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Pustulan and branched β-galactofuranan from the phytopathogenic fungus *Guignardia citricarpa*, excreted from media containing glucose and sucrose

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Abstract

Guignardia citricarpa is a phytopathogenic fungus and the causal agent of citrus black spot. Incubation in a semi-defined media resulted in formation of exopolysaccharides [EPS(s)]. A medium containing glucose gave rise to a (1 → 6)-linked β-glucan (200 kD), pustulan, which was characterized by NMR and methylation analysis. A sucrose-containing medium provided a homogalactan (376 kD) and methylation analysis showed nonreducing end- (20%), 6-*O*- (53%) and 5,6-di-*O*-substituted Gal*f* units (27%). An HMQC spectrum of the homogalactan showed C-1/H-1 signals at δ 108.2/4.820, 108.3/4.820 and 107.1/5.079, corresponding to three types of β-D-Gal*f* units. A DEPT analysis showed inverted signals (CH_2) at δ 67.8 and 67.2, corresponding to 6-*O*-substituted β-D-Gal*f* units, whereas a C-5 signal at δ 77.0 suggests 5-*O*-substitution, confirming a novel structure for a β-galactofuranan.

Keywords: *Guignardia citricarpa*; Exopolysaccharides; Fungus; Citrus pathogen

1. Introduction

The important citrus pathogen *Guignardia citricarpa* is the causal agent of citrus black spot, a disease which has an important impact on world-wide citrus production, since the affected fruit is virtually useless for commercial and industrial use.

We now examine three different isolates of *G. citricarpa*, which form exopolysaccharides (EPS(s)) and have determined their yields when media containing glucose and sucrose as carbon sources were employed. Structural analysis of EPS(s) was carried out in detail for the PC13 strain. While the glucose-based medium gave rise to a glucan, that containing sucrose gave a galactan.

2. Materials and methods

2.1. Media and organisms

The mineral medium (mm), used for obtaining of the *G. citricarpa* isolates, had the following composition (g l^{-1}): $NaNO_3$, 6.0; KH_2PO_4, 1.5; KCl 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4$, 0.01; $ZnSO_4$ 0.01, with its pH adjusted to 5.8 with NaOH. To obtain different EPS(s), MM was complemented with glucose or sucrose as carbon sources (10.0 g). The basic mineral complemented medium (mc), used for mycelia cultivation and EPS production, had the same composition as the MM medium, with 1.5 g l^{-1} of commercial hydrolysed casein and the following vitamins (μg ml^{-1}): nicotinic acid, 1.0; *p*-aminobenzoic acid, 0.1; biotin, 0.002; pyridoxine, 0.5; riboflavin, 1.0; and thiamine 0.5.

Three isolates of *Guignardia citricarpa* were studied. Strain PC13, was kindly supplied by Dr Aguilar-Vildoso from Centro de Citricultura Sylvio Moreira; Instituto Agronômico de Campinas, Cordeiropolis, São Paulo-SP, Brazil. Strains EC145 and ECPR8 were isolated from apparently healthy citrus leaves. They were each aseptically immersed in 70% aq. EtOH for 1 min, followed by 3% aq. NaOCl w/v for 4 min, and washed with 70% EtOH for 30 s. The externally disinfected leaves were then cut into squares of 5 mm, which were transferred to plates containing the MC medium with a tetracycline adjust (100 μg ml^{-1}) and incubated at 28°C for 25 days. EC145, ECPR8 and PC13 isolates, were

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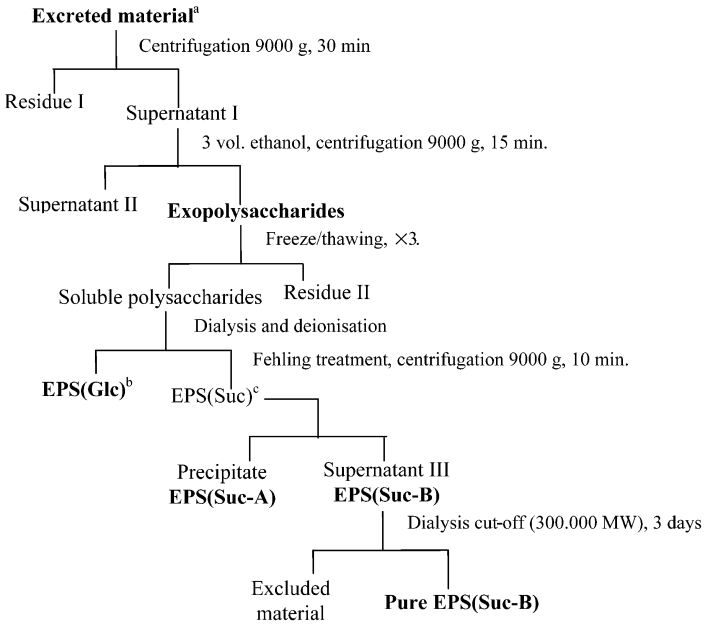

Scheme 1.

classified as *Guignardia citricarpa* by Dr H. A. van der Aa from the Centraalbureau voor Schimmelcultures, PO Box 273, 3740 Ag, Baarn, The Netherlands.

2.2. Inoculum preparation and culture conditions

Colonies grown for 15–25 days on MC were collected, diluted in 5 ml of aseptic 1% Tween 80 v/v, and macerated with a sterile glass rod in order to liberate spores by pycnidia rupture. The resulting suspensions were then filtered through glass wool and the spores counted using a Neubauer chamber. A volume containing 10^3 spores was then inoculated into 2 l Erlenmeyer flasks containing 500 ml of MC medium and incubated at 28°C for 6 days in a rotary shaker at 160 rpm.

2.3. Isolation of polysaccharides

After 6 days' incubation in the MC medium, each sample of mycelia obtained from different carbon sources of each strain were removed by centrifuging for 30 min at 9000 *g* and weighed. In order to investigate the production of EPS(s) from strain PC13, the following cultivation procedures were carried out. The supernatants were collected and added to EtOH (3 v/v), followed by overnight storage at 4°C. The resulting precipitates were recovered following centrifugation at 9000 *g* for 15 min at 4°C, then solubilised in distilled water and recentrifuged. Each supernatant was submitted to freezing followed by gentle thawing at 4°C until precipitates no longer appeared (only sub-milligram quantities of insoluble material were formed), and then dialysed against tap water for 12 h. The solution containing EPS(Glc) from the glucose medium was deionised with mixed-bed ion-exchange resins, evaporated to a small volume and added to an excess of EtOH, which precipitated polysaccharide (85 mg), $[\alpha]_D + 23°$ (*c*, 0.3). Only 1.2 mg of H_2O-insoluble material was present. The supernatant obtained on culture of *G. citricarpa* in a sucrose-containing MC medium, was submitted to the same procedure. An aq. solution was treated with Fehling solution (50 ml), and the insoluble Cu^{++} complex was removed by centrifugation, and the polysaccharide recovered by neutralisation with HOAc, dialysis against tap water and deionisation with mixed ion exchange resins. The resulting material was analysed by SEC-GPC and dialysed (cut-off 300,000 MW) against tap water, the retained material EPS(Suc-B), was finally separated from the residual EPS(Suc-A) (Scheme 1).

2.4. General analytical methods

Evaporations were performed under reduced pressure at 30°C. Gas liquid chromatography–mass spectrometry (GC–MS) was performed using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, with a DB-225 capillary column (30 m × 0.25 mm i.d.) held at 50°C for injection, and then programmed at 40°C min^{-1} to 230°C (constant temperature), with He as carrier gas. Paper chromatography was carried out by the descending method using Whatman no. 1 filter paper (solvent: *n*-BuOH–pyridine–H_2O 5:3:3 v/v), with glucose as standard: sugars were then detected by the acetone–$AgNO_3$ dip method (Trevelyan et al., 1950). The phenol–H_2SO_4 (Dubois et al., 1956) was used for quantitative sugar determinations. Specific rotations were measured in H_2O at 25°C with an Acatec model PDA-8200 polarimeter.

Table 1
Percentage correlation between the components of the biomass produced by the three strains studied under the growth conditions used

Measurement of biomass and excreted material	Isolates					
	PC13[a]	PC13[b]	ECPR8[a]	ECPR8[b]	EC145[a]	EC145[b]
Total produced biomass (%)	95.6	96.5	96.6	97.0	95.7	96.1
Total excreted material (%)	4.41	3.4	3.4	2.5	4.2	3.8
Purified EPS(s) in relation with total excreted material (%)	EPS(Glc) 67.3	EPS(Suc-B) 31.8	EPS(Glc) 69.2	nd[c]	EPS(Glc) 71.1	nd[c]

[a] Cultures grown in CM medium containing solely glucose.
[b] Cultures grown in CM medium containing solely sucrose.
[c] nd, Not determined.

2.5. Monosaccharide composition

The EPS(s) were hydrolysed with 2 M TFA at 100°C for 8 h, followed by evaporation to dryness and successive reduction with $NaBH_4$ and acetylation with Ac_2O pyridine at room temperature for 15 h. The resulting alditol acetates were then examined by GC–MS (Sawardeker et al., 1965).

2.6. Methylation analysis

Per-*O*-methylation of EPS(s) were carried out using powdered NaOH in DMSO-MeI (Ciucanu and Kerek, 1984). The products were converted into partially *O*-methylated aldose acetates by successive treatments with refluxing 2% MeOH–HCl for 2 h and total hydrolysis with 0.5 M H_2SO_4 for 14 h at 100°C, neutralised ($BaCO_3$), filtered, and the filtrate evaporated to dryness. The products were converted into partially *O*-methylated alditol acetates as described above, and analysed by GC–MS.

2.7. Periodate oxidation and Smith degradation of EPS(Glc)

The polysaccharide (50 mg) was oxidised in 0.05M $NaIO_4$ (50 ml) at room temperature for 72 h, samples of the reaction mixture being collected at intervals and periodate uptake and formic acid production determined. 1,2-Ethanediol was then added, the solution dialysed, reduced with $NaBH_4$ for 24 h, neutralised (HOAc), dialysed, and concentrated. The residue was hydrolysed with 1 M TFA at 100°C for 8 h, the solution evaporated, treated to successive $NaBH_4$ reduction and acetylation with Ac_2O–pyridine (1:1 v/v) at 25°C for 12 h, giving polyol acetates, which were analysed by GC–MS.

2.8. Determination of homogeneity and molecular weight of the EPS(s)

The polysaccharides (1.0 mg) were dissolved in distilled water and loaded on to a column of Sepharose CL-4B (Pharmacia) (21.5 × 1.8 cm i.d.), which was then eluted with 0.05 M NaOH, The resulting fractions (2 ml), were tested for carbohydrates by the phenol–H_2SO_4 method (Dubois et al., 1956). The void volume of the column was determined by elution of a standard dextran $M_r = 5 \times 10^6$: the EPS(s) were eluted after the void volume, showing only one peak. The column was calibrated using dextrans of $M_r = 81.6 \times 10^3$, 266×10^3, 5.0×10^5 and 2.0×10^6. In order to economise EPS(Suc-B), homogeneity analyses were carried out using a DAWN DSP (Wyatt Technology) light scattering apparatus with a 0.1% aq. solution employing GPC columns in series of ultrahydrogels—120, 250, 500 and 2000 eluted with 0.1 M $NaNO_3$–0.02% NaN_3. The dn/dc value of EPS(Suc-B) was 0.132 mg ml^{-1}.

2.9. ^1H and ^{13}C nuclear magnetic resonance spectroscopy

^1H and ^{13}C − NMR, ^1H, ^{13}C HMQC, COSY, TOCSY and DEPT spectra were obtained using a 400 MHz Bruker model DRX Avance spectrometer incorporating Fourier transform. Samples were dissolved in D_2O, 1% NaOD in D_2O or in DMSO-d_6 and examined at 30°C. Chemical shifts (δ) are expressed relative to resonance of Me_4Si (TMS; $\delta = 0$).

3. Results

The yields of EPS(s) excreted by *G. citricarpa* under various culture conditions depended on the strain. When grown in glucose, yields varied from 3.4 to 4.4%, of the total produced biomass, whereas cultures growth in a sucrose medium, they were from 2.5 to 3.8% of excreted material (Table 1). All detailed structural analyses were carried out with the PC13 strain.

3.1. Analysis of EPS(Glc), PC13strain

EPS(Glc) contained exclusively glucose as the monosaccharide component. The EPSs obtained from the 3 studied strains were also in pure glucans corresponding to 2.4–3.0% of the total biomass, or approximately 70% of total excreted material (Table 1).

The glucan gave only one peak with M_W 200 kD when submitted to permeation chromatography on Sepharose CL-4B. Methylation–GC–MS analysis gave rise to partially *O*-methylated alditol acetates corresponding to

Table 2
Analysis of *O*-methylated alditol acetates obtained from the methylated exopolysaccharides produced by *G. citricarpa* using sucrose and glucose as carbon sources

Carbon source	% of *O*-Me-alditol acetates			
O-Me-alditol acetates[a]	Glucose		Sucrose	
	T_M[b]	EPS(Glc)	EPS(Suc-B)	Linkage type
2,3,4,6-Me_4-Glc	1.00	0.4	–	Glc*p*-(1 →
2,3,5,6-Me_4-Gal	1.03	–	20.2	Gal*f*-(1 →
2,3,4-Me_3-Glc	1.34	96.6	–	6 →)-Glc*p*-(1 →
2,3,5-Me_3-Gal	1.42	–	53.2	6 →)-Gal*f*-(1 →
2,3-Me_2-Gal	1.82	–	26.6	5,6 →)-Gal*f*-(1 → [c]

[a] The purified exopolysaccharide were methylated as described by Ciucanu and Kerek (1984).
[b] Retentation time (T_M) relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.
[c] Concluded from the NMR experiments.

6-*O*-substituted (96.6%) and nonreducing end units of glucopyranose (0.4%), showing a linear structure having nonreducing end-units which show a molecular weight in the same range (Table 2). Oxidation of the polysaccharide with periodate yielded 0.98 mol mol^{-1} of formic acid and Smith degradation yielded only glycerol, consistent with the (1 → 6)-linked structure.

^{1}H NMR spectra showed the (1 → 6)-linked glucan to have a β-configuration, by virtue of a high-field H-1 signal at δ 4.25, $J = 7.8$ Hz. This was confirmed by a typical low field C-1 signal in its ^{13}C NMR spectrum at δ 103.1 (Fig. 1A). Typical of a hexose homopolymer, its ^{13}C spectra contained six signals (Table 3). These were assigned on COSY, TOCSY, HMQC and DEPT examination.

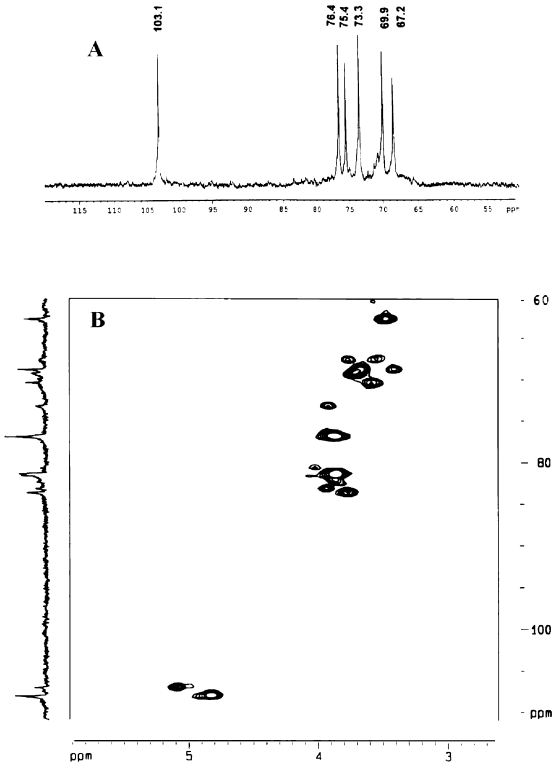

Fig. 1. A. ^{13}C-NMR spectrum (D_2O; chemical shifts in δ, PPM) of EPS(Glc), B. HMQC spectrum of (DMSO-d_6; chemical shifts in δ, PPM) EPS(Suc-B) obtained from the *Guignardia citricarpa* PC13 isolate.

3.2. Analysis of EPS(Suc-B), PC13 strain

EPS(Suc-B) contained only galactose and when submitted to GPC analysis, it gave a single peak with M_W 376.0 kD. Methylation analysis showed the presence of non reducing ends (20%), 6-*O*-substituted (53%), and 5,6-di-*O*-substituted Gal*f* units (27%) (Table 2). The presence of three different Gal*f* structures was confirmed by its HMQC spectrum with three C-1/H-1 signals at δ 108.2/4.820, 108.3/4.820 and 107.1/5.079. The low-field C-1 signals showed the Gal*f* units to have β-linked furanosyl forms (Fig. 1B). The DEPT NMR spectrum of the galactan contained an inverted CH_2 signal at δ 62.8, arising from unsubstituted C-6 moieties and which correlated (HMQC) with H-6 signals at δ 3.990 and 3.590. 6-*O*-substituted C-6 signals were at δ 67.8 (3.762, 3.542) and 67.2 (3.990, 3.590). Also, the ^{13}C and HMQC NMR spectra showed the presence of a signal to low field at δ 77.0/3.849, which could indicate *O*-substitution in C-5 (Gorin, 1981) (Table 3).

4. Discussion

The results show that the use of glucose or sucrose as carbon sources did not alter the production of mycelial biomass for the studied strains, although they were capable of providing different EPS(s) structures in the excreted material

The GPC, methylation and NMR data showed the presence of a glucan and galactofuranan from *G. citricarpa*, strain PC13. The former had the (1 → 6)-linked β-glucan structure of a pustulan, first isolated from the lichen *Umbilicaris pustulata* (Gorin and Barreto-Bergter, 1983). It has also been isolated in the *O*-acetylated form from other lichens *Gyrophora esculenta*, *Lasallia papulosa* (Shibata

Table 3
Assignments of ^{1}H and ^{13}C signals in the β-glucan and galactofuranan

Proton	EPS(Glc)				EPS(Suc-B)			
	6 →)-Glc*p*-(1 →		6 →)-Gal*f*-(1 →		6,5 →)-Gal*f*-(1 →		Gal*f*-(1 →	
	δ H/C		δ H/C		δ H/C		δ H/C	
H-1	4.25	103.3	4.820	108.2	5.079	107.1	4.820	108.3
H-2	3.01	73.3	3.762	83.7	3.911	83.2	3.762	83.7
H-3	3.17	76.6	3.849	81.4	3.849	81.4	3.849	81.4
H-4	3.10	69.9	–	–	–	–	–	–
H-5	3.30	75.4	3.911	73.3	3.849	77.0	3.911	73.3
H-6[a]	3.99[a]	67.2	3.713	68.9	3.762	67.8	3.440	62.8
H-6[a]	3.59[a]		3.341		3.542		3.440	

[a] Chemical shifts of protons linked directly to the carbon nucleus. Assignments based on ^{13}C NMR spectra of the galactocarolose reported by Gorin (1981), in correlation with our data from 1D and 2D NMR experiments.

et al., 1968) and *Sticta* sp. (Corradi da Silva et al., 1993) and as a malonic ester from *Penicillium luteum* (Anderson et al., 1939), among other fungi.

The β-galactofuranan had an unusual branched structure with mainly 6-*O*-substituted β-Gal*f* units and smaller amounts of 5,6-di-*O*-substituted and nonreducing end β-Gal*f* residues, which indicates a previously unidentified structure. β-Gal*f* units can exist as (1 → 5)-linked chains released from galactomannans, as in galactocarolose. Work on the final elucidation of the *G. citricarpa* galactan is in progress.

Formation of more than one exocellular polysaccharide can depend on the composition of the medium. For example, *Hansenula capsulata* and *Hansenula* (now *Pichia*) *holstii* normally elaborate phosphonomannans, but with the omission of orthophosphate from the growth medium, these yeasts produce a mannan (Slodki et al., 1970), similar to that of a cell wall (Gorin and Spencer, 1972). Whether our glucan and galactan are true exocellular polysaccharides, or whether one is derived from the cell wall remains to be determined.

Many functions have been attributed to the exopolysaccharides, and among them was the observation that some attachments of plant pathogens are largely mediated by them. In view of this, there is a possibility that one or more of the *G. citricarpa* EPS may be involved in biological activities, although confirmation of this should be carried out. The involvement of an extracellular mucilaginous material, called 'spore tip mucilage', in the adhesion of spores has been described by Hamer et al. (1988). Experiments involving the establishment of optimal physiological conditions for EPS(s) production by *Guignardia citricarpa*, and their possible biological functions are being carried out.

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