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Structural analysis of mucoidan, an acidic extracellular polysaccharide produced by a pristane-assimilating marine bacterium, *Rhodococcus erythropolis* PR4

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Abstract—*Rhodococcus erythropolis* PR4 is a marine bacterium that can degrade various alkanes including pristane, a C_{19} branched alkane. This strain produces a large quantity of extracellular polysaccharides (EPS), which are assumed to play an important role in the hydrocarbon tolerance of *R. erythropolis* PR4. The strain produced an acidic EPS, mucoidan, together with a fatty acid-containing EPS, PR4 FACEPS. The chemical structure of the mucoidan was determined using ^{1}H and ^{13}C NMR spectroscopy and by conducting 2D DQF-COSY, TOCSY, HMQC, HMBC, and NOESY experiments. The mucoidan was shown to consist of a pentasaccharide repeating unit with the following structure:

Mucoidan
β-D-Glc*p*
1
↓
3
[4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*A-(1→3)-α-L-Fuc*p*-(1→]$_n$

Keywords: *Rhodococcus*; Bioremediation; Pristane; Extracellular polysaccharide; Structure; NMR spectroscopy

Abbreviations: EPS, extracellular polysaccharide; FACEPS, fatty acid-containing extracellular polysaccharides; CTAB, cetyltrimethylammomium bromide; TFA, trifluoroacetic acid; ABEE, 4-aminobenzoic acid ethyl ester; DQF-COSY, double quantum filtered-correlation spectroscopy; TOCSY, total correlation spectroscopy; HMQC, heteronuclear multiple-quantum coherence; HMBC, heteronuclear multiple-bond coherence; NOESY, nuclear Overhauser effect spectroscopy

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1. Introduction

Rhodococci are Gram-positive bacteria showing a wide variety of metabolic activities and are known to produce a family of fatty acid-containing extracellular polysaccharides (FACEPS). Previous studies[1–3] have suggested the possibility of FACEPS for use as sustainable, environmentally friendly products such as emulsifiers, moisture adsorbents, or thickeners. In the accompanying paper, we reported the chemical composition and structure of the FACEPS produced by *Rhodococcus erythropolis* PR4 (PR4 FACEPS), because the genome sequence of the strain will greatly increase our understanding of the mechanisms involved in the biosynthesis of FACEPS. During that study we showed that the strain produced another acidic extracellular polysaccharide containing little or no fatty acid, which we named 'mucoidan'. The latter EPS was composed of glucose, *N*-acetylglucosamine, glucuronic acid, and fucose at a molar ratio of 2:1:1:1.

In this study we determined the chemical structure of mucoidan based on the data obtained from ^{1}H and ^{13}C NMR spectroscopic analyses as well as 2D DQF-COSY, TOCSY, HMQC, HMBC, and NOESY experiments.

2. Results and discussion

2.1. Purification of mucoidan produced by *R. erythropolis* strain PR4

Mucoidan produced by the *R. erythropolis* strain PR4 was extracted by CTAB method and purified by DEAE-Toyopearl column chromatography, being eluted with 0.2 M NaCl (Fig. 1 of accompanying paper). Mucoidan gave a single band on cellulose acetate membrane electrophoresis (data not shown) and a broad single peak that appeared earlier than that of Dextran T2000 on Sephacryl S1000 gel-filtration chromatography (data not shown). These data indicate that mucoidan had been purified to homogeneity. Mucoidan was a white fibrous material soluble in water and alkalis but not in acids, MeOH, EtOH, or acetone.

2.2. Compositional analysis

The monosaccharide content of mucoidan was determined by TFA hydrolysis, followed by HPLC analysis, and the results showed glucose, *N*-acetylglucosamine, glucuronic acid, and fucose detected at a molar ratio of 2:1:1:1. The absolute configurations of these monosaccharides were determined by GLC of acetylated (−)-2-butyl derivatives, and the data showed that all had the D-configuration except for fucose, which had the L-configuration. Spectrophotometrically, little or no absorption was detected at 280 nm or at 255 nm, suggesting that the mucoidan did not contain proteins or nucleic acids. Fatty acids were either not detected or detected in miniscule amounts from the alkali hydrolyzate of mucoidan or from untreated mucoidan.

2.3. NMR spectral analyses

Five doublet signals were observed at δ 4.55, 4.59, 4.91, 5.00, and 5.17 in the anomeric region of the ^{1}H NMR spectrum of mucoidan (Fig. 1a). In the ^{13}C NMR spectrum of mucoidan (Fig. 1b), four signals were detected in the anomeric region at δ 99.1, 101.4, 102.8, and 103.2. Based on the results of the HMQC experiment, three anomeric carbons at δ 99.1, 102.8, and 103.2 were correlated to three anomeric protons at 5.00, 4.91, and 4.55, respectively, whereas one anomeric carbon at δ 101.4 was correlated to two anomeric protons at δ 4.59 and 5.17 (data not shown), indicating that the anomeric carbon signal at δ 101.4 was an overlapping signal consisting of two anomeric proton signals at δ 4.59 and 5.17. The relative intensities of the five anomeric proton signals at δ 4.55, 4.59, 4.91, 5.00, and 5.17 were almost equal, suggesting mucoidan to consist of a pentasaccharide repeating unit. Other characteristic signals in the

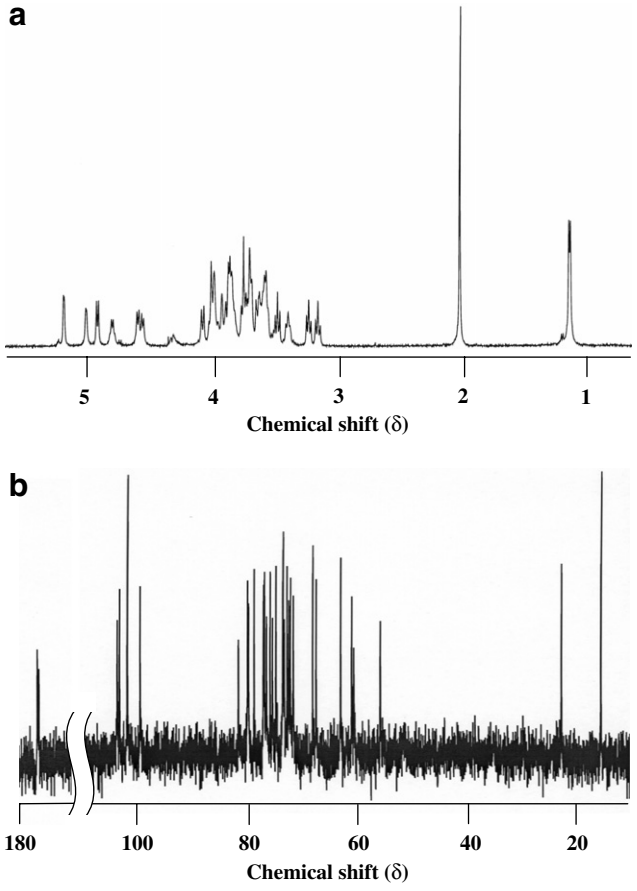

Figure 1. 500-MHz ^{1}H NMR spectrum (a) and 125 MHz ^{13}C NMR spectrum (b) of mucoidan recorded in D_2O at 70 °C.

Table 1. ^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) of mucoidan recorded in D_2O at 70 °C

Glycosyl residue	H-1 ($J_{H-1,H-2}$)	H-2	H-3	H-4	H-5	H-6a	H-6b
A: β-D-Glc*p*-(1→	4.91 (7.5)	3.24	3.49	3.17	3.40	3.61	3.90
B: →3,4)-β-D-Glc*p*-(1→	4.55 (8.3)	3.58	4.03	3.72	3.60	3.84	4.00
C: →3)-β-D-Glc*p*NAc-(1→[a]	4.59 (7.7)	3.75	3.71	3.63	3.76	3.87	4.03
D: →4)-α-D-Glc*p*A-(1→	5.17 (3.2)	3.67	3.89	3.78	4.08		
E: →3)-α-L-Fuc*p*-(1→	5.00 (2.3)	4.02	3.88	3.95	4.79	1.19	

[a] Additional chemical shift: NAc at δ 2.05.

^{13}C and ^{1}H NMR spectra of the mucoidan were as follows: The ^{13}C NMR spectrum of mucoidan (Fig. 1b) revealed one nitrogen-bearing carbon (C-2 of D-Glc*p*NAc) at δ 56.0, two methyl groups (C-6 of L-Fuc*p* and CH_3 of an *N*-acetyl group) at δ 16.3 and 23.4, respectively, and two carboxyl groups at δ 175.3 and 175.6. The ^{1}H NMR spectrum (Fig. 1a) showed two methyl groups (C-6 of L-Fuc*p* and CH_3 of *N*-acetyl group) at δ 1.19 and 2.05, respectively. The identity of the monosaccharides was established on the basis of 1D and 2D ^{1}H and ^{13}C NMR spectroscopy as described below.

The ^{1}H chemical shifts of the mucoidan (Table 1) were assigned by means of 2D TOCSY (mixing times 100–300 ms; Fig. 2, upper panel) and 2D DQF-COSY experiments (data not shown). Starting points for the interpretation of the spectra were the anomeric signals of residues **A**–**E**. Comparison of TOCSY spectra with increasing mixing times allowed the assignment of the sequential order of the chemical shifts belonging to each single spin system. Since a signal in the high-field region (δ 1.19, methyl proton) was assigned to H-6 of spin system **E**, the **E** residues were attributed to L-Fuc*p*. The results of the 2D ^{13}C–^{1}H HMQC experiment showed that the H-2 of spin system **C** was correlated to a carbon signal at 56.0 ppm (nitrogen-bearing carbon). The N-acetylation was proven by the upfield shift of C-2 and by the occurrence of an acetyl methyl group resonance at δ 23.4. The **C** residues were thus attributed to D-Glc*p*NAc. On the basis of the chemical analyses, the remaining spin systems, **A**, **B**, and **D**, were either D-Glc*p* residues or D-Glc*p*A residues. H-6 of spin system **D** was not observed, whereas the H-6's of **A** and **B** were observed, indicating that the **D** residues should be attributed to the D-Glc*p*A, and the **A** and **B** residues to D-Glc*p*. Based on the observed $J_{H-1,H-2}$ values and chemical shifts, residues **A**, **B**, and **C** were assigned the β anomeric configuration, and residues **D** and **E** the α anomeric one. All ^{13}C resonances of the mucoidan could be assigned by inspection of the 2D ^{13}C–^{1}H HMQC spectrum (Table 2).

The sequence of glycosyl residues was determined using long-range ^{13}C–^{1}H correlations obtained from NOESY (Fig. 2, lower panel) and HMBC spectra (Fig. 3). The cross-peaks of both anomeric protons of each of the glycosyl residues were examined, and intra- and inter-residual connectivities were obtained from the NOESY (Table 3). By NOESY analysis, residue **A**

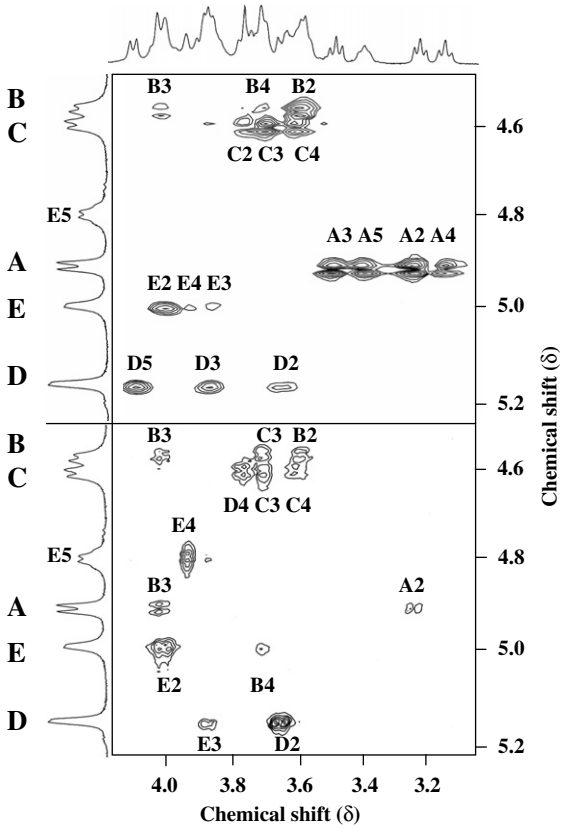

Figure 2. TOCSY (upper panel) and NOESY (lower panel) spectra of mucoidan. The mixing times for the TOCSY and NOESY spectra shown were 200 and 100 ms, respectively.

Table 2. ^{13}C NMR chemical shifts (ppm) of mucoidan recorded in D_2O at 70 °C

Glycosyl residue	C-1	C-2	C-3	C-4	C-5	C-6
A: β-D-Glc*p*-(1→	102.8	74.7	76.8	71.6	77.0	63.1
B: →3,4)-β-D-Glc*p*-(1→	103.2	76.4	79.6	73.2	75.3	61.1
C: →3)-β-D-Glc*p*NAc-(1→[a]	101.4	56.0	79.8	75.8	73.5	60.8
D: →4)-α-D-Glc*p*A-(1→	101.4	72.4	72.1	81.4	73.5	175.3
E: →3)-α-L-Fuc*p*-(1→	99.1	68.1	78.6	72.7	67.5	16.3

[a] Additional chemical shifts: NAc at δ 23.4 (CH_3), 175.6 (CO).

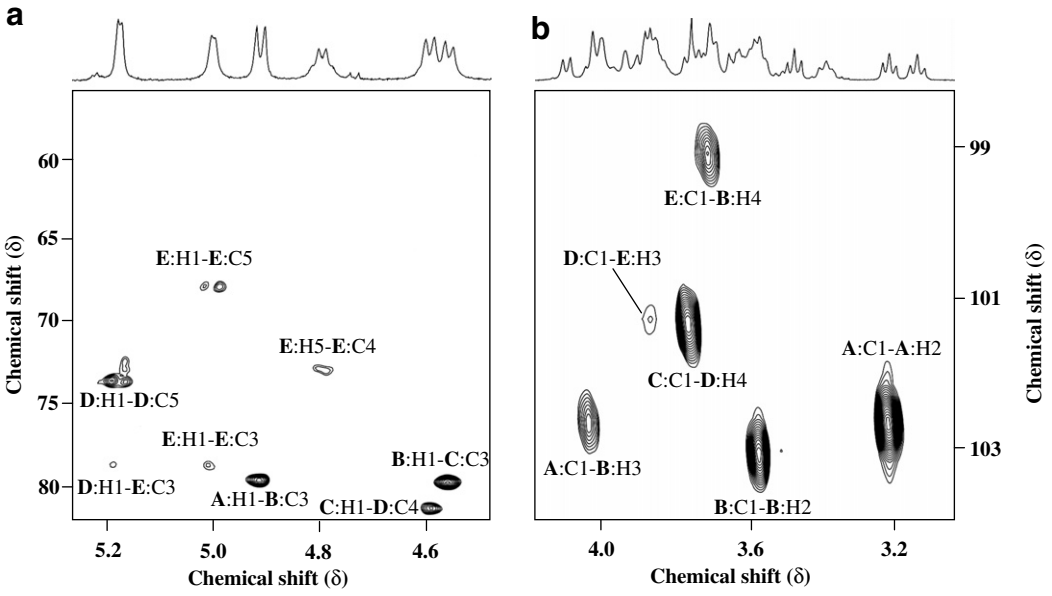

Figure 3. HMBC spectrum of mucoidan: (a) anomeric proton region and (b) anomeric carbon region.

Table 3. NOESY experiments on mucoidan in D_2O at 70 °C

Residue	Anomeric atom δ (^1H)	NOE contact δ (^1H)	Residue atom	Intensity[a]
A: β-D-Glc*p*-(1→	4.91	3.24	**A**: H-2	w
		4.03	**B**: H-3	w
B: →3,4)-β-D-Glc*p*-(1→	4.55	3.58	**B**: H-2	w
		4.03	**B**: H-3	w
		3.71	**C**: H-3	s
C: →3)-β-D-Glc*p*NAc-(1→	4.59	3.71	**C**: H-3	s
		3.63	**C**: H-4	w
		3.78	**D**: H-4	s
D: →4)-α-D-Glc*p*A-(1→	5.17	3.67	**D**: H-2	s
		3.88	**E**: H-3	w
E: →3)-α-L-Fuc*p*-(1→	5.00	4.02	**E**: H-2	s
		3.72	**B**: H-4	w

[a] The intensities were estimated from visual inspection of the NOESY spectra shown in Figure 2, and are given as the following: s = strong and w = weak.

had a weak NOE contact of its H-1 with the H-3 of the **B** residue. The following sequence was thus established:

β-D-Glc*p*-(1→3)-β-D-Glc*p*-(1→

A **B**

A strong NOE contact between H-1 of residue **B** and H-3 of residue **C** was observed, indicating the following sequence:

β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→

B **C**

Residue **C** had a strong NOE contact of its H-1 with the H-4 of **D**. The following sequence was thus established:

β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*A-(1→

C **D**

A weak NOE contact was observed between H-1 of residue **D** and H-3 of residue **E**, thus establishing the following sequence:

α-D-Glc*p*A-(1→3)-α-L-Fuc*p*-(1→

D **E**

Residue **E** had a weak NOE contact of its H-1 with the H-4 of residue **B**. The following sequence was thus established:

α-L-Fuc*p*-(1→4)-β-D-Glc*p*-(1→

E **B**

Thus, the NOESY data suggested the presence of the pentasaccharide repeating unit in the polysaccharide mucoidan with the following structure:

β-D-Glc*p*
1
↓
3
[4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→4)-α-D-Glc*p*A-(1→3)-α-L-Fuc*p*-(1→]$_n$ (1)

HMBC studies (Table 4) confirmed the results of the NOESY experiments (Table 3). Cross-peaks were found between H-1 of residue **A** and C-3 of residue **B** and between C-1 of residue **A** and H-3 of residue **B.** A cross-peak between H-1 of residue **B** and C-3 of residue **C** was observed, as were cross-peaks between H-1 of residue **C** and C-4 of residue **D** and between C-1 of residue **C** and H-4 of residue **D**. We also found cross-peaks between H-1 of residue **D** and C-3 of residue **E** and between C-1 of residue **D** and H-3 of residue **E**, as well as one between C-1 of residue **E** and H-4 of residue B. Thus, the HMBC data clearly support the presence of the pentasaccharide repeating unit in the polysaccharide mucoidan with the structure shown in Formula 1.

In conclusion, all results permit Formula 1 to be assigned to the repeating unit of the mucoidan.

3. Experimental

3.1. Organism, growth conditions, and isolation of extracellular polysaccharides

R. erythropolis strain PR4 (= NBRC 100887 = MBIC 01337) was grown on IB agar plates[4] at 25 °C under aerobic conditions, and EPS was removed from the cells by shaking. EPS was partially purified by enzymatic treatments, use of the CTAB method,[5] and ethanol precipitation as described by Urai et al.[2] This partially purified EPS was further purified by DEAE-Toyopearl 650M column chromatography as described by Urai et al.[3]

Table 4. HMBC experiment on mucoidan in D_2O at 70 °C

Residue	Anomeric atom		$J_{H,C}$ Connectivities to		Connectivities
	δ (^{1}H)	δ (^{13}C)	δ (^{1}H)	δ (^{13}C)	
A	4.91			79.6	**A**: H-1 **B**: C-3
		102.8	3.24		**A**: C-1 **A**: H-2
			4.03		**A**: C-1 **B**: H-3
B	4.55			79.8	**B**: H-1 **C**: C-3
		103.2	3.58		**B**: C-1 **B**: H-2
C	4.59			81.4	**C**: H-1 **D**: C-4
		101.4	3.78		**C**: C-1 **D**: H-4
D	5.17			73.5	**D**: H-1 **D**: C-5
				78.6	**D**: H-1 **E**: C-3
		101.4	3.88		**D**: C-1 **E**: H-3
E	5.00			78.6	**E**: H-1 **E**: C-3
				67.5	**E**: H-1 **E**: C-5
		99.1	3.72		**E**: C-1 **B**: H-4

$J_{H,C}$ Connectivities for the anomeric atoms (H-1 and C-1) of the mucoidan.
A: β-D-Glc*p*-(1→; **B**: →3,4)-β-D-Glc*p*-(1→; **C**: →3)-β-D-Glc*p*NAc-(1→; **D**: →4)-α-D-Glc*p*A-(1→; and **E**: →3)-α-L-Fuc*p*-(1→.

3.2. Monosaccharide analysis

EPS (50 μg) was completely hydrolyzed by 66% TFA at 100 °C for 18 h, and the monosaccharides thus obtained were labeled with ABEE and analyzed by HPLC as described below. A mixture of L-arabinose, L-fucose, D-galactose, D-glucose, D-mannose, L-rhamnose, D-xylose, D-galacturonic acid, D-glucuronic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-mannosamine (1 mg/mL each) was treated as described above and then employed as standard substances.

3.3. Fatty acid analysis

Fatty acids were prepared by alkaline hydrolysis of the EPS and subsequent *n*-hexane extraction under acidic conditions, and their methyl esters were analyzed by gas chromatography/mass spectrometry as described by Urai et al.[1]

3.4. NMR experiments

All NMR spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) with an ECA 500 instrument (JEOL Ltd. Tokyo, Japan). Chemical shifts were given in δ (ppm) with acetone (δ ^{1}H 2.23, δ ^{13}C 31.1) used as an internal reference for samples measured in D_2O solutions. Assignments of signals were made from the findings of the DQF-COSY, TOCSY, HMQC, NOESY, and HMBC experiments. ^{1}H NMR chemical shifts of overlapping signals were obtained from the center of the cross-peaks in the 2D spectra.

3.5. General methods

Gel-filtration column chromatography was performed on a Sephacryl S1000 (850 mm × 15 mm, Amersham Biosciences Ltd., Buckinghamshire, UK) column with 1 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl as the eluent. Fractions containing saccharide were monitored by the phenol–H_2SO_4 method.[6] Purity of EPS was analyzed by cellulose acetate membrane electrophoresis as described by Seno et al.,[7] using 0.2 M barium acetate (pH 7.5) as an electrophoresis buffer, 0.5% Toluizine Blue as a stain, and water for destaining. Monosaccharides were labeled with ABEE by using an ABEE labeling kit (Seikagaku Corporation, Tokyo, Japan), and the ABEE-labeled saccharides were separated on an ODS column (Honenpak C_{18}, 75 mm × 4.60 mm, Seikagaku Corporation, Tokyo, Japan) by HPLC (Shimadzu LC-10; Shimadzu, Kyoto, Japan) according to the supplier's instructions. For determination of the absolute configuration of the EPS, the TFA hydrolyzate of EPS was converted into acetylated (−)-2-butyl glycosides and analyzed by GLC.[8,9] The pyruvic acid content was enzymatically determined with an F-kit Pyruvic acid (J.K. International Co., Tokyo, Japan), as described by the supplier, after hydrolysis with 2% aq TFA at 100 °C for 2 h.

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