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The structure of the exocellular polysaccharide produced by *Rhodococcus* sp. RHA1

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Abstract—*Rhodococcus* sp. RHA1 is a Gram-positive actinomycete capable of metabolizing a wide spectrum of organic compounds whose survival in chemically hostile environments is believed to be in part due to the production of an exocellular polysaccharide (EPS). In order to investigate the functional nature of the EPS, its structure was determined using a combinatory approach including hydrolysis, composition, and methylation, analysis methods, as well as 2D ^{1}H and ^{13}C NMR spectroscopy. The EPS was found to be a high-molecular-mass polymer of a repeating tetrasaccharide unit composed of D-glucuronic acid, D-glucose, D-galactose, L-fucose and *O*-acetyl (1:1:1:1:1), and has the structure:

$$\begin{array}{c} [\rightarrow 3)\text{-}\alpha\text{-L-Fuc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{-}(1\rightarrow]_n \\ \begin{array}{cc} 4 & 2 \\ \uparrow & \uparrow \\ \beta\text{-D-Glc}p\text{A} & \text{Ac} \end{array} \end{array}$$

Keywords: *Rhodococcus*; Exocellular polysaccharide; Structure; NMR spectroscopy

1. Introduction

Interest in exocellular polysaccharides (EPS) of Gram-positive *Rhodococcus* species has been stimulated by two major factors. The first concerns their role in pathogenicity. It was shown that EPS of pathogenic *Rhodococcus equi*[1,2] play a major role in the virulence of this zoonic pathogen, a cause of equine necrotizing pneumonia. The EPS structures of the five capsule-based serotypes of *R. equi* have been fully characterized[3–7] and have provided structural information assisting in the determination of their role in virulence, in diagnostic serology, and in their use as investigative conjugate vaccines. Secondly, EPS was demonstrated to play a role in the bacterial degradation of non-polar aromatic and aliphatic compounds. *Rhodococcus* species have attracted attention because of their extensive catabolic activities toward a wide variety of organic compounds,[8–15] including polychlorinated biphenyls (PCBs), aliphatic and aromatic hydrocarbons, and also crude oil, thus suggesting their use as potential agents for bioremediation of contaminated environments. Mucoid strains of *Rhodococcus rhodochrous* grow well in the presence of hydrocarbons in contrast to rough strains, which do not. However, the rough strains

Abbreviations: EPS, exocellular polysaccharide; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy; NOESY, nuclear Overshauser effect spectroscopy; HMBC, heteronuclear multiple bond correlation; GLC, gas–liquid chromatography; MS, mass spectrometry; PCB, polychlorinated biphenyls; PAH, polyaromatic hydrocarbons

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showed restored growth after the addition of EPS produced by the mucoid strain.[14,16] Furthermore, it was shown that the addition of EPS produced by *R. rhodochrous* strain S-2 to oil-contaminated seawater, resulted in oil emulsification and increased degradation of polyaromatic hydrocarbons (PAH) and the promotion of PAH indigenous degrading marine bacteria.[13] Thus, rhodococcal EPS plays a role, not only in cell viability and survival, but also in biodegradation of reluctant aromatic compounds.

Similar considerations pertain to Gram-negative bacteria, such as *Pseudomonas* sp.[17] and *Acinetobacter* sp.[18] where lipopolysaccharides, rather than EPS expression, are related to effective crude oil degradation.

Rhodococcus sp. strain RHA1 was isolated from hexachlorocyclohexane-contaminated soil[19] and characterized as one of the most effective aerobic PCB-degraders. RHA1 has one of the largest bacterial genomes sequenced to date, comprising 9,702,737 bp (67% G + C) arranged in a linear chromosome and three linear plasmids.[20,21] Reports have described RHA1 as a catabolic powerhouse of industrial interest for its ability to grow on a wide range of aromatic compounds: biphenyl, ethylbenzene, benzoate, phthalate, phenylacetate, phenol, 4-hydroxybenzoate, toluene, *o*-xylene, benzene, terephthalate, dibenzothiophene, 2-ethoxyphenol, guaiacol, 3-hydroxyphenylpropionic acid, 3-(2-hydroxyphenyl)propionic acid, isopropylbenzene, styrene, vanillate, veratrol and polychlorinated biphenyls.[19,22–29] The adaptation of RHA1 to soil environment and its ability to survive multiple environmental stresses make it a promising organism for bioremediation of polluted soil and oil spills.

RHA1 genome analysis predicted three large gene clusters potentially involved in the biosynthesis of polysaccharides with different sugar composition (Patrauchan, not published). The present structural study of RHA1 EPS is a step toward discovering its role in bacterial growth, survival, maintenance of metabolic activities and action as a biosurfactant.

Herein, we report the structural analysis of the *Rhodococcus* sp. RHA1 EPS that revealed it to be a high-molecular-mass polymer of a repeating tetrasaccharide unit composed of the hexopyranosyl residues of D-glucose (D-Glc), D-galactose (D-Gal), D-glucuronic acid (D-GlcA) and L-fucose (L-Fuc) (1:1:1:1) in which the D-Gal*p* residues were stoichiometrically substituted at C-2 by *O*-acetyl groups.

2. Experimental

2.1. Bacterial growth and isolation of EPS

Rhodococcus sp. RHA1 (NRCC 6316) was cultured on Chocolate Agar plates (Oxoid) at 30 °C for 72 h, and the collected cells from 10 plates were used to inoculate 2 × 4 L baffle flasks each containing 1 L of Brain Heart Infusion (BHI, Difco). The cultures were grown overnight in a New Brunswick Scientific G26 Psycrotherm Incubator at 30 °C and 175 RPM. The 2 × 1 L cultures were used to inoculate 22 L BHI medium in a new 30 L MBR fermenter with the addition of 2 mL DF204 (Mazu Chemicals) for foam control. Dissolved oxygen was controlled at 20% saturation and at 250 RPM fixed agitation and variable air/oxygen. After the culture reached A_{600} 8.8, cells were killed by incubation in the presence of 2% phenol for 2 h at 4 °C, and harvested by centrifugation.

The collected cell paste (260 g) was resuspended in water (250 mL), stirred at 60 °C, and following the addition of 90% aq phenol (60 °C, 250 mL) was further stirred for 5 min. The cooled extraction mixture was diluted with water (500 mL) and dialyzed against running tap water at 4 °C until free from phenol, and the retentate was lyophilized. The lyophilizate was taken up in 0.02 M sodium acetate (120 mL, pH 7.0), treated sequentially with RNase, DNase, and proteinase K (37 °C, 4 h each), and following the removal of solids by low speed centrifugation, the clear solution was subjected to ultracentrifugation (105,000*g*, 4 °C, 12 h). The precipitated gel of EPS was dissolved in distilled water (60 mL) and lyophilized (0.42 g).

2.2. Chromatography

Sephadex G-50 gel-filtration column (2.0 × 95 cm) chromatography was performed using 0.05 M pyridinium acetate (pH 4.6) buffer as the mobile phase and eluate monitoring using a Waters 402 refractometer. Preparative paper chromatography was performed on water-washed Whatman 3MM filter paper using *n*-butanol–pyridine–water (10:3:3, solvent A) or ethyl acetate–acetic acid–formic acid–water (18:8:3:6, solvent B) as the mobile phases, and sugars were detected on guide strips using 2% *p*-anisidine HCl in ethanol. Sugar mobilities are quoted relative to D-galactose ($R_{Gal} = 1.0$), and the sugars were collected by water elution of appropriate excised paper strips.

DEAE-Sephacel ion-exchange chromatography was made on the EPS preparation (30 mg) following passage through mixed Rexyn 101 (H^+) and Duolite (OAc^-) ion-exchange resins (1 mL). The concentrated deionized eluant was applied to a column of DEAE-Sephacel (1 × 20 cm) (Pharmacia Fine Chemicals) equilibrated with 0.05 M Tris–HCl buffer (pH 7.00). The column was eluted with the same buffer (20 mL) followed by a 0–1.0 M NaCl gradient in the same buffer. Fractions (1 mL) were collected and monitored colorimetrically for aldose by the phenol–sulfuric acid method,[30] and for uronic acid by the *m*-hydroxydiphenyl method.[31]

Gas chromatography was done using an Agilent 6850 gas-chromatograph fitted with a hydrogen-flame detector and a capillary DB-17 fused silica column (0.25 mm × 30 m). Temperature programs used were (A) 180 °C (delay 2 min) to 240 °C at 2 °C/min for acetylated alditol derivatives, and (B) 200 °C (delay 2 min) to 240 °C at 1 °C/min for methylated alditol derivatives. Retention times are quoted relative to hexa-*O*-acetyl-D-glucitol ($T_G = 1.0$) or to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol ($T_{GM} = 1.0$). The GLC mobilities and MS spectra of all prepared derivatives were identical with those of authentic reference samples.

2.3. NMR spectroscopy

Experimental conditions were the same as those previously recorded.[32] ^1H and ^{13}C NMR spectra were recorded using a Varian Inova 500 MHz spectrometer in D_2O solutions at 60 °C with acetone internal standard (2.225 ppm for ^1H and 31.1 ppm for ^{13}C) using standard pulse sequences for COSY, TOCSY (mixing time 120 ms) NOESY (mixing time 200 ms), HSQC and HMBC (optimized for an 8 Hz coupling constant).

2.4. Carboxyl reduction of EPS

Following previously recorded conditions,[33] EPS (50 mg) dissolved in water (10 mL) was adjusted to pH 4.7 with 0.05 M hydrochloric acid and, following the addition of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (170 mg), the stirred reaction mixture was maintained at pH 4.7 over 4 h by the slow addition of 0.05 M hydrochloric acid. Following the addition of sodium borodeuteride (170 mg) the mixture was stirred for 12 h at 20 °C. The reaction mixture was brought to pH 7 with acetic acid, and after dialysis against running tap water, the reduced polysaccharide (EPS-d_2, 42 mg) was recovered by lyophilization.

2.5. Methylation analysis

Samples of oligo- and polysaccharide (2–3 mg) dissolved in Me_2SO (1 mL) were methylated by the Ciucanu–Kerek procedure[34] and were hydrolyzed with 3 M TFA (105 °C, 2 h), reduced ($NaBD_4$), acetylated and analyzed using a Varian Saturn 200 ion-trap GLC–MS instrument using the GLC columns conditions described in Section 2.2.

2.6. General methods

Hydrolysis of oligo- and polysaccharide samples was made in 2 M TFA for 4 h at 105 °C, followed by concentration to dryness in a stream of nitrogen. Determination of the D- or L-configuration of isolated EPS component glycoses was made by capillary GLC of their acetylated 2-(+)-octyl glycoside derivatives according to the method and directions of Leontein et al.[35] O-Deacetylated EPS was made by the treatment of native EPS (30 mg) with 10% aq ammonia (20 mL, 37 °C, 10 h), followed by concentration and lyophilization from water solution.

3. Results and discussion

RHA1 was fermenter grown in Brain Heart Infusion with agitation and variable air/oxygen, and the cell mass was collected. Since very low yields of EPS were obtained from cell water extraction, EPS was extracted by stirring with hot 50% aqueous phenol. The diluted aqueous mixture was dialyzed against water until free from phenol and then lyophilized. The product was sequentially digested with RNase, DNase, and proteinase K, and the clear solution was ultracentrifuged to yield a precipitated EPS gel that was dissolved in water and lyophilized (yield 3.5% based on dry cell weight).

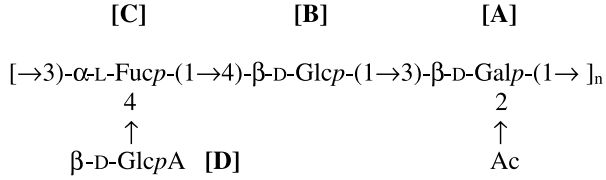
The EPS was fractionated by DEAE-Sephacel ion-exchange column chromatography from which the EPS was eluted as a discrete peak at the beginning of a sodium chloride gradient. Colorimetric analysis of the column eluate indicated that the EPS was composed of hexuronic acid and neutral glycose in the approximate ratio of 1:3. Due to the opalescence of the EPS solution, it was not possible to determine a specific optical rotation. Hydrolysis of the EPS with 2 M TFA, followed by paper chromatography (solvent A) and detection with *p*-anisidine·HCl revealed components tentatively identified as galactose (R_{gal} 1.00), glucose (R_{gal} 1.35), and fucose (R_{gal} 2.84). GLC–MS analysis (conditions A) of the reduced ($NaBD_4$) and acetylated EPS hydrolysate gave elution peaks identified as the acetylated alditol-1-*d* derivatives of galactitol, glucitol, and fucitol (1:1:0.8). A similar hydrolysis of a larger sample of EPS (40 mg), followed by preparative paper chromatographic separation of the neutral glycoses, afforded chromatographically pure samples of D-galactose, D-glucose, and L-fucose with the assigned configurations determined from their specific optical rotations and by GLC analysis of their derived acetylated 2-(+)-octyl glycosides.[35]

The EPS was subjected to two sodium borodeuteride reduction treatments using the carbodiimide procedure[33] to yield a polysaccharide (EPS-d_2), in which the hexuronic acid component was converted to the neutral aldose D-glucose-6-d_2. The latter was identified by GLC–MS as a component of an approximately equimolar mixture of hexa-*O*-acetylglucitol-6-d_2 and hexa-*O*-acetylglucitol present in the eluting peak of the EPS-d_2 acetylated reduced ($NaBH_4$) EPS-d_2 acid hydrolysate. The evidence indicated that the EPS is composed of repeating tetrasaccharide units composed of D-galact-

ose, D-glucose, L-fucose, and D-glucuronic acid, a conclusion substantiated by NMR analysis.

The reduced ($NaBD_4$) and acetylated hydrolyzed methylated EPS-d_2 polysaccharide products were identified by GLC–MS as 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol-1-*d*-6-d_2 (T_{GM} 1.00), 1,3,4,5-tetra-*O*-acetyl-2-*O*-methyl-fucitol-1-*d* (T_G 1.16), 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol-1-*d* (T_G 1.41), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol-1-*d* (T_G 1.47) in a molar ratio 1.0:0.8:0:9:0.9, indicating the repeating unit to be composed of the four glycosidically linked residues: D-Glc*p*A-(1→, →3,4)-L-Fuc*p*(1→, →4)-D-Glc*p*-(1→, and →3)-D-Gal*p*-(1→).

2D NMR analysis of the native EPS and O-deacetylated EPS gave spectra (glycose residues labeled **A**–**D** in order of decreasing anomeric proton chemical shifts) that allowed assignment of the chemical shifts and anomeric configurations of the constituent glycose components in the respective tetrasaccharide repeating units (Tables 1 and 2). The significant change in the proton shift of H-2 (5.09 ppm) in **A** to the H-2 (3.94 ppm) in **A′** in the α-D-Gal*p* (**A′** to **A**) residues in the respective native and O-deacetylated EPS spectra showed that the *O*-acetyl substituent in the native EPS was located at the O-2 of the D-Gal*p* residue. Linkages between monosaccharide residues were identified on the basis of the following NMR TOCSY and NOE cross-peaks **A**1:**C**3, **B**1:**A**3, **C**1:**B**4 and **D**1:**C**4 (Fig. 1), and corresponding HMBC correlations. The combined composition, methylation analysis, and NMR data lead to the characterization of the repeating unit of the EPS as having the structure:



Further confirmation of the proposed structure of the EPS was obtained from the characterization of two oligosaccharides obtained by mild acid hydrolysis of the O-deacetylated EPS. Following hydrolysis of the deacylated EPS with 0.05 M sulfuric acid (100 °C, 4 h), neutralization ($BaCO_3$), filtration, and concentration, the residue was dissolved in water and treated with ethanol (6 volumes). The precipitated material (Ba^{2+} salts of hexuronic acid products) was collected by centrifuga-

Table 1. ^1H and ^{13}C NMR chemical shifts (ppm) and coupling constants (Hz) of *Rhodococcus* sp. RHA1 native EPS[a]

Residue	Chemical shifts $^1H/^{13}C$					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
[**A**] →3)-α-D-Gal*p*-(1→ 2 ↑ Ac	5.31 99.1	5.09 70.9	4.19 77.1	4.22 70.2	4.31 71.7	3.68(a) 3.76(b) 62.6
[**B**] →4)-β-D-Glc*p*-(1→	4.72 104.2	3.30 74.5	3.64 75.3	3.534 78.1	3.72 75.9	3.79(a) 4.00(b) 61.3
[**C**] →3,4)-α-L-Fuc*p*-(1→	4.97 100.1	3.99 68.9	3.97 76.9	4.15 80.6	4.45 69.0	1.30 16.3
[**D**] β-D-Glc*p*A-(1→	4.61 104.1	3.49 74.2	3.54 76.2	3.64 72.7	3.82 77.2	 174.2

[a] Spectra were recorded at 60 °C (δ ppm from acetone 2.225/31.1 ppm $^1H/^{13}C$).

Table 2. ^1H and ^{13}C NMR chemical shifts (ppm) and coupling constants (Hz) of *Rhodococcus* sp. RHA1 O-deacetylated EPS[a]

Residue	Chemical shifts $^1H/^{13}C$					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
[**A′**] →3)-α-D-Gal*p*-(1→	5.25 101.4	3.94 68.8	4.00 80.0	4.22 69.6	4.23 71.7	3.71(a) 3.71(b) 62.6
[**B′**] →4)-β-D-Glc*p*-(1→	4.85 103.3	3.36 74.7	3.68 75.3	3.54 78.0	3.74 75.9	3.79 61.3
[**C′**] →3,4)-α-L-Fuc*p*-(1→	5.00 100.1	4.05 69.1	4.01 76.2	4.17 80.6	4.44 68.8	1.30 16.3
[**D′**] β-D-Glc*p*A-(1→	4.55 104.4	3.41 74.6	3.50 76.4	3.61 72.9	3.62 78.5	 174.2

[a] Spectra were recorded at 60 °C (δ ppm from acetone 2.225/31.1 ppm $^1H/^{13}C$).

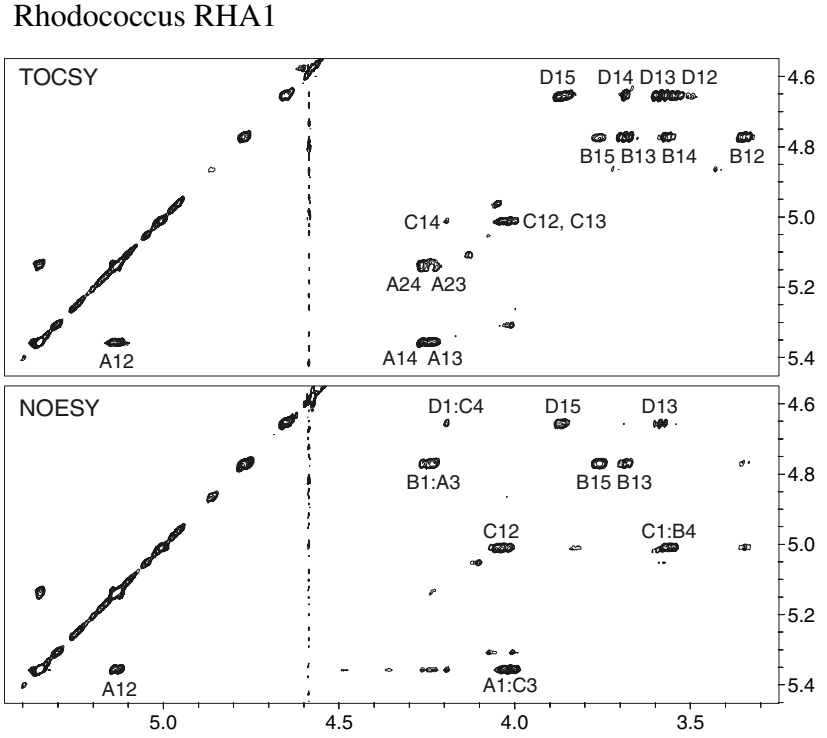


Figure 1. TOCSY and NOESY spectra of *Rhodococcus* sp. RHA1 EPS showing labeled signal correlations of the component monosaccharide residues: [**A**] 2-*O*-Ac-β-D-Gal*p*-, [**B**] β-D-Glc*p*, [**C**] α-L-Fuc*p*, and [**D**] β-D-Glc*p*A. Intraresidue correlations are labeled with the residue designation letter and the numbers of the interacting protons (e.g., **C**12 = correlation between H-1 and H-2 of residue **C**).

tion. The concentrated supernatant (neutral aldose products) was fractionated by preparative paper chromatography (solvent A) to yield the major chromatographically pure disaccharide (OS-1, R_{Gal} 0.40) composed of D-Glc and D-Gal (1:1). The reduced ($NaBH_4$) disaccharide was composed of D-glucose and

Table 3. ^1H and ^{13}C NMR chemical shifts (ppm) and coupling constants (Hz) of *Rhodococcus* sp. RHA1 EPS disaccharide hydrolysis product β-D-Glc*p*-(1→3)-α,β-D-Gal (OS-1)[a]

Residue	Chemical shifts $^1H/^{13}C$					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
→3)-α-D-Gal*p*	5.25	3.95	3.95	4.22	4.08	3.70
	93.8	68.9	81.1	70.6	71.8	62.7
→3)-β-D-Gal	4.60	3.62	3.77	4.16	3.69	3.70
	97.8	72.5	84.2	70.0	76.4	62.7
β-D-Glc*p*-(1→	4.65	3.35	3.47	3.40	3.42	—
	105.4	74.9	77.1	71.0	77.4	—

[a] Spectra were recorded at 25 °C (δ ppm from acetone 2.225/31.1 ppm $^1H/^{13}C$).

Table 4. ^1H and ^{13}C NMR chemical shifts (ppm) and coupling constants (Hz) of *Rhodococcus* sp. RHA1 EPS disaccharide hydrolysis product β-D-Glc*p*A-(1→4)-α,β-L- Fuc*p* (OS-2)[a]

Residue	Chemical shifts $^1H/^{13}C$					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
→3)-α-L-Fuc*p*	5.20	3.75	3.81	3.98	4.26	1.27
	93.9	70.4	70.3	82.8	68.2	17.0
→3)-β-L-Fuc*p*	4.57	3.42	3.58	3.93	43.84	1.30
	97.8	73.5	73.8	81.9	72.4	17.1
β-D-Glc*p*A-(1→	4.49	3.43	3.47	3.40	3.42	—
	104.7	74.8	76.9	73.2	76.9	174.2

[a] Spectra were recorded at 25 °C (δ ppm from acetone 2.225/31.1 ppm $^1H/^{13}C$).

galactitol. This finding combined with the NMR analysis (Table 3) of the disaccharide established its structure as α-D-Glc*p*-(1→3)-D-Gal.

The preparative paper chromatography (solvent B) of the deionized (Rexyn 101 (H^+)) hexuronic acid-containing product gave the chromatographically pure disaccharide (OS-2, R_{Gal} 0.34), identified by hydrolysis and GLC chromatography as being composed of D-GlcA and L-Fuc. The NMR analysis of this disaccharide (Table 4) revealed its structure to be β-D-Glc*p*A-(1→4)-L-Fuc. The characterization of the two major hydrolysis products is consistent with the proposed EPS structure and, in particular, confirms the proposed glycosyl 3-O- and 4-O-substitution positions on the α-L-Fuc*p* branched residue in the EPS backbone chain.

The EPS of other rhodococci so far examined appear to contain sugars associated with carboxylic acid functions[15,36,37] either in the form of a hexuronic acid, 1-carboxyethylidene groups bridging sugar hydroxyl groups, or a 1-carboxyethyl pyruvate group linked to a single sugar hydroxyl group. The determined structure of the RHA1 EPS conforms to that of previously characterized rhodococcal EPS as polymers composed of regularly linked acidic oligosaccharide units.

The deduced structure will facilitate further studies on genomic determinates of EPS biosynthesis in *Rhodococcus* sp. strain RHA1 and will provide insights into understanding of the EPS role in the physiology of soil actinomycetes. More detailed studies are needed to define general structural requirements for EPS optimal stability and effectiveness in bioremediation. It will be of interest to determine whether the EPS is retained by the cell and how it is localized on the cell surface in order to facilitate attachment and subsequent transport of non-polar substrates. Another area of interest is the possible role of EPS in oil dispersion and biodegradation as well as in the solubilization of heavy metals.[38]

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