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

Makoto Urai,[a] Hiroshi Yoshizaki,[a] Hirosi Anzai,[b] Jun Ogihara,[c] Noriyuki Iwabuchi,[a] Shigeaki Harayama,[d,†] Michio Sunairi[a,*] and Mutsuyasu Nakajima[a]

[a]*Laboratory of Molecular Microbiology, Department of Applied Biological Science, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-8510, Japan*
[b]*Laboratory of Applied Biochemistry, Department of Agriculture, Junior College, Nihon University, Fujisawa, Kanagawa 252-8510, Japan*
[c]*Laboratory of Enzymology and Molecular Biology, Department of Agricultural and Biological Chemistry, College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-8510, Japan*
[d]*Marine Biotechnology Institute, Heita, Kamaishi, Iwate 026-0001, Japan*

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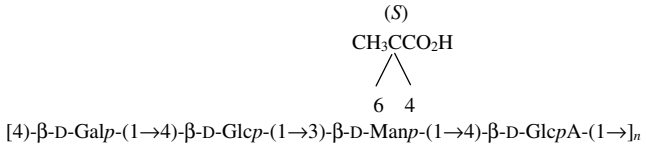
Abstract—*Rhodococcus erythropolis* PR4 is a marine bacterium that can degrade various alkanes including pristane, a C_{19} branched alkane. This strain produces a large quantity of extracellular polysaccharides, which are assumed to play an important role in the hydrocarbon tolerance of this bacterium. The strain produced two acidic extracellular polysaccharides, FR1 and FR2, and the latter showed emulsifying activity toward clove oil, whereas the former did not. FR2 was composed of D-galactose, D-glucose, D-mannose, D-glucuronic acid, and pyruvic acid at a molar ratio of 1:1:1:1:1, and contained 2.9% (w/w) stearic acid and 4.3% (w/w) palmitic acid attached via ester bonds. Therefore, we designated FR2 as a PR4 fatty acid-containing extracellular polysaccharide or FACEPS. The chemical structure of the PR4 FACEPS polysaccharide chain was determined by 1D ^{1}H and ^{13}C NMR spectroscopies as well as by 2D DQF-COSY, TOCSY, HMQC, HMBC, and NOESY experiments. The sugar chain of PR4 FACEPS was shown to consist of tetrasaccharide repeating units having the following structure:

PR4 FACEPS



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

* Corresponding author. Tel.: +81 466 84 3706; fax: +81 466 84 3354; e-mail: sunairi@brs.nihon-u.ac.jp
† Present address: Department of Biotechnology, National Institute of Technology and Evaluation, 2-5-8 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan.

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1. Introduction

Rhodococci show versatile metabolic activities, including the degradation of xenobiotic compounds such as benzene[1] and polychlorinated biphenyls (PCBs).[2–4] This property seems to be useful in bioremediation of polluted environments.[5] Among these Rhodococci, some strains were reported to produce acidic extracellular polysaccharides containing fatty acids attached via ester bonds,[6–9] which we will hereafter refer to as FACEPS (fatty acid-containing extracellular polysaccharides). These FACEPS showed a variety of functions, such as emulsification,[6–9] protection against hydrocarbon toxicity,[6] enhancement of polyaromatic hydrocarbon degradation by marine bacteria consortia,[6] moisture retention and adsorption,[7] and thickening.[8] Therefore, they can be synthesized as bio-based polymers by the bacteria grown on an annually renewable agricultural biomass. Furthermore, being biodegradable, FACEPS can also be utilized by other microorganisms living in the environment. These properties suggest that FACEPS could be used as sustainable, environmentally friendly products such as moisture adsorbents, thickeners, or emulsifiers. To understand the mechanisms of FACEPS biosynthesis, it is necessary to isolate genes and enzymes required for the synthesis and modification of FACEPS. Recently, the whole genomic sequence of *Rhodococcus erythropolis* PR4 was determined by NITE Genome Analysis Center, Department of Biotechnology, National Institute of Technology and Evaluation. This strain was isolated from the Pacific Ocean, south of Okinawa Island, Japan, at a depth of 1000 m and degrades C_8 to C_{20} *n*-alkanes and pristane.[10] Preliminary experiments showed that the strain also produced a FACEPS (PR4 FACEPS). Knowledge of the chemical composition and structure of the PR4 FACEPS would greatly increase, not only our understanding of the relationship between its functions and structure, but also biosynthetic mechanisms when combined with the genomic information. In this study, we report the determination of the chemical structure of PR4 FACEPS by methylation analyses and ^{1}H and ^{13}C NMR spectroscopic analyses, including those conducted by performing 2D DQF-COSY, TOCSY, HMQC, HMBC, and NOESY experiments.

2. Results and discussion

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

EPS produced by the *R. erythropolis* strain PR4 was extracted by the CTAB method and purified by DEAE-Toyopearl column chromatography, being eluted with a linear gradient (0–0.5 M) of NaCl. This procedure gave two peaks, one at ~0.2 M and the other at ~0.3 M NaCl (Fig. 1). For each peak a single band was detected by cellulose acetate membrane electrophoresis, and the mobility of the peak II band was faster than that of the peak I band (data not shown). Each EPS was eluted as a broad single peak earlier than Dextran T2000 by Sephacryl S1000 gel-filtration chromatography, and the EPS in peak II was eluted earlier than that in peak I (data not shown). These data indicate that these EPSs had been purified to homogeneity. Polysaccharides in peak I and peak II were tentatively named as FR1 EPS and FR2 EPS, respectively. Both EPSs were a white fibrous material soluble in water and alkalis but not in acids, MeOH, EtOH, or acetone. The FR2 EPS showed oil-emulsifying activity, whereas the FR1 EPS did not.

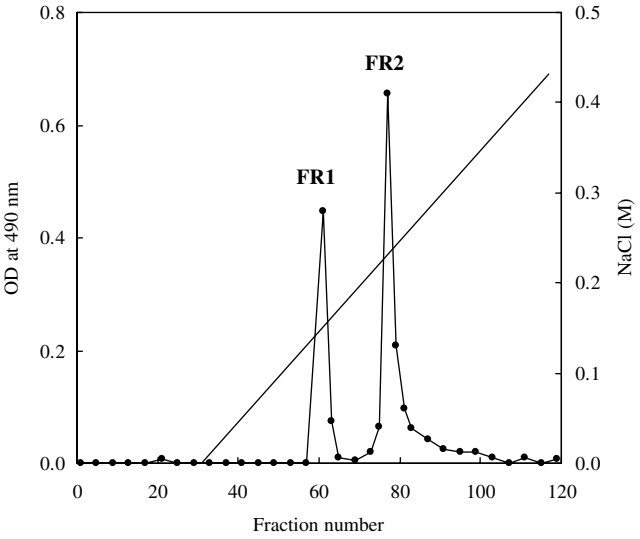

Figure 1. Ion-exchange chromatography of PR4 EPS. PR4 EPS (40 mg) was dissolved in 40 mL of 10 mM Tris–HCl buffer, applied to a DEAE-Toyopearl column (180 mm × 25 mm), and eluted with a 600-mL linear gradient (0–0.5 M) of NaCl.

2.2. Compositional analysis

The monosaccharide contents of these EPSs were determined by trifluoroacetic acid (TFA) hydrolysis followed by HPLC analysis. Consequently, glucose, *N*-acetylglucosamine, glucuronic acid, and fucose at a molar ratio of 2:1:1:1 were detected in FR1, and galactose, glucose, mannose, and glucuronic acid at a molar ratio of 1:1:1:1 were found in FR2. The absolute configurations of these monosaccharides and carboxyl-reduced glucuronic acid were determined by GLC analysis of their acetylated (−)-2-butyl derivatives, which showed that all had the D-configuration except for fucose, which had the L-configuration.

Spectrophotometrically, no or little absorption was detected at 280 nm or at 255 nm, suggesting that these

EPSs did not contain proteins or nucleic acids. Fatty acids were extracted from the alkali hydrolyzate of FR2 EPS, but not from untreated FR2 EPS, suggesting that the fatty acids were bound to FR2 EPS by ester bonds. According to the GLC–MS analysis of methylated fatty acids, FR2 EPS contained 2.9% (w/w) stearic acid and 4.3% (w/w) palmitic acid. Only a small amount or no fatty acids was detected from the alkali hydrolyzate of FR1 EPS or from untreated FR1 EPS. These data indicate that FR2 EPS was the PR4 FACEPS, and we named this FR1 EPS as mucoidan.

The structure of PR4 FACEPS was examined since PR4 FACEPS showed oil-emulsifying activity and contained fatty acid residues attached via ester bonds as did S-2 EPS.[9] For further analysis, depyruvated PR4 FACEPS and carboxyl-reduced PR4 FACEPS were prepared by heating the EPS in dilute acetate and by reduction using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide methyl *p*-toluenesulfonate and $NaBH_4$, respectively.

2.3. Methylation analysis

Table 1 shows the results of the methylation analysis of native, carboxyl-reduced, and depyruvated PR4 FACEPSs. The results of these three PR4 FACEPSs indicate that the D-galactose, D-glucose, and D-glucuronic acid residues were substituted at their O-4 position for the following reasons: both 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol were detected at a molar ratio almost equal to that of the two other partially methylated alditol acetate derivatives, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol and 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methyl-D-mannitol in the methylation analysis of the native PR4 FACEPS, whereas only 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol was detected at a level twice that of the two other alditol acetate derivatives in the methylation analysis of the carboxyl-reduced PR4 FACEPS. A pyruvate residue seemed to be bound to both the O-4 and O-6 positions of every D-mannose residue of the PR4 FACEPS, since 1,3,4,5,6-penta-*O*-acetyl-2-*O*-methyl-D-mannitol was detected in the methylation analysis of native and carboxyl-reduced PR4 FACEPS, whereas 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol was detected in that of depyruvated PR4 FACEPS. These data indicate that the PR4 FACEPS consisted of O-4-linked galactose, O-4-linked glucose, O-4-linked glucuronic acid, and O-3-linked mannose with pyruvic acid linked to its O-4 and O-6.

2.4. NMR analyses

The NMR spectra of the native polysaccharide were complex due to acylation and the high molecular weight. To improve the NMR spectra, we deacylated the PR4 FACEPS and subsequently sonicated it to reduce the molecular weight. A signal of the methyl proton of pyruvate moieties was detected in the high-field region (δ 1.47) of the ^{1}H NMR spectrum of the sonicated PR4 FACEPS (Fig. 2a) but not in that of the depyruvated PR4 FACEPS (Fig. 2b). The signals characteristic of the methyl carbon (δ 25.7), acetal carbon (δ 102.8), and carboxyl carbon (δ 175.9) of the pyruvic acid moiety were detected in the ^{13}C NMR spectrum of sonicated PR4 FACEPS (Fig. 3a and b) but not in the ^{13}C NMR spectrum of depyruvated PR4 FACEPS (Fig. 3c and d). These data confirmed the depyruvation of the EPS by heating in dilute AcOH.

One doublet at δ 4.49 and overlapping signals at $\sim\delta$ 4.7 were observed in the anomeric regions of the ^{1}H NMR spectra of both sonicated and depyruvated PR4 FACEPS (Fig. 2a and b, respectively). The ^{13}C NMR spectrum of sonicated PR4 FACEPS (Fig. 3c and d) revealed four signals in the anomeric region, at δ 100.0, 101.2, 103.9, and 104.1; and these four anomeric carbons were correlated to four anomeric protons, at δ 4.69, 4.72, 4.49, and 4.70, respectively, by conducting an HMQC experiment (data not shown). Similarly, four anomeric carbon signals were observed and correlated to anomeric protons by the 1D and 2D experiments on depyruvated PR4 FACEPS. These data indicate that the overlapping signal consisted of three anomeric proton signals. The relative intensity of the methyl protons (δ 1.47) was three times higher than that of the anomeric proton at δ 4.49 and almost equal to that of the three anomeric protons at δ 4.69, 4.70, and 4.72, indicating one pyruvic acid moiety to be present in every repeating

Table 1. Methylation analysis data on PR4 FACEPS

Derivative	Molar ratio		
	Native EPS[a]	Carboxyl-reduced EPS	Depyruvated EPS
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-galactitol	0.9	1.0	1.0
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol	1.0	2.0	0.9
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol	ND[b]	ND	0.9
1,4,5,6-Tetra-*O*-acetyl-2,3-di-*O*-methyl-D-glucitol	1.0	ND	0.8
1,3,4,5,6-Penta-*O*-acetyl-2-*O*-methyl-D-mannitol	1.0	0.6	ND

[a] The D-glucuronic acid residues contained in EPS were reduced with $NaBH_4$ to D-glucose residues after methylation.
[b] ND indicates not detected.

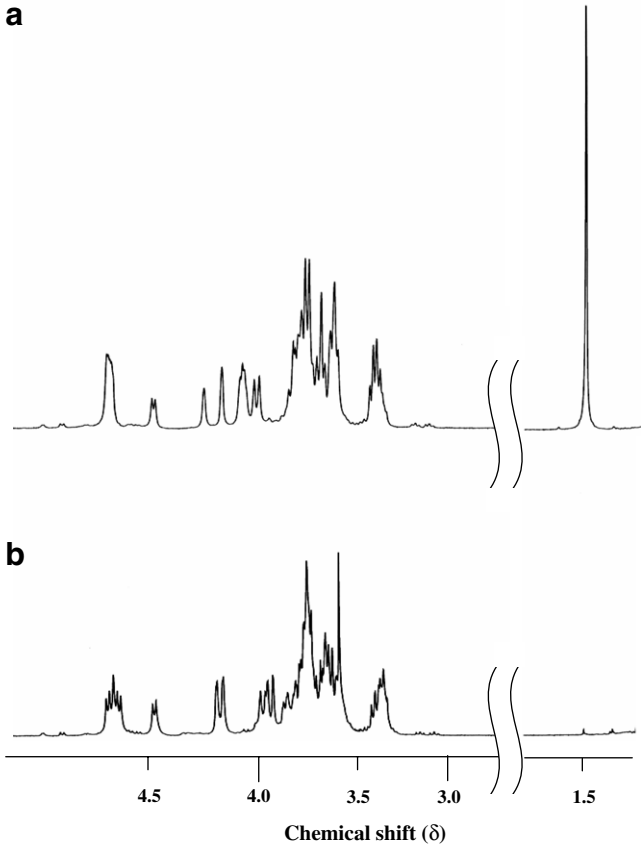

Figure 2. 500-MHz ^{1}H NMR spectra of sonicated PR4 FACEPS (a) and depyruvated PR4 FACEPS (b) recorded in D_2O at 70 °C.

unit. The identity of the monosaccharides was established on the basis of 1D and 2D ^{1}H and ^{13}C NMR spectra as described below.

The ^{1}H chemical shifts of the depyruvated PR4 FACEPS (Table 2) were assigned by means of 2D TOCSY (mixing times 100–300 ms; Fig. 4) and 2D DQF-COSY experiments (data not shown). Starting points for the interpretation of the spectra were the anomeric signals of residues **A**–**D**. Comparison of the TOCSY spectra with increasing mixing times allowed the assignment of the sequential order of the chemical shifts belonging to each single spin system. The signal at δ 4.65 of the depyruvated PR4 FACEPS was assigned to H-1 of the D-mannopyranose residues (**C** residues) on the basis of its broad singlet appearance and its rather high-field chemical shift. Since the spin system of the **A** residues was distinguished from those of the **B** and **D** residues by typically small $J_{4,5}$ values of <3 Hz, which were estimated either from the 1D or 2D NMR spectrum, the **A** residues were attributed to the D-galactopyranose residues. The remaining two spin systems, those of the **B** and **D** residues, were attributed to the D-glucopyranose residue and D-glucuronopyranose residue, respectively. Based on the observed $J_{H\text{-}1,H\text{-}2}$ values and chemical shifts, residues **A**, **B**, and **D** were allocated to the pyranose ring form and β anomeric configuration. All ^{13}C resonances of the depyruvated PR4 FACEPS (Table 2) could be assigned from the 2D ^{13}C–^{1}H HMQC spectrum. All four residues **A**–**D** were allocated to the β

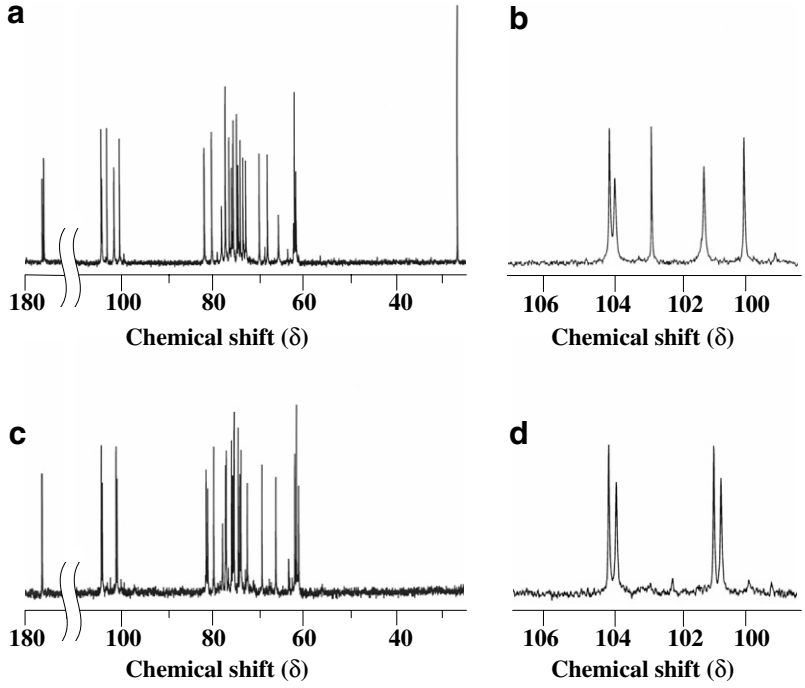

Figure 3. 125-MHz ^{13}C NMR spectra of sonicated PR4 FACEPS (a and b) and depyruvated PR4 FACEPS (c and d) recorded in D_2O at 70 °C. A signal at δ 102.8 (a and b) was assigned to the acetal carbon of the pyruvate moiety.

Table 2. ^{1}H and ^{13}C NMR chemical shifts (δ) of depyruvated PR4 FACEPS recorded in D_2O at 70 °C

Glycosyl residue	H-1 ($J_{H-1,H-2}$) C-1 ($J_{C-1,H-1}$)	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b
A: →4)-β-D-Gal*p*-(1→	4.47 (8.0)	3.64	3.77	4.17	3.79	3.78	3.83
	103.9 (162)	72.3	76.9	77.7	75.7	61.6	
B: →4)-β-D-Glc*p*-(1→	4.63 (8.0)	3.42	3.69	3.67	3.78	3.85	4.00
	100.9 (159)	73.7	75.1	79.6	73.9	61.1	
C: →3)-β-D-Man*p*-(1→	4.65	4.20	3.88	3.72	3.43	3.77	3.95
	100.6 (159)	69.1	80.9	66.1	77.0	62.0	
D: →4)-β-D-Glc*p*A-(1→	4.68 (7.5)	3.46	3.65	3.78	3.74		
	104.1 (160)	74.3	75.2	81.3	75.5	175.6	

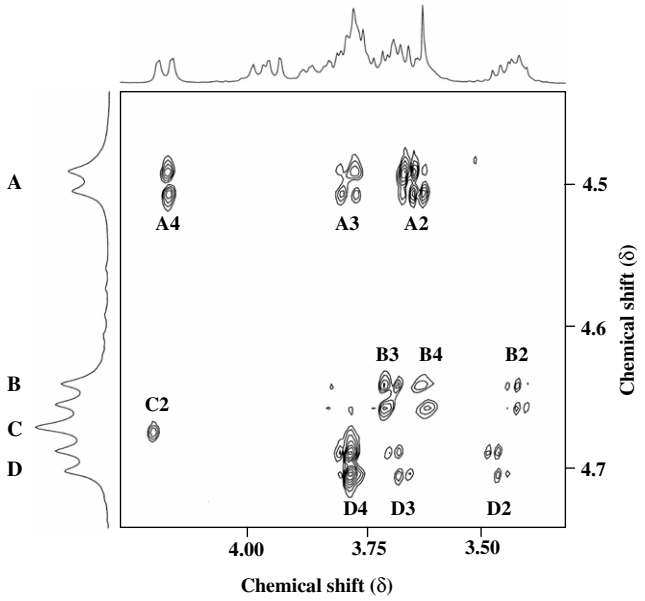

Figure 4. TOCSY spectra of depyruvated PR4 FACEPS. The mixing time for the TOCSY was 100 ms. Complete assignment required several TOCSY experiments having mixing times ranging from 100 to 300 ms.

anomeric configuration based on the observed $J_{C-1,H-1}$ values.[11] Similarly, the ^{1}H and ^{13}C chemical shifts of the sonicated PR4 FACEPS were assigned (Table 3).

A comparison of ^{13}C NMR chemical shift data for the sonicated PR4 FACEPS (Table 3) and the depyruvated PR4 FACEPS (Table 2) revealed that, by depyruvation, the resonances of C-4 and C-6 of residue **C** showed up-field shifts of δ 6.8 (from δ 72.9 to δ 66.1) and δ 3.1 (from δ 65.1 to δ 62.0), respectively, which is indicative of substitution at the corresponding positions.[12] These data concurred with the results of the methylation analysis. The chemical shift of the pyruvate methyl carbon (δ 25.7, Table 3) showed that the pyruvate acetal carbon atom had the (*S*) configuration.[13]

The sequence of glycosyl residues was determined by conducting HMBC and NOESY experiments. As to the HMBC spectrum (evolution times 62.5 ms; Fig. 5), the intra- and inter-residual connectivities of both anomeric protons and carbons of each of the glycosyl residues are summarized in Table 4. Cross-peaks were found between H-1 of residue **A** and C-4 of residue **B** and between C-1 of residue **A** and H-4 of residue **B**. The following sequence was thus established:

β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→

A **B**

Cross-peaks between H-1 of residue **B** and C-3 of residue **C** and between C-1 of residue **B** and H-3 of residue **C** were observed. The following sequence was thus established:

β-D-Glc*p*-(1→3)-β-D-Man*p*-(1→

B **C**

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** were observed, thus establishing the following sequence:

Table 3. ^{1}H and ^{13}C NMR chemical shifts (δ) of sonicated PR4 FACEPS recorded in D_2O at 70 °C

Glycosyl residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6a C-6	H-6b	Pyruvic acid residue CH_3	 C	 CO_2H
A: →4)-β-D-Gal*p*-(1→	4.49	3.65	3.75	4.17	3.76	3.70	3.76			
	103.9	72.3	76.8	77.6	75.5	61.6				
B: →4)-β-D-Glc*p*-(1→	4.69	3.46	3.66	3.67	3.79	3.82	4.01			
	100.0	73.5	75.9	79.8	73.9	61.3				
C: →3)-β-D-Man*p*-(1→ 6 4 \ / CH_3CCO_2H	4.72	4.25	4.08	3.84	3.42	3.78	4.09	1.47		
	101.2	69.3	76.8	72.9	67.5	65.1		25.7	102.8	175.9
D: →4)-β-D-Glc*p*A-(1→	4.70	3.44	3.65	3.74	3.70					
	104.1	74.2	75.0	81.4	75.1	175.6				

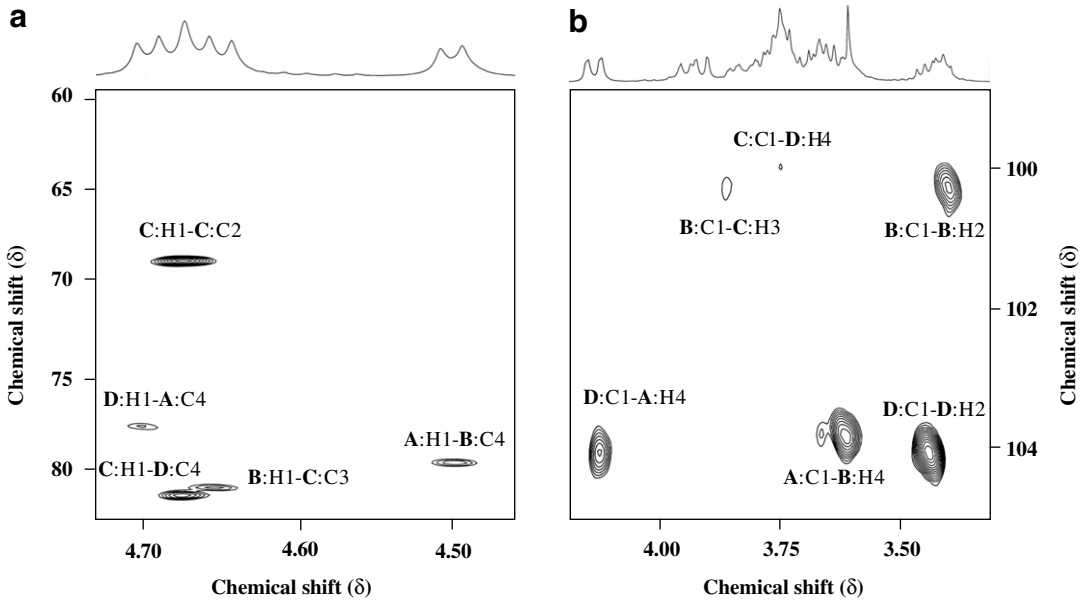

Figure 5. HMBC spectrum of depyruvated PR4 FACEPS: (a) anomeric proton region and (b) anomeric carbon region.

Table 4. HMBC experiments on depyruvated PR4 FACEPS in D_2O at 70 °C[a]

Residue	Anomeric atom		$J_{H,C}$ Connectivities to		Connectivities
	δ (^{1}H)	δ (^{13}C)	δ (^{1}H)	δ (^{13}C)	
A	4.47			79.6	**A**: H-1 **B**: C-4
		103.9	3.67		**A**: C-1 **B**: H-4
B	4.63			80.9	**B**: H-1 **C**: C-3
		100.9	3.42		**B**: C-1 **B**: H-2
			3.88		**B**: C-1 **C**: H-3
C	4.65			69.1	**C**: H-1 **C**: C-2
				81.3	**C**: H-1 **D**: C-4
		100.6	3.78		**C**: C-1 **D**: H-4
D	4.68			77.7	**D**: H-1 **A**: C-4
		104.1	3.46		**D**: C-1 **D**: H-2
			4.17		**D**: C-1 **A**: H-4

A: →4)-β-D-Gal*p*-(1→; **B**: →4)-β-D-Glc*p*-(1→; **C**: →3)-β-D-Man*p*-(1→; and **D**: →4)-β-D-Glc*p*A-(1→.

[a] $J_{H,C}$ Connectivities for the anomeric atoms (H-1 and C-1) of the depyruvated PR4 FACEPS.

β-D-Man*p*-(1→4)-β-D-Glc*p*A-(1→

C **D**

The cross-peaks between H-1 of residue **D** and C-4 of residue **A** and between C-1 of residue **D** and H-4 of residue **A** were also observed. The following sequence was thus established:

β-D-Glc*p*A-(1→4)-β-D-Gal*p*-(1→

D **A**

The tetrasaccharide repeating unit is thus assigned as shown below (Formula 1):

[4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*A-(1→]$_n$

A **B** **C** **D**

(1)

NOESY studies (mixing time 100–300 ms, Table 5) confirmed the results of the HMBC experiments (Table 4). Residue **A** had a weak NOE contact of its H-1 with the H-4 of residue **B**, indicating residue **A** to be linked at the C-4 position of the **B** residue. Residue **B** had a strong inter-residue contact of its H-1 with the H-3 of residue **C**, supporting the linkage between residue **B** to the C-3 position of the **C** residue determined by the HMBC experiments. Residue **C** had a strong contact of its H-1 with the H-4 of residue **D**, suggesting residue **C** to be linked at the C-4 position of the **D** residue. Residue **D** had a strong inter-residue contact between its H-1 and the H-4 of residue **A**, indicating residue **D** to be linked at the C-4 position of residue **A**. Thus, the NOESY data clearly support the presence of the tetrasaccharide repeating unit in the polysaccharide FR2 EPS as described above (Formula 1).

2.5. Partial hydrolysis of EPS

PR4 FACEPS was mildly hydrolyzed with TFA or acetolyzed, and the resulting mixtures of oligosaccharides were fractionated by Bio-Gel P-2 gel-filtration column chromatography, followed by Dowex I-X8 anion-exchange column chromatography, yielding six types of oligosaccharides. These oligosaccharides were analyzed by a combination of quantitative monosaccharide

Table 5. NOESY experiments on depyruvated PR4 FACEPS in D_2O at 70 °C

Residue	Anomeric atom δ (^{1}H)	NOE contact δ (^{1}H)	Residue atom	Intensity[a]
A: →4)-β-D-Gal*p*-(1→	4.47	3.77	**A**: H-3	s
		3.67	**B**: H-4	w
B: →4)-β-D-Glc*p*-(1→	4.63	3.42	**B**: H-2	s
		3.69	**B**: H-3	s
		4.20	**C**: H-2	s
		3.88	**C**: H-3	s
C: →3)-β-D-Man*p*-(1→	4.65	4.20	**C**: H-2	s
		3.88	**C**: H-3	s
		3.78	**D**: H-4	s
D: →4)-β-D-Glc*p*A-(1→	4.68	3.46	**D**: H-2	s
		3.78	**D**: H-4	s
		4.17	**A**: H-4	w

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

Oligosaccharide **1**: β-D-Glc*p*-D-Man

Oligosaccharide **2**: β-D-Glc*p*A-D-Gal

Oligosaccharide **3**: β-D-Gal*p*-D-Glc

Oligosaccharide **4**: β-D-Glc*p*A-β-D-Gal*p*-D-Glc

Oligosaccharide **5**: β-D-Gal*p*-β-D-Glc*p*-D-Man

Oligosaccharide **6**: β-D-Glc*p*-β-D-Man*p*-β-D-Glc*p*A-D-Gal

B C D A B C

Figure 6. The structure of oligosaccharides obtained from partially TFA-hydrolyzed or partially acetolyzed PR4 FACEPS. Oligosaccharides **2**, **3**, and **6** were obtained from the TFA hydrolyzate; and oligosaccharides **1**–**5**, from the acetolyzate.

analysis by HPLC, methylation analysis, glycosidase digestion, and reduction of the reducing ends with $NaBH_4$, and their structures are shown in Figure 6. We briefly explain here how the structures of oligosaccharides were determined by using the structure of oligosaccharides **3** and **6**, because this pair covers the repeating unit of PR4 FACEPS. Oligosaccharide **3** was obtained by both TFA hydrolysis and acetolysis (Fig. 7a), which consisted of D-glucose and D-galactose at an equimolar ratio. After reduction of the reducing end of oligosaccharide **3**, D-glucose was not detected, whereas D-galactose was detected, suggesting that D-glucose is the reducing end of oligosaccharide **3**. Among the four glycosidases tested, α- and β-glucosidase, and α- and β-galactosidase, only β-galactosidase cleaved oligosaccharide **3** into D-glucose and D-galactose (Fig. 7b). Thus, the structure of oligosaccharide **3** was determined to be the following:

β-D-Gal*p*-D-Glc (**3**)

A B

Oilgosaccharide **6** was obtained by TFA hydrolysis (Fig. 7c), which consisted of D-mannose, D-glucose, D-glucuronic acid, and D-galactose at an equimolar ratio. After reduction of the reducing end of oligosaccharide **6**, D-galactose was not detected, whereas D-mannose, D-glucose, and D-glucuronic acid were shown to be present, suggesting D-galactose to be the reducing end of oligosaccharide **6**. Among the seven glycosidases tested, α- and β-mannosidase, α- and β-glucosidase, α- and β-galactosidase, and β-glucuronidase, only β-glucosidase cleaved oligosaccharide **6** into D-glucose and oligosaccharide **7** (Fig. 7d). Oligosaccharide **7** of the cleavage product was obtained by fractionation using Bio-Gel P-2 (Fig. 7e). Oligosaccharide **7** thus obtained was again treated with the glycosidases mentioned above, and only β-mannosidase cleaved this oligosaccharide into D-mannose and oligosaccharide **2** (Fig. 7f). Oligosaccharide **2** of the cleavage product was obtained by fractionation using Bio-Gel P-2 (Fig. 7g). Oligosaccharide **2** thus obtained was again treated with the glycosidases mentioned above, and only β-glucuronidase cleaved this oligosaccharide into D-glucuronic acid and D-galactose (Fig. 7h). Thus, the structure of oligosaccharide **6** was determined to be the following:

β-D-Glc*p*-β-D-Man*p*-β-D-Glc*p*A-D-Gal (**6**)

B C D A

The structures of the other four oligosaccharides (Fig. 6) were determined by similar methods and support the PR4 FACEPS tetrasaccharide repeating unit shown in Formula 1.

The results obtained here indicate that PR4 FACEPS consists of a tetrasaccharide repeating unit containing an acetal-linked pyruvic acid moiety with the following structure:

[4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*A-(1→]$_n$

A B C D

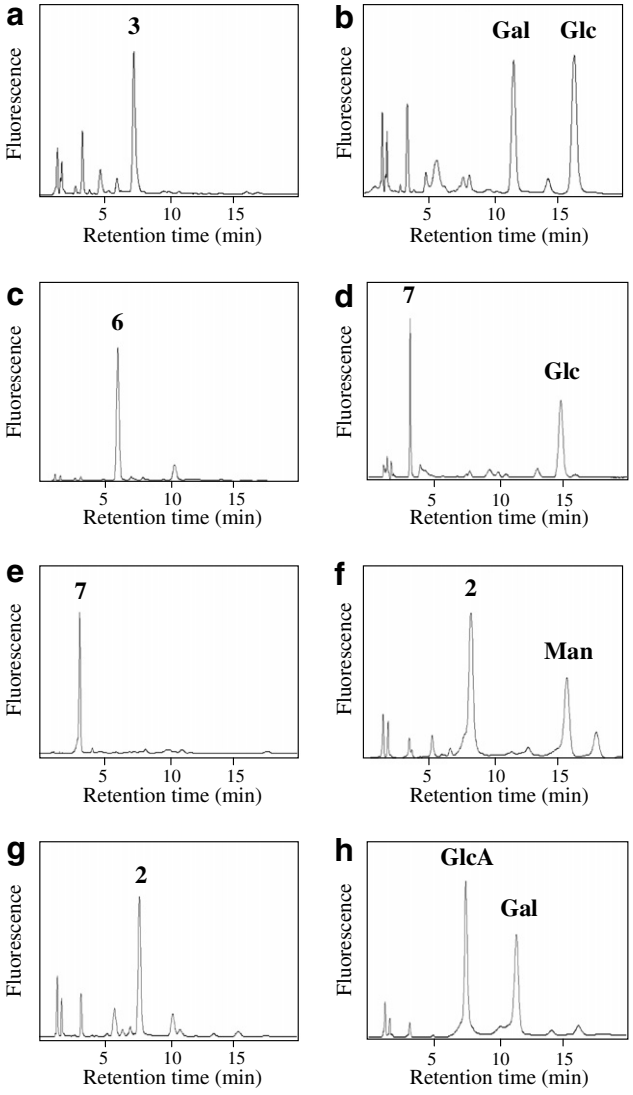

Figure 7. Analysis of oligosaccharides prepared by partial hydrolysis of PR4 FACEPS: the HPLC profile of purified oligosaccharide **3** (a), the β-galactosidase-cleaved products of oligosaccharide **3** (b), the purified oligosaccharide **6** (c), the β-glucosidase-cleaved product of oligosaccharide **6** (d), the purified oligosaccharide **7** from the β-glucosidase-cleaved products of oligosaccharide **6** separated on Bio-Gel P-2 (e), the β-mannosidase-cleaved products of oligosaccharide **7** (f), the purified oligosaccharide **2** from the β-mannosidase-cleaved products of oligosaccharide **7** separated on Bio-Gel P-2 (g), and the β-glucuronidase-cleaved products of oligosaccharide **2** (h).

Among bacterial polysaccharides whose structures have been reported so far, the chemical structure of the EPS produced by the benzene-tolerant bacterium *Rhodococcus* sp. 33 reported by Urai et al.,[14] seems to be most similar to that of PR4 FACEPS determined in this study, except for the fatty acid residues bound to PR4 FACEPS via ester bonds. This pair of bacteria, *Rhodococcus* sp. 33 and *R. erithlopolis* PR4, will, therefore, greatly help our understanding of the biosynthesis, structures, and functions of FACEPS by comparison of their EPS and the genes and the enzyme reactions related to their EPS synthesis. The chemical structure of PR4 FACEPS reported here provides fundamental data for investigating the relationship between structures and functions of FACEPSs.

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[15] at 25 °C under aerobic conditions, and EPS was removed from the cells by shaking. EPS was partially purified by enzymatic treatments, phenol–chloroform treatment or the CTAB method[16] and ethanol precipitation as described by Urai et al.[7] These partially purified EPSs were further purified by DEAE-Toyopearl 650M column chromatography as described by Urai et al.[8]

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.

3.3. Fatty acid analysis

Fatty acids were prepared by alkali 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.[9]

3.4. Modification of EPS

Carboxyl reduction was performed using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methyl-*p*-toluenesulfonate and $NaBH_4$ as the reducing agents.[17] By the monosaccharide content analysis, D-glucuronic acid was obtained from carboxyl-reduced PR4 FACEPS at a molar ratio of less than 1/20 of that of D-glucose. Depyruvation was performed as described by Urai et al.[14] No signals of the pyruvic acid moiety were detected by NMR analysis of depyruvated PR4 FACEPS, and little or no pyruvate was detected by enzymatic analysis after complete hydrolysis of depyruvated PR4 FACEPS, suggesting that depyruvated PR4 FACEPS contained either no or just a few pyruvic acid moieties. Deacylation was performed as described by Urai et al.[9] The EPS solutions (5 mg/mL) were ultrasonicated on ice by using an Astrason model XL2020 (Misonix Inc., NY, USA).

Oligosaccharides were prepared by partial TFA hydrolysis or acetolysis as described by Urai et al.,[9] except that the acetolysis was performed at 30 °C for 18 h. The partial TFA hydrolyzates or acetolyzates were separated by Bio-Gel P-2 gel-filtration chromatography on a column (900 × 15 mm) with 0.2 M HOAc as the eluent. Peaks containing oligosaccharides were pooled, applied to a Dowex I-X8 anion-exchange chromatography column (50 mm × 15 mm), washed with 13 mL of 0.2 M HOAc (to obtain the neutral oligosaccharide), and eluted with 23 mL of 2 M HOAc (to obtain the acidic oligosaccharide).

3.5. Methylation analysis

Methylation of the polysaccharides was performed according to the Hakomori method using the sodium methylsulfinylmethyl anion and CH_3I.[18] The methylated polysaccharides were then hydrolyzed, reduced, and acetylated before analysis by GLC–MS.

3.6. NMR experiments

All NMR spectra were recorded at 500 MHz (^1H) and 125 MHz (^{13}C) with an ECA 500 instrument (JEOL Ltd. Tokyo, Japan). Chemical shifts were given in δ units, with acetone (δ ^1H 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 DQF-COSY, TOCSY, HMQC, NOESY, and HMBC experiments. ^1H NMR chemical shifts of overlapping signals were obtained from the center of the cross-peaks in the 2D spectra.

3.7. General methods

Gel-filtration column chromatography was performed on a Sephacryl S1000 (850 mm × 15 mm; Amersham Biosciences UK Ltd., Buckinghamshire, UK) column for polysaccharides or on a Bio-Gel P-2 column for oligosaccharides with 1 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl or 0.2 M HOAc, respectively, as the eluent. Fractions containing saccharide were monitored by the phenol–H_2SO_4 method.[19] Purity of EPS was analyzed by cellulose acetate membrane electrophoresis as described by Seno et al.,[20] using 0.2 M $Ba(OAc)_2$ (pH 7.5) as an electrophoresis buffer, 0.5% Toluizine Blue as a stain, and water for destaining. Monosaccharides and oligosaccharides 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.[21,22] 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. Glycosidases were purchased from Seikagaku Corporation (Tokyo, Japan) except for α-and β-glucosidases and β-glucuronidase, which were obtained from Wako Pure Chemicals and used as described by the manufacturers.

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