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Note

Three exopolysaccharides of the β-(1→6)-D-glucan type and a β-(1→3;1→6)-D-glucan produced by strains of *Botryosphaeria rhodina* isolated from rotting tropical fruit

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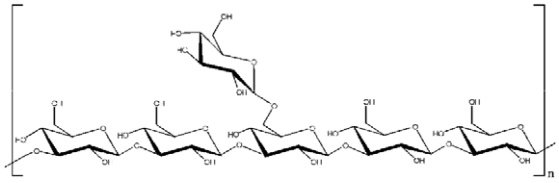
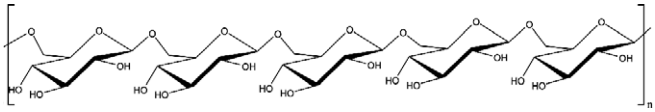
ABSTRACT

Four exopolysaccharides (EPS) obtained from *Botryosphaeria rhodina* strains isolated from rotting tropical fruit (graviola, mango, pinha, and orange) grown on sucrose were purified on Sepharose CL-4B. Total acid hydrolysis of each EPS yielded only glucose. Data from methylation analysis and ^{13}C NMR spectroscopy indicated that the EPS from the graviola isolate consisted of a main chain of glucopyranosyl (1→3) linkages substituted at O-6 as shown in the putative structure below:



The EPS of the other fungal isolates consisted of a linear chain of (1→6)-linked glucopyranosyl residues of the following structure:



FTIR spectra showed one band at 891 cm^{-1}, and ^{13}C NMR spectroscopy showed that all glucosidic linkages were of the β-configuration. Dye-inclusion studies with Congo Red indicated that each EPS existed in a triple-helix conformational state. β-(1→6)-D-Glucans produced as exocellular polysaccharides by fungi are uncommon.

Basidiomycetous[1–3] and ascomycetous fungi[4,5] have been used to obtain extracellular D-glucans, mainly β-glucans, and in particular, the more common (1→3)- and (1→3,1→6)-linked polysaccharides.[6] Among them are scleroglucan, schizophyllan, and lentinan, the most common fungal D-glucans.[7,8] It is now well recognized that β-(1→3)-D-glucans, including those of mixed β-(1→3,1→6) glucosidic linkages, are biological response modifiers (BRMs) as they are able to stimulate the nonspecific (innate) immune system of animals.[9] According to some reports, the presence of branches on C-6 and a triple-helix conformation are important factors influencing BRM activities.[9–11]

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β-(1→6)-D-Glucans play a central role in the molecular organization of the cell wall of yeast and filamentous fungi.[12–14] In *Saccharomyces cerevisiae*, β-(1→6)-D-glucans are important because they anchor the mannoproteins in the cell wall and are interconnected to the β-1,3-glucans and chitin.[15] The literature records few extracellular produced β-(1→6)-D-glucans. Among them is a β-(1→6)-D-glucan (lutean) produced by *Penicillium luteum* Zukal,[16] which although having a β-(1→6)-D-glucan backbone, carries *O*-malonyl groups. However, its chemical analysis suggests that linkages other (1→6) may be present. Sassaki et al.[17] on the other hand found a typical unbranched β-(1→6)-D-glucan produced by the causal agent of citrus black spot, *Guignardia citricarpa*, when grown on glucose as the carbon source.

The ascomycete, *Botryosphaeria rhodina* MAMB-05, produces a family of botryosphaerans (β-(1→3,1→6)-D-glucans[5]) when cultured on different carbohydrate substrates with highest yields produced on sucrose.[18,19] The cell wall of this fungal isolate furthermore produced a series of D-glucans including a mixed-linked β-(1→3,1→6)-glucan and a β-(1→6)-glucan, in addition to a starch-like glucan.[20]

We now report on the exopolysaccharides produced by four isolates of *B. rhodina* obtained from rotting tropical fruits (graviola, mango, pinha, and orange). These strains were grown on sucrose as sole carbon source, and are designated EPS_{SUC-1}, EPS_{SUC-2}, EPS_{SUC-3}, and EPS_{SUC-4}, respectively. With the exception of the graviola isolate, three *B. rhodina* isolates produced β-(1→6)-D-glucans as the sole exopolysaccharide. This finding is uncommon considering that these D-glucans are usually found as cell-wall components in yeasts and fungi, but nevertheless are common in lichens[21] (e.g., pustulan).

Table 1
GC–MS data arising from the methylation analyses of EPS produced by *Botryosphaeria rhodina* strains isolated from graviola, mango, pinha, and orange fruits

Polysaccharide	Components[a]	t_R[b]	Mole percent (%)	Linkage
	2,3,4,6-Me_4-Glc	1.00	23	Glc*p*-(1→
EPS_{SUC-1}	2,4,6-Me_3-Glc	1.82	56	→3)-Glc*p*-(1→
	2,4-Me_2-Glc	4.20	21	→3,6)-Glc*p*-(1→
EPS_{SUC-2}	2,3,4,6-Me_4-Glc	1.00	0.6	Glc*p*-(1→
	2,3,4-Me_3-Glc	2.20	99.4	→6)-Glc*p*-(1→
EPS_{SUC-3}	2,3,4,6-Me_4-Glc	1.00	0.7	Glc*p*-(1→
	2,3,4-Me_3-Glc	2.20	99.3	→6)-Glc*p*-(1→
EPS_{SUC-4}	2,3,4,6-Me_4-Glc	1.00	0.5	Glc*p*-(1→
	2,3,4-Me_3-Glc	2.20	99.5	→6)-Glc*p*-(1→

[a] 2,3,4,6-tetra-*O*-Me-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucose, etc.
[b] Retention times of the corresponding alditol acetates compared to 2,3,4,6-tetra-*O*-methyl-D-glucose.

The amount of EPS produced by each *B. rhodina* isolate when grown on sucrose was 1.5, 0.4, 1.3, and 1.8 g/L for EPS_{SUC-1}, EPS_{SUC-2}, EPS_{SUC-3}, and EPS_{SUC-4}, respectively, and with the exception of EPS_{SUC-2}, these yields were similar to that of botryosphaeran (1.2 g/L)[5,18] produced under the same growth conditions. The crude EPS preparations contained mainly carbohydrate (>95%) and lesser amounts of protein (<5%). The EPS produced by each of the fungal isolates was purified by gel-permeation chromatography on Sepharose CL-4B with each polysaccharide sample eluting as a single peak after the void volume, indicating that they were homogeneous. Total acid hydrolysis showed only glucose as the monosaccharide for each EPS sample.

Methylation analysis of EPS_{SUC-1} (Table 1) showed 2,3,4,6-tetra-*O*-methyl-glucose, 2,4,6-tri-*O*-methyl-glucose, and 2,4-di-*O*-methyl-glucose as the main methylated sugar derivatives in the molar percentages of 23:56:21, respectively. These results demonstrated that EPS_{SUC-1} was a glucan consisting of a (1→3)-linked glucosyl backbone substituted with approx. 20% branching at C-6. By contrast, methylation and GC–MS analyses of EPS_{SUC-2}, EPS_{SUC-3}, and EPS_{SUC-4} (Table 1) gave rise to partially O-methylated alditol acetates corresponding to 6-*O*-substituted (~99%), and nonreducing end units of glucopyranose (~1%), indicating a linear structure. This evidence indicated that these exopolysaccharides were (1→6)-glucans.

FTIR spectra of each of the four EPS samples showed bands at 891 cm^{-1} and 1371 cm^{-1} indicating that all the glucosidic linkages were of the β configuration (see Supplementary data, Fig. S1). Bands at 1150, 1110, and 1040 cm^{-1} are indicative of glucose.[22,23] These results demonstrated that the four EPS samples produced by isolates of *B. rhodina* were β-glucans.

The ^{13}C NMR spectrum (Fig. 2a) of EPS_{SUC-1} showed two anomeric signals: one at 103.2 ppm that corresponded to nonreducing end units (Fig. 1, residue F), while the other at 103.0 ppm was due to 3-*O*-substituted and 3,6-di-*O*-substituted units (Fig. 1, residues A, B, C, D, and E). The β-anomeric configuration for glucopyranosyl units was shown by the C-1 signals at low field.[24] The signals at 86.6 (residues A and E) and 86.3 ppm (residues B and D) ppm were attributable to substitutions at O-3, while that at 86.0 ppm arose from 3,6-di-O-substituted units (residue C) and that at 76.9 ppm corresponded to C-3 of the nonreducing end units (residue F). Resonances at 68.8 ppm arose from substitutions at O-6 (residue C), while those at 61.2 ppm (residues A and E), 61.0 (residues B and D) and 60.9 ppm (residue F) arose from nonsubstituted C-6. The C-6 signals were confirmed from the inverted signal of the DEPT spectrum (data not shown). The signals were similar to those found in other polysaccharides.[24–26] Methylation and NMR data suggested that the degree of branching along the β-1,3-glucan chain at C-6 was about 20%. From this, we conclude that EPS_{SUC-1} has a backbone of (1→3)-linked β-D-glucopyranosyl residues, with

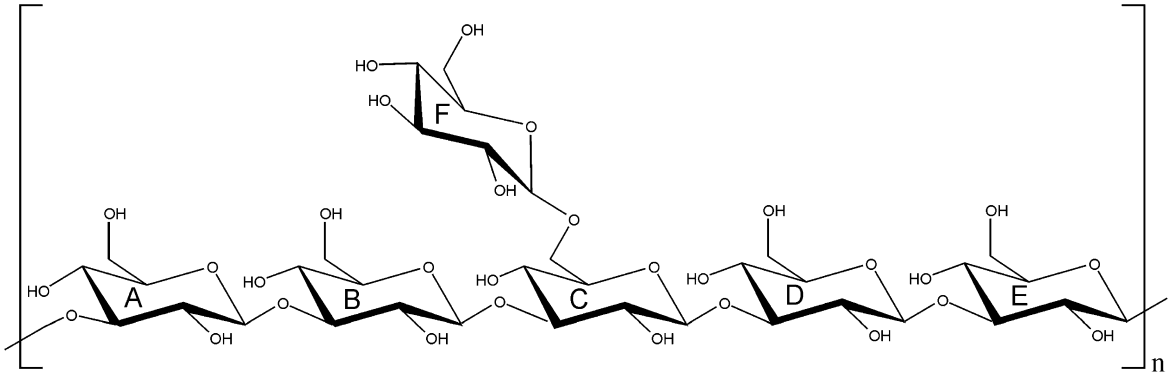

Figure 1. Putative structure of β-(1→3,1→6)-D-glucan (**1**).

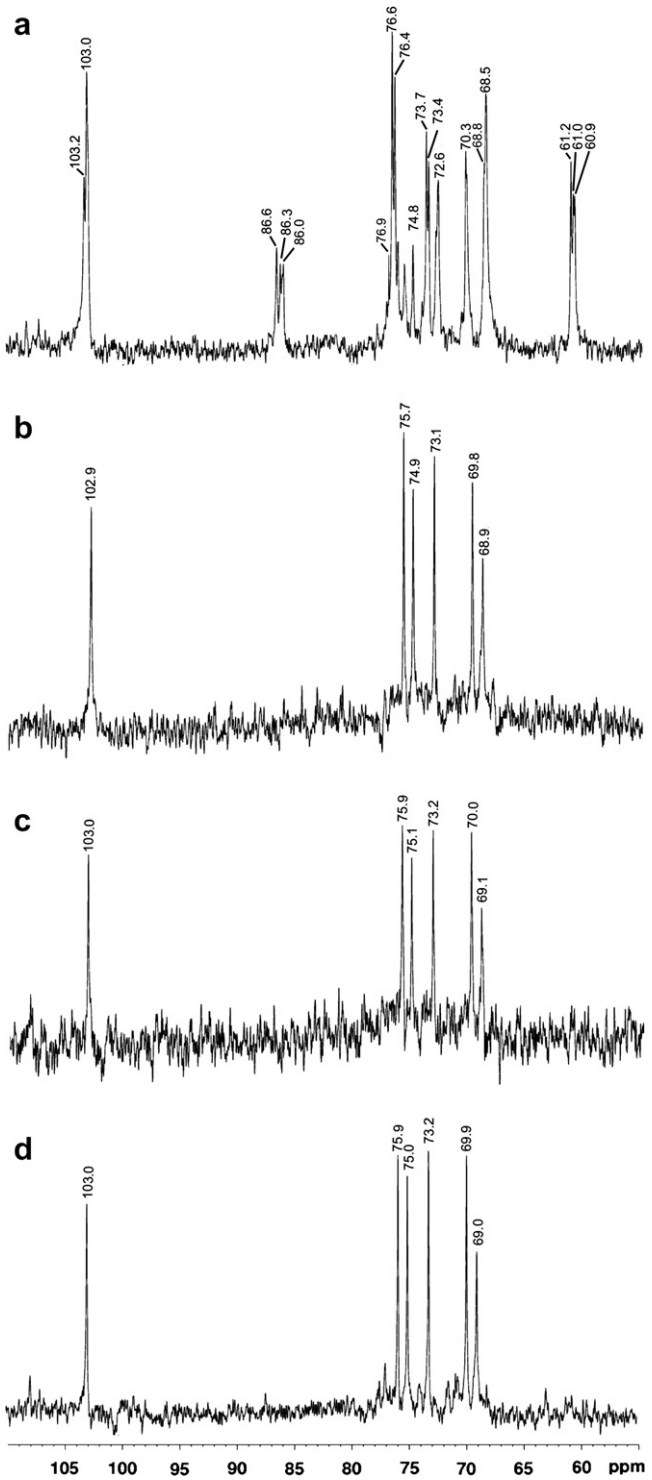

Figure 2. ^{13}C NMR spectra of exopolysaccharides from *Botryosphaeria rhodina* isolates (a): EPS_{SUC-1}; (b): EPS_{SUC-2}; (c): EPS_{SUC-3}, and (d): EPS_{SUC-4}.

one single β-D-glucopyranosyl branch substituted at O-6 on average for every five backbone units, giving rise to a putative structure as shown in Figure 1. In this respect, its structure is somewhat similar to botryosphaeran from another *B. rhodina* strain (MAMB-05, isolated from a stem canker on a eucalyptus tree) in branching frequency, but having different substituent appendages (viz., glucose and gentiobiose residues).[5]

The ^{13}C NMR spectra of EPS_{SUC-2}, EPS_{SUC-3}, and EPS_{SUC-4} showed them to be polymers with the β-configuration by virtue of a typical low-field C-1 signal at 103.0 ppm for EPS_{SUC-3} and EPS_{SUC-4}, and 102.9 ppm for EPS_{SUC-2}. The signal corresponding to the anomeric carbon of α(1→6) linkages (~99 ppm)[27] was not detected. Typical of glucose homopolymers, their ^{13}C NMR spectra contained six signals (Fig. 2b–d). The chemical shifts 75.7/75.9/75.9 ppm, 74.9/75.1/75.0 ppm, 73.1/73.2/73.2, and 69.8/70.0/69.9 ppm were attributed, respectively, to C-3, C-5, C-2, and C-4 to EPS_{SUC-2}/EPS_{SUC-3}/EPS_{SUC-4}. The absence of a signal close to 60.0–61.0 ppm, and the presence of chemical shifts between 68.9 and 69.1 ppm, indicates that these exopolysaccharides are (1→6)-linked glucans[17,20] (see Fig. 3). Preliminary experiments using a β-glucanase preparation from *Trichoderma harzianum* Rifai containing β-1,6-glucanase activity demonstrated that these exopolysaccharides were hydrolyzed, liberating glucose and gentiobiose.

Sassaki et al.[17] isolated and identified a similar exocellular β-(1→6)-glucan from *G. citricarpa* grown on a nutrient medium containing glucose. But when the glucose was replaced by sucrose, it produced an exocellular β-galactan in the furanoside form. Fungal exopolysaccharides of the β-(1→6)-glucan type are uncommon, if not rare, according to a literature search, as these polysaccharides exist mainly as constituents of the cell wall. The reason why the *B. rhodina* isolates from mango, pinha, and orange produce exocellular soluble β-(1→6)-glucans is unknown. These could arise during synthesis and remodeling processes executed during cell growth[15] and are exported into the extracellular fluid. The fact that they are produced in water-soluble form by these isolates is novel, and this will open up avenues for their production and applications as BRM polysaccharides in view of recent developments by Rubin-Bejerano et al.[28] In their study, β-(1→6)-glucans were demonstrated against expectation to be more active immunostimulants of human neutrophils than the β-(1→3)-glucans. Furthermore, they revealed that soluble β-(1→6)-glucans were a major stimulatory component of both phagocytosis and reactive oxygen species production.

Many (1→3)- and mixed-linked (1→3,1→6)-β-D-glucans adopt ordered helical conformations in aqueous solution,[29,30] and this property is important for BRM activity.[9] The intramolecular force maintaining a single-helical conformation and the intermolecular force that maintains the triple-helical conformation are hydrogen bonds.[9] Increasing the temperature in aqueous solutions above transition temperature, or dissolving the molecules in either dimethyl sulfoxide or aqueous alkali (>0.18–0.25 M NaOH/KOH) will disrupt hydrogen bonding causing transition from triple-helix to single-helix, and single-helix to random coil conformational states.[9,31]

According to Ogawa et al.,[32] polysaccharides existing in an ordered conformation form a complex with Congo Red in dilute NaOH solution as denoted by a shift in the λ_{max}, whereas they

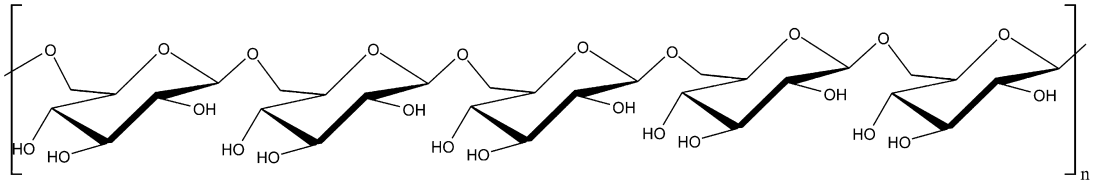

Figure 3. Structure of β-(1→6)-D-glucan (**2**).

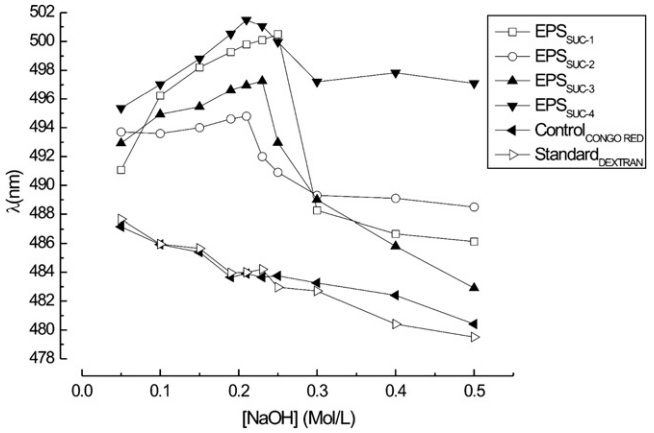

Figure 4. Helix–coil transition analysis of EPS isolated from strains of *Botryosphaeria rhodina* according to the change in the absorption maximum of Congo red-polysaccharide complex at various concentrations of NaOH. Congo red in NaOH and Dextran served as controls.

practically behave as a random coil at higher concentrations. Figure 4 shows the shift of λ_{max} at different concentrations of NaOH in the presence of the exopolysaccharides. At low concentrations (0.05–0.25 M), the λ_{max} shifted to a longer wavelength; 501.5 nm for EPS_{SUC-4}, and 500.5 nm for EPS_{SUC-1}. When the NaOH concentration was increased by more than 0.20 M, the λ_{max} dropped for EPS_{SUC-2}, EPS_{SUC-3}, and EPS_{SUC-4} (all β(1→6)-glucans), and only after 0.25 M for EPS_{SUC-1} (a β(1→3,1→6)-D-glucan). This suggests that they probably adopted a highly ordered conformation, which remained stable even under strong alkaline conditions. This finding was in agreement with that observed for botryosphaeran, an EPS produced by another *B. rhodina* strain, viz., MAMB-05, which also existed in a triple-helix conformation.[33] At various NaOH concentrations, the shift of maximum wavelength of the control and dextran (standard) was observed. But the phenomenon was different from those of the samples. So no shift was observed for dextran, an α-(1→6)-D-glucan that does not exist in a helix conformation.

1. Experimental

1.1. Microorganism and growth conditions

Four fungal isolates of ascomycetes, morphologically identified as *Lasiodiplodia theobromae*, and molecularly classified as *B. rhodina* (anamorphic form)[34] were obtained from different Brazilian tropical fruit rots. They included: *B. rhodina* MMGR (isolated from graviola, *Annona muricata*), *B. rhodina* MMMFR (mango, *Mangifera* sp.), *B. rhodina* MMPI (pinha, *Annona squamosa*), and *B. rhodina* MMLR (orange, *Citrus* sp.). The isolates were maintained at 4 °C on potato–dextrose agar.[35] The conditions of fungal growth were those as previously described for the production of botryosphaeran by *B. rhodina* MAMB-05,[5] except that commercial sucrose (50 g/L) was used as the sole carbon source for exopolysaccharide production according to Steluti et al.[18]

1.2. Preparation of exopolysaccharides

Cell-free culture fluid was obtained after removal of the mycelium by centrifugation (5500g/20 min) at 4 °C. The supernatants were treated with three volumes of absolute ethanol. The precipitated material was recovered and dissolved in distilled water, followed by extensive dialysis (MW cut-off 12,000 Da) against frequent changes of distilled water over 48 h, and then freeze-dried. The EPS produced by *B. rhodina* MMGR, *B. rhodina* MMMFR, *B. rhodina* MMPI, and *B. rhodina* MMLR were designated, respectively, as EPS_{SUC-1}, EPS_{SUC-2}, EPS_{SUC-3}, and EPS_{SUC-4}.

1.3. Analytical techniques

Total sugars were determined by the phenol–sulfuric acid method[36] with D-glucose as the standard. Protein was measured by the method of Bradford[37] using bovine serum albumin as the standard.

1.4. Homogeneity of the exopolysaccharides

The homogeneity of the exopolysaccharides was determined by gel-permeation chromatography on a Sepharose CL-4B column (1.5 × 30 cm) by applying 1 mg of each of the 4 crude exopolysaccharide samples dissolved in water (1.5 mL). The columns were eluted with distilled water at a flow rate of 0.5 mL/min. Fractions (1.5 mL) were collected and analyzed for carbohydrate and monitored for protein at 280 nm.

1.5. Composition analysis of exopolysaccharides

Each of the four purified exopolysaccharide samples (50 μg total sugar) were hydrolyzed with 5 M trifluoroacetic acid at 100 °C in a sealed-tube for 15 h.[20] Excess acid was removed by co-distillation with water after hydrolysis was completed, and the aliquots were analyzed by HPAEC–PAD (high-performance anion-exchange chromatography–pulsed amperometric detection).

1.6. HPAEC–PAD analysis

Acid hydrolysates (50 μg) of each EPS sample were dissolved in pure water (1.25 mL). Twenty-five microlitres of these solutions were used for anion-exchange chromatographic analysis by HPAEC–PAD using a Dionex DX-500 chromatography system equipped with a CarboPac PA-1 column (4 × 250 mm) and a PA-1 guard-column at a flow rate of 1.0 mL/mim, eluting with 14 mM NaOH (a mixture of water and 200 mM NaOH) as described by Corradi da Silva et al.[19]

1.7. Methylation analysis of exopolysaccharides

Each of the four EPS samples (10 mg) was methylated (3 times) using the procedure described by Haworth[38] and outlined by Ciucanu and Kerek.[39] The methylated products were isolated by partitioning in a $CHCl_3$–H_2O mixture, and the organic phase containing the methylated sugars was washed ten times with 4 mL of water and dried. The methylated products were hydrolyzed using 45% formic acid (1 mL) at 100 °C for 15 h, then reduced with sodium borohydride and acetylated with 1:1 acetic anhydride–pyridine as described by Corradi da Silva et al.[19] The products were analyzed by gas chromatography–mass spectrometry (GC–MS) using a Varian model 3300 gas chromatograph linked to a Finnigan Ion-Trap, model 810 ITD R-12 mass spectrometer. The capillary columns (30 m × 0.25 i.d.) used were (a) OV-225, and (b) DB-210, and were held at 50 °C for 1 min during injection, and then programmed at 40 °C/min to 220 °C (constant temperature).

1.8. Spectroscopic analyses

^{13}C DEPT and ^{13}C NMR determinations were carried out using a 400-MHz Bruker model DRX Avance Fourier transform spectrometer. Samples (~20 mg) were dissolved in Me_2SO-d_6 and examined at 50 or 70 °C. Chemical shifts are expressed in ppm relative to the resonance of Me_2SO-d_6 at 39.70 for samples examined in this solvent. FTIR analyses were run using discs containing polysaccha-

ride (1 mg) and KBr (250 mg). FTIR spectra were recorded on a Bruker Vector 22 Model FTIR Spectrometer. Scans were conducted in the range of 1800–500 cm^{-1}.

1.9. Helix–coil transition assay

Solutions of each EPS sample (1 mg/mL) in 0.0–0.4 M NaOH (increasing stepwise by 0.05 M increments) were prepared containing 91 μM of Congo Red.[33,40,41] After a 3-h reaction, visible absorption spectra were recorded from 400 to 700 nm at 25 °C with a Shimadzu 1601 UV–vis spectrophotometer.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.06.013.

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