



## 1. Introduction <sup>1</sup>

*Pseudomonas* “gingeri” is a mushroom (*Agaricus bisporus*) pathogen which causes a disease called ginger blotch characterized by the formation of ginger-coloured lesions on the mushroom caps. The disease was first reported to occur in the UK [1], later in Australia [2], and more recently in Pennsylvania, USA [3].

Eight of the ten strains of *P. “gingeri”* isolated by our group [3] exhibited a mucoid phenotype when grown on Difco *Pseudomonas* agar F at room temperature. Preliminary characterization of the “gingeri” EPS indicated the presence of glucose, mannose and glucuronic acid [4]. In this paper, we report the structure of the exopolysaccharide produced by one mucoid strain, Pf9, as part of our research program on the identification and structural elucidation of bacterial exopolysaccharides which may have various industrial and biotechnological uses.

## 2. Results and discussion

*Isolation and composition of the Pf9 exopolysaccharide.*—From 40 agar plates the strain yielded about 400 mg of crude EPS. The polysaccharide eluted as a single peak between 0.5 and 0.9 M NaCl on a DEAE-Sepharose column run under dissociative conditions. The neutral sugars identified by GLC of their aldononitrile or alditol acetate derivatives were glucose and mannose in a molar ratio of 1:2. The uronic acid was identified as glucuronic acid after carboxyl reduction of the EPS [5] followed by derivatization and GLC analysis. Methanolysis of the EPS followed by trimethylsilylation and subsequent GLC analysis showed the presence of glucuronic acid, glucose and mannose, together with a hexose substituted with a pyruvate group, as revealed by GLC–EI–MS and GLC–CI–MS (NH<sub>4</sub>) analyses. All of the constituent sugars were shown to have the D-configuration by GLC analysis of their trimethylsilylated (–)-2-octyl glycosides [6]. The EPS contained 6.2% (w/w) of acetyl groups and 9.2% (w/w) of pyruvate groups, as determined by colorimetric [7] and enzymatic tests [8], respectively.

The <sup>1</sup>H NMR spectrum of the EPS showed two signals at high field (2.03 and 1.36 ppm), attributed to an acetyl and a pyruvate group, respectively. Integration indicated that these substituents are present in equimolar amounts. The <sup>13</sup>C NMR spectrum showed two signals at 25.4 and 21.0 ppm, which are consistent with the presence of a pyruvate methyl group in the S-configuration [9] and an acetyl group, respectively.

*Methylation analysis and related experiments.*—The native and depyruvated EPS were methylated and the derived alditol acetates were analysed by GLC–MS (Table 1). A sample of the depyruvated, methylated EPS was carboxyl-reduced and subsequently analysed with and without prior remethylation. GLC–MS of the derived alditol acetates gave the results shown in Table 1. These methylation analyses revealed that the EPS is

<sup>1</sup> Reference to brand or firm names does not constitute an endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Table 1

Methylation analysis of *Pseudomonas* “gingeri” EPS

Methylated sugar <sup>a</sup> (as alditol acetate)	$t_R$ <sup>b</sup>	Molar ratio <sup>c</sup>			
		I	II	III	IV
2,4,6-Man	0.92		1.3	0.9	0.3
2,3,6-Glc	1.00	1.0	1.0	1.0	1.0 <sup>e</sup>
2,3-Glc <sup>d</sup>	1.17			0.8	
2-Man	1.22	0.9			

<sup>a</sup> 2,4,6-Man = 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol, etc.<sup>b</sup> Retention time relative to that of 2,3,6-Glc.<sup>c</sup> I, methylated EPS; II, depyruvated methylated EPS; III, depyruvated–methylated then carboxyl-reduced EPS; IV, depyruvated–methylated then carboxyl-reduced and remethylated EPS.<sup>d</sup> C-6 dideuterated according to GLC–MS.<sup>e</sup> 50% of 2,3,6-Glc is C-6 dideuterated, as revealed by the presence of equal amounts of the primary fragments at  $m/z$  233 and 235 and of the secondary fragments at  $m/z$  173 and 175 in the GC mass spectrum.

composed of 4-linked glucuronic acid, 4-linked glucose, and 3-linked mannose. The pyruvic acid is linked to O-4 and O-6 (acetal form) of the mannose residue.

The  $\beta$ -elimination reaction was performed on the native methylated EPS followed by realkylation with CD<sub>3</sub>I. GLC–MS of the alditol acetate derivatives indicated the presence of a new component, namely a terminal glucose substituted with a CD<sub>3</sub> group on O-4, thus revealing that the glucuronic acid is linked to the glucose residue. The greater stability of the glycosiduronic linkage explains the uneven molar ratio of the glucose and mannose residues found in the neutral sugar analysis.

In order to determine the position of acetyl substitution, a sample of depyruvated EPS was methylated under neutral conditions [10] and the derived alditol acetates were analysed by GLC–MS. A new derivative, indicative of a 2,3-linked mannose, was present together with 4-linked glucose and a small amount of 3-linked mannose, indicating that the O-2 of the mannopyranosyl residue bears the acetyl group.

**NMR studies of the deacetylated–depyruvated exopolysaccharide.**—The <sup>1</sup>H NMR spectrum (not shown) showed three anomeric signals in the region 4.80–4.50 ppm, suggesting a trisaccharide repeating unit. The sugars giving rise to these <sup>1</sup>H resonances were named **a** to **c** in order of decreasing chemical shift. The **b** anomeric signal had a very small coupling constant (< 3 Hz), typical of hexopyranosides in the *manno* configuration, while the **a** and **c** signals had <sup>3</sup>*J*<sub>H1,H2</sub> values of 7.4 and 7.6 Hz, respectively, indicating two  $\beta$ -anomers.

The <sup>13</sup>C NMR spectrum contained C-1 signals at 103.78, 101.36, and 101.15 ppm and a total of 18 signals, thus confirming the presence of a trisaccharide repeating unit. The <sup>1</sup>*J*<sub>C1,H1</sub> values obtained from a coupled <sup>13</sup>C experiment were characteristic of three  $\beta$ -linkages (161.5 < *J*<sub>C1,H1</sub> < 166.0) [11]. Furthermore, the carboxyl resonance at 176.50 ppm confirmed the presence of a uronic acid residue.

Complete assignment of the <sup>1</sup>H resonances was obtained from <sup>1</sup>H–<sup>1</sup>H correlation (COSY [12], RELAY COSY [13], TOCSY [14]) experiments, while the corresponding <sup>13</sup>C chemical shifts were deduced from <sup>1</sup>H–<sup>13</sup>C correlation (HETCOR [15] and HMBC [16,17]) experiments (Fig. 1). The chemical shifts for each spin system are reported in

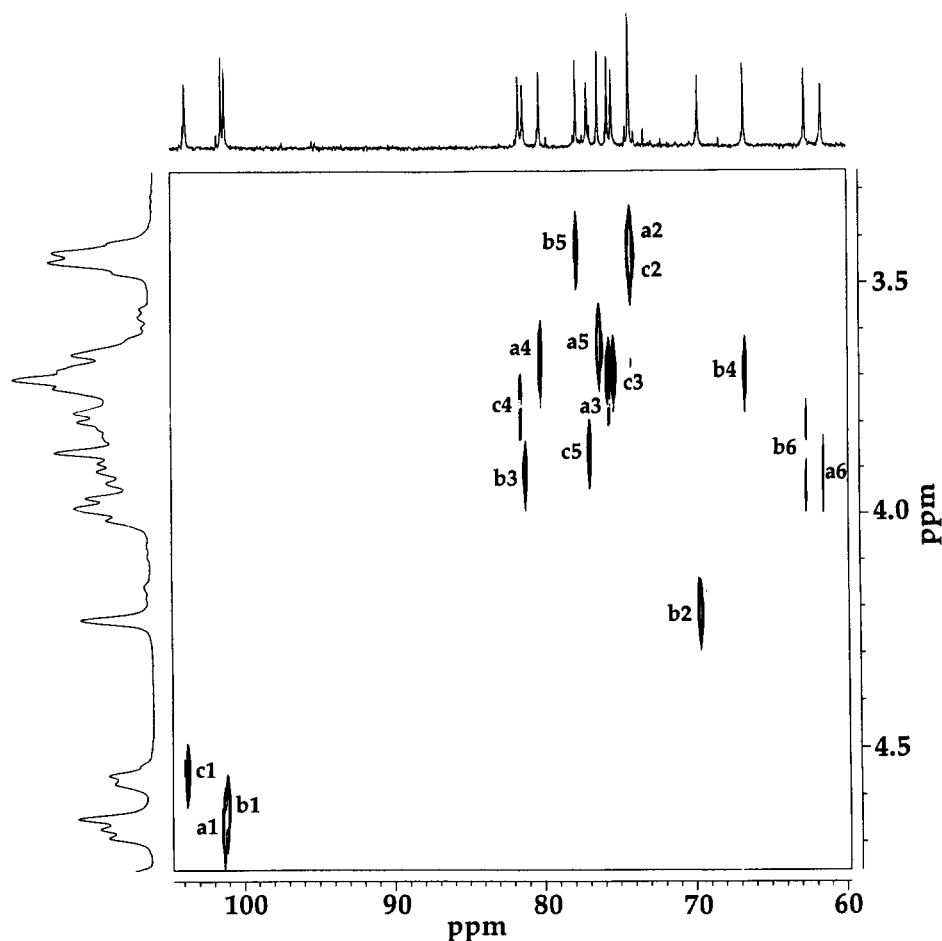


Fig. 1.  $^1\text{H}$ – $^{13}\text{C}$  correlation map of the deacetylated–depyruvated EPS. **a1** indicates the cross-peak between H-1 and C-1 of residue **a**, etc.

Table 2. On the basis of its small  $^3J_{\text{H1,H2}}$ , spin system **b** was assigned to the mannopyranosyl residue; it followed that **a** is the glucopyranosyl residue and **c** the glucopyranosiduronic acid residue. The assignments for the three spin systems are in good agreement with literature data [18–20].

*NMR studies of the depyruvated exopolysaccharide.*—The anomeric region of the  $^1\text{H}$  NMR spectrum contained two new resonances at 5.57 and 4.85 ppm together with a group of signals in the interval 4.68–4.39 ppm which were not resolved. A high-field singlet at 2.21 ppm indicated the presence of an *O*-acetyl group in the approximate amount of one every two repeating units, thus suggesting that about 50% of the substituent was lost in the acidic removal of the pyruvate groups.

The  $^{13}\text{C}$  NMR spectrum was complicated, with five signals in the region 103.89–99.91 ppm; comparison with the  $^{13}\text{C}$  NMR spectrum of the deacetylated–depyruvated EPS

Table 2  
 $^1\text{H}$  and  $^{13}\text{C}$  data for the deacetylated–depyruvated EPS at 27°C

Residue		$^1\text{H}$ or $^{13}\text{C}$ chemical shifts (ppm) <sup>a</sup>						
		1	2	3	4	5	6	6'
<b>a</b>	$^1\text{H}$	4.68	3.43	3.71	3.67	3.63	3.84	4.01
4)- $\beta$ -Glc	$^{13}\text{C}$	101.36	74.24	75.67 <sup>b</sup>	80.19	76.3	61.46	
<b>b</b>	$^1\text{H}$	4.64	4.22	3.92	3.71	3.46	3.96	3.78
3)- $\beta$ -Man	$^{13}\text{C}$	101.15	69.64	81.28	66.60	77.77	62.57	
<b>c</b>	$^1\text{H}$	4.56	3.46	3.70	3.78	3.88		
4)- $\beta$ -GlcA	$^{13}\text{C}$	103.78	74.24	75.40 <sup>b</sup>	81.56	77.02	176.5	

<sup>a</sup> Relative to internal acetone at 2.23 ppm and 31.07 ppm for  $^1\text{H}$  and  $^{13}\text{C}$ , respectively; 1–6' are atom numbers.

<sup>b</sup> Assignments may be reversed.

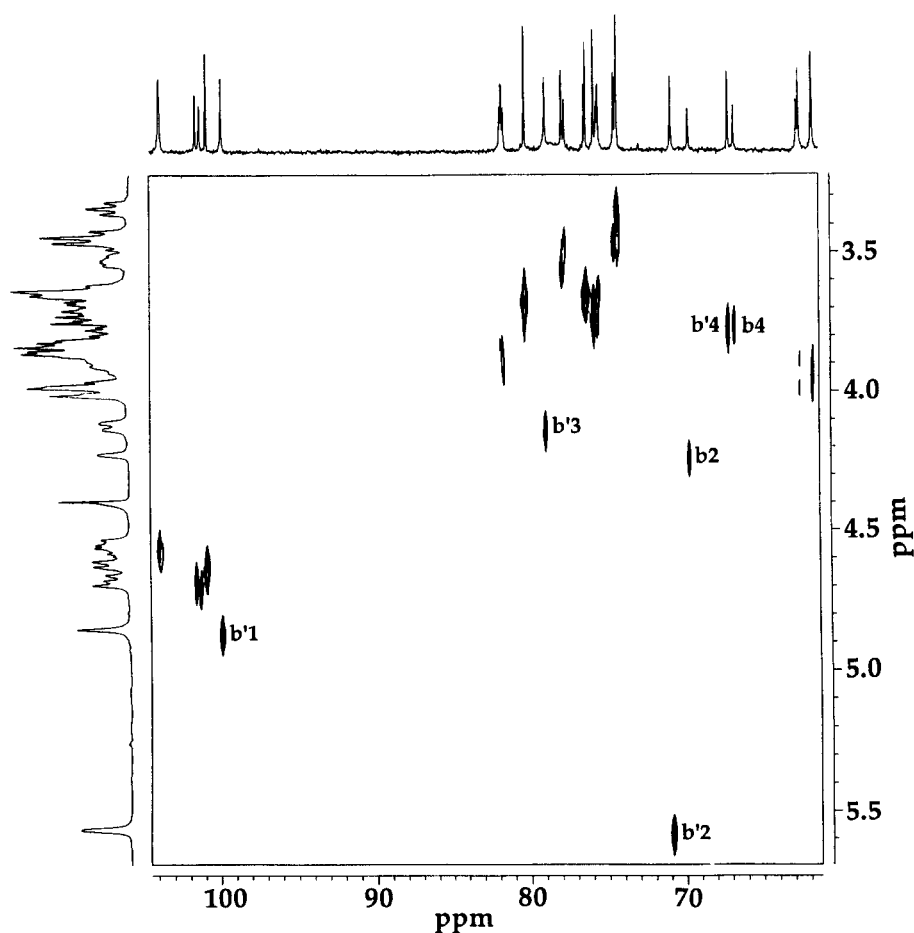


Fig. 2.  $^1\text{H}$ – $^{13}\text{C}$  correlation map of the depyruvated EPS. Some of the C–H correlations are shown: **b** and **b'** indicate the non-acetylated and the acetylated mannose residues, respectively.

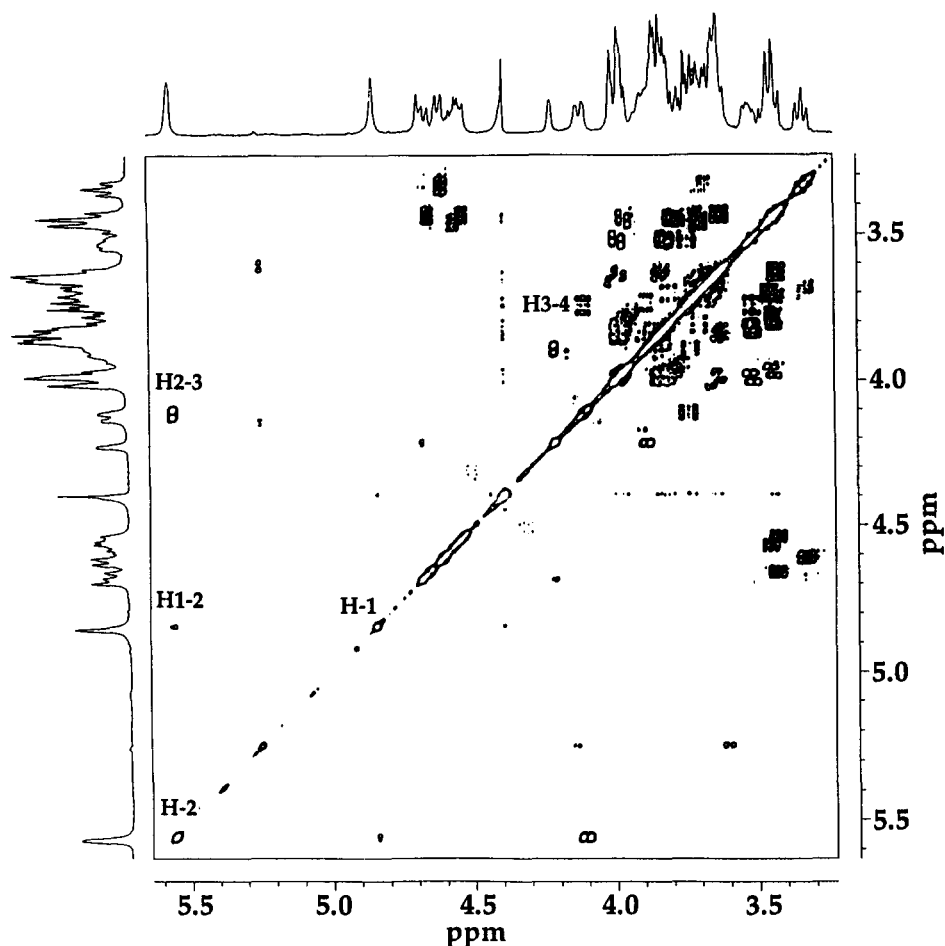


Fig. 3. COSY contour plot of the region 5.65–3.20 ppm for the depyruvated EPS. Some of the  $^1\text{H}$  resonances for the acetylated mannose residue are shown.

revealed the presence of new resonances also in the ring region. Inspection of the HETCOR diagram (Fig. 2) showed that the signals at 5.57 and 4.85 ppm were connected to carbons not present in the  $^{13}\text{C}$  NMR spectrum of the deacetylated–depyruvated EPS and resonating at 70.89 and 99.91 ppm, respectively. The signal at 5.57 ppm was assigned to the ring proton geminal to the attached *O*-acetyl group [21]. This signal shifted into the anomeric region as a result of the *O*-acetylation chemical shift effect. Because of its very small  $^3J_{\text{H}_1, \text{H}_2}$  the new anomeric signal at 4.85 ppm was ascribed to H-1 of a mannose residue. In the COSY plot (Fig. 3) the anomeric signal at 4.85 ppm connected in sequence to the resonances at 5.57 ppm (H-2), 4.12 ppm (H-3) and 3.74 ppm (H-4), thus showing that the mannose residue is the one substituted with an acetyl group on O-2. The chemical shifts of the  $^{13}\text{C}$  resonances for the acetylated mannose residue were obtained from inspection of the HETCOR plot (C-1 to C-4) and of the  $^{13}\text{C}$



(Hewlett–Packard) using the same temperature programme as for the alditol acetate derivatives. GLC–MS was performed on a Hewlett–Packard model 5995B instrument using the same columns and temperature programme.

*Pseudomonas* “gingeri” strain Pf9 was grown on Difco *Pseudomonas* agar F (PAF) contained in culture dishes (100 × 15 mm) for 2–3 days at room temperature and the polysaccharide was isolated as described previously [4].

The EPS was methanolysed with 2 M HCl in MeOH for 24 h at 75°C. Hydrolyses were carried out with 2 M CF<sub>3</sub>CO<sub>2</sub>H at 120°C for 1 h. Alditol acetates were prepared as described previously [25]. Aldonitrile acetates were prepared according to Varma et al. [26].

Anion-exchange chromatography of the EPS was performed on a DEAE-Sepharose column with 0.05 M Tris–HCl buffer (pH 6.5) containing 7 M urea [27]. A salt gradient (0–2 M NaCl) in the same buffer was used as the eluent.

*Methylation analysis and related experiments.*—Methylations were performed according to the modified Hakomori [28] method using potassium methylsulfinylmethanide [29]. The methyl-esterified carboxyl groups were reduced with LiAlD<sub>4</sub> [30]. β-Elimination reaction of the methylated polysaccharide was carried out using a modification [31] of the method of Lindberg et al. [32]. The partially methylated alditol acetates obtained from these reactions were analysed by GLC–MS.

*Removal of the non-carbohydrate substituents.*—The pyruvic acid substituent was hydrolysed [33] with 0.5 M oxalic acid at 100°C for 90 min. The *O*-acetyl groups were removed [34] by treatment with 0.01 M NaOH at room temperature for 5 h.

*NMR spectroscopy.*—The sample was dissolved in 99.96% deuterium oxide containing a trace of acetone as internal reference (2.23 ppm for <sup>1</sup>H and 31.07 ppm for <sup>13</sup>C). Spectra were recorded at 300 K for the deacetylated–depyruvated EPS and at 330 K for the depyruvated EPS on a Bruker ARX-400 spectrometer, equipped with a 5 mm <sup>13</sup>C/<sup>1</sup>H dual probe. Standard Bruker pulse programs (UXNMR 930601.5) were used.

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