

## Novel Acetylated Linear Periplasmic Glucans Isolated from *Pseudomonas syringae*

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Periplasmic glucans, which exist in periplasmic space of many gram negative bacteria, are biologically important molecules in osmoregulation or pathogenesis.<sup>1</sup> In fact, mutants unable to synthesize the periplasmic glucans are impaired for growth in low osmotic condition or virulence.<sup>2,3</sup> These glucans also have biotechnological applications such as chiral additives,<sup>4</sup> solubility enhancers,<sup>5</sup> and catalysts.<sup>6</sup> Those applications of glucans are based on the structure having a complexation property with other compounds containing hydrophobic molecules.<sup>7,8</sup> Their structures vary according to the glycosidic linkages, Degree of polymerization (DP), and the cyclic or non-cyclic forms.<sup>1</sup> Since there has been structural analysis on various glucans,<sup>9-14</sup> functional studies depending on structural variation have been expected. Herein, we report structural analysis of newly found periplasmic glucans isolated from *Pseudomonas syringae*.

*P. syringae*<sup>9</sup> produces linear structured periplasmic glucans which have  $\beta$ -1,2 polyglucose chain branched with  $\beta$ -1,6 linkage, together with *Escherichia coli*,<sup>10</sup> *Erwinia chrysanthemi*,<sup>11</sup> and *Pseudomonas aeruginosa*.<sup>12</sup> The periplasmic glucans of *E. coli* were firstly discovered in 1973.<sup>13</sup> Since the discovery, many studies on the components in periplasmic spaces of gram negative bacteria have been reported where *P. syringae* produced periplasmic glucans which have similar structures to those of *E. coli*. The molecules can also be substituted by non-glucose residues originating from intermediary metabolisms such as acetyl, succinyl and methylmalonyl or the membrane phospholipids such as phosphoglycerol, phosphocholine, and phosphoethanolamine.<sup>1</sup> For the acetyl, succinyl and methylmalonyl substituents, acyl-coenzymes A is suspected to be the donor. Since the function of substituents in periplasmic glucans is not clear, the physiological role of those is needed to be studied.

It has been reported so far that periplasmic glucans of *E. coli* have phosphoglycerol, phosphoethanolamine and succinyl substituents,<sup>10</sup> while *E. chrysanthemi* produce succinyl and acetylated periplasmic glucans.<sup>11</sup> It also has been known that succinyl substituents exist in periplasmic glucans of *P. aeruginosa*.<sup>12</sup> Recently, we have reported succinylated and large sized periplasmic glucans isolated from *P. syringae* in low osmolarity (LO) media.<sup>14</sup> In this study, we describe for the first time that *P. syringae* produces one or two acetylated linear periplasmic glucans in high osmolarity (HO) media.

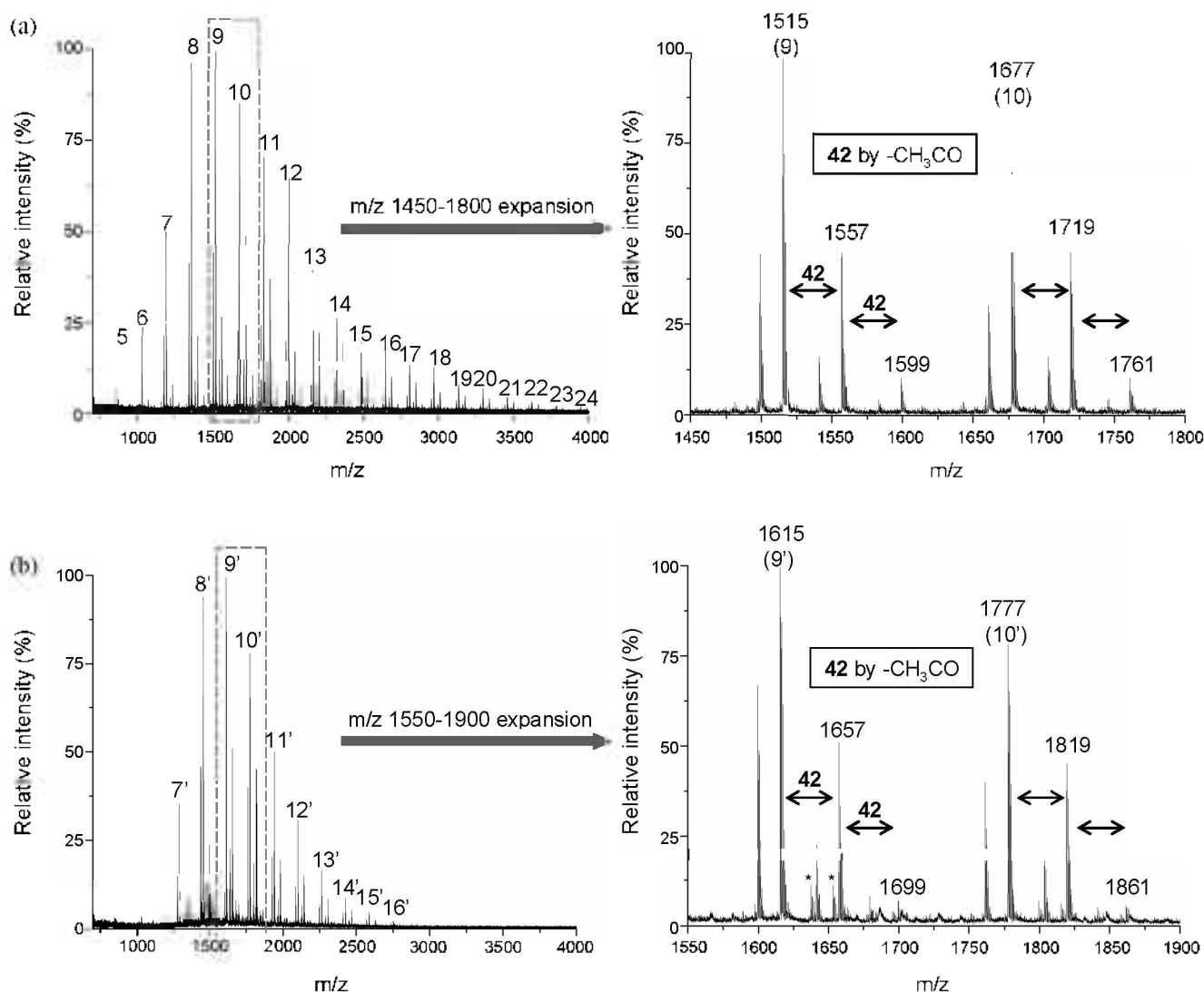
*P. syringae* which is a plant pathogen was grown in HO media and the glucans were isolated and purified through chromatographic techniques from the cell extract. After anion

exchange chromatography through the DEAE column, neutral and anionic glucans were separated and analyzed, respectively. The mass spectrum of the left side shown in Figure 1a represents neutral linear glucans, and there are 20 potassium and sodium-cationized molecular ions ([unsubstituted linear periplasmic glucans + K<sup>+</sup>] and [unsubstituted linear periplasmic glucans + Na<sup>+</sup>]). The potassium adduct ions at 867, 1029, 1191, 1353, 1515, 1677, 1839, 2001, 2163, 2326, 2488, 2650, 2812, 2974, 3136, 3298, 3460, 3622, and 3784 are identical to those of unsubstituted linear glucans that contain 5 to 24 glucose residues, together with sodium adduct ions with  $m/z$  reduced by 16 below the masses of the corresponding potassium adduct ions.

Peaks at  $m/z$  1072, 1233, 1395, 1557, 1719, 1881, 2043, 2205, 2367, 2530, 2692, 2854, 3017, 3178, 3341, 3503 and 3664 that also correspond to one acetylated DP 6-22 glucans were newly detected with half of the intensity of unsubstituted glucans. Smaller peaks at  $m/z$  1275, 1437, 1599, 1761, 1923, 2085, 2248, 2410, 2572, 2734 and 2895 indicate the presence of two acetylated DP 7-17 glucans. An upper enlarged spectrum between  $m/z$  1450 and 1800 clearly shows the presence of one or two acetylated glucans with the respective mass difference of 42.<sup>19,20</sup> This result indicates that one or two acetylated glucans as a neutral form are synthesized from *P. syringae* cultured in HO media.

Compared to Figure 1a, Figure 1b shows a  $m/z$  shift of 100 and it represents anionic glucans with one succinyl moiety. Their peaks which are  $m/z$  1291, 1453, 1615, 1777, 1939, 2101, 2263, 2426, 2588, and 2750 are assigned to potassium adducts. Sodium adducts with  $m/z$  reduced by 16 are also present and the DP range is 7 - 16. In the enlarged mass spectrum shown in the right side of Figure 1b, two other mass differences of 42 are shown. This fact indicates that these anionic glucans have complexed forms composed of one succinylated glucans with an addition of one or two acetyl residues. The linear nature of these glucans was confirmed by the hydrated molecular weight ( $m/z$  18 added molecular weight) compared with the cyclic form.

Additionally, we could detect the presence of reducing glucoses by doublets near 5.4 and 4.7 ppm in proton peaks of <sup>1</sup>H-<sup>13</sup>C-heteronuclear single quantum correlation (HSQC) spectrum (Figure 2a).<sup>15</sup> A cross peak at 5.4 and 91.8 ppm is indicative of  $\alpha$ -anomeric <sup>1</sup>H-<sup>13</sup>C correlation of reducing glucose and a cross peak at 4.7 and 94.9 ppm represents  $\beta$ -anomeric <sup>1</sup>H-<sup>13</sup>C correlation of reducing glucose. Previous study on the unsubstituted periplasmic glucans of *P. syringae* that used glycosidic linkage analysis was reported to be highly

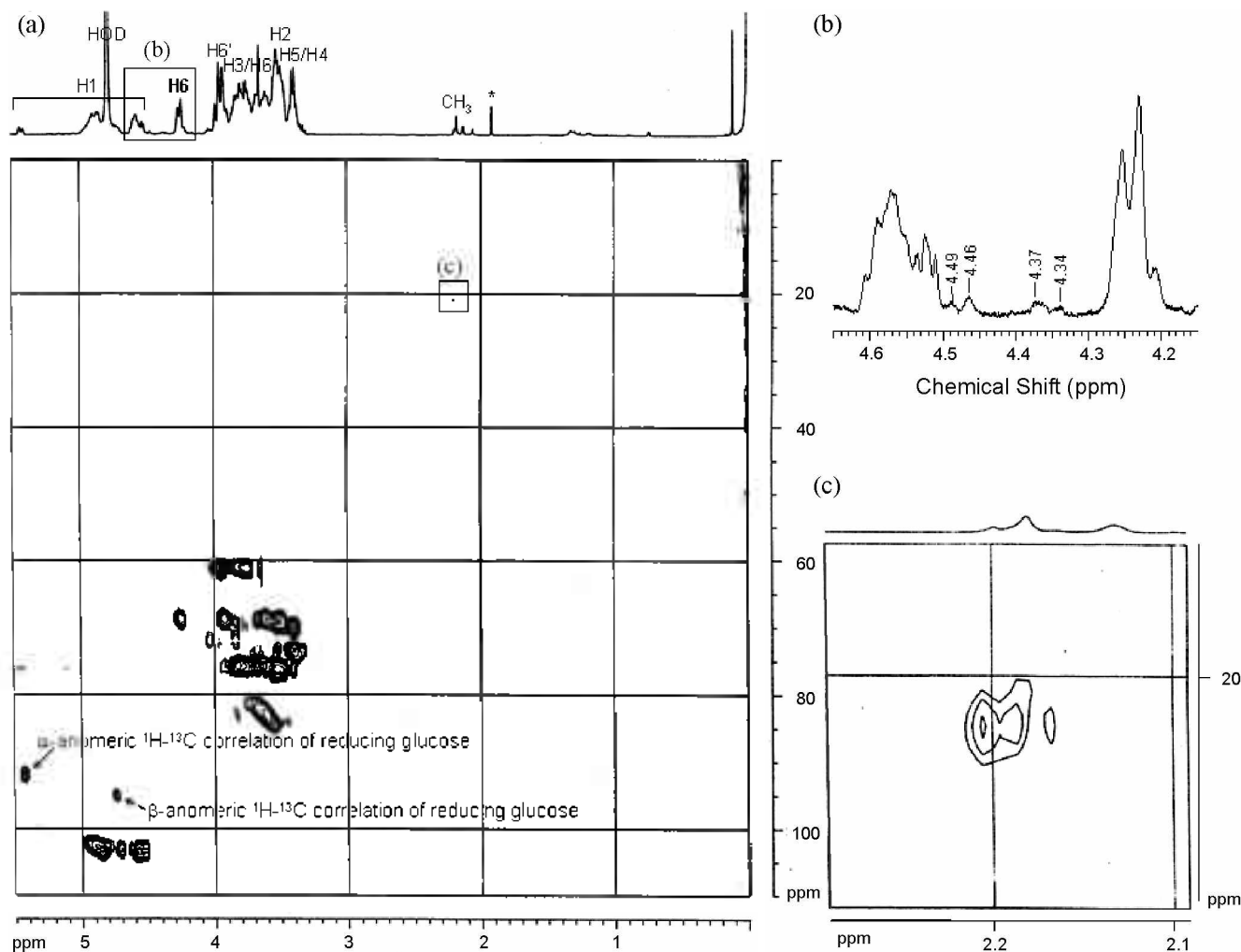


**Figure 1.** MALDI-TOF MS spectra of neutral (a) and anionic glucans (b) isolated from *P. syringae* pv. *syringae*. Number 5 - 24 in (a) and number 7' - 16' in (b) mean DP of neutral and anionic glucans, respectively. Enlarged spectra of  $m/z$  1450 - 1800 and 1550 - 1900 are shown in the right side of Figure 1a and b, respectively. \*: impurity.

$\beta$ -1,6 branched  $\beta$ -1,2 glucans.<sup>8</sup> In this report, the presence of  $\beta$ -1,6 linkages is shown in Figure 2a. C-6 peaks of  $\beta$ -1,6 linkages are assigned near 69.0 ppm and correlated with proton peaks near 4.2 ppm. Two weakly observed doublets ranging from 4.3 to 4.5 ppm in the partial  $^1\text{H}$ -NMR spectrum as shown in Figure 2b are attributed to H-6 and H-6' of glucose residues having an acetate linked at C-6 via an ester bond.<sup>16</sup> However, the  $^1\text{H}$ - $^{13}\text{C}$  correlation of the protons is not detected in the HSQC spectrum (Figure 2a) because there is a relatively low concentration of acetylated glucans, compared to unsubstituted linear periplasmic glucans (Figure 1a). In another enlarged spectrum (Figure 2c), the correlation at 2.18 ppm for  $^1\text{H}$  and at 21.0 ppm for  $^{13}\text{C}$  is identified due to methyl group of substituted acetyl residue. Other signals from H-1 to H-6 in the proton peak area are designated as shown in Figure 2a.

In this study through matrix-assisted laser desorption/ionization Time of flight (MALDI-TOF) mass spectrometric and 1D or 2D NMR spectroscopic analysis, we have described

that *P. syringae* produces one or two acetylated linear periplasmic glucans in HO media, which are different from LO media. This fact offers evidence that the substituent structure of periplasmic glucans can be derivatised according to cultural conditions. The produced amount of periplasmic glucans also decreased and instead, trehalose as another osmoregulator responsible for high-osmotic adaptation was newly synthesized (data not shown). This structural analysis of the changed periplasmic glucans shows the possibility for biological and biotechnological functions of the acetylated linear periplasmic glucans. Some enzymes that transfer substituents (phosphoglycerol or succinate) to backbone glucan have been reported,<sup>17,18</sup> but there is no study on enzyme for acetylation to periplasmic glucans. In *E. coli*, *mdoB* and *mdoC* encode a membrane bound proteins which transfer phosphoglycerol and succinyl residues, respectively.<sup>17,18</sup> Gene for transferring acetyl residues has not demonstrated only, acetyl coA suspects to be the donor molecule. Thus, the exact mechanism for substituting the acetyl



**Figure 2.**  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra (a) of purified neutral linear glucans isolated from *P. syringae*. Protons by the presence of  $\beta$ -1,6 linked and  $\beta$ -1,2 linked glucoses, respectively, are designated in bold and ordinary type. HOD means partially deuterated water. \*: impurity by the methyl group of free acetate. Partial  $^1\text{H}$ -NMR spectrum (b) ranges from 4.2 to 4.6 ppm and an expanded HSQC spectrum (c) for the correlation at 2.18 ppm for  $^1\text{H}$  and at 21.0 ppm for  $^{13}\text{C}$  is shown.

group to periplasmic glucans does not exist. Further investigation for this acetylation mechanism is in progress.

### Experimental Sections

**Purification of linear glucans.** *P. syringae* pv. *syringae* (ATCC 19310) was grown on a rotary shaker at 26 °C in HO medium.<sup>10</sup> To obtain HO medium, 0.3 M NaCl was added to the LO medium. The microorganisms were collected after 1 day by centrifugation at 8,000 rpm for 10 minute. The cell pellets were extracted with 5% trichloroacetic acid, and the extract was neutralized with ammonia water. The neutralized one was concentrated and applied to a Sephadex G-25 column. The fractions of the putative glucans detected by thin-layer chromatography (TLC) were pooled and concentrated by rotary evaporation. The sample was applied to a DEAE-Sephadex column (2.5 × 17.5 cm) to separate the neutral and anionic glucans, which were respectively collected and desalted on a Sephadex G-10 column. The desalted molecule that dissolved

in water was finally lyophilized and analyzed.

**TLC.** The analytes were spotted on the silica Gel G-60 (E. Merck, 400 - 240 mesh) TLC plates. The plates were developed under the solvent (butanol : ethanol : water = 5 : 5 : 4) and dried on the hot plate that measured 120 °C.<sup>18</sup>

**MALDI-TOF MS.** For MALDI-TOF MS, the glucans were dissolved in water and mixed with the matrix (2,5-dihydroxybenzoic acid). Mass spectra were recorded on a mass spectrometer (Voyager-DE<sup>TM</sup> STR BioSpectrometry, PerSeptive Biosystems, Framingham, MA, USA) in positive-ion mode.

**NMR spectroscopy.** For NMR spectroscopic analyses, the glucans were dissolved in  $\text{D}_2\text{O}$  at room temperature. A Bruker Avance 500 spectrometer was used to record the  $^1\text{H}$ -NMR and HSQC spectra. The HSQC spectrum was recorded using 256/2048 complex data points and 29898/4844 Hz spectral widths in t1 and t2, respectively.

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