Effect on lipopolysaccharide structure of aeration during growth of a plum isolate of *Pseudomonas syringae* pv. *morsprunorum*

A. R. W. Smith,¹ S. M. Munro,¹ R. Wait² and R. C. Hignett^{1†}

Author for correspondence: A. R. W. Smith. Tel: +44 81 316 8221. Fax: +44 81 316 8305.

¹ School of Biological and Chemical Sciences, University of Greenwich, Wellington Street, London SE18 6PF, UK

² Public Health Laboratory Service, Centre for Applied Microbiology Research, Porton Down, Salisbury SP4 0JG, UK

The composition of lipopolysaccharide (LPS) extracted with aqueous phenol from a virulent English plum isolate of *Pseudomonas syringae* pv. *morsprunorum* varied according to the partial pressure of oxygen (pO₂) in the culture medium at the time of harvest. When pO₂ was low, the organism grew slowly and produced smooth LPS bearing rhamnan sidechains. As pO₂ was raised, the rate of growth increased and smooth LPS was replaced by a rough species deficient in rhamnose, which co-extracted with a D-glucan. Organization of rhamnose and glucose into separate polymers was shown by the selective susceptibility of the rhamnose-containing polymer to hydrolysis by rhamnanase of the phage A7. By methylation analysis, GC-MS, and ¹H- and ¹³C-NMR spectroscopy, the glucan was shown to consist of α(1 → 4)-linked residues with α(1 → 4,6)-branch points and non-reducing terminal residues in the approximate ratio 4:1:1, resembling glycogen in composition. A glucan which co-extracted with LPS using phenol/water from an avirulent plum isolate that was resistant to lysis by phages A1 and A7 was shown by methylation analysis to have a similar structure. Whether the effect on LPS composition was due directly to pO₂, or was dependent on the rate of growth, has not been established. It is suggested that, because epiphytic growth would entail exposure to high pO₂, English plum isolates growing on the surfaces of host plants might be unable to produce smooth LPS. Since cell surface composition affects virulence in plant-pathogenic pseudomonads, this effect could account for the observed failure of the English plum isolates to enter the host via leaf scars.

Keywords: *Pseudomonas syringae* pv. *morsprunorum*, lipopolysaccharide, glycogen, aeration

INTRODUCTION

Pseudomonas syringae pv. *morsprunorum* causes bacterial canker in cherry and plum. The side-chain of lipopolysaccharide (LPS) from the English cherry isolate C28 is composed of a rhamnan linked in the repeat sequence

3)-α-D-Rhap(1 → 3)-α-D-Rhap(1 → 2)-α-D-Rhap-(1 →

(Smith *et al.*, 1985b). Glycosidic bonds at 2)-α-D-Rhap(1 → 3 are hydrolysed by the phage A7, which uses LPS as

its binding site, releasing tri- and hexasaccharides in equimolar proportion (Smith *et al.*, 1994).

Garrett *et al.* (1974) showed that phage-resistant mutants of cherry isolates of the pv. *morsprunorum* displayed diminished virulence. Mutants resistant to phages binding LPS, such as A7, have altered LPS structure. Zamze *et al.* (1985) found that three phage A7-resistant mutants of strain C28 contained rough LPS. A fourth mutant, D17-A1R, derived from the cherry-type plum isolate D17 by selection for resistance to the related phage A1, apparently bore side-chains composed entirely of glucose.

Zamze *et al.* (1985) found that the side-chains of LPS from the plum isolate D10, which was cultured in stirred 1 l conical flasks, appeared to be composed of rhamnose with a small amount of glucose. It was later found that when the organism was cultured in a baffled aerated fermenter,

† **Present address:** Department of Science, Mid-Kent College of Higher and Further Education, Horstead, Maidstone Road, Chatham ME5 9UQ, UK.

Abbreviations: KDO, 3-deoxy-2-octulosonic acid; PMAA, partially-methylated alditol acetate; TMS, tetramethylsilane.

the LPS recovered apparently bore side-chains composed of a glucan. In this paper, we describe this effect and compare the structures of the glucans from strains D10 and D17-A1R. A preliminary report of this work was given by Munro *et al.* (1986).

METHODS

Organisms. D10, a typical virulent English plum isolate of *P. syringae* pv. *morsprunorum*, was originally supplied by Dr C. M. E. Garrett, Horticultural Research International, East Malling, Kent, UK. D17-A1R, a mutant of diminished virulence, was selected by Garrett *et al.* (1974) for resistance to phage A1 from strain D17, an atypical virulent English plum isolate sensitive to phages A1 and A7 (Crosse & Garrett, 1963). D17-A1R was also resistant to phage A7. Both bacterial strains, and bacteriophage A7 (Smith *et al.*, 1994), were propagated, assayed and stored as described by Smith *et al.* (1985a).

Bulk culture of bacteria and extraction of LPS. Bacteria were cultured at 18 °C in glucose-minimal salts medium (Smith *et al.*, 1985a). Dissolved oxygen tension was measured using a steam-sterilizable galvanic lead electrode (Borkowski & Johnson, 1967).

Strain D10 was grown in stirred, baffled 1 l Erlenmeyer flasks containing 250 ml portions of medium, and harvested at late exponential phase ($OD_{540} = 2.0$), as described by Smith *et al.* (1985a). The organism was also grown in 8 l batches by draw-and-fill technique, in a type 1/1000 LHE fermenter (LH Engineering) fitted with a 10 l stirred, baffled vessel, with agitation and aeration rates of 750 r.p.m. and 2 l air min⁻¹ respectively (described below as moderate aeration). Growth under conditions of vigorous aeration was provided by raising the agitation and aeration rates to 1250 r.p.m. and 5 l air min⁻¹, respectively. Strain D17-A1R was routinely grown with moderate aeration.

Fermenter cultures were checked daily for purity by testing for characteristic colony morphology and levan production (Garrett *et al.*, 1966), and for induction of the hypersensitive response in tobacco (Klement, 1963).

LPSs were extracted from cells using aqueous phenol and purified as described previously (Smith *et al.*, 1985a, b).

Partial hydrolysis of LPS by phage A7. High- and low- M_r products generated by the action of phage A7 on LPS from cells of strain D10 grown with poor or moderate aeration were separated by ultracentrifugation. Oligosaccharides in the low- M_r fraction were then resolved chromatographically using Sephadex G-25 (Smith *et al.*, 1994).

Analytical and chromatographic methods. Most procedures employed for the analysis of LPS, the hydrolysis with 1% (v/v) acetic acid and de-O-acylation of LPS in alkali, and gel permeation chromatography of the products, were those of Smith *et al.* (1985a, b). Total carbohydrate, KDO and glucose were determined in the free polysaccharide moiety or in whole LPS after acid hydrolysis. Samples were treated with 0.5 M H₂SO₄ for 3.5 h at 105 °C under N₂ in sealed glass ampoules, neutralized with Dowex-1 resin (OH⁻ form), and clarified by passage through a sintered glass filter. Total carbohydrate was determined by the phenol/sulphuric acid reaction (Dubois *et al.*, 1956) using L-rhamnose or D-glucose as the standard. Rhamnose was determined separately using the cysteine/sulphuric acid method (Osborn, 1963); D-glucose was estimated enzymically with glucose oxidase (kit no. 510; Sigma) after hydrolysis with 2 M HCl.

Neutral sugars released by hydrolysis of side-chain polysaccharides were identified by descending paper

chromatography (Smith *et al.*, 1985a), and were determined by gas chromatography (GC) as their alditol acetates (Zamze *et al.*, 1986), using a 1.83 × 0.64 cm column of GP 3% SP-2340 on Supelcoport 100/120 (Supelco) with temperature programming from 170 to 270 °C at 2 °C min⁻¹. Response factors for flame ionization detection were calculated using derivatized mixtures of standard sugars in equimolar proportion.

Polyacrylamide gel electrophoresis of LPSs. Intact LPSs were submitted to PAGE and silver-stained by the procedure of Aucken & Pitt (1993).

Absolute configuration of rhamnose and glucose. The absolute configuration of rhamnose was determined by GC of the acetylated oct-2-yl rhamnosides (Smith *et al.*, 1985b). The configuration of glucose was determined by submitting hydrolysed samples of the glucan from LPS of strain D10 to estimation by D-glucose oxidase, and comparing the values with those from determinations made with phenol/sulphuric acid.

Methylation analysis of oligo- and polysaccharides. Polysaccharides (6–10 mg), or oligosaccharide derived by digestion with phage A7 (3–6 mg), were methylated in dimethyl sulphoxide (DMSO) and recovered and analysed as described by Smith *et al.* (1985b, 1994). GC-mass spectrometry was performed as described by Ray *et al.* (1987), except that chromatography on SE30 was performed with temperature programming from 120 to 200 °C at 10 °C min⁻¹.

NMR spectroscopy. NMR spectra of oligo- and polysaccharides were recorded in 100% D₂O (2.5 ml) in a Bruker WH-400 spectrometer (Smith *et al.*, 1994). ¹H-NMR spectra of the polysaccharides (20 mg) liberated by mild acid hydrolysis from LPSs of cells of strain D10 grown with moderate and vigorous aeration were recorded at 400.13 MHz, at 358K, relative to internal DOH ($\delta = 4.30$ p.p.m.), with a pulse width of 4 μ s, and acquisition times of 3.998 s and 1.524 s for the two respective polysaccharides.

The ¹³C-NMR spectrum of the polysaccharide (20 mg) from strain D10 cells grown with vigorous aeration was recorded at 100.62 MHz, at 358K with complete proton decoupling, relative to TMS ($\delta = -2.167$ p.p.m.) in a capillary tube, and 36503 accumulations of free induction decay were made, with a pulse width of 10 μ s and an acquisition time of 0.655 s. A completely coupled ¹³C-NMR spectrum was also obtained from 33596 accumulations of free induction decay, with a pulse width of 10 μ s, an acquisition time of 0.655 s and an interpulse spacing of 1.655 s.

RESULTS

Extraction and purity of LPS from strains D10 and D17-A1R

Yields of acetone-dried cells from strains D10 and D17-A1R grown in 8 l batches in the fermenter were typically 1.1–1.3 g (l medium)⁻¹. Dried cells of strains D10 and D17-A1R yielded 2.5–3.0% and 1.0% (w/w) respectively of LPS, which contained approximately 1.5% (w/w) nucleic acid and 1–2% (w/w) protein.

Culture pO₂ and sugar content of whole LPS from strain D10

As found previously by Zamze *et al.* (1985), strain D10 yielded LPS containing side-chains composed almost exclusively of rhamnose (type A LPS) when grown in stirred, baffled 1 l flasks. During the present investigation, this polymer was shown by methylation analysis, ¹H- and

Fig. 1. Changes in pO_2 of culture medium of strain D10 grown under different conditions. Partial pressures of oxygen were measured using a galvanic lead electrode. A, cultures grown in stirred, baffled Erlenmeyer flasks yielding low pO_2 conditions; B, C, cultures grown in a baffled fermenter stirred at 750 r.p.m. and aerated at 2 l air min^{-1} , and 1250 r.p.m. and 5 l air min^{-1} (medium and high pO_2 conditions respectively). Vertical arrows indicate routine times of harvest.

^{13}C -NMR spectroscopy to be structurally identical with the rhamnan from the cherry isolate C28 described by Smith *et al.* (1985a, b).

When cultured in the fermenter with vigorous aeration, strain D10 yielded a second form of LPS (type B) in which glucose had apparently replaced rhamnose. With moderate aeration, the organism produced an intermediate form (type AB LPS) containing both sugars.

The organisms recovered from fermenter cultures were invariably indistinguishable from strain D10 on the basis of their colony morphology, ability to produce levan on nutrient sucrose agar, induction of the hypersensitive

response in tobacco, and sensitivity to phage A7. Production of the novel LPS was therefore not due to the emergence of a contaminant during bulk culture. Nor could the altered composition be ascribed to differences in growth medium, temperature or pH, since the same medium and temperature were used throughout, and the same pH (6.4) was reached at the time of harvest. However, the terminal pO_2 in the various cultures differed considerably. Production of type A LPS correlated with low terminal pO_2 ; types AB and B LPSs were produced at progressively higher pO_2 values.

Fig. 1 shows the changes in culture pO_2 during growth of strain D10 in stirred, baffled flasks, and in moderately- and vigorously-aerated fermenter cultures. The approximate times of harvesting for routine extraction of LPS are indicated in each case. In flask culture, pO_2 during growth fell at a uniform rate to 0% saturation, and returned sharply to the original level when growth ceased. In fermenter cultures given moderate aeration, pO_2 fell to 45% saturation before returning to the initial value when growth ceased. With vigorous aeration, the minimal value in culture was 77%. Since the medium was carbon-limited, the cessation of O_2 consumption apparently corresponded in each case with glucose depletion. Typical times taken to reach the break-point were 67 h for growth in flasks, and 20 h and 12 h in moderately- and vigorously-aerated fermenter cultures, respectively, indicating (not unexpectedly) that the growth rate was lower when oxygen became a limiting factor.

Cultures were routinely harvested for extracting LPS when the OD_{540} reached 2.0. At this point, typical values for pO_2 were 25%, 53% and 84% saturation for flask cultures, and moderately- and vigorously-aerated fermenter cultures respectively.

Table 1 shows the content of rhamnose and glucose in LPSs extracted from cells grown under each set of

Table 1. Rhamnose and glucose content of intact LPS from strain D10 grown at differing pO_2 values

Organisms were cultured as described in Methods, using a galvanic electrode to determine dissolved pO_2 values. Glucose and rhamnose were estimated using glucose oxidase and cysteine/ H_2SO_4 respectively.

Culture conditions	pO_2 of culture at harvest (% saturation)	Carbohydrate [$\mu\text{mol (mg LPS)}^{-1}$]				Glc:Rha ratio in polysaccharide fraction	Type of LPS
		Glucose	Rhamnose	Total*	Glc:Rha		
Stirred baffled flask	25	0.18	1.36	1.54	0.13	0.16	A
	ND	0.29	1.33	1.62	0.22	ND	ND
Stirred aerated baffled fermenter 750 r.p.m.; 2 l air min^{-1}	53	0.24	0.55	0.79	0.44	0.63	AB
	ND	0.37	0.64	1.01	0.58	ND	ND
	84	1.02	0.38	1.40	2.68	33.6	B
	ND	1.41	0.31	1.72	4.55	ND	ND

ND, Not determined.

* Total glucose + rhamnose.

Fig. 2. SDS-PAGE of LPSs from strains of *P. syringae* pv. *morsprunorum*. LPS samples (30 µl, denatured by heat treatment with SDS) were loaded onto a 10% separating polyacrylamide gel slab (13×18 cm) surmounted by 5% stacking gel, submitted to electrophoresis in a discontinuous Tris/glycine buffer containing SDS, then fixed and stained with silver as described in Methods. Lanes contain LPS of: 1, strain C28 (a cherry isolate: see Smith *et al.*, 1985a); 2, cells of strain D10 harvested at low pO_2 ; 3, cells of strain D10 harvested at high pO_2 ; 4, strain D17-A1R. Origins are situated at the top of the photograph.

conditions, in relation to the pO_2 at the point of harvest. Glucose:rhamnose ratios increased with increasing dissolved pO_2 , but for ratios of 0.13, 0.44 and 2.68 the combined quantities of the two sugars per mg of LPS were 1.54, 0.79 and 1.40 µmol respectively. The rhamnose content therefore fell before the glucose content was appreciably increased.

Structure of type B LPS from strain D10

To determine whether glucose was attached to the lipid A in type B LPS, samples (20 mg) were de-O-acylated with ethanolic alkali and fractionated on Sephadex G-200. The carbohydrate content of the deacylated LPS was completely recovered in a single peak that co-eluted with the single peak of KDO. The molar ratio of KDO:glucose in the combined peak fractions was 1:27, which corresponded closely with that for native LPS (1:23). Co-elution indicated that the glucose content was covalently joined with the KDO; but this interpretation was not supported by an examination of silver-stained SDS-polyacrylamide electrophoretograms of intact LPSs (Fig. 2). The banding pattern of smooth LPS typically appears on gels as a ladder-like profile, due to the presence of multiple molecular species of differing side-chain length (Helander, 1985); rough LPSs give only a fast-moving band formed by lipid A units bearing only core oligo-

saccharide. In the present comparison, only LPS from cells grown with poor aeration yielded the ladder pattern. LPS from cells grown with vigorous aeration gave only the fast-moving band formed by rough LPS. With the latter sample, a small amount of silver-stained material, possibly uncharged polysaccharide, was detected at the site of application to the gel.

Composition of LPSs from strain D10

Analysis of the fatty acids in lipid A from type B LPS revealed the presence of 3-OH 10:0, 12:0, 2-OH 12:0 and 3-OH 12:0, in molar ratio 1:30:0.51:1.00:1.74. The same four components were found in type A LPS (Zamze *et al.*, 1985), though in differing ratios.

After mild acid hydrolysis, each LPS yielded three carbohydrate peaks from gel filtration on Sephadex G-75 or G-50. In each case a polysaccharide was eluted at the void volume of the column, followed sequentially by two peaks (both of which also contained phosphate) representing core oligosaccharide and liberated KDO respectively, as described by Smith *et al.* (1985a) for the hydrolysis products of LPS from strain C28.

Complete hydrolysis of the components of the void-volume peaks yielded glucose and rhamnose, which were demonstrated by paper chromatography in all samples except those derived from cells grown with vigorous aeration, which contained only glucose. Analysis of the sugars by GC after conversion to alditol acetates confirmed that only a small quantity of rhamnose was present in the LPS of cells grown at high pO_2 . The molar ratio of glucose:rhamnose, determined as alditol acetates, increased with an increase in terminal pO_2 in the culture (Table 1).

The changes in rhamnose and glucose content were localized in the polysaccharide fraction. Analysis by GC of the acetylated oct-2-yl rhamnosides derived from type A LPS demonstrated that the sugar was in the D-configuration. Identical amounts of glucose were detected in hydrolysed polysaccharide from type B LPS using glucose oxidase and phenol/sulphuric acid reagent, indicating that this sugar was also of the D-form.

Although the gluco-polysaccharide from type B LPS eluted from Sephadex G-75 in the position expected of liberated side-chain material, the behaviour of intact type B LPS on PAGE described above indicated that this LPS was rough. The polysaccharide was apparently unattached to the LPS, and was not a species of side-chain.

LPS from strain D17-A1R

The LPS produced by strain D17-A1R contained glucose regardless of the pO_2 sustained during growth. The ratio of KDO to glucose (1:16) was identical with the value found by Zamze *et al.* (1985). In silver-stained SDS-polyacrylamide gel electrophoretograms of the intact LPS (Fig. 2), the fast-moving band of rough LPS was accompanied by a short ladder of relatively fast-running bands in a pattern characteristic of molecular species with

Table 2. PMAAs derived from the side-chains of LPSs from strains D10 and D17-A1R

PMAAs were separated by GC on 3% ECNSS-M at 180 °C, as described in Methods. Retention times, t_R , are quoted relative to the PMAAs derived from glycogen. Molar ratios were derived from peak areas after allowing for differences in response factors by applying the correction factors of Sweet *et al.* (1975). GC-MS was performed as described in Methods, using a 200 × 0.2 cm silanized glass column of 3% SE30 for chromatographic separation of components.

Identity of peak	Published Glycogen		D10 polysaccharide				D17-A1R polysaccharide		Mass spectrometry primary ions (<i>m/z</i>)
	<i>t</i> _R [*]	<i>t</i> _R	From type AB LPS		From type B LPS		<i>t</i> _R	Molar ratio	
			<i>t</i> _R	Molar ratio	<i>t</i> _R	Molar ratio			
3,4-Me ₂ Rha	0.92	—	0.92	2.89	—	—	—	—	131, 189
2,3,4,6-Me ₄ Glc	1.00	1.00	1.01†	6.18	1.00	0.80	1.00	0.83	45, 117, 161, 205
2,4-Me ₂ Rha	1.01	—			—	—	—	—	117, 131, 233
2,3,6-Me ₃ Glc	2.50	2.46	2.35	4.10	2.46	4.28	2.42	4.79	45, 117, 233
2,3-Me ₂ Glc	5.39	4.95	4.95	1.00	4.96	1.00	4.95	1.00	117, 261

* Published retention times for authentic standards are quoted from Jansson *et al.* (1976) and Smith *et al.* (1985b).

† These two peaks were unresolved on ECNSS-M.

truncated side-chains. A trace of silver-stained material was detected at the load origin.

Samples (6–10 mg) of the polysaccharide recovered by gel permeation chromatography after mild acid hydrolysis of LPS from this strain revealed glucose and rhamnose present in the ratio 20:1 (as their alditol acetates).

Methylation analysis of polysaccharides from LPSs of D10 and D17-A1R

PMAAs derived from samples of the methylated polysaccharides (6–10 mg) from type B LPS of strain D10 and LPS from D17-A1R were identified by comparing their retention times (t_R) on ECNSS-M with published values, and their mass spectra with those of authentic standards derived from permethylated glycogen. The major PMAAs detected in both samples (Table 2) were 2,3,4,6-tetra-*O*-methylglucitol 1,5-diacetate (2,3,4,6-Me₄Glc), 2,3,6-tri-*O*-methylglucitol 1,4,5-triacetate (2,3,6-Me₃Glc) and 2,3-di-*O*-methylglucitol 1,4,5,6-tetraacetate (2,3-Me₂Glc). For the polysaccharide from D10, the approximate molar ratios of the products were respectively 1:4:1, which (allowing for some undermethylation of the non-reducing terminal residues) indicated a side-chain composed of 16.6% Glc(1 → (terminal) groups, 66.8% 4)Glc(1 →, and 16.6% of 4,6)Glc(1 → (branch) residues. For D17-A1R, the ratio was approximately 1:4.5:1.

NMR spectroscopy of the polysaccharide from type B LPS of strain D10

The ¹H-NMR spectrum for the anomeric region of polysaccharide (20 mg) from type B LPS of strain D10 (Fig. 3) revealed an intense doublet prominent at 5.38–5.39 p.p.m. ($J = 3.5$ Hz; assigned to H-1 of 4)-α-

Fig. 3. ¹H-NMR spectrum, with integration, of the anomeric region of polysaccharide from type B LPS of strain D10. The spectrum was recorded at 400.13 MHz from 20 mg type B polysaccharide derived from LPS of strain D10 grown in the fermenter with vigorous aeration, in 2.5 ml 100% D₂O at 358K. The pulse width was 4 μs and the acquisition time 1.524 s. Chemical shifts were calculated relative to internal D₂O ($\delta = 4.30$ p.p.m.).

Glc(1 →), with a pronounced shoulder (minor peak) at 5.35 p.p.m. (H-1 of non-reducing terminal residues, α-Glc(1 →) (van der Veen, 1962), and a further minor peak at 5.01 p.p.m. in the resonance region for α(1 → 6)-linkages (4.7–4.8 p.p.m.; Meyer *et al.*, 1978), which was assigned to 4,6)-α-Glc(1 →. Ratios of the peak areas were 4:2:1:1 respectively, consistent with the ratios of residues indicated by methylation analysis. Signals ascribable to rhamnose H-6 (approx. 1.3 p.p.m.) were almost absent from this spectrum.

The ¹³C-NMR spectrum of the same polysaccharide (20 mg) contained eleven peaks (Fig. 4). The assignment

Fig. 4. ^{13}C -NMR spectrum, with integration, of polysaccharide from type B LPS of strain D10. The spectrum was recorded at 100.62 MHz from 20 mg polysaccharide derived from type B LPS of strain D10 grown in the fermenter with vigorous aeration, in 2.5 ml 100% D_2O at 358K. A total of 36503 accumulations were recorded, with a pulse width of 10 μs and an acquisition time of 0.655 s. Chemical shifts were calculated relative to TMS ($\delta = -2.167$ p.p.m.) in a capillary tube.

of resonances is given in Table 3. Peaks at 101.06 p.p.m. (doublet, $^1J_{\text{CH}} = 181$ Hz) and 101.28 p.p.m. (doublet, $^1J_{\text{CH}} = 181$ Hz) were attributed to α -anomeric C atoms (Seymour *et al.*, 1976; Usui *et al.*, 1973; Bock & Pederson, 1974).

The intense asymmetric signal at 62.11 p.p.m. was attributed to unsubstituted Glc C-6 (triplet; $^1J_{\text{CH}} = 141$ Hz). Three major signals (doublets, $^1J_{\text{CH}} = 140$ –152 Hz) between 70 and 80 p.p.m. were assigned to C-2, C-3 and C-5 of the predominant 4)- α -Glc(1 $\rightarrow$ residues (Seymour *et al.*, 1976; Usui *et al.*, 1973), and a

signal at 78.93 p.p.m. was assigned to C-4 in linkage. Four resonances of lower intensity were assigned to the corresponding carbons of non-reducing terminal residues.

No signals were found that were uniquely attributable to the branch residue. The peak at 71.01 p.p.m., attributed above to C-4 of α -Glc(1 $\rightarrow$ 4), may have been shared by C-5 of the branch residue, which resonates at a similar value (Gorin, 1981). Resonances anticipated at 67.3 p.p.m. for C-6 in linkage, and at 99.5 p.p.m. for C-1 of residues linked α (1 $\rightarrow$ 6) to branch residues, were not detected. Gorin (1981) reported similar difficulty in detecting these signals in spectra of β -limit dextrans from rabbit liver glycogen and plant amylopectin, and ascribed the problem to low segmental motion of the 4,6-di-O-substituted branch points.

Phage A7-mediated hydrolysis of type AB LPS from strain D10

To establish whether rhamnose and glucose were co-polymerized or formed separate polysaccharides in type AB LPS, a sample (90 mg, with a glucose:rhamnose ratio of 0.63:1 for the polysaccharide fraction) was incubated with phage A7 for 2 h, and the mixture was centrifuged to deposit liberated core-lipid A together with any remaining intact LPS. The low- M_r hydrolysis products recovered in the supernatant gave $\lambda_{\text{max}} = 480$ nm when assayed with phenol/sulphuric acid reagent, consistent with a pre-dominance of rhamnose, and amounted to approximately 6 mg of rhamnose equivalents.

This material was separated by chromatography on a column of Sephadex G-25 to yield two peaks eluting in

Table 3. ^{13}C -NMR spectroscopy: chemical shifts assigned to glucose residues in the polysaccharide from type B LPS of strain D10

Source	Residue	Chemical shift (p.p.m.)					
		C-1	C-2	C-3	C-4	C-5	C-6
Polysaccharide from type B LPS	4)- α -Glc(1 $\rightarrow$	101.06	73.03	74.67	78.93	72.77	62.11
	α -Glc(1 $\rightarrow$ 4)	101.28	74.14	74.45	71.01	73.26	62.11
	4,6)- α -Glc(1 $\rightarrow$	ND	?	?	?	71.01?	ND
Maltotriose*	4)- α -Glc(1 $\rightarrow$	101.1	73.1	74.7	78.5	72.7	62.4
	α -Glc(1 $\rightarrow$ 4)	101.1	74.3	75.1	71.2	73.4	62.4
Pullulan†	4)- α -Glc(1 $\rightarrow$	100.76	?	?	78.45	?	61.45
		101.24	72.51	74.38	78.80	71.30	61.65
D-glucan‡	4)- α -Glc(1 $\rightarrow$	101.03	72.42	74.33	79.54	71.26	61.55
Panose§	α -Glc(1 $\rightarrow$ 6)	99.3	73.1	74.6	71.2	73.1	62.4
β -Limit dextrin	4,6)- α -Glc(1 $\rightarrow$	?	?	?	?	71.0	67.3

ND, No plausible signal detected.

* Maltotriose, α -Glc(1 $\rightarrow$ 4)- α -Glc(1 $\rightarrow$ 4)-Glc (Usui *et al.*, 1973).

† Pullulan Y6992, containing α -Glc(1 $\rightarrow$ 4)- and α -Glc(1 $\rightarrow$ 6)-linked residues (Seymour *et al.*, 1976).

‡ Glucan B1254, containing α -Glc(1 $\rightarrow$ 4), α -Glc(1 $\rightarrow$ 4), α -Glc(1 $\rightarrow$ 6) and α -Glc(1 $\rightarrow$ 4,6)-linked residues (Seymour *et al.*, 1976).

§ Panose, α -Glc(1 $\rightarrow$ 6)- α -Glc(1 $\rightarrow$ 4)-Glc (Usui *et al.*, 1973).

|| β -Limit dextrin from rabbit liver glycogen (see Gorin, 1981).

Table 4. ^{13}C -NMR spectroscopic chemical shifts of polysaccharide from type AB LPS of strain D10

Chemical shift (p.p.m.)*	Possible identity
16.95 (L)	C-6 of 3)- α -Rha(1 $\rightarrow$
17.05 (M)	C-6 of 2)- α -Rha(1 $\rightarrow$
61.10 ((S)†)	C-6 of 4)- α -Glc(1 $\rightarrow$ and α -Glc(1 $\rightarrow$
69.55 (L)	C-5 of 3)- α -Rha(1 $\rightarrow$
69.75 (M)	C-5 of 2)- α -Rha(1 $\rightarrow$
70.30 (L)	C-3 of 2)- α -Rha(1 $\rightarrow$
70.40 (L)	C-2 of 3)- α -Rha(1 $\rightarrow$
70.55 (L)	C-4 of α -Glc(1 $\rightarrow$ and C-5 of 4,6)- α -Glc(1 $\rightarrow$?
71.80 (L)	C-4 of 3)- α -Rha(1 $\rightarrow$
72.00 (L)	C-4 of 3)- α -Rha(1 $\rightarrow$
72.75 (M)	C-5 of 4)- α -Glc(1 $\rightarrow$ and C-4 of 2)- α -Rha(1 $\rightarrow$
73.40 (S)	C-2 of 4)- α -Glc(1 $\rightarrow$ and/or C-5 of α -Glc(1 $\rightarrow$?
73.65 (S)	?
78.20 (M)	C-3 of 3)- α -Rha(1 $\rightarrow$
78.35 (M)	C-2 of 2)- α -Rha(1 $\rightarrow$
101.10 (M)	C-1 of 2)- α -Rha(1 $\rightarrow$ or 4)- α -Glc(1 $\rightarrow$
102.20 (L)	C-1 of 3)- α -Rha(1 $\rightarrow$ or α -Glc(1 $\rightarrow$

*(L), (M), (S) refer to large, medium and small peaks (based on visual estimates of intensities). In this polysaccharide, rhamnose:glucose was 1:0.63; therefore glucose signals were expected to be the weaker.

† Broad grouping of weak signals.

the positions of a hexa- and a trisaccharide respectively. The monosaccharides liberated by hydrolysis of the presumed hexasaccharide were converted to the corresponding alditol acetates and analysed by GC to reveal rhamnitol pentaacetate and glucitol hexaacetate in molar ratio 16:1. The presumed trisaccharide was not examined, although the reaction product with phenol/sulphuric acid also gave $\lambda_{\text{max}} = 480 \text{ nm}$.

Methylation analysis of the presumed hexasaccharide confirmed its identity with the rhamnose hexasaccharide recovered from the digestion of side-chains from LPS of strain C28 by phage A7 (Smith *et al.*, 1994). The presumed trisaccharide was not examined.

The pellet recovered by ultracentrifugation of the LPS after digestion with phage A7 contained a total of 3.1 mg of glucose and 1.2 mg of rhamnose (glucose:rhamnose $\approx$ 2.6:1).

On a column of Sepharose 4B, the products formed by the action of phage A7 on 5 mg of type B LPS yielded a large carbohydrate peak eluting at the void volume. Only a small quantity of rhamnose oligosaccharide emerged at the totally-included volume. The elution profile was almost identical with that produced by Zamze (1983) after treatment of LPS from strain D17-A1R with phage A7.

Taken together, the data indicate that rhamnose and glucose in type AB LPS did not form a co-polymer.

Fig. 5. ^1H -NMR spectrum, with integration, of the anomeric region of polysaccharide from type AB LPS of strain D10. The spectrum was recorded at 400.13 MHz from 20 mg polysaccharide derived from type AB LPS of strain D10 grown in the fermenter with moderate aeration, in 2.5 ml 100% D_2O at 358K. The pulse width was 4 μs and the acquisition time 3.998 s. Chemical shifts were calculated relative to internal D_2O ($\delta = 4.30 \text{ p.p.m.}$).

Methylation analysis of type AB LPS from strain D10

PMAAs derived from the methylated polysaccharide derived from type AB LPS of strain D10, when analysed by GC on a column of ECNSS-M, yielded four major peaks (Table 2), the first of which was 3,4-di-O-methylrhamnitol 1,2,5-triacetate (3,4-Me₂Rha; $t_R = 0.92$). The second ($t_R = 1.01$) contained 2,3,4,6-Me₄Glc and 2,4-di-O-methylrhamnitol 1,3,5-triacetate (2,4-Me₂Rha) as minor and major unresolved components respectively. The third and fourth peaks were 2,3,6-Me₃Glc ($t_R = 2.35$) and 2,3-Me₂Glc ($t_R = 4.95$). All components, including the two unresolved PMAAs of the second peak, were subsequently separated on 3% SE30 and identified by mass spectrometry.

The demonstration of 2,4- and 3,4-Me₂Rha as the major PMAAs in this sample indicated that the rhamnan in type AB polysaccharide was structurally identical with the side-chain of strain C28 (Smith *et al.*, 1985b).

NMR spectroscopy of polysaccharide from type AB LPS of strain D10

The anomeric region of the ^1H -NMR spectrum of type AB polysaccharide (20 mg) from strain D10 (Fig. 5) displayed three singlet signals at 5.23, 5.08 and 5.01 p.p.m., which were of approximately equal intensity and similar to those found in the spectrum of the rhamnan from strain C28 (Smith *et al.*, 1985b). Also present were the resonances assigned above to anomeric protons of α -glucopyranose residues: a broad peak at 5.39 p.p.m. with a shoulder at 5.36 p.p.m., and a shoulder at 5.00 p.p.m. to

the signal at 5.01 p.p.m. The ratio of the combined peak areas of signals attributed to rhamnose and to glucose accorded with the glucose:rhamnose ratio (1:1.59) for the polysaccharide mixture. The two doublets centred on 1.3 p.p.m. associated with rhamnose H-6 amounted to three protons per rhamnose H-1. The spectrum was therefore derived additively from the spectra of the two individual polymers.

A ^{13}C -NMR spectrum obtained from the same sample was of poor quality, but likewise appeared to be derived from the spectra of two separate polymers. Major peaks detected are identified in Table 4.

DISCUSSION

Extraction of cells of the plum isolate *P. syringae* pv. *morsprunorum* strain D10 and the phage-resistant mutant D17-A1R with aqueous phenol yielded LPS preparations containing glucans which resembled glycogen in residue composition. The ^{13}C -NMR spectrum of the glucan from strain D10 was also glycogen-like, but neither the precise structure of this material, nor its original cellular location, has been determined.

Unlike D17-A1R, strain D10 also synthesized a D-rhamnan identical with the side-chain produced by the cherry isolate C28. The loss of the rhamnan and appearance of the glucan occurred when the pO_2 during culture was increased. At intermediate pO_2 values, both polymers were present; from batch to batch of LPS a continuous variation was observed in the ratio of the two sugars. Evidence for covalent attachment of the glucan to lipid A was sought by examining the chromatographic behaviour of the alkaline de-O-acylation products of LPS. As evidence of structural association, the co-elution of these two components during chromatography was almost certainly misleading; SDS-PAGE of intact type B LPS did not give the ladder pattern characteristic of smooth LPS. It is possible that the glucan did not react with silver strain, but that is unlikely since the predominant residue in the polymer is (1 → 4)-linked glucose, which should be susceptible to periodate oxidation and should therefore have reacted with silver once oxidized. Glucan not attached to the LPS would have remained at the origin during electrophoresis; traces of stained material were in fact detected around this position on the electrophoretogram of type B LPS. The available evidence therefore indicates that type B LPS was rough. Since rough LPS is not normally easily extracted from cells with aqueous phenol, the physical association between glucan and LPS, established before or during extraction, may have enhanced the solubility of the LPS in this reagent.

Nor was there any evidence in type AB LPS that strain D10 produced a heteropolysaccharide containing both sugars. Both methylation analysis and NMR spectroscopy indicated the simultaneous presence of two distinct polymers. Further evidence was provided by demonstrating that rhamnose was selectively liberated by the action of phage A7 rhamnanase, yielding oligosaccharides similar to those derived from the side-chain of strain C28 by the same treatment (Smith *et al.*, 1994).

The effect of pO_2 on LPS structure requires further investigation. Since *P. syringae* is an obligate aerobe, the effect may simply be to limit the growth rate, which more directly determines LPS type. This possibility could be examined by comparing the LPSs recovered from organisms grown with constant pO_2 at different dilution rates in a chemostat. Alternatively, the controlling factor could be pCO_2 , since the rate at which carbon dioxide is swept from liquid culture is dependent on agitation and aeration intensity (Lilly, 1983). Chester & Meadow (1975) found that LPS of *P. aeruginosa* varied in composition depending on the growth vessel employed, but did not investigate the influence of partial pressure of dissolved gases.

Although the core regions of types AB and B LPS have not yet been rigorously examined, Zamze *et al.* (1985) demonstrated that the cores of LPSs from cherry and plum isolates of *P. syringae* pv. *morsprunorum* were similar in composition. In the present investigation the same four fatty acids were found in the lipid A of type B as in type A LPS.

The ability to produce two distinct species of LPS may have ecological or pathological significance, since there is evidence that the composition of the cell surface in plant-pathogenic pseudomonads affects virulence. Mutants of cherry isolates of pv. *morsprunorum* resistant to LPS-binding phages such as A7 have rough LPS (Zamze *et al.*, 1985) and display diminished virulence (Garrett *et al.*, 1974). The role of LPS in plant disease is by no means clear; for example, Hignett *et al.* (1986) found that pretreatment of cherry leaf scars with the side-chain from LPS of strain C28 inhibited the development of canker after subsequent inoculation with cells of strain C28.

Both cherry and plum isolates of *P. syringae* pv. *morsprunorum* are epiphytic during the summer, entering the plant in the autumn to overwinter (Crosse & Garrett, 1970). In cherry isolates such as C28, pO_2 does not affect the type of LPS formed in culture, but in view of the observed influence of environmental factors on LPS structure in strain D10, it is conceivable that plum isolates could elaborate rough LPS when growing epiphytically under conditions of high pO_2 , and smooth LPS during growth within the host. Cherry strains enter the plant primarily through the leaf scars; plum isolates are predominantly wound pathogens. It is possible that plum strains do not establish infections via leaf scars because the rough LPS that they present on entry triggers plant defence mechanisms. Conversely, moribund tissue at wound sites might offer no resistance to saprophytic growth of the organism, which would cause a local fall in pO_2 , allowing in turn the synthesis of the side-chain, and enabling the organism to penetrate into neighbouring healthy tissue. By contrast, when plum isolates grow as epiphytes, they are exposed to attack by LPS-binding phages. In such conditions, presentation of rough LPS (and glucan?) at the cell surface might assist in ensuring their survival.

The production of glucan by strain D17-A1R was unaffected by pO_2 in culture. As a mutant resistant to an

LPS-binding phage, this organism should be rough. In the parent strain (D17), Zamze *et al.* (1985) found that the rhamnose-bearing polysaccharide fraction recovered after mild acid hydrolysis of the LPS also contained 27% glucose. Strain D17 may therefore be capable of producing substantial quantities of glucan. Since this biosynthetic capability should not have been affected by mutation to phage resistance, a mutant such as D17-A1R might plausibly synthesize the polymer regardless of growth conditions.

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