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Carbohydrate Research 315 (1999) 159–168

CARBOHYDRATE RESEARCH

Structural determination of the acidic exopolysaccharide produced by a *Pseudomonas* sp. strain 1.15

Paola Cescutti [a,b,*], Renato Toffanin [b], Piero Pollesello [c], Ian W. Sutherland [d]

[a] *Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, via L. Giorgieri 1, I-34127 Trieste, Italy*

[b] *Centro Ricerche POLY-biòs, Area di Ricerca, Padriciano 99, I-34012 Trieste, Italy*

[c] *Cardiovascular Drug Discovery, Department of Pharmacology, Orion Pharma, Orion Corporation, PO Box 65, FIN-02101 Espoo, Finland*

[d] *Institute of Cell and Molecular Biology, Edinburgh University, Edinburgh EH9 3JH, UK*

Received 12 October 1998; revised 25 November 1998; accepted 27 November 1998

Abstract

Pseudomonas strain 1.15 was isolated from a freshwater biofilm and shown to produce considerable amounts of an acidic polysaccharide which was investigated by methylation analysis, NMR spectroscopy and ionspray mass spectrometry (ISMS). The polysaccharide was depolymerised by a bacteriophage-associated endoglucosidase and by autohydrolysis, and the resulting oligosaccharides were investigated by NMR spectroscopy and mass spectrometry. The resulting data showed that the parent repeating unit of the 1.15 exopolysaccharide (EPS) is a branched hexasaccharide. The main chain is constituted of the trisaccharide →4)-α-L-Fuc*p*-(1→4)-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→ and the side chain α-D-Gal*p*-(1→4)-β-D-GlcA*p*-(1→3)-α-D-Gal*p*-(1→ is linked to O-3 of the first Fuc residue. The terminal non-reducing Gal carries a 1-carboxyethylidene acetal in the R configuration at the positions 4 and 6. Of the four different *O*-acetyl groups present in non-stoichiometric amounts, two were established to be on O-2 of the 3-linked Gal and on O-2 of the 4-linked Fuc.

Keywords: *Pseudomonas*; Exopolysaccharide; Structure; NMR; Mass spectrometry

1. Introduction

The existence of micro-organisms in biofilms is widespread in both natural and man-made environments. The biofilms are composed of microbial cells and cell products together with extensive amounts of exopolysaccharides (EPSs) which form a matrix binding the whole complex to solid surfaces. Although EPSs from biofilm-forming species in clinical situations such as *Pseudomonas aeruginosa* [1] and *Staphylococcus epidermidis* [2] have received considerable attention, few studies report on the structure or composition of bacterial isolates from natural biofilms. One exception was a marine *Hyphomonas* sp. in which the EPS was shown to play a major role in adhesion to solid surfaces [3]. We now report on the characterisation of the EPS structure of a pseudomonad from a temperate freshwater habitat. Bacteriophage lysing this strain proved to be of widespread occurrence

Abbreviations: EPS, exopolysaccharide; O-Ac, O-acetyl group; pyr, pyruvate substituent.

* Corresponding author. Tel.: +39-040-6763685; fax: +39-040-6763691.

E-mail address: cescutti@bbcm.univ.trieste.it (P. Cescutti)

0008-6215/99/$ - see front matter

PII: S0008-6215(98)00318-8

in raw sewage from southern Scotland and Northern Ireland [4]. The polysaccharide depolymerase induced by one such virus proved to be a useful adjunct to our study.

2. Results and discussion

Isolation and composition of the 1.15 exopolysaccharide.—Strain 1.15 was isolated from a glass surface placed in a local stream (K.M. Bell, unpublished results). The polysaccharide was prepared from shaken flask cultures [4]. An aqueous solution of the polysaccharide was loaded on a DEAE Sephadex A-50 column and a neutral polymer eluted with water, while an acidic EPS eluted with 2 M NaCl solution. The two polysaccharides were hydrolysed, their neutral sugars converted to alditol acetates which were then analysed by GLC. The results indicated that the neutral polymer was composed of mannose and glucose in the molar ratio 1.0:2.0, while the acidic EPS contained fucose, galactose and glucose in the molar ratio 2.0:2.2:1.5. The acidic EPS, being the most abundant, was named 1.15 and was further investigated. The absolute configuration was shown to be L for the fucose residues and D for all the other sugars by GLC analysis of their trimethylsilylated (−)-2-butyl glycosides [5,6]. The EPS contained 4.9–5.8% (w/w) of acetyl groups and 6.2–8.7% (w/w) of pyruvate groups, as determined by the methods of Hestrin [7] and Sloneker [8], respectively.

The ^{1}H NMR spectrum of the native EPS (data not shown) exhibited signals in the high-field region attributed to the methyl protons of acetyl (2.12 ppm) and pyruvate (1.42 ppm) substituents as well as fucose residues (1.23 ppm). Integration data of the methyl signals indicated the presence of two fucose residues and 1.4 acetyl groups for each pyruvate substituent. The ^{13}C NMR spectrum showed signals at 26.04, 21.17 and 16.18 ppm, which are consistent with the proton data.

Methylation analysis and related experiments.—The native and depyruvated EPS (EPS-depyr) were methylated and the derived alditol acetates were analysed by GLC–MS (Table 1). A sample of the methylated EPS and of the methylated EPS-depyr were carboxyl-reduced and subsequently analysed by GLC–MS of the derived alditol acetates (Table 1). The removal of the pyruvyl substituents was not complete, as revealed by the concurrent presence of terminal galactose and 4,6-substituted Gal. The methylation analyses indicated that the 1.15 EPS is constituted of 4-linked Fuc, 3,4-linked Fuc, 3-linked Glc, 3-linked Gal, 4-linked GlcA and terminal Gal substituted with pyruvic acid linked to O-4 and O-6.

Oligosaccharides from autohydrolysis.—The native EPS was subjected to autohydrolysis and the products, fucose, glucose and three

Table 1
Methylation analysis of 1.15 EPS and depyruvated 1.15 EPS

Methylated sugar[a] (as alditol acetate)	rrt[b]	Molar ratios[c]			
		EPS	EPS-R	EPS-depyr	EPS-depyr-R
2,3-Fuc	0.84	1.23	0.73	1.02	0.87
2,3,4,6-Gal	0.85			0.55	0.38
2-Fuc	0.93	1.27	0.94	1.13	0.96
2,4,6-Glc	0.97	1.31	0.93	1.10	0.91
2,4,6-Gal	1.00	1.00	1.00	1.00	1.00
2,3-Glc[d]	1.24		0.60		0.55
2,3-Gal	1.25	0.59	0.51	0.33	0.26

[a] 2,3-Fuc = 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylfucitol, etc.
[b] Retention time relative to that of 2,4,6-Gal.
[c] R indicates methylated then carboxyl-reduced sample.
[d] C-6 dideuterated according to GLC–MS.

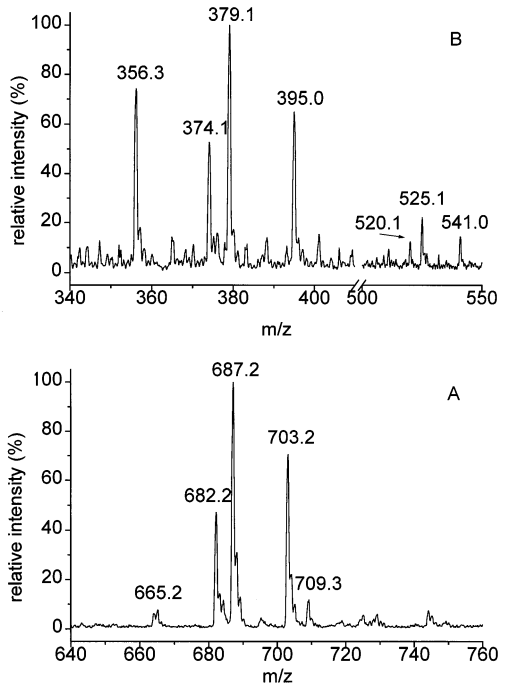


Fig. 1. Positive-mode ionspray mass spectra of FrA (A) and FrB (B).

fractions, were isolated by paper chromatography. The oligosaccharide fractions were named FrA, FrB and FrC in order of increasing Rglc.

Ionspray mass spectrometry (ISMS) analysis indicated that FrA contained mainly only one oligosaccharide. In fact, the ions detected in the positive mode ionspray mass spectrum (Fig. 1(A)) at m/z 665.2, 682.2, 687.2 and 703.2 correspond to $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$, where M is the monoisotopic mass of a tetrasaccharide containing two hexoses, one fucose and one uronic acid. FrA was thoroughly investigated by 1D and 2D NMR spectroscopy. The ^1H NMR spectrum (Fig. 2(A)) showed signals for two α- and three β-linked anomeric protons and integration data confirmed that FrA is a tetrasaccharide. The signals at 5.22 and 4.65 ppm were attributed to the α- and β-fucose residue (Fuc^a) at the reducing end, respectively. The anomeric signals at 5.38, 4.65 and 4.58 ppm were assigned to α-galactose (Gal^a), β-glucose and β-glucuronic acid, respectively, after inspection of the HMQC plot (plot not shown). The twinning of the signal at 5.38 ppm was ascribed to the anomerisation of the reducing end, thus suggesting the sequence Gal–Fuc. In the resolution-enhanced ^{13}C spectrum (not shown), the twinning of the anomeric signal belonging to the Glc residue (103.7 ppm) suggested its linkage to the fucose residue. TOCSY experiments of FrA (not shown) afforded the assignment of all the ^1H resonances, and the ^{13}C assignment followed from the HMQC spectrum. The ^1H and ^{13}C chemical shifts for FrA are reported in Table 2. The HMQC of FrA, optimised for long-range couplings (plot not shown), revealed the following interresidue connectivities: GlcA(1→3)Gal^a, Glc(1→4)Fuc^a. The results indicate that FrA has the following structure:

β-D-Glc*p*-(1→4)-L-Fuc
3
↑
1
α-D-Gal*p*
3
↑
1
β-D-GlcA*p*

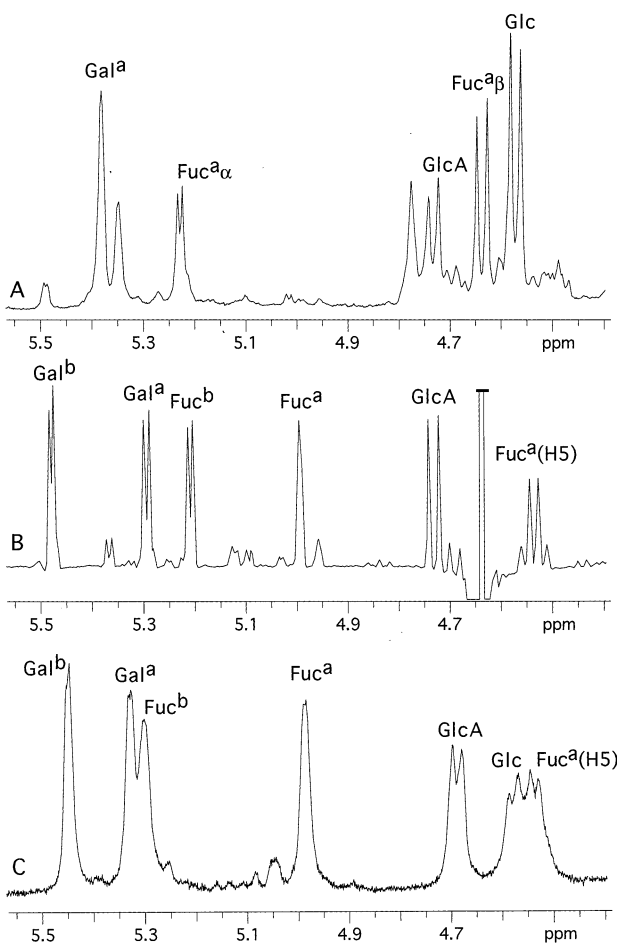


Fig. 2. 400 MHz ^1H NMR spectra of the region 5.55–4.40 ppm for FrA (A), Fr1-ol (B) and deacetylated 1.15 EPS (C).

Table 2
^1H and ^{13}C NMR assignments for the oligosaccharide FrA

Residue		^1H or ^{13}C chemical shifts (ppm)[a]					
		1	2	3	4	5	6
α-Gal[a]	^1H	5.38	3.97	3.97	4.22	4.46	3.84
	^{13}C	100.3	68.5	80.2	70.2	71.8	62.3
α-Fuc[a]	^1H	5.22	4.01	4.06	4.19	4.28	1.30
	^{13}C	93.1	69.2	73.5	79.4	67.9	16.4
β-GlcA	^1H	4.74	3.41	3.52	3.52	3.80	
	^{13}C	104.5	73.9	76.1	72.4	76.2	
β-Fuc[a]	^1H	4.65	3.66	3.86	4.15	3.87	1.35
	^{13}C	96.9	72.9	76.6	78.7	72.0	16.5
β-Glc	^1H	4.58	3.36	3.51	53.56	3.41	3.97–3.71
	^{13}C	103.7	74.5	76.1	70.5	77.2	61.9

[a] Relative to internal acetone at 2.225 ppm and 31.07 ppm for ^1H and ^{13}C, respectively.

The oligosaccharide was reduced with $NaBD_4$ (FrA-ol), methylated and the derived alditol acetates, with and without prior carboxyl-reduction, were analysed by GLC–MS (Table 3). The results were consistent with the NMR findings.

Sugar analysis of FrB indicated that it contained uronic acid and galactose in equimolar amounts, while the ionspray mass spectrum (Fig. 1(B)) indicated the presence of an aldobiuronic acid containing a hexose residue, and an aldotriuronic acid containing a hexose and a deoxyhexose residue, together with some unidentified peaks. The collected data for FrB showed that it is a mixture of at least two oligosaccharides with the following sequence, GlcA–Gal and GlcA–Gal–Fuc.

ISMS of FrC revealed that it is a complicated mixture of oligosaccharides and therefore it was not further investigated.

Oligosaccharide from phage degradation.— Oligosaccharides were also obtained through digestion of the EPS with a phage-induced polysaccharide depolymerase. The oligosaccharide Fr1 was subjected to positive-mode ISMS with the orifice potential set at 50 V, to determine its molecular mass (Fig. 3(A)), and at 180 V to obtain fragmentation (Fig. 3(B)), respectively. The spectrum reported in Fig. 3(A) is rather complicated by the presence of ammonium, sodium and potassium (both as counterions and as ionising species) and of acetyl groups in non-stoichiometric amounts. From this spectrum it can be inferred that oligosaccharide Fr1 corresponds to the hexasaccharide repeating unit of the EPS, in that it contained two fucoses, three hexoses, one uronic acid, one pyruvate substituent, and up to four acetyl groups. The assignment of the ions obtained upon collision-induced dissociation experiment (Fig. 3(B)) is reported in Table 4. The fragmentation pattern indicated that at least two *O*-acetyl substituents are located on the fragment Fuc–Fuc–Hex.

Oligosaccharide Fr1 was then reduced with $NaBH_4$ at 4 °C in order to avoid peeling of the reducing end. ISMS of the reduced oligosaccharide (Fr1-ol) showed a 100% reduction and a partial removal of the *O*-acetyl groups (spectrum not shown). A sample of Fr1-ol was used for methylation analysis and another sample was investigated by 1D and 2D NMR spectroscopy. Reduction of the methyl ester groups was achieved with $LiAlD_4$, and the results of GLC–MS analysis after each step of the derived alditol acetates are reported in Table 3.

The ^1H NMR spectrum of Fr1-ol (Fig. 2(B)) showed resonances attributable to four α- and one β-linked sugar residues. The lower-intensity signals confirmed the presence of residual acetyl groups. Comparison of the ^1H NMR spectra of FrA and Fr1-ol permitted the assignment of the resonance at 4.74 ppm to the H-1 of the glucuronic acid residue (GlcA), while the remaining anomeric resonances were assigned after inspection of the HMQC spectrum optimised for long-range couplings (Fig. 4). Among all the intra-residue connectivities, some were particularly useful for the correct assignment of the anomeric signals. The proton resonance at

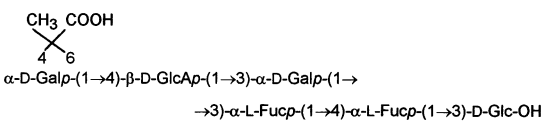
Table 3
Methylation analysis of FrA-ol and Fr1-ol

Methylated sugar[a] (as alditol acetate)	rrt[b]	Molar ratios[c]			
		FrA-ol	FrA-ol-R	Fr1-ol	Fr1-ol-R
1,2,4,5,6-Glc[d]	0.52			0.91	0.78
1,2,5-Fuc[d]	0.57	0.58	0.69		
2,3,4,6-Glc	0.80	1.02	1.02		
2,4-Fuc	0.82			1.23	0.82
2,3-Fuc	0.86			1.16	0.93
2,4,6-Gal	1.00	1.00	1.00	1.00	1.00
2,3,4-Glc[e]	1.03		0.88		
2,3-Glc[e]	1.24				0.61
2,3-Gal	1.25			0.85	0.51

[a] 1,2,4,5,6-Glc = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylglucitol, etc.
[b] Retention time relative to that of 2,4,6-Gal.
[c] R indicates methylated then carboxyl-reduced sample.
[d] C-1 deuterated according to GLC–MS.
[e] C-6 dideuterated according to GLC–MS.

5.48 ppm showed a correlation with a carbon resonance at 63.4 ppm, which is typical of C-5 of the α-Gal bearing a pyruvic acetal substituent on O-4 and O-6 [9]. The proton resonances at 5.21 and 5.00 ppm correlated with carbon resonances at 68.7 and 67.6 ppm, respectively, which are characteristic of C-5 of deoxy hexose residues. Based on these observations, the proton anomeric resonances were assigned as follows: galactose substituted with pyruvate (Gal[b]) at 5.48 ppm; galactose (Gal[a]) at 5.29 ppm; fucose (Fuc[b]) at 5.21 ppm; fucose (Fuc[a]) at 5.00 ppm. The ^{13}C NMR spectrum of Fr1-ol (not shown), obtained by using the maximum entropy method, showed five C-1 signals in the region 105–100 ppm and their assignment followed from inspection of the HMQC plot (not shown) optimised for one-bond couplings. TOCSY spectra of Fr1-ol (not shown) afforded the assignment of most of the proton resonances, except those belonging to the glucitol spin system due to signal overlap. The complete assignment of the Fr1-ol resonances derived from analysis of the HMQC spectrum. The ^{1}H and ^{13}C chemical shifts for Fr1-ol are presented in Table 5. The HMQC experiment optimised for long-range couplings (Fig. 4) confirmed some of the assignments and established the following inter-residue linkages: Gal[a](1→3)Fuc[a], Fuc[a](1→4)-Fuc[b], Fuc[b](1→3)Glc-ol, Gal[b](1→4)GlcA and GlcA(1→3)Gal[a].

All the results indicate the following linear structure for Fr1-ol:



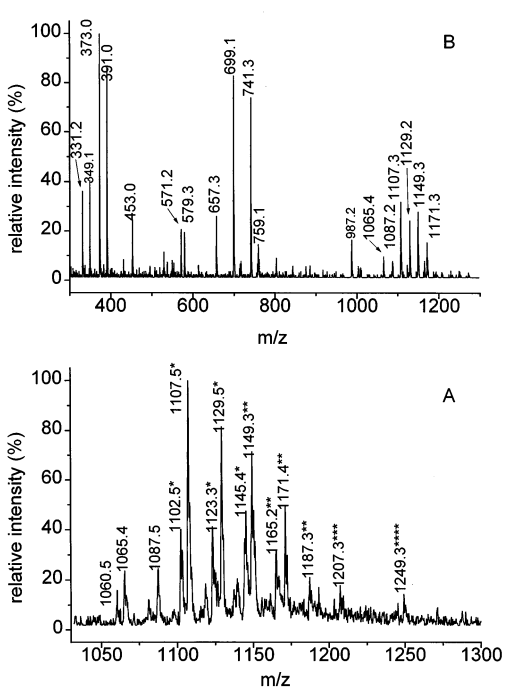


Fig. 3. Positive-mode ionspray mass spectrum of Fr1 (A) and CID mass spectrum of Fr1 (B). In (A), the number of asterisks (*) indicates the number of *O*-acetyl groups.

Table 4
Assignment of the positive ions obtained with CID experiment of Fr1 oligosaccharide

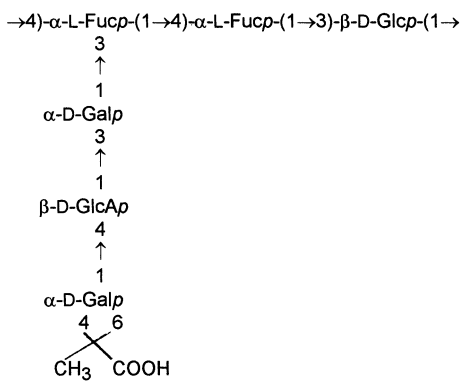
Observed ions (m/z)	Proposed sequence[a]	OAc[b]
331.2	[Fuc–Hex($-H_2O$)+Na]+	0
349.1	[Fuc–Hex+Na]+	0
373.0	[Fuc–Hex($-H_2O$)+Na]+	1
391.0	[Fuc–Hex+Na]+	1
453.0	[pyr(Na)–Hex–GlcA(Na)]+	0
571.2	[pyr–Hex–GlcA–Hex]+	0
579.2	[Fuc–Fuc–Hex+Na]+	2
657.3	[Hex–Fuc–Fuc–Hex+Na]+	0
699.1	[Hex–Fuc–Fuc–Hex+Na]+	1
741.3	[Hex–Fuc–Fuc–Hex+Na]+	2
759.1	[Hex–Fuc–Fuc–Hex+H_2O+Na]+	2
987.2	[pyr–Hex–GlcA–Hex–Fuc–Fuc+Na]+	2
1065.4	[pyr–Hex–GlcA–Hex–Fuc–Fuc–Hex+Na]+	0
1087.2	[pyr–Hex–GlcA(Na)–Hex–Fuc–Fuc–Hex+Na]+	0
1107.3	[pyr–Hex–GlcA–Hex–Fuc–Fuc–Hex+Na]+	1
1129.2	[pyr–Hex–GlcA(Na)–Hex–Fuc–Fuc–Hex+Na]+	1
1149.3	[pyr–Hex–GlcA–Hex–Fuc–Fuc–Hex+Na]+	2
1171.3	[pyr–Hex–GlcA(Na)–Hex–Fuc–Fuc–Hex+Na]+	2

[a] pyr, pyruvate substituent; (Na), sodium as counterion; ($-H_2O$), dehydrated fragment ion.
[b] OAc, number of *O*-acetyl groups.

The COSY spectrum of Fr1 (not shown) revealed correlations not present for Fr1-ol which were ascribed to signals associated with acetylation. The strong deshielding of H-2 of both Gal[a] and Fuc[b] (+0.89 and +1.19 ppm, respectively) indicates that acetylation partially occurred on O-2 of these residues. At the same time, the COSY spectrum revealed the involvement of other residues in the substitution pattern, but the absence of resolved cross-peaks aside from the H-1/H-2 correlations prevented the exact positioning of the remaining acetyl substituents. In addition to this, it is known that the acetyl substitution may cause a perturbation of the signals of both the substituted residue and the one adjacent to it [10,11] and therefore, the perturbation of the H-1 and H-2 signals alone was not sufficient for a precise location of the substitution. On these bases, the COSY spectrum together with the ^{13}C NMR spectrum (not shown) excluded the presence of *O*-acetyl groups on the Gal[b] residue.

NMR studies of the deacetylated exopolysaccharide.—The ^1H NMR spectrum of the deacetylated EPS (Fig. 2(C)) revealed four α- and two β-linked anomeric protons, which were assigned as follows after comparison with the ^1H NMR spectrum of Fr1-ol: Gal[b] at 5.45 ppm; Gal[a] at 5.32 ppm; Fuc[b] at 5.30 ppm; Fuc[a] at 4.98 ppm; GlcA at 4.68 ppm and Glc at 4.58 ppm. The assignment of most of the proton resonances derived from TOCSY experiments (Fig. 5) and the ^{13}C assignment followed from the HSQC spectrum (Fig. 6). The presence of two anomeric ^{13}C correlations for Gal[b] (data not shown) and a larger number of cross-peaks than expected in the HSQC spectrum was explained with the co-existence of pyruvated and depyruvated galactose (depyr-α-Gal) residues. The partial loss of this substituent was probably due only to the high temperature (70 °C) applied during the NMR experiments, and not to a concurrent effect of heat and acidic pH, as found in the literature [12]. The chemical shifts attributed to both the pyruvated and depyruvated galactose residues of the EPS are in good agreement with literature values for the corresponding methyl galactosides [9]. The proton and carbon NMR data for the deacetylated 1.15 EPS are reported in Table 6.

Based on the data presented in this report, the structure of the 1.15 exopolysaccharide is as follows:



The four *O*-acetyl groups are present in non-stoichiometric amounts and none of the residues appears fully substituted. The substituents whose positions were determined

are located on the O-2 of 3-linked galactose and O-2 of 4-linked fucose.

This structure is identical to that of *Erwinia chrysanthemi* Ech6 exopolysaccharide [13], except for the presence of acetyl substituents and for the anomeric configuration of the glucuronic residue. At the same time, 1.15 EPS shares several structural features with the mucous polysaccharide known as M-antigen or colanic acid, which is produced by different Enterobacteriaceae species [14–16]. Although the carbohydrate sequence is the same, the major differences are the anomeric configuration of both the galactose residues and of the branched fucose residue, the interchange of the carbon atoms involved in the linkage of the side chain and main chain at the branch point and the degree and position of acetylation.

3. Experimental

Isolation of the exopolysaccharide.—*Pseudomonas* was grown in 2 L Erlenmeyer flasks of yeast extract medium [17] on a rotary shaker at 200 rpm at 30 °C for 48 h. Bacterial cells were removed by centrifugation at 50,000*g* for 25 min and the EPS was recovered from the supernatant fluid by the addition of two volumes of cold (0 °C) acetone. The polymer was redissolved in water, dialysed extensively against running tap water, recentrifuged and lyophilised. Treatment with DNAse, RNAse and protease was used to remove nucleic acids and proteins. The EPS was subjected to anion-exchange chromatography on a DEAE-Sephadex A-25 column in water. Elution was performed first with water and then with a 2 M NaCl solution. The fractions were tested for the presence of neutral sugars using a colorimetric reaction [18].

General methods.—Analytical GLC was performed with a Hewlett–Packard 5890 gas chromatograph equipped with a flame ionisation detector and an SP2330 capillary column (Supelco, 30 m), using He as the carrier gas. The following temperature programs were used: for alditol acetates, 150–250 °C at 4 °C/min; for methylated alditol acetates, 125–250 °C at 4 °C/min. GLC–MS analyses were carried out on a Hewlett–Packard 5890 gas chromatograph coupled to a Hewlett–Packard 5971 mass selective detector.

Hydrolyses of the EPS and of oligosaccharides were carried out with 2 M CF_3CO_2H at 120 °C for 1 h and at 100 °C for 6 h, respectively. Alditol acetates were prepared as described previously [19]. The absolute configuration of the sugar residues was established via GLC analysis of the derived trimethylsilylated (−)-2-butyl glycosides which were separated on a ULTRA-1 capillary column (50 m × 0.2 mm, 0.33-μm film) using the temperature program 50 °C (2 min) to 135 °C at 20 °C/min, 135 °C (2 min) to 240 °C at 1 °C/min.

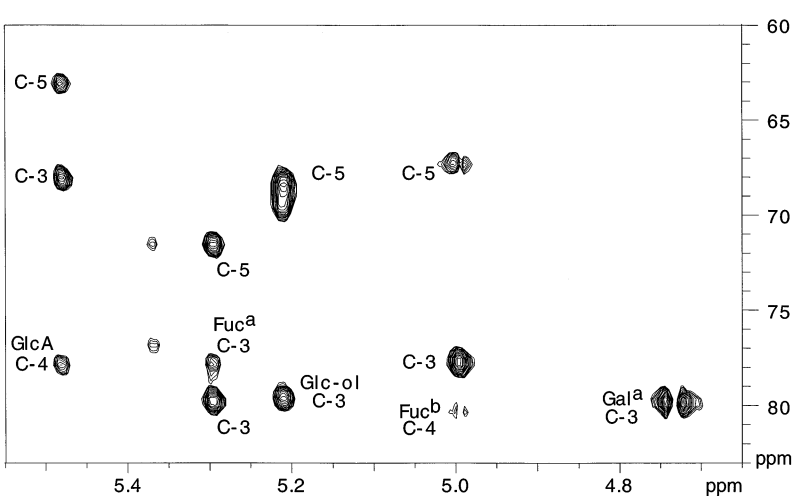


Fig. 4. Expansion of the HMQC spectrum optimised for long-range couplings of Fr1-ol.

Table 5
^1H and ^{13}C NMR assignments for the oligosaccharide Fr1-ol

Residue		^1H or ^{13}C chemical shifts (ppm)[a]					
		1	2	3	4	5	6
α-Gal[b]	^1H	5.48	3.94	3.94	4.22	3.79	3.90–4.03
	^{13}C	100.4	68.4	68.4	69.7	63.4	65.8
α-Gal[a]	^1H	5.29	4.02	4.10	4.21	4.11	n.r.
	^{13}C	101.1	68.8	80.0	72.2	71.9	62.0
α-Fuc[b]	^1H	5.21	4.03	3.95	3.90	4.27	1.32
	^{13}C	101.0	69.2	69.7	80.7	68.7	15.9
α-Fuc[a]	^1H	5.00	4.04	4.03	4.05	4.55	1.18
	^{13}C	101.2	72.5	77.9	72.9	67.6	16.1
β-GlcA	^1H	4.74	3.48	3.80	3.82	3.94	
	^{13}C	104.6	73.8	76.5	78.1	76.2	175.9
Glc-ol	^1H	3.83–3.67	4.01	4.01	3.71	3.71	3.85–3.68
	^{13}C	62.6	72.9	80.2	70.2	71.8	63.7
pyr	^1H	1.48					
	^{13}C	25.8	n.r.	174.8			

[a] Relative to internal acetone at 2.225 ppm and 31.07 ppm for ^1H and ^{13}C, respectively; n.r., not resolved.

The pyruvic acid substituent was hydrolysed [20] with 0.5 M oxalic acid at 100 °C for 90 min. The *O*-acetyl groups were removed [21] by treatment with 0.01 M NaOH at rt for 5 h.

Methylation analysis and related experiments.—Methylations were performed according to the modified Hakomori [22] method using potassium methylsulphinylmethanide [23]. The methyl-esterified carboxyl groups were reduced with $LiAlD_4$ [24]. The partially methylated alditol acetates obtained from these reactions were analysed by GLC–MS.

Autohydrolysis.—An aqueous solution of the native polysaccharide (0.1% w/v) was converted to its acid form by passage through an Amberlite IR 120 (H^+) column and subsequently subjected to autohydrolysis by heating in a sealed tube at 100 °C for 6 h. The diffusable products were recovered after dialysis against 10 volumes of distilled water and reduced to a syrup by rotary evaporation under reduced pressure. The products were separated by preparative paper chromatography in ethyl acetate–acetic acid–formic acid–water, 18:3:1:4, by volume.

Phage degradation.—1.15 EPS (approx 1 g) was dissolved in 100 mL of water containing 0.02% (w/v) sodium azide. Sufficient phage-induced polysaccharide depolymerase [4] was added to ensure 70–80% digestion of the EPS at 30 °C in 24 h. The oligosaccharides were recovered by dialysis against 20 volumes of distilled water at 0 °C and concentrated to a syrup by rotary evaporation at 45 °C. Finally separation on a Biogel P2 column (55 × 1 cm) eluted with distilled water at 6 mL/h gave one product, oligosaccharide Fr1. Further purification was achieved by means of ion exchange chromatography on DEAE Sephadex A-25. Elution was performed with milliQ water followed by a salt gradient (0–2 M NaCl) in which the oligosaccharide Fr1 was recovered. The sample was desalted on a Biogel P2 column using water acidified with formic acid (pH 3.5) as eluent.

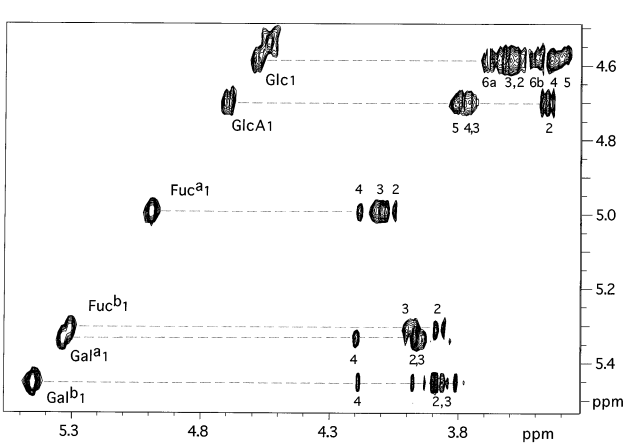


Fig. 5. Expansion of the 400 MHz TOCSY spectrum for deacetylated 1.15 EPS.

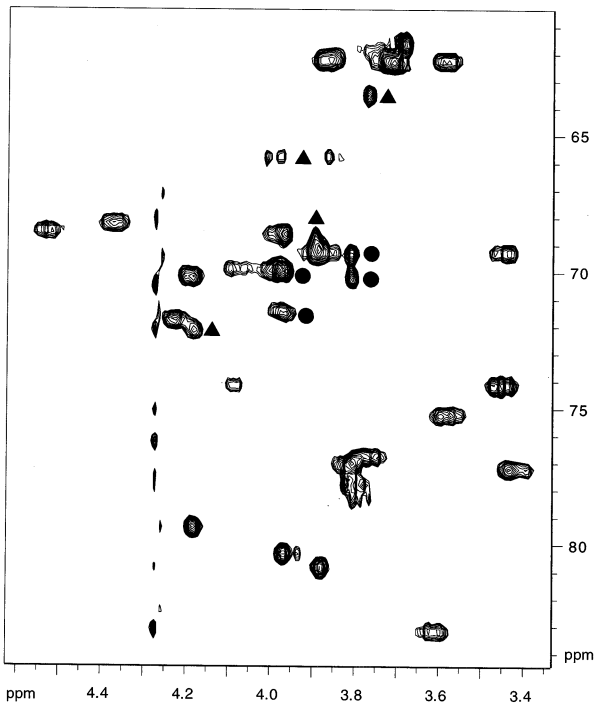


Fig. 6. Part of the HSQC spectrum for deacetylated 1.15 EPS. ▲ and ● indicate some of the cross-peaks of the pyruvated and depyuvated galactose residues, respectively.

Ionspray mass spectrometry.—The mass spectra were recorded on a API-I PE SCIEX quadrupole mass spectrometer equipped with an articulated ion spray and connected to a syringe pump for the injection of the samples. The instrument was calibrated using polypropylene glycol (PPG) mixture (3.3×10^{-5} M PPG 425, 1×10^{-4} M PPG 1000 and 2×10^{-4} M PPG 2000), 0.1% (v/v) acetonitrile and 2 mM ammonium formate in 50% (v/v) aqueous methanol. The oligosaccharides were dissolved in 50% aqueous acetonitrile and ammonium acetate at 0.63×10^{-4} M was used as ionising agent. The sample flow rate was 5 μL/min. The ionspray voltage (ISV) was 5000 V and the orifice potential (OR) was 50 V, and when fragmentation was needed, the OR was set at 180 V. The spectra were recorded using a step size of 0.1 amu.

NMR spectroscopy.—NMR experiments were performed at a probe temperature of 60 °C for the deacetylated EPS and 25 or 40 °C for the derived oligosaccharides. ^{1}H NMR experiments were performed at 4.69 and 9.40 T on Bruker AC 200 and ARX 400 instruments (^{1}H: 200 and 400 MHz), respectively. COSY spectra were recorded at 200 MHz using a spectral width of 1 kHz and a repetition time of 1.0 s. In the F_2 and F_1 dimensions, 1k and 512 data points were used, respectively, with zero-filling in F_1 dimension.

Table 6
^{1}H and ^{13}C NMR assignments for the deacetylated 1.15 EPS

Residue		^{1}H or ^{13}C chemical shifts (ppm)[a]					
		1	2	3	4	5	6
α-Gal[b]	^{1}H	5.45	3.90	3.90	4.18	3.77	3.99–3.84
	^{13}C	100.4	69.3	69.3	72.2	63.5	65.8
α-Gal[a]	^{1}H	5.32	3.98	3.97	4.20	4.23	n.r.
	^{13}C	100.4	68.7	80.4	70.2	71.8	62.4
α-Fuc[b]	^{1}H	5.30	3.87	3.98	3.88	4.37	1.27
	^{13}C	100.2	69.3	70.0	80.9	68.2	16.3
α-Fuc[a]	^{1}H	4.98	4.09	4.09	4.19	4.53	1.29
	^{13}C	101.2	70.0	74.0	79.4	68.5	16.2
β-GlcA	^{1}H	4.68	3.45	3.76	3.80	3.82	
	^{13}C	104.6	74.2	76.9	78.2*	77.2	175.3
β-Glc	^{1}H	4.58	3.57	3.61	3.45	3.42	n.r.
	^{13}C	103.5	75.3	83.1	69.3	77.3	61.6
pyr	^{1}H	1.42					
	^{13}C	26.0	n.r.	174.1			
depyr-α-Gal	^{1}H	5.45	3.82	3.82	4.02	3.97	n.r.
	^{13}C	99.3	69.3	70.3	70.0	71.5	62.4

[a] Relative to internal acetone at 2.225 ppm and 31.07 ppm for ^{1}H and ^{13}C, respectively; n.r., not resolved.
* The chemical shift for the C-4 of GlcA adjacent to depyruvated galactose is 77.6.

TOCSY spectra (τ_m 60–120 ms) were recorded using a spectral width of 4 kHz and a repetition time of 2.0 s. In the F_2 and F_1 dimensions, 2k and 512 data points were used, respectively, with zero-filling in both dimensions. A shifted Qsine window multiplication was applied in each dimension before Fourier transformation. ^{1}H Chemical shifts were referenced to internal acetone (2.225 ppm at all temperatures).

One-dimensional ^{13}C NMR data were recorded at 4.70 T (50.33 MHz) on a Bruker AC 200 spectrometer using a spectral width of 16 kHz and 32k or 16k data points. The resolution enhanced ^{13}C NMR spectra were obtained by using the maximum entropy method implemented in the Gifa program [25]. Two-dimensional ^{1}H-detected heterocorrelated NMR data were acquired at 9.40 T on a Bruker ARX instrument. The heteronuclear multiple quantum coherence experiments, optimised for one-bond ($J_{CH} = 140$ Hz) or long-range couplings ($^3J_{CH} = 7$ Hz), were performed by using a pulse sequence with z-gradients derived from the HMQC scheme described by Hurd and John [26]. The maximum amplitudes of the three sine-bell shaped gradients (G1, G2 and G3), calculated from the gyromagnetic ratios of proton and carbon, were 9, 9 and 4.5 G/cm, respectively. The gradient pulses were of 2 ms and the relaxation delay 1.5 s. The number of transients was 96 per increment and 512 increments were collected. Data were expressed in magnitude mode after Fourier transformation. In the case of the deacetylated EPS, a heteronuclear single-quantum coherence (HSQC) [27] experiment was performed using 512 points in F_2 and 2.0k points in F_1, with spectral widths of 2 and 15 kHz, respectively. A shifted Qsine window multiplication was applied in each dimension, prior to Fourier transformation. ^{13}C Chemical shifts were referenced to internal acetone (31.07 ppm at all temperatures).

Acknowledgements

The authors wish to thank Drs M. Bosco, H. Parolis, and G. Widmalm for their useful discussion. Mass spectra were run at the Central Facility for Mass Spectrometry of the University of Trieste. POLY-tech Scarl is gratefully acknowledged for the use of the Hewlett–Packard 5890 gas chromatograph and of the Hewlett–Packard 5971 mass selective detector.

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