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Osmoregulated Periplasmic Glucans of *Erwinia chrysanthemi*

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We report the initial characterization of the osmoregulated periplasmic glucans (OPGs) of *Erwinia chrysanthemi*. OPGs are intrinsic components of the bacterial envelope necessary to the pathogenicity of this phytopathogenic enterobacterium (F. Page, S. Altabe, N. Hugouvieux-Cotte-Pattat, J.-M. Lacroix, J. Robert-Baudouy and J.-P. Bohin, J. Bacteriol. 183:0000–0000, 2001 [companion in this issue]). OPGs were isolated by trichloracetic acid treatment and gel permeation chromatography. The synthesis of these compounds appeared to be osmoregulated, since lower amounts of OPGs were produced when bacteria were grown in media of higher osmolarities. However, a large fraction of these OPGs were recovered in the culture medium. Then, these compounds were characterized by compositional analysis, high-performance anion-exchange chromatography, matrix-assisted laser desorption mass spectrometry, and ^{1}H and ^{13}C nuclear magnetic resonance analyses. OPGs produced by *E. chrysanthemi* are very heterogeneous at the level of both backbone structure and substitution of these structures. The degree of polymerization of the glucose units ranges from 5 to 12. The structures are branched, with a linear backbone consisting of β-1,2-linked glucose units to which a variable number of branches, composed of one glucose residue, are attached by β-1,6 linkages in a random way. This glucan backbone may be substituted by *O*-acetyl and *O*-succinyl ester-linked residues.

Osmoregulated periplasmic glucans (OPGs) are general constituents of the envelopes of gram-negative bacteria (4). Glucose is the sole constitutive sugar, and their abundance in the periplasmic compartment is osmoregulated, with the highest levels synthesized during growth at very low osmolarity. Four families of OPGs have been described on the basis of structural features of the polyglucose backbone. In family I, OPGs appear to range from 5 to 12 glucose residues, with the principal species containing 8 or 9 glucose residues. 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. In family II, OPGs are composed of a cyclic β-1,2-glucan backbone containing 17 to 25 glucose residues. In family III, OPGs are β-1,6 and β-1,3 cyclic glucans containing 10 to 13 glucose units per ring. In family IV, OPGs are cyclic and have a unique degree of polymerization (DP, = 13, 16, or 18). One linkage is α-1,6 whereas all the other glucose residues are linked by β-1,2 linkages. Depending on the species considered, OPGs can be modified to various extents by a variety of substituents. Mutations at the loci *ndvA* and *ndvB* in *Sinorhizobium meliloti* (8), *ndvB* and *ndvC* in *Bradyrhizobium japonicum* (3), *chvA* and *chvB* in *Agrobacterium tumefaciens* (17), and *hrpM* in *Pseudomonas syringae* (11, 13) impair OPG biosynthesis, and these mutants fail to interact properly with a host plant as a symbiont or a pathogen. However, beyond this functional homology, the OPGs synthesized by these different bacteria are very different in structure. The OPGs of *S. meliloti* and *A. tumefaciens* are cyclic structures of family II that may be modified with anionic substituents such as phosphoglycerol and/or succinyl moieties (5), the OPGs of *B. japonicum* are cyclic structures of family III that may be modified by substitution with phosphocholine (18), while the OPGs of *P. syringae* are linear and highly branched and devoid of any substituents (24).

Except for substitution, the OPGs of *P. syringae* are very similar to the OPGs synthesized by the enterobacterium *Escherichia coli*, which colonizes animals. In this bacterium, OPGs are a heterogeneous family of oligosaccharides ranging from 5 to 12 glucose residues (9). The structure is highly branched and consists of a backbone of β-1,2-linked glucose units to which the branches are attached by β-1,6 linkages. They may be substituted to various degrees with *sn*-1-phosphoglycerol, phosphoethanolamine, and *O*-succinyl ester residues.

Erwinia chrysanthemi is a pathogenic enterobacterium responsible for the soft rot disease of a wide range of plants. The pathogenicity of the bacterium is due in part to its ability to produce extracellular enzymes, such as pectinases, cellulases, and proteases, which are able to degrade constituents of the plant cell wall (1, 19).

Recently, the *opgGH* operon of *E. chrysanthemi* was cloned and inactivated on the chromosome (15). The mutants, defective in OPG synthesis, exhibit a pleiotropic phenotype and a complete loss of virulence. In an attempt to understand the function of OPGs at the molecular level in the plant-bacterium interaction, we examined the structures of the OPGs synthesized by *E. chrysanthemi*. OPGs produced by *E. chrysanthemi* are heterogeneous in size and possess structures similar to those of *E. coli* and *P. syringae*. In addition, they may be substituted by *O*-acetyl and *O*-succinyl ester residues.

MATERIALS AND METHODS

Bacterial strains and growth. *E. chrysanthemi* 3937 was grown on a rotary shaker at 30°C in LOS (low osmolarity) medium [4 g of casein hydrolysate, 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)] supplemented with 2 mg of thiamine and 2 g of glycerol. The LOS medium osmolarity is 85 mosM. To obtain high-osmolarity medium, NaCl was added to LOS medium up to 0.3 M.

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Cellular compartmentalization of OPGs. One hundred-milliliter cultures were harvested during the stationary phase of growth. After centrifugation at 4°C for 20 min at 4,000 × *g*, the supernatant was extracted with 50% ethanol, and cells from the pellet were gently resuspended at 4°C in 3 ml of 200 mM Tris-HCl (pH 8)–8.5% sucrose–10 μg of lysozyme per ml–20 mM EDTA and 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. After concentration in a rotary evaporator, the extracts were fractionated by gel filtration on a Bio-Gel P-4 (Bio-Rad). The column (1.6 cm in cross section, 55 cm in height) was eluted at room temperature with 0.5% acetic acid at a flow rate of 15 ml/h, and fractions of 2.5 ml were collected. The oligosaccharides emerged in a peak of intermediate weight detected by the phenol-sulfuric acid procedure (6).

Large-scale isolation and purification of OPGs. Bacteria were collected during the exponential phase of growth by centrifugation at 4°C for 15 min at 8,000 × *g*. Cell pellets were extracted with 5% trichloroacetic acid (TCA), and the TCA extracts were neutralized with ammonium hydroxide and desalted on a Sephadex G-15 column. The desalted material was then fractionated by gel filtration on a Bio-Gel P-4 as described above. Fractions containing oligosaccharides were pooled and lyophilized.

Determination of neutral and anionic characteristics of OPGs. Glucans extracted as described above were desalted on a Bio-Gel P-2 column (Bio-Rad), lyophilized, and resuspended in 2 ml of 10 mM Tris-HCl (pH 7.4) buffer. OPG-containing fractions were pooled and chromatographed on a DEAE-Sephacel column (1.5 cm in cross section, 38 cm in height; Pharmacia) equilibrated with 10 mM Tris-HCl (pH 7.4) and eluted with the same buffer containing increasing concentrations of NaCl ranging from 0 to 0.2 M in steps of 0.05 M. A volume of 60 ml was used for each NaCl concentration, and the volume of each collected fraction was 4 ml.

Determination of succinate and acetate content from OPGs. OPGs were prepared as described above except that formic acid (0.5%) was used in place of acetic acid for Bio-Gel P-4 elution. OPGs were then desalted on Bio-Gel P-2 and separated by DEAE-Sephacel chromatography as described above. Fractions containing oligosaccharides were pooled, desalted, and lyophilized. One milligram of OPGs was dissolved in 0.2 ml of 0.5 M NaOH and incubated at 100°C for 30 min to liberate the succinyl and acetyl residues from OPGs. Glucosidic backbones were removed by absorption on 50 mg of charcoal suspended in 0.3 ml of water, and the charcoal was then washed three times with 0.5 ml of water. The four supernatants were pooled (2 ml) and neutralized with Dowex AG 50W-X8 (Bio-Rad) on H^+ form. Succinic acid and acetic acid contents were determined with a succinic acid kit and an acetic acid kit, respectively (Boehringer Mannheim).

Deesterification of OPGs. OPGs were treated with 0.1 M KOH at 37°C for 1 h. After neutralization with AG 50W-X8 (Bio-Rad) on H^+ form, the samples were desalted on a Bio-Gel P-2 column.

HPAEC-PAD. For high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), analysis and preparation of oligosaccharides were performed on a CarboPac PA-100 anion-exchange column (4 by 250 mm; Dionex, Sunnyvale, Calif.) equipped with a CarboPac PA guard column (3 by 25 mm; Dionex). Oligosaccharides were detected with a PAD II pulsed amperometric detector with a gold electrode (Dionex). The chromatographic data were integrated and plotted with an SP 4270 integrator (Spectra-Physic, San Jose, Calif.). Oligosaccharides were eluted at a flow rate of 1 ml/min by a two-step procedure consisting of (i) 0.05 M sodium acetate in 0.15 M NaOH for 5 min and (ii) a linear gradient of 0.05 to 0.2 M sodium acetate in 0.15 M NaOH for 35 min. After every run, the column was reequilibrated in 0.15 M NaOH for 15 min.

The oligosaccharides were prepared in the same way. Fractions were collected and separated on a Dowex AG 50W-X8 column on H^+ form (5 by 1 cm; Bio-Rad) eluted with water. The acetic acid produced was neutralized by NH_4OH. Some Na^+ was left, which was subsequently removed by desalting on a Bio-Gel P-2 column.

MALDI-MS. For matrix-assisted laser desorption-ionization (MALDI)-mass spectrometry (MS), experiments were carried out on a Vision 2000 (Finnigan MAT, Bremen, Germany) time-of-flight mass spectrometer equipped with a nitrogen laser (337-nm wavelength and 3-ns pulse width). After selection of the appropriate site on the target by a microscope, the laser light was focused onto the sample-matrix mixture at an angle of 15° and a power level of 10^6 to 10^7 W/cm^2. Positive ions were extracted by a 5- to 10-keV acceleration potential, focused by a lens, and the masses were separated by a Reflectron time-of-flight instrument. At the detector, ions were postaccelerated to 20 keV for maximum detection efficiency. The resulting signals were recorded with a fast transient digitizer with a maximum of 2.5 ns channel resolution and transferred to a personal computer for accumulation, calibration, and storage. All MALDI mass spectra are the result of 20 single-shot accumulations.

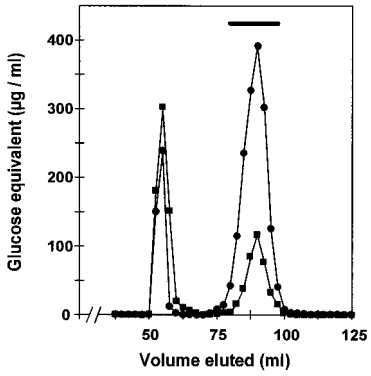

FIG. 1. Bio-Gel P-4 elution profile of OPGs of *E. chrysanthemi* grown in LOS medium (●) or in LOS medium supplemented with 0.3 M NaCl (■). The column (1.6 by 55 cm) was eluted with 0.5% acetic acid, and aliquots were analyzed for total carbohydrates (see Materials and Methods). Results are expressed as glucose equivalents per milliliter of eluant. In both cases, fractions indicated by the horizontal bar were lyophilized.

The following matrices for carbohydrate analysis were used: 2,5-dihydroxybenzoic acid, (10 g/liter in water [22]) and 3-aminoquinoline (10 g/liter in water [23]). Lyophilized oligosaccharides samples were redissolved in double-distilled water and then diluted with an appropriate volume of the matrix solution (1:5, vol/vol), 1 μl of the resulting solution was deposited onto a stainless steel target, and the solvent was evaporated under a gentle stream of warm air.

Methylation analysis. The oligosaccharides were methylated according to Paz-Parente et al. (16). The methyl ethers were obtained after methanolysis (0.5 M HCl in methanol at 80°C for 24 h) and analyzed as partially methylated methyl glycosides by gas-liquid chromatography (GLC)-MS (7). GLC was performed using a Delsi apparatus with a capillary column (25 m by 0.2 mm) coated with DB-1 (0.5-μm film thickness), applying a temperature gradient of 110 to 240°C at 3°C/min and a helium pressure of 40 kPa. The mass spectra were recorded on a Nermag 10-10B mass spectrometer (Rueil-Malmaison, France) using an electron energy of 70 eV and an ionizing current of 0.2 mA.

NMR spectroscopy. Prior to nuclear magnetic resonance (NMR) spectroscopic analysis, the oligosaccharides were twice treated with ^2H_2O at room temperature. After each exchange treatment, the materials were lyophilized. Finally, each sample was redissolved in 0.5 ml of ^2H_2O (99.96 atom% ^2H; Aldrich). The NMR experiments were performed on a Bruker AM-400 spectrometer controlled by an Aspect 3000 computer with an array processor and equipped with a 5-mm mixed ^1H-^{13}C probe head at a temperature of 25°C. Chemical shifts (δ) were referenced to acetone, the internal standard. Two-dimensional homonuclear COSY (correlation spectroscopy) 90, relayed and double-relayed COSY experiments, and two-dimensional heteronuclear multiple quantum coherence (HMQC) were performed by using standard Bruker pulse programs.

Other methods. Protein concentrations were determined by the method of Lowry et al. (12) with bovine serum albumin as the reference protein. Total carbohydrate concentrations were determined by the anthrone method of Spiro (22) with D-glucose as the standard. Sugar analysis was carried out by GLC of trimethylsilyl derivatives of methyl glycosides formed by methanolysis in 0.5 M HCl in methanol at 80°C for 24 h (14). Reducing sugars were measured by the same method after reduction of the oligosaccharides with $NaBH_4$.

RESULTS

Isolation and characterization of OPGs. OPGs were extracted from cells of *E. chrysanthemi* by previously described procedures which involved TCA extraction. Fractionation on a Bio-Gel P-4 column allowed the separation of two main sugar-containing compounds (Fig. 1). The compounds eluting in the void volume of the column were probably high-molecular-weight lipo- or exopolysaccharides, as previously observed

(24). The second peak represented the OPGs. When cells of *E. chrysanthemi* were grown in LOS medium supplemented with 0.3 M NaCl, the amount of cell-associated oligosaccharides was reduced (Fig. 1). With no addition of NaCl, the OPG content (micrograms of glucose per milligram of cell protein) was 76 ± 5. With the addition of 0.3 M NaCl, the OPG content was 7 ± 3. Thus, cells grown in a medium of low osmolarity synthesized approximately 10 times more OPGs than cells grown in the same medium with 0.3 M NaCl.

Gas chromatographic analysis of the two OPG preparations after methanolysis, re-*N*-acetylation, and trimethylsilylation reactions revealed that glucose was the only monosaccharide present, indicating the absence of contaminating lipo- or exopolysaccharide material. The same analysis was performed after reduction of the OPGs and showed in both cases an average of 6.7 glucose residues per glucitol unit in the reduced glucans, consistent with an average of 7.7 glucose units/mol of glucans. Moreover, this result indicates that the glucans of *E. chrysanthemi* are not cyclic.

Cellular compartmentalization of OPGs. Export of OPGs to the extracellular medium has been observed for members of the family *Rhizobiaceae*, and extracellular glucans may play a fundamental role in plant-bacterium interactions (3, 5). However, this export varies greatly among different species and strains and is dependent on growth stage and culture conditions. Thus, it was important to determine whether the OPGs produced by *E. chrysanthemi* can be recovered in the external medium, at least under certain circumstances. Actually, 75% of the OPGs were found in the growth medium from stationary-phase cultures at low osmolarity (see Materials and Methods). Of the remaining 25%, 80% were liberated by EDTA treatment, which is known to release periplasmic content. Similar results were obtained when cells were separated by filtration.

OPG substitution. Thin-layer chromatography analysis showed different patterns for native and KOH-treated OPGs (see Materials and Methods; data not shown). This was indicative of a substitution of OPGs by ester-linked residues (alkaline-sensitive substitution). Therefore, OPGs were analyzed by anion-exchange chromatography (DEAE-Sephacel). When the OPGs were extracted from cells grown at low osmolarity, practically no material was retained by the column (Fig. 2), but when the OPGs were extracted from cells grown at high osmolarity, 20% of the total amount was retained and subsequently eluted with low-ionic-strength buffer (10 mM Tris-HCl [pH 7.4], 50 mM NaCl).

Succinyl esters were previously found to give an acidic character to OPGs synthesized by different bacterial species. A new substituent, acetyl ester, was recently found in association with succinyl ester in *Rhodobacter sphaeroides* OPGs (P. Talaga, V. Cogez, J.-M. Wieruszeski, et al., unpublished data). For these reasons, succinic and acetic acids liberated after alkaline treatment of OPGs were determined using commercial kits (see Materials and Methods). A total of 28.5 ± 5.1 μg of succinic acid per mg of glucose was found in the anionic fraction of OPGs extracted from cells grown in the high-osmolarity medium, whereas the amount was under the limit of detection (2.1 μg/mg of glucose) when cells were grown in the low-osmolarity medium. A constant amount (6.1 ± 1.9 μg) of acetic acid per mg of glucose was found whatever the growth conditions or faction of OPGs considered.

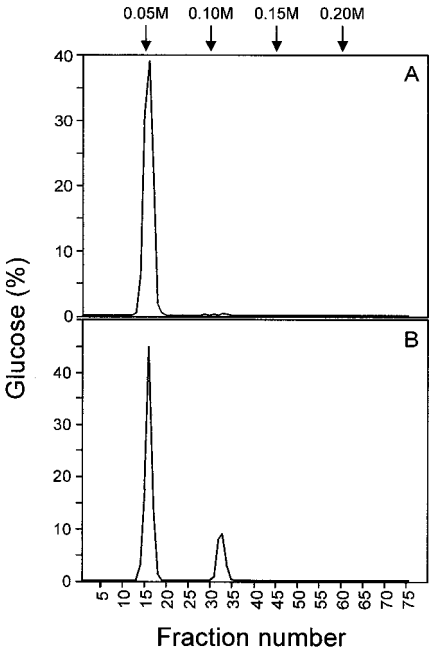

FIG. 2. DEAE-Sephacel anion-exchange column chromatography profiles of OPGs from *E. chrysanthemi* grown in LOS medium (A) or LOS medium with 0.3 M NaCl (B). Ionic strength was increased by steps of 0.05 M NaCl at the fractions indicated by the arrows. Fractions (4 ml) were collected, and total carbohydrate concentrations were determined by the anthrone method (see Materials and Methods). The amount of glucans in each fraction is expressed as a percentage of the total amount loaded on the column.

^{1}H-NMR analysis. The OPGs of *E. chrysanthemi* grown in the high-osmolarity medium (Fig. 3) were analyzed by ^{1}H-NMR analysis. Peaks at 5.4 ppm are indicative of the H-1 of reducing glucose residues with the α-anomeric configuration. Peaks near 4.9 and 4.55 ppm are indicative of the H-1 of glucose residues engaged in β-1,2 and β-1,6 linkages, respectively. Those at 4.2 ppm were assigned to the H-6 of glucose residues linked by a β-1,6 linkage. The two triplets present between 2.4 and 2.8 ppm confirmed the presence of succinyl substituents.

Methylation analysis. OPGs extracted from cells grown at low or high osmolarity were first subjected to alkaline treat-

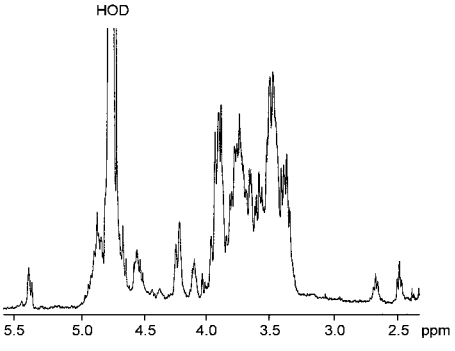

FIG. 3. ^{1}H-NMR analysis of OPGs of *E. chrysanthemi* grown in LOS with 0.3 M NaCl. NMR spectra were recorded as described in Materials and Methods. HOD, partially deuterated water.

TABLE 1. Methylation analysis of OPGs of *E. chrysanthemi*

Carbohydrate[a]	Relative amt detected[b] after growth in LOS medium with:	
	No NaCl	0.3 M NaCl
Terminal Glc (2,3,4,6-tetra-*O*-methylglucose)	1.1	1.2
2-Linked Glc (3,4,6-tri-*O*-methyl Glc)	1.9	1.8
6-Linked Glc (2,3,4-tri-*O*-methylglucose)	0.2	0.2
2,6-Linked Glc (3,4-di-*O*-methylglucose)	1.0	1.0

[a] 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 the amount of 3,4-di-*O*-methylglucose as an arbitrary standard (assigned a value of 1).

ment to remove all succinic and acetic substituents. Then, the OPGs were methanolized and, after acetylation, subjected to GLC-MS analysis. In both cases, the results of the methylation analysis revealed the presence of 3,4-di-, 3,4,6-tri-, and 2,3,4,6-tetra-*O*-methylglucose (Table 1). This indicated that the glucans were branched structures, with branch points doubly substituted in positions 2 and 6. The ratio of 2-linked Glc to 2,6-linked Glc was approximately 2. This suggested that about two glucose residues were linked through positions 1 and 2 for every internal glucose residue linked through positions 1, 2, and 6. This indicated that the glucans were highly branched structures.

^{13}C-NMR analysis. KOH-treated OPGs of *E. chrysanthemi* grown in low-osmolarity medium were analyzed by ^{13}C-NMR analysis (Fig. 4A). This analysis revealed that the C-1 resonance peaks all clustered near 104 ppm. This shift is indicative of the β-glycosidic linkage. The peaks near 70 ppm are indicative of β-1,6 linkages and represent the resonances for both C-4 and C-6 (β-1,6) carbons. The C-6 assignment was confirmed by a nonselective polarization transfer technique, showing the characteristic CH_2 negative signal. The resonances near 83 ppm were 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, while those at 74.5 ppm are indicative of C-2 carbons not involved in glycosidic linkages. The resonances near 77.5 ppm may be assigned to both C-5 and C-3

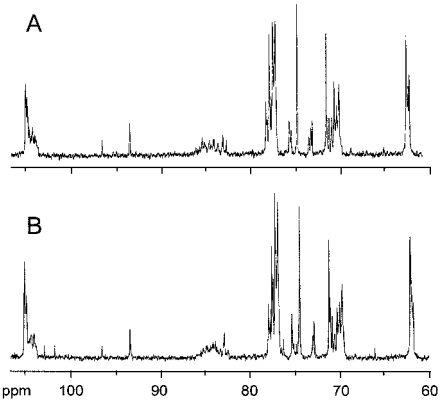

FIG. 4. ^{13}C-NMR analysis of KOH-treated OPGs of *E. chrysanthemi* (A) and *P. syringae* (B) grown in LOS medium. NMR spectra were recorded as described in Materials and Methods.

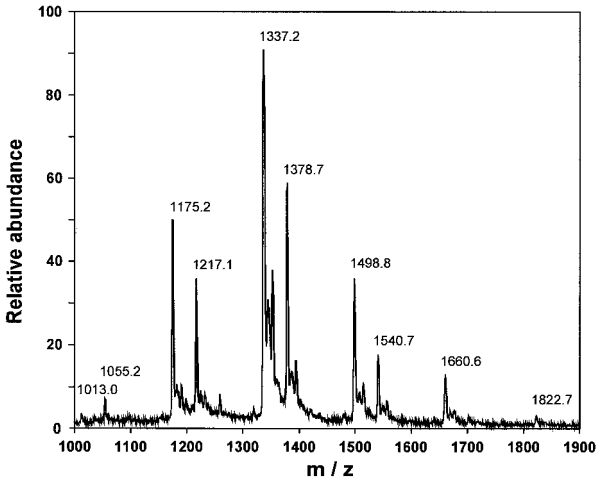

FIG. 5. Positive-ion MALDI mass spectra of OPGs of *E. chrysanthemi* grown in LOS medium without addition. Mass assignments are based on an external calibration.

carbons not involved in glycosidic linkages. The acyclic nature of the glucans was confirmed by the presence of resonances at 96 and 92.5 ppm, corresponding to the β and α anomeric carbons, respectively, of the reducing-glucose residues. Furthermore, the resonances at 72.7 ppm could be assigned to C-3 and C-5 of the α anomer of the reducing glucose, while the corresponding signals of the β anomer were part of the complex of signals at 75 ppm. All these assignments were confirmed by a ^{13}C-decoupled, ^{1}H-detected HMQC analysis.

The ^{13}C-NMR spectra of the OPGs of *E. chrysanthemi* and those of *P. syringae* are very similar (Fig. 4A and B). We can conclude that both glucans possess essentially the same structure: a backbone consisting of β-1,2-linked glucose units, to which branches, composed of one glucose residue, are attached by β-1,6 linkages.

MALDI-MS. Quasimolecular ions were obtained by the MALDI-MS method for the OPGs of *E. chrysanthemi* grown in LOS medium without NaCl. This analysis revealed the presence of six sodium-cationized molecular ions, $[M + Na]^+$, at approximately *m/z* 1,013, 1,175, 1,337, 1,499, 1,661, and 1,823 (Fig. 5). These molecular ion species had masses identical to those expected for linear glucans composed of 6 to 11 glucose residues, with the principal species containing 8 glucose residues. Four sodium-cationized molecular ions, $[M + Na]^+$, at approximately *m/z* 1,055, 1,217, 1,379, and 1,541, were detected and correspond to glucans of 6, 7, 8, and 9 degrees of polymerization, respectively, with a mass increment of 42, which is expected for an *O*-ester-linked acetyl residue (Fig. 5). Peaks corresponding to minor species with *m/z* decreased by 18 below the values of the main molecular ions were due to in-source loss of water occurring during ionization, a phenomenon frequently observed in MS of carbohydrates. Peaks corresponding to the $[M + K]^+$ ions were also present, with *m/z* increased by 16 above the masses of the corresponding sodiated ions. This analysis confirmed that each species of OPGs can be substituted by one acetyl residue.

MALDI-MS analyses of KOH-treated OPGs extracted from

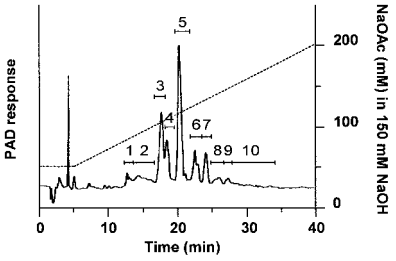

FIG. 6. HPAEC elution profile of KOH-treated OPGs of *E. chrysanthemi*. Oligosaccharide peaks were detected by PAD. The number above each peak indicates the fraction number. NaOAc, sodium acetate.

cells grown in the presence or absence of NaCl revealed identical spectra (data not shown).

HPAEC-PAD analysis. In HPAEC-PAD analysis of KOH-treated OPGs (Fig. 6), the number of peaks was greater than the number of carbohydrate-based signals determined by MALDI-MS. In order to understand this phenomenon, we performed HPAEC separation and subsequent MALDI-MS and methylation analyses of the predominant fractions. The MALDI-MS analysis (Table 2) revealed a molecular weight distribution slightly larger (5 to 12 glucose residues) than observed before separation (Fig. 5) and indicated that the retention volume increased as the degree of polymerization increased. Moreover, the presence of several isomeric oligomers was also revealed, as the component of fractions 3 and 4 contained 7 glucose units (Fig. 7A and B) and components of fractions 5 and 6 contained 8 glucose units (Fig. 7C and D). The presence of minor signals in the spectra (Fig. 7) could result from some contamination of the neighboring peaks or from coelution with other compounds. Based on the MALDI-MS data, methylation analyses were performed on the components of fractions 3, 4, 5, and 6 (Table 3). These analyses clearly indicate that the OPGs of fraction 3 possess a higher number of β-1,6 branches than the OPGs of fraction 4 (Table 3). The same result was obtained for the OPGs of fractions 5 and 6 (Table 3). This clearly indicates that for a given isomeric OPG, the retention volume decreased when the number of β-1,6 branches increased.

Since ^{1}H-NMR analyses of OPGs from fractions 3, 4, 5, and 6 (data not shown) are as complex as the NMR analysis of the complete mixture, we can conclude that there is no repeat unit, such as alternating substituted and unsubstituted glucose units.

TABLE 2. HPAEC-PAD analysis of OPGs of *E. chrysanthemi*

Fraction no.	Retention time (min)	Measured mass(es) $[M + Na]^+$	No. of glucose residues
1	13	851	5
2	15	1,013	6
3	17.7	1,175	7
4	18.5	1,175	7
5	20.3	1,337	8
6	22.7	1,337	8
7	24.3	1,499	9
8	25.9	1,499, 1,662	9, 10
9	27.6	1,662	10
10	28–34	1,824, 1,986	11, 12

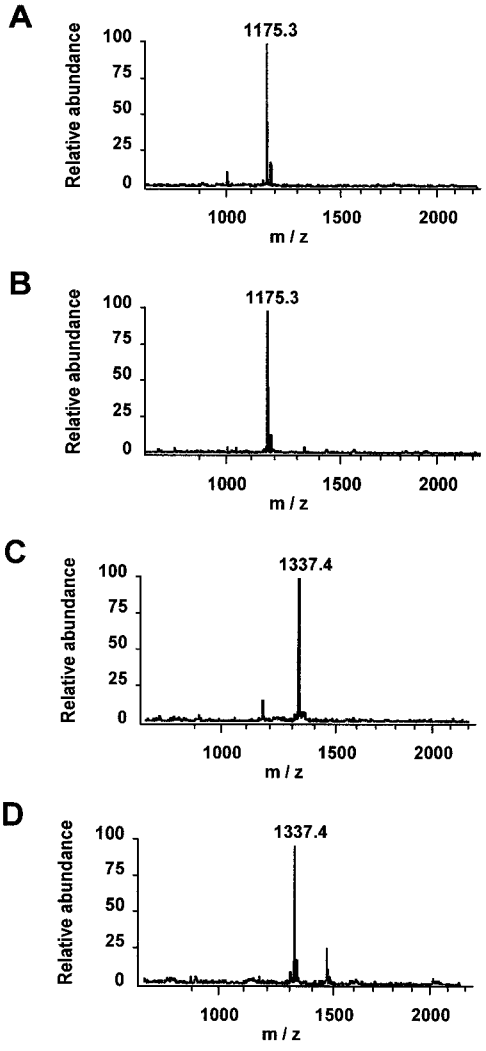

FIG. 7. Positive-ion MALDI mass spectra of OPGs of *E. chrysanthemi* HPAEC-PAD fractions 3 (A), 4 (B), 5 (C), and 6 (D).

The β-1,6 branches are situated randomly along the β-1,2 linear chains.

DISCUSSION

OPGs of *E. chrysanthemi* are found in larger amounts when the osmolarity of the growth medium decreases. The ratio observed (10-fold more in low osmolerity) is similar to those

TABLE 3. Methylation analysis of HPAEC fractions 3, 4, 5, and 6

Fraction no.	% Methylated sugar[a] (molar ratio)			
	Terminal Glc	2-Linked Glc	6-Linked Glc	2,6-Linked Glc
3	35 (2.4)	32 (2.2)	7 (0.5)	26 (1.9)
4	25 (1.8)	55 (3.8)	3 (0.2)	17 (1.2)
5	36 (2.9)	27 (2.2)	8 (0.6)	29 (2.3)
6	31 (2.5)	44 (3.5)	5 (0.4)	20 (1.6)

[a] As methyl-*O*-acetylglucosides after methanolysis and peracetylation as described in Materials and Methods. The molar ratio is based on the degree of polymerization observed in the MALDI spectrum for each fraction.

already reported for *E. coli* and *P. syringae* (12, 14, 24). The structure determination of *E. chrysanthemi* OPGs showed that they are small glucans ranging in size from 5 to 12 glucose residues, made of a β-1,2-linked glucose backbone to which branches are attached by β-1,6 linkages. Thus, the OPG backbone structures synthesized by the two *Enterobacteriaceae* and the pseudomonad can be considered identical. One noticeable difference is at the level of cell concentration (about 30, 50, and 75 μg of glucose per mg of cell protein for *E. coli, P. syringae*, and *E. chrysanthemi*, respectively). But the main differences reside in the number and variety of substituents, from the no-substituted OPGs of *P. syringae* to the highly substituted anionic OPGs of *E. coli*.

Further analyses by HPAEC-PAD revealed the high level of heterogeneity of these OPGs: a variable number of glucose residues are arranged with a variable number of branches whose position on the backbone is random. In no way can the OPGs of this family be described as repetitions of a conserved unit. This characteristic marks the demarcation between OPGs and other polysaccharidic structures found in bacteria.

Recently, the *E. chrysanthemi* genes involved in OPG biosynthesis (*opgGH*, accession number AJ294718) have been cloned. They exhibit a very high degree of similarity (86%) to their homologues *mdoGH* and *hrpM*, respectively, in *E. coli* and *P. syringae* (15). The fact that highly similar genes control the biosynthesis of OPGs belonging to the same structural family may seem obvious. However, the *Rhodobacter sphaeroides opgGH* genes (accession number AF016298) were recently isolated and characterized (A. Puskas, V. Cogez, E. Gak, J.-P. Bohin, and S. Kaplan, unpublished data). They show a significant, although lower (55%), level of similarity to *mdoGH*, but they control the biosynthesis of OPGs belonging to family IV (Talaga et al., unpublished). Thus, structural analysis of OPGs will remain necessary even when gene sequences are known.

Moreover, this structural analysis revealed differences at the level of backbone substitution. *E. chrysanthemi* OPGs can be substituted by acetyl and succinyl residues, and the presence of succinyl depends on the growth conditions. Thus, while *P. syringae* OPGs are neutral and *E. coli* OPGs are highly anionic (10), *E. chrysanthemi* OPGs could have a mild anionic character when growth occurs at high osmolarity. The function of glucan substitution, which varies to a large extent from species to species, remains obscure. A highly attenuated *Salmonella enterica* mutant with a high immunogenic and protective potential in mice was found to have a transposon insertion in a gene similar to *mdoB* (25). In *E. coli*, the product of this gene is a membrane-bound P-glycerol transferase, which transfers residues from phospholipids to OPGs. Thus, this observation supports the conclusion that OPG substitution could play a role in bacterial virulence. *E. chrysanthemi opgG* and *opgH* mutants were recently obtained by cassette insertion into the chromosome (15). These mutants, which are defective in OPG synthesis, exhibit a pleiotropic phenotype and a complete loss of virulence (15). In an attempt to isolate mutants defective in OPG substitution, we screened several collections of Tn*5* insertions in the strain 3937, as previously described for *E. coli* (10). However, despite several attempts, we were unable to obtain such mutants.

A large fraction of the OPGs synthesized by *E. chrysanthemi* were recovered in the supernatant of centrifuged cultures when the cultures were collected in the stationary phase after growth at low osmolarity. However, it was not possible to determine the fraction of OPGs released (or secreted) during growth and the fraction released during the separation procedure. OPG detection in the medium is not possible without cell separation, and whatever the treatment, if one considers that outer membrane stability may be weakened under certain circumstances, shearing forces could release OPGs from the periplasmic space. No similar phenomenon was observed with *E. coli* cultures, but *E. chrysanthemi* envelope properties are clearly different, since, for example, OPG-defective mutants exhibit bile salt hypersensitivity, while similar mutants of *E. coli* have normal bile salt resistance (15). Similarly, strain T83 of *Erwinia amylovora* released a tightly complexed mixture of oligosaccharides and lipopolysaccharide in place of extracellular polysaccharide (20). The oligosaccharides, being branched structures with an average of 11 β-1,2- and β-1,6-linked glucose units, are most probably the OPGs produced by this particular strain. These observations open the questions of the actual organization of extracellular OPGs and of their role in pathogenesis. Membrane vesicles containing outer membrane protein, lipopolysaccharide, phospholipids, and periplasmic constituents are constantly extruded from the surface of a number of gram-negative bacteria (for a recent review, see Beveridge [2]). It was possible that OPGs released in the medium by *E. chrysanthemi* were included in such membrane vesicles. Nevertheless, we were unable to separate any fraction of the released OPGs when culture supernatants were further ultracentrifuged (100,000 × *g*, 3 h; data not shown). Thus, released OPGs are probably in a soluble form.

Growth conditions in plant tissues are certainly very different from those in a liquid low-osmolarity medium on a rotary shaker. Thus, any extrapolation should be taken with caution. However, if OPGs are released during the infection of plants by *E. chrysanthemi*, they could play a fundamental role in pathogenesis. To test this possibility, potato tubers were inoculated with a mixture of Opg^+ and Opg^- bacteria (15), and growth of the different bacteria was compared. *E. chrysanthemi* strains defective in OPG synthesis were unable to grow in planta, and growth could not be restored by the OPGs eventually provided by the neighboring wild-type bacteria. OPGs must be present in the periplasmic space of *E. chrysanthemi* to allow its growth in the plant host.

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