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Structural analysis of an extracellular polysaccharide produced by a benzene tolerant bacterium, *Rhodococcus* sp. 33

Makoto Urai,[a] Tomoko Aizawa,[a] Hirosi Anzai,[b] Jun Ogihara,[c] Noriyuki Iwabuchi,[a] Brett Neilan,[d] Iain Couperwhite,[d] Mutsuyasu Nakajima[a] and Michio Sunairi[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]*School of Biotechnology and Biomolecular Sciences, Faculty of Science, University of New South Wales, Sydney, NSW 2052, Australia*

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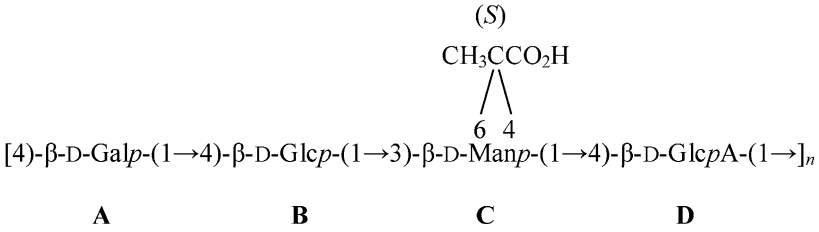
Abstract—*Rhodococcus* sp. 33 can tolerate and efficiently degrade various concentrations of benzene, one of the most toxic and prevailing environmental pollutants. This strain produces a large quantity of extracellular polysaccharide (33 EPS), which plays an important role in the benzene tolerance in *Rhodococcus* sp. 33, especially by helping the cells to survive an initial challenge with benzene. This EPS has been reported to be composed of D-galactose, D-glucose, D-mannose, D-glucuronic acid, and pyruvic acid at a molar ratio of 1:1:1:1:1. To understand the protective effect of 33 EPS, we determined its chemical structure by using ^{1}H and ^{13}C NMR spectroscopy including 2D DQF-COSY, TOCSY, HMQC, HMBC, and NOESY experiments. The polysaccharide was shown to consist of tetrasaccharide repeating units with the following structure:



Keywords: *Rhodococcus*; Bioremediation; Benzene tolerance; Extracellular polysaccharide; Structure; NMR spectroscopy

1. Introduction

Rhodococcus sp. 33 was reported to tolerate and to efficiently degrade various concentrations of benzene up to its maximal solubility (1.8 g/L) in aqueous media.[1] To elucidate the mechanism by which strain 33 tolerates such high concentrations of benzene, Gutierrez et al. examined the effect of benzene on the cellular fatty acid composition and the fluidity of the membranes of strain 33.[2,3] The results showed an increase in the ratio of saturated/unsaturated cellular fatty acids in the presence of

* Corresponding author. Tel.: +81 466 84 3706; fax: +81 466 84 3354; e-mail: sunairi@brs.nihon-u.ac.jp

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benzene from 1.3 (for mannitol-grown cells) to 1.5 (for benzene-grown cells).[2] The fluidity of the membranes of strain 33 decreased after 18–20 h incubation in the presence of benzene,[3] suggesting this decrease in fluidity, caused by changes in the cellular fatty acid composition, as a possible mechanism for the benzene tolerance. Although the fluidity of the membranes was observed to decrease after the cells had been exposed to benzene for approximately 18–20 h, the immediate effect of benzene on the membrane was increased fluidity, which was observed for more than 6 h. These data suggest that an adaptative response, set to mitigate benzene toxicity, was induced only after long exposure and that with respect to the organism's tolerance during the first 6 h, *Rhodococcus* sp. 33 may have a well-adapted inherent mechanism to survive an initial challenge with benzene.

Recently, Aizawa et al. examined the effect of 33 EPS on the benzene tolerance of *Rhodococcus* sp. 33.[4] They showed that under resting or growing conditions, rough mutant strains derived from strain 33 were more sensitive to benzene than the mucoidal parent strain. The rough strains produced no or little EPS, whereas the parental strain 33 did so in large quantity (33 EPS). By the addition of 33 EPS to the rough strains, both the survival and growth of the rough strains in media containing benzene were improved. These data indicate that 33 EPS plays an important role in the benzene tolerance in *Rhodococcus* sp. 33, especially by helping the cells to survive an initial challenge with benzene. They also reported 33 EPS to be an acidic polysaccharide containing D-galactose, D-glucose, D-mannose, D-glucuronic acid, and pyruvic acid at a molar ratio of 1:1:1:1:1.

The elucidation of the chemical structure of 33 EPS as well as the isolation of genes and enzymes required for the synthesis and modification of it should lead to a better understanding of the principles underlying the protection from benzene toxicity. Such knowledge would undoubtedly expand the possibilities of bioremediation of benzene-contaminated environments. Therefore, in this study we determined the chemical structure of 33 EPS by methylation analyses and ^{1}H and ^{13}C NMR spectroscopic analyses including 2D DQF-COSY, TOCSY, HMQC, HMBC, and NOESY experiments.

2. Results and discussion

2.1. Depyruvylated 33 EPS

By heating 33 EPS in dilute AcOH, we depyruvylated it and then examined this form for its protective effect against benzene toxicity. No signals of the pyruvic acid moiety were detected by NMR analysis of depyruvylated 33 EPS, as described below, and no pyruvic acid was detected enzymatically after complete hydrolysis of depyruvylated 33, indicating that the depyruvylated 33 EPS contained no or just few pyruvic acid moieties. After the addition of depyruvylated 33 EPS to the medium, the benzene-sensitive rough mutants derived from *Rhodococcus* sp. 33 showed no growth in the presence of benzene; whereas by the addition of native 33 EPS they showed good growth, indicating that the pyruvic acid moiety of 33 EPS might be involved in its protective effect against benzene toxicity.

2.2. Methylation analysis

Native, carboxyl-reduced, and depyruvylated 33 EPSs were methylated, and the derived alditol acetates were analyzed by GLC–MS (Table 1). For the 3 EPSs, the data shown in Table 1 indicate that D-galactose and D-glucose were substituted at their O-4 position. In the methylated native 33 EPS, 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 in a molar ratio almost equal to that of the two other 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-mono-*O*-methyl-D-mannitol. On the other hand, in the methylation analysis of the carboxyl-reduced 33 EPS, 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 detected. These data indicate that D-glucuronic acid residues were also substituted at their O-4 position. In the methylation analysis of native 33 EPS, 1,3,4,5,6-penta-*O*-acetyl-2-mono-*O*-methyl-D-mannitol was detected; whereas 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol was detected in that of depyruvylated 33 EPS, indicating that a pyruvic acid residue was bound to both O-4

Table 1. Methylation analysis data on 33 EPS

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

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

and O-6 positions of every D-mannose residue of the 33 EPS. The methylation analyses thus indicate that the 33 EPS is composed of 4-linked galactose, 4-linked glucose, 4-linked glucuronic acid, and 3-linked mannose with pyruvic acid linked to its O-4 and O-6.

2.3. NMR analyses

A signal in the high-field region (1.47 ppm) was detected in the ^{1}H NMR spectrum of the sonicated 33 EPS (Fig. 1A) but not in that of depyruvylated 33 EPS (Fig. 1B), which was attributed to the methyl proton of pyruvic acid moieties. In the ^{13}C NMR spectrum of sonicated 33 EPS (Fig. 2A and B) signals at 25.6, 102.8, and 175.9 ppm were detected, which are, respectively, characteristic of the methyl carbon, acetal carbon, and carboxyl carbon of the pyruvic acid moiety. These signals were not detected in the ^{13}C NMR spectrum of depyruvylated 33 EPS (Fig. 2C and D). These data indicate that the 33 EPS were depyruvylated by heating in dilute AcOH.

In the anomeric region of the ^{1}H NMR spectrum of either sonicated or depyruvylated 33 EPS (Fig. 1), one well-resolved signal at 4.48 ppm and overlapping signals around 4.6 ppm were observed. In the ^{13}C NMR spectrum of depyruvylated 33 EPS (Fig. 2C and D) revealed 4 signals in the anomeric region at δ 100.6, 100.8, 103.9, and 104.1 ppm, and these four anomeric carbons were correlated to four anomeric proton at δ 4.66, 4.63, 4.48, and 4.69 ppm, respectively, by HMQC experiment (data not shown). Similarly, four anomeric carbon signals were observed and correlated to anomeric protons

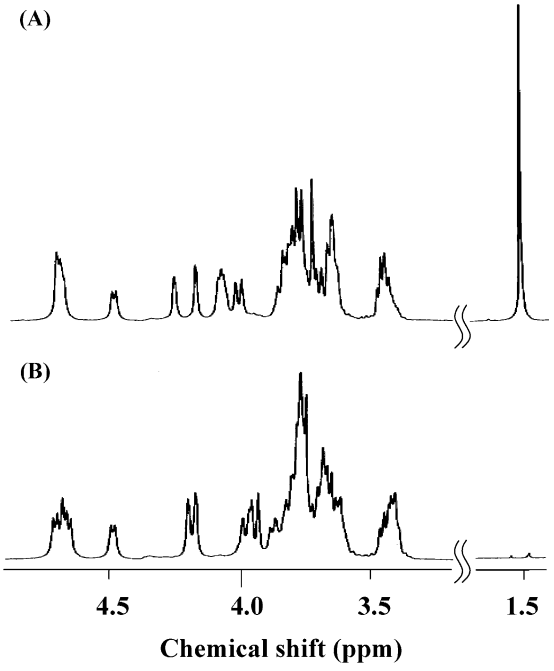


Figure 1. 500 MHz ^{1}H NMR spectra of sonicated 33 EPS (A) and depyruvylated 33 EPS (B) recorded in D_2O at 70 °C.

by the 1D and 2D experiments of sonicated 33 EPS. These data indicate the presence of four anomers in the 33 EPS. The relative intensity of the methyl protons (1.47 ppm) was three times higher than that of the anomeric proton at δ 4.48 ppm and almost equal to that of the three anomeric protons at δ 4.68, 4.69, and 4.71 ppm, indicating one pyruvic acid moiety to be present in every repeating 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 depyruvylated 33 EPS (Table 2) were assigned by means of 2D TOCSY (mixing times 100–300 ms; Fig. 3) 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 TOCSY spectra with increasing mixing times allowed the assignment of the sequential order of the chemical shifts belonging to a single spin system. The signal at δ 4.66 ppm was assigned to H-1 of the D-mannopyranose residue (**C** residue) on the basis of its broad singlet appearance and its rather high-field chemical shift. Since the spin system of the **A** residue was distinguished from those of **B** and **D** residues by typically small $J_{4,5}$ value of <3 Hz, the **A** residue was assigned to the D-galactopyranose residue. The remaining two spin systems, **B** and **D** residues, were attributed to the D-glucopyranose 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 depyruvylated 33 EPS (Table 2) could be assigned by using the 2D ^{13}C–^{1}H HMQC spectrum. All four residues **A**–**D** were allocated to a β anomeric configuration based on the observed $J_{C\text{-}1,H\text{-}1}$ values.[5] Similarly, the ^{1}H and ^{13}C chemical shifts of the sonicated 33 EPS were assigned (Table 3).

A comparison of ^{13}C NMR chemical shift data for the sonicated 33 EPS (Table 3) and the depyruvylated 33 EPS (Table 2) revealed that, following the depyruvylation, the resonances of C-4 and C-6 of residue **C** showed upfield shifts of 6.8 ppm (from 72.9 to 66.1 ppm) and 3.1 ppm (from 65.1 to 62.0 ppm), respectively, which is indicative of substitution at the corresponding positions.[6] These data are in agreement with the results of the methylation analysis. The chemical shift of the pyruvate methyl carbon (25.6 ppm, Table 3) showed that the pyruvate acetal carbon atom had the (*S*) configuration.[7]

The sequence of glycosyl residues was determined by using long-range ^{13}C–^{1}H correlations obtained from an HMBC spectrum (Fig. 4). The cross-peaks of both anomeric protons and carbons of each of the glycosyl residues were examined, and intra- and inter-residual connectivities were obtained from the HMBC experiment (Table 4). Cross-peaks were found between H-1 of **A** residue and C-4 of **B** residue and between C-1 of

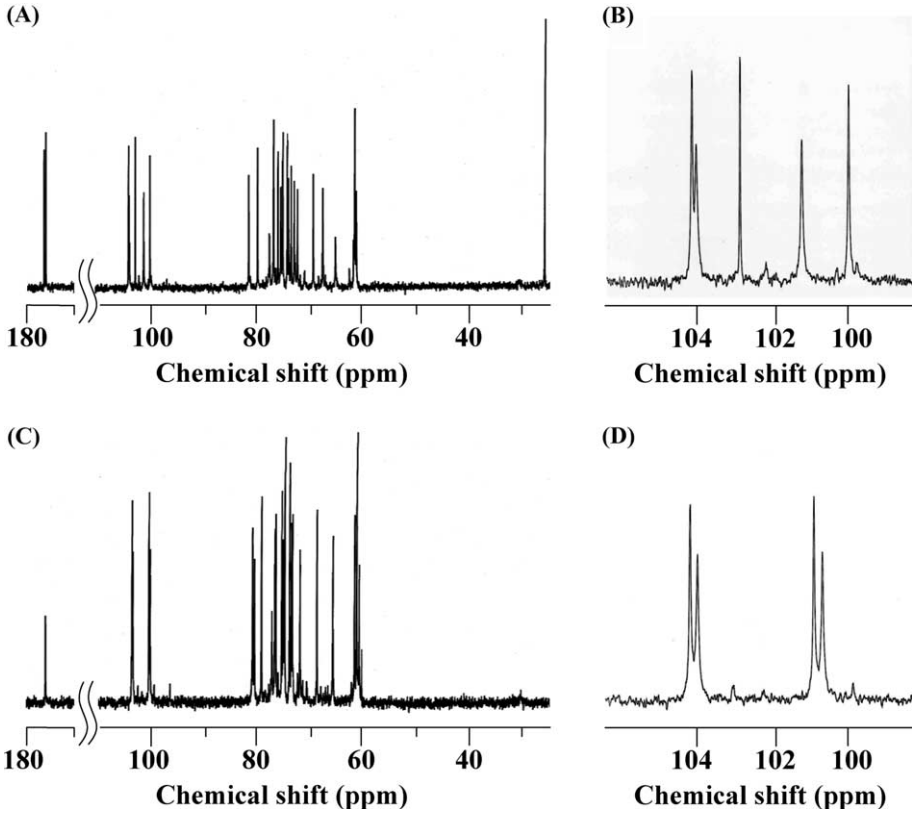


Figure 2. 125 MHz ^{13}C NMR spectra of sonicated 33 EPS (A and B) and depyruvylated 33 EPS (C and D) recorded in D_2O at 70 °C. A signal at 102.8 ppm (A and B) was assigned to the acetal carbon of the pyruvic acid moiety.

Table 2. ^{1}H and ^{13}C NMR chemical shifts (ppm) of depyruvylated 33 EPS 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
→4)-β-D-Gal*p*-(1→	4.48 (7.5)	3.64	3.78	4.16	3.76	3.74	3.81
A	103.9 (163)	72.3	76.8	77.6	75.7	61.6	
→4)-β-D-Glc*p*-(1→	4.63 (8.0)	3.41	3.67	3.63	3.77	3.84	3.99
B	100.8 (161)	73.7	75.1	79.6	73.9	61.1	
→3)-β-D-Man*p*-(1→	4.66	4.18	3.87	3.71	3.42	3.78	3.94
C	100.6 (161)	69.1	80.9	66.1	77.0	62.0	
→4)-β-D-Glc*p*A-(1→	4.69 (8.0)	3.46	3.65	3.75	3.73		
D	104.1 (163)	74.3	75.2	81.2	75.5	175.6	

A residue and H-4 of **B** residue. 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 **B** residue and C-3 of **C** residue and between C-1 of **B** residue and H-3 of **C** residue were observed. The following sequence was thus established:

β-D-Glc*p*-(1→3)-β-D-Man*p*-(1→

B **C**

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

β-D-Man*p*-(1→4)-β-D-Glc*p*A-(1→

C **D**

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

β-D-Glc*p*A-(1→4)-β-D-Gal*p*-(1→

D **A**

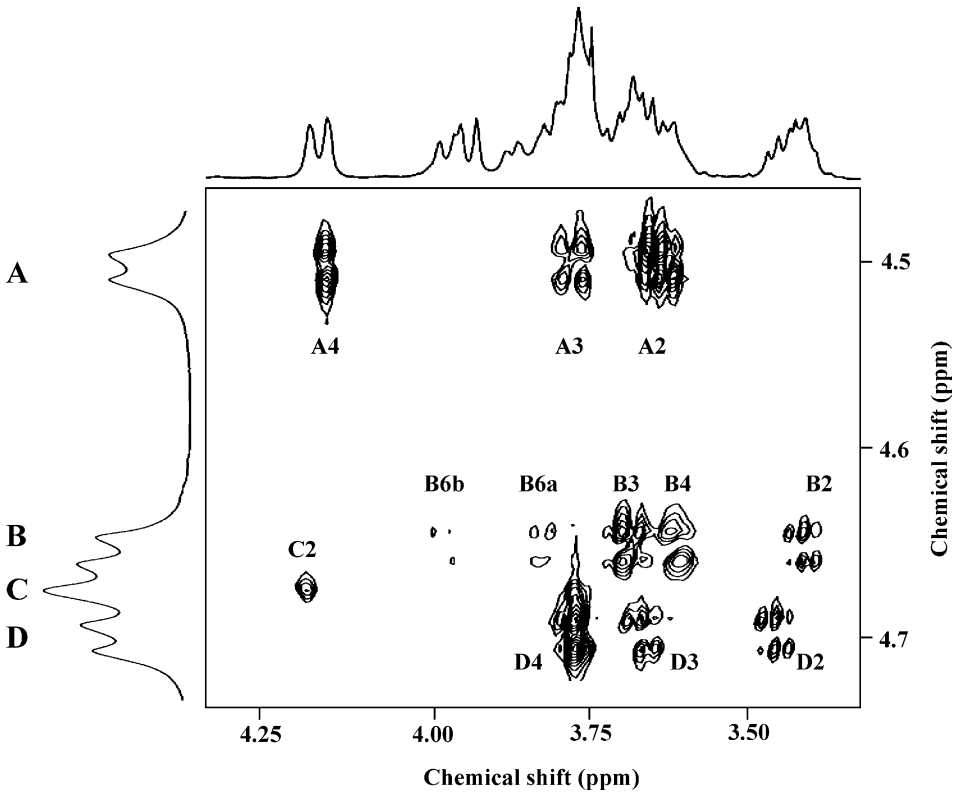


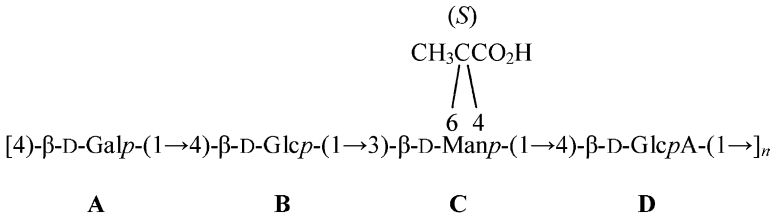
Figure 3. TOCSY spectrum of depyruvylated 33 EPS. Complete assignment required several TOCSY experiments having mixing times ranging from 100 to 300 ms.

Table 3. ^{1}H and ^{13}C NMR chemical shifts (ppm) of sonicated 33 EPS recorded in D_2O at 70 °C

Glycosyl residue	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	Pyruvic acid residue		
	C-1 ($J_{C-1,H-1}$)	C-2	C-3	C-4	C-5	C-6		CH_3	C	CO_2H
→4)-β-D-Gal*p*-(1→	4.48	3.64	3.76	4.16	3.74	3.72	3.79			
A	104.0 (164)	72.3	76.7	77.6	75.4	61.5				
→4)-β-D-Glc*p*-(1→	4.68	3.45	3.67	3.66	3.78	3.84	4.00			
B	100.0 (160)	73.5	75.9	79.8	73.9	61.2				
→3)-β-D-Man*p*-(1→										
6 4										
\ /	4.71	4.25	4.07	3.84	3.41	3.75	4.06	1.47		
CH_3CCO_2H	101.2 (160)	69.3	76.8	72.9	67.5	65.1		25.6	102.8	175.9
C										
→4)-β-D-Glc*p*A-(1→	4.69	3.44	3.62	3.73	3.70					
D	104.1 (162)	74.2	75.0	81.4	75.1	175.6				

NOESY studies (Table 5) confirmed the results of the HMBC experiments (Table 4). Residue **A** had a strong 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**, indicating that residue **B** was linked at the C-3 position of the **C** residue. 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 the residue **A**. Thus, the NOESY data clearly support the presence of the tetrasaccharide repeating unit in the polysaccharide 33 EPS as described above.

The results obtained here indicate that 33 EPS consists of tetrasaccharide repeating unit containing an acetal-linked pyruvic acid moiety with the following structure:



Among bacterial polysaccharides whose structures have been reported so far, the chemical structure of the capsular polysaccharide produced by *R. equi* serotype 3 reported by Severn and Richards[8] seems to be most similar to that of 33 EPS determined in this study, except for the modification of mannose residue and the substitutional position of pyruvate. This study shows that the pyruvic acid moiety of 33 EPS may be involved in the protective effect of 33 EPS against benzene toxicity. Aizawa et al. suggested that carboxyl residues and the high molecular weight of these 33 EPS should contribute to the protective effect of 33 EPS against benzene toxicity based on the data obtained from similar experiments using carboxyl-reduced or partially hydrolyzed 33 EPS.[4]

The chemical structure of 33 EPS reported here provides fundamental data for investigating its roles in the protection against benzene toxicity.

Table 4. HMBC experiments on depyruvylated 33 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	4.48			79.6	**A**:H-1 **B**:C-4
		103.9	3.63		**A**:C-1 **B**:H-4
B	4.63			80.9	**B**:H-1 **C**:C-3
		100.8	3.41		**B**:C-1 **B**:H-2
			3.87		**B**:C-1 **C**:H-3
C	4.66			69.1	**C**:H-1 **C**:C-2
				81.2	**C**:H-1 **D**:C-4
		100.6	3.75		**C**:C-1 **D**:H-4
D	4.69			77.6	**D**:H-1 **A**:C-4
		104.1	3.46		**D**:C-1 **D**:H-2
			4.16		**D**:C-1 **A**:H-4

$J_{H,C}$ connectivities for the anomeric atoms (H-1 and C-1) of the depyruvylated 33 EPS.
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→.

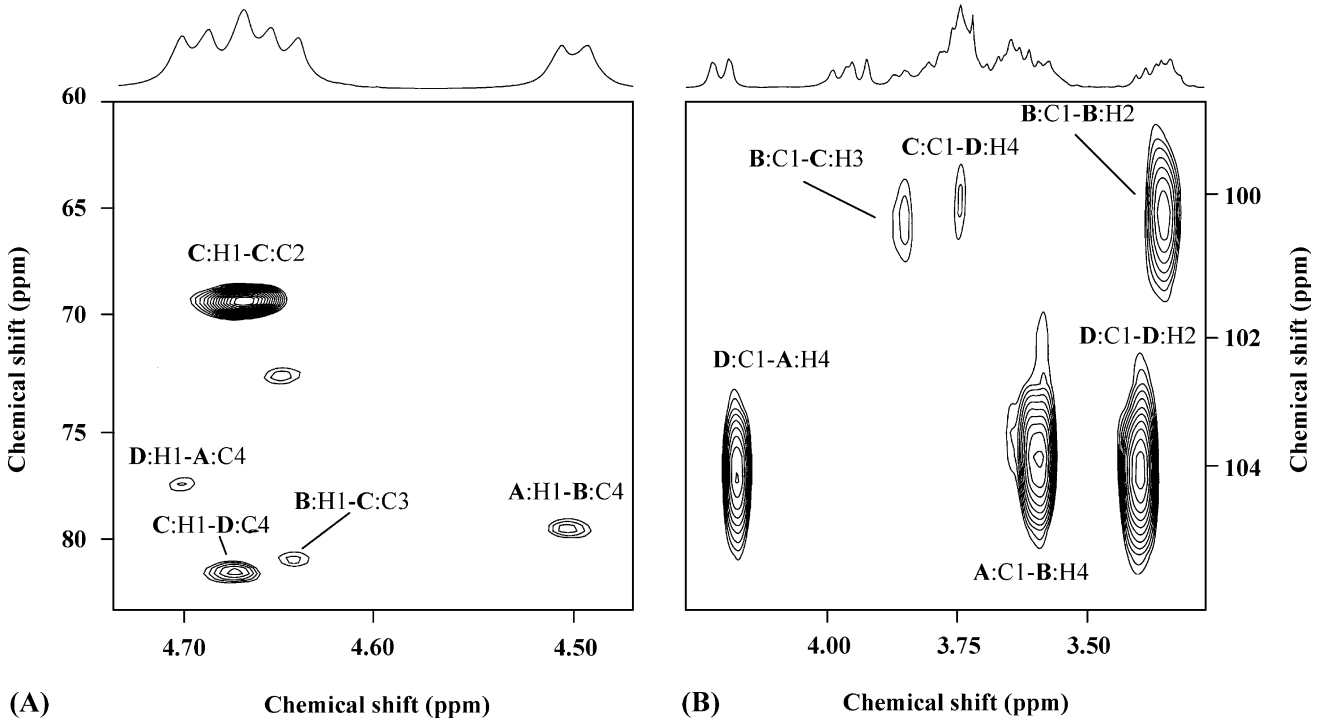


Figure 4. HMBC spectra of depyruvylated 33 EPS: (A) anomeric proton region and (B) anomeric carbon region.

Table 5. NOESY experiments on depyruvylated 33 EPS in D_2O at 70 °C

Residue	Anomeric atom δ (^{1}H)	NOE contact δ (^{1}H)	Residue atom	Intensity[a]
→4)-β-D-Gal*p*-(1→	4.48	3.64	**A**:H-2	s
A		3.78	**A**:H-3	s
		4.16	**A**:H-4	w
		3.63	**B**:H-4	s
		3.84	**B**:H-6a	w
		3.99	**B**:H-6b	w
→4)-β-D-Glc*p*-(1→	4.63	3.41	**B**:H-2	s
B		3.67	**B**:H-3	s
		4.18	**C**:H-2	w
		3.87	**C**:H-3	s
→3)-β-D-Man*p*-(1→	4.66	4.18	**C**:H-2	s
C		3.87	**C**:H-3	s
		3.75	**D**:H-4	s
→4)-β-D-Glc*p*A-(1→	4.69	3.46	**D**:H-2	s
D		3.65	**D**:H-3	w
		3.75	**D**:H-4	s
		4.16	**A**:H-4	s

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

3. Experimental

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

Rhodococcus sp. 33[1] and its benzene-sensitive rough mutants, R0, R1, R2, R3, R4, and R5,[4] were grown on PAS agar media containing 1% mannitol as a carbon source at 28 °C under aerobic conditions for 3 days, as described previously.[1] The 33 EPS was extracted from the harvested cells and purified as described earlier.[9,10]

3.2. Benzene tolerance test

A loopful of *Rhodococcus* sp. 33 cells grown on nutrient agar plates was inoculated into 50 mL of PAS medium containing 1% mannitol in a 250-mL shake flask and incubated with shaking for 3 days. After incubation, 2.5 mL of this culture was inoculated into 50 mL of fresh PAS medium containing 1% mannitol and incubated for 24 h. Then, 2.5 mL of this culture was transferred into 50 mL of PAS medium equilibrated with benzene in a 250-mL shake flask; and, in the case of depyruvylated 33 EPS, it was added to the medium at 0.1 mg/mL as the final concentration. The media were incubated with shaking. PAS medium equilibrated with benzene was prepared as follows: Fifty milliliters of PAS medium was dispensed into a 250-mL shake flask, and an empty test tube (100 mm × 10 mm∅) was placed inside the flask. Benzene was added to the test tube to allow its vapor to diffuse into the medium and the headspace. The flask was plugged with cotton to allow diffusion of air into the system. The flask was preincubated with shaking (100 rpm) for 1 h before inoculation. All procedures were performed at 28 °C.

3.3. Modification of 33 EPS

Carboxyl reduction was performed by using 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-*p*-toluenesulfonate and $NaBH_4$ as the reducing agents.[11] 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. Depyruvation was performed as follows: 33 EPS (30 mg) was treated with 6 mL of 2% AcOH for 2 h at 100 °C, neutralized, and dialyzed against water. To lower the molecular weight, we ultrasonicated the 33 EPS solution (5 mg/mL) on ice by using an Astrason model XL2020 (Misonix Inc., NY, USA).

3.4. Methylation analysis

Methylation of the polysaccharides was performed according to the Hakomori method using sodium methylsulfinyl anion and CH_3I.[12] The methylated polysaccharides were then hydrolyzed, reduced, and acetylated before analysis by GLC–MS.

3.5. 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 ppm, with acetone (δ ^{1}H 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.

3.6. General methods

Sugar content was determined by the phenol–H_2SO_4 method.[13] Purity of EPS was analyzed by cellulose acetate membrane electrophoresis as described by Seno et al.,[14] using 0.2 M barium acetate (pH 7.5) as an electrophoresis buffer, 0.5% Toluizine blue as a stain, and water for destaining. For determination of the absolute configuration of the EPS, the TFA hydrolysate of carboxyl-reduced 33 EPS was converted into acetylated (+)-2-octyl glycoside and analyzed by GLC.[15] 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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