS preparations had identical hexose substitution patterns. Avirulent strains of *X. c.* pv. *glycines* produced as much or more acidic EPS as did virulent strains in vitro. None of the EPS preparations were active as elicitors of the soybean pterocarpanoid phytoalexin glyceollin as determined by a soybean cotyledon bioassay.

Key words: *Xanthomonas*; exopolysaccharide; elicitor activity; xanthan gum; glyceollin; bacterial pustule

Introduction

The bacterium *X. c.* pv. *glycines* is the causal agent of bacterial pustule disease of soybean [1]. This pathogen primarily invades leaves of susceptible soybean cultivars resulting in the formation of small erumpent blisters or pustules. Occasionally, non-pathogenic mutants arise after culture in the laboratory which do not cause pustular lesions to form and show reduced growth in vivo [2].

Cell surface components (exopolysaccharides and lipopolysaccharides) of plant pathogenic bacteria are thought to play a role as virulence factors and in host-pathogen recognition mechanisms [3,4]. Often avirulent mutants of plant pathogenic bacteria

lack the ability to form EPS and are immobilized by electron-dense material of unknown composition in their host [4].

We have found avirulent strains of *X. c.* pv. *glycines* and the crucifer pathogen (soybean non-pathogen) *X. c.* pv. *campestris* strain Xc42 to be immobilized by electron-dense material in the intercellular leaf spaces of soybean cv. Clark, which is susceptible to bacterial pustule disease [5]. However, these strains did not induce accumulation of appreciable levels of the soybean pterocarpanoid phytoalexin glyceollin [2]. Virulent strains of *X. c.* pv. *glycines* are not immobilized [5], but some virulent strains did induce glyceollin accumulation in leaves [2].

The purpose of this study was to determine if avirulent strains of *X. c.* pv. *glycines* produce exopolysaccharide and if so to compare the chemical composition, and the phytoalexin elicitor activity of purified EPS preparations from virulent and avirulent strains of *X. c.* pv. *glycines* and *X. c.* pv. *campestris* strain Xc42.

Abbreviations: DMSO, dimethyl sulfoxide; EPS, exopolysaccharides; GC, gas-liquid chromatography; MC, mass spectrometry; TLC, thin-layer chromatography.

Materials and methods

Bacterial strains

The source and origin of bacterial strains used in this study have been reported previously [2]. *X. c. pv. glycines* strains XP175 and A are virulent on susceptible soybean cultivar Clark while strains 1136 and S-9-8 are naturally occurring avirulent mutant strains [2]. *X. c. pv. campestris* strain Xc42 is pathogenic towards cabbage but not towards soybean [2].

Isolation and purification of bacterial EPS

Bacteria were grown overnight on Difco* nutrient agar (Difco Laboratories, Detroit, MI, U.S.A.) at 28°C. Cells were scraped off the agar surface and suspended in sterile water to give an $A_{600\text{nm}}$ of 1.0. Ten milliliters of bacterial suspension were added to 1 l of a completely-defined medium contained in a 2800-ml Fernbach flask. The completely defined medium used was based on the study of Souw and Demain [6] and had the following composition: KH_2PO_4 , 6.8 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; citric acid, 2.0 g; H_3BO_3 , 0.006 g; ZnO, 0.006 g; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.0024 g; CaCO_3 , 0.02 g; HCl, 0.13 ml; L-glutamic acid, 0.22 g; distilled water, 1 l. The pH of the medium was adjusted to 7.0 with NaOH before sterilization. Sucrose in water was sterilized separately and added to the cooled medium to give a 4% (w/v) final concentration. Fernbach flasks were shaken (150–200 oscillations/min) at 28°C. Bacterial growth was monitored by following the culture $A_{660\text{nm}}$.

When cultures reached early stationary phase, samples were removed and checked by serial dilution plating for the absence of microbial contaminants. Cultures were then centrifuged at $25\,400 \times g$ for 1 h. The supernatant fluids were collected and reduced in volume by a factor of 5–10 by rotary evapor-

ation under vacuum at 65°C. Concentrated fluids were dialyzed extensively against distilled water at 4°C. The dialyzed culture fluids were centrifuged at $27\,000 \times g$ for 20 min to remove any particulates. Purification of EPS was done according to Sutherland [7].

Characterization of bacterial EPS

Protein was measured by the colorimetric procedure of Markwell et al. [8] with bovine plasma albumin as the standard. Pyruvate content was determined by an enzymic method which utilized lactate dehydrogenase [9]. Content of acetyl groups was determined by the colorimetric hydroxamic acid method [10] with glucose pentaacetate as standard.

Neutral sugar composition was determined by preparation of aldonitrile derivatives [11]. Analyses were done by gas-liquid chromatography (GC) using a Hewlett-Packard model 5880 GC with a 25-m SP-2330 capillary column.

Permethylation analysis on selected EPS preparations was done to compare hexose substitution patterns. Two milligram of EPS was dissolved in 1 ml of dimethyl sulfoxide (DMSO) to which 3 ml of dimethyl K (prepared by dissolving 17 g of potassium *t*-butoxide in 50 ml DMSO) was added. The mixture was sonicated (Bransonic 12) for 30 min and then stirred for 18 h. Methyl iodide (1 ml) was added to the water-cooled solution with stirring. After 1 h the reaction mixture was diluted carefully with H_2O to a volume of 20 ml, the solution was extensively dialyzed against distilled water for 24 h, and was then lyophilized. The lyophilized product was hydrolyzed in two stages; first in 90% (v/v) formic acid for 1 h at 95°C, followed by 0.25 M H_2SO_4 for 16 h at 95°C. The formic acid was removed by evaporation under a stream of N_2 , the H_2SO_4 was neutralized with BaCO_3 , and the BaSO_4 removed by centrifugation. The hydrolyzed samples were taken up in water and reduced with NaBH_4 (25 mg) for 2 h. After neutralization with Dowex-50 (H^+ form) and separation

*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

from the resin by decanting, the samples were concentrated under a stream of nitrogen, taken up in 1 ml methanol containing 10% (v/v) acetic acid and again concentrated. This last step was repeated three times. The permethylated alditols were acetylated by heating at 70°C in the presence of pyridine (1 part) and acetic anhydride (1 part) by volume. GC/mass spectrometry (MS) analysis of the methylated, acetylated alditols was done on a Hewlett-Packard model 5995 GC/MS fitted with a 25-m OV-101 capillary column.

Uronic acid content was determined using the colorimetric procedure of Blumenkrantz and Asboe-Hansen [12] with D-glucuronic acid as standard.

Assay of EPS for phytoalexin elicitor activity

Phytoalexin elicitor activity was determined by a cotyledon bioassay [13]. Cotyledons from 8- to 10-day-old plants of cv. Clark were harvested and surface-sterilized by stirring gently in 0.525% (v/v) sodium hypochlorite for 5 min. Cotyledons were then washed several times with sterile water then left to dry in a sterile laminar flowhood. Cotyledons were transferred to sterile polystyrene culture dishes lined with filter paper with five cotyledons per dish. The filter paper in each dish was wetted with 2 ml of sterile water. Using a sterilized scalpel, a portion of the lower epidermal tissues and underlying tissue was removed, forming a shallow depression. Assay solutions (20 µl vols. for EPS and buffer solutions, 2 µl for fungal elicitor preparation) were placed onto the wounded area of each cotyledon. Two sets of five cotyledons each were treated per sample. Culture dishes were sealed with parafilm and wrapped in aluminum foil. After 24 h of incubation at room temperature, each set of cotyledons was weighed and then placed into 125-ml Erlenmeyer flasks containing 25 ml 95% (v/v) EtOH. Flasks were kept at room temperature overnight. The 95% EtOH extract was separated from the cotyledons by filtration, and the cotyledons were rinsed with 95%

EtOH. The combined filtrate and wash were evaporated to dryness under a stream of nitrogen. Methanol (5 ml) was added to each flask and the mixture was then transferred to conical glass centrifuge tubes. Insoluble material was removed by centrifugation, the fluid was transferred to glass vials, and evaporated to dryness under a stream of nitrogen.

Glyceollin content of the samples was determined by a bioassay on thin layer chromatogram plates using *Cladosporium* sp. as the bioassay organism [14] as described previously [2]. This assay can detect as little as 0.5 µg of glyceollin [15].

Results

There were no significant differences in culture generation times between virulent and avirulent strains of *X. c. pv. glycines* with the generation times ranging between 7.5 and 9.5 h. The soybean nonpathogen *X. c. pv. campestris* strain Xc42 had a generation time of 7.0 h.

The culture fluids from *X. c. pv. campestris* and *X. c. pv. glycines* strains all gave a gelatinous clear pellet after acetone precipitation and centrifugation. Protein content of all purified EPS preparations was at most 2%, but usually less than 1%. Yields of purified EPS based on the amount of sucrose initially added to the culture medium were quite low,

Table I. Acidic group composition of bacterial EPS.

Source of EPS	% by weight		
	Uronic acid	Pyruvate	Acetate
<i>X. c. pv. campestris</i> strain 42	18 ± 1 ^a	2 ± 0	3
<i>X. c. pv. glycines</i> strain XP175	17 ± 1	4 ± 2	5
A	22 ± 1	7 ± 3	3
1136	23 ± 6	4 ± 0	3
S-9-8	23 ± 1	6 ± 1	4

^aValues for uronic acid and pyruvate are M. ± S.D. of data for two separate EPS preparations. Acetate values are for a single preparation.

ranging from a high of 1.6% for avirulent *X. c. pv. glycines* strain 1136 to a low of 0.1% for virulent *X. c. pv. glycines* strain XP175.

Examination of the aldonitrile derivatives by GC showed the neutral sugar composition of all the bacterial EPS preparations to be the same as commercial xanthan gum, namely glucose/mannose in a 1:1 molar ratio. Colorimetric assays for uronic acid content showed a range of 17–23% on a dry wt. basis for the bacterial EPS preparations (Table I). Pyruvate and acetate content ranged from 2 to 7 and 3 to 5%, respectively (Table I).

Permethylolation analysis was done on commercial xanthan gum (Sigma Chemical Co., St. Louis, MO, U.S.A.) and EPS preparations from *X. c. pv. campestris* strain 42 and *X. c. pv. glycines* strains XP175 and 1136. All samples contained unsubstituted hexose and hexose substituted at the 2; 4; 3,4; or 4,6 positions which is consistent with the known structure of xanthan gum [16,17].

The uronic acid present in acid hydrolyzed (2-*N*-trifluoroacetic acid, 90 min at 120°C) EPS samples was identified as glucuronic acid by thin-layer chromatography (TLC) on aminopropyl bonded-phase silica plates impregnated with monosodium phosphate [18]. Plates were irrigated with ethanol/water (50:50, v/v) containing 0.1% (v/v) *n*-butylamine. Sugars were visualized by spraying the plates with bromocresol green prepared by adding 0.04 g to 100 ml of ethanol and adjusting the pH with NaOH until the solution just turned blue, followed by heating the plates at 60°C for 5 min.

Phytoalexin elicitor activity

Purified EPS from virulent *X. c. pv. glycines* strain XP 175 and avirulent strain 1136 was tested for ability to elicit the accumulation of the soybean phytoalexin glyceollin by a cotyledon bioassay. The presence of glyceollin in cotyledon extracts was detected by a TLC *Cladosporium* sp. bioassay. EPS samples were prepared at 2 mg ml⁻¹ and a fungal mycelial elicitor preparation from

Phytophthora megasperma f. sp. *glycinea* was prepared by the method of Alves et al. [19] with an added final filtration step through an 0.45 µm filter before autoclaving (supplied by R. Zacharius) at 7.5 mg dry wt. ml⁻¹ in either 0.05 M citrate buffer (pH 5.0); 0.1 M phosphate buffer (pH 7.0); or 0.05 M glycine–NaOH buffer (pH 9.0).

Results of the *Cladosporium* sp. bioassay indicated that all EPS preparations lacked elicitor activity at all pH values tested, while the fungal mycelial elicitor was active at all 3 pH values. The sensitivity of the assay should have allowed the detection of 10 µg glyceollin g⁻¹ fresh wt. [15].

Discussion

The exopolysaccharide of *X. c. pv. campestris* has been extensively studied due to its various commercial applications [20]. The exopolysaccharide of *X. c. pv. campestris* is commonly referred to as xanthan gum. The structure of xanthan gum consists of a repeating pentasaccharide unit with a cellulosic backbone and trisaccharide side chains on alternating glycosyl residues [16,17]. The side chains are composed of two mannosyl residues often with *O*-acetyl or pyruvate ketal substituents and an intervening D-glucuronic acid residue. Thus, the carbohydrate composition consists of glucose/mannose/glucuronic acid at a molar ratio of 2:2:1.

The exopolysaccharides produced by virulent and avirulent strains of *X. c. pv. glycines* and by *X. c. pv. campestris* strain Xc42 in a completely defined medium were all found to be of the xanthan gum type containing glucose, mannose, and glucuronic acid. These exopolysaccharides also contained acetyl and pyruvyl substituents in varying amounts. No correlation between the amounts of acetyl and pyruvyl substituents and virulence of *X. c. pv. glycines* was found. The levels of pyruvate and acetate in xanthan gum preparations is known to be highly variable depending on the culture conditions and on the bacterial strain of *X. c. pv.*

campestris employed [21]. All exopolysaccharides from members of the species *X. campestris* which have been studied to date have very similar carbohydrate compositions [7]. Strains of the separate species *X. albilineans* and *X. fragaria* produce exopolysaccharides which differ from xanthan gum in that they contain galactose [7].

Avirulence of *X. c. pv. glycines* strains 1136 and S-9-8 in vivo also appears not to be due to a lowered ability to synthesize acidic exopolysaccharide, as avirulent strain 1136 gave the highest yields in vitro.

None of the exopolysaccharide preparations tested by the cotyledon bioassay were active elicitors of glyceollin accumulation. This is in agreement with the results of Ebel et al. [22] who found that commercial xanthan gum induced enzymes of general phenylpropanoid metabolism (phenylalanine ammonia lyase) and of the flavonoid pathway (chalcone synthase) when added to soybean cell suspension cultures, but glyceollin did not accumulate. In leaves of soybean cv. Clark, virulent *X. c. pv. glycines* strain XP175 induced accumulation of moderate levels of glyceollin while avirulent *X. c. pv. glycines* strain 1136 and the soybean non-pathogen *X. c. pv. campestris* strain Xc42 induced low levels or no accumulation, respectively [2]. Thus, the differential induction of glyceollin accumulation in soybean leaves by these strains does not appear to be due to the differential production of elicitor active or nonactive acidic exopolysaccharides. Acidic exopolysaccharide production may be important for bacterial adhesion to leaf surfaces and for maintenance of a favorable environment for bacterial growth in leaf intercellular spaces during early stages of pathogenesis [23].

Acknowledgments

The authors wish to thank Ms. Grace Maher, Mr. Bob Seaner, and Mr. Dave Hilber for their excellent technical assistance; Dr. Landis Doner for performing the TLC analyses

for glucuronic acid, and Dr. R. Zacharius for supplying the *Phytophthora* elicitor preparation.

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