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Structural analysis of an extracellular polysaccharide produced by *Rhodococcus rhodochrous* strain S-2

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Abstract—A possibility has been suggested of applying the EPS produced by *Rhodococcus rhodochrous* strain S-2 (S-2 EPS) to the bioremediation of oil-contaminated environments, because its addition, together with minerals, to oil-contaminated seawater resulted in emulsification of the oil, increased the degradation of polyaromatic hydrocarbons (PAH) of the oil, and led to the dominance of PAH-degrading marine bacteria. To understand the underlying principles of these phenomena, we determined the chemical structure of the sugar chain of S-2 EPS. The EPS was found to be composed of D-galactose, D-mannose, D-glucose, and D-glucuronic acid, in a molar ratio of 1:1:1:1. In addition, 0.8% (w/w) of octadecanoic acid and 2.7% (w/w) of hexadecanoic acid were also contained in its structure. By ^{1}H and ^{13}C NMR spectroscopy, including 2D DQF-COSY, TOCSY, HMQC, HMBC, and NOESY experiments, as well as chemical and enzymatic analyses, the polysaccharide was shown to consist of tetrasaccharide repeating units with the following structure:

β-D-Glc*p*A
1
↓
2
→3)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→

Keywords: *Rhodococcus rhodochrous*; Extracellular polysaccharide; Bioremediation; Emulsification; Structure; NMR spectroscopy

1. Introduction

There have been a number of reports on the catabolic activities of *Rhodococcus* toward a wide variety of organic compounds, including such xenobiotics as polychlorinated biphenyls, aliphatic and aromatic hydrocarbons,

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and complex mixtures thereof, including crude oil.[1–4] It has been reported that mucoid strains of *Rhodococcus* showed good growth in the presence of hydrocarbons, whereas rough strains did not, but that the rough strains could grow in the presence of hydrocarbons by addition of the extracellular polysaccharides (EPS) produced by these mucoid strains.[5,6] Recently, we reported that the addition of EPS produced by *Rhodococcus rhodochrous* strain S-2 (S-2 EPS) together with minerals to oil-contaminated seawater resulted in emulsification of the oil, increased the degradation of polyaromatic hydrocarbons (PAH) of the oil, and led to the dominance of a species of PAH-degrading marine bacteria, *Cycloclusticus* sp., in the samples, suggesting the possibility of its application to the bioremediation of oil-contaminated environments.[7]

An understanding of the biochemical and biophysical properties of S-2 EPS and the isolation of genes and enzymes required for the synthesis and modification of it should lead to a better grasp of the principles underlying the protection from hydrocarbon toxicity and the promotion of oil-, especially PAH-, degradation by marine bacteria. Such knowledge will undoubtedly expand the possibilities of bioremediation for oil-contaminated marine environments. In this study, we present the composition of S-2 EPS and the chemical structure of its sugar chain.

2. Results and discussion

2.1. Purification of S-2 EPS

EPS produced by *R. rhodochrous* strain S-2 was extracted and purified by DEAE-Toyopearl column chromatography, being eluted with a linear gradient (0–1 M) of NaCl. This procedure gave a major single peak at approximately 0.3 M concentration of NaCl, and the fractions contained in this peak were combined as the S-2 EPS. Two minor peaks were also detected at 0 M (non-absorbed fraction) and approximately 0.1 M NaCl, and the former and the later peaks contained glucomannan and mannan, respectively (data to be presented elsewhere), which did not show the activities of S-2 EPS reported previously,[6,7] such as emulsification of the oil. Spectrophotometrically, no absorption was detected at 280 nm or at 255 nm, suggesting that the S-2 EPS did not contain proteins or nucleic acids. A single band was detected by cellulose acetate membrane electrophoresis of S-2 EPS (data not shown). The S-2 EPS was eluted as a broad single peak earlier than Dextran T2000 by Sephacryl S1000 gel-filtration chromatography (data not shown), suggesting its apparent molecular weight to be greater than 2,000,000. These data indicate that the S-2 EPS had been purified to homogeneity. S-2 EPS 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 S-2 EPS was determined by both H_2SO_4 hydrolysis followed by gas–liquid chromatography (GLC) analysis and trifluoroacetic acid (TFA) hydrolysis followed by HPLC analysis. Consequently, galactose, glucose, mannose, and glucuronic acid were detected in the molar ratios of 1:1:1:1. The absolute configurations of these three monosaccharides and carboxyl-reduced glucuronic acid were determined by GLC of acetylated (+)-2-octyl derivatives and all were shown to have the D configurations.

Methylation analysis was performed on both native and carboxyl-reduced S-2 EPS (Table 1). For both EPS, the data indicated that the D-galactose and D-glucose were substituted at the O-3 positions. The presence of 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl-D-mannitol in both methylated EPSs indicated that the S-2 EPS was branched at the D-mannose residues. In the methylation analysis of carboxyl-reduced S-2 EPS, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol was detected. In contrast, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol was detected in the methylated native S-2 EPS after carboxyl-reduction. These data indicate that D-glucuronic acid residues in the S-2 EPS were present at the non-reducing ends of side chains and that D-mannose residues were side-chain branching points.

Fatty acids were extracted from the alkali-hydrolyzate of S-2 EPS, but not from untreated S-2 EPS, suggesting that the fatty acids were bound to EPS by ester bonds. According to the GLC–MS analysis of methylated fatty

Table 1. Methylation analysis data of S-2 EPS

Derivatives	Molar ratio	
	Native EPS[a]	Carboxyl-reduced EPS
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol	0.75	N.D.
1,2,3,5-Tetra-*O*-acetyl-4,6-di-*O*-methyl-D-mannitol	0.87	0.69
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol	0.69	0.82
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol	1.00	1.00
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol	N.D.	0.52

[a] The D-glucuronic acid residues contained in native EPS were reduced with $NaBH_4$ to the D-glucose residues after methylation.

acids, S-2 EPS contained 0.8% (w/w) octadecanoic acid and 2.7% (w/w) hexadecanoic acid.

2.3. NMR analyses

The NMR spectra of the native polysaccharide were complex due to partial acylation and the high molecular weight. The ^{1}H NMR spectrum (Fig. 1) and the ^{13}C NMR spectrum (Fig. 2) were greatly improved by deacylation and reduction of the molecular weight of S-2 EPS. By Sepharose CL-2B gel-filtration chromatography, the apparent molecular weight was reduced to approximately 2,000,000. By NMR analysis, no acyl residues were detected in the S-2 EPS.

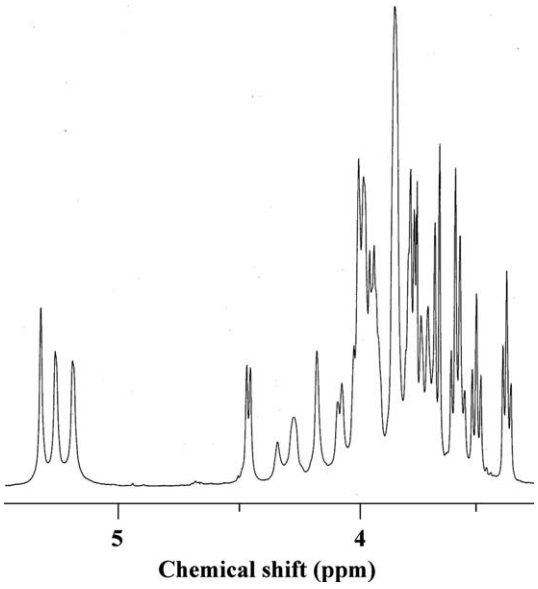

Figure 1. Spectrum of 500-MHz ^{1}H NMR of S-2 EPS recorded in D_2O at 70 °C.

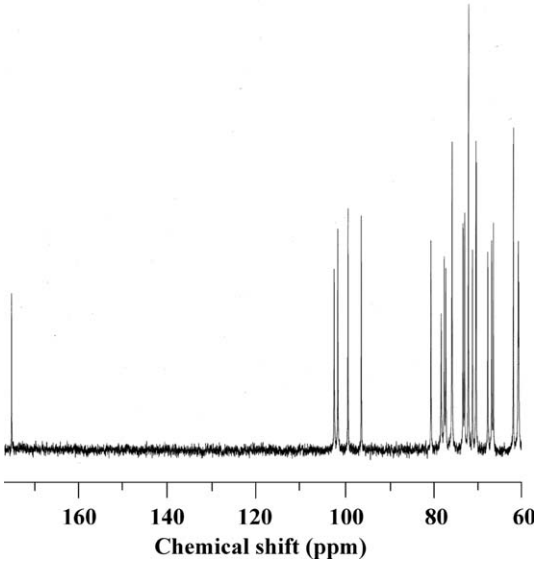

Figure 2. Spectrum of 125-MHz ^{13}C NMR of S-2 EPS recorded in D_2O at 70 °C.

The 1D ^{1}H NMR spectrum of the S-2 EPS (Fig. 1) showed that the repeating unit contained four kinds of sugars identified by the compositional analysis. In the anomeric region, four proton signals were observed between δ 4.46 and 5.34 ppm. The identity of the monosaccharides was established on the basis of 1D and 2D ^{1}H and ^{13}C NMR. One of these signals at δ 4.46 ppm, having a $J_{H\text{-}1,H\text{-}2}$ value of 8.0 Hz, was attributed to H-1 of the β-D-glucopyranuronic residues (**A** residues). Two of them, at δ 5.21 and 5.28 ppm and having a $J_{H\text{-}1,H\text{-}2}$ value of 2.3 Hz, were attributed to H-1 of the α-D-glucopyranose residues (**C** residues) and α-D-galactopyranose residues (**D** residues), respectively. One singlet signal at δ 5.34 ppm was assigned to H-1 of the D-mannopyranose residues (**B** residues). Based on the observed $J_{H\text{-}1,H\text{-}2}$ values and chemical shifts, residues **C** ($J_{H\text{-}1,H\text{-}2}$ 2.3 Hz) and **D** ($J_{H\text{-}1,H\text{-}2}$ 2.3 Hz) were allocated to the pyranose ring form and α anomeric configuration, and residue **A** ($J_{H\text{-}1,H\text{-}2}$ 8.0 Hz) was assigned to the pyranose form and β anomeric configuration.

The ^{1}H chemical shifts of the S-2 EPS (Table 2) were assigned by means of 2D TOCSY (mixing times 100–300 ms; Fig. 3, upper panel) and 2D DQF-COSY experiments. Starting points for the interpretation of the spectra were the anomeric signals of residues **A**–**D**. Comparison of TOCSY spectra with increasing mixing times allowed the assignment of the sequential order of the chemical shifts belonging to a single spin-system.

The ^{13}C NMR spectrum (Fig. 2) revealed four signals in the anomeric region, at δ 96.8, 99.8, 102.1, and 102.9 ppm. These data are in agreement with the presence of a repeating unit composed of four sugars as already described. The signal at δ 99.8 ppm, with a $J_{C\text{-}1,H\text{-}1}$ value of 175 Hz, corresponded to C-1 of the α-mannopyranose sugar (**B** residues); whereas that at δ 102.9 ppm, with a $J_{C\text{-}1,H\text{-}1}$ value of 154 Hz, was assigned to C-1 of the β-glucopyranuronic residue (**A** residues). The signal at δ 102.1 ppm, having a $J_{C\text{-}1,H\text{-}1}$ value of 175 Hz, was assigned to the α-galactopyranose sugar (**D** residues). The fourth signal, at δ 96.8 ppm and with a $J_{C\text{-}1,H\text{-}1}$ value of 171 Hz, was assigned to the α-glucopyranose sugar (**C** residues). All ^{13}C resonances of the S-2 EPS could be assigned as shown in Table 3 by using the 2D ^{13}C–^{1}H HMQC spectrum. The $J_{C\text{-}1,H\text{-}1}$ values of the anomeric ^{13}C atoms of residues **B** ($J_{C\text{-}1,H\text{-}1}$ 175 Hz), **C** ($J_{C\text{-}1,H\text{-}1}$ 171 Hz), and **D** ($J_{C\text{-}1,H\text{-}1}$ 175 Hz) were in agreement with α anomeric configurations; and that value of residue **A** ($J_{C\text{-}1,H\text{-}1}$ 154 Hz), with the β anomeric configuration. The configurations of **A**, **C**, and **D** residues were in accordance with those determined based on $J_{H\text{-}1,H\text{-}2}$ values and chemical shifts of anomeric protons.[8]

Table 2. ^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) of S-2 EPS 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-6
β-D-Glc*p*A-(1→ **A**	4.46 (8.0)	3.37	3.50	3.59	3.67	
→2,3)-α-D-Man*p*-(1→ **B**	5.34	4.35	4.08	3.94	4.01	3.85
→3)-α-D-Glc*p*-(1→ **C**	5.21 (2.3)	3.69	3.93	3.57	3.99	3.77
→3)-α-D-Gal*p*-(1→ **D**	5.28 (2.3)	3.96	3.99	4.18	4.26	3.72

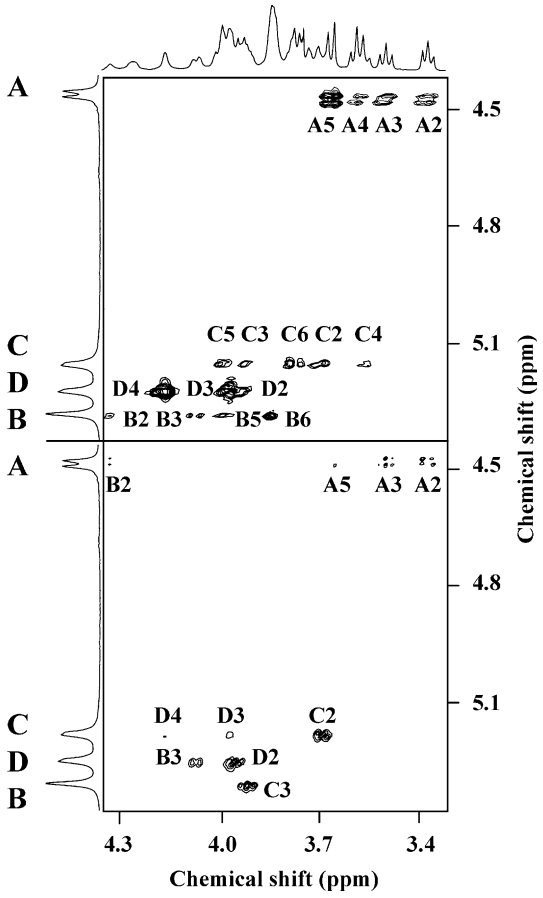

Figure 3. TOCSY (upper panel) and NOESY (lower panel) spectra of S-2 EPS. The mixing times for the TOCSY and NOESY spectra shown were 200 and 100 ms, respectively. Complete assignment required several TOCSY and NOESY experiments having mixing times ranging from 100 to 300 ms and 100 to 500 ms, respectively.

The sequence of glycosyl residues was determined from NOESY studies (Table 4; Fig. 3, lower panel), followed by confirmation with HMBC (Table 5) experiments. Residue **A** (β-D-glucuronic acid) had a weak NOE contact of its H-1 with the H-2 of residue **B** (α-D-mannose) in addition to weak intra-residue NOE contacts with H-2, H-3, and H-5, indicating residue **A** to be linked at the C-2 position of the **B** residue. The following sequence was thus established:

β-D-Glc*p*A-(1→2)-α-D-Man*p*-(1→

A **B**

Residue **B** (α-D-mannose) had a strong inter-residue contact of its H-1 with the H-3 of residue **C** (α-D-glucose), indicating that residue **B** was linked at the C-3 position of the **C** residue:

α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→

B **C**

Residue **C** (α-D-glucose) had a strong inter-residue contact of its H-1 with the H-3, and a weak one with the H-4, of residue **D** (α-D-galactose) in addition to a weak intra-residue NOE contact with its H-2, suggesting residue **C** to be linked at the C-3 position of the **D** residue. The linkage was also supported by the results of HMBC experiments as well as methylation analysis of native S-2 EPS and oligosaccharides obtained by partial hydrolysis:

α-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→

C **D**

Residue **D** (α-D-galactose) had a strong inter-residue contact between its H-1 and the H-3 of residue **B** (α-D-mannose) in addition to strong intra-residue NOE contacts with H-2, indicating following sequence:

Table 3. ^{13}C NMR chemical shifts (ppm) of S-2 EPS recorded in D_2O at 70 °C

Glycosyl residue	C-1 ($J_{C-1,H-1}$)	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*A-(1→ **A**	102.9 (154)	73.6	76.4	72.7	78.2	175.6
→2,3)-α-D-Man*p*-(1→ **B**	99.8 (175)	78.8	77.8	67.0	73.9	61.3
→3)-α-D-Glc*p*-(1→ **C**	96.8 (171)	70.9	81.1	71.0	72.7	61.4
→3)-α-D-Gal*p*-(1→ **D**	102.1 (175)	68.3	76.4	67.4	71.8	62.5

Table 4. NOESY experiments on S-2 EPS in D_2O at 70 °C

Residue	Anomeric atom δ (^1H)	NOE contact δ (^1H)	Residue atom	Intensity[a]
β-D-Glc*p*A-(1→ **A**	4.46	3.37	**A**: H-2	w
		3.50	**A**: H-3	w
		3.67	**A**: H-5	w
		4.35	**B**: H-2	w
→2,3)-α-D-Man*p*-(1→ **B**	5.34	3.93	**C**: H-3	s
→3)-α-D-Glc*p*-(1→ **C**	5.21	3.69	**C**: H-2	s
		3.99	**D**: H-3	s
		4.18	**D**: H-4	w
→3)-α-D-Gal*p*-(1→ **D**	5.28	3.96	**D**: H-2	s
		4.08	**B**: H-3	s

[a] The intensities are estimated from visual inspection of the NOESY spectra shown in Figure 3, and are given as the following: s = strong and w = weak.

α-D-Gal*p*-(1→3)-α-D-Man*p*-(1→

D **B**

Residue **B** is a 1,2,3-linked residue, and the tetrasaccharide repeating unit is assigned as shown below (Formula 1):

A
β-D-Glc*p*A
1
↓
2
→3)-α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Gal*p*-(1→

B **C** **D**

Long-range ^{13}C–^1H correlations (Table 5) obtained from an HMBC spectrum corroborated the assigned four disaccharides as well as the tetrasaccharide elements deduced from the NOESY experiment. The cross-peaks of both anomeric protons and carbons of each of the glycosyl residues were examined, and intra- and inter-residual connectivities were observed from the HMBC experiment (Table 5). Cross-peaks were found between H-1 (δ 4.46 ppm) of the D-glucuronic acid residue and C-2 of the D-mannose residue (**A** H-1, **B** C-2); between C-1 (δ 102.9 ppm) of the D-glucuronic acid residue and H-2 of the D-mannose residue (**A** C-1, **B** H-2), along with other intra-residual couplings between C-1 of D-glucuronic acid and its own H-2 and H-5 atoms. Similarly, cross-peaks between H-1 (δ 5.34 ppm) of D-mannose and C-3 of D-glucose (**B** H-1, **C** C-3) and between C-1 (δ 99.8 ppm) of D-mannose and H-3 of D-glucose (**B** C-1, **C** H-3) were observed.

Table 5. HMBC experiments on S-2 EPS 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	4.46			78.8	**A**: H-1 **B**: C-2
		102.9	3.37		**A**: C-1 **A**: H-2
			3.67		**A**: C-1 **A**: H-5
			4.35		**A**: C-1 **B**: H-2
B	5.34			77.8	**B**: H-1 **B**: C-3
				73.9	**B**: H-1 **B**: C-5
				81.1	**B**: H-1 **C**: C-3
		99.8	3.93		**B**: C-1 **C**: H-3
C	5.21			81.1	**C**: H-1 **C**: C-3
				72.7	**C**: H-1 **C**: C-5
				76.4	**C**: H-1 **D**: C-3
		96.8	3.99		**C**: C-1 **D**: H-3
D	5.28			71.8	**D**: H-1 **D**: C-5
				77.8	**D**: H-1 **B**: C-3
		102.1	4.08		**D**: C-1 **B**: H-3

$J_{H,C}$ connectivities for the anomeric atoms (H-1 and C-1) of the S-2 EPS.
A: β-D-Glc*p*A-(1→; **B**: →2,3)-α-D-Man*p*-(1→; **C**: →3)-α-D-Glc*p*-(1→; and **D**: →3)-α-D-Gal*p*-(1→.

Other intra-residual couplings between H-1 of D-mannose with its C-3 and C-5 were also detected. The cross-peaks between H-1 (δ 5.21 ppm) of D-glucose and C-3 of D-galactose (**C** H-1, **D** C-3) and between C-1 (δ 96.8 ppm) of D-glucose and H-3 of D-galactose (**C** C-1, **D** H-3) were observed, together with two intra-residual couplings between H-1 of D-glucose and its C-3 and C-5. The cross-peaks between H-1 (δ 5.28 ppm) of D-galactose and C-3 of D-mannose (**D** H-1, **B** C-3) and between C-1 (δ 102.1 ppm) of D-galactose and H-3 of D-mannose (**D** C-1, **B** H-3) were also observed. One intra-residual coupling was found between H-1 of D-galactose and its C-5. Thus, the HMBC data clearly support the presence of the tetrasaccharide repeating-unit already described in the polysaccharide S-2 EPS.

2.4. Partially hydrolysis of S-2 EPS

The S-2 EPS was mildly degraded by hydrolysis with TFA or by acetolysis. The resulting mixtures of oligosaccharides were fractionated on Bio-Gel P-2, yielding fractions H-I to H-III (Fig. 4A) and A-I to A-III (Fig. 4D), respectively. Six kinds of oligosaccharides were obtained from these fractions and analyzed by a combination of quantitative monosaccharide analysis by HPLC, methylation analysis, glycosidase digestion, and reduction of reducing ends with $NaBH_4$. We briefly explain here how the structures of oligosaccharides were determined by using the structure of oligosaccharide **3** as an example. Fraction A-III contained only 1 oligosaccharide (Fig. 4E), consisting of D-mannose, D-glucose, and D-galactose at an equimolar ratio, which was named oligosaccharide **3**. After a reduction of the reducing end of oligosaccharide **3**, D-galactose was not detected, whereas D-mannose and D-glucose were, suggesting that D-galactose is the reducing end of oligosaccharide **3**. Among the six glycosidases tested, α- and β-mannosidases, α- and β-glucosidases, and α- and β-galactosidases, only α-mannosidase cleaved the oligosaccharide **3** into D-mannose and an oligosaccharide **2** (Fig. 4F). The oligosaccharide **2** of the cleavage product was obtained by fractionation using Bio-Gel P-2 (Fig. 4G). The obtained oligosaccharide **2** was again treated with the glycosidases mentioned above, and only α-glucosidase cleaved this oligosaccharide into D-glucose and D-galactose (Fig. 4H). Methylation analyses of the oligosaccharides revealed that D-mannose, D-glucose, and D-galactose were substituted at the O-3 positions. The structure of oligosaccharide **2** derived from oligosaccharide **3** was the same to that of oligosaccharide **2** obtained by partial acetolysis of S-2 EPS. Thus, the structure of oligosaccharide **3** was determined to be the following:

α-D-Man*p*-(1→3)-α-D-Glc*p*-(1→3)-D-Gal

B **C** **D**

Structures of the other five oligosaccharides were determined by similar methods, and the results are shown in Figure 5.

Fractions containing oligosaccharides longer than pentaoligosaccharide (fractions before the point indicated by the arrow in Fig. 4A) were combined, mildly hydrolyzed with TFA, and subjected to Bio-Gel P-2 gel filtration column chromatography (Fig. 4B). This procedure was repeated (Fig. 4C), and the same three oligosaccharides (oligosaccharides **4**–**6**) and no other oligosaccharides shorter than pentaoligosaccharides were obtained by the TFA-hydrolysis. The results obtained here also indicate that S-2 EPS consists of tetrasaccharide repeating units shown in Formula 1.

As far as we know, the structure of S-2 EPS is novel since there have been no reports on EPS having the same structure in databases, such as Chemical Abstracts, KEGG GLYCAN, and Glycosciences. There have been several reports by Richards' research group on the chemical structures of five EPSs produced by different serotypes of the equine pathogen *R. equi*,[9–13] which are different from the structure of S-2 EPS and were not reported to contain fatty acids. There have been several reports on bacterial EPS containing long-chain fatty acids (mainly C_{16}, C_{18}) attached via alkali-labile ester bonds, such as emulsan produced by *Acinetobacter lwoffii* RAG-1,[14] simusan produced by *Arthrobacter* sp. strain CE-17,[15] and two EPS's produced by *R. rhodochrous* ATCC 53968[16] and SM-1.[17] These EPS are reported to contain similar amounts of fatty acids and

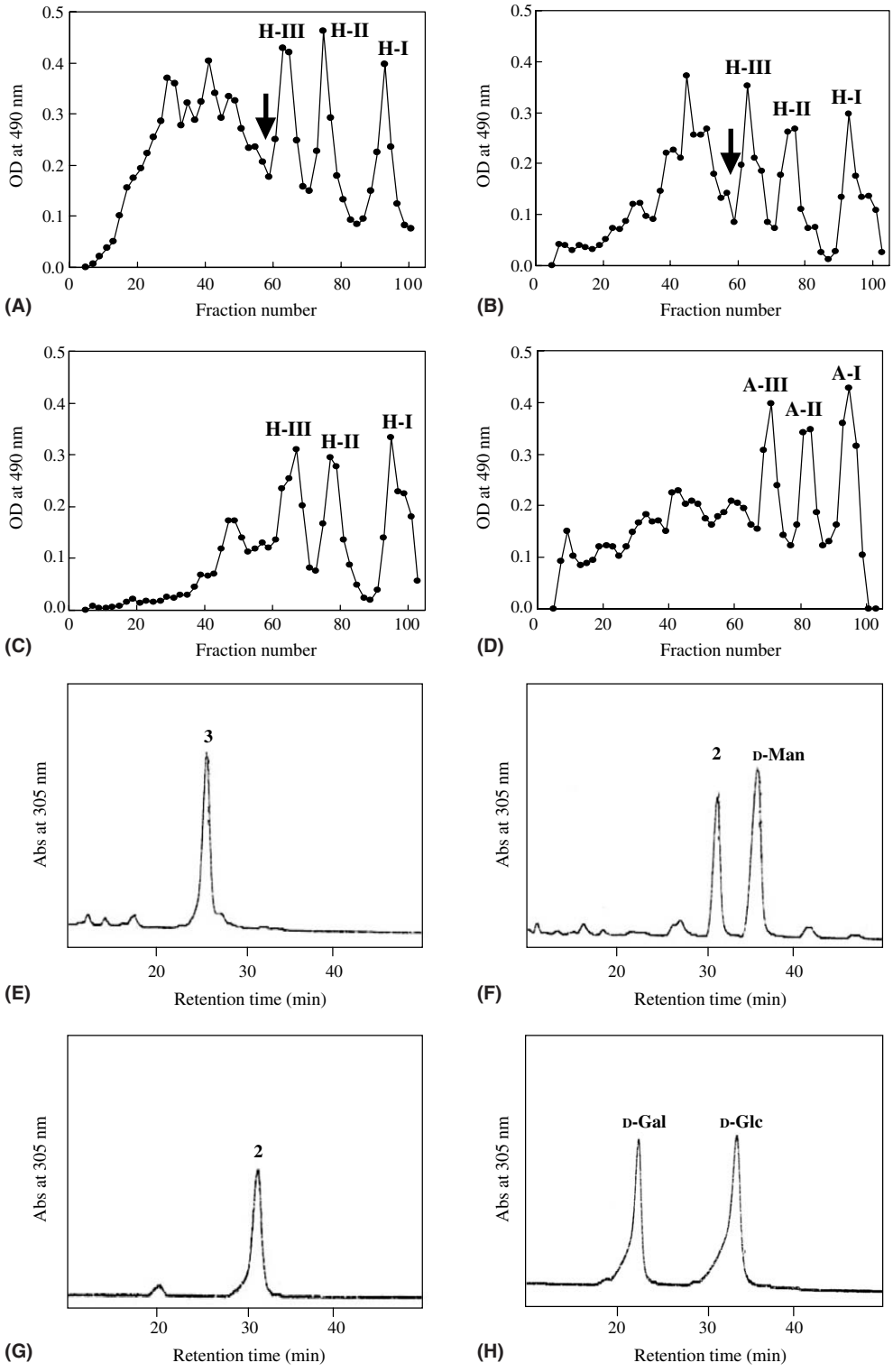

Figure 4. Analysis of oligosaccharides prepared by partial TFA-hydrolysis (A–C) and acetolysis of S-2 EPS (D): Bio-Gel P-2 elution profile of first round (A), second round (B), and third round (C) of TFA-hydrolysis, and partial acetolysis (D) of S-2 EPS are shown. HPLC profiles of ABEE-labeled fraction A-III (E), α-mannosidase-cleaved products of oligosaccharide **3** (F), purified oligosaccharide **2** from cleavage products using Bio-Gel P-2 (G), and α-galactosidase-cleaved products of the oligosaccharide **2** (H) are also given.

to function as a biosurfactant, moisture absorbent or thickener. We are now studying the function of fatty acid residues of S-2 EPS as well as the positions of bindings between the polysaccharide chain and the fatty acid residues. The chemical structure of sugar chain of S-2 EPS reported here provides fundamental data for inves-

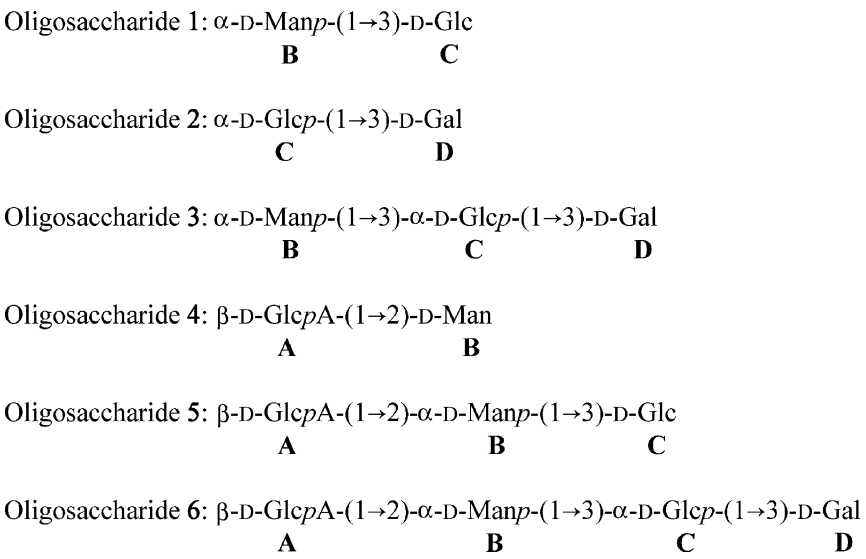

Figure 5. Structures of oligosaccharides obtained from partially acid-hydrolyzed or partially acetolyzed S-2 EPS. Oligosaccharides **1**–**6** were obtained from the fractions A-II, A-II, A-III, H-I, H-II, and H-III, respectively.

tigating its roles in the protection against hydrocarbon toxicity and the promotion of oil degradation by marine bacteria.

3. Experimental

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

R. rhodochrous strain S-2[18] was grown on IB agar plates[19] at 37 °C under aerobic conditions. After a 3-day incubation the cells were collected by scraping and suspended in saline. The cell suspensions were vigorously vortexed and then shaken at 110 rpm for 30 min at room temperature and centrifuged at 10,000 × *g* for 20 min. The supernatant was saved, and the pellet was resuspended in saline, after which the extraction process was repeated.

3.2. Purification of the EPS

The above supernatants were mixed, and then treated with DNase I and RNase at the concentration of 7 units/mL and 2 μg/mL, respectively. After overnight incubation at 37 °C the mixtures were digested with proteinase K (2 μg/mL) at 37 °C for 1 h and then purified by phenol–$CHCl_3$ extraction or the CTAB method[9] followed by EtOH precipitation. The pellets were dissolved in water, and again treated with the enzymes and purified as described above. After at least four times dialysis treatments, each against 5 L of water overnight at 4 °C, the samples were lyophilized.

The lyophilized powder (40 mg) was suspended in 10 mM Tris–HCl buffer (pH 8.0) at the concentration of 1 mg/mL and subjected to DEAE-Toyopearl 650M column chromatography (250 mm × 25 mm∅; Tosoh Co., Tokyo, Japan). After the column had been washed with 120 mL of 10 mM Tris–HCl buffer (pH 8.0), the EPS was eluted with a 600-mL linear gradient (0–1 M) of NaCl in the same buffer. Fractions containing EPS were monitored by the phenol–H_2SO_4 method,[20] combined, dialyzed against water, and lyophilized.

3.3. Modification of S-2 EPS

Carboxyl-reduction was performed by using 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and $NaBH_4$ as the reducing agents.[21] The procedure resulted in more than 90% of the D-glucuronic acid being converted to the D-glucose, as judged by the determination of the monosaccharide content. Deacylation was performed as follows: S-2 EPS (25 mg) was treated with 5 mL of 0.1 M aq NaOH for 16 h at 37 °C, neutralized with aq HCl, and dialyzed against water. To lower the molecular weight, we ultrasonicated the S-2 EPS solution (5 mg/mL) on ice by using an Astrason model XL2020 (Misonix Inc., NY, USA).

3.4. Gel-filtration column chromatography

Gel-filtration column chromatography for determination of the molecular weight was performed on a Sephacryl S1000 or Sepharose CL-2B (850 mm × 15 mm∅; Amersham Biosciences UK Ltd., Buckinghamshire, UK) column, with 1 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl or MilliQ water, respectively, as the eluent, and Dextran T2000 (Amersham Biosciences UK Ltd., Buckinghamshire, UK) as a size marker. Gel filtration for preparation of oligosaccharides was

performed by using a column (900 × 15 mm∅) of Bio-Gel P-2 with 0.2 M acetic acid as the eluent. Fractions containing saccharide were monitored by the phenol–H_2SO_4 method.

3.5. Hydrolysis of S-2 EPS

S-2 EPS was completely hydrolyzed by H_2SO_4 or TFA. In the case of hydrolysis by H_2SO_4, S-2 EPS (10 mg) was dissolved in 0.5 mL of chilled 80% aq H_2SO_4, and then incubated in an ice-cold water bath for 30 min. The mixture was subsequently incubated at 30 °C for 3 h. After the acid treatment, the mixture was diluted with 6.5 mL of chilled water to a final concentration of 1 M H_2SO_4. Next it was incubated at 100 °C for 2 h, and then neutralized by the addition of 0.8 g $CaCO_3$. The reaction mixture was thereafter filtered to remove the precipitate. After the filtrate had been dried and redissolved in MilliQ water, it was subjected to Amberlite IR-120 chromatography to remove the calcium ions. In the case of hydrolysis by TFA, S-2 EPS (50 μg) was treated in 66% TFA (50 μL) at 100 °C for 18 h.

S-2 EPS was partially hydrolyzed or acetolyzed as follows: A sample (10 mg) was partially hydrolyzed in 2% aq TFA (10 mL) at 100 °C for 3 h. The partial hydrolyzates were then lyophilized and dissolved in 0.2 M acetic acid. For partial acetolysis, a sample (5 mg) was dissolved in a 4.3-mL mixture of Ac_2O–acetic acid–concd H_2SO_4 (24:15:3 (v/v/v)) on ice and incubated for 18 h at 60 °C. The reaction mixture was diluted with 50 mL of ice-water and neutralized with 5 g of Na_2CO_3. The resulted acetate was recovered by $CHCl_3$ extraction, lyophilized, and then deacetylated by treatment with 0.05 M NaOMe in MeOH (50 mL). The solution of oligosaccharides was de-ionized with Amberlite IR120 (H-form) resin.

3.6. GLC analysis

The H_2SO_4 hydrolyzates were analyzed by GLC as follows: Prior to GLC analysis, uronic acids and neutral sugars were separated by Dowex I-X8 anion-exchange chromatography. Trifluoroacetylalditol derivatives of neutral sugars[22] and trimethylsilyl derivatives of uronic acids[23] were analyzed by GLC (Shimadzu GC-6A; Shimadzu, Kyoto, Japan) using a DC QF-1 column and an NPGSE column, respectively. A mixture of L-arabinose, L-fucose, D-galactose, D-glucose, D-mannose, L-rhamnose, D-xylose, D-galacturonic acid, and D-glucuronic acid (1 mg/mL each) was treated as described above and then employed as a standard.

3.7. HPLC analysis

Monosaccharides and oligosaccharides were labeled with 4-aminobenzoic acid ethyl ester (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.

3.8. Methylation analysis

Methylation of the polysaccharides was performed according to Hakomori's method using sodium methylsulfinyl anion and CH_3I.[24] Methylation of oligosaccharides was performed as described by Hakomori except that *N*,*N*-dimethylformamide was used instead of Me_2SO. The methylated polysaccharides and oligosaccharides were then hydrolyzed, reduced with $NaBH_4$, and acetylated before analysis on GLC–MS.

3.9. Fatty acid analysis

S-2 EPS (3 mg) was hydrolyzed at 100 °C for 90 min in 3 mL of MeOH containing 10% KOH. After extraction with *n*-hexane the hydrolyzate was acidified by the addition of aq HCl, and then the fatty acids were extracted with *n*-hexane. The fatty acids were dried in vacuo and then methylated by using trimethylsilyldiazomethane reagent (Nakalai Tesque Co., Kyoto, Japan) according to the supplier's instructions. The samples were subsequently subjected to gas chromatography–mass spectrometry (GLC–MS) analysis. The GLC–MS analysis was performed with a QP-5050 instrument (Shimadzu, Kyoto, Japan) fitted with a fused silica capillary column (DB-1, 30 m × 0.25 mm∅; Agilent Technologies, Palo Alto, CA, USA). The operating temperature of the injection port and the interface was 300 °C. The column temperature was set at 150 °C for 2 min and increased to 300 °C at the rate of 10 °C/min.

3.10. 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 parts per million, with acetone (δ^1H 2.23 ppm, δ^{13}C 31.1 ppm) used as internal reference for samples measured in D_2O solutions. Assignments of signals were made from 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.

3.11. General methods

Purity of EPS was analyzed by cellulose acetate membrane electrophoresis as described by Seno et al.,[25] using 0.2 M barium acetate (pH 7.5) as an electro-

phoresis buffer, 0.5% Toluizine blue as a stain, and water for destaining. For determination of absolute configuration, the TFA hydrolyzate of carboxyl-reduced S-2 EPS was converted into the acetylated (+)-2-octyl glycosides and analyzed by GLC.[26] Glycosidases were purchased from Seikagaku Corporation (Tokyo, Japan) except for α- and β-glucosidases and β-glucuronidase, which were purchased from Wako Pure Chemicals, and used as described by the manufacturers.

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