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Structure of the O-specific polysaccharides of the lipopolysaccharides of *Xanthomonas campestris* pv. *vignicola* GSPB 2795 and GSPB 2796

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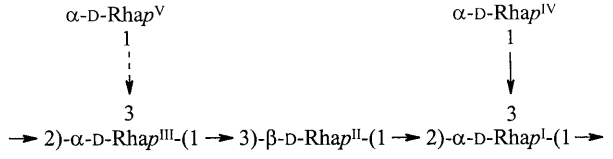
Abstract

The O-specific polysaccharides of *Xanthomonas campestris* pv. *vignicola* GSPB 2795 and GSPB 2796 were studied by sugar and methylation analyses, Smith degradation, 1D, 2D ^{1}H and ^{13}C NMR spectroscopy. It was found that the polysaccharides are similar branched D-rhamnans lacking strict regularity, and their structures can be described as follows:



where Rha^{V} is present in a non-stoichiometric amount, which varies from strain to strain.

Keywords: Phytopathogenic bacteria; *Xanthomonas campestris*; O-Chain polysaccharide; Lipopolysaccharide; Structure

1. Introduction

Phytopathogenic xanthomonads are distributed into a number of different 'pathovars', each of which is mainly found in a very narrow and specific range of host plants [1]. Recently, some pathovars of *Xanthomonas campestris* have been gathered together on the basis of genetic data [2,3]. However, the biochemical mechanisms responsible for the narrow host specificity still have not been clarified. Specific structures of the outer-membrane lipopolysaccharides (LPS) of phytopathogenic pseudomonads and xanthomonads have been suggested to play a decisive role in host/parasite interactions [4]. Unique structures were established in a few cases when the O-specific polysaccharide chain of the LPS of xanthomonads was analysed (*X. campestris* pvs. *campestris* [5], *begoniae* [6] and *manihotis* [7], and a strain of *X. campestris* isolated from strawberry [8]).

In the present paper, we report on the structure of the OPS of *X. campestris* pv. *vignicola*

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GSPB 2796 (no. 56a, from Niger) and GSPB 2795 (no. 28a2, from Nigeria), which represent high and low virulent strains of the same pathovar, respectively [9].

2. Results and discussion

LPS was isolated from bacterial cells of *X. campestris* pv. *vignicola* GSPB 2795 and degraded with mild acid to give a high-molecular-mass O-specific polysaccharide (OPS). Sugar analysis using GLC of acetylated alditol acetates revealed rhamnose as the sole sugar component of both OPS. GLC of acetylated (−)-2-octyl glycosides showed that rhamnose has the D configuration.

Methylation analysis of OPS revealed the presence of 2,3,4-tri-*O*-methylrhamnose, 3,4-di-*O*-methylrhamnose, 2,4-di-*O*-methylrhamnose, and 4-*O*-methylrhamnose in the ratios 0.9:0.8:1:1.2, respectively. Therefore, OPS is branched with terminal rhamnose residues, 2,3-disubstituted rhamnose residues at the branching point, and the remaining 2- and 3-substituted rhamnose residues. These data showed also that all rhamnose residues are in the pyranose form.

The ^{1}H and ^{13}C NMR spectra of the OPS showed two series of signals with a ratio of integral intensities $\sim$3:2. This indicated that OPS includes two different polysaccharide chains or is built up of two different repeating units. The major series in the ^{13}C NMR spectrum (Fig. 1, bottom) contained signals for four anomeric carbons at δ 99.3–103.5, four CH_3–C groups (C-6 of Rha) at δ 17.8–18.2, and 16 other sugar ring carbons in the region δ 70.5–81.3. Accordingly, the major series in the ^{1}H NMR spectrum of OPS (Fig. 2, bottom) contained signals for four anomeric protons at δ 4.72–5.17 and four CH_3–C groups (H-6 of Rha) at δ 1.28–1.34 (each 3H, d, $J_{5,6} \sim 6$ Hz). Therefore, the major repeating unit of OPS is a rhamnose tetrasaccharide. In the minor series, there were signals for one more rhamnose residue; hence, it was suggested that the minor repeating unit is a pentasaccharide.

The rhamnose residues were characterized as shown below, and the ^{1}H and ^{13}C NMR spectra of the OPS were assigned using 2D COSY, TOCSY, and H-detected $^{1}H,^{13}C$ HMQC experiments (Tables 1 and 2). The ^{13}C NMR chemical shift data showed the presence in the major tetrasaccharide repeating unit of 2,3-disubstituted, 3-substituted, 2-substituted, and terminal rhamnose residues (Rha^{I}–Rha^{IV}, respectively). The substituted rhamnose residues were characterised by downfield displacements of the corresponding carbons (2, 3, or both 2 and 3) to δ 77.4–81.3, compared with their positions at δ 71.3–74.0 in nonsubstituted rhamnose [10]. The ^{13}C NMR chemical shifts of the fourth residue (Rha^{IV}) were close to those of α-rhamnopyranose [10]. In the minor series, there was one 3-substituted residue, two 2,3-disubstituted and two terminal residues of rhamnose, both terminal residues (Rha^{IV} and Rha^{V}) being α-linked. A 2D rotating-frame NOE (ROESY) experiment revealed intraresidue H-1,H-2 correlations for all rhamnose residues, except for Rha^{II}, in both series. Hence, two more residues, Rha^{I} and Rha^{III}, are α-linked. The absence of H-1,H-2 correlation and the presence of H-1,H-3,5 correlations showed that Rha^{II} is β-linked in both major and minor repeating units. These conclusions were confirmed by the positions of the signals for H-5 and C-5 at δ_H 3.67–3.86 and δ_C 70.4–71.1 for α-Rha and δ_H 3.36–3.41 and δ_C 73.5 for β-Rha. The substitution pattern established by the ^{13}C NMR chemical shifts was in agreement with methylation analysis data.

The ROESY spectrum of the OPS showed the following interresidue correlations between transglycosidic protons in the major series: Rha^{I} H-1,Rha^{III} H-2 at δ 5.10/4.09, Rha^{III} H-1,Rha^{II} H-3 at δ 5.17/3.60, Rha^{II} H-1,Rha^{I} H-2 at δ 4.72/4.24, and Rha^{IV} H-1,Rha^{I} H-3 at δ 5.07/3.94. In addition, there were cross-peaks between the H-1 signals of Rha^{II} and Rha^{I} at δ 4.72/5.10 (strong) and Rha^{I} and Rha^{III} at δ 5.10/5.17 (weak), which are typical of (1→2)-linked sugar residues. A H-detected $^{1}H,^{13}C$ heteronuclear multiple-bond correlation (HMBC) experiment revealed Rha^{I} H-1,Rha^{III} C-2, Rha^{III} H-1,Rha^{II} C-3, Rha^{II} H-1,Rha^{I} C-2, and Rha^{IV} H-1,Rha^{I} C-3 correlations at δ 5.10/79.6, 5.17/81.3, 4.72/77.7, and 5.07/77.4, respectively. These data confirmed the glycosylation pattern and

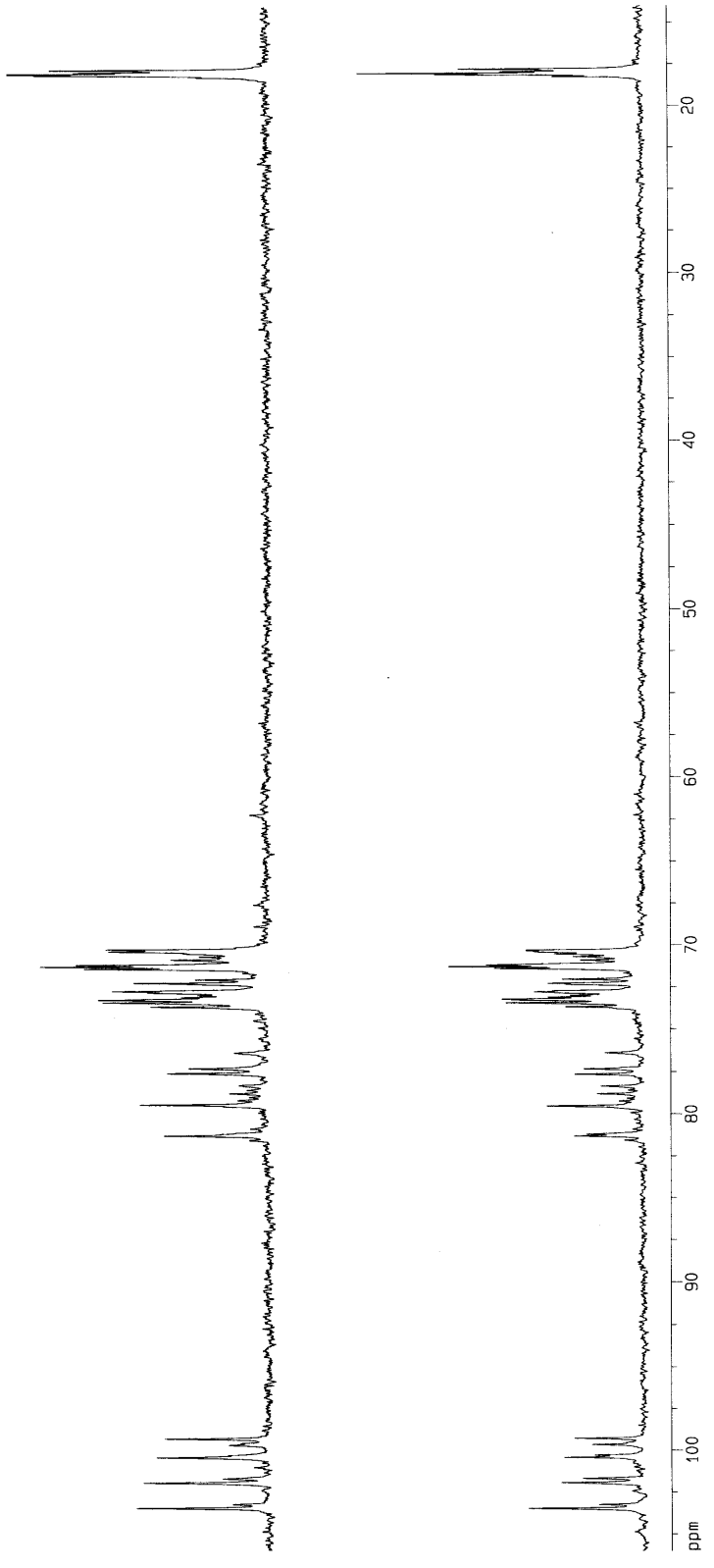


Fig. 1. ^{13}C NMR spectra of the OPS from *X. campestris* pv. *vignicola* GSPB 2795 (bottom) and GSPB 2796 (top).

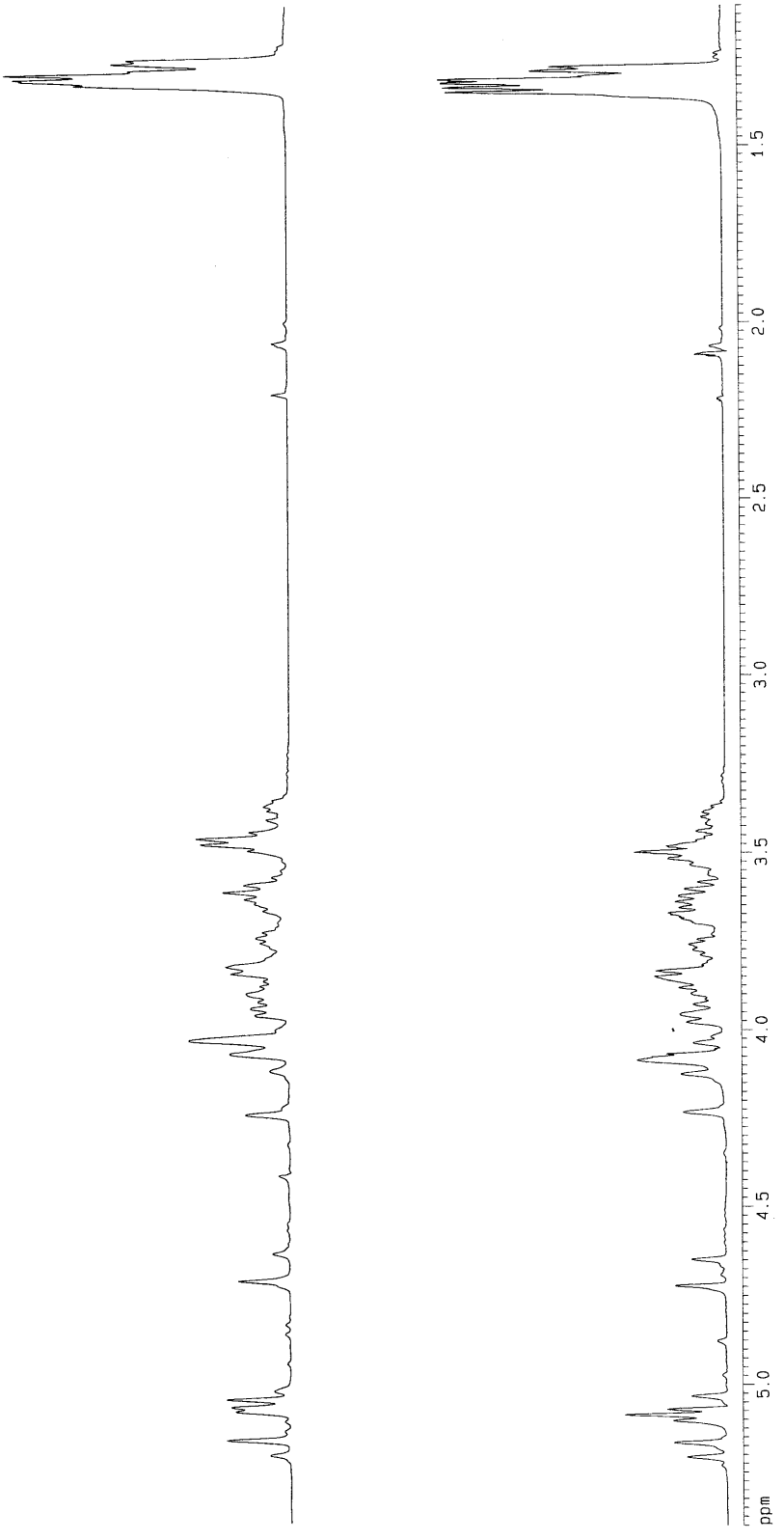
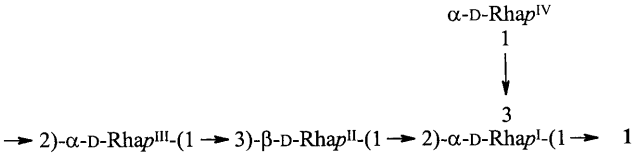
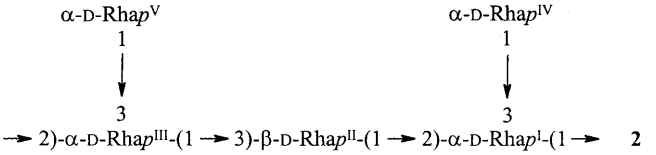


Fig. 2. ^{1}H NMR spectrum of the OPS from *X. campestris* pv. *vignicola* GSPB 2795 (bottom) and GSPB 2796 (top).

established the sugar sequence in the major repeating unit of the OPS, which has thus structure **1**.



For the minor repeating unit, only a partial sugar sequence could be determined from the ROESY data owing to insufficient resolution of some signals. In particular, there were Rha^{I} H-1,Rha^{III} H-2, Rha^{III} H-1,Rha^{II} H-3, and Rha^{II} H-1,Rha^{I} H-2 correlations at δ 5.03/4.13, 5.21/3.62, and 4.65/4.08, as well as Rha^{II} H-1,Rha^{I} H-1 and Rha^{I} H-1,Rha^{III} H-1 correlations at δ 4.65/5.03 and 5.03/5.21, respectively. These data indicated that the main chain in the minor repeating unit has the same structure as in the major one. The sites of attachment of the two terminal residues, Rha^{IV} and Rha^{V}, were determined from the HMBC spectrum, which showed Rha^{IV} H-1, Rha^{I} C-3 and Rha^{V} H-1,Rha^{III} C-3 correlations at δ 5.09/76.4 and 5.09/78.8, respectively. On the basis of these data, it was suggested that the minor repeating unit of the OPS has structure **2**.



Therefore, the OPS studied lacks the strict regularity owing to the nonstoichiometric substitution with a terminal rhamnose residue (Rha^{V}). In order to confirm the structures of the repeating units **1** and **2**, Smith degradation of the OPS was carried out, which resulted in an oligosaccharide (**3**) and a modified polysaccharide (**4**).

Table 1
500 MHz ^{1}H NMR data (δ in ppm)

	H-1	H-2	H-3	H-4	H-5	H-6
O-Specific polysaccharide						
Major repeating unit ***1***						
→2)-α-D-Rha*p*^{I}-(1→ 3 ↑	5.10	4.24	3.94	3.63	3.76	1.28
→3)-β-D-Rha*p*^{II}-(1→	4.72	4.05	3.60	3.47	3.36	1.34
→2)-α-D-Rha*p*^{III}-(1→	5.17	4.09	3.97	3.48	3.82	1.32
α-D-Rha*p*^{IV}-(1→	5.07	4.06	3.84	3.49	3.86	1.33
Minor repeating unit ***2***						
→2)-α-D-Rha*p*^{I}-(1→ 3 ↑	5.03	4.08	3.97	3.67	3.78	1.29
→3)-β-D-Rha*p*^{II}-(1→	4.65	4.07	3.62	3.49	3.41	1.34
→2)-α-D-Rha*p*^{III}-(1→ 3 ↑	5.21	4.13	4.03	3.59	3.86	1.31
α-D-Rha*p*^{IV}-(1→	5.09	4.06	3.84	3.50	3.67	1.33
α-D-Rha*p*^{V}-(1→	5.09	4.06	3.84	3.50	3.67	1.32
Oligosaccharide-glyceraldehyde ***3***						
→2)-α-D-Rha*p*^{I}-(1→	5.10	4.18	3.84	3.48	3.92	1.28
β-D-Rha*p*^{II}-(1→	4.72	4.05	3.60	3.40	3.40	1.33
→2)-Gro-al	5.10	3.67	3.74 [a]			
Smith-degraded polysaccharide ***4***						
→2)-α-D-Rha*p*^{I}-(1→	5.09	4.25	3.79	3.48	3.72	1.27
→3)-β-D-Rha*p*^{II}-(1→	4.75	4.08	3.64	3.51	3.44	1.33
→2)-α-D-Rha*p*^{III}-(1→	5.19	4.09	3.65	3.49	3.82	1.30

[a] H-3a; H-3b at δ 3.87.

Table 2
125 MHz ^{13}C NMR data (δ in ppm)

	C-1	C-2	C-3	C-4	C-5	C-6
O-Specific polysaccharide						
Major repeating unit **1**						
→2)-α-D-Rha*p*^{I}-(1→	100.5	77.7	77.4	72.8	70.9	17.8
3 ↑						
→3)-β-D-Rha*p*^{II}-(1→	99.3	72.3	81.3	72.9	73.5	18.2
→2)-α-D-Rha*p*^{III}-(1→	102.0	79.6	71.2	73.4	70.6	18.0
α-D-Rha*p*^{IV}-(1→	103.5	71.4	71.3	73.7	70.5 [a]	18.1
Minor repeating unit **2**						
→2)-α-D-Rha*p*^{I}-(1→	100.3	78.3	76.4	73.0	71.1	17.9
3 ↑						
→3)-β-D-Rha*p*^{II}-(1→	99.7	72.4	81.2	72.7	73.5	18.3
→2)-α-D-Rha*p*^{III}-(1→	101.7	79.6	78.8	73.3	70.7	17.9
3 ↑						
α-D-Rha*p*^{IV}-(1→	103.5	71.2	71.5	73.3	70.4 [a]	18.1
α-D-Rha*p*^{V}-(1→	103.3	71.3	71.5	73.2	70.4 [a]	18.1
Oligosaccharide-glyceraldehyde **3**						
→2)-α-D-Rha*p*^{I}-(1→	98.8	79.1	70.9	73.9	70.3	17.7
β-D-Rha*p*^{II}-(1→	100.0	72.2	73.9	73.3	73.6	17.9
→2)-Gro-al	90.5	81.5	61.0			
Smith-degraded polysaccharide **4**						
→2)-α-D-Rha*p*^{I}-(1→	100.9	78.5	70.7	73.4	70.4	17.8
→3)-β-D-Rha*p*^{II}-(1→	99.4	72.0	81.0	72.7	73.3	17.8
→2)-α-D-Rha*p*^{III}-(1→	101.9	79.5	71.1	73.7	70.3	17.8

[a] Assignment could be interchanged.

Analysis of the ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) showed that oligosaccharide **3** contains two rhamnose residues (δ_{H-1} 4.72 and 5.10; δ_{C-1} 98.8 and 100.0) and a glyceraldehyde (Gro-al) residue in the hydrated form [δ_{H-1} 5.10; δ_{C-1} 90.5 and δ_{C-3} 61.0]. The latter formed evidently by oxidation of a 2-substituted rhamnose residue, and, therefore, **3** was derived from the major repeating unit **1** of the OPS. The following structure of oligosaccharide **3** was established:

β-D-Rha*p*^{II}-(1→2)-α-D-Rha*p*^{I}-(1→2)-Gro-al **3**

The ^{1}H and ^{13}C NMR spectra of the polysaccharide **4** were completely assigned using 2D COSY, TOCSY, and ^{1}H,^{13}C HMQC experiments (Tables 1 and 2), and it was found that **4** has a trisaccharide repeating unit (δ_{H-1} 4.75, 5.09, and 5.19; δ_{C-1} 99.4, 100.9, and 101.9). A 2D ROESY experiment revealed intraresidue H-1,H-2 correlations for Rha^{I} and Rha^{III} in **3**, and, hence, they are α-linked. The presence of H-1,H-3,5 correlations indicated that Rha^{II} is β-linked. The ROESY spectrum of **4** showed strong interresidue cross-peaks between the following transglycosidic protons: Rha^{I} H-1,Rha^{III} H-2 at δ 5.09/4.09, Rha^{III} H-1,Rha^{II} H-3 at δ 5.19/3.64, and Rha^{II} H-1,Rha^{I} H-2 at δ 4.75/4.25. These data demonstrated that polysaccharide **4** is linear and has the following structure:

→2)-α-D-Rha*p*^{III}-(1→3)-β-D-Rha*p*^{II}-(1→2)-α-D-Rha*p*^{I}-(1→ **4**

This product was evidently derived from the minor repeating unit **2** of the OPS by oxidation of both terminal rhamnose residues. In GPC on Sephadex G-50, it eluted in a broad range later than the initial OPS, and, hence, both types of the repeating units, **1** and **2**, coexist in the same polysaccharide chain.

The ^{1}H and ^{13}C NMR spectra of the OPS of *X. campestris* pv. *vignicola* GSPB 2796 (Figs. 1 and 2, top), as well as 2D $^{1}H,^{1}H$ and $^{1}H,^{13}C$ shift-correlated spectra, showed a high degree of similarity to the OPS from strain GSPB 2795. The OPS of strain GSPB 2796 was not strictly regular and contained the same two types of repeating units **1** and **2**, but their ratio was different (~ 3:1 in strain GSPB 2796 compared with ~ 3:2 in strain GSPB 2795). As mentioned above, *X. campestris* pv. *vignicola* GSPB 2796 and GSPB 2795 are high and low virulent strains, respectively [9]. However, it does not seem probable that such a minor difference in the ratio of the two repeating units of the OPS can explain the different degree of disease symptoms caused in plants. Therefore, more strains of *X. campestris* pv. *vignicola* should be studied in respect to the OPS structure and other features.

A similar type of structural heterogeneity was demonstrated in the OPS of a representative of another *Xanthomonas* species, *X. campestris* pv. *begoniae* GSPB 525 [6], and in that of *Pseudomonas fluorescens* IMV4125 (ATCC 13525) [11]. These polysaccharides have an L-rhamnan backbone and two lateral sugar substituents in each repeating unit, L-xylose or 3-acetamido-3,6-dideoxy-D-galactose, respectively, one of which being present in the stoichiometric and the other in a non-stoichiometric amount. It is also worth noting that D-rhamnose is a commonly occurring OPS component of phytopathogenic bacteria, including *Xanthomonas* and the related genera *Stenotrophomonas* and *Pseudomonas* [12,13]. Like the polysaccharide studied in this work, some of them are D-rhamnans, and the repeating unit of *Pseudomonas syringae* pv. *cerasi* 435 [14] shows much similarity to the repeating unit **1**.

3. Experimental

Growth of bacteria and digestion of cells.—*X. campestris* pv. *vignicola* GSPB 2795 and GSPB 2796 from the culture collection 'Göttinger Sammlung Phytopathogener Bakterien' (Göttingen, Germany) were cultivated in a 100-L fermenter at 28 °C on King's Medium B [15] with glycerol as carbon source. The cultures were stirred at 70 rpm at an aeration rate of 60 L/min. Cells were harvested at the late exponential phase by centrifugation, washed three times with 3 L EDTA-saline at 2 °C and lyophilised. Bacterial cells were extracted subsequently with EtOH for 2 h and acetone for 1.5 h to remove phospholipids, and then sonicated for 10 min to disintegrate the cell walls. Nucleic acids were removed by digestion with DNAse and RNAse (5 mg per g lyophilised bacteria) for 8 h. Proteins were removed by digestion with proteinase K for 12 h. The resultant cell material was dialysed against deionised water and lyophilised.

Isolation of LPS and OPS.—LPS was isolated by the procedure of Galanos et al. [16]. The bacterial mass was suspended in 250 mL warm aq 90% phenol (70 °C), stirred for 30 min at 70 °C and then overnight at ambient temperature. Chloroform (250 mL) and petrol ether (250 mL) was added; the suspension was stirred for 30 min at ambient temperature, and centrifuged (4 °C, 20 min, 9600 rpm). The pellet was extracted twice with 500 mL of a 2:5:8 phenol–$CHCl_3$–petrol ether mixture. After solvent evaporation, the remaining solution was kept overnight in a cool room and the LPS was precipitated by adding water while the suspension was cooled on ice. Within 1 h, the LPS precipitate was separated by centrifugation (4 °C, 10 min, 5000 rpm), washed three times with acetone, and dried on air.

The OPS were prepared by degradation of the LPS with aq 2% AcOH for 1.5 h at 100 °C, followed by GPC on a column (70 × 2.6 cm) of Sephadex G-50 using 0.05 M pyridinium acetate buffer (pH 4.5) as eluent and monitoring with a Knauer differential refractometer.

Sugar analysis.—The OPS (0.5 mg) was hydrolysed with 2 M CF_3CO_2H (120 °C, 2 h), rhamnose was identified by GLC as its alditol acetate [17] using a Hewlett–Packard 5880 instrument with an Ultra 2 capillary column and a temperature gradient of 150 °C (1 min) to 290 °C at 10 °C/min. The absolute configurations were determined by GLC of acetylated glycosides with (−)-2-octanol by the method [18] under the same chromatographic conditions as above.

Methylation analysis.—Methylation was carried out with MeI in Me_2SO in the presence of solid NaOH [19]. Hydrolysis was per-

formed as for the sugar analysis, partially methylated monosaccharides were reduced with $NaBH_4$, acetylated, and analysed by GLC/MS on a Hewlett–Packard 5890 chromatograph equipped with a DB-5 fused-silica capillary column and a NERMAG R10-10L mass spectrometer, using a temperature gradient of 160 °C (1 min) to 250 °C at 3 °C/min.

Smith degradation.—The OPS (37 mg) was oxidized with 0.1 M $NaIO_4$ in the dark for 48 h at 20 °C, after $NaBH_4$ reduction and desalting by dialysis against distilled water, the product was hydrolyzed with aq 1% AcOH for 1 h at 100 °C and fractionated by GPC on a column (80 × 1.6 cm) of TSK HW-40 in aq 1% AcOH to give a polysaccharide **4** (8.5 mg) and an oligosaccharide-glyceraldehyde **3** (5.8 mg).

NMR spectroscopy.—OPS samples were deuterium-exchanged by freeze-drying three times from D_2O and then examined in a solution of 99.96% D_2O. Spectra were recorded using a Bruker DRX-500 spectrometer at 50 °C. Chemical shifts are reported with internal acetone (δ_H 2.225, δ_C 31.45). Data were acquired and processed using the XWINNMR 2.1 program. A mixing time of 200 and 100 ms was used in 2D TOCSY and ROESY experiments, respectively. A 60 ms delay was used for the evolution of long-range connectivities in a 2D H-detected ^1H,^{13}C HMBC experiment.

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