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Note

# O-specific polysaccharide structure of the aqueous lipopolysaccharide fraction from *Xanthomonas campestris* pv. *vitians* strain 1839

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**Abstract**

The structure of *Xanthomonas campestris* pv. *vitians* O-specific polysaccharide of the lipopolysaccharide fraction, extracted from the aqueous phase, was defined, on the basis of chemical and spectroscopical methods, as constituted by the following repeating unit:

$$[\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow]_n 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow$$

where $n$ is more frequently equal to 2, but it also assumes values equal to 1 and to 3. 

*Keywords:* *Xanthomonas*; Lipopolysaccharide; Rhamnan; Phytopathogen; Lettuce

*Xanthomonas campestris* pv. *vitians* (proposed new names: *X. hortorum* pv. *vitians*/*X. axonopodis* pv. *vitians* [1]) is the causal agent of leaf spot and headrot of lettuce. In a previous study two main phytotoxic metabolites, identified as the 3-methylthiopropanoic acid and its dihydroderivative, were isolated from the culture filtrate. In the same work the phytotoxic activity of the two acids was investigated also in relation to their role in the phytopathogenic process [2]. On considering that other bacterial metabolites and constitutive components may be involved in the early events of pathogenicity [3], studies on the isolation and chemical characterization of the lipopolysaccharides produced in vitro from *X. campestris* pv. *vitians* strain NCPPB 1839 were undertaken.

From the aqueous phase of the phenol/water treatment of dried *Xanthomonas campestris* pv. *vitians* cells a glucan rich lipopolysaccha-

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ride fraction was extracted, which, after purification gave only a small amount of LPS. Its mild acetic acid hydrolysis gave the O-specific polysaccharide (O-PS) moiety, which was constituted of only 3-linked L-Rha residues on the basis of methanolysis, methylation and absolute configuration analyses. This datum indicated a linear 3-linked rhamnan, but the appearance of its $^{1}H$ spectrum (Fig. 1(A)) suggested a more complicated structure, showing, in the anomeric region, three major broad singlets at $\delta$ 5.106, 5.033 and 4.832 and two minor ones at $\delta$ 5.090 and 5.050. The $^{13}C$ NMR (Fig. 2(A)) spectrum gave two anomeric signals at $\delta$ 102.9 and 97.4 in about 2:1 ratio. The chemical shift value of this last signal together with that of its correlated anomeric proton at $\delta$ 4.832 suggested the presence of β-rhamnosyl units, besides more abundant α residues.

The small amount available of O-PS prevented a detailed NMR analysis. However, we were able to gain further material from the more abundant phenol phase LPS fraction [4], the O-PS of which consisted of *t*-Fuc3NAc, 3-linked L-Rha*p* and 2,3-linked L-Rha*p*, with both α- and β-rhamnose units. By a Smith degradation of this N-deacetylated polysaccharide, a rhamnan was obtained, which was built of only 3-linked Rha and showed both $^{1}H$ and $^{13}C$ NMR spectra identical to those of the O-PS from aqueous LPS (Figs. 1(B) and 2(B)).

Starting from the three more intense proton anomeric signals the complete assignment of both carbon and proton NMR signals of their residues, indicated by **A**–**C** (Table 1), was accomplished by 2D NMR experiments. The $^{1}J_{CH}$ value of 163 Hz for the signal at 97.4, correlated to proton at $\delta$ 4.832, confirmed the presence of β-rhamnosyl units. The low-field chemical shifts of glycosylated carbon signals ($\delta$ 77.9 for **A**, 79.2 for **B** and 81.2, for **C**) with respect to those of the unsubstituted corresponding monosaccharide residues [5] confirmed the presence of only 3-linked residues, in agreement with methylation data.

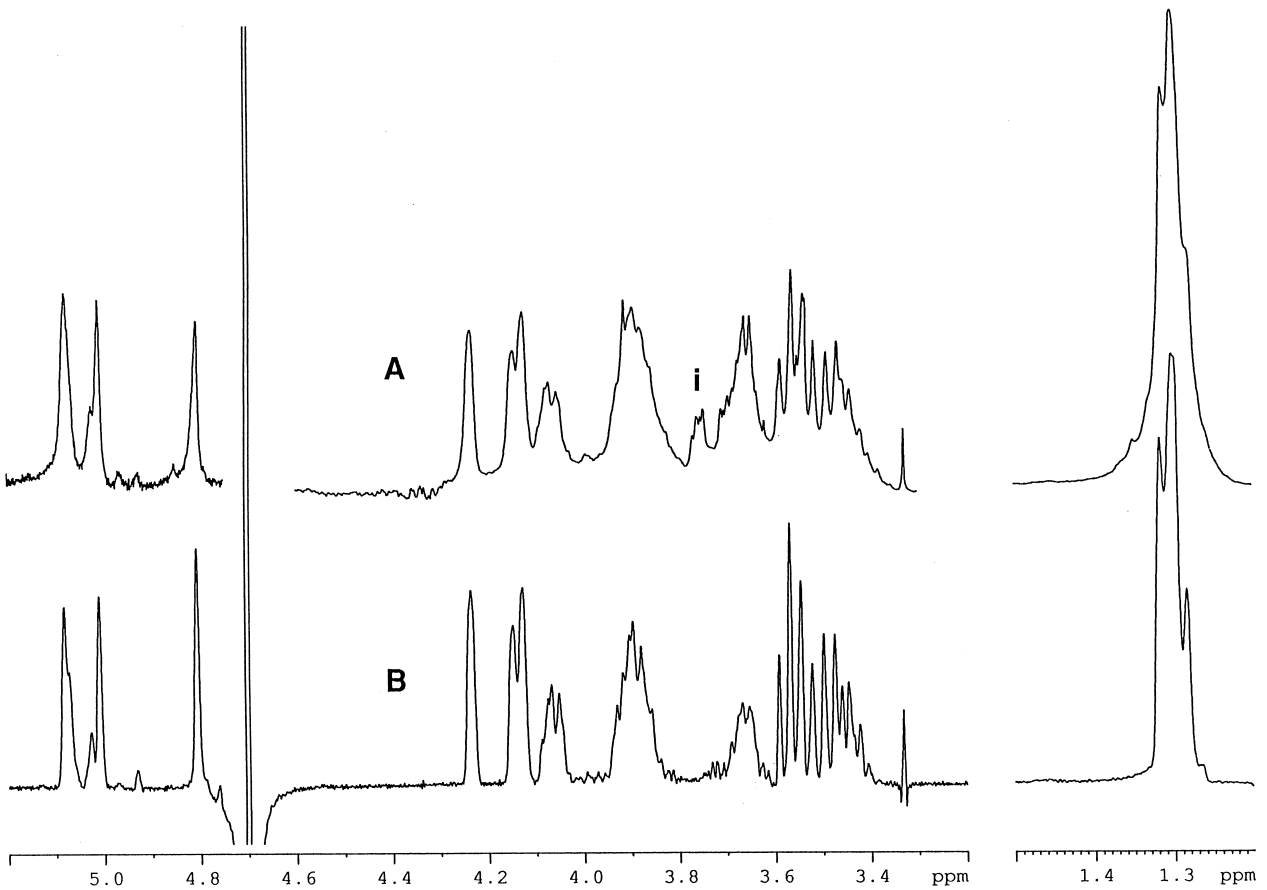


Fig. 1. $^{1}H$ NMR spectra: (A) O-PS of the aqueous LPS, and (B) Smith degraded O-PS of phenol LPS.

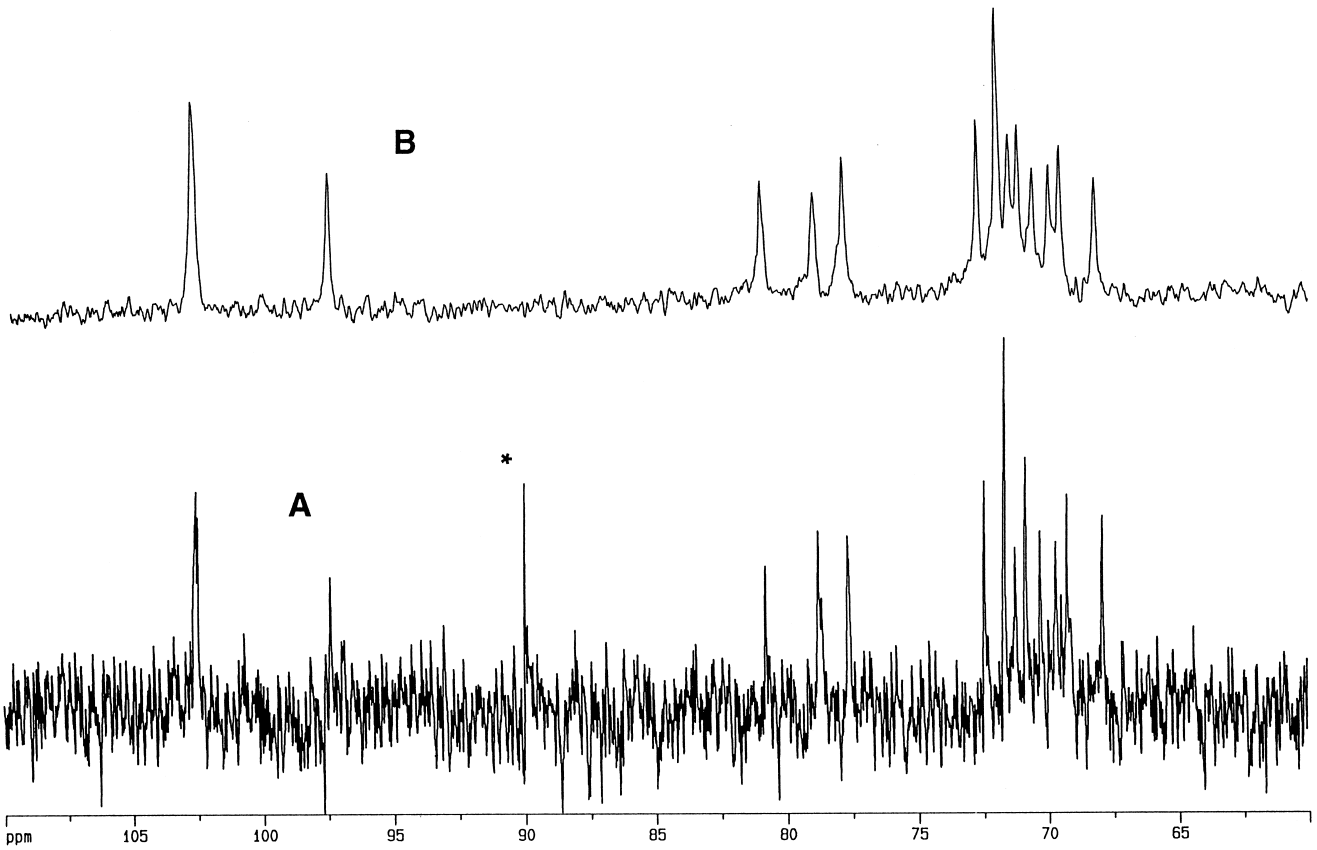


Fig. 2. $^{13}C$ NMR spectra: (A) O-PS of the aqueous LPS, and (B) Smith degraded O-PS of phenol LPS. * The transmitter offset signal.

Table 1
$^{1}H$ and $^{13}C$ NMR data of the most abundant substructure: $\rightarrow 3\mathbf{A}1\rightarrow 3\mathbf{B}1\rightarrow 3\mathbf{C}1\rightarrow$ present in the Smith degraded O-PS

| Sugar residue | Chemical shift ($\delta$) | | | | | |
|---|---|---|---|---|---|---|
| | H-1<br>C-1 | H-2<br>C-2 | H-3<br>C-3 | H-4<br>C-4 | H-5<br>C-5 | H-6<br>C-6 |
| 3-α-Rha*p* **A** | 5.106<br>102.9 | 4.263<br>68.4 | 3.094<br>77.9 | 3.596<br>71.3 | 3.923<br>69.6 | 1.329<br>17.4 |
| 3-α-Rha*p* **B** | 5.033<br>102.9 | 4.167<br>71.0 | 3.950<br>79.2 | 3.580<br>71.4 | 3.961<br>70.0 | 1.329<br>17.4 |
| 3-β-Rha*p* **C** | 4.832<br>97.4 | 4.151<br>72.2 | 3.688<br>81.2 | 3.524<br>72.2 | 3.490<br>72.4 | 1.329<br>17.4 |

The following residue sequence

$$\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}$$
$$\quad\mathbf{A}\qquad\qquad\mathbf{B}$$
$$\beta\text{-L-Rha}p\text{-}(1\rightarrow$$
$$\mathbf{C}$$

was determined by HMBC and NOESY experiments (Table 2). In particular, the anomeric signal of **A** unit at $\delta$ 5.106 showed dipolar coupling with H-3 and a heterocorrelated scalar long-range coupling with C-3 of **B** residue, indicating the linkage of this last position with the anomeric position of **A** residue. In addition, the heterocorrelated long-range coupling of the H-3 at $\delta$ 3.094 of this last unit with C-1 of **C** indicated the linkage $\mathbf{C}1\rightarrow 3\mathbf{A}$. Analogous correlations were found for the other residues (Table 2). In particular, the NOE measured between the anomeric proton of **C** and the H-2 of **A** was justified with the β configuration of this last residue [6].

The above trisaccharide sequence, taking into account the integration of the proton anomeric signals, was representative of the most part of the rhamnan structure, but minor substructures were also present. Information about these latter could be drawn by the minor anomeric signals. The signal at $\delta$ 5.090 gave a NOE with H-3 and a scalar long-range scalar coupling with C-3 of **C** residue, suggesting the presence of an **A′** (Table 2) residue belonging to substructures:

$$\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\underset{\mathbf{A'}}{}\qquad \beta\text{-L-Rha}p\text{-}(1\rightarrow \underset{\mathbf{C}}{}$$

where β units were spaced out with a single α residue.

On the other hand, the signal at $\delta$ 5.050 showed a NOE with H-3 of **B** suggesting the presence of **B′** residue (Table 2), belonging to substructures:

$$\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\underset{\mathbf{B'}}{\alpha\text{-L-Rha}p}\text{-}(1\rightarrow 3)\text{-}$$

$$\underset{\mathbf{B}}{\alpha\text{-L-Rha}p}\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow$$

i.e., β residues were spaced out with three α residues.

In conclusion it is possible to suggest for the structure of the O-specific polysaccharide of the *Xanthomonas campestris* pv. *vitians* LPS, extracted from the aqueous phase, the following repeating unit:

$$[\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow]_n 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow$$

where *n* is more frequently equal to 2, but it also assumes values equal to 1 and to 3.

Table 2
Interresidual connectivities from HMBC and NOESY experiments of the Smith degraded O-PS

| Sugar residue | Proton | $\delta$ | HMBC to atom | NOE to proton |
|---|---|---|---|---|
| 3-α-Rha*p* **A** | H-1 | 5.106 | C-3 **B** | H-3 **B** |
| | H-3 | 3.094 | C-1 **C** | |
| 3-α-Rha*p* **B** | H-1 | 5.033 | C-3 **C** | H-3 **C** |
| | H-3 | 3.950 | C-1 **A** | |
| 3-β-Rha*p* **C** | H-1 | 4.832 | C-3 **A** | H-2 and H-3 **A** |
| | H-3 | 3.688 | C-1 **B** | |
| 3-α-Rha*p* **A′** | H-1 | 5.090 | C-3 **C** | H-3 **C** |
| 3-α-Rha*p* **B′** | H-1 | 5.050 | | H-3 **B** |

## 1. Experimental

*General methods.*—$^1$H and $^{13}$C NMR spectra were obtained in $D_2O$ at 400 and 100 MHz, respectively, with a Bruker DRX 400 spectrometer equipped with a reverse probe, in the FT mode at 30 °C. $^{13}$C and $^1$H chemical shifts are expressed in $\delta$ relative to internal 1,4-dioxane (67.4 ppm) and TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-$d_4$), 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 and GLC–MS separations were obtained with a Hewlett–Packard 5890 instrument fitted with a 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.

*Bacterial strain and lipolysaccharide fractions.*—The *X. campestris* pv. *vitians*. strain NCPPB (National Collection of Plant Pathogenic Bacteria, Harpenden, UK) 1839 was grown in the same conditions previously reported [2]. Dry cells (4.45 g) obtained from 12 L of culture filtrate, were extracted using the phenol/water procedure [7] to give two LPS fractions: one from the phenol phase (370 mg) [4], and the another from the aqueous phase (44.3 mg).

The water phase LPS was contaminated by a large amount of a low molecular weight glucan, so it was further purified by GPC, with Bio-Gel A-15m column (3 × 150 cm) with triethylamine–EDTA buffer (pH 7) as eluent, monitored with a Waters differential refractometer. The amount of pure LPS, eluted in the void volume, was very low (3 mg) while in the phenol phase a more abundant fraction of crude LPS was recovered (370 mg, 8% of cell dry mass) which was submitted to further purification [4]

The product released by mild acid hydrolysis (aq 1% HOAc, 100 °C, 2 h) of the water phase LPS was applied to a GPC, Bio-Gel P-10 column (3 × 90 cm) with ammonium bicarbonate buffer (pH 9) as eluent. The polymeric fraction eluted in the void volume was the *O*-polysaccharide (2.1 mg). The same treatment was applied to an aliquot of the phenol phase LPS to obtain 20 mg of *O*-polysaccharide.

*Sugar analysis.*—Monosaccharides were analyzed by GLC and GLC–MS as acetylated *O*-methyl glycosides derivatives: briefly, samples were methanolyzed with 1 M HCl/MeOH at 80 °C 20 h, dried under reduced pressure and then acetylated with acetic anhydride in pyridine at 80 °C 30 m. The absolute configuration of monosaccharides was determined by the published method [8], using GLC of acetylated (*S*)-2-octyl glycosides, 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 of polysaccharides was carried out by standard procedure [9] and monitored by GLC–MS of the partially methylated alditol acetates [10].

*Smith degradation.*—The phenol phase *O*-polysaccharide (20 mg) was N-deacetylated at 120 °C with KOH 4 M overnight with stirring. After neutralization, dialysis (cut-off 3500 Da) and lyophilization the sample (18 mg) was submitted to Smith degradation: it was treated with 50 mM $NaIO_4$ at 4 °C for 7 days, followed by addition of ethane-1,2-diol, reduction ($NaBH_4$), acidification (2 M HOAc), dialysis and freeze-drying. Then the oxidized polymer was hydrolyzed with 1% HOAc at 100 °C, 1.5 h, and acid was removed by freeze-drying. The product was purified by Bio-Gel P2 ($2 \times 100$ cm), eluted in the void volume with ammonium bicarbonate buffer (pH 9), monitored with a Waters differential refractometer, and dried (15 mg).

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