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Periplasmic Glucans of *Pseudomonas syringae* pv. syringae†

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We report the initial characterization of glucans present in the periplasmic space of *Pseudomonas syringae* pv. syringae (strain R32). These compounds were found to be neutral, unsubstituted, and composed solely of glucose. Their size ranges from 6 to 13 glucose units/mol. Linkage studies and nuclear magnetic resonance analyses demonstrated that the glucans are linked by β-1,2 and β-1,6 glycosidic bonds. In contrast to the periplasmic glucans found in other plant pathogenic bacteria, the glucans of *P. syringae* pv. syringae are not cyclic but are highly branched structures. Acetolysis studies demonstrated that the backbone consists of β-1,2-linked glucose units to which the branches are attached by β-1,6 linkages. These periplasmic glucans were more abundant when the osmolarity of the growth medium was lower. Thus, *P. syringae* pv. syringae appears to synthesize periplasmic glucans in response to the osmolarity of the medium. The structural characteristics of these glucans are very similar to the membrane-derived oligosaccharides of *Escherichia coli*, apart from the neutral character, which contrasts with the highly anionic *E. coli* membrane-derived oligosaccharides.

The *Pseudomonas syringae* group of plant pathogenic bacteria contains various pathogens that cause diseases on the foliage of plants. These bacteria are mainly differentiated by the host plants in which they are capable of multiplying and causing disease. *P. syringae* pv. syringae is the causal agent of brown spot disease of *Phaseolus vulgaris*, the common bean. Isolation of mutants that were affected in their behavior on plants led to the identification of different types of genes involved in plant-pathogen interactions. The genes required for both the expression of disease symptoms on host plants and the development of the hypersensitive reaction on non-host plants have been designated *hrp* for hypersensitive reaction and pathogenicity (41).

The first *P. syringae* pv. syringae *hrp* mutant was obtained by Tn*5* mutagenesis of a streptomycin-resistant derivative of strain R32 (2). Complementation of the mutant phenotype and transposon mutagenesis allowed delimitation of the *hrpM* locus to a length of 3.9 kb (29), while sequence analysis revealed two open reading frames, ORF1 and ORF2 (31). The gene for ORF2 was designated *hrpM*. The *hrpM* gene product was predicted to be an 83-kDa protein spanning the cell membrane. Its function was not elucidated; this protein is not required for growth in minimal medium but is required for growth of bacteria in planta (28).

Recently, Loubens and coworkers (20) have discovered considerable homology (69% identical nucleotides out of 2,816) between the *P. syringae* pv. syringae *hrpM* locus and the *Escherichia coli mdoGH* operon. In *E. coli*, both genes are required for the synthesis of periplasmic glucans (17). The *mdoG* gene product is a 56-kDa periplasmic protein whose function remains to be determined, while the *mdoH* gene product, known to be necessary for glucosyl transferase activity, is a 97-kDa protein spanning the cell membrane (20).

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† We dedicate this article to the memory of our coauthor, Bernard Fournet, who died on 6 January 1993. We will miss his friendship and enthusiasm for science.

The high degree of homology between these two sets of genes suggested that these genes could have similar functions in both organisms. This was partially confirmed by introducing a multicopy plasmid bearing the *hrpM* gene into an *mdoH* mutant of *E. coli* and restoring the periplasmic glucan synthesis capability (21). Thus, the pathogenicity function of the *hrpM* locus appeared to be related to the synthesis of periplasmic glucans in *P. syringae* pv. syringae.

Periplasmic glucans are general components of gram-negative bacteria (37) and share the following features: glucose as the sole sugar, glucose units linked by β-glycosidic bonds, and synthesis under osmotic control and that is inversely correlated to the osmolarity of the growth medium of the cells (26).

Among members of the family *Rhizobiaceae*, periplasmic glucans are cyclic (4). Studies have demonstrated that *Agrobacterium* and *Rhizobium* species synthesize periplasmic glucans with similar structures (3, 10, 16). In both genera, periplasmic glucans are composed of a cyclic β-1,2-glucan backbone containing 17 to 24 glucose residues. Extracts of *Bradyrhizobium* spp. revealed the presence of β-1,6- and β-1,3 cyclic glucans containing 10 to 13 glucose units per ring (25). Periplasmic glucans of *E. coli*, also known as MDOs (membrane-derived oligosaccharides), appear to range from 6 to 12 glucose residues, with the principal species containing 8 or 9 glucose residues (13). The structure is highly branched, the backbone consisting of β-1,2-linked glucose units to which the branches are attached by β-1,6 linkages (36).

In *E. coli*, periplasmic glucans are highly substituted with *sn*-1-phosphoglycerol, phosphoethanolamine, and succinyl ester residues (14, 40). In *Agrobacterium tumefaciens*, one or more phosphoglycerol moieties are substituted for approximately 50% of the total periplasmic glucans (27). In *Rhizobium meliloti*, anionic moieties may be substituted for as much as 90% of the periplasmic cyclic β-1,2 glucans and the predominant anionic substituent present is phosphoglycerol (24). Other substituents can be methylmalonic or succinic acid residues (11). Periplasmic glucans of *Bradyrhizobium* spp. are predominantly neutral (25), but Rolin and coworkers (34) have purified phosphocholine-substituted β-1,6- and β-1,3-glucans from *Bradyrhizobium japonicum* USDA 110.

Studies by numerous authors (7, 9, 33) have provided

evidence for a role of periplasmic glucans in the plant infection process by members of the family *Rhizobiaceae* . The fact that the pathogenicity locus *hrpM* of *P. syringae* pv. syringae appears to be implicated in periplasmic glucan synthesis is consistent with a general role of these compounds in plant infection. We report the results of our analysis of the periplasmic glucans of *P. syringae* pv. syringae.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. syringae* pv. syringae R32 (provided by D. Mills, Department of Botany and Plant Pathology, Oregon State University, Corvallis) and *E. coli* JM101 (42) were grown in LOS medium [4 g of casein hydrolysate, 2 mg of thiamine, 0.5 mg of $FeSO_4$, 18 mg of $MgCl_2$, 200 mg of $(NH_4)_2SO_4$ and 175 mg of K_2HPO_4 per liter (pH 7.2)] on a rotary shaker at 24°C for *P. syringae* and at 37°C for *E. coli*. This low-osmolarity medium was estimated to be 70 mosM (18). When necessary, the osmolarity of the medium was increased by the addition of NaCl.

Protein content. The protein content of cell fractions was determined by the method of Lowry et al. (22) with bovine serum albumin as a standard.

Cellular localization. One-hundred-milliliter cultures were harvested during the mid-log phase of growth. Oligosaccharides were released by an adaptation of the procedure of Schulman and Kennedy (37). After centrifugation at 4°C, the cells were gently resuspended at 4°C in 100 ml of 10 mM Tris-HCl (pH 7.3) containing 20% sucrose and 5 mM EDTA and were immediately centrifuged at 4°C. Both the supernatant, containing the EDTA-released material, and the cell pellet, containing the retained fraction, were extracted with 50% ethanol.

Extraction of cell-associated oligosaccharides. Two-liter cultures were harvested during the late stationary phase of growth. Oligosaccharides were obtained by an adaptation of the procedure of Schneider et al. (36). This involved extraction with 50% ethanol, centrifugation at 10,000 × *g* for 20 min to obtain cell pellets, and concentration of the supernatant by rotary evaporation. The concentrated supernatant was extracted with 2:1 chloroform-methanol and centrifuged. The supernatant was collected as the water-methanol-soluble fraction.

Column chromatographic analysis of cell-associated oligosaccharides. Aqueous methanol extracts were concentrated to a minimum volume and desalted on a Sephadex G-15 (Pharmacia) column (2.2 by 150 cm) at room temperature with H_2O as the eluant at a flow rate of 20 ml/h. Fractions of 4 ml were collected and assayed for total carbohydrates by the phenol-sulfuric acid method (5). Fractions containing sugars eluted in the void volume and were pooled, concentrated, and analyzed by gel filtration chromatography on a Biogel P-6 (Bio-Rad) column (1.6 by 70 cm) as described in the legend to Fig. 1. Fractions containing oligosaccharides, eluting in the volume between 100 and 130 ml, were pooled and lyophilized.

Fifty micrograms of the oligosaccharides was analyzed by high-performance anion-exchange chromatography (HPAE) on a Dionex CarboPac PA-100 column (4 by 250 mm) and monitored with a pulsed amperometric detector. Separations were carried out at pH 6.0 at a flow rate of 1 ml/min. The program used was as follows: isocratic elution at 100% eluant 1 (10 mM sodium acetate buffered to pH 6.0 with acetic acid) for 5 min, followed by a linear increase to 10% eluant 2 (1 M acetate, pH 6.0) at 30 min to 50% eluant 2 at 70 min, followed by isocratic elution for 5 min under the latter condition.

Compositional analysis of cell-associated oligosaccharides. Oligosaccharide preparations were subjected to a variety of chemical analyses. Total monosaccharide compositional analysis was performed by gas-liquid chromatography after methanolysis, acetylation, and trimethylsilylation reactions (30). Reducing sugars were measured with the same method after reduction of the oligosaccharides with sodium borohydride. Succinate, glycerol, and monosaccharides were analyzed by high-performance liquid chromatography on a Biorad HPX-87H column (7.8 by 300 mm) after hydrolysis for 4 h in 4.0 N trifluoroacetic acid at 100°C. Total phosphorus was measured as P_i by HPAE with a conductimetry detector equipped with a Dionex IonPac AG4A-SC column (4 mm in diameter [10–32]) after hydrolysis for 6 h in 6.0 M HCl at 100°C.

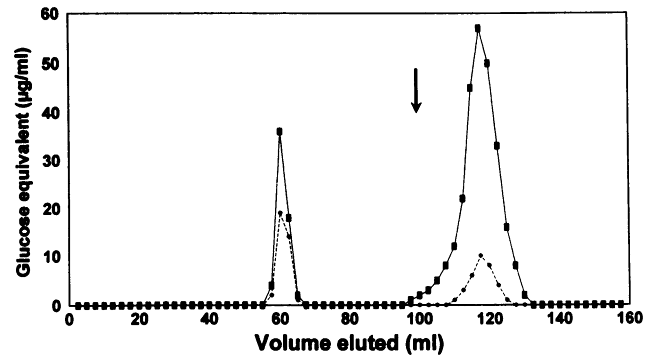

FIG. 1. Cellular localization of the oligosaccharides in *P. syringae* pv. syringae. The oligosaccharides were released as described in Materials and Methods. Both the supernatant (■), containing the EDTA-released material, and the cell pellet (●), containing the EDTA-retained material, were extracted in 50% ethanol. The desalted aqueous methanol extracts were concentrated and applied to a column (70 by 1.6 cm) of Biogel-P6. The column was eluted at room temperature at a flow rate of 15 ml/h with 0.5% acetic acid. Fractions (2.5 ml) were collected and assayed for total carbohydrates by the phenol-sulfuric acid method. The arrow indicates the position expected for MDOs as determined by calibration with a purified standard derived from *E. coli* JM101. Results are expressed as micrograms of glucose equivalent per milliliter of eluant.

Partial acid hydrolysis. Partial acid hydrolysis of the oligosaccharides (1 mg) was performed with trifluoroacetic acid (0.2 N for 3 h at 100°C). After elimination of trifluoroacetic acid in a desiccator, 100 µg of the hydrolysate was directly analyzed by HPAE with pulsed amperometric detection on a Dionex CarboPac PA-1 (4 by 250 mm) by using isocratic elution with 50 mM NaOH at a flow rate of 1 ml/min.

Acetolysis. Acetolysis of the oligosaccharides (2 mg) was performed according to the method of Dubourdieu et al. (6) in 10:10:1 acetic anhydride-acetic acid-H_2SO_4 (1 ml). After acetolysis, the carbohydrates present in the residue were eluted from a column (1.6 by 65 cm) of Biogel-P2 (Bio-Rad) with water as the eluant at a flow rate of 10 ml/h. Fractions of 2 ml were collected, and those containing sugars were pooled and lyophilized.

Matrix-assisted UV-LD-MS. For matrix-assisted UV laser desorption mass spectrometry (UV-LD-MS), oligosaccharides were dissolved in water at a concentration of about 1 g/liter and mixed in a ratio of 1:5 with the matrix (2,5-dihydroxybenzoic acid, 10 g/liter dissolved in 10% aqueous ethanol solution) and mass spectra were recorded on a VISION 2000 instrument (FINNIGAN MAT Ltd., Paradise, United Kingdom), which consisted of an LD ion source coupled to a time-of-flight mass analyzer. Light from a frequency-quadrupled Nd-YAG laser, which operated at a wavelength of 355 nm, was focused onto

the sample target with a single fused silica lens. Each pulse of desorbed ions was accelerated to 20 keV of energy for detection. The spectra acquired consisted of the sum of 20 acquisitions to increase the signal/noise ratio.

SFC. Five hundred micrograms of the oligosaccharides dissolved in 100 μl of pyridine was silylated in 200 μl of HMDS-TMCS (5:1 [vol/vol] 1,1,1,3,3,3-hexamethyldisilazan [Merck]–trimethylchlorosilan [Fluka]) for 1 h at 80°C and left overnight at room temperature. Supercritical fluid chromatography (SFC) of the silylated oligosaccharides was carried out with a Brownlee syringe pump equipped with a 60-nl Valco injection valve. All separations were performed on a DB 5 (diphenyl-dimethylpolysiloxane [1:1]) capillary column (0.1-μm-film thickness, 50-μm internal diameter by 10 m; J&W Scientific, Folsom, Calif.) placed in a gas chromatography oven (Carlo Erba), with flame ionization detection. The mobile phase was CO_2 (SFC grade; Prodair, Lille, France). During chromatography, the oven temperature was maintained at 120°C. Sample elution was achieved with the following programmed pressure increase: 5-min hold at 150 atm (15,193.50 kPa) and then a linear increase to 410 atm (41,528.90 kPa) at a rate of 6.5 atm (658.39 kPa)/min.

NMR analysis. The 100-MHz ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectra were recorded at 25°C on a Bruker AM-400 WB spectrometer equipped with a 5-mm mixed ^{1}H-^{13}C probe head operating in the pulsed F.t. mode and controlled by an Aspect 3000 computer with an array processor. The ^{13}C experiments were performed with the standard Bruker pulse program, POWGATE, with ^{1}H broadband composite pulse decoupling. Nonselective polarization transfer spectroscopy was also used to assign ^{13}C spectra. Twenty milligrams of the oligosaccharides was dissolved in 0.5 ml of D_2O. A 90° pulse (4.8 μs) and a 1.0-s recycle delay were used in each experiment. The chemical shifts are given relative to the methyl of internal acetone (δCH_3 = 31.55 ppm under the conditions used).

Methylation analysis. Glycosidic linkage analysis was performed by methylation according to the method of Paz Parente et al. (32). The methyl ether compounds were analyzed as the partially methylated and acetylated alditols obtained after hydrolysis (trifluoroacetic acid [4 N at 100°C for 4 h]), reduction with sodium borohydride, and peracetylation and as the partially methylated and acetylated methylglucosides obtained after methanolysis (MetOH-HCl [0.5 N at 80°C for 24 h]) of permethylated oligosaccharides and peracetylation, by gas chromatography MS according to the method of Fournet et al. (8).

Fast atom bombardment MS. Mass spectra of permethylated oligosaccharide samples were recorded on a concept 2 H-H mass spectrometer (Kratos Analytical Instrument, Urmstom, Manchester, United Kingdom) equipped with a DS 90 (DG DG/30) data system. The mass spectrometer was operated at an 8-keV accelerating potential. An Ion Tech model B 11 NF saddle field fast atom source energized with the B50 current-regulator power supply was used with xenon as the bombarding atom (operation condition, 7.3 kV, 1.2 mA). The mass range 3,000 to 400 was scanned at 10 s/decade. The methylated oligosaccharides were analyzed in positive mode with thioglycerol in the presence of 0.5 M sodium acetate as matrix.

RESULTS

Cell-associated oligosaccharides of *P. syringae* pv. syringae are localized in the periplasmic space. Cells of *P. syringae* pv. syringae were grown in a medium of low osmolarity, and the cultures were treated with 50% ethanol (see Materials and Methods). Analysis of the ethanol-soluble fraction by Biogel P-6 chromatography revealed the presence of a large peak of glucose-containing material (data not shown). A compositional analysis of this material was performed and revealed the presence of glucose and the absence of succinic acid, glycerol, or phosphorus. In contrast, when the cells were harvested by centrifugation, the supernatant contained only 22% of these oligosaccharides. Treatment of the cells with EDTA in the presence of 20% sucrose led to the release of 88% of the total cell-associated oligosaccharides (fractions 100 to 130 [Fig. 1]). Under these conditions, which avoid osmotic shock, these oligosaccharides could be readily extracted. This finding indicated that cell-associated oligosaccharides are localized in the periplasmic space of the bacteria, in common with the MDOs of *E. coli* (37) and the cyclic glucans of members of the family *Rhizobiaceae* (23, 26). It should be noted that a low level of oligosaccharides was found in the culture medium, comparable with that observed for *E. coli*. Comparison of elution volumes on Biogel-P6 indicated that the oligosaccharides extracted from cultures of *P. syringae* pv. syringae were smaller than the MDOs of *E. coli* (Fig. 1).

Biosynthesis of the periplasmic oligosaccharides of *P. syringae* pv. syringae is regulated by the osmolarity of the growth medium. When cells of *P. syringae* pv. syringae were grown in LOS medium supplemented with 0.2 or 0.4 M NaCl, the amount of periplasmic oligosaccharides recovered in the Biogel-P6 peak (Fig. 1) was strikingly reduced as follows. With no addition, the periplasmic oligosaccharide content (micrograms of glucose per milligram of cell protein) was 52; with 0.2 and 0.4 M NaCl added, the periplasmic oligosaccharide contents were 15.5 and 9, respectively. Synthesis of the periplasmic glucans in cells grown in medium of low osmolarity was approximately 3.5 times higher than that in cells grown in the same medium including 0.2 M NaCl and 6 times higher than that in cells grown in the same medium including 0.4 M NaCl.

Periplasmic oligosaccharides of *P. syringae* pv. syringae are neutral glucans, are acyclic, and are the same size as the MDOs of *E. coli*. The apparent difference in size between the periplasmic oligosaccharides of *P. syringae* pv. syringae and the MDOs of *E. coli* was resolved after further fractionation of these compounds by HPAE at pH 6.0 as described in Materials and Methods. The periplasmic oligosaccharides of *P. syringae* pv. syringae were found to elute in the void volume (data not shown), which is indicative of an unsubstituted, neutral character. This is in contrast to the highly anionic character of the MDOs of *E. coli* (14). Gas chromatographic analysis of the oligosaccharide preparations after methanolysis, re-N acetylation, and trimethylsilylation reactions confirmed that glucose was the only monosaccharide present in the preparations, indicating the absence of contaminating lipo- or extracellular polysaccharide material. In contrast, a compositional analysis of the material eluting in the void volume of the Biogel-P6 column (fractions 55 to 65 [Fig. 1]) revealed the presence of rhamnose, a monosaccharide found in the O-specific antigen of *P. syringae* pv. tomato (15). The same analysis was performed after reduction of the periplasmic glucans and showed a ratio of 7.8 glucose residues per glucitol unit in the reduced oligosaccharides, consistent with an average of 8.8 glucose units/mol of the oligosaccharides. Moreover, this result demonstrates that the periplasmic glucans of *P. syringae* pv. syringae are not cyclic in nature and thus differ from the glucans of the family *Rhizobiaceae* but are similar to the MDOs of *E. coli*.

The molecular weight distribution of the glucans of *P. syringae* pv. syringae was determined by matrix-assisted UV-LD-MS as described in Materials and Methods. This analysis

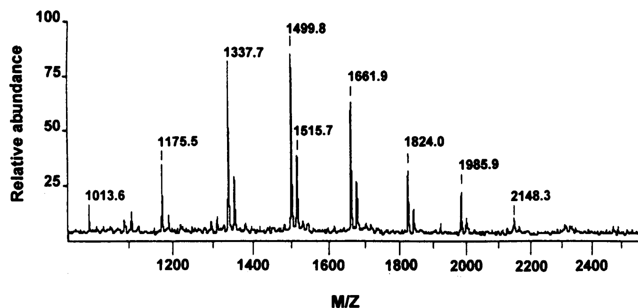

FIG. 2. Matrix-assisted UV-LD-MS of the periplasmic glucans of *P. syringae* pv. syringae. The mass spectra were recorded as described in Materials and Methods. Molecular weights measured by this technique correspond to the chemical molecular weight of the cationated species $[M + Na]^+$ or $[M + K]^+$.

revealed the presence of eight sodium-cationized molecular ions, $[M + Na]^+$, at *m/z* 1,013.6, 1,175.5, 1,337.7, 1,499.8, 1,661.9, 1,824.0, 1,985.9, and 2,148.3 respectively (Fig. 2). These molecular ion species have the same masses as would be expected for linear glucans composed of 6 to 13 glucose residues with the principal species containing 8, 9, and 10 glucose residues. The minor peaks in front of the sodium-cationized ions are attributed to $[M + K]^+$ ions.

The detection of these species by matrix-assisted UV-LD-MS is consistent with the results obtained by SFC with flame ionization detection. The SFC chromatogram of the silylated glucans of *P. syringae* pv. syringae (Fig. 3B) showed numerous peaks with the same retention time distribution as that expected for silylated glucans composed of 6 to 10 glucose residues (Fig. 3A). These results are concordant with those obtained by Kennedy and coworkers for the MDOs of *E. coli* (13).

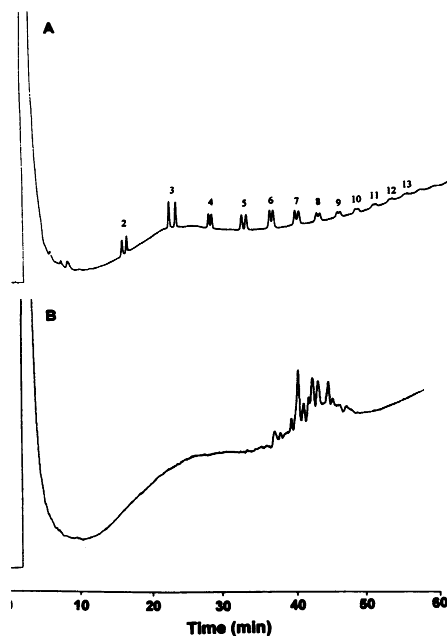

FIG. 3. SFC analysis. Oligosaccharides were examined by SFC as described in Materials and Methods. (A) Maltooligosaccharide standards. (B) Periplasmic glucans of *P. syringae* pv. syringae. The number beside each peak indicates its degree of polymerization.

Periplasmic glucans of *P. syringae* pv. syringae are linked primarily by β-1,2 and β-1,6 glycosidic bonds, and the structures are highly branched. ^{13}C-NMR analysis of the periplasmic glucans of *P. syringae* pv. syringae revealed the presence of β glycosidic linkages and the absence of α glycosidic linkages (Fig. 4). The NMR spectra of the periplasmic glucan preparations contained C-1 resonance peaks clustered near 104 ppm. This shift is indicative of the β glycosidic linkage (39). The peaks near 70 ppm are indicative of the presence of β-1,6 linkages and represent the resonances for both C-4 and C-6 (β-1,6) carbons (39). The C-6 assignment was confirmed by a nonselective polarization transfer (INEPT) technique, showing the characteristic CH_2 negative signal. The resonances near 83 ppm can be assigned to C-2 in β-1,2 glycosidic linkages. Peaks near 62 ppm are indicative of C-6 carbons not involved in glycosidic linkages (39).

The noncyclic nature of the glucans was confirmed by the presence of resonances at 96 and 92.5 ppm, which correspond to the β and α anomeric carbons, respectively, of the reducing-glucose residues. Furthermore, the resonances at 72.7 ppm could be assigned to the C-3 and C-5 of the α anomer of the reducing-glucose residue, while the corresponding smaller signals of the β anomer should be part of the complex of signals at ca. 75 ppm. Peaks near 75 ppm are indicative of C-2 not involved in glycosidic linkages, and the resonances near 77.5 ppm may be assigned to both C-5 and C-3 not involved in glycosidic linkages.

Characteristic of the NMR spectra of all the glucan preparations is the presence of irregular multiple resonances for all carbons. This may be due to the variation in size of the glucan molecules, as previously observed by Dell and coworkers (4) for the neutral cyclic β-1,2-glucans, and to the fact that there is no repetition unit in the sequences of these oligosaccharides.

The presence of 1,6 and 1,2 glycosidic linkages within *P. syringae* pv. syringae periplasmic glucans, determined by ^{13}C-NMR, was confirmed by methylation followed by gas chromatography MS analysis. The results of the methylation analysis (Table 1) revealed equivalent amounts of 3,4-di-, 3,4,6-tri-, and 2,3,4,6,tetra-*O*-methylglucose. This fact indicated that the glucans are highly branched structures with the branch points doubly substituted at positions 2 and 6. The presence of a minor amount of 1,3 glycosidic bonds should be noted.

HPAE analysis of the partial hydrolysate of the glucan preparations revealed the presence of two disaccharide components (Fig. 5). These components were shown to coelute with sophorose (2-*O*-β-D-glucopyranosyl-D-glucose; 60% of the total disaccharides) and gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucose; 40% of the total disaccharide). Thus, the glucose units are joined by 1,2 and 1,6 glycosidic linkages in an apparent ratio of 1.5:1.

The periplasmic glucans of *P. syringae* pv. syringae consist of β-1,2-linked glucose units to which the branches are attached by β-1,6 linkages. 1,6 Glycosidic linkages in oligo- and polysaccharides are preferentially cleaved during acetolysis, and the relative rate for β-1,3 and β-1,6 linkages in disaccharides is 1:41 (35). After acetolysis of the periplasmic glucan preparations, gel filtration separation on Biogel-P2 gave only two fractions. The first (with the highest molecular weight) was eluted near the void volume, and the glycosidic linkage analysis showed the presence of 1,2 (95%) and 1,6 (5%) glycosidic bonds. This is consistent with (1,2)-linked oligosaccharides resulting from almost complete cleavage of the branched chains. Fast atom bombardment MS analysis revealed the presence of four major sodium-cationized molecular ions, $[M + Na]^+$, with masses of 885, 1,089, 1,293, and 1,497 (Fig. 6). These molecular ion species had the same

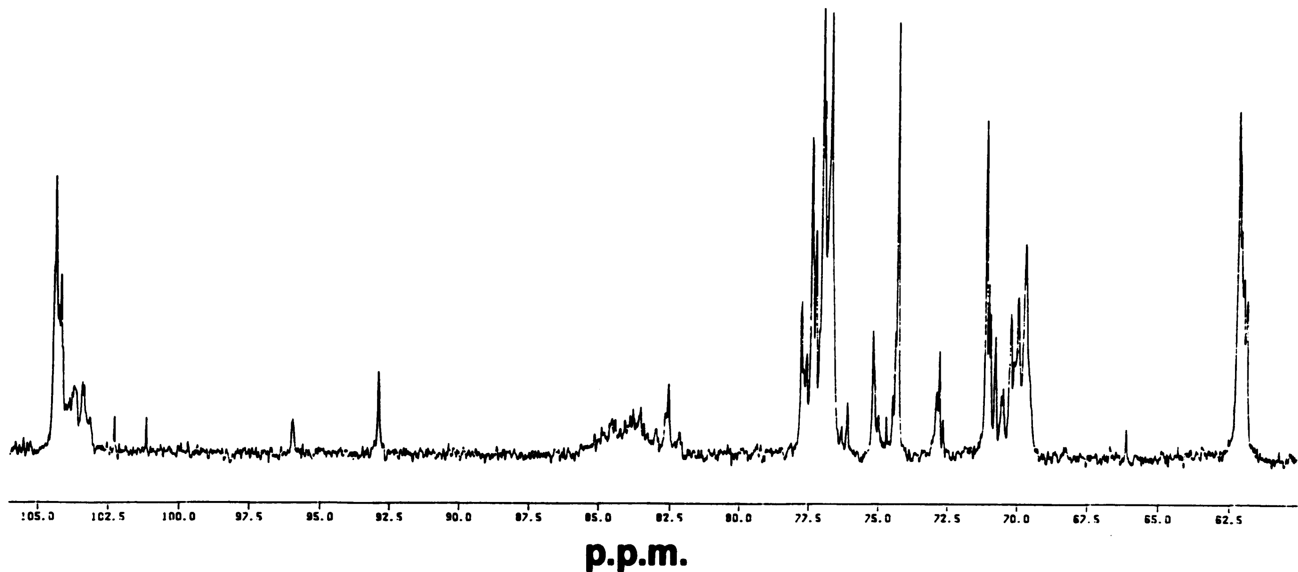

FIG. 4. ^{13}C-NMR analysis of periplasmic glucans of *P. syringae* pv. syringae. NMR spectra were recorded as described in Materials and Methods.

masses as would be expected for linear glucans consisting of four to seven glucose residues. In addition to the major ions, there were two less-abundant sodium-cationized ions with masses of 1,701 and 1,905, which correspond to those expected for linear glucans composed of eight and nine glucose residues, respectively. The minor peaks in front of the sodium-cationized ions are attributed to the thioglycerol-adducted ions.

The second fraction, which eluted at the total volume, was composed solely of glucose monomers, suggesting the presence of single D-glucosyl groups as side chains. Thus, the glucans of *P. syringae* pv. syringae are organized as a β-1,2-linked glucose backbone—with a size ranging from four to seven glucose residues—to which the branches are attached by β-1,6 linkages.

DISCUSSION

As expected from the strong similarity observed between the *hrpM* gene product and the MdoH glucosyltransferase of *E. coli* (20, 21), *P. syringae* pv. syringae produces glucans fundamentally similar to the MDOs of *E. coli*, with a small size, ranging from 6 to 13 glucose units, and a β-1,2-linked glucose backbone to which branches are attached by β-1,6 linkages. Methylation analysis indicated the presence of a small amount of β-1,3 linkages. Other methods did not confirm this fact, and we can assume that the formation of 4,6-di-*O*-methylglucose resulted from undermethylation of a small part of the molecules.

As with MDOs, these compounds are mainly found associated with the cell envelope and are probably localized within the periplasmic compartment of the bacterium. The small amount of glucans found in the culture media could be accounted for by moderate cell lysis. Periplasmic glucans of *P. syringae* pv. syringae are found in larger amounts when the

TABLE 1. Methylation analysis of periplasmic glucans from *P. syringae* pv. syringae

Carbohydrate[a]	Amt detected after[b]:	
	Acid hydrolysis and peracetylation	Methanolysis and peracetylation
Terminal Glc (2,3,4,6-tetra-*O*-methylglucose)	1.5	1.0
2-Linked Glc (3,4,6-tri-*O*-methylglucose)	1.2	1.0
6-Linked Glc (2,3,4-tri-*O*-methylglucose)	0.2	0.1
2,6-Linked Glc (3,4-di-*O*-methylglucose)	1.0	1.0
2,3-Linked Glc (4,6-di-*O*-methylglucose)	0.1	0.1

[a] As glucitol acetates after acid hydrolysis and peracetylation and as methyl-*O*-acetylglucosides after methanolysis and peracetylation as described in Materials and Methods.

[b] The amounts (as determined from peak areas in the gas chromatographic profiles) were compared by taking 3,4-di-*O*-methylglucose as an arbitrary standard (assigned a value of 1).

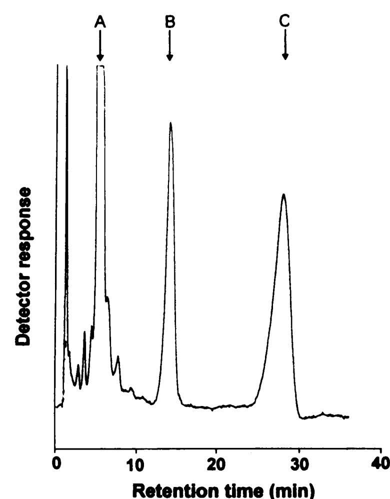

FIG. 5. HPAE analysis of the disaccharides obtained by partial acid hydrolysis of the periplasmic glucans of *P. syringae* pv. syringae. A fraction of the partial hydrolysate was analyzed by HPAE as described in Materials and Methods. The arrows indicate the retention time for the following standards: A, glucose; B, gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucose); C, sophorose (2-*O*-β-D-glucopyranosyl-D-glucose).

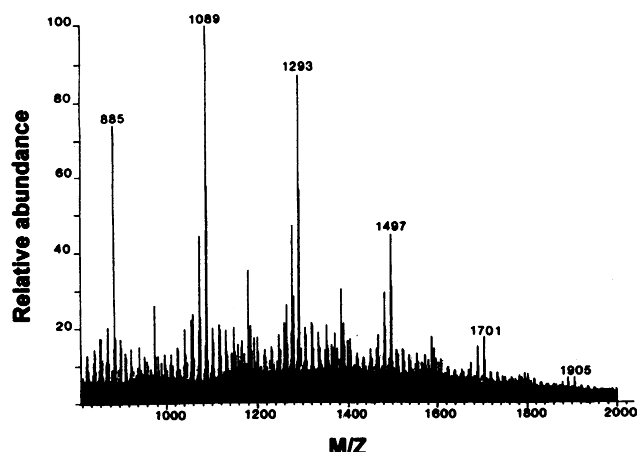

FIG. 6. Positive-ion fast atom bombardment MS of the highest-molecular-weight glucans produced by acetolysis of the periplasmic glucans of *P. syringae* pv. syringae as described in Materials and Methods. The mass spectra were recorded as described in Materials and Methods. The *m/z* values are recorded as the nominal masses of the sodium-cationized species $[M + Na]^+$.

osmolarity of the growth medium decreases. The ratios observed are very similar to those reported in the case of MDO biosynthesis in *E. coli* (12, 18).

MDOs were discovered by Van Golde et al. while measuring phospholipid turnover in *E. coli* (40). Further investigations revealed the highly anionic character of these oligosaccharides, and Kennedy (13) emphasized that these molecules with their net negative charge must contribute substantially to the Donnan equilibrium shown to be present across the outer membrane of *E. coli* (38). Thus, the increase in MDO content observed when the bacteria were grown in media of low osmolarity was interpreted as a mechanism for controlling the osmolarity of the periplasmic space (12). Besides their strong structural similarity to MDOs, the periplasmic glucans found in *P. syringae* pv. syringae are neutral in character. Thus, one may envisage either that different functions have evolved for those closely related compounds or that the neutral or charged character of these glucans does not play a part in a common function. This function, which cannot be directly related to the Donnan equilibrium, remains a mystery.

Since cyclic β-glucans are produced by bacteria of the family *Rhizobiaceae*, and because these bacteria are well known for their ability to infect higher plants, it is possible that the cyclic character of these compounds is specific for plant infection (23, 25). In other families of plant-interacting bacteria, emphasis has been put on extracellular carbohydrates (exopolysaccharides and lipopolysaccharides) as factors implicated in cell-cell surface interactions and signalling (see reference 19 for a recent short review). Thus, very few data are available concerning the periplasmic glucans of bacteria other than those already mentioned in this paper (1, 37). The present study and previous data (21) demonstrate that the pathogenicity and elicitation of the hypersensitive reaction by *P. syringae* pv. syringae are related to the osmotically regulated synthesis of MDO-like glucans in the periplasm of this bacterium. This observation supports the notion that these compounds are functional analogs of the cyclic glucans of the family *Rhizobiaceae*. The differences observed between the structures may be attributable to the phylogenetic positions of these organisms among proteobacteria rather than host specificity. Further investigations of periplasmic glucans are aimed at improving our knowledge of the structural diversity of these compounds in relation to the phylogenetic position and ecology of the bacteria that produce them.

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