Original Article

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Characterization of the chemical structure and innate immune-stimulating activity of an extracellular polysaccharide from *Rhizobium* sp. strain M2 screened using a silkworm muscle contraction assay

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Summary **We screened innate immunostimulant-producing bacteria using a silkworm muscle contraction assay, and isolated *Rhizobium* sp. strain M2 from soil. We purified the innate immunostimulant from strain M2, and characterized the chemical structure by nuclear magnetic resonance spectroscopy and chemical analyses. The innate immunostimulant (M2 EPS) comprised glucose, galactose, pyruvic acid, and succinic acid with a molar ratio of 6.8:1.0:0.9:0.4, and had a succinoglycan-like high molecular-weight heteropolysaccharide structure. To determine the structural motif involved in the innate immunostimulating activity, we modified the M2 EPS structure chemically, and found that the activity was increased by removal of the succinic and pyruvic acid substitutions. Strong acid hydrolysis completely inactivated the M2 EPS. Unmasking of the β-1,3/6-glucan structure of the side-chain by deacylation and depyruvylation may enhance the innate immune-stimulating activity of M2 EPS. These findings suggest that the succinoglycan-like polysaccharide purified from strain M2 has innate immune-stimulating activity, and its glycan structure is necessary for the activity.**

Keywords: *Rhizobium*, extracellular polysaccharide, structure, innate immune-stimulating activity, silkworm muscle contraction assay

1. Introduction

Innate immunity is an important defense mechanism against microbe infection and tumor development. Natural agents that stimulate innate immunity may contribute to maintain human health. Medicinal herbs and mushrooms are sources of antitumor and immunomodulating agents based on their empirical drug actions, and many active polysaccharides have been extracted (*1*). The structures of these active polysaccharides, however, are limited to β-glucans and pectins because they are mainly extracted from the cell walls of fungi and plants, respectively (*1*). Because polysaccharides extracted from cell walls are generally heterogeneous, the structural motif involved in their immune-stimulating activities is difficult to determine. Chemical synthesis of the highly polymerized glycan structure to imitate the active polysaccharide is also difficult.

Several applications of extracellular polysaccharide (EPS) secreted by bacteria have been proposed,

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including those taking advantage of their antitumor or immunomodulating activities (*2*). Bacteria produce EPS with diverse structures made up of various kinds of monosaccharides, and are frequently substituted with organic or inorganic substances, such as organic acids, phosphate, and sulfate (*2*). EPS secreted to an ambient environment by bacteria are usually homogeneous compared to the cell wall polysaccharides extracted from fungi or plants, and their structures are constructed of regularly repeating units (*2*). This homogeneous and consistent structure may have advantages for determining the structure-activity relations. Wide screening of innate immune stimulants from bacterial EPS using a convenient method to evaluate innate immune-stimulating activities has not been performed.

We previously proposed using silkworm larvae, *Bombyx mori*, as an animal model for the discovery of drug candidates (*3*). We isolated lysocin E, a bactericidal antibiotic from the soil bacterium *Lysobacter* sp. RH2180-5, using a silkworm model of infection, and characterized its structure and mechanism of action (*4*). Injection of β-glucans from yeast and peptidoglycans from bacteria into the silkworm *Bombyx mori* induces maturation of the insect cytokine paralytic peptide, which results in muscle contraction of the larvae (*5,6*). We established an assay using the silkworm based on muscle contraction, which is associated with the activation of innate immunity (*7*), and isolated a polysaccharide with innate immune-stimulating activity from green tea (*8*). We also evaluated the activities of natural polysaccharides from various origins using the silkworm muscle contraction assay (*9*). This assay system does not respond to lipopolysaccharides (LPS) due to the presence of LPS-absorbing proteins in the silkworm hemolymph, which may be highly advantageous for screening innate immune-stimulating compounds from Gram negative bacteria, as contamination of the assay system by LPS during the first screening is negligible.

In the present study, we screened bacteria that produce EPS with innate immunostimulating activity using the silkworm muscle contraction assay system, and isolated *Rhizobium* sp. strain M2 from soil. Furthermore, we purified the EPS from culture of strain M2, and characterized the chemical structure. The structural motif involved in the innate immunostimulating activity is also discussed.

2. Materials and Methods

2.1. *Silkworm muscle contraction assay*

Eggs of silkworms (*Bombyx mori*, HuYo Tukuba Ne) were purchased from Ehime Sanshu (Ehime, Japan), and larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan, Yokohama, Japan) at 27°C. A silkworm muscle contraction assay was performed to evaluate innate-immunity activation, as described previously (*5*). Samples were dissolved in sterile saline, and 100 μL of each sample was injected into the body fluid of a specimen. The muscle contraction value was calculated by measuring the maximum length of each specimen before (x cm) and after (y cm) the injection using the formula (x – y)/x. One unit of activity was defined as that causing muscle contraction with a value of 0.15. The specific activities of the samples were determined by creating a titration curve with diluted samples.

2.2. *Screening of innate immune-stimulant producing bacteria*

First, we screened bacteria that form mucoid colonies on a glucose-asparagine (GA) agar plate [containing (l^{-1}): 10 g glucose, 0.5 g L-asparagine, 0.5 g K_2HPO_4, and 15 g agar (pH 7)] from diluted soil samples collected from various sites in Japan. Isolated mucoid strains were grown on GA agar plates at 30°C under aerobic conditions. After a 5-day incubation, the cells were collected by scraping and suspended in saline. The cell suspensions were vigorously vortexed and centrifuged at 10,000× g for 10 min. The supernatant was saved, two volumes of ethanol were added, and the mixture was centrifuged. Precipitates were lyophilized, and the innate immunostimulating activity was evaluated using the silkworm muscle contraction assay.

Taxonomic analysis of the isolated strain was performed as described previously (*10*). The 16S rRNA gene of the isolated strains (~1,500 bp) was amplified by polymerase chain reaction using universal primers (*11*). The phylogenetic relationship with closely related species was determined using MEGA version 7 (*12*) and CLUSTAL_X (*13*). Evolutionary distances were computed as described previously (*14*), and the phylogenetic tree was constructed using the neighbor-joining method (*15*). The reliability of the tree topology was evaluated by bootstrap analysis with 1000 replicates (*16*).

2.3. *Purification of M2 EPS*

Strain M2 was grown on GA agar plates at 30°C for 5 days under aerobic conditions, and the cells were collected by scraping and suspended in saline. M2 EPS was extracted from the supernatant of the suspension as described previously (*17*), except the Sevag method was used instead of phenol–chloroform treatment (*18*). M2 EPS was further purified by DEAE-Toyopearl 650 M column chromatography as described previously (*19*). Fractions containing saccharides were monitored using the phenol-H_2SO_4 method (*20*). Two volumes of ethanol were added to each fraction, and the mixture was centrifuged. The precipitates were dissolved in saline, and the innate immunostimulating activity was evaluated using the silkworm muscle contraction

assay. The fractions contained in the activity peak were combined, dialyzed against MilliQ water, and lyophilized.

To confirm the homogeneity of M2 EPS, gel-filtration column chromatography was performed on a Sephacryl S1000 (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 as the eluent (*17*). Dextran T2000 (Amersham Biosciences UK Ltd., Buckinghamshire, UK) was used as a size marker. Fractions containing saccharide were monitored using the phenol-H_2SO_4 method.

2.4. *Monosaccharide analysis*

Monosaccharide analysis was performed as described previously (*17*). Briefly, M2 EPS was hydrolyzed by 4 M trifluoroacetic acid at 100°C for 3 h, and the obtained monosaccharides were labeled with aminobenzoic acid ethyl ester (ABEE) and analyzed by high performance liquid chromatography (HPLC; 1500 HPLC system, Waters, Milford, MA).

2.5. *Nuclear magnetic resonance (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 units, with acetone (δ ^{1}H 2.23, δ ^{13}C 31.1) was used as an internal reference for samples measured in D_2O solutions. Two-dimensional (2D) homonuclear correlation spectroscopy, total correlated spectroscopy, heteronuclear multiple quantum coherence, and heteronuclear multiple bond coherence experiments were used to assign signals. ^{1}H NMR chemical shifts of overlapping signals were obtained from the center of the cross-peaks in the 2D spectra.

2.6. *Methylation analysis*

Polysaccharide methylation was performed according to the Ciucanu method using sodium hydroxide and CH_3I (*21*). The methylated polysaccharide was then hydrolyzed, reduced, and acetylated before analysis by gas chromatography/mass spectroscopy (GLC-MS). The GLC-MS analysis was performed with a QP-2010 plus instrument (Shimadzu, Kyoto, Japan) fitted with a fused silica capillary column (Rtx-5, 30 m × 0.25 mm × 0.25 μm; Restek, Shimadzu).

2.7. *Structural modifications of M2 EPS*

O-Deacylation and depyruvylation of M2 EPS were performed as described previously (*17,22*). Acid hydrolysis was performed under the same conditions described above (*Monosaccharide analysis*). The modified structures of M2 EPS were confirmed by NMR analysis, and the innate immunostimulating activity was evaluated in the silkworm muscle contraction assay.

3. Results

3.1. *Isolation of Rhizobium sp. strain M2, innate immune stimulant-producing bacteria*

First, we screened bacteria from soil that formed mucoid colonies on an agar plate. To prevent contamination of the innate immune stimulant by components of the medium, (*e.g.*, β-glucan from yeast extract), we used a GA agar plate, a chemically defined media. Next, EPS were roughly extracted from the isolated mucoid strains, and the innate immunostimulating activity was evaluated using the silkworm muscle contraction assay. Crude EPS from a strain named M2 exhibited immune-stimulating activity (data not shown). The nearly complete 16S rRNA gene sequence (1479 bp) of strain M2 showed high similarities to sequences of other strains of species belonging to the genus *Rhizobium*, and the highest sequence similarities were found with the strain of *R. pusense* (98% similarity). The phylogenetic tree constructed using the neighbor-joining method is shown in Figure 1. *Rhizobium* sp. strain M2 was deposited as NBRC 108899 to the National Institute of Technology and Evaluation, Japan. Next, we purified the active compound from the crude EPS fraction of strain M2.

3.2. *Purification of innate immune stimulant from Rhizobium sp. strain M2*

The innate immune stimulant produced by *Rhizobium* sp. strain M2 was extracted and purified by DEAE-Toyopearl column chromatography, eluted with a linear gradient (0-1 M) of NaCl. This procedure gave a single peak of carbohydrate at ~0.5 M concentration of NaCl detected by the phenol-H_2SO_4 method (Figure 2). The silkworm muscle contraction activity in each fraction was also evaluated, and a single peak of activity was detected in the carbohydrate-eluted fractions. The fractions contained in this peak were combined as the M2 EPS.

M2 EPS was eluted as a symmetric single peak earlier than Dextran T2000 by Sephacryl S1000 gel-filtration chromatography (Figure 3), suggesting an apparent molecular weight greater than 2,000,000. These data indicate that the innate immune stimulant from *Rhizobium* sp. strain M2 was purified to homogeneity as M2 EPS.

M2 EPS induced silkworm muscle contraction in a dose-dependent manner, with a specific activity of 1.4 units/mg (Figure 4). Spectrophotometrically, no absorption was detected at 280 nm or at 255 nm, suggesting that the M2 EPS did not contain proteins or nucleic acids.

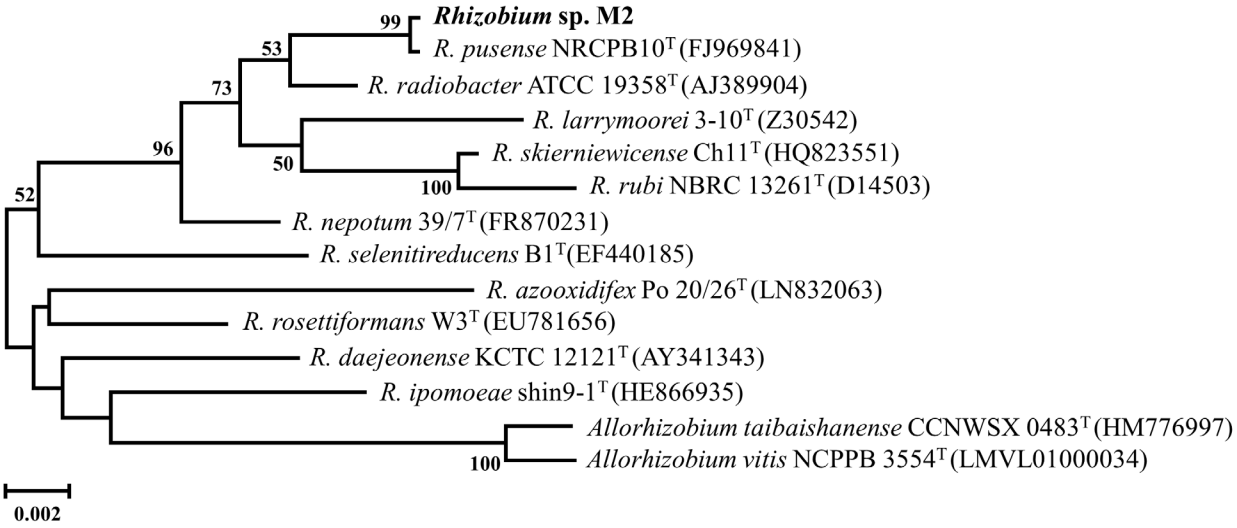

Figure 1. Neighbor-joining tree based on nearly complete 16S rRNA gene sequences, showing the position of the strain M2 among its phylogenetic neighbors. Numbers at branch nodes are percentages of bootstrap support based on 1000 resampling; only values over 50% are given. Bar, 0.002 substitutions per nucleotide position.

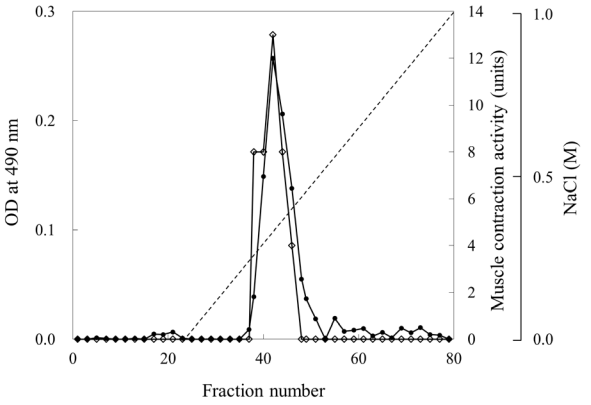

Figure 2. Ion-exchange chromatography of M2 EPS. M2 EPS was dissolved in 10 mM Tris-HCl buffer (pH 8.0), applied to a DEAE-Toyopearl column (100 mm × 25 mm ϕ), and eluted with a 300-mL linear gradient (0-1 M) of NaCl. Closed circles, OD at 490 nm; opened diamonds, muscle contraction activity.

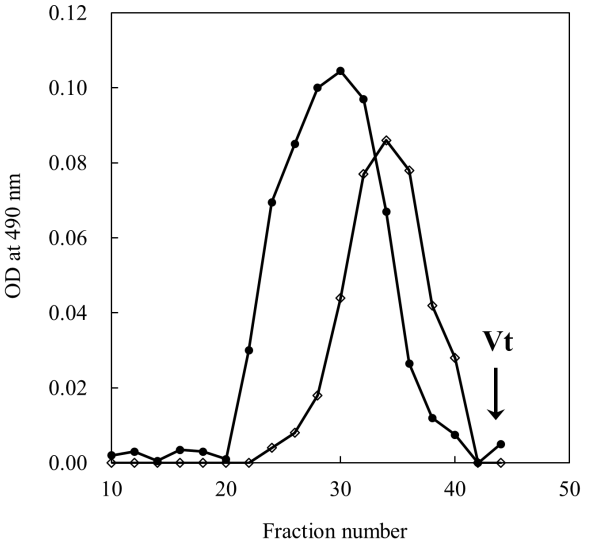

Figure 3. Gel filtration column chromatography of M2 EPS. M2 EPS was applied to a Sephacryl S1000 gel filtration column (1150 mm × 15 mm ϕ) and 1 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl as an eluate. Blue dextran 2000 was used as a size marker. The arrow indicates the total volume of the gel (Vt). Closed circles, M2 EPS; opened diamonds, Blue dextran 2000.

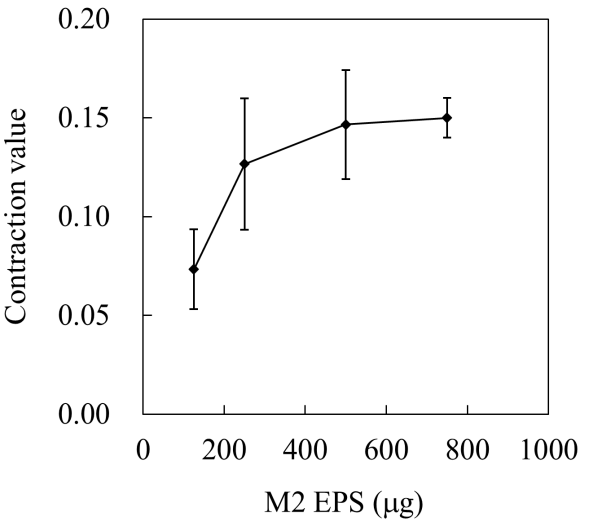

Figure 4. Muscle contraction of silkworm larval specimens by injecting of M2 EPS. The data-points represent the means ± S.D., n = 3.

3.3. *Structural analysis of M2 EPS*

The monosaccharide composition of M2 EPS, determined by trifluoroacetic acid hydrolysis and HPLC analysis, revealed glucose (Glc) and galactose (Gal) in a molar ratio of 6.8:1.0 (Figure 5). ^{1}H and ^{13}C NMR analysis showed that the pattern of signals detected in M2 EPS was characteristic of a polysaccharide, not a protein or lipid (Figure 6). Although the signals detected in anomeric regions of both 1D spectra overlapped (δ 4.54-4.82 for ^{1}H and δ 101.7-104.4 for ^{13}C), all anomeric signals detected were allocated to a

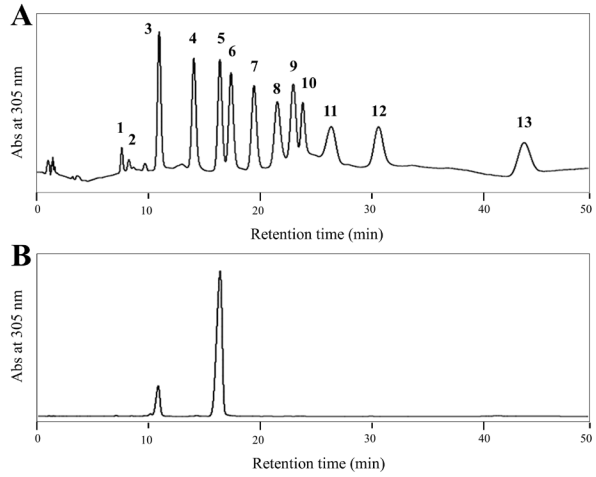

Figure 5. HPLC elution profile of acid hydrolysate of M2 EPS. Panel **A**, standard monosaccharides; and **B**, acid hydrolysate of M2 EPS. Standard monosaccharides: **1**, D-glucuronic acid; **2**, D-galacturonic acid; **3**, D-galactose; **4**, D-mannose; **5**, D-glucose; **6**, L-arabinose; **7**, D-ribose; **8**, *N*-acetyl-D-mannosamine; **9**, D-xylose; **10**, *N*-acetyl-D-glucosamine; **11**, L-fucose; **12**, L-rhamnose; and **13**, *N*-acetyl-D-galactosamine.

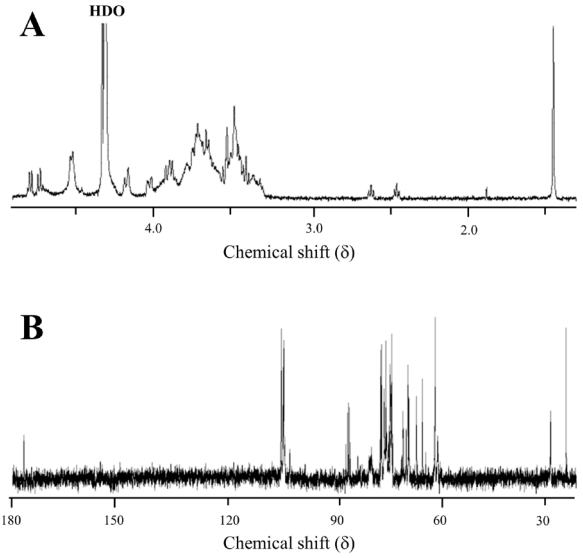

Figure 6. NMR spectra of M2 EPS. A, ^{1}H NMR; **B,** ^{13}C NMR spectrum.

Table 1. Methylation analysis of M2 EPS

Peak	Derivatives	Structural feature	Native[a]	Depyruvylated[a]	Succinoglycan[b]
1	1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol	Glc*p*-(1→	0.1	0.6	
2	1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol	→3)-Glc*p*-(1→	1.5	2.1	2.0
3	1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol	→4)-Glc*p*-(1→	1.6	1.6	2.0
4	1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol	→3)-Gal*p*-(1→	1.2	1.1	1.0
5	1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol	→6)-Glc*p*-(1→	1.0	1.0	1.0
6	1,4,5,6-Tetra-*O*-acetyl-2,3-di-*O*-methyl-glucitol	→4,6)-Glc*p*-(1→	1.9	1.3	2.0

[a] Ratio of peak area. [b] Theoretical ratio (*24*).

β configuration based on the observed chemical shift values (*23*). Next, we performed methylation analysis to elucidate the details of the connectivity of the sugar residues detected by the monosaccharide analysis. Five major peaks of the partially methylated alditol acetate (PMAA) derivatives were detected from M2 EPS by GLC-MS analysis (Supplementary Figure 1A). The connectivity of the sugar residues was determined based on the retention times and mass fragmentation patterns of the detected peaks (Table 1). M2 EPS mainly comprised →3)-Glc*p*-(1→, →4)-Glc*p*-(1→, →3)-Gal*p*-(1→, →6)-Glc*p*-(1→, and →4,6)-Glc*p*-(1→ in an approximate molar ratio of 2:2:1:1:2. These results indicate that M2 EPS consists of octasaccharide repeating units. The molar ratio of the detected sugar connectivity was in good agreement with the theoretical ratio of PMAA from succinoglycan, a representative polysaccharide produced by genus *Rhizobium* (*24*).

Signals of the glycan structure detected in the ^{1}H and ^{13}C NMR spectra of M2 EPS (Figure 6) were almost the same as those previously reported for succinoglycan of *R. meliloti* (*25*). The glycan chain of succinoglycan is substituted by succinic acid, pyruvic acid, and acetic acid (*25*). Succinyl-methylene (δ 2.46 and 2.62 for ^{1}H and δ 29.9 for ^{13}C) and pyruvate-methyl (δ 1.46 for ^{1}H and δ 26.1 for ^{13}C) signals were detected in the one-dimensional NMR spectra of M2 EPS (Figure 6). The contents of the succinate and pyruvate residues were 40% and 85%, respectively, as estimated by the ratio of succinyl-methylene and pyruvate-methyl resonances to anomeric protons of the glycan backbone in the ^{1}H NMR spectra. No methyl signal of an acetyl residue was detected in M2 EPS.

To determine the pyruvylated position of the M2 EPS, we prepared the depyruvylated M2 EPS by mild acid hydrolysis. In the methylation analysis of depyruvylated M2 EPS, the molar ratio of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-glucitol was decreased, and that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol was increased compared with the native M2 EPS (Supplementary Figure 1, Table 1). These results indicate that the pyruvate residue was bound to both the *O*-4 and *O*-6 positions of terminal glucose residue of side-chain structure. The chemical shift of the pyruvate-methyl carbon (26.1 ppm) indicated that the pyruvate acetal carbon atom had the (*S*) configuration

(*26*). These findings revealed that M2 EPS consists of branching octasaccharide repeating units substituted by succinate and pyruvate residues shown in Figure 7.

3.4. *Effects of structural modifications on the innate immune-stimulating activity of M2 EPS*

To determine the structural motif involved in the innate immunostimulating activity of M2 EPS, we modified its structure by chemical treatment. We prepared deacylated (weak alkali hydrolyzed), depyruvylated (weak acid hydrolyzed), deacylated and depyruvylated, and strong acid hydrolyzed M2 EPS. The modified structures of the M2 EPS were confirmed by ^{1}H NMR analysis (Figure 8), and the succinate and pyruvate residue content was estimated by the ratio of succinyl-methylene and pyruvate-methyl resonances to anomeric protons of the glycan backbone in the spectra (Table 2). Succinate was successfully removed from M2 EPS by the weak alkali hydrolysis, but a small amount of pyruvate remained in the weak acid-hydrolyzed M2 EPS.

The innate immunostimulating activities of chemically modified M2 EPSs were evaluated using the silkworm muscle contraction assay. The activities were increased by removal of the substitution of succinate and pyruvate. Strong acid hydrolysis completely inactivated the M2 EPS.

4. Discussion

In this study, we screened innate immunostimulant-producing bacteria using the silkworm muscle contraction assay, and isolated the bacterial strain M2 from soil. The 16S rRNA gene sequence of strain M2 had the highest similarities with sequences of *R. pusense*. *Rhizobium* is a genus of Gram-negative soil bacteria frequently isolated from plant rhizospheres or root nodules (*27*). *R. pusense* is a remarkable species as an opportunistic human pathogen in the genus *Agrobacterium/Rhizobium* (*28*). Many bacterial strains producing EPS belong to this genus (*27*). Recently, an extracellular β-glucan possessing antitumor activity was purified from the *Rhizobium* sp. N613 (*29*). To our knowledge, however, this is the first report of the screening of innate immune stimulants from bacterial EPS using a convenient method such as the silkworm muscle contraction assay. Because the silkworm muscle contraction assay does not respond to LPS, the innate immune stimulant produced by strain M2, which is a Gram-negative bacterium, is not LPS.

We purified the innate immunostimulant from the strain M2, and characterized the chemical structure as a succinoglycan like high-molecular weight heteropolysaccharide (structural model is shown in Figure 7). Succinoglycans are acidic heteropolysaccharides produced by a variety of bacteria belonging to the *Rhizobium*, *Agrobacterium*, *Alcaligenes*, and *Pseudomonas* (*30*). Succinoglycan is important for plant living rhizobia to evade plant immunity (*31*). A succinoglycan was also isolated from *Agrobacterium*

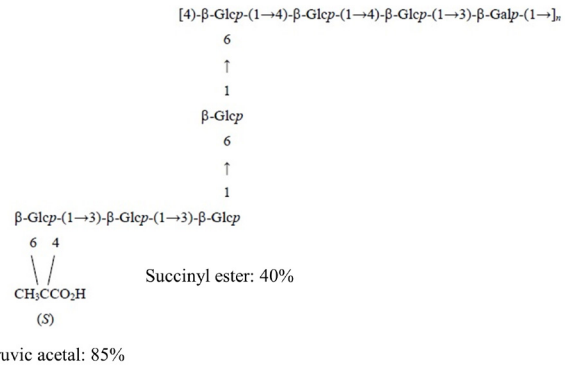

Figure 7. Predicted structure of M2 EPS from *Rhizobium* sp. strain M2

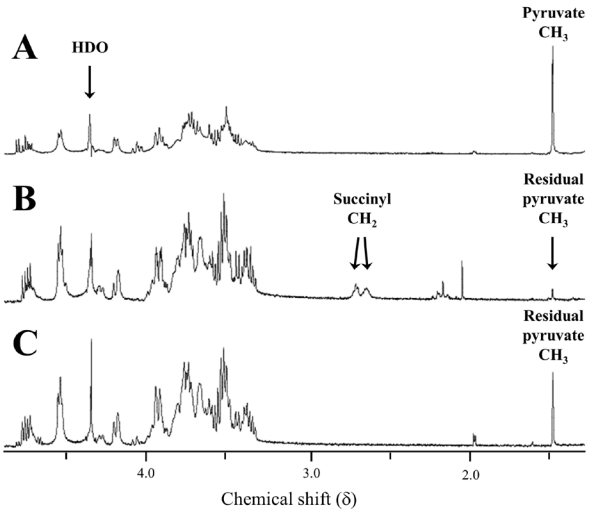

Figure 8. ^{1}H NMR spectra of chemically modified M2 EPS. A, deacylated; **B,** depyruvylated; and **C,** deacylated and depyruvylated M2 EPS.

Table 2. Effects of structural modifications on the innate immune-stimulating activity of M2 EPS

Samples	Succinate contents (%)[a]	Pyruvate contents (%)[a]	Specific activity (units/mg)
M2 EPS	40	85	1.4
Deacylated M2 EPS	ND[b]	100	1.6
Depyruvylated M2 EPS	66	7.0	2.6
Deacylated- depyruvylated M2 EPS	ND[b]	32	3.4
Acid hydrolyzed M2 EPS	NA[c]	NA[c]	< 1

[a] Percentage of repeating unit substituted by the residue. [b] Not detected. [c] Not analyzed.

radiobacter, a clinical isolate from a patient with cystic fibrosis (*24*). Innate immunostimulating activity of succinoglycans, however, has not been reported. Further studies are necessary to evaluate whether M2 EPS exerts innate immunostimulating activity in a mammalian system.

To determine the structural motif involved in the innate immunostimulating activity of M2 EPS, we modified its structure by chemical treatment. The activities were increased by the removal of the substitution of succinic and pyruvic acid. Because the deacylation (alkaline hydrolysis) of M2 EPS did not attenuate the innate immune stimulating activity, the active substance is not LPS contaminating the culture of strain M2, reliably. Strong acid hydrolysis completely inactivated the M2 EPS. These results suggest that the acidic residues present in M2 EPS do not participate in the innate immunostimulating activity, and the polysaccharide structure is necessary for the activity. To our knowledge, this is the first report of the glycan chain of succinoglycan possessing innate immune-stimulating activity. A pyruvate residue was bound to both *O*-4 and *O*-6 positions of the terminal glucose residue of the side-chain of M2 EPS. Although we could not determine the binding position of the succinate in the M2 EPS, succinate groups bind to glucose residues in the side-chains of succinoglycan from various microbial sources (*24,25*). The glycan structure of the side-chain of deacylated and depyruvylated M2 EPS is a β-1,3/6-glucan with the following structure:

β-Glc*p*-(1→3)-β-Glc*p*-(1→3)-β-Glc*p*-(1→6)-β-Glc*p*-(1→6)-β-Glc*p*-(1→

Previously, the innate immunostimulating activities of β-1,3/6-glucan from various sources were evaluated using the silkworm muscle contraction assay (*9*), and the activities varied according to the origin. Unmasking of the β-1,3/6-glucan structure of the side chain by deacylation and depyruvylation may enhance the innate immune-stimulating activity of M2 EPS. Detailed analysis of the structure-activity relations of the β-1,3/6-glucan will be an interesting topic for future studies.

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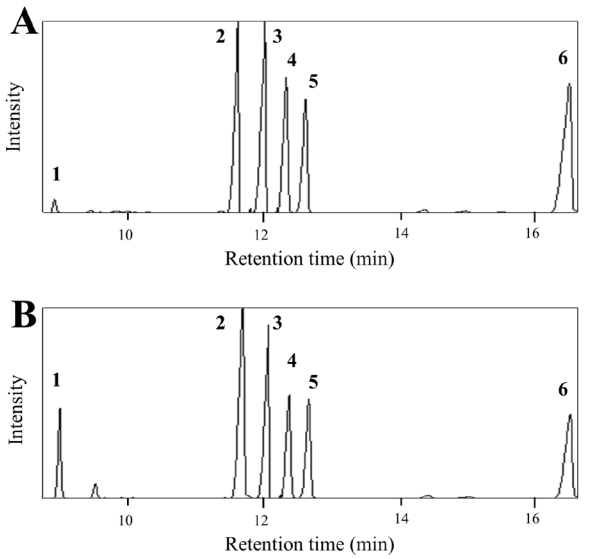
Supplemental Data



Supplementary Figure 1. GLC patterns of partially methylated alditol acetate derivatives from M2 EPS. Panel **A**, native M2 EPS; and **B**, depyruvylated M2 EPS. 1, 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucitol; 2, 1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucitol; 3, 1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol; 4, 1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-galactitol; 5, 1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucitol; and 6, 1,4,5,6-Tetra-*O*-acetyl-2,3-di-*O*-methyl-glucitol.