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Structural analyses of novel glycerophosphorylated α-cyclosophorohexadecaoses isolated from *X. campestris* pv. *campestris*

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Abstract—Novel periplasmic anionic cyclic glucans produced by *Xanthomonas campestris* pv. *campestris* were isolated by trichloroacetic acid treatment and various chromatographic techniques. No report has been made on the presence of substituted cyclic glucans of the *Xanthomonas species*. We show, for the first time, that *X. campestris* pv. *campestris* produces the anionic cyclic glucans with phosphoglycerol residues, the presence of which can be predicted by analyzing the sequence database with the aid of the NCBI RefSeq database. To analyze the structure of isolated anionic cyclic glucans analyses, we used NMR spectroscopy, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOFMS) and electrospray–ionization mass spectrometry (ESIMS). The results suggest that the novel anionic forms of the cyclic glucans of *X. campestris* pv. *campestris* are glycerophosphorylated α-cyclosophorohexadecaose with one or two phosphoglycerol substituents at the C-6 positions of the glucose residues.

Keywords: *Xanthomonas campestris* pv. *campestris*; Cyclic glucan; Osmoregulated periplasmic glucan; Glycerophosphorylated α-cyclosophorohexadecaose; Structural analyses

1. Introduction

Osmoregulated periplasmic glucans (OPGs) are general components of the periplasm of Gram-negative bacteria. A number of studies have demonstrated that soil bacteria, namely the *Agrobacterium* or *Rhizobium* species synthesize OPGs composed of cyclic β-(1→2)-D-glucans with 17–40 glucose residues. In general, OPGs are found in neutral or anionic forms in which some glucose residues keep the anionic functional groups such as phosphoglycerol,[1] methylmalonic acid,[2] succinic acid,[3] or phosphocholine.[4] These anionic oligosaccharides within the periplasmic place of microorganisms are directly involved in the osmotic adaptation of microorganisms as well as neutral ones.[5]

The *Xanthomonas* genus is one of the most ubiquitous groups of plant-associated bacteria. *Xanthomonas campestris* pv. *campestris* is the causative agent of black rot in cruciferous plants.[6] Furthermore, the cell-surface carbohydrates of this microorganism are generally involved in bacterium–plant interactions in pathogenesis. The *Xanthomonas* species reportedly produce a unique neutral cyclic β-(1→2)-D-glucan with one α-(1→6)-linkage,[7,8] namely α-cyclosophorohexadecaose (α-C16), though there are no reports of it producing any anionic derivatives attached to this neutral α-C16.[9]

2. Results and discussion

While recently analyzing the RefSeq databases[10] for the *mdoB* (*opg*B) gene that encodes the phosphoglycerol-transferase I in *E. coli*,[11,12] we detected the presence of the *mdoB* (*opg*B) gene in *X. campestris* pv. *campestris*,

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which belongs to the osmoregulated periplasmic glucan B (*opg*B) family. The *opg*B encodes the membrane-bound protein, phosphoglyceroltransferase I, which transfers phosphoglycerol residues at the OPGs. Based on this analysis, we report, for the first time, that we have experimentally confirmed the presence of novel glycerophosphorylated α-C16 synthesized by *X. campestris* pv. *campestris* (ATCC 13951).

A thin-layer chromatogram (TLC) indicates the presence of anionic and neutral α-C16s isolated from *X. campestris* pv. *campestris* (data not shown). With yields of anionic and neutral glucans at 19 and 3 mg/10 L, respectively, the anionic α-C16 was produced up to 86% of all the α-C16s isolated from *X. campestris* pv. *campestris*.

The presence of phosphorus was confirmed by ^{31}P NMR spectroscopy (Fig. 1(a)), and the resonances at 1.11 and 1.21 ppm indicate two kinds of phosphoglycerol substitution. Figure 1(b) shows a ^{1}H NMR spectrum. The peak at 5.08 ppm is indicative of the anomeric proton (H-1) of the α-glucose residue. Furthermore, the peaks from 4.56 to 5.01 ppm are indicative of the H-1 protons of the glucose residues engaged in β-(1→2)-linkages, whereas those at 4.03 and 4.10 ppm are assigned to the H-6 and H-6′ protons of the glucose residues with phosphoglycerol linked at the C-6 carbons.

We also analyzed the structure with the aid of ^{13}C NMR and ^{1}H–^{13}C-heteronuclear single quantum coherence (HSQC) spectroscopy. As shown in Figure 1(c), no peak in the range of 92–96 ppm indicates the presence of nonreducing terminal glucosyl groups in this molecule: that is, there is cyclic backbone structure.[13]

The peak assignments of neutral α-C16 are partially based on a previous report.[8b] Consequently, we assigned the resonances of anomeric carbon (C-1) to a range of 101.00–104.00 ppm, and we assigned the chemical shift

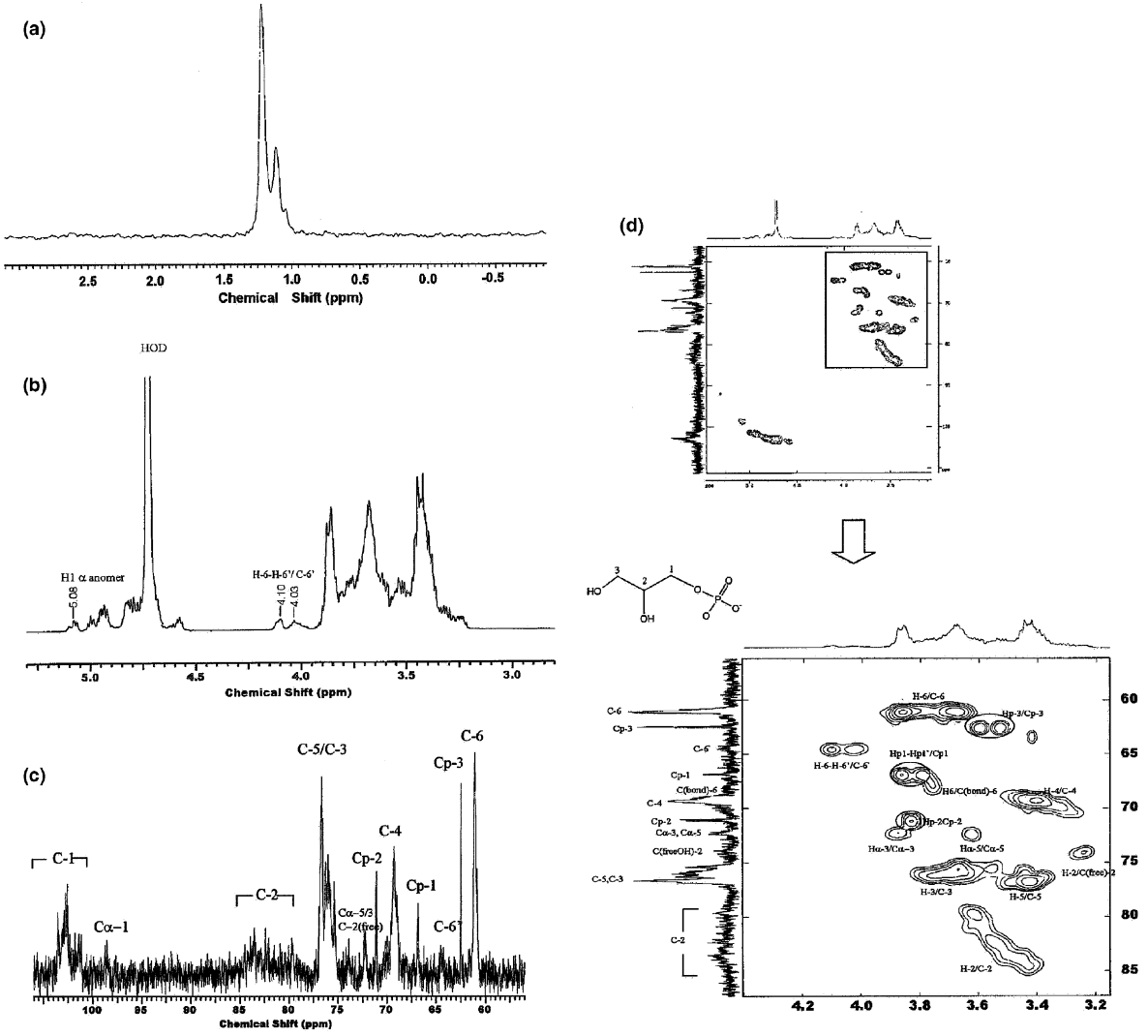

Figure 1. NMR spectra of purified glycerophosphorylated α-cyclosophorohexadecaoses from *X. campestris* pv. *campestris*. (a) ^{31}P NMR spectrum. The resonances at 1.21 and 1.11 ppm indicate the phosphoglycerol substitution, (b) ^{1}H NMR spectrum, (c) ^{13}C NMR spectrum and (d) ^{1}H–^{13}C HSQC spectrum. Cα; carbons of α-anomeric glucose residue Cp; carbons of phosphoglycerol.

in the C-1 carbons of the α-glucose residue to 98.79 ppm. We assigned the weaker and broader signals, which ranged from 79.68 to 83.50 ppm, to the C-2 carbons induced by phosphoglycerol substituents, whereas we assigned the C-2 carbons not involved in glycosidic linkages to those at 73.88 ppm. The peaks at 61.05 and 68.07 ppm correspond to the C-6 carbons with a free OH group and to those involving α-(1→6)-linkages, respectively. The C-4 carbons of the β-glucose residues were assigned to peaks ranging from 68.85 to 69.95 ppm, the C-3 and C-5 carbons of the β-glucose residues to peaks ranging from 75.36 to 76.66 ppm, and the C-3 and C-5 carbons of the α-glucose residues to the peaks at 72.18 and 72.34 ppm, respectively.

We detected four novel signals at 62.48, 66.82, 71.90 and 62.52 ppm where these signals were absent in the ^{13}C NMR spectrum of the neutral α-C16 reported previously.[7,8] Three peaks at 62.48, 66.82 and 71.9 ppm were then assigned, respectively, to the resonances of C-3, C-1 and C-2 carbons of the phosphoglycerol substituent attached to the α-C16.

Another peak at 64.52 ppm was assigned to the resonance of the phosphoglycerol-substituted C-6 in the α-C16. We designated this particular C-6 as C-6′, and its presence was confirmed in an HSQC spectrum (Fig. 1(d)). The C-6′ without the free OH group shifted downfield to 64.52 ppm, at which point the H-6 and H-6′ resonances attached to the C-6′ carbons were correlated.

The H-1 and H-1′ resonances of phosphoglycerol were correlated to the C-1 carbons of the phosphoglycerol at 66.82 ppm of phosphoglycerol, and the correlations at 3.84 ppm for ^{1}H and at 71.04 ppm for ^{13}C were assigned to the C-2 carbons of phosphoglycerol. The H-3 and H-3′ resonance of phosphoglycerol were also correlated to the C-3 carbons of the phosphoglycerol at 62.48 ppm. The strong cross peaks in the HSQC spectrum confirm that phosphoglycerol was attached to the neutral α-C16. Moreover, similar peaks of phosphoglycerol were also present in the ^{13}C spectrum of glycerolphosphated cyclic glucans of *Rhizobium meliloti*.[14]

These neutral and anionic α-C16s purified from *X. campestris* pv. *campestris* were identified with the aid of matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry in which we used 2,5-dihydroxybenzoic acid (DHB) as a matrix in the positive-ion mode. The mass spectrometric analysis confirms the presence of neutral (Fig. 2(a)) and glycerophosphorylated α-C16 (Fig. 2(b)) from *X. campestris* pv. *campestris*. The spectrum (Fig. 2(b)) in the positive-ion mode shows the base peak that corresponds to α-C16 with one phosphoglycerol substituent.

We also detected the anionic cyclic glucan could be detected in various forms such as $[M_1+Na]^+$, $[M_1-H+2Na]^+$ $[M_2-H+Na+K]^+$ and $[M_2-H+2K]^+$ where M_1 and M_2 indicate α-C16 with one or two phosphoglycerol substituents,[15] respectively. Other anionic

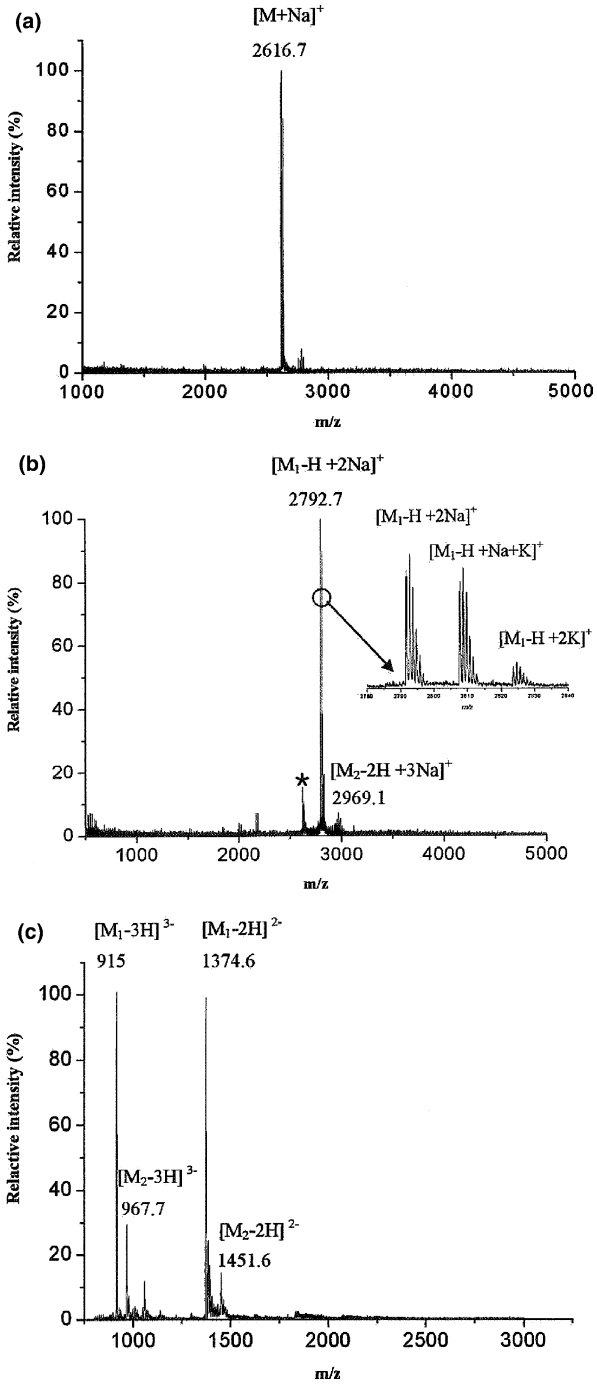

Figure 2. Mass spectra of neutral and anionic glycerophosphorylated α-cyclosophorohexadecaose (α-C16). MALDI-TOFMS in the positive-ion mode with 2,5-DHB as matrix. (a) MALDI-TOFMS of neutral α-C16 of purified from *X. campestris* pv. *campestris*. (b) MALDI-TOFMS of anionic α-C16 of purified from *X. campestris* pv. *campestris*. The base peak is at *m/z* 2792.7 corresponding to 16 glucose residues with one phosphoglycerol cationized with two sodium such as $[M_1-H+2Na]^+$. (c) ESIMS in the negative-ion mode of anionic glucans. Each mass unit at *m/z* 915.9, 967.2, 1347.6 and 1451.6 corresponds to the $[M_1-3H]^{3-}$, $[M_2-3H]^{3-}$, $[M_1-2H]^{2-}$, and $[M_2-2H]^{2-}$, respectively. *M*: molecular weight of unsubstituted α-C16, M_1: molecular weight of α-C16 with one phosphoglycerol substituent, M_2: molecular weight of α-C16 with two phosphoglycerol substituents, *: neutral cyclic glucan, impurity.

peaks were also detected as $[M_2+Na]^+$, $[M_2-2H+3Na]^+$, $[M_2-2H+2Na+K]^+$, $[M_2-2H+Na+2K]^+$ and $[M_2-2H+3K]^+$.

The α-16 with the single phosphoglycerol substituent $[M_1-H+2Na]^+$, was the dominant product. The base peak at *m*/*z* 2792.7 corresponds to the calculated mass for α-16 with one phosphoglyserol substituent, cationized with two sodium substituents such as $[M_1-H+2Na]^+$. Furthermore, there were other peaks that corresponded to the $[M_1-H+Na+K]^+$ and $[M_1-H+2K]^+$.

The small peak at *m*/*z* 2969.1 is the calculated mass for α-16 with two phosphoglycerols cationized with three sodium substituents such as $[M_2-2H+3Na]^+$. Although the peak of *m*/*z* 2969.1 has a small intensity, the induced peaks appear as various cationized forms such as $[M_2-H+2Na]^+$, $[M_2-H+Na+K]^+$, $[M_2-2H+3Na]^+$, $[M_2-2H+3Na]^+$ and $[M_2-2H+3K]^+$ with their own isotope patterns. These peaks suggest two phosphoglycerol units are attached to α-C16, and the suggestion is confirmed by ESIMS analysis in the negative-ion mode as shown in Figure 2(c). The spectrum in Figure 2(c) contains −2 ($[M_1-2H]^{2-}$) and −3 ($[M_1-3H]^{3-}$) charged ions of one glycerophosphorylated α-C16 at *m*/*z* 1374.6 and at *m*/*z* 915.9, respectively. The peaks at *m*/*z* 967.7 and *m*/*z* 1451.6, which correspond to $[M_2-3H]^{3-}$ and $[M_2-2H]^{2-}$, indicate the α-C16 with two phosphoglycerol substituents.

In conclusion, we experimentally confirmed the presence of novel anionic cyclic glucans of *X. campestris* pv. *campestris* as one or two glycerophosphorylated α-C16s, and that one substituted form seems to be major. *Xanthomonas campestris* OPGs are known to possess no substituent. Recently, complete genomic sequences of *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri* have been reported. When analyzing the RefSeq database for the *mdo*B (*opg*B) gene that encodes phosphoglyceroltransferase I, we expected to find anionic forms. On the basis of this expectation, we seriously tried to determine the anionic forms of α-C16. As a result, we successfully isolated acidic α-C16 in *X. campestris* pv. *campestris* (ATCC 13951), even though other strains such as *X. campestris* pv. *citri* N17, *X. campestris* pv. *campestris* 8004[8c] and *X. campestris* pv. *glycines*[8a] were not known to produce any anionic OPGs. Unlike the succinyl substituent for *Erwinia chrysanthemi*, phosphoglycerol substituents in *X. campestris* pv. *campestris* (ATCC13951) are not dependent on a growth condition. We deduced that the synthesis of the anionic substituent could be due to the diversity of the strain. However, further studies are needed to confirm this viewpoint.

The physiological functions of the OPGs of Gram-negative bacteria can regulate the osmotic pressure between the cell and external medium. However, the exact molecular mechanism for the difference in osmotic regulation between neutral and anionic OPGs is unknown.

3. Experimental

3.1. Bacterial cultures and conditions

X. campestris pv. *campestris* ATCC 13951 were from the Korean Collection for Type Cultures (KCTC), and they were grown in a low-osmolarity medium[16] at 24 °C with agitation.

3.2. Preparation of neutral and anionic glucans from *X. campestris* pv. *campestris*

Xanthomonas campestris pv. *campestris* was cultured in 1-L Erlenmeyer flasks containing low-osmolarity medium as previously reported.[7] The microorganisms were collected during the early exponential growth phase by centrifugation at 4 °C for 10 min at 8000 rpm. The cell pellets were extracted with 1% trichloroacetic acid, and after centrifugation, the supernatant was neutralized with NH_4OH[5] and desalted on a Sephadex G-25 column (2 cm × 40 cm). The desalted material was then, fractionated by gel filtration on a Bio-Gel P4 column (Bio-Rad). The column (2 cm × 42 cm) was eluted at room temperature with 0.5% HOAc at a flow rate of 20 mL/h, and 3-mL fractions were collected and then applied to a column (2 cm × 35 cm) of DEAE-cellulose to separate the neutral and anionic form of the α-C16.

3.3. TLC analysis

Using TLC, we analyzed the samples on Silica Gel G-60 (E. Merck, 400–240 mesh) in a mixture of butanol, ethanol and water (5:5:4 v/v). To aid detection, we sprayed the plates with a solution of 5% ethanolic sulfuric acid and heated them at 200 °C.

3.4. NMR spectroscopic analyses

For NMR spectroscopic analysis, we used a Bruker AMX spectrometer to record the ^1H NMR spectra (at 600 MHz), the ^{13}C NMR spectra (at 150 MHz) and the HSQC spectra. The ^{31}P NMR spectra were recorded with a Bruker DRX-500 NMR spectrometer operated at a ^{31}P frequency of 202 MHz. Before the NMR spectroscopic analysis, we treated the oligosaccharide twice with D_2O at room temperature.

3.5. MALDI-TOF mass spectrometry

The mass spectra of the oligosaccharides were obtained with a MALDI-TOF mass spectrometer (Voyager-DE STR BioSpectrometry, PerSeptive Biosystems, Framingham, MA, USA) in the positive-ion mode using 2,5-dihydroxybenzoic acid (DHB) as the matrix. The mass spectrometer was a reflector-type time-of-flight spectrometer, and it was operated in the positive-ion mode.

3.6. ESI mass spectrometry

ESIMSs were recorded with a QUATTRO LC Triple Quadrupole Tandem Mass Spectrometer (Waters-Micromass, Manchester, UK). The capillary voltage was 2.50 kV, and the cone voltage was 30 V. The source and desolvation temperature were kept at 70 and 150 °C, respectively. Nitrogen was used as the drying and nebulizing gas at a flow rate of 256 and 90 L/h, respectively. The sample was dissolved in a mixture of water and acetonitrile (1:1 v/v).

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