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Note

Structure elucidation of the O-chain from the major lipopolysaccharide of the *Xanthomonas campestris* strain 642

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Abstract

A novel O-polysaccharide consisting of D-Xyl*p* and L-Rha*p* in the molar ratio of 1:2.5 was identified as the major component in the lipopolysaccharide fraction of *Xanthomonas campestris* strain 642, which is responsible for a new bacterial disease of the strawberry plant. Its structure was mainly determined using chemical analysis, Smith degradation and 1D and 2D NMR spectroscopy experiments as:

β-D-Xyl*p*
1
↓
2
→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→
4
↑
1
β-D-Xyl*p*

Keywords: *Xanthomonas campestris*; Lipopolysaccharide; Xylorhamnan; Phytopathogen; Strawberry

Strawberry is attacked by several pathogens including *Xanthomonas fragariae*, the agent of the strawberry angular leaf spot, the only phytopathogenic bacterium so far recognised to cause a serious disease on this culture [1].

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However, recently a new bacterial disease of strawberry has been reported [2]. The observed symptoms, which occur mainly on leaves, differ from those caused by either *X. fragariae* or plant pathogenic fungi, causal agents of leaf diseases on strawberry. Preliminary investigations on the characterisation of the bacterium, consistently isolated from lesions, showed that on the basis of the pathogenetic and biochemical features it may be ascribed to *X. campestris*. Since it has been suggested [2] that mixed infections (*X. fragariae* and *X. campestris*) may occur on the same plant/leaf, it seems of interest to gain further information on this phytopatogenic bacterium. Here the chemical characterisation of the O-chain polysaccharide of strain 642 of the *X. campestris* isolated from leaf lesions of strawberry is reported.

The lipopolysaccharide (LPS) fraction of *X. campestris* was obtained by the aqueous phase of phenol–water treatment of dried cells. Electrophoresis behaviour on SDS-PAGE, KDO assay and fatty acids analysis indicated the LPS nature of the extract material, which contained only a trace of proteins (2%).

The mild acetic acid hydrolysis of the LPS fraction yielded the lipid moiety, as a precipitate, and in the liquid phase the O-chain polysaccharide, which was purified by elution in the void volume on a Bio-Gel P-10 column. Further gel permeation chromatographies (Bio-Gel P100, Bio-Gel A 0.5 m, Sephacryl S-300) showed the presence of a single peak in all cases, suggesting a homogeneous product whose average molecular weight was determined to be 63 kDa. The O-chain methanolysis data indicated a monosaccharide composition consisting only of rhamnose and xylose in the 2.5:1 molar ratio. The absolute configuration of these sugars was measured to be D and L for xylose and rhamnose, respectively, on the basis of the GLC analysis of their 2-octyl glycoside acetates. The ^{1}H NMR spectrum (Fig. 1) showed signals of different intensities, thus suggesting the presence of a minor polysaccharide component. However, six main signals were clearly visible; five of these appeared as broad singlets in the range 5.316–4.835 ppm, whereas the last one, centred at δ 4.450, consisted of two overlapping doublets. Furthermore, very intense methyl signals at δ 1.305–1.368 were in agreement with the presence of rhamnose. The ^{13}C NMR spectrum (Fig. 2) showed five anomeric signals, of which those at 104.0 and 100.4 ppm were more intense than others. The methylation analysis revealed the presence of terminal Xyl*p*, 2-substituted Rha*p*, 3-substituted Rha*p* and 2,3,4-trisubstituted Rha*p* in about 2:2:2:1 ratios, respectively. These data suggested a heptasaccharide structure as the repeating unit of the major O-chain polysaccharide component. Starting from the anomeric protons the homo- and heteroscalar connectivities for each residue were established allowing the complete assignment of all proton and carbon signals (Table 1) by 2D NMR experiments (COSY, TOCSY, HSQC and HMBC). In particular the anomeric protons at δ 4.450 (**F**) and δ 4.440 (**G**), appearing as doublets with a $^3J_{H,H}$ of 7.0 Hz and both correlated to the carbon signal at δ 104.0, were assigned to the two terminal β-xylopyranose residues, whereas all of the other anomeric signals, appearing as broad singlets at δ 5.316 (**A**), 5.216 (**B**), 5.153 (**C**), 4.990 (**D**) and 4.835 (**E**), were attributed to the α-rhamnopyranose units on the basis of the chemical shift values of their H-5 and C-5 signals occurring at lower (δ 4.033–3.642) and higher field (δ 69.7–69.0) respectively, than those of β-Rha*p* (δ 3.390 and δ 72.3) [3,4]. The anomeric configurations were confirmed by the $^1J_{C,H}$ values of 161 and 171 Hz for the anomeric carbon signals of xylopyranose and rhamnopyranose residues, respectively, measured by a 2D NMR coupled heterocorrelated experiment.

The low-field chemical shifts of glycosylated carbon signals (δ 80.5 for **A**, 78.6 for **B**, 79.4, 74.2 and 80.5 for **C**, 79.4 for **D** and 79.5 for **E**) with respect to those of the unsubstituted corresponding monosaccharide residues [5] confirmed the presence of two 3-linked, two 2-linked and one 2,3,4-trisubstituted rhamnopyranose units in addition to two terminal non-reducing xylopyranose residues, in agreement with the methylation data. Sequence residue analysis of the O-chain was obtained from HMBC and NOESY experiments. The results are given in Table 2.

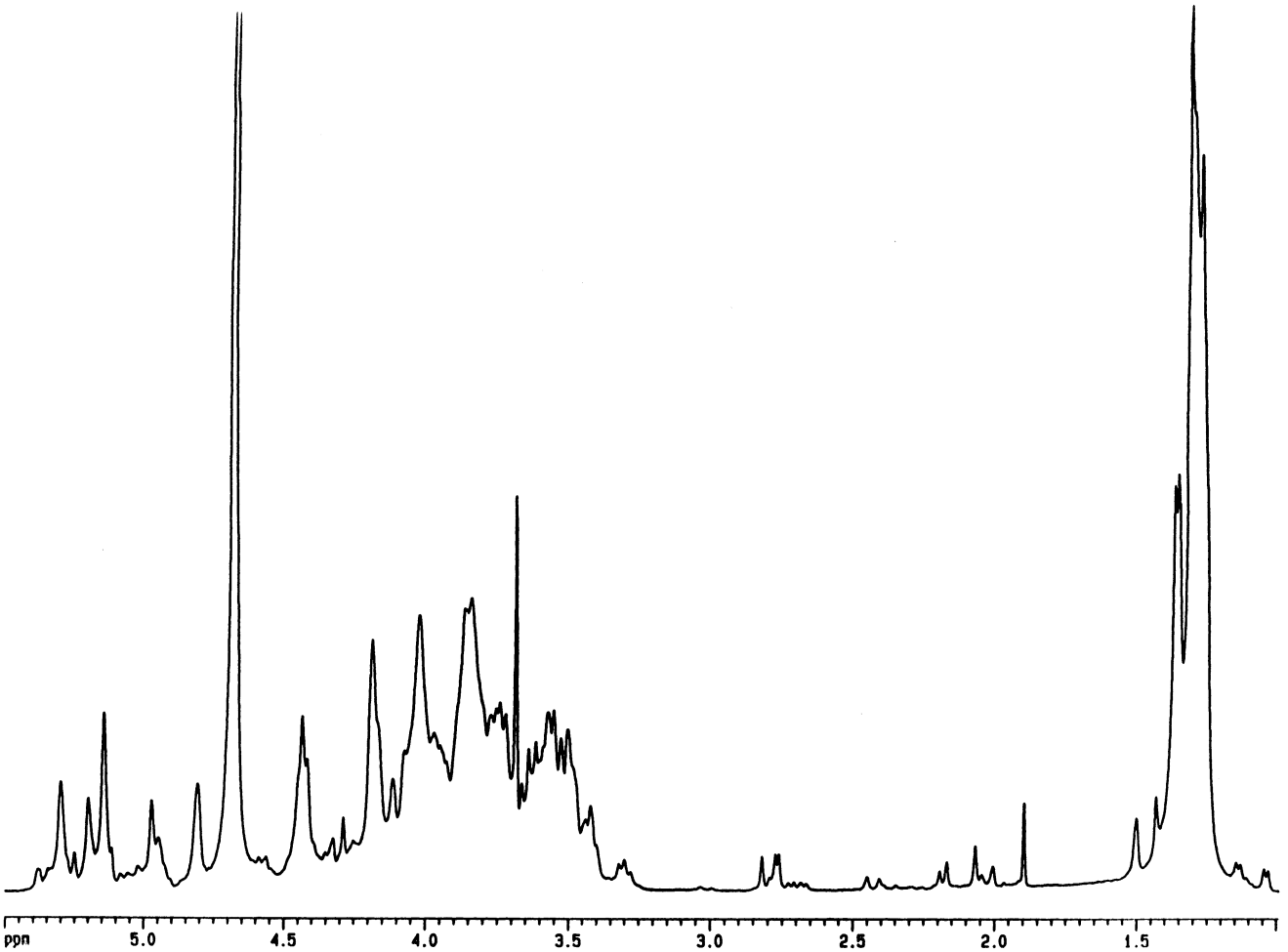


Fig. 1. ^{1}H NMR spectrum of the O-polysaccharide of *Xanthomonas campestris*.

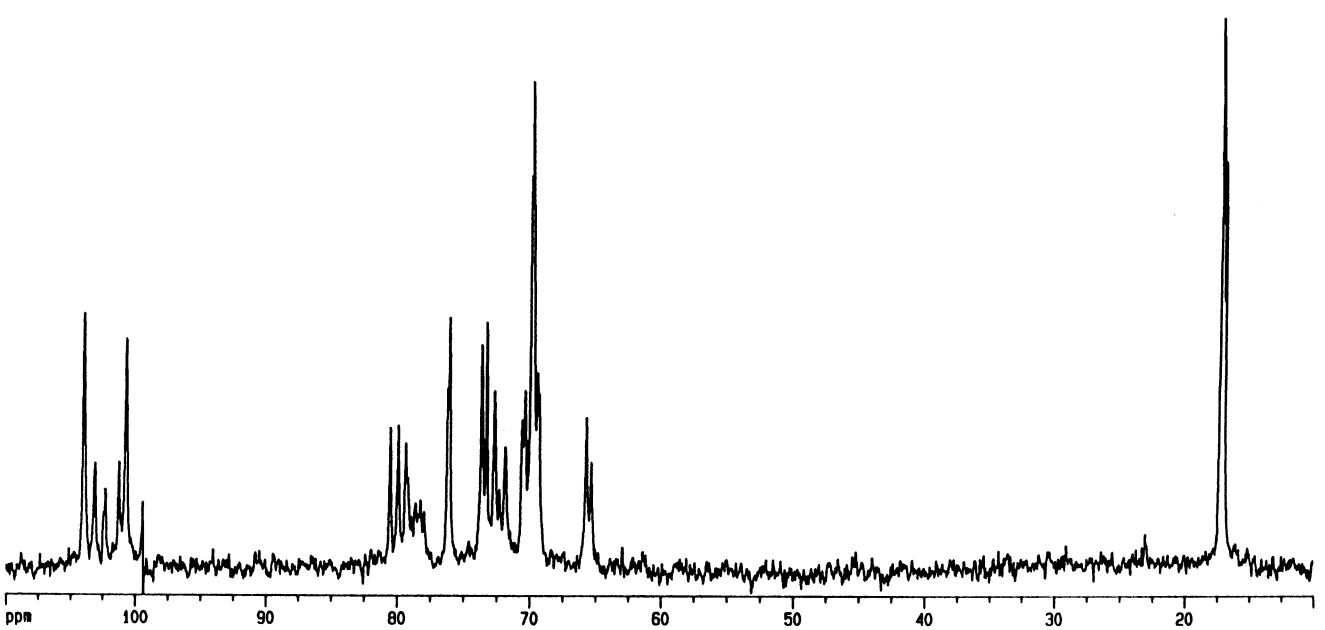


Fig. 2. ^{13}C NMR spectrum of the O-polysaccharide of *Xanthomonas campestris*.

Table 1
^{1}H and ^{13}C NMR data of the polysaccharide [a]

Sugar residue	Chemical shift (ppm)					
	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5,H-5′ C-5	H-6 C-6
2-Rha*p* **A**	5.316 100.4	4.049 80.5	3.849 70.2	3.855 70.0	3.870 69.5	1.305 17.1
2-Rha*p* **B**	5.216 101.2	4.080 78.6	3.967 70.3	3.510 72.6	3.798 69.2	1.305 17.1
2,3,4-Rha*p* **C**	5.153 100.4	4.204 79.4	4.058 74.2	3.740 80.5	4.033 69.0	1.368 16.9
3-Rha*p* **D**	4.990 102.2	4.219 70.6	3.895 79.4	3.870 70.0	3.957 69.7	1.305 17.1
3-Rha*p* **E**	4.835 103.2	4.150 70.9	3.839 79.5	3.574 72.0	3.642 69.4	1.305 17.1
t-Xyl*p* **F**	4.450 104.0	3.516 74.0	3.434 76.3	3.640 70.1	3.299, 4.007 65.7	
t-Xyl*p* **G**	4.440 104.0	3.312 73.5	3.434 76.3	3.640 70.1	3.299, 4.007 65.6	

[a] The assignments were given on the basis of two-dimensional NMR experiments (COSY, TOCSY, NOESY, HSQC and HMBC).

The inter-glycosidic three-bond proton–carbon correlations also permitted further support of the substitution positions, except for unit **E** (Fig. 3). The correlations of H-1/C-1 of xylose residues **F** and **G** to C/H position 2 and 4, respectively, of the same residue **C** allowed us to identify this one as the nodal unit. Then the correlations from H-1/C-1 in **C** to C-3/H-3 in **D**, from H-1/C-1 in **D** to C-2/H-2 in **B**, and those from H-1/C-1 in **E** to C-2/H-2 in **A** and from H-1/C-1 in **A** to C-3/H-3 in **C** led us to establish the sequences **C**–**D**–**B** and **E**–**A**–**C**, respectively. The linkage between these substructures was indicated by the dipolar coupling correlation between H-1 of **B** at δ 5.216 and H-3 of **E** at δ 3.839 in the NOESY spectrum (Fig. 4). The inter-residue NOEs measured for the anomeric protons of all other residues were in complete accordance with the unit sequence indicated from the HMBC data.

Confirmatory evidence of the sequence arose from Smith degradation results. The periodate oxidation of the O-chain, followed by mild acid hydrolysis, yielded, after chromatography on Bio-Gel P-2, two main fractions. The ^{1}H NMR spectrum of the less-retained fraction showed at high field two doublets at δ 1.301 and 1.292, whereas in the anomeric region three signals occurred, two as singlets at δ 4.970 and δ 5.053 and the third one as a doublet at δ 5.133. The coupling constant value of 4.7 Hz of this last ruled out its assignment to a rhamnose unit and suggested its attribution to the proton of a hydroformyl group of the glyceraldehyde aglycone arisen from the degradation of a 2-substituted Rha*p*. Accordingly, the ^{13}C NMR spectrum

Table 2
Inter-residual connectivities from HMBC and NOESY experiments

Sugar residue	Anomeric atom	HMBC to atom	NOE to proton
	δ_H δ_C	δ_C δ_H	δ_H
2-Rha*p* **A**	5.316 100.4	C-3 **C** 74.2 H-3 **C** 4.058	H-3 **C** 4.058
2-Rha*p* **B**	5.216 101.2		H-3 **E** 3.839
2,3,4-Rha*p* **C**	5.153 100.7	C-3 **D** 79.4 H-3 **D** 3.895	H-3 **D** 3.895
3-Rha*p* **D**	4.990 102.2	C-2 **B** 78.6 H-2 **B** 4.080	H-2 **B** 4.080
3-Rha*p* **E**	4.835 103.2	C-2 **A** 80.5 H-2 **A** 4.049	H-2 **A** 4.049
t-Xyl*p* **F**	4.450 104.0	C-2 **C** 79.4 H-2 **C** 4.204	H-2 **C** 4.204
t-Xyl*p* **G**	4.440 104.0	C-4 **C** 80.5 H-4 **C** 3.740	H-4 **C** 3.740

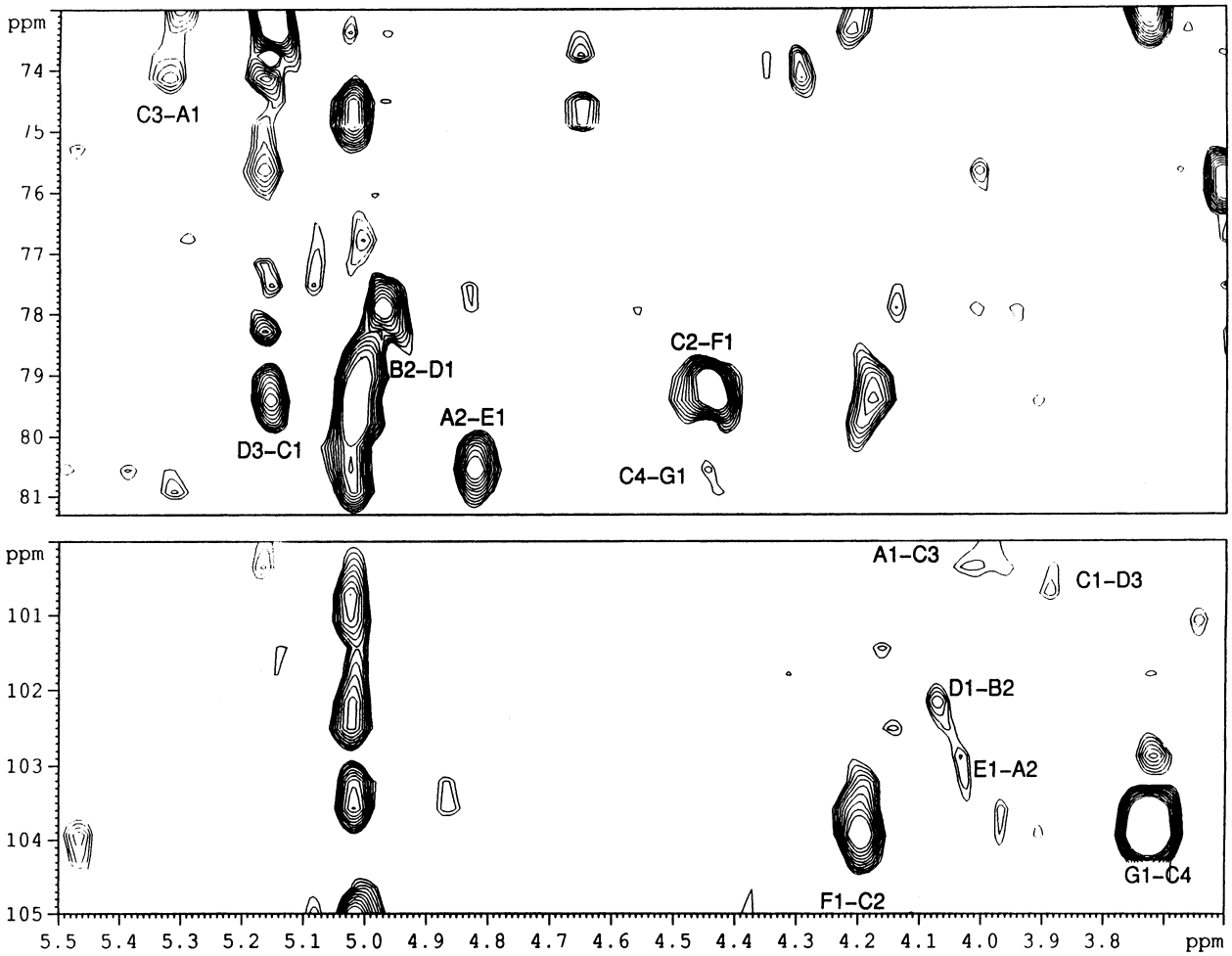
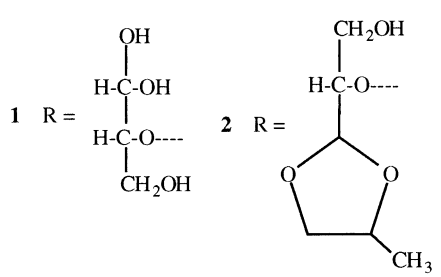


Fig. 3. Part of the $^{1}H,^{13}C$ HMBC spectrum of the O-polysaccharide. The corresponding parts of the ^{1}H and ^{13}C NMR spectra are displayed along the horizontal and vertical axes, respectively; the most important inter-glycosidic three-bond carbon–proton correlations in the carbinolic and anomeric region are shown.

showed a hydroxymethyl signal at δ 60.0 and the hydrate methine signal at δ 89.7. Further diagnostic signals were the two anomeric carbons occurring at δ 103.1 and 100.1, the low-field shifted signals at δ 80.6 and 79.1 of two glycosylate carbons and the methyl signal at δ 17.2, integrate for two methyls, all in agreement with disaccharide structure **1**.

α-L-Rha*p*-(1→3)-α-L-Rha*p*-R



This structure was substantiated by $NaBH_4$ reduction, which gave a product whose ^{1}H NMR spectrum showed only two anomeric singlets at δ 5.060 and 4.973 and the ^{13}C NMR spectrum missed the signal at δ 89.7, whereas it showed an additional hydroxymethyl signal at δ 61.9. All of these spectroscopic variations indicated the reduction of the hydrate aldehyde function of aglycone to the hydroxymethyl group. The methylation analysis indicated the presence of 3-rhamnose and the EIMS data of the permethylated product were in full agreement with this hypothesis, showing, inter alia, peaks at m/z 363 due to the disaccharide glycosydic cation and m/z 189, due to a loss of rhamnose unit from m/z 363 and in addition a peak at m/z 103 corresponding to the permethylated glycerol aglycone.

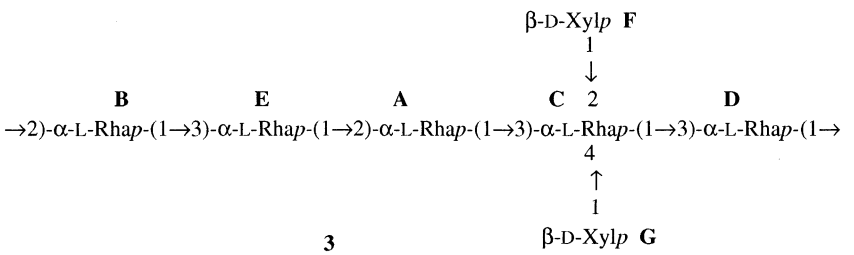
The ^{1}H NMR and GLC–MS of the second fraction of the Smith degradation more re-

tained on Bio-Gel P-2 chromatography indicated that it mainly consisted of a rhamnoside whose aglycone was still the hydrate glyceraldehyde.

A minor fraction containing the same two rhamnosyl moieties with a different aglycone was also obtained from Smith degradation. Because of its small amount, it was investigated as a mixture, only by GLC–MS both as methylated and acetylated derivatives. In both spectra, together with the signals due to the monosaccharidic and disaccharidic fragments, the signals of a methylated or acetylated aglycone at m/z 145 and 173 were present, which suggested the presence of structure **2**, a substituted 1,3-dioxolane, which could derive from a transacetalation of the glyceraldehyde moiety with 1,2-propandiol fragment obtained as a Smith degradation product [6].

Combining both chemical and spectroscopic data, it was possible to suggest the following structure **3** for the O-chain repeating unit of the major LPS component of *X. campestris* strain.

Xanthomonas campestris strain



3

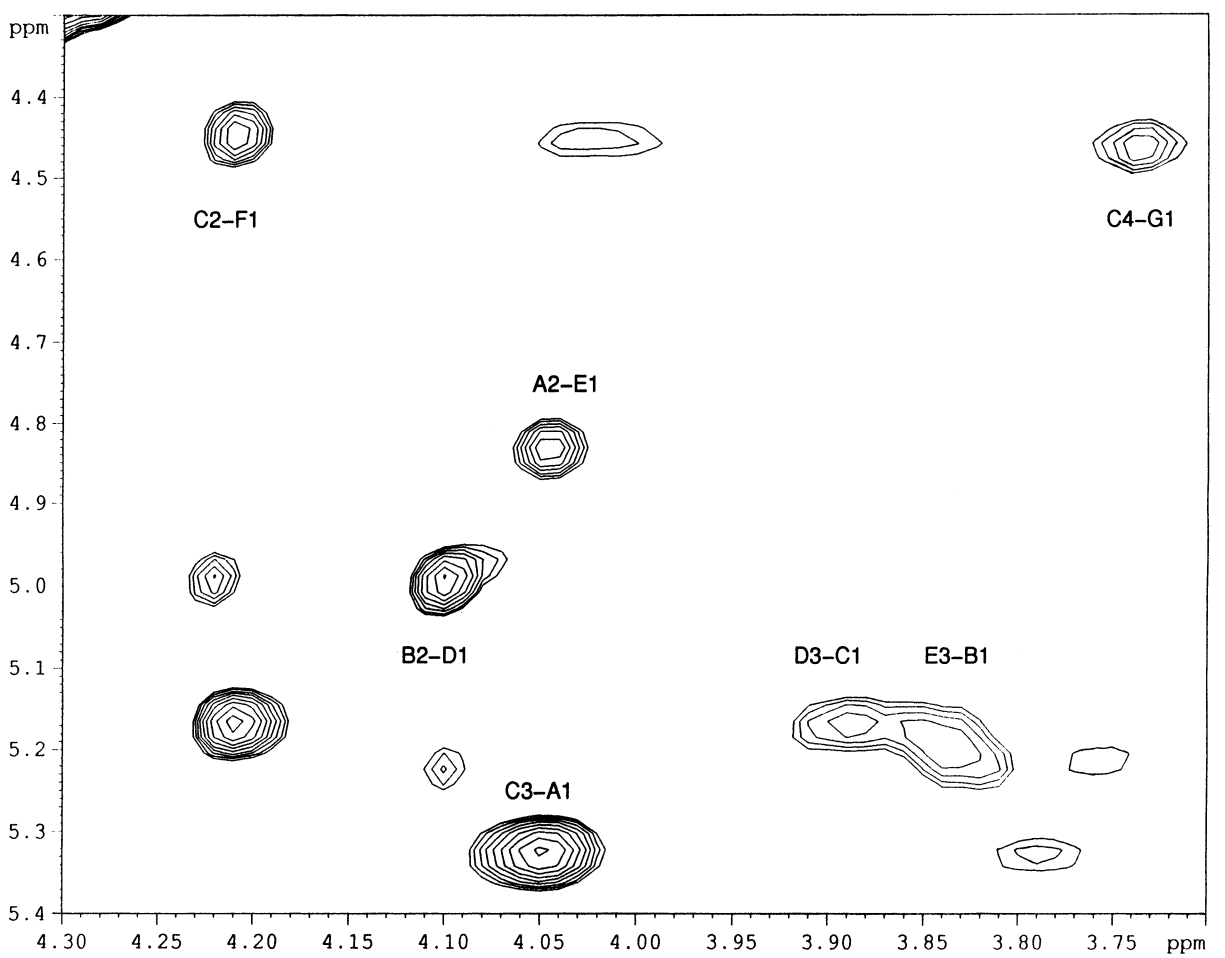


Fig. 4. Part of the NOESY spectrum of the O-polysaccharide. All the inter-residual cross-peaks of the anomeric protons are shown, which contributed to the establishment of the sequence of the sugar residues.

In conclusion, a new O-polysaccharide chain has been isolated and identified from the LPS fraction of the bacterium *X. campestris*, whose repeating unit can be considered peculiar because of the presence of the rare 2,3,4-substituted rhamnose and because of the high number of sugar residues in it. In order to establish a putative correlation between the *X. fragariae* and *X. campestris* strains work is in progress to define the O-chain structure of the *X. fragariae* strain.

1. Experimental

General.—The ^{1}H and ^{13}C NMR spectra were obtained in D_2O at 400 and 100 MHz, respectively, with a Bruker AM 400 spectrometer equipped with a reverse probe, in the FT mode at 30 °C. ^{13}C and ^{1}H chemical shifts are expressed in ppm relative to internal 1,4-dioxane (67.4 ppm) and sodium 3-trimethylsilylpropionate-2,2,3,3-d_4 (TSP), respectively. Two-dimensional spectra (COSY, TOCSY, NOESY, HSQC and HMBC) were measured using standard Bruker software. A mixing time of 200 ms was used in the NOESY experiment. GLC was performed with a Dani instrument equipped with a flame ionisation detector and GLC–MS with a Hewlett–Packard 5890 instrument.

Growth of bacteria, isolation of LPS and OPS.—Strain 642 of *X. campestris*, isolated from strawberry and generously supplied by Dr M. Scortichini, was maintained for long-term storage at −70 °C in 30% glycerol and routinely grown on Wilbrink's medium (WB) slants at 25 °C [7]. Bacterial cells for LPS extraction were obtained by growing the above strain in 500 mL Erlenmeyer flasks filled with 200 mL of liquid WB on a rotary shaker at 150 rpm at 25 °C for 96 h. Cultures were centrifuged (12,000 rpm, 15 min), the pellet washed twice with saline (0.8% NaCl) and then cells were freeze-dried (3.35 g).

The LPS fraction was obtained by phenol–water treatment as described (313 mg) [8], checked by SDS-PAGE electrophoresis [9], and then it was analysed for KDO [10], fatty acids [11] and protein content [12]. Its acid hydrolysis with aq 1% AcOH for 2 h at 100 °C gave, after centrifugation (11,000 rpm at 4 °C, for 1 h), as supernatant the OPS fraction (200 mg). This was purified by gel permeation chromatography on a Bio-Gel P-10 column (90 × 1.5 cm) using 0.05 M ammonium bicarbonate as eluent and monitored with a Waters differential refractometer. The fraction eluted in the void volume was further chromatographed on Bio-Gel P100, Bio-Gel A 0.5 m, Sephacryl S-300 columns in the above conditions.

Sugar and methylation analysis.—The monosaccharides were analysed as acetylated O-methyl glycoside derivatives; samples were methanolysed with 1 M HCl–MeOH at 80 °C 20 h, dried under reduced pressure and then acetylated with Ac_2O in pyridine at 80 °C for 30 min. After work-up, the sample was analysed by GLC–MS, Hewlett–Packard 5890 instrument, SPB-5 capillary column (0.25 mm × 30 m, Supelco), temperature program: 150 °C for 5 min, then 5 °C min^{-1} to 300 °C. Absolute configuration was determined by GLC of acetylated glycosides with (+)-2-octanol according to the published method [13], temperature program: 150 °C for 8 min, then 2 °C min^{-1} to 200 °C for 0 min, then 6 °C min^{-1} to 260 °C for 5 min.

Methylation analysis for the O-polysaccharide sample and the oligosaccharide samples was carried out with methyl iodide in dimethylsulfoxide in the presence of sodium hydroxide [14]. The hydrolysis of the methylated O polysaccharide was performed with 2 M TFA (120 °C, 1 h) and the partially methylated monosaccharides, reduced with $NaBD_4$, were converted into alditol acetates with Ac_2O in pyridine at 80 °C for 30 min and analysed by GLC–MS using the above conditions. The methylated oligosaccharides were analysed by GLC–MS using a temperature program: 230 °C for 10 min, then 5 °C min^{-1} to 280 °C for 10 min. The peracetylated oligosaccharides were obtained with Ac_2O in pyridine at 80 °C for 30 min and analysed by GLC–MS in the same conditions as the methylated oligosaccharides.

Size determination.—OPS (1 mg) was dissolved in 0.5 mL of 50 mM NH_4HCO_3 and chromatographed on a column (30 × 1 cm) of

Sephacryl S 300 HR (Pharmacia) eluted with 50 mM NH_4HCO_3 and monitored using a differential refractometer. The column was previously calibrated using dextran standards (molecular masses: 670, 150, 80, 12, and 5 kDa).

Smith degradation.—The OPS fraction (100 mg) was treated with 0.05 M $NaIO_4$ (30 mL) at 4 °C for 96 h in the dark with stirring. The reaction was quenched with ethylene glycol and the crude mixture was reduced at room temperature overnight by addition of $NaBH_4$. The mixture was neutralised with AcOH, dialysed (cut-off 10 kDa) and lyophilised. The sample was then treated with 1% AcOH (20 mL) at 100 °C for 1.5 h. After evaporation, the crude mixture was purified on a Bio-Gel P-2 (Bio-Rad, 96 × 1.5 cm) column, eluted with water and monitored with a Waters differential refractometer. Two main fractions were obtained, the first one containing a disaccharide glycoside product (15 mg), the second one was composed of a monosaccharide glycoside component (13 mg) and a third minor fraction (5 mg) eluted between these two, which contained a mixture of glycosides with different aglycones.

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